Detailed Methods

Transgenic mice

All mice used in this study are in the FVB/N inbred background. The creation and characterization of the tg mice with expression of a modified green fluorescence protein (GFPdgn) have been previously described.[1] GFPdgn was engineered via carboxyl fusion of degron CL1 to an enhanced green fluorescence protein (GFP) and is a proven substrate for the UPS.[1, 2]

To establish a genetic model of CR-PSMI, we created stable tg mouse lines that overexpress a missense mutation (T60A) of the murine precursor of β5 subunit of the 20S proteasome (hereafter known as T60A-β5) under the control of an attenuated murine *mhc6* promoter that was generously donated by Dr. Jeffrey Robbins of University of Cincinnati (Cincinnati, OH). The promoter consists of a full-length murine *mhc6* promoter in which 3 GATA sites and 2 TREs (thyroid response elements) were ablated but other *cis*-acting regions important for cardiac-specific expression were left intact.[3]

LV pressure–volume relationship analysis

For the invasive assessment of pressure–volume (P-V) relationship, mice were anesthetized with isoflurane (2%) in medical grade oxygen, then intubated and ventilated using a pressure controlled respirator at a tidal volume of 200 μl and a frequency of 130 strokes/minute. Body temperature was monitored with a rectal thermometer and maintained at 37°C. The LV was catheterized in a retrograde fashion via the right carotid artery with a 1.2-F mouse P-V catheter (Scisense, London, Ontario). The instrumented animal was stabilized for 10min and the data were recorded with a sampling rate of 1,500 Hz with Ponemah software (Data Sciences International, Valley View, OH) during steady-state conditions. For subsequent analysis of P-V loops Ponemah software was used. The raw conductance volumes were corrected for parallel conductance by the hypertonic saline bolus.

Proteasome peptidase activity assays

Myocardial samples were homogenized at 4°C in 10 volumes HEPES buffer (50 mM, pH 7.5) containing: KCl 20 mM, $MgCl_2$ 5mM, DTT 1mM. Cell debris was removed by centrifugation for 15 minutes at 12,000 g, and the supernatants were immediately used for protein concentration assay and then determination of peptidase activities. The following synthetic fluorogenic substrates: Suc-LLVY-AMC (18μM), Z-LLE-AMC (45μM) (CALBIOCHEM, San Diego, CA), and Ac-RLR-AMC (40μM, BIOMOL Plymouth Meeting, PA) were used respectively for measuring chymotrypsin-like, caspase-like, and trypsin-like peptidase activities in the absence or presence of a proteasome inhibitor, MG-132 (20μM, A.G. Scientific, Inc. San Diego, CA) for chymotrypsin-like and caspase-like activities, or epoxomycin (5μM, CALBIOCHEM, San Diego, CA) for trypsin-like activity. Measurements of each specimen are performed in triplicates. A 5μg of crude protein extract is added to 200μl of the HEPES buffer containing the fluorogenic substrate to each well in 96-well plates, and incubated at 37°C. The fluorescence intensity was measured at 60min of incubation using a Perkin Elmer 2030 Multilabel Microplate Reader with the excitation wave length of 380nm and emission wave length at 460nm. The portion of peptidase activity inhibited by the proteasome inhibitor is attributed to the proteasome.

Western blot analysis

This was performed as previously described.[4] Crude proteins were extracted from myocardial tissues. The protein concentration was determined using bicinchoninic acid (BCA) reagents (Pierce biotechnology, Rockford, IL). Equal amounts of samples were subjected to sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF

membrane using a Trans-blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat-dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 hour at room temperature and then probed with appropriate primary antibodies overnight at 4˚C. Primary antibodies include: ubiquitin (U5379, Sigma-Aldrich, Saint Louis, MO), GAPDH (G8795, Sigma-Aldrich), GFP(B2) (sc-9996, Santa Cruz Biotechnology), β-tubulin (sc-55529, Santa Cruz), murine proteasome β5 subunit (customized), c-Myc (MB600-336, Novus Biological, Littleton, CO), cleaved caspase 3, total Akt (9272S, Cell Signaling), Ser473-phosporylated Akt (9271S, Cell Signaling), and PKCδ (sc-213, Santa Cruz) and GAPDH (G8795, Sigma). The corresponding horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were used respectively for chemiluminescence-based western blot analyses. The bound secondary antibodies were detected using either enhanced chemiluminescence (ECL-Plus) detection reagents (GE Healthcare, Piscataway, NJ) or, for weak signals, ECL Advance Western Blotting Kit (GE Healthcare) and visualized with a VersaDoc3000 imaging system (Bio-Rad). The signal was quantified with the Quantity One software (Bio-Rad).

Immunostaining and fluorescence confocal microscopy

These were performed as previously described.[4] Mouse tissues were fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) by perfusion fixation, saturated with 40% sucrose solution and embedded in Tissue-Tek O.C.T. (Sakura Finetek. USA, Inc, Torrance, CA), and then underwent tissue sectioning at 5 micron thickness. The tissue cryosections were permeabilized with 1% of Triton-X100 in PBS for 1 hour, quenched with 0.1M glycine in PBS for 1 hour, and blocked with 0.5% BSA for 1 hour. The specimens were then incubated with primary antibodies overnight at 4°C. The primary antibodies used for immunostaining include: rabbit anti-c-Myc (MB600-336, Novus Biological) and mouse monoclonal anti-sarcomeric α-actinin (A5044, Sigma). The secondary antibodies used are the Alexa-Fluor 488 donkey anti-rabbit Ig, and the Alexa-Fluor 568 donkey anti-mouse Ig (Molecular Probes). The immunostaining was visualized using a fluorescence confocal microscope (Olympus Fluoview 500) and the images were captured and digitalized using the associated software.

Semi-quantitative reverse transcription-(RT-) polymerase chain reaction (PCR) analyses

These were performed as we previously described.[5] Total RNA was isolated from ventricular myocardium tissue using the TRI-Reagent (Molecular Research Center, Inc., Cincinnati, CA) following the manufacturer's protocol. The concentration of RNA was determined using Agilent RNA 6000 Nano assay (Agilent technologies, Inc. Germany) following the manufacturer's protocol.

Semi-quantitative RT-PCR is a PCR amplification technique that employs both reverse transcriptase and thermostable Taq DNA polymerase. The first step is reverse transcription which synthesizes complementary DNA (cDNA) from the total mRNA template. The subsequent step is traditional PCR amplifying the synthesized cDNA to detectable levels. For reverse transcription reaction, 1μg of RNA was used as a template to generate cDNA using the SuperScript III First-Strand Synthesis kit (Invitrogen) and was carried out according to the manufacturer's instructions. For PCR amplification reaction, 2μl of solution resulting from the reverse transcription reaction and specific primers towards the gene of interest were used. The transcript levels of GPFdgn were semi-quantitated by PCR at the minimum of cycles that can detect PCR products. The sequences of the specific primers were as follows, GFPdgn: forward 5'-TCT ATA TCA TGG CCG ACA AGC AGA-3' and reverse 5'-ACT GGG TGC TCA GGT AGT GGT TGT-3'; GAPDH: forward 5'-GCC GTA TTG GGC GCC TGG TCA-3' and reverse 5'-AAC

ATA CTC AGC ACC GGC CTT ACCC-3'. Relative mRNA levels were normalized with GAPDH mRNA levels.

In vivo myocardial I/R and assessment of Infarct Size:
These were carried out as we previously described.[6] Mice were anesthetized with isoflurane and ventilated through intubation. A thoracotomy was performed at the fourth intercostal space to expose the heart. I/R was produced in mice by surgical ligation of the left anterior descending coronary artery (LAD) for 30min followed by releasing of the ligation for 24h. At the end of reperfusion, the LAD was re-ligated at the original position and the heart was perfused with phthalocyanine blue and cut transversely into 6 slices. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) at 37^{o}C for 15 minutes. Each section was weighed and digitally photographed. The phthalocyanine blue–stained area is defined as the remote area (RA), the area unstained by phthalo blue is known as the area at risk (AAR), the TTC unstained area (white) is the infarct area. The RA, the AAR, the infarct area, and the total ventricular areas from both sides of each section were measured using Image-Pro plus software. The AAR was expressed as the percentage of the total ventricular weight and the infarct size was expressed as a percentage of the AAR. For the sham surgery group, the same surgical procedures were performed except for the LAD ligation part.

