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1 **Identification of Coa2 as an assembly factor for cytochrome *c* oxidase biogenesis**

2

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4

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7

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**ABSTRACT**

The assembly of cytochrome *c* oxidase (CcO) in yeast mitochondria is dependent on a new assembly factor designated Coa2. Coa2 was identified from its ability to suppress the respiratory deficiency of *coa1*Δ and *shy1*Δ cells. Coa1 and Shy1 function at an early step in maturation of the Cox1 subunit of CcO. Coa2 functions downstream of Coa1 and the heme *a*<sub>3</sub> related function of Shy1. Cells lacking Coa2 exhibit a specific defect in CcO assembly and show a rapid degradation of newly synthesized Cox1. Rapid Cox1 proteolysis also occurs in *shy1*Δ cells; suggesting that in the absence of Coa2 or Shy1, Cox1 forms an unstable conformer. Overexpression of Cox5a and Cox6 or attenuation of the proteolytic activity of the m-AAA protease partially restores respiration in *coa2*Δ cells. The matrix localized Coa2 protein may aid in the assembly of an early Cox1 intermediate containing the nuclear subunits Cox5a and Cox6. We propose that Coa2 functions at a Cox1 assembly checkpoint where Cox1 assembly and degradation of stalled Cox1 intermediates are coupled.

## 1 INTRODUCTION

2 Cytochrome *c* oxidase (CcO) is the terminal enzyme of the energy transducing  
3 respiratory chain in mitochondria of eukaryotes. Eukaryotic CcO consists of 12-13 subunits,  
4 with three mitochondrial encoded subunits (Cox1-Cox3) forming the core enzyme embedded  
5 within the mitochondrial inner membrane (IM). The remaining nuclear encoded subunits pack  
6 on the periphery of the catalytic core (37). The core enzyme contains three copper atoms and  
7 two heme *a* cofactors (36).

8 The biogenesis of CcO, occurring within the IM, commences with the mitochondrial  
9 synthesis of the Cox1 subunit followed by the insertion of heme *a* and copper cofactors and  
10 the addition of the remaining subunits. The translation of Cox1 on mitoribosomes occurs in  
11 juxtaposition to the IM and is mediated in yeast by the IM-tethered translational activator  
12 Pet309 along with the IM-associated Mss51 (25, 26, 30, 32). The translational activators may  
13 recruit mitoribosomes and promote co-translational IM insertion together with the Oxa1  
14 translocase (9, 18).

15 Insights have also been gleaned on the assembly process of CcO through  
16 characterization of stalled intermediate complexes in human cells of patients with mutations  
17 in assembly factors. One intermediate that accumulates in Leigh's syndrome patients with  
18 *SURF1* mutations consists of Cox1, CoxIV and CoxVa (34, 38, 41). This intermediate fails to  
19 accumulate in patient cells with mutations in Cox10 or Cox15, two enzymes that function in  
20 heme *a* biosynthesis. Thus, the prediction is that heme *a* insertion occurs prior to the addition  
21 of CoxIV and CoxVa (corresponding to yeast Cox5a/b and Cox6) (1, 2). Two heme *a*  
22 moieties and a copper ion are buried inside a barrel within Cox1 (36). These cofactors may be  
23 inserted through an interface obstructed by the association of the Cox2 subunit. Consistent  
24 with this postulate is the fact that Cox2 associates downstream of the Cox1, Cox5a, Cox6  
25 complex (19).

26 Mss51, in conjunction with Cox14, has a post-translational function in yeast Cox1  
27 maturation (8, 30). Mss51 and Cox14 form a complex that interacts with Cox1 and regulates  
28 Cox1 translation/elongation. In cells lacking the yeast *SURF1*, *shy1*Δ cells, Cox1 translation is  
29 attenuated (8). We recently reported that Coa1 functions in an early step of Cox1 assembly in  
30 yeast (31). Coa1 is part of the Mss51, Cox14, Cox1 complex and in addition binds to Shy1  
31 (27, 31). Coa1 appears to coordinate the transition of newly synthesized Cox1 from the Mss51

1 complex to a later component involving Shy1 that likely functions in heme  $a_3$  insertion. We  
2 isolated a series of extragenic suppressors of *coa1*Δ cells. High copy *MSS51*, *MDJ1* and  
3 *COX10* suppress the respiratory defect of *coa1*Δ cells. In addition, we observed that  
4 respiration in both *coa1*Δ and *shy1*Δ cells is enhanced when *MSS51* and *COX10* are co-  
5 expressed. The synergistic suppression is consistent with Mss51 and Cox10 affecting Cox1  
6 translation and heme  $a_3$  insertion suggesting that Coa1 links co-translational insertion and  
7 heme addition to Cox1.

8 A fourth extragenic suppressor of *coa1*Δ cells was found. High copy expression of the  
9 un-annotated yeast ORF, YPL189C-A, designated Coa2 for cytochrome oxidase assembly 2,  
10 is shown presently to effectively restore respiratory growth to *coa1*Δ and *shy1*Δ cells. We  
11 report that Coa2 functions in Cox1 maturation downstream from the Mss51 translational  
12 elongation step and at the heme  $a_3$  insertion step involving Shy1.

13

## 14 MATERIALS AND METHODS

15

16 **Yeast strains and vectors.** Yeast strains used in this study are described in Table I. The  
17 deletions strains were created by disruption using homologous recombination of KanMX4 or  
18 *Candida albicans* *URA3*. Proper integration was checked by PCR of the locus and reversion  
19 of the phenotype induced by the deletion was insured by complementation with a WT copy of  
20 the gene on a plasmid. Cells were cultured in either rich medium or synthetic complete (SC)  
21 medium lacking the appropriate nutrients for plasmid selection. The carbon source used was  
22 either 2% glucose, 2% glycerol-2% lactate or 2% raffinose. *COA2* was cloned into YEep  
23 lac181 with 400 bp of its own promoter and terminator using BamHI and SalI. *COA2* ORF  
24 was cloned into pRS416 under the control of the *MET25* promoter and the *CYCI* terminator  
25 (28) using BamHI and SalI. *COX5a* and *COX6* were inserted in pRS426 and pRS423 under  
26 the control of their own promoter (600 and 430 bp respectively) and terminator (500 bp) by  
27 restriction with BamHI and XhoI. The vector containing Yta12 E614Q was a gift from Dr.  
28 Thomas Langer. Sequencing was used to confirm cloning products in all created vectors.  
29 Yeast strains were transformed using lithium acetate.

30

1 **Mitochondria purification and assays.** Intact mitochondria were isolated from yeast as  
2 described previously (13, 15). Total mitochondrial protein concentration was determined by  
3 Bradford assay (11). Cytochrome *c* oxidase activity was assessed by monitoring the oxidation  
4 of reduced cytochrome *c* and reaction rates were normalized to total mitochondrial protein  
5 (12). The oxygen consumption of the cells grown to stationary phase was determined on a  
6 5300A Biological Oxygen Monitor (Yellow Springs Instrument Co.). The linear response was  
7 considered in calculating the rate of oxygen consumption. Heme analysis was conducted on  
8 purified mitochondria (1-2 mg protein) extracted with 0.5 mL of acetone containing 2.5% HCl  
9 as described previously (31). The pH of the extract was adjusted to 4 by addition of 1  $\mu$ L  
10 formic acid and titration of a KOH solution. The sample was clarified by centrifugation at  
11 13000 rpm for 5 min and 1 mL was injected onto a 3.9 x 300 mm C18 Bondclone column  
12 (Phenomex, USA).

13

14 **Hydrogen peroxide sensitivity, respiratory growth assay and arginine auxotrophy.**

15 Sensitivity of the strains and transformants to hydrogen peroxide was assessed as previously  
16 described (22). To test respiratory competence, the strains were grown overnight in selective  
17 medium containing 2% raffinose - 0.2% glucose, serially diluted and spotted on 2% glucose  
18 containing medium (control) and 2% glycerol-2% lactate rich (YPLG) or synthetic complete  
19 medium (SCLG). The *cox1 $\Delta$ ::ARG8*, *COX1(1-512)::ARG8* strains and their derivatives were  
20 grown overnight in selective medium containing 2% glucose and 1x arginine (20 mg/mL Arg)  
21 or 0.2x Arg as indicated. The cultures were serially diluted and dropped onto synthetic  
22 complete medium 2% glucose containing no Arg or 1x Arg (control).

23

24 ***In vivo* mitochondrial protein translation assay.** The cells were grown overnight in  
25 selective medium containing 2% raffinose and then re-inoculated in YP-2% raffinose to  
26 grow to an absorbance of 1 at 600 nm. The labeling and preparation of the samples for 12%  
27 SDS-PAGE or 15% SDS-PAGE (to visualize mp15) was as described in (7). The gel was  
28 dried and radiolabeled proteins were visualized by exposing autoradiographic films at -80°C.

29

1 ***In vitro* import into isolated mitochondria.** *In vitro* import of Cox5a into isolated purified  
2 mitochondria was carried out as described (23) using Cox5a precursor protein labeled in a  
3 rabbit reticulocyte lysate (Promega, WI) with [<sup>35</sup>S]-Met.

4  
5 **Blue native gel electrophoresis (BN-PAGE).** BN-PAGE analysis was performed essentially  
6 as described (39) except that 1.5% digitonin was used. After incubation for 15 min on ice and  
7 centrifugation (20,000 g for 10 min at 2°C), supernatants were mixed with sample buffer (5%  
8 Coomassie brilliant blue G250, 0.5 M 6-aminocaproic acid, pH 7.0) and loaded on 4-13% or  
9 6-16% gradient polyacrylamide gel. Separated complexes were detected by immunoblotting  
10 on PVDF membrane or by autoradiography of dried gels.

11  
12 **Immunoblotting.** Protein samples were separated on 15% acrylamide gels and transferred to  
13 nitrocellulose. Proteins were visualized using ECL reagents with horseradish peroxidase-  
14 conjugated secondary antibodies. Anti-Myc and HA antisera were purchased from Santa  
15 Cruz, anti-porin was from Molecular Probes and antisera to Cox1-Cox3 were from  
16 Mitosciences. Antiserum to Sod2 was provided by Dr. Val Culotta, and antisera to Cyb2 and  
17 Cyt1 were provided by Dr. Carla Koehler. Dr. Alex Tzagoloff provided antiserum to F1 ATP  
18 synthase and Dr. Peter Rehling provided antiserum to Cyt1.

