COA4 IS REQUIRED FOR COPPER DELIVERY TO CYTOCHROME *C* OXIDASE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Coa4 is Required for Copper Delivery to Cytochrome *c* Oxidase

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Copper is required for the assembly and activity of cytochrome *c* oxidase (CcO), the terminal enzyme of the mitochondrial respiratory chain. As a multi-subunit metalloprotein, CcO biogenesis involves many assembly factors necessary for the proper association of protein subunits and insertion of cofactors. Although the identity of these assembly factors has long been known, their precise roles in CcO biogenesis have remained unknown. Here we investigated the role of Coa4, an evolutionarily conserved CcO assembly factor with unknown function. We found that *coa4Δ* yeast cells have a respiratory growth phenotype correlated with impaired CcO assembly and activity, as well as reduced mitochondrial copper levels, all of which can be alleviated by exogenous copper supplementation. These results suggest that disrupted copper transport to CcO is the biochemical basis of the respiratory deficiency of *coa4Δ* cells. Copper delivery to CcO occurs in a bucket brigade fashion via copper delivery pathway proteins. To place Coa4 in the copper delivery pathway, we performed a candidate-based suppression screen with 15 genes implicated in CcO assembly. Overexpression of *COX11*, a metallochaperone involved in copper delivery to the CcO subunit Cox1, rescued the respiratory growth of *coa4Δ* cells, suggesting that Coa4 acts upstream of Cox11. The sub-mitochondrial localization and

sequence analyses of Coa4 suggested a redox role in the copper delivery process. In support of this idea, we found that glutathione supplementation and hypoxia treatment each rescued the respiratory growth of *coa4Δ* cells. Taken together, these results uncover Coa4 as a novel member of the copper delivery pathway to the Cox1 subunit of CcO.

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NOMENCLATURE

COA4	Cytochrome Oxidase Assembly factor 4
MRC	Mitochondrial respiratory chain
ATP	Adenosine triphosphate
CcO	Cytochrome *c* oxidase
IMS	Intermembrane space
ROS	Reactive oxygen species
GSH	Glutathione
DTT	Dithiothreitol
WT	Wild type
bp	Base pairs
CHCH	Coiled coil-helix-coiled coil-helix
MISS	Mitochondrial intermembrane space sorting signal
ITS	Intermembrane space-targeting signal

CHAPTER I

INTRODUCTION

The mitochondrial respiratory chain (MRC) is responsible for generating the majority of energy in the cell. The MRC consists of four enzyme complexes in the inner membrane of the mitochondria that sequentially transport electrons derived from the food we eat to oxygen from the air we breathe. Electron transport via the MRC is coupled to proton translocation across the inner mitochondrial membrane, which generates an electrochemical potential gradient that drives ATP synthesis. Cytochrome *c* oxidase (CcO), or Complex IV, is the terminal enzyme complex of the MRC and the main site of cellular respiration. CcO is a multimeric complex containing 14 protein subunits, two of which contain copper ions that function as catalytic cofactors to transfer electrons from cytochrome *c* to O_2, reducing it to H_2O (Timon-Gomez et al., 2018; Tsukihara et al., 1995). Copper is not only essential for CcO activity but is also required for its assembly; thus, impaired delivery of copper to CcO results in deficient respiration, which causes fatal infantile diseases in humans (Calvo et al., 2012; Pecina et al., 2004).

Copper delivery and insertion to CcO require many proteins that function in a bucket-brigade fashion (Timon-Gomez et al., 2018). These proteins are localized to the mitochondrial intermembrane space (IMS). In this process, Cu(I) from the mitochondrial matrix copper pool is transported to the IMS, where it is delivered to Cox1 and Cox2, the two copper-coordinated subunits of CcO (Cobine et al., 2004). The process of delivering Cu(I) to Cox1 and Cox2 is mediated by a dedicated set of proteins that function as metallochaperones and thiol-disulfide oxidoreductases (Soma et al., 2019). It has been shown that Cox17, a metallochaperone, transfers

copper to downstream metallochaperones, Cox11 and Sco1, which facilitate its insertion into Cox1 and Cox2, respectively (Horng et al., 2004; Leary et al., 2004).

While the biochemical function of metallochaperones Cox11, Cox17, and Sco1 have been well documented (Hiser et al., 2000; Horng et al., 2004; Morgada et al., 2015), the functions of many mitochondrial IMS proteins involved in CcO biogenesis remain unresolved (Longen et al., 2009). Some of these proteins may also be involved in copper delivery to CcO. Recently, our lab has elucidated the role of one such protein, Coa6, in mitochondrial copper delivery to CcO (Ghosh et al., 2014). Coa6 was shown to physically and genetically interact with Sco1 and Sco2 in copper delivery to Cox2 (Ghosh et al., 2016). Specifically, Coa6 facilitates copper delivery to the Cu_A site in Cox2 by reducing disulfides in Sco1 and Cox2, allowing these proteins to coordinate copper (Soma et al., 2019).

To build on this discovery, we focused on finding other members of the mitochondrial copper delivery pathway by identifying proteins that shared key features with Coa6, such as a respiratory growth deficiency, requirement for CcO assembly, localization to the IMS, and the presence of a characteristic cysteine-rich motif. One protein that possessed all four of these characteristics was Coa4, an evolutionarily conserved IMS protein known to be required for CcO assembly and activity but with no previously established link to copper delivery (Bestwick et al., 2010; Bode et al., 2013; Longen et al., 2009).

Coa4 was first characterized in 2009 as a newly identified member of the twin Cx_9C family of small mitochondrial IMS proteins (Longen et al., 2009). The cysteine-rich motif of these proteins is required for their localization to the IMS and stabilizes a helix-turn-helix structure by forming a pair of parallel disulfide bonds (Longen et al., 2009). Many of these are known to play a role in CcO biogenesis and/or activity, and a subset of them—including Cox17,

Cox19, Cox23, and Cmc1—have been linked to copper delivery to CcO (Longen et al., 2009). Yeast cells lacking Coa4 have been shown to have a respiratory growth deficiency associated with a lower oxygen consumption rate, diminished CcO activity, decreased levels of CcO subunits, and reduced formation of CcO-containing MRC supercomplexes (Bestwick et al., 2010; Bode et al., 2013; Longen et al., 2009), implying that Coa4 is required for CcO biogenesis and function. In addition, *coa4Δ* yeast cells exhibit elevated production of H_2O_2, and their respiratory growth defect can be rescued by treatment with the reducing agents glutathione (GSH), dithiothreitol (DTT), and ascorbic acid, suggesting that the respiratory growth deficiency of *coa4Δ* cells may be related to production of reactive oxygen species (ROS) by cytotoxic CcO assembly intermediates (Bode et al., 2013). However, the molecular function of Coa4 remains elusive.

