Supporting Information

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SI Methods

Generation of Gene-Trap (*Ndufs6*$^{gt/gt}$) Mice. The *Ndufs6* gene-trap embryonic stem cell (ESC) line (AR01380) was purchased from the International Gene Trap Consortium (www.genetrap.org). *Ndufs6*$^{gt/gt}$ mice were generated at the Transgenic Animal Service of Queensland (Brisbane, Australia) using standard methods (1). Mice were genotyped by duplex PCR on genomic DNA isolated from tail biopsies. The following primers were used: primer A, GAGTGAGGACGAGGAGAGTTG; primer B, CCATGGCCTTCTAAATTCAGGT; and primer C, AAGTGGTGGCCTAACTACGG. Primers A and B flank the genomic insertion site of the gene-trap vector in intron 2 of the *Ndufs6* gene and amplify a product for the wild-type allele (489 bp). The forward primer (primer C), complementary to the gene-trap vector, was used in conjunction with primer B to specifically amplify the mutated allele (240 bp). The gene-trap ES cells were derived from 129/Ola strain. The chimeric mice were bred to C57BL/6 background. All mice were produced in the mixed genetic background of C57BL/6 and 129/Ola. Mice were bred and maintained at the University of Melbourne following an approved protocol by the Murdoch Childrens Research Institute (MCRI) Animal Ethics Committee. Mice were housed on 12-h light/dark cycles and provided ad libitum access to food and water.

Longevity Study. Mice (10–12 per group) were weighed weekly for up to 1 y. Health was monitored and scored by signs of illness including weight loss, abnormal gait, fur condition, abnormal posture, lethargy, abdominal distension, and labored/rapid breathing. Mice were killed when their condition deteriorated significantly on the basis of this scoring system.

Western and Blue-Native PAGE Blot. For Western blotting, 15 μg total mitochondrial proteins was loaded per lane for 12.5% (wt/vol) SDS/PAGE analysis, as described in ref. 2. Ndufs6 was detected using a Ndufs6 antibody (1:250) that was generated by injecting a mixture of two Ndufs6 peptides synthesized by GL Biochem into rabbits. The peptides were conjugated to keyhole limpet hemocyanin (KLH). The Ndufs6 peptide sequences were KLH-Cys-Gly-Leu-Gln-Phe-Lys-Gln-His-His-His-OH and H-Thr-His-Thr-Gly-Gln-Val-Tyr-Asp-Glu-Lys-Asp-Tyr-Arg-KLH. Fusion protein was detected with mouse anti–β-gal monoclonal antibody (1:2,000; Promega). Blots were also analyzed for CII 70-kDa subunit (Sdha) as loading control using a mouse monoclonal antibody (A-11142; Invitrogen). BN-PAGE used a rabbit polyclonal antibody to the CI 39-kDa (Ndufa9) subunit (3) and an antibody to the 70-kDa CII subunit.

RNA Isolation and Quantitative PCR Analysis. Total RNA was isolated from liver, kidney, and brain using Illustra RNA Spin mini kit (GE Healthcare). For heart and skeletal muscle, total RNA was extracted by the TRIzol method (Astral Scientific). RNA was subsequently treated with DNaseI using Ambion Turbo DNA freeTM kit. cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR was performed using TaqMan assays and the signal was detected by Biorad iQ 5 Multicolor real-time PCR detection system. Primer and probe for *Ndufs6* were designed with Primerexpress software (Applied Biosystems) with the probe spanning across the exon 2/3 boundary. Primers and probes for *Gapdh*, *Anp*, and β-*MHC* were purchased from Applied Biosystems. Samples were run in triplicate for both the gene of interest and *Gapdh* as the internal control.

Acylcarnitine Measurement. Acylcarnitine species were analyzed from ~10 mg of heart tissue using an electrospray tandem mass spectrometry approach similar to van Vlies et al. (4). To semiquantify the amount of acylcarnitine species in heart tissue, internal standards were added: $^2[H]_9$-carnitine was used as internal standard for C0–C2 carnitines, $^2[H]_3$-isovaleryl carnitine for C3–C6 carnitines, $^2[H]_3$-octanoyl-carnitine for C8–C10 carnitines, and $2[H]_3$-tetradecanoyl carnitine for C12–C20 carnitines. The dried 80% (vol/vol) acetonitrile supernatant was butylated with 100 μL 9:1 (vol/vol) mixture of butanol and acetyl chloride at 65 °C for 15 min. Samples were measured in duplicate by electrospray tandem mass spectrometry using a Waters Quattro LC instrument.

