SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Generation of an inducible double transgenic mouse model with cardiac-specific expression of a truncated, active form of inhibitor-1 (I-1c)

The T35D-I-1 mutant was derived from the full-length mouse I-1, using site-directed mutagenesis. Subsequently, the truncated, constitutively active I-1 was generated by polymerase chain reaction (PCR) from the I-1-T35D cDNA, using the forward primer: 5' – CAGA GGATCC ATG GAG CCC GAC AAC AGC CC – 3'; and the reverse primer: 5' – CAGA GGATCC TCA TGA CAA GGT GGA CTT GAG AAG – 3' (BamHI restriction enzyme sites underlined). The I-1c cDNA was cloned into a vector bearing an attenuated α-myosin heavy chain promoter (α-MHC_p) in which the three GATA sites and the two thyroid responsive elements were replaced with seven repeats of the TetO sequence [α-MHC_{min}-$(TetO)_7$]. The completed construct was submitted to the University of Cincinnati Transgenic Mouse Core for pronuclear microinjection. The I-1c single transgenic mice (TG2) were crossed with mice carrying the tetracycline-controlled transactivator (tTA) gene, which is under the regulation of the traditional α-MHC_p (TG1). Mice carrying both transgenes are designated double transgenic (DTG). I-1c expression was effectively suppressed, using doxycycline (Dox) chow (625 mg/kg). Removal of Dox from their diet allowed for I-1c expression. The α-MHC_{min}-$(TetO)_7$ promoter plasmid as well as the single transgenic TG1 mice were provided by Dr. Jeffrey Robbins of Cincinnati Children's Hospital, OH. Animals were handled as approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Detection of inhibitor-1

Cardiac tissue from mice was enriched in I-1, using the trichloroacetic acid (TCA) method, as previously detailed (1). Briefly, 400 mg of frozen tissue was pulverized and

homogenized in ice-cold phosphate-buffered solution. Subsequently, TCA was added to a final concentration of 1.5% (w/v) and rotated for 1 hour at 4^{o}C before centrifugation at 9,000 rpm for 30 min. The supernatant was adjusted to 15% TCA and rocked overnight at 4^{o}C. This was centrifuged at 18,000 rpm and the pellet was resuspended in 0.5 mmol/L Tris-HCl, pH=8.0 and subjected to Western blotting, as described below.

Immunoblotting

Alterations in the phosphorylation and/or total level of proteins were analyzed from whole heart homogenates, with the exception of MyBP-C, which was analyzed from a myofibril protein preparation (2), using Western blotting. Briefly, 2-70 μg of protein were separated on polyacrylamide gels (6-15%) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies, which were visualized by peroxidase-conjugated secondary antibodies (Amersham Biosciences) and enhanced chemiluminescence (Supersignal West Pico Chemiluminescent, Pierce). The bands were then quantified with densitometry, using ImageQuant 5.2. The antibodies were obtained from the sources listed: pSer16-PLN, pThr17-PLN and pSer2808-RyR (Badrilla), PLN (Upstate), RyR and actin (Sigma), pSer22/pSer23-TnI and TnI (Research Diagnostics Inc.), pSer282-MyBP-C (custom-made commercially, ProSci Inc.), MyBP-C (home-made, references 2, 3), I-1 and SERCA2a (custom-made commercially, Affinity Bioreagents), CSQ (Affinity Bioreagents), PDI (Alexis Biochemicals), Grp78 (Santa Cruz Biotechnology), Ire1α (Abcam), pSer116-Bad, pSer136-Bad and Bad (Cell Signaling).

