Extended Experimental Procedure

Echocardiography

Left ventricular systolic function was determined echocardiographically on conscious mice using the Visual Sonics Vevo 2100, equipped with a 40 MHz mouse ultrasound probe. Ejection fraction and fractional shortening were calculated based on end diastolic and end systolic dimensions obtained from M-mode ultrasound.

Reactive oxygen species determination

Dihydrorhodamine 123 (Life Technologies, D-23806) or CM-H2DCFDA (Life Technologies, C6827) was reconstituted in DMSO. Freshly made cryosections of unfixed hearts were incubated in 10μM dihydrorhodamine 123 or CM-H2DCFDA in PBS for 20 min in dark and then mounted with vectashield.

HPLC detection of DHE oxidation products

The hearts were harvested and cut into 3 pieces for p1 and 4 pieces for p4 and p7 and weighed. Each piece was immediately incubated with DHE (100μM in PBS) at 37°C for 30min. The buffer was removed and the sample washed once with PBS. DHE and oxidized products were extracted with acetonitrile (500μl), briefly sonicated (3x30sec, 8W). Samples were spun down (14,000rpm, 10 min at 4°C), the supernatant were collected, and dried under vacuum. The samples were further dissolved in 120μl PBS-DTPA and injected into the HPLC system as described previously (Fernandes et al., 2007; Laurindo et al., 2008; Ray et al., 2011)

Real-time PCR

Total RNA was isolated using Qiagen's RNeasy Mini Kit according to manufacturer's instruction. cDNA was synthesized from 2μg of RNA using

SuperScript II RT (Invitrogen). Quantitative real time PCR was performed with SyberGreen (Applied Biosystems) on ABI Prism 7700 Sequence Detector (Applied Biosystems) with primers (table S1). Beta actin, TBP, GAPDH and Cyclophilin A were used as housekeeping control to normalize gene expression using the $\Delta\Delta$Ct method.

For mtDNA quantification, DNA was extracted from ventricles with Proteinase K digestion and subsequent phenol/chloroform extraction. Mitochondrial DNA (mtDNA) was quantified with real-time PCR with primers shown in Table S1 using SYBR green PCR Master Mix and a 7000 Sequence Detection System (Applied Biosystems). The relative mtDNA copy number was calculated from the ratio of mtDNA copies to nuclear DNA (nucDNA) copies. The relative fold change was then calculated based on the $\Delta\Delta$Ct method.

Short-run SDS-PAGE and In-gel Tryptic Digestion

Before analyses, freshly frozen hearts were minced and homogenized in 1.0 mL of ice-cold homogenization buffer (25 mM Mops, 1.0 mM EDTA, pH7.4) using a Polytron homogenizer. Total heart homogenate was then sonicated and frozen at -70˚C until further analysis. Protein concentrations were determined using the bicinchoninic acid method (Pierce) with bovine serum albumin as a standard. Aliquots of the homogenates containing 60 μg total protein were mixed with SDS and an internal standard containing 8pmol bovine serum albumin. The samples were mixed well and heated at 70°C to assure complete dissolution before desalting by precipitation in 1mL of acetone overnight at −20°C. The protein pellet was solubilized in 60 μL Laemmli sample buffer and 20 μg protein loaded in a 12.5% SDS-PAGE gel (BioRad Criterion system). The gel was run for approximately 15 min at 150 V to give a 1.5 cm gel. The gel was fixed, washed with several changes of water, and stained for 5 min with Coomassie blue (GelCode blue, Pierce Chemical Company). Each lane was cut as a single sample and the gel piece divided roughly into 8–10 pieces. The gel pieces were destained

in 50% ethanol, 40% water, 10% acetic acid overnight at 50°C with several changes as needed for complete destaining. A standard in-gel digestion method was used (Kinter M., 2000). Briefly, proteins were reduced with DTT and alkylated with iodoacetamide (15 mg/mL and 30 mg/mL in 10 mM ammonium bicarbonate, respectively) for 20 min each. The reduction and alkylation reagents were removed and digestion was carried out by adding 1 µg trypsin (Promega) in 200 µL 10 mM ammonium bicarbonate for overnight at room temperature. The peptides produced were collected by extraction in 200 µL 50% ethanol, 50% water with 1% formic acid. The extract was evaporated to dryness and reconstituted in 150 µL 1% acetic acid in water for LC-tandem MS analysis.

