EXPANDED METHODS

All studies were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. [NIH] 85-23, revised 1996).

Mouse models. Male mice (10-16 weeks of age, weighing 25-30 g) were used. Cardiac-specific HO-1 transgenic (TG) mice were obtained from Dr. Shaw-Fang Yet (Harvard Medical School). These mice have been well-characterized previously [1]. We used HO-1 TG mice expressing 4 copies of a human HO-1 transgene under the control of the α-MyHC promoter [1]. Originally bred on a FVB background, HO-1 TG mice lines were backcrossed for a minimum of 7 generations into C57BL/6. NTG littermates were used as controls. For *in vivo* CORM-3 supplementation studies, C57BL/6 mice were used.

Coronary ligation. Permanent coronary ligation was performed as previously described [2]. After induction of anesthesia with tribromoethanol (0.25 mg/g i.p.), mice were intubated and supported with a MiniVent Mouse Ventilator (Type 845, Harvard Apparatus) at 125-150 breaths/minute depending on body weight (tidal volume 6.4 µL/g, PEEP 5-7 cm H_2O). Anesthesia was maintained with 1% isoflurane. Heat lamps and heating pads were used to maintain body temperature at 37°C. Under sterile conditions, a left thoracotomy was performed in the 4th intercostal space, the heart exposed, and the pericardium opened. An 8.0 prolene ligature was passed and tied around the proximal left coronary artery, 1 mm distal to the left atrial appendage border. Successful occlusion was confirmed by the production of pallor and dyskinesia in the distal myocardium. In sham animals, the suture was passed but not tied. The chest was then closed in layers using 5.0 silk, and the mice were allowed to recover and followed for 4 weeks.

Echocardiography. Under tribromoethanol sedation (0.25 mg/g i.p.), echocardiography (M-mode, 2D, and Doppler) was performed at baseline and 4 weeks post-operatively using a Philips Sonos 5500, 15 MHz linear array transducer, and 120 Hz frame rate. Measured parameters included the short-axis end-diastolic (ED) and end-systolic (ES) diameter (D) and

wall thickness (WT), and long-axis end-diastolic and end-systolic volume (EDV and ESV) using the modified Simpson's method. LV systolic function was indexed by single plane planimetered LV ejection fraction (EF = EDV-ESV]/EDV).

LV pressure measurement. In a subset of animals, LV catheterization was performed 4 weeks after coronary ligation or sham operation as previously described [3]. Mice were anesthetized with tribromoethanol (0.25 mg/g i.p.), intubated and ventilated with anesthesia maintained using 1% isoflurane. Body temperature was maintained at 37°C using a heating pad and heat lamps. The left jugular vein was cannulated for fluid/drug administration. A Millar 1.4 Fr conductance catheter (Model SPR-719) was inserted via the right carotid artery into the ascending aorta and subsequently advanced retrogradely into the LV for pressure measurement using a Powerlab/Chart system (ADInstruments). Correct catheter positioning was confirmed by on-line visualization of the pressure and conductance signals. LV pressure (P) and conductance were A:D converted at 500 Hz and recorded at steady-state after equilibration for at least 10-15 minutes. At the end of the study, IV hypertonic saline (0.5-1 μL/g) was given to determine parallel conductance. LV volume (μL) was derived from the measured conductance, parallel conductance, and *ex vivo* cuvette calibration with heparinized, warm blood. Systolic function was indexed by dP/dt_{max}, and dP/dt_{max} normalized for either instantaneous LVP (IP) or EDV as relatively load-independent indices. Diastolic function was assessed by LV end-diastolic pressure (LVEDP) and tau, the time constant of LV relaxation, as determined from the regression of dP/dt versus LVP.

Tissue harvest. Following the final echocardiographic or hemodynamic study, mice were given additional anesthesia with sodium pentobarbital (50 mg/kg i.p.). The heart was arrested in diastole with IV KCl and rapidly excised and rinsed in ice-cold physiological saline. The ventricles were dissected and weighed separately. A short-axis slice of the LV was fixed in formalin for 16 h, dehydrated in ethanol, and paraffin-embedded for subsequent histological

studies. The remaining LV tissue was separated into infarcted (scar) and non-infarcted regions, snap-frozen in liquid nitrogen, and stored at -80°C for biochemical and molecular studies. Unless otherwise specified, non-infarcted tissue was used for molecular analyses.

Isolation of mouse cardiomyocytes. Mice were deeply anesthetized with pentobarbital (80 mg/kg IM) and given heparin 10 U/g i.p. The heart was rapidly excised, and Ca^{2+}-tolerant mouse ventricular myocytes were isolated by modified Langendorff perfusion and collagenase digestion as previously described [3]. Cell viability was typically 75-80%, as assessed by trypan blue exclusion. Cells were maintained at a density of 10^4 rod-shaped cells/cm^2 in serum-free supplemented DMEM medium (with albumin 0.2%, L-carnitine 2mM, creatine 5mM, taurine 5mM, L-glutamine 1.3 mM, insulin 0.1 mM, triiodothyronine 0.1 nM, pyruvate 2.5 mM, BDM 10 mM, and penicillin/streptomycin 0.1%) at 37ºC in 5% CO_2 until experimentation.

MTT assay. Myocyte cell death was determined using the MTT assay. Myocytes were incubated with 500 μM H_2O_2 for 1 h. Some groups were pre-incubated and co-incubated with 50 μM hemoglobin (Hgb, CO scavenger) or 100 μM desferoxamine (DFO, iron chelator) starting 30 minutes before H_2O_2. MTT solution (5 mg/ml) was added to the media 30 minutes before the end of incubation. The cells were centrifuged for 5 minutes and the media removed. Any crystals were dissolved by adding 200 μL DMSO and pipette mixing, and the plate was incubated at 37°C for 5 minutes to dissolve air bubbles. Absorbance was read at 550 nm. Cell viability was calculated as the percent ratio of absorbance of the sample to the reference control.

