Supplemental Material

Materials and Methods

Animal models

HAX-1 transgenic, HAX-1 heterozygous knockout, PLN knockout (HAX-1 OE, HAX-1 +/- and PLNKO, FVB/N) and their wild type littermates were used in this study, as described previously[1-3]. To avoid the complication of gender differences, only male mice at 3 months of age were used for these studies, which were performed according to the National Institutes of Health Publication No. 8523: *Guide for the Care and Use of Laboratory Animals.*

Global ischemia/reperfusion injury *ex vivo*

The cellular and functional responses to ischemia/reperfusion were assessed, using an isolated perfused heart model, as previously described[4]. Briefly, hearts were mounted on a Langendorff apparatus, and perfused with Krebs–Henseleit buffer. Temperature was maintained constant at 37 °C by water-jacketed glassware for the heart chamber, buffer reservoirs, and perfusion lines. In addition, an overhead light source was used to ensure maintenance of temperature during ischemia, which was monitored by a thermometer placed close to the perfused heart in the glass chamber. A water-filled balloon made of plastic film was inserted into the left ventricle and adjusted to achieve a left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mmHg. The distal end of the catheter was connected to a Heart Performance Analyzer (Micro-Med) via a pressure transducer. Hearts were paced at 400 bpm except during ischemia, and pacing was reinitiated 2 minutes after reperfusion. After a 20-minute equilibration period, hearts were subjected to 40 minutes of no flow global ischemia, followed by 60 minutes of reperfusion. Maximum rate of contraction (+dP/dt), and maximum rate of relaxation (−dP/dt) were monitored during this process.

Regional ischemia *in vivo*

In vivo myocardial ischemia/reperfusion was performed by ligation of the left anterior descending coronary artery (LAD) and releasing the ligature, as previously described[4]. ECG electrodes were placed subcutaneously, and data were recorded by the PowerLab system (Australia AD Instruments). The LAD was ligated at 2 mm distal from the tip of the left appendix for a period of 30 min, and this was followed by releasing the ligation and closing the chest. Ischemia was confirmed by visual observation (cyanosis) and continuous ECG monitoring. After 24 hours of reperfusion, the aorta was cannulated, and the heart was perfused with 1% 2,3,5-Triphenyltetrazolium chloride (TTC) to stain the viable myocardium. The LAD was then re-occluded, and each heart was perfused with 5% phthalo blue to delineate the area at risk, i.e. perfusion area of the LAD. The hearts were frozen and cut into 5 to 6 transverse slices. The images of the slices were digitally analyzed for infarct area, ischemic area (area at risk), and total left ventricular (LV) area, using NIH image software. The ratio of area at risk/total LV and the ratio of infarct area/area at risk were calculated and expressed as percentages, as previously described[4].

Rat myocyte isolation and virus infection

Rat cardiomyocytes were isolated as described previously[1]. Adult male Sprague-Dawley rats (8-10 weeks, 250-350g) were anesthetized using pentobarbital solution (100 mg/kg, i.p.) supplemented with heparin (5000 U/kg). Hearts were quickly removed and the aorta was cannulated on Langendorff system and perfused with modified Krebs-Henseleit buffer (KHB: 118 mmol/L NaCl, 4.8mmol/L KCl, 25mmol/L HEPES, 1.25mmol/L K_2HPO_4, 1.25 mmol/L $MgSO_4$, 11 mmol/L Glucose, 5mmol/L Taurine and 10mmol/L Butanedione Monoxime; pH 7.4) for 5-10 min. Subsequently, hearts were perfused with digestion buffer containing collagenase type II (278 U/mg), 0.2g/L hyaluronidase, 0.1% BSA, and 25μmol/L calcium (Ca). Following 15 min digestion, the left ventricular tissue was excised and the tissue was dispersed into a cellular suspension. Cells were centrifuged and re-suspended in KHB solution supplemented with 1% BSA. The cell suspension was exposed to gradual increases of Ca concentrations in KHB solution to a final concentration of 1.0mmol/L. Cells were then re-suspended in DMEM supplemented with ATCC (0.2%BSA, 2mmol/L L-carnitine, 5mmol/L creatine, 5mmol/L taurine, 100 IU penicillin and 100mg/L streptomycin), counted and plated on laminin-coated dishes. After 2 hours of plating, isolated rat cardiomyocytes were infected with adenovirus carrying the HAX-1 gene linked to a green fluorescent protein (GFP) at a multiplicity of infection (MOI) of 200, as described previously[1] The efficiency of adenoviral gene transfer was evaluated as the number of cells showing green fluorescence signal corresponding to GFP expression. Cardiomyocytes infected with Ad-GFP were used as control. Nearly 100% of myocytes appeared infected after 24 hours. The cell phenotype and morphology remained similar among non-infected and adenoviral-infected groups after 24 hours of infection. Pharmacological agents, such as Brefeldin A (Sigma) and 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG; Selleck Chemicals) were applied to the medium after 24 hours of infection. The myocytes were washed with PBS and harvested for quantitative immunoblotting or RT-PCR, as outlined in the results.

Mouse myocyte isolation and calcium kinetics measurements

Mouse myocytes from WT and HAX-1 OE hearts were isolated as described previously[5]. Briefly, adult mouse hearts were excised following mouse anesthesia with sodium pentobarbital (70mg/kg, i.p.) and cannulated on a Langendorff system. Ca-free Tyrode solution (140nmol/L NaCl, 4mmol/L KCl, 1mmol/L MgCl2, 10mmol/L glucose, and 5nmol/L HEPES, pH 7.4) was used to perfuse the heart for 3 minutes at 37℃. The perfusion was then switched to a digestion solution containing 75units/mL of type 1 collagenase (Worthington). Following digestion, the left ventricular tissue was excised, minced, and dissociated into a cell suspension. Calcium was serially added to the cellular suspension until final calcium concentration in the Tyrode solution was 1.8mmol/L.

To record intracellular calcium kinetics, cells attached to a glass cover-slip were loaded with the cell-permeable calcium sensitive dye fura-2 AM (2μmol/L) for 30 minutes. After 20 minutes washing in 1.8μmol/L Ca-Tyrode buffer to allow de-esterification, cover-slips were mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200). Myocytes were field stimulated at 0.5Hz

using platinum electrodes connected to Grass S5 stimulator that generated rectangular voltage pulses of 1ms duration. The applied voltage was twice the threshold voltage intensity. Cells were continuously perfused with 1.8mmol/L Ca-Tyrode solution for 1 minute, followed by 5 minutes perfusion with Tyrode solution, containing 0.1μmol/L of 17-AAG. Fura 2 AM loaded cells were excited at both 340 and 380 nm and the emission fluorescence signal was collected at 510nm using a Delta Scan dual-beam spectrophotofluorimeter (Photon Technology International). Intracellular calcium changes were expressed as changes in ratio $R=F_{340}/F_{380}$ and analyzed using Felix software (Photon Technology International).

Cardiomyocyte apoptosis treatment and Annexin V staining

To induce apoptosis by ER stress, isolated WT and HAX-1 OE mouse cardiomyocytes in suspension were treated with Brefeldin A for 4 hours in 1.8mmol/L Ca-Tyrode solution at 37℃, in the absence or presence of Irestatin (Axon Medchem) or 17AAG.

To induce apoptosis by simulated ischemia/reperfusion[6], mouse cardiomyocytes were first incubated with ischemia buffer (128mmol/L NaCl, 4mmol/L $NaHCO_3$, 1.8mmol/L $CaCl_2$, 5mmol/L KCl, 0.3mmol/L KH_2PO_4, 0.5mmol/L $MgCl_2$, 0.4mmol/L $MgSO_4$, 10mmol/L HEPES) at 20% CO_2, 1% O_2 and 37℃ for 1 hour. The solution was switched to 1.8mmol/L Ca-Tyrode solution and cell suspensions were kept at 5% CO_2, 20%O_2 at 37℃ for 3 hours, in the absence or presence of Irestatin.