SR Ca^{2+}-uptake

Initial SR Ca^{2+}-uptake rates were determined in cardiac homogenates, using the Millipore filtration technique and $^{45}CaCl_2$, as previously described (4). Briefly, 100-250 μg of cardiac homogenate were incubated at 37°C in a reaction buffer containing: 40 mmol/L imidazole, pH=7.0, 95 mmol/L KCl, 5 mmol/L NaN_3, 5 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, and 5 mmol/L potassium oxalate. The initial uptake rates were determined over a wide range of Ca^{2+} concentrations (pCa 8 to 5). Ca^{2+}-uptake into SR vesicles was initiated by addition of 5 mmol/L ATP, and aliquots were filtered through a 0.45 μm Millipore filter after 0, 30, 60 and 90 seconds. The specific $^{45}Ca^{2+}$-uptake values were analyzed by non-linear regression, using the OriginLab 5.1 program to obtain the Ca^{2+} affinity (EC_{50}) and the maximal Ca^{2+}-uptake rate (V_{max}).

In vivo catheterization

To assess the effects of I-1c expression on left ventricular contractile parameters, hemodynamic and pressure volume loops were recorded during steady state, using a 1.4-French scale Millar catheter. Mice were anesthetized with sodium pentobarbital (80 mg/kg) and the right carotid artery was cannulated with a microtip pressure transducer catheter (SPR-839, Millar Instruments) connected to the MPVS-300 pressure-volume signal conditioning hardware, which provided analog outputs of ventricular pressure and volume signals for data acquisition over time. Analysis of the data was carried out, using Millar's PVAN software (Version 3.5).

Two dimensional (2D) gel electrophoresis and image analysis

Hearts were dounce-homogenized at 4°C in a buffer consisting of 10 mmol/L Tris, supplemented with protease (Roche) and phosphatase inhibitor cocktails (Sigma). Cardiac homogenates were centrifuged at 800 x g for 10 min at 4°C and the supernatant was subsequently

diluted 1:1 with extraction/lysis buffer (9 mol/L urea, 4% CHAPS, 0.1% SDS). 150 μg of protein per sample was then separated by 2D electrophoresis, using pre-cast immobilized pH gradient strips (Amersham), with pH ranges 3-10, as previously described (5). Protein concentration was determined, using the Non-Interfering Protein Assay (G-Biosciences, St Louis, USA). For isoelectric focusing (IEF), the samples were loaded on the immobilized pH gradient strip by in-gel sample rehydration, using GE healthcare destreak rehydration solution (GE healthcare) as a solubilizing agent overnight. Isoelectric focusing on an multhiphor II isoelectrofocusing unit (Amersham/GE Healthcare) was conducted at 20°C, focused for 18:31 hours (0-300 V in 1 min (gradient), 300 V for 6 h; 300-3500 V in 1:30 h (gradient), 3500 V for 12 h) for a total of 47.5 kVhr. IPG strips were equilibrated 2 times for 15 min in 40 ml of equilibration buffer with DTT and 1 time for 15 min with equilibration buffer plus iodoacetic acid (IAA). The equilibration buffer consisted of: 6 mol/L urea, 112 mmol/L Tris/acetate, 30% v/v glycerol, 5% w/v SDS, 0.01% w/v bromophenol blue and freshly added DTT (2% w/v) or IAA (2.5% w/v); pH= 8.8). Strips were embedded on top of 10% T (total monomer), 2.2% C (cross-linking agent) duracryl double gels (220 x 220 x 1.5 mm; Nextgen Sciences Inc) after filling the chambers of the vertical SDS-PAGE unit (Genomic Solution) with appropriate buffers (anode buffer: 210 mmol/L Tris/acetate, pH=8.9; cathode buffer: 100 mmol/L Tris, 100 mmol/L tricine, 0.1% w/v SDS). The second dimensional electrophoresis was carried out at 8°C with constant current (50 mA per gel) for approximately 6 hours. The protein spots were visualized by either silver staining, using a Silver stain kit (Nextgen Sciences Inc) or ProQ-Diamond staining (Invitrogen/Molecular Probes). ProQ-stained gels were scanned with a FUJI FLA-5100 fluorescence imager (532nm excitation laser, FUJI LPG filter at 100 μm resolution). Silver-stained gels were scanned as 16 bit grayscale TIF images, using Image Scanner II, (Amersham/GE Healthcare) and digital

quantified with the differential display image analysis software Progenesis Same Spots (Nonlinear Dynamics Ltd). The value for each spot was calculated as a proportion of the total spot volume of all spots in the gel, following background subtraction and removal of other artifacts. Differential expression between the groups was determined as a fold-change and proteins with the most appropriated change in expression between the groups were selected for analysis by mass spectrometry.

