Supplemental Material

Detailed Methods

Cultured cardiac myocytes

Neonatal rat ventricular myocytes (NRVM) were isolated by enzymatic digestion of neonatal rat hearts and purified by Percoll density gradient centrifugation, as described[1]. Briefly, NRVM were prepared from 1 to 3 day-old Sprague-Dawley rat hearts using a neonatal cardiomyocyte isolation system (cat# LK003300, Worthington Biochemical Corp, Lakewood, NJ). Isolated cells were counted and collected by centrifugation at 250 xg for 5 min. Forty to 60 million cells were then resuspended in 2 ml of red (with phenol red) 1x ADS buffer (116 mM NaCl, 20 mM HEPES, 769 µM NaH_2PO_4, 5.55 mM glucose, 5.37 mM KCl, 831 µM $MgSO_4$, 0.002% phenol red, pH 7.35 ± 0.5). Stock Percoll was prepared by combining 9 parts of Percoll (cat# 17-0891-02, GE healthcare, Piscataway, NJ) with 1 part of clear (without phenol red) 10x ADS. The stock Percoll was used to make the Percoll gradient for the top (density= 1.059 g/ml; 1 part Percoll stock added to 1.2 parts clear 1x ADS) and bottom (density= 1.082 g/ml; 1 part Percoll stock added to 0.54 parts red 1x ADS) layers. The gradient, consisting of 4 ml top Percoll and 3 ml bottom Percoll, was set in a 15 ml conical tube by pipetting the top Percoll first, and layering the bottom Percoll gently underneath, and the cells (in 2 ml red 1x ADS buffer) were layered on the top. Subsequently, the Percoll gradient was centrifuged at 1500×g for 30 min with no deceleration brake at 4°C. The isolated myocytes, which concentrated in the layer located between the lower red ADS layer and the middle clear ADS layer, were carefully collected and washed twice with 50 ml of 1x ADS, and were then resuspended in plating medium and counted. Following Percoll purification, myocytes were plated at the desired density on plastic culture plates that had been pre-treated with 5 µg/ml fibronectin for one hour. Cultures were then maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

Adenovirus

Construction of plasmid vectors encoding FLAG-tagged constitutively active ATF6 [ATF6α(1-373)], called form 1 in this paper, partially active ATF6 [ATF6α(39-373)], i.e. form 2, inactive ATF6 [ATF6α(94-373)], i.e. form 3, and empty vector AdV-control (AdV-Con) has been previously described[2]. The ATF6 vectors were used to generate recombinant adenovirus (AdV) expressing different forms of ATF6 using AdEasy system as previously described[3]. AdV-mito-Hyper containing a mitochondria-targeted Hyper protein was a generous gift from Dr. Junichi Sadoshima (New Jersey Medical School, NJ). Transduction was performed by incubating cultures overnight with the appropriate AdV at a multiplicity of infection of five.

Immunoblotting

NRVM were lysed in RIPA buffer comprising 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor cocktail (Roche Diagnostics). Mouse heart tissues were homogenized in modified RIPA buffer with 1 % SDS. Lysates were clarified by centrifugation at 15,000 x g for 15 min at 4°C, and the protein concentration was determined using DC protein assay (Bio-Rad, Hercules, CA). Samples usually comprising 40 μg of protein were mixed with Laemmli sample buffer, boiled for 5 min, then subjected to SDS-PAGE followed by transferring onto PVDF membrane for immunoblotting analysis. To detect HMGB1 released from cells as a result of necrosis, 20 μl of culture media were analyzed by SDS-PAGE followed by immunoblotting for HMGB1. For ATF6 antibody validation, antibodies raised against the N-terminal of ATF6 were purchased from Proteintech (1:1000, cat# 24169-1-AP, Rosemont, IL), Abcam (1:1000, cat# ab37149, Cambridge, MA), Novus Biologicals (1:1000, cat# NBP1-40256, Littleton, CO), and Santa Cruz Biotechnology (1:500, cat# sc-14250, Dallas, TX). An antibody raised against the C-terminal of ATF6 was purchased from Signalway (1:1000, cat# 32008, College Park, MD). An anti-KDEL antibody (1:8,000, cat# ADI-SPA-827 , Enzo Life Sciences, Farmingdale, NY) was used to detect GRP94, GRP78 and PDIA6, all of which have C-terminal KDEL sequences that cross-react with this antibody. Other antibodies used included anti-PARP (1:1000, cat# 9542, Cell Signaling, Danvers, MA), anti-catalase (1:1000, cat# ab16731, Abcam), anti-FLAG M2 (1:10,000, cat# F1804, Sigma-Aldrich, St. Louis, MO), anti-HMGB1 (1:1000, cat# ab18256 Abcam), anti-CHOP (1:1000, cat# 5554, Cell Signaling), anti-IRE1 (1:500, cat# sc-390960, Santa Cruz), anti-XBP1s (1:1000, cat# 619502, BioLegend, San Diego, CA), anti-phospho-PERK (1:1000, cat# 3179, Cell Signaling), anti-PERK (1:1000, cat# 3192, Cell Signaling), and anti-β-actin (1:1000, cat# sc-47778, Santa Cruz).

MTT and lactate dehydrogenase (LDH) assays

NRVM were plated in 96-well plates (1.5 x 10^4 cells/well) and treated with tunicamycin (TM) at 40 μg/ml for 48h, or with hydrogen peroxide (H_2O_2) at 37.5 μM for 8h. Dose response experiments were carried out to determine that these were the optimal doses of TM and H_2O_2 for measuring the effects of ATF6 gain- and loss-of-function on cell viability (MTT) and necrosis (medium LDH activity). Cell viability was measured using an MTT assay (Cell Proliferation Kit I, Roche Diagnostics), as previously described[1]. Media samples from H_2O_2-treated NRVM were collected for LDH activity assays, which were done using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), according to the manufacturer's instructions.

Isolation of adult mouse cardiac myocytes

Adult mouse ventricular myocytes (AMVM) were isolated essentially as previously described [4, 5]. Briefly, hearts were rapidly cannulated via the ascending aorta, mounted on a perfusion apparatus and retrograde perfused at 3 ml/min for 4 min at 37°C with

heart medium (Joklik Modified Minimum Essential Medium; cat# M-0518, Sigma-Aldrich, supplemented with 10 mM HEPES, 30 mM taurine, 2 mM D-L-carnitine, 20 mM creatine, 5 mM inosine, 5 mM adenosine, and 10 mM butanedione monoxime (BDM), pH 7.36). Collagenase digestion of hearts was performed by perfusing for 13 min with heart medium supplemented with type 2 collagenase (50-60 mg; ~320U/ml, cat# LS004176, Worthington) and 12.5 μM $CaCl_2$. Hearts were removed from the cannula and submerged in 2.5 ml of effluent collected off the heart during the collagenase digestion, and dissociated using forceps. Collagenase was neutralized by adding 2.5 ml of heart medium supplemented with 10% FCS, and the final concentration of $CaCl_2$ was adjusted to 12.5 μM. Cells were dissociated further by gently triturating for 4 min. The cell suspension was then filtered through a 100μm mesh filter and myocytes were allowed to sediment by gravity for 6 min at room temperature. The supernatant containing non-viable cells and non-myocytes was discarded and the remaining myocytes were resuspended in 5 ml of heart medium containing 5% FCS and 37.5 μM $CaCl_2$. The concentration of $CaCl_2$ in this suspension was slowly increased in a careful stepwise manner as follows: Step 1- add 50 μl of 10 mM $CaCl_2$, mix gently, allow to sit for 4 min; Step 2- repeat Step 1; Step 3- add 100 μl of 10 mM $CaCl_2$ and wait 4 min; Step 4- add 80 μl of 100 mM $CaCl_2$ and wait 4 min. Cells were resuspended in plating medium (MEM medium; cat# 12350-039, Thermo Fisher Scientific, Waltham, MA, 1x insulin-transferrin-selenium; cat# 41400-045, Thermo Fisher, 10 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin, 10 mM BDM and 4% FCS). Cells were plated at 5 x 10^4 cells per well in 12-well culture plates coated with laminin (10 μg/ml). After at least 2h, the medium was changed to maintaining medium (MEM medium, 1x insulin-transferrin-selenium, 10 mM HEPES, 1.2 mM $CaCl_2$ and 0.01% bovine serum albumin, 25 μM blebbistatin. Cells were used for experiments 12-18h later.

Simulated ischemia/reperfusion

NRVM or AMVM were subjected to simulated ischemia (sI) or simulated ischemia followed by simulated reperfusion (sI/R), as previously described[6]. Briefly, cells were placed into 12-well plates at 2 x 10^5 cells/well for NRVM, or 5 x 10^4 cells/well for AMVM. For sI of NRVM, medium was replaced with 0.5 ml of glucose-free DMEM containing 2% dialyzed FCS. Cells were incubated at 0.1% O_2 in a hypoxia chamber with an oxygen controller (ProOx P110 oxygen controller, Biospherix, Parish, NY). To simulate reperfusion, medium was replaced with medium containing 17.5 mM glucose and cells were incubated at 21% O_2. In some experiments, 5 mM N-acetyl cysteine (pH 7.5), a ROS scavenger, was added to the reperfusion medium. To examine the effects of sI/R on NRVM viability, cells were incubated in sI for 16h, followed by reperfusion for 24h. To examine the effects of sI/R on ROS generation in NRVM, cells were incubated in sI for 8h, followed by reperfusion for 1h. To examine the effects of sI/R on AMVM viability, cells were incubated in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml glutamine for 3h, followed by reperfusion with maintaining medium for 24h. To examine the effects of catalase on sI/R-mediated death of AMVM, cultures were pre-treated with 100U/mL of PEG (polyethylene glycol)-conjugated-Catalase, PEG-Catalase (Sigma cat# C4963) overnight, then PEG-Catalase was added into sI and sI/R media. Viable AMVM and were identified as calcein blue AM-positive

(Thermo Fisher) and images were obtained using an IX70 fluorescence microscope (Olympus, Melville, NY). Numbers of viable, calcein blue AM-positive cells were counted using ImageJ or Image-Pro Plus software (Medium Cybernetics, Rockville, MD).

