Supplement Material

Methods and Materials

Morphological Analysis

Mouse hearts were fixed in 10% neutralized formalin and embedded in paraffin. Serial sections (5μm) were routinely stained with hematoxylin–eosin and Masson's Trichrome, examined under a light microscope (×400) and photographed for morphological analysis. Paraffin-embedded tissue was also examined for sarcolemma staining labeled with wheat germ agglutinin (WGA, Invitrogen), according to manufacturer's instruction.

2-Dimensional (2-D) Gel Electrophoresis

Mouse left ventricular tissue was isolated and proteins were extracted for 2-D gel electrophoresis by the Proteomics Lab of the Genome Research Institute, University of Cincinnati. 2-D gel images were analyzed using ImageMaster 2D Elite software. The protein spots of interest were excised and their tryptic peptides were subjected to MALDI-TOF or LC-MS/MS for identification.

Perfused Heart Experiments

Isolation and perfusion of mouse hearts by the Langendorff method was carried out, as described previously.[1] Briefly, hearts were retrograde-perfused with a phosphate-free Krebs-Henseleit buffer equilibrated at 37°C with 5% CO_2/95% O_2, pH 7.4. First, hearts were perfused at constant pressure (65cm H_2O) for a 30-minute stabilization period. Global ischemia was applied by eliminating flow for 45min, followed by 2h reperfusion. Contractility was assessed with a fluid-filled intraventricular balloon connected to a pressure transducer (Micro-Med Ltd). The end-diastolic pressure (EDP) was set to 5 to 10 mmHg. A bipolar electrode (NuMed) was inserted into the right atrium, and atrial pacing was performed at 400 bpm with a Grass S-5 stimulator. At the end of the perfusion period, hearts were either frozen rapidly in liquid nitrogen and stored at -80°C or immersed in 10% buffered formalin.

Pretreatment of Mouse Hearts with the Autophagy Inducer

$Hsp20^{S16A}$ or non-TG mice were divided into two groups (n=6): controls and rapamycin treated (autophagy inducer). Rapamycin (5ng total in 0.5ml saline[2]), or the corresponding volume of saline (controls) was administered intraperitoneally (i.p.), and 60min after injection animals were subjected to *ex vivo* ischemia/reperfusion, as described above.

In Vivo Ischemia/Reperfusion and Assessment of Myocardial Infarction

Ischemia/reperfusion was produced in $Hsp20^{S16A}$ and non-TG controls by transiently ligating the left anterior descending coronary artery (LAD). The mice underwent 30min of occlusion,

followed by 24 hours of reperfusion. Infarct assessment was performed, as described in our previous studies.[3] The heart was perfused with 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC). Afterwards, the occluder was retied and the heart was perfused with 5% phthalo blue. Hearts were transversely cut into 5 to 6 sections. Infarct size was expressed as the percentage of the infarct area (unstained by TTC) relative to the total area at risk (AAR, unstained by phthalo blue). The total AAR was calculated by adding all sections together, and then presented as percentage of the whole left ventricle in each heart.

Evaluation of Cardiac Injury and Apoptosis

Cardiac injury was assessed by measuring lactate dehydrogenase (LDH) release. Perfusion effluent was collected every 10 minutes of pre-ischemia and during reperfusion. Total LDH released from the heart was determined, using an In Vitro Toxicology Assay Kit (Sigma) and expressed as units per gram of wet heart weight. DNA fragmentation was analyzed using a Cell Death Detection ELISA plus kit (Roche), which quantified the cytoplasmic histone-associated DNA fragments (180 base pair nucleotides or multiples) in cardiac lysates. Results were normalized to the standard, provided in the kit, and expressed as a fold increase over control. For terminal dUTP nick end-labeling (TUNEL) assays, hearts were removed from the apparatus after ischemia/reperfusion, and the atrial tissue was dissected away. The ventricles were fixed in 10% buffered formalin and later embedded in paraffin according to standard procedures and 5 μm-thick sections were obtained to perform TUNEL assays using the DeadEnd™ Fluorometric TUNEL system (Promega, Madison, WI), according to the manufacturer's instructions. TUNEL-positive nuclei (green) were determined by randomly counting 10 fields of the midventricular section and were expressed as a percentage of the total nuclei population. Cardiomyocytes were stained with α-sarcomeric actin (1:50 dilution; Sigma) labeling; nuclei were stained by DAPI (Invitrogen). Sections were analyzed with a fluorescence microscope. Changes in the caspase-3 activity were studied using Western blot analysis. The presence of caspase-3 activation was assessed by the observation of a 17-kDa subunit, which was derived from the cleavage of the 32-kDa proenzyme caspase-3.

