Supplemental Figures and Legends

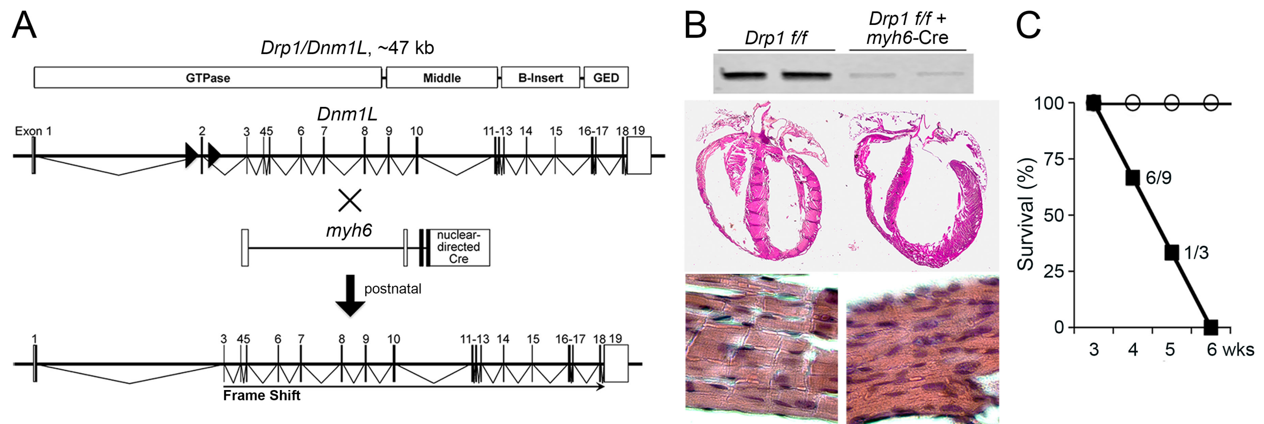


Figure S1, related to Figure 1. *Premature lethality from postnatal ablation of cardiac Drp1.* **A.** Schematic of *myh6*-Cre mediated Drp1 ablation in the early postnatal mouse hearts. **B.** Characteristics of Drp1-deficient hearts: Immunoblot analysis (top) is shown with gross and histological pathology of hematoxylin and eosin stained hearts. **C.** Survival curve; open circles are *Drp1f/f* controls, closed squares are *Drp1f/f* + *myh6*-Cre knockouts.

