SUPPLEMENTAL MATERIAL

EXTENDED MATERIALS AND METHODS

Research Objectives - To test the efficacy of genetic MPTP inhibition or mitochondrial-specific ROS suppression to prevent mitochondrial dysfunction and mitophagic cardiomyopathy in mice with cardiomyocyte-specific deficiency of Mfn2.

Animal models - All experiments were approved by the Washington University Institutional Animal Care and Use Committee and adhered to the NIH Guide for the Care and Use of Laboratory Animals. $Mfn2^{loxp/loxp}$ mice crossed onto *myh6*-driven nuclear-directed Cre (cardiac Mfn2 KO), *ppif* null (cyclophilin D KO), and lowCAT and hi-CAT transgenic mice have been described previously [1-3]. Mice were interbred to achieve the desired allelic combinations and genotyped using PCR. M-mode echocardiography, cardiac morphometric and histological analyses were performed using standard techniques [4].

Experimental design - These were unblinded case-control laboratory studies using littermate controls. Phenotypically normal "Ctrl" mice carried floxed alleles without Cre.

Immunoblot analyses were performed on mouse hearts snap-frozen in liquid nitrogen. Frozen hearts were homogenized (4 ^{o}C) in homogenization buffer (10 mM HEPES (pH 7.2), 320 mM sucrose, 3 mM $MgCl_2$, 25 mM $Na_2P_4O_7$, 1 mM DTT, 5 mM EGTA, 1 mM PMSF and complete mini protease inhibitor cocktail tablet (Roche)) using an electric tissue homogenizer. Myocardial homogenate was collected from the supernatant after centrifugation at 3,800g (10 min) and a mitochondrial fraction the pellet after centrifugation at 10,000g (10 min). Samples were quantified and solubilized in Laemmli sample buffer for size-separation on 4-15% SDS-PAGE mini-gels (Bio-Rad, Hercules, CA). After transfer to PVDF membranes and blocking for 1 hr with 5% nonfat dry milk in phosphate-buffered saline (PBS) and 0.1% Tween-20 (PBS-T), primary antibody was applied for two hrs, washed, and secondary antibody (horseradish peroxidase conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:3000, Cell Signaling)) applied for 1 hr. After developing with the ECL chemiluminescence reagent (Bio-Rad, Hercules, CA), bands were visualized and quantified by chemiluminescence electronic scanning on an LI−COR Odyssey infrared imaging system (LI−COR Corporate, Lincoln, NE). Primary antibodies used are: mouse monoclonal anti-Mfn2 [1:500 dilution, Abcam], mouse monoclonal anti-p62 [1:1000 dilution, Abcam], rabbit polyclonal anti-LC3 I/II [1:1000 dilution, Abcam], rabbit polyclonal anti-CAT [1:2000 dilution, Abcam], mouse monoclonal anti-poly Ub [1:100 dilution, Santa Cruz], mouse monoclonal anti-Parkin [1:500 dilution, Cell Signaling], rabbit polyclonal anti-S-nitrosocysteine [1:500 dilution, Abcam], mouse monoclonal anti-GAPDH [1:3000 dilution, Abcam], mouse monoclonal anti-COX IV [1:3000 dilution, Abcam], mouse monoclonal anti-β-actin [1:2000 dilution, Sigma], and MitoProfile total OXPHOS rodent WB antibody cocktail [1:500 dilution, Abcam]. Myocardial protein oxidation was measured using Oxyblot protein oxidation detection kit according to the manufacturer's protocol (Millipore, Billerica, MA).