Reactive oxygen species (ROS) production

The levels of intracellular ROS were determined with the CellROX Orange fluorescent dye (Thermo Fisher). After sI/R, NRVM were incubated with 5 μM CellROX Orange for 20 min at 37°C, and then washed with PBS. Images were obtained using a fluorescence microscope at a magnification of 20X. Fluorescence intensity (absorbance units) was determined using Image-Pro Plus software. To detect intracellular H_2O_2, NRVM were permeabilized with 40 μM digitonin. The resulting media containing intracellular H_2O_2 were transferred to black 96-well plates. The levels of H_2O_2 were measured using Amplex Red Hydrogen Peroxide/peroxidase Assay Kit (Thermo Fisher) according to manufacturer's instructions. Mitochondrial-specific H_2O_2 production was detected through the expression of mitochondria-specific HyPer protein using AdV mito-Hyper as previously described[7].

Small interfering RNA (siRNA) transfection

Reverse transfection of siRNA duplexes into NRVM using TransMessenger Transfection Reagent (Qiagen, Valencia, CA) has been described previously[1]. Briefly, Percoll-purified NRVM (3 x 10^5 cells) were suspended in medium containing 2% FCS, incubated with 100 nM siRNA and transfection reagent followed by plating in 12-well plates for overnight. Medium was changed the next day. The sequence of siRNA targeting rat ATF6 was 5-GCUCUCUUUGUUGUUGCUUAGUGGA-3, and the sequence targeting rat catalase was 5-GGAACCCAAUAGGAGAUAAACUUAA-3 (cat# CatRSS302058, Stealth siRNA, Thermo Fisher). A non-targeting sequence (cat# 12935300, Thermo Fisher) was used as a control siRNA.

Malondialdehyde assay

Lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA). Briefly, sI/R treated NRVM (6 x 10^5 cells) were scrapped into 0.5 ml of PBS, and sonicated on ice. MDA levels in cell lysates were measured using a TBARS assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Immunocytofluorescence (ICF)

NRVM- NRVM were plated and on fibronectin-coated glass chamber slides (Nunc Lab-Tek II Chamber Slide) as previously described[1]. After treatment, cells were fixed with -20°C methanol for 10 min, then blocked for 1h with SuperBlock blocking buffer (Thermo Fisher). Slides were then incubated overnight with an anti-ATF6 antibody (1:50, cat# sc-166659, Santa Cruz)[8], and an anti-α-actinin antibody (1:50, cat# GTX103219, GeneTex, Irvine, CA). Slides were subsequently incubated at room temperature for 90

min with the appropriate fluorophore-conjugated a secondary antibody including: Cy3-conjugated anti-mouse IgG (1:100), FITC-conjugated anti-goat IgG (1:100), or FITC-conjugated anti-rabbit IgG (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were counterstained for 1 min with Topro-3 (1:1000, Thermo Fisher). Images were obtained using laser scanning confocal microscopy on an LSM 710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

AMVM- were plated in plating medium at $1.0x10^5$ cells per chamber on laminin-coated 4-chamber glass slides (Falcon). After 3h, the medium was changed to maintaining medium containing 25 μM blebbistatin. After 16h, the medium was replaced with 0.5 ml of medium appropriate for sI/R for the appropriate times (see above), or with medium containing tunicamycin (10 μg/ml) for 24h. After each treatment, AMVM were washed with PBS, fixed for 15 min with 4% paraformaldehyde, followed by permeabilization for 10 min with 0.5% Triton-X. For ATF6 staining, AMVM were fixed for 10 min with -20°C methanol. Slides were blocked for 1h with SuperBlock, and then incubated with primary antibodies for 16h at 4°C. Primary antibodies used for staining AMVM were anti-α-actinin (1:200, cat# A7811, Sigma-Aldrich), anti-GRP78 (C-20, 1:30, cat# SC-1051, Santa Cruz), anti-catalase (1:100, Abcam), and anti-ATF6 (targeting to N-terminus of ATF6, 1:50, cat# sc-14250, Santa Cruz). Slides were incubated with appropriate fluorophore-conjugated secondary antibodies, followed by nuclei counter stain, as described above.

Adult mouse hearts were embedded and sectioned as previously described[11]. Briefly, mice were anesthetized and a catheter was inserted into the abdominal aorta. The aorta was retro-perfused with PBS followed by relaxation buffer containing $CdCl_2$ and KCl, fixed in neutral buffered 10% formalin. After 24h in formalin, the hearts were dehydrated in ethanol and embedded in paraffin. Five-μm sections of paraffin-embedded hearts were heated in antigen retrieval citrate buffer (10mM; pH 6.0). The heart sections were then treated with blocking buffer (Thermo Scientific, cat# 37528) for 1h at room temperature and then incubated with primary antibodies overnight at 4°C. Primary antibodies in this study included: anti-GRP78 (C-20, 1:30, cat# SC-1051, Santa Cruz), anti-catalase (1:50, Abcam), anti-ATF6 (targeting to N-terminus of ATF6, 1:50, cat# sc-14250, Santa Cruz), and anti-tropomyosin (1:200, cat# T9283, Sigma-Aldrich). Slides were incubated with appropriate fluorophore-conjugated secondary antibodies as described above for 90 min at room temperature (all at 1:100 dilutions). Nuclear marker counterstain, TO-PRO-3 (Thermo Fisher; 1:10000), was incubated for 15min at room temperature prior to application of coverslips fixed with Vectashield Hardset (Vector Labs, H1400).

Echocardiography

Echocardiography of WT and ATF6 KO mice was performed using an ultrasound imaging system (Vevo 2100 System, Fujifilm VisualSonics, Toronto, Ontario, Canada) as described[1].

In Vivo ischemia/reperfusion

Myocardial ischemia/reperfusion (I/R) was performed *in vivo* by 30 min of ligation of the left anterior descending coronary artery followed by 24h of reperfusion, as previously described[9]. Following I/R, 1% of Evans Blue was injected apically to determine the area at risk (AAR). Hearts were harvested and 1-mm sections of the hearts were stained with 1% triphenyl tetrazolium chloride (TTC) to measure infarcted area (INF) as previously described[9]. The AAR, INF and left ventricle area (LV) of digital images of heart sections were analyzed using ImageJ software.

Ex vivo ischemia/reperfusion

Mouse hearts were isolated and subjected to global I/R as previously described[10]. Briefly, isolated hearts were mounted onto a Langendorff perfused heart apparatus. Global no-flow ischemia was performed for 20 min followed by reperfusion for 1 h. Left ventricular developed pressure (LVDP) was measured using a pressure sensor balloon and analyzed using Powerlab software (ADInstruments, Colorado Springs, CO).

Intravenous Injections

For AAV9, C57/BL6 mice were anesthetized with 2% isoflurane and then injected via the lateral tail vein with 100 μl of AAV9-control or AAV9-3xFlag-ATF6α(1-373) containing $1x10^{11}$ viral particles, and then hearts were obtained after either 2 weeks for immunoblots **(Fig. 6E)**, or after 2d for *ex vivo* I/R **(Fig. 7J)**. In other experiments, C57/BL6 mice were anesthetized with 2% isoflurane and then injected via the lateral tail vein with 100 μl of 1000u/kg PEG-Catalase (Sigma cat# C4963), or vehicle 16h before hearts were obtained and subjected to *ex vivo* I/R **(Fig. 7G)**.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from NRVM or hearts using Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA) or RNeasy Mini kit (Qiagen), respectively. cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Thermo Fisher). qRT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix in a StepOnePlus RT-PCR System (Thermo Fisher). The following primers were used:

Rat	Forward 5' to 3'	Reverse 5' to 3'
β-Actin	CTTCCTTCCTGGGTATGGAATC	CTGTGTTGGCATAGAGGTCTT
Cat	CTTTGAGGTCACCCACGATATT	GTGGGTTTCTCTTCTGGCTATG
Gpx1	TGAGAAGTGCGAGGTGAATG	CCAGATACCAGGAATGCCTTAG
Gpx3	CATTCGGCCTGGTCATTCT	CAGCGGATGTCATGGATCTT