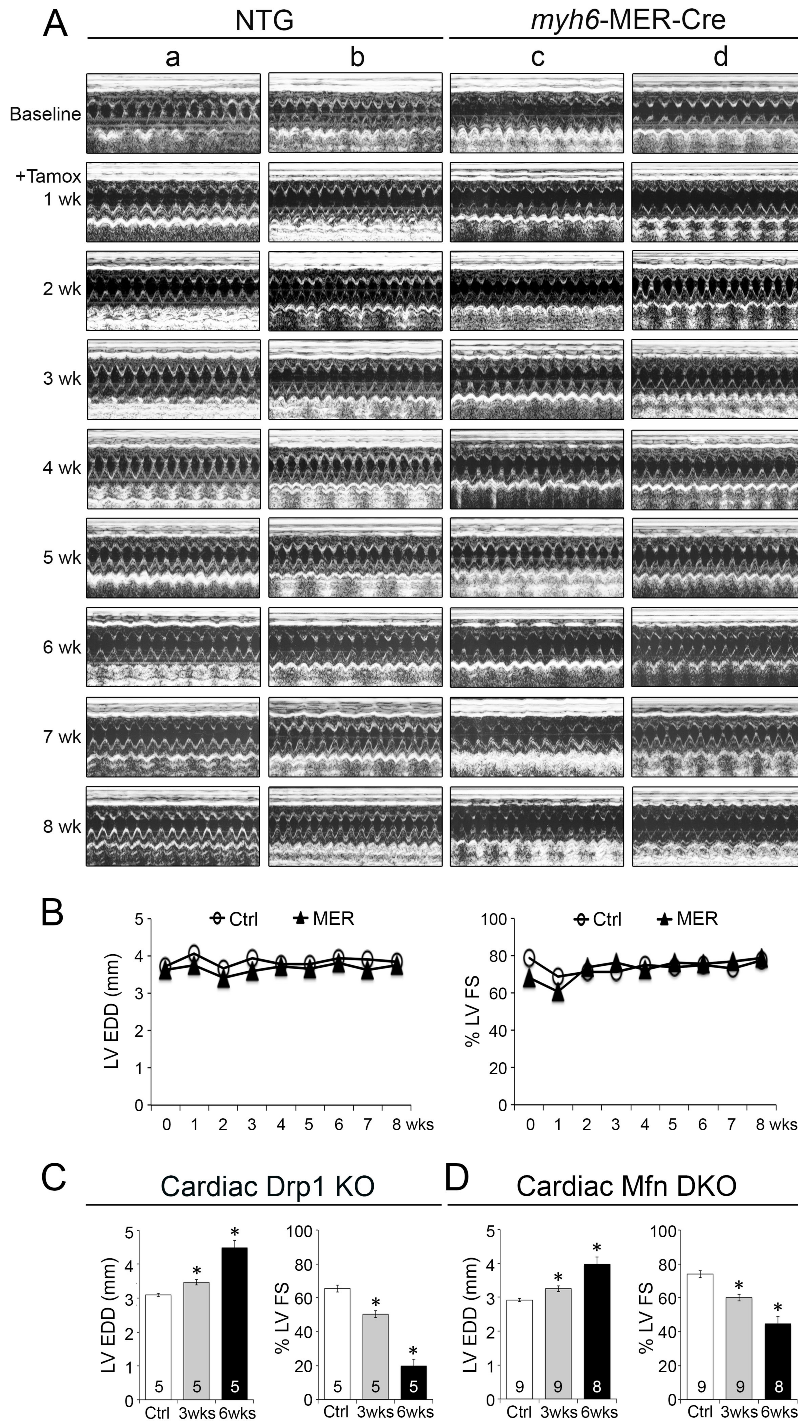


Figure S2, related to Figure 1. *Serial echocardiographic analysis of cardiac function of myh6-MER-Cre transgenic, Drp1 KO, or Mfn DKO mice.* **A-B.** Serial M-mode echocardiographic analysis of cardiac function in *myh6*-MER-Cre transgenic mice. Tamoxifen, with or without the *myh6*-MER-Cre transgene, exhibits no baseline effects on cardiac function within 8 weeks compared to the non-transgenic littermate controls (N=2). Echocardiographic images for each mouse (**A**) and group quantitative data for left ventricular (LV) end-diastolic dimension (EDD) and fractional shortening (FS) (**B**) were shown at each time point. **C-D.** Quantitative data for LV EDD and FS before, at 3 and 6 weeks after Drp1 ablation (**C**) or Mfn1/Mfn2 ablation (**D**). Data are mean ± SEM; * = P<0.05 vs identically treated controls.

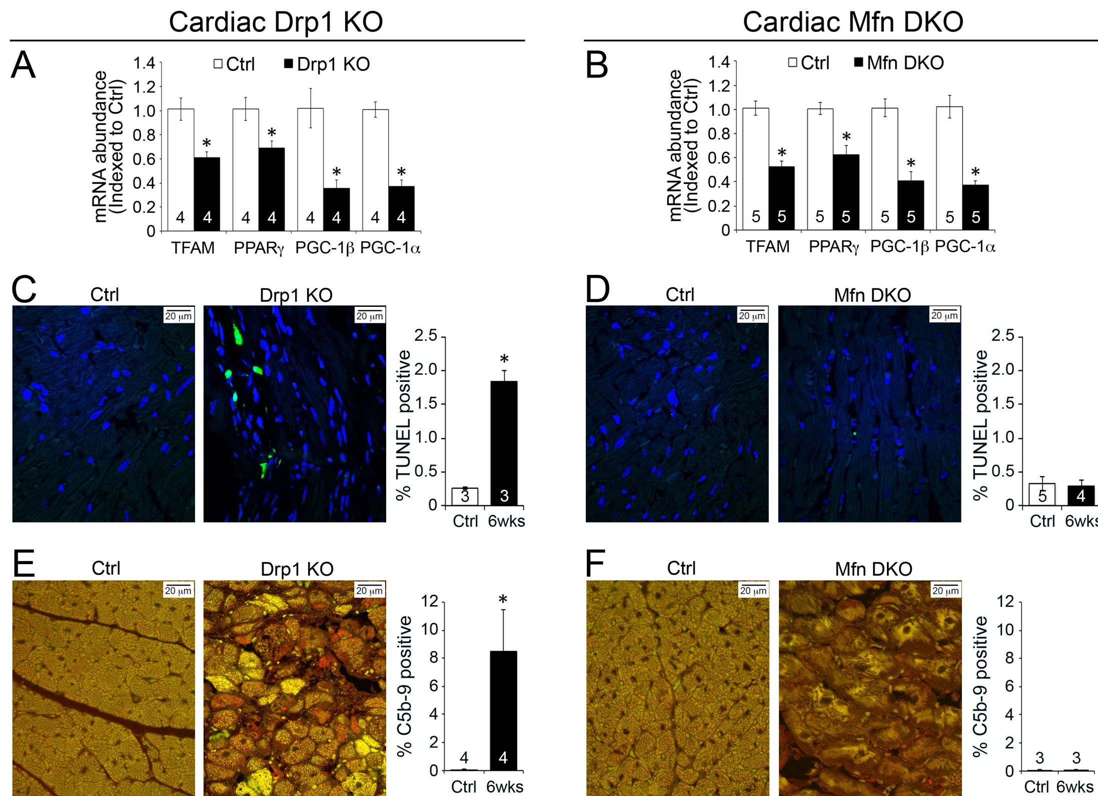


Figure S3, related to Figure 2. *Effects of Drp1 or Mfn1/Mfn2 ablation on mitochondrial biogenesis and cardiomyocyte death.* **A-B.** Quantitative RT-PCR analysis of mitochondrial biogenic gene expressions in Drp1 KO (**A**) or Mfn DKO (**B**) hearts. TFAM is transcription factor A, mitochondrial; PPARγ is peroxisome proliferator activated receptor gamma; PGC-1β is peroxisome proliferative activated receptor, gamma, coactivator 1 beta; PGC-1α is peroxisome proliferative activated receptor, gamma, coactivator 1 alpha. **C-D.** TUNEL assay in Drp1 KO (**C**) or Mfn DKO (**D**) hearts. Green dots indicate the nuclei of apoptotic/necrotic cardiomyocytes; counterstained with DAPI. **E-F.** Fluorescence microscopy of C5b-9 stained (green) Drp1 KO (**E**) or Mfn DKO (**F**) hearts; counterstained against cardiac heavy chain myosin (red). Data are mean ± SEM; * = $P<0.05$ vs identically treated controls.

