H11 Kinase/Hsp22 deletion impairs both nuclear and mitochondrial functions of STAT3 and accelerates the transition into heart failure upon cardiac overload

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SUPPLEMENTAL MATERIAL

Material and Methods

Surgical procedure in the mouse

Surgery was performed on 3 to 4 months-old WT and KO mice, anesthetized (ketamine 65 mg/ kg, xylazine 1.2 mg/kg, and acepromazine 2.17 mg/kg) and ventilated via tracheal intubation connected to a rodent ventilator with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths per minute. To generate pressure overload, mice were submitted to thoracic aortic constriction for three days or two weeks. The left chest was opened at the second intercostal space. The transverse thoracic aorta between the innominate artery and left common carotid artery was dissected. A 7-0 braided polyester suture was then tied around the aorta against a 28-gauge needle under microscopic control[1]. The chest was closed and the pneumothorax reduced. All mice survived the surgery and were included in the study. Age-matched sham-operated control animals underwent a similar surgery without constricting the aorta. Heart weight, LV weight, body weight and tibial length were measured in all cases. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Protein extraction and subcellular fractionation

Total protein extraction was performed at 4 ^{o}C in a lysis buffer (25 mmol/L Tris-HCl-pH 8.0, 150 mmol/L NaCl, 15 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.5% Triton X-100 and 5% glycerol) supplemented with protease, kinase and phosphatase inhibitors, followed by centrifugation at 12,000 x *g* for 20 min at 4^{o}C. Subcellular fractions were prepared by differential centrifugation of tissue homogenized manually in hypotonic buffer. After an initial spin at 100*g* to discard the cellular debris and unbroken cells, the nuclear fraction was obtained from low-speed centrifugation (500 *g*), followed by three washes with phosphate-buffered saline. Mitochondrial fraction was isolated as described below. The cytosolic fraction was collected as the supernatant resulting from the ultracentrifugation (100,000 *g*) of the homogenates. Proteins were denatured by boiling, resolved on SDS-PAGE gels, and transferred

to membranes. Immunoblottings were performed with the following antibodies (Cell Signaling, Danvers, MA): phospho-Akt (S473), phospho-Smad 1/5/8, phospho-ERK1/2, phospho-STAT1 (Y702), phospho-STAT3 (Y705 and S727), Akt, Smad 1, ERK 1/2, STAT1, STAT3, Hsp70, iNOS, glucose-6-phosphate dehydrogenase, lamin A/C and glyceraldehyde-3-phosphate dehydrogenase. The Hsp22 antibody was generated as reported before[3]. Primary antibodies were added at 1/500-1/1,000 dilution and incubated overnight. After washing and incubation with the secondary antibody, detection was performed by chemiluminescence (Dupont/NEN, Boston, MA), and quantified by densitometry.

Histopathology

Samples were fixed in 10% formalin and cut in 7 μm-thick sections. Tissue sections were stained with Picric Acid Sirius Red to identify specifically collagen[4]. The surface of tissue section covered by collagen was measured on a Nikon E800 Eclipse microscope by video-based Metamorph Image analyzer system (Universal Imaging Corporation, Westchester, PA), and reported as a percentage of the total surface of the tissue section. Myocyte cross sectional area was determined on digitized images of TRITC-labeled wheat germ agglutinin stained sections[1, 5]. Myocyte outlines were traced and the cell areas measured using Image-ProPlus Software System (Silver Springs, MD). At least 100 myocytes were measured in each region. TUNEL was performed as described[6] on sections treated with 2% H_2O_2 to inactivate peroxidases and with 20 μg/ml proteinase K for permeabilization. DNA fragments were labeled with 2 nM biotin-conjugated dUTP and 0.1 U/μl deoxynucleotidyl transferase for 1 h at 37°C[7]. Incorporation of biotin-16-dUTP was measured with FITC-ExtrAvidin (Sigma Aldrich, St. Louis, MO). Slides were mounted in a Vector DAPI medium for fluorescent microscopic observation at x40-objective field. Nuclear counterstaining was performed with DAPI. TUNEL-positive and DAPI-positive cells were counted on at least ten different fields and are reported per tissue surface.

