SUPPLEMENTAL MATERIAL

Supplemental Materials and Methods

In vitro profiling of G-protein signaling and β-arrestin 2 recruitment

HEK-293 cells were stably transfected to overexpress β-arrestin 2 fused to a β-galactosidase fragment and human AT1R fused to a complementary β-galactosidase fragment. The growth medium used was MEM with 10% FBS, 1% penicillin/streptomycin, 150 µg/liter of neomycin, and 150 µg/liter of hygromycin. β-arrestin 2 recruitment and inositol monophosphate (IP1) accumulation were measured in small-volume, 384-well plates on a BMG PheraStar multimodal reader (Durham, NC). IP1 was measured with the IP-One Tb HTRF kit per manufacturer's instructions (Cisbio, Bedford, MA) using a time-resolved fluorescence ratio (665 nm/620 nm). The PathHunter protein complementation assay (DiscoveRx Corporation, Fremont, CA) was performed according to the manufacturer's protocol and read for chemiluminescent

signaling, as described previously.[1]

Osmotic Pump Implantation and Drugs

The biased ligand, TRV120067 (Ser-Arg-Val-Tyr-Lys-His-Pro-Ala-OH) (synthesized by GenScript, Piscataway, NJ) was administered at 28.8 mg/kg/day, based on the dose used in our previous study optimized for treatment with the osmotic pumps, and the ARB, losartan (Sigma, St. Louis, MO.) administered at 10.0 mg/kg/day were administered by osmotic mini pump (Alzet Model 2004, Durect Inc., Cupertino, CA).[2] Both drugs were diluted in sterile saline. Osmotic mini pumps were incubated in sterile saline at 37°C for 48 hours prior to use. Mice were anesthetized in a closed Plexiglas chamber using 2% isoflurane delivered by vaporizer and surgical anesthesia was maintained using 1-2 % isoflurane delivered through a nose cone supplying 100% oxygen. One dose of

buprenorphine (0.1 mg/kg) was administered before the start of the procedure, after induction of anesthesia, for post-operative analgesia. The level of surgical anesthesia was monitored by lack of toe-pinch reflex throughout the procedure. The pumps were then implanted subcutaneously along the dorsal surface of the neck. The skin was closed with 6-0 prolene monofilament sutures (Covidien LLC, Mansfield, MA), which were removed 7-10 days after surgery, as described previously.[3] This procedure was repeated three times per mouse (once per month per three months) to replace empty pumps as to accommodate the length of the treatment protocol.

High-Resolution Echocardiography

Mice were anesthetized as described above for osmotic pump implantation. The mouse was then secured in the supine position on a warming plate and hair removed from the chest using Nair™ depilating agent. Body temperature was monitored and kept to 37°C throughout the procedure. Transthoracic echocardiography was performed using a Vevo 2100 High-Resolution In Vivo Imaging System and MS400 scan head with a frequency of 40 MHz (VisualSonics, Toronto, ON). Anatomical M-Mode images of the left ventricle (LV) outflow tract (LVOT), ascending aorta (AO) and left atrium (LA) were taken from the parasternal long axis view. The parasternal short axis view at the level of the papillary muscles was used to measure the LV internal dimension (LVID), inter-ventricular septum (IVS) and posterior wall (PW) thicknesses. Pulsed Doppler was performed in the apical four-chamber view. The mitral inflow was recorded with the Doppler sample volume at the tip level of the mitral valve leaflets to obtain the peak velocities of flow in the early phase of diastole (E) and after LA contraction (A). The Doppler sample volume was

moved toward the LVOT and both the mitral inflow and LV outflow were simultaneously recorded to measure the isovolumic relaxation time (IVRT). Additional information about the diastolic function was obtained with tissue Doppler imaging. Peak myocardial velocities in the early phase of diastole (E') and after LA contraction (A') were obtained with the sample volume at the septal side of the mitral annulus in the four-chamber view. All measurements and calculations were averaged from three consecutive cycles and performed according to the American Society of Echocardiography guidelines. Data analysis was performed offline with the Vevo 2100 Analytic Software (VisualSonics, Toronto, ON).[3]

Histological Analysis

Mice at the end of the treatment protocol were heparinized, and euthanized as described above. Their hearts were excised and immediately retrogradely perfused with saline, followed by 10% neutral buffered formalin. The hearts were allowed to sit in formalin for 48 hours, after which the hearts were sectioned into four sections using a mouse heart-sectioning guide (Zivic Instruments, Pittsburgh, PA). The sections were then sent to Veterinary Diagnostic Laboratory at the University of Illinois at Urbana-Champaign College of Veterinary Medicine for paraffin-embedding and further sectioning to 3 μm-thick sections. These sections were then deparafinized and re-hydrated, antigen retrieval performed using boiling sodium citrate buffer (10 mM sodium citrate, 0.05% tween-20, pH 6.0) for one hour, and washed three times for 10 mins in PBS. The sections were then stained using wheat germ agglutinin conjugated to FITC (Sigma, St. Louis, MO) at a dilution of 1:200 in PBS for two hours. The slides were washed three times for 10 mins in PBS and imaged on a Carl Zeiss Observer.Z1 with

attached AxioCam MRm imager (Carl Zeiss AG, Oberkochen, Germany). Cell size was determined by outlining the cell area of sharply cross-sectioned cells and area calculated using ImageJ (National Institutes of Health, Bethesda, MD), as described previously.[4]

