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Fabien Pierrel, Megan L. Bestwick, Paul A. Cobine, Oleh Khalimonchuk, Julia A. Cricco and Dennis R. Winge*

From the University of Utah Health Sciences Center, Departments of Medicine and Biochemistry, Salt Lake City, Utah 84132

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*To whom correspondence should be addressed

Tel: 801-585-5103 Fax: 801-585-5469

Email: dennis.winge@hsc.utah.edu

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ABSTRACT

The assembly of cytochrome *c* oxidase (CcO) in yeast mitochondria is shown to be dependent on a new assembly factor designated Coa1 that associates with the mitochondrial inner membrane. Translation of the mitochondrial-encoded subunits of CcO occurs normally in *coa1*Δ cells, but these subunits fail to accumulate. The respiratory defect in *coa1*Δ cells is suppressed by high copy *MSS51*, *MDJ1* and *COX10*. Mss51 functions in Cox1 translation and elongation, whereas Cox10 participates in the biosynthesis of heme *a*, a key cofactor of CcO. Respiration in *coa1*Δ and *shy1*Δ cells, is enhanced when Mss51 and Cox10 are co-expressed. Shy1 has been implicated in formation of the heme a_3-Cu_B site in Cox1. The interaction between Coa1 and nascent Cox1 and the physical and genetic interactions between Coa1 and Mss51, Shy1 and Cox14 suggest that Coa1 coordinates the transition of nascent Cox1 from the Mss51:Cox14 complex to the heme *a* cofactor insertion involving Shy1. *coa1*Δ cells also display a mitochondrial copper defect suggesting that Coa1 may have a direct link to copper metallation of CcO.

INTRODUCTION

Cytochrome *c* oxidase (CcO) is the terminal enzyme of the energy transducing respiratory chain in mitochondria of eukaryotes, coupling the reduction of molecular oxygen to proton translocation across the inner membrane (IM) which drives ATP synthesis. Eukaryotic CcO consists of 12-13 subunits, with three mitochondrial encoded subunits (Cox1-Cox3) forming the core enzyme. The remaining subunits are encoded by the nuclear genome and consist of small polypeptides that surround the catalytic core (Tsukihara et al., 1995). The core enzyme contains three copper atoms and two heme *a*, a cofactor specific to CcO (Tsukihara et al., 1995). The enzyme is embedded within the IM with a portion of the molecule protruding into the intermembrane space (IMS) and a separate portion extending into the matrix compartment.

Assembly of CcO requires a myriad of steps including the assembly of subunits translated on cytoplasmic and mitochondrial ribosomes, modification of protoheme to heme *a* and the subsequent insertion of this moiety, along with that of copper, zinc and magnesium ions, into the nascent enzyme complex (Carr and Winge, 2003; Khalimonchuk and Rodel, 2005). Two separate copper centers Cu_A and Cu_B are functionally important in both Cox2 and Cox1, respectively. The assembly process of CcO requires over 30 accessory proteins (Barrientos et al., 2002a; Tzagoloff and Dieckmann, 1990) and appears to follow a sequential order of subassembly intermediates in mammalian tissues (Nijtmans et al., 1998; Williams et al., 2004).

Translation of mitochondrial-encoded subunits occurs within the matrix on ribosomes associated with both the inner boundary membrane and cristae membrane (Vogel et al., 2006). Transcripts for *COX1-COX3* are recruited to the IM in yeast by a series of IM-associated translational activators (Sanchirico et al., 1998). These activators bind sequences in the 5' untranslated regions of the mRNA and promote co-translational membrane insertion (Naithani et al., 2003). Translation of the *COX1* transcript is activated by Pet309 (Manthey and McEwen, 1995), the *COX2* transcript by Pet111 (Green-Willms et al., 2001), and the *COX3* transcript by Pet54, Pet122 and Pet494 (McMullin and Fox, 1993). In addition to Pet309, Mss51 is required for

COX1 translational initiation and has an additional role in translational elongation (Barrientos et al., 2004; Perez-Martinez et al., 2003; Siep et al., 2000). Mss51 also binds Cox1 nascent chains and collaborates with Cox14 in a post-translational step in CcO assembly (Barrientos et al., 2004). Cox1-Cox3 are inserted into the IM by translocases, one of which is Oxa1 (He and Fox, 1997; Hell et al., 2001).

Heme *a* biogenesis involves farnesylation and oxidation of protoheme catalyzed by Cox10 and Cox15, respectively. Although eukaryotic heme *a* insertion is not well understood, heme *a* is likely inserted in Cox1 prior to its association with other subunits (Williams et al., 2004). In the absence of Cox2, Cox1 accumulates in *Rhodobacter sphaeroides* with a single heme *a* moiety in the heme *a* site leaving the a_3 site unpopulated (Bratton et al., 2000). Heme a_3 formation likely occurs concurrent with Cox11-mediated Cu_B site formation in Cox1. Shy1 appears to be important for heme a_3 insertion or stabilization of the intermediate prior to Cox2 binding because *R. sphaeroides* lacking *SHY1* have a CcO assembly defect in heme a_3 and Cu_B (Smith et al., 2005). Shy1 is not absolutely required for CcO assembly in yeast, since *shy1*Δ cells retain 10-15% residual CcO activity. Mutations in *SURF1,* the human ortholog of *SHY1*, cause a CcO deficiency resulting in Leigh syndrome (Zhu et al., 1998).

Copper insertion is required in the maturation of both Cox1 and Cox2. A number of accessory factors are important in the copper metallation of CcO within the IMS. The source of copper for the formation of the Cu_A and Cu_B cofactor sites in CcO derives from a copper pool within the mitochondrial matrix. This pool of copper is present in a ligand complex (CuL) that is conserved in eukaryotic cells (Cobine et al., 2006a). Targeting of heterologous copper-binding proteins to the matrix attenuates this CuL complex resulting in an impairment of CcO assembly. This predicts the presence of mitochondrial IM proteins for the transport of the CuL complex (Cobine et al., 2006b).

A group of mitochondrial proteins of unknown function were designated as the Found in Mitochondrial Proteome (FMP) family in a yeast proteomic study (Sickmann et al., 2003). In an

attempt to identify proteins involved in the translocation of the CuL complex to mitochondria, we screened a collection of FMP gene deletion strains for those exhibiting respiratory defects and abnormal mitochondrial copper concentrations. In the present study, we identify one FMP protein (Yil157c/Fmp35) that when deleted from cells results in low mitochondrial copper levels and attenuated CcO activity. This IM protein, renamed Coa1 for Cytochrome Oxidase Assembly 1, is shown presently to be important for Cox1 assembly. Deletion of *COA1* or *SHY1* leads to very similar phenotypes that can be suppressed by *MSS51* and *COX10* arguing that Coa1 and Shy1 function at a closely related step in Cox1 assembly. Genetic and physical interaction of Coa1 with Mss51, Cox14 and Shy1 suggest that Coa1 is important for coordinating Cox1 translation and cofactor assembly in this subunit.

RESULTS

*coa1*Δ cells are respiratory deficient and have low level of mitochondrial copper

Mitochondria isolated from 54 FMP deletion strains were screened by ICP/OES analysis for abnormal mitochondrial copper levels. One strain, *coa1*Δ (*yil157c*Δ), showed reduced mitochondrial copper (Fig. 1A). Copper levels were 43% of WT in gradient purified mitochondria, yet total cellular copper was unaffected. This defect was copper specific as zinc, iron and manganese levels were not altered in *coa1*Δ cells. Mitochondria isolated from the deletion cells cultured in 0.5 mM $CuSO_4$ contained the same elevated levels of copper as WT cells (Fig. 1A). As copper is required for CcO function, we quantified oxygen consumption of *coa1*Δ cells. This deletion strain consumed less oxygen than the WT and the addition of 1 mM $CuSO_4$ to the growth medium partially enhanced respiration (Fig. 1B). Consistent with the reduced oxygen consumption of *coa1*Δ cells, the mutant cells were compromised for growth on non-fermentable medium containing glycerol/lactate (Fig. 1C). The deletion of *COA1* in the BY or W303 backgrounds resulted in an inability to grow on minimal glycerol/lactate medium.

However, weak growth could be observed for W303 *coa1*Δ cells on rich glycerol/lactate medium, whereas BY4743 *coa1*Δ cells failed to grow (data not shown and Fig. 1C). The addition of copper to rich glycerol/lactate medium enhanced the growth of *coa1*Δ cells (Fig. 1C). The partial rescue in growth by copper salts was specific for copper, as high concentrations of iron or zinc salts failed to yield cell growth (Fig. 1C).

_coa1_Δ cells have a specific defect in CcO assembly that is partially rescued by copper

Mitochondria purified from *coa1*Δ cells exhibit low CcO activity (30% of WT) that can be partially restored by addition of copper to the growth medium (Fig. 2A). Complex II and complex III activities were unaffected by the deletion of *COA1* (data not shown). Cells lacking *COA1* had reduced steady state levels of CcO subunits Cox1-3 (Fig. 2B) and the level of heme *a* in mitochondria from *coa1*Δ cells was only 20% of WT (Fig. 2C) consistent with the low CcO activity. Heme *b* levels were comparable in mitochondria from *coa1*Δ or WT cells (Fig. 2C), in accordance with the normal complex III activity.

Since Coa1 was reported to interact with Atp14 in a global two-hybrid interaction study (Ito et al., 2001), we assessed the level of complex V in *coa1*Δ cells. The migration and levels of monomeric and dimeric complex V on Blue Native PAGE (BN-PAGE) were comparable to WT (data not shown), making it unlikely that Coa1 is important for complex V assembly.

To investigate whether Coa1 has a role in the translation of mitochondrial encoded subunits of CcO, [^{35}S]-Met incorporation into mitochondrial translation products was monitored *in vivo*. *coa1*Δ cells exhibit a labeling profile of mitochondrial encoded proteins similar to WT cells after the 15 min pulse (Fig 2D). During the 90 min chase, the levels of Cox1-Cox3 diminished markedly in *coa1*Δ cells, whereas Var1 and Cyt *b* remained stable as in the WT cells. This selective degradation of Cox1-Cox3 is consistent with an assembly defect in CcO. Taken together, these data show that *coa1*Δ cells have a post-translational CcO specific defect.

