Super-Suppression of Mitochondrial Reactive Oxygen Species Signaling Impairs Compensatory Autophagy in Primary Mitophagic Cardiomyopathy

Moshi Song, Yun Chen, Guohua Gong, Elizabeth Murphy, Peter S. Rabinovitch, Gerald W. Dorn II

**Rationale:** Mitochondrial reactive oxygen species (ROS) are implicated in aging, chronic degenerative neurological syndromes, and myopathies. On the basis of free radical hypothesis, dietary, pharmacological, and genetic ROS suppression has been tested to minimize tissue damage, with remarkable therapeutic efficacy. The effects of mitochondrial-specific ROS suppression in primary mitophagic dysfunction are unknown.

**Objective:** An in vivo dose-ranging analysis of ROS suppression in an experimental cardiomyopathy provoked by defective mitophagic clearance.

**Methods and Results:** Mice lacking mitofusin 2 (Mfn2) in hearts have impaired parkin-mediated mitophagy leading to accumulation of damaged ROS-producing organelles and progressive heart failure. As expected, cardiomyocyte-directed expression of mitochondrial-targeted catalase at modest levels normalized mitochondrial ROS production and prevented mitochondrial depolarization, respiratory impairment, and structural degeneration in Mfn2 null hearts. In contrast, catalase expression at higher levels that supersuppressed mitochondrial ROS failed to improve either mitochondrial fitness or cardiomyopathy, revealing that ROS toxicity is not the primary mechanism for cardiac degeneration. Lack of benefit from supersuppressing ROS was associated with failure to invoke secondary autophagic pathways of mitochondrial quality control, revealing a role for ROS signaling in mitophagic clearance. Mitochondrial permeability transition pore function was normal, and genetic inhibition of mitochondrial permeability transition pore function did not alter mitochondrial or cardiac degeneration, in Mfn2 null hearts.

**Conclusions:** Local mitochondrial ROS (1) contribute to mitochondrial degeneration and (2) activate mitochondrial quality control mechanisms. A therapeutic window for mitochondrial ROS suppression should minimize the former while retaining the latter, which we achieved by expressing lower levels of catalase. (Circ Res. 2014;115:348-353.)

**Key Words:** cardiomyopathies ■ catalase ■ mitochondria ■ mitochondrial degradation ■ mitofusin 1

Mitochondria produce ATP that fuels excitation–contraction coupling. They are also sources of damaging reactive oxygen species (ROS). Preserving cardiac mitochondrial health is, therefore, essential to maintaining normal cardiac function. We recently identified a central role for the mitochondrial fusion protein mitofusin 2 (Mfn2) in mitochondrial quality control signaling. Ablation of Mfn2 in mouse hearts interrupts parkin-mediated mitophagy of damaged organelles, impairing cardiomyocyte respiration and inducing cardiomyopathy.

Mfn2 dimers form molecular bridges linking cardiomyocyte mitochondria to sarcoplasmic reticulum, which is independent of mitophagy. Calcium microdomains thus created are essential to regulatory bioenergetic feedback. When mitochondrial uptake of sarcoplasmic reticulum–derived calcium and the calcium-sensitive generation of reduced NADPH are delayed by Mfn2 ablation, mitochondrial production of superoxide (O$_2^-$) is transiently increased. However, whether increased ROS contribute to the mitophagic cardiomyopathy provoked by Mfn2 deficiency is unknown. Accordingly, we suppressed mitochondrial ROS in Mfn2 null hearts using transgenically expressed mitochondrial-targeted catalase (mCAT). Remarkably, we observed an inverse dose–response relationship: low mCAT expression...
improved all aspects of cardiac and mitochondrial function, whereas high mCAT expression evoked persistent organ and organelle dysfunction, despite supersuppression of ROS. These results validate the notion that local mitochondrial ROS contribute to mitochondrial degeneration and also uncover an essential role for ROS in mitochondrial quality control signaling.

**Methods**

*Mfn2* loxP/loxP mice crossed onto *myh6*-driven nuclear-directed Cre (cardiac *Mfn2* knockout), *ppif* null (cyclophilin D knockout), and lowCAT and hiCAT transgenic mice have been described previously.1,3,4 Details of experimental and analytic protocols are in the Online Data Supplement.

