Supplemental Material

Loss of Myocardial Retinoic Acid Receptor α Induces Diastolic Dysfunction by Promoting Intracellular Oxidative Stress and Calcium Mishandling in Adult Mice

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Expanded Methods Section

Experimental Model

Tamoxifen-inducible cardiomyocyte specific RARα gene knockout mice (α-MHC-Cre-RARα$^{fl/fl}$) were generated by crossing the floxed RARα mice (RARα$^{fl/fl}$, kindly provided by Dr. P. Chambon)[1] with α-MHC-MerCreMer mice at the Jackson Laboratory (Bar Harbor, ME). All mice were on a C57BL/6 and B6. FVB129 mixed background and fed a regular chow diet. Male α-MHC-Cre-RARα$^{fl/fl}$ mice received tamoxifen (0.5 mg/day, 2 mg total) at the age of 6 wks, to induce gene deletion (RARαKO). Age-matched α-MHC-Cre-RARα$^{fl/fl}$ mice treated with vehicle or RARα$^{fl/fl}$ mice, received tamoxifen at the same age, were used as WT control. One set of mice (n=20) were sacrificed at 20 wks and another set at 64 wks (n=20) after tamoxifen injection, to analyze cardiac functional and structural changes which developed at different stages. Type 1 diabetic model was induced by streptozotocin (STZ) injection, 4 wks after tamoxifen treatment. STZ (50 mg/kg/day) was injected intraperitoneally for 5 days, while those in the control group received 0.1 M sodium citrate buffer (pH 4.5). After 2 wks, mice with a blood glucose value of $\geq$ 250 mg/dl were considered diabetic. Mice were sacrificed at 16 wks after STZ injection. Another set of WT and RARαKO mice (n=8) were fed with HFD (60% of calories from fat; Harlan Teklad, WI) or standard rodent chow for 16 wks. Gene deletion was induced the same day as initiation of HFD feeding (age of 6 wks).

Echocardiographic measurements

Transthoracic echocardiography was performed on anesthetized mice, using a VisualSonicVevo 2100 and a 40-MHz probe, before injection with tamoxifen (0) and every 4 wks until 64 wks after gene deletion. Briefly, mice were anesthetized with 3–5% isoflurane that was reduced to 1.5% to maintain the heart rate between 400–450 beats per minute. The heart was imaged in the 2-dimensional, short-axis and 4 chamber views[2]. All mice recovered from the procedure without signs of distress. LV posterior wall end diastole (LVPWd), interventricular septal end diastole (IVSd), fractional shortening (FS%), ejection fraction (LVEF%), cardiac output (CO), heart rate (HR), LV internal dimension at end-diastole and end-systole (LVIDd and LVIDs), ratio of mitral valve flow velocities (E/A), isovolumic relaxation time (IVRT), deceleration time of the E-wave (DT), ratio of transmitral Doppler early filling velocity to tissue Doppler early diastolic mitral annular velocity (E/E' ratio) and tissue Doppler early diastolic mitral annular velocity (TDI E') were measured.

Hemodynamic studies

After 16 wks of gene deletion, LV catheterization was performed using a 1.2-F microconductance pressure-volume catheter (Scisense, Transonic Systems Inc, NY) to evaluate LV systolic and diastolic function. Systolic function was quantified by LV end systolic pressure, contractility (dP/dt max), end-systolic elastance (LV dP/dt_{max}/end-diastolic volume relation) and LVEF. Global cardiac function was quantified by the end systolic volume, end diastolic volume, stroke volume, cardiac output and heart rate. Diastolic function was measured by LV end diastolic pressure (LVEDP), dP/dt_{min} and tau.

Histological analysis and immunofluorescence staining

Paraffin embedded heart sections (5 μmol/L) were used for H&E and Masson's trichrome (Sigma Aldrich) staining[3]. Images were scanned and acquired using a Leica SCN400 image system. Interstitial and perivascular fibrosis were measured, using NIH Image J software. Frozen LV sections embedded in OCT compound (Tissue-Tec) were co-stained with phalloidin (1:100), 4′,6-diamidino-2-phenylindole and wheat germ agglutinin to visualize cytoplasm actin filament, nuclei and cell boundaries, respectively. Images were acquired on a confocal microscope (TCS SP5; Leica) with Leica LAS AF software. The following lenses were used: HC PL APO 20×/0.70, HCX PL APO 40×/1.25-0.75 oil, and HCX PL APO 63×/1.40-0.60 oil. All images were taken at room temperature and processed in Image J for CSA (cross-sectional area) analysis. CSA was calculated from 20-30 cells per section and 6 sections per group. Dihydroethidium (DHE) staining (Sigma Aldrich) was performed to identify intracellular ROS production[4]. Mean DHE fluorescence was calculated by subtracting integrated density of the background signal from the integrated density of the fluorescent staining from 10 fields/heart, 5 hearts/group and normalized to control.

Hydroxyproline Analysis

Hydroxyproline (the hydroxylation of proline) measurements accurately reflect the amount of collagen in the tissue. To determine the effect of RARα deletion on cardiac collagen production, hydroxyproline assay was performed, according to the manufacturer's instructions (Sigma Aldrich, MAK008). LVs (5 mg) collected from WT and RARαKO at 20 and 64 wks after gene deletion were homogenized and acid hydrolysis completed by adding hydrochloric acid. The standards and samples were read in a 96-well plate at 560 nm on a spectrophotometer, with results reported as μg hydroxyproline/mg LV.