Liquid Chromatography-tandem Mass Spectrometry

The LC-tandem MS system was a TSQ Vantage triple quadrupole mass spectrometry system (ThermoScientific) with a splitless nanoflow HPLC system with autoinjector (Eksigent). A 10 cm C18 column (Phenomenex Jupiter) packed in a fused silica electrospray tip (New Objective) was used. 10 µL Volumes of the samples were injected and loaded onto the column at 2 µL/min with 0.1% formic acid. The column was eluted at 160 nL/min with a linear gradient of CH3CN in water with 0.1% formic acid (3% CH3CN to 63% CH3CN in 30 min). The triple quadrupole mass spectrometer was operated in the selected reaction monitoring (SRM) mode. Ion source conditions were: spray voltage = 2.5 kV, ion transfer tube temperature = 300°C, positive ions. Collision induced dissociation conditions were: Q1 and Q3 resolution = 0.7Da, collision cell pressure = 1mTorr, collision energy dependent on the m/z of the parent ion and optimized for each reaction, and cycle time was set for 1.0 sec. SRM conditions were managed through the program Pinpoint (ThermoScientific) and included 2 to 3 peptides from each protein with 6 to 8 fragmentation reactions per peptide. Scheduling was used to monitor each peptide in a 4 min time window centered on the elution time of the peptide. Integrated chromatographic peak areas for each peptide were determined using the Pinpoint program. The response for each protein was calculated as the total

integrated area for all peptides monitored for that protein. Data were analyzed as either this raw total integrated area and after normalization to the internal standard protein.

NADH Oxidase Assay

Electron transport chain (ETC) activity was measured as rotenone inhibitable NADH oxidation (340, $\varepsilon = 6{,}200\ M^{-1}\ cm^{-1}$). Total sonicated heart homogenate (25 μg/mL) was analyzed in buffer containing 10 mM Mops, 10 mM KCl, at pH =7.4. NADH (200 μM) was added to initiate electron transport and activity is normalized to total protein analyzed.

Quantification of GSH and GSSG

The levels of GSH in cardiac tissue were quantified using reverse-phase HPLC and electrochemical detection (Rebrin et al., 2003). GSH and GSSG were extracted from total heart homogenate by treatment with 5% metaphosphoric acid. Proteins were precipitated upon incubation on ice (5.0 min) followed by centrifugation (10 min at 16,000 g). The supernatant was filtered (0.45-μm syringe filters) and GSH and GSSG were resolved by HPLC and quantified by electrochemical detection (Shimadzu HPLC system, ESA Coularray electrochemical detector 5600A set at 750 mV). GSH and GSSG were eluted through a C18 column (Phenomenex Luna C18(2), 100 Å, 3 μm, 150×4.6 mm) at 0.5 ml/min using an isocratic mobile phase consisting of 25 mM NaH_2PO_4, 0.5 mM 1-octane sulfonic acid, 4% acetonitrile, pH 2.7. GSH and GSSG concentrations were calculated employing GSH and GSSG standard curves constructed from peak areas.

Cell cycle analysis

P1 neonatal cardiomyocytes were plated on 24-well plates. The day after, cells were exposed to either 0.5 or 2 μM for 16hr. Cells were then washed in 1× PBS at room temperature, fixed in cold 50% ethanol, and stained with anti-alpha-

actinin primary antibody (Abcam #ab9465), followed by AlexaFluor488, anti-mouse secondary antibody (Molecular Probes). After washing, cells were resuspended in 1× PBS containing 0.2% NP40, 0.2 mg/ml RNase-A (Roche) and 40 ug/ml PI (Sigma-Aldrich) and incubated for at least 10 min before analysis. PI incorporation in cardiomyocytes was evaluated by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and BD CellQuest Pro software (Becton Dickinson).