Gpx4	GCAGGAGCCAGGAAGTAATC	ACGCAGCCGTTCTTATCAA
Prdx1	TGTAGCTCGACTCTGCTGATA	GTCCCAATCCTCCTTGTTTCT
Prdx2	ATGATGAGGGCATCGCTTAC	TCAGGCTCACCGATGTTTAC
Prdx3	CGCTCAGAGGTCTCTTCATTATT	GTACTGGTGCTATGTGCTACTT
Prdx4	GGGAAGGAACAGCTGTGATAA	GATCCAGGCCAAGTGAGTAAA
Prdx5	CAGAGCTGTTCAAGGACAAGA	CCCAAAGAGAGACACCAAAGA
Prdx6	CCTGGAGCAAGGACATCAAT	GGAGTCAACCACTCTGAGAATC
Sod1	TGGGTTCCATGTCCATCAATA	CAATCCCAATCACACCACAAG
Sod2	CTGACCTGCCTTACGACTATG	CTTGCAGTGGGTCCTGATTA
Vimp	GACCTTCTACTTCATCGGTCATC	TCAGAACAGAAATCAGCCCTAC
Mouse		
β-Actin	GACGGCCAGGTCATCACTAT	GTACTTGCGCTCAGGAGGAG
Cat	AACTGGGATCTTGTGGGAAAC	GTGGGTTTCTCTTCTGGCTATG
Grp78	CACGTCCAACCCCGAGAA	ATTCCAAGTGCGTCCGATG
Grp94	TCGTCAGAGCTGATGATGAAGT	GCGTTTAACCCATCCAACTGAAT
Pdia6	TGCCACCATGAATCAGGTTCT	TCGTCCGACCACCATCATAGT

For the RT-PCR array, total RNA was isolated from Ad-Con or Ad-ATF6 form 1 treated NRVM (5 x 10^5 cells) using miRNeasy Mini Kit (Qiagen). Synthesis of cDNA was performed using 500 ng of RNA and RT^2 First Strand Kit (Qiagen). Rat Oxidative Stress RT^2 Profiler PCR Arrays (Qiagen) were performed using RT^2 SYBR Green ROX qPCR Mastermix in a StepOnePlus RT-PCR System according to the manufacturer's instructions.

Adeno-Associated Virus Serotype 9 (AAV9)

AAV9 preparation was carried out essentially as previously described[1]. For generation of recombinant AAV9-control and AAV9-3xFlag-ATF6α(1-373), shuttle vectors for these recombinants were constructed and co-transfected with AAV9 helper, pDG-9 (a gift from Dr. Roger Hajjar) into HEK293T cells to produce virus. Two different expression constructs were prepared; AAV9-CMV-3xFlag-ATF6α(1-373) and AAV9-

CMV_{enh}MLC800-3xFlag-ATF6α(1-373). Similar results were obtained with each vector, except the AAV9-CMV-3xFlag-ATF6α(1-373) results in more robust expression than AAV9-CMV_{enh}MLC800-3xFlag-ATF6α(1-373). Since the recombinant AAV9 were prepared using similar strategies, only the preparation of AAV9-CMV_{enh}MLC800-3xFlag-ATF6α(1-373) is described here. The shuttle vector, pTRUF-CMV_{enh}MLC800, was constructed by modifying pTRUF12 (a gift from Dr. Roger Hajjar) by first removing the region encoding GFP that was down-stream of the IRES. New restriction sites were inserted into the multiple cloning site to include Nhe1, Pme1, Xho1, and Mlu1. The CMV promoter was replaced with a composite promoter comprised of an 800bp fragment of the MLC2v promoter downstream of a minimal region of the CMV enhancer (a gift from Dr. Oliver J. Muller). AAV9 vectors with wild-type capsids were generated by co-transfection of the helper plasmid, pDG-9. pTRUF-CMV_{enh}MLC800-3xFlag-ATF6α(1-373) was created by sub-cloning the human 3xFlag-ATF6 a (1-373) cDNA from pcDNA3.1-3xFlag-ATF6α(1-373). To prepare the recombinant AAV9, HEK293T cells were plated a density of $8x10^6$ per T-175 flask and maintained in DMEM/F12 containing 10%FBS, penicillin/streptomycin at 37°C and 5% CO_2. For each virus preparation, 48 flasks were used. Twenty-four hours after plating, cultures were transfected using Polyethylenimine "Max" (MW 40,000, Polysciences, cat# 24765) as follows: For each T-175 flask, 15µg of helper plasmid and 5µg of pTRUF plasmid were mixed with 1 ml of DMEM:F12 (no antibiotics) and 160 µl of polyethylenimine (0.517 mg/ml), vortexed for 30 seconds, and incubated for 15 min at room temperature. This was then mixed with 18 ml DMEM/F12 containing 2% FBS, penicillin/streptomycin then used to replace the media on the cultures. The cultures were then rocked intermittently for 15 minutes before incubation. Three days after transfection, the cells collected from six T-175 flasks were centrifuged at 500xg for 10 min, then resuspended in 10 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCL). The resuspended cells were then subjected to three rounds of freeze-thaw, followed by treatment with benzonase (1500 U of benzonase; Novagen) and 1 mM $MgCl_2$ at 37°C for 30 min. The cell debris was collected by centrifugation at 3,400 x g for 20 min. The supernatant obtained from six T-175 flasks containing the AAV9 was then purified on an iodixanol gradient comprised of the following four phases: 7.3 ml of 15%, 4.9 ml of 25%, 4 ml of 40%, and 4 ml of 60% iodixanol (Optiprep; Sigma-Aldrich) overlayed with 10 ml of cell supernatant. The gradients were centrifuged in a 70Ti rotor (Beckman Coulter) at 69,000 rpm for 1h using OptiSeal Polyallomer Tubes (Beckman Coulter). Virus was collected by inserting a needle 2 mm below the 40%-60% interface and collecting 4 or 5 fractions (~4ml) of this interface and most of the 40% layer. The fractions were analyzed for viral content and purity by examining 10 µl of each fraction on a 12% SDS-PAGE gel (BioRad), followed by staining with InstantBlue (Expedeon) to visualize the viral capsid proteins, VP1, VP2 and VP3. The virus was then collected from the fractions of several gradients and the buffer was exchanged with lactated Ringer's using an ultrafiltration device, Vivaspin 20, 100kDa MWCO (GE Healthcare). The final viral preparation was then fractionated on a 12% SDS-PAGE gel, stained with InstantBlue, and then compared with a similarly stained gel of a virus of a known titer. Alternatively, a qPCR was performed using a forward primer (AAGTCTCCACCCCATTGACGT) and reverse primer (AGGAGCCTGAGCTTTGATTCC), which spans the CMV_{enh}MLC800 composite promoter. A pTRUF vector containing the CMV/MLC800 promoter was used as a

standard to determine copy number. pTRUF-CMV$_{enh}$MLC-empty was used to generate an analogous control virus.

Cloning and mutagenesis

The promoter region of the rat catalase gene spanning nucleotides -1161 to +131 was amplified by PCR using ggaacgGGTACCTCACTGCCTTTATGGGCTTC as the forward primer, which introduced a Kpn1 site (lower case) just 5' of rat catalase -1161, and ggaacgCTCGAGGTGTAGGATTGCGGAGCTG as the reverse primer, which introduced an Xho1 site just 3' of rat catalase +131. Upper case nucleotides match those in the rat catalase gene. The amplified product was then cloned into pGL2p to generate rat-cat(-1161/+131)-Luc. Truncated versions of rat-cat luciferase were cloned into pGL2p using a similar strategy and the same reverse primer coupled with the following forward primers:

ggaacgGGTACCAAAGGAGCCATGAAGCTGAA (-689),
ggaacgGGTACCACAGTGGGCCAAGTGACAAG (-410), and
ggaacgGGTACCGTCCCCGAACTGTGACTCTC (-191)

to generate rat-cat(-689/+131)-Luc, rat-cat(-410/+131)-Luc and rat-cat(-191/+131)-Luc, respectively. The underlined regions of these primers correspond to rat catalase gene sequences. Informatics analyses identified putative ER stress response elements in the rat catalase gene at nucleotide positions -979 to -962 and -194 to -184 in the rat catalase gene, which we called ERSE-2 and ERSE-1, respectively. These elements in rat-cat(-1161/+131)-Luc were mutated by site-directed mutagenes in ways predicted to ablate ATF6 binding using cat-ERSE-2 mut sense primer,

CCCAAGGGATTGCAAACTTACAATTTTACCccgCTCTGTTACCcacTCTTTGTCAAATC
AAGAACAAGTTTTGGAGT

and cat-ERSE-2 mut antisense primer,

ACTCCAAAACTTGTTCTTGATTTGACAAAgagTGGGTAACAGAGcggGGTAAAATTGT
AAGTTTTGCAATCCCTTGGG

cat-ERSE-1 mut sense primer,

CGTTGCACAGAGGAcggttTaaCagAACTGTGACTCTCAG, and

cat-ERSE-1 mut antisense primer,

CTGAGAGTCACAGTTctGttAaccgTCCTCTGTGCAACG.

PCR-based mutagenesis was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Lower case letters represent mutated nucleotides; upper case letters represent nucleotides that are identical to those in the rat catalase gene.

Luciferase reporter assay

Suspended NRVMs were co-transfected by electroporation with one of the above reporter constructs along with pCH110 plasmids encoding SV40-beta-galactosidase

and pGL2B, as described previously.[5] NRVMs were plated overnight and then infected with different forms of AdV-ATF6, as described above. NRVMs were lysed after 48 h, and the activities of luciferase and beta-galactosidase were measured using an Optocompt II luminometer (MGM Instruments, Hamden, CT) as described previously[6].

