SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

Animal procedures

Myocardial infarction was produced by ligating the left anterior descending (LAD) branch of the coronary artery using a 8-0 suture (Ethicon). Control mice underwent a sham operation. No differences in peri-operative mortality rates (within 24h) were observed between Control and PRAS40 mice. For assessment of post MI survival, mice that had survived the first 24h after surgery were followed for 6w and were inspected daily.

Echocardiography and Analysis

Echocardiography was used to evaluate cardiac function after MI using a Vevo 770 High-Resolution *In-Vivo* Imaging system. Mice during echocardiographic analysis were subjected to 1-2% isoflourane before assessing cardiac function. End diastolic diameter (LVEDD) and end systolic diameter (LVESD) were measured in order to calculate LV fractional shortening (FS) and LV ejection fraction (EF).

Hemodynamics

Mice under chloral hydrate sedation were subjected to catheterization of the right carotid artery in order to enter the left ventricle of the heart. The catheter, a 1.2F PV- 4.5mm Electrode, was supplied by Scisense (Ontario, Canada) and data acquisition and analysis was performed using LabScribe2 software, compatible with Scisense equipment. LabScribe2 software assisted in determining left ventricular volume and pressure measurements. Mice after catheterization were immediately subjected to heart retroperfusion or hearts were removed for protein and mRNA analysis.

Tissue collection

We sacrificed mice at various time points after MI or sham operation and removed the hearts. For later isolation of RNA and protein in the MI study, left ventricles were divided into the

infarcted area (apex, distal to the ligation site) and the non-infarcted remote myocardium (basal part of the interventricular septum) and stored in in liquid N2.

Retroperfusions

Mice were sacrificed under chloral hydrate sedation. Before the procedure of removing the hearts, mice were weighed in grams. Thereafter, hearts were arrested in diastole by catheterizing the abdominal aorta and flushing the heart with a high-potassium/cadmium solution. Phosphate buffered formalin fixative was perfused into the coronary arteries at systolic pressure while the left ventricle was filled with formalin at diastolic pressure. Retroperfused hearts were then removed from the chest cavity and placed in formalin for at least 24h, weighed in milligram and processed for paraffin embedding.

Paraffin heart sections were deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Antigen retrieval was achieved by boiling the slides in 10mmol/L citrate pH 6.0 for 12–15 min. Slides were washed several times with distilled water and once with TN buffer (100 mmol/L Tris, 150 mmol/L NaCl). Endogenous tissue peroxidase activity was quenched with TN buffer supplemented with 3% H2O2 for 20 min whenever necessary. Slides were then washed in TN buffer and blocked in TNB buffer (TSATM kit from Perkin-Elmer) at room temperature for at least 30 min. Primary antibodies were applied overnight at 4°C in TNB buffer. The next day, samples were washed in TN buffer and incubated with secondary anti- bodies at room temperature in the dark for 2h. When amplification of signal was needed, slides were washed in TN buffer and incubated with streptavidin horseradish peroxidase-conjugated diluted 1:100 vol/vol in TNB buffer for 30 min at room temperature, and signals were developed using Tyramide substrate diluted 1:50 vol/vol in Amplification Diluent (Perkin-Elmer) for 10 min. Slides were washed in TN buffer and coverslipped using Vectashield eventually in the presence of DNA staining. List of primary and secondary antibodies is reported in Supplemental Table 2.

Immunohistochemistry

Immunostaining of myocytes was performed on cells grown on permanox or glass chamber

slides (gelatin-coated). Cells were fixed by 4% paraformaldehyde (PFA) for 20 min at room temperature, permeabilized in PBS supplemented by 0.1% Triton-X for 10 min, and blocked in PBS supplemented with 10% horse serum for at least 30 min. Before starting the protocol and at the end of each successive step, cells were washed in PBS at room temperature. Primary antibodies diluted in blocking solution (PBS with 10% horse serum) were applied overnight at 4 °C. The next day, cells were washed with PBS and incubated for 1h at room temperature and in the dark, with the secondary antibody (Jackson Laboratories) diluted in blocking solution. Conjugated phalloidin 633 (Jackson Laboratories) was diluted in the same buffer at 1:100 vol/vol. Sytox Blue or To-Pro (Molecular Probes) was diluted in Vectashield (Vectra Labs) mounting media at 1:500 vol/vol and used as nuclear staining.

Infarct size measurements

Paraffin sections were stained with tropomyosin to visualize live myocardium and TO-PRO-3 iodide to determine nuclei distribution and area of infarction. Area of live myocardium was measured using an outlining tool supplied by the Leica Imaging software and normalized to the total area of the left ventricular free wall and converted to a percentage.

Myocyte size measurements

Paraffin sections were stained for tropomyosin to visualize live myocardium wheat germ agglutinin-488 (Carlsbad, Invitrogen) to outline cardiomyocyte membrane and TO-PRO-3 to visualize nuclei. Myocytes were measured using a ImageJ to measure area as μm^3. An n=4 per group was measured.

Cardiomyocyte cell culture and treatment

Neonatal Ventricular cardiomyocytes from 1- to 2d-old rat neonatal hearts (NRCMs) were prepared by trypsin digestion using standard procedures. Cell suspensions were pre-plated for 2h in M199 medium (Cell-Gro) supplemented with 15% fetal bovine serum (FBS) to reduce non-myocyte cell contamination. Myocytes were plated in gelatin (Sigma) precoated 10cm dishes or in laminin (Sigma) precoated glass slides using the preplating media.

