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
W303-1A	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+	A
aW303I^o	**MATa** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	ρ^+ (intronless)	Barros *et al*, 2006
MR6	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (S288C)	Rak *et al.* 2007
MRS-3A	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (S288C)	This study
MRS-3B	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (S288C)	This study
aMRSI^o	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (intronless)	This study
MRSI^o	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (intronless)	This study
DFK160	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52*	ρ^o	Steele *et al*, 1996
DFKρ^o	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^o	This study
DFKΔCOX1ρ^-	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^- (pCOX1/ST2)	This study
DFKΔCOX1-HAρ^-	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^- (pCOX1/ST10)	This study
DFKΔCOX1-HAHρ^-	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^- (pCOX1/ST11)	This study
DFKΔCOX1-HACρ^-	**MAT**α *kar1-1 ade2-10 arg8::URA3 leu2Δ ura3-52 lys2*	ρ^- (pCOX1/ST12)	This study
MRSI^{o}ΔCOX1	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (intronless, *Δcox1::ARG8^m*)	This study
aMRSI^{o}ΔCOX1	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ (intronless, *Δcox1::ARG8^m)*	This study
MRS/COX1-HAH	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^+ *COX1-HAH*	This study

(Table I cont)

MRSI^{o}/COX1-HA	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^{+} *COX1-HA*	This study
MRI^{o}/COX1-HAH	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^{+} (intronless, *COX1-HAH*)	This study
aMRSI^{o}/COX1-HAC	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^{+} (intronless, *COX1-HAC*)	This study
aMRS/COX1-HAC	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3*	ρ^{+} (*COX1-HAC*)	This study
W303ΔCOX4-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3 leu2::COX4-HAC*	ρ^{+}	This study
W303ΔCOX5a-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox5::HIS3 trp1::COX5-HAC*	ρ^{+}	This study
W303ΔCOX6-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3 trp1::COX6-HAC*	ρ^{+}	This study
W303ΔCOX9-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox9::HIS3 trp1::COX9-HAC*	ρ^{+}	This study
W303ΔSHY1-CH	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 shy1::HIS3 leu2::SHY1-CH*	ρ^{+}	This study
W303ΔCOX14-CH	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1cox14::trp1 URA3::COX14-CH*	ρ^{+}	This study
W303ΔMSS51-CH	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 mss51::HIS3 URA3::MSS51-CH*	ρ^{+}	This study
W303ΔCOA1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coa1::HIS3 URA3::COA1-HAC*	ρ^{+}	This study
W303ΔCOX5a/COX1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox5a::HIS3*	ρ^{+} (*COX1-HAC*)	This study
W303ΔPET494/COX1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet494::HIS3*	ρ^{+} (*COX1-HAC*)	This study
W303ΔPET111/COX1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet111::HIS3*	ρ^{+} (*COX1-HAC*)	This study

W303ΔSHY1/COX1-HAC	**MAT**α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 shy1::HIS3*	ρ^+ (*COX1-HAC*)	This study
aMRSI^{o}ΔCBP6/COX1-HAC	**MAT**a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 arg8::HIS3 cbp6::LEU2*	ρ^+ (introless, *COX1-HAC*)	This study

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Table S2 Primers used to construct different *cox1* alleles and genes expressing tagged proteins

