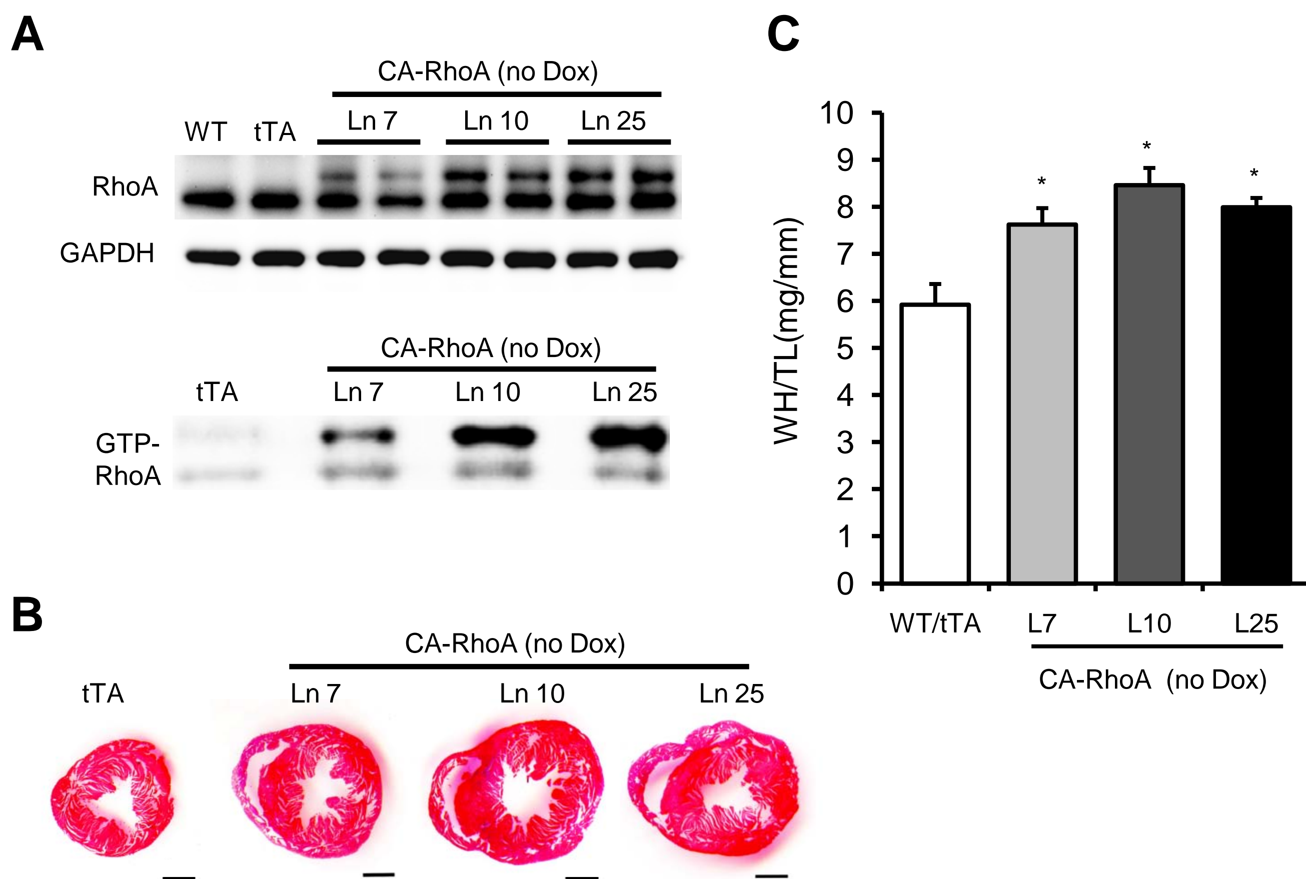
Supplemental Materials and Methods

Supplemental Table

Baseline echocardiograpic characteristics of inducible RhoA transgenic mice

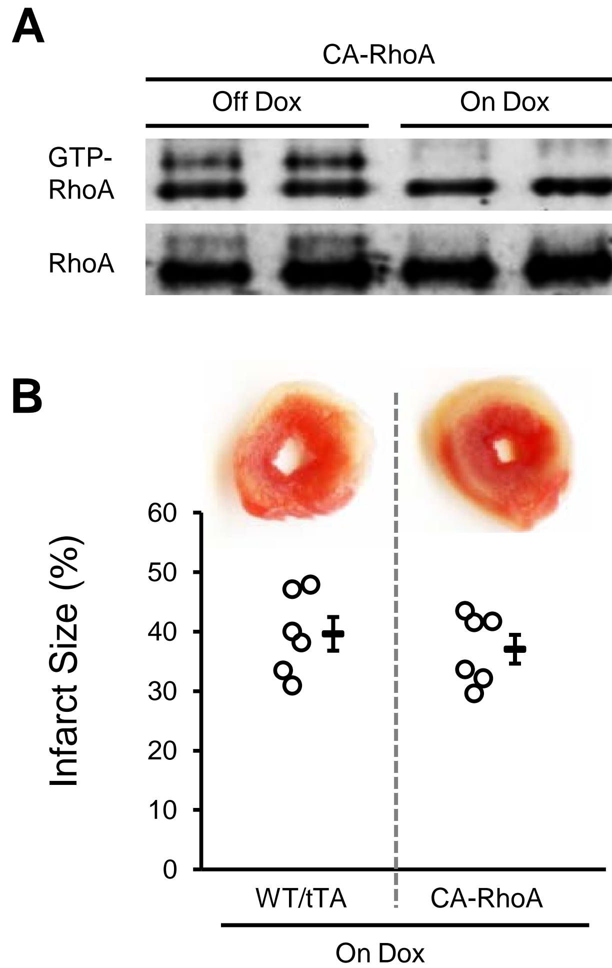
		WT/tTA	CA-RhoA
Diastolic	LVID	3.7 0.3	3.6 0.2
(mm)	LVPW	0.9 0.1	0.9 0.2
	LVAW	0.8 0.1	0.9 0.1
Systolic	LVID	2.1 0.4	2.1 0.3
(mm)	LVPW	1.3 0.2	1.4 0.2
	LVAW	1.5 0.1	1.4 0.2
	%EF	74.7 8.6	74.6 5.3
	%FS	43.6 7.5	42.9 4.4

Mice were kept on Dox until 3 weeks after birth and analysis was carried out at 8 weeks after Dox removal. LVID, Left Ventricular Internal Dimension; LVPW, Left Ventricular Posterior Wall; LVAW, left ventricular anterior wall; EF, ejection fraction; FS, fractional shortening. There was no significant difference in any of the parameter measured between WT and tTA, data was pooled. Data expressed as mean ± SEM; n=6.



