Supporting Information

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SI Text

SI Materials and Methods

Experimental Animals. The strategy to keep cGMP-dependent protein kinase I (cGKI) KO animals alive and healthy by expressing cGKIβ selectively in smooth muscle (SM) was described earlier (1). To create these animals, the heterozygous cGKI mouse line (genotype: $cGKI^{+/L-}$) (2, 3) was crossed to double-transgenic animals (genotype: $SM22\alpha^{+/I\beta}$; $cGKI^{+/L-}$) that carry the cGKIβ knock-in in the SM22α gene (Iβ) to generate "rescue" mice (βRM; genotype: $SM22\alpha^{+/I\beta}$; $cGKI^{L-/L-}$) for the experiments. Offspring from the same litters were used as controls (CTR; genotype $SM22\alpha^{+/I\beta}$; $cGKI^{+/L-}$ or $SM22\alpha^{+/+}$; $cGKI^{+/L}$). The SM22α and cGKI control (+), cGKI KO (L–) and the modified SM22α knock-in (Iβ) cGKI alleles were detected by PCR analysis of tail-tip DNA as described (1, 2). If not stated otherwise, in vivo experiments were performed in 10- to 17-wk-old male mice on a 129/Sv genetic background. All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München, and had free access to tap water and standard chow. All experimental procedures were conducted according to the local government's committee on animal care and welfare in Munich.

Angiotensin II, Sildenafil, Heparin, and Proton Pump Inhibitor Administration. Osmotic minipumps (model 1007D; Alzet) were implanted s.c. in the interscapular region to deliver angiotensin II (AII) in saline solution containing 0.0025 M acetic acid at a constant rate of 2 $mg \cdot kg^{-1} \cdot d^{-1}$ for 7 d. Pumps loaded with vehicle alone were implanted in the control group animals. Sildenafil citrate (purchased as powder from Molekula) was dissolved in drinking water (tap water acidified with citric acid to pH 5.2) at a concentration of 400 mg/L (0.6 mM) and given ad libitum to mice, resulting in the ingestion of ∼60–100 $mg \cdot kg^{-1} \cdot d^{-1}$ (based on an average water consumption of 3–5 mL per d for an adult mouse) (4, 5). The sildenafil water solution was stored at 4 °C for a maximum of 3 wk. Stability of the preparation was assayed by spectrophotometric reading at 289 nm as described (6). The light absorbance during this time span was constant, indicating that the solution was stable and stayed in solution. Heparin was diluted 1:20 in physiological saline. Eighty microliters of diluted heparin containing 20 units were daily injected s.c. in the back of each mouse. Seven days after the minipump implant, the mice were killed and their hearts were harvested, weighed, and subjected to further analysis. For administration of sildenafil by oral gavages, the drug was given at a dosage of 100 $mg \cdot kg^{-1} \cdot d^{-1}$ in a single daily administration, as a suspension [vehicle: 1% (wt/vol) carboxymethyl-cellulose in water]. Where indicated, animals were fed a solid pellet diet that contained 8 mg/kg esomeprazol (a proton pump inhibitor that reduces acid secretion through inhibition of the H^+/K^+ ATPase in gastric parietal cells) encapsulated in a multiple unit pellet system and dispersed in the normal chow by the manufacturer (Altromin) (7).

Echocardiography. Images were obtained using a Vevo 770 Visual Sonics scanner equipped with a 30-MHz probe (Visual Sonics). The mice were lightly anesthetized (1.5% isoflurane), anchored to a warming platform in a dorsal position, and fitted with ECG limb electrodes. The chests were shaved and cleaned to minimize ultrasound attenuation. Left ventricle systolic and diastolic diameters and fractional shortening (FS) (reduction of the length of the end-diastolic diameter that occurs by the end of systole) was assessed from the M mode of the parasternal short axis view.

Heart Histology and Fibrosis. Hearts were dissected, rinsed in PBS, fixed overnight in 4% (wt/vol) paraformaldehyde/PBS solution, dehydrated successively in an alcohol series with increasing concentrations of ethanol, and then embedded in paraffin. Sectioning was performed at 8-μm intervals. H&E staining was performed using standard procedures. For fibrosis measurements, sections were stained with Sirius red and Fast Green. Whole-section images were taken with a digital camera mounted on an optic microscope, and then the percentage fibrosis was measured using software-assisted image analysis (MetaMorph).

Cardiomyocyte Cross-Sectional Area. Cardiomyocyte membrane staining with wheat germ agglutinin (WGA) was used as a method to measure cell areas. Paraffin-embedded 8-μm-thick heart sections were deparaffinized and rehydrated, treated with microwave exposure in Na-citrate buffer for 20 min, and then incubated with WGA conjugated to Alexa Fluor 647 (Invitrogen–Molecular Probes; 1:500 dilution in PBS), and SYTOX Green for nuclear staining (Invitrogen; 1:1,000 in PBS). After three washes in PBS, sections were mounted in Vectashield HardSet Mounting Medium (Vector Labs) and observed under a 20× magnification objective at a confocal microscope (Zeiss). A minimum of five images from each heart sample was taken, spanning different regions of the left ventricle. Cardiomyocyte area was measured using software-assisted image analysis (MetaMorph), with a minimum of 500 cells measured for each sample.