Studies of isolated mouse heart mitochondria were performed as previously described [2]. Briefly, hearts were rapidly removed from sacrificed animals, immersed in ice-cold PBS supplemented with 10 mM EDTA and minced into small pieces. Minced heart tissues were resuspended in PBS supplemented with 10 mM EDTA and 0.05% trypsin (Sigma, Saint Louis, MO) for 15 min on ice and neutralized by addition of 0.025% trypsin inhibitor (Sigma, Saint Louis, MO). Digested heart tissues were resuspended with 5 mL of freshly prepared ice-cold IB_m1 (0.067 M sucrose, 0.05 M Tris-HCl, 0.05 M KCl, 0.01 M EDTA and 0.2% BSA, adjust pH to 7.4) and homogenized by using 10-15 strokes of a Teflon pestle operated at 1,600 r.p.m. Homogenates were centrifuged at 700*g* for 10 min at 4 °C. Supernatants were transferred to new tubes and centrifuged at 8,000*g* for 10 min at 4 °C. Pellets were resuspended in ice-cold IB_m2 (0.25 M sucrose, 3 mM EGTA, 0.01 M Tris-HCl, and 0.01 M KCl, adjust pH to 7.4), and centrifuged at 8,000*g*

for 10 min at 4 °C. Pellets containing mitochondria were resuspended in 500 µl of IB_m2, and assayed immediately.

Mitochondrial oxygen consumption was measured in freshly isolated cardiac mitochondria as described [5] using a Strathkelvin 782 apparatus (Strathkelvin Instruments Limited, North Lanarkshire, Scotland). Briefly, 100 µL of IB_m3 (0.25 M sucrose, 0.01 M Tris-HCl, 5 mM $MgCl_2$, 20 µM EGTA and 2 mM KH_2PO_4, adjust pH to 7.4) with substrate glutamate/malate (5 mM/2.5 mM, Sigma, Saint Louis, MO) was added into MT200/MT200A Respirometer Cell. Upon stabilization, 20 µg of mitochondria were added, basal oxygen consumption rate (state 2) and ADP (400 µM) stimulated oxygen consumption rate (state 3) were recorded. ATP synthesis was inhibited by adding 0.6 µg/ml of oligomycin (Sigma, Saint Louis, MO), and final uncoupled oxygen consumption rate assessed after addition of 50 nM CCCP (Sigma, Saint Louis, MO).

Flow cytometric analysis of mitochondrial size and polarization state was performed after labeling with 2.5 µM MitoTracker Green and 10 µM Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) (Invitrogen, Carlsbad, CA) for 15 min at room temperature, then washed twice with ice-cold PBS and resuspended in 500 µL of ice-cold IBm3. Fluorescent signal intensities of stained mitochondria were read on a BD LSR II instrument (BD Biosciences, San Jose, CA). Data are presented as histograms for 100,000 ungated events.

Mitochondrial H_2O_2 production was assessed with the Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Briefly, 87.5 µg mitochondria were mixed with Amplex Red reagent in 1x reaction buffer and fluorescent signal intensities measured on a Spectra MAX M5 (Molecular devices, Sunnyvale, CA) 96 well plate reader with excitation at 544 nm and emission at 590 nm.

Calcium-induced mitochondrial permeability transition pore opening was determined in freshly isolated cardiac mitochondria. Mitochondria were suspended in 200 µl reaction buffer (120 mM KCl, 10 mM Tris (pH 7.6) and 5 mM KH_2PO_4) at a concentration of 250 µg/ml and stimulated by the addition of 25 µM and 250 µM $CaCl_2$. The absorbance was continuously measured using a Spectra MAX M5 (Molecular devices, Sunnyvale, CA) 96 well plate reader at 540 nm.

