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Supplemental Data

Article

Mice with Mitochondrial Complex I Deficiency

Develop a Fatal Encephalomyopathy

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Supplemental Experimental Procedures

Generation of *Ndufs4* KO Mice

To create an animal model for CI deficiency the second exon of *Ndufs4* was flanked by loxP sites using standard gene-targeting techniques in AK18.1 ES cell (129/Sv), the *Sv-Neo* gene was removed by crossing the mice with *Rosa26-FlPer*, and then the second exon was deleted by crossing the mice with *Mox2-Cre* (Figure S1A), which removes exon 2 in the germline (Tallquist, M.D. and Soriano, P. (2000). Epiblast-Restricted Cre Expression in MORE Mice: A Tool to Distinguish Embryonic vs. Extra-Embryonic Gene Function, Genesis 26:113–115). Mice heterozygous for the *Ndufs4* null allele *(Ndufs4*$^{\Delta/+}$*)* on a mixed 129/Sv:C57Bl/6 genetic background were interbred to create the homozygous mutant (KO). Absence of NDUFS4 protein was verified by western analyses with antibodies against NDUFS4 (Mitosciences, Eugene OR). KO mice were born from HET crosses at almost the expected Mendelian ratio (WT:HET:KO; 1.4:1.9:1.0, n =790) and could usually be recognized as early as postnatal day 16 (P16) by their sparse fur or small size.

Animal Experiments

All animal experiments were performed with the approval of the Animal Care and Use Committee of the University of Washington. Mice were maintained with rodent diet (5053, Picolab, Hubbard, OR) and water available ad libitum in a vivarium with 12-hr light-dark cycle at 22^{o} C.

Metabolic Activity

WT and KO mice were placed in metabolic cages (Columbus Instruments, Columbus, OH) and measurements of O_2, CO_2, food and water were made for three consecutive days. VO_2 was normalized to lean body mass, which was measured using EcoMRI-100 system (Houston, TX). The ages of mice ranged from P26 to P37. Mice were fed ad libitum with ground chow (D5053M, PicoLab), with the exception of the second night when they were food-deprived. The experiment was conducted by The Clinical Nutrition Research Unit - Body Composition and Energy Expenditure Core facility at Harborview Hospital, University of Washington, Seattle, WA.

Locomotor Activity
Locomotor activity was measured as laser-beam breaks in locomotor chambers (Columbus Instruments, Columbus, OH). Each experiment lasted 48-72 h, after at least 24 h habituation to the chamber.

Body Temperature Monitoring
WT, HET and KO mice were subdermally implanted with temperature-sensitive probes (IPTT300 probe, Bio Medic Data Systems, Seaford, DE) in the scapular region and allowed to recover for at least 7 days prior to monitoring using telemetric receivers. Body temperature was recorded during two weeks of 12:12 h light:dark cycles at 25° C ambient temperature. Each day several measurements at different times were taken from each animal to avoid variability of temperature due to inconsistencies in feeding and locomotor activities.

Blood Glucose and Lactate Measurements
Blood was taken from the saphenous vein after an overnight fast. Glucose was measured using B-Glucose HemoCue microcuvettes (Ängelholm, Sweden). Lactate was measured using a lactic acid assay kit (State University of New York, Buffalo NY) after plasma isolation using Becton Dickinson (Franklin Lakes, NJ) serum-separator tubes.

Hearing
Gross hearing ability was assessed by an automated acoustic startle system (San Diego Instruments) that records the amplitude of whole body flinch in response to bursts of white noise (Davis et al., 1982). In a single session, the startle response to bursts of 80, 90, 100, 105, 110, and 120 dB were recorded. Ten rounds of successive sound bursts increasing in amplitude were performed per session.