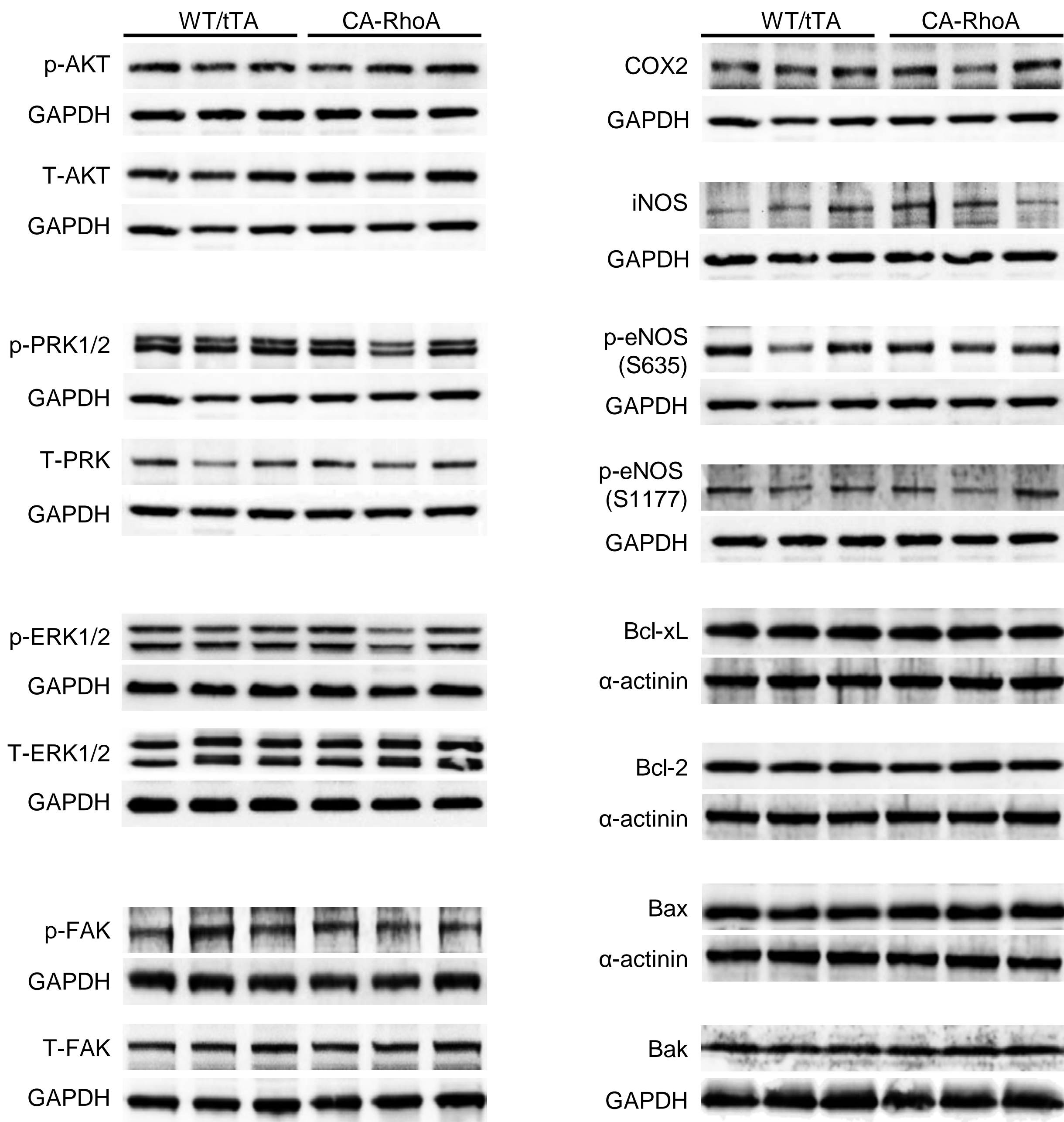
Supplemental Figure 1

Characterization of cardiac-specific RhoA transgenic mouse lines without Dox treatment (no Dox). (**A**) Representative blots showing RhoA expression (top) and RhoA activation (bottom) in age-matched (4 months) WT/tTA and three lines of CA-RhoA mice (line7, 10, 25). (**B**) Representative Masson's trichrome staining of heart sections from a tTA and CA-RhoA mice. Scale bars: 1 mm. (**C**) Heart weight (HW) to tibial length (TL) ratio from WT/tTA and CA-RhoA mice. * $P < 0.001$ vs. WT/tTA (n=7-10).