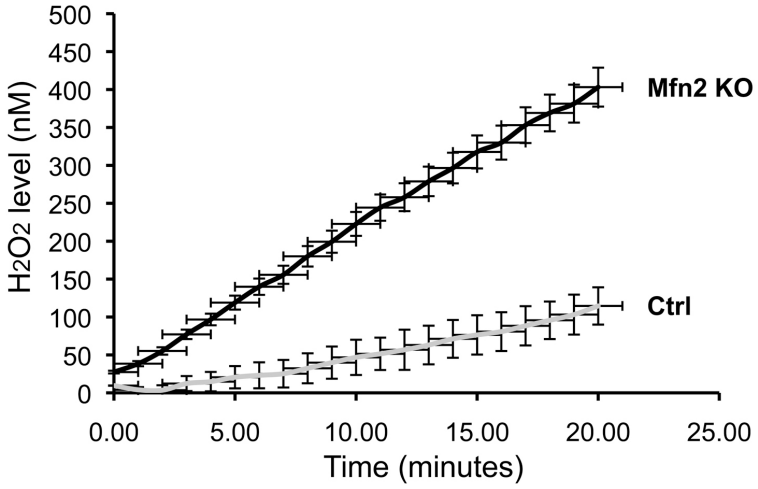
Histological analyses were performed on mouse hearts fixed with 4% paraformaldehyde in situ, paraffin embedded, and sectioned at 5 µm. Cardiomyocyte cross sectional area was measured on sections stained with Alexa fluor 488 conjugate of wheat germ agglutinin (WGA) (Invitrogen, Carlsbad, CA) and imaged on a Nikon Eclipse Ti confocal microscope using a 60x oil immersion objective, exciting at 488 nm and monitoring emission at 510-560 nm. Myocyte cross-sectional area analysis was performed using Image J (NIH) measurements of 40-60 cells per section.

TUNEL positivity used the same confocal instrumentation and the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI), with excitation at 488 nm and emission monitored at 510-560 nm.

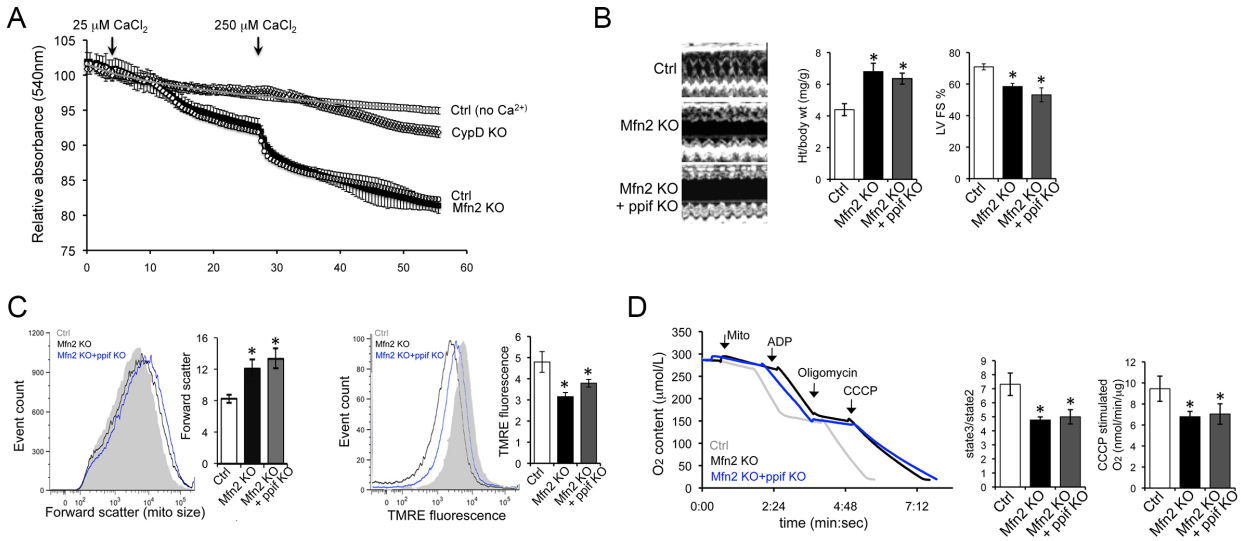
For ultrastructural examination, samples of left ventricular free wall were fixed in EM fixative buffer (4% paraformaldehyde, 0.1 M sodium cacodylate and 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, CA)) prior to sequential staining with osmium tetroxide and uranyl acetate, dehydrated, and embedded in Polybed 812. Tissue was thin-sectioned on a Reichert-Jung Ultra-Cut microtome (90 nm thickness), post-stained in uranyl acetate and lead citrate, and viewed on a Jeol electron microscope (JEM-1400) at 5,000x direct magnification (JEOL, Tokyo, Japan).