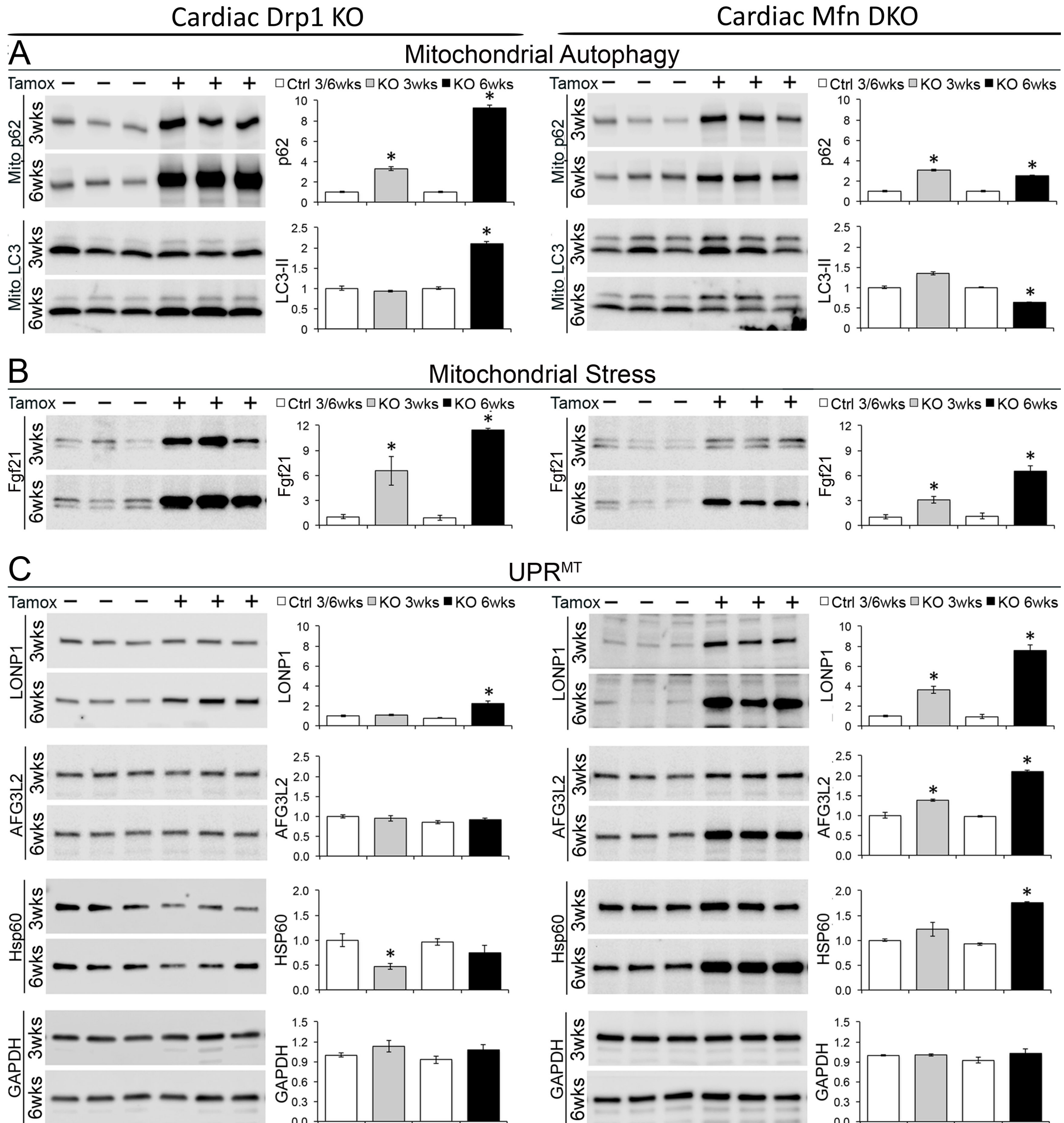


Figure S4, related to Figure 3. *Immunoblot analyses of protein abundance of markers for mitochondrial autophagy, mitochondrial stress, and mitochondrial unfolded protein response (UPR^{mt}) in Drp1 KO or Mfn DKO hearts.* **A.** Immunoblot analysis of autophagy markers p62 and LC3-II in mitochondrial fractions from Drp1 KO (left) or Mfn DKO (right) hearts. **B.** Immunoblot analysis of a mitochondrial stress marker Fgf21 in cardiac homogenates. **C**. Immunoblot analysis of UPR^{mt} markers LONP1, AFG3L2, Hsp60 in cardiac homogenates. Each lane is a different mouse heart. p62 is Sequestosome1; LC3-II is microtubule associated protein 1 light chain 3, processed form; Fgf21 is fibroblast growth factor 21; LONP1 is Lon peptidase 1; AFG3L2 is ATPase family member 3-like 2; Hsp60 is heat shock protein chaperone 60; GAPDH is shown as loading control. Quantitative group data are to the right. White columns indicate Cre-negative controls; grey columns indicate data 3 weeks, and black columns 6 weeks, after tamoxifen injection. Data are mean ± SEM of 4 hearts per group; * = P<0.05 vs identically treated controls.