Blood Pressure Radiotelemetry. A telemetry transmitter (PA-C10 device from Data Sciences International) surgical insertion was performed following the manufacturer's guidelines and instructions. In brief, anesthesia was induced and maintained by continuous oxygen/isoflurane inhalation. A ventral midline incision of the upper chest was performed to implant the blood pressure transmitter into the abdominal cavity. The pressure measuring catheter was inserted into the left carotid artery. Each mouse was allowed at least 7 d of recovery before the start of recording. The signals from the implanted transmitter were collected by a DSI acquisition system. Basal blood pressure of animals that had recovered from the transmitter surgery was continuously recorded for 6 d and nights. Thereafter, the same animals were subjected to implantation of an osmotic minipump delivering AII or saline, and the recording was continued for an additional 6 d and nights. Mean arterial pressure (MAP) was calculated as the average between systolic and diastolic blood pressure for any given time point recording.

mRNA Expression Analysis. Total RNA was purified from isolated heart ventricles with peqGOLD RNA pure reagent (PEQLabBiotech) according to the manufacturer's instructions. The purified RNA was quantified by UV photometry in a NanoDrop spectrophotometer (ND-1000; Perkin-Elmer) and diluted in diethylpyrocarbonate-treated ddH_2O to a final concentration of 0.5 μg/μL. cDNA was synthesized from 200 ng of total RNA using M-MLV reverse transcriptase and random hexadeoxynucleotides (Promega) as primers for the first-strand synthesis. Singleplex TaqMan real-time PCR was performed using the Universal Probe Library System (Roche) and specific primers for each target (Table S2) 18S rRNA was used as endogenous control to normalize target expression levels. Amplification was performed on 384-well plates using the 7900 HT Fast Real Time system (Applied Biosystems). Relative

quantification of gene expression levels was analyzed using RQ Manager software (Applied Biosystems).

Immunoblotting of Proteins. For Western blot analysis, hearts were isolated and thoroughly rinsed with cold PBS injected via the aorta by a 25-gauge needle. The ventricles were dissected, frozen in liquid nitrogen, and stored at −80 °C until homogenization was carried out for extraction of the total protein in lysis buffer [RIPA buffer, 0.2 mM PMSF, protease and phosphatase inhibitor mixture (Sigma)]. Proteins (40 μg) were separated by their molecular weight using denaturing 10% SDS/PAGE electrophoresis and transferred to PVDF membrane. Immunodetection was performed using the cGKI common antibody(1:200 dilution) (8) and primary antibodies specific for the following: phospho-p42/p44 mitogen-activated protein kinase (MAPK), phosphoAkt(Ser473), Akt (all used at 1:1,000 dilution; Cell Signaling Technology), p42/p44 MAPK(Thr202/Tyr204) (1:2,000; Sigma), GAPDH (1:10,000; Fitzgerald Industries International), vimentin (Abcam), pPLB(Ser16), phospholamban (PLB) (all used at 1:5,000 dilution; Badrilla). To identify the primary antibody protein complexes, secondary anti-mouse (Santa Cruz Biotechnology), or anti-rabbit (Dianova), antibody coupled to horseradish peroxidase was used. Digital images of the developed signal were taken and analyzed with Science Lab Multi Gauge software (Fujifilm).

Cardiac Fibroblast Isolation. Cardiac fibroblasts (CFs) were prepared from freshly isolated mouse hearts that have been attached to a Langendorff retrograde perfusion system and perfused with a buffer solution containing collagenase and protease (for more details, refer to the online protocols for cardiomyocyte isolation at www.signaling-gateway.org/data/cgi-bin/ProtocolFile.cgi/afcs_PP00000125.pdf?pid=PP00000125). After the perfusion, the hearts were mechanically dissolved using a disposable plastic pipette or a 1-mL syringe and the cell suspension transferred in a new 15-mL tube. To get rid of cardiomyocyte and undigested tissue debris, the suspension was centrifuged four times at 100 × *g* for 2 min, and each time the supernatant was transferred in a new tube. After these centrifugation steps, the supernatant contained mostly only CF (as verified by visualization at the microscope). The cells were then either lysed for Western blot analysis or otherwise plated in culture dishes with DMEM supplemented with 10% of FCS and incubated at 37 °C. After 2 h, culture medium was changed to remove nonadhering cells, to further purify the fibroblast from other contaminating cell types like macrophages, residual myocytes, or blood components.

Generation and Analysis of Neuronal-Specific cGKI KO Mice. Neuronal-specific ablation of cGKI was obtained by crossing a mouse line that carry floxed alleles for the cGKI gene ($cGKI^{flox/flox}$) on a SV129 background, with a mouse line that expresses Cre recombinase under the control of the rat nestin promoter and enhancer ($Nestin\text{-}Cre^{+/Tg}$) on a C57BL/6J background (9). Neuronal-specific KO mice (Neu-KO) (genotype: $Nestin\text{-}Cre^{+/Tg}$; $cGKI^{flox/flox}$) and littermates controls (genotype: $Nestin\text{-}Cre^{+/Tg}$; $cGKI^{+/flox}$) were bred in our animal facility (*Experimental Animals*) and used for the experiments at the age of 13–15 wk. At this age, the Neu-KO animals present a slightly reduced body weight compared with littermate controls but comparable tibia length (Fig. S3 *A* and *B*).