Ataxia Assays
The ledge test measured the amount of time a mouse could remain on a 7-mm ledge during a 60 s trial. The negative geotaxis test measured the time it took a mouse place nose down on a vertical wire grid to turn 180º. To measure grip, mice were place on a wire grid that was then turned upside-down; the time to fall during a 180 sec test was recorded. For the rotarod test, mice were placed on a rotating drum (Rotarod, San Diego Instruments) that gradually accelerated from 4 rpm to 50 rpm during a 3-min test. The results of 3-5 replicates of each tested were averaged after removing the lowest score for each mouse (to reduce the variance due to an inadvertent slip, and because the KO mice, being blind, often fell before learning that they were ~2 ft above the padded floor). Clasping was observed during a 10 s tail suspension. The older KO mice were usually too weak to clasp hind limbs; attempted clasping was considered equivalent.

Electroretinogram
ERGs were recorded with a gold wire-contact lens electrode on the cornea that was pre-treated with a 2.5% methylcellulose solution. Briefly, animals were dark adapted overnight and then anesthetized with xylazine/ketamine (8/130 mg/kg body weight) and their pupils dilated with a drop of phenylephrine (2.5%, wt/vol) and tropicamide (0.5%, wt/vol). Body temperature was maintained at 37° C throughout the experiment with a heating block. ERGs were evoked by flashes of light at intensities ranging from 10^{-4} to 20 cd m^{-2} s^{-1}. To minimize the variance, three responses evoked by light were averaged for each luminance step. Electrodes on each eye were

connected to a reference electrode inserted subcutaneously at the level of the corresponding frontal region and connected to a two-channel amplifier. For each group of mice, the mean wave amplitude was plotted as a function of time.

Respiratory Complex Assays of Maximal Enzyme Activity

Enriched mitochondrial extracts were made by Dounce homogenization of MEFs or mouse tissue in 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5% BSA, 5 mM HEPES pH7.2. The homogenate was centrifuged (twice if >500 mg tissue) for 10 to 20 min at 2000 rpm using an HB6 rotor (Sorvall RC-5B, DuPont Instruments) and the supernatant was removed and centrifuged again at 9800 rpm for 15 to 30 min (the longer times were used for larger amounts of tissue). The mitochondrial pellet was suspended in the same buffer (lacking BSA) and centrifuged again. The final mitochondrial pellet was suspended in the same buffer without BSA and the protein content determined. Sucrose, Ficoll and Percoll gradients were also tested, but these methods did not improve the quality of mitochondria from KO mice, as measured by CI activity. Submitochondrial particles (SMPs) were most often made from the enriched mitochondria preparations by sonication for 10 s on ice, using a Branson 250 sonifier (Danbury, CT) at 50% pulse and 30% output. The freeze-thaw method and Triton X-100 lysis were also used to create SMPs with similar results. Mitochondria and SMPs were kept on ice prior to assay. For CI, II, III and IV assays, 10 to 30 μg of SMPs (depending on the assay) were added to 0.5 ml total volume in cuvettes maintained at 30° C and the absorbance was measured for 1 to 6 min using a spectrophotometer (Beckman DU 640, Fullerton, CA). CI activity is the rotenone-sensitive oxygen consumption in the presence of pyruvate and malate. CI activity was measured by following the oxidation of NADH at 340 nm (minus background of 380 nm), or most often, by the reduction of decylubiquinone (DB) at 272 nm (minus background at 243 nm). The reaction mixture consisted of 250 mM sucrose, 1 mM EDTA, 50 mM Tris HCl pH 7.4, 50 μM DB, and 2 mM KCN. After a 3 min equilibration 50 μM NADH was used to initiate the reaction and DB reduction (or NADH oxidation) was monitored for 1 min. Rotenone (5 μg/ml, Sigma) was used to confirm CI-specific activity. Similar results were acquired with SMPs from brain and mouse embryonic fibroblasts (MEFs) (data not shown). The results were not influenced by the method of mitochondrial isolation or SMP preparation, or the substrate used in the assay [reduction of the ubiquinone analogue decylubiquinone (DB) versus oxidation of nicotinamide adenine dinucleotide (NADH)]. CII activity was measured by following the reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm (minus 750 nm) by DB in the presence of succinate. The buffer contained 50 mM $KHPO_4$, pH 7.4, 20 mM succinate. After 10 min of incubation at 30^{o}C DCPIP, KCN, rotenone, and antimycin A were added and any residual change in absorbance was recorded to subtract from the complex II-driven reaction. DB was added to initiate reaction and reduction of DCPIP was measured for 1 min. CIII activity was measured by monitoring the reduction of cytochrome C at 550 minus 540 nm, using decylubiquinol (DBH_2) as substrate. DBH_2 was made by reduction of DB with KBH_4 in an acidic ethanol solution. The reaction mix contained 25 mM KH_2PO_4 pH 7.4, 80 μM ferricytochrome C, 2 mM KCN; the reaction was initiated by addition of 50 μM DBH_2, and terminated with 2.5 μM antimycin A to verify complex III activity. CIV activity was measured by recording for 1 min the oxidation ferrocytochrome C (fully reduced with $Na_2S_2O_4$ and then dialysed) at 550 minus 540 nm. The reaction mix consisted of 50 mM ferrocytochrome C and 10 mM KH_2PO_4; the reaction was started by addition of SMPs and terminated with 5 μM KCN to verify CIV activity.