Western Blot

Western blot analysis was performed as previously described.[1] After blocking, the membranes were probed with specific antibodies against Hsp20 (Research Diagnostics, Inc), phospho-Ser16-Hsp20 (customized antibody[4] from Affinity BioReagents, Inc), αB-crystallin (Affinity BioReagents Inc), Hsp25 (Research Diagnostics, Inc), Beclin 1(BD Biosciences) and LC3 (Medical & Biological Laboratories). The ECL system (Amersham Biosciences, Inc) was used for detection.

Sucrose Gradient Electrophoresis

Frozen heart samples were homogenized in 10 mmol/L HEPES-buffered saline (pH=7.4) containing phosphatase inhibitor and proteinase inhibitor cocktail sets (Calbiochem) and

0.5% Triton X-100. After protein quantification, 100 μg of protein was loaded on a 2mL sucrose gradient (5% to 40%) and spun at 166,180 g for 5 hours with a TLS 55 rotor. Fractions (100 μL) were collected from the top and mixed with Laemmli buffer directly before denaturation and analysis by 12% SDS-PAGE for immunoblot analysis, as described.[5]

Co-immunoprecipitation

Association of phospho-Ser16-Hsp20 with Beclin-1 was studied by co-immunoprecipitation, as described previously.[6, 7] Briefly, protein lysates were extracted from wild type mouse cardiac homogenates with 1 × cell lysis buffer (Cell Signaling, #9803), which was supplemented with 1mM PMSF and protease inhibitor cocktail (Sigma), and centrifuged at 13,000rpm for 30 min at 4°C. The phospho-Ser16-Hsp20 or anti-Beclin 1 (BD Biosciences) antibody (4μg) was added into 1ml diluted cell lysates (1μg/μl) and incubated overnight on a rotary wheel at 4 ºC. Protein G PLUS agarose beads (Santa Cruz Bitotech) (1μg antibody/10 μl agarose beads) were added into the above mixture, and incubated for an additional 1-2 h at 4 ºC. Beads were sedimented and washed 6 times with the cell lysis buffer. Beads-bound proteins were dissolved in 2xSDS sample buffer, and boiled at 95 ºC for 5 min. Finally, the identity of proteins was determined by immunoblotting. Preimmunoprecipitated WT heart homogenate was used as positive control (+), and immunoprecipitate with anti-IgG PLUS agarose was used as negative control (–).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using a 2-tailed Student t-test for unpaired observations and ANOVA followed by the Bonferroni *post hoc* test for multiple comparisons (Systat 11). $P<0.05$ was considered statistically significant.