Culture of H9c2 cardiomyocytes and HO-1 transfection. H9c2 cells (rat embryonic cardiomyoblasts) were obtained from ATCC and cultured in DMEM (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin for 7-10 passages. For transfection studies, Ad5/HO-1, the replication-deficient adenoviral vector containing the entire coding region of rat HO-1 cDNA, was generated in 293 cells by homologous recombination of pCMV/HO-1 and pJM17, a circularized adenovirus genome lacking its E1 region and a portion of the E3 region. Plaque-

isolated viral clones were propagated in 293 cells and then purified over two CsCl gradients and titered by plaque assay as previously described [4]. Ad5/LacZ with the same adenoviral backbone containing the β-galactosidase gene driven by CMV promoter was used as a control vector. For transient HO-1 transfections, cells were seeded in 100 mm tissue culture dishes and transfected with Ad5/HO-1 or Ad5/LacZ for 45 min at an MOI of 10 for 72 h prior to treatments.

Histomorphometry, immunohistochemistry, and apoptosis assessment. Paraffin-embedded tissue sections (5 μm) were de-paraffinized, rehydrated and stained with Masson Trichrome (for collagen) using standard histologic techniques [2]. To determine myocyte cross-sectional area, rhodamine-conjugated wheat germ agglutinin (Molecular Probes) staining was performed at 1:1000 dilution for 45 min at room temperature (RT). The sections were then rinsed with PBS and mounted with SlowFade Gold antifade reagent with DAPI (Molecular Probes). Myocyte area (average of ~100 cross sectional cells with centrally located round nuclei) and the degree of tissue fibrosis (blue-green stain) were assessed using Metamorph Imaging Software. Oxidative stress was indexed by immunostaining for malondialdehyde (MDA)-adducted proteins as previously described, using IgG-purified anti-MDA primary antibody (Academy Bio-Med) [5]. For quantitation of protein-MDA adducts by immunostaining, 4-6 mice per group were used. For each heart, using the short axis section of the LV, digital images were acquired from six different fields at standard distance intervals in the remote zone and analyzed by MetaMorph 4.5 image quantitation software. The threshold for protein-MDA adduct staining was predetermined, and held constant for all sections analyzed. For immunohistological analyses, we typically analyzed 6 fields/heart with an area of 66 mm^2/field (total area 396 mm^2/heart).

For HO-1 immunofluorescent staining, tissue sections were blocked in PBS containing 0.1% Triton X-100, 7% goat serum, and 5% nonfat dry milk for 10 min at 37°C and then again with mouse IgG Blocking Reagent (FMK-2201, Vector Laboratories) for an additional 1 h at RT. Anti-mouse HO-1 antibody (Stressgen) was used as the primary antibody at a 1:100 dilution,

and TRITC-conjugated goat anti-mouse IgG was used as the secondary antibody at 1:200 dilution. Negative controls were performed by omission of primary antibody. Isolectin staining was performed to identify capillaries; FITC-conjugated isolectin B4 (Vector Labs) was used as primary antibody without secondary antibody. Apoptosis in tissue and cells was determined using the DeadEnd Fluorometric TUNEL System (Promega), which catalytically incorporates fluorescein-12-dUTP at DNA strand breaks. Tissue sections were further incubated with anti-troponin I antibody (Santa Cruz Biotechnology) 1:1000 dilution and Texas Red-conjugated secondary antibody at 1:10,000 (Molecular Probes) to identify cardiomyocytes. All sections were counterstained with DAPI (Molecular Probes) at a final concentration of 2 μM. Optical sections were obtained with a Zeiss LSM510 inverted confocal scanning laser microscope equipped with Enterprise/argon/HeNe lasers and excitation wavelengths appropriate for multi-channel scanning in the individual tracks to allow co-localization. Images were recorded within 24 h and analyzed with Adobe PhotoShop 5.5 software.

HO-1 gene expression. Total RNA was isolated from cardiac tissue as previously described [2,5]. HO-1 mRNA was quantified and normalized to mouse beta-actin mRNA by real-time PCR with LUX gene-specific primers (Invitrogen) after reverse transcription (Superscript III, Invitrogen) of 250 ng total RNA purified from sham and failing hearts (TRIzol method). The forward labeled primer was CACGATCCAAGTTCAAACAGCTCTATCG, and reverse unlabeled primer was CTGTCACCCTGTGCTTGACCTC.

Western immunoblotting. Protein extraction, Western immunoblotting, and densitometry were performed as previously described [2,3,5]. Protein (50 μg) was denatured with SDS sample buffer (2% SDS, 10% glycerol, 5 mM DTT in Tris buffer, pH 6.8) at 90 ºC for 5 min and separated on 12% polyacrylamide gel with a 4% stacking gel in SDS-Tris Glycine running buffer. The proteins then were electro-transferred to a PVDF membrane using a Bio-Rad Mini Trans-Blot Cell in Tris-glycine-methanol buffer at 350 mA for 90 min. Immunodetection then followed the Amersham ECL Western Blotting Protocols. Primary antibodies used included

anti-p53, anti-poly-ADP ribose polymerase (PARP), anti-Bax, anti-Bcl-2, anti-β-actin, and anti-α-tubulin from Santa Cruz Biotechnology, and anti-HO-1 from StressGen.

Measurement of free MDA by gas chromatography-negative ionization chemical ionization-mass spectrometry (GC-NICI-MS). Tissue MDA concentration was measured by GC-NICI-MS as previously described [6]. Heart tissue homogenate was derivatized with pentfluorobenzylhydroxylamine (PFBHA) and the PFB-oxime derivatives of MDA were measured by GC-NICI-MS using select ion monitoring mode. Benzaldehyde ring d_5 was used as the internal standard. The following ions were monitored for the indicated aldehyde: benzaldehyde d_5 - m/z 286 (M^+-HF) and MDA m/z 204 (M+-$C_7H_2F_5$-HFNO-C_2H_3).