Primer name	Primer sequence (5' -> 3')
	Primers for construction of the *cox1* null allele
COX1-1	GTCGAGCTCAAGTAATATATAATATTAAATAATAAG
COX1-2	GTCGGATCCTTTTACTTTTTTTTTATATATTAATTTTAATAATATTATTATTATTATATTC
COX1-3	GTCGGATCCGTTATAAAATTTAATTATTTAC
COX1-4	GTCTCTAGATACAATTAATTTAATCCGTTT
	Primers used to construct COX1-HA, COX1-HAH and COX1-HAC alleles
COX1-11	GGCGGATCCTGCAGCTAATTCTAATCTAAT
COX1-12	GGCGAATTCGGTACCAAGTAATATATAATATTAAATAATAAG
COX1-13	GGCGTGCACCTGGTTCACAATATTTAC
COX1-16	GGCGGTGACCCAATCTTATACGAGCATTTATTTTGATTCTTTGGACACCCTGAAGTA
COX1-HA	GGCGGATCCTTAAGCGTAGTCTGGGACGTCGTATGGGTAAGATTGTACAGCTGGTGTATT
COX1-H	GGCGGATCCTTAATGATGATGATGATGATGACCTCCAGCGTAGTCTGGGACGTCGTA
COX1-C	GGCGGATCCTTACTTACCATCGATTAACCGTGGATCTACCTGATCTTCACCTCCAGCGTAGTCT GGGACGTCGTA
	Primers used to construct nuclear gene products with HA and HAC tags
COA1-5'	AAGAGATCTTGGCCGTGCCCTTATCATCGT
COA1-HA	GGCGTCGACTCAAGCGTAGTCTGGGACGTCGTATGGAATTGGTGTCTTGGTATTTTC
COA1-C	GGCGAGCTCTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTG GGACGTCGTATGG
COX4-5'	GGCGAGCTCGCTTCTTCCAAGTATAAACAC
COX4-HA	GGCGGATCCTCAAGCGTAGTCTGGGACGTCGTATGGGTAGTGATGGTGGTCATCATTTG
COX4-C	GGCGGATCCTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTG GGACGTCGTATGG
COX5a-5'	GGCGAATTCCCTACGCTTCTAAATAG
COX5a-HA	GGCGACCTCTCAAGCGTAGTCTGGGACGTCGTATGGGTATTTAGATTGGACCTGAGAATA
COX5a-C	GGCGAGCTCTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTG GGACGTCGTATGG
COX6-5'	GGCGAGCTCCATACGAGCCAATCAG
COX6-HA	GGCGGTACCGGTTCAAGCGTAGTCTGGGACGTCGTATGGGTAAGAAGAGCTTGGAAATAGCTC
COX6-C	GGCCTGCAGTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTG GGACGTCGTATGG
COX9-2	GGCCTGCAGTCAATGGTGATGGTGATGGTGGTTCTCTTGCTTTTTCCTCTC
COX9-HA	GGCCTGCAGTCAAGCGTAGTCTGGGACGTCGTATGGGTAGTTCTCTTGCTTTTTCCTCTC
COX9-C	GGCCTGCAGTCATTTACCATCGATTAATCTTGGATCTACTTGATCTTCTCCTCCAGCGTAGTCTG

	GGACGTCGTATGG
	Primers used to construct nuclear gene products with CH tags
COX14-5’	GGCGAGCTCGATTGGGGTGTCAACGTAATGTAC
COX14-3’	GGCCTGCAGCTCGGTAGGAGGTGCAGTGGGACTTTG
MSS51-5’	GGCGAGCTCGGTTCTGTTAAAGTAGGACCCGC
MSS51-3’	GGCCTGCAGTTGTCTCTTGATTGTATGGTACC
SHY1-5’	GGCGAGCTCCCCAAAGGATATACAGCCGAGCAGG
SHY1-3’	GGCCTGCAGCATATATTTCCTTGAATGCTTCAG
	Complementary oligonucleotides used to construct YIp351-CH and YIp352-CH
	GGAAGATCAGGTAGATCCACGGTTAATCGATGGTAAGGGAGGAGGACACCATCACCATCA ACGTCCTTCTAGTCCATCTAGGTGCCAATTAGCTACCATTCCCTCCTCCTGTGGTAGTGGTAGT TCACTAAA AGTGATTTTCGA

The primers used to add the sequences for the HA, poly histidine, and protein C tags terminate with HA, H, and C, respectively. The upstream and downstream primers used to amplify the genes for cloning into YIp351-CH and YIp352-CH terminate with the suffixes 5’ and 3’, respectively.

