Nrf2 Enhances Myocardial Clearance of Toxic Ubiquitinated Proteins

Wenjuan Wang[1, 2], Siying Li[1, 2], Hui Wang[1, 2], Bin Li[1, 2], Lei Shao[1, 2], Yimu Lai[2], Gary Horvath[3], Qian Wang[3], Masayuki Yamamoto[4], Joseph S. Janicki[2], Xing Li Wang[1], Dongqi Tang[1,2], Taixing Cui[1,2]

[1]Shandong University Qilu Hospotial Research Center for Cell Therapy, Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu Hospital of Shandong University, Jinan 250012, China

[2]Department of Cell Biology and Anatomy, University of South Carolina, SC 29208, USA

[3]Department of Chemistry and Biochemistry, University of South Carolina, SC 29208, USA

[4]Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Miyagi, 980-8575, Japan

ONLINE SUPPLEMENTARY MATERIALS

I. A Full Description of Methods

Animals

Animals were kept on a 12-h light/dark cycle in a temperature-controlled room with ad libitum access to food and water. Transgenic mice with cardiac myocyte-specific overexpression of Nrf2 ($Nrf2^{ctg}$) were produced in a FVB/NJ background. Full-length human Nrf2 cDNA was cloned into a vector containing the alpha-myosin heavy chain (α-MHC) promoter [1] (a gift from Dr. Jeffrey Robbins at Cincinnati Children's Hospital Medical Center, Cincinnati, Oho) and was used for microinjection at Medical University of South Carolina (MUSC) Transgenic Core (Supplementary figure 1). $Nrf2^{ctg}$ mice were screened by PCR of tail DNA using a two-primer reaction that identified a 1058–bp product from the transgene. The forward primer was 5'- CGG CAC TCT TAG CAA ACC TC -3' (corresponding to the a-MHC promoter). The reverse primer was 5'- ACT GGT TGG GGT CTT CTG TG 3' (corresponding to the transgene of human Nrf2). Nrf2 knockout ($Nrf2^{-/-}$) mice were generated using heterozygote breeding pairs ($Nrf2^{+/-}$ in ICR/Sv129 background) as previously described [2]. FVB/NJ wild type (WT) mice of the transgene negative siblings served as controls for $Nrf2^{ctg}$ mice. WT litter mates of $Nrf2^{-/-}$ mice served as controls for $Nrf2^{-/-}$ mice. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health), and all protocols were approved by the University of South Carolina Institutional Animal Care and Use Committee.

Transverse Aortic Arch Constriction (TAC)

Male mice at 8-9 weeks of age were subjected to sham or TAC operations as previously described [2]. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The use of a horizontal incision at the level of the suprasternal notch allows direct visualization of the transverse aorta without entering the pleural space and thus obviates the need for mechanical ventilation. The transverse aorta was constricted between the right innominate and left carotid arteries to the diameter of a 27-gauge needle using a 7-0 silk suture. Sham operations on sex- and age-matched mice were identical with the exception of omitting the actual aortic banding and served as controls for all experimental groups. Cardiac hypertrophy was determined by the heart weight–to–tibia length (HW/Tibia) ratio, myocardial cross-sectional area, and expression levels of cardiac hypertrophy marker genes including atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), alpha-myosin heavy chain (αMHC), beta-myosin heavy chain (βMHC), sarcoplasmic reticulum calcium ATPase2a (SERCA).

Echocardiography and Blood Pressure Measurement

Echocardiography was performed on anesthetized (isoflurane) mice, using a Vevo 770 High-Resolution Imaging System (VisualSonics Inc.) with a 37.5-MHz high-frequency transducer, as previously described [2]. Briefly, mice were anesthetized with 3% isoflurane and maintained with 1.5% isoflurane in room air supplemented with 100% O_2. After the anterior chest was shaved, the animals were placed on a warming pad to maintain normothermia. The echocardiographic gel was warmed before use to avoid hypothermia. Care was taken to avoid excessive pressure on the thorax, which can induce bradycardia. Two-dimensional (2D) long axis images of the left ventricle (LV) were obtained at the plane of the aortic and mitral valves where the LV cavity is largest and visualization of the LV apex is adequate, and a short-axis image was recorded at the level of the papillary muscles. A 2D guided M-mode echocardiogram was recorded through the anterior and posterior LV walls at a speed of 200 mm/s. Images were obtained at the level of the papillary muscle tips, and measurements in mm were then performed to obtain the LV internal dimension (LVID), intraventricular septal wall thickness (IVS), and LV posterior wall thickness (LVPW) according to the leading-edge method of the American Society of Echocardiography. LV percent fractional shortening FS (%) was calculated via VisualSonics Measurement Software. Blood pressures were measured in conditioned, unanesthetized mice using the tail-cuff method (Hatteras Instruments, MC4000 Blood Pressure Analysis System).

Pathology

Mice were anesthetized and perfused via the abdominal aorta with a saline solution to wash out blood in the heart. Then, the hearts were harvested, dried on gauze, weighed, dissected, and frozen. Lungs and tibias were dissected. Lungs were dried on gauze and weighed. The length of the tibias from the condyles to the tip of the medial malleolus was measured by micrometer calipers.

Histology and Immunochemistry

Hearts were cannulated via the left ventricular apex, cleared by perfusion with saline (0.9% NaCl) at 90 mmHg, arrested in diastole with 60 mM KCl, fixed by perfusion with 10% formalin, and embedded in paraffin. Paraffin sections were prepared (5 μm, Leica RM2030, rotary microtome) and stored at room temperature until staining. Histology and immunohistochemistry was performed as previously described [2]. Briefly, cardiomyocyte cross-sectional area was measured via Texas Red-X conjugated wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA) staining. For myocardial fibrosis, coronal sections were stained for collagen with a Masson's Trichrome Kit (Poly Scientific, Bay Shore, NY). Apoptosis was measured by TUNEL assays on tissue sections using an In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN). Myocardial oxidative stress was assessed with 4-Hydroxy-2-Nonenal (4-HNE) and 8-hydroxydeoxyguanosine (8-OHdG) staining; primary antibodies included a

mouse 4-HNE antibody (ab48506, Abcam Inc.), and a mouse 8-OHdG antibody (sc-660369, Santa Cruz Biotechnology, Inc.), respectively. Cardiomyocytes were stained with a rabbit tropomyosin I antibody (ab55915, Abcam Inc.) or green Alexa Fluor® 488 phalloidin (SKU# A12379, Invitrogen) to identify myocardial F-actin. Nuclei were labeled with 4', 6-Diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time (qPCR). Total LV RNA was extracted, and RT, PCR and qPCR were performed as previously described [2]. Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels or other internal control gene mRNAs according to the protocol provided by the manufacturer. Primers that were used for qPCR are summarized in Supplementary Table 4.

