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Supplemental Information

Rcf1 Mediates Cytochrome Oxidase Assembly and Respirasome Formation, Revealing Heterogeneity of the Enzyme Complex

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Supplemental Information Inventory:

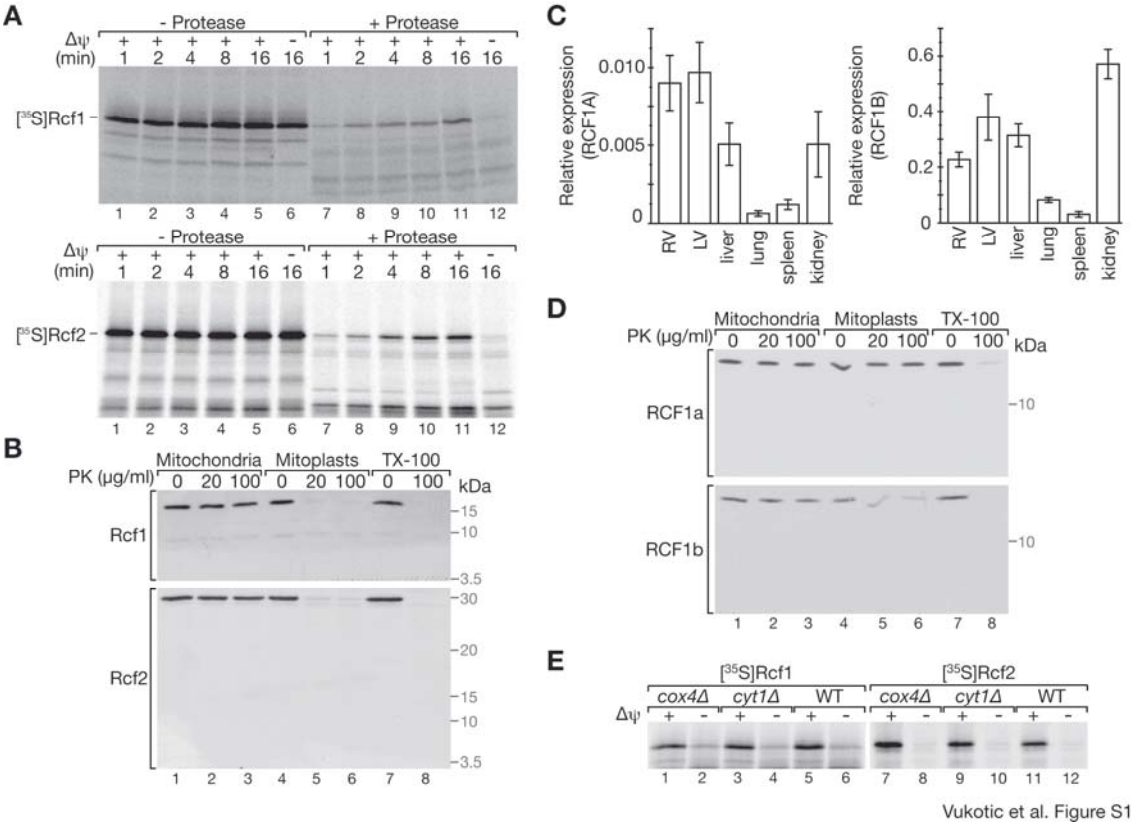

Figure S1. **Rcf1 and Rcf2 are mitochondrial inner membrane proteins.**

(A) Radiolabeled Rcf1 and Rcf2 were imported into isolated mitochondria in the presence or absence of a membrane potential ($\Delta\psi$) for indicated times. Samples were split and left untreated or treated with protease. Samples were analyzed by SDS-PAGE and digital autoradiography. (B) Protease protection assays were performed as described in Figure 1E. Presented are sections of the blots below Rcf1 and Rcf2. No proteolytic fragments of Rcf1 and Rcf2 were detected with antibodies directed against the C-termini. (C) mRNA expression of RCF1A and RCF1B in indicated mouse tissue samples expressed as a relative expression to the housekeeping gene mS12 ($2^{-\Delta Ct}$). Right ventricle (RV), Left ventricle (LV). (D) Mitochondria isolated from HEK-293 cells were subjected to the protease protection assay. Presented is the section of the blot below RCF1a and RCF1b. No proteolytic fragments of RCF1a and RCF1b were detected. (E) Samples from Figure 2, Panel B (BN-PAGE), were analyzed by SDS-PAGE and digital autoradioagraphy.