References

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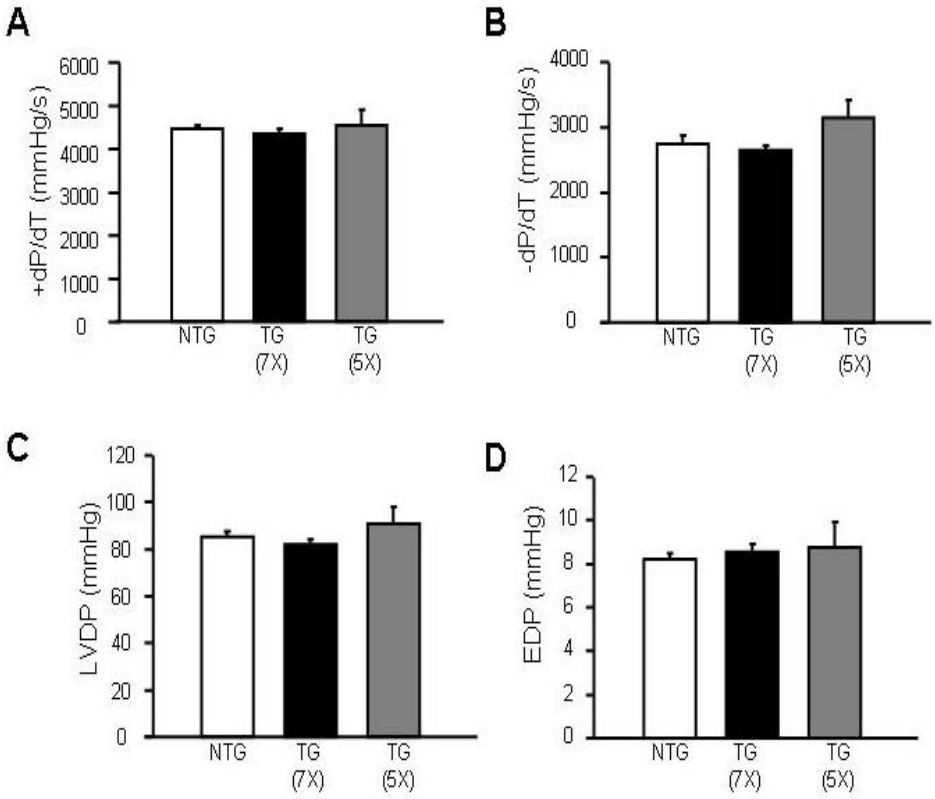
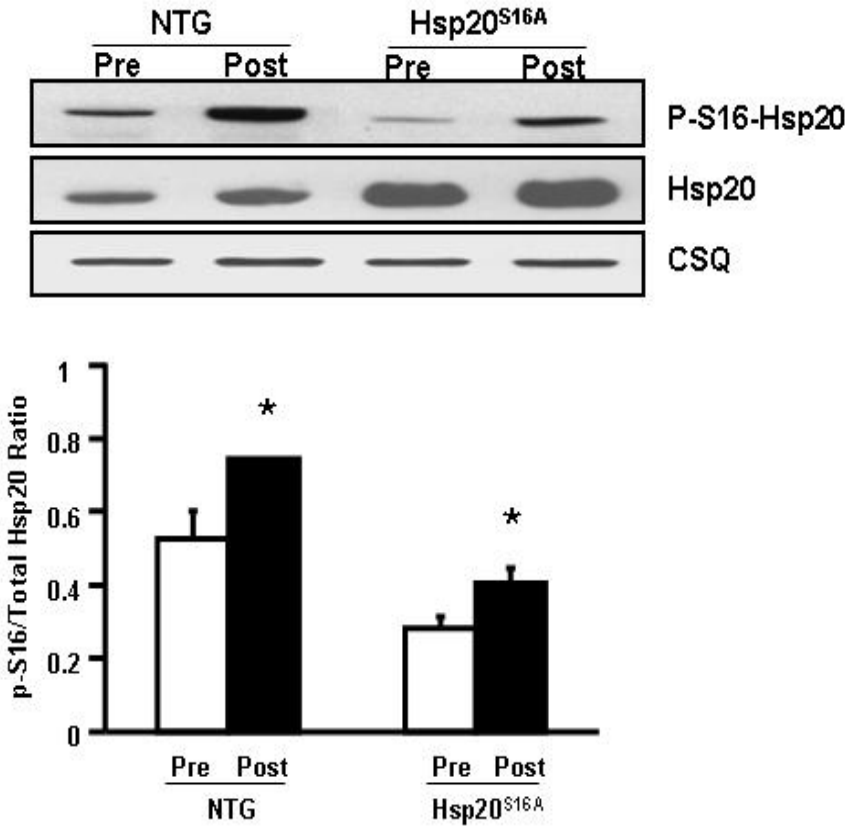
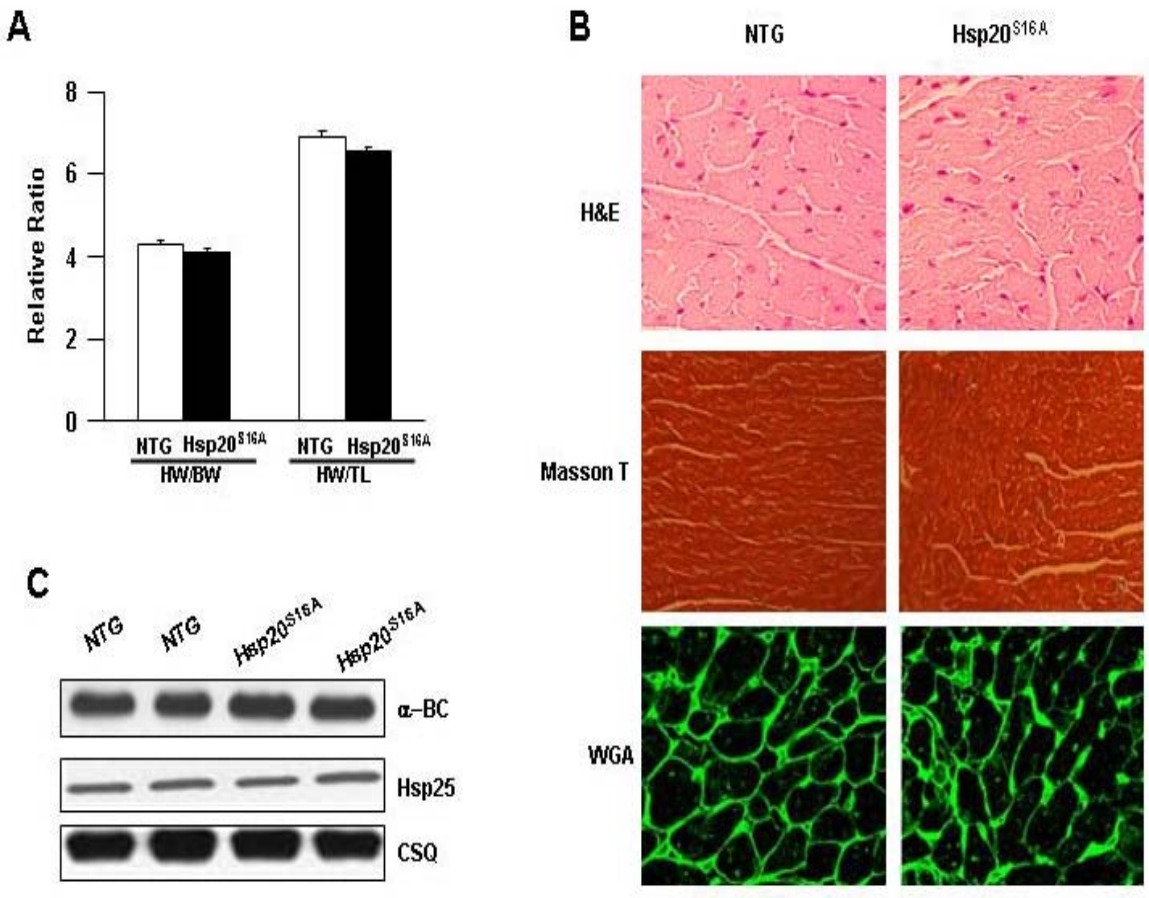