Statistical Analysis - Unless otherwise specified all tests used ANOVA with Tukey's post hoc test; $P<0.05$ was considered significant.

SUPPLEMENTAL FIGURES

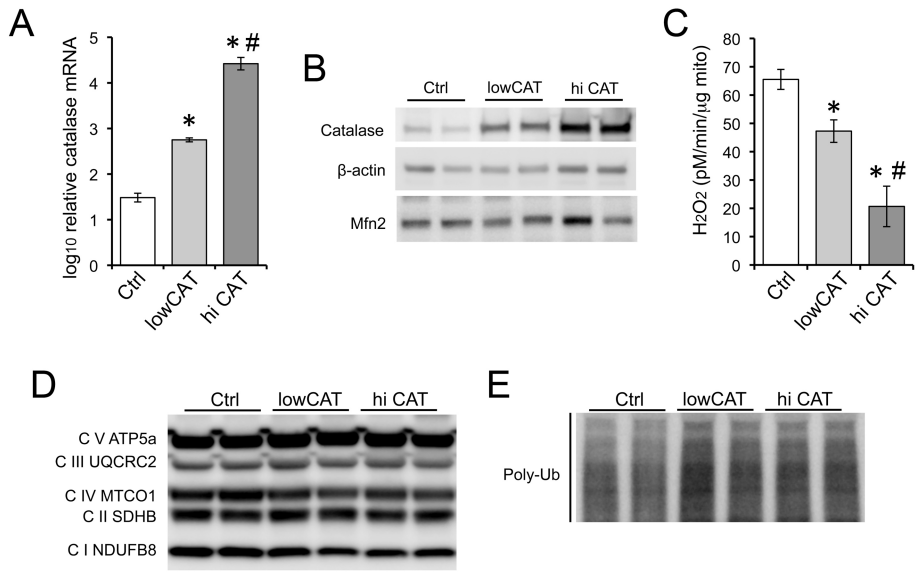


Online Figure I. *Basal H_2O_2 production by control and Mfn2 KO heart mitochondria assessed by Amplex Red fluorescence.* Each point is mean±SEM of 6 (Mfn2 KO) or 7 (Ctrl) independent determinations.

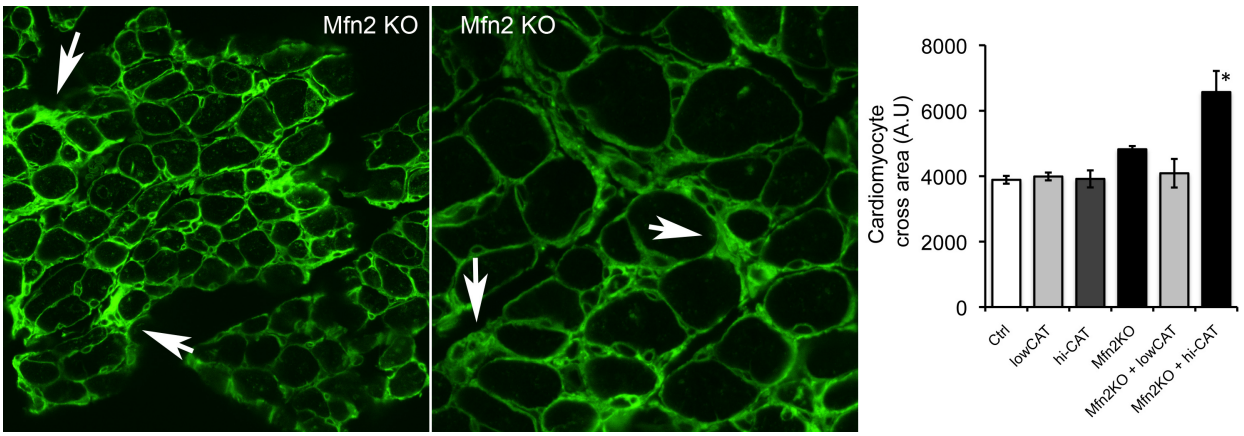


Online Figure II. *MPTP function is normal in Mfn2 null hearts and MPTP inhibition does not improve cardiomyopathy induced by Mfn2 ablation.*