Mitochondrial membrane permeability transition (MPT). In adult cardiomyocytes and H9c2 cells, changes in mitochondrial membrane potential ($\Delta\psi_m$) were assessed with tetramethylrhodamine methyl ester (TMRM). TMRM fluorescence was measured at 5-minute intervals for 30 minutes in live cells using a 585-nm long pass filter and laser-scanning confocal microscopy (LSM 510). TMRM is a cationic dye that accumulates in mitochondria in proportion to $\Delta\psi_m$. The TMRM concentration chosen (100 nM) does not suppress mitochondrial respiration [7] and is nonquenching so that mitochondrial depolarization is accompanied by a decrease in cellular TMRM fluorescence over time followed by cell shortening due to ATP depletion [8].The time required to cause at least a 2-fold decrease in TMRM fluorescence was taken as an endpoint for the development of MPT.

Mitochondrial isolation. Mitochondria were isolated from adult mouse hearts by differential centrifugation as described previously [9]. Isolated mitochondria were resuspended in EGTA-free homogenization buffer (250 mM sucrose, 10 mM HEPES, pH 7.4 with Tris·HCl) to yield 3–5 mg/ml of mitochondrial protein. Mitochondria were kept on ice and used within 4 h.

Mitochondrial MPT pore opening. MPT pore (MPTP) opening in isolated cardiac mitochondria was induced by Ca^{2+} as previously described [9]. Isolated cardiac mitochondria

were resuspended in swelling buffer (containing in mmol/L: 120 KCl, 10 Tris·HCl (pH 7.4), 20 MOPS, and 5 KH_2PO_4) to a final protein concentration of 0.25 mg/ml. Mitochondrial swelling induced by pore opening was measured spectrophotometrically as a reduction in absorbance at 520 nm (A_{520}).

Mitochondrial respiration. Freshly isolated mitochondria were resuspended in respiration buffer (pH 7.2) containing in mmol/L: 225 mannitol, 70 sucrose, 10 KH_2PO_4, 1 EGTA. A Clark electrode was used to measure the oxygen content of mitochondrial suspension with 10 mM pyruvate and 5 mM malate. State 4 respiration was measured at baseline. 330μM ADP was added to stimulate state 3 respiration. The respiratory control ratio (RCR) was defined as the ratio of state 3:state 4 respiration. The ADP:O ratio was calculated using total oxygen consumption during state 3 respiration.

Preparation of the CO-donor tricarbonylchloro(glycinato)ruthenium(II) (CORM-3). CORM-3, a water-soluble transition metal carbonyl drug that stably releases CO, was synthesized following a published protocol [10]. The structure of CORM-3 was confirmed by infrared spectroscopy and NMR. To detect CO release, 25 μM CORM-3 was added to PBS containing myoglobin (50 μM) and the shift in the absorbance spectrum of deoxymyoglobin to carbonmonoxy myoglobin was recorded. The synthesized drug was stored at -20°C and fresh solution was made just before use. For control experiments, CORM-3 was inactivated by dissolving it in Krebs-Henseleit buffer and allowing CO liberation overnight at RT.

Statistical analysis. Several statistical techniques were employed. For two-group comparisons, we used the unpaired two sample t test. For comparisons of more than two groups, we used one-way ANOVA if there was one independent variable, two-way ANOVA if there were two independent variables (*e.g.,* genotype and ligation status), and two-way repeated measures ANOVA for matched observations over time with two independent variables. To adjust for multiple comparisons, we performed Student-Newman-Keuls post-test for one-way

ANOVA and Bonferroni post-test for two-way ANOVA. Pair-wise comparisons were made between sham groups across genotypes, sham versus HF within each genotype, and HF groups across genotypes. Animal survival was evaluated by the Kaplan-Meier method, and the log-rank test was used to compare survival curves between NTG sham and HF, HO-1 TG sham and HF, and NTG and HO-1 TG HF. A p value of < 0.05 was considered significant. Continuous data are summarized as mean ± SD.

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Supplemental Table 1. Baseline Echocardiography in WT and HO-1 TG mice

	NTG mice	HO-1 TG mice
HR (bpm)	545 ± 80	542 ± 55
LVEDD (mm)	3.6 ± 0.3	3.5 ± 0.4
LVESD (mm)	2.0 ± 0.3	1.9 ± 0.4
FS (%)	45 ± 5	45 ± 8
LVEDV (μL)	25 ± 5	29 ± 9
LVESV (μL)	6 ± 1	6 ± 3
LVEF (%)	78 ± 4	78 ± 7
AWT (mm)	0.75 ± 0.11	0.69 ± 0.07
PWT (mm)	0.75 ± 0.08	0.76 ± 0.18
RWT	0.42 ± 0.05	0.43 ± 0.11
Body weight (g)	27.8 ± 2.3	26.0 ± 2.2

HR, heart rate; LV, left ventricular; EDD and ESD, end-diastolic and end-systolic diameter; FS, fractional shortening; EDV and ESV, end-diastolic and end-systolic volume; EF, ejection fraction; AWT, PWT, and RWT, anterior, posterior, and relative wall thickness. All values mean ± SD. n = 10 per group.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. HO-1 is upregulated in the failing heart. ***A,*** Representative mouse LV sections 4 weeks after sham operation or coronary ligation and myocardial infarction (MI) and M-mode echocardiograms at baseline and 4 weeks post-MI. Large infarction is demonstrated with marked remodeling and systolic dysfunction. ***B,*** HO-1 mRNA analysis (real-time PCR) and HO-1 and HO-2 protein analysis (Western blotting) showing two-fold upregulation of HO-1 in heart failure (HF) (n = 4-6/group). ***C,*** Representative HO-1 immunostaining in sham and HF hearts using anti-HO-1 antibody and TRITC-conjugated secondary antibody (red immunofluorescence) indicating increased myocyte expression of HO-1 in HF (scale bar 20 μm). Examples of immunostaining from non-transgenic (NTG) and HO-1 transgenic (TG) hearts are shown for comparison (scale bar 50 μm).