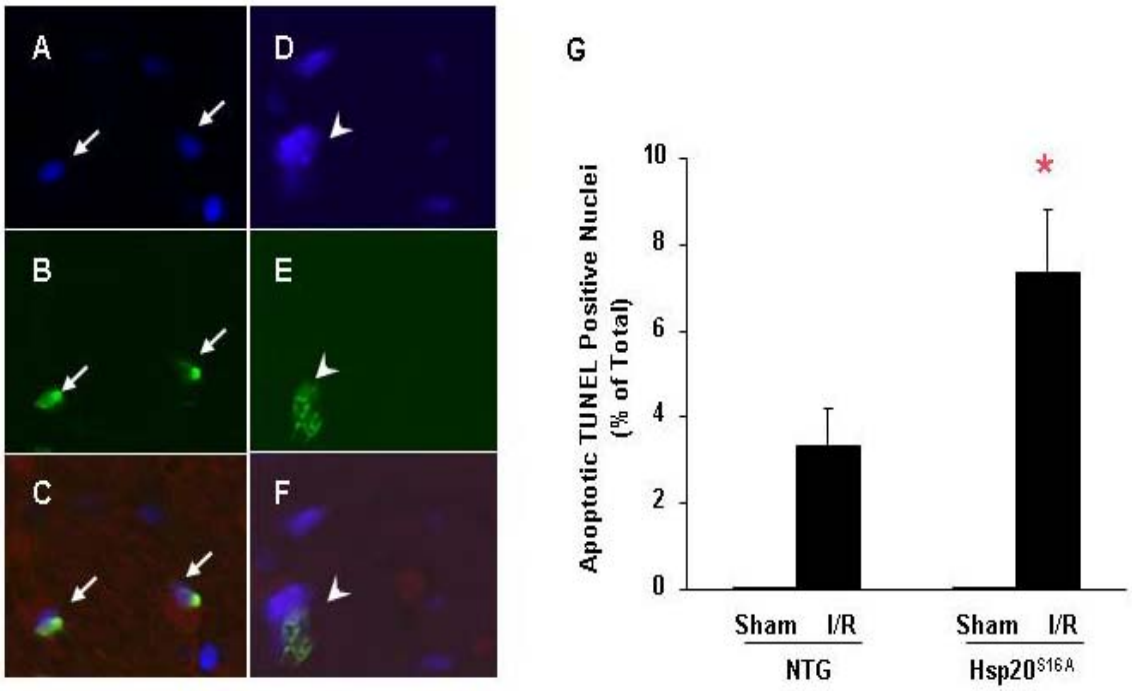
Online Figure I. **Basal function of non-transgenic and Hsp20^{S16A} hearts.** ± dP/dt (A, B), LVDP (C) and EDP (D) in Hsp20^{S16A} TG (7x or 5x overexpressing) hearts were not significantly different than those of non-TGs under basal conditions (non-TGs: n=10, TG: n=8; P>0.05, Hsp20^{S16A} vs. non-TG)**.** NTG: non-transgenic, TG: transgenic.



