Supporting Information

Supekova et al. 10.1073/pnas.1000735107

SI Materials and Methods

Yeast Strains, Media, and Transformation. *S. cerevisiae* strains used in this study were NB80 (*MAT***a** *lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG* [rho+]) and its isonuclear derivative NB97 (*MAT***a** *lys2 leu2-3,112 ura3-52 his3ΔHinDIII arg8::hisG* [cox2-60 mit$^-$]), which contains a 129-bp deletion located around the *COX2* initiation codon (1). Yeast cells were routinely grown at 30 °C in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose). Transformations with plasmid DNA were performed by the lithium acetate method (2) followed by transformant selection on synthetic complete (SC) medium plates lacking uracil. The respiration-competent phenotype was identified by testing the transformed yeast cells for the ability to grow on YPG medium plates (1% Bacto yeast extract, 2% Bacto peptone, 3% glycerol). To measure cellular respiration and to isolate mitochondrial membranes, yeast cells were grown in liquid SC medium lacking uracil and containing 2% raffinose as the sole carbon source.

Synthetic DNAs. The following oligonucleotides (Integrated DNA Technologies) were used for the generation of the DNA constructs described in this report: ACT1rev, 5′-CACCGGTAAATTTTC-GATCTTGGGAAGAAAAAAGC-3′; ACT1for, 5′-CCTCGAGT CTTCCTTAT CGGATCCTCAAAAC-3′; ADH1 for, 5′-GCGAA TTTCTTATGATTTATG-3′; ADH1rev, 5′-CGGCCGGCTCTA GAACTAGTGGATCCTACAATTGGGTGAAATGG-3′; ATP 2for, 5′-GGAA TTCGAAATAAAGCTTAAACCAAGGG-3′; ATP2rev, 5′-GCGGCCGGACATGTCCAGTGGGAAA GCGA-3′; AURfor, 5′-AAACTCACAAATTAGAGC-3′; and AURrev, 5′-CAAATATGAACACTGA TG-3′. The coding sequences of the *S. cerevisiae COX2* gene and various MTSs were codon optimized for *S. cerevisiae* cytoplasmic expression with Gene Designer software (DNA2.0), and the synthesized genes were inserted into pJ201 vector (DNA2.0.) The yeast *ACT1* promoter, $ADH1^{3'\text{-}UTR}$, and $ATP2^{3'\text{-}UTR}$ were amplified by PCR from *S. cerevisiae* N97 strain chromosomal DNA and cloned into pCRII-TOPO vector (Invitrogen).

Generation of *COX2* Expression Constructs. Standard techniques were used for DNA cloning, transformation, and plasmid DNA purification from *Escherichia coli*. To generate individual expression vectors, $ATP2^{3'\text{-}UTR}$ and $ADH1^{3'\text{-}UTR}$ were separately inserted into the pJ201-*COX2* and pJ201-$COX2^{W56R}$ genes, using EcoRI/EagI restriction endonucleases. In the next step, four *COX2*/$COX2^{W56R}$-*3′-UTR* constructs were inserted into six pJ201-*MTS* vectors through ClaI/EagI restriction sites to generate 24 *MTS-COX2*/$COX2^{W56R}$-*3′-UTR* plasmids. To generate the final expression plasmids, the *ACT1* promoter was inserted into YEp352 vector using EcoRI/XbaI restriction sites, and, subsequently, 24 *MTS-COX2*/$COX2^{W56R}$-*3′-UTR* coding DNA fragments were inserted downstream of the *ACT1* promoter using AgeI/EagI restriction sites.