Polarography

Activities of complexes I, II, and IV were measured by monitoring the rate of oxygen consumption in the presence of the complex-specific substrates and were calculated as the fraction that was sensitive to complex-specific inhibitors. Complex activity measured in this manner implies coupling through a functional respiratory chain with oxygen as the final electron acceptor. Freshly dissected, minced tissue fragments were stirred at 37^{o} C in a 3-ml vessel of an oxygen monitoring apparatus (5300A system, YSI, Yellow Springs, OH) and provided with substrates (in mM) for measuring CI (10 pyruvate/2 malate/1 ADP), CII (10 succinate), or CIV (0.4 N,N,N',N'-tetramethyl-ρ-phenylenediamine (TMPD)/0.4 ascorbate). Specific inhibitors: 1.25 mM rotenone (CI), 2.5 μM antimycin A (CIII), and 2 mM potassium cyanide (CIV) were used to confirm complex-specific activity. The amount of oxygen consumed was calculated by assuming the initial oxygen concentration in the buffer to be 0.223 μmol O_2/ml. Similar results were obtained with brain, liver, or muscle. Permeabilization of cells with digitonin or saponins, utilization of isolated mitochondria, utilization of glutamate as substrate (for CI), utilization of different reaction buffers, or addition of the mitochondrial uncoupler, carbonylcyanide-ρ-trifluoromethoxyphenylhydrazone (FCCP), did not change the extent of CI deficiency measured in tissue from KO mice.

Gel Electrophoresis of Protein and DNA

Mitochondria-enriched extracts were prepared as in respiratory assay description. PAGE gels was performed by standard techniques (Bio-Rad). BNGE gels were electrophoresed as recommended by Mitosciences (Eugene, OR). Briefly, mitochondria-enriched samples were suspended in 0.75 M aminocaproic acid in 50 mM Bis-Tris, pH 7.0. n-dodecyl-β-D-maltopyranoside was added for to a final concentration of 1.6%. After centrifugation at 16,000 x g, the supernatant was collected and 2.5 μmol of 5% Coomassie blue G (in 0.5 M aminocaproic acid) was added. PMSF (1 mM), 1 μg/ml leupeptin and 1 μg/ml pepstatin were added to inhibit proteases. Samples of equivalent protein content were loaded onto 10% gels and separated at 150 V for about 2 h. After transfer to nylon membranes (Hybond P, Amersham), the presence of CI was assessed using antibodies against NDUFS3, NDUFB8, and NDUFA9 (Mitosciences), followed by ECL development (Source).