cDNA microarrays

Messenger RNA was purified from phenol-extracted[8] total RNA with the NucTrap oligo-dT affinity column (BD Biosciences, San Jose, CA). After quantitation by the A_{260} absorbance, 10 micrograms of

mRNA were used for first strand cDNA synthesis with the SuperScript reverse transcriptase and a T7-oligo(24)dT primer[9, 10]. Second-strand synthesis was performed with DNA polymerase I. Products were cleaned-up by Phase-Lock Gels/Phenol-Choloroform extraction. The DNA was subsequently transcribed into biotin-labeled synthetic antisense RNA, using the Bioarray RNA Labeling Kit (ENZO, New York, NY) with the T7 polymerase[10]. This synthetic sRNA was used for hybridization (GeneChip 430.2.0, Affymetrix, Santa Clara, CA), and data analysis was performed with the appropriate software (Microarray Analysis Suite, GenePix Pro). Bioinformatics analysis of the microarray data was performed with the Welch's t-Test, which assumes independent variation, with the Benjamin & Hochberg method, which adjusts P-values in order to control the overall false discovery rate (FDR), and with the Significance Analysis of Microarray (SAM) method. We used a P value<0.05 after adjustment, and a change ratio cutoff>1.2 to select genes[10].

Gene density map

Genes were distributed in a 20x20 table based on fold changes for two comparisons. The number of genes in each cell was normalized to an expected value, which was obtained from randomized data. Randomization was carried out by assigning genes to the table using randomly selected fold change values. The process was carried out 100 times and the average number of genes in each cell was used. The ratio to expected value for each cell represents depletion or enrichment of genes for the cell, and is shown by a color indicating its value.

Isolation of fresh cardiac mitochondria

Left ventricular tissue was homogenized in a buffer (220 nmol/L mannitol 220, 70 nmol/L sucrose, 10 nmol/L HEPES, 2 nmol/L EGTA, pH 7.4 at 4°C) supplemented with 0.25% bovine serum albumin, using a Potter-Elvehjem glass homogenizer in a final volume of 10 ml. The homogenate was filtered through cheese cloth and centrifuged at 1, 000 x *g* for 5 min at 4°C. The supernatant was centrifuged at 10, 000 x *g* for 10 min at 4°C. The mitochondrial pellet was resuspended in 50 µl of homogenization buffer

without EGTA and bovine serum albumin. protein concentration was determined by the method of Lowry[11].

Mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was measured at 30°C with a Clark-type electrode fitted to a water-jacketed reaction chamber of 0.5 ml (Hansatech, Cergy, France). Mitochondria (0.4 mg/ml) were incubated in a respiration buffer containing 100 mmol/L KCl, 50 mmol/L sucrose, 10 mmol/L HEPES, 5 mmol/L KH_2PO_4, pH 7.4 at 30°C. The following parameters of mitochondrial respiration were evaluated: 1. Substrate-dependent respiration rate (state 2 and state 4, oxygen consumption with 5 mmol/L pyruvate/ 5 mmol/L malate) in the absence of exogenous ADP; 2. ADP-stimulated respiration rate (state 3, oxygen consumption) in presence of 250 μmol/L ADP; 3. Respiration uncoupling in presence of 0.2 μmol/L carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP).

Isolation of adult mouse myocytes

Ventricular myocytes were enzymatically isolated from left ventricle of mouse hearts, either sham-operated or submitted to two weeks TAC. Briefly, the hearts were removed from mice anesthetized with intravenous pentobarbital sodium, and perfused retrogradely in Langendorff mode at 37°C with Ca^{2+}-free Tyrode solution containing ~1.4 mg/ml collagenase (type II; Worthington) and 0.1 mg/ml protease (type XIV, Sigma) for 11 min[12]. After the enzyme solution was washed out, the hearts were removed from the perfusion apparatus and swirled in a culture dish. The cell length, defined as the longest length parallel to the longitudinal axis of the myocytes, was directly measured by imaging system (Imaging Workbench software, INDEC Biosystems).

Culture of rat neonatal cardiac myocytes

Cultures of ventricular cardiac myocytes were prepared from Sprague-Dawley rat pups as before[3, 13, 14]. Myocytes were dispersed from the ventricles by digestion with 0.1% collagenase type IV (Worthington),

