Supplemental Materials

Molecular Biology of the Cell

Su et al.

Supplementary material

Table S1. Genotypes and Sources of *Saccharomyces cerevisiae* Strains

Strain	Genotype	mtDNA	Source
W303-1A	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+	A
W303-1B	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+	A
MR6	**MAT**a *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ (S288C)	Rak *et al.* 2007
MRS-3A	**MAT**a *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ (S288C)	(McStay *et al.*, 2013)
MRS-3B	**MAT**α *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ (S288C)	(McStay *et al.*, 2013)
aDFKρ^o	**MAT**a *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^o	(McStay *et al.*, 2013)
DFKρ^o	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^o	(McStay *et al.*, 2013)
MRSI^o/COX1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3*	Intronless with *COX1-HAC*	(McStay *et al.*, 2013)
MRS/COX2-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3*	*COX2-HAC*	This study
aMRSI^o/COX3-HAC	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3*	Intronless with *COX3-HAC*	This study
aMRS/COX3-HAC	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3*	*COX3-HAC*	This study
MRSI^o/COX3-pH	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3*	Intronless with *COX3-pH*	This study

W303/COX4-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3 trp1::pG66/ST19*	ρ^+	(McStay *et al.*, 2013b)
aW303/COX7-HAC	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox7::URA3 trp1::pG199/ST10*	ρ^+	(McStay *et al.*, 2013b)
aW303ΔCOX7/COX1-HAC	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox7::URA3*	*COX1-HAC*	(McStay *et al.*, 2013b)
W303/COX9-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox9::HIS3 ura3::pG16/ST16*	ρ^+	(McStay *et al.*, 2013b)
aW303/COX12-HAC	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox8::HIS3 ura3::pCOX12/ST4*	ρ^+	(McStay *et al.*, 2013b)
W303/COX13-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox13::HIS3 trp1::pCOX13/ST4*	ρ^+	(McStay *et al.*, 2013b)
aW303/RCF1-HAC	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rcf1::HIS3 trp1::pRCF1/ST4*	ρ^+	(McStay *et al.*, 2013b)
W303ΔRCF1/COX4-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rcf1::HIS3 trp1::pG66/ST19*	ρ^+	W303/COX4-HAC x aW303/RCF1-HAC
aMRSΔPET111ρ^0	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1arg8::HIS3 pet111::HIS3*	ρ^0	(McStay *et al.*, 2013b)
W303ΔPET111/COX4-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet111::HIS3 cox4::URA3 trp1::pG66/ST19*	ρ^+	W303/COX4-HAC x aMRSΔPET111ρ^0
aMRSΔPET494ρ^0	**MATa** *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet494::HIS3*	ρ^0	(McStay *et al.*, 2013b)
aW303ΔPET494/ COX4-HAC	**MATa** *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3- 1 pet494::HIS3 trp1::pG66/ST4*	ρ^+	W303/COX4-HAC x aMRSΔPET494ρ^0
aMRSΔMSS51/ρ^0	**MATa** *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 mss51::HIS3*	ρ^0	(McStay *et al.*, 2013b)

	trp1::pG66/ST4		
W303ΔMSS51/COX4-HAC	**MAT**α *ade2-1 arg8::HIS3 his3-1,15 leu2-3,112 trp1-1 ura3-1 mss51::HIS3*	ρ^+	W303/COX4-HAC x aMRSΔMSS51ρ^0

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Table S2. Sequences of primers

Primer name	Primer sequence
	Primers used to amplify *COX3* 5' and 3' regions
cox3-9	ggcgagctcgaagatattatcaatgatttatattaa
cox3-5n	ggcggatccaaatttattgttatatatatattatat
cox3-7	ggccgatccggctatagaattatatatcta
cox3-10	ggctctagacatattaaaaaagtcccgctggag
	Primers used to introduce the poly histidine tag at the C-terminus
cox3-9n	ggcggtaccttattaatgatgatggtgatgatggactcctcatcagtagaagac
cox3-10n	ggcggtaccggctatagaattatatatctaaatg
	Primers used to indtroduce the protein C followed by HA tag at the N-terminus
cox3-20	ggcgagctcgaataaataatccggtcgaaagagatattaattcg
cox3-21	ggcatcgattaatcttggatctacttgatcttccataaatttattgttatatatatattatatatataatataattaaat
cox3-15	ggcatcgatggtaaaggtggttacccatacgacgtcccagactacgctacacatttagaaagaagtagacatcaa
cox3-4	ggcctgcagtcctctctcatggaggtt