After apoptosis treatment, cells were gently washed once and stained with Annexin V specific APC dye (eBioscience) for 25 minutes. Stained cardiomyocytes were then washed gently once and re-suspended in solution containing propidium iodide (eBioscience) for exclusion of cells without membrane integrity. Fluorescence signals from APC and propidium iodide excited at 633nm and 488nm were collected at emission of 660nm and 610nm, respectively, in >10000 cardiomyocytes per sample group. FlowJo software (Tree Star Inc.) was used to generate diagram of cell distribution according to fluorescence intensity, and to calculate the percent of Annexin V positive population as an indication of apoptosis.

Western blot analysis

Hearts were snap frozen in liquid nitrogen at the end of the Langendorff perfusion period and homogenized in 1× Cell Lysis Buffer (Cell Signaling Technology) supplemented with 1mM PMSF and complete protease inhibitor cocktail (Roche Applied Science). For each protein, equal amounts of samples (5–120 μg) from each heart were analyzed by SDS-PAGE, as previously described[7]. After transfer to membranes, immunoblotting analysis was performed with the corresponding primary antibodies [HAX-1 from BD Biosciences; CHOP, phospho-eIf2α, total eIf2α and Hsp90 from Abcam; IRE-1 from Abgent; ATF6 from Enzo; PDI and XBP-1 from Santa Cruz; caspase-12 from Sigma; GRP94, GAPDH and PLN from Thermo Scientific; SERCA2a (custom-made commercially, Affinity Bioreagents)]. This was followed by incubation with secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. Visualization was achieved using SuperSignal West Pico chemiluminescent substrate (Pierce) or ECLPLUS Western Blotting Detection kit (Amersham Pharmacia Biotech). The intensities of bands were determined by the AlphaEaseFC™ software. For

each protein, the densitometric values from pre-I/R WT controls were arbitrarily converted to 1.0, and the values of samples from the other groups were normalized accordingly and expressed as fold changes. GAPDH was used as an internal standard.

Caspase-3 and calpain activities measurement

The activities of caspase-3 and calpain were assessed using a fluorometric assay, according to the manufacturer's instructions (Biovision). For caspase-3 activity, 100μg of protein from each heart were incubated on ice for 10 minutes, followed by the addition of 50μl of 2X Reaction buffer (containing 10mmol/L DDT). The reaction was initiated by addition of 5μl of the caspase-specific fluorescent substrate. The mixture was incubated at 37℃ for 2 hours in the dark. For calpain activity, 100μg of protein from each heart were diluted to 85μl of Extraction buffer provided by the manufacturer, followed by addition of 10μl of 10X Reaction Buffer. The reaction was started by addition of calpain fluorescent substrate and carried out at 37℃ for 1 hour in the dark. The caspase-3 or calpain activity was quantified by a fluorometer (excitation: 400nm, emission: 505 nm)

DNA fragmentation measurement

DNA fragmentation, measured by the level of cytosolic mono- and oligonucleosomes, was examined using an ELISA assay, according to the manufacturer's instructions (Roche Applied Science). Briefly, homogenates were centrifuged at 13000 rpm for 10 minutes to remove the nuclei. 50ug of cytosolic fraction were incubated with immunoreagent for 2 hours in a streptavidin coated 96-well plate. 100ul of ABTS solution was applied for color development after washing out the immunoreagent. The extent of DNA fragmentation was assessed in a plate reader at 405nm.

Terminal dUTP nick end labeling (TUNEL) assays

After *ex vivo* I/R procedure, the hearts were fixed in 10% buffered formalin and subsequently embedded in paraffin to obtain 5μm-thick sections. DeadEnd™ Fluorometric TUNEL system (Promega) was used to stain for apoptotic nuclei. TUNEL-positive nuclei were counted by randomly selecting 10 fields of the midventricular section. α-sarcomeric actin (1:50 dilution; Sigma) labeling was used to show myocyte cytoplasm. Nuclei were stained by DAPI (Invitrogen). Sections were analyzed with a fluorescence microscope.

Plasma troponin I (TnI) measurement

At 24 hours post-I/R surgery, blood was drawn directly from the aorta from anesthetized mice after thoracic dissection and heart excision. 50 μL of 20mmol/L EDTA was added into 450uL of blood sample to prevent clotting. After gently inverting for ten times, blood samples were centrifuged at 4000 rpm for 10 minutes at room temperature. Only clear supernatant was collected and stored at -80℃.

Cardiac TnI level in each plasma sample was measured using an ELISA kit (Life Diagnostics Inc.), according to manufacturer's instructions. Briefly, 100μL of cTnI HRP conjugates were mixed with 100μL of standard provided in the kit or diluted plasma sample in each anti-cTnI coated well. Mixtures were incubated on a shaker (150 rpm) at room temperature for 60 minutes. After washing the wells six times with 1x wash solution, 100 μL of TMB reagent was added to initiate the reaction.

Following the 20 minute shaking, 100 µL of stop solution was added and the absorbance in each well was measured at 450nm with a microtiter well reader. TnI concentration of each plasma sample was derived from the absorbance value by linear regression, using the values from standard solution, and normalized to the amount of protein loaded, based on the protein concentration of the sample.

Quantitative real-time PCR assay

Total RNA was extracted and purified from heart tissue or cultured cardiomyocytes with miRNeasy Mini Kit (QIAGEN 133220834). The first strand cDNA was generated from total RNA (1µg) with reverse trascriptase kit (Invitrogen Cat. No.11752). PCR was then performed with Bio-Rad real time thermal cycler, using the following specific primer sequences: Mouse full length Xbp1: (Forward) 5'-TCCGCAGCACTCAGACTATGT-3' , (Reverse) 5'-ATGCCCAAAAGGATATCAG ACTC-3'; Mouse spliced Xbp1: (Forward) 5'-GAGTCCGCAGCAGGTG-3', (Reverse) 5' –GTGTCAGAGTCCATGGGA-3'; Mouse GAPDH (Forward) 5'-TCAACAGCAACTCCCACTCTT-3', (Reverse) 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'. Rat spliced Xbp1: (forward) 5'- GAGTCCGCAGCAGGTGC-3', (reverse) 5'-GGTCCAACTTGTCCAGAATGC-3'; Rat GAPDH: (Forward) 5'-CCTAAATGATACCCCACCGTG-3', (Reverse) 5'-TGTCACAAGAGAAGGCAGC-3'. The values were normalized to those obtained with GAPDH.

Generation of Recombinant Proteins and Blot Overlay Assay

To evaluate in vitro the interactions between Hsp90 and HAX-1, appropriate constructs for protein expression were generated by PCR amplification. The HAX-1 construct (amino acids 1-260) was generated by PCR amplification using sense primer 1 (5' GAATTCCTGGAGGGGTTCAAAGGT 3') and antisense primer 1 (5' GTCGACGCTGGAGGTCTTGGTGATTC 3'). The Hsp90 construct (amino acids 1-724) was generated using sense primer 2 (5' CAAGATGCCTGAGGAAGTG 3') and antisense primer 2 (5' ACAAGGGCACAAGTTTTCCA 3'). The Hsp90 deletion constructs of Hsp90 were produced as outlined below: Hsp90-A (amino acids 1-199) using sense primer 2 and antisense primer 3 (5' TTTGACCCGCCTCTCTTCTA 3'); Hsp90-B (amino acids 194-448) using sense primer 4 (5' AGAAGAGAGGCGGGTCAAA 3') and antisense primer 4 (5' GCGGTTAGTGGAGTCTTCGT 3'); and Hsp90-C (amino acids 440-724) with sense primer 5 (5' TGGAATCCACGAAGACTCCAC 3') and antisense primer 2. PCR products were subcloned in the EcoRI/SalI sites of the pMAL-c2x vector (New Englands Biolabs) or the SmaI/NotI sites of pGEX5x-1 vector (Amersham Biosciences) and the sequence of all generated constructs was verified by sequencing analysis. For the generation of the construct containing the cytoplasmic domain of IRE1α, sense primer 6 (5' ATCATCACCTATCCCCTGAGC 3') and antisense primer 6 (5' CTCAGAGGGCGTCTGGAGT 3') were used and the resulting PCR product was cloned in the EcoRI/XhoI sites of the pET28 vector (Novagen, Nottingham, UK). Expression of recombinant proteins was performed as previously described[8] and proteins were purified by affinity chromatography on Glutathione Sepharose™ 4B

(Amersham Biosciences) beads, amylose resin (New England Biolabs) or Ni-NTA agarose (QIAGEN, Hilden, Germany) according to manufacturer's instructions.

