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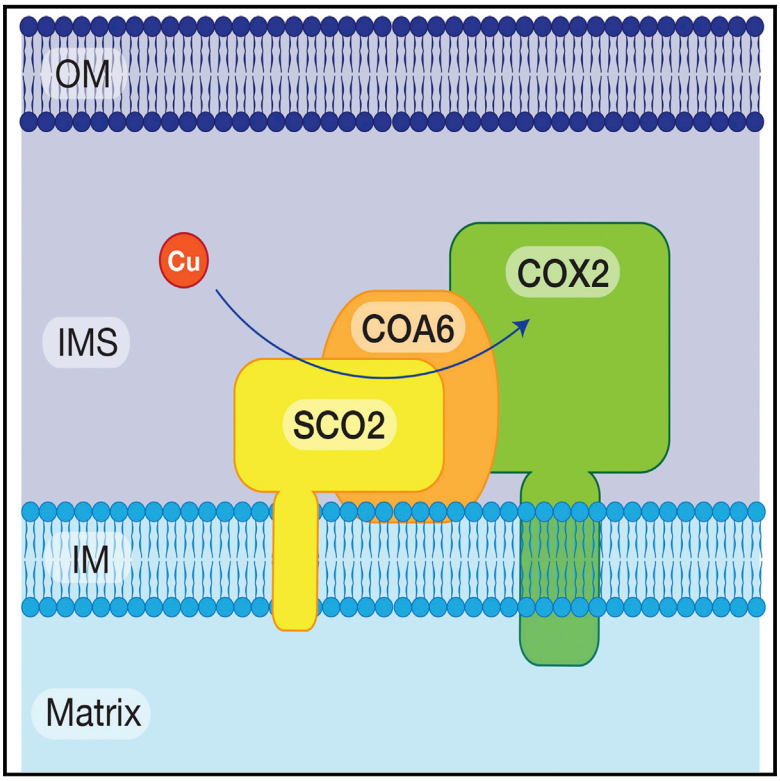
Cell Metabolism

Cooperation between COA6 and SCO2 in COX2 Maturation during Cytochrome *c* Oxidase Assembly Links Two Mitochondrial Cardiomyopathies

Graphical Abstract



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In Brief

Numerous assembly and chaperone proteins are involved in the biogenesis of cytochrome c oxidase (Complex IV), including COX2, which houses its redox-active copper center. Pacheu-Grau et al. show that cytochrome c oxidase assembly factor 6 (COA6) is part of the COX2 copper relay system involving the copper metallochaperone SCO2. Mutations preventing SCO2/COA6 interactions underlie patient mitochondrial cardiomyopathies.

Highlights

- COA6 is required for COX2 stability
- COA6 interacts transiently with the catalytic domain of newly synthesized COX2
- COA6 interacts with the copper metallochaperone SCO2
- Mutations in SCO2 or COA6 link defects in COX2 metallation to COX deficiency in heart

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Cooperation between COA6 and SCO2 in COX2 Maturation during Cytochrome *c* Oxidase Assembly Links Two Mitochondrial Cardiomyopathies

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SUMMARY

Three mitochondria-encoded subunits form the catalytic core of cytochrome *c* oxidase, the terminal enzyme of the respiratory chain. COX1 and COX2 contain heme and copper redox centers, which are integrated during assembly of the enzyme. Defects in this process lead to an enzyme deficiency and manifest as mitochondrial disorders in humans. Here we demonstrate that COA6 is specifically required for COX2 biogenesis. Absence of COA6 leads to fast turnover of newly synthesized COX2 and a concomitant reduction in cytochrome *c* oxidase levels. COA6 interacts transiently with the copper-containing catalytic domain of newly synthesized COX2. Interestingly, similar to the copper metallochaperone SCO2, loss of COA6 causes cardiomyopathy in humans. We show that COA6 and SCO2 interact and that corresponding pathogenic mutations in each protein affect complex formation. Our analyses define COA6 as a constituent of the mitochondrial copper relay system, linking defects in COX2 metallation to cardiac cytochrome *c* oxidase deficiency.

INTRODUCTION

The mitochondrial oxidative phosphorylation system generates the bulk of cellular ATP, fueling the energetic demands of most eukaryotes. Five multi-subunit protein complexes in the mitochondrial inner membrane, termed complexes I to V, comprise the oxidative phosphorylation system. These complexes are genetic hybrids. Thirteen core subunits of complexes I, III, IV, and V are encoded in the mitochondrial genome, while the remaining subunits are nuclear-encoded and imported into mitochondria (Mick et al., 2011; Soto et al., 2012). Hence, enzyme complexes assemble from subunits that reach the inner membrane via different transport routes. Moreover, redox-active cofactors have to be inserted during maturation of respiratory chain complexes. In the case of cytochrome *c* oxidase (complex IV), which oxidizes cytochrome *c* and transfers electrons to molecular oxygen, the mitochondria-encoded COX1 and COX2 house the redox-active heme and copper centers. While COX1 contains two heme moieties (*a* and a_3) and the Cu_B site, the Cu_A site is positioned in the intermembrane space domain of COX2. A plethora of factors participate in the process of protein assembly and cofactor insertion, and perturbation of the assembly process causes a cytochrome *c* oxidase deficiency and severe disorders in humans.

Cytochrome *c* oxidase assembly initiates with the synthesis of COX1, which associates with assembly factors in the inner mitochondrial membrane, forming the MITRAC intermediate in humans. This association stabilizes COX1 and feedback-regulates the translation of *COX1* mRNA. Insertion of heme, a process in which the SURF1 protein is implicated, is thought to occur once the first nuclear-encoded subunits have engaged with COX1. COX2 also spans the inner mitochondrial membrane, and contains soluble N- and C-terminal tails that protrude into the intermembrane space. OXA1 mediates export of the N-terminal tail of COX2 (Hell et al., 1997, 1998), while COX18 is required for export of its C terminus (Fiumera et al., 2007; Saracco and Fox, 2002). Delivery of copper to the Cu_A site contained within the C-terminal tail of COX2 depends on a relay of metallochaperones in the intermembrane space. COX17 is crucial for copper delivery to both COX1 and COX2. The transfer of copper from COX17 to COX1 is facilitated by COX11 (Herrmann and Funes, 2005). In the case of copper transfer from COX17 to the Cu_A site, two related proteins, SCO1 and SCO2, are involved (Herrmann and Funes, 2005). SCO1 participates in delivering copper to COX2 (Krummeck and Rödel, 1990; Rigby et al., 2008; Schulze and Rödel, 1988), whereas the function of SCO2 has remained elusive. In yeast, Sco2 is dispensable for cytochrome *c* oxidase biogenesis but is nonetheless able to partially complement Sco1 deficiency, indicating that Sco1 and Sco2 have overlapping functions (Banci et al., 2011). In contrast to yeast, human mitochondria possess two Sco1 homologs, termed SCO1 and SCO2, both of which are essential and have non-overlapping functions in the biogenesis of the Cu_A site (Horng et al., 2005). SCO2 has been proposed to act upstream of SCO1, hinting at a sequential copper delivery process for metallation of COX2 (Leary et al., 2004, 2009). After COX2 metallation, the COX1

and COX2 assembly lines merge and biogenesis of the enzyme complex continues by addition of COX3 and the other imported subunits.

Defects in heme *a* synthesis or delivery, as well as in copper chaperone function, have been linked to severe human disorders with cytochrome *c* oxidase deficiency. Patients with *SCO1* mutations display cytochrome *c* oxidase deficiency, leading to fatal encephalopathy, hypertrophic cardiomyophathy, neonatal hepatopathy, and ketoacidotic comas (Stiburek et al., 2009; Valnot et al., 2000; Leary et al., 2013). In contrast, mutations in *SCO2* are associated with neonatal encephalocardiomyopathy (Papadopoulou et al., 1999) or hypertrophic cardiomyopathy (Jaksch et al., 2000). Surprisingly, these distinct clinical phenotypes are not due to tissue-specific expression of SCO1 and SCO2, since both are ubiquitously expressed (Papadopoulou et al., 1999). One explanation for this could be that other factors involved in COX2 maturation contribute to the tissue selective phenotype.

Exome sequencing approaches have now significantly extended the number of known pathogenic mutations affecting complexes of the oxidative phosphorylation system, expanding the set of factors putatively involved in the biogenesis of its enzyme complexes. One example for this is COA6. A recent sequencing study on infants with mitochondrial disorders identified a mutation in *COA6* (*C1orf31*). The patient presented with hypertrophic cardiomyopathy, severe complex IV deficiency, and a mild reduction in complex I activity in heart tissue (Calvo et al., 2012). A second *COA6* patient, also with fatal hypertrophic cardiomyopathy, was recently reported (Baertling et al., 2015). The yeast homolog of COA6 had been identified previously in a proteomic study as a mitochondrial intermembrane space protein (Ghosh et al., 2014; Vögtle et al., 2012). *coa6*Δ cells display reduced levels of cytochrome *c* oxidase (Ghosh et al., 2014; Vögtle et al., 2012). While a partial suppression of mutant phenotypes in the presence of high copper concentrations has been reported, the function of COA6 and why its loss leads to cardiomyopathy remains unknown (Baertling et al., 2015; Calvo et al., 2012; Ghosh et al., 2014).

Here, we define the molecular function of COA6. We demonstrate that COA6 interacts with newly synthesized COX2 and that this interaction is conserved from yeast to man. In yeast mitochondria, Coa6 interacts with Sco1 and Sco2 preferentially in the absence of Cox2. In human mitochondria, COA6 selectively interacts with SCO2 in a COX2-dependent manner. A loss of COA6 leads to fast turnover of COX2, explaining the reduction in cytochrome *c* oxidase levels. Interestingly, pathogenic amino acid substitutions in both SCO2 and COA6 destabilize complex formation, thus linking the SCO2/COA6 interaction with the observed cardiac defect. We conclude that COA6 is a new constituent in the COX2 copper relay system, promoting SCO2 function during the metallation process.