Fig. S1

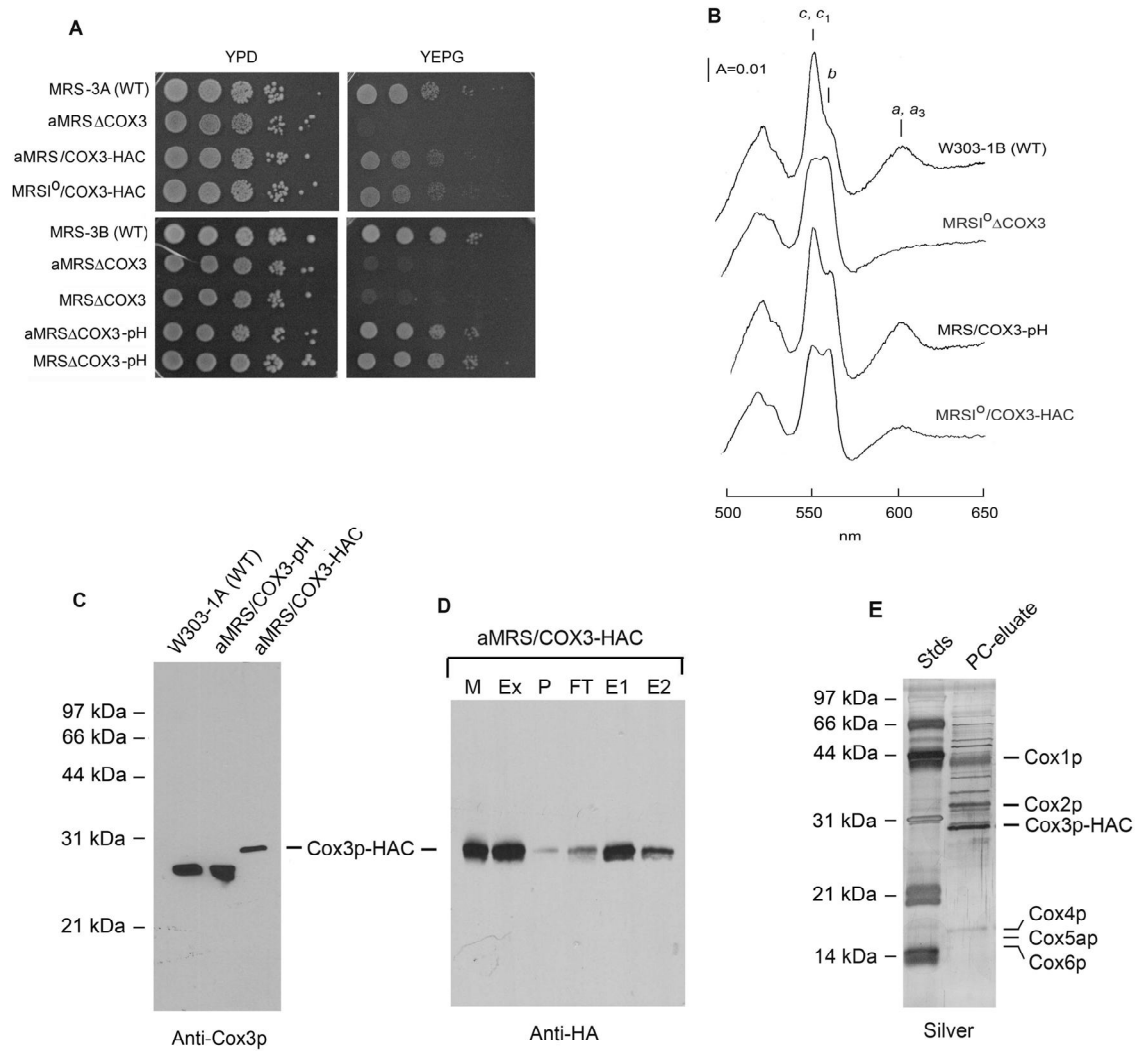

Fig. S1**.** Properties of yeast expressing Cox3p with a C-terminal poly histidine or N-terminal HAC tag. (A) The parental respiratory competent strains MRS-3A and MRS-3B, the *cox3* null mutants aMRSΔCOX1 and the recombinant strains MRS/COX3-pH, aMRS/COX3-pH, MRSI^{o}/COX3-HAC and aMRS/COX3-HAC expressing Cox3p with a single poly histidine and a double HA plus protein C tag , respectively, were serial diluted and spotted on YPD and YEPG media and incubated at 30^{o}C for 2 days. (B**)** Mitochondria of the respiratory competent strain W303-1B, of the *cox3* null mutant MRSI^{o}ΔCOX3 and of the recombinants MRS/COX3-pH and MRSI^{o}/COX3-HAC expressing Cox3p with the poly histidine and HAC tags, respectively, were extracted with 1% potassium deoxycholate in the presence of 1M KCl (Tzagoloff *et al.*., 1975). The difference spectra of the extract reduced with dithionite and oxidized with ferricyanide were recorded at room temperature. The positions of the α absorption bands of cytochrome *a*, a_3, *b*, *c*, and c_1 are marked. (C) Total mitochondrial proteins (25 μg) from the indicated strains were

separated on a 12% polyacrylamide gel by SDS-PAGE, transferred to a nitrocellulose membrane and probed with a monoclonal antibody against Cox3p (Mitosciences, Eugene, OR). Following reaction with a peroxidase coupled rabbit anti mouse