For DNA analysis, DNA was isolated by phenol-chloroform extraction. The DNA concentration was determined by H33258 fluorescence, and 5 μg was digested with specified restriction enzyme then electrophoresed through agarose, transferred to Zeta-Probe membrane (Bio-Rad) and hybridized with radioactive probes made by random-priming specific DNA fragments by standard techniques.

Nuclear Magnetic Resonance Imaging and Optical Spectroscopy

Mice were anesthetized with an intraperitoneal injection of Avertin (0.55 mg/g body wt). The respiration rate was monitored and additional doses of anesthetic were given subcutaneously as needed. Mice were immobilized with their bodies in a horizontal position in a custom-made probe. A hind leg was inserted into an inflatable cuff to initiate and then dissipate ischemia, and a thermostat was inserted to monitor leg temperature. The animals were kept warm with forced air throughout the experiments, adjusted as necessary to maintain the temperature of the leg between 35.5 and 37.5° C. The magnetic resonance experiments were performed in a 7 T Oxford vertical bore magnet. The mouse leg was probed using a solenoidal coil tuned to ^{31}P resonance frequency (121.65 MHz). Static magnetic field (B_o) homogeneity was optimized by shimming

using the proton peak from tissue water. Fully-relaxed spectra were acquired by summing 16 free induction decay signals (FIDS) with a 20-s interpulse delay, 2048 points and 5000 Hz sweep width. During the experimental procedure, spectra were acquired by summing 16 FIDS with a standard one-pulse sequence with a 1.5 s interpulse delay, a 45° flip angle, 2048 points, and 5000 Hz sweep width. Data were line-broadened with a 10 Hz exponential filter. Resting PCr concentrations were calculated with the PCr:ATP peak ratio from the fully relaxed spectra and the ATP concentrations measured after HPLC separation of extracts from frozen hind limbs. PCr concentrations throughout the dynamic experiments were determined by comparing the relative peak areas with the resting PCr concentrations. pH was determined from the chemical shift of P_i relative to PCr in each spectrum. Animals were allowed to recover from anesthesia overnight between optical and NMR experiments.

For optical spectroscopy, the hair was removed from the lower hind limb with a commercial hair removal cream (Neet). The leg was held between the fiber-optic bundles so that light traveled through the leg. During the optical experiments the animals breathed 100% O_2 to maintain high hemoglobin saturation of the blood. Ischemia was induced as in the NMR experiment. Optical transmission spectra were acquired with illumination from a constant-intensity, cooled, quartz-tungsten-halogen white light source (model 66184, Oriel Instruments) passed through a 7-mm optical fiber bundle (K42-347, Edmund Scientific), the mouse leg, and was then transmitted to a 2-mm fiber bundle. Constant light intensity was insured by a photo-feedback system (68850, Oriel Instruments). Spectra from 450 to 950 nm were acquired via a diffraction spectrograph (100S, American Holographics) with a 512-pixel photodiode array (C4350, Hamamatsu) using a 200-ms exposure time. Spectral acquisition was gated to acquire data at 1-s intervals, and the data were converted into digital form with a 16-bit analog-to-digital converter (no. AT-MIO-16X, National Instruments). Myoglobin and hemoglobin saturations were measured by partial least squares analysis of the spectra. Total oxygen consumption was computed from the slope of declining myoglobin and hemoglobin saturations at onset of ischemia. After the spectra were acquired, the hind limb was removed and frozen in liquid nitrogen.

Animals were euthanized with an overdose of anesthetic. HPLC (Waters Corporation, Milford, MA, USA) equipped with a 996 photodiode array was used to determine PCr and ATP levels. Resting ATPase was determined from the slopes of the least squares regression lines through the plots of PCr concentration during the initial phase of PCr breakdown during ischemia. Phosphorylation capacity was calculated by fitting the PCr recovery data to a monoexponential function as described (Blei et al., 1993). Two-tailed t-tests were used for comparisons between control and KO mice. Data are presented as means ± SEM.

