Supplement Material

Methods

Morphometry/Histology

After the specified drug treatment schedule, mini-pumps were removed where necessary and tissues were isolated from anesthetized mice immediately following conscious echocardiography Briefly, anesthetized mice were weighed, sacrificed and then tissues including heart, lung and liver were isolated and individually weighed. The heart was bisected horizontally and the upper half including the atria and portions of the left and right ventricles were fixed in fresh 4% paraformaldehyde while the lower portion was snap frozen in liquid nitrogen for protein isolation. For histology, hearts were embedded in paraffin and then serial 5 mm sections were collected. Sections were used for routine hematoxylin and eosin staining or Masson's trichrome staining.

Protein Preparation and Immunoblotting

Isolated cardiomyocytes

Proteins were isolated from cardiomyocytes using a modification of the method of DeFea et al[1]. Briefly, cardiomyocytes are plated onto laminin coated 6 cm plates at 2-3x10^5 cells per plate and maintained for 2 hours in plating medium (MEM + 1% serum, 2 mM L-glutamine, 1X penicillin/streptomycin) prior to treatment. Cells were treated with 10 μM M119 or vehicle for 5 minutes followed by treatment with 1μM Iso or PBS (vehicle) for 5 minutes. Immediately following, cells were rinsed once with 2 mL of Tyrode's buffer, lysed in 300 μl of HES buffer (10 mM HEPES, 1 mM EDTA, 250 mM Sucrose) containing protease inhibitors (Complete Mini; Sigma-Aldrich) and phosphatase inhibitors (20 μM β-glycerophosphate, 1 μM sodium orthovanadate, 50 nM okadaic acid), scraped from the plate and homogenized with 20 strokes in an ice cold Dounce homogenizer. The homogenate was centrifuged at 700Xg for 10 min and the supernatant was then cleared by centrifugation at 100,000Xg for 60 min to separate cytosolic and high-speed membrane fractions. Membrane pellets were resuspended in HES buffer + 1% Triton containing protease and phosphatase inhibitors.

Whole heart tissue

For whole-heart lysates, the nitrogen-frozen heart tissue was pulverized and mechanically homogenized in an equal volume of RIPA (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium doedecyl sulfate, 150mM NaCl, 50mM Tris pH8.0). Following a clearing spin at 3000 rpm for 10 min at 4ºC, the supernatants were sonicated for 10 seconds and centrifuged at 15,000 rpm for 20 min at 4ºC. The supernatant was carefully removed and used for immunoblotting.

β-AR binding studies

Myocardial membrane and cytosol fractionation were performed, membranes were assayed with [^{125}I]-CYP, non-specific binding determined with 1 μM alprenolol. Specific binding (Bmax) was normalized to membrane protein concentration[2, 3].

Immunoblotting

Protein concentrations were determined via the Bradford method with the BioRad DC protein assay (BioRad Inc.). Equal amounts of either cardiomyocyte membranes (10 mg) or whole heart lysate (50 μg) were loaded onto pre-cast NuPAGE 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and reacted with primary antibodies to GRK2 (rabbit anti-GRK2, mouse anti-β-Arrestin, 1:500, Santa Cruz Biotechnology). Primary antibodies

were then detected using either HRP conjugated goat anti-rabbit secondary antibodies and ECL detection (Roche) or fluorescent-conjugated secondary antibodies (Rockland Immunochemicals, Molecular Probes) and a Li-COR fluorescent detection system (Li-COR Biosciences).

RNA extraction and Real-Time PCR

RNA was isolated and analyzed as we and others have previously described[4, 5]. Briefly, Total RNA extraction from murine left ventricle tissue was performed with the RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA integrity was analyzed using the Agilent RNA 6000 Nano assay for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse Transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA).