**Results**

**Mitochondrial ROS Production Is Increased in Mitophagically Impaired *Mfn2*-Deficient Hearts**

Because superoxide production is transiently increased in hemodynamically stressed young *Mfn2*-deficient cardiomyocytes,2 we asked whether ROS are chronically increased because these mice develop their characteristic delayed cardiomyopathy.1 Basal hydrogen peroxide (*H₂O₂*) production by *Mfn2*-deficient cardiac mitochondria (Amplex Red)5 was increased ≈3-fold over control values (Online Figure I). Immunoreactive catalase also increased with *Mfn2* deficiency (Figure 4A and 4B), consistent with an adaptive antioxidant response to chronically increased mitochondrial ROS.6

**Mitochondrial Permeability Transition Pore Inhibition Does Not Prevent Cardiomyopathy in Cardiac *Mfn2* Null Mice**

Both ROS and altered mitochondrial–sarcoplasmic reticulum interactions can open mitochondrial permeability transition pores (MPTP).7–9 In contrast to a previous report,10 here (Online Figure IIA) and previously,2 we do not observe intrinsic abnormalities in calcium-stimulated MPTP opening in *Mfn2* null cardiac mitochondria. Nevertheless, we tested whether MPTP opening contributed to the cardiomyopathy of *Mfn2* deficiency by genetically deleting the MPTP regulatory protein cyclophilin D in cardiac *Mfn2* knockout mice. Compound knockout mice followed up for 30 weeks exhibited no difference in cardiac hypertrophy, left ventricular contractile impairment, mitochondrial enlargement and depolarization, or mitochondrial respiratory dysfunction when compared with cardiac *Mfn2* null mice (Online Figure IIB–IID). Thus, inhibiting MPTP opening by genetic ablation of cyclophilin D does not improve the cardiomyopathy.

**Mitochondrial ROS Negatively and Positively Contribute to the Cardiomyopathy of *Mfn2* Insufficiency**

*H₂O₂* is generated during oxidative phosphorylation as the normal reaction product of *O₂* and superoxide dismutase; its toxic effects are managed in part by catalase-mediated decomposition of *H₂O₂* into oxygen and water. Expression of mitochondrial-targeted human catalase (mCAT) moderates the toxic effects of ROS.11,12 Accordingly, we expressed mCAT in hearts at 2 different expression levels, both of which have improved other murine cardiac disease models.3 In normal hearts, the mCAT chicken β-actin–driven transgene expressed at ≈10-fold higher levels than the mCAT floxed-stop bacterial artificial chromosome (here in combination with *myh6*-driven nuclear-targeted Cre),13 with corresponding dose-dependent effects on substrate-stimulated mitochondrial *H₂O₂* production (Online Figure IIIA–IIIC). We, therefore, called these as hiCAT and lowCAT, respectively. Alone, neither hiCAT nor lowCAT affected levels of myocardial *Mfn2*, electron transport chain proteins, ubiquitinated proteins (Online Figure...
IIIB, IIID, and IIIE), or any measured metric of cardiac or mitochondrial function (vide infra).

We expressed lowCAT on the cardiac Mfn2 null background to determine how mitochondrial ROS contributes to the cardiomyopathy caused by cardiac Mfn2 deficiency. As with other cardiac disease models,1 lowCAT attenuated cardiac enlargement (Figure 1A) and cardiac cardiomyocyte hypertrophy (Figure 1B) and improved left ventricular dysfunction (Figure 1C). These benefits were associated with normalization of mitochondrial ROS production (Figure 1D).

Ignoring Voltaire’s aphorism that “better is the enemy of good,” we tested ROS supersuppression in Mfn2 deficiency by coexpressing hiCAT. Unexpectedly, reduction of mitochondrial ROS production in Mfn2 null hearts to well below normal levels (Figure 1H) failed to improve any aspect of the hallmark cardiomyopathy. Rather, cardiac enlargement in 30-week-old mice was increased further (Figure 1E and 1F), and the time-dependent decline in ventricular pump function was accelerated (Figure 1G). These results reveal an inverse dose–response relationship for catalase-mediated ROS suppression in this mitophagic cardiomyopathy.