Detergent-Extracted ("Skinned") Myofiber Bundles

Mice were heparinized (100 IU/kg), anesthetized using ketamine/xylazine for euthanasia as described above, and hearts extracted by cardiectomy. Left ventricular papillary muscles were then isolated, dissected into fiber bundles approximately 200 μm in width and 3-4 mm in length and detergent-extracted in a high relaxing (HR) solution (10 mM EGTA, 41.89 mM K-Propinate, 100 mM BES, 6.75 mM $MgCl_2$, 6.22 mM Na_2ATP, 10mM Na_2CrP, 5mM NaN_3, pH 7.0) with 1% v/v Triton X-100 for 3-4 hours at 4°C. The HR solution was then replaced with HR solution without Triton X-100. Free Ca^{2+} concentrations were calculated using WEBMAXC STANDARD, and ranged from pCa (-log [Ca^{2+}]) values of 8.0 to 4.5. Free Ca^{2+} concentrations were generated by mixing varying ratios of HR solution with HR solution containing 9.99 mM $CaCl_2$. Fiber bundles were mounted between a micromanipulator and a force transducer and bathed in HR solution. The sarcomere length, measured using He-Ne laser diffraction and adjusted to 2.3 μm, was set using a micromanipulator and kept constant throughout the experiment. The fibers were initially contracted at pCa 4.5, placed back into HR solution, and the width and diameter measured along three points. Fibers were then subjected to sequential increases in Ca^{2+} concentration; their developed force was recorded on a chart recorder, as described previously.[5] All experiments were carried out at 23°C.

Neonatal Rat Ventricular Myocyte Isolation, Culture, and Treatment

Hearts were removed from one to two-day-old neonatal Sprague-Dawley rats and placed in cold Dulbecco's modified Eagle's medium (DMEM) (Catalog 11-995-065, Gibco, Waltham, MA) and washed several times in cold DMEM to remove blood. The hearts were then placed in fresh, cold DMEM, atria removed, and ventricles minced. The minced tissue was suspended in 1.0 mg/mL Collagenase type 2 (Worthington Biochemical, Lakewood, NJ) in phosphate-buffered saline. The tissue was allowed to digest at 37°C in a shaking water bath and titurated every 10 mins through a cannula attached to a syringe. The supernatant was placed in an equal volume of fetal bovine serum (Denville Scientific, Inc., Holliston, MA) and the pellet resuspended in fresh collagenase solution. This was reputed until the tissue was completely digested. The supernatant was then centrifuged for 10 min at 1,000 x g, and resuspended in DMEM containing 10% FBS, filtered through a 70 μm cell strainer to remove large material and allowed to pre-plate in a 5% CO_2 incubator for one-hour. Non-adherent cells were collected and centrifuged for 10 min at 1,000 x g and resuspended in PC-1 media (Lonza Chemical, Basel, Switzerland). The cells were plated in fibronectin-coated (10 μg/ml) six-well plates at a density 1,500,000 cells per dish or 35 mm glass-bottom dishes at a density of 200,000 cells per dish and allowed to plate for 48 hours and maintained in a 5% CO_2 incubator, as described previously.[6] After 48 hours, the cells were placed in serum-free DMEM for 12 hours before treatment. For inhibitor studies, 10 μM BI-D1870 (Cayman Chemical, Ann Arbor, MI) and 15 μM FR180204 (Cayman Chemical, Ann Arbor, MI) were added to the media one hour prior to 500 nM losartan (Sigma, St. Louis, MO), 15 μM TRV067, or 1 μM ang II (Sigma, St. Louis, MO) and

treated for one hour. Cells were then prepared for biochemical and microscopic analysis by sub-cellular fractionation or harvested for immunoblotting.

Subcellular Fractionation

The Calbiochem ProteoExtract Subcellular Proteome Extraction Kit was used (EMD Millipore, Darmstadt, Germany) according to manufacturer's instructions with the addition of phosphatase inhibitors (EMD Millepore, Darmstadt, Germany) at a 1:100 dilution, as described previously.[6] The media was removed from the cells and the cells washed two times in ice-cold wash buffer. The cells were then incubated in buffer I for 10 min at 4°C on a rocker, the buffer was removed and saved as the cytosolic fraction. The cells were briefly washed once in ice-cold wash buffer and buffer II was added for 30 min at 4°C on a rocker. The buffer was removed and saved as the membrane fraction. The cells were briefly washed once in ice-cold wash buffer and buffer III was added for 30 min at 4°C on a rocker. The buffer was removed and saved as the nuclear fraction. The cells were briefly washed once in ice-cold wash buffer and room temperature buffer IV was added and the cells scraped and harvested as the sarcomeric fraction. The cells were then prepared for western blot by adding 4X Laemlli sample buffer (BioRad, Inc., Hercules, CA) and protein concentration determined described below. Subcellular fractionation for staining and microscopy was performed as described above, with the exception that the cells were fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO) after the cytosolic membrane fractions were collected so that the nuclear and sarcomeric fractions were left on the dish.