For isolation of adult myocytes, following chloral hydrate anesthesia (400 mg/kg body weight, i.p.), the heart was excised and left ventricular (LV) myocytes were enzymatically dissociated 11. The myocardium was perfused retrogradely through the aorta at 37°C with a Ca^{2+} free solution gassed with 85% O_2 and 15% N_2. After 5 min, 0.1mM $Ca^{2+}Cl_2$, 274 units/mL collagenase (type 2, Worthington Biochemical Corp, Lakewood, NJ) and 0.57 units/mL protease (type XIV, Sigma, St. Louis, MO) were added to the solution which contained (mM): NaCl 126, KCl 4.4, MgCl 5, HEPES 5, Glucose 22, Taurine 20, Creatine 5, Na Pyruvate 5 and NaH2PO4 5 (pH 7.4, adjusted with NaOH). At completion of digestion, the LV was cut in small pieces and re-suspended in Ca^{2+} 0.1mM solution. Adenoviral transfection of isolated ventricular cardiomyocytes was carried out 2h after plating in HEPES- modified medium 199 (M199 Sigma M 7528 supplemented with 5mM taurine, 5mM carnitine, 5mM creatine, 5mM N-mercaptoproprionyl glycine, 0.1μM insulin, 10,000 U/ml penicillin and 10mg/ml streptomycin, pH 7.25) with a multiplicity of infection/cell of 20. Cells were maintained in M199 for 24h at 37°C, 95%O_2, 5%CO_2 and used for further analysis after medium exchange.

Plasmid construction, adenoviral infection and siRNA transfection

PRAS40 was PCR amplified from murine cardiac cDNA with the following primer For: 5' ATGGCGTCTGGGCGGCCAGA 3' with an EcoRI attached to the 5' end; Rev: 5' AGCTGAAGCGGAAATATTAA 3' with a Flag and NheI site attached at the end. Generated PRAS40 was subcloned into the Pshuttle CMV adenoviral shuttle vector (Agilent Technologies) and AAV9 shuttle vector. Expression was confirmed by transfection with Fugene (Roche) into Hela cells followed by immunoblot. Recombinant adenovirus was produced and generated using the AdEasy system. The gene of interest was cloned into the pShuttle-CMV vector and a recombinant adenoviral plasmid was produced by homologous recombination in E. coli. The recombinant vector was linearized and transfected into low passage HEK293 cells. After 7-10d, transfected cells were harvested and subjected to 4 freeze/thaw cycles. Cells were pelleted and the supernatant was used to infect additional HEK293 cultures to expand and amplify the virus.

Supernatant and cells were harvested from 20, 15cm infected plates of HEK293 cells. The cells were pelleted and resuspended in 30 mL of reserved infected media and subjected to 4 freeze thaw cycles. DNAse was added at a concentration10ug/mL and incubated for 30 min at 37°C. Cells were then pelleted and the supernatant was loaded on top of a CsCl gradient and spun in a SW41 rotor at 22.5k rpm overnight. The next morning, the viral band was extracted with a syringe and the virus was diluted with buffer. This mixture was loaded on top of a second CsCl gradient and spun at 22.5k rpm overnight. The following day, the viral band was extracted and the OD260 was measured. The virus was loaded into a 10,000 MWC dialysis cassette and was dialyzed for 4h, with the buffer being changed every hour. The virus was extracted from the cassette with a syringe and the OD260 was measured. The adenovirus is aliquoted and stored in the -80. The titer of the virus was obtained by performing a standard plaque assay.

NRCMs were infected for 2h with purified virus, then fed with M199 media containing 0.5% FBS. On the following day cells were either fixed for immunofluorescent staining or used for further experiments.

NRCM were transfected with small interfering RNAs (siRNAs, 100nM) by using HiPerfect transfection reagent (Qiagen) as per the manufacturer's recommendations. Briefly, 3µl siRNA and 12µl HiPerfect were diluted in 100µl serum-free M199 medium. After incubation for 5-10 min, transfection complexes were added to the cells for 48h. The following siRNA sequences were used: rat PRAS40 siRNA, 5'-GGGCGGUAGCGAUAAUGGAtt3'; The scrambled siRNA was obtained from Ambion.

Adeno-associated virus serotype 9 generation and systemic in vivo AAV9 cardiac-targeted gene transfer protocol

For generation of recombinant AAV vector pTRUF-CMVenhMLC800 was constructed by modifying pTRUF12(a gift from R Hajjar) by first removing the region encoding GFP that was down-stream of the IRES. New restriction sites were inserted into the multiple cloning site to include Nhe1, Pme1, Xho1, and Mlu1. The CMV promoter was replaced with a composite

promoter comprised of an 800bp fragment of the MLC2v promoter downstream of a CMV enhancer (a gift from Dr. Oliver J. Muller; see Muller OJ et al. Cardiovasc Res (2006) 70 (1): 70-78). AAV9 vectors with wild-type capsids were generated by cotransfection the helper plasmid pDG-9 (a gift from Dr. Roger Hajjar). A MLC2v-GFP virus was used to test the efficiency of the gene transfer.

pTRUF-CMVenhMLC800-PRAS40 was created by subcloning the mouse PRAS40 cDNA from pcDNA3.1-PRAS40.