Coa1 is associated with the IM

Glycerol/lactate growth was fully restored in *coa1*Δ cells when transformed with 3' Myc-tagged *COA1* on a YCp vector under the control of the *MET25* promoter and *CYC1* terminator (Fig. 3A). Mitochondria isolated from *coa1*Δ *COA1*-Myc cells showed a prominent Coa1-Myc band at the expected mass of 22 kDa (Fig. 3B). The protein is retained in mitoplasts and is not extracted by carbonate buffer (Fig. 3C), low or high salt concentrations and sonication (Fig. 3D) suggesting a tight association with either the inner or outer membrane. Digestion of Coa1-Myc by proteinase K in mitoplasts was slow compared to OM or IMS proteins and was most similar to the degradation pattern of the matrix protein Sod2 (Fig. 3E). Coa1-Myc was degraded if the mitoplasts were initially solubilized with 1% deoxycholate (DOC) or disrupted by sonication, excluding the possibility that Coa1 is somehow resistant to degradation by proteinase K (Fig. 3E). The hydropathy profile of Coa1 reveals a central, conserved hydrophobic stretch whose length is sufficient to form a transmembrane domain. Overall, these data strongly suggest that Coa1 spans the IM.

***MSS51, COX10* and *MDJ1* are high copy suppressors of the respiratory defect of *coa1*Δ cells**

To address the role of Coa1 in CcO function, we screened for high copy suppressors of the respiratory growth defect of *coa1*Δ cells. Cells transformed with a high copy DNA bank prepared from *coa1*Δ cells were screened for growth on glycerol/lactate medium. From about 200,000 colonies replicated on glycerol/lactate medium, 51 colonies showed significant growth after 5-7 days of incubation at 30°C. The growth of most of these colonies was shown to be vector dependent based on the loss of respiratory growth when the *URA3*-based vector was shed by 5-fluoroorotic acid selection. Sequencing showed that the plasmids contained different fragments of genomic DNA that encompassed one of three genes. *MSS51* was present in 21

plasmids, *COX10* in 5 and *MDJ1* in 6. *MSS51* was a stronger suppressor relative to *MDJ1* and *COX10* (Fig. 4A).

Due to the low mitochondrial copper in *coa1*Δ cells (Fig. 1A), we assessed the efficiency of the high copy suppressors upon modulation of the copper concentration in the growth medium. At 6 mM copper, the suppression of the respiratory defect of *coa1*Δ by Mdj1 or Cox10 is weakly enhanced (Fig. 4B), but this may arise from the general copper suppression occurring in *coa1*Δ cells as shown in Fig.1C. When copper was limited by the chelator bathocuproine sulfonate (BCS), the growth of *coa1*Δ cells covered with *COA1* on a low copy plasmid was slightly attenuated compared to the YPLG medium; however respiratory growth conferred by the high copy *MSS51* was more strongly attenuated (Fig. 4B). Thus, the *MSS51* suppression of *coa1*Δ cells appears linked to copper status.

Mdj1 is a DnaJ protein in the mitochondrial matrix that activates the ATPase activity of mtHsp70. Over-expression of mtHsp70 did not rescue the respiratory deficiency of *coa1*Δ cells, nor did it improve the suppression observed with *MDJ1* when the two genes were over-expressed together (data not shown).

Cox10 is the farnesyl transferase in heme *a* biogenesis, an obligate cofactor bound to Cox1. The suppression by *COX10* is dependent on the catalytic function of the Cox10 farnesyl transferase, as a mutant allele that lacked catalytic activity (R212A, R216A) failed to act as a suppressor (data not shown).

MSS51 restores the CcO activity in *coa1*Δ cells without enhancing Cox1 translation

Mss51 is a multi-function protein acting as a translational activator of *COX1* along with Pet309, as a translational elongation factor for *COX1* and, in addition, binds the Cox1 nascent chain (Barrientos et al., 2004; Perez-Martinez et al., 2003; Siep et al., 2000). *MSS51* is a known suppressor of the CcO deficiency in *shy1*Δ cells by reversing the Cox1 translation defect

(Barrientos et al., 2002b). As mentioned, Cox1 translation was normal in *coa1*Δ cells (Fig. 2D). Over-expression of Mss51 in *coa1*Δ cells did not increase *COX1* translation in an *in vivo* mitochondrial translation study during the pulse phase of the reaction (data not shown). Both oxygen consumption measured on intact *coa1*Δ cells over-expressing Mss51 and CcO activity of mitochondria purified from this strain increased to about 50% of WT (Fig. 5A,B). Steady-state levels of Cox1-3 (Fig. 5C) and heme *a* levels (Fig. 5D) were also elevated in *coa1*Δ cells over-expressing Mss51. These data show that over-expression of Mss51 partially reverses the low CcO activity in *coa1*Δ cells presumably through a post-translational action of binding to nascent Cox1.

Since both Mss51 and Cox10 are related to Cox1 synthesis/maturation, we over-expressed these two proteins together in *coa1*Δ cells. Although *COX10* and *MDJ1* are only weak suppressors of *coa1*Δ cells, *COX10,* but not *MDJ1,* had a strong synergistic effect in combination with *MSS51* (Fig. 5E). The strong synergism between *MSS51* and *COX10* suggests that the two jointly correct a defect in *coa1*Δ cells relating to Cox1 through separate steps.

Shy1 and Coa1 function at a related step of CcO assembly

Given the additive effect of Mss51 and Cox10 co-expression in *coa1*Δ cells, we tested whether *COX10* also had a synergistic effect with *MSS51* in *shy1*Δ cells. The combination of *MSS51* and *COX10* clearly enhances respiratory growth in *shy1*Δ (Fig. 6A). However, this phenotype is Mss51-dependent, as over-expression of *COX10* by itself could not rescue the respiratory growth defect of *shy1*Δ cells (data not shown). While *COX10* enhanced the stimulatory effect of *MSS51* in *shy1*Δ cells, over-expression of *COA1* abrogated it (Fig. 6A). Over-expression of Coa1 in *shy1*Δ cells by itself did not diminish the residual CcO activity as assessed by oxygen consumption and CcO activity measurements (data not shown). Therefore, the negative effect of Coa1 seen in *shy1*Δ cells over-expressing Mss51 is likely due to an impairment of Mss51 function. The level of expression of Coa1 had no effect on the steady state

level of Mss51 (data not shown). To determine whether over-expression of Coa1 attenuated the translational stimulation by Mss51 in *shy1*Δ cells, we evaluated the incorporation of [^{35}S]-Met into mitochondrial translation products. The enhanced translation of Cox1 seen in *shy1*Δ cells over-expressing Mss51 was unaffected by the over-expression of Coa1 (Fig. 6B), making it likely that Coa1 exerts its negative effect at a post-translational step. Similarly, we assessed whether Shy1 could have a negative effect on the Mss51 suppression of *coa1*Δ cells. Over-expression of Shy1 neither rescued the respiratory growth defect of *coa1*Δ cells nor affected the suppression of this defect by high levels of Mss51 (supplemental Fig. 1).

The similarities in residual CcO activity and in *MSS51*/*COX10* suppression of *shy1*Δ and *coa1*Δ strains led us to investigate whether *shy1*Δ cells resembles *coa1*Δ strains in having a mitochondrial copper defect. *shy1*Δ cells are deficient in mitochondrial copper (Fig. 6C), yet total cellular copper is normal (data not shown). In addition, supplementation of *shy1*Δ cultures with exogenous copper partially restores growth on glycerol/lactate medium (Fig. 6D). The copper defect observed in *coa1*Δ and *shy1*Δ cells suggested that Cu_B site formation could be impaired. Since Cox11 is implicated in Cu_B site formation, we tested whether over-expression of *COX11* had a growth stimulatory effect. High copy *COX11* neither stimulated respiratory growth of *coa1*Δ or *shy1*Δ cells nor was synergistic with *MSS51* in suppressing *shy1*Δ cells (data not shown). Given the similarities between *shy1*Δ and *coa1*Δ strains, Shy1 and Coa1 probably act at a closely related step in CcO assembly.

Coa1 is part of a high molecular weight complex and interacts with Mss51

The genetic interaction between *COA1* and *MSS51* in *shy1*Δ suggested that the two proteins might interact physically. We constructed a strain in which we integrated 13 copies of the Myc epitope at the 3'end of *COA1* and 3 copies of the HA epitope at the 3'end of *MSS51*. These tags did not affect the ability of the strain to grow on respiratory medium. Mitochondria

isolated from the *COA1*-13Myc *MSS51*-3HA cells were solubilized with 0.1% DOC and fractionated on a size exclusion column (Fig. 7A). Analysis of the distribution of Coa1 by immunoblotting of the elution fractions revealed the presence of Coa1-Myc in fractions corresponding to high molecular mass components near 400 kDa. Similarly, BN-PAGE of *COA1*-13Myc mitochondria revealed the presence of a Coa1 complex of approximately 400 kDa (Fig. 7B).

A fraction of Mss51 was found in a similar mass range as Coa1 (Fig. 7A, complex A in Fig. 7B), in addition to a smaller mass component (B in Fig. 7B). Immunoprecipitation of Mss51-HA from solubilized mitochondria resulted in the co-adsorption of Coa1-Myc demonstrating that the two proteins physically interact (Fig. 7C). Together, these data show that Coa1 and Mss51 are part of a ~400 kDa complex.

Coa1 interacts with Cox14 and nascent Cox1

Mss51 was reported previously to interact with Cox14 (Barrientos et al., 2004). To assess whether Coa1 interacts with Cox14 in addition to Mss51, we constructed a strain with 13Myc integrated at the 3' end of *COX14* in addition to 3HA integrated on *COA1*. Immunoprecipitation of Cox14-Myc resulted in the co-precipitation of Coa1-HA (Fig. 8A). Likewise, a strain with a 3HA tag at the 3' end of *COX14* in addition to the integrated 13 Myc tag on *COA1* enabled the co-precipitation of Cox14 and Coa1 (Fig. 8B). Thus, Coa1 interacts with both Mss51 and Cox14. Deletion of *COX14* disrupted the interaction between Coa1 and Mss51 (Fig. 8C) whereas the interaction between Mss51 and Cox14 was independent of the presence of Coa1 (Fig. 8D). These results suggest that Cox14 may mediate the interaction between Mss51 and Coa1. BN-PAGE confirmed that the 400 kDa Coa1:Mss51 complex is abrogated in *cox14*Δ cells (Fig. 7B). Attempts were made to visualize Cox14-Myc after BN-PAGE but were unsuccessful. One

possibility is that Cox14 occupies a central position in the complex that may mask the Myc tags, therefore precluding detection by immunoblotting under native conditions.