RESULTS

COA6 Is Required for Cytochrome *c* Oxidase Biogenesis

In *Saccharomyces cerevisiae*, Coa6 is an intermembrane space protein that is released from mitochondria upon outer membrane opening and that is characterized by the presence of a $Cx_9Cx_nCx_{10}C$ motif (Ghosh et al., 2014; Vögtle et al., 2012). Interestingly, COA6 is highly conserved from yeast to metazoa (Figure S1). To assess the function of human COA6 (C1orf31) and understand how its malfunction leads to cardiomyopathy, we determined the localization of human COA6 by transfecting U2OS cells with COA6 containing a C-terminal FLAG-tag. Immunofluorescence microscopy demonstrated colocalization of $COA6^{FLAG}$ with mitotracker labeling (Figure 1A). Next, we generated a stable HEK293T cell line expressing COA6 with a C-terminal FLAG tag. Carbonate extraction and detergent treatment released COA6 into the soluble fraction, while the integral membrane proteins TIM23 and MITRAC12 were only solubilized by detergent treatment (Figure 1B). Moreover, $COA6^{FLAG}$ was present in isolated mitochondria where it only became accessible to externally added protease under conditions of outer membrane disruption, such as hypo-osmotic treatment (mitoplasts) or sonication (Figure 1C). Since defects in COA6 function cause cardiomyopathy in humans, we assessed expression levels of COA6 isoform 1 in different mouse tissues by qPCR. COA6 displayed increased expression in tissues with a high energy demand, such as heart, liver, and kidney (Figure 1D). Hence, no tissue-specific expression of COA6 was apparent, but rather an expression pattern resembling typical mitochondrial proteins (Vukotic et al., 2012; Yao and Shoubridge, 1999).

To assess COA6 function, we established a cellular model in which we downregulated the amount of COA6 by siRNA treatment. Upon treatment of HEK293T cells with siRNA targeted against COA6, doubling of cells was significantly affected after 3 days and exacerbated after that (Figure 1E). To reduce nonspecific effects due to the loss of COA6, we harvested cells after 4 days of treatment for further analyses.

Since *coa6*Δ yeast cells display reduced levels of cytochrome *c* oxidase similar to patients carrying mutations in *COA6*, we assessed respiratory chain function upon COA6 knock-down. We analyzed the oxygen consumption rate of COA6 knock-down cells by real-time respirometry and observed drastically decreased oxygen consumption at basal and maximal capacity compared to control cells (Figure 1F). This phenotype was explained by significantly decreased cytochrome *c* oxidase activity and reduced overall levels of the enzyme complex (Figure 1G). Loss of activity and reduced cytochrome *c* oxidase content correlated with each other, indicating that enzyme activity directly reflects complex abundance (Figure 1H). Since defects in complex I activity had been described for one *COA6* patient but were not apparent in a second pedigree (Baertling et al., 2015; Calvo et al., 2012), we also measured the enzymatic activity and the total amount of complex I. Cells with reduced COA6 levels displayed a significant decrease in complex I activity and content (Figure 1I). Accordingly, a lack of COA6 caused complex I and complex IV defects in HEK293T cells.

COA6 Interacts with Newly Synthesized COX2 in Human and Yeast Mitochondria

Our results demonstrate that human COA6, similar to its yeast homolog, is required for cytochrome *c* oxidase function and biogenesis. However, how COA6 contributes to cytochrome *c* oxidase assembly remained to be assessed.

Assembly of cytochrome *c* oxidase is initiated with the synthesis of COX1, which engages with a multitude of assembly factors in the inner mitochondrial membrane to form an early assembly intermediate called MITRAC (Mick et al., 2012). To address if

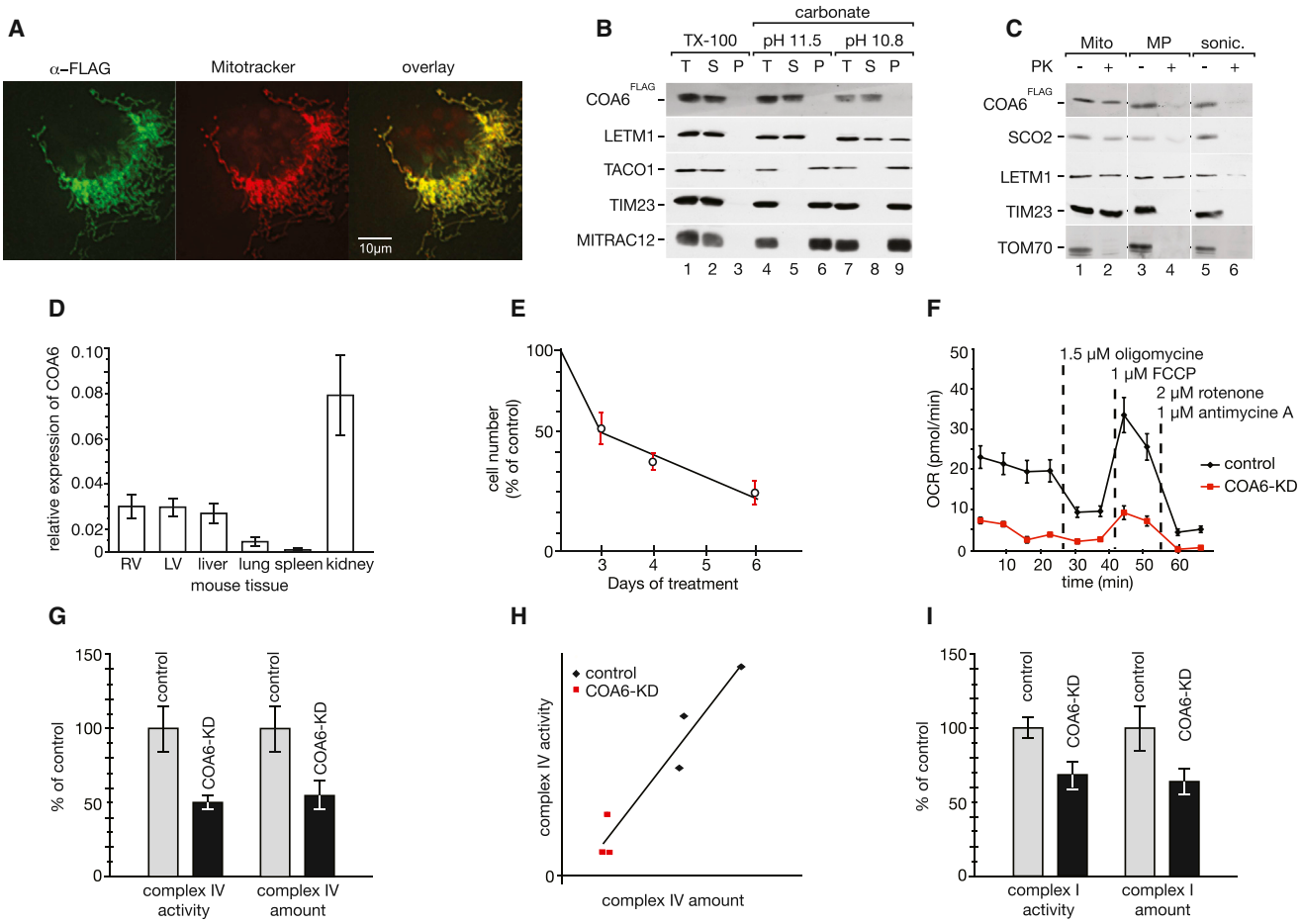

Figure 1. COA6 Is Required for Cytochrome *c* Oxidase Biogenesis

(A) Immunofluorescence analysis of U2OS cells after transient transfection with $COA6^{FLAG}$ using anti-FLAG antibodies and Mitotracker.

(B) Mitochondria containing $COA6^{FLAG}$ were subjected to carbonate extraction or detergent lysis, followed by differential centrifugation; pellet, P; supernatant, S; total, T. Samples were analyzed by SDS-PAGE and western blotting.

(C) Submitochondrial localization analysis using $COA6^{FLAG}$ mitochondria. Mito, mitochondria; MP, mitoplasts; Sonic., sonication; PK, proteinase K. Samples were analyzed by SDS-PAGE and western blotting.

(D) mRNA levels of *COA6* in indicated mouse tissue samples. Right ventricle, RV; left ventricle, LV.

(E) Cell count after treatment of cells with siRNA directed against COA6 compared to control cells.

(F) Real-time respirometry of COA6 knock-down and control cells; oxygen consumption rates, OCR.

(G) Cytochrome *c* oxidase activity and content in COA6 knock-down and control cells (SEM, n = 3) was determined by spectrophotometric measurement and affinity capture of the complex, respectively, as described in Experimental Procedures.

(H) Regression plot of activity values versus cytochrome *c* oxidase content (from [G]) (r = 0.92).

(I) Complex I activity and content in COA6 knock-down and control cells (SEM, n = 4). Enzymatic activity was determined by spectrophotometry, and amount of complexes was determined by ELISA.

See also Figure S1.

COA6 was part of MITRAC in human mitochondria, we performed immunoprecipitation of MITRAC12 (Clemente et al., 2013; Mick et al., 2012) from wild-type and $COA6^{FLAG}$ mitochondria. MITRAC12 coimmunoprecipitated known components of the MITRAC complex, such as COX1 and COX4-1. However, COA6 did not copurify with MITRAC12 (Figure 2A), demonstrating that COA6 does not participate in early steps of COX1 assembly.

To analyze if COA6 interacts with other complexes containing newly synthesized mitochondria-encoded proteins, we radiolabelled mitochondrial translation products in $COA6^{FLAG}$ or $MITRAC12^{FLAG}$ cells and performed immunoisolation. As expected, COX1, and to a lesser extent other mitochondria-encoded subunits, copurified with MITRAC12 (Mick et al., 2012). Interestingly, newly synthesized COX2 was isolated together with COA6 (Figures 2B and S2A).