To prepare the recombinant AAV9, HEK293T cells were grown in DMEM/F12 containing 10% FBS, penicillin/streptomycin at 37°C and 5% CO2. HEK293T cells were plated at 8x106 per T-175 flask. Twenty-four hours after plating, cultures were transfected using Polyethylenimine “Max” (MW 40,000; cat. No: 24765; Polysciences, Warrington, PA) as follows 15ug of helper plasmid and 5ug of pTRUF plasmid were mixed with 1 ml of DMEM:F12 and 160ul of polyethylenimine (0.517mg/ml),vortexed for 30 seconds, and incubated for 15 minutes at room temperature. This mixture was then added to the cultures in a drop-wise fashion. The cultures were then rocked intermittently for 15 minutes before incubation. The culture medium was changed 6 to 18hrs later. Three days after transfection, the cells were collected from six flasks and then centrifuged at 500xg for 10 minutes. The cells were resuspended in 10ml of lysis buffer (150 mmol/l NaCl, 50mmol/l Tris-HCL). The resuspended cells were then subjected to three rounds of freeze-thaw followed by treatment with benzonase (1500u of benzonase; Novagen cat. no. 71205) and 1mM MgCl2 at 37°C for 30 minutes. The cell debris was collected by centrifugation at 3,400xg for 20 minutes. The supernatant containing the AAV9 virus was then purified on an iodixanol gradient comprised of the following four phases: 7.3ml of 15%, 4.9ml of 25%, 4ml of 40%, and 4ml of 60% iodixanol (Optiprep; Sigma-Aldrich, cat. No. D1556) overlayed with 10ml of cell supernatant. The gradients were centrifuged in a 70Ti rotor (Beckman Coulter, Brea, CA) at 69,000 rpm for 1 hour using OptiSeal Polyallomer Tubes (cat. no. 361625; Beckman Coulter). The virus was collected by inserting a needle 2 mm below the

40%-60% interphase and collecting 4 or 5 fractions (~4ml) of this interphase and most of the 40% layer. The fractions were analyzed for viral content and purity by analyzing 10µl of each fraction on a 12% SDS-PAGE gel (BioRad, cat. no. 345-0119) followed by staining with InstantBlue (Expedeon; cat. no. ISB1L) to visualize the viral capsid proteins, VP1, VP2 and VP3. The virus was then collected from the fractions of several gradients and the buffer was exchanged with lactated Ringer's using an ultrafiltration device, Vivaspin 20, 100kDa MWCO (GE Healthcare, cat. no. 28-9323-63). The final viral preparation was then fractionated on a 12% SDS-PAGE gel, stained with InstantBlue, and then compared with a similarly stained gel of a virus of a known titer. Alternatively, a qPCR was performed using a forward primer (AAGTCTCCACCCCATTGACGT) and reverse primer (AGGAGCCTGAGCTTTGATTCC), which spans the CMVenhMLC800 composite promoter. A pTRUF vector containing the CMV/MLC800 promoter was used as a standard to determine copy number.

All viruses are available upon request.

Real-time RT-PCR

Total RNA was isolated from frozen heart or cultured cells by using Quick-RNA™ MiniPrep (Zymo Research) and reverse-transcribed into complementary DNA (cDNA) by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on all samples in triplicate using QuantiTect™ SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. All primer sequences are shown in Supplemental Table 1 of the Online Data Supplement.

Immunoblotting

Immunoblotting was performed using standard procedures. Protein lysates from ventricles or cultured cardiomyocytes were loaded onto a 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) for electrophoresis. Separated proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked with 5% skim milk in Tris-Buffered Saline Tween-20 (TBST) for 1h at room temperature, and exposed to primary antibodies. Alkaline phosphatase (AP), horseradish

peroxidase (HRP) or Cy5-conjugated IgG (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies. Fluorescence signal was detected and quantified by using a Typhoon 9400 fluorescence scanner together with ImageQuant 5.0 software (Amersham Biosciences).

Flow cytometry

NRCM were stained with Annexin V (BD Biosciences) and 7-AAD (Invitrogen) according to the manufacturer's instructions. Cell pellet was collected by trypsinization and centrifugation, then incubated with Annexin V (1:50) and 7-AAD (1:1000) for 15min. Annexin V has a high affinity for the membrane phospholipid phosphatidylserine which is exposed to the external environment during early apoptosis. 7-AAD is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but will not cross intact membranes in viable cells. Flow cytometry was performed by using a BD FACSAria Flow Cytometer (BD Biosciences). Adult myocytes were isolated 1 week after AAV9-GFP ene transfer and fixed with 4%PFA for 30 min. Fixed cells in Calcium- and Magnesium-Free PBS were aliquoted into tubes (~200,000 adult myocytes/ml), pelleted at 800xg for 3min, incubated 5min withTriton X-100 and blocked in PBS with 10%FBS and finally incubated overnight with primary antibodies. Flow cytometry was performed by using a BD FACSAria Flow Cytometer (BD Biosciences) to quantify the percentage of GFP positive myocytes.

SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 1

(A) mTORC1 and mTORC2 are activated two days after myocardial infarction (MI) as shown by immunohistochemistry and immunoblotting. Representative confocal scans are shown for pRibS6 (top panel), AKT^{S473} (bottom panel), actin and nuclei (red, green and blue, respectively, in overlays). Bar, 150μm. **(B)** Torin1 blocks dose dependent mTOR kinase. **(C)** Cells treated

with 50μM H_2O_2 for 3h and stained for Annexin V and 7-AAD. Annexin V positive cells were considered to be apoptotic *P<0.05 vs vehicle, #P<0.05 vs Control H_2O_2. n=3 independent experiments. **(D)** Schematic diagram of the Torin1 experiment *in vivo*. **(E)** Hemodynamic measurement of heart rate (HR) two weeks after infarction. HR is unchanged between vehicle and Torin1 treated mice.

Supplemental Figure 2

(A) Cell death assay. AKT decreases cell death after challenge with (50μM for 4h), but mutation of the serine residue at 473 to an alanine (phosphodead) is unable to protect the cells (*p<0.01 versus control, n=2 independent experiments). **(B)** Silencing of Rictor decrease phosphorylation of FoxO, whereas phosphorylation of GSK3, 308AKT and S6K1 is unchanged. Raptor knockdown decreased phosphorylation of S6K1. **(C)** Cells treated with 50μM H_2O_2 for 3h and stained for Annexin V. Annexin V positive cells were considered to be apoptotic *P<0.05 vs vehicle, #P<0.05 vs Control H_2O_2. n=3 independent experiments.

Supplemental Figure 3

(A) Left panel: Cells isolated from an adult mouse LV 1 week after AAV9-GFP gene transfer were fixed, stained, and analyzed by flow cytometry. Cells were identified as myocytes by Troponin I, or as nonmyocytes negative for Troponin I. Myocytes positive for Troponin were also positive for GFP. Quantification of GFP positive cardiomyocytes. Right panel: Confocal microscopy of a paraffin-embedded PRAS40 mouse heart stained for Actin (red), FLAG (green) and nuclei (blue). Quantification of FLAG-tag positive cardiomyocytes 1 week after tail vein delivery. n=4 hearts. **(B)** Prolonged treatment with rapamycin impairs mTORC2 activation. Cell death in NRCMs. Short-term treatment with Rapamycin (20nM) but not prolonged treatment decreases cell death after challenge with H_2O_2 (50μM for 4h). (*p<0.01 versus control). **(C)**

Short term-treatment with rapamycin does not impair mTORC2 function after myocardial infarction (left panel), whereas treatment with rapamycin for three weeks before infarction decreases mTORC2 function after infarction.

Supplemental Figure 4

PRAS40 expressed in myocytes blocks mTORC1 and improves mTORC2 signaling *in vitro*.

(A) PRAS40 phosphorylation is diminished in Akt1KO mice. **(B)** Activation of Akt with insulin (100nM for 15min) is associated with increased PRA40 phosphorylation in isolated adult wild type myocytes. (**C)** PRAS40 overexpression reduces mTORC1 activation in response to H_2O_2 (50μM for 15min). **(D)** PRAS40 overexpression increase Akt phosphorylation in response to H_2O_2. **(E)** Cells treated with 50μM H_2O_2 for 3h and stained for Annexin V. Annexin V positive cells were considered to be apoptotic *P<0.05 vs vehicle, #P<0.05 vs Control H_2O_2. n=3 independent experiments. **(F)** Cell death in NRCMs, PRAS40 overexpression reduces cell death after challenge with H_2O_2 (50μM for 4h). Akt inhibitor V treatment (5μM) abrogates protection in PRAS40 overexpressing myocytes. Overexpression of a Akt^{S473A}, a point mutant of the kinase that cannot be phosphorylated abrogates protection. *p<0.05 versus control; $p<0.05 versus Control H_2O_2, #p,0.05 vs PRAS H_2O_2, n=4 independent experiments.

Supplemental Figure 5

(A) PRAS40 expression and phosphorylation increase in remote and border zone after infarct. *p<0.05 versus sham. **(B)** Confocal microscopy of sham or infarcted hearts stained for pPRAS (red), Actin (green) and nuclei (blue). In Sham operated mice only interstitial cells (non myocytes) show phosphorylation of PRAS40 whereas in infarcted hearts myocytes show strong phosphorylation of PRAS40.

Supplemental Figure 6

(A) Scheme of the AAV9PRAS40 under MLC2 promoter to overexpress PRAS40 in myocytes. **(B)** Immunoblot of whole hearts 4w after tail vein injection with either control or PRAS40 AAV9.

Histogram depicting quantification of PRAS40 expression (*p<0.01 vs Control)**.** Echocardiographic assessment of EF and FS in control and PRAS40 mice. **(C)** Line graph representing echocardiographic assessment of heart rate in the indicated groups. **(D)** Confocal microscopy of paraffin-embedded from PRAS40 mouse hearts after MI surgery (2d) stained for phosphoPRAS40 (red), Actin (green) and nuclei (blue), scale bar 150μm). **(E)** Confocal microscopy of paraffin-embedded from control and PRAS40 mouse hearts after MI in the border zone (6w) stained for phosphoRibS6 (red), Actin (green) and nuclei (blue), scale bar 150μm). **(F)** SERCA2a and NCX1 transcription in hearts of mice of the indicated group 6 weeks after Sham or MI (*p<0.01 versus Control sham; #p<0.05 versus Control MI). Error bars indicate means ± sem. n=6 per group.

Supplemental Figure 7

(**A)** Immunoblots of heart lysates for indicated protein in NTG and $PI3K^{-/-}$ mice after myocardial infarction (**B)** Immunoblots of heart lysates for indicated protein in $AKT1^{-/-}$ mice after myocardial infarction.