Western Blot

Western blot was performed as previously described [2, 3]. Cells were treated with various stimuli as indicated for 6 h unless specified in figure legends and then subjected to Western blot analysis of accumulation of ubiquitinated proteins in soluble and insoluble fractions. Cellular proteins were separated into detergent-soluble and -insoluble fractions with the 2% Triton X-100 buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 2% Triton X-100, a protease inhibitor cocktail (Roche)]. The insoluble fractions were solubilized in the buffer with 1% SDS lysis buffer [50mM Tris (pH8.0); 10 mM EDTA, 1% SDS, protease inhibitor cocktail (Roche) and phosphatase inhibitor (Sigma–Aldrich)]. For autophagy flux assay, a strong 2% SDS buffer (2% SDS, 150 mM NaCl, 10 mM Tris pH 8.0) was applied to analyze total cellular LC3 protein expression in both soluble and insoluble fractions. Primary antibodies included anti-LC3 (L7543, Sigma-Aldrich), anti-Ub (sc-8017, Santa Cruz Biotechnology, Inc.), anti-LAMP-1 (ab24170, Abcam), anti-LAMP-2 (L0668, Sigma-Aldrich), anti-Beclin-1 (MAB5295, R&D System), and anti-NQO1 (ab34173, Abcam). After Western blotting with primary antibodies, the membranes were then stripped and reprobed with anti-GAPDH (G8795, Sigma-Aldrich) or anti-beta-actin (A1978, Sigma-Aldrich) antibodies to measure the expression of GAPDH or β-actin as internal loading controls. Densitometric analysis was performed by using an image scanner and NIH image software.

Filter Trap Assay

Filter retardation assays were performed as described previously [4]. LV tissues were lysed for 30 minutes on ice in Nondinet P-40 (NP-40) buffer [100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2% NP-40, 10 mM EDTA, pH 8.0], supplemented with complete mini protease inhibitor cocktail (Roche) and

phosphatase inhibitor cocktail (Sigma-Aldrich). After centrifugation for 15 min at 20,800 g at 4°C, cell pellets were resuspended in benzonase buffer (1 mM $MgCl_2$, 50 mM Tris-HCl; pH 8.0) and incubated for 1 h at 37°C with 250 U benzonase (Merck). Reactions were stopped by adding 2x termination buffer (40 mM EDTA, 4% SDS, 100 mM DTT). Aliquots of 30 μg protein extract were diluted into 2% SDS buffer (2% SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and filtered through a 0.2 μm cellulose acetate membrane (Invitrogen) pre-equilibrated in 2% SDS buffer. Filters were washed twice with 0.1% SDS buffer (0.1% SDS, 150 mM NaCl, 10 mM Tris pH 8.0) and subsequently blocked in 5% nonfat milk (Sigma-Aldrich) in TBS. Captured aggregates were detected by incubation with FK2 antibody (ENZO, 1:1000) and further treated with goat anti-IgG-HRP (sc-2004, Santa Cruz Biotechnology, Inc., 1:2000) for western blotting.

Virus Preparation

Adenovirus of GFP, murine Nrf2, scramble shRNA, and rat Nrf2 shRNA was generated as previously described [2].

Cell Culture, Virus Infection, and Cytotoxicity Assay

Rat Cardiac H9C2 cells, a myoblast cell line that is derived from embryonic rat hearts that maintains some features of cardiac myocytes, (CRL 1446, American Type Tissue Collection) were cultured as described elsewhere [5]. Rat neonatal cardiac myocytes were isolated and cultured as previously described [2]. After 3 days in culture, cells were infected with adenovirus of Gfp, Nrf2, scramble control shRNA (Ctr sh), or Nrf2 shRNA in serum free PC-1 media (Lonza Walkersville, Inc., Walkersville, MD) for 48 h.

Adult murine cardiac myocytes were isolated as previously described with a slight modification [6, 7]. Briefly, hearts were removed, cannulated and perfused with collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) to dissociate ventricular myocytes. Myocytes were plated at a density of 50 rod-shaped myocytes per square millimeter on laminin-coated culture dishes in minimal essential medium (MEM) containing 2.5% FBS and 1% penicillin-streptomycin. One hour after the plating, the culture medium was changed to FBS-free Hank's Balanced Salt Solution supplemented with 1 mg/ml bovine serum albumin, 10 mM 2,3-butanedione monoxime, and 100 U/ml penicillin in a 5% CO_2 incubator at 37°C. Cell viability was determined by trypan blue staining, which has been considered to be the gold standard for evaluating cell viability of isolated adult cardiomyocytes [8, 9]. Briefly, cultured cells were gently washed once with warm protein-free Hank's Balanced Salt Solution and then stained with trypan blue (Sigma-Aldrich) in the same medium with a final concentration of 0.04% (wt/vol) for 15 min. After washing 3 times with warm protein-free Hank's Balance Salt Solution, the cardiomyocytes were visualized under microscope. At least 300 cells were counted for each experimental group. The

number of viable (unstained) and nonviable (blue stained) cells was recorded and the percentage of viability was defined as the number of unstained myocytes divided by that of total cells. We found that depending on murine strains, 20~40% of isolated adult murine ventricular cells died 24 h after the cell culture as previously reported [6]. Six hour after plating myocytes, puromycin was added for 16 h.

Cell death was also measured using a cytotoxicity detection kit (630117, Clontech). Cells were treated with various stimuli as indicated for 16 h unless specified in figure legends. Cell viability was calculated as follows: cell viability = (LDH activity of cell lysate in *Exp.*)/(LDH activity of cell lysate in *Con.*) × 100%. Alternatively, cell death rate = (LDH activity of supernatant in *Exp*)/(LDH activities of supernatant and cell lysate in *Exp*). *Exp.* and *Con* indicates experimental and control groups, respectively.

Cell culture-based experiments were repeated at least three times.

Autophagy Flux Assay

Rat neonatal cardiomyocytes were treated with or without BafA1 (5 nmol/L) in PC-1 medium for 4 h. In addition, rat neonatal cardiomyocytes were treated with or without rapamycin (0.5 μmol/L) for 16 h in PC-1 medium and additionally with vehicle or BafA1 (5 nmol/L) for the last 4 h. These cells were then subjected to Western blot analysis of LC3 expression as described above. Autophagic flux was calculated by a formulation of BafA1-induced (LC3-II/Gapdh) density – basal (LC3-II/Gapdh) density.

DNA Ladder Assay

Isolation of cellular DNA was performed using the ClassicTM DNA Isolation Kit (D109, Lamda Biotech) according to the protocol provided by the manufacturer. The isolated DNAs were subjected to electrophoresis in 1.5% agarose gel with the logic DNA Marker (M103-1, Lamda Biotech) and a positive control of DNA ladder.