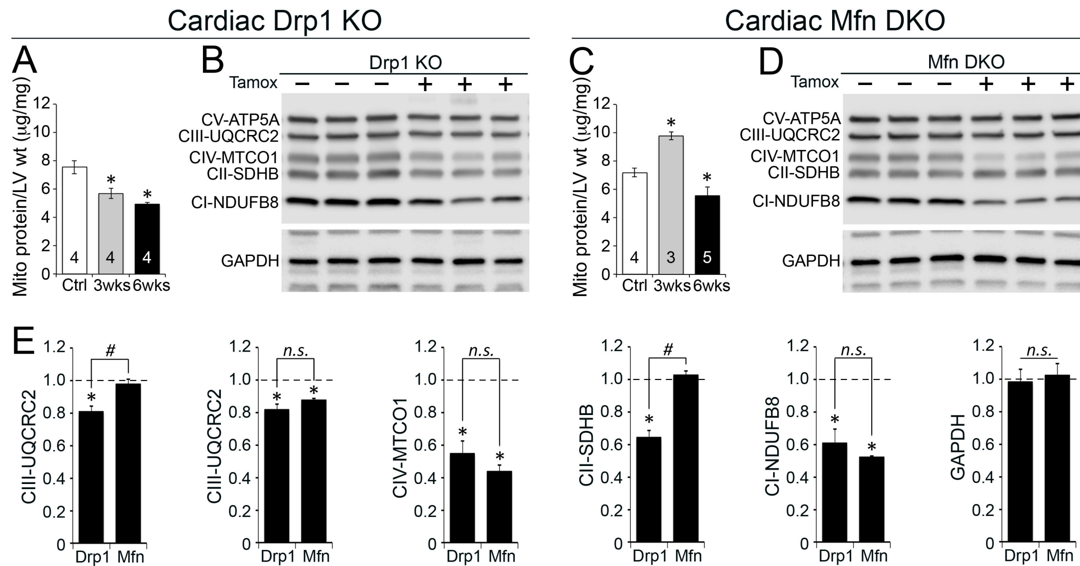


Figure S5, related to Figures 4 and 5. *Effects of Drp1 or Mfn1/Mfn2 ablation on cardiac mitochondrial content.* **A.** Ratio of myocardial mitochondrial proteins to left ventricle (LV) weight in Drp1 KO hearts. **B.** Immunoblot analysis showing depletion of mitochondrial proteins in cardiac homogenates of Drp1 KO hearts 6 weeks after gene deletion. **C-D**, exactly as described for figure S5a and S5b except using Mfn DKO hearts. **E.** Quantifications of immunoblot results shown in figure S5b and S5d. CI-NDUFB8 is NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8; CII-SDHB is succinate dehydrogenase complex, subunit B; CIII-UQCRC2 is ubiquinol-cytochrome c reductase core protein 2; CIV-MTCO1 is cytochrome c oxidase I, mitochondrial; CV-ATP5A is ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit; GAPDH is shown as loading control. Horizontal dashed line represents normal value from Cre-negative controls. Data are mean ± SEM of 4 hearts per group; * = P<0.05 vs identically treated controls; # = P<0.05 vs 6wks Drp1 KO.