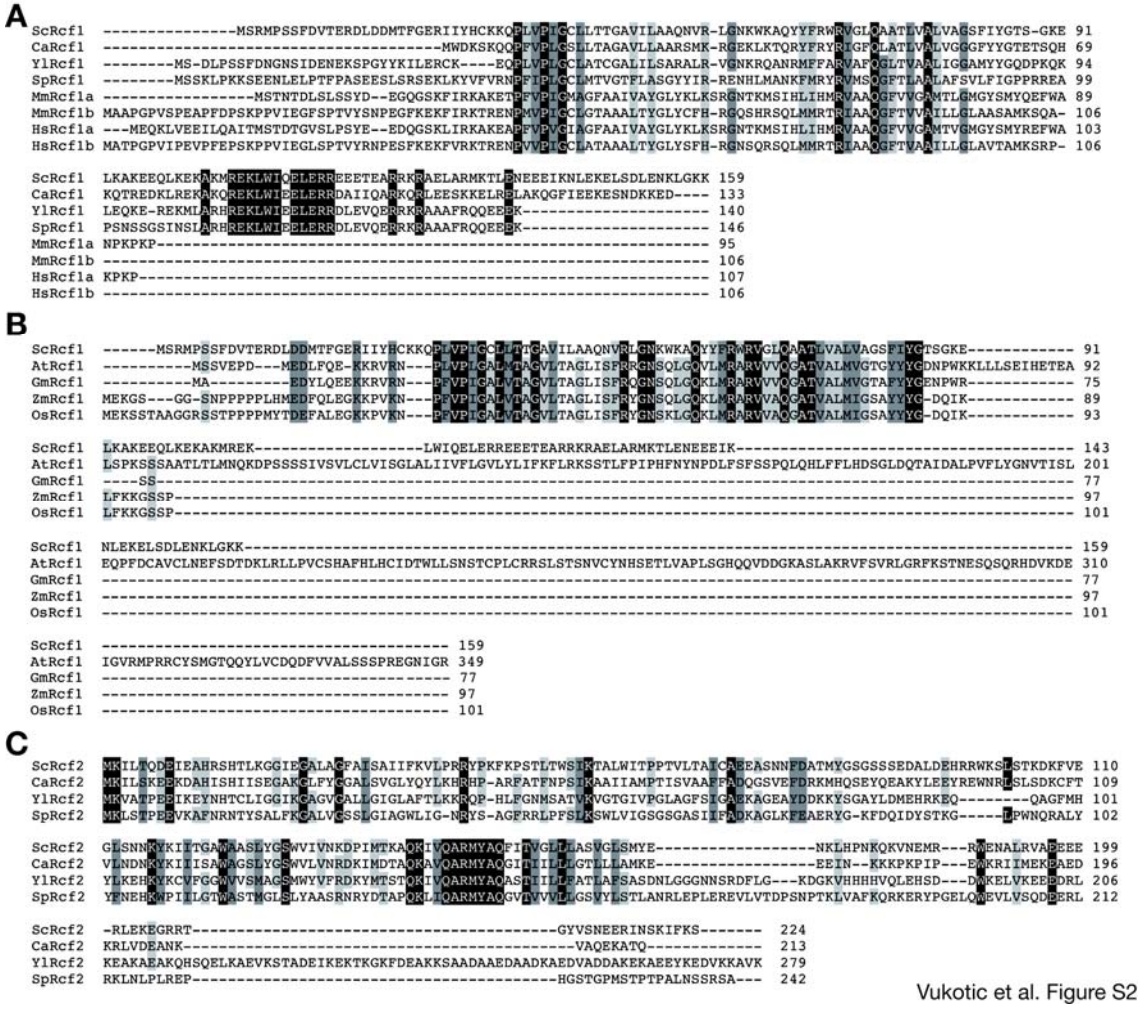

Figure S2. **Rcf1 is conserved among eukaryotes, while Rcf2 is conserved among fungi.** Alignment of Rcf1 homologs and Rcf2 homologs with ClustalW (2.0.12). Black boxes, identical residues in all species, grey boxes similar amino acids. (A) Alignment of Rcf1 with fungal and animal homologs. *Sc S. cerevisiae*; *Ca C. albicans*; *Yl Y. lipolytica*; *Sp S. pombe*; *Mm M. musculus*; Hs *H. sapiens*. (B) Alignment of Rcf1 with plants homologs. *Sc S. cerevisiae*; *A. thaliana; G. max; Z. mays; O. sativa..* (C) Alignment of Rcf2. *Sc S. cerevisiae*; *Ca C. albicans*; *Yl Y. lipolytica*; *Sp S. pombe.*