Protein extraction from heart tissue and western blotting

Whole-cell extracts from pooled heart tissue samples {pooled P1 (n=15); P4 (n=6); P7 (n=3); control/mCAT P4 and P7 (n=3)} were prepared in RIPA buffer by homogenization using a hand held homogenizer (Fisher) on ice for 30 min. Cell extracts were centrifuged at 14000 RPM for 30 min at 4°C, to remove insoluble material. RIPA buffer contained PMSF, aprotinin, leupeptin, pepstatin A, NaF, and $NaVO_3$ at 1 μg/mL each. Aliquots containing 100 µg protein were resolved by 8% SDS-PAGE, transferred onto nitrocellulose membrane at 30 V at 4C overnight. Membranes were blocked with 5% milk in TBS-T (TBS-0.1% Tween 20) at room temperature for 20 min, and reacted with different antibodies in 5% milk in TBS-T at 4°C overnight. Subsequently, membranes were washed three times with TBS-T for 5 min each and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (anti-mouse/rabbit/goat) in 5% milk for two hours at room temperature. The primary antibodies used for western blotting are as follows: pATM (10H11.E12) (Santa Cruz, sc-47732, mouse, 1:500), Wee1 (Abcam, ab137377, rabbit, 1:2000), cardiac troponin T (Fisher Scientific, 13-11, mouse, 1:10,000), Ku70 (M-19) (Santa Cruz, sc-1487, goat, 1:1000), Catalase (Cell Signaling, 8841, rabbit, 1:1000). Quantification analysis of the WB signal was done using ImageJ (NIH).

Supplemental Figure Legends

Figure S1. (A) Comprehensive quantitative mass spectrometry analysis of enzymes involved in glycolysis, Krebs cycle and fatty acid beta-oxidation in P1, P4 and P7 mouse heart. Relative amount of proteins normalized by P1 are presented. (B) Relative enzyme activity of NADH oxidase in P1, P4 and P7 hearts was increased from P1 to P7, indicating the postnatal activation of mitochondrial electron transport chain. Circle graphs indicate percentage of genes showing upregulation, no change or downregulation at P7 compared with P1. (C) Imaging of ROS on cryosections with 5-(and-6)-carboxy-2',7'-dicholorodihydrofluorescein deacetate, acetyl ester (CM-H2DCFDA) indicated an increase in cardiomyocyte ROS level from P1 to P7 (arrows). (D) Cellular GSH and GSSG measured based on HPLC was decreased postnatally. (E) Proteomic analysis of antioxidant enzymes revealed an increase of superoxide dismutase 2 (SOD2) in 7 days postnatally in the heart. (F) Quantitative real time RT-PCR analysis of genes involved in DNA damage response pathway in P1, P7 and P14 heart indicatesd activation of both cell cycle arrest and DNA damage repair after birth. Amongst 11 genes tested, 9 were upregulated at P7 compared to P1. Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

Figure S2. DNA damage is low in adult zebrafish cardiomyocytes compared with mouse P7 cardiomyocytes. Co-immunostaining on cryosections with anti-8-oxoG and alpha actinin antibodies showed oxidated DNA in P7 mouse cardiomyocyte but not in adult zebrafish cardiomyocyte (arrows).

Figure S3. Injection of ROS generator paraquat accelerated cardiomyocyte cell cycle arrest. (A) 5mg/kg of paraquat was injected subcutaneously for 3 days after

birth and hearts were harvested at P3. (B) WGA staining showed significantly increased cardiomyocyte cell size in hearts of paraquat injected neonates. (C) Co-immunostaining with anti- pH3 and anti-TnT antibodies showed a drastic decrease in cardiomyocyte mitosis in paraquat injected neonates. TUNEL assay showed no significant increase in cardiomyocyte apoptosis. (D) Paraquat injection resulted in decreased cardiomyocyte cytokinesis as shown by immunostaining with anti-Aurora B and anti-TnT antibodies. Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

Figure S4. Injection of hydrogen peroxide (H_2O_2) induced post-natal cardiomyocyte cell cycle arrest. (A) 1μM of H_2O_2 was injected into the left ventricle at P1 and hearts were harvested at P3. (B) Immunostaining showed nuclear accumulation of phosphorylated ATM in H_2O_2 injected heart. (C) HW/BW ratio showed no statistically significant difference. (D) Cardiomyocyte cell size was significantly increased confirmed with wheat germ agglutinin (WGA) staining. (E) Co-immunostaining with anti-phospho-histone H3 Ser10 (pH3) and anti-Troponin T (TnT) antibodies showed drastic decrease in cardiomyocyte mitosis in H_2O_2 injected hearts. (F) TUNEL assay showed increased apoptotic cardiomyocyte cell death which occured mostly around the needle track of the H_2O_2 injection (arrows). Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

Figure S5. NAC treatment (500nM, 2μM) increased DNA synthesis and mitosis, and suppressed polyploidization in neonatal rat cardiomyocytes *in vitro*. Cell cycle analysis presented as percentage of viable cardiomyocytes. Blue letters in the table indicate statistically significant increase ($p<0.05$). Error bars indicate SEM.