Since both Cox14 and Mss51 were shown to interact with nascent Cox1 (Barrientos et al., 2004), we tested whether Coa1 also interacts with nascent Cox1. Mitochondrial translation products were labeled *in vitro* in purified mitochondria from *COA1*-13Myc or WT cells. The mitochondria were then solubilized in digitonin-containing buffer and the soluble fraction was immunoprecipitated with anti c-Myc-agarose beads. Cox1 was specifically detected in the eluate of the beads incubated with the extracts from *COA1*-13Myc mitochondria (Fig. 9). This result shows that like Cox14 and Mss51, Coa1 interacts with newly synthesized Cox1.

Coa1 interacts with Shy1 and is necessary for the inhibition of Cox1 translation in *shy1*Δ cells

The similar phenotypes of *coa1*Δ and *shy1*Δ cells suggested that Coa1 might also interact with Shy1. The deletion of *COA1* did not affect the protein level of Shy1 and *vice versa* (data not shown). We constructed a strain with 13Myc integrated at the 3' end of *SHY1* in addition to the 3HA tag on *COA1*. Fractionation of extracts of solubilized mitochondria from this strain showed that Shy1 eluted in fractions similar to those containing Coa1 (supplemental Fig. 2). This result is consistent with the presence of Shy1 in a large molecular weight complex (Nijtmans et al., 2001). Immunoprecipitation of Shy1-Myc resulted in the co-precipitation of Coa1 (Fig. 10A), and the reverse experiment, immunoprecipitation of Coa1-HA led to the recovery of Shy1 with the beads (Fig. 10B) showing that Coa1 and Shy1 are part of a complex. The ~400 kDa Coa1:Mss51 complex observed on BN-PAGE remained intact in *shy1*Δ cells (Fig. 7B), suggesting that Shy1 is not part of this complex. Shy1 was postulated to disassemble the Mss51:Cox14 complex during normal Cox1 translation and IM insertion (Barrientos et al., 2004). As predicted from this model, the abundance of the Coa1:Mss51 complex appears to be enhanced in *shy1*Δ cells (Fig. 7B).

The attenuated Cox1 translation observed in *shy1*Δ cells was postulated to arise from stabilization of a Mss51:Cox14 complex that effectively titrates out the Mss51 translational activator (Barrientos et al., 2004). Because of the normal translation of Cox1 observed in *coa1*Δ cells, *COA1* was deleted in *shy1*Δ cells and Cox1 translation was quantified (Fig. 10C). The absence of Coa1 in *shy1*Δ cells partially reversed the Cox1 translation block. Quantification of the gels from 3 independent *in vivo* translation experiments showed that only the translation of Cox1 was decreased in *shy1*Δ cells and that deletion of *COA1* in this background significantly restored it, although not to WT levels (Fig. 10D). This result suggests that Coa1 has a role in maintaining Mss51 in a state unavailable for translational activation of Cox1 in a *shy1*Δ background. Despite the significant recovery of Cox1 translation, the deletion of *COA1* in *shy1*Δ did not reverse the respiratory growth defect or the low mitochondrial copper level (supplemental Fig. 3).

DISCUSSION

One of the many challenges that cells face in building active CcO is to coordinate the assembly of the hydrophobic mitochondrial encoded subunits with the subunits derived from the nuclear genome. This task is even greater when one considers that Cox1 and Cox2 have to acquire their metallic cofactors essential for activity. We have identified the gene *COA1* as functioning in linking Cox1 translation and the insertion of its cofactors.

CcO biogenesis in the mitochondrial IM commences with the translation of the mitochondrial encoded subunits mediated by a series of IM-associated translational activators. These activators appear to recruit mRNA to the IM for co-translational insertion of the nascent polypeptides. Co-translational insertion of Cox1 is dependent on the Pet309 and Mss51 translational activators (Manthey and McEwen, 1995; Perez-Martinez et al., 2003; Siep et al., 2000). Of the three subunits of CcO encoded by the mitochondrial genome, Cox1 is the first to

join the assembly chain. Oxa1 is likely to play a role in the IM insertion of the twelve transmembrane domains of Cox1, as cells lacking Oxa1 show marked Cox1 instability, as seen in a mitochondrial translation assay (Barrientos et al., 2004; Bonnefoy et al., 1994).

Two heme *a* molecules and a copper atom are buried inside the barrel formed by Cox1 (Tsukihara et al., 1995). Cox1 has an open channel from the IMS side of the IM where heme *a* insertion may occur. However, this channel is obstructed by the association of Cox2. It is therefore probable that Cox1 acquires its cofactors at an early stage of the assembly process. Heme *a* is coordinated by axial ligands on helices 2 and 10 of Cox1, thus heme *a* insertion could have a significant effect on the IM folding of Cox1 which could trigger the assembly process to proceed to the next step. An intermediate consisting of Cox1, Cox4 and Cox5A accumulates in human cells from patients carrying mutations in a number of genes encoding assembly factors like Sco1 and SURF1 (human Shy1) (Stiburek et al., 2005; Williams et al., 2004). The intermediate fails to accumulate in Cox10 or Cox15-deficient patient cells consistent with the idea that heme *a* insertion occurs prior to the addition of Cox4 and Cox5A (Antonicka et al., 2003a; Antonicka et al., 2003b; Williams et al., 2004).

A clear connection between co-translational insertion of Cox1 and cofactor assembly has not been established to date. We show the gene *COA1* is required for efficient CcO assembly in yeast. The respiratory defect of *coa1*Δ cells is suppressed by high copy *MSS51*, *MDJ1* and *COX10* and the suppression by elevated levels of Mss51 and Cox10 is enhanced when the two proteins are co-expressed. This observed synergistic suppression is consistent with the different functions exerted on Cox1 by Mss51 and Cox10 in translation and heme *a* biosynthesis, respectively. These results suggest that Coa1 links co-translational insertion and heme addition to Cox1 (Fig. 11). Coa1, like Mss51, only has robust homologs within fungi (Supplemental Fig. 4). However, the presence of a functional homolog that would link translation of Cox1 to the assembly of its cofactors in higher eukaryotes is likely and genetic screens are ongoing in our laboratory to check this possibility.

*coa1*Δ cells resemble *shy1*Δ cells in many aspects. First, both strains display residual CcO activity that can be suppressed by elevated levels of Mss51. Second, their respiration is enhanced when Mss51 and Cox10 are combined together. Lastly, mitochondria of each strain have low levels of copper. These data and the physical interaction between Coa1 and Shy1 strongly suggest that the two proteins function at a related step of CcO assembly. The enhancement of the respiratory growth of *shy1*Δ cells over-expressing Mss51 by *COX10* is the first indication that Shy1 is linked to a heme *a* step in eukaryotes. Accordingly, Shy1 appears to be important for insertion/stabilization of the Cu_B-heme a_3 in *R. sphaeroides* (Smith et al., 2005).

Mss51 functions in Cox1 translation and elongation of the nascent chain and is known to interact with nascent Cox1 (Siep et al., 2000; Perez-Martinez et al., 2003; Barrientos et al., 2004). Moreover, Mss51 also interacts with Cox14 (Barrientos et al., 2004). Cox1 translation is attenuated in *shy1*Δ cells and this defect can be rescued either by over-expression of Mss51 or deletion of *COX14* (Barrientos et al., 2002b; Barrientos et al., 2004). Shy1 was postulated to disassemble the Cox1:Cox14:Mss51 complex allowing for Mss51 recycling to its translation/elongation function (Fig. 11) (Barrientos et al., 2004). The Cox1:Cox14:Mss51 complex is thought to accumulate in *shy1*Δ cells, therefore titrating out Mss51 from Cox1 translation/elongation and creating a negative feedback loop that may coordinate translation and membrane insertion (Barrientos et al., 2004). The negative feedback loop model may however be more complex as the deletion of *SHY1* in cells containing a *COX1-ARG8* chimera gave normal levels of the Arg8 reporter (Perez-Martinez et al., 2003).

The interaction of Coa1 with Mss51, Cox14 and nascent Cox1 suggests that Coa1 is part of the feedback regulatory loop. Coa1 appears to be a key component of the stalled complex as Cox1 translation defect in *shy1*Δ cells is partially reversed by deleting *COA1* and Cox1 translation is normal in *coa1*Δ cells. The stalled complex that inhibits Cox1 translation could correspond to the 400 kDa Mss51:Coa1 complex that we detected by BN-PAGE, since we

showed that this complex is stabilized in *shy1*Δ cells and is disrupted upon deletion of *COX14* (Fig. 7B). It is likely that the Mss51:Coa1 complex also contains Cox14 and nascent Cox1. We detected a lower molecular weight complex containing Mss51 (Fig. 7A,B). This complex is present at higher level in strains where translation of Cox1 occurs optimally (WT and *cox14*Δ) than in *shy1*Δ cells where translation of Cox1 is compromised. Therefore, we suggest that the low molecular weight Mss51 complex may be involved in Cox1 translation.

We propose that Coa1 stabilizes the Mss51:Cox14:Cox1 complex until Shy1 interacts with Coa1, causing Mss51 and Cox14 to disassemble and Cox1 to proceed to the next assembly step involving the insertion of the cofactors (Fig. 11). Even though we showed that the interaction between Mss51 and Cox14 was not dependent on Coa1, the absence of Coa1 will likely preclude the formation of the 400 kDa complex, resulting in normal Cox1 translation. Cells lacking *COA1* translate Cox1 normally but are compromised for assembly presumably because Cox1 cannot be presented efficiently to Shy1. The partial suppression of the respiratory defect of *coa1*Δ cells by over-expression of *MSS51* is likely due to the post-translational function of Mss51 which could increase the stability of nascent Cox1 upon binding, allowing eventually more assembly over time. As mentioned, overexpression of *MSS51* allows respiratory growth of *shy1*Δ cells, but the effect is nullified by co-expression of *COA1*. This dominant negative effect of *COA1* on the *MSS51*-mediated suppression of *shy1*Δ cells may arise from titrating out a factor such as Cox14 if formation of the Mss51:Cox14:Coa1 complex occurs in a step-wise manner. High levels of *COA1* do not titrate out Mss51, as translation of Cox1 is not attenuated in *shy1*Δ cells over-expressing *COA1* and *MSS51* (Fig. 6B).