0.1% trypsin (GIBCO) and 15 μg/mL DNase I (Sigma). Cell suspensions were applied on a discontinuous Percoll gradient (1.060/1.082 g/ml) with Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1, Invitrogen), 17 mM $NaHCO_3$, 2 mM glutamine and 50 μg/ml gentamycin. Myocytes were plated at a density of 10^6 cells per well. The culture medium was changed to a serum-free medium after 24 hours. A recombinant adenovirus was constructed by the COS-TPC (Terminal Protein Complex) method using the Adeno-X-system (Clontech)[15]. The coding sequence of H11 kinase was first amplified by PCR from a mouse heart cDNA library (Clontech) and ligated into pcDNA3.1 (Invitrogen). The insert was subsequently digested and ligated in the Adx vector (Clontech), with or without a Myc-tag. The recombinant adenovirus was prepared in 293 cells by cotransfection of a cosmid containing the adenovirus type 5 genome (devoid of E1 and E3) with the shuttle vectors, using lipofectamine (GIBCO). The recombinant adenovirus was propagated in HEK 293 cells. Titers were determined on 293 cells overlaid with Dulbecco's Modified Eagle Medium (DMEM) plus 5% equine serum and 0.5% agarose. An adenovirus harboring LacZ was prepared similarly and used as a negative control[16]. For Hsp22 knockdown, a short hairpin RNA was designed from the mouse Hsp22 sequence and a recombinant adenovirus was generated as described previously[16]. An adenovirus harboring a silencing sequence for luciferase was prepared similarly and used as a negative control[16].

DNA binding activity

Cardiac myocytes were transfected with 20 moi Cignal lentivirus particles designed for STAT3 and NF-κB reporter assays (SABiosciences, Frederick, MD), using the SureENTRY Transduction Reagent (SABiosciences, Frederick, MD). After 48 hours, cells were infected with the adenovirus harboring the Hsp22 sequence or the LacZ control. After 36 hours, cells were collected and firefly luciferase activity was measured with the Luciferase Reporter Gene Assay (Roche, Indianapolis, IN) in an automated 96-well plate luminometer. The results were normalized with respect to cell number.

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Supplemental Table 1. Physiological and morphological characteristics of Hsp22 KO mice compared to WT littermates in basal conditions

	WT (n=4)	KO (n=4)
Body weight (g)	28±1	28±1
LV/body weight	3.5±0.2	3.8±0.1
LV/tibial length	5.5±0.4	6.1±0.4
Lung weight/tibial length	7±1	8±1
LV Ejection fraction (%)	72±2	66±2
LV Fractional shortening (%)	34±1	31±2
Heart rate (bpm)	410±35	450±12
LV Septal wall thickness (mm)	0.8±0.07	0.8±0.03
LV Posterior wall thickness (mm)	0.8±0.03	0.8±0.02
LV End-diastolic diameter (mm)	3.9±0.1	3.9±0.2
LV End-systolic diameter (mm)	2.6±0.1	2.7±0.1
LV Systolic pressure (mm Hg)	94±9	84±6
LV Diastolic pressure (mm Hg)	3.0±2.0	4.0 ±1.0
LV +dP/dt (mm Hg/sec)	6100±665	5270±371
LV -dP/dt (mm Hg/sec)	5830±726	4770±145
Systolic blood pressure (mm Hg)	94±9	84±6
Diastolic blood pressure (mm Hg)	63±8	59±7
Myocyte cross-sectional area (μm^2)	278±5	298±16
Apoptosis (positive cells per μm^2)	0.7±0.07	0.8±0.03
Collagen (% surface)	3.5±1.1	3.2±0.7