Fig. S1

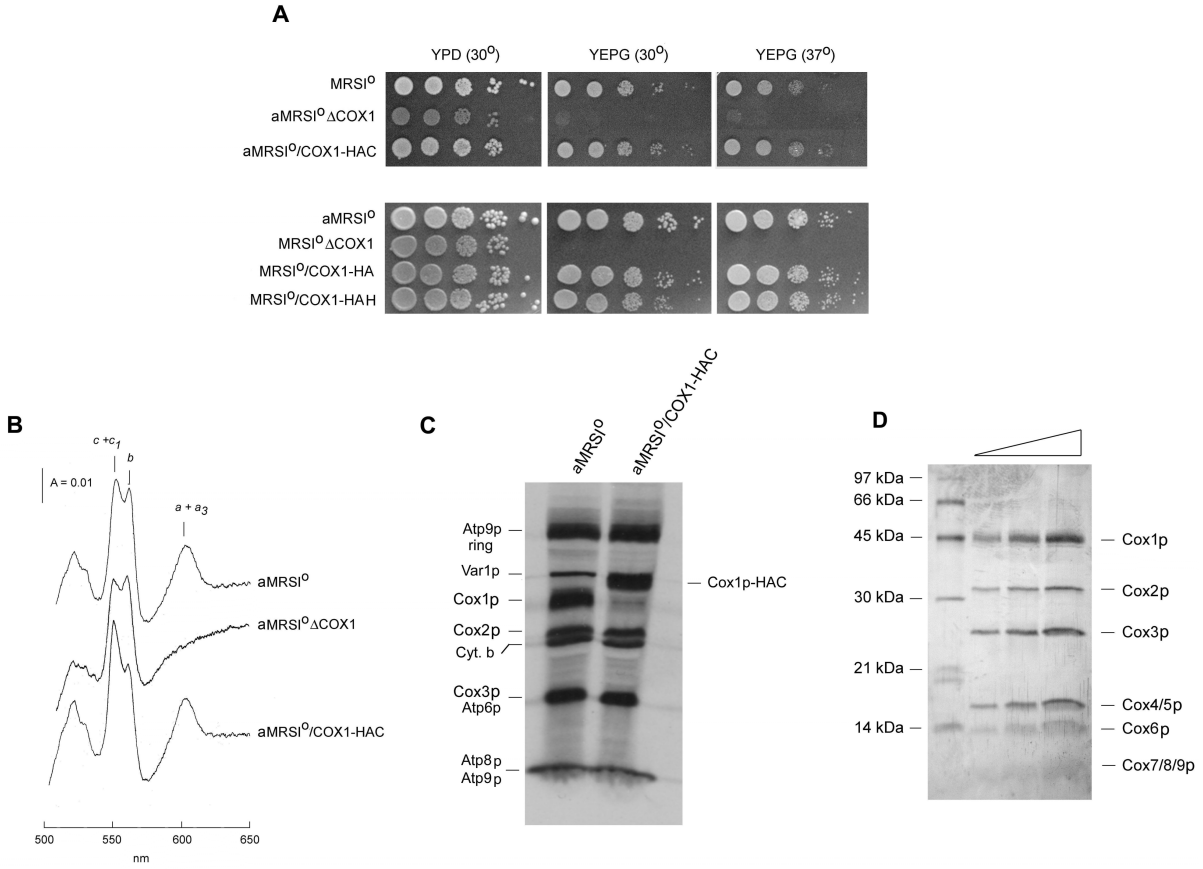

A
YPD (30°)
YEPG (30°)
YEPG (37°)
MRSI°
aMRSI°ΔCOX1
aMRSI°/COX1-HAC
aMRSI°
MRSI°ΔCOX1
MRSI°/COX1-HA
MRSI°/COX1-HAH
B
c + c1
b
A = 0.01
a + a3
aMRSI°
aMRSI°ΔCOX1
aMRSI°/COX1-HAC
500
550
600
650
nm
C
aMRSI°
aMRSI°/COX1-HAC
Atp9p ring
Var1p
Cox1p
Cox2p
Cyt. b
Cox3p Atp6p
Atp8p Atp9p
Cox1p-HAC
D
97 kDa
66 kDa
45 kDa
30 kDa
21 kDa
14 kDa
Cox1p
Cox2p
Cox3p
Cox4/5p
Cox6p
Cox7/8/9p