COX2 is part of the catalytic core of cytochrome *c* oxidase and is conserved among species (Soto et al., 2012). To assess if the COA6/COX2 interaction is conserved between human and yeast mitochondria, we labeled mitochondrial translation products and performed immunoprecipitations using antiserum directed against Coa6. Indeed, newly synthesized Cox2 was specifically

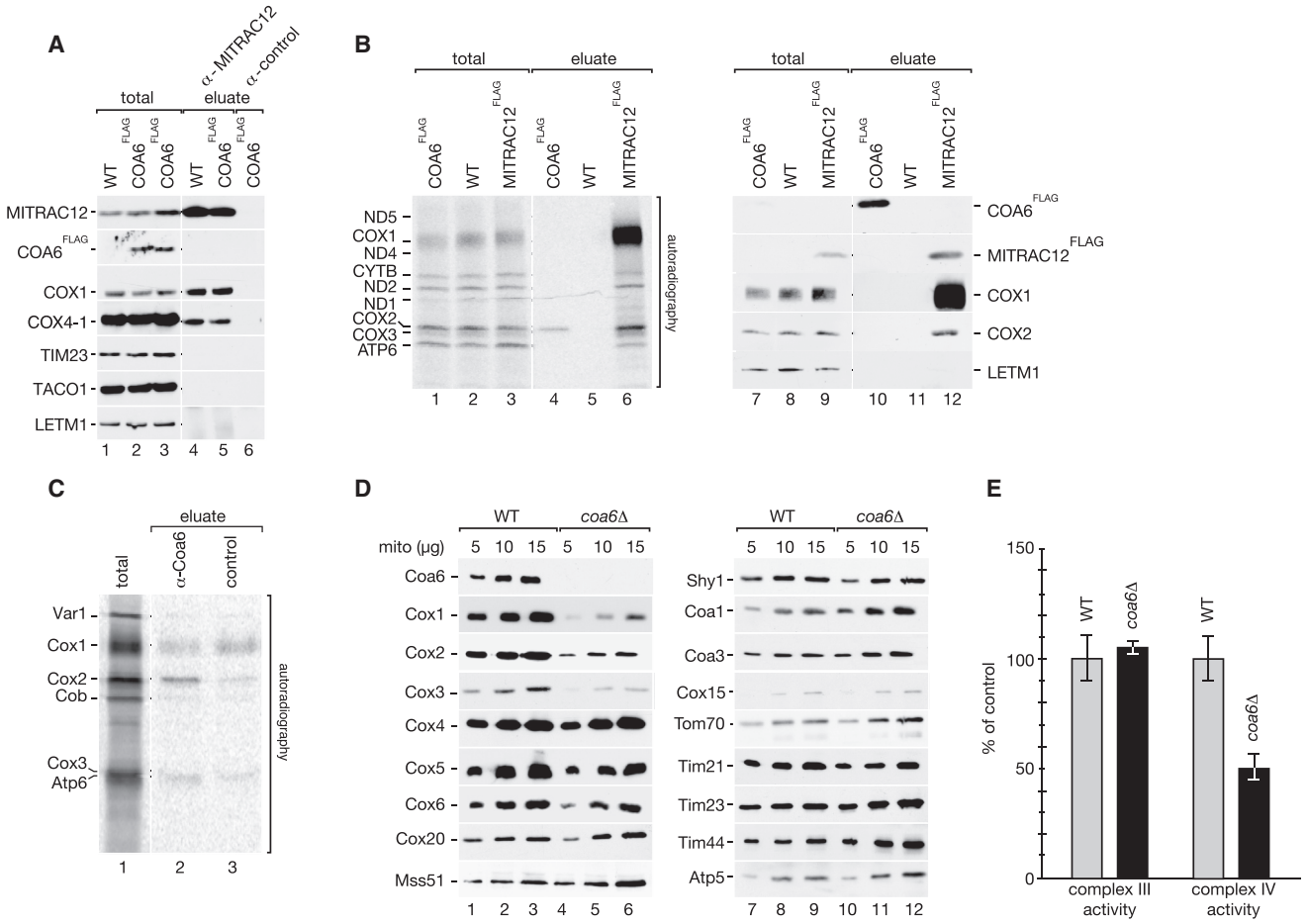

Figure 2. COA6 Interacts with Newly Synthesized COX2

(A) Anti-MITRAC12 immunoprecipitation from control or COA6^{FLAG}-containing mitochondria. Total, 5%; eluate, 100%. Samples were subjected to SDS-PAGE and western blotting.

(B) Anti-FLAG immunoprecipitation from control, COA6^{FLAG}-, or MITRAC12^{FLAG}-containing cells after radiolabeling of mitochondrial translation products. Total, 5%; eluate, 100%. Samples were analyzed by SDS-PAGE and digital autoradiography or western blotting.

(C) Immunoprecipitation from digitonin-solubilized mitochondria after in organello radiolabeling (20 min) of mitochondrial translation products using anti-Coa6 or control antibodies. Total, 4%; eluate, 100%. Samples were analyzed by SDS-PAGE and digital autoradiography. Fold enrichment over control sample: Cox1, 0.7; Cox2, 5.7; Cox3, 1.4.

(D) Isolated wild-type (WT) and *coa6*Δ mitochondria were analyzed by western blotting.

(E) Enzymatic activity of complex III or complex IV in wild-type and *coa6*Δ mitochondria (SEM, n = 3). See also Figure S2.

copurified with Coa6, indicating the conservation of this interaction and potentially COA6 function (Figure 2C). Because Cox2 was not significantly enriched in Coa6 immunoprecipitates by western blotting (Figure S2B), we conclude that the interaction between Cox2 and Coa6 is transient and only occurs early after Cox2 synthesis.

To address if absence of Coa6 in yeast mitochondria has a specific effect on Cox2, we analyzed steady-state protein levels in wild-type and *coa6Δ* mitochondria. The levels of Cox2 and other structural subunits of cytochrome *c* oxidase, such as Cox1 and Cox3, were reduced in *coa6Δ* mitochondria (Figure 2D). The levels of nuclear-encoded COX subunits such as Cox5 and Cox6 were also reduced, albeit to a lesser extent. In contrast, the protein levels of assembly factors such as Coa1, Coa3, Shy1, and the translational regulator of Cox1, Mss51, were not affected (Figure 2D). In agreement with the observed reduction of cytochrome *c* oxidase on blue native PAGE (Ghosh et al., 2014; Vögtle et al., 2012), isolated *coa6Δ* mitochondria showed selectively decreased cytochrome *c* oxidase activity (Figure 2E).

COA6 Interacts with the Copper Insertion Machinery and Is Required for COX2 Stability

To address if Coa6 affects translation of the *COX2* mRNA, we pulse-labeled mitochondrial translation products in yeast cells under conditions of inhibited cytosolic translation. Cox2 was barely detectable in *coa6Δ* cells (Figure 3A). This observation could be interpreted as an indication for reduced translation of Cox2 in cells lacking Coa6. To directly assess this possibility, we pulse-labeled mitochondrial translation products over a short time interval. We observed that less Cox2 was detectable with longer pulses in yeast cells lacking Coa6 (Figure 3B). Since a

direct effect of Coa6 on mitochondrial ribosomes is unlikely, as Coa6 localizes to the intermembrane space, we assessed if newly synthesized Cox2 is destabilized in the absence of Coa6. We pulse-labeled mitochondrial translation products in wild-type and *coa6Δ* cells and monitored the fate of mitochondrial translation products over a brief 5 min chase period with unlabeled methionine. While the stability of Cox3 was largely unaffected (Figure S3A), newly synthesized Cox2 was rapidly turned over in the deletion strain, having a half-life of approximately 3 min (Figure 3C). It is interesting to note that Cox2 was also unstable after labeling translation products in isolated mitochondria. However, the kinetics of Cox2 turnover was slower than in intact cells, suggesting that mitochondrial dynamics or cellular organization may contribute to the quality control process in vivo (data not shown). In conclusion, absence of Coa6 in yeast cells leads to decreased amounts of Cox2 through increased protein turnover, eventually leading to a loss of cytochrome *c* oxidase.

Coa6 and its human homolog, COA6, are localized in the mitochondrial intermembrane space (IMS). Cox2 possesses two transmembrane spans, exposing a short N-terminal and a catalytic C-terminal tail into the intermembrane space (Figure 3D). In yeast, the N terminus of Cox2 undergoes proteolytic processing by the intermembrane space processing peptidase (IMP) (Nunnari et al., 1993). Cox18 is required for export of the Cox2 C terminus into the IMS (Saracco and Fox, 2002). We speculated that Coa6 interacts with either the soluble N-terminal or C-terminal domain of Cox2. Hence, we assessed the interaction of newly synthesized Cox2 with Coa6 in *cox18Δ* and *imp1Δ* mitochondria. In *imp1Δ* mitochondria, the interaction of Cox2 with Coa6 was similar to wild-type, indicating that N-terminal processing is not required for Coa6 binding. In contrast, no interaction between Cox2 and Coa6 was found in *cox18Δ* mitochondria (Figure 3E). In cells lacking Cox18, the N terminus of Cox2 is inserted into the inner membrane and processed, and Cox2 is therefore resistant to protease degradation after swelling (Fiumera et al., 2007; Fiumera et al., 2009) and to carbonate extraction (Figure S3B). Thus, we conclude that Coa6 interacts with Cox2 via its IMS-exposed C-terminal tail.