## 19 20 **RESULTS**

21  
22 **Coa2 is a multicopy suppressor of *coa1Δ* and *shy1Δ* respiratory growth defect.** In a  
23 search for high copy suppressors of the respiratory growth defect of *coa1Δ* cells, we  
24 recovered *MSS51*, *COX10* and *MDJ1* (31). A fourth suppressor, which we designated *COA2*,  
25 was also identified (Fig. 1A). Five plasmids recovered from the respiratory competent clones  
26 contained a DNA fragment encompassing YPL189c-a, an uncharacterized ORF that was  
27 identified by homology to an ORF in *Ashbya gossypii* (10, 20). Our identification of this gene  
28 as an extragenic suppressor and the respiratory deficiency of the deletion of YPL189c-a (10,  
29 20) imply that YPL189c-a is a functional gene. The complementation of the respiratory  
30 growth defect of *coa1Δ* cells by overexpression of *COA2* is similar to that observed by  
31 overexpression of *MSS51* (Fig. 1A). Since Coa1 and Shy1 function at a related step in CcO

1 assembly (31), we tested whether *COA2* overexpression rescued the respiratory defect of  
2 *shy1Δ* cells. Coa2 overexpression was able to partially complement the respiratory growth  
3 defect of *shy1Δ* cells (Fig. 1B).

4

5 ***coa2Δ* cells are respiratory deficient with a CcO specific defect.** The *COA2* ORF was  
6 deleted by homologous recombination with a KanMX4 cassette in BY4741 and W303 strain  
7 backgrounds. The *coa2Δ* strains showed normal growth on glucose medium but were unable  
8 to propagate on respiratory medium containing glycerol and lactate as carbon sources (Fig.  
9 2A). Introduction of a centromeric plasmid expressing *COA2* under the control of the *MET25*  
10 promoter and *CYCI* terminator in the *coa2Δ* strains restored respiratory growth to WT level  
11 (Fig. 2B). Consistent with the growth defect observed on glycerol-lactate medium, oxygen  
12 consumption was strongly reduced in the *coa2Δ* strains compared to their isogenic WT (Fig.  
13 2C). Overall, these results confirm that Coa2 is necessary for respiration in *S. cerevisiae* as  
14 was shown by Kastenmayer and coworkers (Kastenmayer et al., 2006).

15 Since Coa1 and Shy1 are part of the CcO assembly machinery, we assessed whether  
16 the absence of *COA2* led to a CcO defect. CcO activity measured on purified mitochondria  
17 was diminished to about 10% of WT in the *coa2Δ* strains (Fig. 3A). Heme *a*, a cofactor  
18 exclusive to CcO, was undetectable in mitochondrial extracts of *coa2Δ* strains (Fig. 3B). The  
19 *bc<sub>1</sub>* activity was reduced to 50-60% of WT and heme *b* levels were on average 58% of WT.  
20 Numerous CcO assembly mutants have reduced *bc<sub>1</sub>* activity. Immunoblotting showed an  
21 absence of Cox1-Cox3 in mitochondria purified from the *coa2Δ* strain, while cytochrome *c*  
22 and Qcr7, a subunit of complex III, were present at WT levels (Fig. 3C). Finally, CcO was  
23 undetectable by blue native PAGE (BN-PAGE) in the *coa2Δ* mitochondria (Fig. 3D). The  
24 absence of CcO caused complex III to be redistributed from the WT III<sub>2</sub>/IV<sub>2</sub> and III<sub>2</sub>/IV  
25 supercomplexes into the dimeric form (III<sub>2</sub>) and the deletion of *COA2* had no effect on  
26 complex V (Fig. 3D). Overall, these data argue for a specific CcO defect in *coa2Δ* cells.

27

28 **Coa2 is a mitochondrial matrix soluble protein partially associated with the IM.** We  
29 chromosomally integrated three copies of the HA epitope at the 3' end of the *COA2* ORF. The  
30 tag did not affect the function of Coa2 as judged by the WT growth on respiratory medium of



1 the *COA2*-3HA strain (data not shown). Nycodenz gradient purified mitochondria isolated  
2 from the *COA2*-3HA strain showed a single band at ~17 kDa upon immunodetection with HA  
3 antiserum (Fig. 4A). This apparent mass is higher than the expected 10.8 kDa, although the  
4 basic isoelectric point of Coa2 (10.6) may account for its aberrant migration on SDS-PAGE.  
5 Coa2 was detected predominantly in the soluble fraction of sonicated mitochondria like the  
6 soluble proteins Cyc1 and Sod2 (Fig. 4B). When the concentration of sodium chloride was  
7 increased in the sonication buffer, Cyc1 lost its membrane association as expected, whereas  
8 the fraction of Coa2 recovered in the pellet increased (Fig. 4B). This salt effect suggests that  
9 Coa2 may have a hydrophobic interaction with a mitochondrial membrane or with one of its  
10 proteins. This candidate interaction may involve a highly hydrophobic domain between  
11 residues 13 and 33 that was assigned to be a transmembrane helix by several topology  
12 prediction programs. In order to determine the sub-compartmentalization of Coa2, we treated  
13 intact or hypotonically-lysed mitochondria with proteinase K. The inner membrane space  
14 protein Cyc1 was degraded in mitoplasts, whereas Coa2 was protected similarly to the matrix  
15 protein Sod2 (Fig. 4C). However, Coa2 was degraded by proteinase K upon solubilization of  
16 mitochondria with Triton X100 (data not shown). Coa2, like Sod2, was retained in the  
17 insoluble fraction of mitoplasts, whereas it was liberated in the soluble fraction upon  
18 sonication of mitochondria (Fig. 4D). On the contrary, Ccs1, an IMS soluble protein, was  
19 mostly present in the soluble fraction of mitoplasts. Taken together, these results show that  
20 Coa2 is a soluble protein in the mitochondrial matrix and that Coa2 may have an interaction  
21 with an IM anchored protein or the inner membrane itself.

22

23 **The absence of *COA2* leads to low level of newly synthesized Cox1.** *In vivo* labeling of  
24 mitochondrial translation products revealed that very limited amounts of Cox1 were detected  
25 in *coa2* $\Delta$  cells in both W303 and BY4741 backgrounds. Cox2, Cox3 and Var1 were translated  
26 at WT levels while Cob (Cyt *b*) translation was slightly increased (Fig. 5A and Supplementary  
27 Fig. 1). During the 60 min chase, the levels of Var1 and Cyt *b* remained stable, whereas Cox2  
28 and Cox3 diminished markedly in *coa2* $\Delta$  cells, a phenomenon consistent with a CcO  
29 assembly defect. Low abundance of Cox1 during the pulse phase of *in vivo* mitochondrial  
30 translation experiments has been reported for most CcO mutants (8). This has been proposed  
31 to result from the accumulation of a Mss51:Cox14:Cox1 complex which titrates Mss51 out of

1 its Cox1 translational activator role (8). The synthesis of Cox1 can be restored in certain CcO  
2 mutants when Mss51 is overexpressed, when *COX14* is deleted (8) or when *COA1* is deleted  
3 (31). Deletion of *COX14* or *COA1* in *coa2Δ* cells restored WT levels of newly synthesized  
4 Cox1, whereas overexpression of Mss51 only partially returned Cox1 (Fig. 5B). However,  
5 none of these strains recovered respiratory growth (data not shown).

6 Recently, Barrientos and coworkers showed that attenuation of Mss51 levels or  
7 titration of Mss51 out of its Cox1 translational activator role leads to the synthesis of mp15, a  
8 truncated translation product of *COX1* mRNA in the W303 background (40). Mp15 was  
9 apparent in *cox5aΔ* cells, but its abundance was lower in *coa2Δ* cells (Fig. 5C). Consistent  
10 with reported data (40), mp15 synthesis was abrogated when *cox5aΔ* or *coa2Δ* cells were  
11 treated with chloramphenicol prior to labeling and the synthesis of some Cox1 was restored  
12 (Fig. 5C). The low abundance of mp15 in *coa2Δ* cells suggests that Mss51 is still able to  
13 fulfill its translation functions in the absence of *COA2*.

14

15 **Cox1 is translated but rapidly degraded in *coa2Δ* cells.** To evaluate the status of translation  
16 at the *COX1* locus in *coa2Δ* cells, we used Arg8 reporter strains constructed by Fox and  
17 coworkers (30). Arg8 is encoded by a nuclear gene and is imported to the mitochondrial  
18 matrix where it participates in the biosynthesis of arginine. These reporter strains constructed  
19 in an *arg8Δ* background, contain the *ARG8* gene in place of *COX1* ORF (*cox1Δ::ARG8*) or  
20 fused at the 3' end of the 512 codon intronless form of the *COX1* coding sequence (*COX1(1-*  
21 *512)::ARG8*) (30). The growth of these strains in medium lacking arginine is indicative of  
22 translation of *COX1(1-512)::ARG8* mRNA. Deletion of *COA2* in these two strains did not  
23 produce an arginine auxotrophy, implying that translation was occurring at the *COX1* locus  
24 (Fig. 6A). As expected, the *COX1(1-512)::ARG8* strain was unable to respire when *COA2* was  
25 deleted (Fig. 6A). To ascertain that the arginine prototrophy of the *cox1Δ::ARG8* and  
26 *COX1(1-512)::ARG8* strains lacking *COA2* was not sustained by only low amounts of Arg8,  
27 we performed *in vivo* mitochondrial translation assays in these strains. The level of mature  
28 Arg8 detected in the *cox1Δ::ARG8* strain was comparable whether *COA2* was present or not  
29 (Fig. 6B). In the *COX1(1-512)::ARG8* strain, a band corresponding to the unprocessed fusion  
30 was detected during the pulse phase of the reaction independently of the presence of *COA2*  
31 (Fig. 6C). The processing of this band into Cox1 and Arg8 during the chase was variable

1 between experiments. However, the level of total Arg8 (processed Arg8 and Cox1-Arg8  
2 fusion) was comparable between the strains containing or not *COA2*, whereas the amount of  
3 processed Cox1 at the end of the chase was consistently greatly reduced in the absence of  
4 *COA2*. The experiments with the Arg8 reporter strains show that efficient translation occurs at  
5 the *COX1* locus even when the *COA2* gene is deleted.