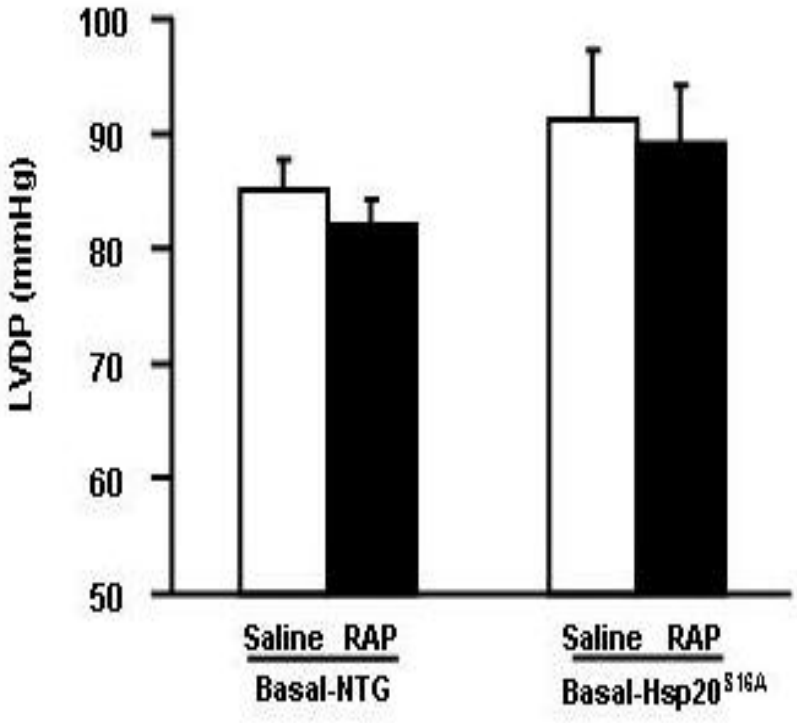
Online Figure II. Phosphorylation of Hsp20 in non-transgenic and Hsp20^{S16A} hearts before or after *ex vivo* ischemia/reperfusion injury. Before I/R, the ratio of phospho-Ser16 /total Hsp20 was decreased in Hsp20^{S16A} hearts, compared with non-transgenics ($P<0.05$). I/R induced higher level of phosphorylation of Hsp20 in non-transgenic, compared with Hsp20^{S16A} hearts myocardium (n=6, *: $P<0.01$)