Cox1 is the central platform for assembly of cytochrome *c* oxidase. A regulatory feedback mechanism couples COX1 translation to its assembly, ensuring that Cox1 is synthesized at an appropriate rate (Fontanesi et al., 2008; Kim et al., 2012; Mick et al., 2011; Ott and Herrmann, 2010). To assess if the interaction between Coa6 and Cox2 is affected in the absence of Cox1, we immunoprecipitated Coa6 after labeling of mitochondrial translation products in isolated mitochondria lacking Cox1. Similar amounts of Cox2 coimmunoprecipitated in *cox1*$^{-}$ and wild-type mitochondria (Figure 3F). This finding indicates that the interaction between Coa6 and Cox2 occurs before incorporation of Cox2 into the Cox1 assembly line.

The C-terminal domain of Cox2 forms a ten-stranded β-barrel containing the binuclear Cu_A center (Tsukihara et al., 1996). Taking this, and the presence of a $Cx_9Cx_nCx_{10}C$ motif in Coa6, into account, we investigated the Cox2/Coa6 interaction in the context of proteins involved in copper delivery to Cox2. The delivery of copper to the Cu_A site involves the metallochaperone Sco1. A related protein in yeast, Sco2, is dispensable for cytochrome *c* oxidase assembly but can compensate for loss of Sco1 upon its overexpression. Serendipitously, we detected Sco1 in Coa6 immunoprecipitations. Interestingly, Sco1 or Sco2 were not significantly recovered when Coa6 was precipitated from wild-type mitochondria, but only in cox2^{-} mutant mitochondria (Figure 3F). Based on this finding, we performed immunoprecipitation experiments using mitochondria from several mutant strains: *pet111Δ*, defective in Cox2 translation; *rho*^{0}, lacking functional mitochondrial DNA; and *cox18Δ*. Only low amounts of Sco1 and Sco2 copurified with Coa6 in wild-type mitochondria. However, significantly increased copurification of Sco1 and Sco2 was observed in mitochondria lacking Cox2 (*rho*^{0}, *pet111Δ)*. Moreover, increased Sco1 and Sco2 copurification was apparent in *cox18Δ* mitochondria (Figure 3G and Figure S3C). The observed increased association between Coa6 and Sco1/Sco2 in the absence of Cox2 indicates that Coa6 plays a role in copper insertion/delivery. Hence, we analyzed copper binding of Coa6. We expressed and purified GST-Coa6, GST-Sco1, and GST-Sco2 fusion proteins from *E. coli* cells. Copper content was assessed by atomic absorption spectrophotometry. While GST alone did not contain significant amounts of copper, GST-Coa6, GST-Sco1, and GST-Sco2 contained similar amounts of copper in this assay (Figure S3D). These data suggest that Coa6 is able to bind copper with an affinity similar to the Sco proteins when expressed in *E. coli*.

To assess if the interaction between Coa6 and Cox2 depends on the mitochondrial copper-relay system, we performed Coa6 immunoprecipitation after labeling of mitochondrial translation products in mitochondria from mutants lacking proteins involved in mitochondrial copper homeostasis. Cox17 is a copper-binding metallochaperone, containing a CCXC motif, and transfers copper to Sco1 and Cox11, which are responsible for copper insertion into the Cu_A and Cu_B sites, respectively (Carr et al., 2002; Glerum et al., 1996). Sco2 and Sco1 display sequence similarity and have overlapping but not identical functions (Banci et al., 2011; Glerum et al., 1996; Leary, 2010). Cox19 and Cox23 are putative copper chaperones localized to the intermembrane space. However, their function is still not well defined (Barros et al., 2004; Rigby et al., 2007). The normalized amount of newly synthesized Cox2 that coimmunoprecipitated with Coa6 was considerably increased in *cox11Δ*, *cox17Δ*, *cox19Δ*, *cox23Δ*, and *sco1Δ* mitochondria, compared to wild-type (Figures 3H and S3E). In contrast, deletion of *SCO2* resulted in significantly decreased Cox2 copurification (Figures 3H and S3E). Thus, in the absence of copper chaperones involved in copper transfer to Cox2, Coa6 accumulated in a Cox2-associated state. In contrast, a lack of Sco2 led to an apparent loss of the Coa6/Cox2 interaction (Figures 3H and S3E). Accordingly, in yeast, Coa6 interacted with Cox2 in a copper-chaperone-dependent manner. As the interaction between Sco1/2 and Coa6 was increased in the absence of Cox2, we conclude that Coa6 accumulates in a complex with the metallochaperones in the absence of its substrate.

Human SCO2 and COA6 Form a Complex

To address if the interaction between COA6 and SCO proteins is conserved in human cells and depends on COX2, we performed immunoisolation of $COA6^{FLAG}$ from mitochondria isolated from non-treated cells or cells treated with specific inhibitors of cytosolic (emetine) or mitochondrial (thiamphenicol) translation

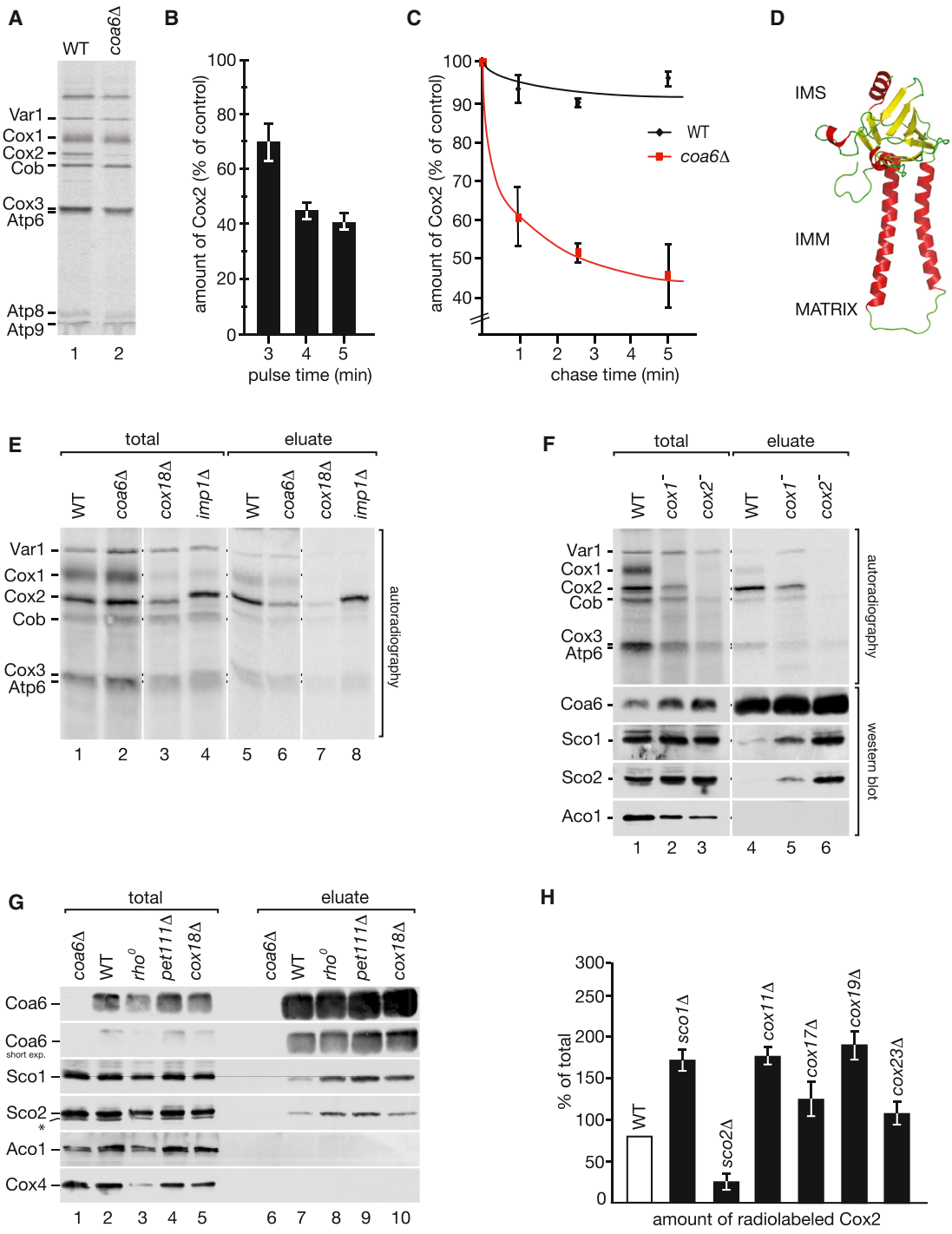

Figure 3. Coa6 Interacts with Copper Insertion Machinery and Affects Stability of Newly Synthesized Cox2

(A) In vivo labeling of mitochondrial translation products in wild-type and *coa6*Δ cells. Samples were pulsed for 10 min and analyzed by SDS-PAGE and digital autoradiography.

(B) Mitochondrial translation products were labeled in vivo for different pulse times. Quantification of newly synthesized Cox2 in wild-type and *coa6*Δ cells, normalized to Cob (SEM, n = 4).

(C) Mitochondrial translation products were labeled in vivo for 10 min and samples taken during the chase. Quantification of newly synthesized Cox2 in wild-type and *coa6*Δ cells, normalized to Cob (SEM, n = 3).

(D) Structure of COX2 in bovine cytochrome *c* oxidase (PDB 3ASN). IMM, inner mitochondrial membrane. IMS, intermembrane space.