Protein digestion and peptide mass spectrometry

Protein spots were excised, destained, dehydrated from the gels and subjected to a tryptic digestion as originally described by Shevchenko (6) with modifications as presented by Jarrold (7). The extracted peptides were concentrated in a Speed-Vac centrifuge (Savant) to a final volume of 10-15 µl. Peptides were desalted and purified utilizing the C18 ZipTips® (Millipore). Purified peptides were eluted with 2.5 µl of 0.3% trifluoroacetic acid in 60% v/v acetonitrile, followed by peptide concentration to a final volume of 0.5 µl by Speed-Vac centrifugation. Subsequently, 1 µl of matrix solution (0.1% trifluoroacetic acid in 50% v/v acetonitrile + 5 mmol/L ammonium phosphate monobasic + 5 µg/µl α-cyano-4-hydroxycinnamic acid) was added to each sample and transferred directly onto a matrix assisted laser desorption/ionization (MALDI) target plate (Applied Biosystems). MALDI-MS/MS analysis was performed on a 4800 MALDI-TOF-TOF instrument from Applied Biosystems operated in reflector positive mode followed by automate transition to a 2kV MS/MS mode to produce fragmentation spectra on the 15 most abundant peptides in each sample. Proteins were subsequently identified from the MS/MS fragmentation spectrum, using the GPSExplorer analysis software (Applied Biosystems) coupled to an in house MASCOT server (Matrix Science). Criteria for protein identification

included peptide composite ion scores of greater than 100 with a minimum of 2 MS/MS spectra per protein.

Global ischemia ex vivo

The role of I-1c in ischemic injury was assessed, using an isolated perfused heart model, as described previously (8, 9). Briefly, WT and DTG hearts were mounted on the Langendorff apparatus and perfused with Krebs-Henseleit physiological solution. A plastic, water-filled balloon was inserted in the left ventricle via the mitral valve and inflated to yield a left ventricular end-diastolic pressure (LVEDP) of 5-10 mmHg. The balloon was attached to a pressure transducer connected to a Heart Performing Analyzer (Micro-Med) and left ventricular developed pressure (LVDP) was monitored continuously. After a 30-min stabilization period, the hearts were subjected to 40 min of no-flow, global ischemia, followed by 15-120 min of reperfusion. Hearts were paced at 400 bpm except during ischemia.

Regional ischemia in vivo

I/R injury was induced *in vivo*, as described previously (8, 9). Briefly, mice were subjected to myocardial I/R by ligating the left anterior descending coronary artery (LAD) for 30 min, followed by release of the ligation. Contractile function was assessed after 60 min of reperfusion, using open-chested catheterization *in vivo*. Analysis of the data was carried out, using SonoSOFT software (Version 3.4.45). In separate experiments, the hearts were subjected to 30 min of ischemia and allowed to reperfuse for 24 hrs to assess infarct size. Specifically, the heart was removed from the animal, the aorta was cannulated and the heart was perfused with 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC). The occluder, which had been left in place, was

re-tied and the heart was perfused with 5% phtalo blue. Hearts were frozen and cut into 5 or 6 transverse sections, with one section made at the site of the ligature. The slices were analyzed for infarct area, area at risk and total left ventricular area, using ImageJ software, available through the National Institute of Health (NIH). The infarct size was then expressed as a percentage of the area at risk.

Lactate dehydrogenase (LDH) release

Release of LDH was measured from the outflow of perfused hearts after I/R, using an assay kit, according to manufacturer's instructions (Sigma). In particular, 100 µl of LDH assay mixture, consisting of equal volumes of LDH assay substrate, cofactor and dye solution, were added to 50 µl of perfusate in a 96-well plate. The plate was incubated in the dark for 30 min. The reaction was quenched by addition of 15 µl of 1N HCl and absorbance was measured in a spectrophotometer at 490 nm. Values were normalized to the volume of the effluent and the heart weight.