Statistical Analysis. GraphPad (Prism) software, version 4, was used for statistical analysis. If not otherwise indicated, all values are presented as the mean ± SEM. To assess statistical significance comparisons between groups, genotypes and/or stimulation conditions were performed by using ANOVA or unpaired Student *t* test. In scatter plot diagrams, bar and whiskers represent mean value and SEM, respectively.

SI Results

Characterization of Used βRM Mice. Under basal conditions, young adult Ctr and βRM animals (8–14 wk old) display a size difference, with the βRM mice being on average smaller (Fig. S1*A*) and leaner than their Ctr littermates as we previously reported (10) and also indicated by the ratio of body weight on tibia length (Ctr, 1.46 ± 0.03, vs. βRM, 1.31 ± 0.04; Fig. S1*B*). Hearts of βRM mice appeared normal during a gross morphological and histological inspection (Fig. S1*C*). They displayed slightly but not significantly reduced mass when normalized to tibia length (Ctr, 6.26 ± 0.11; βRM, 6.06 ± 0.11; Fig. S1*D*).

Some βRM Mice Die During AII Infusion. βRM mice are more fragile and about 40% of the βRM mice died during the 7-d AII infusion, whereas almost all WT mice survived. Coadministration of sildenafil did not alter the survival trends (Fig. S2*A*). Control groups for both βRM and WT animals, which received infusion of vehicle solution, also did not show any difference in survival rate, which were always above 90% both in the presence or absence of sildenafil. We tested several possible explanations for the increased mortality rate of βRM.

With aging, βRM mice suffer from anemia caused, at least in part, by intestinal ulceration and blood loss (7). A subset of animals were given esomeprazol, a gastric proton pump inhibitor (PPI) capable of reducing intestinal pH, for 3 wk before the AII infusion. This treatment was shown to be effective in reducing anemia and restoring normal values for red blood cells in βRM (7). However, it had no improving effect on survival rate to AII infusion of βRM mice (six out of eight βRM survived the AII treatment, vs. six out of six WT). Interestingly although, hearts from PPI-treated βRM mice showed slightly increased hypertrophic growth compared with untreated mice after AII (Fig. S2*B*).

Because βRM mice lack cGKI in the central nervous system (CNS), another possible explanation for the increased mortality rate could be a neuronal signaling defect as observed previously for other physiological process (7). We tested this hypothesis on a neuronal-specific cGKI KO mouse line (Neu-KO). As the βRM, Neu-KO mice were significantly smaller than their littermate controls (Fig. S3*B*). In this case, survival to AII treatment was similar and above 90% in all of the treated groups (Fig. S3*C*). However, the AII-induced cardiac hypertrophy did not differ between Ctr and Neu-KO animals (Fig. S3*D*). Sildenafil reduced in both groups the AII-induced hypertrophy. These results suggest that neither CNS cGKI nor the body weight account for the differences observed between Ctr and βRM mice regarding survival and AII-induced cardiac hypertrophy.

We tested a third possibility in a small group of mice. The βRM mice lack cGKI in the platelets (11), a condition associated with increased platelet adhesion and thrombus formation (12). Hypertension is known to activate platelets and thrombus formation (13). To prevent thrombus formation during the AII infusion, we injected s.c. Ctr and rescue mice with heparin at a dose of 20 U/day, a dose used in humans and mice (14). Heparin treatment induced premature death in one animal in each group that was caused by intestinal bleeding, but no difference between both groups and similar cardiac hypertrophy was found after the 7-d infusion of AII (Fig. S4). Although the number of investigated animals is small, these results support the notion that hypertension-associated thrombosis caused the premature death of the βRM mice.

MAPK and Phosphoinositol 3-Kinase Signaling Pathways Are Not Altered by AII or Sildenafil in Ctr and βRM Mice. It has been reported that cGKI affects the MAPK and the phosphoinositol 3-kinase (PI3K) pathways in heart (15). Therefore, we investigated whether or not these pathways responded to AII infusion and sildenafil treatment in the two genotypes (Figs. S6 and S7). We measured ERK1/2 phosphorylation as a marker of MAPK pathway activation, and Akt phosphorylation as a marker of

PI3K pathway activation. We could not detect any significant change in the phosphorylation levels of the two proteins analyzed, neither upon AII, nor after sildenafil treatment. In the basal condition, the levels of both ERK1/2 and Akt protein were comparable between the two genotypes (Figs. S5 and S6).