Figure S2. Group data for absolute values for state 3 and state 4 respiration measured in mitochondria isolated from NTG and HO-1-TG hearts. Recordings in NTG mitochondria were performed in the absence or presence of co-treatment with 50 μmol/L CORM-3. *$p < 0.05$ vs. NTG; n = 3 per group.

Figure S3. Ca^{2+}-induced mitochondrial swelling (*i.e.,* decline in A_{520}, an index of MPT) in mitochondria isolated from non-transgenic (NTG) hearts treated with or without cyclosporine A (CsA, 30 nM, 5 minutes before Ca^{2+} was added) or recombinant HO-1 protein (rHO-1, 2μg given 15 min before Ca^{2+}).

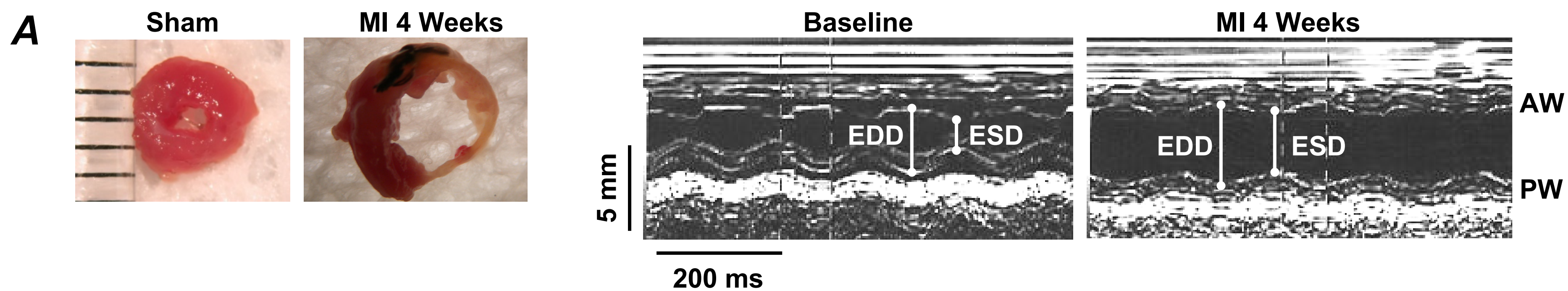
Figure S4. Representative high magnification confocal images of TMRM-loaded adult cardiomyocyte (***Left***) and H9c2 cells (***Right***). Note clearly delineated punctuate mitochondrial staining at baseline in H9c2 cells and the typical linear organization of mitochondria along the longitudinal axis in the adult cardiomyocyte example.

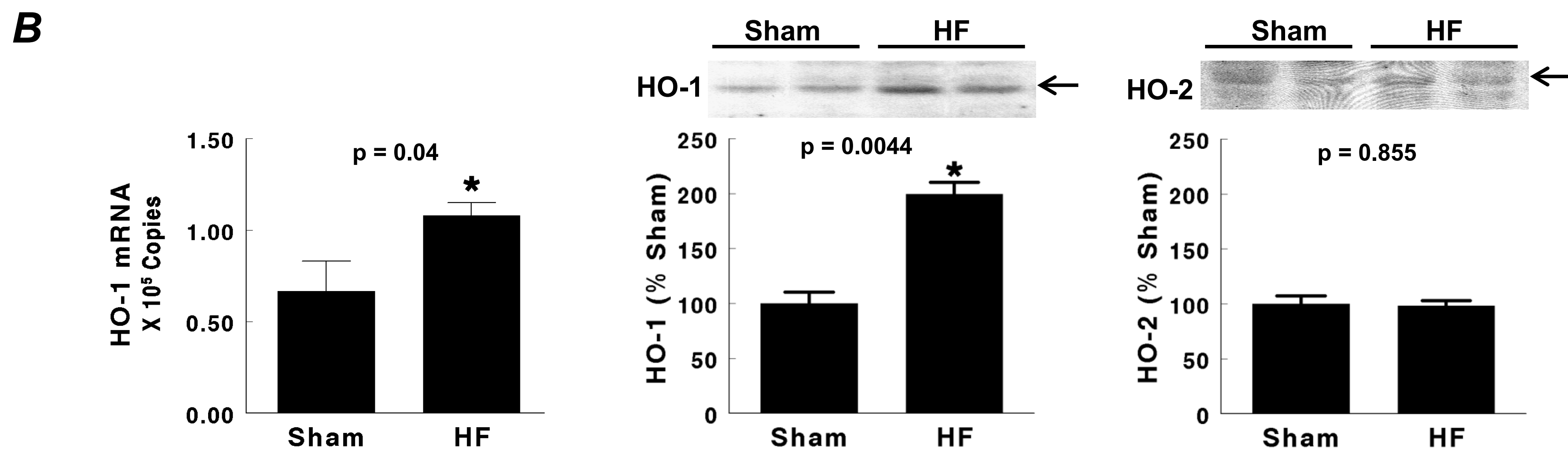
Figure S5. TMRM fluorescence in H9c2 cells 30 min after exposure to PAO alone (control) or after pre-treatment with CsA (100 nM, given 5 min prior to PAO), CORM-3 or

iCORM-3 (50 μM, given 30 min prior to PAO). The panels are representative of 3-4 experiments per condition. The scale bar is 10 μm.