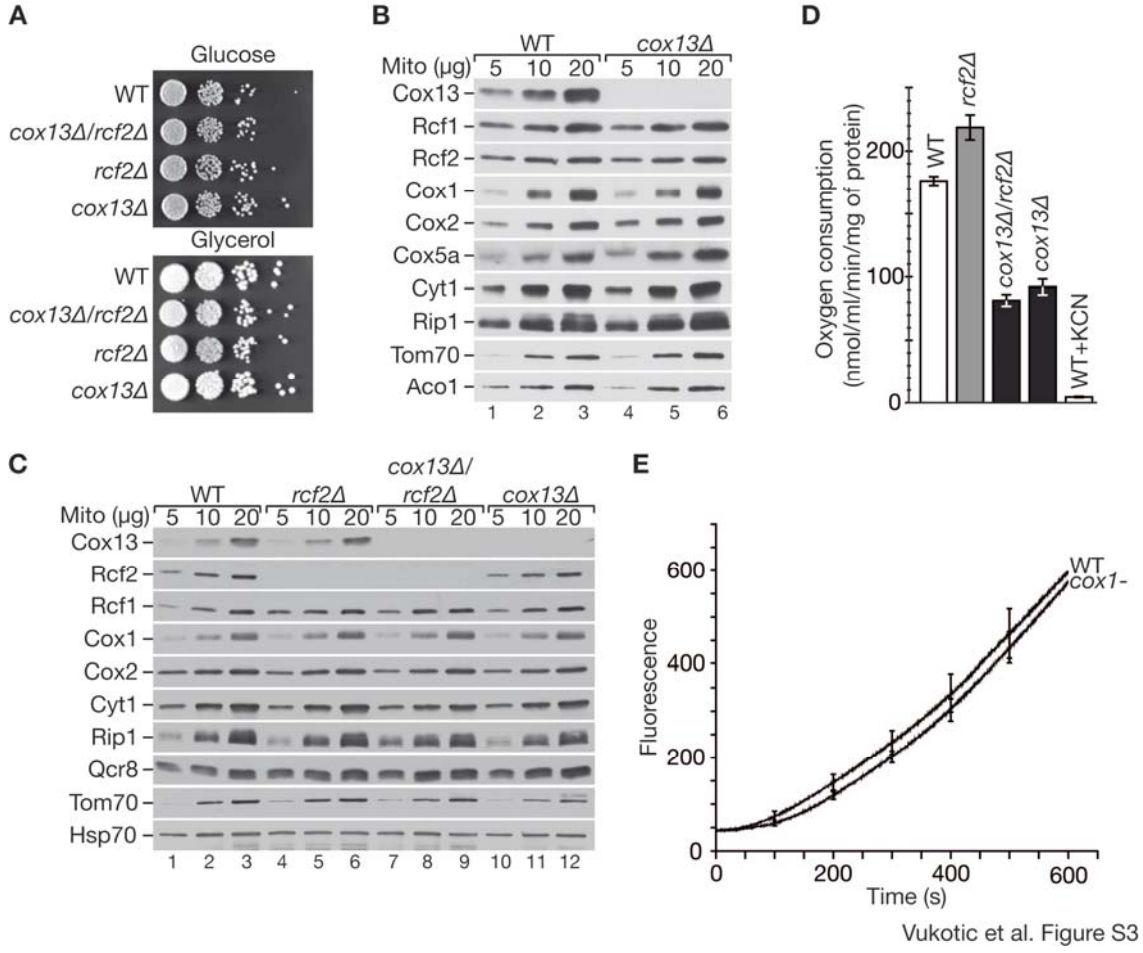

Figure S3. ***cox13Δ/rcf2Δ* phenotype is not distinct to that of *cox13Δ***

(A) Wild type (WT), *rcf2Δ*, *cox13Δ/rcf2Δ* and *cox13Δ* yeast cells were grown over night in liquid YPD media, washed, spotted in serial 10-fold dilutions on fermentable (glucose) and non-fermentable (glycerol) media and incubated at 30°C. (B) Isolated wild type (WT) and *cox13Δ* mitochondria were subjected to SDS-PAGE and analyzed by Western-blotting and immunodecoration with indicated antisera. (C) Mitochondria isolated from wild type (WT), *rcf2Δ*, *cox13Δ/rcf2Δ* and *cox13Δ* mitochondria were subjected to SDS-PAGE and analyzed by Western-blotting and immunodecoration with indicated antisera. (D) Oxygen consumption measurements of mitochondria isolated from indicated strains. (E) Mitochondrial ROS production in indicated strains. (D), (E) (SEM, n=3).