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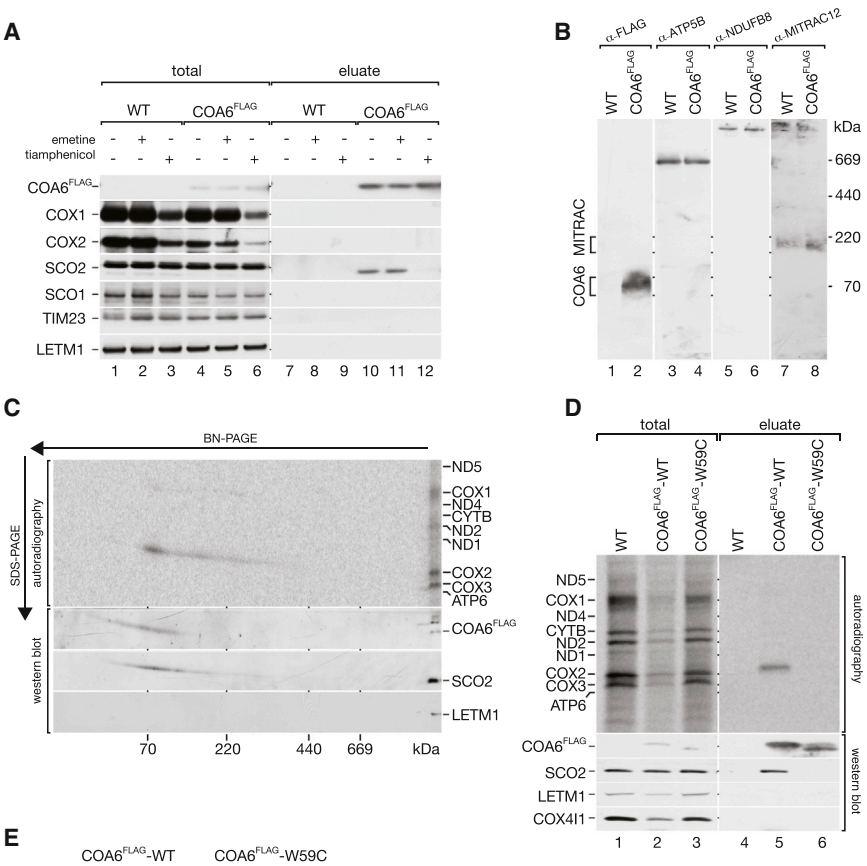

Figure 4. COA6 Interacts Specifically with SCO2

(A) Anti-FLAG immunoprecipitation from control and COA6^{FLAG} mitochondria. Mitochondria were isolated from non-treated cells or from cells treated with emetine or thiamphenicol. Total, 5%; eluate, 100%. Samples were subjected to SDS-PAGE and analyzed by western blotting.

(B) BN-PAGE analyses from control and COA6^{FLAG} mitochondria. Samples were analyzed by western blotting.

(C) After radiolabeling of mitochondrial translation products, COA6^{FLAG} mitochondria were subjected to anti-FLAG immunoprecipitation and eluates subsequently analyzed by 1D BN- and 2D SDS-PAGE. Samples were analyzed by digital autoradiography or western blotting.

(D) After transient transfection with wild-type or mutant COA6^{FLAG}, mitochondrial translation products were radiolabeled and anti-FLAG immunoprecipitation performed. Total, 5%; eluate, 100%. Samples were analyzed by SDS-PAGE digital autoradiography or western blotting.

(E) Immunofluorescence analysis of U2OS cells after transient transfection with COA6^{FLAG} and COA6^{FLAG}-W59C expressing plasmids using anti-FLAG antibodies and Mitotracker (Bar = 10 μm).

(F) Submitochondrial localization analysis using mitochondria derived from COA6^{FLAG} expressing cells and cells transfected with COA6^{FLAG}-W59C. Mito, mitochondria; MP, mitoplasts; Sonic., sonication; PK, proteinase K. Samples were analyzed by SDS-PAGE and western blotting.

(Chomyn, 1996; Mick et al., 2012). Human mitochondria have two homologs of yeast Sco1, termed SCO1 and SCO2 (Soto et al., 2012). Interestingly, COA6^{FLAG} specifically coisolated with SCO2, but not with SCO1. Moreover, treatment with thiamphenicol strongly affected the COA6/SCO2 interaction (Figure 4A). Accordingly, also in human mitochondria, COA6 interacted with metallochaperones of the SCO family. However, COA6 displayed SCO2 specificity and did not interact significantly with SCO1. Moreover, in human cells, COX2 is required for the interaction between COA6 and SCO2. In BN-PAGE analyses, COA6 formed a single complex of approximately 70 kDa at steady-state (Figure 4B). We radiolabelled mitochondrial translation products and isolated the COA6^{FLAG}-containing complex under native conditions. The purified complex was first separated on BN-PAGE and then subjected to a 2D separation on SDS-PAGE. Radiolabelled COX2 copurified with COA6^{FLAG} and comigrated together with COA6 and SCO2, suggesting that these proteins form a ternary complex (Figure 4C).

Patients with mutations in *SCO1* and *SCO2* present with early-onset mitochondrial disease in which affected tissues exhibit a cytochrome *c* oxidase deficiency and a profound reduction of their total copper content. Interestingly, mutations in *SCO1* have been reported to cause fatal encephalopathy, hypertrophic cardiomyophathy, neonatal hepatopathy, and ketoacidotic comas (Stiburek et al., 2009; Valnot et al., 2000; Leary et al.,

(E and F) Immunoprecipitation of Coa6 from digitonin-solubilized mitochondria isolated from indicated strains after in organello radiolabeling of mitochondrial translation products. Total, 4%; eluate, 100%. Samples were analyzed by SDS-PAGE and digital autoradiography ([E] and [F]) or western blotting (F).

(G) Immunoprecipitation of Coa6 from digitonin-solubilized wild type and mutant mitochondria. Samples were analyzed by SDS-PAGE and western blotting.

(H) Quantification of radiolabeled Cox2 co-immunoprecipitated with Coa6. Samples were normalized to the amount present in the total (SEM, n = 3). See also Figure S3.

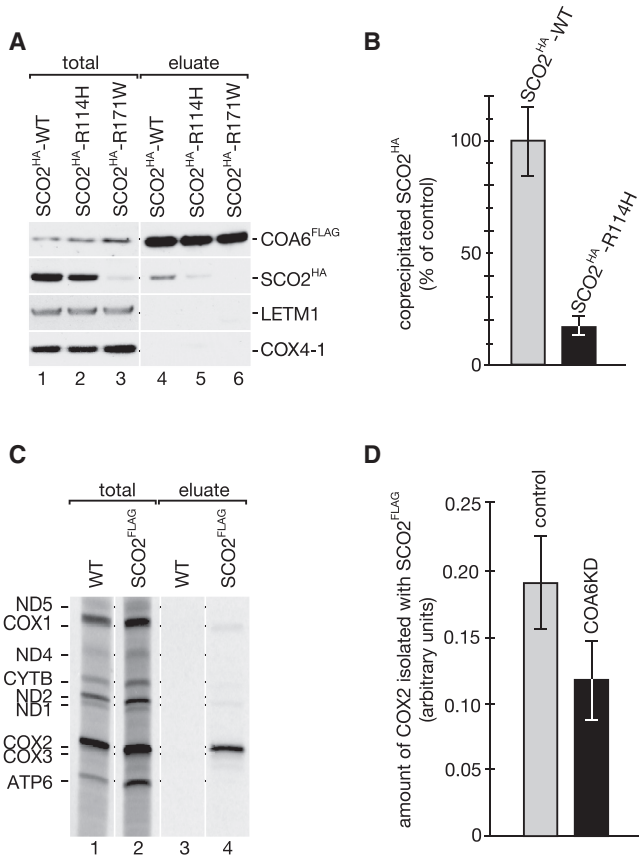

Figure 5. Mutations in SCO2 and COA6 Affect Protein Association

(A) After transient transfection with wild-type or mutant $SCO2^{HA}$, cells expressing $COA6^{FLAG}$ were solubilized in digitonin and analyzed by western blotting. Total, 5%; eluate, 100%.

(B) Quantification of the amount of $SCO2^{HA}$ coimmunoprecipitated with COA6, normalized to the total amount of SCO2 and of COA6 immunoprecipitated in each sample (SEM, n = 3).

(C) After radiolabeling of mitochondrial translation products, $SCO2^{FLAG}$ mitochondria were subjected to anti-FLAG immunoprecipitation and eluates subsequently analyzed by SDS-PAGE and digital autoradiography.

(D) Following siRNA treatment and radiolabelling of mitochondrial translation products, $SCO2^{FLAG}$ was immunoprecipitated and eluates analyzed by SDS-PAGE, western blotting, and digital autoradiography. The amount of newly synthesized COX2 was quantified and standardized to the precipitated amount of SCO2 (range, n = 2).

2013), whereas mutations in *SCO2* are primarily associated with neonatal hypertrophic cardiomyopathy (Jaksch et al., 2000). Hence, SCO2 defects appear to cause a clinical phenotype resembling that seen in *COA6* patients. In the case of COA6, two alleles have been identified in patients that encode COA6 with amino acid substitutions within, or in proximity to, the $Cx_9Cx_nCx_{10}C$ motif (Baertling et al., 2015; Ghosh et al., 2014). We therefore expressed a $COA6^{FLAG}$ variant harboring the pathogenic W59C substitution and analyzed whether it affected COA6 interactions with COX2 and SCO2. After radiolabeling of mitochondrial translation products, COX2 coisolated with wild-type COA6 but not with the COA6-W59C variant (Figure 4D). Moreover, western blot analyses revealed that the mutant COA6 was no longer able to interact with SCO2 (Figure 4D). Coa6 in yeast was reported to be imported via the MIA pathway, in agreement with the presence of a $Cx_9Cx_nCx_{10}C$ motif (Vögtle et al., 2012). The W59C substitution introduces an additional cysteine residue into this motif that may affect the mitochondrial localization of the mutant protein. To address this possibility, we expressed $COA6^{FLAG}$ with a W59C substitution in U2OS cells and assessed mitochondrial localization by fluorescence microscopy. Both $COA6^{FLAG}$ and $COA6^{FLAG}$-W59C localized to mitochondria (Figure 4E). To further analyze the submitochondrial localization of the W59C variant, we performed protease accessibility assays after swelling of mitochondria expressing wild-type COA6 or the mutant protein. While wild-type COA6 was sensitive to protease treatment in mitoplasts, the W59C variant was resistant, indicating protection by the inner mitochondrial membrane. These analyses point to the mislocalization of the COA6-W59C variant to the mitochondrial matrix (Figure 4F). Accordingly, the molecular basis for a loss of COA6-W59C function is an intramitochondrial transport defect caused by a single amino acid substitution.