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed essentially as previously described[11]. Briefly, AdV-FLAG-ATF6 form 1-infected NRVM (2 x 10^6 cells) were treated with fixing buffer (50 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 1% formaldehyde) for 10 min, quenched with 125 mM glycine, and scraped into ice-cold PBS. Cells were centrifuged, resuspended in lysis buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, and protease inhibitor cocktail), and incubated on ice for 10 min. After centrifugation at 1,800 x g for 10 min, the pellets were washed with buffer containing 10 mM Tris, pH 8.1, 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA, resuspended in shearing buffer (0.1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.1), and then transferred to microTUBEs (Covaris, Woburn, MA). Chromatin was sheared by sonication for 15 min using an M220 focused-ultrasonicator (Covaris). Triton X-100 and NaCl were added to the final concentration of 1% Triton and 150 mM NaCl followed by centrifugation at 16,000 x g for 10 min. Immunoprecipitation was performed by incubated 140 µl of sheared chromatin with 5 µg of anti-FLAG antibody (cat# F1804, Sigma-Aldrich) and 260 µl of immunoprecipitation buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris, pH 8.1, 1% Triton X-100, and 150 mM NaCl) at 4°C overnight. Protein A/G magnetic beads (5 µl, BcMag, Bioclone, San Diego, CA) were added to the mixtures and incubated at 4°C for 1.5 h. Magnetic beads were sequentially washed with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM HEPES-KOH, pH7.9, and 150 mM NaCl), high salt wash buffer with 500 mM NaCl, LiCl wash buffer (100 mM Tris-HCl, pH 7.5, 0.5 M LiCl, 1% NP-40, and 1% deoxycholate acid), and TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). Immune complexes were eluted by incubating beads with proteinase K digestion buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.5% SDS, and 0.4 mg/ml proteinase K) at 50°C for 15 min. Formaldehyde crosslinking was reversed by incubating with 0.3 M NaCl and 0.3 mg/ml RNase A at 65°C overnight. Samples were further incubated with 550 µg/ml proteinase K at 50°C for 1h. DNA was purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Bethlehem, PA) and eluted by 30 µl of water. Two µl of DNA was used for qRT-PCR analysis with primers targeting rat Cat ERSE-1 (5'-CTACCCACCAATTAGTACCAAATAA-3' and 5'-AGAAGGGACAGGATTGGAAG-3'), rat Cat ERSE-2 (5'-CACATTCTAGGGACAGTGTAGATG-3' and 5'-ACCTTGATTATGGGCTGTGG-3') or the rat Pdia6 ERSE (5'-CACATGAGCGAAATCCACAGA-3' and 5'-ACTAGTCGAGCCATGCTGAT-3'). Pdia6 served as a positive control for a known ATF6 target gene in cardiac myocytes[11]. ChIP signals obtained from the qRT-PCR were normalized to the input DNA.

Supplemental References:

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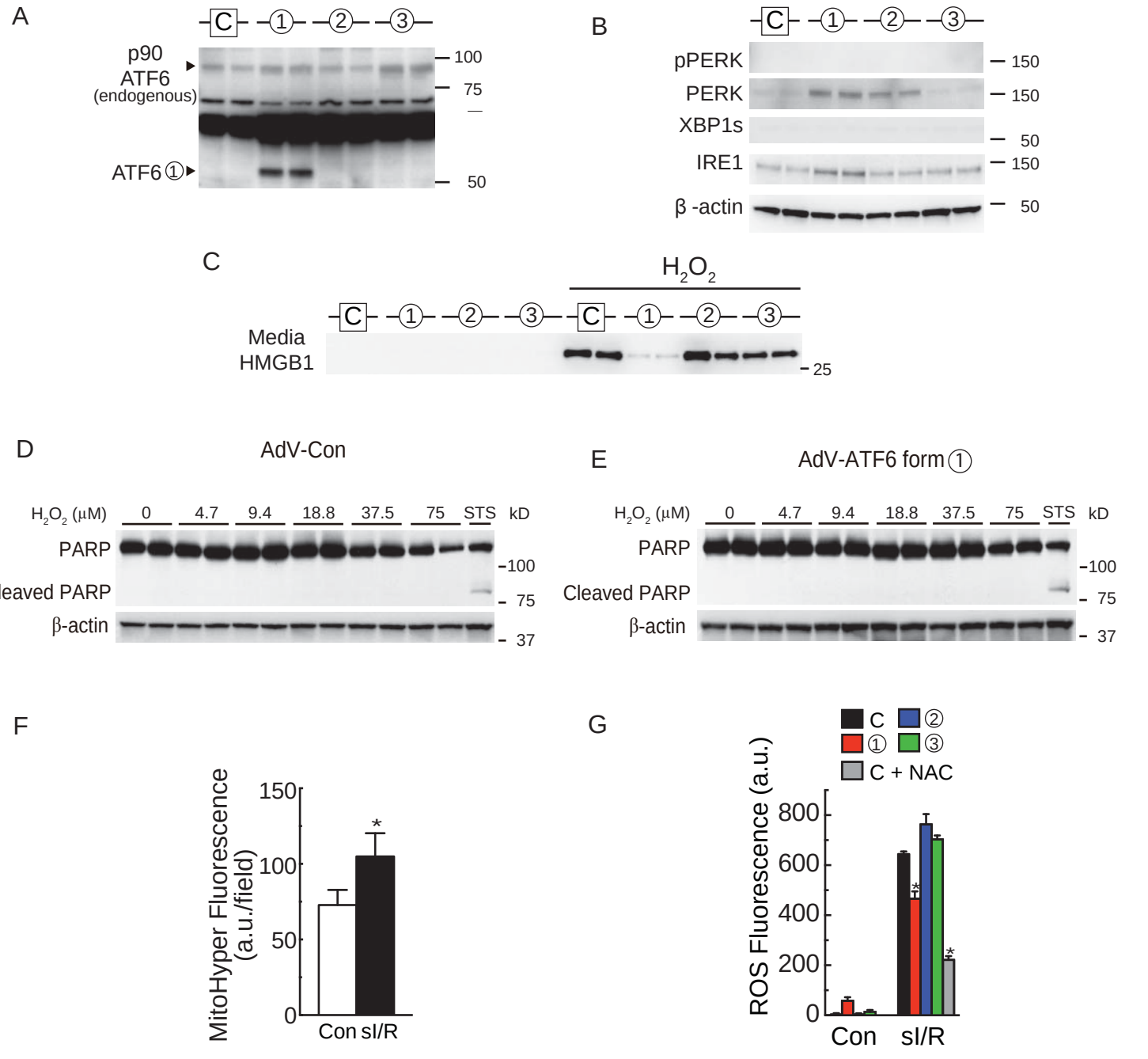
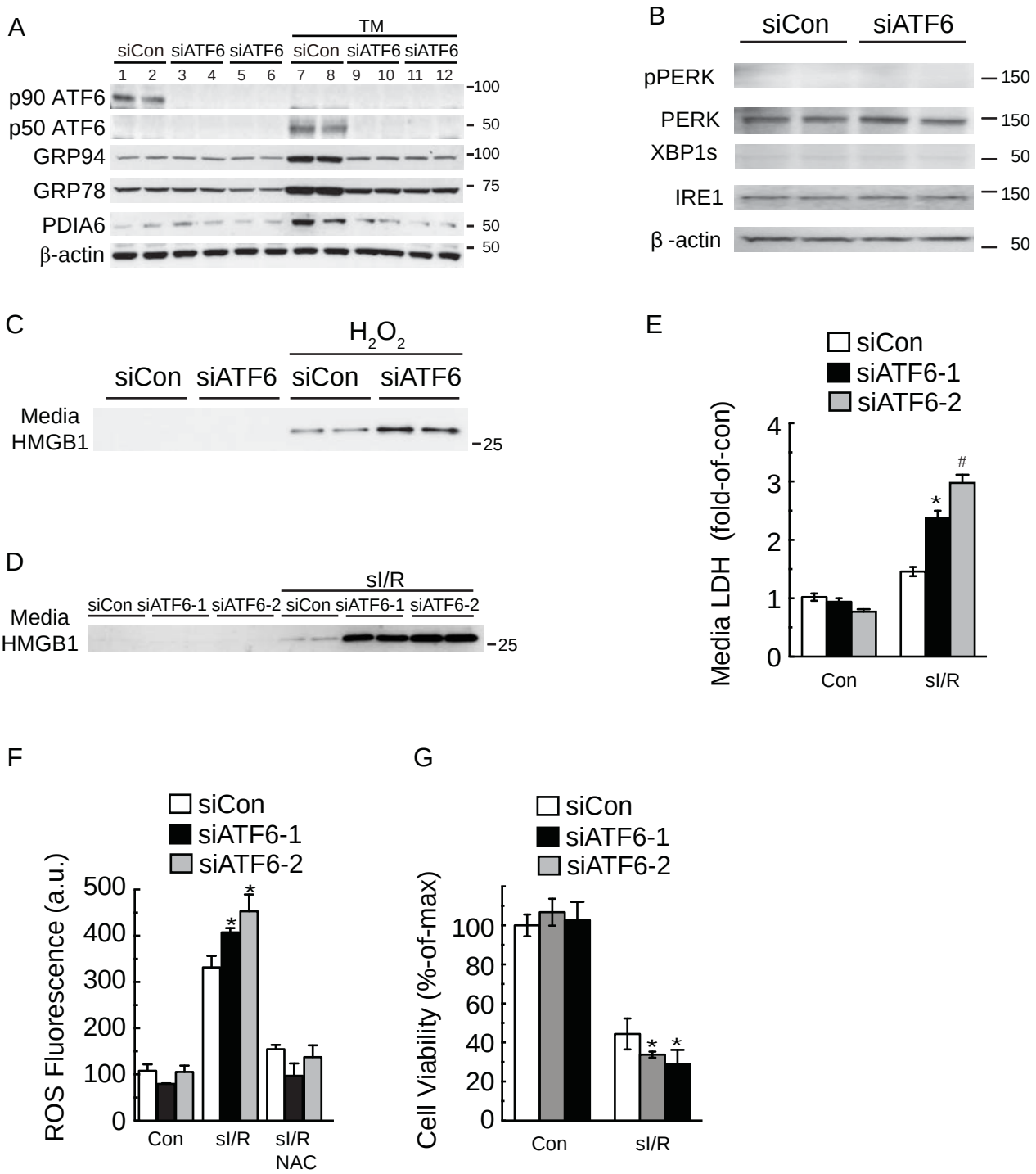
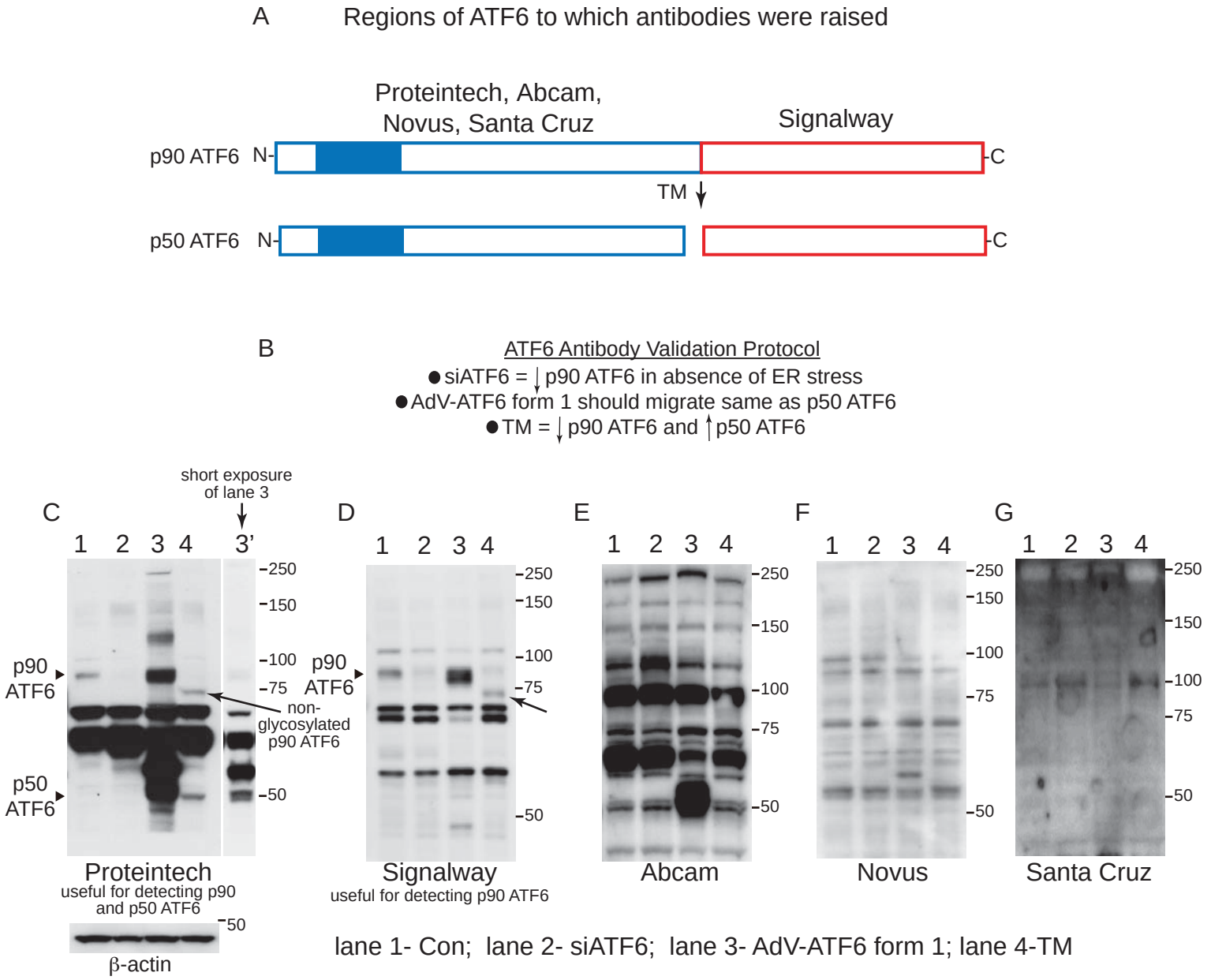