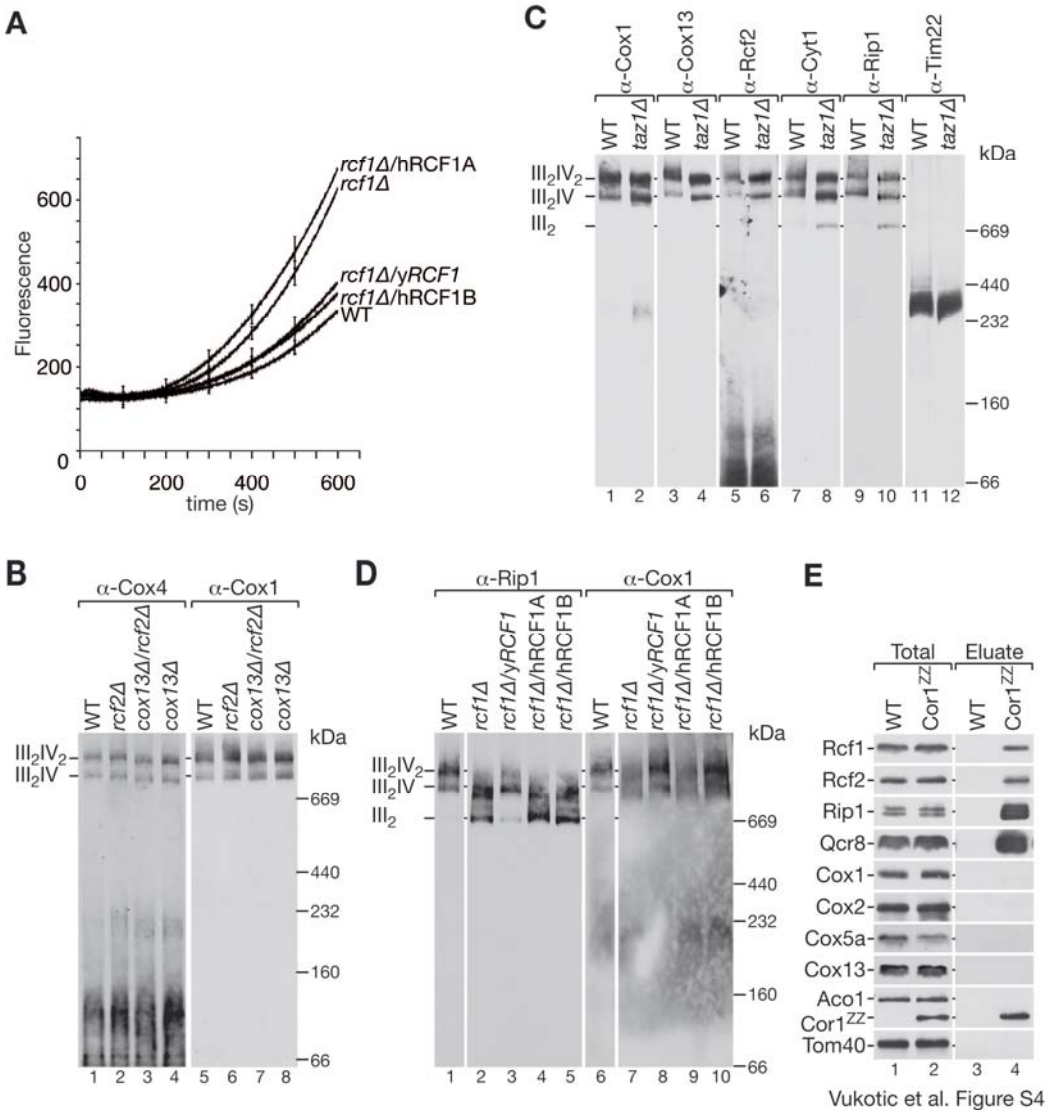

Figure S4. **hRCF1B complements the ROS phenotype in *rcf1Δ***

(A) ROS production in mitochondria isolated from indicated strains was assayed as described in Experimental Procedures. (SEM, n=3). (B) Mitochondria isolated from indicated strains were lysed in 1% digitonin buffer, analyzed by BN-PAGE and Western-blotting. (C) Mitochondria isolated from wild type (WT) and *taz1Δ* mitochondria were solubilized in 1% digitonin buffer, analyzed by BN-PAGE and Western-blotting. (D) Mitochondria isolated from indicated strains were lysed in 1% digitonin buffer, analyzed by BN-PAGE and immunodecoration. (E) $Cor1^{ZZ}$ – containing complexes were isolated from DDM-solubilized mitochondria via IgG chromatography, analyzed by SDS-PAGE and Western-blotting. Total 10%; Elution 100%.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast strains, growth conditions and isolation of mitochondria

Yeast strains used in this study are described below. For mitochondrial isolation, yeast deletion mutants and corresponding wild type cells were grown at 30°C in medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) galactose or 3% (v/v) glycerol as a carbon source. Mitochondria were isolated by differential centrifugation as described (Meisinger et al., 2006). For growth analysis, yeast cultures were adjusted to an OD_{600} of 1, diluted to a series of 1:10 and spotted onto agar plates containing 3% glycerol