γ globulin antibody Cox3p was visualized with with SuperSignal chemiluminescent substrate kit (Pierce, Rockford, IL). (D) Purification of COX from aMRS/COX3-HAC. Mitochondria were suspended in 0.6M sorbitol, 20 mM Hepes, pH 7 at a protein concentration of 10 mg/ml and 300 μl was extracted with 0.1 volumes of 10% lauryl maltoside. The soluble fraction obtained after centrifugation at 100,000 x g_{av} for 10 min was added to 100 μl of packed Anti Protein C Affinity Matrix (Roche) that had been washed with binding buffer (10 mM Tris-Cl, pH 7.5, 200 mM NaCl, 2 mM $CaCl_2$). The mixture was rotated at 4^{o}C for 90 min and centrifuged 1,000 x g_{av} for 1 min. Unbound proteins were removed and the beads were washed 3 times with 1 ml of binding buffer containing 0.2% lauryl maltoside. The beads were eluted for 30 min successively two times with 100 μl of elution buffer (10 mM Tris-Cl, pH 7.5, 200 mM NaCl, 0.5% lauryl maltoside, 7 mM EDTA). Samples of the mitochondria (M), extract (Ex), pellet after extractions (P), proteins that were not adsorbed to the beads (FT) and proteins eluted from the beads (E1, E2), adjusted for volume were separated by SDS-PAGE on a 15% polyacrylamide gel and Cox3p-HAC detected as in C. (E) The eluate from the protein C antibody beads (PC eluate) and protein standards (stds) were separated on a 17% polyacrylamide gel by SDS-PAGE and stained with silver.