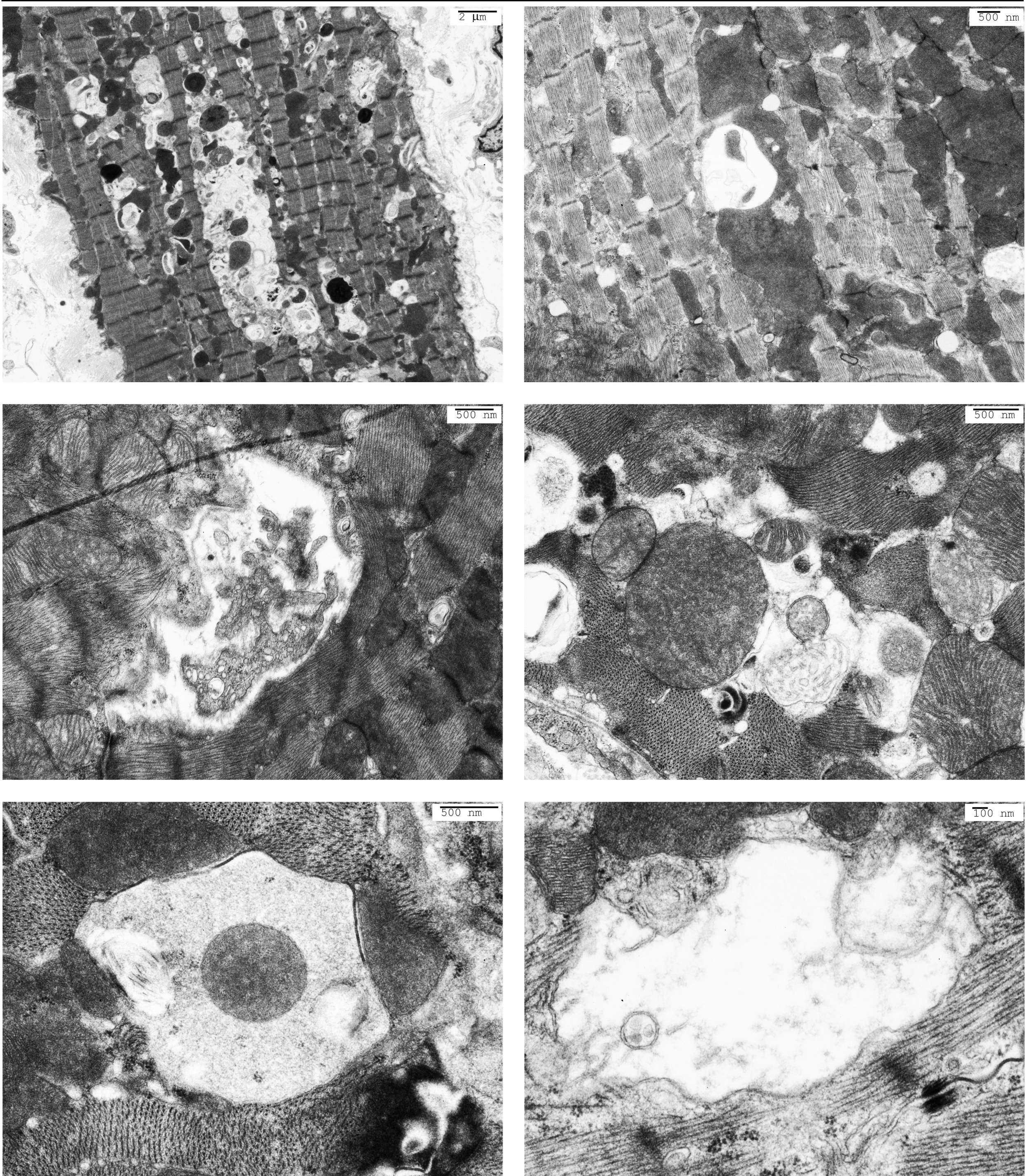
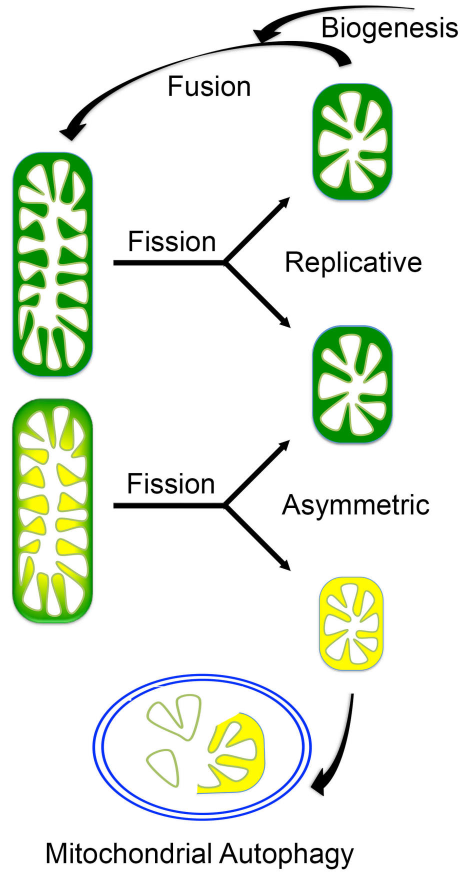
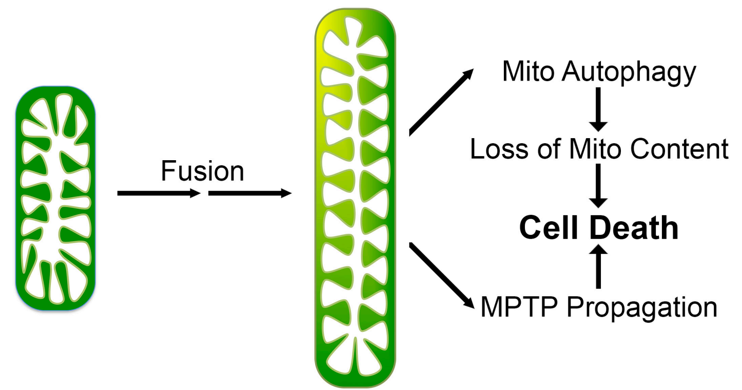


Figure S6, related to Figure 6. *Mitochondrial autophagy in Drp1 null hearts.* Transmission electron microscopic images (2,500x-20,000x) showing mitochondria inside cardiomyocyte autophagosomes in Drp1-deficient hearts.