Online Figure I- Effect of AdV-ATF6 on ER Stress Signaling, HMGB1, H_2O_2 on PARP cleavage, and ROS in cultured cardiac myocytes: A, B, C, NRVM were infected with AdV encoding the forms of ATF6 shown then immunoblotted for ATF6 (A), phospho-PERK (pPERK), PERK, spliced XBP1 (XBP1s), IRE-1, and β-actin (B), or treated with H_2O_2 then immunoblotted for HMGB1 (C). **D, E,** NRVM were infected with AdV-Con, or AdV-ATF6 form 1, then treated for 8h with various concentrations of H_2O_2, as shown. As a positive control, NRVM were treated with staurosporine (STS; 1 μM) for 24h, which is known to induce apoptosis and not necrosis. Cultures were then extracted and subjected to immunoblotting for PARP using an antibody that detects intact and cleaved PARP. Blots were also probed for β-actin as a loading control. **F,** NRVM were infected with AdV-mito-HyPer, then subjected to either Control (Con) conditions, or sI/R after which fluorescence was measured. * p<0.05 different from Con. **G,** NRVM were infected with AdV-Con, or AdV encoding the forms of ATF6 shown, then subjected to either Con or sI/R and ROS were measured using Amplex Red. * p<0.05 different from all other values, by ANOVA.

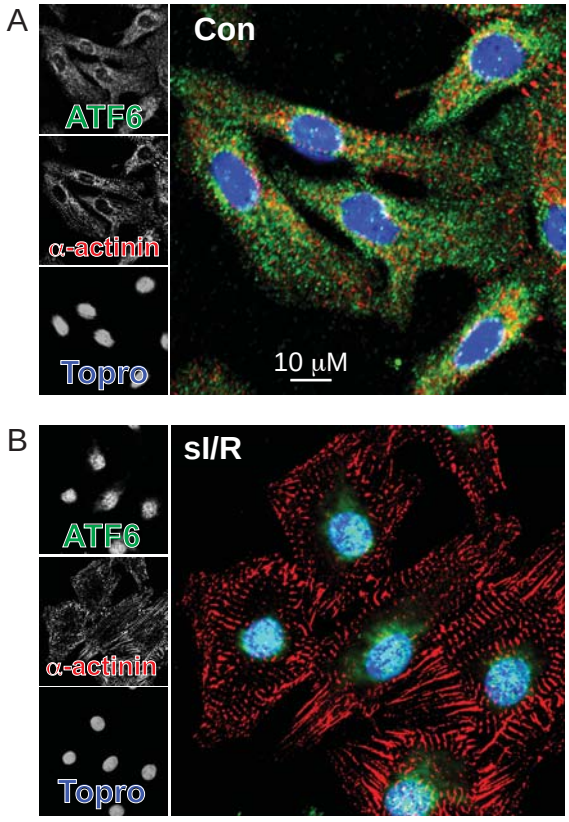


Online Figure II- Effect of ATF6 siRNA in NRVM: NRVM were transfected with siCon or two different ATF6-targeted siRNAs, siATF6-1 or siATF6-2 separately, where indicated, then either untreated, or treated with TM, sI/R or H_2O_2, as shown, then immunoblotted for the proteins shown, media measured for HMGB1 or LDH levels, ROS measured using an Amplex Red assay, or cell viability was measured using calcein AM blue staining. In E-G, * $p<0.05$ different from all other values in that treatment group by ANOVA.