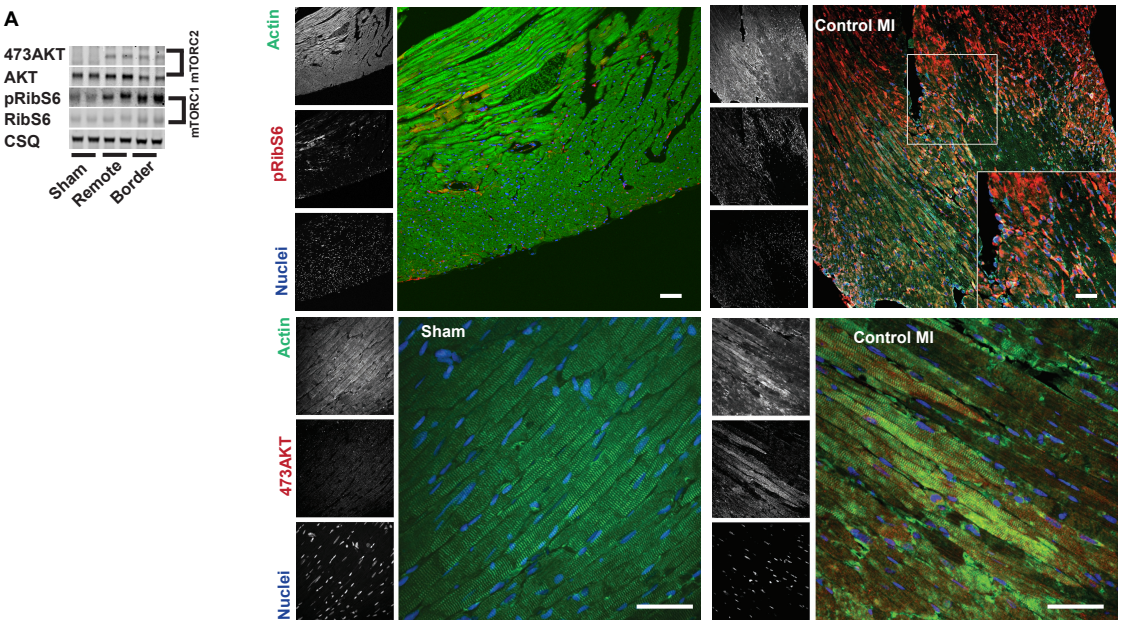
Supplemental Table 1-2

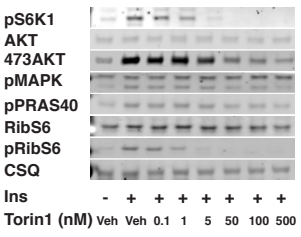
List of primers and antibodies

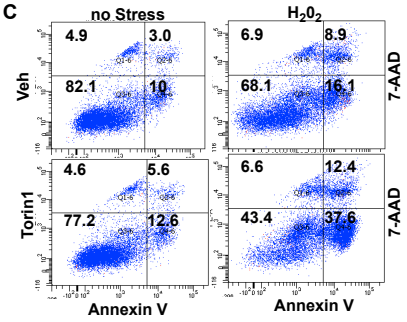
Supplemental Table 3-5

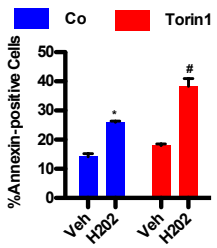
Characterization of mice after Sham or MI by morphometry and echocardiography. Abbreviations: n: number of mice analyzed; LVEDV,μl: left ventricular end diastolic volume; LVESV,μl: left ventricular end systolic volume; EF,%: ejection fraction. HR,bpm: Heart rate; BW, body weight; HW: heart weight; HW/BW: heart weight/body weight ratio

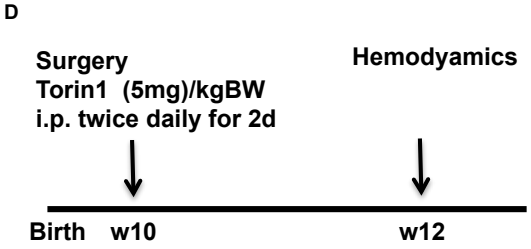
Supplemental Figure 1

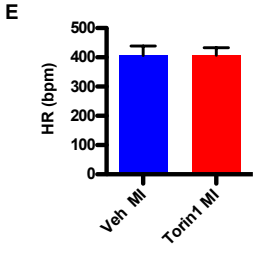






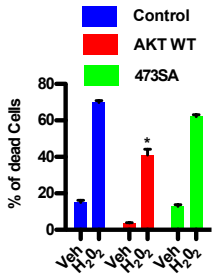






Supplemental Figure 2

A



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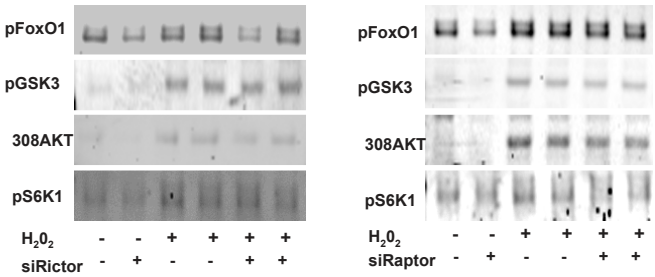