Previous studies have indicated that activated cGKI can regulate target proteins that are involved in calcium handling in cardiomyocytes, namely PLB and sarcoplasmic-reticulum calcium ATPase (SERCA) (16). Therefore, we quantified mRNA expression and protein phosphorylation levels of PLB in cardiac extracts in each experimental group but did not find any obvious change following AII and/or sildenafil treatment, between corresponding Ctr and βRM hearts (Figs. S7 and S8).

cGKI Expression Is Absent in Native Isolated Fibroblasts. To better understand whether or not CFs express cGKI, we cultured CFs from Ctr and βRM mice that were infused with AII for 7 d. Expression of cGKI was detected only in Ctr cells, whereas βRM fibroblasts do not contain virtually any amount of the enzyme (Fig. S9*A*). cGKI expression was reactivated in the cultured βRM fibroblast, as soon as after 24 h after plating, likely due to activation of the SM22a promoter (17) in these cells (Fig. S9*B*).

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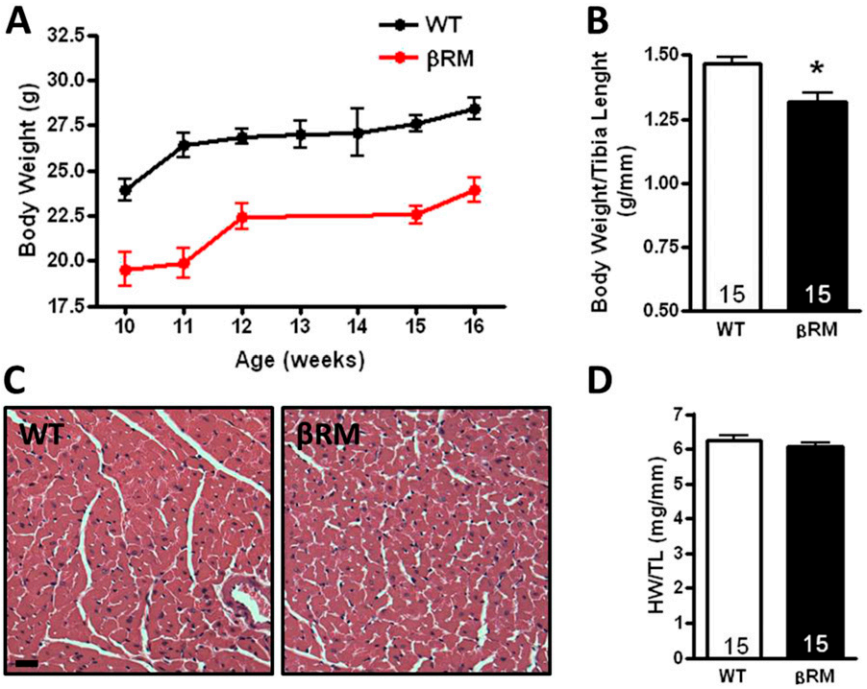

Fig. S1. Comparison between βRM and Ctr/WT littermates. Average body weight (*A*; $n = 6$–17 for each time point), and body weight/tibia length (BW/TL) ratio (*B*). **t* test: $P < 0.05$ vs. Ctr group. (*C*) H&E staining of heart sections [a 20× magnification of the left ventricle is pictured (scale bar: 50 μm)] showed normal tissue morphology in βRM compared with Ctr littermates. (*D*) Heart mass measured as total heart weight/tibia length (HW/TL) ratio in basal conditions. Numbers in the bars represent *n* values (this is valid for all of the following figures).