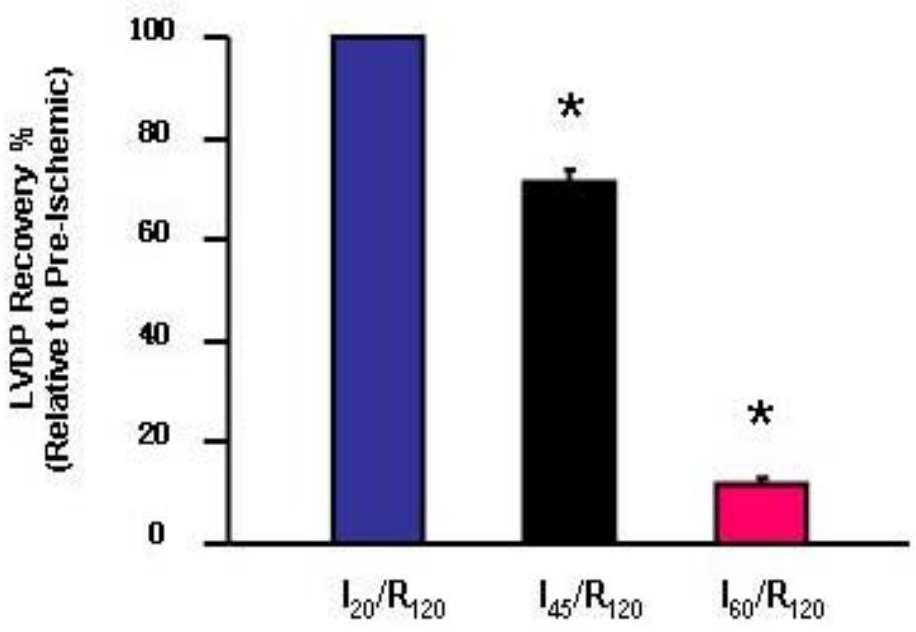
Online Figure III. **Characterization of Hsp20^{S16A} hearts.** (A) There is no difference in body weight or tibia length between non-TGs and Hsp20^{S16A} TGs (P>0.05). Heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios were similar between Hsp20^{S16A} TG mice and non-TGs at 12 weeks of age (n=10, P>0.05). (B) Ventricular sections from 12 week- old Hsp20^{S16A} mice stained with HE and Masson's T indicated no inflammation or cardiac fibrosis. WGA staining of Hsp20^{S16A} hearts showed similar myocyte size with non-TGs (n=6, P>0.05). (C) Hsp25 and αB-crystallin (αBC) were not altered in Hsp20^{S16A} hearts, compared with non-TGs (n=6, P>0.05).