Protein binding between Hsp90 and HAX-1 was assessed by blot overlay assays, as previously described[7]. Briefly, affinity-purified GST and GST-Hsp90 recombinant proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific sites on the membrane were blocked in buffer A (50mmol/L Tris-HCl pH 7.5, 150mmol/L NaCl, 0.05% (v/v) Tween-20, 2mmol/L dithiothreitol, 0.5% NP-40, 5% nonfat milk) for 16 h at 4°C. The membrane was then incubated with MBP-HAX-1 or His-IRE-1 recombinant protein for 4 h at 25° C in buffer A that also contained 1 mmol/L ATP. Following three washes in buffer C (50mmol/L Tris-HCl pH 7.5, 150mmol/L NaCl, 0.05% (v/v) Tween-20, 2mmol/L dithiothreitol, 1% NP-40), the membrane was probed with anti-MBP (New England Biolabs) or anti-His antibody (Sigma-Aldrich) and bands were visualized using ECL reagents.

GST-pull down assays

GST-pull down assays were performed as previously described[8, 9]. In brief, mouse cardiac homogenates were prepared in 10mmol/L $NaPO_4$ (pH 7.2), 2mmol/L EDTA, 10mmol/L NaN_3, 120mmol/L NaCl and 1% NP-40, supplemented with protease inhibitors (Sigma-Aldrich). Equal amounts of recombinant GST and GST-Hsp90 proteins were mixed with 0.5mg of cardiac homogenates at 4^0C for 16 hours. The samples were then washed three times with 10mmol/L $NaPO_4$ (pH 7.2), 10mmol/L NaN_3, 120mmol/L NaCl and 0.1% Tween-20. Following SDS-PAGE analysis and transfer to nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH), the membranes were incubated with monoclonal antibodies to PLN, SERCA2 (Affinity Bioreagents) or HAX-1 (BD Transduction Laboratories) and were subsequently washed in 50mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl and 0.05% Tween-20 before incubating with a peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich). Immunoreactive bands were detected using ECL reagents (Amersham Biosciences Europe). Similar experiments were also performed using GST-PLN recombinant protein, expressed from a PLN construct (amino acids 1-37) that has been previously described[8]. GST-pull down assays were again performed using equivalent amounts of GST and GST-PLN recombinant proteins and samples were analyzed by western blot analysis using Hsp90 (Abcam), SERCA2 or HAX-1 antibodies.

Competitive protein binding ELISA assay

Competitive binding assay using recombinant proteins was performed as described[10]. Briefly, each well on a 96-well high affinity binding plate (BD Falcon Catalog# 351172) was coated with 200ng of recombinant Hsp90β protein (StressMarq) or GST protein (Novus Biologicals, as control) in 100µL coating buffer (85mmol/L NaHCO3 and 15mmol/L Na2CO3, pH 9.5) overnight at 4℃. After five washes with PBS and 0.05% tween-20 (PBS-T), blocking solution (PBS containing 1% BSA) was added for 1 hour at room temperature. Following another five PBS-T washes, 200ng of HAX-1 protein (Novus Biologicals) was added along with 200ng of competition protein mixture, which contained various compositions of recombinant GST protein and the cytosolic fragment of IRE-1

(Sino Biological Inc.). Incubation proceeded for 2 hours at room temperature. Each well was then washed with PBS-T six times, and the HAX-1 antibody, which was dissolved in blocking solution, was applied for 2 hours at room temperature. After six washes with PBS-T, the secondary antibody conjugated with horseradish peroxidase was added for 1 hour at room temperature. Following eight PBS-T washes, TMB substrate reagent (BD Biosciences) was applied to develop signal in blue color. After color was developed, 1mol/L of HCl was added to stop the reaction. The colorimetric intensity of each well was read at 450nm with a microplate reader.

Co-immunoprecipitations

Hearts from WT, HAXOE and PLNKO mice were homogenized with 1X cell lysis buffer (Cell Signaling Technology), supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail. The homogenate was centrifuged at 10000 rpm for 30 minutes at 4°C. The centrifuged homogenate was diluted to 1g/L (1mL of final volume) and incubated with anti-HAX-1 (BD Biosciences), anti-Hsp90 (Abcam), anti-IRE-1, anti-PLN (Thermo Scientific), or IgG antibody (Santa Cruz Biotech) at 4°C overnight with rotation. 100µL of protein G PLUS agarose beads (Santa Cruz Biotech) were added into the mixture and incubated for an additional 5 hours. Agarose beads were sedimented and washed 6 times with the cell lysis buffer. Beads-bound proteins were dissociated in 2X SDS at room temperature for 30 minutes with vortexing every 5 minute-intervals. The identification of the associated proteins was detected by western blots. Homogenates from WT heart were used as positive controls.

Immuno-fluorescence staining

WT or HAX-1 OE cardiomyocytes were isolated as described above, and plated in a laminin-coated Lab-Tek chamber slide (Thermo Scientific). Cardiomyocytes were fixed with 1.8mmol/L Ca-Tyrode solution containing 4% Paraformaldehyde (Electron Microscopy Sciences) for 30 minutes, and permeabilized with saline solution containing 0.1% Triton-X at 4°C overnight. Non-specific binding was limited by 4 hours incubation with blocking solution containing 5% of goat serum. Cells were stained with anti-SERCA2a (custom-made commercially, Affinity Bioreagents) and anti-Hsp90 (Abcam), subsequently followed by incubation with Alex flour 488 goat anti-rabbit (Invitrogen) and DyLight 594 goat anti-rat (Abcam) in blocking solution. Images were obtained using Zeiss confocal microscopy.

Statistical analysis

Data were expressed as mean±SEM. Comparisons between the means of two groups were performed by unpaired Student's t-test. Multiple groups were analyzed, using a one-way ANOVA with a Bonferroni test for post hoc analysis. Results were considered statistically significant at $P<0.05$.

References

1. Zhao W, Waggoner JR, Zhang ZG, Lam CK, Han P, Qian J, Schroder PM, Mitton B, Kontrogianni-Konstantopoulos A, Robia SL, Kranias EG. The anti-apoptotic protein HAX-1 is a regulator of cardiac function. *Proc Natl Acad Sci U S A.* 2009;106:20776-20781.

2. Chao JR, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. *Nature.* 2008;452:98-102.

3. Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. *Circ Res.* 1994;75:401-409.

4. Fan GC, Ren X, Qian J, Yuan Q, Nicolaou P, Wang Y, Jones WK, Chu G, Kranias EG. Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. *Circulation.* 2005;111:1792-1799.

5. Yuan Q, Fan GC, Dong M, Altschafl B, Diwan A, Ren X, Hahn HH, Zhao W, Waggoner JR, Jones LR, Jones WK, Bers DM, Dorn GW, 2nd, Wang HS, Valdivia HH, Chu G, Kranias EG. Sarcoplasmic reticulum calcium overloading in junctin deficiency enhances cardiac contractility but increases ventricular automaticity. *Circulation.* 2007;115:300-309.

6. Nicolaou P, Knoll R, Haghighi K, Fan GC, Dorn GW, 2nd, Hasenfub G, Kranias EG. Human mutation in the anti-apoptotic heat shock protein 20 abrogates its cardioprotective effects. *J Biol Chem.* 2008;283:33465-33471.

7. Qian J, Vafiadaki E, Florea SM, Singh VP, Song W, Lam CK, Wang Y, Yuan Q, Pritchard TJ, Cai W, Haghighi K, Rodriguez P, Wang HS, Sanoudou D, Fan GC, Kranias EG. Small heat shock protein 20 interacts with protein phosphatase-1 and enhances sarcoplasmic reticulum calcium cycling. *Circ Res.* 2011;108:1429-1438.