Supplemental Table 2. Gene ontology analysis of the microarrays

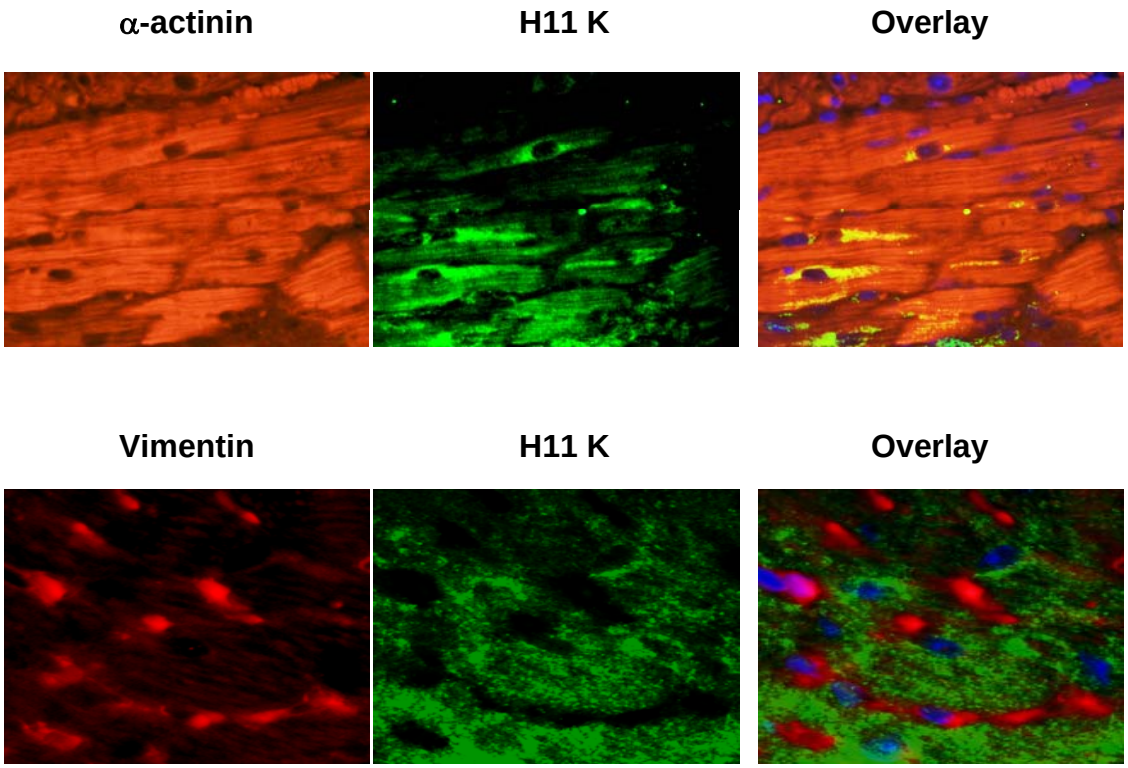
Sham			3 day TAC		
GO ID	***GO name***	***P value***	***GO ID***	***GO name***	***P value***
	Up-regulated			**Up-regulated**	
35050	heart tube development	7.30E-04	16055	Wnt receptor signaling pathway	1.80E-05
51640	organelle localization	6.50E-03	7389	pattern specification process	6.60E-05
35239	tube morphogenesis	6.60E-03	35295	tube development	1.20E-04
51170	nuclear import	7.80E-03	3002	regionalization	2.30E-04
7050	cell cycle arrest	1.00E-02	7166	cell surface signal transduction	3.90E-04
9581	detection of external stimulus	1.00E-02	7155	cell adhesion	6.20E-04
6913	nucleocytoplasmic transport	1.30E-02	35239	tube morphogenesis	6.80E-04
51169	nuclear transport	1.30E-02	6817	phosphate transport	1.00E-03
17038	protein import	1.30E-02	30198	extracellular matrix	1.00E-03
51656	organelle localization	1.40E-02	7275	multicellular organismal development	1.00E-03
51647	nucleus localization	1.50E-02	35282	segmentation	1.20E-03
16055	Wnt receptor signaling pathway	2.30E-02	48754	morphogenesis of a tube	1.30E-03
6270	DNA replication initiation	2.40E-02	9887	organ morphogenesis	1.30E-03
34620	response to unfolded protein	2.40E-02	15698	inorganic anion transport	1.50E-03
30705	intracellular transport	2.40E-02	48514	blood vessel morphogenesis	1.60E-03
31585	IP3receptor	2.40E-02	48513	organ development	2.40E-03
9653	structure morphogenesis	2.80E-02	7165	signal transduction	2.70E-03
7512	adult heart development	2.80E-02	1525	angiogenesis	4.70E-03
30261	chromosome condensation	2.80E-02	8283	cell proliferation	4.80E-03
30518	steroid hormone receptor	2.80E-02	48731	system development	5.40E-03
42176	protein catabolic process	2.80E-02	7154	cell communication	5.40E-03
7017	microtubule-based process	3.00E-02	14855	striated muscle cell proliferation	6.20E-03
			60038	cardiac muscle cell proliferation	6.20E-03
			9952	anterior/posterior pattern formation	6.50E-03
			6801	superoxide metabolic process	8.20E-03
	Down-regulated			**Down-regulated**	
7156	homophilic cell adhesion	1.50E-03	43170	macromolecule metabolic process	9.60E-05
48015	phosphoinositide signaling	7.30E-03	43283	biopolymer metabolic process	4.20E-04
7205	protein kinase C activity	8.00E-03	44238	primary metabolic process	1.10E-03
6844	acyl carnitine transport	1.40E-02	16071	mRNA metabolic process	1.30E-03
45747	Notch signaling pathway	1.40E-02	44237	cellular metabolic process	2.20E-03
46340	diacylglycerol catabolic process	1.40E-02	10467	gene expression	3.70E-03
6289	nucleotide-excision repair	2.50E-02	44267	cellular protein metabolic process	3.80E-03
7200	G-protein signaling, coupled to IP3	2.50E-02	6913	nucleocytoplasmic transport	4.70E-03
720	pyrimidine dimer repair	2.80E-02	51169	nuclear transport	5.10E-03
1302	replicative cell aging	2.80E-02	51168	nuclear export	6.20E-03
6003	fructose 2,6-bisphosphate	2.80E-02	8152	metabolic process	7.00E-03
6649	phospholipid transfer to membrane	2.80E-02	30384	phosphoinositide metabolic process	7.00E-03
7263	nitric oxide mediated signaling	2.80E-02	19538	protein metabolic process	7.10E-03
35166	post-embryonic hemopoiesis	2.80E-02	6396	RNA processing	7.90E-03
16337	cell-cell adhesion	3.00E-02			
6919	caspase activation	3.10E-02			
9968	negative regulation of signaling	3.40E-02			
43280	positive regulation of caspase	3.70E-02			