Statistics

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance with the Student t test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by ANOVA followed by Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

Reference

[1] Subramaniam A, Jones WK, Gulick J, Wert S, Neumann J, Robbins J. Tissue-specific regulation of the alpha-myosin heavy chain gene promoter in transgenic mice. J Biol Chem. 1991;266:24613-20.

[2] Li J, Ichikawa T, Villacorta L, Janicki JS, Brower GL, Yamamoto M, et al. Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. Arterioscler Thromb Vasc Biol. 2009;29:1843-50.

[3] Li J, Zhang C, Xing Y, Janicki JS, Yamamoto M, Wang XL, et al. Up-regulation of p27(kip1) contributes to Nrf2-mediated protection against angiotensin II-induced cardiac hypertrophy. Cardiovascular research. 2011;90:315-24.

[4] Menzies FM, Hourez R, Imarisio S, Raspe M, Sadiq O, Chandraratna D, et al. Puromycin-sensitive aminopeptidase protects against aggregation-prone proteins via autophagy. Hum Mol Genet. 2010;19:4573-86.

[5] Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes. 2002;51:1938-48.

[6] Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobilka BK, Ziman B, et al. Culture and adenoviral infection of adult mouse cardiac myocytes: methods for cellular genetic physiology. Am J Physiol Heart Circ Physiol. 2000;279:H429-36.

[7] O'Connell TD, Ishizaka S, Nakamura A, Swigart PM, Rodrigo MC, Simpson GL, et al. The alpha(1A/C)- and alpha(1B)-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse. The Journal of clinical investigation. 2003;111:1783-91.

[8] Portal L, Martin V, Assaly R, d'Anglemont de Tassigny A, Michineau S, Berdeaux A, et al. A model of hypoxia-reoxygenation on isolated adult mouse cardiomyocytes: characterization, comparison with ischemia-reperfusion, and application to the cardioprotective effect of regular treadmill exercise. Journal of cardiovascular pharmacology and therapeutics. 2013;18:367-75.

[9] Diaz RJ, Wilson GJ. Studying ischemic preconditioning in isolated cardiomyocyte models. Cardiovascular research. 2006;70:286-96.

II. Supplementary Tables

Supplementary Table 1. Baseline body weight, heart rate, and blood pressure of wild type (WT) and Nrf2^{ctg} mice

(n)	WT	Nrf2^{ctg}
BW (g)	27.6 ± 0.8 (14)	26.0 ± 2.1 (22)
HR (beat/min)	649.3 ± 49.2 (16)	660.0 ± 68.8 (13)
SBP (mmHg)	113.3 ± 7.2 (16)	114.1 ± 11.0 (13)
DBP (mmHg)	96.4 ± 11.3 (16)	98.2 ± 9.4 (13)
MAP (mmHg)	101.7 ± 9.7 (16)	103.2 ± 9.5 (13)

Male WT and Nrf2^{ctg} mice at age of 8 weeks were weighed and then subjected to blood pressure measurement via the tail cuff method (Tail cuff measurement Hatteras 4000). BW, body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. Animal numbers for each group are indicated in the parentheses.

Supplementary Table 2. Baseline echocardiography of WT and Nrf2^{ctg} mice

(n)	WT	Nrf2^{ctg}
	(24)	(22)
LVID;d (mm)	3.55 ± 0.68	3.52 ± 0.18
LVID;s (mm)	2.10 ± 0.41	1.97 ± 0.20
LVPW;d (mm)	0.99 ± 0.60	0.87 ± 0.08
FS (%)	41.50 ± 5.30	44.06 ± 4.52

Male WT and Nrf2^{ctg} mice at age of 8 weeks were subjected to echocardiography using Visualsonics Vevo 770 system. LVID;d, left ventricular internal dimension diastolic; LVID;s, left ventricular internal dimension systolic; LVPW;d, left ventricular posterior wall thickness diastolic; FS, fractional shortening. Animal numbers for each group are indicated in the parentheses.

Supplementary Table 3. Time-course study of echocardiography in WT and Nrf2^{ctg} mice after TAC

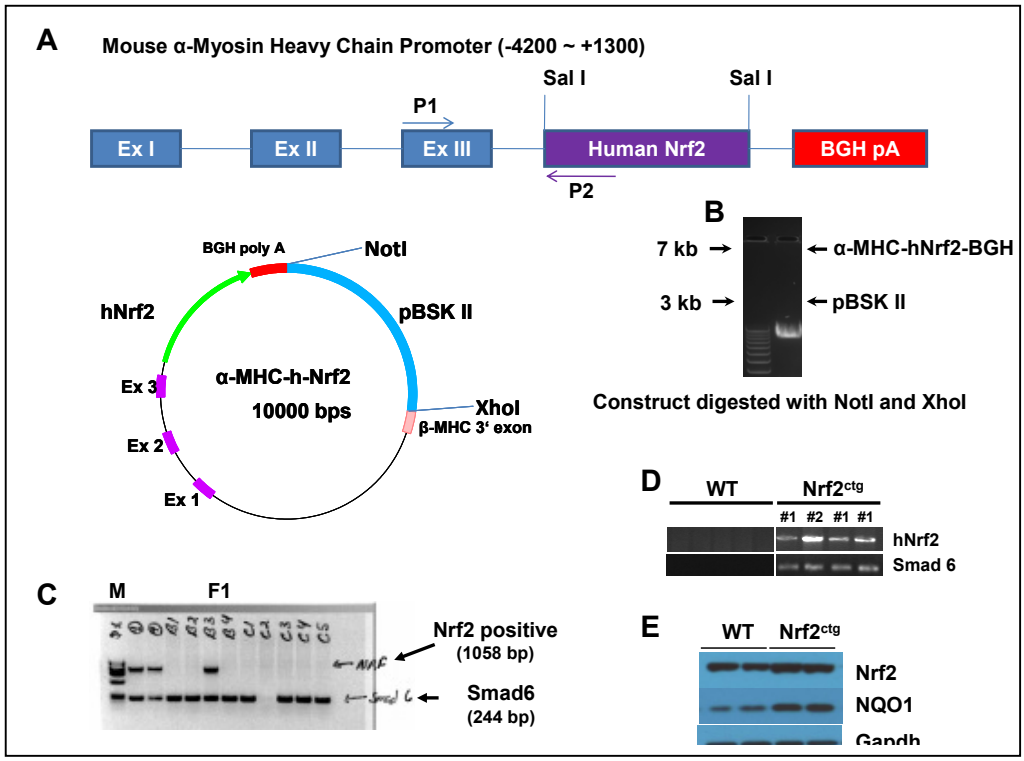
	WT		Nrf2^{ctg}	
	Sham (11)	**TAC (10)**	**Sham (11)**	**TAC (10)**
Day 0				
LVID;d (mm)	3.88 ± 0.41	3.55 ± 0.30	3.35 ± 0.29	3.67 ± 0.43
LVID;s (mm)	2.36 ± 0.38	2.02 ± 0.24	1.95 ± 0.29	2.18 ± 0.36
LVPW;d (mm)	0.77 ± 0.13	0.88 ± 0.13	0.82 ± 0.11	0.80 ± 0.13
FS (%)	39.43 ± 2.45	43.10 ± 2.23	41.73 ± 3.49	40.60 ± 1.93
Day 14				
LVID;d (mm)	3.88 ± 0.58	3.50 ± 0.23	3.59 ± 0.42	3.1 ± 0.53
LVID;s (mm)	2.27 ± 0.66	2.46 ± 0.24	1.97 ± 0.42	1.88 ± 0.25
LVPW;d (mm)	0.87 ± 0.12	1.30 ± 0.15^{A}	0.86 ± 0.12	1.22 ± 0.14^{B}
LVFS (%)	41.56 ± 5.04	29.60 ± 2.96^{A}	45.10 ± 4.61	39.47 ± 4.38^{B,C}
Day 28				
LVID;d (mm)	3.83 ± 0.34	3.60 ± 0.32	3.57 ± 0.38	3.32 ± 0.20
LVID;s (mm)	2.18 ± 0.48	2.63 ± 0.30^{A}	2.17 ± 0.33	2.18 ± 0.20^{C}
LVPW;d (mm)	0.81 ± 0.13	1.37 ± 0.15^{A}	0.93 ± 0.08	1.20 ± 0.10^{B,C}
FS (%)	42.90 ± 5.20	27.26 ± 2.79^{A}	39.25 ± 2.19	33.89 ± 3.02^{B,C}