Neonatal Rat Ventricular Myocyte Staining and Microscopy

After subcellular fractionation, the nuclear and sarcomeric fractions remaining were washed for 10 min in PBS containing 2% triton-X100 and then washed three times for 10 mins in PBS. The cells were blocked in 1% BSA in PBS for 30 mins, and washed three times for 10 mins in PBS. The cells were immunostained with mouse MF-20 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at a dilution of 1:600 in 1% BSA in PBS and β-arrestin 2 antibody, which actually recognizes both beta-arrestin 1 and 2, at a dilution of 1:200 in 1% BSA in PBS (Boster Biological Technology, Pleasanton, CA) overnight. The next day, cells were washed three times for 10 mins in PBS and incubated for one hour in secondary fluorescent-labeled goat-anti-mouse Alexa Fluor 568 and goat-anti-rabbit Alexa Fluor 488 antibodies (Thermo Fisher Scientific, Waltham, MA) shielded from light. After one hour, the cells were washed three times for 10 mins in PBS and then left in PBS for imaging. Cells were imaged on a Carl Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Left Ventricular Preparations

Snap-frozen left ventricular tissue (15.0-20.0 mg) was homogenized using a Duall ground-glass homogenizer in urea-thiourea (UT) sample buffer (8 M urea, 2 M thiourea, 0.05 M tris base, 75 mM DTT, 3% SDS, 0.005% bromophenol blue, adjusted to pH 6.8) containing protease (Sigma, St. Louis, MO) and phosphatase inhibitor cocktails (Calbiochem, Darmstadt, Germany) at a 1:100 dilution and 1 μM calyculin A (Cell Signaling Technology, Danvers, MA) and transferred to a microcentrifuge tube. Lysates were then vortexed for 30 min, sonicated in a water-bath sonicator for 10 minutes, and vortexed for a final 15 min. The samples were then spin-clarified by centrifugation at

15,000 x g for four min. Protein concentrations of all the samples were determined using the RC-DC assay (Bio-Rad, Inc., Hercules, CA) and samples were stored at -80°C until used.

β-Myosin Heavy Chain Expression

To determine the myosin heavy chain isoform population of mouse hearts in our study, we loaded 2.0 µg of left ventricular tissue onto a 16 cm x 16 cm 6% SDS-PAGE gel, prepared as described previously.[7] Heart tissue from a post-natal day zero mouse (3.0 µg) served as the standard. Electrophoresis was carried out at constant amperage of 16 mA until the dye front ran off. To visualize total protein, Coomassie blue R-250 stain (Bio-Rad, Inc., Hercules, CA) was added for 30 min, then de-stained with 10% methanol, 10% acetic acid, and imaged on a ChemiDoc XRS+ (Bio-Rad, Inc., Hercules, CA), using a Coomassie blue filter set. Band densities from gels were determined using ImageLab 5.0 software (Bio-Rad, Inc., Hercules, CA).

Myofibrillar Preparations

Snap-frozen left ventricular tissue (10.0 to 15.0 mg) was homogenized twice using glass Dounce homogenizers in standard relax buffer (10 mM Imidazole pH 7.2, 75 mM KCL, 2 mM MgCl2, 2 mM EDTA, and 1 mM NaN_3) with 1% (v/v) Triton X-100, as described previously.[8] Myofribrils were centrifuged and the supernatant fraction was removed. The pellets were then washed once in standard relax buffer to remove the Triton X-100. The standard relax buffers contained both the protease (Sigma, St. Louis, MO) and phosphatase (Calbiochem, Darmsdat, Germany) inhibitors at a 1:100 dilution. The pellet was solubilized in 2X Laemlli sample buffer (Bio-Rad, Inc., Hercules, CA) containing 50

mM DTT and the protein concentration of the samples was determined with an RC-DC assay kit (Bio-Rad, Inc., Hercules, CA). Samples were stored at -80°C until used.

In Vitro Myofibril Kinase Assay

Hearts were isolated from heparinized, anesthetized four-month old NTG mice and Langendorff perfused in a continuously oxygenated Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM D-glucose and adjusted to pH 7.4 with $NaHCO_3$) for 10 mins and then for 1 hr in Krebs-Henseleit buffer that contained 5 μM propranolol (Sigma, St. Louis, MO) and 5 μM ML-7 (Cayman Chemical, Ann Arbor, MI). At the end of perfusion, the hearts were snap-frozen and stored at -80°C until used. Myofibrils were isolated as described above, except that they were washed twice in and resuspended in kinase assay buffer (25 mM MOPS, 25 mM $MgCl_2$, 12.4 mM glycerol-2-phosphate, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, pH 7.2) before use. 3.0 μg of myofibrils were added to the reaction tube with 0.3 μg of active RSK3 (Lot F385-3, 90% purity, 151 nmol/min/mg specific activity) (SignalChem, Richmond, BC, Canada). ATP dissolved in kinase assay buffer was used at a final concentration of 10 mM to start the reaction. Controls were tubes that contained myofibrils and ATP, and kinase assay buffer was added in place of RSK3. The reactions were carried out at 30°C on a dry heating plate from 0 to 20 mins. The reaction was stopped by adding an equal volume of UT buffer containing protease (Sigma, St. Louis, MO) and phosphatase inhibitor cocktails (Calbiochem, Darmstadt, Germany) at a 1:100 dilution and 1 μM calyculin A (Cell Signaling Technology, Danvers, MA) followed by heating at 95°C for 1.5 min. The samples were run on a 12% SDS-PAGE gel the same day.

One-dimensional Isoelectric Focusing PAGE

Samples were prepared as described in left ventricular homogenates but the samples were homogenized in 8 M urea, 2 M thiourea, 4% CHAPS. The isoelectric focusing gel consisted of 5% acrylamide, 6 M urea, 1.5% pH 4.2-4.9 pharmalyte (GE Healthcare, Chicago, IL), 0.5% pH 4-6 ampholyte (SERVA Electrophoresis GmbH, Heidelberg, Germany), 0.02% ammonium persulfate (APS), and 0.02% TEMED. The solution was filtered through a 0.2 µm syringe-driven filter and degassed for 30 min before the addition of APS and TEMED, where it was then cast to a mini-gel format. The gel was allowed to polymerize for 2 hrs at room temperature. The upper buffer consisted of 20 mM lysine and 20 mM arginine and the lower buffer consisted of 15 mM phosphoric acid. The gel was run at 4°C at 100 V for 15 mins and 500 V for 3 hours. The gel was then washed in 10 mM CAPS, pH 11, twice for 10 mins and transferred to PVDF membrane for immunoblotting as described below.