or 2% glucose as a carbon source. Alternatively, plates containing synthetic medium were used: 0.67% yeast nitrogen base (Difco, BD), 0.7 g/l drop-out mix of CSM-URA (MP biomedicals), supplemented with 2% glucose (SD) or 3% gylcerol (SG) (Reinhold *et al.,* 2011). For growth analysis under low oxygen conditions, plates were incubated in an INVIVO$_2$ 400 Hypoxic Workstation (Ruskinn Life Science), at 30°C, 1% oxygen, for 48 hours. Followed by an incubation at 30°C, 20% oxygen, for 48 hours.

To analyse the growth rate of yeast cells during hypoxia and normoxia, yeast cells were grown in a semisynthetic galactose medium, (SSG-TEA; per liter: 10 g of galactose, 3 g of yeast nitrogen base, 0.8 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of NaCl, 0.34 g of $MgSO_4$, 0.4 g of $CaCl_2$, 5 µg of $FeCl_3$, 0.1% Tween 80 (v/v)), supplemented with amino acids, nucleotides and 20µg/ml ergosterol, in a BIOSTAT Aplus (Sartorius) fermentor system. The temperature (30°C), pH (7.0) and stirring speed (300rpm) were controlled automatically by the fermentor system software. The dissolved oxygen concentration in the fermentor was monitored with a Clark type oxygen electrode (Hamilton). The fermentor was inoculated at a low cell concentration with mid-exponential precultures and grown either aerobically (20% oxygen) or hypoxic (~ 0% oxygen). To monitor the cell density, every four hours samples were taken and the OD_{600} was measured.