DNA fragmentation

DNA fragmentation was assessed, using a commercially available ELISA kit (Roche Applied Science), which measures cytosolic mono- and oligo-nucleosomes, as described previously (8, 9). Briefly, 50 µg of heart homogenate was combined with the immunoreagent in streptavidin-coated plates and incubated for 2 hours at room temperature. After extensive washing, color development was initiated with 100 µl of the colorimetric substrate, ABTS. The extent of apoptosis was quantified, using an ELISA plate reader at 405 nm.

Caspase activity

The activities of caspase-9 and 12 were assessed in a fluorometric assay, according to the manufacturer's instructions (Biovision). In particular, 100 μg of protein were diluted in 50 μl of cell lysis buffer and incubated on ice for 10 min. 50 μl of 2X Reaction buffer (containing 10 mmol/L DDT) were added to the samples. The reaction was initiated with addition of 5 μl of the caspase-specific fluorescent substrate and the reaction was carried out at 37^{o}C for 2 hours in the dark. Caspase activity was quantified in a fluorometer (excitation: 400 nm, emission: 505 nm).

Isolation of adult rat cardiomyocytes and simulated ischemia/reperfusion

Animals were handled according to the Institutional Animal Care and Use Committee at the University of Cincinnati. Myocytes from adult male Sprague-Dawley rats (~300 grams) were isolated by collagenase digestion, as previously described (1, 10). Myocytes were resuspended in modified culture medium (M199, Sigma), counted and plated on laminin-coated plates or dishes for 2 hours at 37°C in a humidified, 5% CO_2 incubator and subsequently infected with the adenoviruses at a multiplicity of infection (MOI) of 500 for 2 hours. Construction of the viruses has been previously described (11). At 24 hours post-infection, the cells were subjected to 1 hour of ischemia followed by 3 hours of reperfusion, as previously described (10). Specifically, the media was replaced with ischemic buffer containing 1.13 mmol/L $CaCl_2$, 5 mmol/L KCl, 0.3 mmol/L KH_2PO_4, 0.5 mmol/L $MgCl_2$, 0.4 mmol/L MgSO4, 128 mmol/L NaCl, 4 mmol/L $NaHCO_3$, 10 mmol/L HEPES, pH=6.8, and placed in a chamber mimicking the hypoxic (1% O_2) and hypercapnic conditions (20% CO_2), observed during ischemia. The ischemic buffer was replaced with normal media and the cells were placed back in the humidified chamber at

atmospheric conditions, to allow reperfusion for 3 hours. The cells were harvested and stored at -80^{o}C until further analysis.

Statistical Analysis

All the values are expressed as mean ± SEM for n experiments. Comparisons between two groups were evaluated by Student's t-test for unpaired data. Statistical analysis of multiple groups was carried out by one-way ANOVA, with a Tukey test for *post-hoc* analysis. Results were considered statistically significant at $p< 0.05$.

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Online Figure I: Expression of I-1c in the adult mouse heart enhances basal contractility.

Analysis of contractile parameters indicated that the maximum rates of contraction **(A)** and relaxation **(B)** were increased, while the time constant of relaxation (Tau) was decreased **(C)** by I-1c expression, in a temporal manner. 4 weeks of expression elicited siginificant increases in contractile parameters, similar to DTG mice without Dox. TG2 mice, carrying only the I-1c transgene and DTG mice kept on a Dox regimen displayed contractile parameters similar to WT, suggesting no leakage of the TG2 attenuated promoter. WT, n=8; TG2, n=7 and DTG, n=6, *$p<0.05$ vs. WT; #: $p<0.05$ DTG vs. DTG with no Dox.