Co-immunoprecipitation

Using a commercially available kit (Thermo Scientific, Rockford, IL), primary antibodies against proteins of interest were cross-linked to protein A/G magnetic beads and washed to remove unbound antibody per manufacturer's instructions. Cross-linked antibodies are incubated with samples suspended in IP Lysis buffer provided for one hour at 4°C to bind to protein complexes on a tube rotator. The beads were collected using a magnetic stand and the unbound sample saved and complexes are eluted from the beads using the provided elution buffer. The eluate was precipitated using the 2D clean-up kit (GE healthcare, Chicago, IL) and then suspended in UT buffer and run on an SDS-PAGE gel

and transferred to PVDF membrane for immunoblotting as described below.

Immunoblotting

Following gel electrophoresis, proteins were transferred to 0.2 μm PVDF membranes (Bio-Rad, Inc., Hercules, CA) in 10 mM CAPS, pH 11.0, using the Criterion tank blotter system (Bio-Rad, Inc., Hercules, CA) for 90 min at 25 V, constant voltage. After transfer, total protein was visualized using SWIFT total protein stain (G-Biosciences, St. Louis, MO). The blots were then blocked for one hour in 5% non-fat skim milk at room temperature and washed three times for 10 min each in tris-buffered saline, pH 7.5, containing 0.1% Tween-20 (TBS-T). Blots were then probed in primary antibody overnight at 4°C. Antibodies were diluted in 1% bovine serum albumin and used at the following dilutions: 1:1000 for total total RSK3 (Abcam, Inc. Cambridge, MA) β-catenin, p-ERK1/2, total ERK1/2, p-RSK3, SERCA2, p-SRF, total SRF (Cell Signaling Technology, Danvers, MA), total MLC2v (Enzo Life Sciences, Farmindale, NY), p-MYPT1/2 and p-PLN S16 (Upstate Biotechnology, Darmsdat, Germany), and total MYPT1/2 (BD Transduction Laboratories, Franklin Lakes, NJ); 1:2000 for p-Akt and total Akt (Cell Signaling Technology, Danvers, MA), β-actin (Proteintech Group, Chicago, IL); 1:4000 for p-PLN T17 (Badrilla, Leeds, UK), total PLN (Upstate Biotechnology, Darmsdat, Germany), and total cTnI (Fitzgerald Industries Intl., Acton, MA), 1:1000 for total Tm (CH1) (Developmental Studies Hybridoma Bank, Iowa City, Iowa). The phospho-specific (S273, S282, and S302) cMyBP-C antibodies were generated by Sakthivel Sadayappan, PhD. The total cMyBP-C antibody was generated by Richard L. Moss, PhD. The p-Tm (S283) antibody was generated by our lab. The

membranes were then washed three times for 10 minutes each in TBS-T and incubated in secondary antibody diluted in 2.5% non-fat dry skim milk, 1:20,000-40,000 for anti-rabbit (Cell Signaling Technology, Danvers MA) and 1:50,000 for anti-mouse (Sigma, St. Louis, MO). The membranes were washed again three times for 10 minutes each in TBS-T, incubated for four minutes in either Clarity ECL Substrate (Bio-Rad, Inc., Hercules, CA) or SuperSignal West Femto Maximum Sensitivity ECL Substrate (Thermo Fisher Scientific, Waltham, MA), imaged on imaged on a ChemiDoc XRS+ (Bio-Rad, Inc., Hercules, CA), and band densities determined using Image Lab 5.0 software (Bio-Rad, Inc., Hercules, CA).

Supplemental References

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Supplemental Figure Legends

Supplemental Table 1. Cardiac function and morphology was improved in Tm-E54K mice after chronic treatment with TRV067. After three months TRV067 treatment, Tm-E54K animals had improved ejection fraction and fractional shortening compared to untreated and losartan treated Tm-E54K animals. TRV067 and losartan had no significant effect on NTG animals. LA, Left Atrial; LVAW;d, Left Ventricular Anterior Wall Dimension at diastole; LVID;d; Left Ventricular Internal Diameter at diastole; LVID;s, Left Ventricular Internal Diameter at systole; LVPW;s, Left Ventricular Posterior Wall Dimension at diastole; LVPW;s, Left Ventricular Posterior Wall dimension at Systole; E/A, early to late mitral inflow velocities; E/Em, early transmitral flow velocity to early diastolic tissue velocity; EF, Ejection Fraction; FS, Fractional Shortening; Vcf, Velocity of circumferential fiber shortening; Sm, Systolic Myocardial Velocity. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.

Supplemental Figure 1. TRV067 is a β-arrestin 2 biased ligand. β-arrestin 2 recruitment (black filled circles) was measured by chemiluminescent β-galactosidase activity upon enzyme fragment complementation and G-protein coupling was determined by measuring IP1 accumulation (grey open squares) in HEK-293 cells expressing human AT1R for **(A)** angiotensin II and **(B)** TRV067. Data are means ± SEM of 20 or more independent experiments.