6 We previously reported that Mss51 is distributed into 2 complexes when analyzed by  
7 BN-PAGE (31). The largest of this complex (complex A) has been postulated to contain  
8 Cox14, Coa1 and newly synthesized Cox1 based on the observations that this complex is  
9 disrupted upon deletion of *COX14* and is stabilized in *shy1Δ* cells (31). Complex A was  
10 resolved into two different complexes A1 and A2 when a gel with a 4-13% gradient was used  
11 (Fig. 6D). These two complexes were abrogated upon deletion of *PET309*, a *COX1*  
12 translational activator (Fig. 6D), supporting the assumption that newly synthesized Cox1 is  
13 required for the formation of complexes A1 and A2. These complexes were not significantly  
14 perturbed by the deletion of *COA2*, suggesting that newly synthesized Cox1 is present in this  
15 strain. Altogether, the data strongly suggest that the CcO deficiency in *coa2Δ* cells is not a  
16 result of a translation defect of Cox1.

17 The m-AAA protease composed of Yta10-Yta12 is known to degrade non-assembled  
18 mitochondrially encoded proteins within the IM (4). Additionally, the m-AAA protease is  
19 essential for mitoribosome assembly through processing of MrpL32 (35). To evaluate whether  
20 the m-AAA protease was responsible for the low level of newly synthesized Cox1 in *coa2Δ*  
21 cells, we used a mutant m-AAA containing a E614Q substitution in Yta12 that attenuates its  
22 proteolytic activity yet maintains its processing function (3, 35). Respiratory growth was  
23 partially restored in *coa2Δ* cells when *YTA12* was deleted and replaced with the Yta12 E614Q  
24 allele (Fig. 6E). Therefore, rapid degradation of Cox1 contributes to the CcO deficiency in  
25 *coa2Δ* cells.

26

27 **Coa2 acts downstream of Shy1, Coa1 and Cox14 in CcO assembly pathway.** Deletion of  
28 certain CcO assembly factors, such as Cox11 and Sco1, render yeast cell sensitive to  
29 hydrogen peroxide (6), a phenotype generated by a pro-oxidant heme *a*<sub>3</sub>-containing Cox1  
30 assembly intermediate (22). We found that *coa2Δ* cells are also sensitive to hydrogen  
31 peroxide in a Cox1-dependent way, as the *cox1Δ::ARG8* strain did not exhibit sensitivity in

1 the absence of *COA2* (Fig. 7A). Thus, despite low levels of Cox1 observed in the [<sup>35</sup>S]  
2 translation assay in *coa2Δ* cells, sufficient Cox1 was present to cause the peroxide sensitivity.  
3 This highlights the sensitivity of the hydrogen peroxide assay. We showed previously that  
4 peroxide sensitivity was dependent on heme *a*<sub>3</sub> insertion into Cox1 (22). We took advantage  
5 of this phenotype to define the step in CcO assembly at which Coa2 is involved. Deletion of  
6 *COA1*, *SHY1* or *COX14* in *coa2Δ* cells abrogated the peroxide sensitivity (Fig. 7B), despite  
7 the elevated levels of newly synthesized Cox1 detected in *coa2Δ,coa1Δ* and *coa2Δ,cox14Δ*  
8 cells (Fig. 5B). Deletion of *COA1* or *COX14* must preclude heme *a*<sub>3</sub> insertion as is the case  
9 with *SHY1* disruption. These data together with the fact that overexpression of Coa2 can  
10 suppress *coa1Δ* and *shy1Δ* cells strongly support a role for Coa2 downstream of Coa1, Cox14  
11 and Shy1 in the assembly pathway of CcO.  
12

13 **The respiratory defect of *coa2Δ* cells is suppressed by overexpression of Cox5a and**  
14 **Cox6.** Cox1 occupies a central position in the mature CcO structure (37). We reasoned that  
15 the rapid degradation of Cox1 in *coa2Δ* cells likely occurs at an early stage in CcO assembly.  
16 In humans, CoxIV and CoxVa (yeast Cox5a and Cox6) associate with Cox1 as the first  
17 subassembly intermediate (29, 38) and these subunits are in close contact with Cox1 in the  
18 bovine CcO (Fig. 8A). We addressed whether Cox5a and Cox6 have a significant effect on  
19 Cox1 stability at early stages of CcO assembly. We performed *in vivo* mitochondrial  
20 translation experiments in strains carrying deletion of different CcO subunits, early assembled  
21 subunits like Cox5a and Cox6 and subunits assembled at a latter stage like Cox7 and Cox12.  
22 Only minimal levels of Cox1 were detected in *cox5aΔ* and *cox6Δ* cells, whereas *cox7Δ* and  
23 *cox12Δ* cells showed WT levels of Cox1 during the pulse of the reaction (Fig. 8B). This result  
24 prompted us to overexpress Cox5a and Cox6 in *coa2Δ* cells. The two genes, when  
25 overexpressed individually, showed suppression of *coa2Δ* respiratory defect at 37°C (Fig.  
26 8C). However, Cox5a and Cox6 had to be overexpressed together to produce significant  
27 growth of *coa2Δ* cells at 30°C (Fig. 8C). Coa2 is probably not required for Cox5a stability  
28 *per se*, since the same residual level of Cox5a was detected in *coa2Δ* or in *cox19Δ* cells (Fig.  
29 8D). Cox19 is another CcO assembly factor. Coa2 may therefore aid in complex formation  
30 between Cox5a and newly synthesized Cox1.

1 Genomically tagged Coa2 migrated on BN-PAGE as a single complex whose mass  
2 was approximately 100 kDa (Fig. 8E). This is in contrast to the predicted mass of 27 kDa for  
3 Coa2-13Myc. The observed Coa2 complex is smaller than any of the Cox5a containing  
4 complexes described recently (27) suggesting that Coa2 may not form a stable complex with  
5 Cox5a. Immunoprecipitation experiments between Coa2 and Cox5a failed to show an  
6 interaction between the two proteins (data not shown). In addition, import of radiolabeled  
7 Cox5a into purified mitochondria from WT cells and cells containing Coa2-13Myc showed  
8 the same mass complexes (Supplemental Fig. 2). A mass shift was shown to occur in the  
9 import of radiolabeled Cox5a in mitochondria containing an epitope-tagged Shy1 relative to  
10 mitochondria with an untagged Shy1 (27). Due to the low Met and Cys content of Coa2, we  
11 engineered a C-terminal extension with 6 methionine residues. However, attempts to  
12 radiolabel Coa2 *in vitro* with <sup>35</sup>S were unsuccessful, precluding experiments to assess the  
13 association of Coa2 in transient complexes upon *in vitro* import. The 100 kDa Coa2 complex  
14 does not appear to contain Cox1, since this complex on BN-PAGE was apparent in *pet309Δ*  
15 cells in which Cox1 is not translated (data not shown). We failed to detect an interaction  
16 between Coa2 and newly synthesized Cox1 when Coa2 was immunoprecipitated from  
17 radiolabeled mitochondria (data not shown). However, not all IM complexes are stably  
18 maintained under BN-PAGE conditions (14).

19

20 **COA2 and MSS51 have non-redundant complementary functions.** As in *coa2Δ* cells,  
21 Cox1 synthesis is also unusually low in *cox5aΔ* cells. In order to test whether the low level of  
22 Cox1 detected in *cox5aΔ* (Fig. 8A) was a result of lack of translation or rapid degradation, we  
23 disrupted *COX5a* in the *COX1(1-512)::ARG8* strain. This strain showed a partial arginine  
24 auxotrophy which was reversed by overexpression of *MSS51* or *COA2* (Fig. 9A). Consistent  
25 with the Cox1 translation block model (8), overexpression of *MSS51* in a *cox5aΔ* strain  
26 partially returned the level of newly synthesized Cox1 while *COA2* overexpression did not  
27 (Fig. 9B). These results suggest that *MSS51* probably increases translation of the Cox1-Arg8  
28 fusion to rescue the arginine auxotrophy of the *COX1(1-512)::ARG8 cox5aΔ* strain, whereas  
29 *COA2* suppression may arise in a post-translational step through stabilization of the translated  
30 Cox1-Arg8. The low intensity of this band by *in vivo* mitochondrial translation assay in the  
31 *COX1(1-512)::ARG8 cox5aΔ* strain prevented us from directly testing this hypothesis.

1 We subsequently assayed the peroxide sensitivity of the *cox5a*Δ strain to assess heme  
2 *a*<sub>3</sub>:Cox1 levels. Cells lacking Cox5a were not sensitive unless *MSS51* or *COA2* were  
3 overexpressed (Fig. 9C). The coexpression of the two proteins further increased the peroxide  
4 sensitivity (Fig. 9C), arguing for non-redundant functions of *MSS51* and *COA2*. High levels  
5 of Mss51 and Coa2 appear to enhance Cox1 levels, via translational and post-translational  
6 effects respectively.

7 A synergistic effect of *MSS51* and *COA2* was also found in the suppression of the  
8 respiratory defect of *coa1*Δ, a strain that has impaired CcO assembly due to a post-  
9 translational defect (Fig. 10A). Similarly, co-overexpression of *MSS51* and *COA2* improved  
10 the growth of a *shy1*Δ strain (Fig. 10B). Taken together, these results suggest that Coa2 is  
11 involved in Cox1 maturation at a post-translational step after the heme *a*<sub>3</sub> insertion mediated  
12 by Shy1. The respiratory defect of a *shy1*Δ strain was recently shown to be partially  
13 suppressed by overexpression of Cox5a and Cox6 (16). We did not observed any synergism in  
14 the suppression of a *shy1*Δ strain when these 2 subunits were overexpressed in combination  
15 with Coa2 (data not shown) suggesting that Coa2, Cox5a and Cox6 function at a related step  
16 in CcO assembly.

## 17 18 **DISCUSSION**

19  
20 Coa2 is a 68 residue mitochondrial protein shown to be required for respiration and  
21 having a specific role in CcO assembly. The most striking aspect of the CcO deficiency of  
22 *coa2*Δ cells is a rapid degradation of newly synthesized Cox1. Only limited quantity of newly  
23 synthesized Cox1 is observed with *in vivo* mitochondrial translation and this limitation results  
24 from rapid degradation rather than impaired synthesis. Two lines of evidence support this  
25 conclusion. First, deletion of *COA2* in a *COX1(1-512)::ARG8* fusion strain resulted in normal  
26 growth on medium lacking arginine, whereas deletion of *COX5a* in the same background  
27 induced an Arg auxotrophy partially due to restricted translation. Second, respiration was  
28 partially recovered in *coa2*Δ cells by diminishing the activity of the m-AAA protease, a  
29 complex which has been shown to degrade misfolded inner membrane proteins (5).