Pathogenic Mutations in SCO2 Affect Its Interaction with COA6

Since expression of the pathogenic W59C allele of *COA6* resulted in mislocalization of the protein, we analyzed if pathogenic mutations in SCO2 affected its ability to associate with COA6. We generated HA-tagged variants of SCO2 ($SCO2^{HA}$-R114H, $SCO2^{HA}$-R171W), mimicking mutations found in *SCO2* patients. After transfection into $COA6^{FLAG}$-expressing cells, we assessed SCO2/COA6 association. While the R171W substitution rendered SCO2 unstable, $SCO2^{HA}$-R114H remained stable in mitochondria (Figure 5A, lanes 1–3). The presence of the R114H substitution reduced the level of SCO2 copurifying with COA6 by 80% (Figures 5A and 5B). Thus, expression of a pathogenic allele of *SCO2* significantly affected its interaction with COA6.

Newly synthesized COX2 can be specifically coisolated together with SCO2 (Figure 5C). To address if a loss of COA6 affected this association, we carried out $SCO2^{FLAG}$ immunoisolations after COA6 knock-down or treatment of cells with a non-targeting control. Upon reduction of COA6, the amount of newly synthesized COX2, which coisolated together with $SCO2^{FLAG}$, was significantly decreased (Figure 5D), indicating that COA6 is required for SCO2 to bind to COX2 or to stabilize this association.

We conclude that COA6 and SCO2 interact with each other under physiological conditions to cooperate in COX2 maturation. A loss of COA6 interaction with SCO2 and/or COX2 causes cytochrome *c* oxidase deficiency (Figure 6). Moreover, the SCO2/COX2 interaction depends on COA6. Since defects in COA6 and SCO2 function cause cardiomyopathy in humans, we suggest that the pathology of the patients is linked to tissue-specific requirements during copper incorporation into COX2.

DISCUSSION

Cytochrome *c* oxidase deficiency leads to a variety of severe human disorders (Ghezzi and Zeviani, 2012; Shoubridge, 2001). Here we find that cells lacking COA6 display a significant reduction in cytochrome *c* oxidase activity and total content.

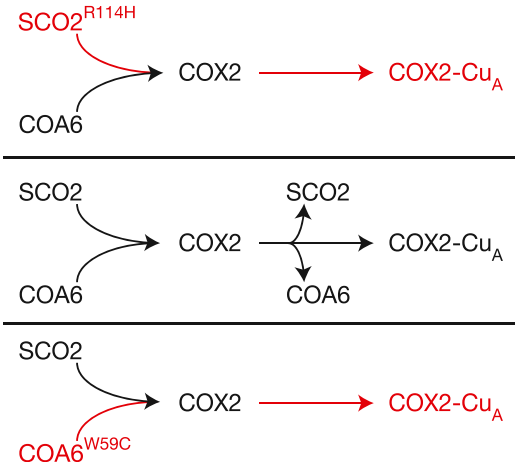

Figure 6. COA6 Cooperates with SCO2 in COX2 Metallation and Assembly of Cytochrome c Oxidase

COA6 and SCO2 cooperate in Cu_A site formation of COX2. Mutations in COA6 or SCO2 affecting their interaction cause defects in the maturation of the COX2 Cu_A site. Consequently, assembly of the cytochrome c oxidase is disturbed.

Moreover, SCO2 and COA6, which have been causally linked to severe cardiac disorders, physically interact. Our analyses indicate that a loss of complex formation contributes to the molecular pathology of the disorders, since pathogenic variants of SCO2 and COA6 lose their ability to interact with one another. In the case of the W59C variant of COA6, we find that the mutant protein is misrouted into the mitochondrial matrix, instead of being physiologically directed into the intermembrane space by the MIA pathway. Hence, in patient mitochondria, mutant COA6 is no longer available to its interaction partners. It is tempting to speculate that the introduction of an additional cysteine residue affects recognition of COA6 by the MIA transport machinery. However, the mechanism by which the pathogenic W59C variant is directed into the matrix will require detailed analyses on cryptic targeting signals within the COA6 protein.

SCO2 is a core constituent of the mitochondrial copper relay system that is required for metallation of COX2. Similar to SCO2 (Horng et al., 2005), heterologously expressed COA6 purified from *E. coli* is copper loaded. While it is tempting to interpret this as evidence that COA6 is an active constituent of the copper relay system, direct proof that COA6 binds copper within the mitochondrial intermembrane space is still lacking. It is interesting to note, however, that the interaction between SCO2 and its substrate COX2 is affected in cells with reduced COA6 levels. Moreover, we find that the interaction between SCO2 and COA6 is lost in human cells upon treatment with thiamphenicol to block mitochondrial translation. These observations suggest that newly synthesized COX2 is required for complex formation between SCO2 and COA6. In agreement with this, BN-PAGE analyses indicate that SCO2, COX2, and COA6 form a ternary complex in human mitochondria. However, the order of events that lead to the formation of this complex remains unclear. While the interaction between COA6 and SCO proteins and the interaction of COA6 and COX2 are conserved between yeast and humans, there are a number of notable differences. Unlike the human situation, binding of Coa6 to Sco1/2 is enhanced in the absence of Cox2 in yeast, while a lack of Sco1 promotes the association between Cox2 and Coa6. Hence, the interactions between Coa6 and Sco1/2 or Cox2 appear to be mutually exclusive. Since yeast Sco1, the homolog of human SCO1 and SCO2, is dispensable for Coa6 binding to Cox2, it is conceivable that human COA6 associates with COX2 prior to SCO2 recruitment into the ternary COX2/COA6/SCO2 complex. In summary, our observations in the yeast model are in agreement with a scenario in which Coa6 acts in two distinct reaction steps, on the one hand interacting with Sco1/2 and on the other forming a complex with newly synthesized Cox2. In humans, these mutually exclusive interactions have evolved into a ternary complex formation indicative of a coupled activity that links SCO2 directly to the COX2 substrate.

We suggest a model in which in human mitochondria COA6 promotes copper transfer to COX2. This process appears to occur directly on newly synthesized COX2. In yeast, the process seems to be constituted of independent binding reactions and to occur in a sequential manner. Our data suggest that one possibility is that COA6 could transfer copper directly to COX2 after being loaded with copper by SCO proteins. Alternatively, COA6 may need to be copper-loaded to act as a chaperone to maintain the COX2 tail in a competent state for receiving copper ions from SCO proteins. Reconstitution of this process in vitro will be required to discriminate between these two mechanistic possibilities.

EXPERIMENTAL PROCEDURES

Yeast Strains, Handling, and Mitochondrial Preparation

All *S. cerevisiae* strains used in this study are listed in Table S1. Chromosomal deletion of COA6 was generated by introduction of the HIS3MX6 cassette (Knop et al., 1999). Yeast strains were transformed with PCR-amplified integration-cassettes by the lithium acetate method (Ito et al., 1983) and confirmed by PCR and western blot analysis. In general, yeast cells were grown in liquid media containing 1% yeast extract, 2% peptone, and 2% galactose at 30°C with shaking (150–220 rpm). Mitochondria were isolated essentially as previously described (Meisinger et al., 2006). For steady-state analysis of mitochondrial proteins, different amounts were subjected to SDS-PAGE, followed by western blot analysis.

Cell Culturing, siRNA Constructs, Mutagenesis, and Transfection

Human embryonic kidney cell lines (HEK293T and Flp-In™ T-REx™ 293) were cultured using DMEM supplemented with 10% (v/v) fetal bovine serum (GIBCO, Invitrogen), 2 mM L-glutamine, and 50 μg/ml uridine at 37°C under a 5% CO_2-humidified atmosphere. For inhibition of cytosolic or mitochondrial translation, DMEM medium was supplemented with 20 μg/ml emetine (Sigma-Aldrich) for 6 hr or with 50 μg/ml thiamphenicol (Sigma-Aldrich) for 3 days, respectively. Constructs for the inducible expression of C-terminally FLAG-tagged COA6 and SCO2 were amplified by PCR using cDNA templates obtained from HEK293 cells. Primers were designed based on the sequences, Genbank: NM_001012985.2, NM_005138.2 (COA6 and SCO2, respectively). FLAG peptide sequence was introduced 5′ of the stop codon in the reverse primer. Amplicons and pcDNA5/FRT/TO (Invitrogen) were digested with appropriate enzymes, ligated and confirmed by sequencing. Cell lines expressing the transcript of interest were generated as previously described (Dennerlein et al., 2010). For COA6 knock-down, siRNA (5′-GCUUCAUCGCAGUAGGAAU-3′) was used at 33 nM. Reverse transfections were performed as previously described (Mick et al., 2012) using approximately 250,000 cells/25 cm^2, lipofectamine RNAiMAX (Invitrogen) and OPTIMEM-I medium. Cells were harvested and analyzed after 3, 4 and 6 days for growth kinetic experiments and after 4 days for biochemical experiments. Site-directed mutagenesis was carried out using the Quickchange Lightning Site-Directed

Mutagenesis Kit (Agilent Technologies), according to manufacturer instructions. To generate COA6 mutants, $COA6^{FLAG}$ was cloned into pCDNA5 and used as template. For SCO2 mutants, the $SCO2^{HA}$ in pcDNA3.1 was used as template ($SCO2^{HA}$ was generated by PCR amplification from cDNA using primers designed based on the sequence, Genbank: NM_005138.2, and HA was introduced 5′ of the stop codon in the reverse primer). Transient transfections were performed according to manufacturer's instructions using GeneJuice (Novagen). Briefly, approximately 300,000 cells/25 cm^2 were transfected using 4 μg of transfection reagent and 1 μg of DNA. Cells were collected 48 hr after transfection.