Mdj1 was also recovered as a suppressor of *coa1*Δ cells. This DnaJ protein associated with mitochondrial Hsp70, may enhance the chaperoning activity of Hsp70. Cells lacking the Mba1 ribosomal receptor or harboring a mutant Oxa1 variant showed an association of Cox1 and Hsp70 (Ott et al., 2006). Thus, the suppression by Mdj1 in *coa1*Δ cells may occur through an

enhanced chaperoning activity of Hsp70 that may stabilize/refold nascent Cox1 polypeptides stalled for insertion. This effect would be similar to the suppression of *coa1*Δ by *MSS51* discussed above. However, since Mdj1 has been shown to interact with mitochondrial nascent translation products (Westermann et al., 1996), a more direct role of Mdj1 in CcO assembly can not be excluded.

Cells lacking Coa1 or Shy1 have mitochondrial copper and respiratory growth defects that can be partially restored with supplemental copper. Other CcO assembly mutants do not show a similar mitochondrial copper deficiency. The source of copper used for metallation of CcO arises from the matrix copper-ligand complex conserved in eukaryotic cells (Cobine et al., 2004; Cobine et al., 2006a). The observed diminution in matrix copper levels in *coa1*Δ and *shy1*Δ cells may suggest that the assembly step at which these two proteins are involved could influence the function of the transporter responsible for trafficking the CuL complex. Studies are underway to identify the copper transporter of the IM.

MATERIALS AND METHODS

Yeast strains and vectors. Yeast strains used in this study are described in supplementary Table I. All FMP deletions screened were in the BY4743 background and were grown in liquid YPD at 30^{o}C. The c*oa1*Δ strains were in the BY4743 background or in W303 created by disruption using KanMX. Cells were cultured in either rich medium or synthetic complete (SC) medium lacking the appropriate nutrients for plasmid selection. The carbon source used was either 2% glucose or a 2% glycerol-2% lactate. A 3'-Myc tagged version of *COA1* was cloned into a yeast low-copy vector under the control of the *MET25* promoter and the *CYC1* terminator (Mumberg et al., 1994). *COA1*-Myc was created using PCR, where the 3' oligonucleotide removed the stop codon and added the coding sequence for a 3' Myc tag (LEQKLISEEDL). Sequencing was used to

confirm cloning products in all created vectors. Yeast strains were transformed using lithium acetate.

Mitochondria purification and assays. Intact mitochondria were isolated from yeast as described previously (Diekert et al., 2001). Total mitochondrial protein concentration was determined by standard Bradford assay (Bradford, 1976). Cytochrome *c* oxidase activity was assessed by monitoring the oxidation of reduced cytochrome *c* and reaction rates were normalized to total mitochondrial protein (Capaldi et al., 1995). The oxygen consumption of the cells grown to stationary phase was determined on a 5300A Biological Oxygen Monitor (Yellow Springs Instrument Co.). Only the linear response was considered in calculating the rate of oxygen consumption (Horng et al., 2005). Metals were quantified in isolated mitochondria using a Perkin-Elmer Optima 3100XL inductively coupled plasma optical emission spectrometer (ICP-OES) after nitric acid digestion at 95^{o}C. A standard curve was determined from commercially available standards (Perkin-Elmer), and buffer samples were run in parallel as a control. Heme analysis was conducted on 1-2 mg of mitochondria extracted with 0.5 mL of acetone containing 2.5% HCl as described previously (Barros et al., 2001). The pH of the extract was adjusted to 4 by addition of 1 μL formic acid and titration of a KOH solution. The sample was clarified by centrifugation at 13000 rpm for 5 min and 1 mL was injected onto a 3.9 x 300 mm C18 Bondclone column (Phenomex, USA).

***In vitro* and *in vivo* mitochondrial protein synthesis.** Purified mitochondria were labeled *in vitro* as described (Westermann et al., 2001). For *in vivo* labeling, the cells were grown overnight in selective medium containing 2% raffinose and then re-inocculated in YP-2% raffinose to grow to an absorbance of 1 at 600 nm. The labeling and preparation of the samples for 12% SDS-PAGE was as described in (Barrientos et al., 2002b). The gel was dried and radiolabeled proteins were visualized by exposing autoradiographic films at -80°C.

Blue native gel electrophoresis (BN-PAGE). BN-PAGE was performed essentially as described (Wittig et al., 2006) except 1.5% digitonin was used. After incubation for 20 min on ice and

centrifugation (20000g for 10 min at 2°C) supernatants were mixed with sample buffer (5% Coomassie brilliant blue G250, 0.5 M 6-aminocaproic acid, pH 7.0) and loaded on 4-13% gradient polyacrylamide gel. Separated complexes were detected by immunoblotting on PVDF membrane.

Immunoblotting. Proteins samples were separated on 15% acrylamide gels and transferred to nitrocellulose. ECL reagents were used to visualize proteins with horseradish peroxidase-conjugated secondary antibodies. Anti-Myc and HA antisera were purchased from Santa Cruz, anti-porin was from Molecular Probes and antisera to Cox1-Cox3 were from Mitosciences. Antiserum to Sod2 was provided by Dr. Val Culotta, and antisera to Cyb2, Cyt1 and Cyc1 provided by Dr. Carla Koehler.

Localization Experiments. Proteinase K treatment was done as previously described (Diekert et al., 2001). Carbonate extraction experiments used mitoplasts made by diluting 125 μg intact mitochondria 20-fold in 10 mM Hepes, pH 7.4. Mitoplasts were then incubated in 100 mM sodium carbonate, pH 11.3 for 30 min on ice and centrifuged at 100000g. The supernatant and pellet were analyzed by immunoblotting. High salt extraction experiments also used mitoplasts that were sonicated three times for 30 sec in 25 mM, 100 mM or 500 mM NaCl.

Construction of the high copy library. 36 μg of genomic DNA purified from *coa1*Δ cells was partially digested with Sau3A. DNA fragments between 3 and 10 kb were purified from a 1% agarose gel. The plasmid YEP352 was digested to completion with SalI. The plasmid DNA was then treated with Klenow polymerase in the presence of dCTP and dTTP while the genomic DNA was treated with dATP and dGTP**.** The resulting plasmid DNA was treated with 1 unit of calf intestinal alkaline phosphatase for 30 min at 37°C. Ligation of YEP352 with the genomic DNA was conducted overnight at 16°C. The total number of colonies obtained was estimated to be about 20,000, representing a theoretical 5-fold coverage of the yeast genome.

Gel Filtration. Mitochondria (0.3-0.5 mg) were resuspended in solubilization buffer (PBS, 0.1% DOC). After incubation on ice for 10 min, lysates were centrifuged at 16000 g for 25 min at 4°C, passed through a 0.45 μm filter and loaded onto a Superdex 200 (GE Healthcare) column equilibrated in solubilization buffer.

Immunoprecipitation. 0.3-0.5 mg of purified mitochondria were solubilized by incubation in 0.5 mL Tris 20mM pH 7.4, 100 mM NaCl, 1mM PMSF and either 0.1% lauryl-maltoside or 1% digitonin (IP buffer) for 30 min at 4°C. Lysates were centrifuged at 16000 g for 15 min at 4°C. Immunoprecipitation was performed by incubating overnight at 4°C the clarified lysates with 40 μL anti-Myc or HA rabbit polyclonal antisera. The antibodies were precipitated by the addition of 20 μL of recombinant protein A agarose beads (Pierce) for 4 h at 4°C. The beads were pelleted by centrifugation at 2000 g for 2 min and washed with 0.8 mL of IP buffer for 5 min. This step was repeated 3-4 times. Beads were eluted by boiling for 5 min in 50 μL SDS-PAGE loading buffer. The clarified extracts and wash fractions were TCA precipitated prior to electrophoresis and immunoblotting.

Statistical procedures. Descriptive statistics are represented as average ± standard error. The unpaired student t-test (two tail) was used to compare differences between experimental and control groups.

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FIGURE LEGENDS

Fig. 1. *coa1Δ* cells are respiratory deficient and have low mitochondrial copper. A) Copper levels were assessed by ICP-OES in Nycodenz purified mitochondria of *coa1*Δ cells and the isogenic WT BY4743 grown in YPD. **B)** *coa1*Δ cells and the WT BY4743 were grown in YPD liquid media at 30°C with 1 mM $CuSO_4$ where indicated and oxygen consumption (% O_2/sec/OD_{600}) was measured . **C)** BY4743 and *coa1Δ* cells were grown in YPD then serially diluted and spotted on YP 2% glycerol-2% lactate (G/L) or 2% glucose plates. $CuSO_4$, $FeCl_2$, and $ZnSO_4$ were added to the plate media at specified concentrations. Plates were kept at 30°C for 5 days.

Fig. 2. Deletion of *COA1* results in a CcO specific defect. **A)** *coa1*Δ cells and the isogenic WT BY4743 were grown in YPD with or without 1 mM $CuSO_4$. Purified mitochondria were assayed for CcO activity (ΔOD_{550}/min/10 µg protein). The data represent the average of 3 independent experiments. **B)** Immunoblot of 30 µg of mitochondria from *coa1*Δ cells or from BY4743 for

Cox1-Cox3. Porin is shown as a loading control. **C)** Heme was extracted from 2 mg of mitochondria purified from *coa1*Δ or BY4743 cells and separated by reverse phase HPLC. The area under the curve of the peaks of heme *b* (B) and heme *a* (A) were calculated and used to calculate a heme *a* to heme *b* ratio as the levels of heme *b* were unaffected by the deletion of *COA1*. Protoporphyrin peaks (P) were found to vary as reported previously (Barros and Tzagoloff, 2002). **D)** *In vivo* labeling of mitochondrial translation products. *coa1*Δ and BY4743 cells were pulsed for 15 min with ^{35}S-methionine at which point 20 mM of cold methionine was added. The reaction was either stopped (P) or allowed to chase (C) for 90 min at 30°C. The samples were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film.