For complementation of the yeast *rcf1*Δ mutant, the *RCF1* open reading frame were amplified from yeast genomic DNA by PCR and cloned EcoRI/XhoI into pMaD1 yeast expression vector, derived from pUG35/pUG36 (Niedenthal et al.,

1996) or into p426-TEF yeast expression vector (Dualsystems Biotech). hRCF1A and hRCF1B coding sequences were amplified from HEK293T cDNA by PCR and cloned EcoRI/XhoI into pMaD1 or into p426-TEF. The expression vectors pMaD1, pMaD2 (pMaD1-*RCF1*), pMaD10 (pMaD1-hRCF1B), pMaD11 (pMaD1-hRCF1A), p426-TEF, pMaD12 (p426-TEF-*RCF1*), pMaD13 (p426-TEF-hRCF1B), pMaD14 (p426-TEF-hRCF1A), were transformed into the *rcf1*Δ strain. Resulting clones were cultured and supplied for growth analysis and mitochondria isolation.

Human mitochondria were isolated from HEK293T cells cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Gibco, Invitrogen) at 37°C and 5% CO_2 as previously described (Lazarou *et al.,* 2009; Reinhold *et al.,* 2011). Cells were harvested at 80-85% confluency in 1x PBS and 1 mM EDTA and homogenized in (0.1% BSA, 300 mM Trehalose, 10 mM HEPES-KOH pH 7.7, 10 mM KCl, 1 mM EDTA, 1 mM EDTA) (Yamaguchi *et al*., 2007). Mitochondria were isolated by differential centifugation of homogenized cells and concentrated in homogenization buffer without BSA.

Yeast strains used in this study

Yeast strains used in this study are derivatives of *S. cerevisisae* strain YPH499, except for $Cor1^{TAP}$ (BYB4741) (Open Biosystems, van der Laan *et al*., 2006). Deletions of *RCF1*, *RCF2*, *COX13* and *CYT1* were achieved by homologues recombination of *HISMX6* or *KANMX6* cassettes into the corresponding locus (Knop et al., 1999; Longtine et al., 1998). Generation of protein A-tagged strains was performed by chromosomal integration in YPH499. *cox4Δ* was described previously (Frazier et al., 2006). YPH499 - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801* (Sikorski and Hieter, 1989); BY4741 - *Mat a, his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0* (Euroscarf); 777-A3 - *Mat α, ade1 op1* (Netter et al., 1982); *$Cor1^{TAP}$- Mat a, his3-Δ1 leu2Δ0 met15Δ0 ura3Δ0; cor1*::*cor1-TAP* (Euroscarf); AFY10 (*cyt1Δ*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YOR065w*::*HISMX6* (This study); AFY11 (*cox4Δ*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YGL187c*::*HISMX6* (Frazier et al., 2006); MVY2 (*rcf2Δ*) - *Mat a, ade2-101 ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YNR018w*:: *HISMX6* (This study); yMaD2 (*rcf1Δ*) - *Mat a, ade2-101 his3-*

Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YML030w:: *loxP* (This study); MVY5 (*cox13Δ*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YGL191w*:: *HISMX6* (This study); HCY01 (*cox13^{ZZ}*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; cox13*::*cox13-ZZ* (This study); HCY04 (*cox4^{ZZ}*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; cox4*::*cox4-ZZ* (This study); MVY9 (*cor1^{ZZ}*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; cor1*::*cor1-ZZ* (This study); G421 (*cox1^{-}*) - *Mat α, ade1 op1; cox1-G421* (Netter et al., 1982); DaMY02 (*taz1Δ*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YPR140w*:: *kanMX4* (Brandner et al., 2005); MVY13 (*cox13Δ/rcf2 Δ*) - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YNR018w*:: *HISMX6; YGL191w*::*kanMX4* (This study); *rcf1Δ+*p*RCF1* - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YML030w*:: *loxP* + p426-TEF-*RCF1* (This study); *rcf1Δ+*phRCF1A - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YML030w*:: *loxP* + p426-TEF-hRCF1A(This study); *rcf1Δ+*phRCF1B - *Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; YML030w*:: *loxP* + p426-TEF-hRCF1B (This study).