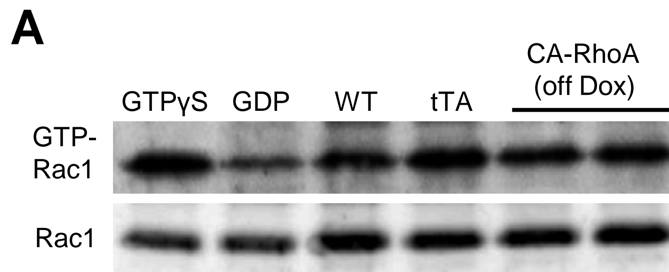
Supplemental Figure 2

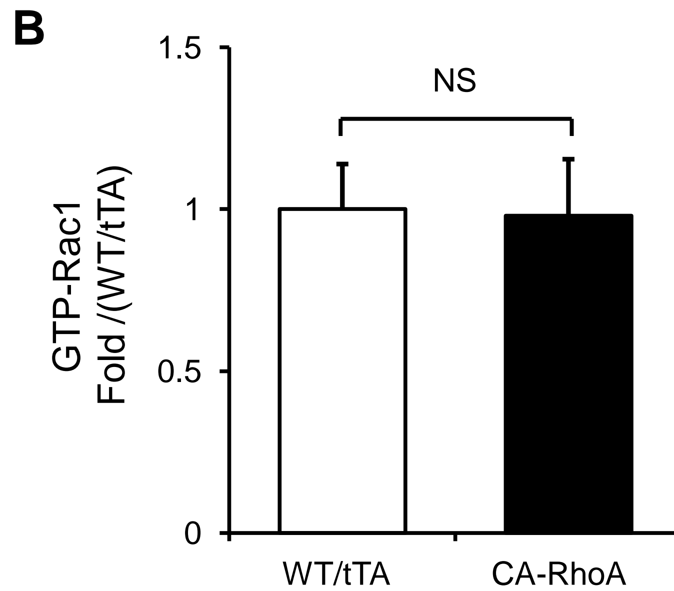
CA-RhoA mice kept on Dox do not express transgenic RhoA and do not protect against I/R injury in the isolated perfused heart. (**A**) Representative western blots of total and GTP-RhoA in the CA-RhoA heart either off Dox or kept on Dox; (**B**) Representative TTC stained heart sections and calculated infarct size of WT/tTA and CA-RhoA mouse (on Dox) hearts subjected to global I/R.



Supplemental Figure 3

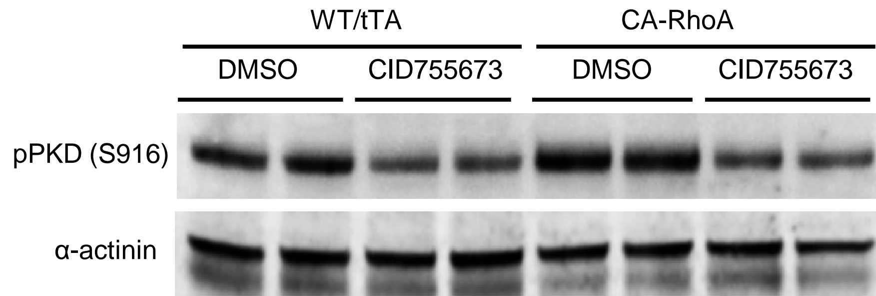
Phosphorylation, expression and activation of anticipated RhoA effector and pro-survival or pro-death signals are not increased in the CA-RhoA mouse heart. Left ventricular lysates were prepared from 8 weeks off Dox CA-RhoA mouse and their littermate WT/tTA controls. Representative blots showing phosphorylation of AKT (Ser473), FAK, ERK1/2, PRK1 (Thr774) / PRK2 (Thr816) and eNOS (Ser635 and Ser1177); as well as expression of AKT, FAK, ERK, PRK, COX2, iNOS, Bcl-2, Bcl-xL, Bax and Bak, (n=3-6).