Fig. 3. Mitochondrial localization of Coa1. A) *coa1*Δ cells expressing a low copy version of *COA1*-Myc were serially diluted on SC 2% glycerol-2% lactate or 2% glucose plates (control) and compared to the same strain containing an empty vector. **B)** Purified mitochondria and the cytosolic fraction from the *coa1Δ* + *COA1*-Myc strain were isolated and probed by immunoblotting for c-Myc and for the mitochondrial markers porin (Por1) and cytochrome b_2 (Cyb2) and the cytosolic marker phosphoglycerate kinase 1 (Pgk1). **C)** Membrane association was assessed by carbonate extraction of Coa1-Myc from whole mitochondria or mitoplasts purified from the *coa1*Δ + *COA1*-Myc strain. The soluble and pellet fractions were assayed by immunoblotting with the c-Myc antibody and for the soluble matrix protein superoxide dismutase 2 (Sod2) or the IM protein cytochrome c_1 (Cyt1). **D)** The soluble and pellet fractions of Coa1-Myc mitoplasts were separated by centrifugation after sonication in buffer containing 25, 100 or 500 mM NaCl. The fractions were assayed by immunoblotting for c-Myc, Sod2 (soluble matrix protein), Cyt1 (integral IM protein), and Cyc1 (IM associated protein). **E)** Mitoplasts were treated with proteinase K (PK) over a time course and analyzed by immunoblotting. As a control, whole mitochondria, mitoplasts, mitoplasts solubilized in 1% deoxycholate (DOC) and sonicated

mitoplasts were treated with proteinase K (right panel). Note: OM fragments remain associated with mitoplasts, hence the presence of some porin (Por1) in mitoplasts.

Fig. 4. High copy suppressors of the respiratory defect of *coa1*Δ cells. A) *coa1*Δ cells transformed with high copy vectors isolated from the suppressor screen were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate or 2% glucose (control) with selection. The plates were incubated at 30°C for 2 days (glucose) or 7 days (glycerol-lactate). **B)** The same cells as in A) were spotted on rich media (YP) supplemented with 50 μM $FeCl_2$ containing 2% glycerol-2% lactate (YPLG) or 2% glucose (YPD) and either the copper specific chelator BCS or $CuSO_4$. The plates were incubated at 30°C for 2 days (YPD) or 6 days (YPLG).

Fig. 5. Restoration of CcO by high copy *MSS51* in *coa1*Δ cells. A) *coa1*Δ cells with high copy *MSS51* or an empty vector (vec) were grown in SC-2% raffinose and assayed for oxygen consumption. **B)** Mitochondria were purified from these cells and assayed for CcO activity. **C**) Immunoblot of 30 μg of mitochondria from *coa1*Δ cells containing an empty vector (vec), a low copy vector expressing *COA1* or a high copy vector expressing *MSS51*. **D)** Heme profile from 2 mg of mitochondria purified from *coa1*Δ containing high copy *MSS51* or an empty vector. A/B represents the ratio of the area under the curve for the peaks of heme *b* (B) and heme *a* (A). **E**) *coa1*Δ cells expressing high copy *MSS51* and containing an empty vector (vec) or high copy vectors with *MDJ1* or *COX10* were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate or 2% glucose with selection. The plates were incubated at 30°C for 2 days (glucose) or 7 days (glycerol-lactate).

Fig. 6. High copy suppression of *shy1*Δ cells and low mitochondrial copper levels. A) *shy1*Δ cells expressing high copy *MSS51* and an empty vector (vec) or high copy vectors containing *COX10* or *COA1* were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate or 2% glucose with selection. The plates were incubated as in Fig. 5E. **B**) *In vivo* labeling of mitochondrial translation products of *shy1*Δ cells containing an empty vector, high

copy *MSS51* in combination or not with high copy *COA1*. The labeling was stopped after 5 and 15 min of pulse. **C**) Copper levels in Nycodenz purified mitochondria of *shy1*Δ cells or the isogenic WT BY4743 grown in YP- 2% raffinose were measured by ICP-OES. The data are the average of 3 independent experiments. **D**) *shy1*Δ cells or WT cells were grown in SC-2% raffinose, serially diluted and spotted on YP 2% glucose (YPD) or YP 2% glycerol-2% lactate (YPLG) supplemented with 1 or 4 mM $CuSO_4$ as indicated.

Fig. 7. Coa1 and Mss51 form similar sized complexes and interact. A) Extracts of 0.5 mg of solubilized mitochondria from *COA1*-13Myc *MSS51*-3HA cells were fractionated over Superdex 200 in solubilization buffer (PBS, 0.1% DOC). The fractions were analyzed by immunoblot. **B)** Mitochondria isolated from either wild-type (WT), *shy1*Δ or *cox14*Δ strains containing genomically tagged *COA1* (*COA1*-13Myc, lanes 1-3) or *MSS51* (*MSS51*-13Myc, lanes 4-6) were solubilized in buffer containing 1.5% digitonin. Lysates were loaded onto a continuous 4-13% gradient gel and protein complexes were separated by BN-PAGE. The distribution of complexes was analyzed by immunoblotting with mouse monoclonal anti-Myc antibody. Monomeric (V_1) and dimeric (V_2) forms of respiratory chain complex V served as a control and were visualized using antisera against F_1-subunit. **C**) 0.3 mg of mitochondria from *COA1*-13Myc or *COA1*-13Myc *MSS51*-3HA cells were solubilized in Tris 20 mM pH 7.4, 100 mM NaCl, 1 mM PMSF, 0.1% lauryl maltoside and clarified extracts were immunoprecipitated with rabbit polyclonal HA antiserum and protein A agarose beads. The load representing 5% of the extracts and the entire fraction of the last wash and bead eluate were analyzed by immunoblotting.

Fig. 8. Coa1 interacts with Cox14 which is required for the Coa1:Mss51 interaction. A) 0.3 mg of mitochondria from *COA1*-3HA *or COA1*-3HA *COX14*-13Myc cells were solubilized in Tris 20 mM pH 7.4, 100 mM NaCl, 1 mM PMSF, 0.1% lauryl maltoside. Clarified extracts were immunoprecipitated with rabbit polyclonal anti-Myc antiserum and protein A agarose beads. The load representing 7% of the extracts and the entire fraction of the last wash and bead eluate were analyzed by immunoblotting with mouse monoclonal antibodies. **B**) Extracts of 0.3 mg of

mitochondria from *COX14*-3HA or *COA1*-13Myc *COX14*-3HA cells were treated and analyzed as in A). **C**) Extracts of 0.3 mg of mitochondria from *COA1*-3HA *MSS51*-13Myc or *COA1*-3HA *MSS51*-13Myc *cox14*Δ cells were treated and analyzed as in A. **D**) Extracts of 0.3 mg of mitochondria from *MSS51*-3HA *COX14*-13Myc or *MSS51*-3HA *COX14*-13Myc *coa1*Δ cells were treated and analyzed as in A.

Fig. 9. Coa1 interacts with newly translated Cox1. Purified mitochondria from WT cells or cells containing a chromosomally tagged *COA1*-13Myc were labeled with ^{35}S-Met for 20 min. Mitochondria were solubilized in Tris 20 mM pH 7.4, 100 mM NaCl, 1mM PMSF, 1% digitonin and clarified extracts were immunoprecipitated with agarose anti c-Myc beads for 3 h at 4°C. The beads were washed three times with 20 volumes of buffer and resuspended in 1 volume of SDS loading dye. The mitochondria (Mito) and the soluble extracts (Soluble) represent 10% of the total sample, the last wash and beads elution correspond to the entire sample. The fractions were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film.

Fig. 10. Coa1 interacts with Shy1 and deletion of *COA1* relieves the repression of Cox1 translation in *shy1Δ* cells. A) 0.3 mg of mitochondria from *COA1*-3HA or *COA1*-3HA *SHY1*-13Myc cells were solubilized in Tris 20 mM pH 7.4, 100 mM NaCl, 1mM PMSF, 1% digitonin and clarified extracts were immunoprecipitated with rabbit polyclonal anti c-Myc antiserum and protein A agarose beads. The load representing 4% of the extracts and the entire fraction of the last wash and bead eluate were analyzed by immunoblotting. **B**) 0.3 mg of mitochondria from *SHY1*-13Myc or *COA1*-3HA, *SHY1*-13Myc cells were solubilized as in A). Clarified extracts were immunoprecipitated with rabbit polyclonal anti HA antiserum and protein A agarose beads and analyzed as in A). **C**) *In vivo* labeling of mitochondrial translation products. Cells were incubated with ^{35}S-Met for 8 or 20 min before stopping the reaction. The samples were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film. **D**) The gel from C) and 2 other gels from similar experiments were quantified using the software Quantity One (Biorad). The intensity of the bands corresponding to Cox1, Cox2 and Cox3 were normalized to

the intensities of the bands of Var1 and Cyt *b*. ** indicates a high statistical significance (P value < 0.0001) of the decrease of Cox1 translation in *shy1*Δ compared to WT. * Indicates statistical significance (P value = 0.0177) of the increase of Cox1 translation in *coa1*Δ *shy1*Δ compared to *shy1*Δ.

Fig. 11. Model for Coa1 linking Cox1 translation to cofactors assembly. After the translation of Cox1 mRNA which depends on Pet309 and Mss51, Mss51 interacts with nascent Cox1 and associates with Cox14 (Barrientos et al., 2004). This ternary complex is recruited by Coa1 and likely forms the ~400 kDa complex that we detected by BN-PAGE. Membrane insertion of Cox1 probably occurs via Oxa1. Shy1 joins the complex and promotes the dissociation of Cox14 and Mss51 that is recycled to its translational function. The cofactor insertion in Cox1 can then proceed via Cox10, Cox15 (heme *a* biosynthesis) and Cox11 (Cu_B insertion) leading to holo-Cox1.

