Supporting Information

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SI Materials and Methods

Generation of Stable Uncoupling Protein 2-Silenced Clones. The four identified oligos, si-Uncoupling protein (UCP)2-1 5′-GCCC-ACTTGTCATCCATAA-3′, siUCP2-2 5′-GTTCCTCTATCT-CGTCTTG-3′, siUCP2-3 5′-TTACCATGCTCCAGAAGGA-3′, and siUCP2-4 5′-CCACTTCACTTCTGCCTTTGG-3′, were cloned into the pLKO1 lentiviral vector (Sigma). The four constructs were sequenced and used for transfection of HEK293T cells. Lentiviral particle production was performed with the Mission Lentiviral Packaging Mix (Sigma) according to the manufacturer's protocol. Lentiviral particles were collected 48 and 72 h after transfection. The silencing efficiency of the four constructs was tested in experiments of transient RNA interference; the lentiviral particles, obtained from HEK293T cells transfected with the pLKO1 empty vector, were used as control. Briefly, 2×10^5 human hepatocellular carcinoma (HepG2) cells were incubated overnight with the lentiviral particles in the presence of 8 μg/mL hexadimetrine bromide (Sigma). The UCP2 transcript was assayed by real-time PCR 72 h after infection. The construct showing the strongest silencing effect was used for the selection of the stable clones, which was accomplished in HepG2 cells in the presence of 5 μg/mL puromycin (Sigma). Due to the random integration of the lentiviral vector in the genome, 20 colonies were selected for further UCP2 expression analysis. HepG2 mitochondria were isolated by using a kit (Pierce) with the Halt Protease Inhibitor Mixture (Pierce; 78415) according to the manufacturer's instructions.

Expression Analysis by Real-Time PCR. Total RNA was extracted from HepG2 cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed with the GeneAmp RNA PCR Core Kit (Applied Biosystems) with random hexamers as primers according to the manufacturer's instructions. Real-time PCR was performed in a MicroAmp optical 96-well plate using the automated ABI Prism 7000 sequence detector system (Applied Biosystems). Twenty microliters of reaction volume contained 200 ng of template (reverse-transcribed, first-strand cDNA), 1× TaqMan Universal Master Mix, and 1× TaqMan UCP2-, *SLC25A4*, *SLC25A5*, and *SLC25A6*-specific probe and primer sets (hs00163349, hs00154037, hs00854499, and hs00745067, respectively; TaqMan Gene Expression Assays). To correct for differences in the amount of starting first-strand cDNAs, human 18S rRNA and cyclophilin A genes were amplified in parallel as reference endogenous housekeeping genes. The relative quantification of UCP2 expression was performed according to the comparative method ($2^{-\Delta\Delta Ct}$, according to Applied Biosystems User Bulletin 2 P/N 4303859, http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Metabolite and Nucleotide Determinations by Mass Spectrometry. For metabolite quantification, cells and cytosolic and mitochondrial fractions were extracted with phenol/chloroform (50/50), and the aqueous phase was centrifuged at $22,800 \times g$ for 20 min at 4 °C to precipitate the protein fraction. A Quattro Premier mass spectrometer with an Acquity UPLC system (Waters) was used for electrospray ionization LC-MS/MS analysis in the multiple reaction monitoring (MRM) mode. Quantification was achieved through calibration curves set at four concentrations of standards processed under the same conditions as the samples. The best fit was determined using regression analysis of the peak analyte area. The MRM transitions monitored in the negative-ion mode were *m/z* 190.95 > 110.89 for citrate, *m/z* 116.88 > 73.20 for succinate, *m/z* 115.07 > 71.31 for fumarate, *m/z* 132.95 > 115.20 for malate, and *m/z* 144.9 > 101.10 for 2-oxoglutarate. The MRM transitions in the positive-ion mode were *m/z* 134.16 > 73.76 for aspartate, *m/z* 148.20 > 83.80 for glutamate, *m/z* 90.00 > 44.24 for alanine, *m/z* 308.28 > 179.02 for reduced glutathione, and *m/z* 613.20 > 484.20 for oxidized glutathione. For ATP, ADP, and AMP quantification in the negative-ion mode, the selected ion monitored *m/z* values were 506.2, 426.2, and 346.2, respectively.