Preparation of the MTS-COX2 Mutagenized Library by PCR. Random mutagenesis of the *2×SU9-COX2* gene was performed by mutagenic PCR. Two 50-μL PCR reactions contained (in addition to other standard components) 1 μM of both AURfor and AURrev primers, 1 ng of the pYEp352-*ACT1-2×*$SU9^{MTS}$-*COX2-*$ADH1^{3'\text{-}UTR}$ DNA, and three dNTPs at 200 μM concentration each (dATP + dGTP + TTP and dATP + dCTP + dGTP). The reactions were further supplemented with the corresponding fourth dNTP (dCTP or TTP) at 2 μM concentration. The PCR reactions were incubated at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min for 30 cycles, followed by incubation for 10 min at 72 °C. The resulting PCR products were cloned into the pCRII-TOPO vector (Invitrogen). Approximately 10,000 *E. coli* transformants per PCR were pooled and used for the plasmid DNA isolation. Resulting DNA pools were digested with AgeI/EagI restriction endonucleases, and the DNA fragments corresponding to the mutagenized *2×*$SU9^{MTS}$-*COX2-*$ADH1^{3'\text{-}UTR}$ were purified. In parallel, pYEp352-*ACT1-2×*$SU9^{MTS}$-*COX2-*$ADH1^{3'\text{-}UTR}$ plasmid DNA was also digested with Age1/Eag1, and the DNA fragment containing the vector backbone together with the *ACT1* promoter was purified. The isolated vector and the insert DNAs were ligated and transformed into *E. coli*. Approximately 20,000 transformants per original PCR were pooled and used for plasmid DNA isolation with subsequent transformation into yeast.

Immunoblot Analysis. The whole cell yeast lysates were prepared from overnight cultures grown in SC −Ura medium containing 2% raffinose as the carbon source according to previously published procedure (3). Cell lysates were subjected to SDS/PAGE and then transferred to a nitrocellulose membrane (Invitrogen). The blots were probed with monoclonal antibodies against yeast Cox2 and Cox3 (both from Invitrogen), an antibody against F1 sector of yeast F-type ATP synthase (raised in chicken, generous gift of David Mueller) (4), and an antibody against Act1 (rabbit, Sigma). Immunoreactive proteins were detected with the ECL plus Western Blotting Detection System (Amersham Biosciences).

Measurement of Cellular and Mitochondrial Respiration. Oxygen consumption experiments were conducted with a single-channel fiber-optic oxygen monitor (Instech Laboratories) model FO/110, equipped with a 250-μL chamber. Measurements were done using either stationary yeast cultures grown in the SC −Ura medium containing raffinose as the carbon source or mitochondrial membranes (20 μg of total protein per reaction) that were resuspended in the respiratory buffer (250 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 30 mM K_2HPO_4, pH 7.4). Respiratory substrates [ascorbic acid, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD)] were purchased from Sigma. The isolation of mitochondria was performed by lysis of yeast spheroplasts and subsequent differential centrifugation according to a previously described protocol (5). Freshly prepared mitochondria were either used immediately or frozen in liquid nitrogen and stored at −80 °C. Total protein concentration in mitochondrial preparations was determined using the BCA method (Pierce).

1. Bonnefoy N, Fox TD (2000) In vivo analysis of mutated initiation codons in the mitochondrial COX2 gene of Saccharomyces cerevisiae fused to the reporter gene ARG8m reveals lack of downstream reinitiation. *Mol Gen Genet* 262:1036–1046.
2. Schiestl RH, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* 16:339–346.
3. Supek F, Supekova L, Nelson H, Nelson N (1996) A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. *Proc Natl Acad Sci USA* 93:5105–5110.
4. Lai-Zhang J, Mueller DM (2000) Complementation of deletion mutants in the genes encoding the F1-ATPase by expression of the corresponding bovine subunits in yeast S. cerevisiae. *Eur J Biochem* 267:2409–2418.
5. Yaffe MP (1991) *Guide to Yeast Genetics and Molecular Biology*, eds Guthrie C, Fink GR (Academic Press, San Diego), pp 627–643.

Table S1. Identified *2×SU9-COX2* alleles able to correct the *cox2-60* yeast mutant growth phenotype

Mutant no.	Mutations
4	W198R T107A
10	W198R A8V, A324V
12	W198R T120S, R128K, I140V
16	W198R
21	W198R S72STOP
26	W198R M1V
27	W198R S72P
29	W198R S3P