In this study, we investigate the role of Coa4 in CcO biogenesis. Based on its localization to the IMS and twin Cx_9C motif, we hypothesized that Coa4 is required for copper delivery to CcO. We performed a variety of microbiological, genetic, and biochemical assays to test this hypothesis and found that copper supplementation can rescue the respiratory defects of *coa4Δ* cells. A targeted genetic suppression screen placed Coa4 upstream of Cox11 in the copper delivery pathway to Cox1. Additionally, we found that hypoxia treatment rescues the respiratory growth of *coa4Δ* cells. Based on these findings, we propose a model in which Coa4 plays a redox role in facilitating copper transfer from Cox17 to Cox11, allowing its proper insertion to the Cox1 subunit of CcO. Gaining insights into the mitochondrial copper delivery pathway to CcO will elucidate the basic cell biology of intracellular copper transport and advance our understanding of the mechanisms of diseases of copper metabolism, which may pave the way for potential treatments in the future.

CHAPTER II

MATERIALS AND METHODS

Yeast strains and culture conditions

Saccharomyces cerevisiae strains used in this study are listed in Table 1. Strains were cultured in YPD (1% yeast extract, 2% peptone, 2% glucose), YPGE (1% yeast extract, 2% peptone, 3% glycerol, 1% ethanol), or YPGal (1% yeast extract, 2% peptone, 2% galactose). After transformation, strains carrying plasmids were maintained on synthetic media lacking uracil (SC glucose –ura).

Table 1. Yeast strains used in this study.

Strain	**Genotype**	**Source**
BY4742 WT	MATα, *his 3Δ1*, *leu 2Δ0*, *lys2Δ0*, *ura3Δ0*	Greenberg lab
BY4742 *coa4Δ*	MATα, *his 3Δ1*, *leu 2Δ0*, *lys2Δ0*, *ura3Δ0*, *coa4Δ::hphNT1*	Gohil lab
BY4742 *coa4(T)*	MATα, *his 3Δ1*, *leu 2Δ0*, *lys2Δ0*, *ura3Δ0, coa4(T):: hphNT1*	This study*
BY4741 WT	MATa, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Greenberg lab
BY4741 *cox11Δ*	MATa, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *cox11Δ::KanMX4*	Open Biosystems
BY4741 *cox19Δ*	MATa, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *cox19Δ::KanMX4*	Open Biosystems

*Note: See "Construction of *COA4* truncation strain."

Yeast growth assays

For solid media spotting assays, yeast strains were cultured overnight at 30°C in 2 mL YPD. After measuring optical density at 600 nm, serial dilutions of approximately 10^4, 10^3, 10^2, and 10^1 cells per 3 µL were prepared in a 96-well plate. 3 µL of each dilution was spotted on the indicated plates. For the stamping assay, the overnight pre-culture was grown in SC glucose –ura medium. Serial dilutions of approximately 10^4, 10^3, 10^2, and 10^1 cells per 1.5 µL were prepared in a 96-well deep well plate and stamped onto solid media in rectangular plates using a 96 pin deep-well replicator. Plates were incubated for up to 7 days at 30 or 37°C, with images taken daily.

Liquid growth assays were performed by culturing a single colony of each yeast strain in 2 mL YPD at 30°C overnight. Pre-cultures were diluted to an OD_{600nm} of 0.1 in 7 mL YPGE and incubated at 30 or 37°C. Growth was quantified by measuring OD_{600nm} at the indicated time points.

Harvesting cells and isolating mitochondria

Yeast strains were cultured at 30°C for 12 hours in 2 mL YPD and then diluted to an OD_{600nm} of 0.1 in 30 mL YPD. These cultures were incubated for 10 hours at 30°C, diluted to an OD of 0.1 in 250 mL YPGal, and incubated at 30°C until reaching early stationary phase (approximately OD_{600nm} of 7). Cells were harvested by centrifuging for 5 minutes at 3000g, washed with water, and stored at -80°C until use. Mitochondria were isolated as described previously (Meisinger et al., 2006), and protein concentration was measured with the BCA assay (Thermo Fisher Scientific). Isolated mitochondria were stored at -80°C before performing any biochemical analysis.

Cytochrome *c* oxidase activity assay

CcO activity was quantified by measuring the rate of decrease in absorbance at 550 nm caused by the oxidation of reduced cytochrome *c* (Barrientos et al., 2009). Isolated mitochondria were suspended in "COX" buffer containing 10 mM KH_2PO_4, 0.25 M sucrose, and 1 mg/ml BSA, adjusted to a pH of 6.5. Mitochondria were added to wells in a 96-well plate with 15 μg mitochondrial protein per well. 200 μM cytochrome *c* was prepared in COX buffer and was fully reduced by adding 2 mM sodium dithionite. 60 μL reduced cytochrome *c* solution was added to each well, followed by COX buffer to a total volume of 174.5 μL. After adding 25.5 μL 1% n-dodecyl-β-D-maltoside (DDM) to permeabilize the outer membrane of the mitochondria, absorbance at 550 nm was immediately measured by a plate reader for 5 minutes. 240 μM KCN was added and absorbance was measured for 5 more minutes. CcO activity was the slope of the linear portion of the line representing absorbance over time for the first five minutes, relative to WT. Data are representative of 3 biological replicates.

SDS-PAGE, BN-PAGE, and immunoblotting

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) based separation of MRC subunits, isolated mitochondria were lysed by heating at 70°C for 10 minutes in NuPAGE LDS sample buffer with NuPAGE reducing agent (Thermo Fisher Scientific). 20 μg mitochondrial protein per sample was separated on a 12% NuPAGE gel. For blue native polyacrylamide gel electrophoresis (BN-PAGE), mitochondrial samples were solubilized by incubating on ice for 15 minutes in buffer with 1% digitonin. Native protein complexes were separated on a 3-12% Native-PAGE gel (Thermo Fisher Scientific).

Following gel electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using semi-dry transfer system (Bio-Rad). Membranes were incubated at

room temperature in blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% milk) for 1 hour. Primary antibodies were added and membranes were incubated overnight at 4°C. Primary antibodies were diluted as follows: COX1, 1:5,000 (Abcam 110270); COX2, 1:100,000 (Abcam 110271); RIP1, 1:100,000 (from Dr. Vincenzo Zara); SDH2, 1:5,000 (from Dr. Dennis Winge); ATP5, 1:40,000 (Abcam 14748); VDAC1, 1:100,000 (Abcam 110326). After five washes for five minutes each in Tris-buffered saline + 0.1% Tween, membranes were incubated in blocking buffer with the appropriate secondary antibody (rabbit or mouse) at a 1:5,000 dilution for 1 hour at room temperature. Membranes were developed using a BioRad ChemiDoc imaging system.

Measurement of cellular and mitochondrial copper levels

Yeast cells and isolated mitochondria were washed with 100 μM EDTA-containing water and digested for 18 hours with 40% nitric acid (TraceSELECT; Sigma) at 90°C. Samples were diluted in ultrapure metal-free water (TraceSELECT; Sigma), and copper levels were measured with inductively coupled plasma mass spectrometry (ICP-MS) using a PerkinElmer DRC II instrument. Copper levels were quantified by comparing with prepared standards.