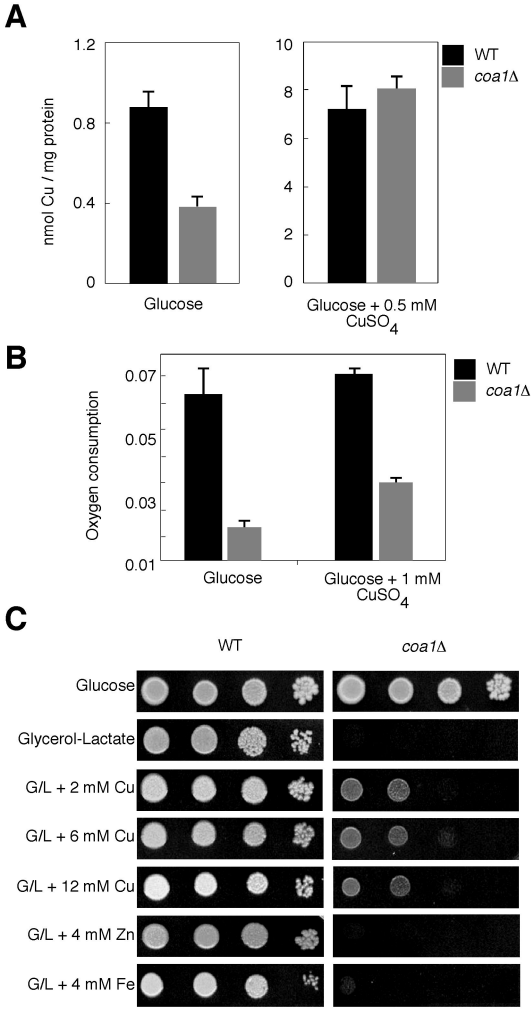
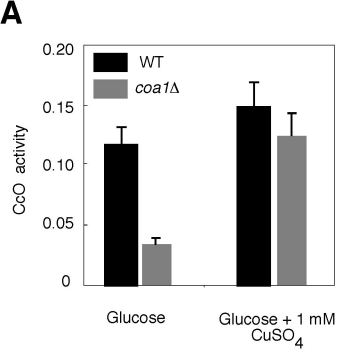
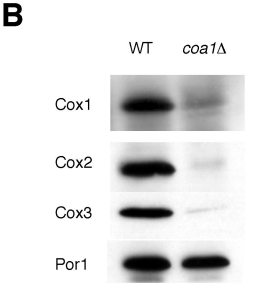
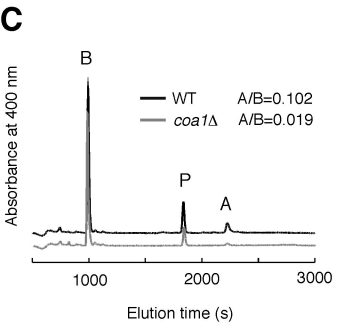

A
1.2
0.8
0.4
0
nmol Cu / mg protein
Glucose
10
8
6
4
2
0
Glucose + 0.5 mM CuSO4
WT
coa1Δ
B
0.07
0.05
0.03
0.01
Oxygen consumption
Glucose
Glucose + 1 mM CuSO4
WT
coa1Δ
C
WT
coa1Δ
Glucose
Glycerol-Lactate
G/L + 2 mM Cu
G/L + 6 mM Cu
G/L + 12 mM Cu
G/L + 4 mM Zn
G/L + 4 mM Fe

Figure 1


A
0.20
0.15
0.10
0.05
0
CcO activity
WT
coa1Δ
Glucose
Glucose + 1 mM
CuSO4


B
WT
coa1Δ
Cox1
Cox2
Cox3
Por1


C
B
WT
A/B=0.102
coa1Δ
A/B=0.019
P
A
Absorbance at 400 nm
1000
2000
3000
Elution time (s)

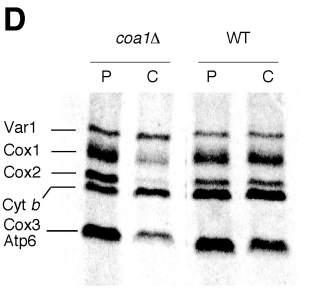

D
coa1Δ
WT
P
C
P
C
Var1
Cox1
Cox2
Cyt b
Cox3
Atp6

Figure 2

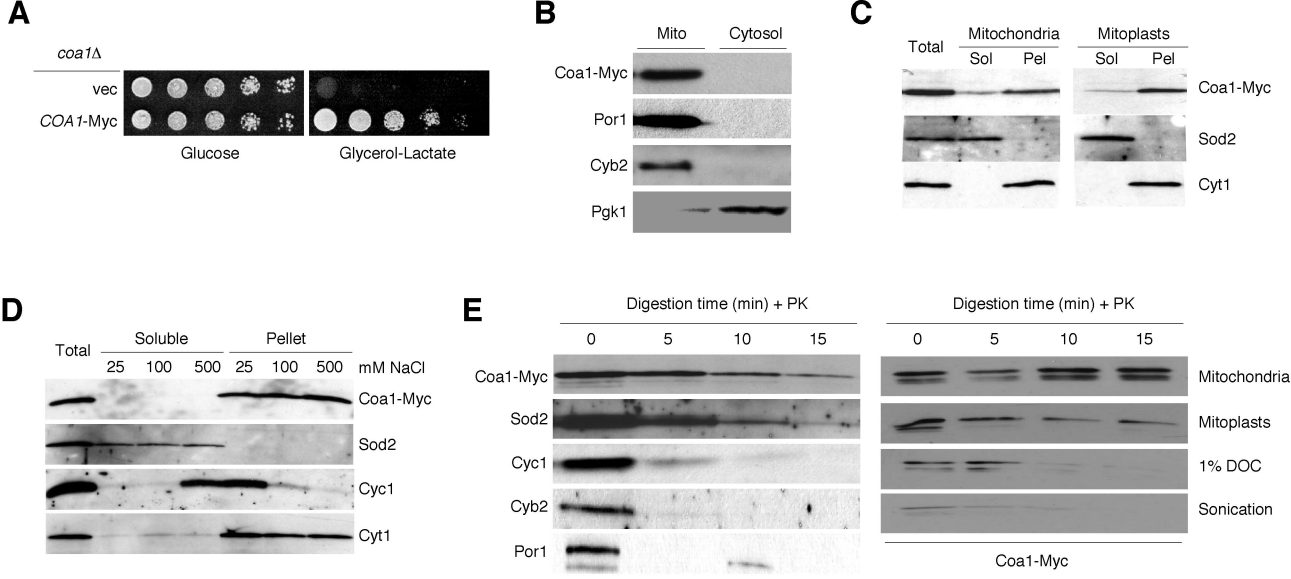
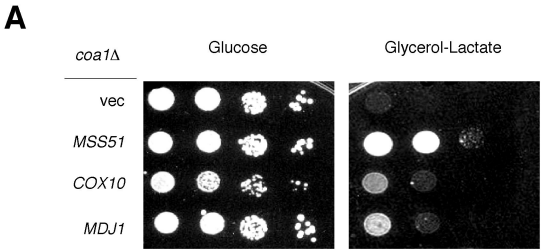

A
coa1Δ
vec
COA1-Myc
Glucose
Glycerol-Lactate
B
Mito
Cytosol
Coa1-Myc
Por1
Cyb2
Pgk1
C
Total
Mitochondria
Mitoplasts
Sol
Pel
Sol
Pel
Coa1-Myc
Sod2
Cyt1
D
Total
Soluble
Pellet
25
100
500
25
100
500
mM NaCl
Coa1-Myc
Sod2
Cyc1
Cyt1
E
Digestion time (min) + PK
0
5
10
15
Coa1-Myc
Sod2
Cyc1
Cyb2
Por1
Digestion time (min) + PK
0
5
10
15
Mitochondria
Mitoplasts
1% DOC
Sonication
Coa1-Myc

Figure 3


A
coa1Δ
Glucose
Glycerol-Lactate
vec
MSS51
COX10
MDJ1

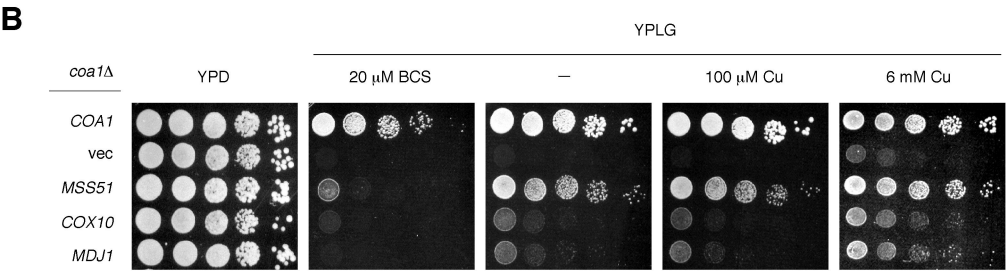
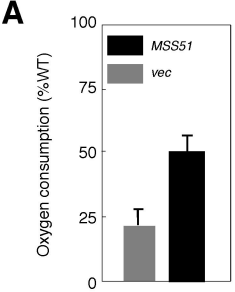
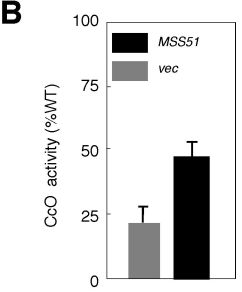
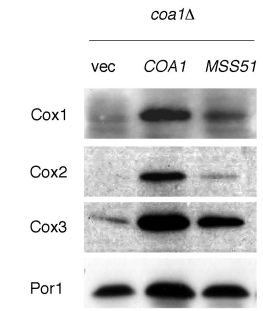
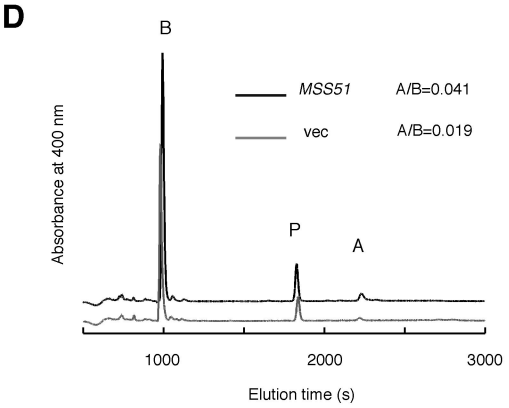

B
YPLG
coa1Δ
YPD
20 μM BCS
—
100 μM Cu
6 mM Cu
COA1
vec
MSS51
COX10
MDJ1

Figure 4


A
Oxygen consumption (%WT)
100
75
50
25
0
MSS51
vec


B
CcO activity (%WT)
100
75
50
25
0
MSS51
vec


C
coa1Δ
vec
COA1
MSS51
Cox1
Cox2
Cox3
Por1


D
B
P
A
MSS51
A/B=0.041
vec
A/B=0.019
Absorbance at 400 nm
1000
2000
3000
Elution time (s)