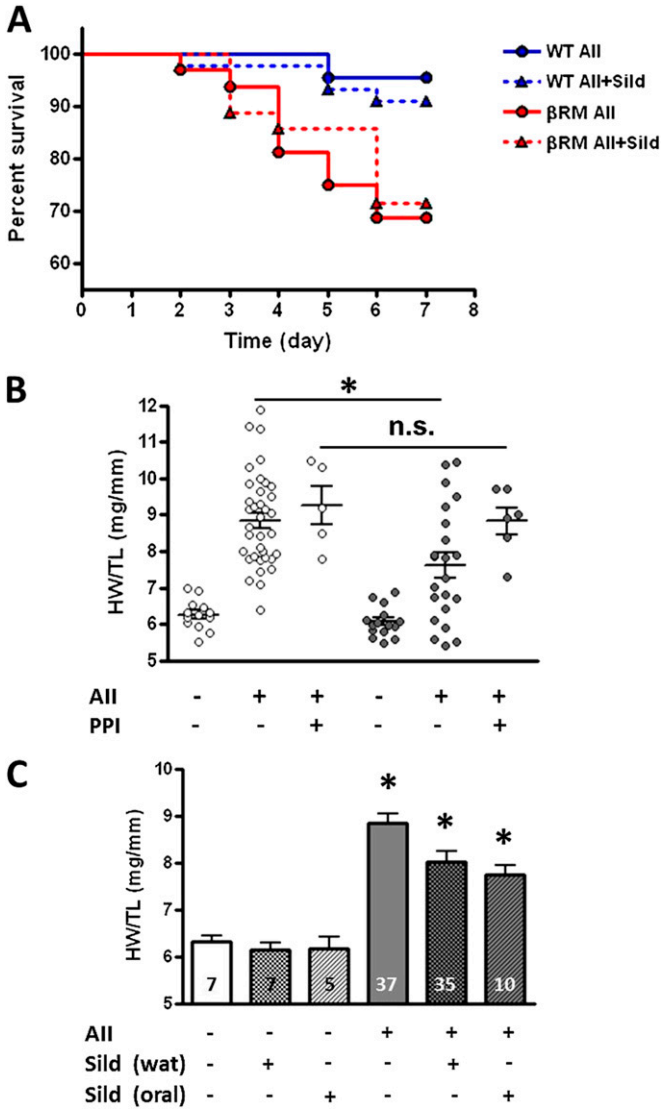

Fig. S2. (*A*) Survival rate of Ctr/WT and βRM mice after AII infusion (number of surviving mice per experimental group are as follows: Ctr AII, 41 of 43; Ctr AII+Sild, 40 of 44; βRM AII, 22 of 32; βRM AII+Sild, 25 of 35). (*B*) Cardiac hypertrophy, expressed as heart weight/tibia length, of Ctr and βRM mice treated with PPI and then AII infusion. The data have been plotted together with those of mice that did not receive PPI treatment as a comparison (these data are taken from Fig. 1). (*C*) Cardiac hypertrophy, expressed as heart weight/tibia length, of Ctr mice that have been infused with AII for 7 d, and treated with sildenafil via drinking water (sild wat) or oral gavages (sild oral); numbers in bars indicate *n* values. **P* < 0.05 vs. control group, one-way ANOVA with Tukey's multiple-comparison test; n.s., nonsignificant.

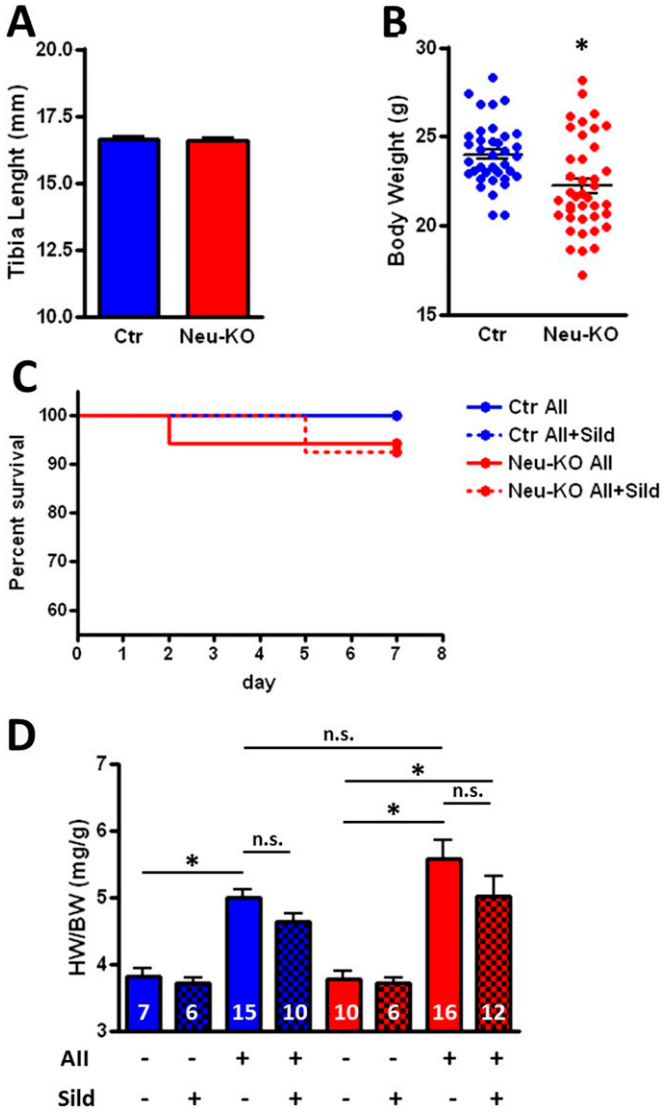

Fig. S3. (*A*) Tibia length and (*B*) body weight of neuronal-specific cGKI KO (Neu-KO; in red) mice and their littermate controls (Ctr; in blue) (n = Ctr: 38, Neu-KO: 40; *$P < 0.05$ Student's t test). (*C*) Survival rate of Neu-KO and Ctr mice after AII infusion (2 mg·kg^{-1}·d^{-1}; number of surviving mice per experimental group are as follows: Ctr AII, 7 of 7; Ctr AII+Sild, 6 of 6; Neu-KO AII, 16 of 17; Neu-KO AII+Sild, 12 of 13). (*D*) Cardiac hypertrophy, expressed as heart weight/body weight ratio (HW/BW), of Neu-KO and Ctr mice after AII infusion with or without sildenafil administration via drinking water. Numbers in bars indicate n values. *$P < 0.05$ calculated with one-way ANOVA with Tukey's multiple-comparison test; n.s., nonsignificant.