Mass spectrometry analysis

Following acetone precipitation of proteins in eluates from IgG-Sepharose affinity purification experiments of solubilised wild type (WT) mitochondria or mitochondria carrying Cor1^{TAP}, tryptic digests were performed in 60% (v/v) methanol and 20 mM NH_4HCO_3. Peptide mixtures were analysed by nano-HPLC/ESI-MS/MS as described (Kaller et al., 2011). For peptide and protein identification, mass spectrometric data were searched against a decoy version of the *Saccharomyces* Genome Database using Mascot (www.matrixscience.com). The ProteinExtractor Tool (version 1.0) in ProteinScape (version 1.3, Bruker Daltonics) was used to assemble proteins based on peptide identifications. Proteins were identified based on at least one unique peptide and a false discovery rate of 5% was applied.

For label-free quantitative analysis, spectral counts (SC) of proteins identified in six independent experiments were determined using in-house written software. A one-sided Student's t-test was performed. Proteins with a p-value < 0.05 and a Cor1^{TAP}/WT ratio > 5 were considered as potential interaction partners of Cor1p

or found to non-specifically copurify with Cor1^{TAP} during affinity purification (AP). Components of respiratory chain supercomplexes identified by AP-MS experiments had to be additionally found in two out of three Blue-Native PAGE analyses using Cor1^{TAP} as bait.

Determination of mitochondrial enzymes activities

Enzymatic activities were assayed spectrophotometrically. Malate dehydrogenase activity was determined by following the oxaloacetate-dependent oxidation of NADH at 340 nm. Assay buffer contained 100 mM potassium phosphate buffer, 0.1 mM NADH and 0.2 mM oxaloacetate. The reaction was initiated by addition of Triton X-100 lysed mitochondria. The extinction coefficient of NADH at 340 nm was 6.3 mM^{-1} cm^{-1}.

Cytochrome *c* oxidase activity was followed by measuring the decrease in absorbance at 550nm. Assay system contained 40 mM potassium phosphate buffer, pH 7.5 and 0.02% reduced cytochrome *c*. Reactions were started by addition of an appropriate amount of Triton X-100 lysed mitochondria. Cytochrome *c*, obtained from Sigma, was reduced by addition of dithionite. Excess dithionite was removed by aeration of the solution. NADH – cytochrome *c* reductase activity was assessed by measuring the increase in absorbance at 550 nm. Assay system contained 40 mM potassium phosphate buffer, pH 7.5, 0.02% oxidized cytochrome *c*, 0.5 mM NADH and 0.1 mM KCN (Tzagoloff et al., 1975). The reaction was initiated by addition of mitochondria. Concentrations of reduced/oxidized cytochrome *c* were determined using the extinction coefficient at 550 nm of 21.84 mM^{-1} cm^{-1}.

Aconitase activity was assayed by measuring the conversion of *cis*-aconitate to isocitrate (Fansler and Lowenstein, 1969) at 240 nm as a function of time. Reaction was performed in quartz cuvette and reaction buffer contains 100 mM NaCl, 20 mM TRIS pH 7.4, 0.1 mM *cis*-aconitate (Sigma). The reaction was started by addition of mitochondria solubilized in 0.5% Triton X-100. The extinction coefficient of *cis*-aconitate at 240 nm was 4.28 mM^{-1} cm^{-1}.

Oxygen consumption measurements in isolated mitochondria

Respiration efficiency was assayed in isolated yeast mitochondria using a Clark type polarographic oxygen electrode from Hansatech Instruments. Measurements were performed at 30°C in a final reaction volume of 2 ml, in a thermostated chamber with a close-fitting lid, in a respiratory media (0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM K_2HPO_4 pH 7.4) in the presence of 2 mM NADH, as a substrate, and 2 mM ADP (causes sudden burst in oxygen uptake as the ADP is converted to ATP) (Barrientos et al., 2009).

ROS-generating activity

The ROS-generating activities were monitored using H_2DFFDA (Invitrogen) (Giorgio et al. 2005). Fluorescence [λ(ex)=498nm, λ(em)=525nm] changes of 10 μM H_2DDFDA in 250 μl assay buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) were recorded with HITACHI F-7000 fluorescence spectrophotometer at 0.2 seconds intervals for 10 minutes.