Figure S6. Effect of ROS scavenging on mitochondrial enzymes and DDR. (A) HPLC detection of fluorescent product from DHE showed decreased superoxides

(EOH), and other ROS including H_2O_2 (E) in the heart of NAC treated mice. (B) HPLC detection of fluorescent product from DHE showed no significant change in the level of superoxides (EOH), but decreased H_2O_2 and other ROS (E) in the mCAT heart. (C) Enzymes related to glycolysis, Krebs cycle and beta-oxidation were mostly unchanged in control and NAC-treated hearts at P7. (D) NADH oxidase activity in control and NAC-treated P7 hearts similarly increased compared with those in P1. (E) Western blot analysis showed increased catalase and reduced pATM and wee1 level in mCAT hearts. Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

References

Fernandes, D.C., Wosniak, J., Jr., Pescatore, L.A., Bertoline, M.A., Liberman, M., Laurindo, F.R., and Santos, C.X. (2007). Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems. Am J Physiol Cell Physiol *292*, C413-422.

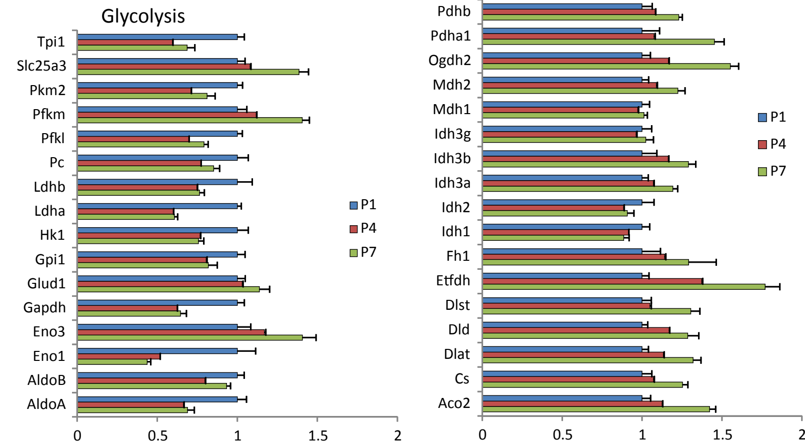
Kinter M., S., N. E. (2000). Protein Sequencing and Identification Using Tandem Mass Spectrometry. John Wiley and Sons, New York.

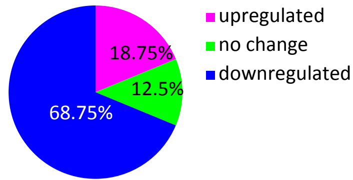
Laurindo, F.R., Fernandes, D.C., and Santos, C.X. (2008). Assessment of superoxide production and NADPH oxidase activity by HPLC analysis of dihydroethidium oxidation products. Methods Enzymol *441*, 237-260.

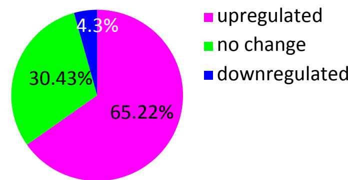
Ray, R., Murdoch, C.E., Wang, M., Santos, C.X., Zhang, M., Alom-Ruiz, S., Anilkumar, N., Ouattara, A., Cave, A.C., Walker, S.J.*, et al.* (2011). Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo. Arterioscler Thromb Vasc Biol *31*, 1368-1376.

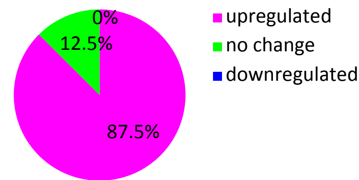
Rebrin, I., Kamzalov, S., and Sohal, R.S. (2003). Effects of age and caloric restriction on glutathione redox state in mice. Free Radic Biol Med *35*, 626-635.