Real Time PCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA). All assays were performed in a 384 well plate with each reaction volume mixture having a final volume of 10mL. All samples were run in triplicate. The reaction mixture for the ANF and b -MHC assays was comprised of 2.5ul of RT products diluted 1:100 in nuclease free water, 900nM forward primers, 900nM reverse primer, 1X Power SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA) and nuclease free water. The reaction conditions for the ANF and β-MHC assays consisted of an initial denaturing step of 95^{O}C for 10 minutes, followed by 40 cycles of amplification and quantitation steps of 95^{O}C for 30 seconds, 60^{O}C for 30 seconds and 72^{O}C for 1 minute. Following cycling a dissociation curve analysis was run consisting of conditions of 95^{O}C for 15 seconds, 60^{O}C for 15 seconds and 95^{O}C for 15 seconds. Primers for the ANF and β-MHC assays were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc., Coralville, IA). The reaction mixture for GAPDH was comprised of 1X Mouse GAPDH Endogenous Control Assay P/N 4352932E (Applied Biosystems Inc, Foster City, CA), TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems Inc, Foster City, CA) and nuclease free water. The reaction conditions for the GAPDH assay consisted of an initial denaturing step of 95^{O}C for 10 minutes, followed by 40 cycles of amplification and quantitation steps of 95^{O}C for 15 seconds, 60^{O}C for 1 minute.

Threshold values were set automatically by the SDS2.3 software (Applied Biosystems Inc, Foster City, CA) and the threshold cycle number (Ct) was determined for each gene of interest and GAPDH. The ΔCt value was calculated (Ctgene of interest – Ct GAPDH) and the ratio of gene of interest to GAPDH was calculated using the formula 2-ΔCt . Fold changes were calculated by dividing the ratio of gene of interest to GAPDH for the experimental group by the ratio of gene of interest to GAPDH for the control group.

<u>cAMP Assay</u>

Freshly isolated cardiomyocytes were resuspended at a final concentration of ~20,000 rod-shaped cardiomyocytes per 100 mL of Krebs buffer containing 750mM IBMX to inhibit endogenous phosphodiesterase activity. Cells were pretreated with 10 mM M119 or vehicle for 5 minutes then stimulated for 15 minutes with 100 nM Iso (Sigma) or 100 mM Forskolin (Sigma) while rotating at room temperature. cAMP was extracted from the cells by adding ice cold 100% ethanol to a final concentration of 70% and incubating on ice for 15 minutes. Cells were then vortexed briefly, centrifuged for 10 minutes at 12,000 rpm and the resulting supernatant containing cAMP was transferred to a fresh eppendorff tube for concentration in a Speed-Vac. Resulting pellets were then assayed for cAMP using the Bridge-It cAMP Designer Fluorescence Assay (Mediomics Inc.) according to manufacturer's recommendations. To control for interexperimental variability in cell number and viability, cAMP measurements were represented as relative fluorescence (RF) calculated as follows: RF= (fluorescence (baseline) – fluorescence

(sample)) / fluorescence (baseline). Results were derived from at least five experiments for each treatment.

Statistical Analysis: For single biochemical/physiological observations, unpaired students t-tests were used. Multiple responses of various physiological and biochemical and assays were analyzed using one-way or repeated measures ANOVA. Post-hoc analysis (ie. Newman-Keuls) was performed if significance was achieved, with $P<0.05$ required for significance in all tests.

Small molecules
M119 (NSC119910) kindly provided by the National Cancer Institute repository. Gallein from Acros Organics, Geel, Belgium.

Online Table I. **Echocardiographic analysis of Iso-pumped, M119-treated animals +/- SEM.**

Mouse ID	V-V	V-M	I-V	I-M
LVID;d (mm)	3.04±0.11	2.87±0.10	3.42±0.16	3.03±0.18
LVID;s (mm)	1.40±0.02	1.29±0.08	**2.39±0.22***	**1.47±0.15**$^{\#\#}$
IVS;d (mm)	0.87±0.03	0.90±0.04	0.88±.04	0.90±0.03
LVPW;d (mm)	0.78±0.01	0.83±0.02	0.80±0.03	0.84±0.03
IVS;s (mm)	1.17±0.08	1.19±0.04	1.10±0.05	**1.25±0.03**$^{\#}$
LVPW;s (mm)	1.30±0.07	1.30±0.04	**1.06±0.03***	**1.18±0.04**$^{\#}$
LV Vol;d (μl)	42.46±2.45	36.89±3.98	**53.68±5.14**	47.49±6.06
LV Vol;s(μl)	5.50±0.26	5.44±0.45	**25.48±4.92***	**10.76±2.64**$^{\#}$
%EF	86.83±0.54	84.51±1.67	**55.24±4.34****	**79.73±2.82**$^{\#\#}$
% FS	54.35±1.05	51.63±1.85	**28.56±2.71*****	**48.03±2.60**$^{\#\#\#}$
AoV ET (msec)	49.25±3.38	47.31±0.92	53.91±2.67	46.09±2.40
HR (beats/min)	570±21	586±39	549±25	509±26
mVcf (circ/sec)	1123±55	1101±57	**555±41*****	**1084±85**$^{\#\#\#}$