Immunoprecipitation of 20S proteasomes
Ventricular myocardial samples from T60A-β5 Ntg and Tg littermate mice at the baseline condition were individually homogenized in RIPA buffer and centrifuged at 10,000xg in 4^{o}C for 10 minutes. The supernatant (the crude protein extracts) was collected and protein concentration is determined using the BCA reagents. The antibody specifically against α3-subunit of the 20S proteasome, generously donated by Dr. Peipei Ping of UCLA,[7] was used to immunoprecipitate the 20S proteasome.[7] The precipitates were then subject to SDS-PAGE and immunoblot using a primary antibody specifically against murine β5 (binds to both endogenous and tg T60A-β5; customized) subunits of the 20S proteasome, c-Myc (binds to tg myc-tagged T60A-β5; MB600-336, Novus Biological, Littleton, CO), or α4 subunit of the 20S (Enzo Life Sciences, Inc. Farmingdale, NY). The reason to probe α4 is to make sure a comparable amount of immunoprecipitated 20S proteasomes are compared between the Tg and Ntg groups.

Terminal deoxynucleotidyl transferase end-labeling (TUNEL) assay
This was performed as previously described.[4] The heart was excised 24 hours after reperfusion. Two tissue samples corresponding respectively to the AAR and the remote area (RA) from the LV free wall of each animal were collected (n=6mice/group), fixed in 4% paraformaldehyde in PBS for 24h at 4°C, embedded in Tissue-Tek O.C.T. Compound (Sakura finetek. USA, Inc, Torrance, CA), and frozen and stored at -80°C before sectioning.

TUNEL staining was performed on 5μm cryosections using the In Situ Cell Death Detection kit (Roche Diagnostics) as previously described.[4] Images were captured with a confocal fluorescence microscope (Olympus Fluoview 500) in 3 fields for each zone of each animal using a 40x objective. The number of TUNEL positive cells (green) was analyzed using Image-pro Plus 4.5 software, expressed as a percentage of the total nuclei population. Cardiomyocytes (red) were stained with phalloidin labeling, and nuclei (blue) were stained by DAPI.

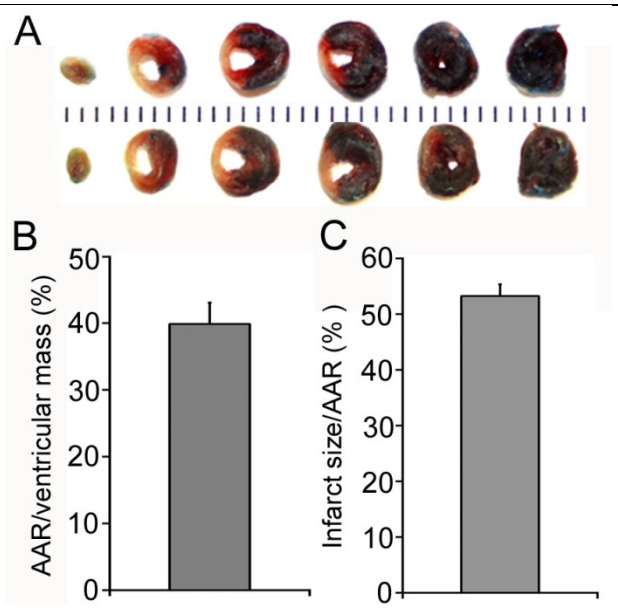
Statistical Analysis
All quantitative data were presented as mean ± S.D. Differences between groups were evaluated for significance using Student's t-test for unpaired two group comparison or one-way

or two-way ANOVA followed by the Scheffé's test when appropriate. The p-value <0.05 is considered statistically significant.