8. Vafiadaki E, Sanoudou D, Arvanitis DA, Catino DH, Kranias EG, Kontrogianni-Konstantopoulos A. Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function. *J Mol Biol.* 2007;367:65-79.

9. Vafiadaki E, Arvanitis DA, Pagakis SN, Papalouka V, Sanoudou D, Kontrogianni-Konstantopoulos A, Kranias EG. The anti-apoptotic protein HAX-1 interacts with SERCA2 and regulates its protein levels to promote cell survival. *Mol Biol Cell.* 2009;20:306-318.

10. Zhang X, Wang X, Zhu H, Kranias EG, Tang Y, Peng T, Chang J, Fan GC. Hsp20 functions as a novel cardiokine in promoting angiogenesis via activation of VEGFR2. *PLoS One.*7:e32765.

Online Figure I

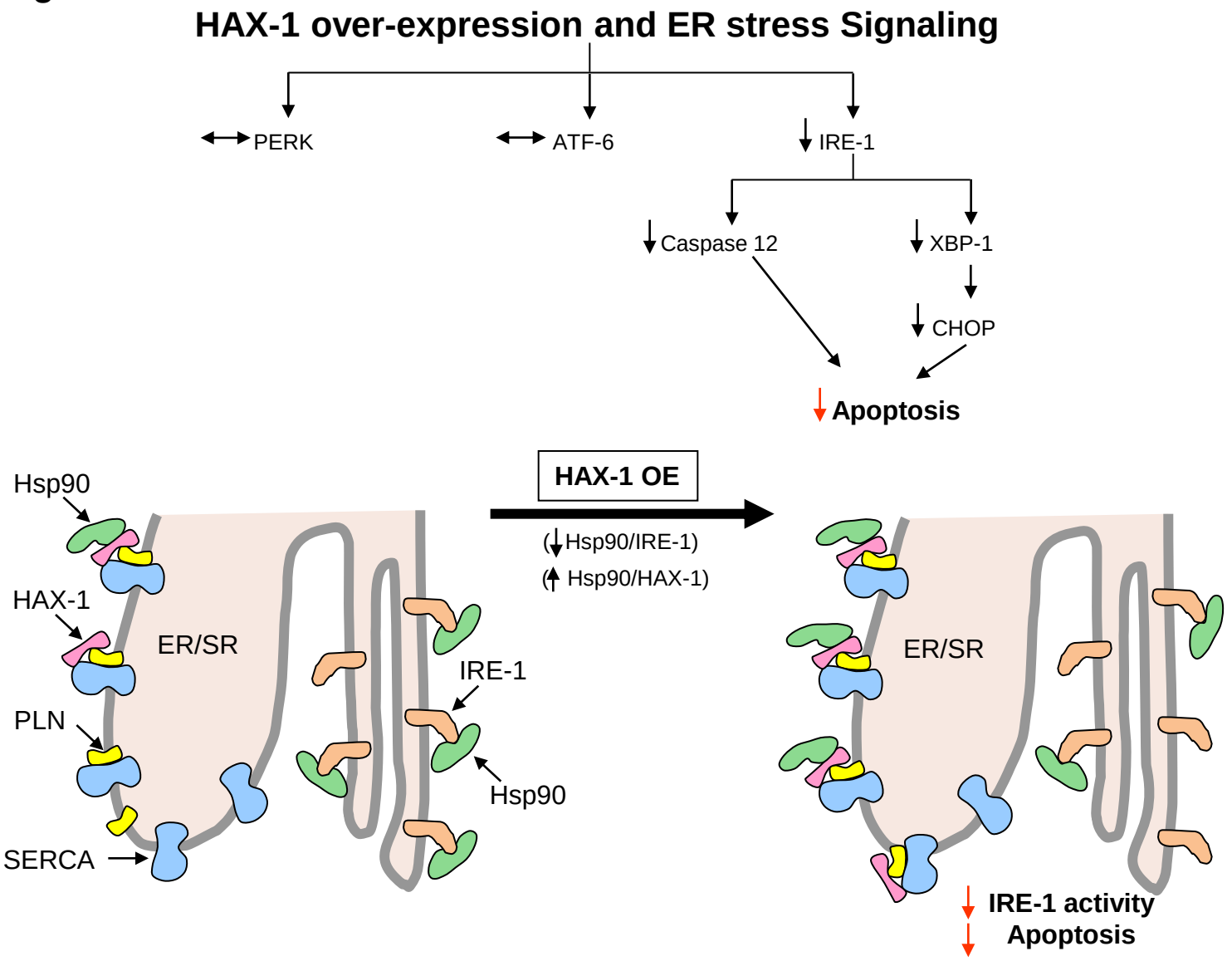

Figure I. Proposed mechanism of HAX-1 regulating ER stress response. IRE-1 activity, promoted by Hsp90, can induce apoptosis in ischemia/reperfusion injury. Over-expressing HAX-1 can displace Hsp90 from IRE-1, attenuating its activity, and recruit Hsp90 to the PLN/SERCA complex.

Online Figure II

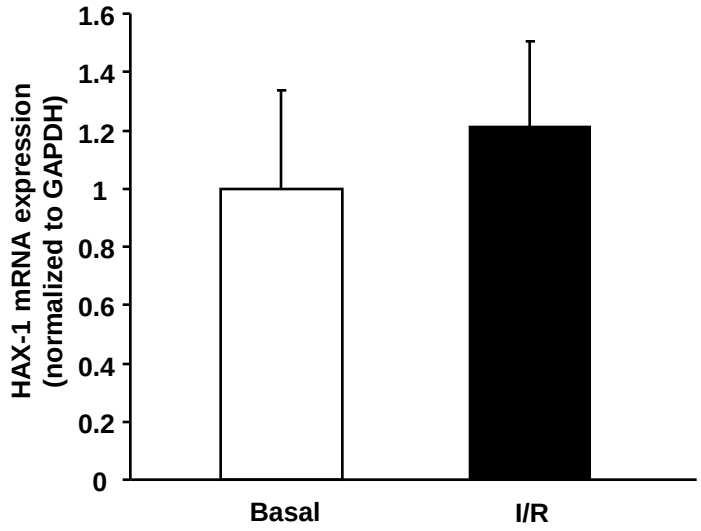

Figure II. HAX-1mRNA levels using real-time PCR. GAPDH was used as control (n = 4 for basal and I/R hearts). Data are presented as mean±SEM.

Online Figure III

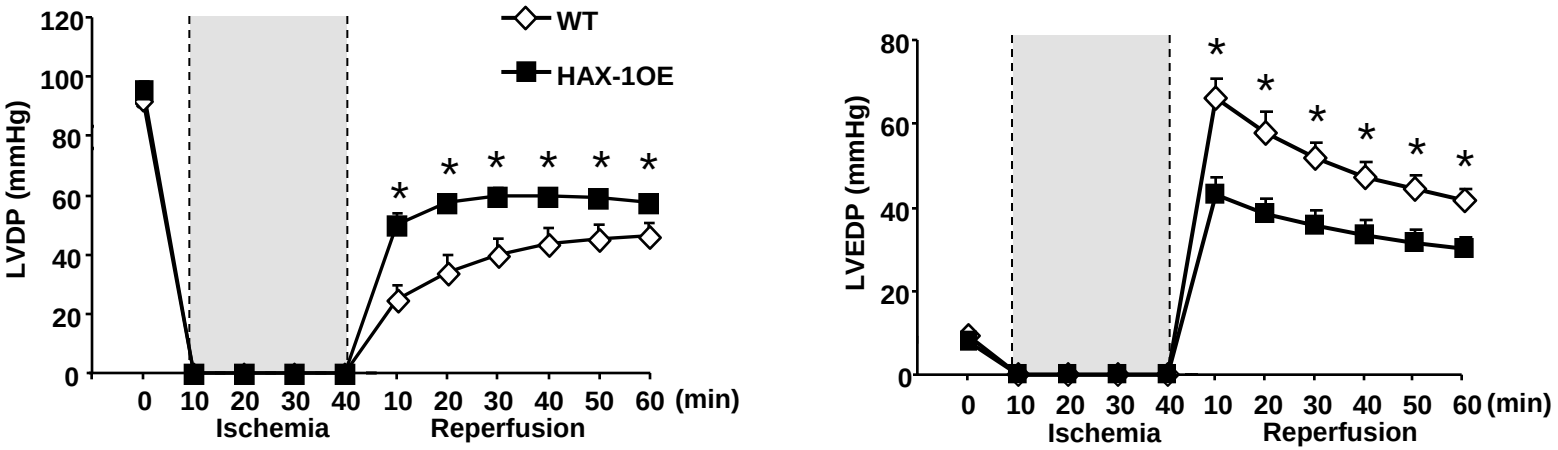

Figure III. HAX-1 OE hearts showed improved left ventricular developed pressure (LVDP) and reduced left ventricular end diastolic pressure (LVEDP) after *ex vivo* ischemia/reperfusion injury. n=6 for WT and 8 for HAX-1 OE hearts. *P<0.05, compared to WTs. Data are presented as mean±SEM.