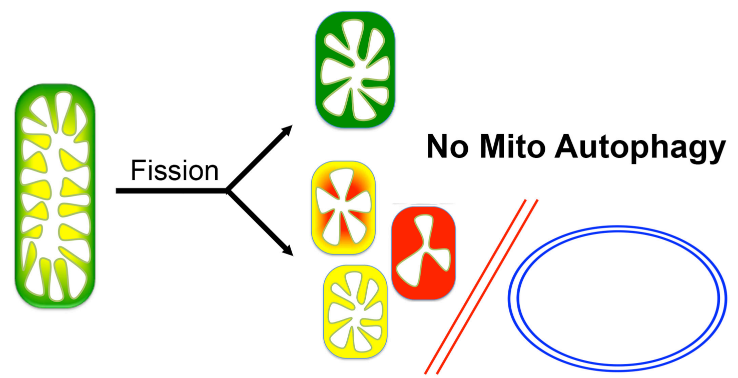


Figure S7, related to Figures 2-7. *Schematic depiction of mechanisms elucidated by conditional cardiac Drp1 or Mfn1/Mfn2 deficiency in the current studies.* Green mitochondria are fully polarized and "healthy"; orange mitochondria are depolarized and "damaged"; red mitochondria are ROS-producing and "toxic".

Supplemental Experimental Procedures

Mouse Generation and Phenotypic Analyses

Drp1$^{loxp/loxp}$ (Ishihara et al., 2009), Mfn1$^{loxp/loxp}$ and Mfn2$^{loxp/loxp}$ (Chen et al., 2003; Chen et al., 2007), and *ppif* null (Baines et al., 2005) mice have been described. Drp1$^{loxp/loxp}$ mice were crossed onto the *myh6*-nuclear-directed Cre transgenic line (Oka et al., 2006) for cardiomyocyte-specific gene deletion after birth. For conditional cardiac ablation, floxed mouse lines were crossed onto the *myh6*-MER-Cre-Mer mice; gene recombination was induced by intraperitoneal (i.p.) administration of tamoxifen (Sohal et al., 2001). Weekly M-mode echocardiography was performed on unsedated mice (Chen et al., 2011); controls were sex- and age-matched Drp1$^{loxp/loxp}$ ± *ppif*$^{-/-}$ littermates, Mfn1$^{loxp/loxp}$+Mfn2$^{loxp/loxp}$ littermates and *myh6*-MER-Cre-Mer mice with or without tamoxifen administration. All of the controls exhibited normal cardiac function over the period of 6-week monitoring (Chen et al., 2011). All experimental procedures were approved by the Washington University Institutional Animal Care and Use Committee.

Western Blotting

Cardiac tissues were harvested, snap-frozen in liquid nitrogen, and homogenized in tissue extraction reagent (Invitrogen, FNN0071) with protease inhibitor (Roche, 05892970001) and phosphatase inhibitor (Roche, 04906837001) as previously described (Chen et al., 2010). Myocardial homogenate was collected from supernatant after centrifugation at 3,800 *g* and myocardial mitochondrial fraction was pelleted and resuspended after centrifugation at 10,000 *g*. Proteins were size-separated by 4-15% pre-cast gradient SDS-PAGE (Bio-Rad, 456-1086), transferred to PVDF membranes (GE Healthcare, 10600021), and blocked with phosphate-buffered saline (PBS, Invitrogen, 70011-069) containing 0.1% Tween-20 (Promega, H5152) plus 5% nonfat dry milk (Bio-Rad, 170-6404) and/or 5% bovine serum albumin (BSA, Santa Cruz Biotech, 9048-46-8), incubated with primary antibodies at room temperature for 2 hours and horseradish peroxidase (HRP) conjugated secondary antibodies for 1 hour, and visualized using the ECL chemiluminescence reagent (Bio-Rad, 170-5060).

Histological Studies

Mice were deeply anesthetized and the hearts were transcardially perfused and immediately fixed with 4% formaldehyde solution in PBS. Tissues were paraffin embedded and sectioned at a thickness of 5 μm on a Leica RM2255 rotary microtome. For chemical staining, the slices were de-paraffinized in xylene and rehydrated with a gradient (100-50%) of ethanol, washed in distilled water. Hematoxylin-eosin stain (Sigma, GHS116 & HT110116), Masson's trichrome stain (Sigma, HT15, HT10132 & 34256), and TUNEL assay (Promega, G3250) were performed according to the manufacturers' protocols.

For Evans blue studies, mice were intraperitoneally administered 1% Evans blue dye (Sigma, E2129) solution in PBS at 1% volume relative (ml) to body mass (g) ~24 h prior to tissue sampling (Hamer et al., 2002). Heart tissues were frozen in optimal cutting temperature (OCT) compound (Andwin Scientific, 14-373-65) and stored at -80 °C before being sectioned at a thickness of 10 μm on a Leica Cryocut 1800 microtome. Sections were pre-warmed at room temperature and then fixed with pre-chilled 100% methanol for 10 min, and stained with FITC conjugated-wheat germ agglutinin (Invitrogen, W834) for 30 min before nuclear counterstaining with DAPI (Vector Laboratories, H-1200). Evans blue positive cardiomyocytes fluoresced in red.