Supplemental Table 3: TFBS analysis of down-regulated genes in KO versus WT 3 days after TAC

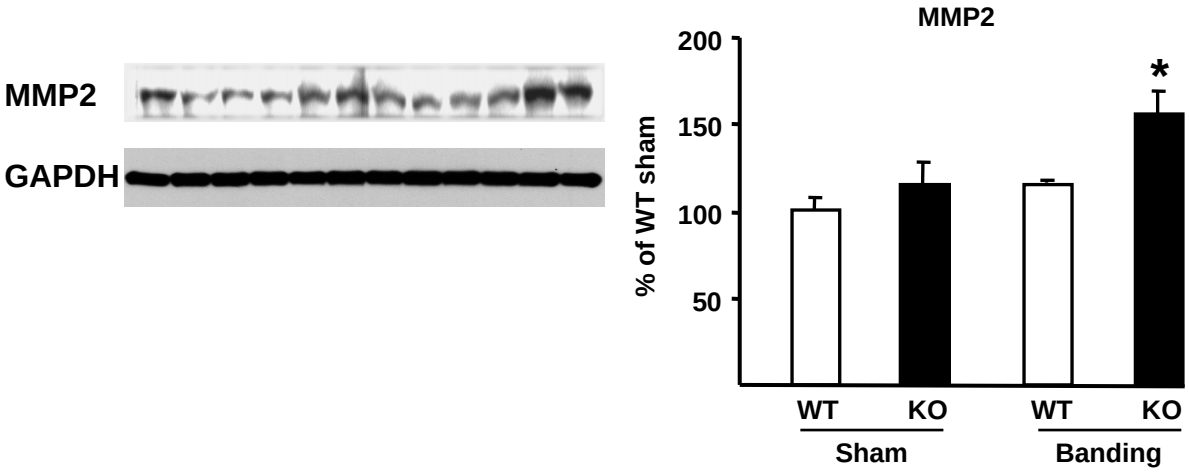
Gene Set	P-value	Description
V$PBX1_01	7.63E-05	Pbx-1,Pbx-1
V$TITF1_Q3	3.76E-03	TTF1 (thyroid transcription factor 1)
V$ELK1_02	1.34E-02	Elk-1,Elk-1
V$OCT1_03	1.42E-02	Oct-1,octamer factor 1
V$EGR3_01	1.60E-02	Egr-3,early growth response gene 3 product
V$ISRE_01	2.01E-02	ISRE, interferon-stimulated response element
V$WHN_B	2.01E-02	Whn, winged-helix factor nude
V$AR_Q6	2.15E-02	AR,half-site matrix
V$AHRARNT_01	2.46E-02	AhR: Arnt,aryl hydrocarbon receptor:
V$MEF2_03	2.46E-02	MEF-2,myogenic MADS factor MEF-2