Histology and Electron Microscopy

Cytochrome c oxidase staining was performed as follows: samples were incubated for 60 min at 25°C in sucrose (7.5 mg/ml), 50 mM phosphate buffer, 3, 3' diaminobenzidine tetrahydrochloride (0.5 mg/ml), cytochrome C (1 mg/ml), with a few crystals of catalase. Samples were then dehydrated in increasing concentrations of ethyl alcohol and finally xylenes, followed by mounting in Permount.

The myofibrillar ATPase assay was used for simultaneous identification of type I, IIA, and IIB muscle fibers, samples were incubated 8 min in buffer containing 50 mM potassium acetate, 17 mM $CaCl_2$, adjusted to pH 4.4 with acetic acid. Samples were then washed 3 x 2 min each, in buffer containing 300 mM Tris-HCl, 53 mM $CaCl_2$, adjusted to pH 7.8 with HCl. ATP (44

μg/ml) was add to 52 mM glycine, 65 mM NaCl, adjusted to pH 9.4 with NaOH, just before use. Samples were incubated in this medium 30 to 60 min, then washed with 1% $CaCl_2$ 3 x 2 min and incubated in 0.1% toluidine blue for 1 min. Samples were briefly soaked in water then quickly dehydrated in ethyl alcohol, followed by xylenes before mounting in organic mounting medium.

To assess succinate dehydrogenase activity, samples were incubated in 0.2 M phosphate buffer, pH 7.6 with nitro-blue tetrazolium (1 mg/ml, Sigma), and sodium succinate (27 mg/ml) for 15 to 60 min (usually 30 min) at 37° C. After 3 washes in deionized water, samples were incubated in 4% paraformaldehyde for 5 min, then rinsed several times with deionized water and mounted with aqueous mounting medium.

For the Gomori trichrome method, samples were stained in hematoxylin solution 5 min, rinsed in water, and then incubated in Gomori trichrome [Chromotrope 2R (6 mg/ml, Sigma), Fast green FCF (3 mg/ml, Sigma), phosphotungstic acid (6 mg/ml), and 1% glacial acetic acid in deionized water, pH 3.4 with NaOH] for 10 min. Samples were dipped 2 to 3 times in 0.2% glacial acetic acid before dehydration in ethyl alcohol/xylene and mounting.

NADH oxidoreductase was assayed by incubating muscle sections in 50 mM Tris pH 7.4, 0.03 mg/ml nitro blue tetrazolium, and 0.024 mg/ml NADH for 25 min at 37° C. After three 3-min washes in distilled water, samples were washed (3 min each) in 30%, 60%, 90% acetone and then 30% acetone. After a rinse in distilled water, slides were mounted with DPX (Fluka).

For electron microscopy, EDL and soleus muscles were fixed by immersion in 3% glutaraldehyde, embedded in plastic resin, sectioned, post-fixed with 2% osmium tetroxide and stained with 2% uranyl acetate. Samples were coded evaluated in a “blinded” manner using a Zeiss 910 electron microscope. The distribution and ultrastructural morphology of mitochondria were examined at a range of magnifications and then digital images were taken of 12 randomly selected muscle fibers from each genotype. They were recorded at a standard magnification to assess the depth of subsarcolemmal mitochondrial aggregates, which were measured as the maximal number of mitochondrial crossed by an imaginary line drawn into the fiber perpendicular to the sarcolemma up to underlying myofibrils.