1 *COX1* translation is impaired in many CcO assembly mutants due to the titration of  
2 Mss51 into a post-translational complex, preventing its function as a translational activator of  
3 *COX1* (8). This *COX1* translation block is unlikely or at least limited in *coa2Δ* cells, since  
4 Arg8 is translated essentially normally in *COX1(1-512)::ARG8 coa2Δ* cells. The attenuation  
5 of Mss51 as a translational activator is also known to result in synthesis of a truncated Cox1  
6 translation product called mp15 (40). Whereas mp15 synthesis occurs in *cox5aΔ* cells, only  
7 limited synthesis of mp15 occurs in *coa2Δ* cells.

8 A translation block of Cox1 likely exists in the absence of *COX5a* as shown by the  
9 rescue of the Arg auxotrophy of *COX1(1-512)::ARG8 cox5aΔ* cells by *MSS51* overexpression  
10 and by the recovery of some level of newly synthesized Cox1 in a *cox5aΔ* strain  
11 overexpressing *MSS51*. The translation block occurring in most CcO mutants can be obviated  
12 in cells lacking Coa1 or Cox14. The deletion of *COX14* leads to a marked enhancement in  
13 Cox1 synthesis, but the newly synthesized Cox1 does not progress to assembled CcO (8).  
14 Deletion of either *COA1* or *COX14* in *coa2Δ* cells leads to a pronounced increase in newly  
15 synthesized Cox1 in an *in vivo* mitochondrial translation assay. The accumulated Cox1 must  
16 be in an environment where it is protected from the protease responsible of its rapid  
17 degradation in *coa2Δ* cells. Cox14 and Coa1 are essential to coordinate Mss51's post-  
18 translational function leading to cofactor insertion in Cox1 (31). Therefore, disruption of  
19 *COX14* or *COA1* likely prevents newly synthesized Cox1 from joining the assembly chain.  
20 Indeed the reversal of the peroxide sensitivity of *coa2Δ* cells by deletion of *COX14* or *COA1*,  
21 even though it increases greatly the amount of newly synthesized Cox1 visible in the  
22 translation assay, suggests that this Cox1 is devoid of heme *a*<sub>3</sub>. Shy1 has been shown to be  
23 important for heme *a*<sub>3</sub> insertion in *Rhodobacter* (33), and the fact that deletion of *SHY1* in  
24 *coa2Δ* cells also reverses the peroxide sensitivity supports this point.

25 At what step in CcO assembly is Coa2 important? Three lines of evidence suggest that  
26 Coa2 functions downstream of Coa1 and the heme *a*<sub>3</sub> related function of Shy1. First, the  
27 Coa1:Mss51:Cox14:Cox1 complex is unaffected in *coa2Δ* cells. Second, Cox1 can acquire  
28 heme *a*<sub>3</sub> in the absence of *COA2*, a step that is abrogated by the deletion of *COA1* and *SHY1*  
29 as shown by the reversal of the peroxide sensitivity of *coa2Δ* cells. Third, overexpression of  
30 *COA2* suppresses the respiratory phenotype of *coa1Δ* and *shy1Δ* cells. These results strongly  
31 point to Coa2 acting downstream of these two assembly factors. Although Shy1 appears to be

1 important in the heme  $a_3$  insertion step, Shy1 stays associated with CcO until the  
2 supercomplex stage (27). It remains unclear whether Shy1 has any function downstream of  
3 the proposed heme  $a_3$  assembly step.

4 Proteolytic degradation of Cox1 may be a protective response to the assembly of the  
5 pro-oxidant heme  $a_3$ :Cox1 intermediate. The enhanced activity in *coa2* $\Delta$  cells may suggest  
6 that Coa2 functions at a Cox1 assembly checkpoint that couples the progression of Cox1  
7 assembly and degradation of stalled heme  $a_3$ :Cox1 intermediates. In the absence of heme  $a_3$   
8 insertion, Cox1 assembly intermediates, e.g. in *cox14* $\Delta$  cells, are not deleterious and perhaps  
9 not a priority for the cell to remove proteolytically.

10 We show that *COX5a* and *COX6* partially suppress the respiratory defect of *coa2* $\Delta$ ,  
11 arguing for Coa2 functioning upstream or proximate to these two subunits joining Cox1.  
12 *COX5a* and *COX6* were recently shown to partially suppress the respiratory defect of *shy1* $\Delta$   
13 cells (16). In this case, the authors suggested that Cox5a and Cox6 protect newly synthesized  
14 Cox1 from proteolytic degradation. Therefore, it is possible that at least part of the decrease in  
15 the amount of newly synthesized Cox1 observed in *shy1* $\Delta$  actually results from rapid  
16 degradation of Cox1 and not entirely from a translation block. This argument is supported by  
17 the observation that a *COX1(1-512)::ARG8 shy1* $\Delta$  strain has no growth defect on medium  
18 lacking Arg (30) and that normal level of Arg8 are present in the *COX1(1-512)::ARG8* strain  
19 in the absence of *SHY1* (30). Therefore, the same rapid Cox1 proteolysis may occur in *shy1* $\Delta$   
20 as well as in *coa2* $\Delta$  cells suggesting that in the absence of these factors Cox1 forms an  
21 unstable conformer. Given the close packing of Cox5a against Cox1, Cox5a may have a  
22 stabilizing effect on newly synthesized Cox1.

23 An early step in Cox1 maturation is formation of the Cox1:Cox5a:Cox6 subassembly  
24 complex (34, 38). Cox5a packs against transmembrane helices 11 and 12 in Cox1 and  
25 together with Cox6 packs onto the matrix face of the Cox1 helical barrel stabilizing loops  
26 connecting the 12 membrane helices. This complex accumulates in mammalian cells with  
27 mutations in SURF1 (Shy1) (34, 38). This assembly intermediate likely contains heme  $a$ ,  
28 since mutant Cox10 or Cox15 fibroblast lines fail to form this intermediate (1, 2). Heme  $a$  is  
29 axially coordinated by histidyl residues from Cox1 helices 2 and 10 and its farnesyl tail packs  
30 between helices 1, 11 and 12 (36). Thus, heme  $a$  insertion may assist in organizing the Cox1  
31 helices creating the Cox5a interface.



1           It is conceivable that heme *a* produced by Cox10/Cox15 and inserted into newly  
2 synthesized Cox1 creates a Cox1 conformer competent for Cox5a,Cox6 binding. The heme  
3  $a_3$ :Cu<sub>B</sub> site may be formed in a second step mediated by Shy1 and Cox11. These two steps  
4 may occur concurrently rather than sequentially. In support of concurrent steps, a 350 kDa  
5 Cox1 assembly intermediate contains both Shy1 and Cox5a, although distinct 250 kDa  
6 complexes exist with either Shy1 or Cox5a (27). Coa2 may have a chaperone activity in  
7 promoting Cox5a/Cox6 association with Cox1, but appears independent of the Shy1 step. The  
8 inability to efficiently form the Cox1:Cox5a:Cox6 intermediate in *coa2Δ* cells could expose  
9 Cox1 to rapid proteolysis. In *shy1Δ* cells the unstable Cox1 intermediate may lack heme  $a_3$ .  
10 Coa2 likely suppress *shy1Δ* respiratory defect via a post-translational effect on Cox1. We did  
11 not detect any enhancement of the suppression of *shy1Δ* by Coa2 when Cox5a and Cox6 were  
12 also overexpressed. Therefore, it is probable that Coa2 suppresses the respiratory defect of  
13 *shy1Δ* cells by a similar mechanism as Cox5a and Cox6 which was proposed to be due to  
14 increasing the stability of newly synthesized Cox1 (16). Such a role of Coa2 would also  
15 explain the H<sub>2</sub>O<sub>2</sub> sensitivity of a *cox5aΔ* strain overexpressing Coa2. Even though we failed to  
16 detect any stable interaction between Coa2 and Cox1 or Cox5a, a transient interaction may  
17 occur. Coa2 showed a salt-dependent association with the membrane fraction consistent with  
18 a hydrophobic interaction that could involve one of these protein. Future studies will focus on  
19 the components in the Coa2 complex.

20

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27 (17) (21) (24)

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- 11  
12

### 13 **Figure Legends**

#### 14 **Fig. 1. Overexpression of *COA2* can suppress the respiratory growth defect of *coa1Δ***

15 **and *shy1Δ* cells.** A) *coa1Δ* cells (BY4743 background) transformed with episomal vectors  
16 expressing *MSS51*, *MDJ1*, *COX10* or *COA2* were grown in SC-2% raffinose, serially diluted  
17 and spotted on SC 2% glycerol-2% lactate (SCLG) or 2% glucose. The plates were incubated  
18 at 30°C for 2 days (glucose) or 7 days (SCLG). B) *shy1Δ* cells (W303 background)  
19 transformed with an episomal vector expressing *COA2* or an empty vector were grown as in  
20 A) and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose. The plates were  
21 incubated at 30°C for 2 days (glucose) or 4 days (YPLG).  
22  
23

#### 24 **Fig. 2. *coa2Δ* cells are respiratory deficient.** A) *coa2Δ* cells and the isogenic WT (BY4741

25 or W303) were grown in liquid YPD then serially diluted and spotted on YP 2% glycerol-2%  
26 lactate (YPLG) or 2% glucose plates (YPD). The plates were incubated at 30°C for 2 days  
27 (YPD) or 4 days (YPLG). B) *coa2Δ*, the isogenic WT and *coa2Δ* cells transformed with a  
28 centromeric vector expressing *COA2* ORF under the control of the *MET25* promoter and the  
29 *CYCI* terminator were grown in SC-2% glucose, serially diluted and spotted on YPD or SC-  
30 2% glycerol-2% lactate (SCLG). The plates were incubated as in A). C) *coa2Δ* cells and the

1 WT were grown in liquid YPD at 30°C and oxygen consumption (% O<sub>2</sub>/sec/OD<sub>600</sub>) was  
2 measured. The data represent the average of 4 independent experiments.