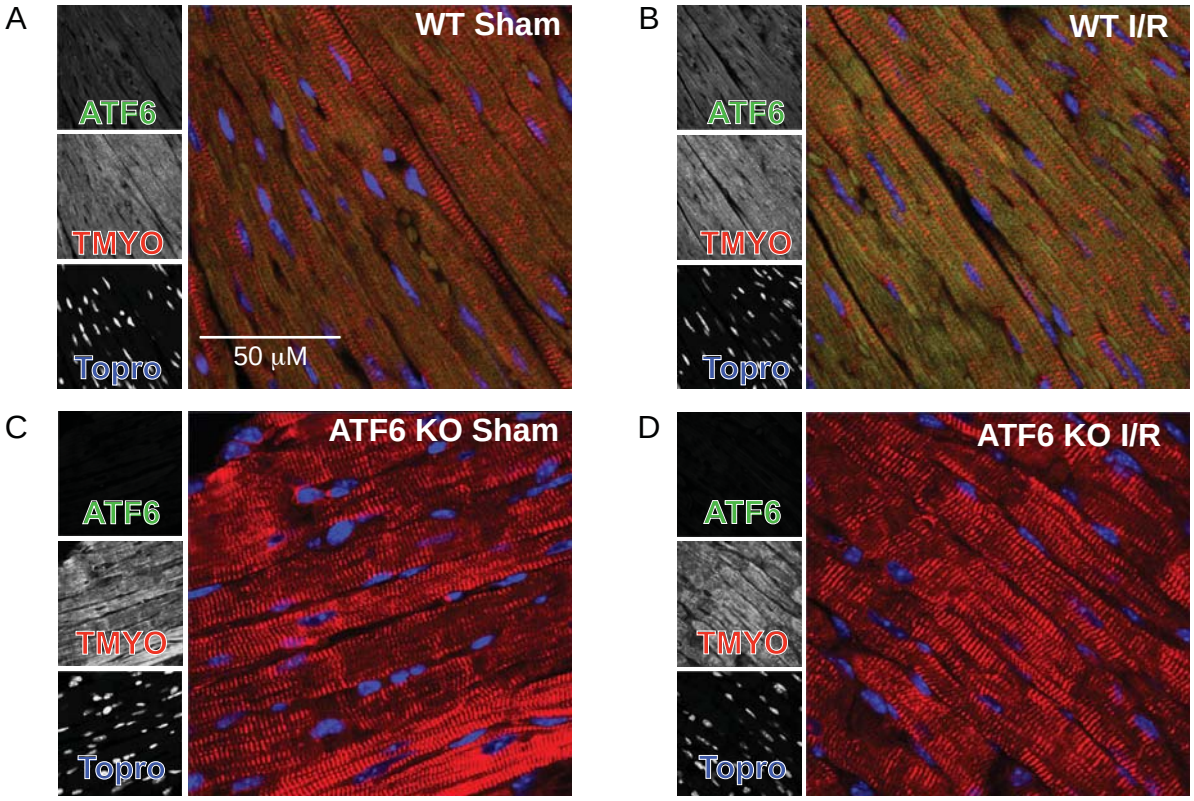


Online Figure III- Validation of ATF6 antibodies used for immunoblotting: A, Locations on ATF6 to which the antibodies used in this study were raised. **B,** ATF6 Antibody Validation Protocol. **C-G,** NRVM were either not transfected (Con; **lane 1**), or transfected with siRNA to rat ATF6 (siATF6; **lane 2**), infected with AdV-ATF6 form 1 (**lane 3**), or treated for 16h with TM (10 μg/ml; **lane 4**). Extracts were then analyzed for ATF6 by immunoblotting using the antibodies shown. The identities of p90 ATF6 and p50 ATF6 were based on the blot results and the ATF6 Antibody Validation Protocol. Note that overexpression of ATF6 form 1 serves as a control (C, lane 3), and a shorter exposure of this lane is shown as 3'. The arrow in C and D indicates the unglycosylated form of p90 ATF6 that appears when cells are treated with TM, an inhibitor of ER protein glycosylation. The identities of bands other than those marked as p90 or p50 ATF6, or unglycosylated p90 ATF6 are not known. The Abcam antibody detected p90 and p50 ATF6 but also crossreacted with other proteins at these same locations, so was not used in this study. The Novus and Stanta Cruz antibodies did not specifically detect p90 or p50 ATF6, so they were not used in this study.

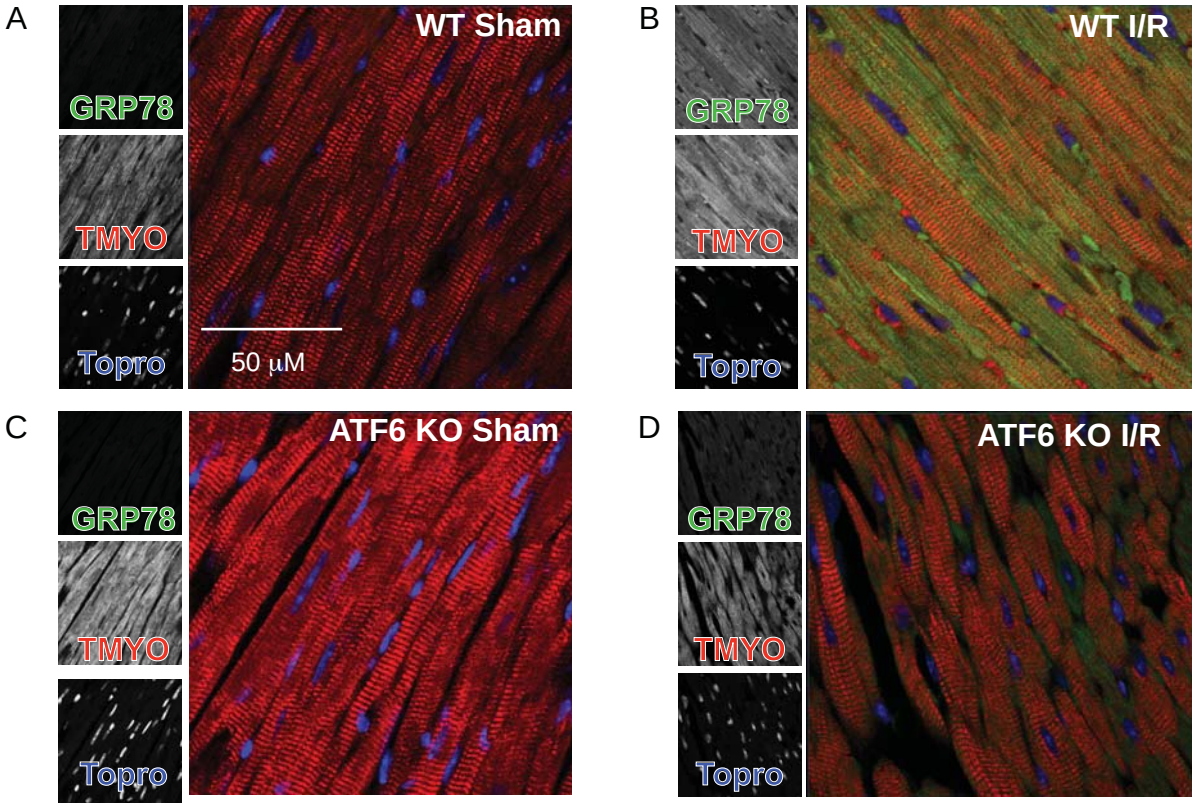
Online Figure III



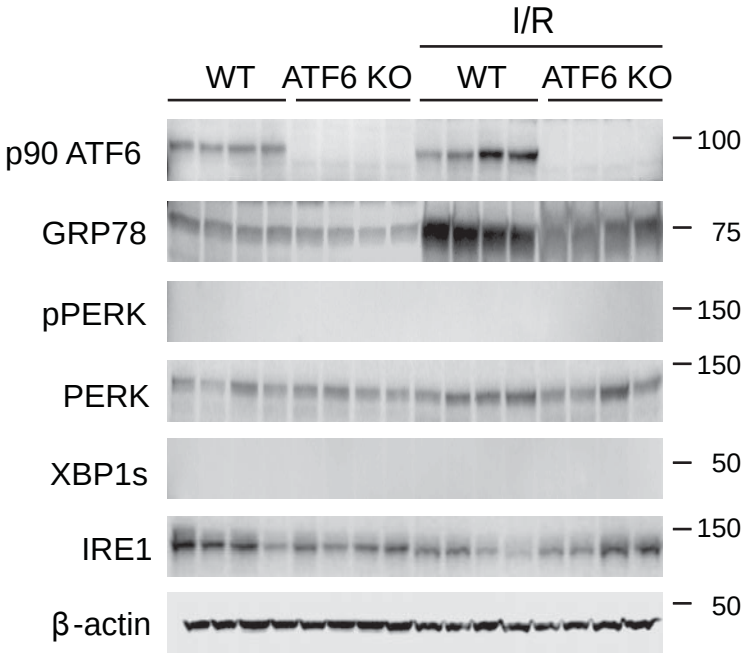
Online Figure IV- Effect of sI/R on ATF6 expression and localization in cardiac myocytes: NRVM were either untreated (Con; A), or treated for 4h with simulated ischemia followed by 24h of simulated reperfusion (sI/R; B), then fixed and stained for ATF6 (ATF6; green), α-actinin (red), or TOPRO (blue).