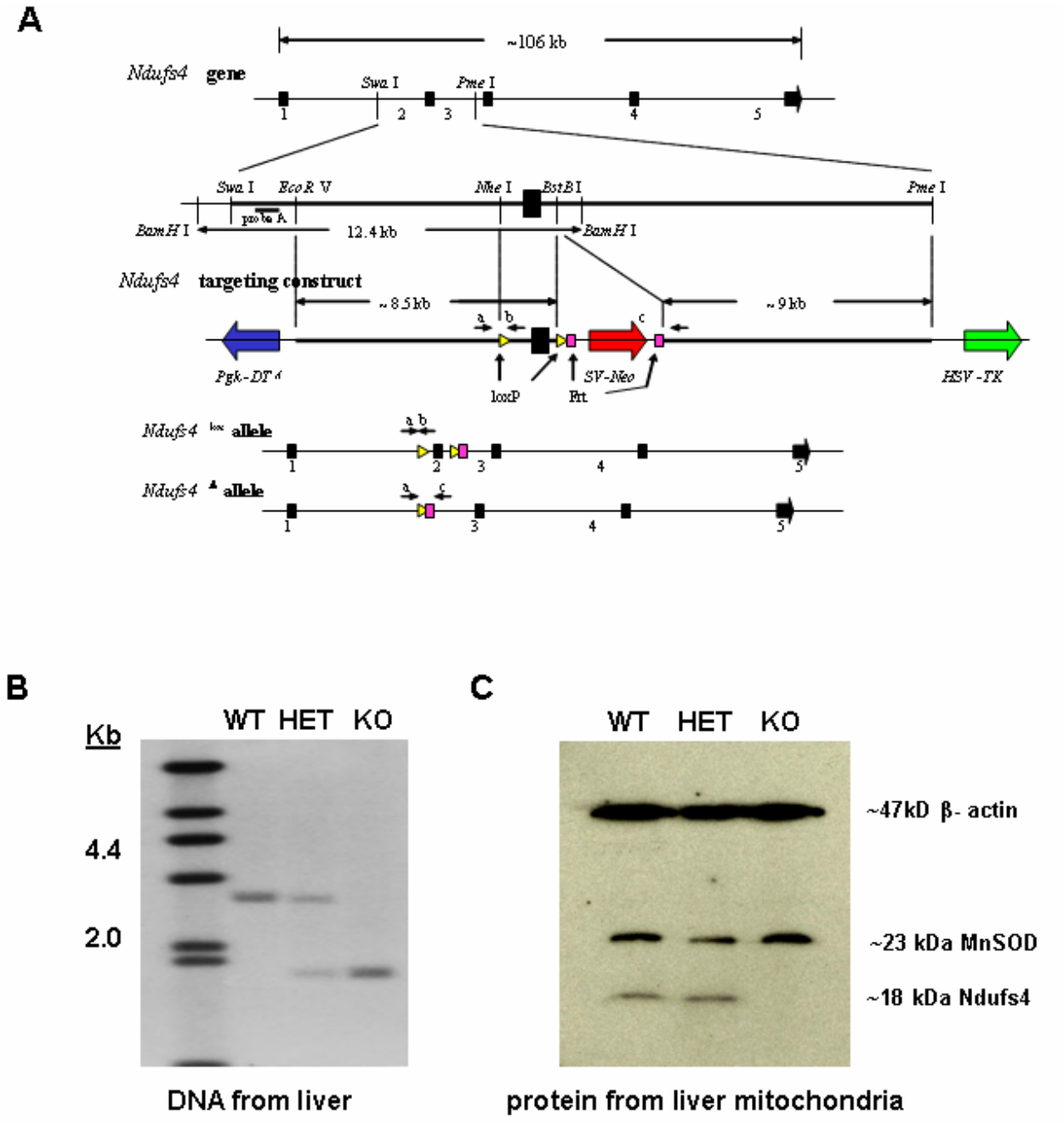

Figure S1. Gene Targeting Strategy; Southern Blot of KO, HET, and WT; and Western Blot Showing Loss of NDUFS4 Protein