Fig. S2

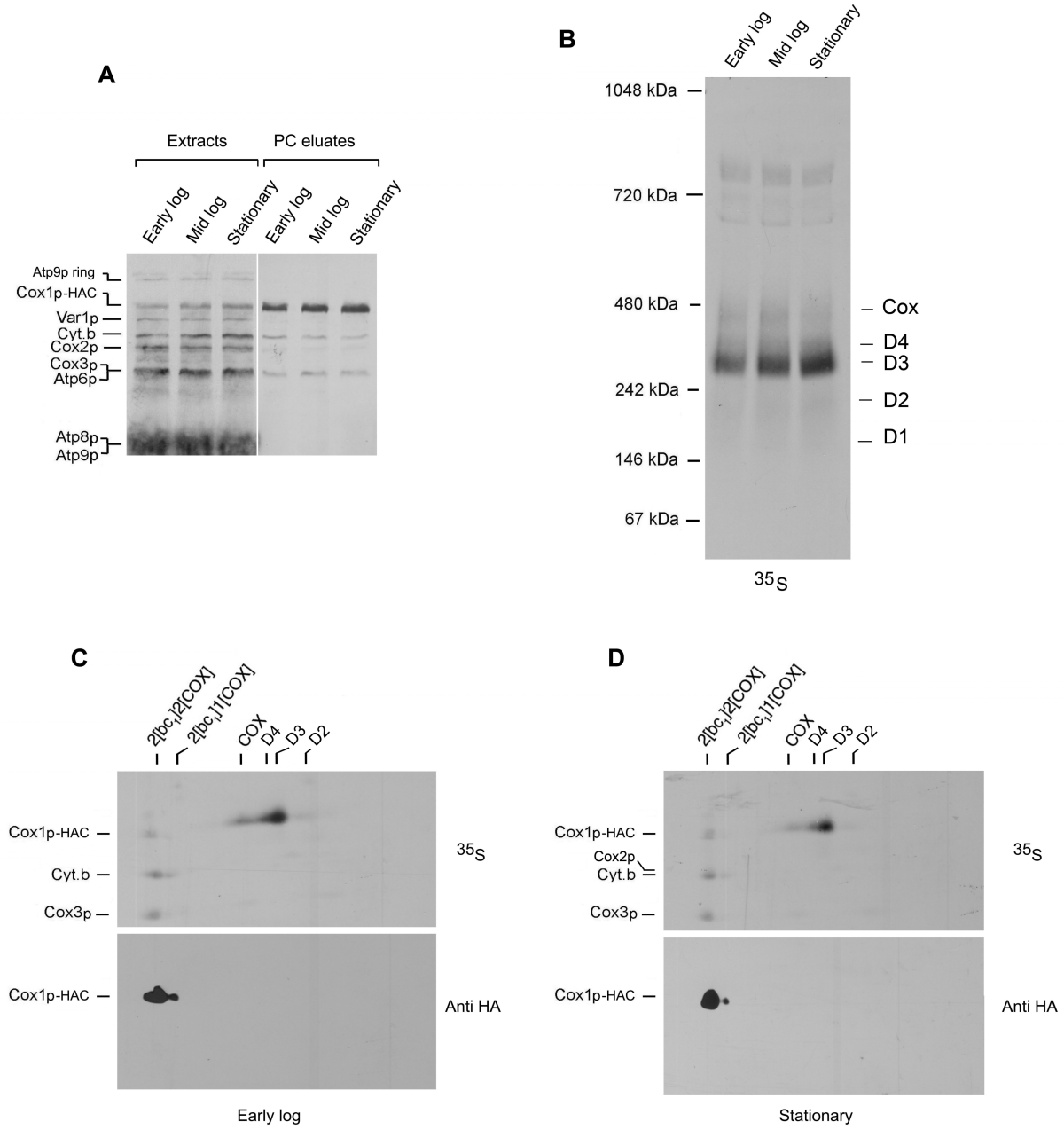

A
Extracts
PC eluates
Early log
Mid log
Stationary
Early log
Mid log
Stationary
Atp9p ring
Cox1p-HAC
Var1p
Cyt.b
Cox2p
Cox3p
Atp6p
Atp8p
Atp9p
B
Early log
Mid log
Stationary
1048 kDa
720 kDa
480 kDa
242 kDa
146 kDa
67 kDa
Cox
D4
D3
D2
D1
35S
C
2[bc,i2[COX]
2[bc,i1[COX]
COX
D4
D3
D2
Cox1p-HAC
Cyt.b
Cox3p
35S
Cox1p-HAC
Anti HA
Early log
D
2[bc,i2[COX]
2[bc,i1[COX]
COX
D4
D3
D2
Cox1p-HAC
Cox2p
Cyt.b
Cox3p
35S
Cox1p-HAC
Anti HA
Stationary