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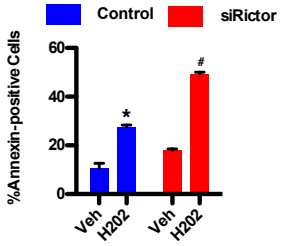
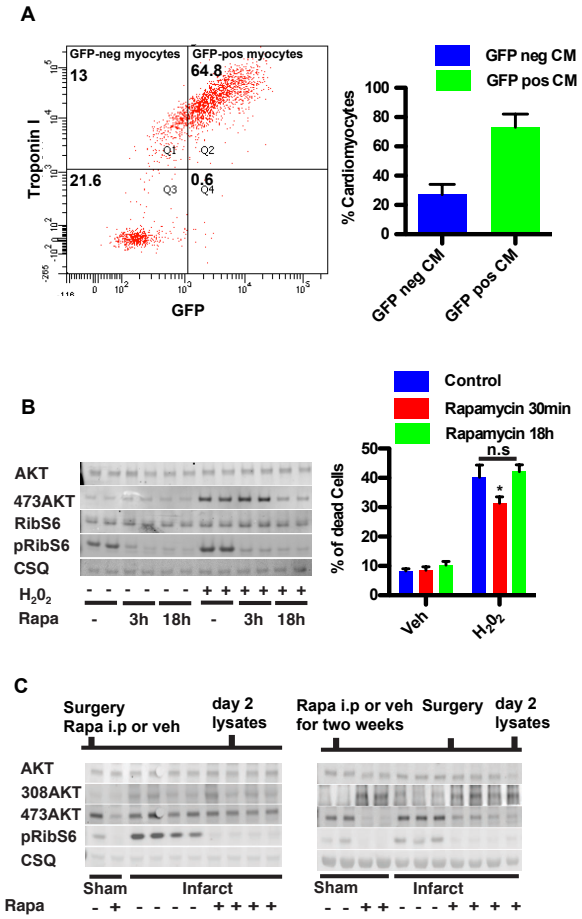
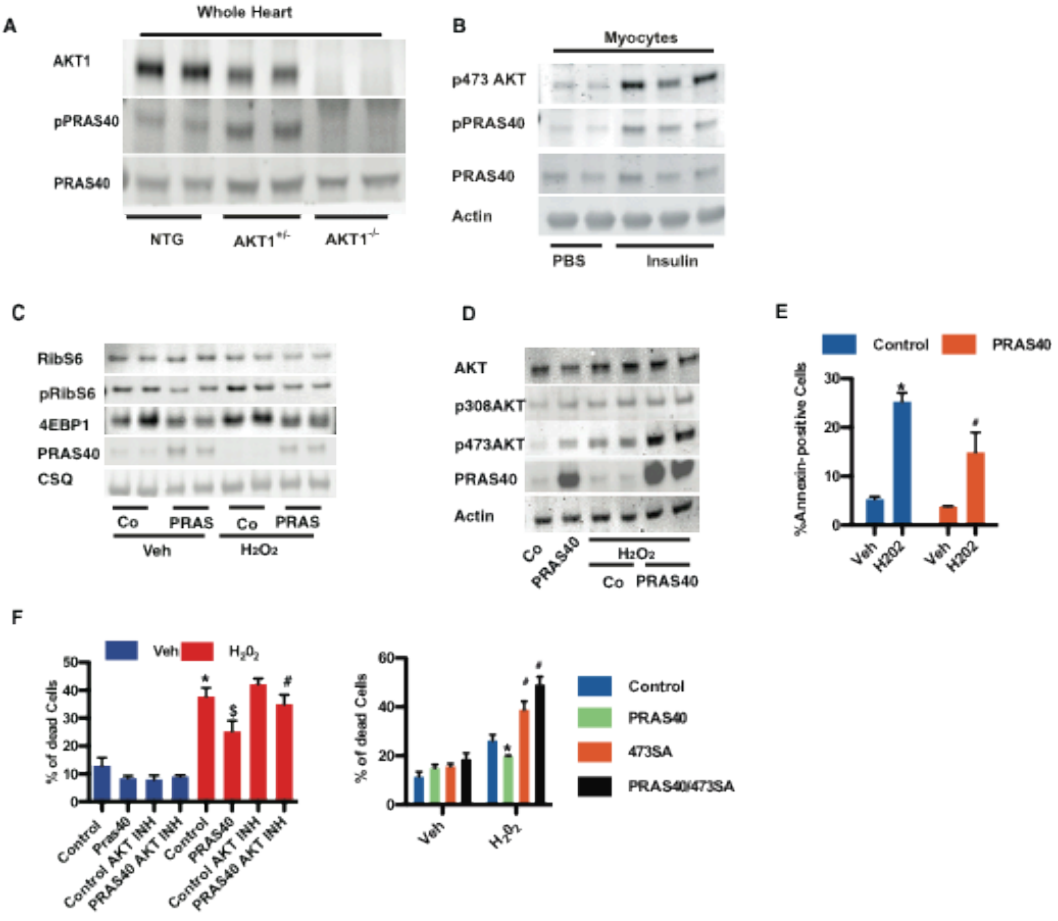