Mini-osmotic pumps containing Iso in saline with .002% ascorbic acid (Iso vehicle) or Iso vehicle alone were implanted into 12-week old C57B6-J mice and Iso was delivered at a rate of 30mg/kg/day. Mice were simultaneously given daily 200μl injections of either M119 (100 mg/kg/day) or 1 x PBS pH8.6 (M119 vehicle). Treatment groups are abbreviated as follows: V-V (Iso vehicle/ M119 vehicle), V-M (Iso vehicle/ M119), I-V (Iso/ M119 vehicle), and I-M (Iso/ M119). Abbreviations used are as follows; LVID;d, left ventricular internal dimension at diastole; LVID;s, left ventricular internal dimension at systole; IVS;d, intraventricular septal thickness at diastole; LVPW;d, left ventricular posterior wall thickness at diastole; IVS;s, intraventricular septal thickness at systole; LVPW;s, left ventricular posterior wall thickness at systole; LV Vol;d, left ventricular volume at diastole; LV Vol;s, left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, percent fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of fractional shortening. *P<.05, **P<.01, ***P<.001 vs. V-V. $^{\#}P$<.05, $^{\#\#}P$<.01, $^{\#\#\#}P$<.001 vs. I-V. All other pair wise comparisons, including I-M vs. V-V and I-M vs. V-M, were not significantly different.

Online Table II. Echocardiographic data in CSQ mice at baseline and following four weeks daily vehicle or Gallein.

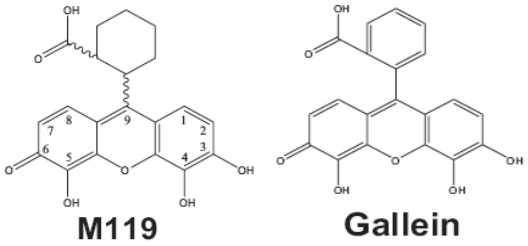
Measurement	Vehicle			Gallein		
	Baseline	**4 Weeks**	**% Change at 4wks**	**Baseline**	**4 Weeks**	**% Change at 4wks**
LVID;d (mm)	4.6±0.16	5.04±0.2	9.65	4.16±0.29	4.37±0.11*	4.88
LVID;s (mm)	3.15±0.13	3.99±0.22	26.65	2.86±0.23	2.94±0.24*	2.64
IVS;d (mm)	0.86±0.05	0.68±0.04	-20.88	0.8±0.05	0.66±0.02	-17.65
LVPW;d (mm)	0.86±0.05	0.67±0.03	-21.81	0.78±0.05	0.68±0.03	-13.15
IVS;s (mm)	1.13±0.03	0.89±0.03	-21.2	1.02±0.04	0.91±0.04	-11.67
LVPW;s (mm)	1.14±0.03	0.88±0.02	-22.71	1.02±0.07	0.93±0.04	-8.43
LV Vol;d (µl)	98.51±7.85	122.21±10.78	24.06	88.4±14.39	86.5±4.96*	-2.15
LV Vol;s (µl)	40.24±3.9	71.42±8.81	77.49	36.42±7.01	34.72±5.92*	-4.69
%EF	58.92±2.92	42.37±3.24	-28.09	54.26±3	60.87±5.51*	12.18
%FS	31.39±2.08	21.08±1.87	-32.84	28.89±2.08	33.07±4.2*	14.46
AoV ET (msec)	46.25±2.35	43.25±0.67	-6.49	41.97±1.71	39.38±1.68	-6.17
HR (beats/min)	363±34.33	252.75±9.5	-30.37	364.44±32.01	260.5±4.32	-28.52