Echocardiography. Echocardiography was performed on six *Ndufs6*$^{gt/gt}$ and nine wild-type mice using a 12-MHz phased array transducer and Vivid I ultrasound machine (GE Medical Systems) or a 40-MHz linear array transducer and the Vevo 2100 ultrasound system (VisualSonics). Anesthesia was induced with 1.5–4% (vol/vol) isoflurane and maintained with 1–1.5% (vol/vol) isoflurane delivered via facemask. Short-axis 2D image-guided M-mode images of the left ventricle were acquired and stored digitally. Offline analysis was performed using EchoPAC (GE Medical Systems) or on-board Vevo 2100 analysis software. After measurement of end-diastolic posterior wall thickness, end-diastolic diameter (ED), and end-systolic diameter (ES) as the average of three cardiac cycles, fractional shortening (%) was calculated as $[(ED - ES)/ED] \times 100$.

Functional Measurement of Isolated Working Mouse Hearts. Mice were anesthetized with an i.p. injection of Avertin (Sigma). After excision of hearts, aortic cannulation was performed under magnification and aortic perfusion commenced within 60 s. Hearts were then cannulated for left atrial perfusion and commenced in working mode at a constant preload of 10 mmHg and an afterload of 50 mmHg. Hearts were perfused with modified Krebs-Henseleit bicarbonate buffer (in mM: 118 NaCl, 4.7 KCl, 2.25 $CaCl_2$, 2.25 $MgSO_4$, 1.2 KH_2PO_4, 0.32 EGTA, 25 $NaHCO_3$, 11 D-glucose) gassed with 95% O_2, 5%CO_2 (vol/vol) at 37 °C (pH 7.4). Following equilibration of the spontaneous heart rate, hearts were electrically paced at 490 beats per minute (bpm). After 20 min of steady-state performance, aortic output and coronary flow rate were measured by timed collection of effluent throughout the protocols. Systolic and diastolic pressures, heart rate, and the left ventricular maximum rate of pressure development ($+\delta P/\delta t$) and relaxation ($-\delta P/\delta t$) were measured using a pressure transducer (CP844; Capto) attached to a needle cannula inserted through the apex of the left ventricle. The analog signal was amplified with an ML110 bridge amplifier (AD Instruments) and sampled at 1,000 Hz using a digital data archiving system (Powerlab8SP; AD Instruments). Stroke volume, external work, and other related parameters were calculated from the left ventricular pressure record as previously reported (5). To assess dynamic left ventricular cardiac functional capacity, Frank-Starling curves were generated by increasing left ventricular end diastolic volume and pressure (preload) between 5, 10, 15, 20, and 25 mmHg by altering the height of the left atrial perfusion reservoir chamber. Coronary perfusion pressure remained set at 50 mmHg throughout the study. Hearts were stabilized for 2–5 min at each preload level before functional measures were assessed (5).

1. Hansen GM, et al. (2008) Large-scale gene trapping in C57BL/6N mouse embryonic stem cells. *Genome Res* 18:1670–1679.
2. Koutsopoulos OS, et al. (2010) Human Miltons associate with mitochondria and induce microtubule-dependent remodeling of mitochondrial networks. *Biochim Biophys Acta* 1803:564–574.
3. Lazarou M, McKenzie M, Ohtake A, Thorburn DR, Ryan MT (2007) Analysis of the assembly profiles for mitochondrial- and nuclear-DNA-encoded subunits into complex I. *Mol Cell Biol* 27:4228–4237.
4. van Vlies N, et al. (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. *Biochem J* 387:185–193.
5. Pepe S, McLennan PL (2002) Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function. *Circulation* 105:2303–2308.