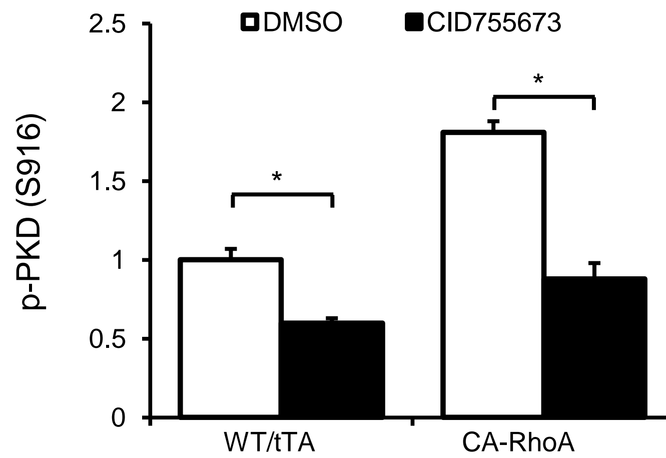




Supplemental Figure 4

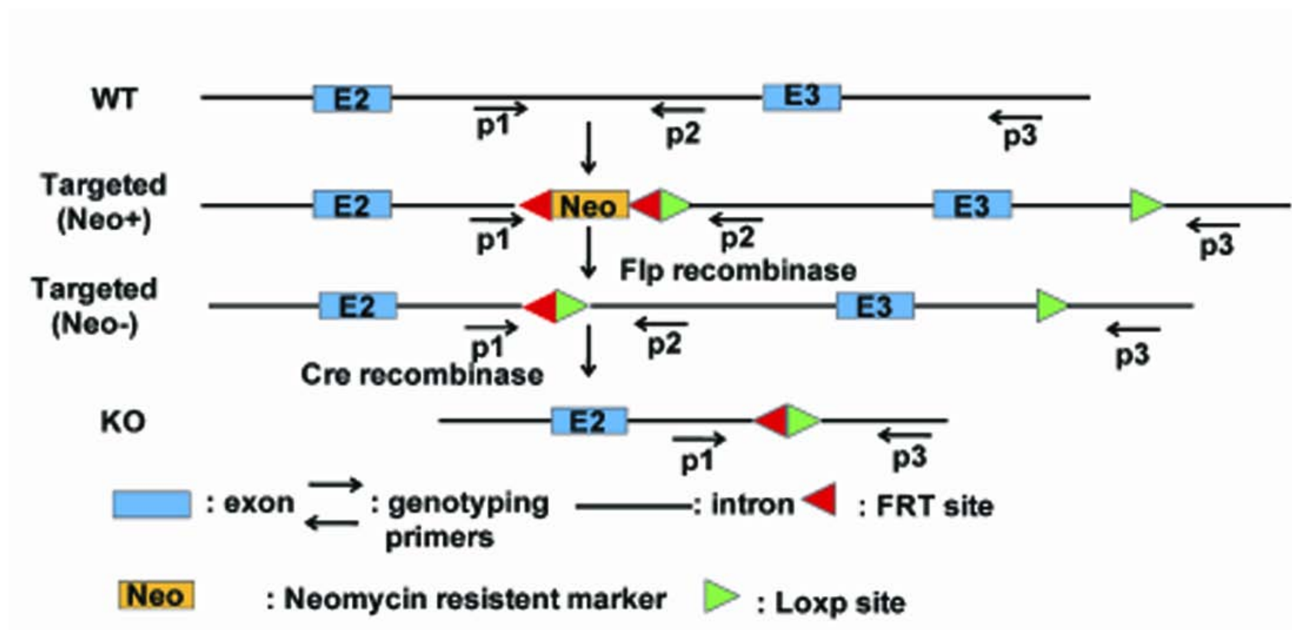
Activation and expression of Rac1 are not different in CA-RhoA hearts. (**A**) Representative blots showing GTP-Rac1 level in WT, tTA and CA-RhoA hearts; GTPγS and GDP incubated lysates (WT) were used as positive and negative controls, respectively. (**B**) Quantification of GTP-Rac1 in the WT/tTA and CA-RhoA mouse hearts (n = 4-5).