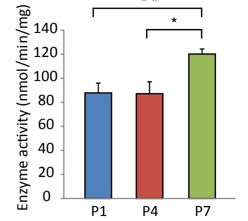
Supplemental Figure 1

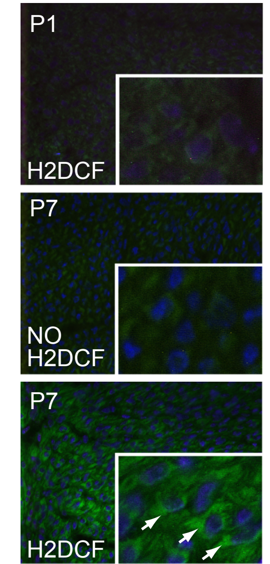


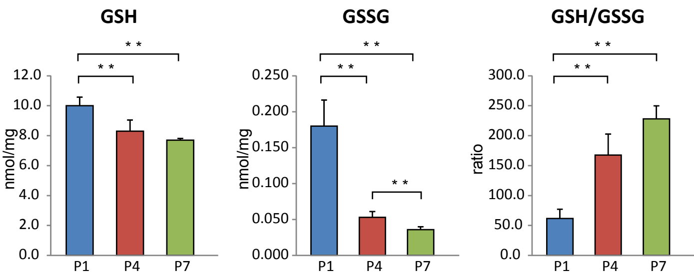


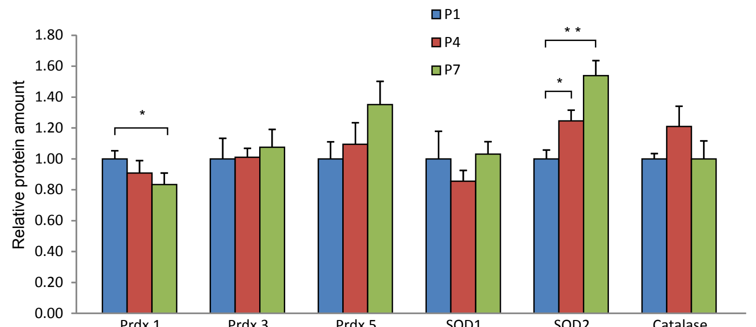


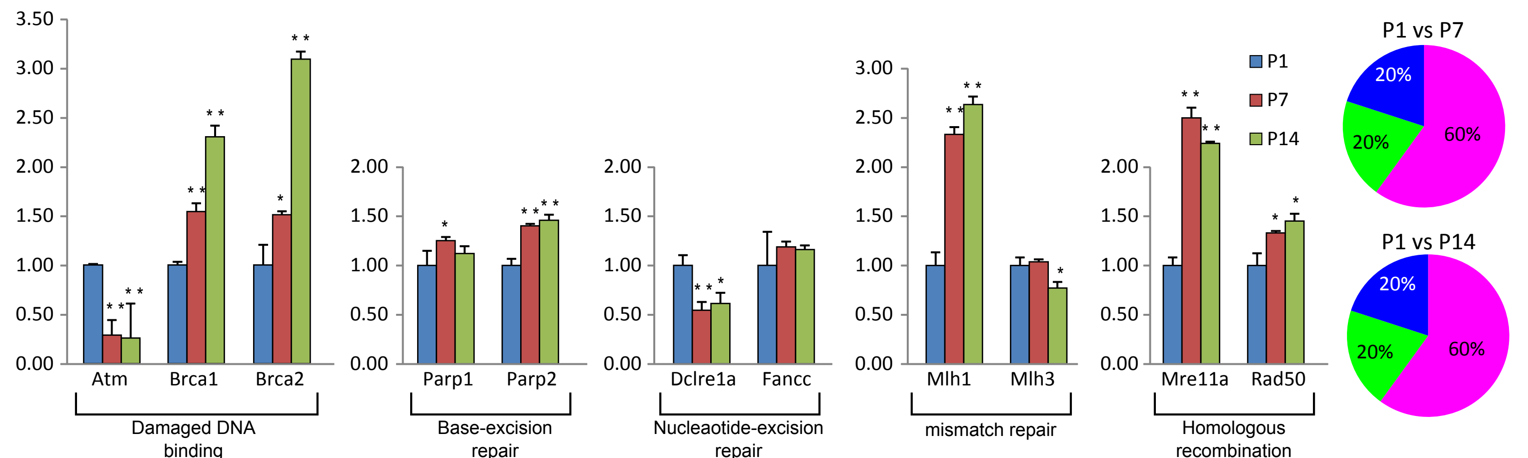




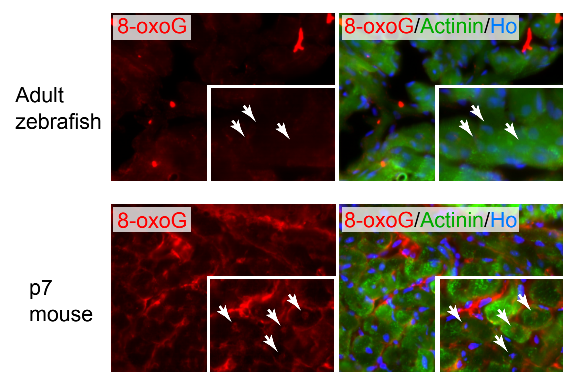






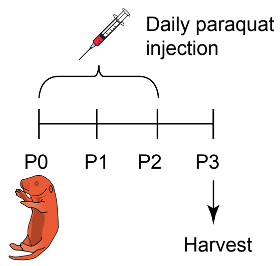


Supplemental Figure 2

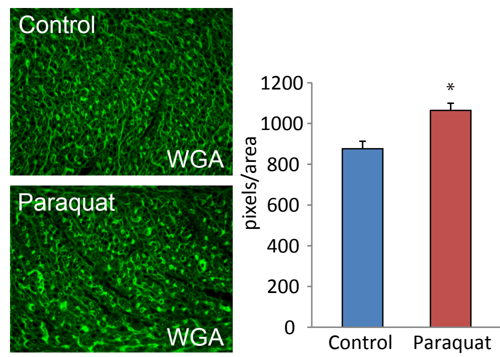


Supplemental Figure 3

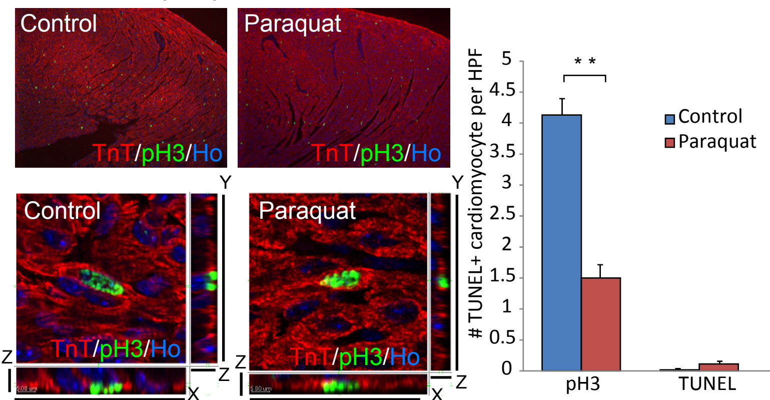
A. Schematic



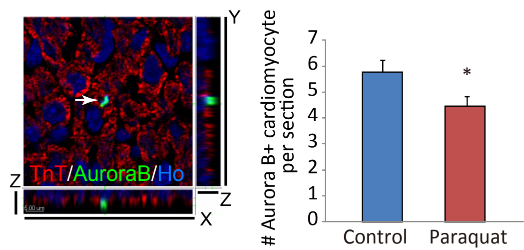