Supplemental Table 2. Analysis of sex-related functional differences in NTG and Tm-E54K Mice. NTG and Tm-E54K four-month-old mice showed no significant

difference in functional or morphological parameters due to sex within genotype. LA, Left Atrial; LVAW;d, Left Ventricular Anterior Wall Dimension at diastole; LVID;d; Left Ventricular Internal Diameter at diastole; LVID;s, Left Ventricular Internal Diameter at systole; LVPW;s, Left Ventricular Posterior Wall Dimension at diastole; LVPW;s, Left Ventricular Posterior Wall dimension at Systole; E/A, early to late mitral inflow velocities; E/Em, early transmitral flow velocity to early diastolic tissue velocity; EF, Ejection Fraction; FS, Fractional Shortening; Vcf, Velocity of circumferential fiber shortening; Sm, Systolic Myocardial Velocity. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.

Supplemental Figure 2. Analysis of mutant tropomyosin expression in Tm-E54K mice. Representative **(A)** Western blot of narrow-range 1D isoelectric focusing gel (pH 4.2-4.9) showing 23.08% ± 7.92 expression of the more basic mutant Tm in Tm-E54K mice and **(B)** no change in total tropomyosin expression assessed by Western blot. Data were analyzed by unpaired Student's t-test and represented as means ± SEM. N = 6 hearts per group.

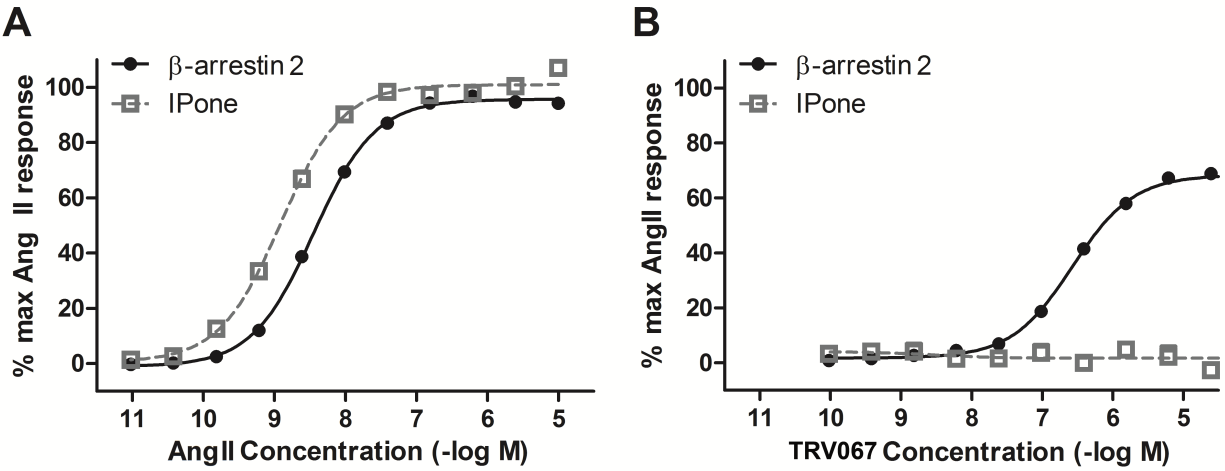
Supplemental Figure 3. Representative M-Mode and tissue Doppler images. Representative **(A)** M-Mode images where red dashed lines indicate left ventricular internal dimension at systole and green dashed lines indicated left ventricular internal diameter at diastole taken from untreated and treated mice after three months treatment. **(B)** Tissue Doppler images taken from untreated and treated mice after three months treatment.

Supplemental Figure 4. Cardiomyocyte cross-sectional area was increased by TRV067 in Tm-E54K mice. Images taken from wheat germ agglutinin (WGA)-stained sections of hearts and summarized quantification of cell cross-sectional area taken from mice treated for three months Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test for multiple comparisons and represented as means ± SEM. N = 4 separate cultures. * $p \leq 0.05$, ** $p \leq 0.01$.

Supplemental Figure 5. TRV067 and losartan reduce maladaptive β-myosin heavy chain expression. Representative SDS-PAGE gel and summarized quantification showing β-myosin heavy chain expression was increased in Tm-E54K mice, but significantly reduced in TRV067 and losartan-treated mice. No expression of β-myosin heavy chain was found in any NTG group. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test for multiple comparisons and represented as means ± SEM. N = 4-6 hearts per group. ** $p \leq 0.01$, *** $p \leq 0.001$.

Supplemental Figure 6. Signaling activated by TRV067 is RSK and ERK1/2-dependent. Representative Western blot images and quantification showing **(A)** RSK3, **(B)** MYPT1/2 and **(C)** SRF phosphorylation is increased in NRVMs when treated with TRV067 but not losartan. This increase was abolished with pre-treatment for one hour with BI-D1870, a pan-RSK inhibitor, or FR180204, a specific ERK1/2 inhibitor. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test for multiple comparisons and represented as means ± SEM. N = 4 separate cultures. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