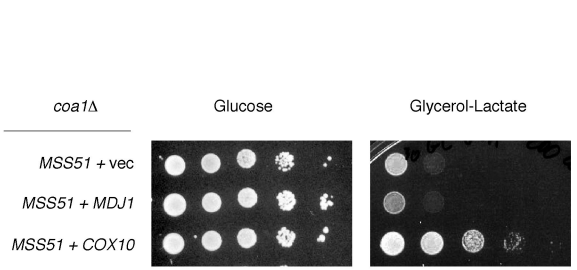

E
coa1Δ
Glucose
Glycerol-Lactate
MSS51 + vec
MSS51 + MDJ1
MSS51 + COX10

Figure 5

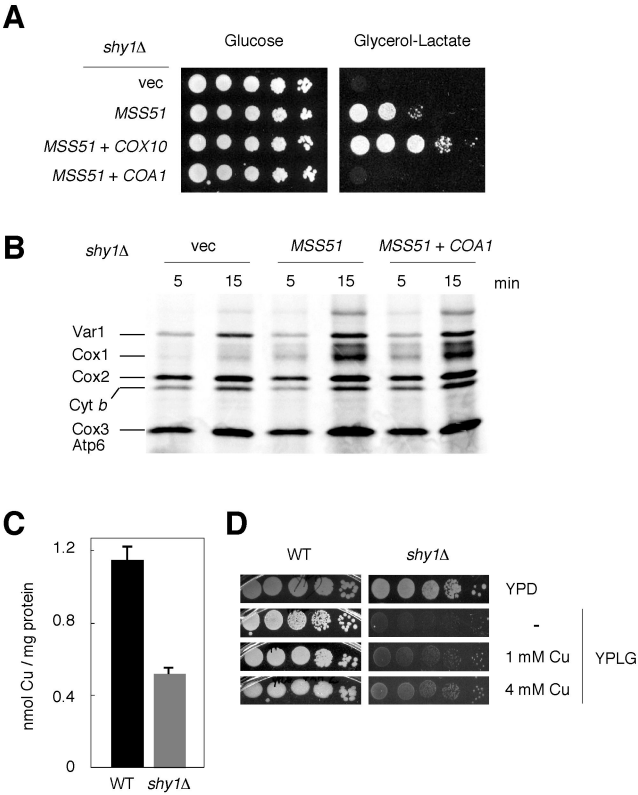

A
shy1Δ
Glucose
Glycerol-Lactate
vec
MSS51
MSS51 + COX10
MSS51 + COA1
B
shy1Δ
vec
MSS51
MSS51 + COA1
5
15
5
15
5
15
min
Var1
Cox1
Cox2
Cyt b
Cox3
Atp6
C
nmol Cu / mg protein
1.2
0.8
0.4
0
WT
shy1Δ
D
WT
shy1Δ
YPD
-
1 mM Cu
4 mM Cu
YPLG

Figure 6

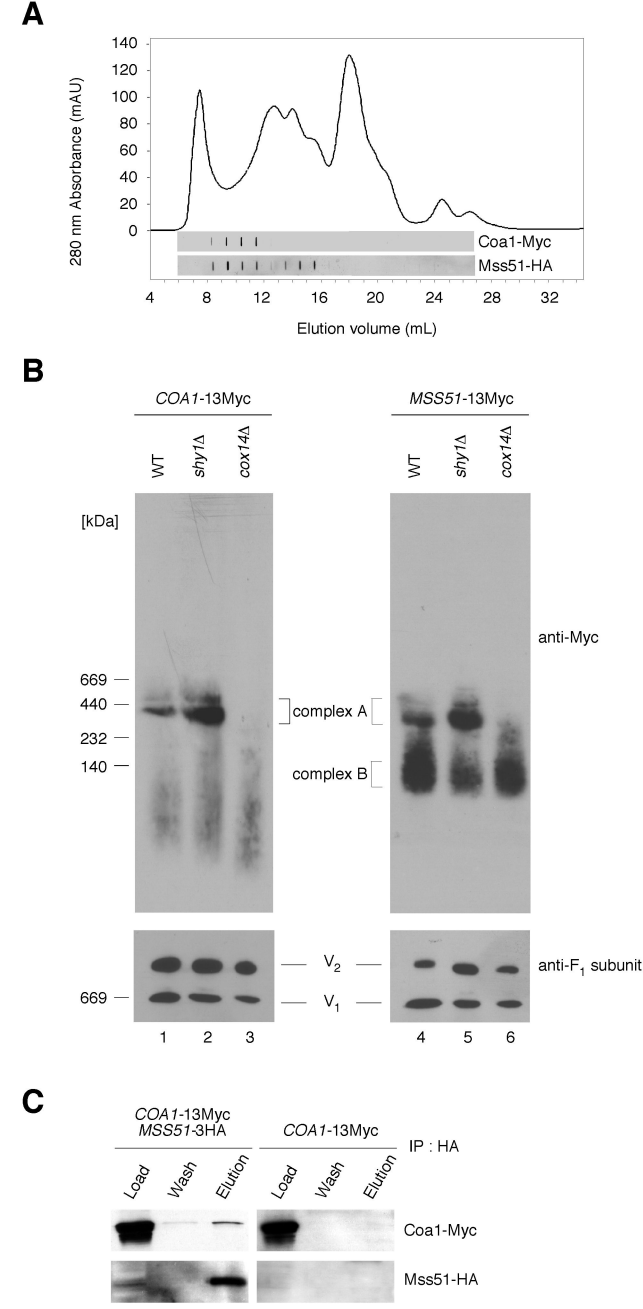

A
280 nm Absorbance (mAU)
140
120
100
80
60
40
20
0
Coa1-Myc
Mss51-HA
4
8
12
16
20
24
28
32
Elution volume (mL)
B
COA1-13Myc
MSS51-13Myc
WT
shy1Δ
cox14Δ
WT
shy1Δ
cox14Δ
[kDa]
669
440
232
140
complex A
complex B
anti-Myc
669
V2
V1
anti-F1 subunit
1
2
3
4
5
6
C
COA1-13Myc
MSS51-3HA
COA1-13Myc
IP : HA
Load
Wash
Elution
Load
Wash
Elution
Coa1-Myc
Mss51-HA

Figure 7

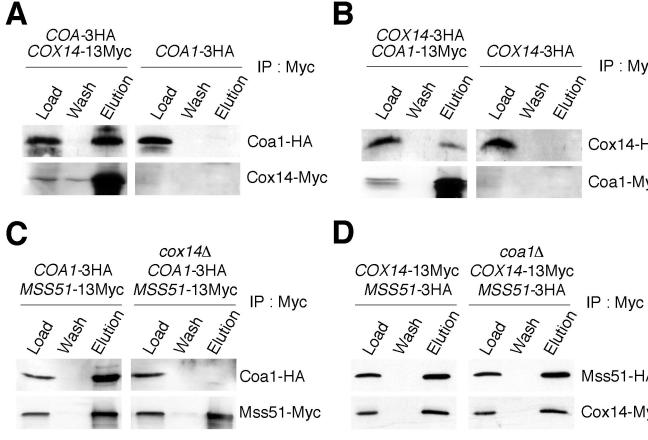

A
COA-3HA
COX14-13Myc
COA1-3HA
IP : Myc
Load
Wash
Elution
Load
Wash
Elution
Coa1-HA
Cox14-Myc
B
COX14-3HA
COA1-13Myc
COX14-3HA
IP : Myc
Load
Wash
Elution
Load
Wash
Elution
Cox14-HA
Coa1-Myc
C
COA1-3HA
MSS51-13Myc
cox14Δ
COA1-3HA
MSS51-13Myc
IP : Myc
Load
Wash
Elution
Load
Wash
Elution
Coa1-HA
Mss51-Myc
D
COX14-13Myc
MSS51-3HA
coa1Δ
COX14-13Myc
MSS51-3HA
IP : Myc
Load
Wash
Elution
Load
Wash
Elution
Mss51-HA
Cox14-Myc

Figure 8

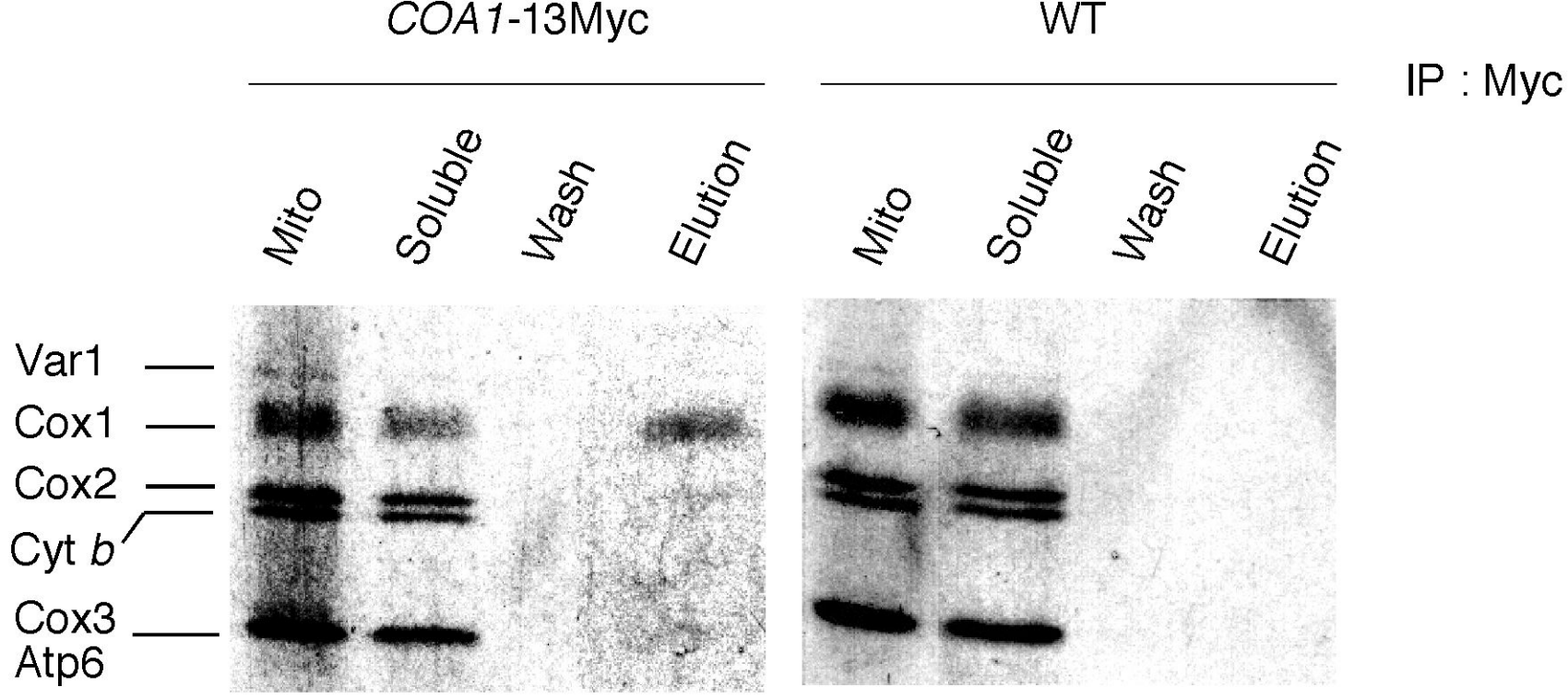

COA1-13Myc
WT
IP : Myc
Mito
Soluble
Wash
Elution
Mito
Soluble
Wash
Elution
Var1
Cox1
Cox2
Cyt b
Cox3
Atp6