WT and Nrf2^{ctg} mice at age of 8-9 weeks were subjected to sham or TAC operations. Echocardiography was performed before or after operation as indicated using Visualsonics Vevo 770 system. LVID;d, left ventricular internal dimension diastolic; LVID;s, left ventricular internal dimension systolic; LVPW;d, left ventricular posterior wall thickness diastolic; FS, fractional shortening. Animal numbers for each group are indicated in the parentheses. ^{A} $p < 0.05$ vs. WT sham; ^{B} $p < 0.05$ vs. Nrf2^{ctg} sham; ^{C} $p < 0.05$ vs. WT TAC

Supplementary Table 4. Primers for qPCR

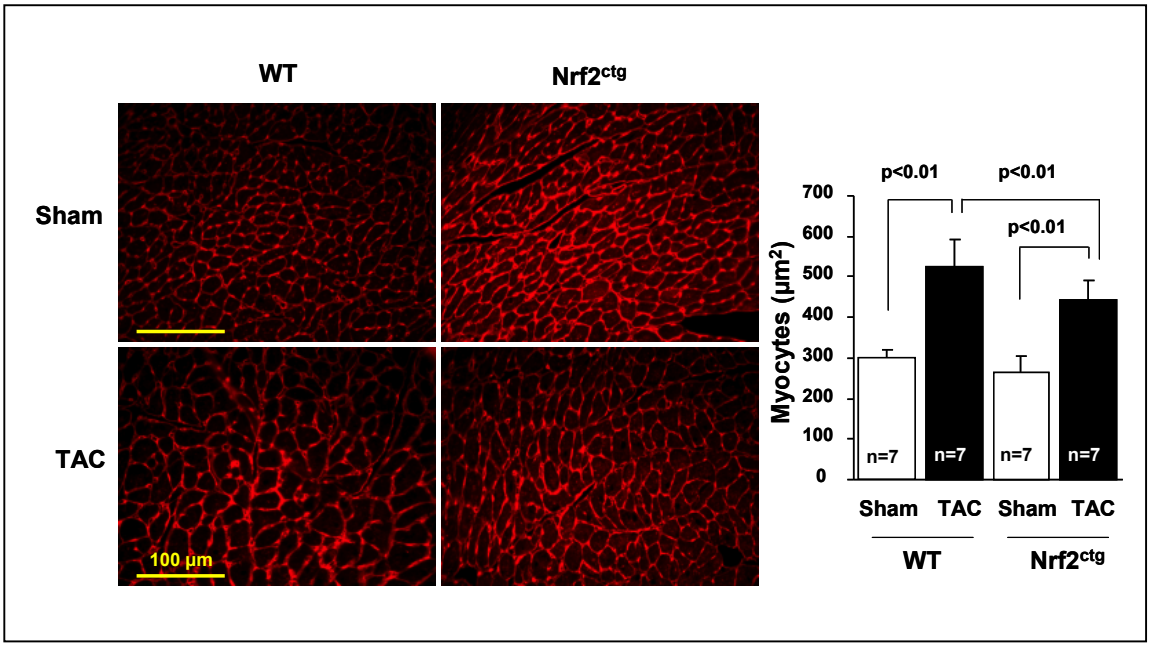
Primers	Gene access#	Forward (5'--3')	Reverse (3'--5')	Product length
ANF	NM_008725.2	CATCACCCTGGGCTTCTTCCT	TGGGCTCCAATCCTGTCAATC	405
BNP	NM_008726.4	GCGGCATGGATCTCCTGAAGG	CCCAGGCAGAGTCAGAAACTG	418
a-MHC	NM_010856.4	CCAATGAGTACCGCGTGAA	ACAGTCATGCCGGGATGAT	254
ß-MHC	NM_080728.2	ATGTGCCGGACCTTGGAA	CCTCGGGTTAGCTGAGAGATCA	170
SERCA2a	NM_009722.3	CCATCTGCTTGTCCATGTCACT	CAAATGGTTTAGGAAGCGGTTACT	213
GAPDH	XM_001479371.4	ATGTTCCAGTATGACTCCACTCACG	GAAGACACCAGTAGACTCCACGACA	171
Nrf2	NM_010902.3	ATGATGGACTTGGAGTTGCC	TCCTGTTCCTTCTGGAGTTA	200
NQO-1	NM_008706.5	CGGTATTACGATCCTCCCTCAACA	AGCCTCTACAGCAGCCTCCTTCAT	120
Txn-1	NM_011660.3	CCCCCACCTTTTGACCCTTTTTAT	AGCCCTTCTTCCATTCCCTCTGT	147
ß-actin	NM_007393.3	TGTCACCAACTGGGACGATA	GGGGTGTTAAGGTCTCAAA	97