**Oxidative Protein Damage Is Minimal in Mfn2 Null Hearts**

The observation that hiCAT is more effective than lowCAT in suppressing mitochondrial ROS production in Mfn2 null hearts, but is ineffective in rescuing the cardiomyopathy, suggested that ROS cytoxicity is not causing cardiac failure. Indeed, Mfn2 null cardiac homogenates show no evidence of increased protein S-nitrosylation or oxidation, whereas both are evident in Mfn1/Mfn2 double knockout hearts used as a positive control (Figure 2A). We confirmed that the hallmark defect in parkin-mediated mitophagy was not affected by lowCAT or hiCAT (Figure 2B; positive control is cardiac parkin transgenic mouse heart).

**Mitochondrial ROS Production Contributes to Mitochondrial Quality Control**

Next, we tested whether mitochondrial-derived ROS were having localized effects on mitochondrial fitness in Mfn2 null hearts. ROS normalization with lowCAT (Figure 1D) improved mitochondrial dysmorphology (Figure 3A; Online Figure IV), normalized mitochondrial polarization status (a marker of mitochondrial health14,15; Figure 3B), and corrected respiratory dysfunction (Figure 3C). In contrast, hiCAT expression in Mfn2 null hearts actually exaggerated the characteristic abnormalities in mitochondrial size (Figure 3D), depolarization (Figure 3E), and respiration (Figure 3F). Ultrastructural examination of Mfn2 knockout+hiCAT hearts also revealed giant mitochondria in various states of degeneration (Online Figure V). Thus, lowCAT improved overall mitochondrial quality control in Mfn2–deficient hearts, whereas hiCAT further impeded it.

We posited that the general benefits of lowCAT on mitochondrial fitness, which can be explained, in part, by modulating mitochondrial damage, might also accrue from relieving the load on compensatory clearance mechanisms invoked by mitophagic dysfunction. Higher levels of autophagosome-associated p62/sequestosome and increased processing of the microtubule-associated protein 1 light chain 3 (LC3) (Figure 4A and 4B) in Mfn2–deficient hearts indicated that cell-wide autophagy, a potential secondary mechanism for culling damaged mitochondria,16 is increased. Whereas neither lowCAT nor hiCAT alone affected autophagy, lowCAT expression further increased the ratio of LC3-II/LC3-I when compared with Mfn2 null alone (Figure 4A).

By contrast, supersuppression of mitochondrial ROS with
hiCAT did not improve LC3 processing in Mfn2 knockout hearts (Figure 4B), revealing a role for mitochondrial ROS in provoking secondary mitochondrial autophagy. In this context, combined suppression of both primary mitophagy and secondary autophagy is a likely reason why the Mfn2 null cardiomyopathy is aggravated by hiCAT (Figure 4C).

**Discussion**

Hydrogen peroxide can function as a signaling molecule during metabolic stress. Here, we show that the mitophagic cardiomyopathy evoked by cardiomyocyte-specific Mfn2 deletion can benefit from suppression, but not virtual elimination, of mitochondrial H$_2$O$_2$. There are 3 major findings: (1) ROS-mediated cytotoxicity is not the primary driver of mitophagic cardiomyopathy, which is likely caused by many different forms of mitochondrial dysfunction (including loss of polarization and respiratory impairment that we dissociated from ROS levels using hiCAT) evoked by interrupted culling; (2) high levels of ROS found in Mfn2 null cardiac mitochondria contribute to ongoing mitochondrial damage when clearance is suppressed (because lowCAT normalized these and enhanced mitochondrial fitness); and (3) Some threshold level of mitochondrial ROS is required, likely in combination with other signals, to initiate autophagy as a secondary mechanism for mitochondrial clearance when mitophagy fails. Our discovery of a therapeutic window for mitochondrial ROS suppression validates the idea that low levels of mitochondrial-derived ROS can be therapeutic by promoting mitophagy/autophagy signaling, whereas high levels are deleterious because they produce mitochondrial and cellular toxicity.