B. Cell size

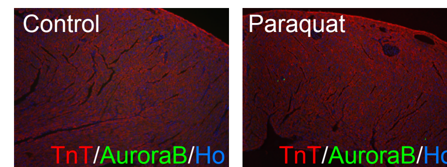


C. Cardiomyocyte mitosis and apoptosis

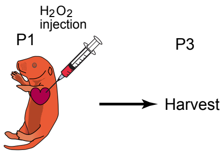


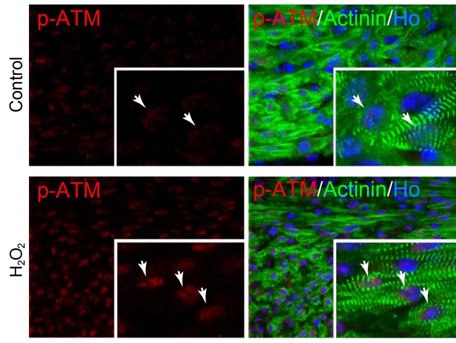
D. Cardiomyocyte cytokinesis

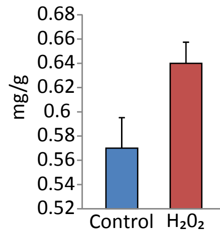


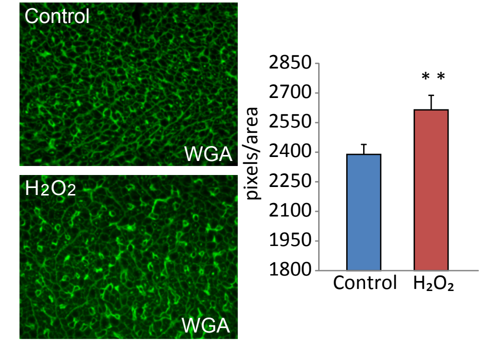


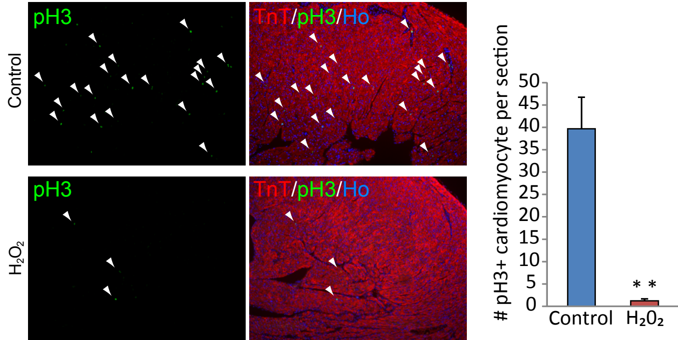
Supplemental Figure 4

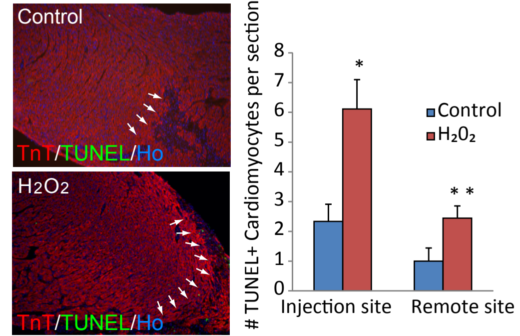




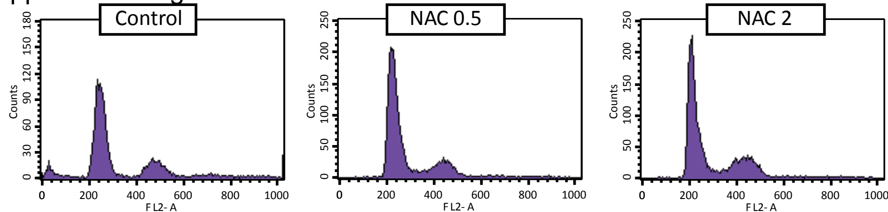


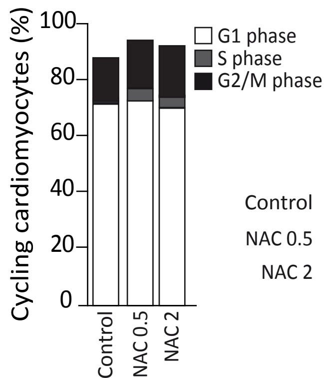