Fig. S2

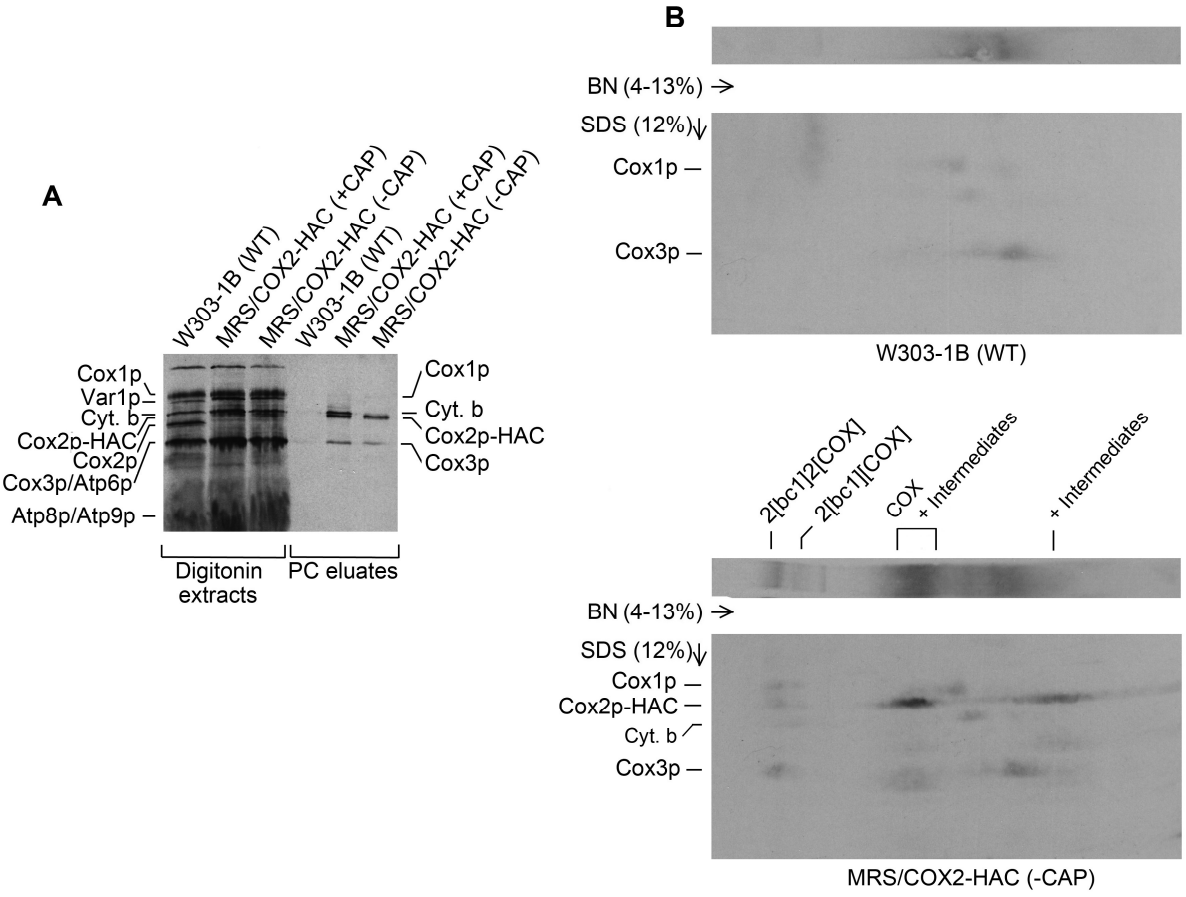

Fig. S2. Co-immunoprecipitation of newly translated Cox1p, Cox2p-HAC and Cox3p with supercomplexes. (A) Mitochondria (50 μg protein) from the wild type W303-1B and from MRS/COX2-HAC, a strain that expresses Cox2p with a C-terminal HAC tag, were labeled for 20 min with ^{35}S-methionine plus ^{35}S-cysteine. The mitochondria were extracted with and equal volume of 4% digitonin in extraction buffer (Wittig *et al*, 2006). The digitonin extracts were further purified on protein C antibody beads as described previously (McStay *et al*, 2013). Equivalent samples of the digitonin extracts and eluates from the protein C beads (PC eluates) were separated on a 17% polyacrylamide gel. Proteins were transferred to nitrocellulose and exposed to X-ray film. The radiolabeled mitochondrial gene products are identified in the margins (B) The remaining eluate (90% of total) was split in half and separated by BN-PAGE on a 4-13% polyacrylamide gel. Strips of the BN-PAGE gel were layered on a 12% polyacrylamide gel and separated by SDS-PAGE. Proteins separated in a single dimension and in two dimensions were transferred to a PVDF membrane and exposed to X-ray film. The migration of Cox1p, Cox2p-HAC and Cox3p in the second SDS-PAGE dimension are indicated in the left-hand margin.