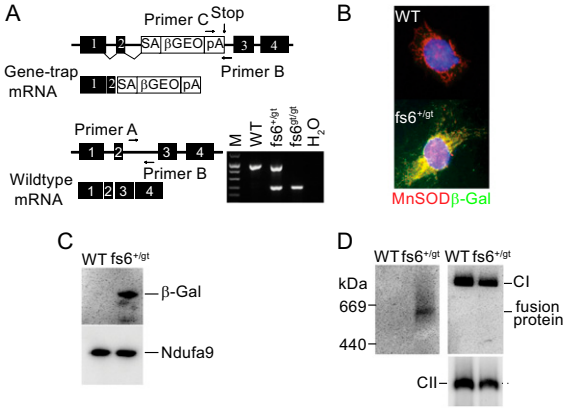

Fig. S1. Generation of *Ndufs6*$^{gt/gt}$ mice. (*A*) Insertion of the gene-trap vector into intron 2 of *Ndufs6* results in the generation of a fusion protein. Duplex PCR screen of mice for the presence of wild-type allele (489 bp) and gene-trap allele (240 bp). Black boxes, exons; SA, splice acceptor site; βGEO, β-galactosidase–neomycin resistance cassette; pA, polyadenylation signal. (*B*) Immunocytochemistry shows colocalization of fusion protein detected by β-Gal antibody (green) with the mitochondrial protein, MnSOD (red). (*C*) SDS/PAGE immunoblotting shows the expression of fusion protein detected by anti–β-Gal antibody. Ndufa9 (CI 39-kDa subunit) was used as the loading control. (*D*) BN-PAGE immunoblotting of isolated mitochondria to detect the fusion protein probed with β-Gal antibody or CI probed with CI 39-kDa subunit antibody. CII was used as the loading control. M, marker; WT, wild-type mice or ESC; fs6$^{+/gt}$, heterozygous *Ndufs6* mice or cells; fs6$^{gt/gt}$, homozygous *Ndufs6* mice.

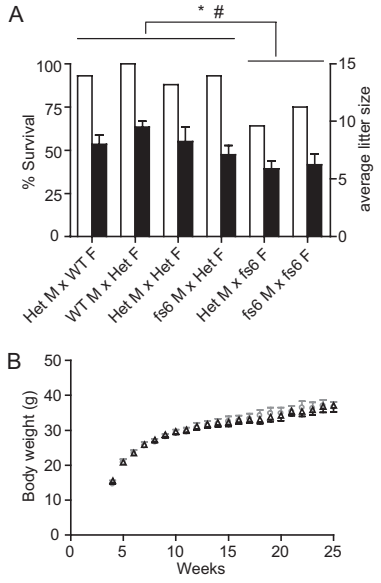

Fig. S2. Phenotype of *Ndufs6*$^{gt/gt}$ mice. (*A*) Survival rate of newborn pups (*Left y* axis, white bar) and average litter sizes of different matings (*Right y* axis, black bar); $n = 2–14$. *$P < 0.05$, survival rate of pups from nonaffected vs. affected females; $^{\#}P < 0.05$, litter sizes of nonaffected vs. affected females. M, male; F, female; WT, wild-type mice; Het, *Ndufs6*$^{+/gt}$ mice; fs6, *Ndufs6*$^{gt/gt}$ mice. (*B*) Growth curve of wild-type and *Ndufs6*$^{gt/gt}$ male mice; $n = 10–11$, expressed as mean body weight ± SEM. Gray open circle, wild-type mice; black open triangle, *Ndufs6*$^{gt/gt}$ mice.

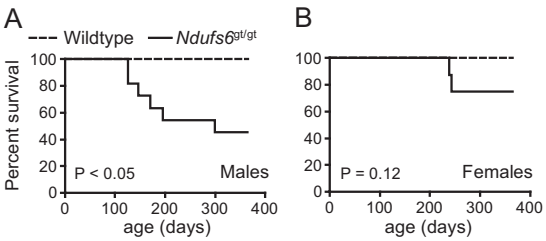

Fig. S3. Kaplan-Meier survival curves. Survival of wild-type and *Ndufs6*$^{gt/gt}$ male (*A*) and female (*B*) mice; n = 10–11 for each group.