Two groups of five male CSQ mice at 8 weeks of age were initiated on once daily IP injections of vehicle or 30 mg/kg/day gallein for one month, and were followed by serial conscious echocardiography. Abbreviations: LVID;d, left ventricular internal dimension at diastole; LVID;s, left ventricular internal dimension at systole; IVS;d, intraventricular septal thickness at diastole; LVPW;d, left ventricular posterior wall thickness at diastole; IVS;s, intraventricular septal thickness at systole; LVPW;s, left ventricular posterior wall thickness at systole; LV Vol;d, left ventricular volume at diastole; LV Vol;s, left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, % fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of circumferential shortening. *P<.05 4wk Vehicle vs. Gallein. No significant differences between groups at baseline.

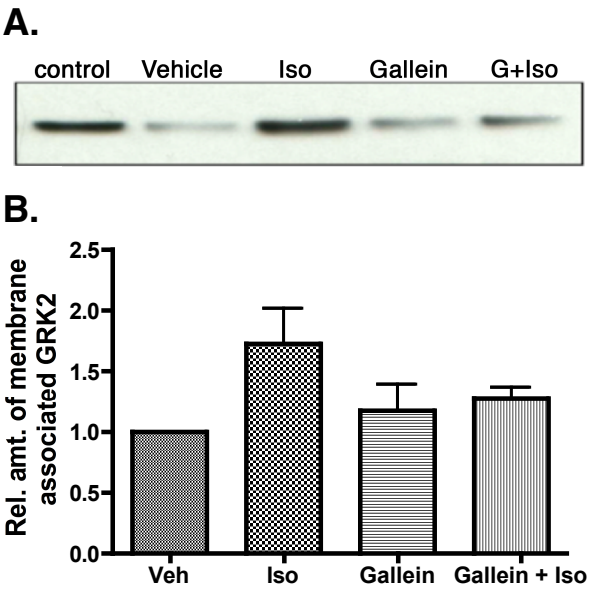
Online Table III. Echocardiographic data in wild-type DBA mice at baseline and following four weeks daily vehicle or Gallein.

Measurement	Vehicle			Gallein		
	Baseline	**4 Weeks**	**% Change**	**Baseline**	**4 Weeks**	**% Change**
LVID;d (mm)	2.85±0.11	2.89±0.11	1.52	2.96±0.17	2.75±0.17	-7.04
LVID;s (mm)	1.37±0.06	1.49±0.12	9.04	1.49±0.12	1.2±0.11	-19.48
IVS;d (mm)	0.96±0.03	0.89±0.02	-6.77	0.92±0.03	0.95±0.03	2.76
LVPW;d (mm)	1.13±0.05	1.16±0.07	2.17	1.1±0.04	1.18±0.06	7.23
IVS;s (mm)	1.34±0.06	1.29±0.04	-3.53	1.34±0.05	1.34±0.05	-0.44
LVPW;s (mm)	1.51±0.05	1.46±0.04	-3.39	1.37±0.05	1.49±0.02	8.99
LV Vol;d (µl)	31.19±3.12	32.34±2.89	3.68	34.76±4.57	29.23±4.16	-15.9
LV Vol;s (µl)	4.87±0.57	6.36±1.33	30.52	6.27±1.12	3.63±0.73	-42.12
%EF	84.44±1.02	80.96±2.66	-4.13	82.53±1.56	88.33±1.17	7.02
%FS	51.93±1.16	48.57±2.73	-6.46	50.01±1.71	56.93±1.62*	13.85
AoV ET (msec)	42.33±1.01	39.33±1.28	-7.09	43.33±3.03	39±1.32	-10
HR (beats/min)	537.8±9.03	564.91±22.5	5.04	508.09±21.69	564.13±8.99	11.03