Fig. S3

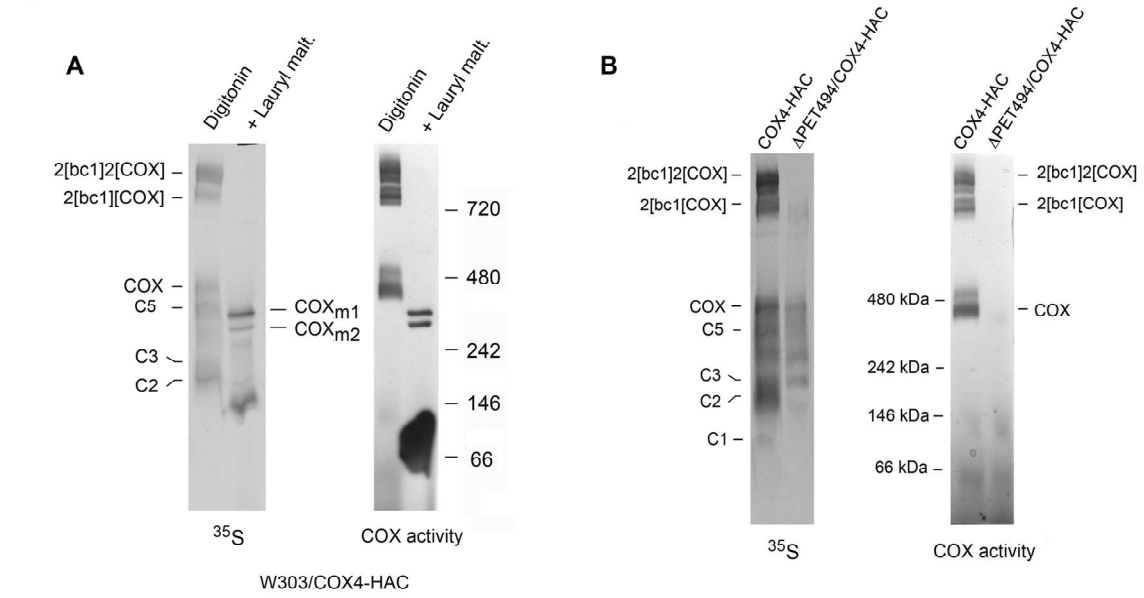

Fig. S3. In-gel cytochrome oxidase activities in respiratory competent and pet494 mutant strains expressing Cox4-HAC. (A) Mitochondria of W303/COX4-HAC were labeled for 20 min with ^{35}S-methionine plus ^{35}S-cysteine and separated in four equal halves. Two samples were applied directly to a 4-13% polyacrylamide gel without further treatment (digitonin). To the other two samples was added 0.1 volumes of 10% lauryl maltoside prior to application to the gel (Lauryl malt). Following separation by BN-PAGE, one half of the gel (left panel) containing the treated and untreated extract was transferred to a PVDF membrane and exposed to X-ray film. The other half of the gel (right panel) was stained for COX activity in a solution containing 0.5 mg/ml DAB and 1 mg/ml horse heart cytochrome *c* for several hours. (B) Mitochondria (50 μg protein) from W303/COX4-HAC (COX4-HAC) and W303ΔPET494/COX4-HAC (ΔPET494/COX4-HAC were labeled as in (A). Translation was terminated by addition of puromycin and excess cold methionine plus cysteine. The mitochondria were extracted with 1.2 volumes of 4% digitonin and samples of the supernatants after centrifugation were purified on protein C antibody beads as described previously (McStay et al, 2013). Equal halves of the eluates from the antibody beads were separated by BN-PAGE on a 4-13% polyacrylamide gel. One half of the gel (left panel) was transferred to a PVDF membrane and exposed to X-ray film. The other half of the gel (right panel) was stained for COX activity as in (A).