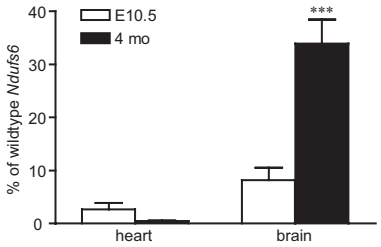

Fig. S4. *Ndufs6* gene expression in embryonic and adult heart and brain tissues. Wild-type *Ndufs6* mRNA levels measured by quantitative real-time PCR in heart and brain tissues from day 10.5 *Ndufs6*$^{gt/gt}$ embryos (E10.5, white bar) and 4-mo-old male *Ndufs6*$^{gt/gt}$ mice (4 mo, black bar). Data from adult tissues are the same as those presented in Fig. 3*A*. Results are expressed as percentage of wild-type levels ± SEM; n = 4–5. ***$P < 0.001$, E10.5 vs. 4 mo brain.

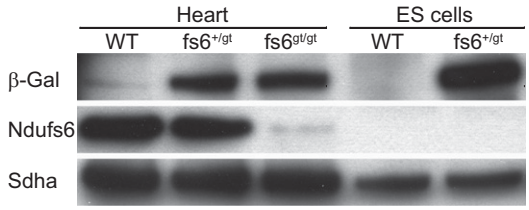

Fig. S5. Fusion protein and Ndufs6 expression in murine hearts and ESC. SDS/PAGE immunoblotting shows expression of fusion protein detected by anti–β-Gal antibody and Ndufs6 protein in both isolated mitochondria of heart tissues and whole cell lysates in ESC. The signal of Ndufs6 protein from ESC is below the threshold of detection. The levels of Sdha (CII 70-kDa subunit) were used as the loading control. WT, wild-type mice or ESC; fs6$^{+/gt}$, heterozygous *Ndufs6*; fs6$^{gt/gt}$, homozygous *Ndufs6* mice.

Table S1. Left ventricular steady-state performance of isolated perfused working hearts with electrical pacing (490 bpm) at left atrial filling pressures (preload) ranging from 5 to 25 mmHg

	WT (n = 7)					*Ndufs6*$^{gt/gt}$ (n = 8)				
Preload, mmHg	5	10	15	20	25	5	10	15	20	25
Peak systolic pressure, mmHg	69.8 ± 1.2	77.6 ± 1.3	81.5 ± 1.2	84.0 ± 1.4	79.5 ± 1.2	61.0 ± 1.3**	65.9 ± 1.5**	67.3 ± 1.6**	68.1 ± 1.5**	65.6 ± 1.7**
End diastolic pressure, mmHg	5.8 ± 0.3	6.0 ± 0.3	8.4 ± 0.3	12.9 ± 1.1	17.0 ± 0.8	9.0 ± 0.4**	13.9 ± 0.5**	15.9 ± 0.9**	20.4 ± 0.6**	24.4 ± 0.6**
Aortic output, mL/min/g	22.7 ± 0.9	37.8 ± 1.4	49.9 ± 1.8	57.2 ± 2.0	57.6 ± 2.1	10.9 ± 1.7**	16.9 ± 2.4**	21.8 ± 3.1**	22.1 ± 3.0**	20.9 ± 2.8**
Coronary flow, mL/min/g	13.7 ± 0.7	13.8 ± 0.6	13.8 ± 0.6	13.2 ± 0.6	13.1 ± 0.5	5.7 ± 0.9**	5.7 ± 1.0**	5.7 ± 0.9**	5.6 ± 0.9**	5.5 ± 0.8**
Stroke volume, mL/g	0.093 ± 0.004	0.132 ± 0.004	0.163 ± 0.008	0.181 ± 1.058	0.181 ± 0.794	0.043 ± 0.006**	0.058 ± 0.008**	0.071 ± 0.010**	0.071 ± 0.010**	0.068 ± 0.010**
+δP/δT, mmHg/s	3,138 ± 78	3,198 ± 85	3,400 ± 33	3,273 ± 23	2,947 ± 37	1,496 ± 106**	1,586 ± 111**	1,639 ± 110**	1,570 ± 101**	1,486 ± 86**
−δP/δT, mmHg/s	2,246 ± 36	2,123 ± 39	1,928 ± 29	2,059 ± 33	1,976 ± 37	1,349 ± 68**	1,287 ± 67**	1,142 ± 40**	1,277 ± 43**	1,374 ± 41**

+δP/δT, peak rate of rise of left ventricular pressure (contraction); −δP/δT, peak rate of decline of left ventricular pressure (relaxation). Data are presented as mean ± SEM; **$P < 0.01$ vs. wild type (WT).