RT-PCR analysis

RNAs were extracted from various organs of FVB mice. Reverse transcription (RT) was performed with 2 μg of RNA and a first strand cDNA synthesis kit (Fermentas, St. Leon-Rot9. Quantification of mRNA levels was performed using 1 μl of the cDNA reaction and SYBR Green qPCR reaction kit (Clontech) in combination with a MX3000P light cycler (Stratagene). The following primers were used for quantification mS12 for 5'-GAAGCTGCCAAGGCCTTAGA-3', mS12 rev 5'-AACTGCAACCAACCACCTTC-3', mRCF1a for GCCCAGGCCTCACGTCTGACA, mRCF1a rev GAGTCTTCCGAGACGCGGGGT, mRCF1b for CCTATAGGCTGTCTGGGGACGG, mRCF1b rev CATGGCGCTTTAAGACCCGGC.

SUPPLEMENTAL REFERENCES

Barrientos, A., Fontanesi, F., and Diaz, F. (2009). Evaluation of the mitochondrial respiratory chain and oxidative phosphorylation system using polarography and spectrophotometric enzyme assays. Curr Protoc Hum Genet. *63*, 19.3.1-19.3.14.

Brandner, K., Mick, D., Frazier, A., Taylor, R., Meisinger, C., and Rehling, P. (2005). Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth Syndrome. Mol Biol Cell *16*, 5202-5214.

Fansler, B., and Lowenstein, J.M. (1969). Methods Enzymol. 13, 26-30.

Frazier, A., Taylor, R., Mick, D., Warscheid, B., Stoepel, N., Meyer, H., Ryan, M., Guiard, B., and Rehling, P. (2006). Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. J Cell Biol *172*, 553-564.

Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., Pelliccia, G., Luzi, L., Minucci, S., Marcaccio, M., Pinton, P., Rizzuto, R., Bernardi, P., Paolucci, F., and Pelicci, P.G. (2005). Cell 122, 221-233.

Kaller, M., Liffers, S.T., Oeljeklaus, S., Kuhlmann, K., Röh, S., Hoffmann, R., Warscheid, B., and Hermeking, H. (2011). Mol Cell Proteomics. 10, Epub. Ahead of print.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast *15*, 963-972.

Longtine, M.S., McKenzie, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953-961.

Lazarou, M., Smith, S.M., Thorburn, D.R., Ryan, M.T., and McKenzie. M. (2009). Assembly of nuclear DNA-encoded subunits into mitochondrial complex IV, and their preferential integration into supercomplex forms in patient mitochondria.FEBS J. 276, 6701–6713.

Meisinger, C., Pfanner, N., and Truscott, K. (2006). Isolation of yeast mitochondria. Methods Mol Biol *313*, 33-39.

Netter P, Carignani G, Jacq C, Groudinsky O, Clavilier L, Slonimski PP. (1982). The cytochrome oxidase subunit I split gene in *Saccharomyces cerevisiae*: genetic and physical studies of the mtDNA segment encompassing the 'cytochrome-*b*-homologous' intron. Mol Gen Genet *188*: 51–59

Niedenthal, R.K., Riles, L., Johnston, M., and Hegemann, J.H. (1996). Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12, 773-786.

Reinhold, R., Bareth, B., Balleininger, M., Wissel, M., Rehling, P., and Mick, D.U. (2011). Mimicking a SURF1 allele reveals uncoupling of cytochrome c oxidase assembly from translational regulation in yeast. Hum Mol Genet *20*, 2379-2393.

Sikorski, RS and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Tzagoloff, A., Akai, A., and Needleman, R.B. (1975). Assembly of the mitochondrial membrane system. Characterization of nuclear mutants of *Saccharomyces cerevisiae* with defects in mitochondrial ATPase and respiratory enzymes. J. Biol. Chem. 250, 8228-8235.

van der Laan, M., Wiedemann, N., Mick, D., Guiard, B., Rehling, P., and Pfanner, N. (2006). A role for Tim21 in membrane-potential-dependent preprotein sorting in mitochondria. Curr Biol *16*, 2271-2276.

Yamaguchi, R., Andreyev, A., Murphy, A.N., Perkins, G.A., Ellisman, M.H., and Newmeyer, D.D. (2007). Cell Death Differ. 14, 616–624.