For immunofluorescent staining, the slices were deparaffinized in xylene and rehydrated with a gradient (100-50%) of ethanol, washed in distilled water. Antigen retrieval was performed by incubating the slices in sodium citrate buffer (Poly Scientific, S2307) at 100 °C for 20 min and the samples were permeabilized by incubating in 1% Triton X-100 (Sigma, T9284) at room temperature for 20 min. The samples were then blocked with 5% goat serum (Jackson ImmunoResearch, 005-000-121) at room temperature for 30 min before incubated with primary antibody against C5b-9 at 4°C overnight followed by secondary fluorescent secondary antibody at room temperature for 1 hour; the samples were then

counterstained using primary antibody against heavy chain cardiac Myosin at room temperature for 2 hours followed by secondary fluorescent secondary antibody at room temperature for 1 hour before mounting in DAPI (Vector Laboratories, H-1200).

Antibodies

For western blotting, primary antibodies against Drp1 (1:1000, ab56788), Mfn1 (1:500, ab57602), Mfn2 (1:1000, ab56889), GAPDH (1:3000, ab8245), p62 (1:1000, ab56416), LC3 I/II (1:1000, ab128025), Fgf21 (1:1000, ab171941), LONP1 (1:500, ab103809), Hsp60 (1:2000, ab46798), and OXPHOS (a premixed cocktail of antibodies against CI-NDUFB8, CII-SDHB, CIII-UQCRC2, CIV-MTCO1 and CV-ATP5A, 1:500, MS604) were from Abcam. Primary antibody against AFG3L2 (1:500, sc-84687) was from Santa Cruz Biotechnology. Horseradish peroxidase (HRP) linked secondary antibodies anti-mouse IgG (1:3000, cs7076) and anti-rabbit IgG (1:3000, cs7074) were from Cell Signaling Technology.

For immunohistochemistry, primary antibodies against C5b-9 (1:200, ab55811) and heavy chain cardiac Myosin (1:200, ab15) were from Abcam. Alexa Fluor conjugated secondary antibodies anti-mouse IgG (1:400, A-11029) and anti-rabbit IgG (1:400, A-11035) were from Invitrogen.

Transmission Electron Microscopy

Mice were deeply anesthetized and cardiac tissues were collected and fixed in EM fixation buffer containing 4% paraformaldehyde (Election Microscope Sciences, 15710), 2.5% glutaraldehyde (Election Microscope Sciences, 16200) in 0.1 M sodium cacodylate buffer, pH 7.4 (Election Microscope Sciences, 12310). A Jeol electron microscope (JEM-1400) at 1,500x - 30,000x direct magnifications (JEOL, Tokyo, Japan) was used for ultrastructural examination of osmium tetroxide/uranyl acetate stained mouse heart thin sections (90 nm). Mitochondrial content was measured as the areas taken by mitochondria compared to those of the cardiomyocytes using the open-source image analysis program ImageJ (NIH) on transmission electron microscopic images.

Isolation of Cardiac Mitochondria

Isolation of cardiac mitochondria used a modification of the standard published protocol (Frezza et al., 2007). Briefly, mouse hearts were collected, minced, and incubated with trypsin before homogenization with a glass/teflon Potter Elvehjem homogenizer. Heart homogenates were centrifuged at 800 *g* x 10 min at 4 °C and the supernatant collected and centrifuged at 8,000 *g* x 10 min at 4 °C; both the pellet and the supernatant were collected. The pellet was again washed and centrifuged at 8,000 *g* x 10 min at 4 °C to obtain normal-size mitochondria, which were resuspended for analyses. The 8,000 *g* supernatant was centrifuged at 16,000 *g* x 10 min at 4 °C to pellet smaller fragmented mitochondria, which were again washed and centrifuged at 16,000 *g* x 10 min at 4 °C before resuspension for analyses.

Measurement of Mitochondrial Protein

Mitochondrial protein concentration was colorimetrically determined using Bio-Rad protein assay dye reagent concentrate (Bio-Rad, 500-0006); the amount of mitochondria was calculated based on the protein concentration and the volume of the mitochondrial suspension.

Mitochondrial Respiration

Respiratory activities of isolated mitochondria were measured using a micro Clark-type electrode in a closed and magnetically stirred glass chamber as previously described (Chen et al., 2011). Non-stimulated (-ADP) (Sigma, A2754) respiration and stimulated (+ADP) respiration were measured, indicated as state 2 and state 3, respectively. Following ADP depletion, oligomycin (Sigma, 75351) was added to check peak respiration. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma, C2920) was added to measure maximal uncoupled respiration.

Determination of Mitochondrial H_2O_2 Production

H_2O_2 production of isolated cardiac mitochondria was determined using Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen, A22188) according to the manufacturer's instructions (Song et al., 2014).