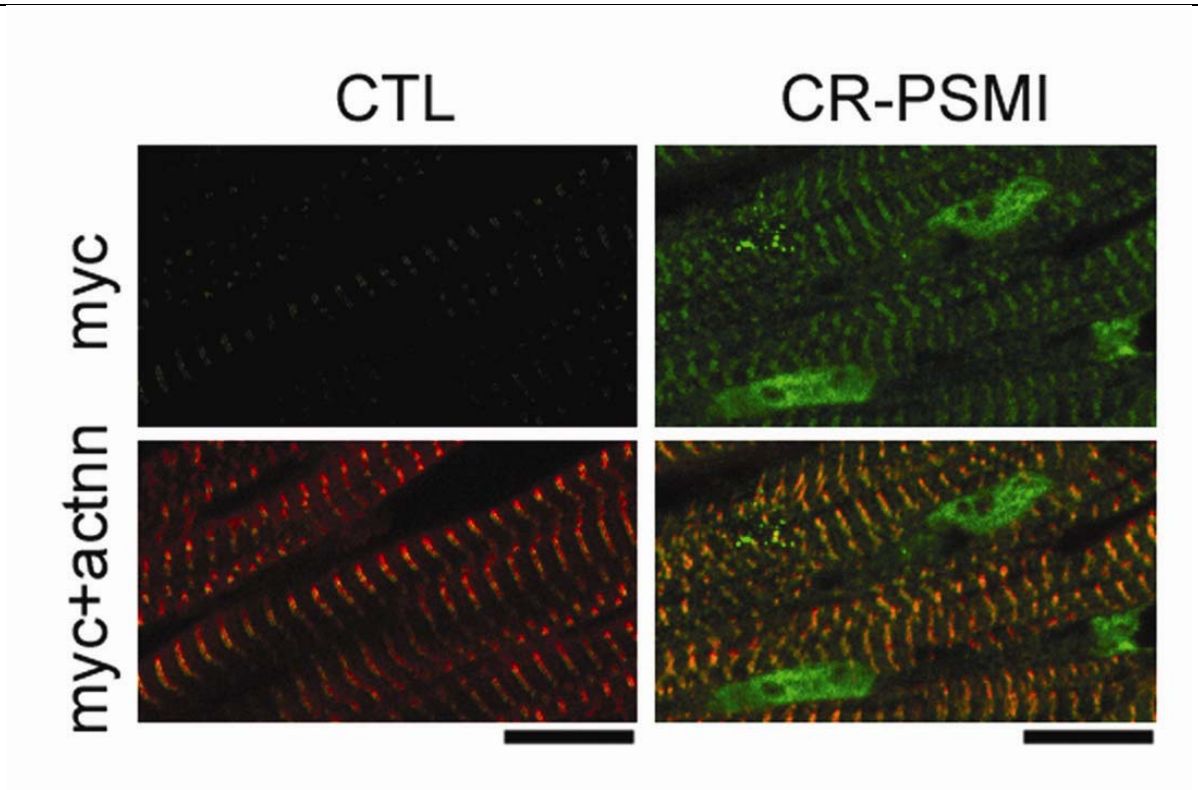
References for Supplemental Methods

1. Kumarapeli AR, Horak KM, Glasford JW, Li J, Chen Q, Liu J, Zheng H, Wang X. A novel transgenic mouse model reveals deregulation of the ubiquitin-proteasome system in the heart by doxorubicin. *FASEB J*. 2005;19:2051-2053
2. Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*. 2001;292:1552-1555
3. Sanbe A, Gulick J, Hanks MC, Liang Q, Osinska H, Robbins J. Reengineering inducible cardiac-specific transgenesis with an attenuated myosin heavy chain promoter. *Circ Res*. 2003;92:609-616
4. Su H, Li J, Menon S, Liu J, Kumarapeli AR, Wei N, Wang X. Perturbation of cullin deneddylation via conditional csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice. *Circ Res*. 2011;108:40-50
5. Zheng Q, Su H, Ranek MJ, Wang X. Autophagy and p62 in cardiac proteinopathy. *Circ Res*. 2011;109:296-308
6. Li J, Horak KM, Su H, Sanbe A, Robbins J, Wang X. Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice. *J Clin Invest*. 2011;121:3689-3700
7. Drews O, Tsukamoto O, Liem D, Streicher J, Wang Y, Ping P. Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. *Circ Res*. 2010;107:1094-1101