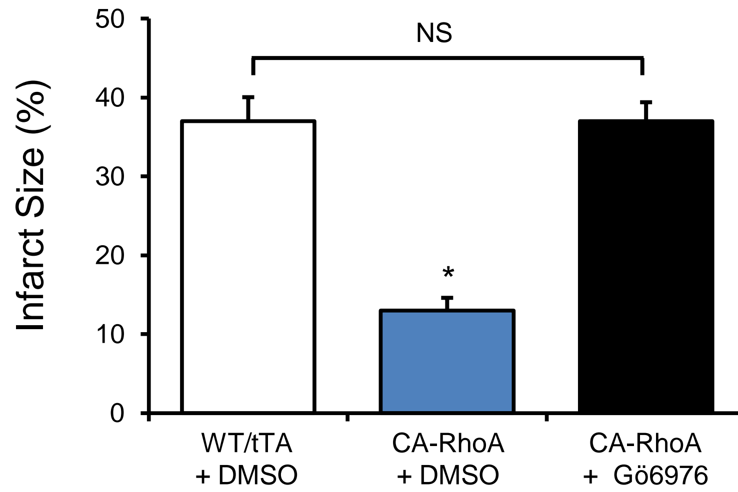
Supplemental Figure 5

PKD inhibitor CID755673 attenuates PKD phosphorylation in WT/tTA and CA-RhoA hearts. Representative blots and quantification of decrease in PKD autophosphorylation by 30 min CID755673 perfusion in the isolated heart. * $P < 0.05$ vs. DMSO (n=4-5).



Supplemental Figure 6

Generation of RhoA loxP-targeted (fl) mice. Exon 3 was flanked by loxP sites to allow tissue specific deletion. A neomycin resistance marker was also introduced. To generate RhoA-null alleles, RhoA loxp/loxp MEFs were infected twice with low titer adenoviral Cre recombinase (adeno-Cre) to achieve complete removal of RhoA alleles without detectable toxicity.



Supplemental Figure 7

Inhibition of PKD (by Gö6976) abolishes cardioprotection against I/R injury in the isolated perfused CA-RhoA heart. Isolated perfused mouse hearts pretreated with PKD inhibitor Gö6976 (1 μM) or DMSO for 30 min prior to I/R (n=5-7). * $P < 0.05$, NS, nonsignificant.

Supplemental Materials and Methods

Western blotting. Freshly removed left ventricles were snap frozen in liquid nitrogen and lysates were prepared with various lysis buffers. Western blot analysis was performed according to protocols described previously (1). The antibodies used for immunoblotting were from the following sources: RhoA, Bak, Bcl-xL, PKC alpha, PKC delta, actin, iNOS, and NF-κB p65 from Santa Cruz Biotechnology, Inc.; HA-tag, GAPDH, RhoGDI, Gβ, AKT, p-AKT (Ser473), ERK, p-ERK1/2, p-PKD (Ser744/748), p-PKD (Ser916), PKD, PKC epsilon, COX2 and Bax from Cell Signaling Technology; Cytochrome *c*, Rac1 and Bcl-2 from BD Biosciences Laboratories; VDAC, p-eNOS antibodies (Ser635 and Ser1177) and iNOS from Millipore.

Echocardiography analysis. Transthoracic echocardiography was performed on mice at 11 wk (adult). Briefly, mice were anesthetized using 2% isoflurane and then moved to a biofeedback warming station that maintained core body temperature, and mice were then kept under anesthesia using1% isoflurane. Ultrasound gel was applied to the chest of the animal, and echocardiography measurements were obtained using the VEVO 770 software package (Visual Sonics). M-mode tracings were taken and measured for posterior and anterior wall thicknesses, as well as the interdimensional space for both systole and diastole of the heart. Ejection fraction and fractional shortening were calculated as previously described (2). An average of seven cardiac cycles per animal was analyzed.