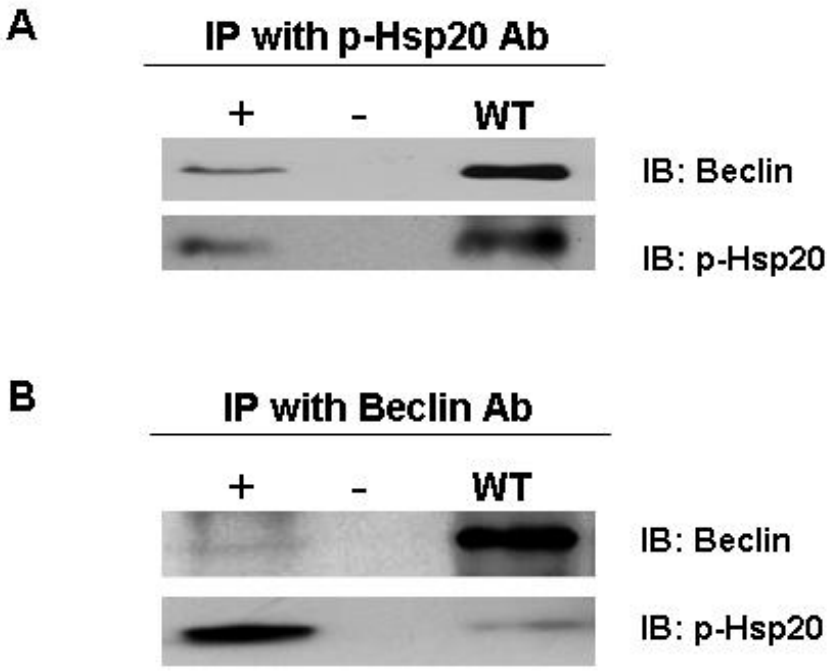
Online Figure IV. Evaluation of apoptosis in non-transgenic and Hsp20^{S16A} transgenic myocardium subjected to *in vivo* 30min ischemia, followed by 24h reperfusion. (A-F) Representative images of TUNEL-positive nuclei according to their apoptosis- and necrosis-like morphology. Arrows indicate TUNEL-positive nuclei with either condensed chromatin (apoptosis, A-C) or diffused chromatin distribution (necrosis, D-F). Blue-DAPI indicates nuclear counterstaining; green indicates TUNEL staining and red indicates actin staining of myocytes by Alexa 594 (Invitrogen). (G) The number of TUNEL- positive nuclei was evaluated in 10 fields for apoptosis-like appearance. Plotted were means±SEM for 3 animals per group.



Online Figure V. Basal function of Hsp20^{S16A} and non-TG hearts was not changed after pretreatment with rapamycin. After 1h of rapamycin administration, mouse hearts were subjected to Langendorff perfusion. After 30min of stabilization, LVDP was measured. There was no significant difference of LVDP between Hsp20^{S16A} and non-TG hearts, whether saline-treated or rapamycin-treated. (n=6, P>0.05)



Online Figure VI. **Functional recovery of wild type hearts under multiple ischemia/reperfusion protocols.** Wild type mouse hearts were subjected to different time courses of ischemia (20min, 45min and 60min) followed by 2h of reperfusion. Recovery of LVDP (expressed as percentage of pre-ischemic value) upon I20min/R120min, I40min/R120min, and I60min/R120min was 100%, 71.5±2.2%, and 11±1.4%, respectively. (n=6, *: P<0.01, vs. I20min/R120min).



Online Figure VII. Protein interaction of phospho-S16-Hsp20 with Beclin-1. Co-immunoprecipitation is performed by using cardiac homogenates of wild type mice and the anti-phosho-S16-Hsp20 (A) or anti-Beclin (B) antibodies. The precipitates were analyzed by immunoblotting with anti-p-Hsp20 or anti-Beclin antibodies, as indicated. Preimmunoprecipitated WT heart homogenate was used as positive control (+), and immunoprecipitate with anti-IgG PLUS agarose was used as negative control (–). IP, immunoprecipitation; IB, immunoblotting.