Fig. S3

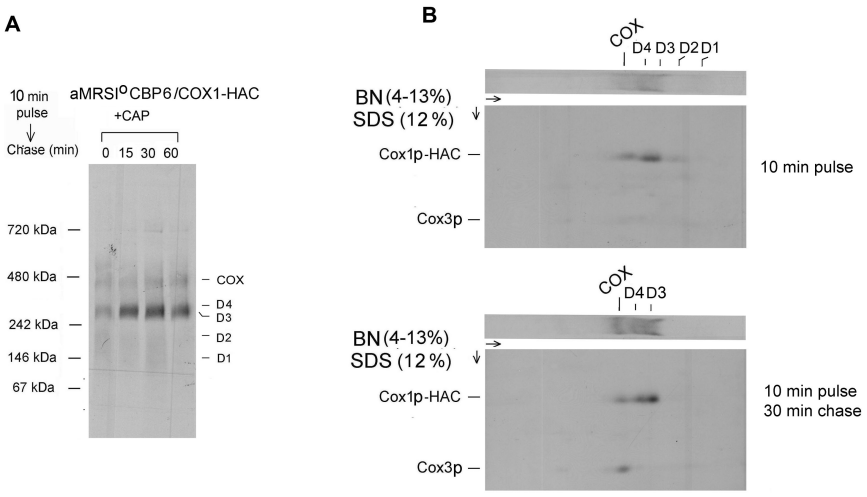

A
10 min pulse
Chase (min)
aMRSI^{O}CBP6/COX1-HAC
+CAP
0 15 30 60
720 kDa
480 kDa
242 kDa
146 kDa
67 kDa
COX
D4
D3
D2
D1
B
COX
D4 D3 D2 D1
BN (4-13%)
SDS (12 %)
Cox1p-HAC
Cox3p
10 min pulse
COX
D4 D3
BN (4-13%)
SDS (12 %)
Cox1p-HAC
Cox3p
10 min pulse
30 min chase