Tissue fractionation and PKC translocation. Cytosolic and particulate fractions were prepared at described before (3). Following isolation and rinsing, left ventricular tissue of WT, tTA and CA-RhoA hearts was minced and homogenized on ice in lysis buffer (20 mM Tris, pH 7.5; 2 mM each of EDTA and EGTA, pH 7.5; 5 mM sodium fluoride; 5 µg/ml each of leupeptin and aprotinin; 0.5 µg/ml pepstatin A; 0.3 mM phenylmethylsulphonyl fluoride; 1 µM vanadate; 0.03% 2-mercaptoethanol) and then centrifuged

at 600 *g* for 10 min. The supernatant was then further centrifuged at 100,000 *g* for 1 h at 4 °C, and this supernatant defined as the cytosolic fraction. Pellets were incubated on ice with frequent vortex in lysis buffer containing 1% Triton X-100 for 1 hour, further cleared by centrifugation at 100,000 *g* for 1 h at 4 °C, and this supernatant defined as the particulate fraction. PKC isoforms distribution in the cytosolic and particulate fractions was then assessed by Western blot, with RhoGDI and Gβ used as cytosolic and particulates protein loading controls, respectively.

Cell culture. Neonatal rat ventricular myocytes were isolated and cultured as described previously (4). Hearts were obtained from 1–2-day-old Sprague–Dawley rat pups, digested with collagenase. Myocytes were purified by passage through a Percoll gradient and plated at a density of 0.4 X 10^6 cells/6-well plate, 0.8 X 10^6 cells/6-cm dish, or 4 X 10^6 cells/10-cm dish and were maintained overnight in 4 : 1 Dulbecco's modified Eagle's medium/medium199, supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin). Cells were then serum starved for 24 h prior to intervention in serum free DMEM supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Rac1 activity assay. Activated Rac1 in the mouse hearts were determined by affinity pull-down assay using a Rac1 activity assay kit (Upstate) according to the manufacturer's instructions. Briefly, left ventricular lysates were prepared from WT, tTA and CA-RhoA hearts, and the same amount of protein was incubated with the agarose beads coupled GST-fusion protein of p21 binding domain (PBD) of the downstream effector of Rac, p21-activated protein kinase (PAK1), to pull-down the active GTP-bound form of Rac1 from the sample lysates. The activated Rac1 was detected with specific Rac1 antibodies using Western Blot analysis.

Cell death enzyme-linked immunosorbent assay (ELISA). DNA fragmentation indicative of apoptosis was assayed using the Cell Death Detection ELISA^{PLUS} (Roche Applied Science) according to the

manufacturer's instructions. Cardiomyocytes were washed in ice-cold PBS and harvested in cytosolic extraction buffer containing (20 mmol/L Tris pH 7.6, 3 mmol/L EDTA, 3 mmol/L EGTA, 125 mmol/L NaCl, 20 mmol/L β-glycerophosphate and 0.4% Nonidet P-40 alternative plus protease and phosphatase inhibitors). Samples were then spun down briefly and the supernatant were incubated with anti-histone-biotin and anti-DNAPOD in a streptavidin-coated microplate for 2 h and washed for 3 times, colorimetric substrate was added, and absorbance was measured at 405 nm.

Transfection of cardiomyocytes with siRNA. Pre-designed PKD1 ON-TARGET*plus* siRNA for rat and control siRNA were purchased from Thermo Scientific. NRVMs were transfected with siRNA using DharmaFECT-I transfection reagent (Thermo Scientific) based on manufacture's instruction. Two μM siRNA were transfected into $1x10^6$ cells. siRNA and DharmaFECT-1 (1:3 ratio) were individually incubated in conical tubes containing OPTI-MEM media (GIBCO) at room temperature for 10 min, mixed and incubated at room temperature for 20 min. Media in culture dishes were replaced with fresh media and siRNA/DharmaFECT-I mixtures were added to culture dishes. Following overnight incubation, cells were washed and cultured for another 48 hrs in serum free DMEM supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

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2. Yang,X.P., Liu,Y.H., Rhaleb,N.E., Kurihara,N., Kim,H.E., and Carretero,O.A. 1999. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. *Am. J. Physiol* **277**:H1967-H1974.

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4. Del Re,D.P., Miyamoto,S., and Brown,J.H. 2008. Focal adhesion kinase as a RhoA-activable signaling scaffold mediating Akt activation and cardiomyocyte protection. *J. Biol. Chem.* **283**:35622-35629.