Genetic suppression screen

Plasmid constructs with genes implicated in CcO biogenesis (*COX11*, *COX12*, *COX17*, *COX19*, *COX23*, *CMC1*, *CMC2*, *COA4*, *CMC4*, *COA5*, *COA6 MIX14*, *MIX17*, *MIX23*, *SCO1*, and *SCO2*) inserted into the pRS416 plasmid were transformed into *coa4Δ* cells. To perform the transformations, yeast strains were cultured in 5 mL YPD at 30°C overnight. 1 mL pre-culture was used for each transformation. Cells were pelleted at 3000g for 6 minutes and resuspended in 100 μL buffer (2N lithium acetate, 60% polyethylene glycol (PEG) 3350, 1M DTT) per transformation. Approximately 600 ng DNA and 50 μg salmon sperm DNA were added for each

transformation. Cells were incubated at room temperature for 30 minutes, in a 42°C water bath for 20 minutes, and on ice for 5 minutes before spreading on SC glu –ura plates.

Transformed strains were grown on YPGE solid media using the stamping assay described above. Growth of the most concentrated spot of each strain relative to that of the WT + EV strain was quantified using ImageJ software.

Construction of *COA4* truncation strain

The *COA4* gene was disrupted in the WT BY4742 background by replacing the codon for Cys92 (5'-TGC-3') with a stop codon (5'-TGA-3') and replacing *COA4* amino acids 93-127 with the hygromycin (hygro) cassette. The hygro cassette was copied from the pFA6-hphNT1 plasmid by using the following PCR primers: 1) 5'-GACCCGGATGTGTGGGACACGAGAAT ATCCAAGACCGGATGACGTACGCTGCAGGTCGAC-3' and 2) 5'-GTCCCATCCACGTCC ACTGTGCTTACGCGCTCTCTATTACCATCGATGAATTCGAGCTCG-3'. The linear cassette was then transformed into WT BY4742 cells with a high efficiency transformation so that the yeast cells could incorporate it at the *COA4* locus via homologous recombination. To prepare competent cells, a single colony was incubated in 3 mL YPD for 8 hours, diluted 10,000x in 50 mL YPD, and grown to an OD_{600nm} of 0.15. Cells were centrifuged at 700g for 5 minutes and resuspended in 100 mL YPD before growing to an OD_{600nm} of 0.5. The cells were harvested by centrifuging at 700g for 5 minutes, washed with sterile water, and washed and resuspended in 1.2 mL 1.1xTE/LiAc solution (11 mM Tris-HCl, pH ~8.0, 1.1 mM EDTA, 110 mM lithium acetate). 50 µL competent cells were transformed using approximately 1 µg hygro cassette DNA and 50 µg salmon sperm DNA in 500 µL PEG/LiAc (40% PEG 3350, 10 mM Tris-HCl, pH ~8.0, 1 mM EDTA, 100 mM lithium acetate) and 20 µL dimethyl sulfoxide (DMSO). Cells were incubated in a 42°C water bath for 15 minutes, resupsended in 1mL YPD,

grown at 30°C for 90 minutes, resuspended in 1 mL 0.9% (w/v) NaCl, and spread on YPD plates containing hygromycin.

Strain confirmation was performed using the following PCR primers: A) 5'-CCAAATAC CATTTTCAACTTTCTTG-3', B) 5'-GGCGACCTCGTATTGGGAAT-3', C) 5'- ACCGCAAG GAATCGGTCAAT-3', and D) 5'-CTAGTCATTTTTACTCCTTGATCGC-3'. The truncation strain, labeled BY4742 *coa4(T)*, was confirmed (Table 2).

Table 2. Strain confirmation for *COA4* truncation.

Strain	**Reaction Primers**	**Expected (bp)**	**Actual (bp)**
BY4742 WT	A-D	958	~950
	A-B	-	-
	C-D	-	-
BY4742 *coa4(T)*	A-D	2588	~2600
	A-B	1649	-
	C-D	1219	~1300

Site directed mutagenesis

Cysteine to Alanine point mutations in *COA4-V5* were made by performing site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit from Agilent Technologies.

CHAPTER III

RESULTS

Copper supplementation rescues the respiratory deficiency of *coa4Δ* cells by restoring CcO assembly and activity

Because of the requirement of Coa4 for CcO assembly, its localization to the IMS, and the presence of a twin Cx_9C motif, we hypothesized that it is involved in copper delivery to CcO. To test this hypothesis, we performed a growth assay to determine if copper supplementation could rescue respiratory growth of *coa4Δ* cells. As expected for mutants with impaired CcO biogenesis, growth of *coa4Δ* cells was comparable to that of wild type (WT) cells in glucose-containing fermentable media; however, on glycerol-ethanol-containing non-fermentable "respiratory" media, *coa4Δ* cells displayed a growth defect, which was most pronounced at 37°C (Figure 1A). Copper supplementation rescued the respiratory growth of the *coa4Δ* cells (Figure 1A). This data suggests that the respiratory growth defect of *coa4Δ* cells is due to a disruption in mitochondrial copper homeostasis.

To determine the biochemical basis of the observed copper rescue of respiratory growth, we measured the effect of copper supplementation on CcO activity. CcO activity is diminished in *coa4Δ* cells, and we found that copper supplementation was able to restore CcO activity to wild type levels (Figure 1B). In the absence of Coa4, steady-state levels of the copper-containing CcO subunits (Cox1 and Cox2) were decreased, while subunits of MRC complexes III (Rip1), II (Sdh2), and V (Atp5) were present in levels comparable to WT (Figure 1C), suggesting that the respiratory defect in *coa4Δ* cells is specific to CcO deficiency. The addition of copper increased Cox1 and Cox2 levels (Figure 1C). Copper supplementation also led to an increase in the

abundance of CcO-containing MRC supercomplexes in *coa4Δ* cells (Figure 1D), which were markedly diminished in *coa4Δ* cells grown without exogenously added copper. These observations indicate that copper supplementation rescues the respiratory growth of *coa4Δ* cells by bypassing the requirement of Coa4 for CcO activity and assembly.

In support of the role of Coa4 in copper homeostasis, we found reduced levels of cellular and mitochondrial copper in *coa4Δ* cells, and these copper levels were increased with copper supplementation (Figure 1E-F). Taken together, these results suggest that Coa4 plays a role in mitochondrial copper delivery to CcO.