III. Supplementary Figures and Legends

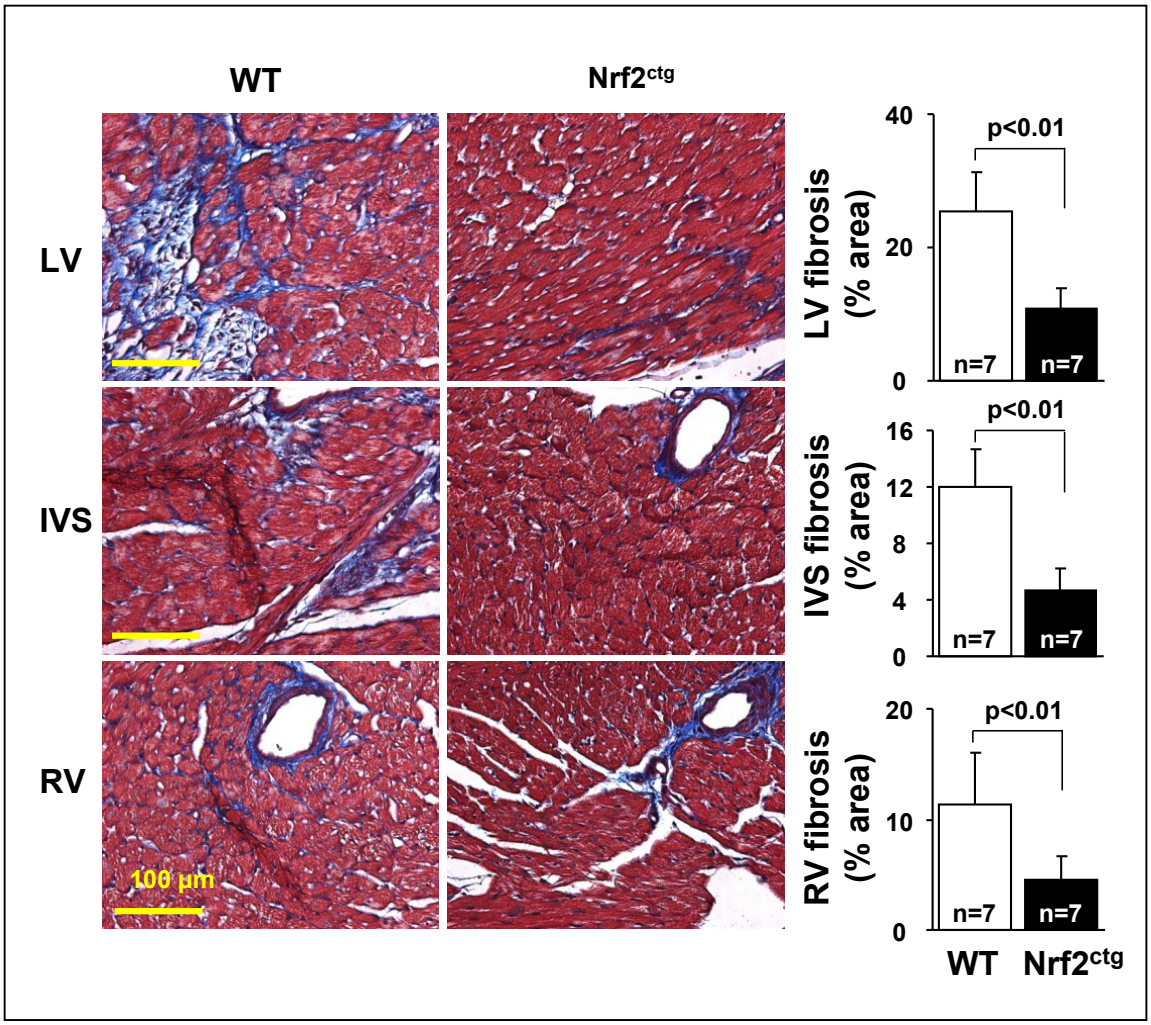


Supplementary Figure 1. Generation of cardiomyocyte specific Nrf2 transgenic mice.

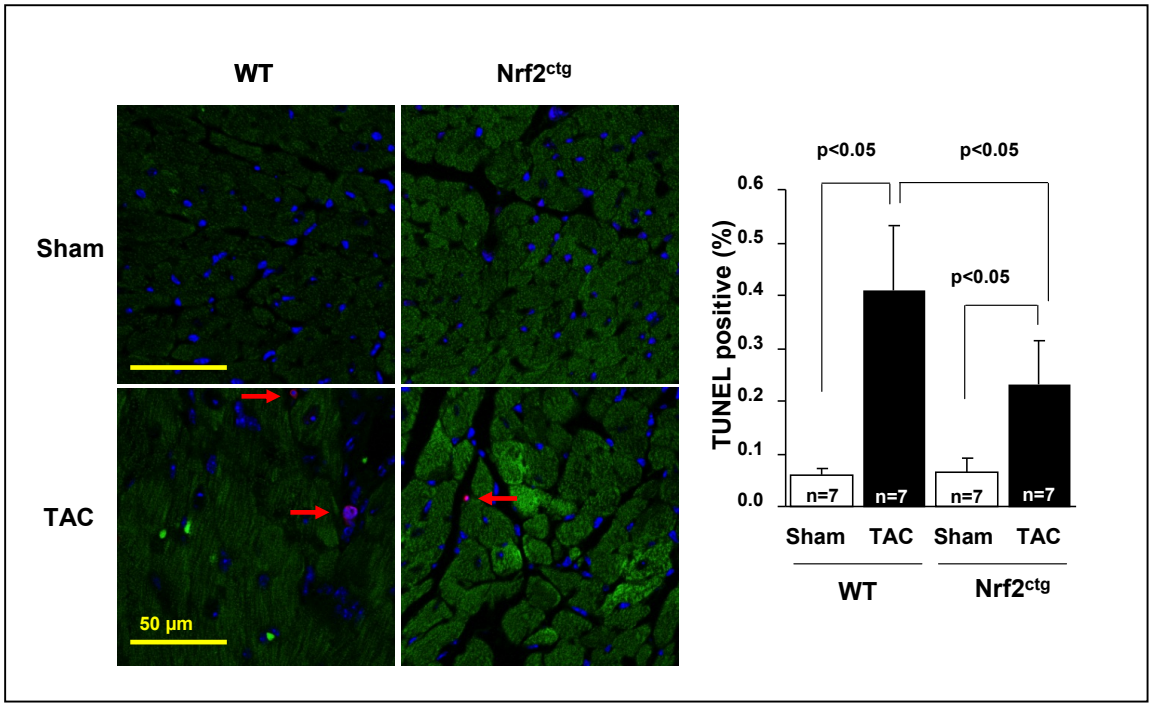
A, Schematic diagram of α-MHC-hNrf2 transgene structure. The human Nrf2 (hNrf2) coding sequence was inserted upstream of the BGH polyadenylation signal. Expression is under the control of the alpha myosin heavy (α-MHC) chain gene promoter (gift from Dr. Jeffrey Robbins at Cincinnati Children's Hospital Medical Center, Cincinnati, Oho). **B,** Enzymatic digestion of transgenic cassette. The generated transgene vectors were digested with restrictive enzymes of Not1 and Xho1 revealing the size of the backbone vector and inserted gene. **C.** Founder genotyping result**.** Tail DNA of first generation (i.e., founder (F1)) of α-MHC-hNrf2 transgenic mice were used for PCR analysis of α-MHC-hNrf2 transgene. α-MHC-hNrf2-forward (P1): 5' – CGG CAC TCT TAG CAA ACC TC - 3' (located in α-MHC promoter) α-MHC-hNrf2-reverse (P2): 5' – ACT GGT TGG GGT CTT CTG TG - 3' (located in Nrf2 coding region) The PCR amplicon size is 1058 bp. Mouse Smad6 gene expression was used as an internal control. Primers of Smad6 are; forward: 5'- CCC CCT CTC CCC CAG CAA TAA -3', reverse: 5'- GCG CCG CAC CGA CTC AC -3'. The PCR product size is 244 bp. M, marker. **D.** Genotyping of transgene lines of $Nrf2^{ctg}$ mice. Regular PCR analysis of relative expression levels of insert Nrf2 in littermates of $Nrf2^{ctg}$ transgene mice**. E.** Western blot analysis. Left ventricles of littermates of wild type (WT) and $Nrf2^{ctg}$ mice were subjected to Western blot analyzing the protein expression of Nrf2 and its downstream gene, NAD(P)H:quinone oxidoreductase (NQO1).