Flow Cytometric Analyses of Isolated Mitochondria

Isolated cardiac mitochondria were stained with 200 nM MitoTracker Green (Invitrogen, M-7514), 2.5 μM MitoSOX red (Invitrogen, M36008), or 100 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC6, Invitrogen, D-273) at room temperature for 20 min and washed twice with PBS. Flow cytometric analyses of mitochondrial size (forward scatter, FSC), mitochondrial superoxide level (MitoSOX red signal intensity detected by PE channel), or mitochondrial membrane potential (DiOC6 signal intensity detected by FITC channel) were performed on a BD LSR II Flow Cytometer (Becton Dickinson, San Jose, CA). Data are shown as histograms for, and as bar graph of average signal intensity of, 50,000 ungated events.

Cell Culture

Drp1 KO MEFs and Mfn1/Mfn2 DKO MEFs were generated from $Drp1^{loxp/loxp}$ and $Mfn1^{loxp/loxp}$+$Mfn2^{loxp/loxp}$ embryos, respectively. Briefly, mouse embryos were aseptically dissected at E13.5. Heads and internal organs were discarded; bodies were rinsed twice with PBS before minced with razor blade. Cells were dissociated from the minced bodies with 10 ml 0.05% trypsin-EDTA (Invitrogen, 25300-054) at 37 °C for 5 min x 3 times and neutralized with fresh medium, pelleted by centrifugation at 200 *g* and plated on 150-mm plastic culture dishes prior to incubation at 37 °C with 5% CO_2. Here, primary MEFs were used within passage 5 to avoid replicative senescence.

MEFs were maintained in Dulbecco's modified Eagle's medium (Invitrogen, 11965-084) supplemented with 10% fetal bovine serum (Invitrogen, 26140-079), 1x non-essential amino acids (Invitrogen, 11130-051), 2 mM L-glutamine (Invitrogen, 25030-081), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, 15140-122).

Adenovirus Infection and Cyclosporin A Treatment

Cre adenovirus was from Vector Biolabs. Adeno-mcherry parkin was a gift from Dr. Åsa Gustafsson, University of California San Diego. MEFs were passaged every 2 days and plated on 25 mm glass round coverslips at a density of $1.0x10^5$ cells per well in 6-well plates. Adeno-Cre was added after cell attaching at a multiplicity of infection (MOI) of 100 at day 0 and kept in the medium for 48 hours; adeno-mcherry-parkin (MOI of 20) and/or cyclosporin A (CsA, 2 μM) were added at 48 hours prior to imaging.

Confocal Microscopy

Live cell imaging used a Nikon Ti confocal microscope equipped with a 60x 1.3NA oil immersion objective.

For visualization of mitochondria and measurement of mitochondrial membrane potential, MEFs were stained with 200 nM MitoTracker Green, 200 nM of tetramethylrhodamine, ethyl ester (TMRE, Invitrogen, T-669) and 10 µg/ml Hoechst (Invitrogen, H3570) at 37 °C for 30 min. The coverslip was loaded onto a chamber (Warner instrument, RC-40LP) in modified *Krebs-Henseleit buffer* (138 mM NaCl, 3.7 mM KCl, 1.2 mM KH_2PO_4, 15 mM Glucose, 20 mM HEPES and 1 mM $CaCl_2$) at room temperature. Fluorescence was excited with a 405 nm laser diode (Hoechst), a 488 nm Argon laser (MitoTracker Green) and a 543 nm HeNe laser (TMRE). For detection of parkin aggregation, MEFs were stained with MitoTracker Green and Hoechst; fluorescence for mcherry-parkin was excited with a 543 nm HeNe laser. For assessment of lysosomal engulfed mitochondria, MEFs were stained with 50 nM LysoTracker Red (Invitrogen, L-7528), MitoTracker Green and Hoechst; fluorescence for LysoTracker Red was excited with a 543 nm HeNe laser.

Image Analysis
Mitochondrial aspect ratio (the ratio of length/width) and content (% of mitochondrial area compared to whole-cell area) was quantified using ImageJ. Mitochondrial depolarization was calculated as % of the area of green mitochondria compared to that of all the mitochondria visualized on MitoTracker Green and TMRE merged images; data are presented as green ÷ (green + yellow mitochondria) × 100%. Parkin aggregation was calculated as % of cells with clumping mcherry-parkin compared to all the cells. Lysosomal engulfment of mitochondria was calculated by counting the number of co-localized lysosomes and mitochondria per cell detected by confocal co-localization of LysoTracker Red and MitoTracker Green.

RNA Expression Analysis
Total RNAs were extracted from cardiac tissues with TRIzol (Invitrogen, 15596-026), and single strand cDNA was prepared using high-capacity cDNA reverse transcription kit with RNase inhibitor (Invitrogen, 4374966) following the manufacturer's protocol. RNA expression analysis was performed using Taqman qRT-PCR master mix (Invitrogen, 4440038) with predesigned primer/probes sets for TFAM (Invitrogen, Mm00447485_m1), PPARγ (Invitrogen, Mm01184322_m1), PGC-1α (Invitrogen, Mm01208835_m1), and PGC-1β (Invitrogen, Mm00504720_m1).

Statistical Analysis
Data represent mean ± SEM of representative experiments, unless otherwise stated. Statistical comparisons used unpaired Student's t-test or one-way ANOVA with Tukey's corrections, as appropriate. $P < 0.05$ was considered statistically significant.