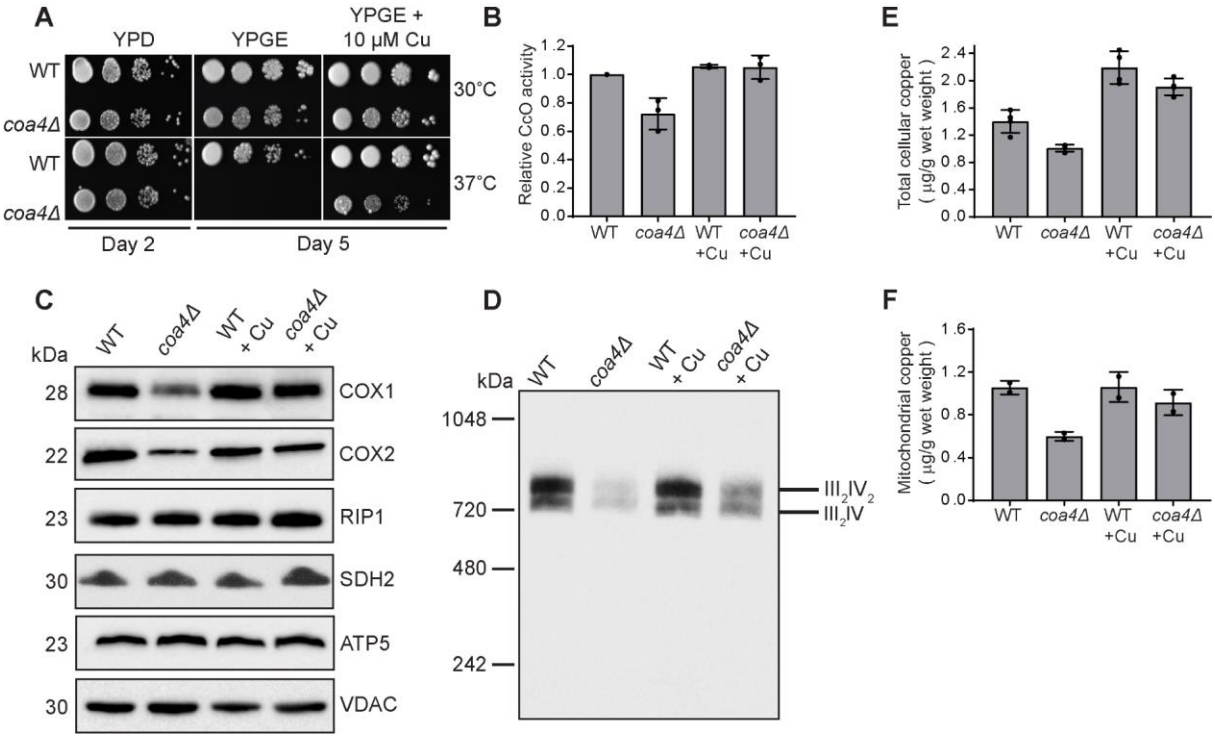

Figure 1. Copper supplementation bypasses the requirement of Coa4 for CcO activity and assembly. (A) Ten-fold serial dilutions of WT and *coa4Δ* cells were spotted on the indicated plates, and images were captured on the indicated day. (B) CcO activity, (C) steady state levels of indicated MRC subunits, (D) abundance of CcO-containing MRC supercomplexes, (E)

cellular copper levels, and (F) mitochondrial copper levels were measured in WT and *coa4Δ* cells as described in the methods section.

Overexpression of *COX11* suppresses the respiratory growth defect of *coa4Δ* cells

In order to place Coa4 in the copper delivery pathway to CcO, we performed a targeted genetic suppression screen. This screen involved overexpression of 15 different IMS proteins, including conserved twin Cx_9C proteins and all other IMS proteins previously linked to copper delivery to CcO, in *coa4Δ* cells to test for their ability to rescue growth on non-fermentable media (Figure 2A). As expected, re-introduction of the *COA4* gene restored growth of the *coa4Δ* knockout strain. Interestingly, overexpression of *COX11* also restored respiratory growth of *coa4Δ* cells. Cox11 is a copper metallochaperone that transfers copper to the Cu_B site on the Cox1 subunit of CcO (Hiser et al., 2000), so this epistatic interaction suggests that Coa4 plays a role in copper delivery to Cox1. To confirm this finding, transformation of *coa4Δ* cells with *COX11* was repeated. Consistent with our previous observation, overexpression of *COX11* suppressed the respiratory growth defect of *coa4Δ* cells (Figure 2B).

This result suggests one of two possibilities: either Coa4 and Cox11 have overlapping functions, or Cox11 acts downstream of Coa4 in the copper delivery pathway to CcO. To distinguish between these two possibilities, we then tested for the reciprocal genetic interaction and found that overexpression of *COA4* cells did not rescue the respiratory growth of *cox11Δ* cells (Figure 2C), indicating that Coa4 and Cox11 have non-redundant roles. Additionally, we found that *COX11* overexpression increased steady state levels of Cox1 and Cox2 in *coa4Δ* cells (Figure 2D), suggesting that overexpression of *COX11* alleviates the respiratory growth defect of

coa4Δ cells by promoting CcO biogenesis. Taken together, these results support the placement of Coa4 upstream of Cox11 in the copper delivery pathway to the CcO Cu_B site.