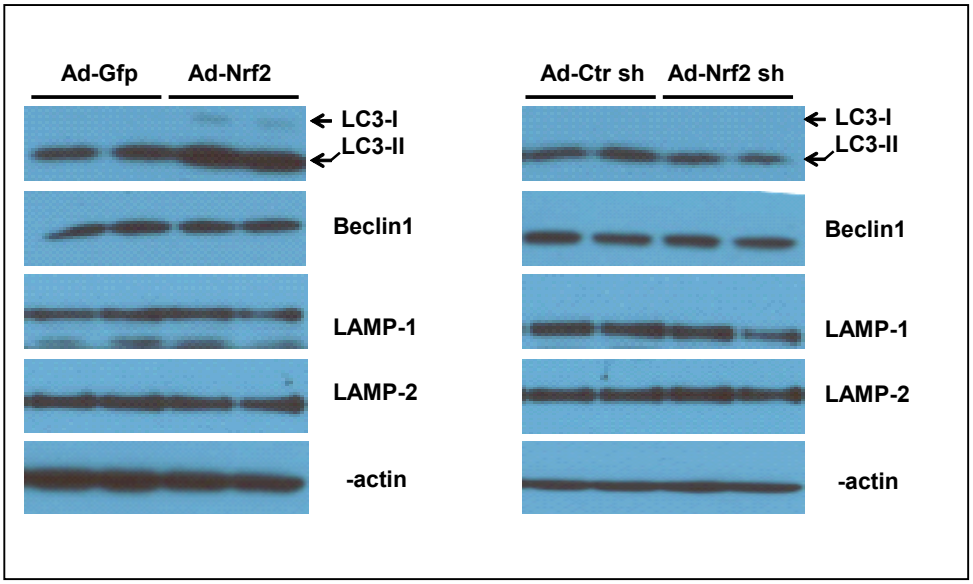
Supplementary Figure 2. TAC-induced myocardial hypertrophy in WT and Nrf2^{ctg} mice. Littermates of male WT and Nrf2^{ctg} mice at ages of 8 ~ 10 weeks were subjected to sham and TAC operations. Left ventricle tissue sections of the hearts from the WT and Nrf2^{ctg} mice 4 weeks after sham or TAC operation were subjected to Wheat germ agglutinin (WGA) staining using WGA-Texas RedR-X conjugate (Cat. W21405, Invitrogen). Images were acquired by E600 fluorescence microscope (Nikon Eclipse E600; Nikon In, Melville, NY) at 400× magnification. Number of hearts for each group (n) is indicated.



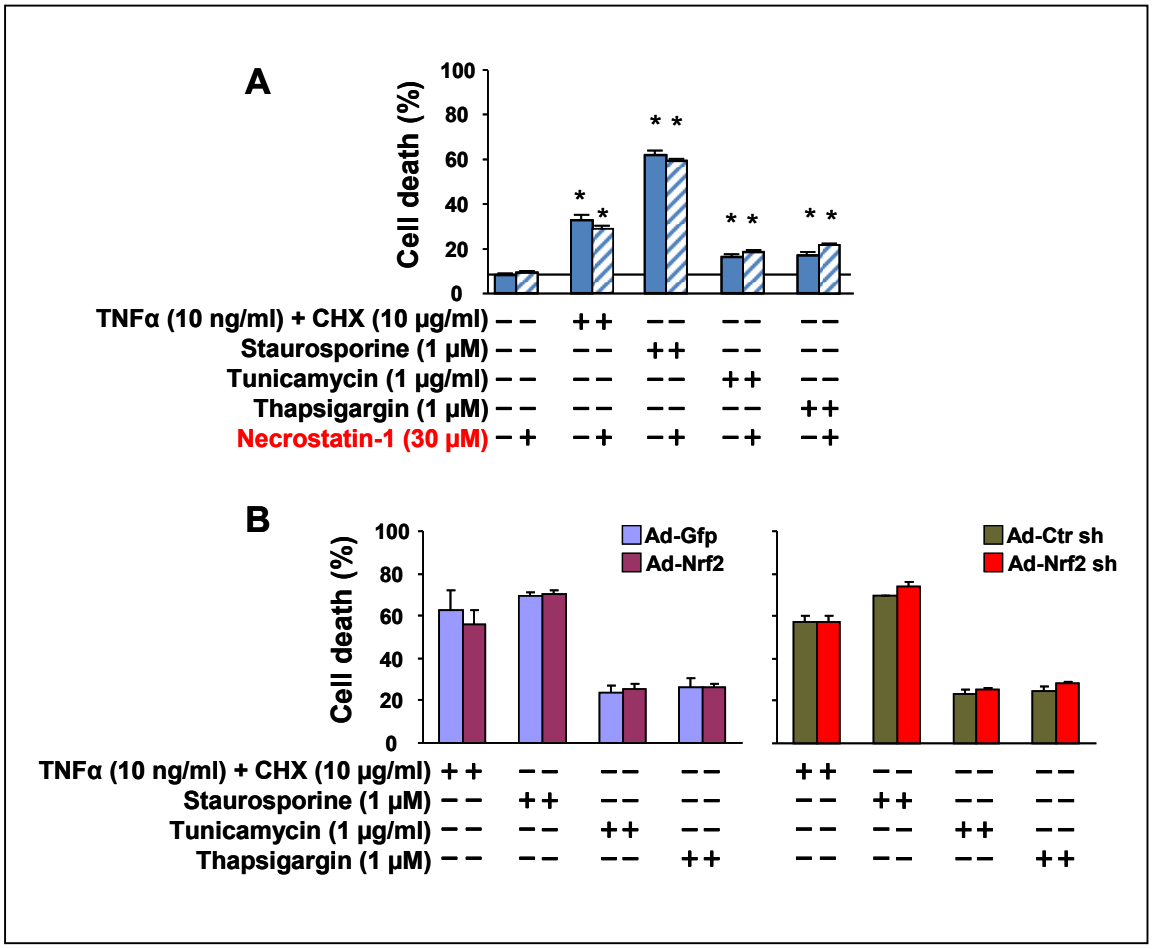
Supplementary Figure 3. TAC-induced cardiac fibrosis in WT and Nrf2^{ctg} mice. Littermates of male WT and Nrf2^{ctg} mice at ages of 8 ~ 10 weeks were subjected to sham and TAC operations. Left ventricle tissue sections of the hearts from the WT and Nrf2ctg mice 4 wk after sham or TAC operation were subjected to collagen staining using Masson's Trichrome Kit (K307, Poly Scientific, Bay Shore, NY). Images were acquired by light microscope (Nikon Optiphot-2; Nikon Inc., Melville, NY) at 200 × magnification. Number of hearts for each group (n) is indicated.



