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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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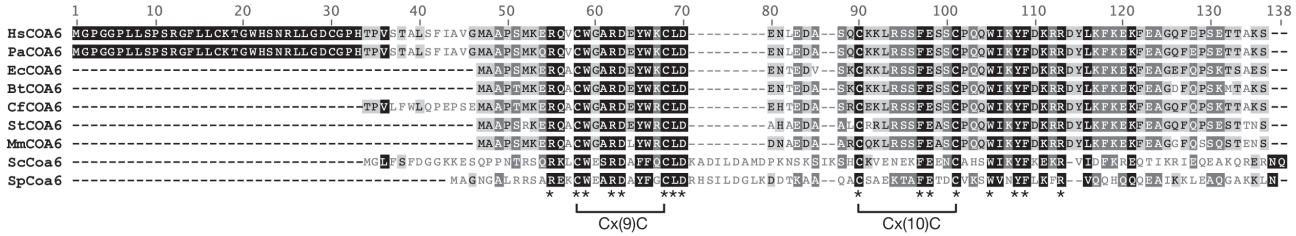

Figure S1, related to figure 1. **COA6 is conserved among eukaryotes.**

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*.

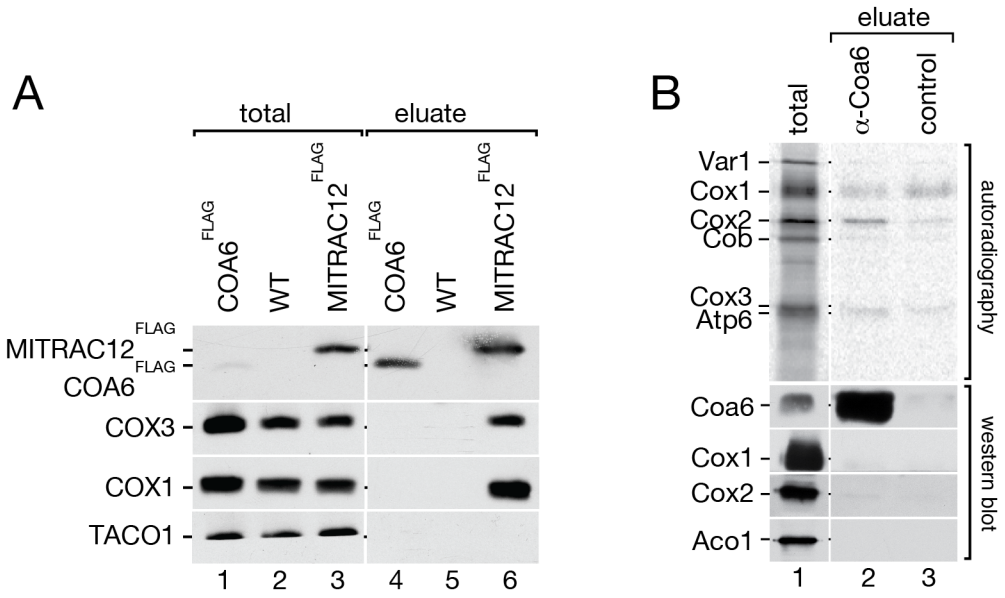

Figure S2, related to figure 2. **COA6 protein interactions on steady state level.**
(A) Anti-FLAG immunoprecipitation from control, MITRAC12^{FLAG} or COA6^{FLAG}-containing mitochondria. Total, 5%; eluate, 100% (WT and COA6^{FLAG}) or 50% (MITRAC12^{FLAG}). Samples were subjected to SDS-PAGE and Western-blotting. (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.

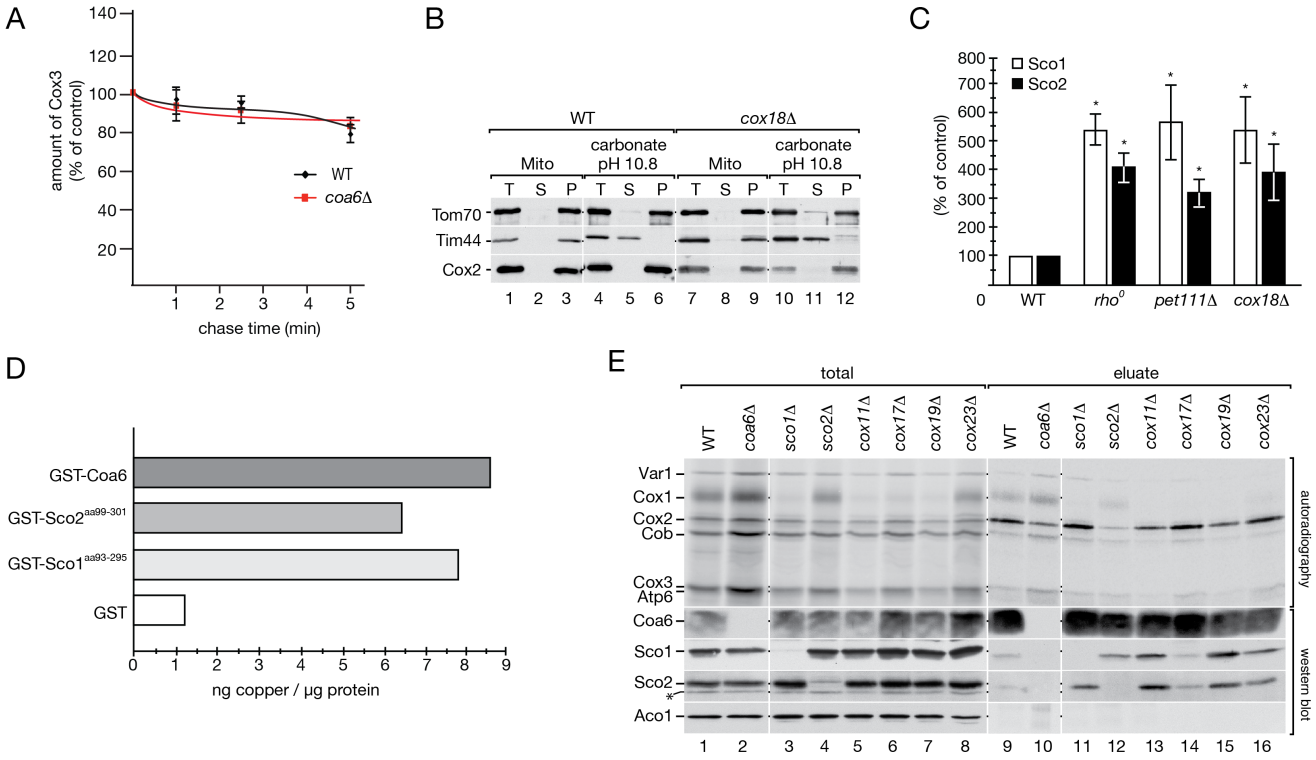

Figure S3, related to figure 3. **Coa6 cooperates with Sco2 in Cox2 binding.**

(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).