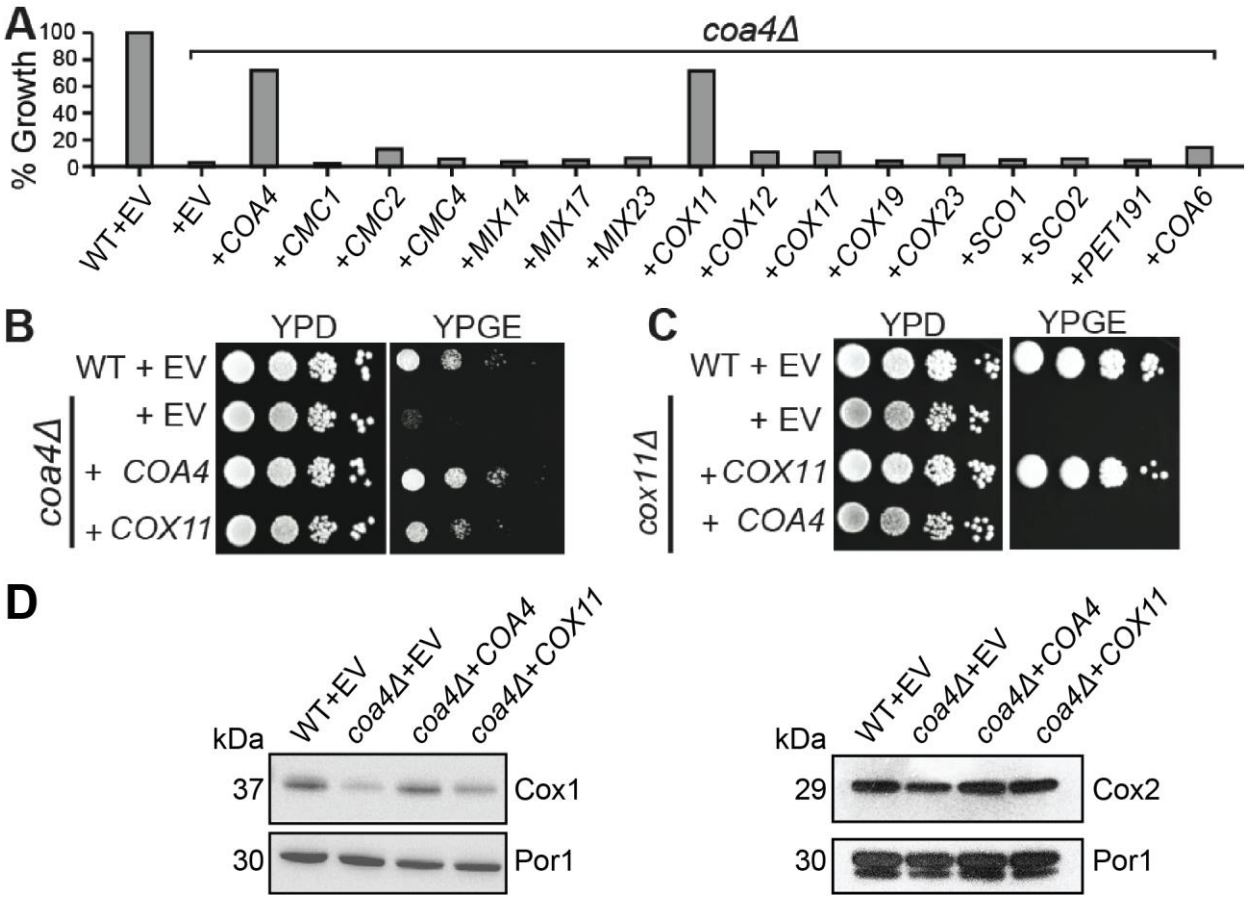

Figure 2. A targeted genetic suppression screen identifies epistatic interaction between *COX11* and *COA4*. (A-C) Ten-fold serial dilutions of the indicated strains were (A) stamped or (B-C) spotted on YPD or YPGE plates and incubated at 30°C. (A) Growth was quantified relative to WT after 3 days using ImageJ software. (B) Images were captured after 3 days. (C) Images were captured after 2 days (YPD) or 6 days (YPGE). (D) Steady state levels of the copper-containing subunits of CcO were measured as described in the methods section.

Reducing conditions rescue the respiratory growth of *coa4Δ* cells

Two disulfide bonds between pairs of cysteines on opposing helices stabilize the coiled coil-helix-coiled coil-helix (CHCH) fold of twin Cx_3C and twin Cx_9C proteins in the mitochondrial IMS. Several of these CHCH proteins take part in redox processes in the IMS involving disulfide exchange (Banci et al., 2009). To test whether Coa4 plays a redox role in copper delivery, we observed the respiratory growth of *coa4Δ* cells when treated with glutathione (GSH) and when grown in hypoxic conditions, as both of these treatments alter the redox milieu of the cell. Both hypoxia and GSH treatment were able to alleviate the respiratory growth defect of *coa4Δ* cells but did not rescue respiratory growth of cells lacking Cox11, a metallochaperone with no known redox role, or cells lacking Cox19, a twin Cx_9C protein that interacts with Cox11 in the CcO copper delivery pathway (Bode et al., 2015) (Figure 3). This suggests hypoxia and GSH treatment rescue the respiratory growth of *coa4Δ* cells by restoring redox processes perturbed in the absence of Coa4.

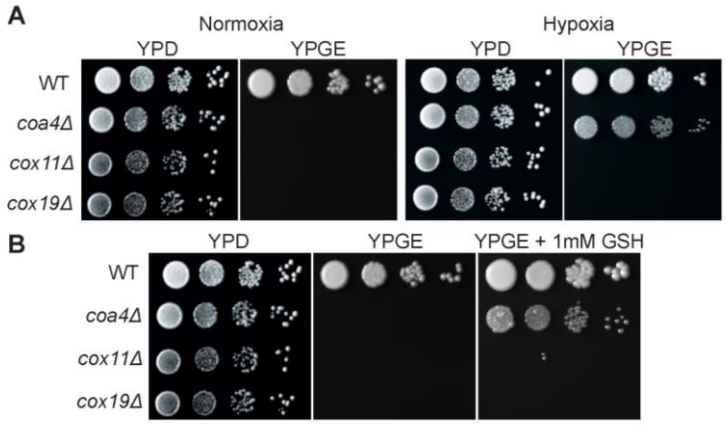

Figure 3. Altering the intracellular redox environment by growth in hypoxia or with exogenous glutathione supplementation specifically improves the respiratory growth of *coa4Δ* cells. (A-B) Ten-fold serial dilutions of the indicated strains were spotted on YPD or YPGE plates and

incubated at 37°C in (A) normoxia or hypoxia, and (B) with or without 1 mM glutathione. Plates were imaged after 2 days (YPD) or 7 days (YPGE).

Expression of *COA4* rescues the respiratory growth deficiency of *coa4(T)* cells

As expected, transforming the *COA4* gene on the pRS416 plasmid into *coa4Δ* cells rescued respiratory growth at 30°C (Figure 2A-B, Figure 4A). However, pRS416-*COA4* did not restore respiratory growth of *coa4Δ* cells at 37°C (Figure 4A). This lack of complementation suggests that the respiratory growth defect at 37°C is caused by an additional mutation in *coa4Δ* cells.

Taking a closer look at the *COA4* gene locus on chromosome XII, we found that the start codon of *COA4* is located 233 bp upstream of the start codon for *MSC3*, which is encoded on the opposite strand, while the stop codon of *COA4* is located 255 bp upstream of *CPR6* (www.yeastgenome.org/locus/S000004208). Because of the small distance between *COA4* and its neighboring genes, we hypothesized that deletion of *COA4* disrupted parts of regulatory elements for these genes. Notably, the null mutant of *CPR6*, which encodes a cyclophilin (Duina et al., 1996), was shown to have increased heat sensitivity in a large-scale screen (Sinha et al., 2008); thus, a perturbation in *CPR6* expression levels could be the basis of the more severe growth phenotype at 37°C which cannot be rescued by *COA4* expression alone. *MSC3* encodes a protein of unknown function (Thompson and Stahl, 1999), and a possible disruption of the *MSC3* promoter in the deletion of *COA4* could have also contributed to the growth phenotype at 37°C.

To minimize potential effects on the expression of neighboring genes, a new *COA4* mutant strain was constructed in which the *COA4* gene was disrupted, rather than completely deleted. The coding sequence for amino acids 93-127, a region of the 150 amino acid protein that

includes the twin Cx_9C motif, was replaced with the hygromycin gene. The resulting strain, labeled *coa4(T)*, encodes a truncated form of Coa4, which is predicted to be non-functional. Indeed, we found that *coa4(T)* cells exhibit a respiratory growth defect, albeit slightly less severe than that of *coa4Δ* cells at 37°C (Figure 4B-C). Transforming *coa4(T)* cells with pRS416-*COA4* restored respiratory growth to WT levels (Figure 4B). Interestingly, in liquid media, *coa4(T)* cells had a less severe respiratory growth phenotype than *coa4Δ* cells even at 30°C, in the absence of temperature stress (Figure 4C).