RT-PCR Analysis

RNA was extracted from various organs of FVB mice. Reverse transcription was performed with 2 μg of RNA and a first strand cDNA synthesis kit (Fermentas). Quantification of mRNA levels was performed using 1 μl of cDNA reaction and SYBR Green qPCR reaction kit (Clontech) and a MX3000P light cycler (Stratagene). Primers used for quantification: mS12 forward 5′-GAAGCTGC CAAGGCCTTAGA-3′, mS12-rev 5′-AACTGCAACCAACCACCTTC-3, COA6-forward 5′-CCTTCCATGAAGGAAAGGCA-3′, and COA6-rev 5′-ACTGGAA TCCTCCTGCTTCAA-3′.

Mitochondrial Isolation from Mammalian Cells and Localization Analysis

Mitochondria were isolated by differential centrifugation as previously described (Lazarou et al., 2009). Protein concentration was measured by Bradford analysis using BSA as a standard. Membrane integration of proteins was determined by incubation of mitochondria with 100 mM Na_2CO_3 (pH 10.8 or 11.5) followed by centrifugation for 30 min at 100,000 × *g* at 4°C. Submitochondrial localization was analyzed by protease protection assay (Mick et al., 2012): mitochondrial membranes were osmotically stabilized in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS [pH 7.2]), outer mitochondrial membrane ruptured by EM buffer (1 mM EDTA and 10 mM MOPS [pH 7.2]), or mitochondrial membranes disrupted by sonication in the presence of proteinase K. After incubation for 10 min on ice, reactions were stopped by addition of 1 mM PMSF.

Labeling of Mitochondrial Translation Products

In vivo labeling in human cells was performed as described previously (Chomyn, 1996). Cytosolic translation was inhibited with 100 μg/ml emetine (Invitrogen) and mitochondrial translation pulsed with 0.2 mCi/ml [^{35}S]methionine for 1 hr. In vivo labeling in whole yeast cells was performed essentially as described (Mick et al., 2010). In brief, yeast cells were resuspended in labeling buffer (40 mM potassium phosphate [pH 6.0], 2% galactose). Cycloheximide (150 μg/ml) was used to stop cytoplasmic translation. Samples were pulse-labeled with 40 μCi [^{35}S]methionine for 10 min at 30°C. To analyze the stability of mitochondrial translation products, mitochondrial translation was stopped by the addition of chloramphenicol (CAP) and excess methionine. Chase samples were taken after different time points during incubation at 30°C. To visualize radiolabeled proteins, whole-cell extracts were prepared as described above and analyzed by SDS-PAGE and digital autoradiography. For coimmunoprecipitations, translation products were labeled in isolated yeast mitochondria for 20 min with [^{35}S]methionine at 30°C, essentially as described previously (Westermann et al., 2001). After labeling was stopped by addition of excess methionine, mitochondria were reisolated and washed with SEM buffer (250 mM saccharose, 1 mM EDTA, and 10 mM MOPS) prior to co-immunoprecipitation.

Miscellaneous

Structure of subunit II of the cytochrome *c* oxidase (COX2) (PDB: 3ASN) was modeled using Swiss-PDB viewer and PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC. Standard methods were used for SDS-PAGE and western blotting of proteins adsorbed to polyvinylidene fluoride membranes (Millipore). Primary antibodies were raised in rabbit or purchased (anti-SCO1, Abcam; anti-SCO2 and anti-COX2, Proteintech). Antigen-antibody complexes were detected by HRP-coupled secondary antibodies and enhanced chemiluminescence detection on X-ray films.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.04.012.

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Supplemental Information

Cooperation between COA6 and SCO2 in COX2 Maturation during Cytochrome *c* Oxidase Assembly Links Two Mitochondrial Cardiomyopathies

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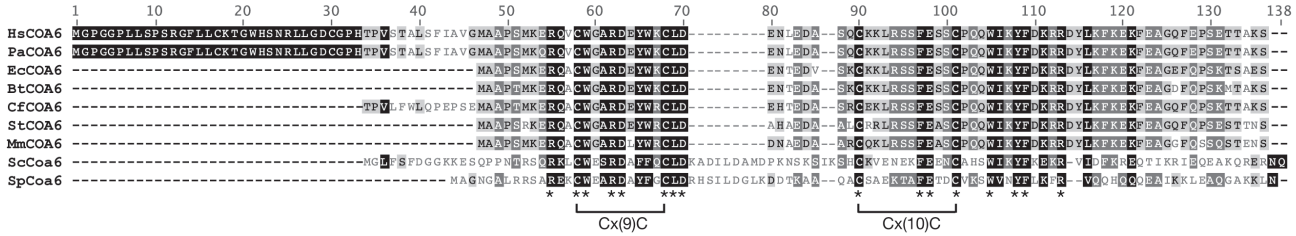

Figure S1, related to figure 1. **COA6 is conserved among eukaryotes.**

Alignment of COA6/Coa6 homolog proteins from different species using ClustalW with Blossum62 score matrix. Black boxes, 100% similarity; dark grey, 80-100% similarity; grey, 60-80% similarity; light grey, 50-60% similarity. Shown are conserved amino acids over all species (*) and the $Cx_9Cx_nCX_{10}C$ motif. *Hs H. sapiens, Pa P. abelii, Ec E. caballus, Bt B. Taurus, Cf C. familiaris, St S. tridecemlineatus, Mm M. musculus, Sc S. cerevisiae, Sp S. pombe*.

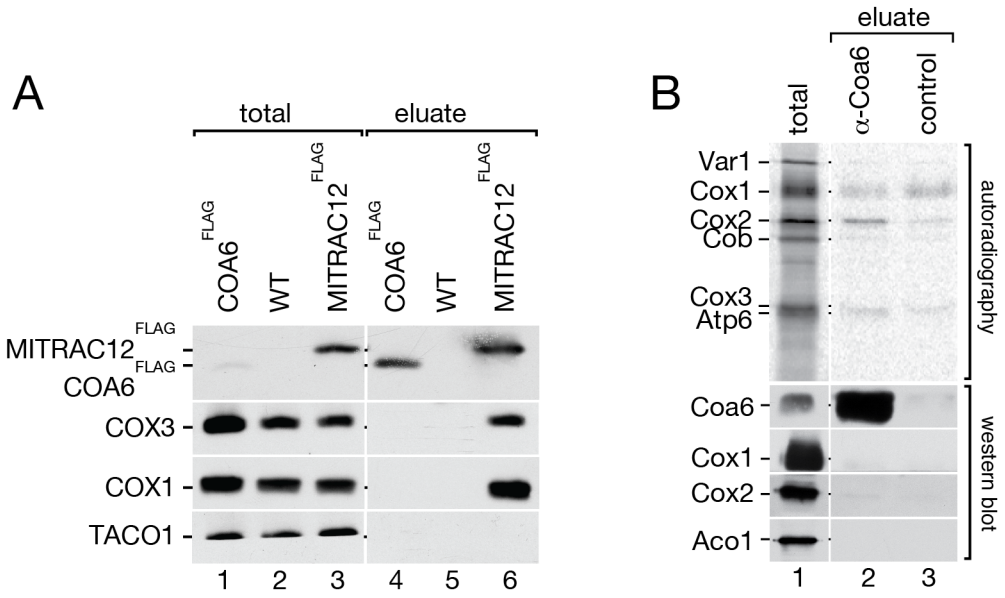

Figure S2, related to figure 2. **COA6 protein interactions on steady state level.**
(A) Anti-FLAG immunoprecipitation from control, MITRAC12^{FLAG} or COA6^{FLAG}-containing mitochondria. Total, 5%; eluate, 100% (WT and COA6^{FLAG}) or 50% (MITRAC12^{FLAG}). Samples were subjected to SDS-PAGE and Western-blotting. (B) Immunoprecipitation from digitonin-solubilized mitochondria after *in organello* radiolabeling of mitochondrial translation products using anti-Coa6 or control antibodies. Total, 4%; eluate, 100%. Samples were analyzed by SDS-PAGE and digital autoradiography or Western-blotting.