Yeast Strains, Growth Conditions, and Swelling of Yeast Mitochondria. BY4742 (wild-type) and *mir1Δ* yeast strains were provided by the EUROFAN resource center EUROSCARF. The *MIR1* gene of the *Saccharomyces cerevisiae* strain BY4742 (MATα, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0) (1) was replaced by kanMX4. The *mir1Δ* yeast strain was transfected with *pYES2* and *pYES2-UCP2*. The WT and mutant strains were grown in synthetic complete medium (1) supplemented with glucose or in yeast peptone (YP) reach medium containing 2% (wt/vol) bactopeptone and 1% (wt/vol) yeast extract supplemented with 2% (wt/vol) glucose or 2% (wt/vol) galactose. The final pH was adjusted to 4.5. Mitochondria were isolated by standard procedures from cells grown in YP medium containing glucose or galactose until the early exponential phase (optical density between 1.0 and 1.5) was reached. The rate of mitochondrial swelling was monitored by recording the decrease in A_{546} with a Varian spectrophotometer, as previously described (2). Yeast mitochondria (0.5 mg of protein) were added to a glass cuvette containing 1 mL of 120 mM ammonium chloride/phosphate/malate, 20 mM Tris, 1 mM EDTA, 5 μM rotenone, and 0.1 μg/mL antimycin (pH 7.4).

Reconstitution of Recombinant UCP2, A268G_UCP2, and UCP1 into Liposomes and Transport Measurements. UCP2, A268G_UCP2, and UCP1 inclusion bodies were solubilized in 1.6% *N*-laurylsarcosine (wt/vol) and then diluted 5.6-fold with a buffer containing 3% Triton X-114, 1 mM EDTA, and 10 mM Pipes·NaOH (pH 7), and a small residue was removed by centrifugation ($258,000 \times g$, 10 min at 4 °C). The solubilized proteins were reconstituted by cyclic removal of detergent with a hydrophobic column as described previously (3). The composition of the initial mixture used for reconstitution was 55 μL solubilized proteins (about 2 μg), 70 μL 10% Triton X-114, 100 μL preformed liposomes [10% (wt/vol) of egg yolk phospholipids in 2 mM Pipes·NaOH, pH 7.0], 20 mM phosphate (except where otherwise indicated), 1 mg/mL cardiolipin, 20 mM Pipes·NaOH (pH 7.0), and water to a final volume of 700 μL. This mixture was recycled 13 times through an Amberlite column (4.0 × 0.5 cm). External substrate was removed from proteoliposomes on a Sephadex G-75 column pre-equilibrated with buffer A (100 mM sucrose, 10 mM Pipes·NaOH, pH 7.0) in the presence of a reversible inhibitor, 10 μM mersalyl, to avoid the efflux of internal substrate. Transport was started by adding 5 mM dithioerythritol (DTE) and 1 mM ^{33}Pi (except where otherwise indicated) and terminated by addition of 10 mM pyridoxal-5′-phosphate (PLP) and 10 mM bathophenanthroline (BAT) according to the "inhibitor-stop" method (3). In controls, the inhibitors were added at the beginning together with the labeled substrate. Finally, the external substrate was removed and the radioactivity entrapped in the liposomes was counted. The experimental values were corrected by subtracting control values. The initial transport rate was calculated from the radioactivity taken up by proteoliposomes af-

ter 1 min (in the initial linear range of substrate uptake). Various other transport activities were assayed by the inhibitor-stop method. For efflux measurements, proteoliposomes containing 5 mM substrate were labeled with 1 mM radioactive substrate by carrier-mediated exchange equilibration (3). After 40 min, the external radioactivity was removed by passing the proteoliposomes through Sephadex G-75 columns in the presence of 10 μM mersalyl. Efflux was started by adding 5 mM DTE and unlabeled external substrate (20 mM) (exchange) or 40 mM sucrose (uniport) or unlabeled external substrate plus inhibitors (20 mM) (exchange-inhibited), and terminated by adding the inhibitors indicated above.