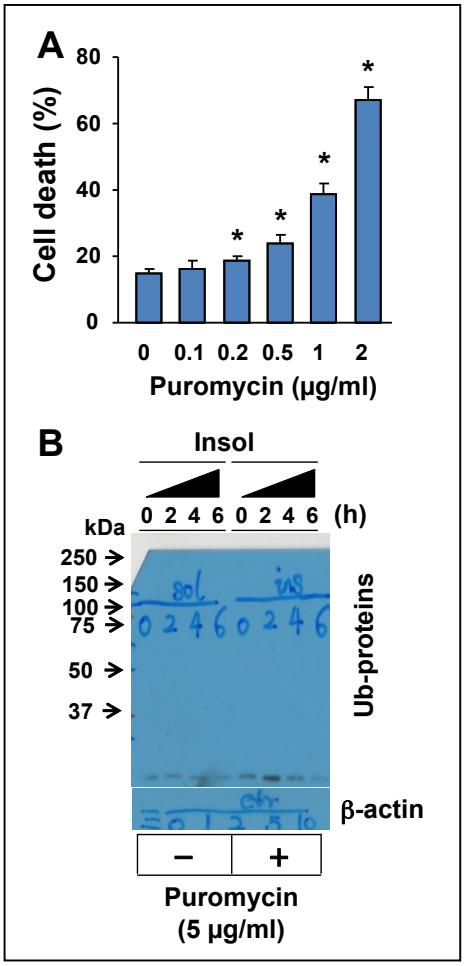
Supplementary Figure 4. TAC-induced myocardial cell death in WT and Nrf2^{ctg} mice. Littermates of male WT and Nrf2^{ctg} mice at ages of 8 ~ 10 weeks were subjected to sham and TAC operations. Left ventricle tissue sections of the hearts from the WT and Nrf2ctg mice 4 weeks after sham or TAC operation were subjected to TUNEL staining using an In Situ Cell Death Detection Kit, TMR red (, Cat#. 12 156 792 910, Roche). Representative images were acquired by confocal microscope (LSM510META, Carl Zeiss Inc., Maple Grove, MN) at 630 × magnification. Myocardial F-actin was highlighted by green Alexa Fluor® 488 phalloidin (SKU# A12379, Invitrogen). Nuclei were counterstained with DAPI (blue). Number of hearts for each group (n) is indicated.



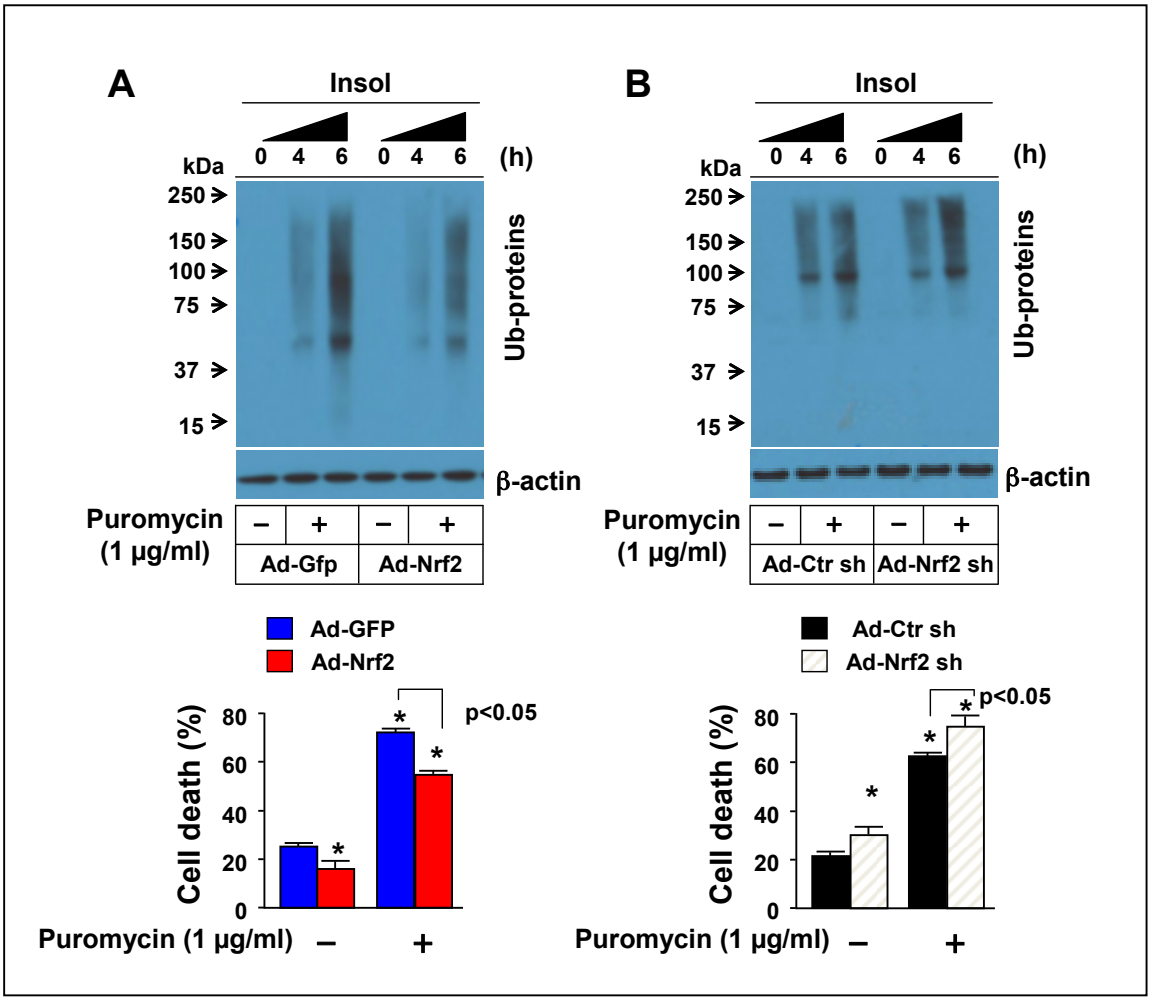
Supplementary Figure 5. Effects of Nrf2 overexpression and knockdown on the expression of Beclin1, LAMP-1, and LAMP-2 in cardiomyocytes. H9C2 cells were infected with Ad-Gfp, Ad-Nrf2, Ad-Ctr sh, or Ad-Nrf2 sh for 48 h and then subjected to Western blot analysis of beclin1, LAMP-1, and LAMP-2 expression. Results are representatives of duplicated immunoblots.