SUPPLEMENTAL FIGURE 1

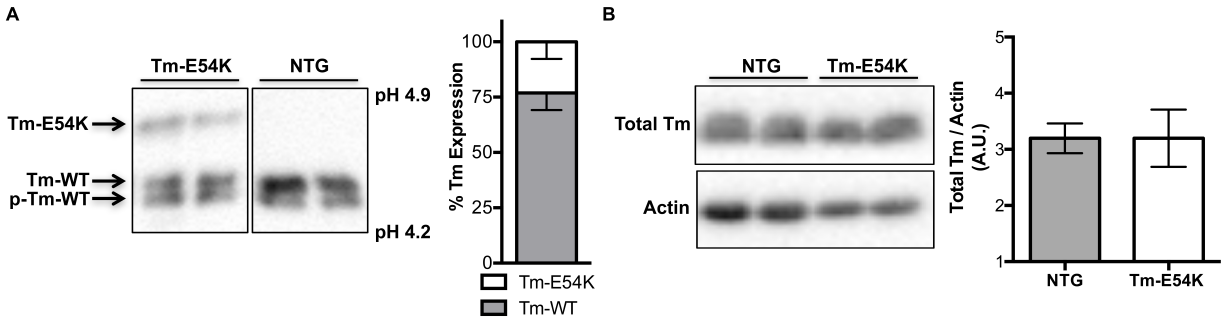


Supplemental Table 1. Assessment of Cardiac Function and Morphology *in vivo* by Echocardiography after Three Months Treatment.

Parameter	NTG Untreated	NTG TRV067	NTG Losartan	Tm-E54K Untreated	Tm-E54K TRV067	Tm-E54K Losartan
Sample Size (n)	13	8	7	11	10	6
Heart Rate (bpm)	483 ± 10	523 ± 17	523 ± 22	510 ± 12	506 ± 13	491 ± 10
LA Size (mm)	1.94 ± 0.08	1.74 ± 0.06	1.76 ± 0.17	2.27 ± 0.08*	2.14 ± 0.07	2.24 ± 0.07
LVAW;d (mm)	0.71 ± 0.04	0.63 ± 0.04	0.63 ± 0.04	0.52 ± 0.03*	0.59 ± 0.03	0.58 ± 0.04
LVAW;s (mm)	1.07 ± 0.04	1.00 ± 0.06	1.04 ± 0.04	0.68 ± 0.05*	0.88 ± 0.03†	0.72 ± 0.05
LVID;d (mm)	3.79 ± 0.08	3.88 ± 0.09	3.79 ± 0.13	4.60 ± 0.11*	4.73 ± 0.07	4.68 ± 0.15
LVID;s (mm)	2.32 ± 0.13	2.40 ± 0.07	2.18 ± 0.11	3.78 ± 0.11*	3.50 ± 0.05	3.88 ± 0.18
LVPW;d (mm)	0.62 ± 0.03	0.57 ± 0.04	0.57 ± 0.01	0.48 ± 0.03*	0.51 ± 0.03	0.53 ± 0.05
LVPW;s (mm)	1.02 ± 0.04	1.00 ± 0.07	0.93 ± 0.05	0.59 ± 0.03*	0.80 ± 0.02†	0.70 ± 0.04†
Ejection Fraction (%)	69.76 ± 2.74	69.84 ± 1.24	73.75 ± 3.70	36.76 ± 1.62*	51.18 ± 1.04†	36.00 ± 2.83‡
Fractional Shortening (%)	38.09 ± 1.92	38.85 ± 3.92	42.44 ± 3.36	17.65 ± 0.86*	26.14 ± 0.65†	17.30 ± 1.50‡
Stoke Volume (μL)	41.51 ± 1.70	45.66 ± 2.36	45.30 ± 4.52	35.85 ± 2.36*	52.05 ± 1.93†	36.14 ± 1.54‡
Cardiac Output (mL/min)	21.81 ± 1.03	24.00 ± 1.63	24.25 ± 4.33	16.77 ± 0.74*	26.27 ± 1.01†	16.94 ± 0.56‡
Vcf (mm/s)	9.45 ± 0.73	11.16 ± 0.61	10.48 ± 1.10	4.07 ± 0.25*	6.51 ± 0.26†	4.10 ± 0.30‡
Sm (mm/s)	28.00 ± 1.07	25.88 ± 1.87	31.56 ± 1.88	18.84 ± 0.68*	24.01 ± 1.14†	21.07 ± 0.92
IVRT (ms)	12.12 ± 0.42	10.83 ± 1.15	10.39 ± 0.46	18.35 ± 0.59*	17.73 ± 1.58	17.62 ± 0.74
E-wave (mm/s)	840 ± 40	869 ± 81	812 ± 62	677 ± 47	685 ± 42	821 ± 56
A-wave (mm/s)	494 ± 33	487 ± 10	467 ± 18	388 ± 61	487 ± 31	407 ± 97
Em-wave (mm/s)	-25.52 ± 2.34	-23.55 ± 3.01	-22.18 ± 0.97	-21.22 ± 1.23	-21.05 ± 1.54	-18.87 ± 0.94
E/A Ratio	1.67 ± 0.09	1.73 ± 0.20	1.76 ± 0.17	1.77 ± 0.21	1.43± 0.07	1.92 ± 0.28
E/Em Ratio	-35.52 ± 2.87	-40.50 ± 2.59	-36.80 ± 3.10	-29.48 ± 2.75	-32.14 ± 2.69	-43.39 ± 2.62

Values are Mean ± SEM.
* $p \leq 0.01$ vs. NTG,
†$p \leq 0.01$ vs. Tm-E54K, ‡$p \leq 0.01$ vs. Tm-E54K TRV067