Fig. S4

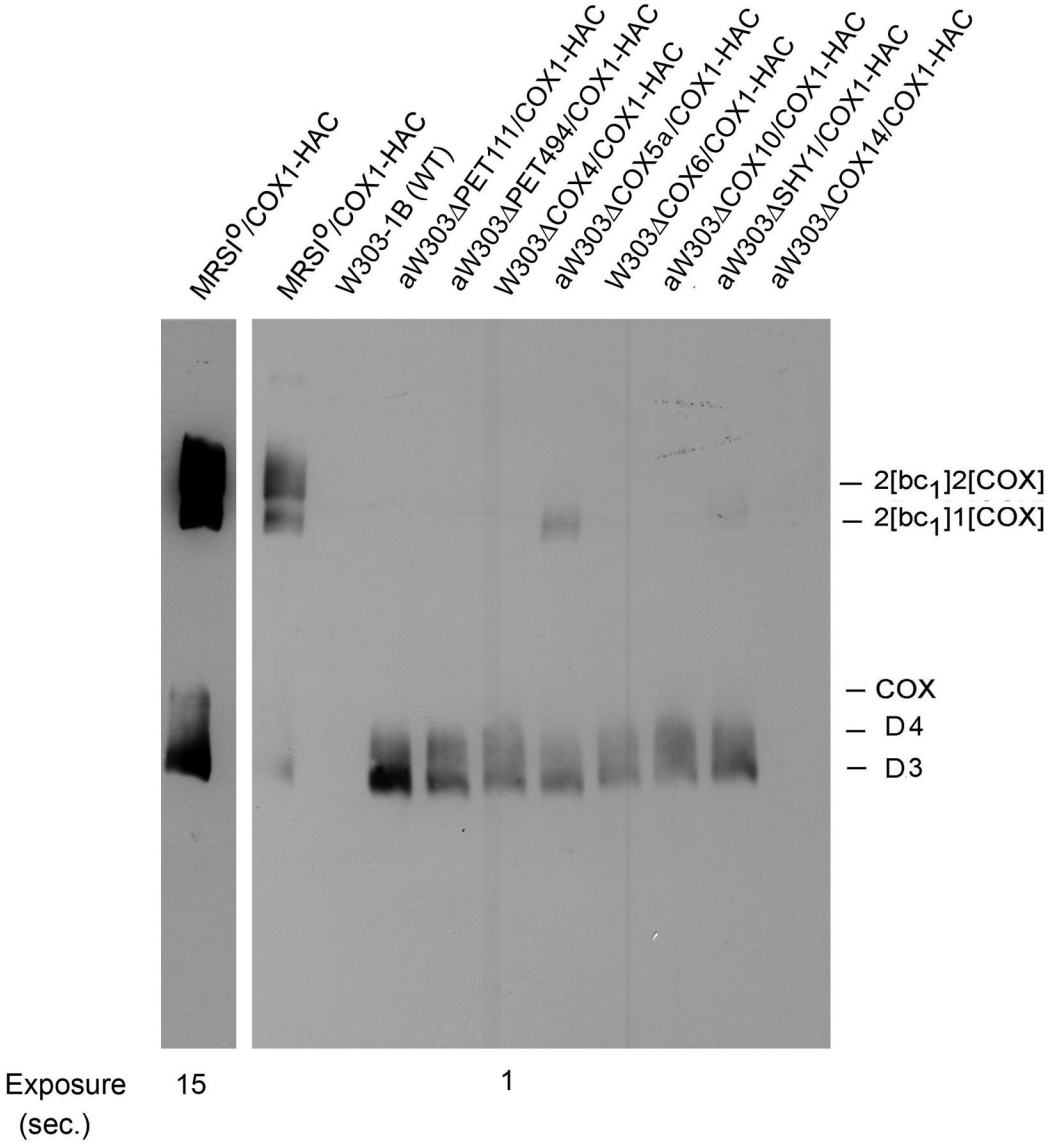
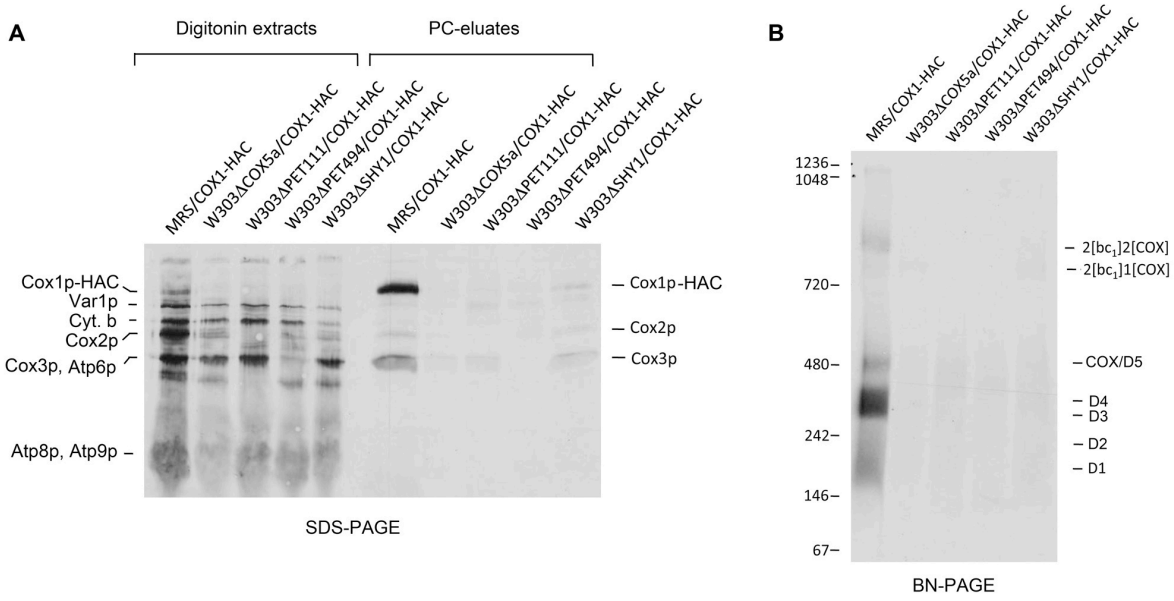