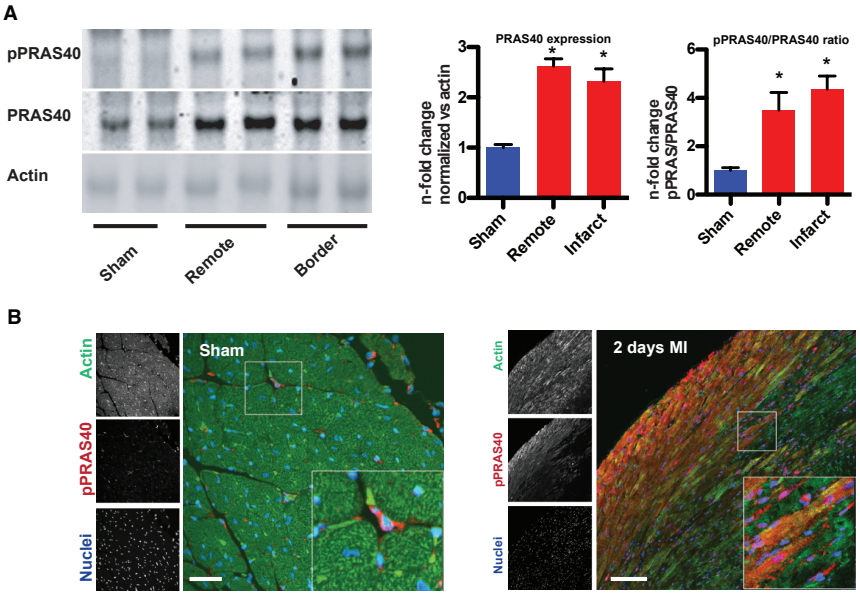
Supplemental Figure 3



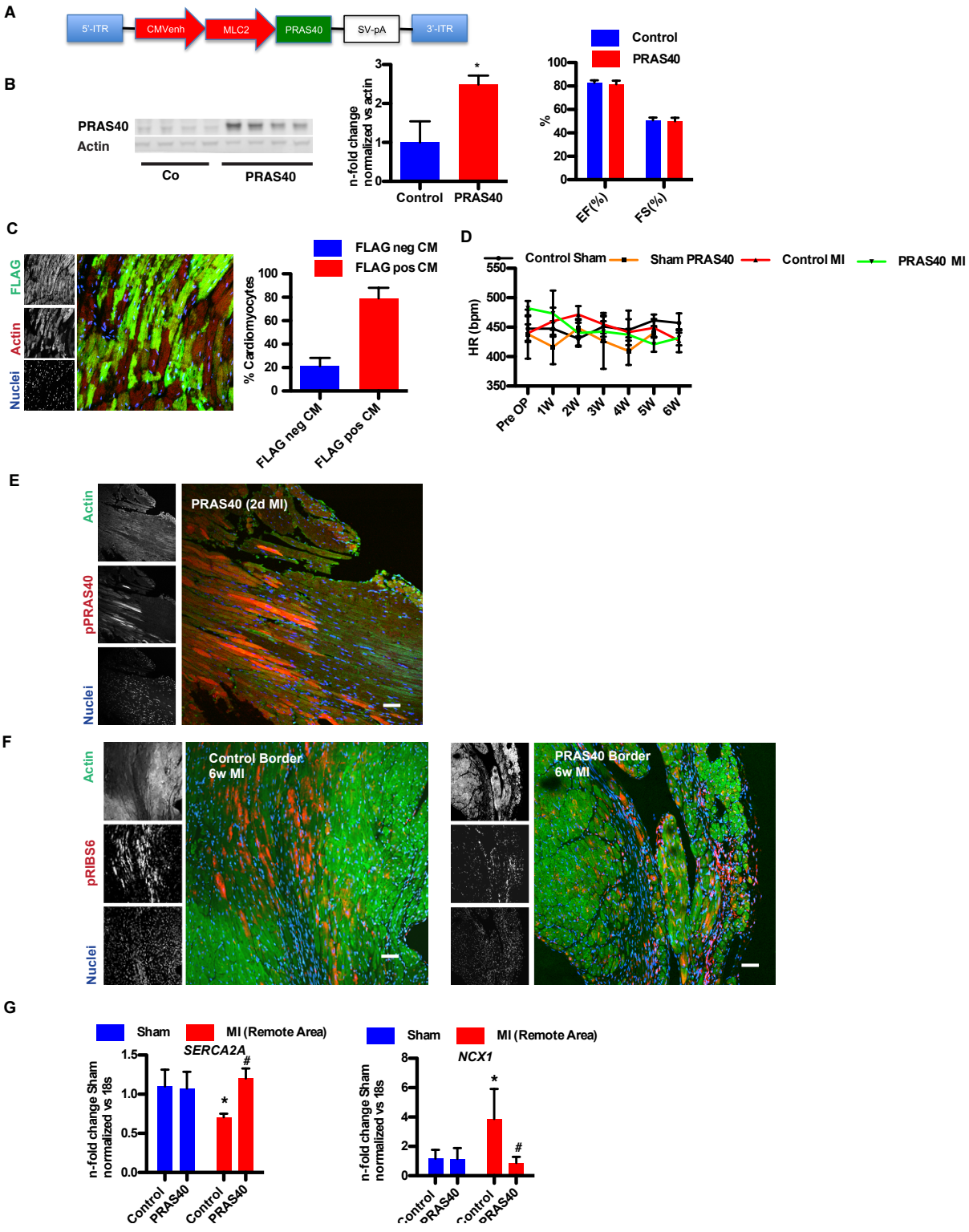
Supplemental Figure 4



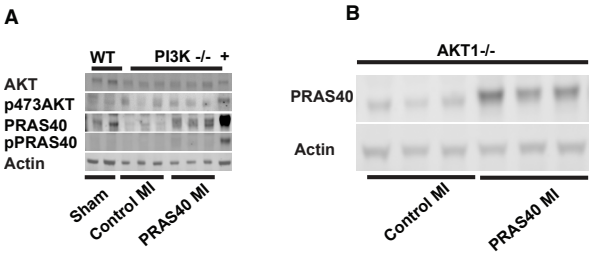
Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7