These results suggest that the previously observed respiratory growth phenotype of *coa4Δ* cells may have been exaggerated by perturbations in expression of neighboring genes. Thus, the *coa4(T)* strain provides a more accurate model to specifically study *COA4* and is used in place of the *coa4Δ* strain in the rest of this study.

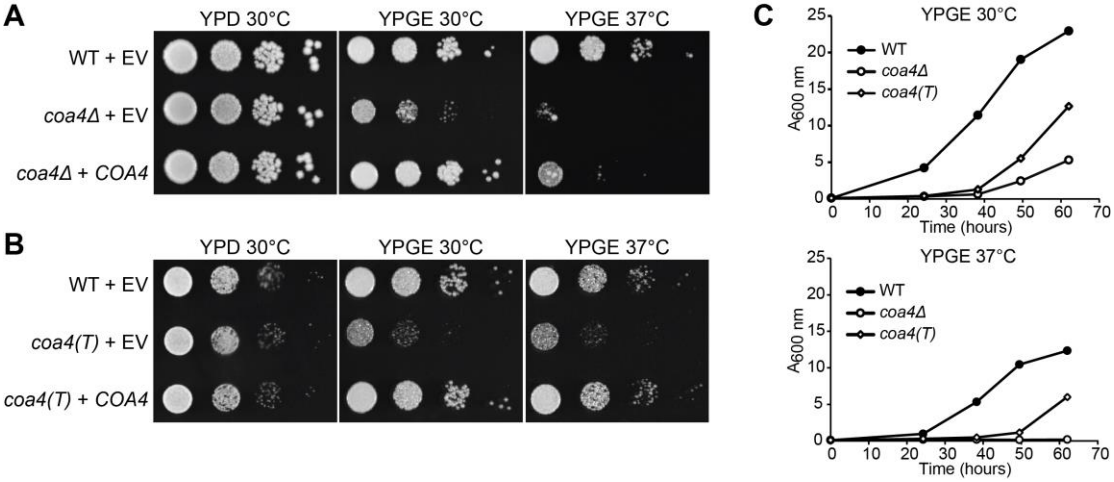

Figure 4. *coa4(T)* cells have a respiratory growth phenotype that is rescued by transformation with pRS416-*COA4*. (A) Ten-fold serial dilutions of WT and *coa4Δ* cells transformed with either the pRS416 empty vector or pRS416-*COA4* were spotted on YPD and YPGE plates and imaged after incubating for 3 days (YPD) or 5 days (YPGE). (B) Ten-fold serial dilutions of WT and

coa4(T) cells transformed with either the pRS416 empty vector or pRS416-*COA4* were spotted on YPD and YPGE plates and imaged after incubating for 1 day (YPD) or 4 days (YPGE). (C) The indicated strains were cultured in YPGE at a starting OD_{600nm} of 0.1 after overnight pre-culture in YPD. Absorbance at 600 nm was measured at the indicated times.

Mutation of any single cysteine in the twin Cx_9C motif does not abrogate the respiratory function of Coa4

Coa4 is evolutionarily conserved across eukaryotes from yeast to humans, with homologues in the rat, fruit fly, and zebrafish, among other organisms (Figure 5A). To test the importance of the conserved cysteines in the characteristic twin Cx_9C motif, we mutated each cysteine to alanine to construct four *COA4* single mutants with the V5 tag and tested the ability of each mutant to rescue respiratory growth of *coa4(T)* cells. Wild type *COA4-V5* and all of the *COA4-V5* mutants were able to complement *coa4Δ* cells, although the C93A mutation led to a slight decrease in respiratory growth at 37°C (Figure 5B).

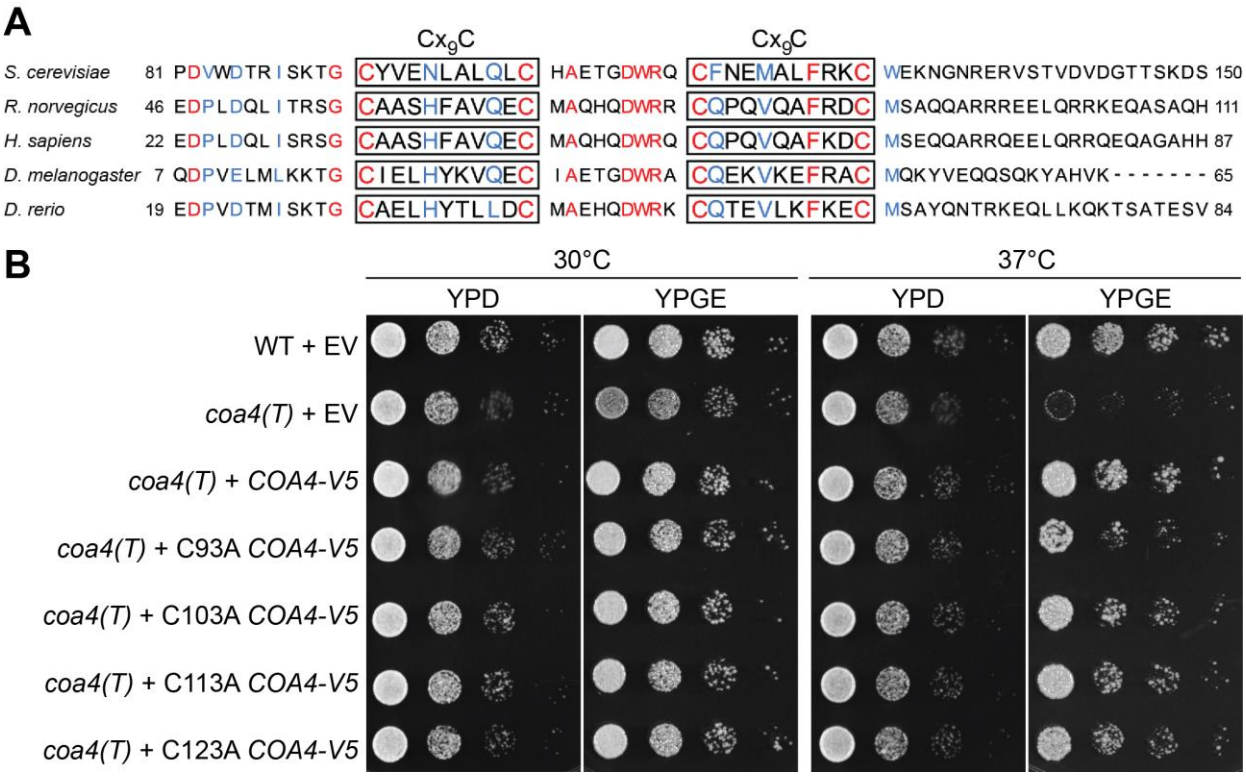

Figure 5. Exploring the role of conserved cysteines of the twin Cx_9C motif in Coa4 function. (A) Sequence alignment of the conserved region of Coa4. The twin Cx_9C motif is shown in rectangular boxes. Red residues are conserved in all five species, and blue residues are conserved in four of these five species. (B) Ten-fold serial dilutions of WT and *coa4(T)* cells transformed with either the pRS416 empty vector or pRS416-*COA4-V5* were spotted on YPD and YPGE plates and imaged after incubating for 1 day (YPD) or 4 days (YPGE).