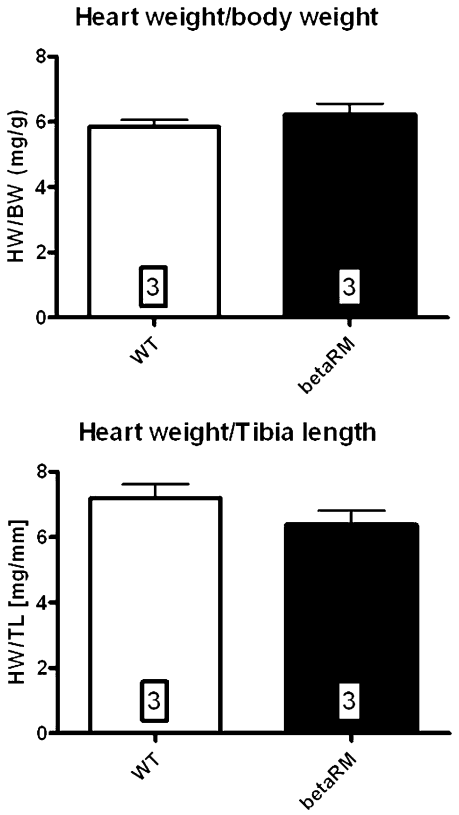

Fig. S4. Heart weight per body weight or tibia length of Ctr (WT) and βRM (betaRM) mice that were infused for 7 d with AII and were injected daily with 20 U of heparin.

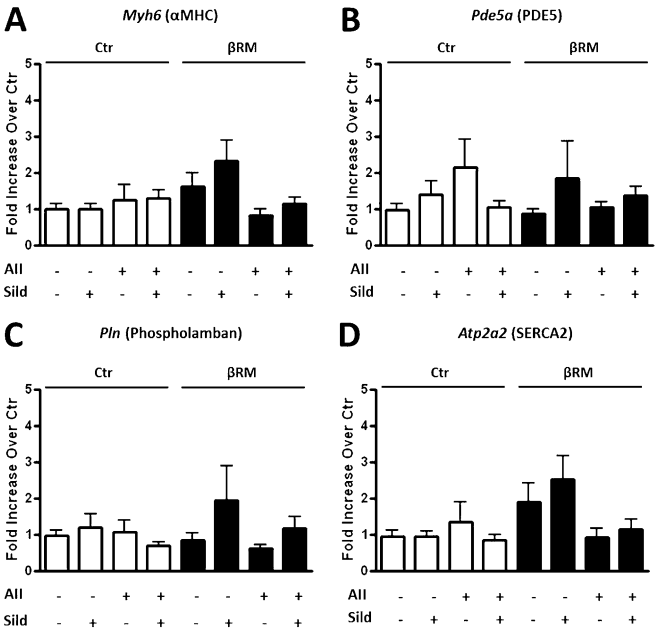

Fig. S5. mRNA expression levels of α-myosin heavy chain (*A*), phosphodiesterase 5 (*B*), phospholamban (*C*), and the sarcoendoplasmic reticulum calcium ATPase (*D*), normalized to 18S rRNA and then to corresponding Ctr group average, assessed by quantitative real-time RT-PCR. The white bars refer to Ctr groups, and the black bars to βRM groups. $n = 5–7$ for each group.

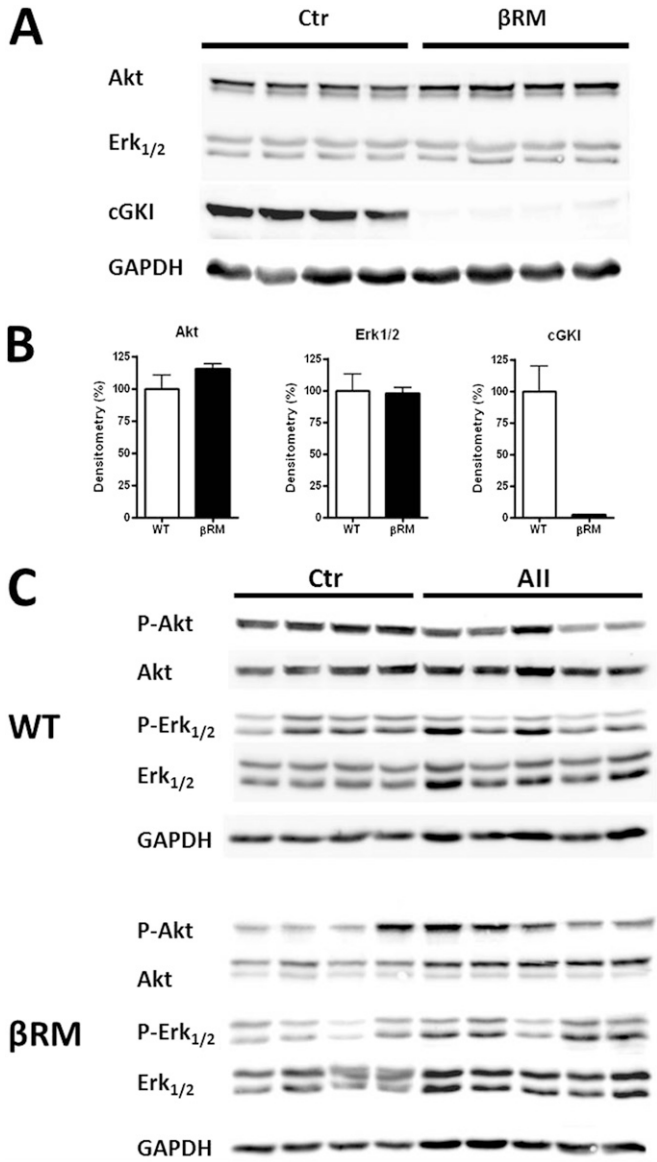

Fig. S6. Signaling molecules expression and activation in Ctr and βRM heart extracts in basal state and after AII treatment. *A* shows a representative Western blot to determine the expression levels of Akt, $Erk_{1/2}$, and cGKI in basal state. The densitometry of a total of six samples for each genotype is depicted in *B*. In *C* are shown two representative Western blots (upper image for WT and lower for βRM samples) of control and AII-treated group (see also Fig. S6 for statistic).