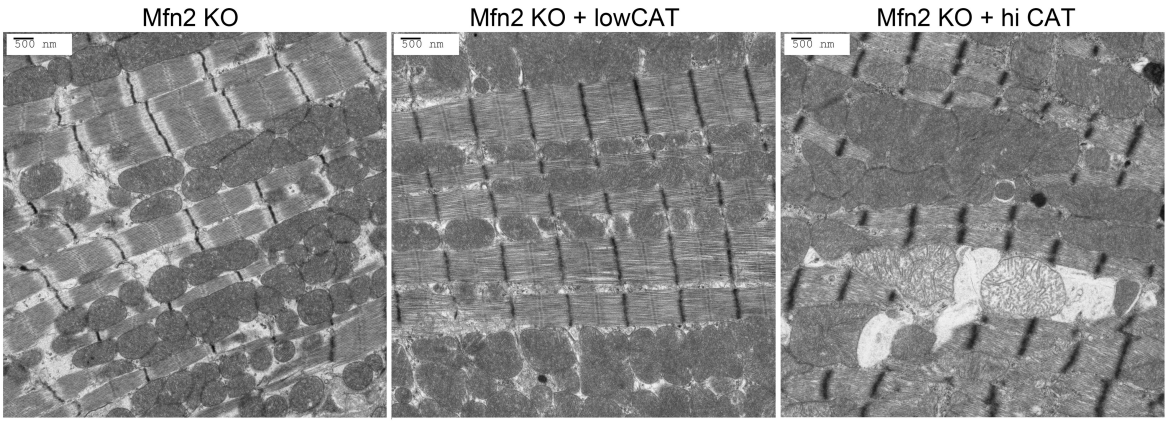
A. Calcium-stimulated swelling of Mfn2 null cardiac mitochondria. CypD null mitochondria and Ctrl mitochondria without calcium stimulation are shown as controls. **B.** Echocardiographic and morphometric studies in 30 week old mice. **C.** Mitochondrial size assessed by flow cytometric forward scatter. **D.** Mitochondrial polarization status assessed by flow cytometric TMRE fluorescence. **E.** Mitochondrial respiration. Mean data are n=4-8/group. In **C**, **D**, and **E**, black tracings are Mfn2 KO and blue tracings are Mfn2 KO + *ppif* KO; grey histograms are normal Ctrl. * = P<0.05 vs Ctrl (ANOVA).



Online Figure III. *Baseline characteristics of human catalase (CAT) overexpressing mouse hearts.* **A.** CAT mRNA levels assessed by RT-qPCR (log_{10} scale). **B.** Immunoblot analysis of CAT protein levels. **C.** H_2O_2 suppression in CAT expressing mitochondria assessed using Amplex Red. **D.** Western blot analysis of mitochondrial respiratory complex proteins. **E.** Immunoblot analysis of mitochondrial protein poly-ubiquitination. * = P<0.05 vs Ctrl; # = P<0.05 vs lowCAT (ANOVA).



Online Figure IV. *Effects of lowCAT and hi-CAT expression on cardiomyocyte hypertrophy and interstitial fibrosis (WGA stain) evoked by cardiac Mfn2 ablation.* Representative confocal micrographs of cardiomyocytes in cross section; interstitial fibrosis is indicated by arrows. Group mean data are n=3/group; * = P<0.05 vs Ctrl (ANOVA).



Online Figure V. *Ultrastructural studies of cardiomyocyte mitochondria.* Original magnification 5,000x. Note severe mitochondrial enlargement and degeneration in Mfn2 KO + hi-CAT.

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