1. Sherman F (1991) Getting started with yeast. *Methods Enzymol* 194:3–21.
2. Palmieri F, Klingenberg M (1967) Inhibition of respiration under the control of azide uptake by mitochondria. *Eur J Biochem* 1(4):439–446.
3. Palmieri F, Indiveri C, Bisaccia F, Iacobazzi V (1995) Mitochondrial metabolite carrier proteins: Purification, reconstitution, and transport studies. *Methods Enzymol* 260: 349–369.

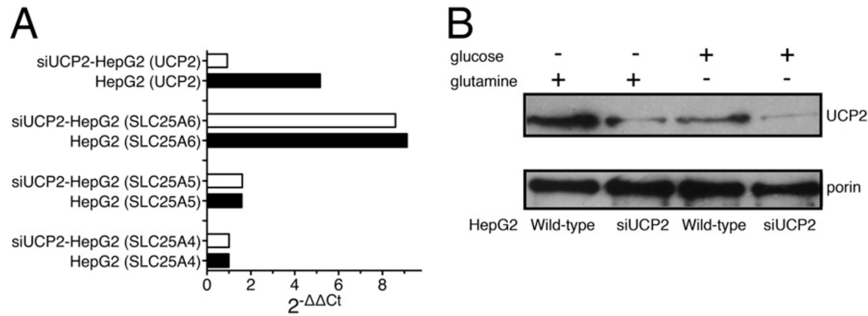

Fig. S1. Expression of human UCP2 in HepG2 and siUCP2-HepG2 cells. (*A*) Real-time PCR was carried out on cDNAs prepared by reverse transcription of total RNAs from wild-type and siUCP2-HepG2 stable clones silenced with the siUCP2-2 oligo. Human 18S rRNA and cyclophilin A genes were used as reference genes to normalize the template. The relative quantification of human UCP2 in HepG2 (black bars) and siUCP2-HepG2 cells (white bars) was performed by the comparative method ($2^{-\Delta\Delta Ct}$). The three isoforms of the human mitochondrial adenine nucleotide carrier encoded by *SLC25A4*, *SLC25A5*, and *SLC25A6* were used as controls. *SLC25A4* was used as an internal calibrator. Similar results were obtained with five siUCP2-HepG2 stable clones. (*B*) Expression of UCP2 in HepG2 mitochondria. Eighty micrograms of mitochondria purified from wild-type and siUCP2-HepG2 cells grown in the presence of glucose (5 mM) or glutamine (2 mM) were examined for UCP2 protein levels by Western blot analysis. An anti-porin antiserum was used as a control.

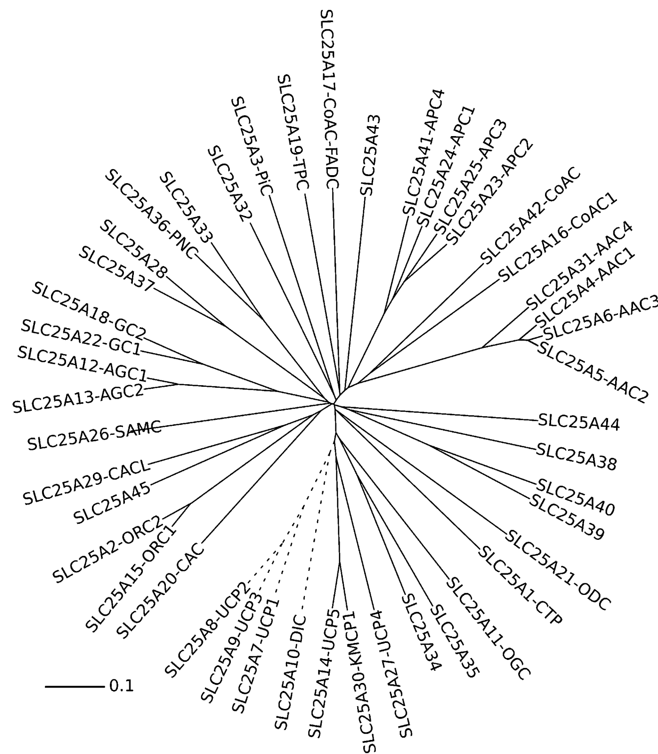

Fig. S2. Phylogenetic tree of *Homo sapiens* mitochondrial carrier family members. The tree was originated by ClustalW (www.clustal.org) multiple-sequence alignment by using the neighbor-joining method implemented in MacVector (http://macvector.com/); gap sites were ignored. Gene names and aliases describe the function of each terminal node. The bar indicates the number of substitutions per residue, with 0.1 corresponding to a distance of 10 substitutions per 100 residues. Dotted lines show the UCP1-3/SLC25A10-dicarboxylate carrier clade.