Figure S6. H9c2 cells were incubated with Ad5/HO-1 or Ad5/LacZ (control) for 45 min at an MOI of 10; 72 h later, cells were loaded with Cationic Dye to localize mitochondria (MITO, red fluorescence) 30 min prior to fixation and immunostaining with anti-HO-1 antibody and FITC-conjugated secondary antibody (green fluorescence). Nuclei were counterstained with DAPI (blue). The panels are representative of 3-4 experiments per condition. The scale bar is 20 μm.

Figure S7. Ad5/HO-1 and Ad5/LacZ transfected H9c2 cells were labeled with TMRM as above and then exposed to H_2O_2 (100 μM) for 60 min and observed under confocal microscopy. The top and bottom panels are low magnification (scale bar 20 μm) and high magnification (scale bar 5 μm), respectively. The panels are representative of 3-4 experiments per condition.





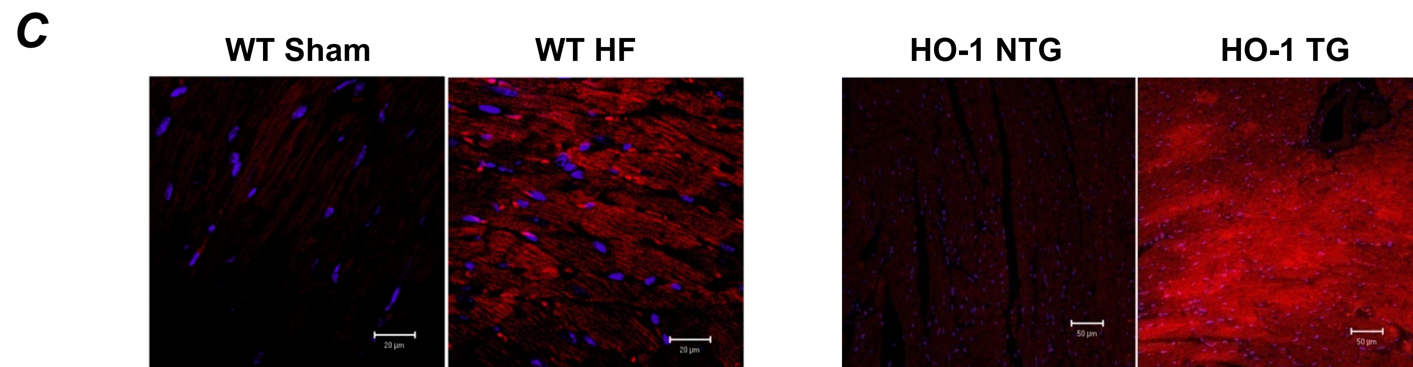


Figure S1

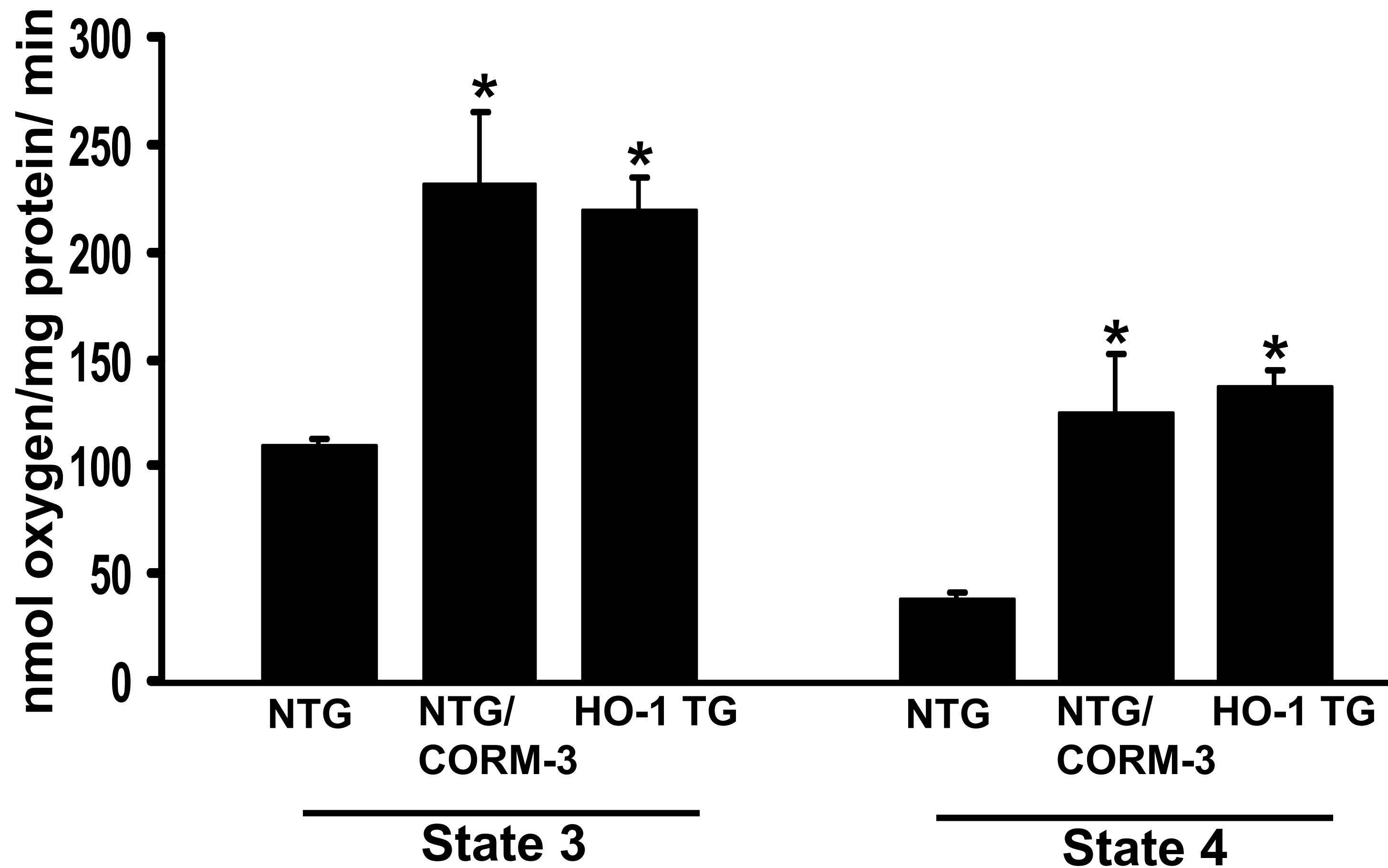


Figure S2

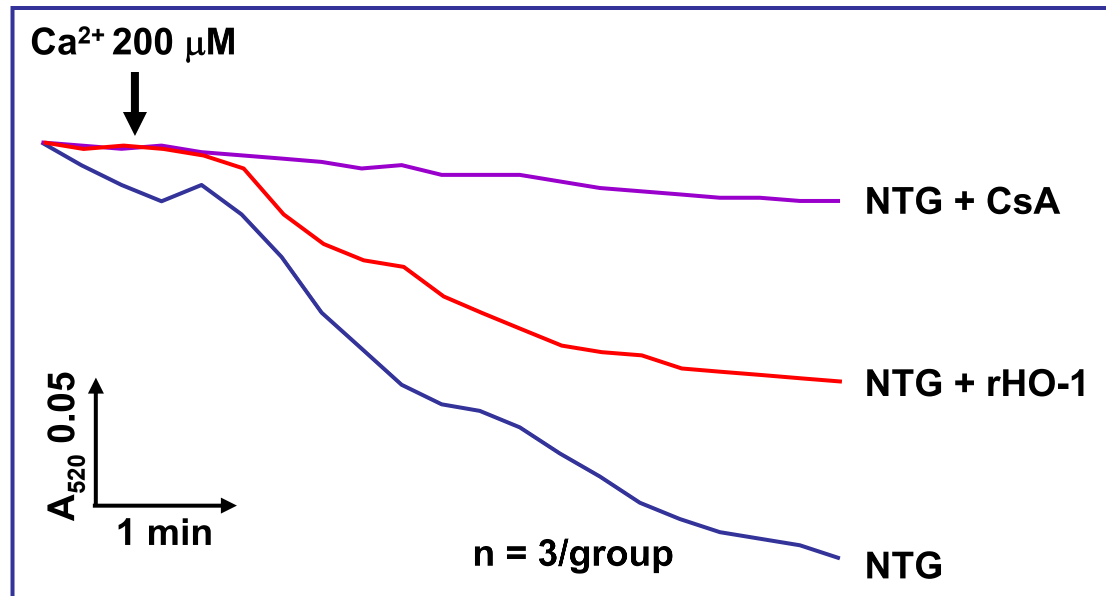
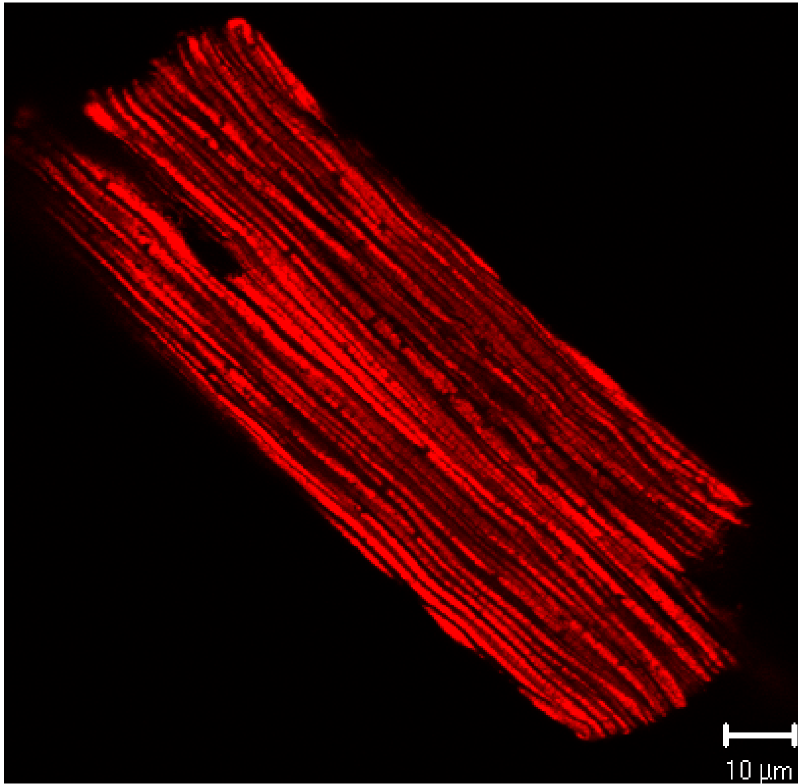
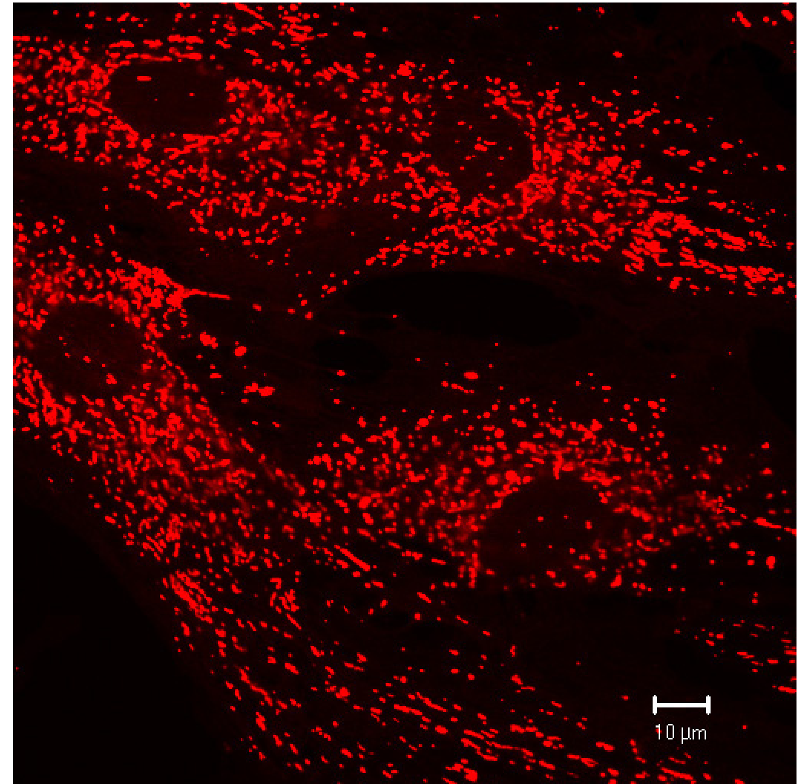