Fig. S4

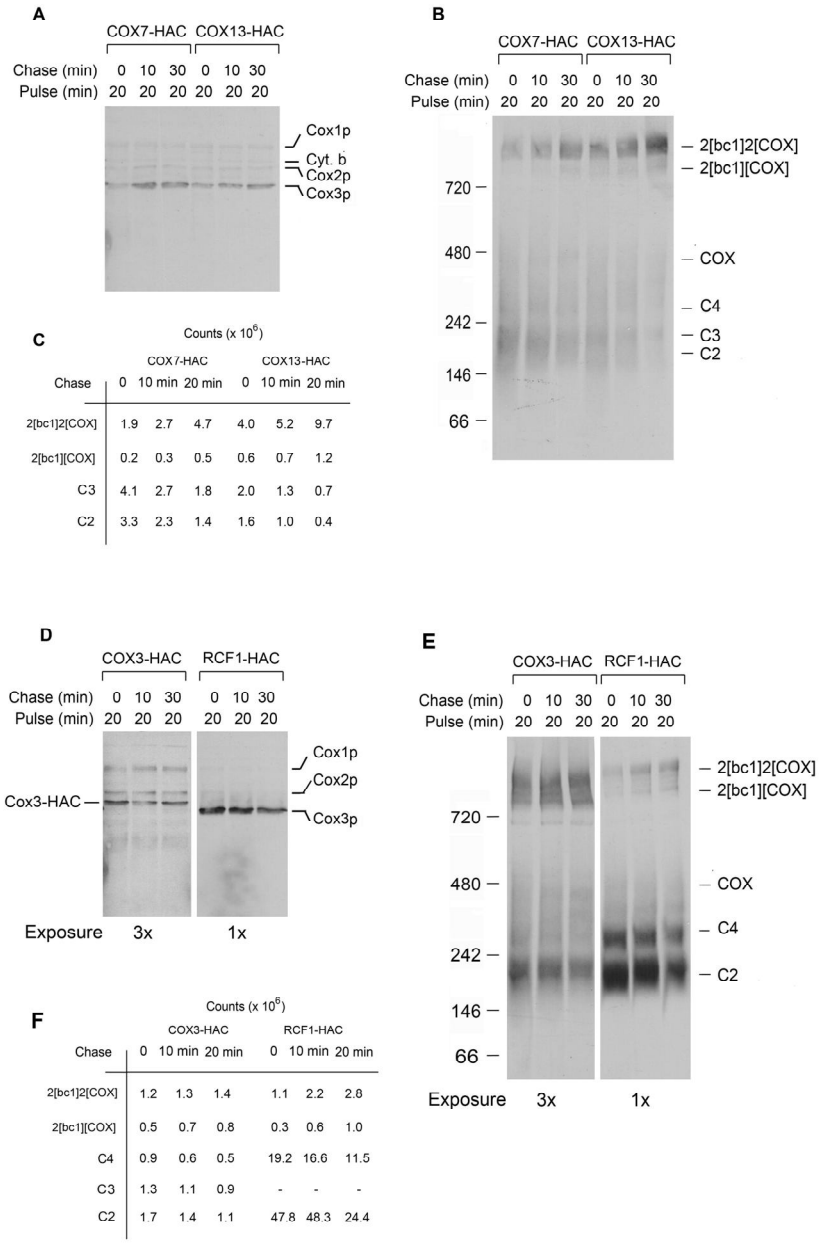

Fig. S4. Pulse-chase analysis of mitochondria expressing Cox7p-HAC, Cox13p-HAC, Cox3p-HAC and Rcf1p-HAC. (A) Mitochondria were labeled for 20 min and samples taken 0, 10 and 30 min after addition of puromycin and excess cold methionine plus cysteine. Mitochondria were extracted with digitonin and affinity purified on protein C antibody beads. Samples of the extracts and eluates from the beads were separated by SDS-PAGE on a 17% polyacrylamide, transferred to nitrocellulose and exposed to X-ray film. (B) The eluates from (A) were separated on a 4-13% polyacrylamide gel, transferred to a PVDF membrane and exposed to X-ray film. (C) Same as (A) except that the tagged proteins were Cox3p and Rcf1p. (D) Samples of eluates from (C) were processed as in (B).