3

4 **Fig. 3. Deletion of *COA2* results in a CcO specific defect.** A) *coa2Δ* cells and the isogenic  
5 WT were grown in YP-2% raffinose, mitochondria were purified and assayed for CcO  
6 activity (ΔOD<sub>550</sub>/min/10 μg protein). The data represent the average of 3 independent  
7 experiments. B) Heme was extracted from mitochondria (2 mg protein) purified from *coa2Δ*  
8 or W303 cells and separated by reverse phase HPLC. The peaks corresponding to heme *b* (B)  
9 and heme *a* (A) and protoporphyrin (P) are shown. C) Immunoblot of mitochondria (30 μg  
10 protein) from *coa2Δ* cells or from wild-type W303. D) Mitochondria (200 μg protein) isolated  
11 from either wild-type (WT) or *coa2Δ* strains were solubilized in buffer containing 1.5%  
12 digitonin. Lysates were loaded onto a continuous 4-13% gradient gel and protein complexes  
13 were separated by BN-PAGE. The distribution of respiratory complexes was analyzed by  
14 immunoblotting with antisera against Cyt1, Cox2 and F<sub>1</sub>-subunit.

15

16 **Fig.4. *Coa2* is a soluble matrix protein partially associated with the inner-membrane.** A)  
17 Immunoblot of mitochondria and post-mitochondrial fraction (PMF) purified from the *COA2*-  
18 3HA strain. Pgc1 is a cytosolic marker and Por1 is a mitochondrial outer-membrane (OM)  
19 protein. B) Purified mitochondria from the *COA2*-3HA strain were sonicated in Hepes 20  
20 mM pH 7.4 and the indicated concentration of NaCl. The soluble and insoluble fractions were  
21 separated by centrifugation at 100,000 g for 1 h and analyzed by immunoblot. Cyt1 is an  
22 integral inner-membrane (IM) protein, Cyc1 is associated with the IM and Sod2 is a matrix  
23 soluble protein. C) Purified mitochondria from the *COA2*-3HA strain were incubated in  
24 Hepes 20 mM pH 7.4 with 1.2 M Sorbitol (intact) or without it (hypotonic) for 30 min on ice,  
25 digested on ice for 40 min with the indicated concentration of proteinase K (PK) (μg/mL) and  
26 analyzed by immunoblotting. D) Purified mitochondria from the *COA2*-3HA strain were  
27 sonicated or incubated in Hepes 20 mM pH 7.4 with 1.2 M Sorbitol (intact) or without it  
28 (hypotonic) for 30 min on ice. The soluble (S) and insoluble fractions (P) were separated by  
29 centrifugation at 15,000 g for 20 min and analyzed by immunoblot. Ccs1 is a soluble IMS  
30 protein.

31

1 **Fig. 5. The absence of COA2 leads to low level of newly synthesized Cox1.** A) *In vivo*  
2 labeling of mitochondrial translation products. *coa2Δ* and WT cells were pulsed for 5 and 15  
3 min with [<sup>35</sup>S]-methionine. After 15 min, 20 mM of cold methionine (Met) was added and  
4 the reaction was chased for 20 and 60 min at 30°C. The samples were run on 12% SDS-  
5 PAGE, the gel was dried and exposed to autoradiographic film. B) Cells were labeled for 8  
6 and 20 min with [<sup>35</sup>S]-Met at 30°C. The samples were analyzed as in A). C) W303 *coa2Δ* or  
7 *cox5aΔ* cells were treated (+) or not (-) for 3 h with 2 mg/mL chloramphenicol (CAP) and  
8 then pulsed for 20 min with [<sup>35</sup>S]-Met. The samples were run on 15% SDS-PAGE, the gel  
9 was dried and exposed to autoradiographic film.

10

11 **Fig. 6. Cox1 is translated in the absence of COA2.** A) *COA2* was deleted in *arg8Δ* strains  
12 in which *ARG8* replaces the *COX1* codons (*cox1Δ::ARG8*) or *ARG8* is fused to 512 codons of  
13 *COX1* (*COX1(1-512)::ARG8*). The strains were grown in SC-2% glucose containing 0.2x  
14 Arg (4 mg/L), serially diluted and spotted on YPD, SC-2% glucose with or without 1x Arg  
15 and YPLG. The plates were incubated at 30°C for 2 days (glucose plates) or 4 days (YPLG).  
16 The *arg4Δ* strain is shown as a control. B) *In vivo* labeling of mitochondrial translation  
17 products of the *cox1Δ::ARG8* strain with or without the *COA2* gene. The cells were treated as  
18 in Fig.5A. The additional band is the mature Arg8. C) The *COX1(1-512)::ARG8* strain with  
19 or without the *COA2* gene was radiolabeled as in Fig.5A. The bands corresponding to the  
20 Cox1::Arg8 fusion and to the mature Arg8 are marked. D) Mitochondria (250 μg protein)  
21 isolated from either wild-type (WT), *coa2Δ* or *pet309Δ* strains containing a genomically  
22 tagged 13Myc *MSS51* (*MSS51::Myc*) were solubilized in buffer containing 1.5% digitonin.  
23 Lysates were loaded onto a continuous 4-13% gradient gel and protein complexes were  
24 separated by BN-PAGE. The distribution of Mss51 complexes was analyzed by  
25 immunoblotting with anti-Myc antibody. E) *coa2Δ yta12Δ* cells transformed with a  
26 centromeric vector expressing Yta12 E614Q were grown in SC-2% raffinose, serially diluted  
27 and spotted on YP 2% glycerol-2% lactate (YPLG) or 2% glucose (YPD). The plates were  
28 incubated at 30°C for 2 days (YPD) or 6 days (YPLG).

29

30 **Fig. 7. Coa2 acts downstream of Coa1 and Shy1 in the assembly pathway of CcO.** A)  
31 Cells were grown to mid-exponential phase and incubated with (+) or without (-) the



1 indicated concentration of H<sub>2</sub>O<sub>2</sub> for 2 h at 30 °C. Serial dilutions were spotted onto YPD  
2 plates and incubated for 36–48 h at 30 °C. **B)** Cells (W303 background) were treated like in  
3 A).

4  
5 **Fig. 8. Overexpression of Cox5a can partially rescue the respiratory deficiency of *coa2Δ***  
6 **cells. A)** View of Cox1, Cox5a and Cox6 based on the structure of bovine CcO (37). Cox1 is  
7 shown in light gray, Cox5a and Cox6 are shown in black and dark gray, respectively. **B)** *In*  
8 *vivo* labeling of mitochondrial translation products in cells lacking CcO subunits. Cells  
9 (BY4743 background) were labeled for 8 and 20 min with [<sup>35</sup>S]-Met at 30°C. The samples  
10 were run on 12% SDS-PAGE, the gel was dried and exposed to autoradiographic film. **C)**  
11 W303 *coa2Δ* cells transformed with episomal vectors expressing *COX5a* or *COX6* were  
12 grown in SC-2% raffinose, serially diluted and spotted on YP 2% glycerol-2% lactate (YPLG)  
13 or 2% glucose (YPD). The plates were incubated at 30°C and 37°C for 2 days (YPD) or 7  
14 days (YPLG). **D)** Immunoblot of TCA extracts from W303, *coa2Δ* and *cox19Δ* cells  
15 containing a centromeric vector expressing a 3HA C-terminal tagged version of *COX5a* under  
16 the control of its own promoter. Porin (Por1) is shown as a loading control. **E)** Mitochondria  
17 isolated from either wild-type (WT) or a strain containing a genomically tagged 13Myc *COA2*  
18 (*COA2::Myc*) were solubilized in buffer containing 1.5% digitonin. Lysates were separated  
19 by BN-PAGE (6-16% gradient gel) and analyzed as in Fig.6D. Monomeric (V<sub>1</sub>) and dimeric  
20 (V<sub>2</sub>) forms of respiratory chain complex V served as a control and were visualized using  
21 antisera against F<sub>1</sub>-subunit.

22  
23 **Fig.9. Coa2 and Mss51 overexpression in *cox5aΔ* cells results in increased levels of Cox1.**  
24 **A)** The *COX1(1-512)::ARG8* strain lacking *COX5a* and with or without episomal vectors  
25 expressing *COA2* or *MSS51* were grown in SC-2% glucose containing 1x Arg (20 mg/L),  
26 serially diluted and spotted on SC-2% glucose containing 1x Arg (+Arg) or not (-Arg). The  
27 plates were incubated at 30°C for 2 days. **B)** W303 *cox5aΔ* cells containing the same plasmids  
28 as in A) were labeled for 8 and 20 min with [<sup>35</sup>S]-Met at 30°C. The samples were run on 12%  
29 SDS-PAGE, the gel was dried and exposed to autoradiographic film. **C)** The same cells as in  
30 B) were grown to midexponential phase and incubated with (+) or without (-) the indicated

1 concentration of H<sub>2</sub>O<sub>2</sub> for 2 h at 30 °C. Serial dilutions were spotted onto YPD plates and  
2 incubated for 36–48 h at 30 °C.

3

4 **Fig.10. Coa2 and Mss51 have a synergistic effect in enhancing respiration of *coa1Δ* and**  
5 ***shy1Δ* cells. A)** BY4743 *coa1Δ* cells transformed with episomal vectors expressing *MSS51*,  
6 *COA2* or both were grown in SC-2% raffinose, serially diluted and spotted on SC 2%  
7 glycerol-2% lactate (SCLG) or 2% glucose. The plates were incubated at 30°C for 2 days  
8 (glucose) or 6 days (SCLG). **B)** BY4743 *shy1Δ* cells transformed with the same vectors as in  
9 A) were grown in SC-2% raffinose, serially diluted and spotted on SC 2% glycerol-2% lactate  
10 (SCLG) or 2% glucose or YP 2% glycerol-2% lactate (YPLG). The plates were incubated at  
11 30°C for 2 days (glucose) or 6 days.