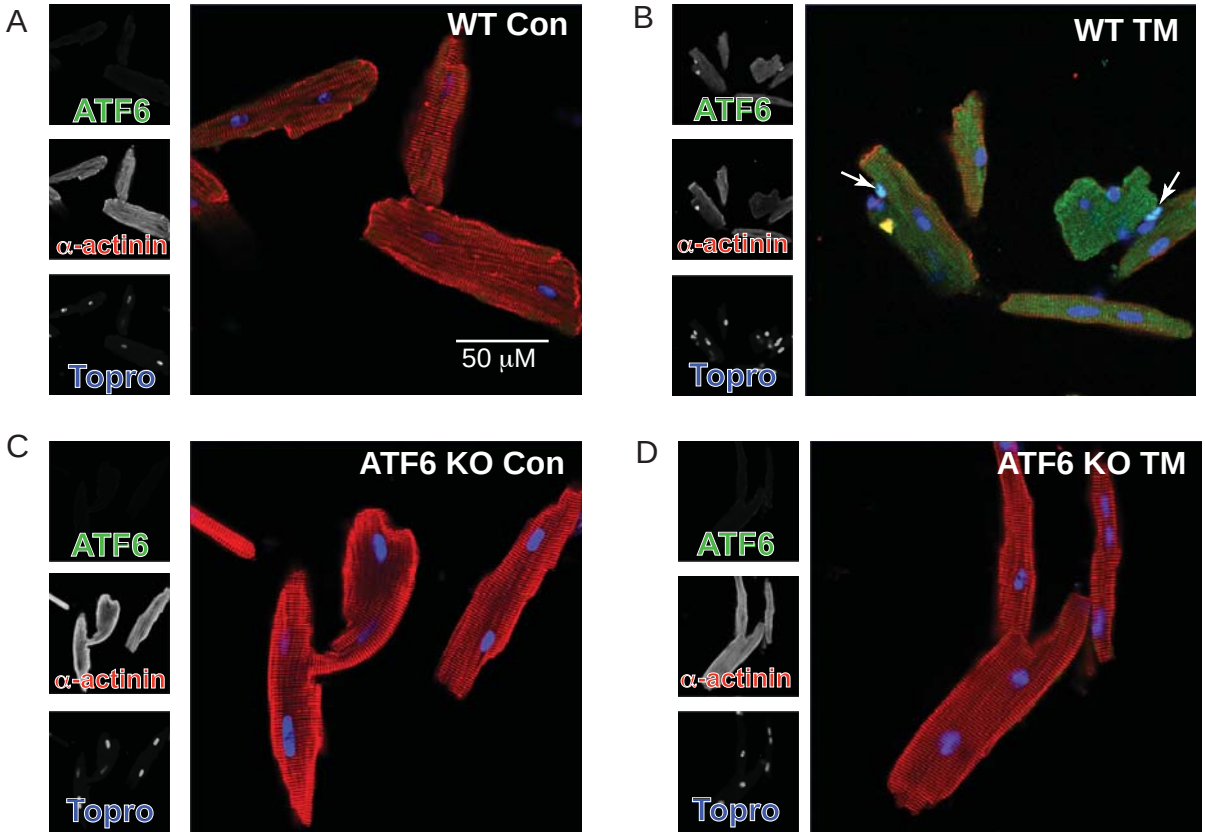
Online Figure V- Effect of I/R on ATF6 expression in WT and ATF6 KO mouse hearts as determined by immunocytofluorescence: A, B, WT or **C, D,** ATF6 KO mice were subjected to I/R *in vivo*, after which hearts were obtained and sections were stained for ATF6 (green), tropomyosin (red), or TOPRO (blue).



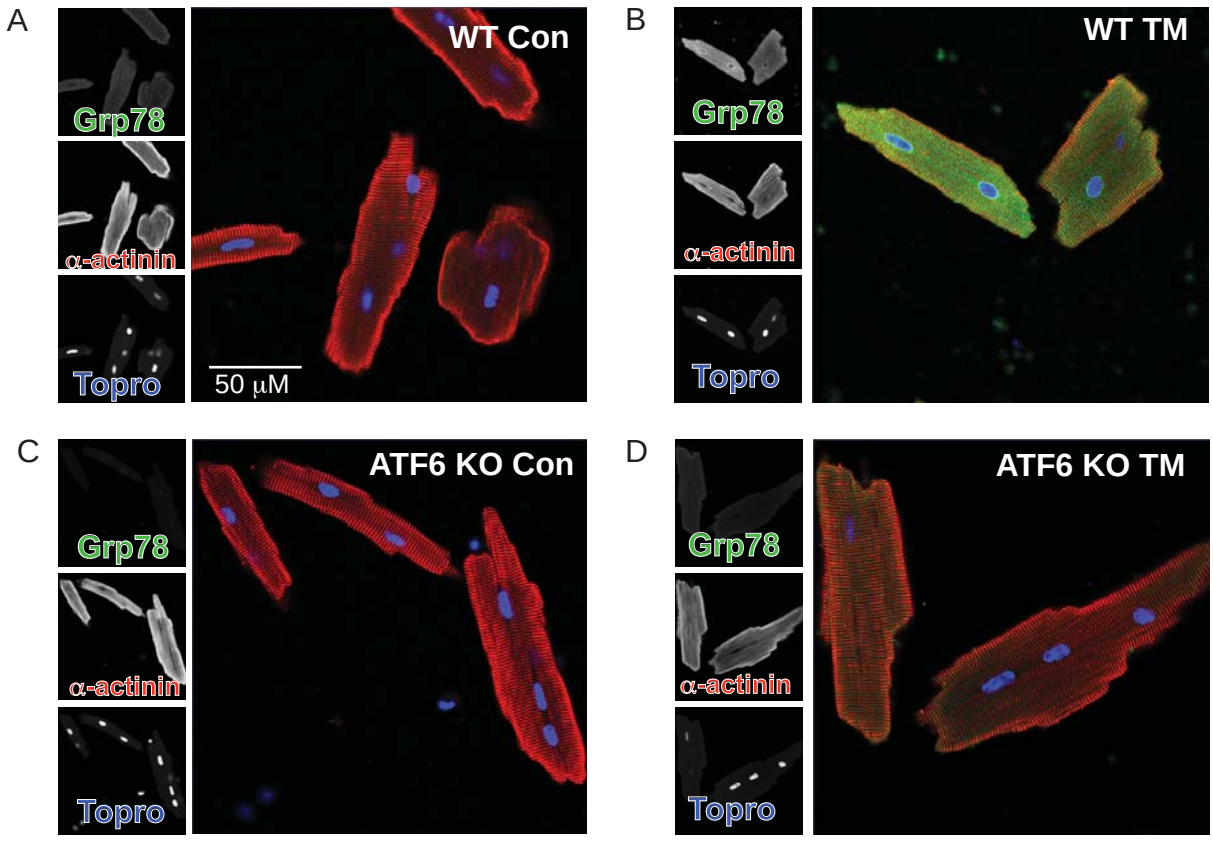
Online Figure VI- Effect of I/R on GRP78 expression in WT and ATF6 KO mouse hearts as determined by immunocytofluorescence: A, B, WT or **C, D,** ATF6 KO mice were subjected to I/R *in vivo*, after which hearts were obtained and sections were stained for GRP78 (green), tropomyosin (red), or TOPRO (blue).



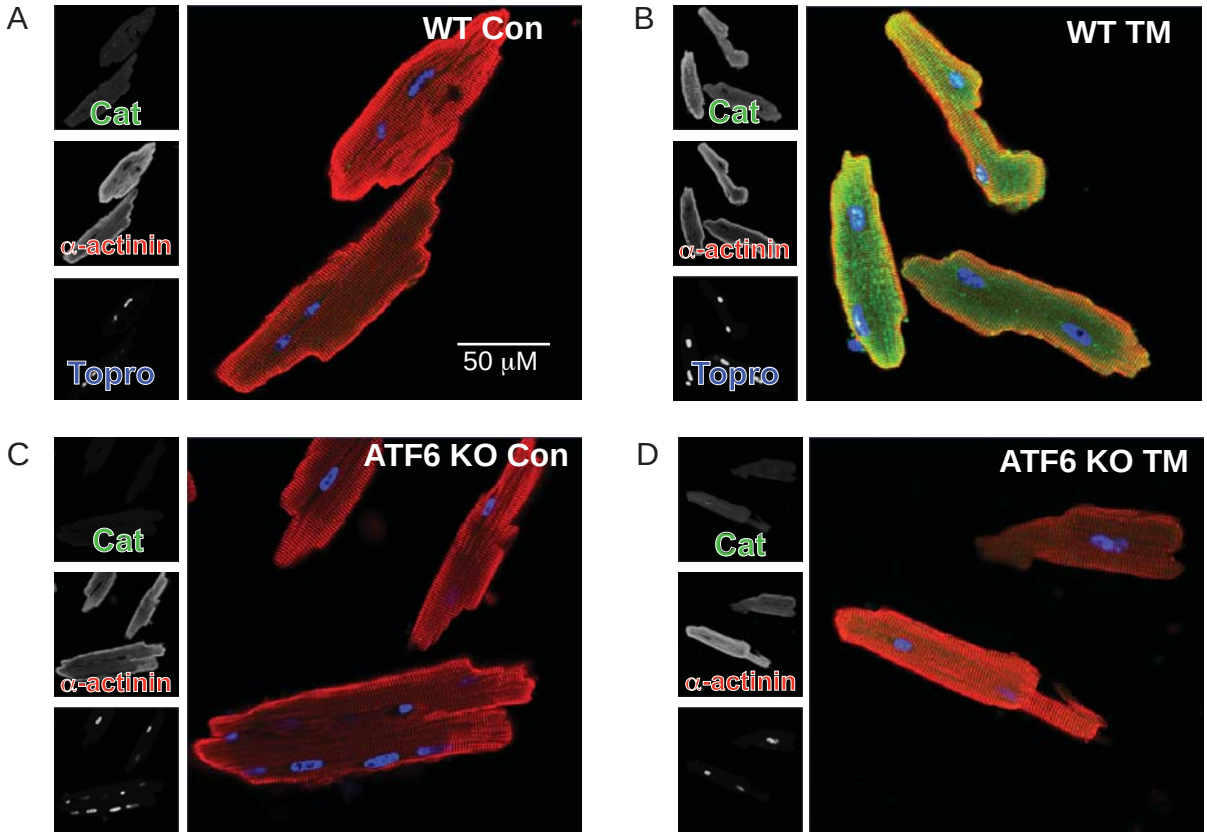
Online Figure VII- Effect of I/R on markers of ER stress in WT and ATF6 KO mouse hearts as determined by immunoblotting: WT or ATF6KO mice were subjected to I/R *in vivo*, after which hearts were obtained and extracts were immunoblotted for p90 ATF6, GRP78, phospho-PERK (pPERK), total PERK (PERK), spliced XBP1 (XBP1s), IRE-1 and β-actin.