(A) The top two lines show the second exon of the *Ndufs4* gene, with flanking SwaI-PmeI sites, subcloned into Bluescript. The location of probe used for Southern analysis of correctly targeted events is indicated as probe A. The third line shows insertion of *SV-Neo* gene (positive selection), and *Pgk-DTA* and *HSV-TK* genes (negative selection). LoxP sites are indicated by triangles flanking *Ndufs4* exon 2; frt sites (squares) flank SvNeo. *Ndufs4*^{lox} allele (fourth line) shows the correctly targeted allele after removal of *SV-Neo* by breeding the mice with *Rosa26-FLPer*. The *Ndufs4*$^{\Delta}$ allele (bottom line) shows the removal of exon 2 after breeding with mice expressing

Mox2-Cre. The excised fragment results in a frameshift mutation. For routine genotyping, the targeted allele was identified by PCR using primers a and b giving a ~200 bp band versus ~150 bp band for the WT allele; The *Ndufs4*$^{\Delta}$ allele was identified using primers a and c.

(B) Southern blot used to distinguish WT, HET and KO mice.

(C) Western blot of protein from WT, HET and KO mice probed with antibodies again NDUFS4 and mitochondrial manganese superoxide dismutase (MnSOD).

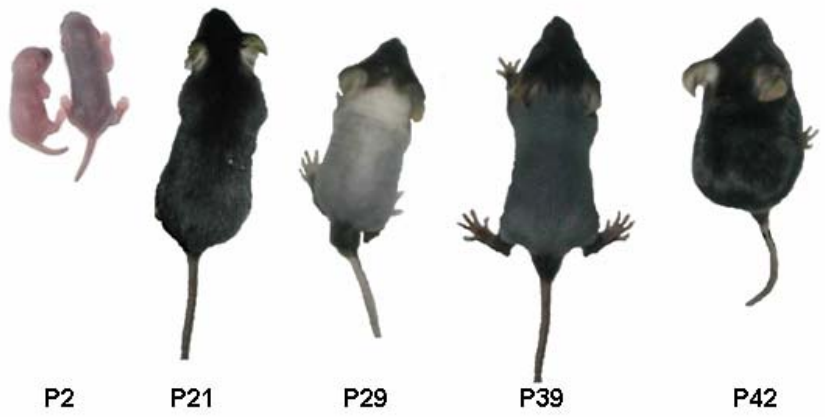

Figure S2

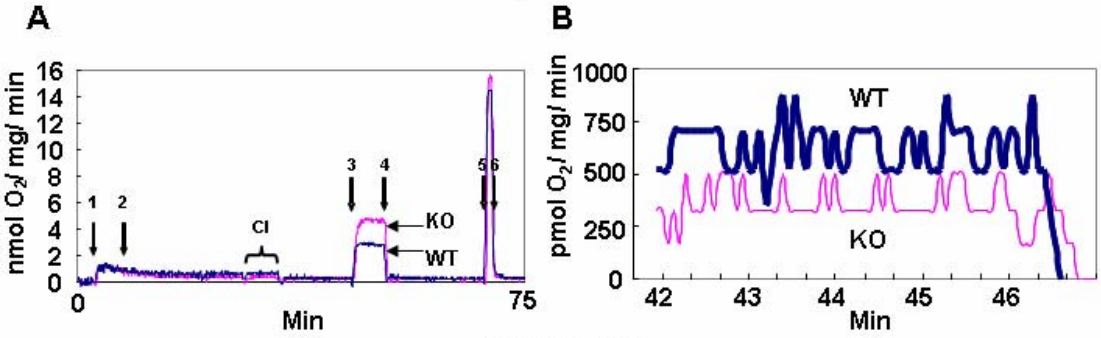

Figure S3

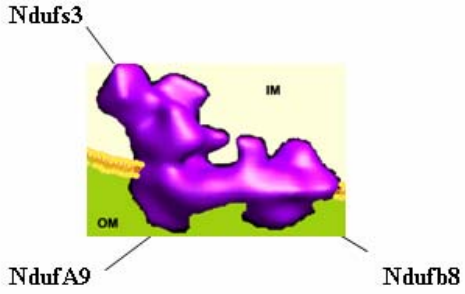

Figure S4

Figure S2. Photographs of KO Mice

Left to right: Photographs of KO mice at P2 (two mice with different weights), P21, P29, P39, and P42. Note loss of hair at P29 and regrowth by P42, and hunched appearance at P42.