Fig. S5

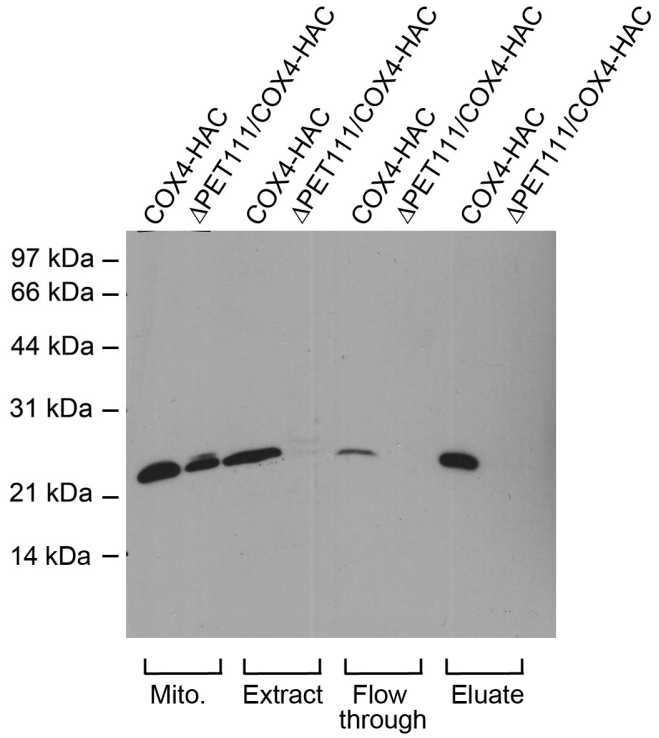

Fig. S5. Fractionation of Cox4p-HAC from a respiratory competent and from a *pet111* mutant. Mitochondria from strains W303/COX4-HAC (*COX4*-HAC) and W303ΔPET11/COX4-HAC (ΔPET11/COX4-HAC) were extracted with digitonin, and fractionated on protein C antibody beads as in Fig. S1D. Mitochondria (Mito), the digitonin extract (extract) the fraction that was not adsorbed on protein C beads (Flow through) and the eluate from the beads were separated by SDS-PAGE on a 15% polyacrylamide gel, transferred to nitrocellulose and probed with an antibody against the HA tag as in Fig. S1D. All the fractions were normalized to the volume of the starting mitochondria.

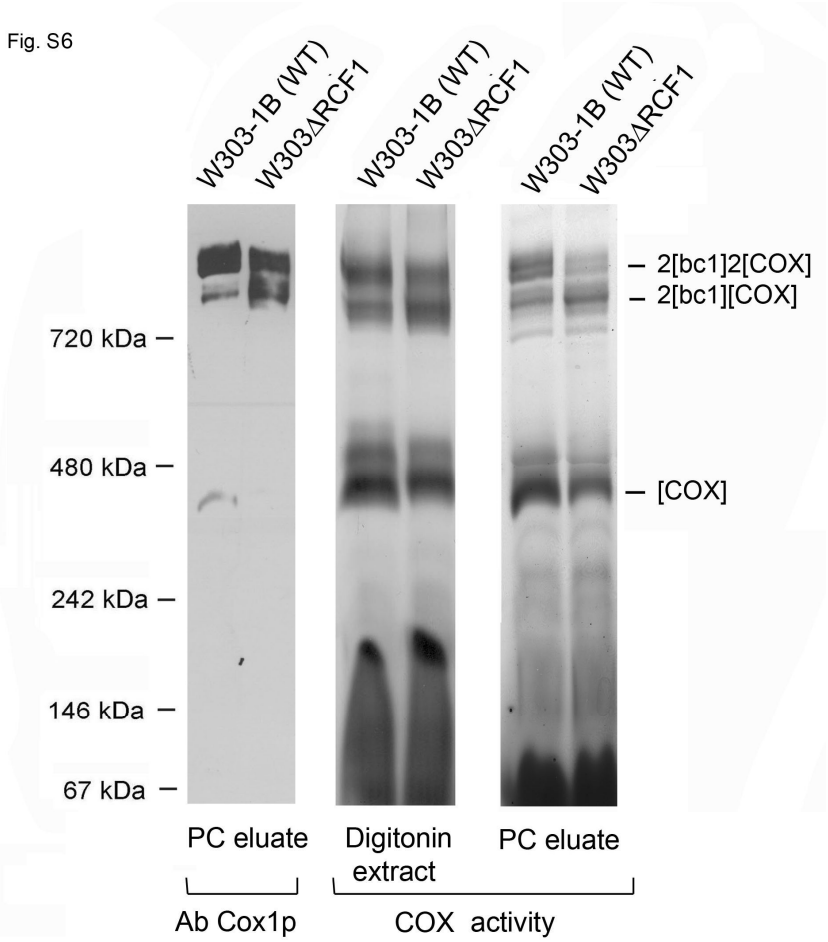

Fig. S6. Western and enzyme analyses of COX in wild type and an *rcf1* null mutant. Mitochondria (50 μg protein) from the indicated strains were extracted with digitonin and part of the extract purified on protein C antibody beads as in Fig. S2A). The digitonin extracts and eluates (PC eluates) were separated by BN-PAGE on a 4-13% polyacrylamide gel and assayed for COX activity as in Fig. S3A. The eluates were also transferred to a PVDF membrane and probed with a monoclonal antibody against Cox1p (Mitosciences, Eugene, OR) as in Fig. S1C.