Online Figure VIII- Effect of TM on ATF6 expression in cardiac myocytes from WT and ATF6 KO mouse hearts: A, B, Myocytes were isolated from WT or **C, D,** ATF6 KO mouse hearts then treated without (A, C) or with TM (10 μg/ml; 24h; B, D), then fixed and stained for ATF6 (ATF6; green), α-actinin (red), or TOPRO (blue). Arrows in B point to ATF6-positive nuclei.

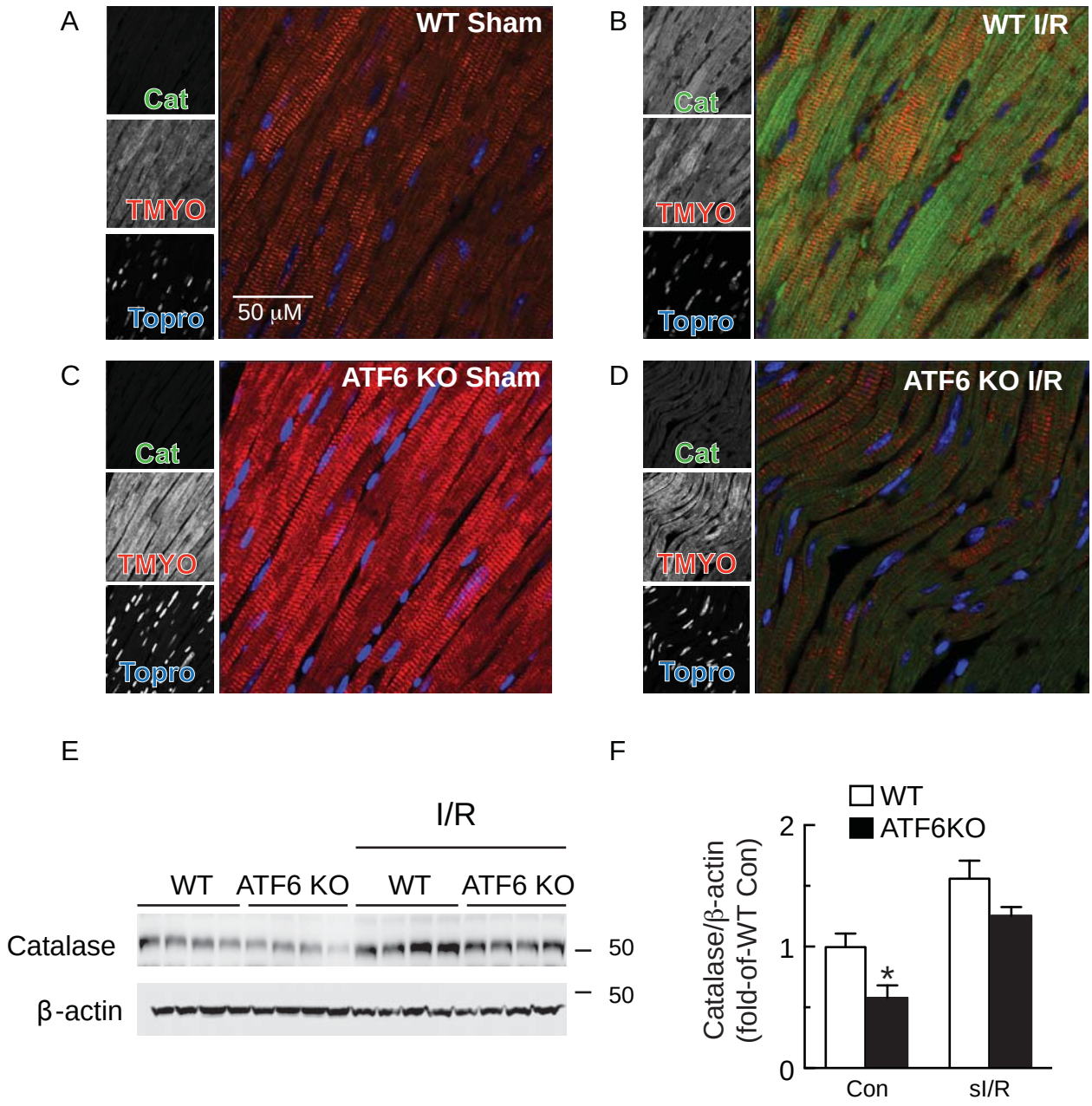


Online Figure IX- Effect of TM on GRP78 expression in cardiac myocytes from WT and ATF6 KO mouse hearts: A, B, Myocytes were isolated from WT or **C, D,** ATF6 KO mouse hearts then treated without (A, C) or with TM (10 μg/ml; 24h; B, D), then fixed and stained for GRP78 (green), α-actinin (red), or TOPRO (blue).



Online Figure X- Effect of TM on Catalase Expression in Cardiac Myocytes from WT and ATF6 KO Mouse Hearts: A, B, Myocytes were isolated from WT or **C, D,** ATF6 KO mouse hearts then treated without (A, C) or with TM (10 μg/ml; 24h; B, D), then fixed and stained for catalase (Cat; green), α-actinin (red), or TOPRO (blue).

Online Figure X



Online Figure XI- Effect of I/R on Catalase expression in WT and ATF6 KO mouse hearts as determined by immunocytofluorescence and immunoblotting: A, B, WT or **C, D,** ATF6 KO mice were subjected to I/R in vivo, after which hearts were obtained and sections were stained for Catalase (green), tropomyosin (red), or TOPRO (blue). **E,** Hearts from WT or ATF6 KO subjected to I/R in vivo were extracted and analyzed for catalase and β-actin by immunoblotting (n = 4 mice per treatment). **F,** The immunoblots shown in E were quantified by densitometry. * $p<0.05$ different from WT Con by t-test.

Online Table I: Echocardiograph results of 10 week-old WT and ATF6 KO mice

	WT 10 week old (n = 8)	ATF6 KO 10 week old (n = 8)
FS (%)	29.89±1.75	30.65±1.03
EF (%)	56.80±2.39	58.81±1.95
LVEDV (μl)	65.70±4.67	62.50±4.47
LVESV (μl)	28.56±3.47	25.88±2.62
LVIDD (mm)	3.87±0.12	3.85±0.11
LVIDS (mm)	2.78±0.12	2.70±0.09
PWTD (mm)	0.71±0.05	0.66±0.02
PWTS (mm)	1.03±0.07	1.04±0.03
AWTD (mm)	0.82±0.10	0.88±0.04
AWTS (mm)	1.19±0.09	1.24±0.06
LV mass (mg)	106.90±14.48	111.90±6.02
HR (bpm)	471.80±6.05	477.9±5.88
SV (μl)	37.11±1.75	35.56±2.38
CO (ml/min)	17.49±0.71	17.54±1.17

FS = fractional shortening
EF = ejection fraction
LVEDV = left ventricular end diastolic volume
LVESV = left ventricular end systolic volume
LVIDD = left ventricular inner diameter in diastole
LVIDS = left ventricular inner diameter in systole
PWTD = left ventricular posterior wall thickness in diastole
PWTS = left ventricular posterior wall thickness in systole
AWTD = left ventricular anterior wall thickness in diastole
AWTS = left ventricular anterior wall thickness in systole
LV mass = left ventricular mass
HR = heart rate in beats per minute
CO = cardiac output
SV = stroke volume

None of the parameters were significantly different between WT and ATF6 KO mice as determined by t-test

Online Table II

Oxidative Stress Response Genes Increased or Decreased by ATF6 in Cardiac Myocytes

No	Gene	Accession	Protein name	Location	Notes	Se[1]	Up/Dn
1	Als	P0C5Y8	Rho guanine nucleotide exchange factor	cytoplasm			Up
2	Cat	P04762	Catalase	peroxisome	metabolizes H_2O_2		Up
3	Dnm2	P39052	Dynamin-2	cytoplasm			Up
4	Epx	D3Z5Y4	Eosinophil peroxidase	extracellular	secreted in exosomes		Up
5	Gpx3	P23764	Glutathione peroxidase 3	ER; secreted	classically secreted metabolizes H_2O_2	X	Up
6	Gpx4	P23764	Glutathione peroxidase 4	mitochondria	mitochondria/cytosol metabolizes peroxylipids	X	Up
7	Nqo1	P05982	NAD(P)H quinone dehydrogenase	cytoplasm			Up
8	Prdx5	Q9R063	Peroxiredoxin-5	mitochondria peroxisomes	has mito and peroxisome targeting sequences	X	Up
9	Vimp	A0G2K953	VCP-interacting membrane Se-protein	cytoplasm; ER	has an ER transmembrane domain	X	Up
10	Slc38a1	Q9JM15	Solute carrier family 38, member 1	cell mem			Up
11	Txnrd1	O89049	Thioredoxin reducatase 1	cytoplasm		X	Up
12	CCl5	P50231	C-C motif chemokine 5	secreted	classically secreted		Dn
13	Cyba	Q62737	Cytochrome b-245 light chain	cell mem	NADHP oxidase component generates ROS		Dn
14	Cygb	Q921A4	Cytoglobin	cytoplasm	metabolizes H_2O_2		Dn
15	Mb	Q9QZ78	Myoglobin	extracellular	secreted in exosomes		Dn
16	Ncf1	F1M707	Neutrophil cytosolic factor 1	cell mem	required for superoxide production		Dn
17	Serpinb1b	F8WGA3	Protein serpinb1b	secreted	serine protease inhibitor		Dn
18	Ucp2	P56500	Uncoupling protein 2	mito			Dn

[1]Se = Selenoprotein