Online Figure IV

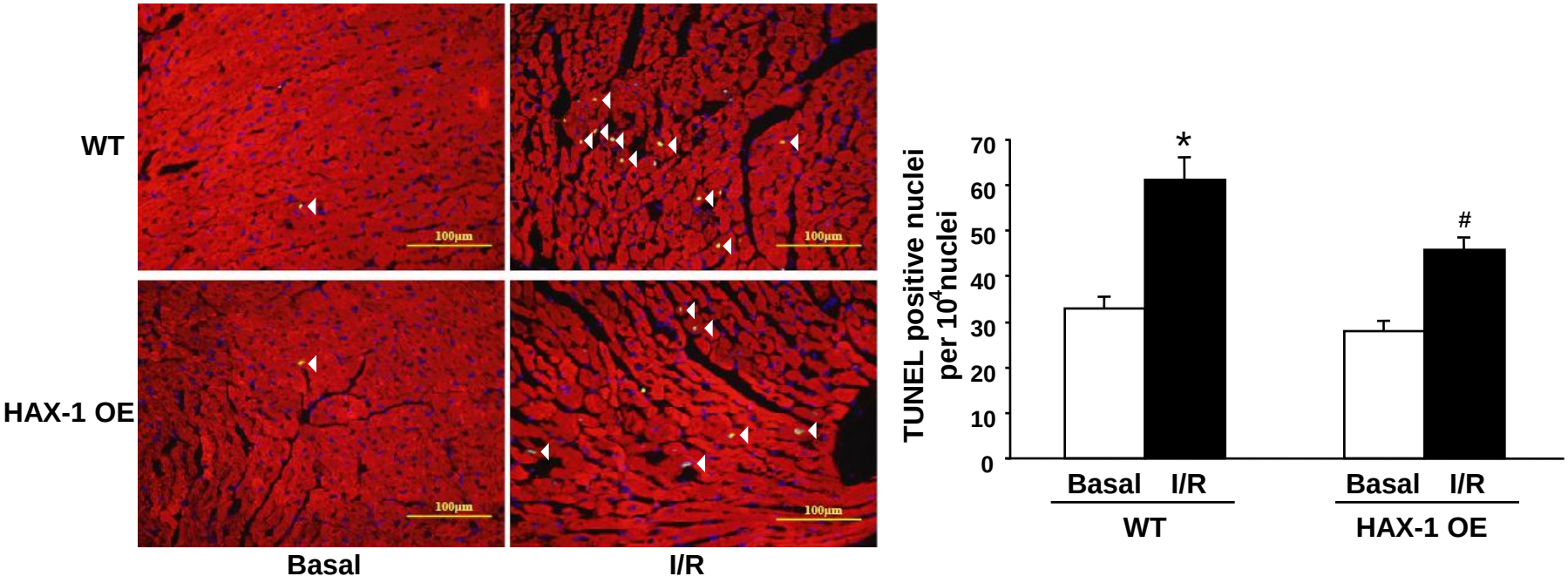

Figure IV. HAX-1 OE hearts showed reduced TUNEL positive nuclei after *ex vivo* ischemia/reperfusion injury. α-actinin was stained in red to reveal cardiomyocytes. All nuclei were stained with DAPI in blue and TUNEL positive nuclei were shown in bright green (indicated by arrowhead). n=4 for WT and HAX-1 OE hearts. *P<0.05, compared to WT basal. #P< 0.05, compared to WT I/R. Data are presented as mean±SEM.

Online Figure V

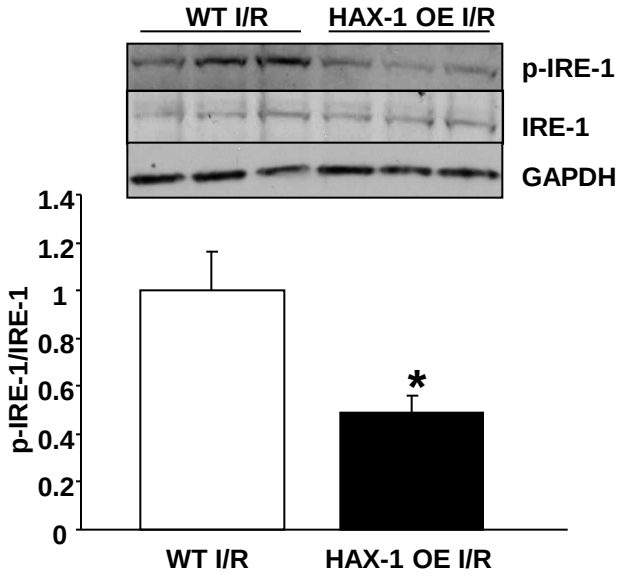

Figure V. p-IRE-1/IRE-1 levels in WT and HAX-1 OE hearts after *ex vivo* ischemia reperfusion injury. GAPDH was used as control. n=4 for both groups. *P<0.05, compared to WT I/R.

Online Figure VI

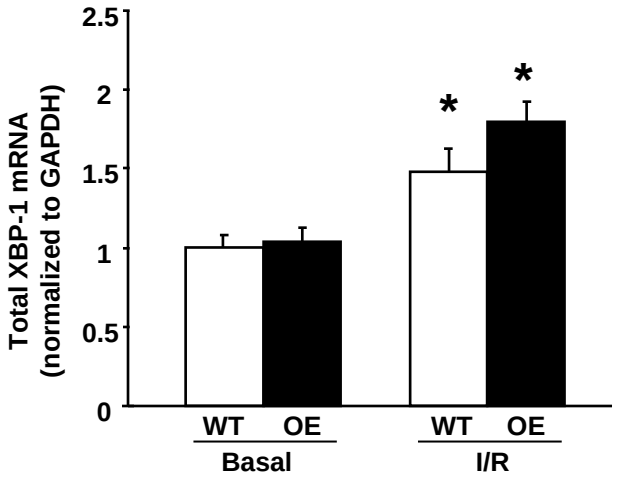

Figure VI. Total (full-length + spliced) xbp1 mRNA levels using real-time PCR. GAPDH was used as control (n = 3 for basals, n=4-6 for I/Rs). *P<0.05, compared to WT basal.

Online Figure VII

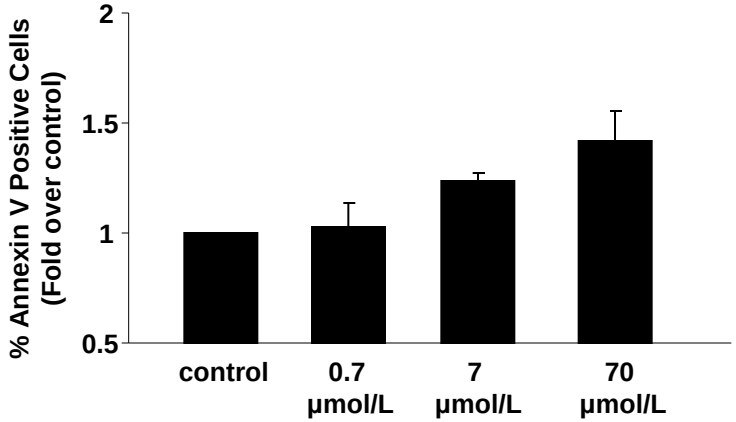

Figure VII. Dose responses for brefeldin A on apoptosis. Isolated WT mouse cardiomyocytes were treated with brefeldin A at various concentrations for 4 hours. Apoptosis was assessed by annexin V staining. n=4 hearts for each group.