MRSI^{o}/COX1-HAC
MRSI^{o}/COX1-HAC
W303-1B (WT)
aW303ΔPET111/COX1-HAC
aW303ΔPET494/COX1-HAC
W303ΔCOX4/COX1-HAC
aW303ΔCOX5a/COX1-HAC
W303ΔCOX6/COX1-HAC
aW303ΔCOX10/COX1-HAC
aW303ΔSHY1/COX1-HAC
aW303ΔCOX14/COX1-HAC
— 2[bc$_1$]2[COX]
— 2[bc$_1$]1[COX]
— COX
— D4
— D3
Exposure (sec.)
15
1

Fig. S5


A
Digitonin extracts
PC-eluates
MRS/COX1-HAC
W303ΔCOX5a/COX1-HAC
W303ΔPET111/COX1-HAC
W303ΔPET494/COX1-HAC
W303ΔSHY1/COX1-HAC
Cox1p-HAC
Var1p
Cyt. b
Cox2p
Cox3p, Atp6p
Atp8p, Atp9p
Cox1p-HAC
Cox2p
Cox3p
SDS-PAGE
B
1236
1048
720
480
242
146
67
2[bc1]2[COX]
2[bc1]1[COX]
COX/D5
D4
D3
D2
D1
BN-PAGE

References

Barros MH, Myers AM, Van Driesche S, Tzagoloff A (2006) *COX24* codes for a mitochondrial protein required for processing of the *COX1* transcript. J Biol Chem 281: 3743-3751.

Rak M, Tetaud E, Godard F, Sagot I, Salin B, Duvezin-Caubet S, Slonimski PP, Rytka J, di Rago JP (2007) Yeast cells lacking the mitochondrial gene encoding the ATP synthase subunit 6 exhibit a selective loss of complex IV and unusual mitochondrial morphology. J Biol Chem 282**:** 10853-10864.

Steele DF, Butler CA, Fox TD (1996) Expression of a recoded nuclear gene inserted into yeast mitochondrial DNA is limited by mRNA-specific translational activation. Proc Natl Acad Sci USA 93**:** 5253–5257.

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.

Legends to Figures

FIGURE S1**.** Properties of yeast expressing Cox1p with different C-terminal tags. **(A)** The parental strains aMRSI^{o} and MRS-3A, the *cox1* null mutants aMRSI^{o}ΔCOX1 and aMRSI^{o}ΔCOX1, and aMRS/COX1-HA, aMRSI^{o}/COX1-HAC and aMRS/COX1-HAH expressing Cox1p with, respectively, the single HA and the double HA plus protein C and HA plus poly histidine tags, were grown in liquid YPD and serial dilutions were spotted on solid YEPG and incubated at 30^{o}C and 37^{o}C for 2 days. The red color resulting from the *ade2* marker in some strains makes them appear to grow less in YPD. (**B)** Cytochromes were extracted from mitochondria of the same strains at a protein concentration of 5 mg/ml and difference spectra of reduced (dithionite) versus oxidized (ferricyanide) were recorded at room temperature (Tzagoloff *et al*, 1975). The α bands of cytochrome *a*, a_3, *b*, *c*, and c_1 are indicated. **(C)** Mitochondria prepared from the respiratory competent haploid strain MRSI^{o} and MRSI^{o}/COX1-HAC were labeled with ^{35}S-methionine plus cysteine. The mitochondria were dissolved in Laemmli sample buffer (Laemmli, 1970) and separated by SDS-PAGE on a 12% polyacrylamide gel. The radiolabeled mitochondrial gene products identified in the margins are the three subunits of cytochrome oxidase (Cox1p or Cox1p-HAC, Cox2p, Cox3p), mitochondrial ribosomal protein (Var1p), cytochrome *b* (Cyt. b), and three subunits of the ATP synthase (Atp6p, Atp8p, Atp9p). The HA and protein C double tag in Cox1p-HAC retards its migration. **(D)** Purification of COX from aMRSI^{o}/COX1-HAC. Mitochondria at a protein concentration of 10 mg/ml in 0.6M sorbitol, 20 mM Hepes, pH 7 were extracted with 1.6 volumes of 10% lauryl maltoside. The soluble fraction (160 μl) obtained after centrifugation at 100,000 x g_{av} for 10 min was diluted with 100 μl of binding buffer (20 mM Tris-Cl, pH 7.5, 2 mM $CaCl_2$ and 100 mM NaCl) and added to 100 μl of packed agarose beads coupled to an antibody against the protein C epitope (Roche Diagnostics, Mannheim) prewashed with binding buffer. 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. COX was eluted from the beads with 80 μl of elution buffer (10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5% lauryl maltoside, 7 mM EDTA). After rotation of the beads at 4°C for 30 min, they were centrifuged and 5, 10 and 20 μl of the supernatant was separated by SDS-PAGE on a 17% polyacrylamide gel. Proteins were detected by staining with silver. Protein standards were loaded in the left lane.