SUPPLEMENTAL FIGURE 2

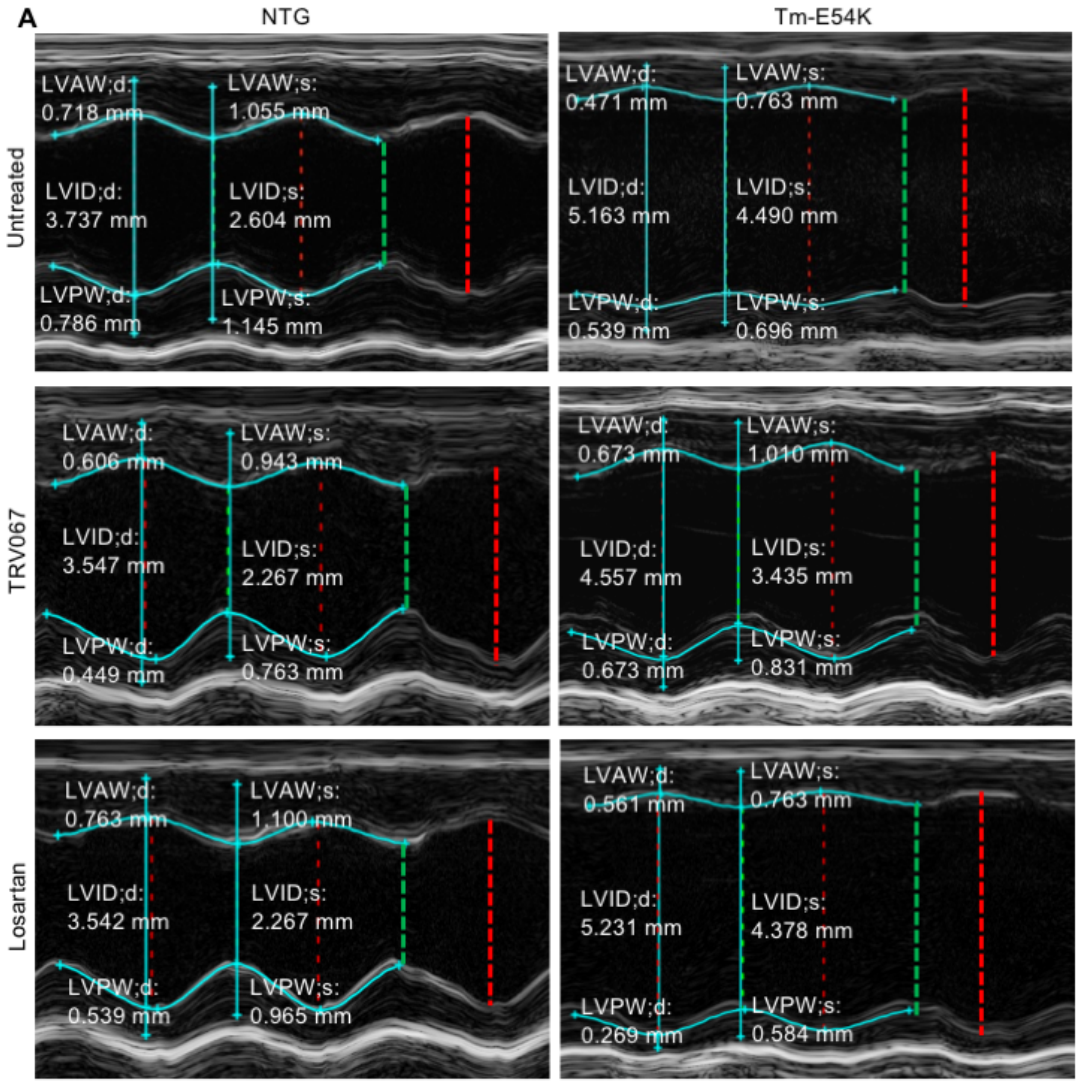


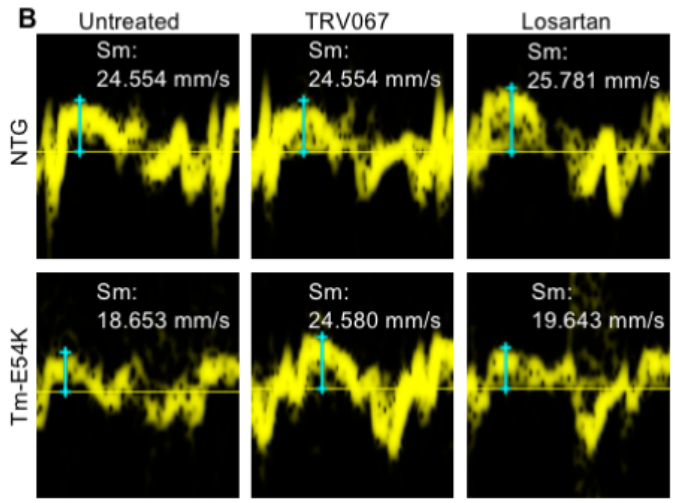
Supplemental Table 2. Assessment of sex-related functional and morphological differences by echocardiography.