Online Figure VIII

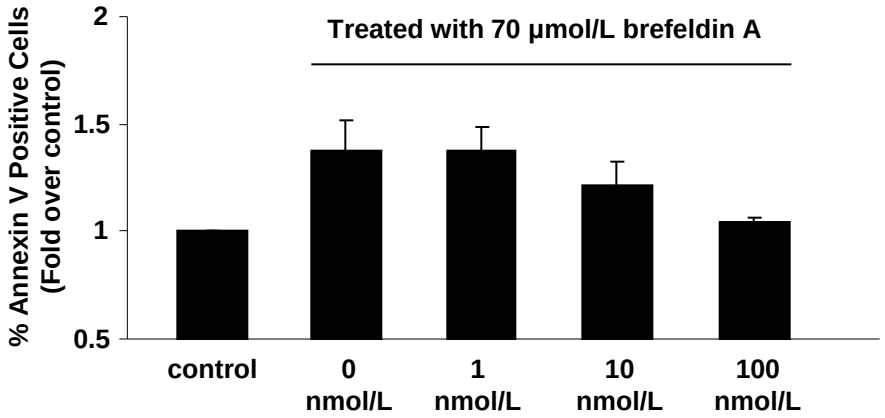

Figure VIII. Dose responses for irestatin, the IRE-1 inhibitor, on the inhibition of brefeldin A induced apoptosis. Isolated WT mouse cardiomyocytes were treated with various concentrations of irestatin and 70 µmol/L brefeldin A for 4 hours. Apoptosis was assessed by annexin V staining. n=4 hearts for each group.

Online Figure IX

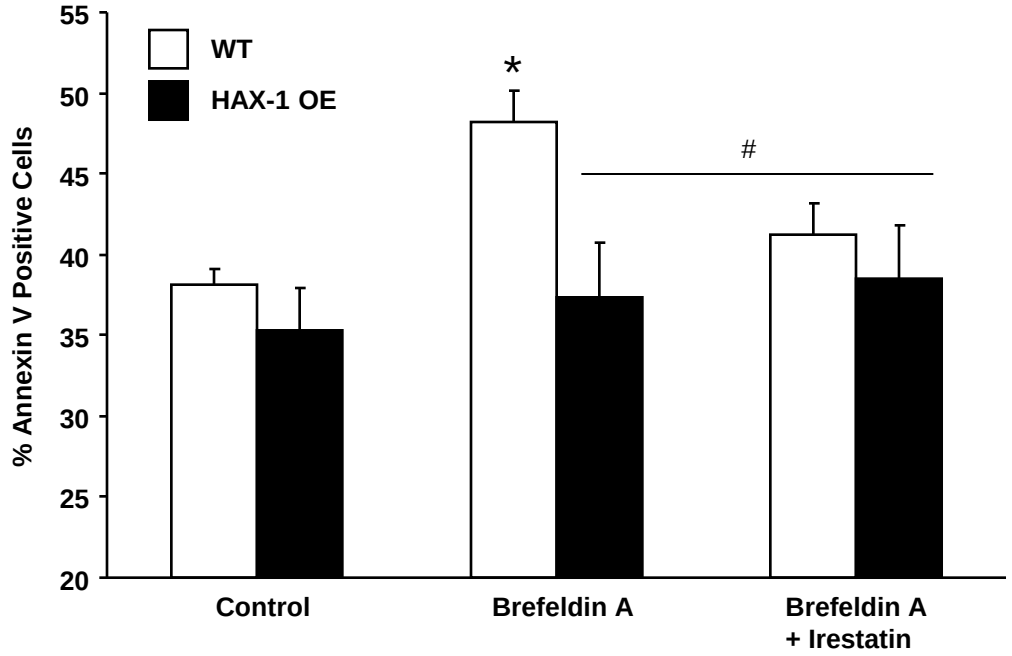

Figure IX. HAX-1 over-expression and IRE-1 inhibition blocked brefeldin A induced apoptosis. n=14 for WT and 6 for HAX-1 OE hearts. Isolated cardiomyocytes were treated with 70 μmol/L brefeldin A in the absence or presence of 100 nmol/L irestatin, IRE-1 inhibitor, in suspension for 4 hours. n=14 for WT and 6 for HAX-1 OE hearts. *P<0.05, compared to WT control. #P<0.05, compared to WT brefeldin A. Data are presented as mean±SEM.

Online Figure X

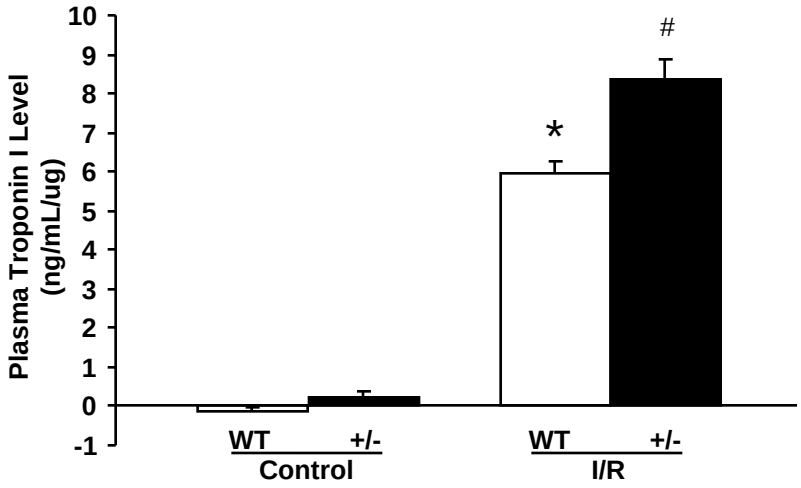

Figure X. HAX-1 heterozygous deficient mice (HAX-1 +/-) exhibited higher plasma troponin I level after *in vivo* ischemia/reperfusion injury. n=3 for control hearts, 4 for WT I/Rs and 5 for HAX-1 +/- I/R hearts. *P<0.05, compared to WT control. #P<0.05, compared to WT I/R. Data are presented as mean±SEM.

Online Figure XI

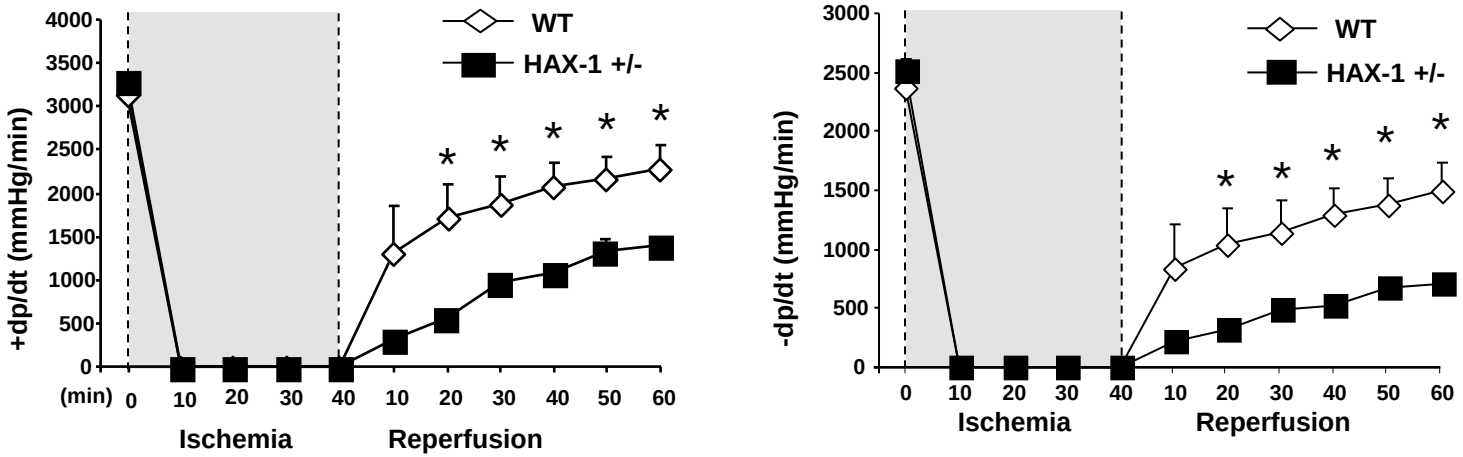

Figure XI. HAX-1 +/- hearts showed decreased contractile recovery after *ex vivo* ischemia/reperfusion injury. Rates of contraction and relaxation (±dP/dt, mmHg/min). n=4 for WTs and 4 for HAX-1 +/- hearts. *P<0.05, compared to WTs. Data are presented as mean±SEM.