Online Table I: Phospho-proteomic analysis of I-1c hearts

Phospho-proteomic analysis revealed that four phospho-protein and eight protein spots were altered in I-1c hearts (Figure 5). Mass spectroscopy analysis positively identified ten out of these twelve spots. The spot number, identity (ID) and fold change are indicated in the table. WT, n=3, DTG, n=3.

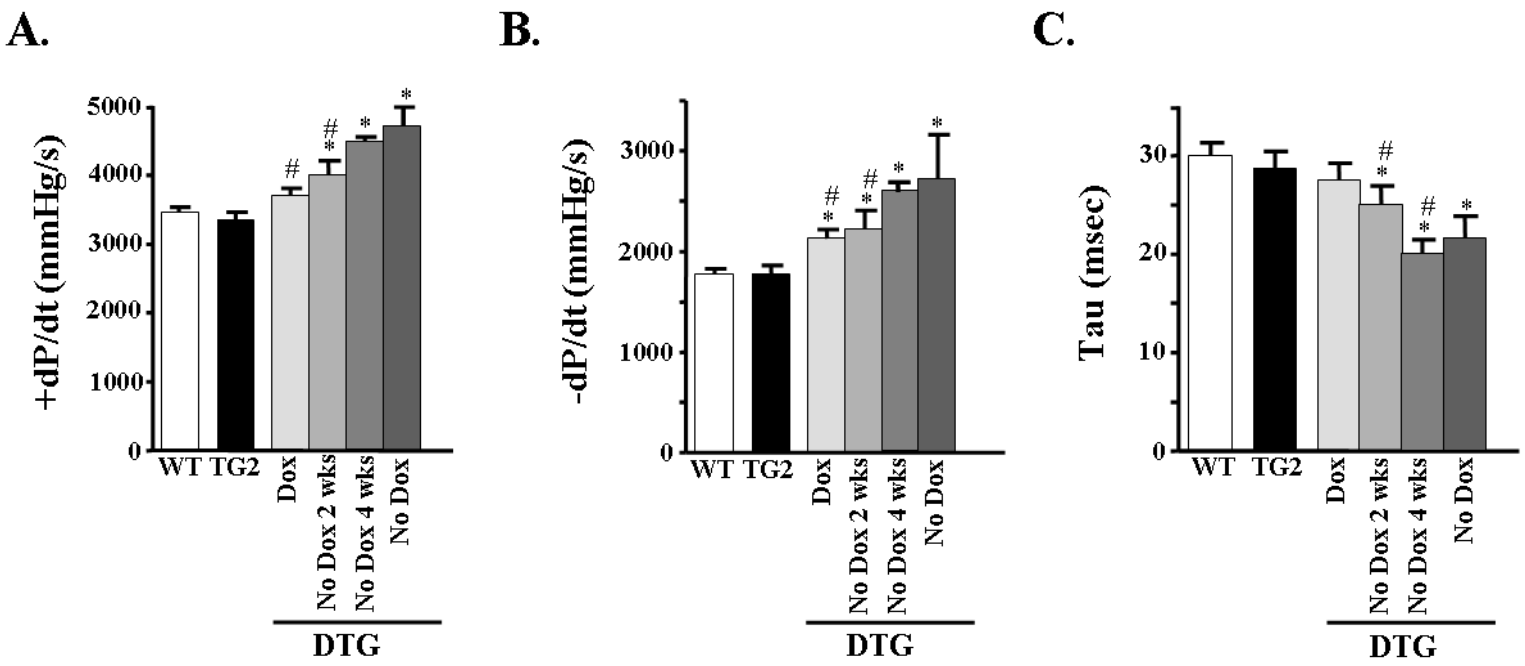
Online Figure II: I-1c expression improves post-ischemic functional recovery, during early reperfusion in isolated perfused hearts.

After 30 min of stabilization, hearts were subjected to 40 min of ischemia followed by 60 min of reperfusion. The graphs represent the percent recovery of the rates of contraction **(A)** and relaxation **(B)**, normalized to pre-ischemic values, in WT and DTG hearts. Values represent mean ± SEM; WT, n=9, DTG, n=10; *$p<0.05$ vs. WT.