CHAPTER IV

CONCLUSION

CcO biogenesis is a complex process involving many assembly factors that are required to facilitate proper assembly of its protein subunits and insertion of cofactors, including the two copper ions and two hemes in its catalytic sites. There are several proteins that have been implicated in CcO assembly and activity whose biochemical functions remain unknown (Timon-Gomez et al., 2018). Many of these assembly factors are twin Cx_9C proteins localized to the mitochondrial IMS (Timon-Gomez et al., 2018). The twin Cx_9C family of small mitochondrial proteins is found in nearly all eukaryotes, and their evolutionary conservation points to their critical roles in mitochondrial function (Cavallaro, 2010; Longen et al., 2009). Most of these proteins are required for mitochondrial respiration, and deletion of many of them is associated with decreased abundance and activity of CcO (Longen et al., 2009). However, aside from the well-characterized copper chaperone Cox17 (Horng et al., 2004), the specific biochemical roles of most of these proteins in CcO assembly have remained unknown.

In this study, we focused on characterizing the function of one such twin Cx_9C protein, Coa4, and placed it in the copper delivery pathway to the Cu_B site of CcO. We found that exogenous copper supplementation can rescue the respiratory growth defect of *coa4Δ* cells, and this growth rescue is correlated with an increase in CcO activity, steady-state levels of CcO subunits, and abundance of CcO-containing MRC supercomplexes (Figure 1A-D). *coa4Δ* cells have diminished intracellular and mitochondrial copper levels, both of which are restored by copper supplementation (Figure 1E-F). Furthermore, genetic epistasis analysis revealed that overexpression of *COX11*, a well-characterized copper metallochaperone, rescues the respiratory

growth of *coa4Δ* cells (Figure 2). Taken together, our results suggest that Coa4 promotes CcO assembly by facilitating copper delivery to its core-catalytic subunit Cox1.

Based on our findings we propose a model placing Coa4 upstream of Cox11 in the copper delivery pathway to Cox1 (Figure 6A). Our model is consistent with previous findings suggesting a link between Coa4 and Cox1 assembly. For example, in a pulse-chase experiment, it was shown that *coa4Δ* cells have decreased synthesis of Cox1, while Cox2 and Cox3—the other CcO subunits encoded by mitochondrial DNA—are synthesized at normal levels but are then degraded rapidly (Bode et al., 2013). Another study identified Coa4 as an allele-specific suppressor of a Shy1 mutant (Bestwick et al., 2010). Shy1 and its human homolog SURF1 are CcO assembly factors required for the formation of the heme a_3:Cu_B bimetallic center of Cox1 (Khalimonchuk et al., 2010). Our findings add to these results by specifically placing Coa4 upstream of Cox11 in the copper delivery pathway to the Cu_B site on the Cox1 subunit.

Our study demonstrates how targeted epistasis studies can be used to link uncharacterized CcO assembly factors to specific pathways. Focused candidate-based screens like the one used here allow a systematic search for genetic interactions with proteins in the pathways of interest, while minimizing the false positive and false negatives often seen in high-throughput approaches. High-throughput genome-wide screens are important in gene discovery, and Coa4 was originally identified as a CcO assembly factor in a genome-wide screen designed to identify Shy1 allele-specific suppressors (Bestwick et al., 2010). The success of using a targeted suppression screen to place Coa4 in the copper delivery pathway to Cox1 demonstrates that our low-throughput approach can complement traditional genome-wide approaches to place genes in cellular pathways.

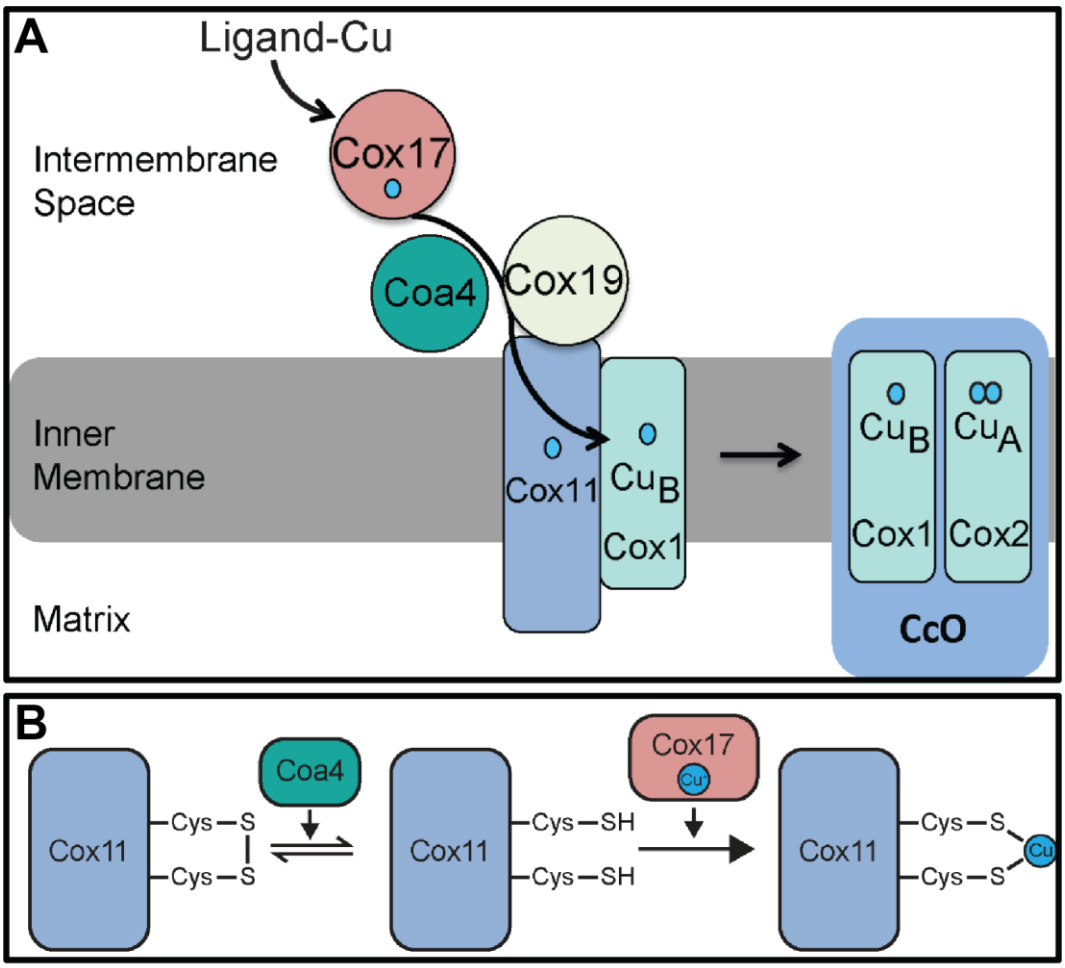

Figure 6. Proposed model for the function of Coa4 in CcO assembly. (A) Coa4 acts upstream of Cox11 in the copper delivery pathway to Cox1. (B) Coa4 may reduce the disulfide bond in the copper-binding site of Cox11, allowing Cox11 to receive copper from Cox17.