**Table I**

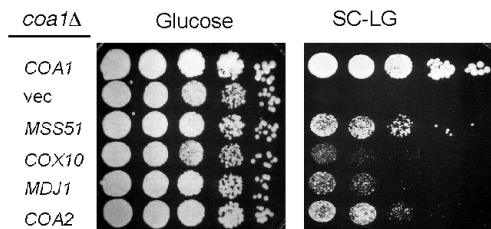
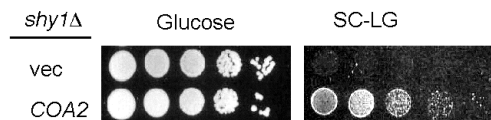
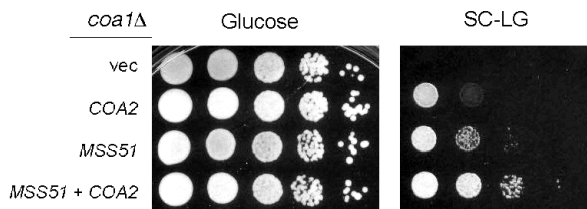
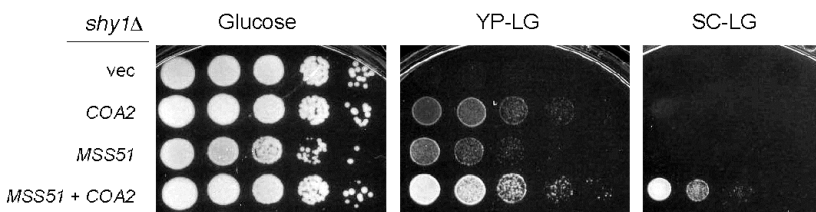
## Yeast strains used in this study

The strains with the 3 HA or 13Myc tags were generated in the DY5113 background by PCR-based gene modification using the template pFA6a-3HA-*TRP1*, pFA6a-13MYC-*TRP1*, pFA6a-3HA-*HIS3MX6* or pFA6a-13MYC-*HIS3MX6* (24).

Strain	Genotype	Reference/Source
BY4741	MATa, <i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Invitrogen Corp.
BY4741 <i>Δcoa2</i>	MATa, <i>his3Δ1, leu2Δ0, met15Δ0 ura3Δ0, Δcoa2:: kanMX4</i>	This work
W303	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1</i>	
W303 <i>Δcoa2</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcoa2:: kanMX4</i>	This work
W303 <i>Δcoa2 Δcox14</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcoa2:: kanMX4, Δcox14:: Ca URA3</i>	This work
W303 <i>Δcoa2</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcoa2:: Ca URA3</i>	This work
W303 <i>Δcoa2 Δyta12</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcoa2:: Ca URA3, Δyta12:: kanMX4</i>	This work
W303 <i>Δcoa2 Δcoa1</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcoa2:: Ca URA3, Δcoa1:: kanMX4</i>	This work
W303 <i>Δshy1</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δshy1::URA3</i>	(7)
W303 <i>Δshy1 Δcoa2</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δshy1::URA3, Δcoa2:: kanMX4</i>	This work
W303 <i>Δcox5a</i>	MATα <i>ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1, Δcox5a:: HIS3</i>	(17)
<i>cox1Δ::ARG8m</i>	MATa, <i>lys2, leu2-3,112, arg8::hisG, ura3-52 [cox1Δ::ARG8m]</i>	(30)
<i>cox1Δ::ARG8m Δcoa2</i>	MATa, <i>lys2, leu2-3,112, arg8::hisG, ura3-52 [cox1Δ::ARG8m], Δcoa2:: kanMX4</i>	This work
<i>COX1(1- 512)::ARG8</i>	MATa, <i>lys2, leu2-3,112, arg8::hisG, ura3-52, [COX1(1- 512)::ARG8m]</i>	(30)
<i>COX1(1- 512)::ARG8 Δcoa2</i>	MATa, <i>lys2, leu2-3,112, arg8::hisG, ura3-52, [COX1(1- 512)::ARG8m], Δcoa2:: kanMX4</i>	This work
<i>COX1(1- 512)::ARG8 Δcox5a</i>	MATa, <i>lys2, leu2-3,112, arg8::hisG, ura3-52, [COX1(1- 512)::ARG8m], Δcox5a:: kanMX4</i>	This work
DY5113	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1</i>	(21)
COA2-3HA	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA2-3HA::TRP1</i>	This work
COA2-13Myc	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA2-13Myc::TRP1</i>	This work
COA2-13Myc <i>Δpet309</i>	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA2-13Myc::TRP1, Δpet309:: CaURA3</i>	This work
MSS51-13Myc COA1-3HA	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA1-3HA::TRP1, MSS51-13Myc::HIS3MX6</i>	(31)
MSS51-13Myc COA1-3HA <i>Δcoa2</i>	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA1-3HA::TRP1, MSS51-13Myc::HIS3MX6, Δcoa2:: kanMX4</i>	This work
MSS51-13Myc COA1-3HA <i>Δpet309</i>	MATa <i>ade2-1 his3-1,15 leu2-3,112 trp1Δ, ura3-1, COA1-3HA::TRP1, MSS51-13Myc::HIS3MX6, Δpet309:: CaURA3</i>	This work
BY4743 <i>Δshy1</i>	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0 Δshy1:: kanMX4</i>	Invitrogen Corp.
BY4743 <i>Δcoal</i>	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0 Δcoal:: kanMX4</i>	Invitrogen Corp.
BY4743	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0</i>	Invitrogen Corp.
BY4743 <i>Δcox5a</i>	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0, Δcox5a:: kanMX4</i>	Invitrogen Corp.
BY4743 <i>Δcox6</i>	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0, Δcox6:: kanMX4</i>	Invitrogen Corp.
BY4743 <i>Δcox7</i>	MATa/α <i>his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0</i>	Invitrogen Corp.

1

	<i>ura3Δ0/ura3Δ0, Δcox7:: kanMX4</i>	
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**A****B****C****D****Figure 1**

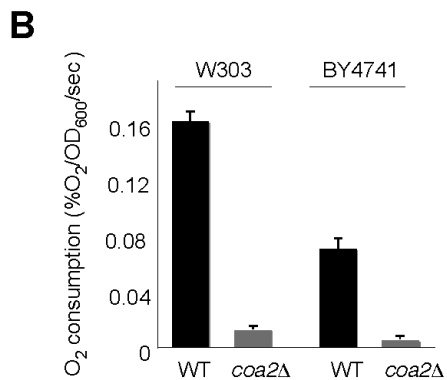
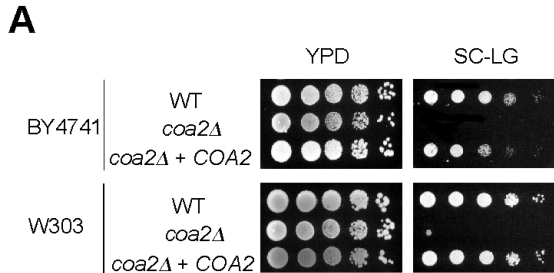


Figure 2

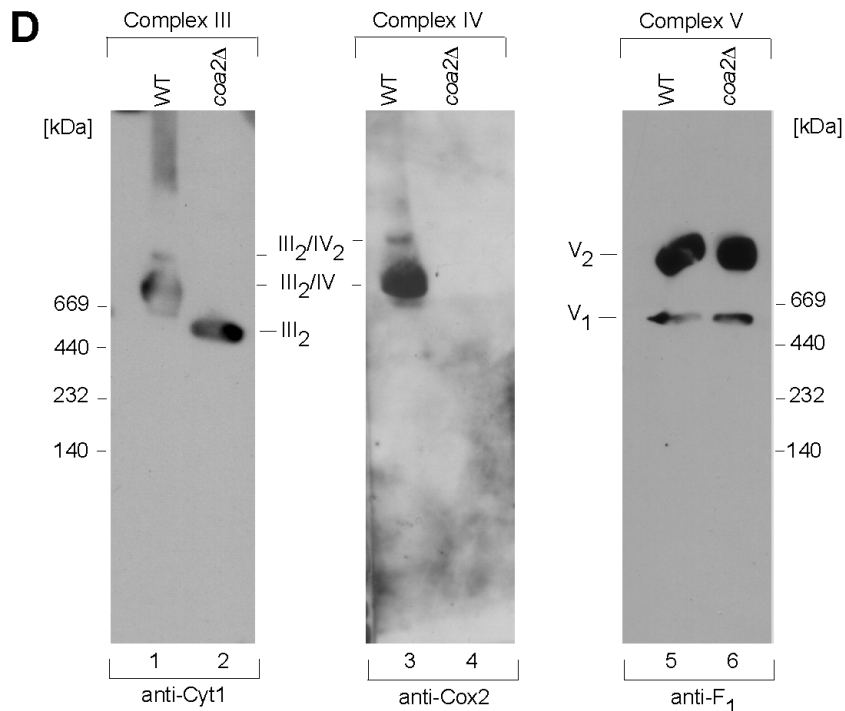
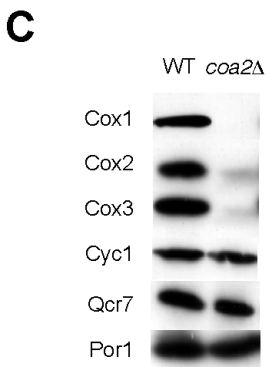
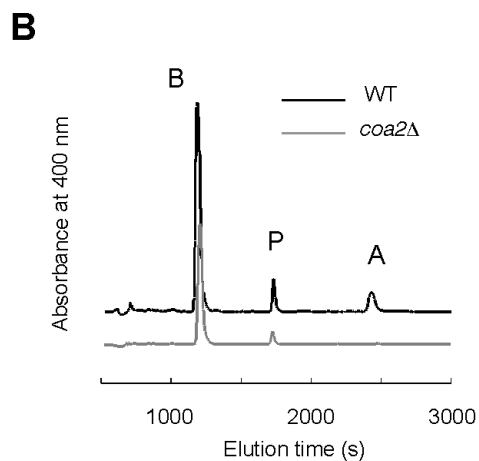
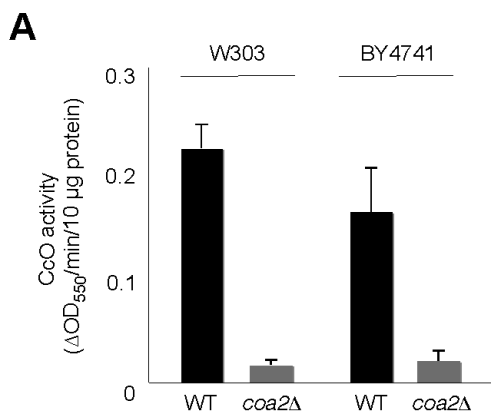