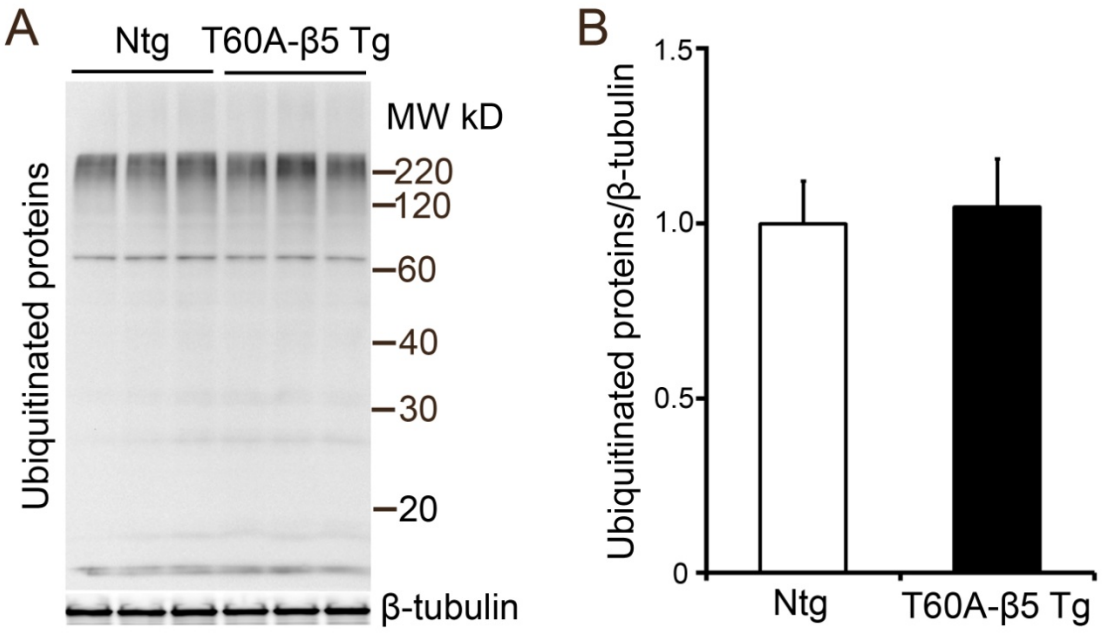
Supplementary Figures I ~ VI



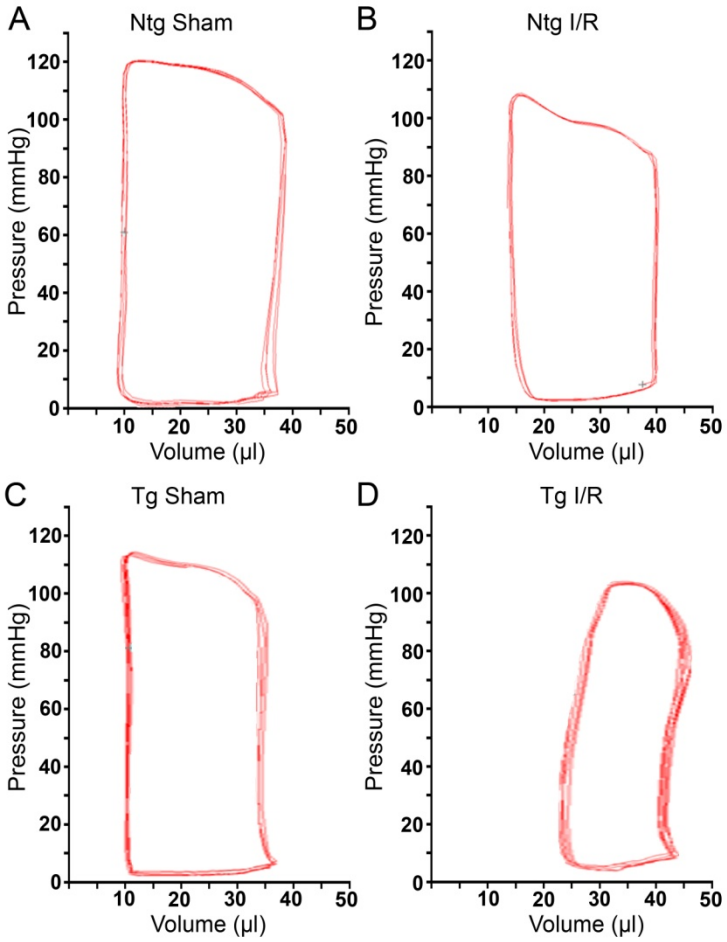
Online Figure I. Average area at risk (AAR) and infarct size in FVB/N mice induced by the ischemia/reperfusion (I/R) protocol used in the present study. Wild type FVB/N mice at ~10 weeks of age were subject to left anterior descending artery (LAD) ligation (ischemia) for 30min and subsequent release of the ligation (reperfusion) for 24 hours. The mice were sacrificed at 24 hours of reperfusion and the AAR and infarct size determination as described in the Methods section. **A**, TTC staining images of two representative hearts. **B** and **C**, A summary of the indicated parameters from a pilot study (n = 4 mice).