Figure S3

TMRM LABELING



Adult Cardiomyocyte



H9c2 Cells

Figure S4

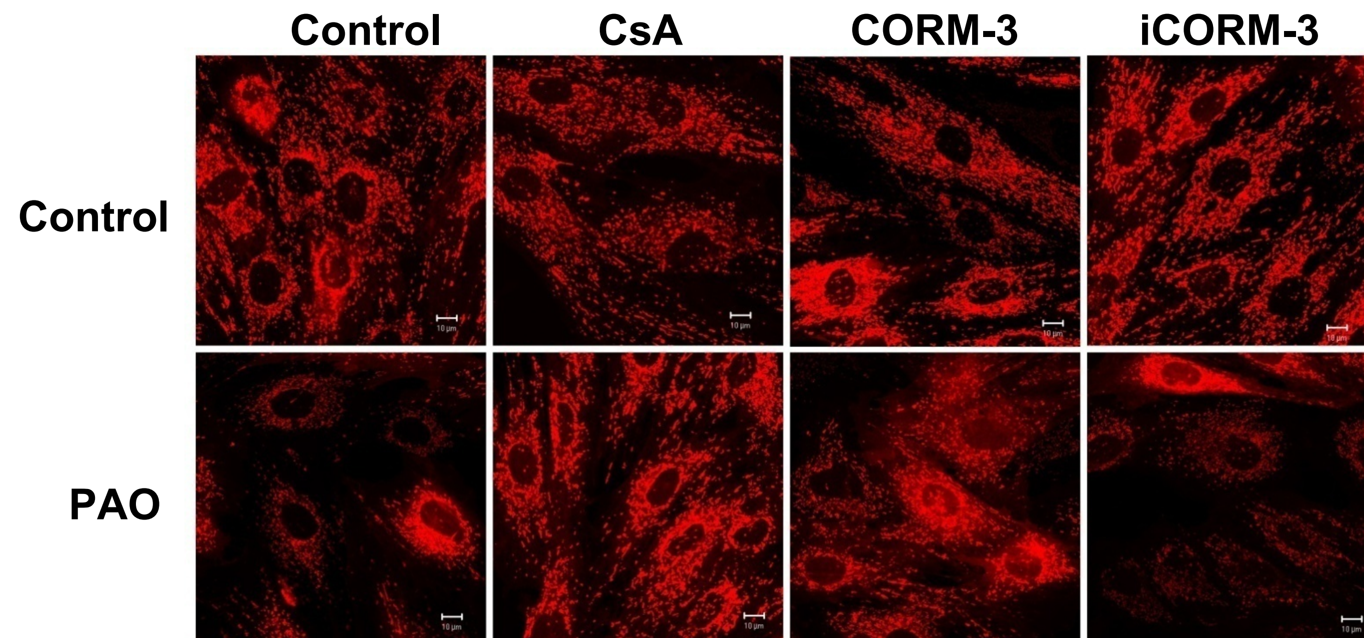