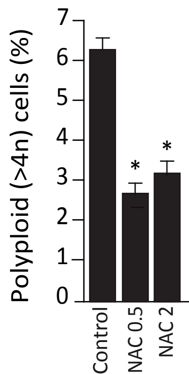


Supplemental Figure 5



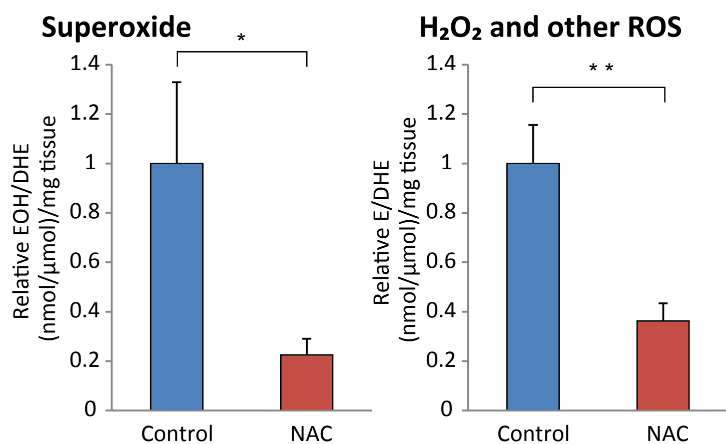


	G1 phase	S phase	G2/M phase
Control	71±5.2	0.45±0.01	16.5±1.2
NAC 0.5	74.4±6.8	4.69±1.11	15.8±2.0
NAC 2	68.1±6.8	3.96±0.57	21.7±2.2

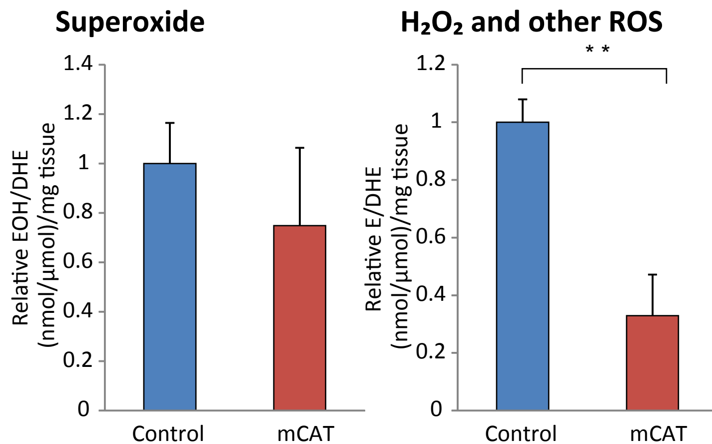


Supplemental Figure 6

A. HPLC-based ROS measurement for NAC



B. HPLC-based ROS measurement for mCAT



C. Quantitative mass spectrometry

Glycolysis

Tpi1
Slc25a3
Pkm2
Pfkm
Pfkl
Pc
Ldhb
Ldha
Hk1