Supplementary Table 1

		Sham		MI	
		Veh n=4	Torin n=4	Veh n=6	Torin n=7
2 week after surgery					
	LVEDV, µl	65.75±6.3	66.1±2.5	103.5±5.04*	132.8±41.8*
	EF,%	41.55±1.71	40.33±2.435	20.21±1.98*	12.69±1.98#
	HR, bpm	475±19	456±19	406±32	413±21

Supplementary Table 2

		Sham	MI	
		shControl n=4	shControl n=6	shRictor n=6
2 week after surgery				
	LVEDV, µl	72.34±7.73	107.9±6.32*	138.6±29.59#
	EF,%	42.48±2.18	19.7±1.71*	10.25±1.53 #
	HR, bpm	427±22	450±14	473±20

Supplementary Table3

		Sham		MI	
		Control n=10	PRAS40 n=7	Control n=13	PRAS40 n=12
1 week after surgery	LVEDV, µl	71.43±6.75	64.76±2.73	115.13±9.54*	101.91±8.4
	LVESV, µl	41.31±2.59	48.12±6.81	93.99±6.48*	77.83±10.17
	EF,%	40.14±1.67	39.82±3.37	15.79±2.43*	25.59±1.36#
	HR, bpm	447±12	416±29	460±22	473±39
		Control n=10	PRAS40 n=10	Control n=13	PRAS40 n=12
6 weeks after surgery	LVEDV, µl	82.58±2.38	83.53±3.47	154.31±14.57*	112.08±8.77#
	LVESV, µl	45.93±3.08	42.32±6.78	139.95±10.94*	98.56±14.73#
	EF,%	43.82±2.15	42.20±0.86	11.96±1.71*	23.94±2.88 #
	HR, bpm	457±16	440±9	427±20	431±12
	HW/BW, mg/g	4.22±0.15	4.35±0.27	6.27±0.48*	5.03±0.0.25 #

Supplemental Table 1

18s Forward	5'-CGAGCCGCCTGGATACC-3'
18s Reverse	5'-CATGGCCTCAGTTCCGAAAA-3'
ANP Forward	5'-TGGGTCTTGTTAGGGCTCAAACCT-3'
ANP Reverse	5'-TGAAACTCAAGGGACACCCATCGT-3'
BNP Forward	5'-AATGGCCCAGAGACAGCTCTTGAA-3'
BNP Reverse	5'-CTTGTGCCCAAAGCAGCTTGAGAT-3'
β-MHC Forward	5'-GAGCCTTGGATTCTCAAACG-3'
β-MHC Reverse	5'-GTGGCTCCGAGAAAGGAAG-3'
mPRAS40 Forward	5'-CGGAGAGCACAGACGACGGC-3'
mPRAS40 Reverse	5'-GCACCGACACGGGCAGAGAC-3'
hPRAS40 Forward	5'-TCACCGTCGCGAGAAAGCGG-3'
hPRAS40 Reverse	5'-GCGACCTGACGTCCCTGACC-3'
Collagen 1a1 Forward	5'-AAGACGGGAGGGCGAGTGCT-3'
Collagen 1a1 Reverse	5'-TCTCACCGGGCAGACCTCGG-3'

Supplemental Table 2

Application	Antibody	Dilution	Amplify	Company
Immunoblot	Actin	1:2000	no	Santa Cruz (sc-81178)
Immunoblot	p246PRAS40	1:1000	no	CST (#2640)
Immunoblot	PRAS40	1:1000	no	CST (#2691)
Immunoblot	pS6Rib	1:2000	no	CST (#4857)
Immunoblot	RibS6	1:500	no	CST (#2317)
Immunoblot	p4EBP1	1:1000	no	CST (#2855)
Immunoblot	p473AKT	1:1000	no	CST (#4058)
Immunoblot	p389S6K	1:500	no	CST (#9234)
Immunoblot	IRS-1	1:1000	no	CST (#2390)
Immunoblot	p389S6K	1:500	no	CST (#9205)

Immunoblot	AKT	1:2000	no	CST (#2966)
IP	Raptor	1:500	no	CST (#2280)
IHC	α-sarcomeric actin	1:100	no	Sigma (A2172)
IHC	p246PRAS40	1:100	yes	CST (#2640)
IHC	PRAS40	1:100	no	CST (#2691)
IHC	pS6Rib	1:100	yes	CST (#4857)
IHC	p473AKT	1:50	yes	CST(#9271)
IHC	FLAG	1:100	yes	Sigma (F3165)
PLA	Raptor	1:50	no	CST (#2280)
PLA	PRAS40	1:50	no	Milipore (05-1070)

1. Völkers, M. *et al.* The inotropic peptide βARKct improves βAR responsiveness in normal and failing cardiomyocytes through G(βγ)-mediated L-type calcium current disinhibition. *Circulation Research* **108**, 27–39 (2011).