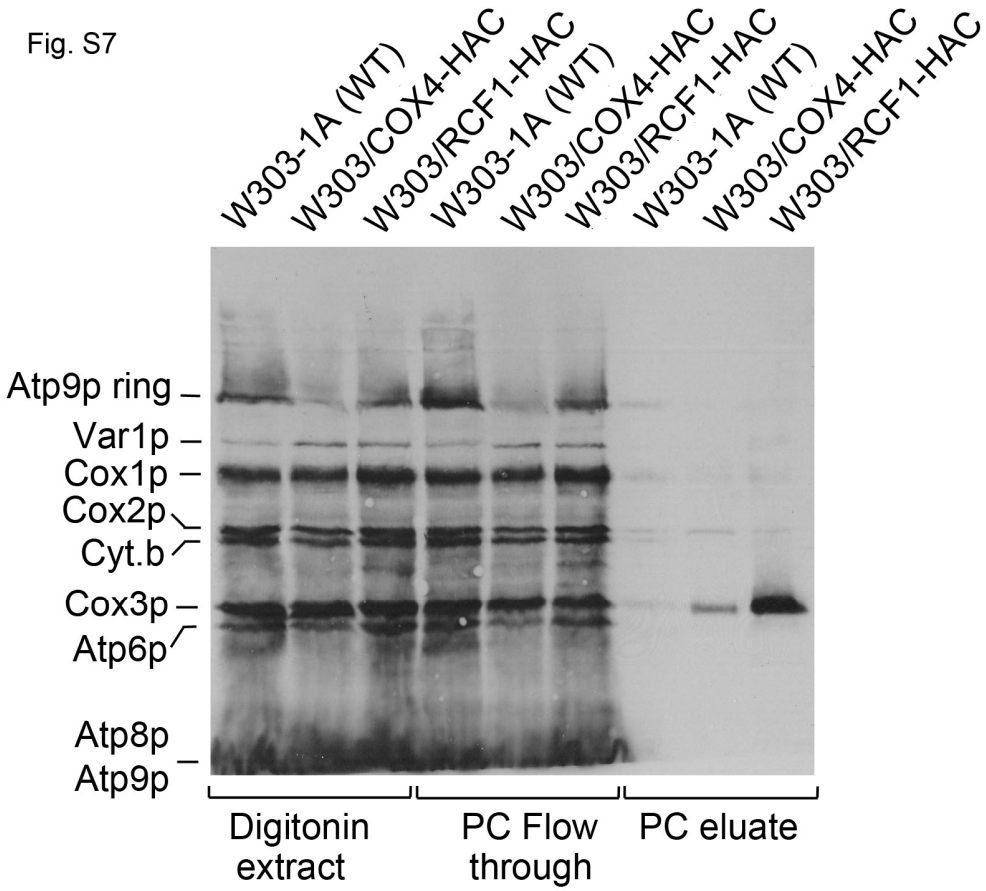

Fig. S7. Recovery of Cox3p by co-immuno adsorption with Cox4p-HAC and Rcf1-HAC. Mitochondria from the indicated strains were labeled, extracted with digitonin and purified on protein C antibody beads as in Fig. S2. Equivalent samples of the extracts, proteins that did not adsorb to the antibody beads (PC Flow through) and twice as much of the fraction eluted from the beads (PC eluate) were separated by SDS-PAGE on a 12% polyacrylamide gel prepared in the presence of 6M urea and 25% glycerol to separate Cox3p from Atp6p.

References

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Tzagoloff A, Akai A, Needleman RB, Zulch G (1975). Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. J Biol Chem 250**:** 8236-8242.

Wittig I, Braun HP, Schagger H (2006) Blue native PAGE. Nat Protoc 1: 418-428.