Supplemental Table 4: Absolute mitochondrial respiration rates

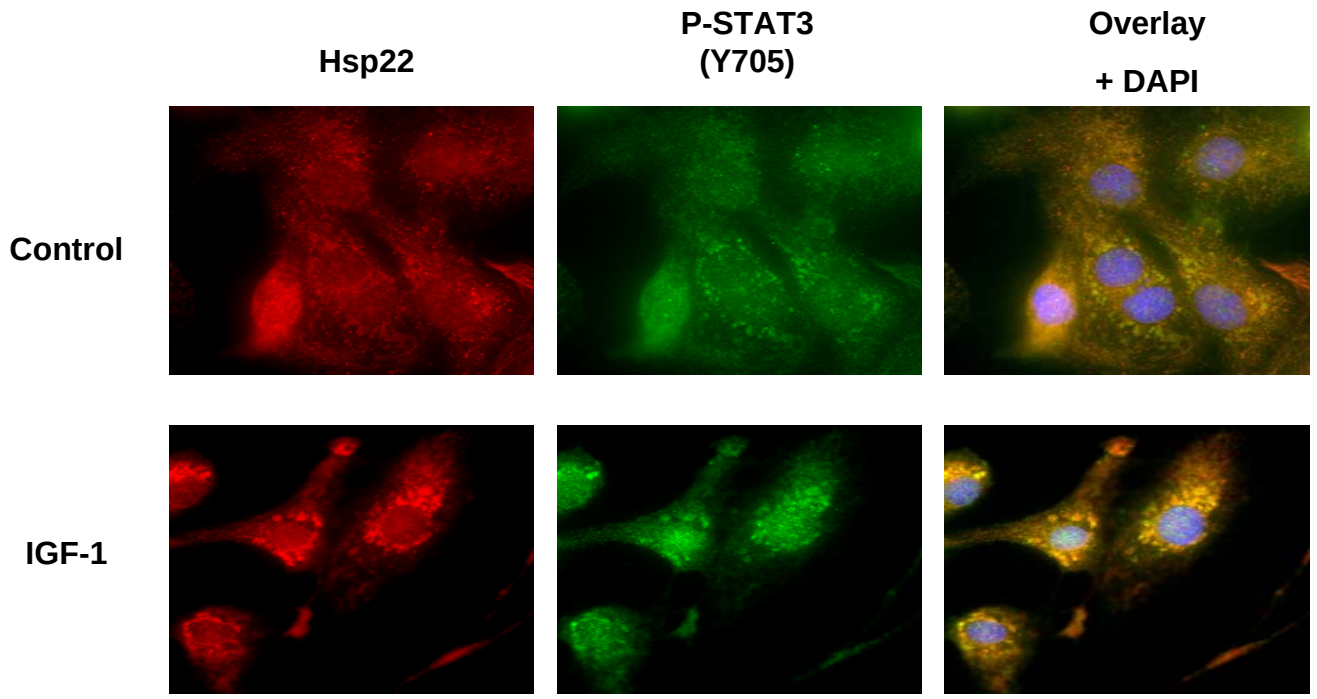
	nmol O_2/min per mg protein				
	State 4	**State3**	**Uncoupling**	**RCR**	**P/O**
WT (C57)					
Mean	40.0	154.1	169.2	4.0	2.3
SEM	2.17	21.26	19.17	0.41	0.06
KO					
Mean	34.7	98.7	93.8	2.9	1.9
SEM	2.29	8.89	9.96	0.23	0.05
P value	0.03	0.007	0.001	0.01	0.0002
WT (FVB)					
Mean	42	173.1	177.2	5.1	2.2
SEM	2.69	5.97	4.12	0.27	0.03
TG					
Mean	56.2	216.6	224.4	5.2	2.4
SEM	2.82	9.95	3.32	0.35	0.08
P value	0.03	0.0004	0.002	0.4	0.25



Supplementary Figure S1. Expression of Hsp22 in myocardium after TAC. Tissue samples were taken from WT mice submitted to three days TAC. Immunofluorescence was performed with antibodies against α-actinin and vimentin to label cardiac myocytes and fibroblasts, respectively, and with an antibody against Hsp22. Overlay of immunostainings was performed after nuclear labeling with DAPI.



Supplementary Figure S2. Regulation of matrix metalloproteinase (MMP) expression. Immunoblotting and quantitation of MMP2 expression in hearts from WT and KO mice submitted to sham operation or three days TAC (banding). *, P<0.05 versus corresponding sham.



Supplementary Figure S3. Nuclear staining of P-STAT3 (Y705) upon addition of insulin-like growth factor-1 (IGF-1). Immunofluorescence was performed in isolated cardiac myocytes using antibodies against Hsp22 and P-STAT3 (Y705), in control conditions or after 24h incubation with IGF-1. Overlay of immunostainings was performed after nuclear labeling with DAPI.