Online Figure XII

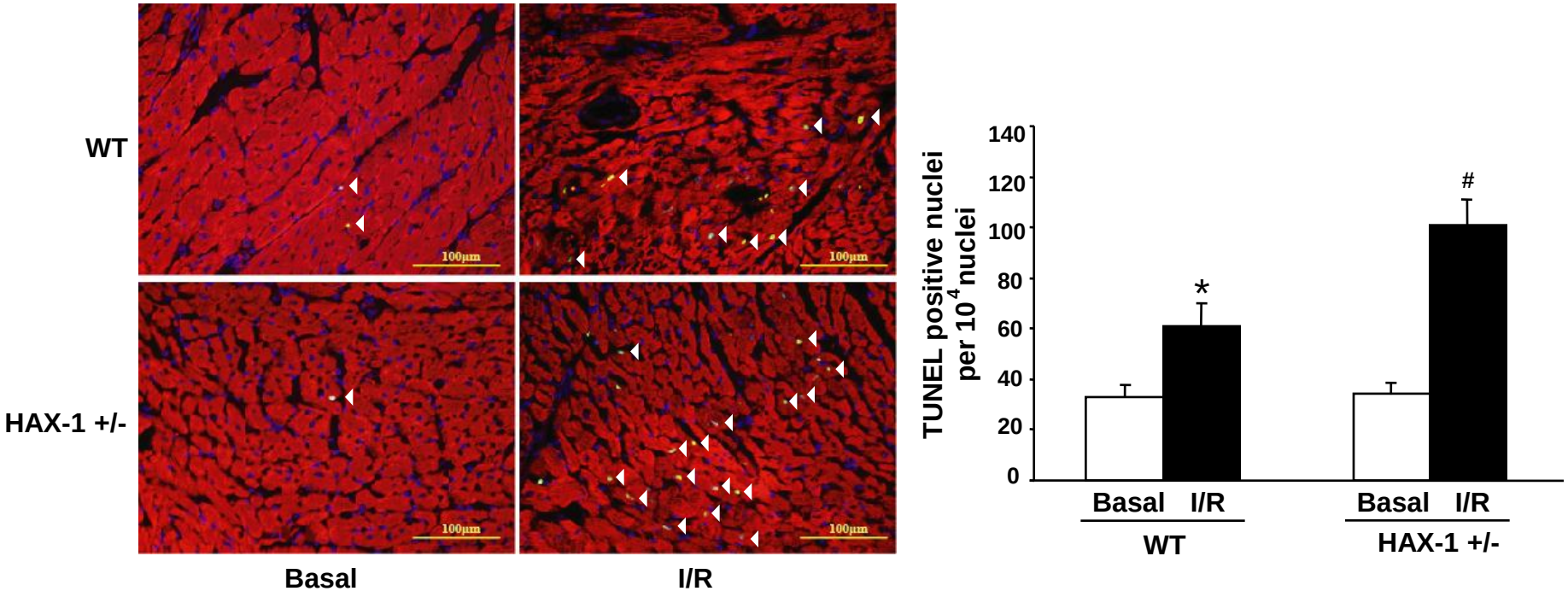

Figure XII. HAX-1 +/- hearts showed increased TUNEL positive nuclei after *ex vivo* ischemia/reperfusion injury. α-actinin was stained in red to reveal cardiomyocytes. All nuclei were stained with DAPI in blue and TUNEL positive nuclei were shown in bright green (indicated by arrowhead). n=4 for WT and HAX-1 OE hearts. *P<0.05, compared to WT basal. #P< 0.05, compared to WT I/R. Data are presented as mean±SEM.

Online Figure XIII

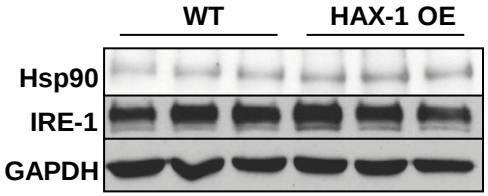

Figure XIII. IRE-1 and Hsp90 expression in WT and HAX-1 OE hearts under basal conditions. GAPDH was used as loading control.

Online Figure XIV

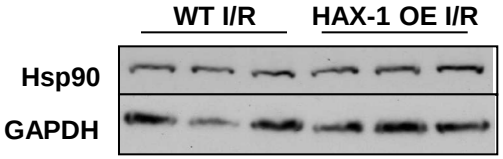

Figure XIV. Hsp90 expression in WT and HAX-1 OE hearts after *ex vivo* ischemia/reperfusion procedure. GAPDH was used as loading control.

Online Figure XV

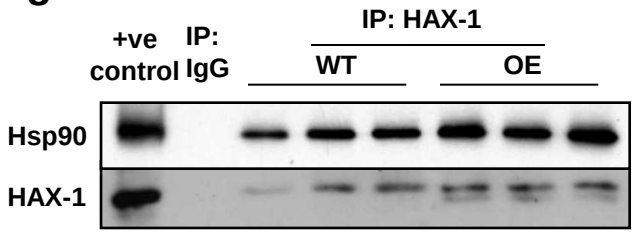

Figure XV. Co-immunoprecipitations were performed with WT and HAX-1 OE homogenates using the HAX-1 antibody. n=5 hearts per group. Eight independent experiments were performed.

Online Figure XVI

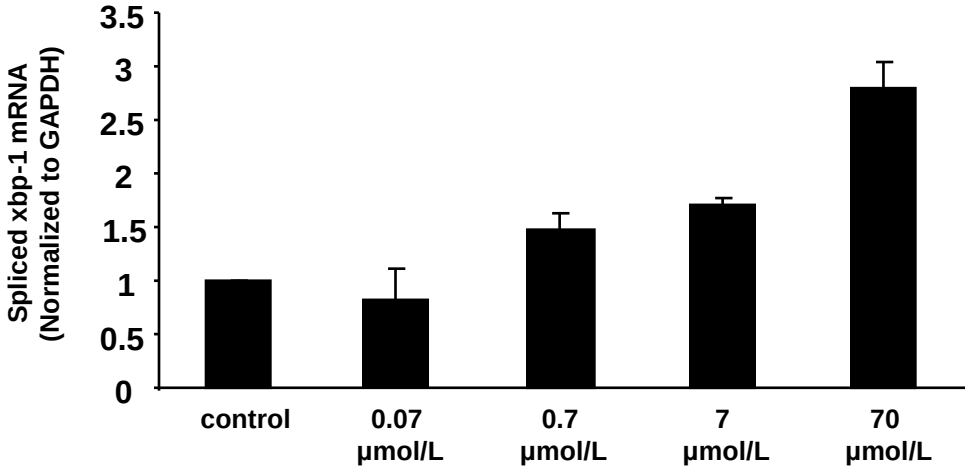

Figure XVI. Dose responses for brefeldin A (BA) on spliced xbp-1 mRNA levels. Rat cardiomyocytes were infected with adenovirus encoding GFP as controls for HAX-1 adenoviral delivery, shown in Figure 7D. BA at various concentrations was administered at 24 hours after infection for 4 hours. The expression of xbp-1 transcript was examined by RT-PCR. n=4 for each group.