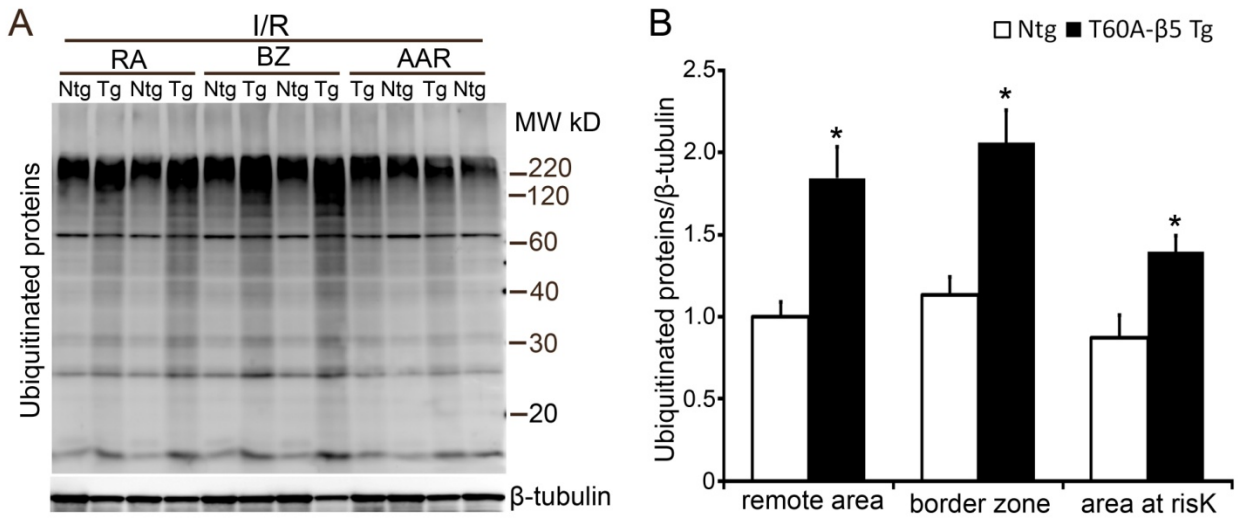
Online Figure II. Immunofluorescence confocal micrographs of the distribution of transgenic T60A-β5 proteins in ventricular myocardium. From a transgenic (tg) mouse line with overexpression of Myc-tagged T60A- β5 precursor under the control of an attenuated *mhc6* promoter and a non-tg (Ntg) littermate, perfusion-fixed ventricular myocardium was collected and subject to cryo-sectioning and immunofluorescence confocal microscopy. Representative confocal micrographs of ventricular myocardium immune-stained for Myc (green) and α-actinin (actnn, red) are shown. Scale bar=10μm.