Figure 3

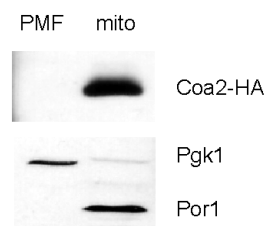
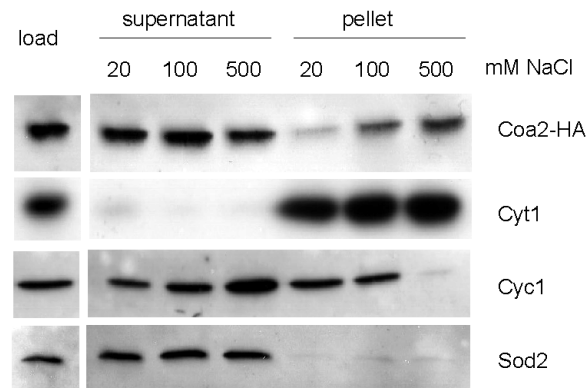
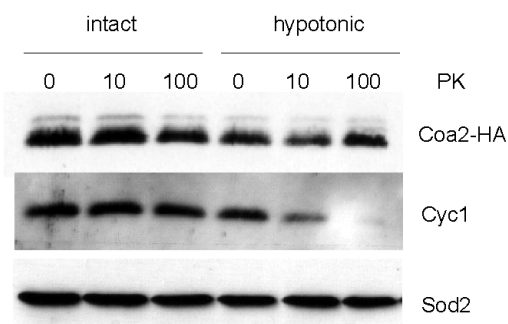
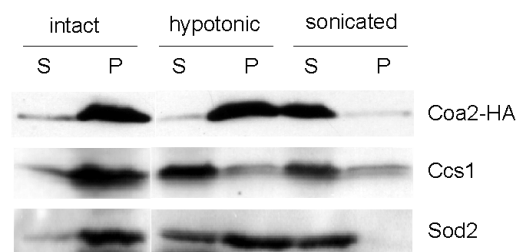
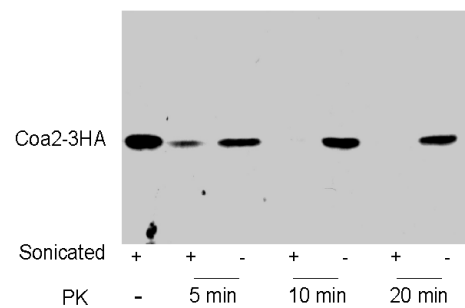
**A****B****C****D****E**

Figure 4



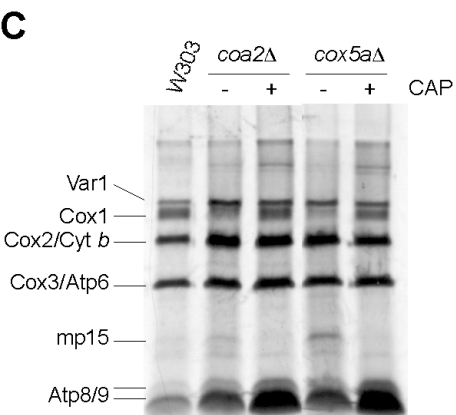
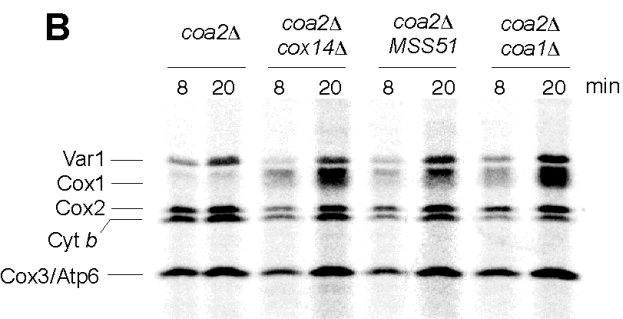
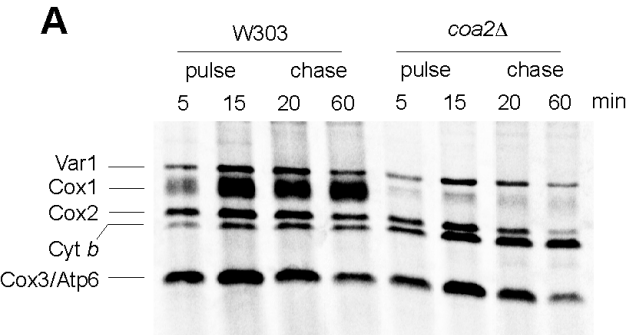


Figure 5

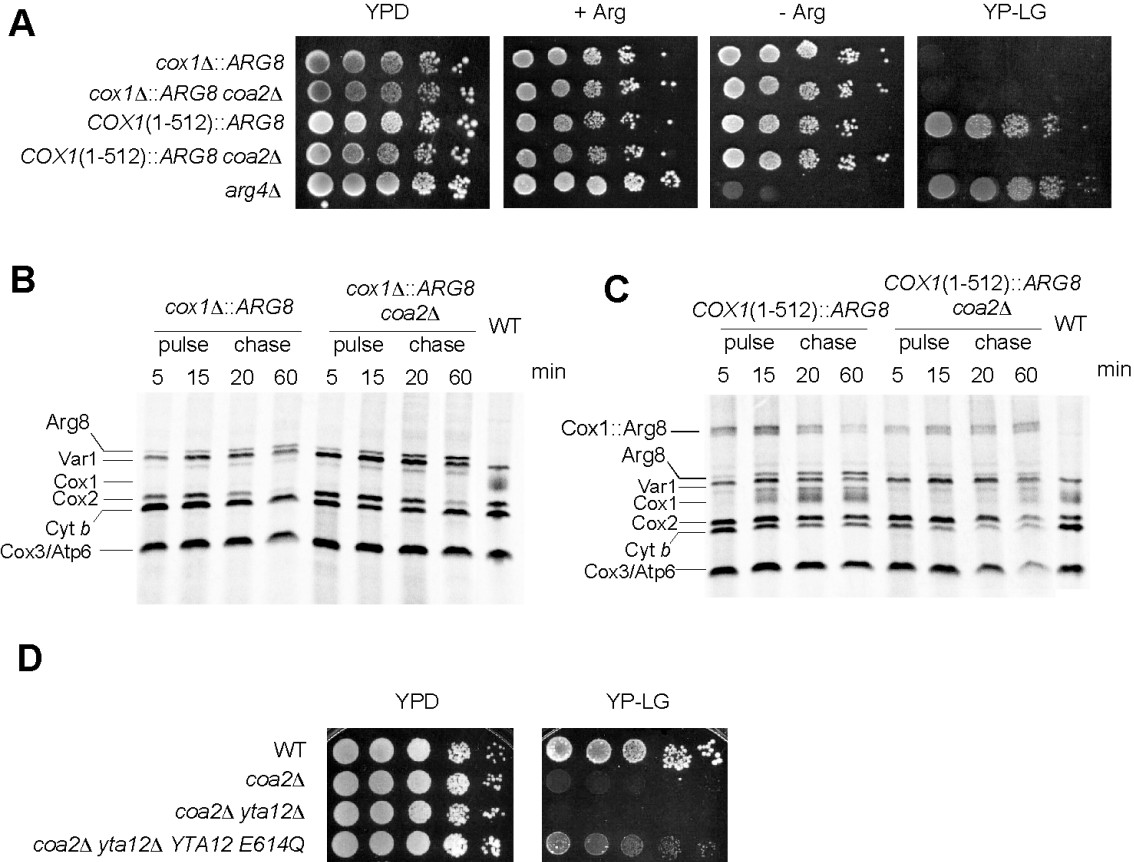


Figure 6

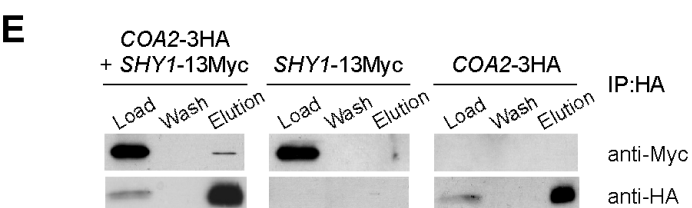
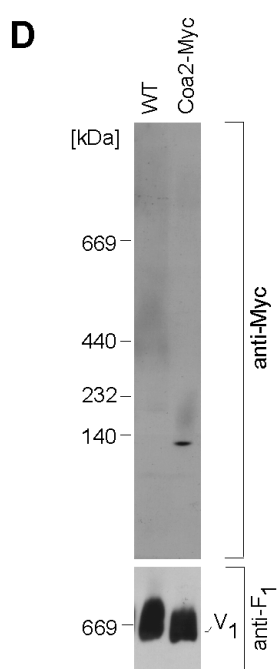
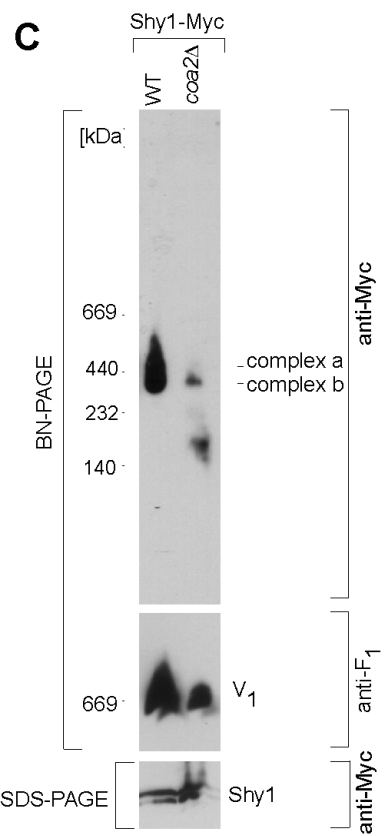
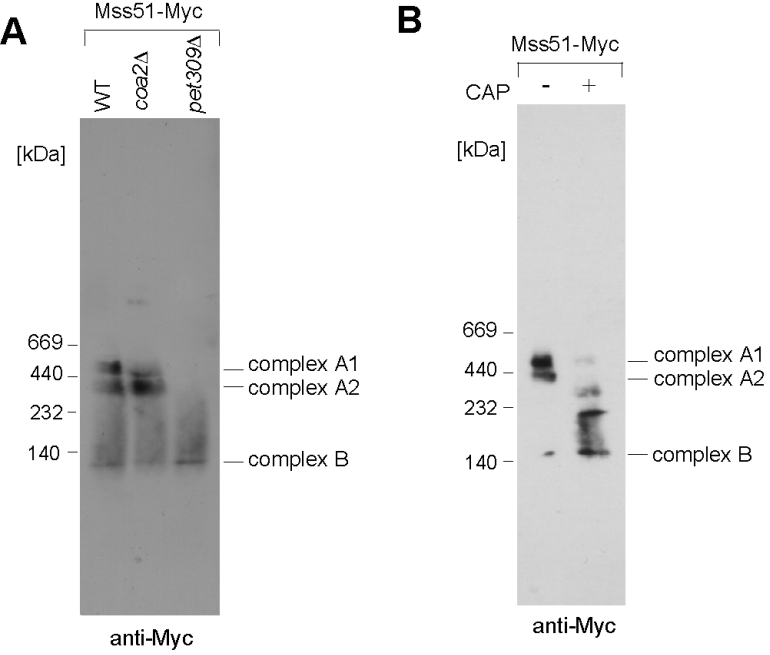