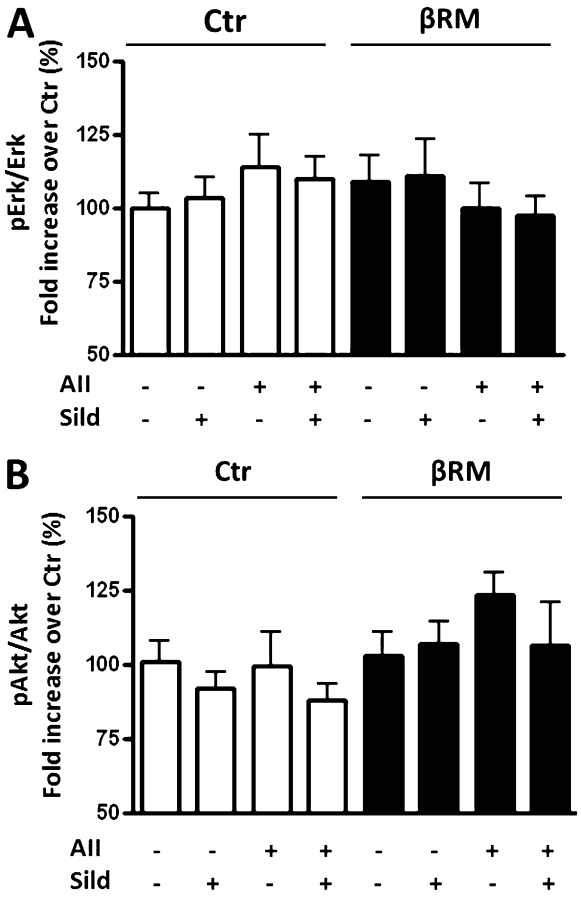

Fig. S7. Activation of MAPK and PI3K pathways, assessed by Western blot on heart homogenates. Graphs report the relative amount of phosphorylated Erk (*A*) or Akt (*B*) in percentage, normalized to total enzyme protein. Densitometry values were normalized on the WT control group average that is set as 100%. $n = 6–8$ for each group.

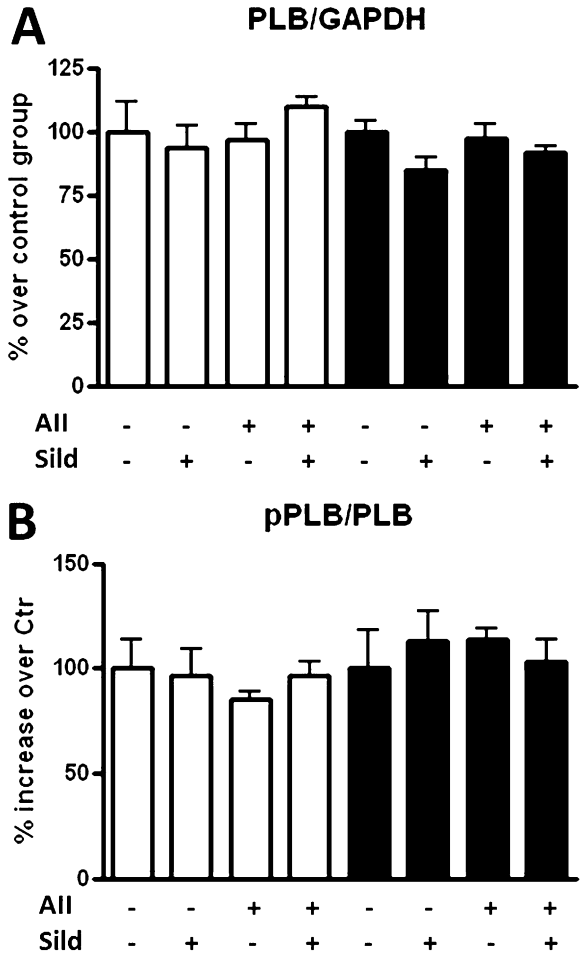

Fig. S8. Graphs report the relative amount of phospholamban protein level, normalized on GAPDH (*A*), or the ratio of phosphorylated over total protein (*B*) in percentage. Densitometry values were normalized on the Ctr control group mean that is set as 100%. The open bars refer to Ctr groups, and the black bars to βRM groups. $n = 6–8$ for each group.