Figure 9

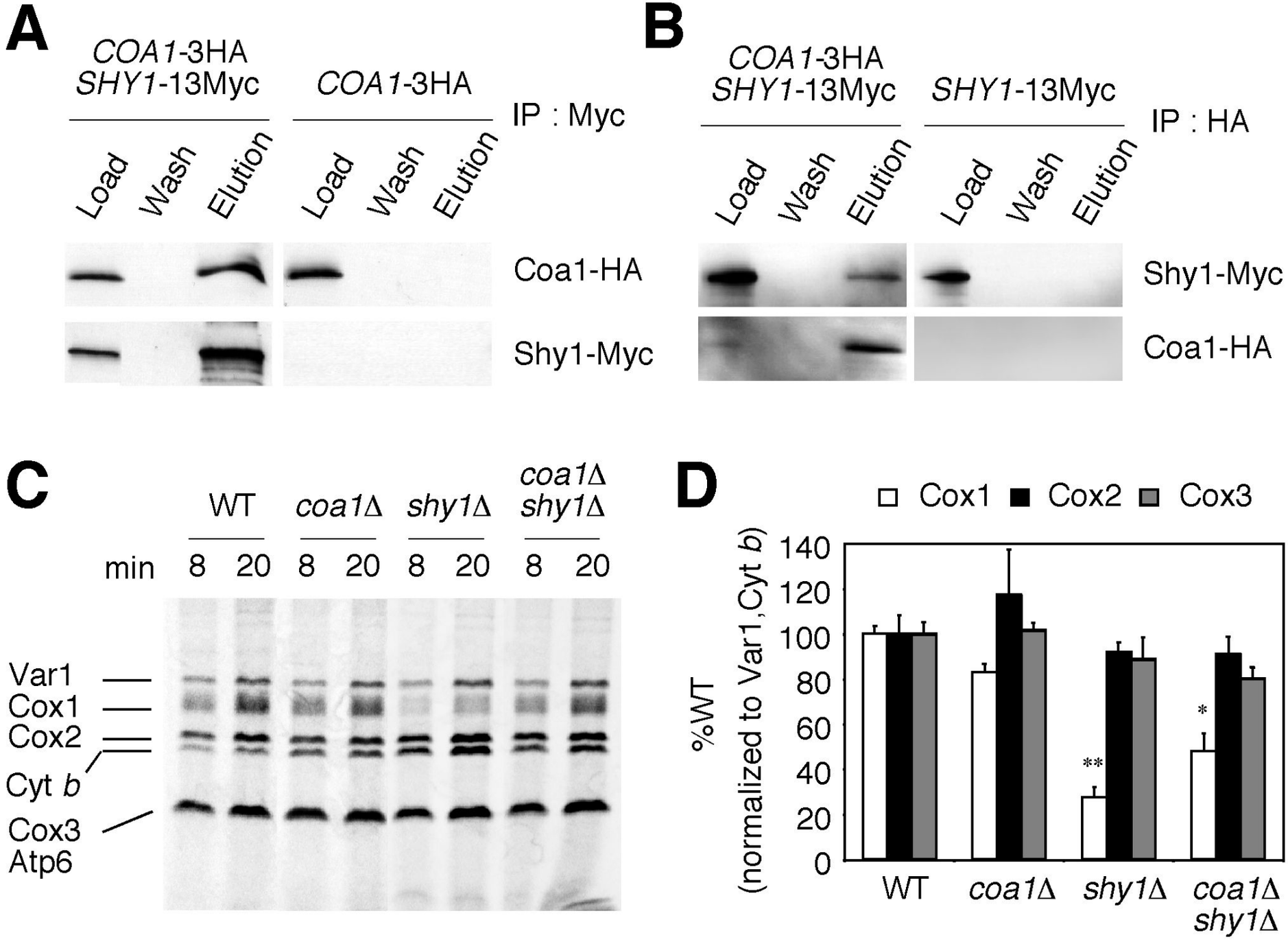
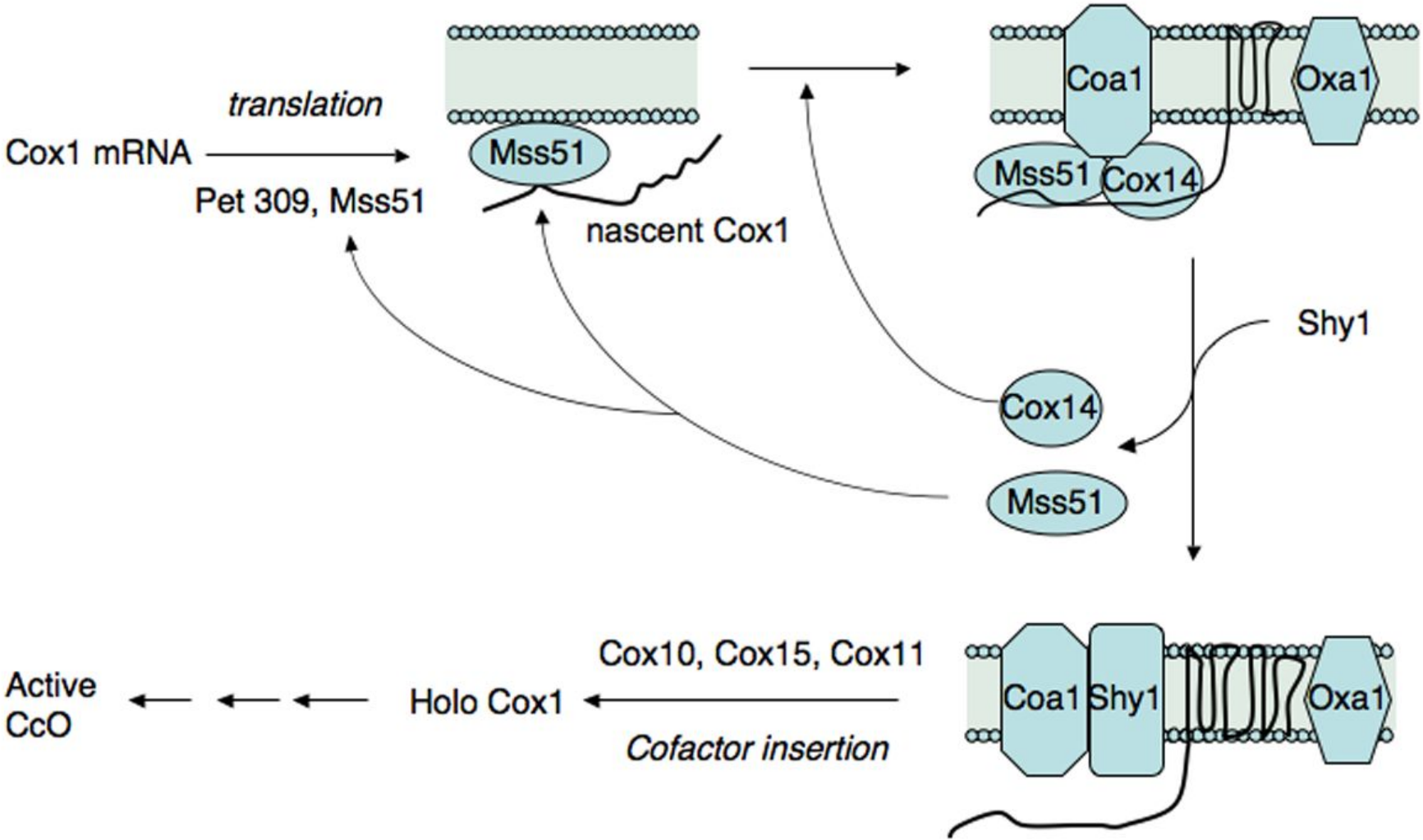

A
COA1-3HA
SHY1-13Myc
COA1-3HA
IP : Myc
Load
Wash
Elution
Load
Wash
Elution
Coa1-HA
Shy1-Myc
B
COA1-3HA
SHY1-13Myc
SHY1-13Myc
IP : HA
Load
Wash
Elution
Load
Wash
Elution
Shy1-Myc
Coa1-HA
C
WT
coa1Δ
shy1Δ
coa1Δ
shy1Δ
min
8
20
8
20
8
20
8
20
Var1
Cox1
Cox2
Cyt b
Cox3
Atp6
D
Cox1
Cox2
Cox3
%WT
(normalized to Var1,Cyt b)
140
120
100
80
60
40
20
0
**
*
WT
coa1Δ
shy1Δ
coa1Δ
shy1Δ

Figure 10


translation
Cox1 mRNA
Pet 309, Mss51
Mss51
nascent Cox1
Coa1
Oxa1
Mss51
Cox14
Shy1
Cox14
Mss51
Active
CcO
Holo Cox1
Cox10, Cox15, Cox11
Cofactor insertion
Coa1
Shy1
Oxa1