Figure 7

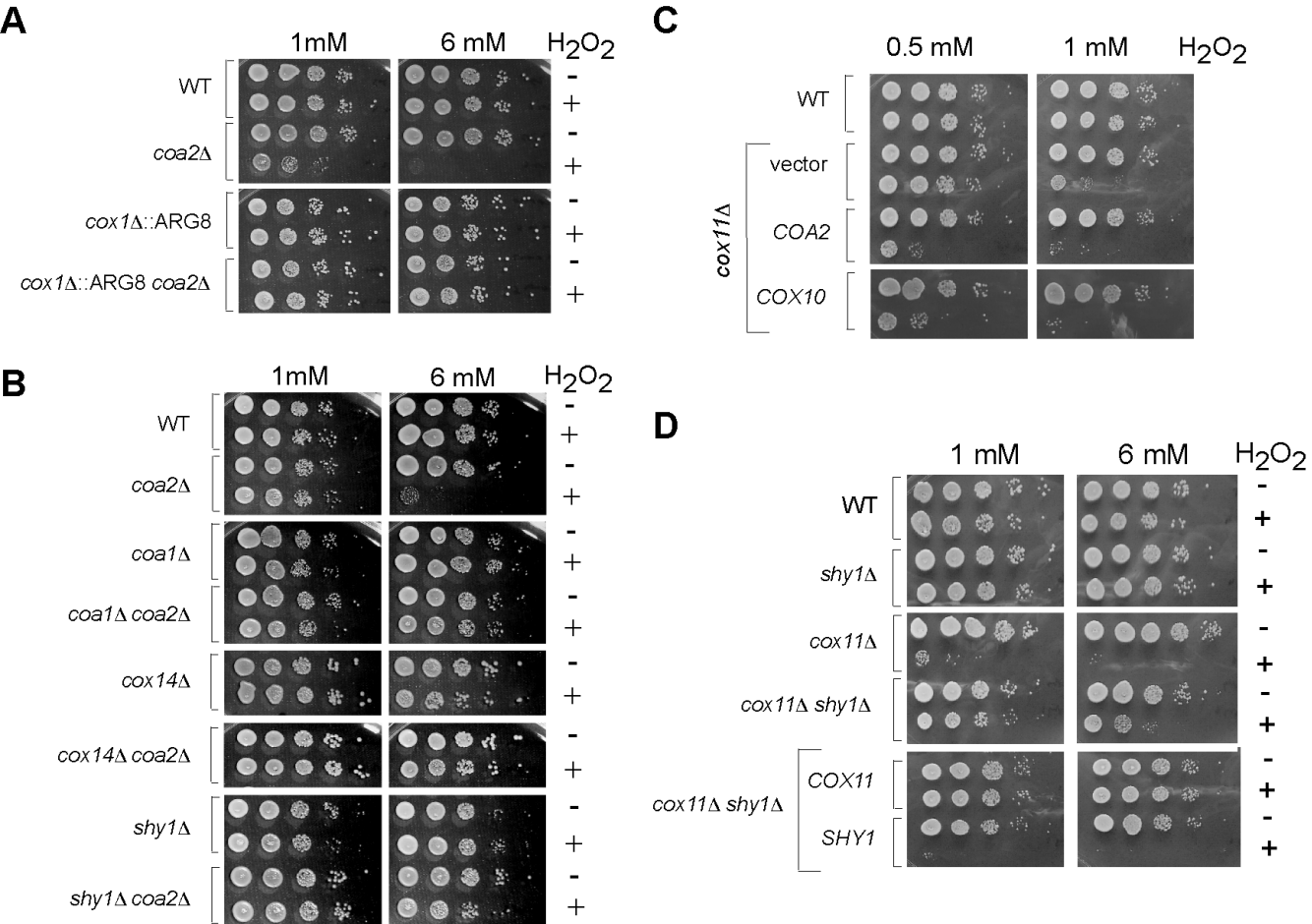


Figure 8

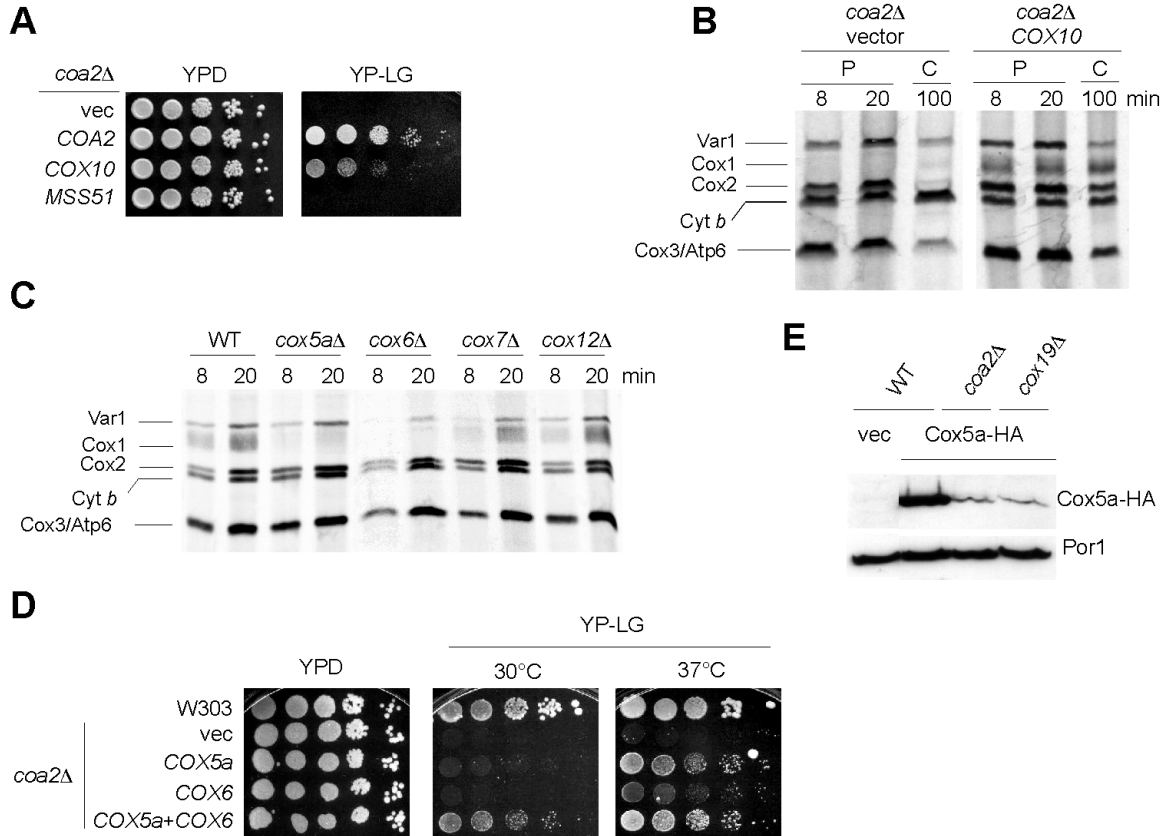


Figure 9

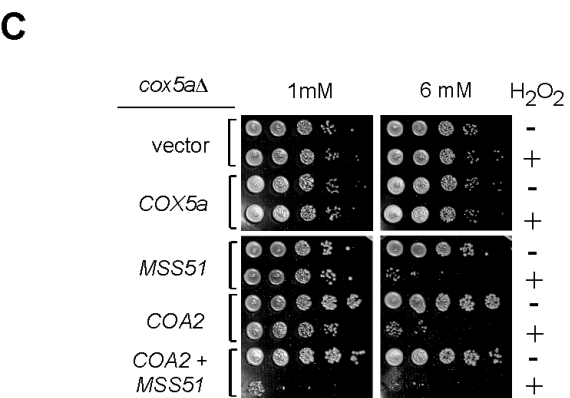
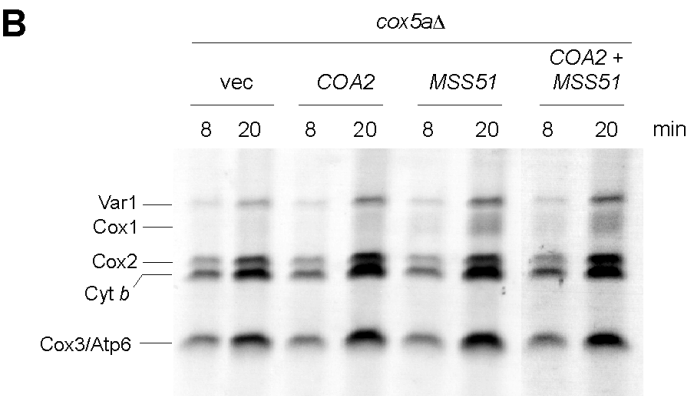
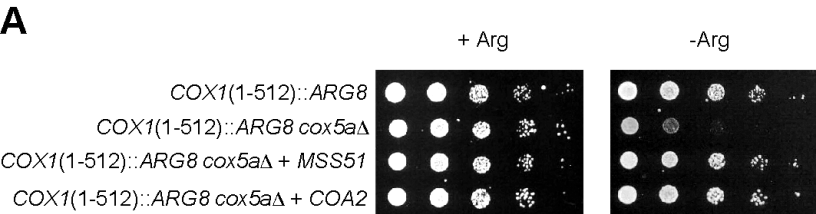


Figure 10