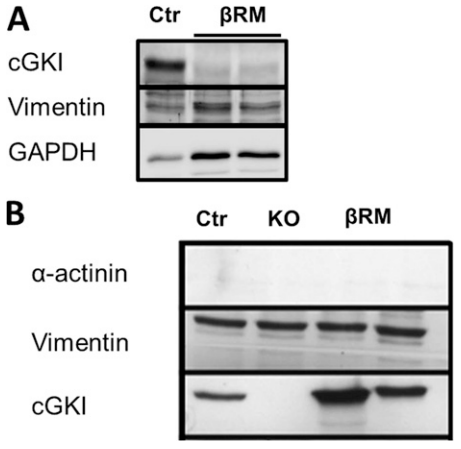

Fig. S9. (*A*) Western blot experiment conducted on CFs isolated from hearts of mice that underwent AII infusion for 7 d. Expression of cGKI is detected only in Ctr cells, whereas βRM fibroblasts do not contain virtually any amount of the enzyme. Vimentin was used as a marker for CF. (*B*) Western blot of CFs (FB) that, after isolation, had been grown in culture dish for 3 d. This incubation is sufficient to drive reexpression of cGKI in the fibroblasts. As controls, CF isolated from Ctr mice (lane 1) and global cGKI-KO mice (lane 2) were used. Staining with anti–α-actinin antibody was done to exclude cardiomyocyte contamination. cGKI expression was also detected in isolated βRM-derived fibroblast that had been cultured for 24 h (data not shown). Both Western blot experiments shown here had been repeated three times.

Table S1. Physical and cardiac values of animal groups

	Group (*n*)							
	Ctr				βRM			
	CTR (13)	Ctr+Sild (17)	AII (37)	AII+Sild (35)	CTR (16)	Ctr+Sild (14)	AII (22)	AII+Sild (22)
Age, wk	13.6 ± 2.0	12.4 ± 4.0	12.7 ± 2.2	13.4 ± 3.4	15.5 ± 1.2	14.2 ± 2.4	13.3 ± 2.5	13.7 ± 2.5
Body weight (at day 0), g	26.2 ± 2.4	28.0 ± 2.0	27.8 ± 3.2	26.9 ± 2.4	22.0 ± 2.2	23.1 ± 2.9	22.0 ± 3.3	22.3 ± 3.0
Body weight (at day 7), g	26.5 ± 2.7	28.7 ± 2.2	24.5 ± 3.1	24.4 ± 2.6	22.7 ± 2.4	22.7 ± 3.4	18.6 ± 3.1	20.3 ± 2.4
Weight loss, g	0.2 ± 0.6	0.7 ± 0.8	−3.1 ± 1.6*	−2,3 ± 1.9*	−0.2 ± 1.5	−0.1 ± 1.2	−3.2 ± 2.4*	−2.4 ± 2.1*
HW, mg	118 ± 1	118 ± 9	152 ± 3	143 ± 2	109 ± 9	103 ± 9	132 ± 3	131 ± 3
TL, mm	18.1 ± 0.4	18.2 ± 0.3	18.2 ± 0.5	18.2 ± 0.4	17.2 ± 0.6	17.4 ± 0.3	17.0 ± 1.4	17.9 ± 0.6
HW/TL, mg/mm	6.3 ± 0.1	6.4 ± 0.1	8.8 ± 0.2*	8.1 ± 0.2*†	6.1 ± 0.1	5.9 ± 0.1	7.6 ± 0.3*	7.7 ± 0.3*
HW/BW, mg/g	4.4 ± 0.3	4.3 ± 0.4	6.2 ± 1.1*	5.9 ± 0.9*	4.9 ± 0.4	4.6 ± 0.7	7.3 ± 1.9*	6.5 ± 1.2*†

Data are presented as average ± SD. Abbreviations: BW, body weight; HW, heart weight; TL, tibia length. *$P < 0.05$ vs. corresponding Ctr group; †$P < 0.05$ vs. corresponding AII group.

Table S2. Primers for quantitative real-time PCR

Gene name	Gene ID	Forward or 5′ primer	Reverse or 3′ primer
Colla1 (Collagen I)	NM_007742.3	catgttcagctttgtggacct	gcagctgacttcagggatgt
Fn1 (Fibronectin)	NM_010233.1	cggagagagtgcccctacta	cgatattggtgaatcgcaga
Tgfb1 (TGF β)	NM_011577.1	tggagcaacatgtggaactc	cagcagccggttaccaag
Nppa (ANP)	NM_008725.2	cacgcagcttggtcacatt	cgtgatagatgaaggcaggaa
Ctgf (CTGF)	NM_010217.2	tgacctggaggaaaacattaaga	agcctgtatgtcttcacactg
Myh6 (αMHC)	NM_010856.4	cgcatcaaggagctcacc	cctgcagccgcattaagt
Myh7 (βMHC)	NM_080728.2	cgcatcaaggagctcacc	ctgcagccgcagtaggtt
Pde5a (PDE5)	NM_153422.1	ccatcattttgaccagtgctt	agaggccactgagaatctgg
Pln (PLB, transcript variant 1)	NM_001141927.1	acgatcaccgaagccaag	tgtaagctggcaagttccttt
Atp2a2 (SERCA2, transcript variant 2)	NM_009722.3	tcgaccagtcaattcttacagg	cagggacagggtcagtatgc