To our knowledge, this is the first evaluation of the dose-dependent effectiveness of ROS suppression in a condition caused by a primary defect in mitochondrial quality control. The bimodal dose–response reveals multiple levels of interconnectedness between mitochondrial dysfunction and culling. To maintain proper cellular homeostasis, mitophagy must selectively target only dysfunctional organelles; polarization status is a central determinant of whether a given mitochondrion will be retained or sequestered and mitophagogically...
Mitochondrial inner membrane potential is normally reciprocally related to mitochondrial ROS production; depolarized mitochondria produce toxic ROS that may mark the organelle for parkin-mediated mitophagy. When lack of Mfn2 interrupts normal parkin signaling, chronically increased mitochondrial ROS contributes to mitochondrial damage, establishing a positive feedback loop. Accumulation of depolarized, ROS-producing organelles invokes a backup mechanism for disposing of damaged cellular components, cell-wide autophagy. Suppressing mitochondrial ROS production to levels that are near-normal (as with lowCAT expression) interrupts this vicious cycle (Figure 4C).

In contrast, hiCAT expression in Mfn2 knockout hearts dissociates mitochondrial polarization status and ROS production; ROS levels drop to a fraction of normal, despite marked organelle depolarization. Thus, any ROS contribution to the signal for mitochondrial elimination via secondary autophagy is lost, and damaged mitochondria continue to accumulate. These findings provide in vivo evidence for an essential role of mitochondrial-derived ROS in mitochondrial quality control via mitophagy and autophagy. The deleterious effects conferred by mitochondrial ROS supersuppression warrant consideration of antioxidant dose-effect in the heart and other organs when evaluating therapeutics for disorders, such as Parkinson disease or aging, wherein mitophagy defects are central to observed pathology.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Damaged or senescent mitochondria produce toxic reactive oxygen species (ROS) and have to be culled through mitophagy.
- When mitophagy is impaired as in aging or some chronic diseases, ROS levels increase.
- Suppressing mitochondrial ROS has been beneficial in experimental aging and disease models.

**What New Information Does This Article Contribute?**

- Mitochondrial ROS toxicity is not a major direct cause of the cardiomyopathy that follows suppression of mitophagy.
- Mitochondrial ROS contribute to mitochondrial dysfunction in mitophagically impaired hearts, which is relieved by ROS normalization with mitochondrial catalase.
- ROS supersuppression with highly expressed catalase interrupts compensatory mitochondrial autophagy, thereby aggravating mitophagic cardiomyopathy.

The free radical hypothesis implicating ROS in aging and numerous chronic diseases is widely accepted and has spawned attempts to prevent senescence or organ degeneration by ROS scavenging. However, ROS may also act as indicators of cell/organelle damage, thereby providing a signal for necessary repair or removal. Under this circumstance, overly aggressive ROS suppression might conceivably interfere with normal adaptive responses. In our study we have, for the first time, validated this scenario. First, we show that mitochondrial ROS production is chronically increased in the mitophagically impaired mitofusin 2–deficient heart model. We then demonstrate that expression of mitochondrial-directed catalase at levels that normalize ROS corrects all mitochondrial abnormalities and prevents the cardiomyopathy evoked by mitofusin 2 deletion. Strikingly however, expression of mitochondrial-directed catalase at higher levels that supersuppress mitochondrial ROS actually exacerbates mitochondrial and cardiac dysfunction. Mechanistically, this is explained by failure to invoke compensatory autophagy when ROS levels are too low. These findings uncover the essential role for mitochondrial-derived ROS in mitochondrial quality control signaling and provide an example of how excessive suppression of mitochondrial ROS can actually cause harm, sounding a cautionary note for any chronic disease being considered for antioxidant therapy.
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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. Mfn2loxP/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. 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.

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 °C) in homogenization buffer (10 mM HEPES (pH 7.2), 320 mM sucrose, 3 mM MgCl2, 25 mM Na2HPO4, 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 were: 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. 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 IBa1 (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 700g for 10 min at 4 °C. Supernatants were transferred to new tubes and centrifuged at 8,000g for 10 min at 4 °C. Pellets were resuspended in ice-cold IBa2 (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 8,000g.
for 10 min at 4 °C. Pellets containing mitochondria were resuspended in 500 µl of IBm2, 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 IBm3 (0.25 M sucrose, 0.01 M Tris-HCl, 5 mM MgCl2, 20 µM EGTA and 2 mM KH2PO4, 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 H2O2 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 KH2PO4) at a concentration of 250 µg/ml and stimulated by the addition of 25 µM and 250 µM CaCl2. 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_{2}O_{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.

REFERENCES