Figure S5

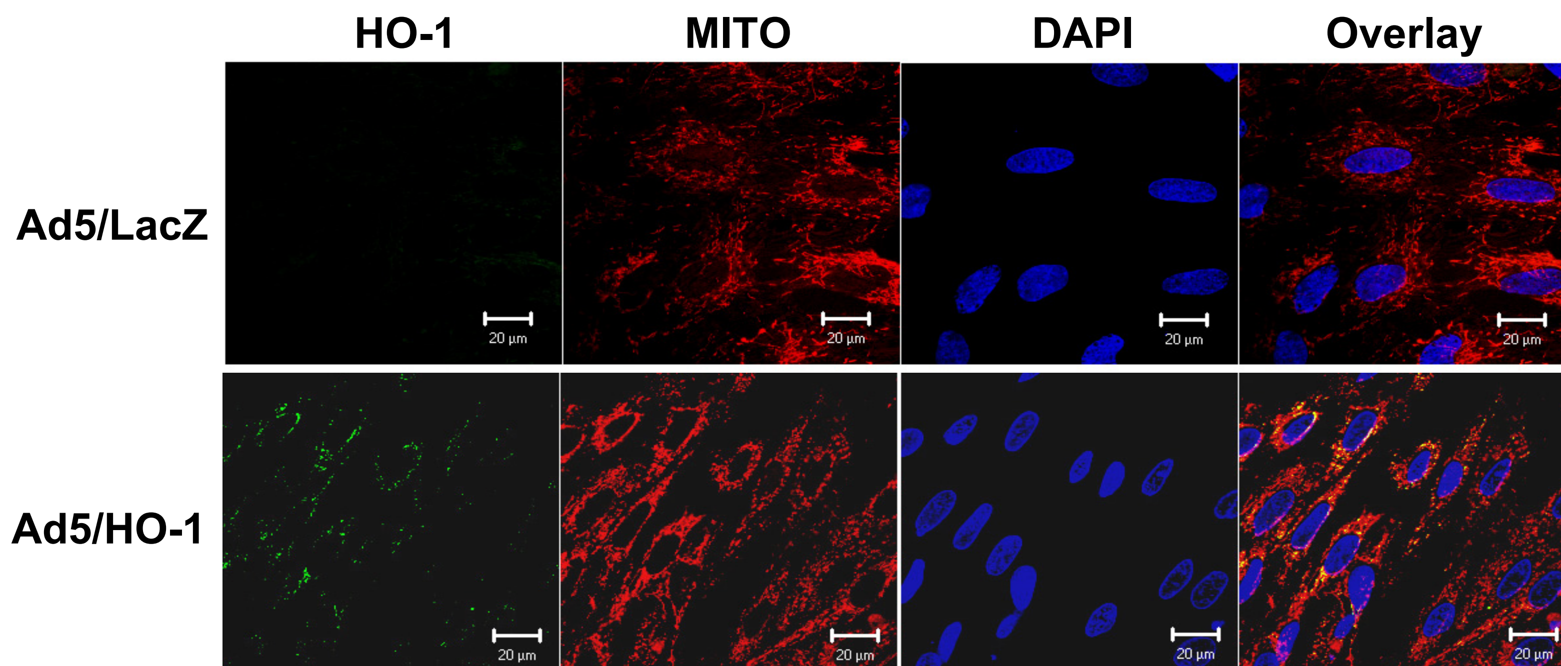


Figure S6

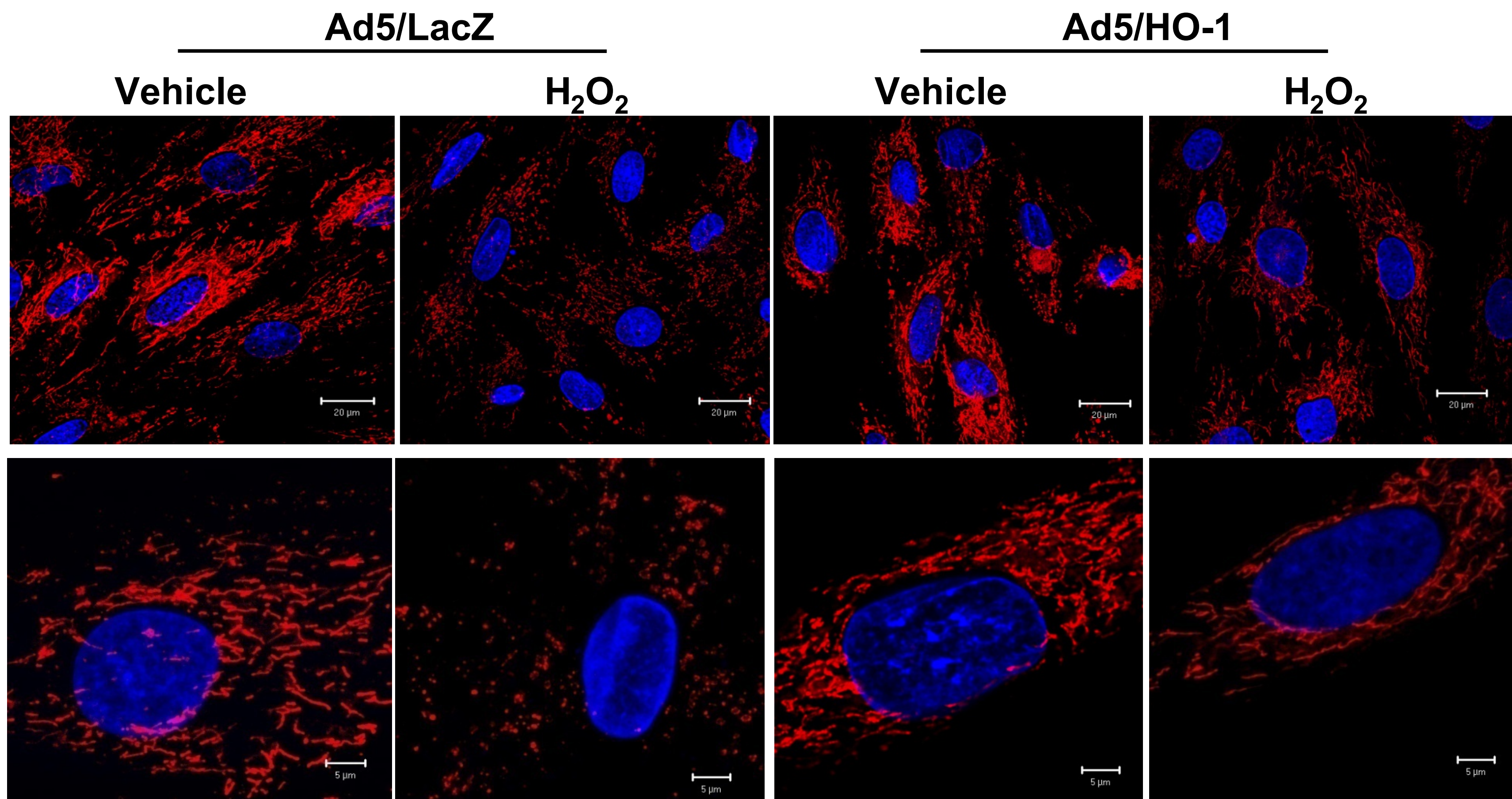


Figure S7