Parameter	NTG Male	NTG Female	Tm-E54K Male	Tm-E54K Female
Sample Size (n)	6	7	5	6
Heart Rate (bpm)	495 ± 7	484 ± 14	511 ± 26	510 ± 9
LA Size (mm)	1.94 ± 0.12	1.91 ± 0.08	2.47 ± 0.13	2.13 ± 0.05
LVAW;d (mm)	0.72 ± 0.02	0.71 ± 0.06	0.56 ± 0.06	0.51 ± 0.03
LVAW;s (mm)	1.07 ± 0.06	1.06 ± 0.06	0.79 ± 0.14	0.65 ± 0.04
LVID;d (mm)	3.91 ± 0.13	3.69 ± 0.07	4.90 ± 0.09	4.35 ± 0.11
LVID;s (mm)	2.43 ± 0.20	2.20 ± 0.17	4.04 ± 0.12	3.57 ± 0.12
LVPW;d (mm)	0.63 ± 0.05	0.62 ± 0.05	0.54 ± 0.05	0.50 ± 0.03
LVPW;s (mm)	1.01 ± 0.07	1.03 ± 0.05	0.67 ± 0.08	0.59 ± 0.03
Ejection Fraction (%)	69.32 ± 4.20	70.14 ± 3.09	36.36 ± 3.23	37.09 ± 1.60
Fractional Shortening (%)	38.76 ± 3.10	39.68 ± 2.61	17.58 ± 1.76	17.72 ± 0.82
Stoke Volume (μL)	41.20 ± 2.90	39.85 ± 0.95	37.15 ± 2.00	31.66 ± 1.37
Cardiac Output (mL/min)	21.12 ± 1.42	21.89 ± 1.45	17.49 ± 1.33	16.29 ± 0.91
Vcf (mm/s)	8.14 ± 0.45	9.31 ± 1.05	3.33 ± 0.33	4.56 ± 0.15
Sm (mm/s)	28.15 ± 1.98	27.87 ± 1.21	15.93 ± 1.33	21.27 ± 0.68
IVRT (ms)	11.99 ± 0.67	12.22 ± 0.60	21.09 ± 2.32	16.56 ± 0.41
E-wave (mm/s)	816 ± 48	863 ± 70	737 ± 45	628 ± 75
A-wave (mm/s)	544 ± 17	445 ± 52	510 ± 57	306 ± 61
Em-wave (mm/s)	-25.90 ± 0.58	-25.18 ± 3.00	-19.81 ± 1.63	-21.22 ± 1.23
E/A Ratio	1.55 ± 0.05	1.77 ± 0.15	1.50± 0.14	1.86 ± 0.34
E/Em Ratio	-31.78 ± 1.83	-37.76 ± 3.63	-38.71 ± 4.48	-29.48 ± 2.75

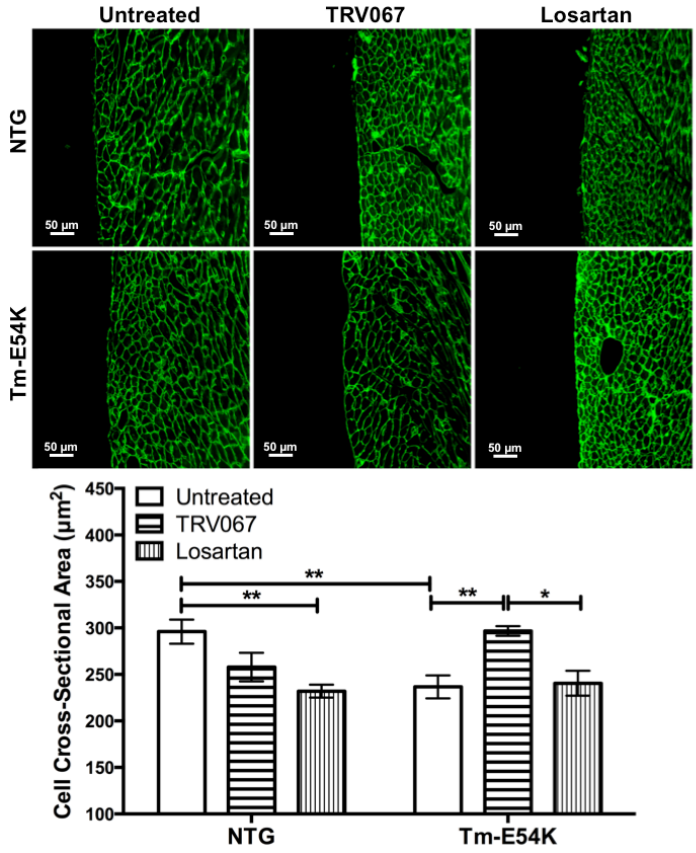
Values are Mean ± SEM.

SUPPLEMENTAL FIGURE 3

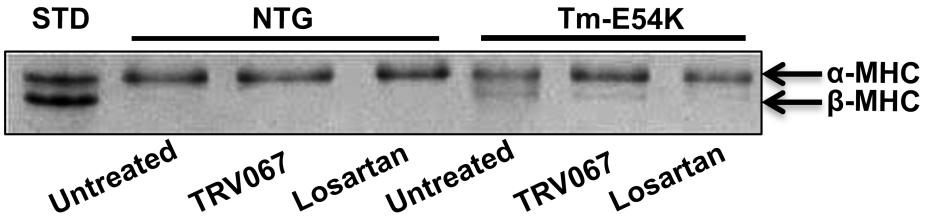


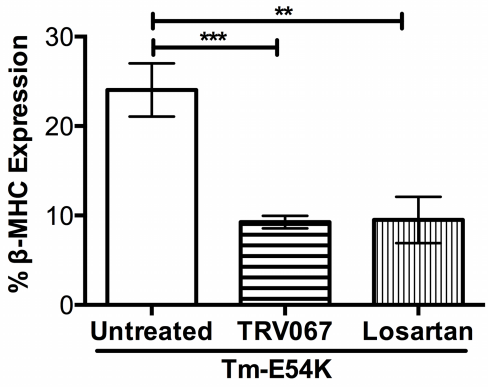


SUPPLEMENTAL FIGURE 4



SUPPLEMENTAL FIGURE 5





SUPPLEMENTAL FIGURE 6