Figure S3.

(A) Representative complete oxygen consumption profile of liver cells taken at P35 from a WT (darker trace) and mildly symptomatic KO mouse (lighter trace). Arrows indicate the addition of: (1) liver cells, (2) ADP, (3) succinate (substrate for complex II), (4) antimycin A, (5) TMPD/ascorbate (substrate for complex IV), and arrow (6) KCN. The beginning of bracket indicates addition of pyruvate/malate (substrate for complex I) and rotenone was added at the end of bracket (see B). O_2 consumption driven by complexes II and IV of KO mice were higher than WT in comparison, but they were usually comparable to that of WT.

(B) Enlargement of bracketed region in (A) showing decreased complex I-driven O_2 consumption. O_2 consumption by brain or liver tissue from KO was always less than half that of WT (n =>20). WT; upper (thicker) trace, KO; lower trace.

Figure S4.

Diagram showing the location of the three subunits used as representative proteins to analyze complex I. NDUFS4 is located in the peripheral arm near NDUFS3; IM, inner mitochondrial membrane, OM, outer mitochondrial membrane.

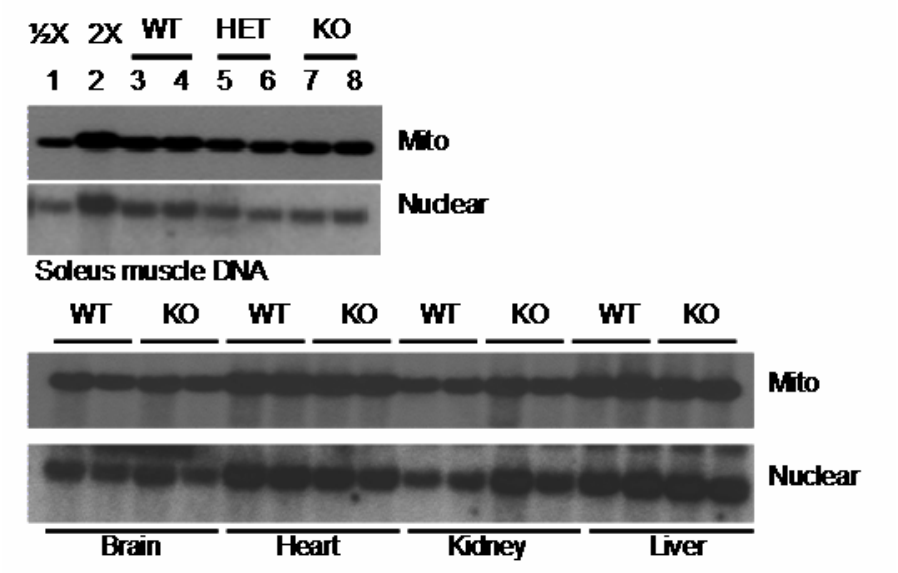

Figure S5.

Southern blot of DNA from various organs hybridized with probe for mitochondrial DNA (Mito) or a single-copy nuclear gene (*Gpr88*). Upper panel: DNA digested with EcoR V from soleus muscle of 2 different wild-type (WT), heterozygous (HET) or *Ndufs4* null (KO) mice; both bands are at ~ 6.6 kb. Lanes 1 and 2 show the results of loading half or twice the amount of DNA in lane 3. Lower panel: DNA digested with EcoR V from brain, heart, kidney and liver of two different WT or KO mice.