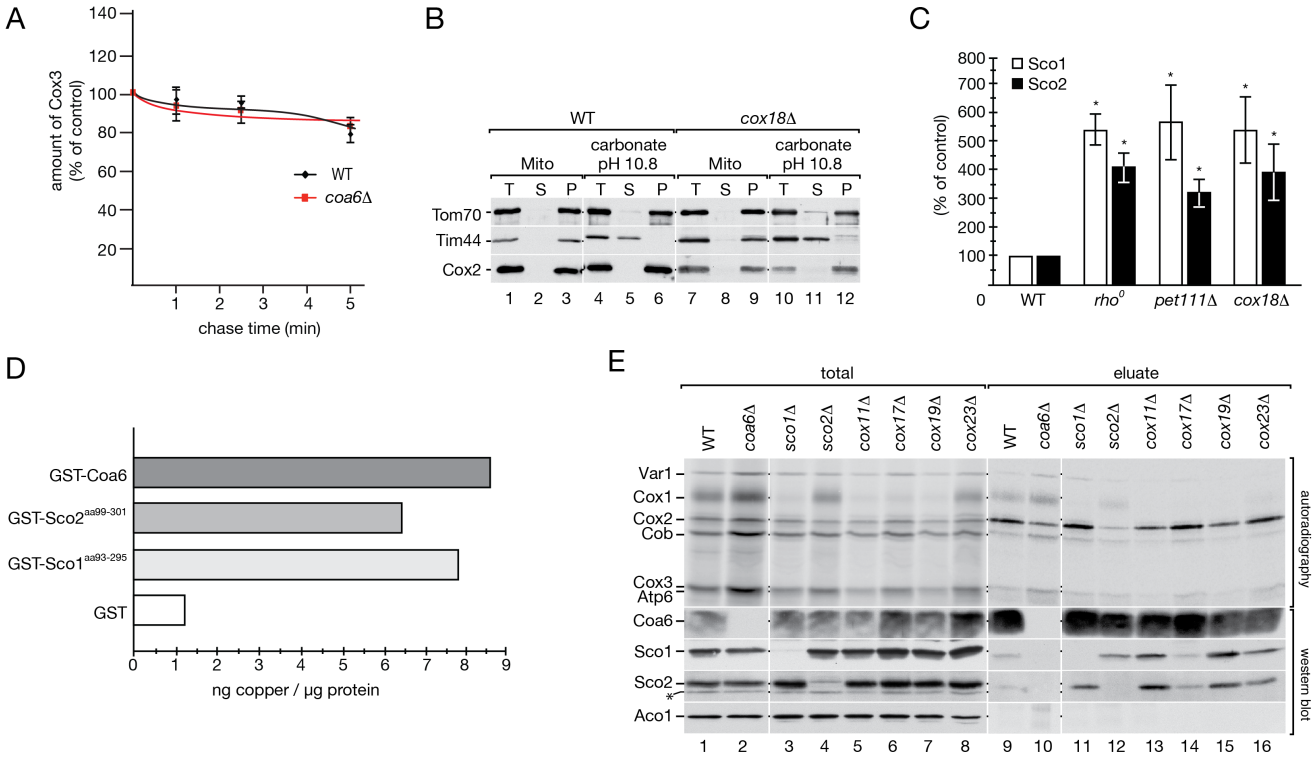

Figure S3, related to figure 3. **Coa6 cooperates with Sco2 in Cox2 binding.**

(A) Mitochondrial translation products were labeled *in vivo* (10min) and samples taken during chase. Quantification of newly synthesized Cox3 in wild type and *coa6*Δ cells, normalyzed by Cob (SEM, n=3). (B) Wild-type and *cox18*Δ mitochondria were subjected to carbonate extraction followed by separation into pellet (P) and supernatant (S); total (T). Samples were subjected to western blot analysis. (C) Quantification of the amounts of Sco1 and Sco2 copurified by Coa6 in wild-type and mutant mitochondria. Samples were normalized to the amount present in the total and the amount of purified Coa6 (SEM, n≥3, * indicates p≤0.05 by unpaired t-test). (D) Coa6 and the IMS domains of Sco1 and Sco2 were expressed as GST fusion proteins and purified from bacteria by GSH-affinity chromatography. Copper and protein were quantified by atomic absorption spectrometry and amino acid analysis, respectively. (E) Immunoprecipitation of Coa6 from digitonin-solubilized wild-type and mutant mitochondria after *in organello* radiolabeling of mitochondrial translation products. Total, 4%; eluate, 100%. Samples were subjected to SDS-PAGE followed by digital autoradiography and Western-blotting.

Supplemental Table T1, related to experimental procedure.

Yeast strains used in this study

strains	genotype	source
YPH499	Mat a, *ade2*-101 *his3*-Δ200 *leu2*-Δ1 *ura3*-52 *trp1*-Δ63 *lys2*-801	Sikorski and Hieter (1989)
BY4741	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	EUROSCARF
cox1^{-}	Mat a, *ade*1 *op1*; *cox1*-G421	Netter *et al* (1982)
cox2^{-}	Mat a, *ade1 op1 met3*; cox2-V25	Kruszewska *et al* (1980)
*coa6*Δ (YPH499)	Mat a, *ade2*-101 *his3*-Δ200 *leu2*-Δ1 *ura3*-52 *trp1*-Δ63 *lys2*-801; YMR224C-A::*HIS3MX6*	This study
*coa6*Δ (BY4741)	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YMR224C-A::*kanMX4*	EUROSCARF
*cox18*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YGR062C*::kanMX4*	EUROSCARF
*imp1*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YMR150C::*kanMX4*	EUROSCARF
*cox17*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YLL009C::*kanMX4*	EUROSCARF
*cox18*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YGR062C::*kanMX4*	EUROSCARF
*cox19*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YLL018C-A::*kanMX4*	EUROSCARF
*cox23*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YHR116W::*kanMX4*	EUROSCARF
*sco1*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YBR037C::*kanMX4*	EUROSCARF
*sco2*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YBR024W::*kanMX4*	EUROSCARF
*pet111*Δ	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YMR257C::*kanMX4*	EUROSCARF
rho^{0}	Mat a, *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0; YER154W::kanMX4/ *rho*^{0}	EUROSCARF

Supplemental experimental procedures

Immunofluorescence microscopy

Prior to fixation, cells were incubated with MitoTracker® Orange CMTMRos (Invitrogen Molecular ProbesTM, 1:2000) for 20min at 37°C. After five washing steps with PBS, cells were fixed with 4% paraformaldehyde (20 min, 37°C). Fixed cells were washed with PBS and subsequently permeabilized with 0.2% Triton-X100. After washing with PBS, fixed cells were incubated with blocking buffer (1% BSA in PBS) for 20min at room temperature, incubated with mouse monoclonal anti-FLAG (Sigma), or mouse monoclonal anti-HA antibody (12CA5, Roche) followed by PBS washing. Subsequently, cells were incubated with the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG). After final washing samples were mounted in histology mounting medium (Fluoroshield^{TM} with DAPI, Sigma).

Images were taken on a DeltaVision Spectris (Applied Precision) fluorescence microscope at 60x magnification equipped with a FITC (excitation 475/28, emission 523/36), TRITC (excitation 542/27, emission 594/45) and DAPI (excitation 390/18, emission 435/48) filter set. A series of 15-20 sections with 0.5µm spacing along the Z-axis were taken. Images were deconvoluted and projections were created from stacks by merging the individual slices using the softWoRx (Applied Precision) software.

Enzymatic activity assays and oxygen consumption

Complex IV enzymatic activity in yeast was determined by measuring the decrease in absorbance at 550 nm in a 96-well plate reader, using reduced cytochrome *c* (1.5 mg/ml), 10 mM Kpi buffer pH 7.0 and yeast mitochondria. Complex III enzymatic activity was determined by measuring the increase in absorbance at 550 nm, using oxidized cytochrome *c* (1.5 mg/ml), 10 mM Kpi buffer pH 7.0, 12.5 mM KCN, 5 mM NADH and

yeast mitochondria. Complex I activity and quantification assay in mammalian cells was performed using the Complex I Enzyme Activity Microplate assay Kit and the NADH Dehydrogenase (Complex I) Human Profiling ELISA Kit respectively (Abcam), according to manufacturer's instructions. Complex IV activity and quantification assay in mammalian cells was performed using the complex IV Human Specific Activity Microplate Assay Kit from Mitosciences (Abcam) and these measurements were normalized to citrate synthase activity, according to manufacturer's instructions..

Oxygen consumption rate (OCR) was measured with a XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). For COA6 knock-down experiments, 4 days siRNA treatment cells were harvested and 50,000 cells/well were seeded into a XF96-well plate. Baseline respiration was measured in DMEM supplemented with 1 mM pyruvate and 25 mM glucose after calibration at 37 °C in an incubator without CO_2. Periodic measurements of oxygen consumption were performed and OCR was calculated from the slopes of change in oxygen concentration over time. Metabolic states were measured after subsequent addition of 1.5 µM oligomycin, 1 µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 1 µM antimycin A, and 1 µM rotenone.

Co-Immunoprecipitation and affinity purification

COA6 or MITRAC12-specific antisera were bound to Protein A-sepharose (GE Healthcare) in 0.1 M potassium phosphate buffer (pH 7.4) and subsequently cross-linked with 5 mg/ml dimethyl pimelimidate solution in 0.1 M sodium borate (pH 9.0) for 30 min. Mitochondria were solubilized on ice in 20 mM Tris/HCl (pH 7.4), 0.1 M NaCl, 10% glycerol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% digitonin for 30 min. After removal of unsolubilized material by centrifugation, a sample was taken and the mitochondrial lysate incubated with anti COA6 or anti MITRAC12 coupled sepharose

respectively at 4 °C under mild agitation. After washing with wash buffer (20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10% glycerol, 5 mM EDTA, 2 mM PMSF, 0.3% digitonin), bound proteins were eluted with 0.1 M Glycine (pH 2.8) and neutralized with 1 M Tris (pH 11.5). Samples were analysed by SDS-PAGE and Western blotting, followed by detection of radiolabeled proteins by digital autoradiography. Quantifications were performed using ImageQuant TL (GE Healthcare).

Isolated mitochondria or whole cells were solubilized in buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 10% (w/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing 1% (w/v) digitonin (Merck) and incubated at 4°C. Lysate was cleared by centrifugation, and supernatants applied to equilibrated anti-FLAG-agarose (Sigma). After washing, bound proteins were eluted with FLAG peptide.

Protein expression and purification

The C-terminal domains of yeast Sco1 and Sco2 and full-length Coa6 were cloned into the *E. coli* expression vector pGEX-6P-2. For expression, all constructs were transformed into BL21 *E. coli* cells. Cells were grown at 30°C to an OD_{600}=0.6, protein expression was induced with 1 mM IPTG and cells were incubated for an additional 4h at 30°C. GST-fusion proteins were purified according to the manufacturer's protocol. In short, cells were resuspended in 10 volumes binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing protease inhibitors and lysed by Emulsiflex treatment. Lysates were cleared by centrifugation at 42,000xg for 30 min. Supernatants were loaded onto 1 ml GSTrap columns, washed with 5 column volumes binding buffer. Bound proteins were eluted with 5 column volumes elution buffer (50 mM Tris-HCl, 10 mM reduced glutathion, pH 8.0).

Copper content determination

Trace mineral analysis was carried out by a commercial provider, Spurenanalytisches Laboratorium (Dr.Heinrich Baumann). Purified proteins from *E.coli* were dialyzed overnight against 20 mM Hespes, pH 7.5 (titrated with Tris base).