FIGURE S2. Labeling of Cox1p intermediates in mitochondria from cells harvested at different stages of growth. aMRSI^0/COX1-HAC cells grown in YPGal were harvested in early log, mid log and early stationary phase. Mitochondria were labeled at 24°C for 20 min with ^{35}S methionine plus cysteine. Puromycin was added and after an additional 30 min incubation they were extracted with digitonin and purified on protein C antibody beads as described in the legend to Fig. 2. **(A)** Samples of the digitonin extracts and antibody eluates were separated by SDS-PAGE on a 17% polyacrylamide gel. **(B)** The antibody eluates were separated by BN-PAGE on a 4-13% polyacrylamide gel. **(C and D)** The antibody eluates obtained from early log and early stationary phase were separated in the first dimension by BN-PAGE and in the second dimension by SDS-PAGE on a 12% polyacrylamide gel. The radiolabeled bands are identified in the margins.

FIGURE S3 Pulse-chase analysis of Cox1p intermediates in a *cbp6* null mutant. **(A)** Mitochondria from aMRSI°ΔCBP6/COX1-HAC harboring the *COX1-HAC* allele were pulsed for 10 min with ^{35}S-methionine plus cysteine at 24°C. Following addition of puromycin incubation was continued for the indicated times. Equal volume samples were extracted with digitonin, purified on protein C antibody beads as described in Fig. 1 and separated by BN-PAGE on a 4-13% polyacrylamide gel. (B) Antibody eluates of the 0 time (pulse) and 30 min pulsed sample were separated in the first dimension by BN-PAGE and in the second dimension by SDS-PAGE on a 12% polyacrylamide gel. The positions of COX and different Cox1p intermediates are marked in the margins.

FIGURE S4. The D3 and D4 intermediates are immunochemically detectable under steady-state conditions in respiratory competent and a panel of COX mutants. Mitochondria from MRSI°/COX1-HAC, W303-1B, aW303ΔPET111/COX1-HAC, aW303ΔPET494/COX1-HAC, aW303ΔCOX4/COX1-HAC, aW303ΔCOX5a/COX1-HAC, W303ΔCOX6/COX1-HAC, aW303ΔCOX10/COX1-HAC, aW303ΔSHY1/COX1-HAC and aW303ΔCOX14/COX1-HAC were solubilized at 10 mg/ml with 3% digitonin (3mg digitonin/1mg mitochondrial protein) in extraction buffer. The extracts (450 μg) were incubated with protein C antibody beads as described in the legend to Fig. 2. The eluates (equivalent to 100 μg starting material) were separated by 4-13% BN-PAGE, transferred to a PVDF membrane and probed with anti-HA antibody.

FIGURE S5. *In organello* translation of Cox1-HAC in a panel of cytochrome oxidase mutants. Mitohondria were prepared from the respiratory competent strain MRS/COX1-HAC and from the COX deficient strains W303ΔCOX5a/COX1-HA, W303ΔPET494/COX1-HAC, W303ΔPET111/COX1-HAC and W303ΔSHY1/COX1-HAC. The mitochondria were labeled for 30 min with ^{35}S-methione plus cysteine and were extracted with 3% digitonin. The digitonin extracts were purified on protein C antibody beads as described in detailed in the legend to Fig. 2. Samples of the extracts (Digitonin extracts) and eluates from the beads (PC-eluates) were

separated by SDS-PAGE on a 17% polyacrylamide gel. The eluates were also separated on a 4-13% gel by BN-PAGE. Radiolabeled proteins were visualized by exposure to X-ray film following transfer to nitrocellulose or PVDF membranes.