Online Figure XVII

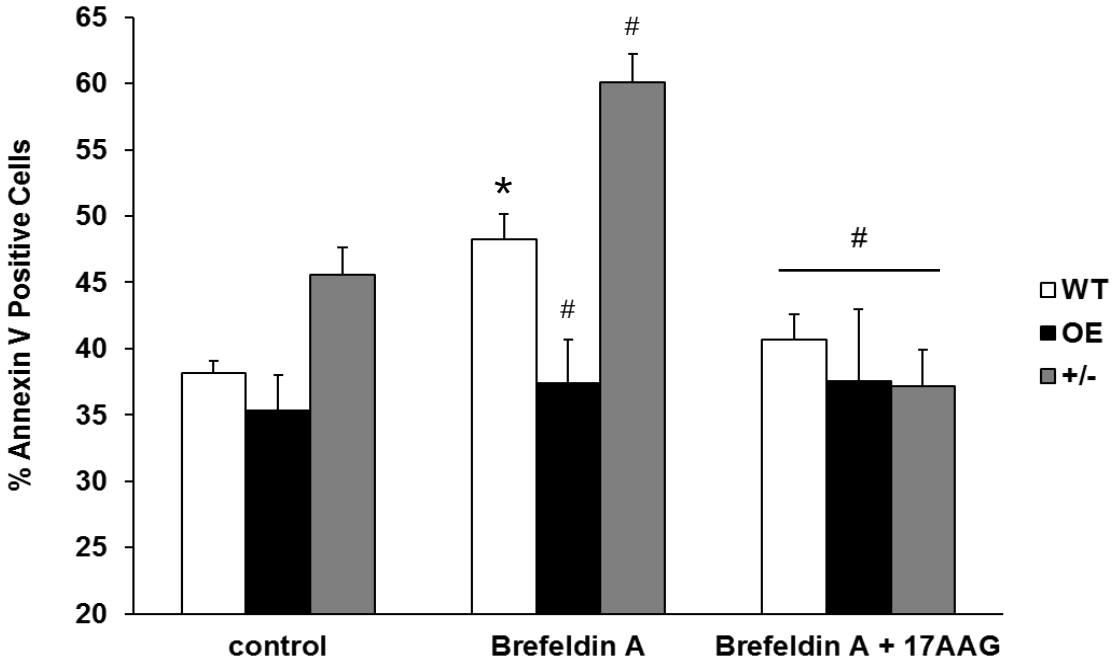

Figure XVII: Quantification of annexin V positive isolated cardiomyocytes after 4 hours of brefeldin A treatment with or without 17AAG administration. n=14 hearts for WT, 6 hearts for HAX-1 OE and HAX-1 +/- in each treatment group. *P<0.05 vs WT control, #P<0.05 vs WT Brefeldin A.

Online Figure XVIII

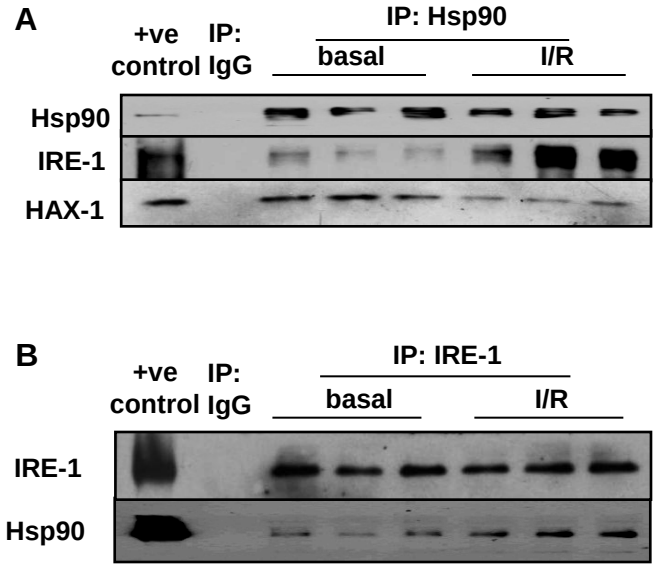

Figure XVIII. Co-immunoprecipitations were performed with WT homogenates under basal conditions or after *ex vivo* I/R treatment using the Hsp90 (A) and IRE-1 (B) antibody. n=4 hearts for both basal and I/R groups. Six independent experiments were performed.

Online Figure XIX

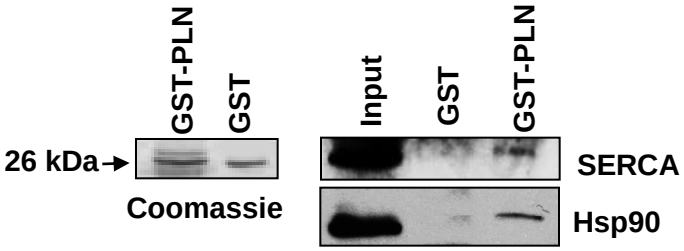

Figure XIX. SDS-gel stained with Coomassie blue showing purified GST and GST-PLN recombinant proteins. GST-pull down assays performed with mouse cardiac homogenates and GST-PLN or GST recombinant proteins.

Online Figure XX

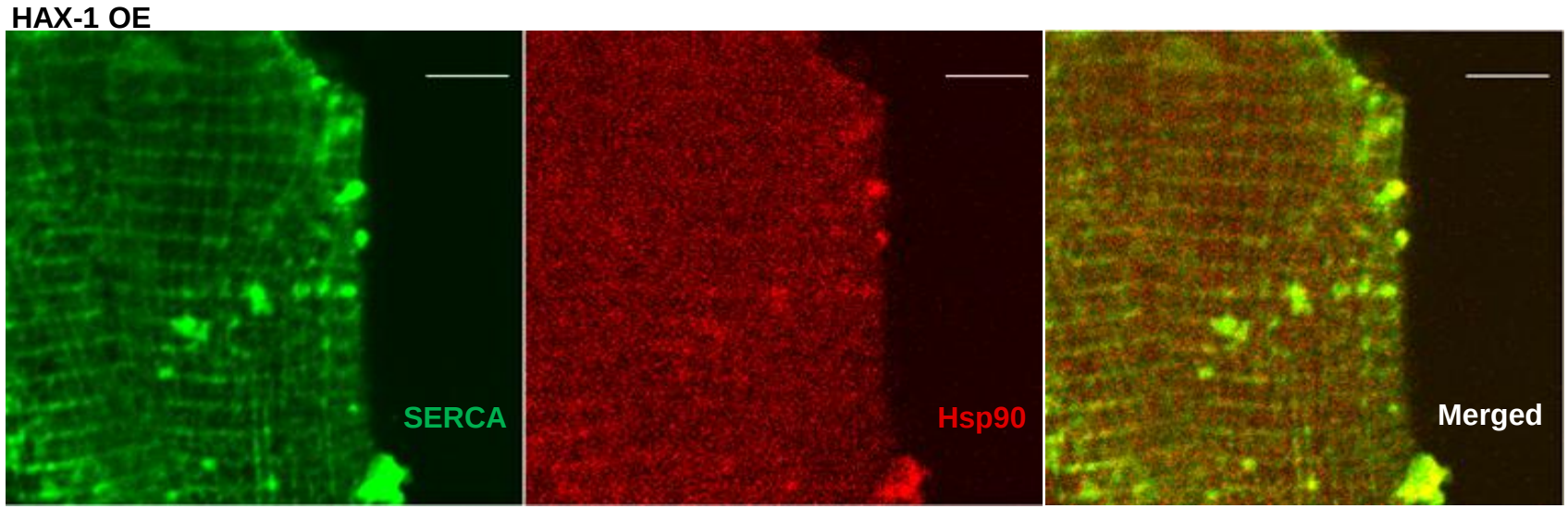

Figure XX. HAX-1 OE cardiomyocytes were immuno-stained for SERCA (green) and Hsp90 (red). Scale bar, 5μm.