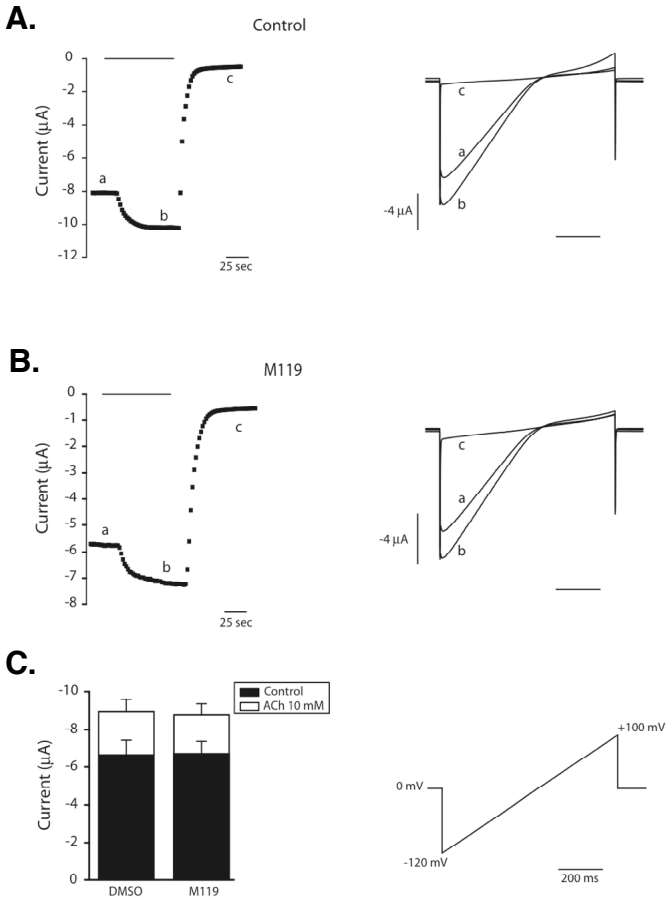
Two groups of five male wild-type DBA mice at 8 weeks of age were initiated on once daily IP injections of vehicle or 30 mg/kg/d gallein for 1 month, and were followed by serial conscious echocardiography. Abbreviations: LVID;d, left ventricular internal dimension at diastole; LVID;s, left ventricular internal dimension at systole; IVS;d, intraventricular septal thickness at diastole; LVPW;d, left ventricular posterior wall thickness at diastole; IVS;s, intraventricular septal thickness at systole; LVPW;s, left ventricular posterior wall thickness at systole; LV Vol;d, left ventricular volume at diastole; LV Vol;s, left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, percent fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of fractional shortening. *P<.05, No significant difference in any measure between groups at



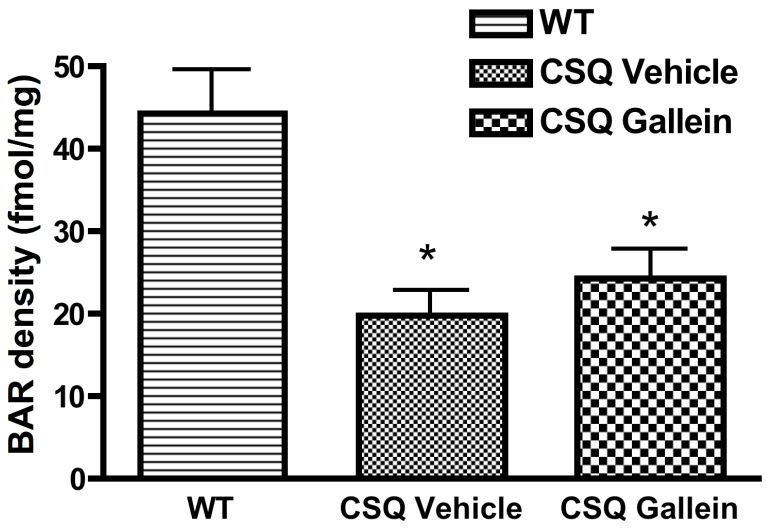
Online Figure I. **Structures of M119 and Gallein**. Gallein and M119 are highly related compounds. Importantly, compounds that duplicate either M119 or gallein but lack the hydroxyl groups at positions 4 and 5 of the tricyclic ring structure are completely inactive (Lehmann et al, Mol Pharm, 2008).