Having placed Coa4 in the copper delivery pathway to Cox1, we next focused on determining its biochemical function. Coa4 is an IMS protein with a CHCH fold, and several IMS proteins with the CHCH fold have been shown to take part in redox processes in the IMS involving disulfide exchange (Banci et al., 2009). Recent studies from our own lab demonstrated that Coa6, another CHCH-fold containing mitochondrial IMS protein, acts as a thiol-disulfide oxidoreductase of Sco1 and Cox2 (Soma et al., 2019). Coa6 facilitates copper binding of Sco1

and Cox2 by reducing disulfides and generating free thiols that are necessary for copper binding. Consistent with this biochemical function of Coa6, it was previously shown that overexpression of *SCO1* could partially suppress the respiratory growth defect of *coa6Δ* cells, suggesting that Coa6 acts upstream of Sco1 (Ghosh et al., 2016). Based on these observations, we hypothesize that Coa4 could play a similar redox role, acting as a disulfide reductase for *COX11* in copper delivery, because the copper-binding site of Cox11 also contains two cysteines which may be prone to forming a disulfide bond (Bode et al., 2015; Carr et al., 2002). In support of this hypothesis, we found that growth in hypoxic conditions and treatment with the reducing agent glutathione could both rescue the respiratory growth defect of *coa4Δ* cells (Figure 3). Thus, one possible biochemical function of Coa4 is to reduce the disulfide bond in the copper-binding site of Cox11, allowing Cox11 to receive copper from Cox17 and then deliver it to Cox1 (Figure 6B). Experiments to test whether Coa4 can reduce the Cox11 disulfide *in vitro* and a co-immunoprecipitation experiment to probe for a physical interaction between Cox11 and Coa4 *in vivo* can be done to test this model of Coa4 function.

The cysteines of twin Cx_3C and twin Cx_9C proteins are thought to be important for import to the mitochondria and localization to the IMS. Rather than containing canonical mitochondria targeting sequences, twin Cx_3C and twin Cx_9C proteins typically contain a mitochondrial intermembrane space sorting (MISS)/IMS-targeting signal (ITS), which is a nine amino acid sequence upstream or downstream of a cysteine that forms a helix with key hydrophobic residues for docking with Mia40 (Cavallaro, 2010; Milenkovic et al., 2009; Sideris et al., 2009). The MISS/ITS motif is recognized by Mia40, which facilitates its import into the mitochondrial IMS and oxidative folding via the formation of intermolecular disulfide bonds between Mia40 and the protein being imported. Formation of the two disulfide bonds between

the two helices of the protein by Mia40, as part of the Mia40-Erv1 disulfide relay, stabilizes their folded structure and plays a role in their import into the IMS (Fischer and Riemer, 2013). Previously, Coa4 import to the mitochondria was shown to depend on the Mia40-Erv1 disulfide relay (Longen et al., 2009). Interestingly, we found that *COA4* single cysteine mutants were able to restore respiratory growth of *coa4(T)* cells, demonstrating that individual cysteines are not critical for Coa4 to perform its function (Figure 5). Further experiments are needed to confirm that the mutated Coa4 proteins are indeed being imported into the mitochondria. If they are, this observation would indicate that there is some "leaky" mitochondrial import of Coa4 in the absence of the canonical twin Cx_9C motif. In this case, perhaps the hydrophobic interactions between helix residues in Coa4 and Mia40 are enough to facilitate import to the mitochondria (Fischer and Riemer, 2013; Peleh et al., 2016). Our result showing the ability of Coa4 single cysteine mutants to complement *coa4(T)* cells also suggests that Coa4 can form a stable folded structure even in the absence of both disulfide bonds.

Further experiments are required to determine the specific role of the conserved cysteines in the function of Coa4. While our results show that Coa4 is functional with any combination of three of the four cysteines in the twin Cx_9C motif, mutations affecting two cysteines simultaneously would demonstrate whether or not having a single pair of cysteines capable of forming a disulfide bond is critical to Coa4 function. Additionally, experiments need to be done to determine whether the Coa4 cysteine mutants are able to rescue CcO assembly and activity.

If the conserved cysteines are not required for Coa4 function, this finding would challenge the model of Coa4 as a thiol-disulfide oxidoreductase. In that case, an alternate model for Coa4 function can be considered, where Coa4 could play a structural role in stabilizing a Cox1 assembly intermediate for formation of the heme a_3:Cu_B bimetallic center. Perhaps Coa4

makes the Cu_B site accessible to Cox11 in order for it to insert copper. Consistent with the finding that Coa4 is an allele-specific suppressor of G137E Shy1 (Bestwick et al., 2010), this model would place Coa4 downstream of Shy1 and upstream of Cox11 in the assembly of Cox1.

A model explaining Coa4 function must also be consistent with previous reports that have identified other genetic and environmental perturbations that compensate for the lack of Coa4. For example, Bestwick et al. have shown that *CYC1* and *CYC7*, two isoforms of cytochrome *c*, can suppress the respiratory growth defect of *coa4Δ* cells (Bestwick et al., 2010). Cytochrome *c* is required for the assembly and stability of CcO by an unknown mechanism (Barrientos et al., 2003; Vempati et al., 2009). These findings argue that Cyc proteins act downstream of Coa4 to compensate for defects in CcO assembly in the absence of Coa4.

In addition, Bode et al. found that GSH, DTT, and ascorbic acid supplementation rescue the respiratory growth of *coa4Δ* cells without rescuing CcO assembly. Based on this finding, it was proposed that the respiratory growth defect in *coa4Δ* cells is caused not by decreased CcO activity directly but by the production of reactive oxygen species (ROS) by reactive CcO assembly intermediates (Bode et al., 2013). In the absence of Cox11, cells accumulate a pro-oxidant heme A-Cox1 intermediate (Khalimonchuk et al., 2007). It is possible that in the absence of Coa4, Cox11 is less efficient in inserting copper into the Cu_B site, leading to accumulation of this cytotoxic assembly intermediate and impairing respiratory growth.

In conclusion, we have identified Coa4 as a novel member of the copper delivery pathway to the Cu_B site on the Cox1 subunit of CcO. Although further experiments are required to clarify the molecular function of Coa4, our results will help us gain a better understanding of the mechanisms of CcO biogenesis and mitochondrial copper metabolism.

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