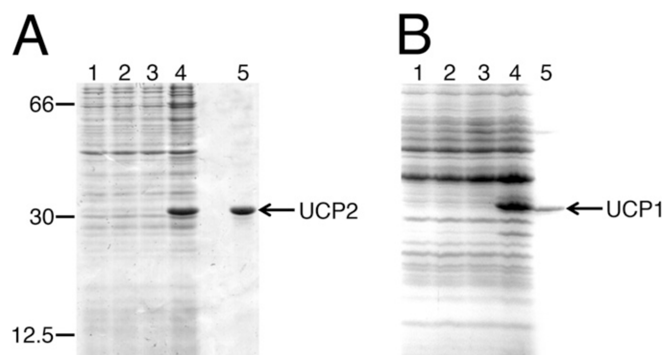

Fig. S3. Expression in *Escherichia coli* of human UCP2 and UCP1. (*A* and *B*) Proteins were separated by SDS/PAGE and stained with Coomassie blue dye. Markers are in the left-hand column [BSA (66 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12.5 kDa)]. (*A*) UCP2 expression in *E. coli* C0214(DE3). (*B*) UCP1 expression in *E. coli* M15(pREP4). Lanes 1–4, *E. coli* containing the expression vector without (lanes 1 and 3) and with (lanes 2 and 4) the coding sequence of UCP2 (*A*) and UCP1 (*B*). Samples were taken at the time of induction (lanes 1 and 2) and 5 h later (lanes 3 and 4). The same number of bacteria was analyzed in each sample. Lane 5, purified UCP2 (6 μg) and UCP1 (1 μg) derived from bacteria shown in lane 4.

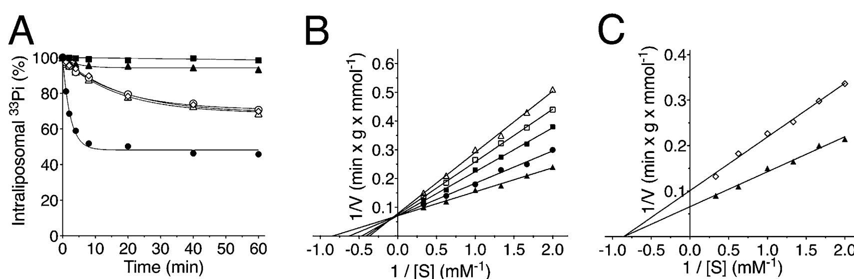

Fig. S4. Efflux of ^{33}Pi from proteoliposomes reconstituted with recombinant UCP2 and kinetic parameter determination. (*A*) The efflux of ^{33}Pi (5 mM) from proteoliposomes was started by the addition of 100 μM unlabeled Pi (●, exchange), 200 μM sucrose (○, uniport), 100 μM laurate (◇), 100 μM propionyl sulfonate (△), 100 μM undecanoyl sulfonate (▲), or 100 μM Pi plus inhibitors (PLP/BAT) (■) (exchange-inhibited). Similar results were obtained in three independent experiments. (*B* and *C*) Dependence of the rate of ^{33}Pi/Pi exchange in UCP2-reconstituted liposomes on the external phosphate concentration (▲). (*B*) Competitive inhibition by L-malate (0.9 mM, □), oxaloacetate (1.2 mM, ■), L-aspartate (2.5 mM, ●), and GDP (10 mM, △). (*C*) Noncompetitive inhibition by undecanesulfonate (0.1 mM, ◇). The transport affinity (K_m) for Pi was 1.12 ± 0.11 mM, and the V_{max} value was 14.2 ± 1.8 mmol/min per gram of protein; the K_i values for malate, oxaloacetate, aspartate, GDP, and undecanesulfonate were 0.86 ± 0.07 mM, 1.39 ± 0.11 mM, 6.84 ± 0.71 mM, 7.68 ± 0.4 mM, and 0.18 ± 0.02 mM, respectively, in at least four independent experiments.