Online Figure III: I-1c expression does not alter the protein or phosphorylation levels of Bad and caspase-9 activity after I/R *ex vivo*.

A. Immunoblot analysis revealed that the protein levels of Bad were reduced after I/R, to a similar extent in WT and DTG Langendorff-perfused hearts. Phosphorylation of Bad was unaltered by the I/R treatment and was similar between the two groups. **B.** Quantitative analysis of the immunoblots shown in (A), normalized to Bad protein levels and actin, for Bad phosphorylation and Bad protein levels, respectively. **C.** Caspase-9 activity was enhanced to a similar extent in both WT and DTG hearts, in the post-ischemic heart. Bars represent mean ± SEM; WT, n=4, DTG, n=4; *$p<0.05$ vs. WT Basal.

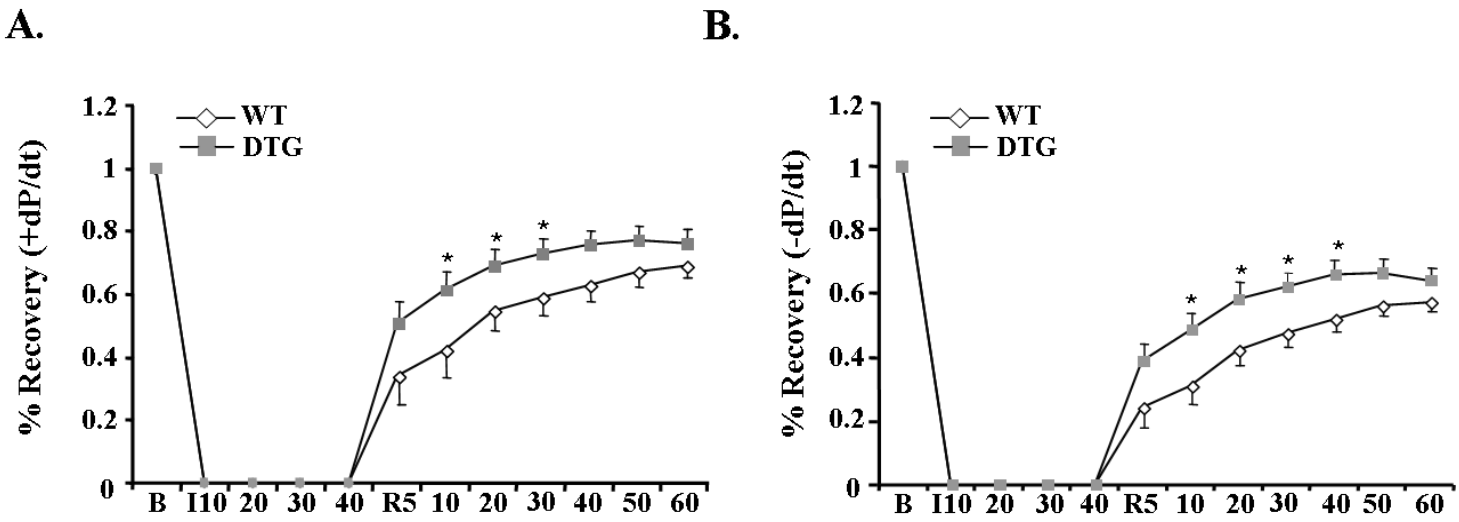
Online Figure I



Online Table I

Spot #	ID	Fold-change
6	contrapsin	+1.52
2	Tu translation elongation factor, mitochondrial	+1.67
34	enoyl coenzyme A hydratase 1, peroxisomal	+75.2
18	inner membrane protein, mitochondrial	+1.96
9	inner membrane protein, mitochondrial	+1.91
8	inner membrane protein, mitochondrial	+1.68
25	electron transferring flavoprotein, dehydrogenase	+1.62
35	glucose phosphate isomerase 1	-1.54
30	creatine kinase, mitochondrial 2	+1.60
4	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	+2.14

Online Figure II



Online Figure III