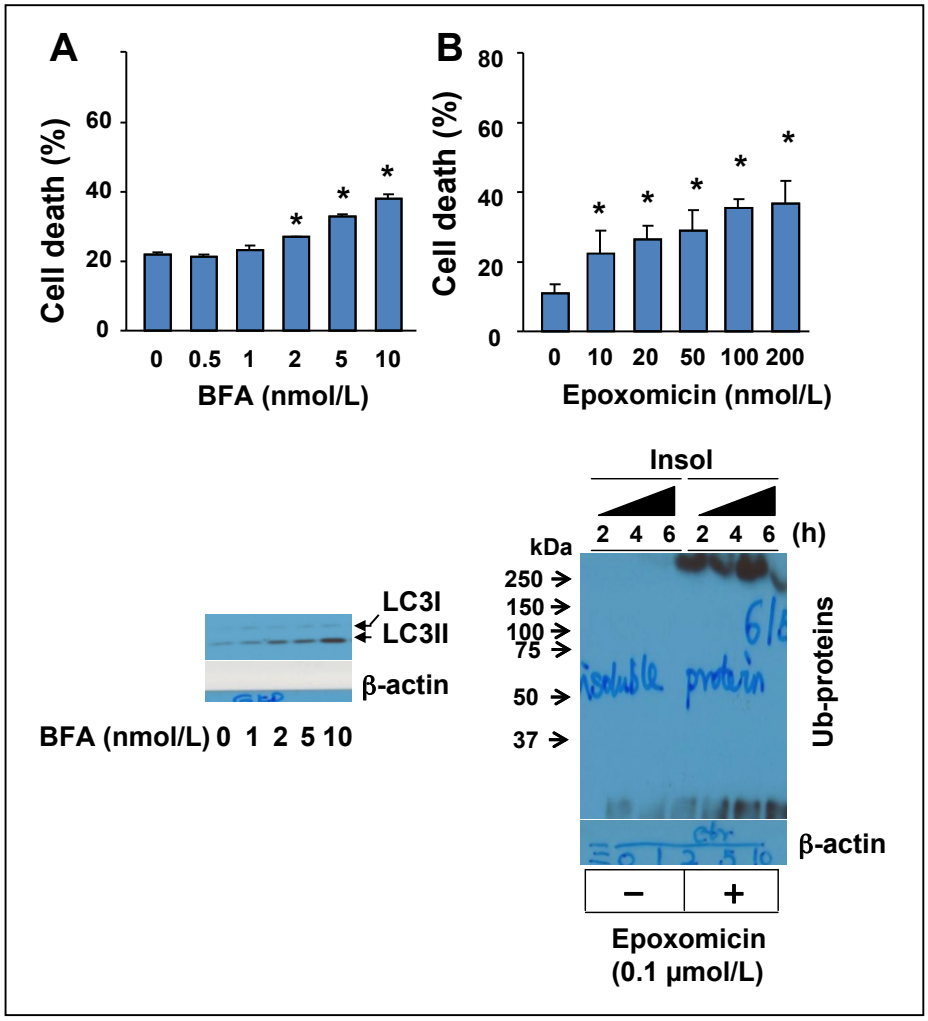
Supplementary Figure 6. Effect of Nrf2 gain- and loss-of-function on apoptosis in H9C2 cardiomyocytes. A, Subconfluent H9C2 cells were treated with various apoptotic inducers with or with necrostatin-1 in DMEM supplemented with 2%FBS as indicated for 24 h. n=6, *p < 0.05 vs. control (-). **B,** Infected H9C2 cells were treated in DMEM supplemented with 1%FBS as indicated for 24 h. n=6. CHX, cycloheximide.



Supplementary Figure 7. Dose responses and time courses of puromycin-induced cell death and protein aggregation in H9C2 cardiomyocytes. A, Sub-confluent cells were treated in DMEM supplemented with 1%FBS in presence or absence of puromycin as indicated for 48 h**.** n=6, *p < 0.05 vs. control (0). **B,** Puromycin-induced accumulation of ubiquitinated protein aggregates. The results are representatives of three independent experiments.



Supplementary Figure 8. Effect of Nrf2 gain- and loss-of-function on puromycin-induced protein aggregation and cell death in H9C2 cardiomyocytes. Infected cells were treated with or without puromycin in DMEM supplemented with 1%FBS for ~ 6h or 48 h and subjected to Western blot analysis of protein aggregation or cell death, respectively. n=6, *p < 0.05 vs. Ad-Gfp or Ad-Ctr sh.



Supplementary Figure 9. Dose responses and time courses of BafA1-, or epoxomicin-induced cell death and protein aggregation in H9C2 cardiomyocytes. A, (Up panel) Sub-confluent cells were treated in DMEM supplemented with 1%FBS in the presence or absence of BafA1 as indicated for 48 h**.** n=6, *p < 0.05 vs. control (0). **(Lower panel)** BafA1-induced cell death and accumulation of LC3. Cells were treated with BafA1 in 1% FBS as indicated for 4 h. The results are representatives of 3 independent experiments. **B, Up panel)** Sub-confluent cells were treated in DMEM supplemented with 1%FBS in the presence or absence of epoxomicin as indicated for 48 h**.** n=6, *p < 0.05 vs. control (0). **(Lower panel)** Epoxomicin-induced accumulation of ubiquitinated protein aggregates. Cells were treated with epoxomicin in 1% FBS as indicated for 4 h. The results are representatives of 3 independent experiments.