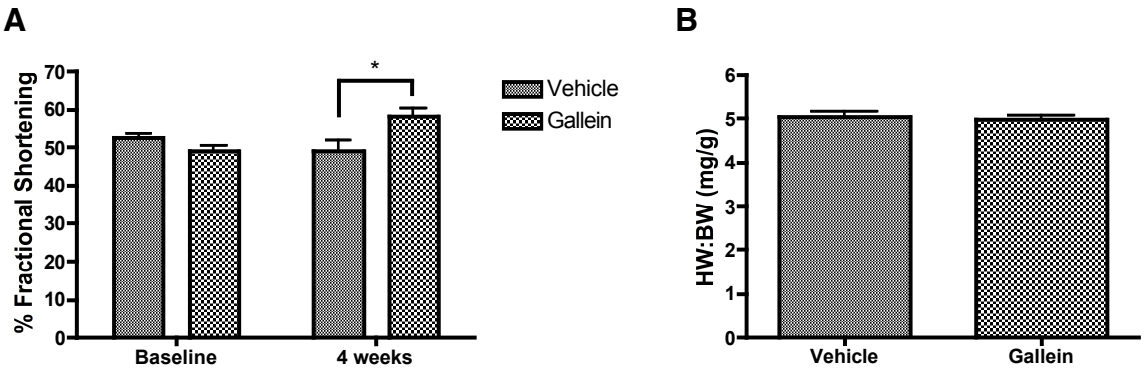
Online Figure II. **Acute gallein treatment can modulate β-AR signaling**. A,B) Gallein interferes with β-AR induced GRK2 membrane recruitment. Representative western blot analysis of GRK2 protein level in membrane fractions of cardiomyocytes treated as indicated. Positive control lane of proteins from HEK293 cells transfected with GRK2 (B). Densitometric analysis of GRK2 membrane recruitment from four independent experiments demonstrates reduction in Iso-induced GRK2 membrane recruitment.



Online Figure III. **M119 does not affect Gβγ-mediated activation of Kir3.1/Kir3.4 channels.** Xenopus ocytes expressing the subunits underlying the cardiac KACh channel, Kir3.1 and Kir3.4, were expressed in oocytes together with the muscarinic type 2 receptor. (A) Typical Kir3.1/Kir3.4 current measured before and after 10 µM ACh application. The K+ channel blocker Ba2+ was applied to determine the zero current level. (B) Effect of M119 on Kir3.1/Kir3.4 currents. Typical Kir3.1/Kir3.4 current before and after ACh application for oocytes treated for 1 hour with M119. (C) Summary data. M119 showed no effect on both the basal and ACh activated Kir3.1/Kir3.4 current.



Online Figure IV. **Effect of Gallein treatment on cardiac β-AR expression.** Radioligand β-AR binding assays determined β-AR density on cardiac membranes isolated from non-transgenic littermate control mice, or from CSQ mice treated with vehicle or gallein once daily (30 mg/kg/day) for one month. *P<.05 vs. wt



Online Figure V. **Gallein treatment of wild-type mice mildly elevates cardiac contractility with no effect on cardiac morphometry.** Two groups of five male wild type DBA mice at 8 weeks of age were initiated on once daily injections of vehicle or 30 mg/kg/day gallein for one month, and were followed by serial conscious echocardiography. A) Quantitation of fractional shortening data. B) Heart weight to body weight ratio at 4 weeks. *P<0.05 vs. vehicle.

References

1. DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol.* 2000;148:1267-1281.

2. Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, Lefkowitz RJ. Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. *Science.* 1995;268:1350-1353.

3. Milano CA, Dolber PC, Rockman HA, Bond RA, Venable ME, Allen LF, Lefkowitz RJ. Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc Natl Acad Sci U S A.* 1994;91:10109-10113.

4. Bullard TA, Protack TL, Aguilar F, Bagwe S, Massey HT, Blaxall BC. Identification of Nogo as a novel indicator of heart failure. *Physiol Genomics.* 2008;32:182-189.

5. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3:1101-1108.