Gpi1
Glud1
Gapdh
Eno3
Eno1
AldoB
AldoA

0 0.5 1 1.5

P7
P7 NAC

Krebs cycle

Suclg1
Sucla2
Slc25a11
Sdhc
Sdhb
Sdha
Pdhb
Pdha1
Ogdh2
Mdh2
Mdh1
Idh3g
Idh3b
Idh3a
Idh2
Idh1
Fh1
Etfdh
Dlst
Dld
Dlat
Cs
Aco2

0 0.5 1 1.5

P7
P7 NAC

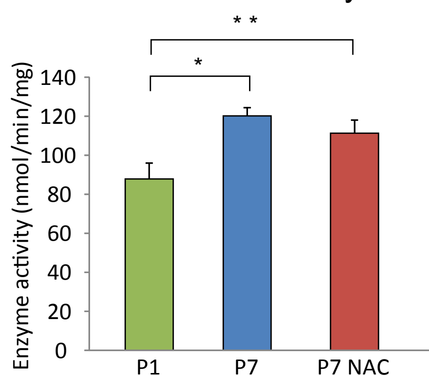
Beta oxidation

Slc25a20
Hadhb
Hadha
Hahd
Fabp4
Fabp3
Etfb
Etfa
Echs1
Ech1
Decr
Dci
Crat
Cpt2
Cpt1b
Cd36
Acsl1
Acox1
Acot13
Acadvl
Acads
Acadm
Acadl
Acca2

0 0.5 1 1.5

P7
P7 NAC

D. NADH Oxidase Activity



E. DDR quantification