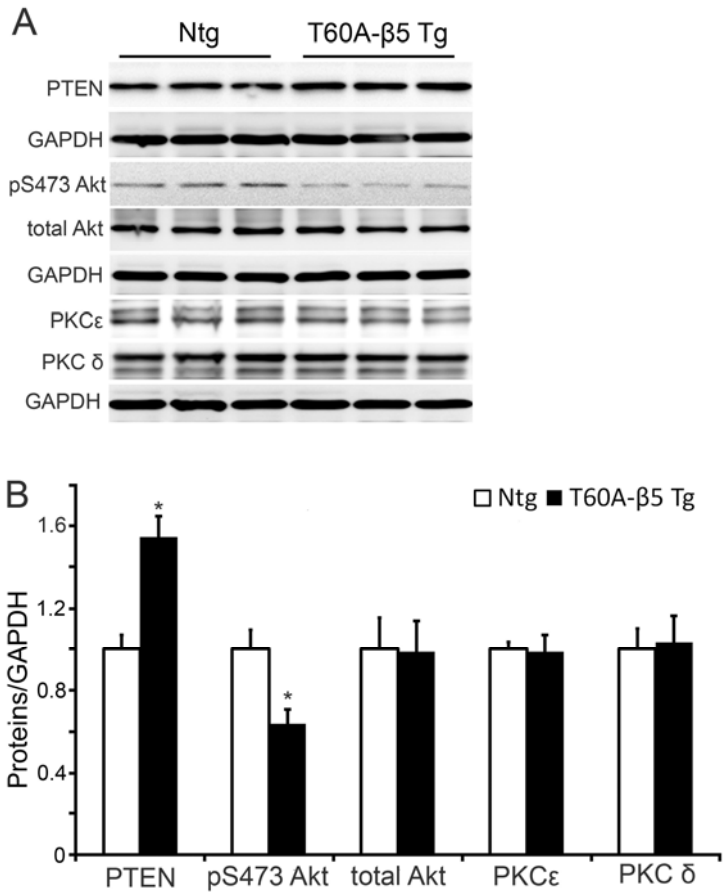
Online Figure III. Western blot analysis for total ubiquitinated proteins in ventricular myocardium of T60A-β5 tg and Ntg mice. Total protein extracts from ventricular myocardium of T60A-β5 Tg and Ntg littermate mice were subject to western blot analyses. β-tubulin was probed as loading controls. The level of total ubiquitinated proteins in ventricular myocardium was not statistically significant between T60A-β5 Tg and Ntg mice ($P>0.25$, t-test, n=6 mice/group).



Online Figure IV. Representative pressure-volume loops of T60A-β5 Ntg (A, B) and Tg (C, D) mice subject to I/R (B, D) or sham surgery (A, C). Data from detailed analyses of P-V loops are presented in Table 1 of the main text.



Online Figure V. Wester n blot analyses for total ubiquitinated proteins in the ventricular myocardium of T60A-β5 Tg and Ntg mice that were subject to myocardial ischemia/reperfusion (I/R). Acute focal I/R were induced as described in the main text. Representative images (**A**) and a bar graph summarizing the pooled densitometry data (**B**) are shown. *p<0.01 vs. Ntg, n=6 mice per group, ANOVA.



Online Figure VI. Wester n blot analyses for the indicated proteins in the ventricular myocardium of T60A-β5 Tg and Ntg mice at the baseline condition. Myocardial tissues were collected from the ventricle of T60A-β5 Tg and Ntg littermate mice at approximately 8 weeks of age. Total protein extracts were used for western blot analyses for the indicated proteins. GAPDH was probed for loading control. Representative images (**A**) and a bar graph showing pooled densitometry data (**B**) are presented. PTEN, phosphatase and tensin homolog; pS473-Akt, Ser473 phosphorylated Akt; PKCδ and PKCε, protein kinase C isoform δ and ε, respectively. *P<0.05 vs. Ntg, n=3 mice for each group, *t*-test.