GSH/GSSG Assay

To determine the oxidative stress, LVs (10 mg) collected from WT and RARαKO mice were homogenized, reduced and oxidized forms of glutathione (GSH and GSSG, respectively) were measured, using a GSH/GSSG Ratio Detection Assay Kit (Abcam), according to the manufacturer's protocol.

Isolation of neonatal and adult mouse cardiomyocytes

Neonatal mouse cardiomyocytes were isolated and cultured from 1-3 day old $RAR\alpha^{fl/fl}$ mice. Neonatal hearts were pre-digested in 0.5 mg/mL Trypsin (Sigma) Hank's Balanced Salt Solution (HBSS), followed by 4 dissociation cycles with 240 U/mL Collagenase type II (Worthington). Fibroblasts were removed by pre-plating for 2 h on plastic tissue culture dishes. Cardiomyocytes were plated on gelatin-coated dishes and maintained in DMEM-M199 medium, 5% Horse Serum and 10% FBS (Gibco). Final cultures contained >90% cardiomyocytes as determined by immunofluorescence staining for sarcomeric alpha-actinin (Sigma). Cardiomyocyte RARα deletion was induced by transfecting cells with adenovirus-mediated

overexpression of Cre recombinase (AdCre, 50 MOI). Cells transfected with AdGFP were used as wild type control.

Adult mouse cardiomyocytes were isolated as previously described with some modifications[5]. Briefly, hearts were rapidly removed from anesthetized WT or RARαKO mice (20 wks after tamoxifen injection) and perfused in a temperature-controlled (37°C) Langendorff's perfusion system. After perfusing with modified calcium-free perfusion buffer containing (in mmol/L) NaCl 120.4, KCl 14.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, 5 $MgSO_4$-$7H_2O$ 1.2, Na-HEPES 10, $NaHCO_3$ 4.6, taurine 30, butanedione monoxime (BDM) 10 and glucose 5.5 for 5 min, hearts were digested with collagenase II (2 mg/ml, Worthington Biochemical Co, NJ) in Ca^{2+}-free perfusion buffer for 10 min. The solution was gassed with 5% CO_2-95% O_2. The digested heart was removed from the cannula and the left ventricle cut into small pieces in a petri dish containing enzyme stopping buffer (perfusion buffer + 5% calf serum and 12.5 μM $CaCl_2$). Tissue pieces were gently agitated and the cell suspension was filtered through a 100-μm nylon mesh and settled by gravity in a 15-ml conical tube for 15 min. Extracellular Ca^{2+} was added incrementally back to 1 mmol/L, over a period of 20 min. The isolated cardiomyocytes were used immediately for Ca^{2+} transient and twitch analysis or cultured for other experiments.

Intracellular Ca^{2+} transient and twitch analysis

Isolated adult mice cardiomyocytes were loaded with 10 μmol/L Fura-2AM (acetoxy-methyl-ester Fura-2) for 10 min and placed on the stage of an inverted microscope and fluorescence measurements recorded with a dual-excitation fluorescence photomultiplier system (IonOptix, MA). Myocytes imaged through an Olympus IX-70 Fluor X40 oil objective were exposed to light emitted by a 75 W lamp and passed through either a 360 or a 380 nm filter (bandwidths were ± 15 nm), while being stimulated to contract at 0.2 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 360 nm for 0.5 sec and then at 380 nm for the duration of the recording protocol. The 360 excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular Ca concentration ($[Ca^{2+}]_i$) were inferred from the ratio of the fluorescence intensity at the two wavelengths. Calcium transient was assessed using the following indices (IonWizard Transient analysis): departure velocity (dep v, characterizing the speed of $[Ca^{2+}]$i goes up); the time to maximal departure velocity (dep v t, characterizing the rate of the departure); baseline as a percentage of peak height (bl% peak h, characterize the magnitude of the transient); time to 50% and 90% of the peak (T_{50}CI and T_{90}CI ,characterizing the speed of calcium increase); return velocity (ret v, the maximal rate of the return phase of the transient, describing the speed of calcium reuptake); time to 50% and 90% of the baseline (T50CR and T90CR characterizing the speed of calcium return); and tau (the exponential decay time constant of the function, characterizing the speed of calcium reuptake). Cardiomyocyte contractile properties were assessed by video-based edge-detection (IonOptix). The following parameters were analyzed: departure velocity (+dL/dt, characterizing the maximal rate of cell contraction); peak shortening (the maximal displacement from baseline); time to 50% and 90% of the peak (TPS50% and TPS90%, characterizing the speed of contraction); return velocity (-dL/dt, the maximal rate of cardiomyocyte relaxation); time to 50% and 90% of the base line (TR50% and TR90%, characterizing cellular relaxation); and tau (the exponential decay time constant of the function, characterizing the speed of relaxation). SR Ca^{2+} loading capacity was assessed by rapid puff of caffeine (10 mmol/L)-induced intracellular Ca^{2+} transient intensity in fura-2-loaded

cardiomyocytes. Data were recorded from at least 15 cells per heart and for at least 5 hearts per group. All parameters were analyzed off-line using IonWizard (IonOptix).

Real-time RT-PCR

Gene expression of ANP, BNP, β-MHC, collagen type I, TGF-β, CaMKIIδ, NOX2 (NADPH oxidase 2) and NOX4 was determined by real-time RT-PCR, as previously described [3].

Western blot analysis

Protein expression and phosphorylation of RARα, CaMKIIδ, PLB, Akt, SERCA2a, SOD1 and SOD2, NOX2 and NOX4, were determined by Western immunoblotting. Briefly, LVs or cardiomyocytes were sonicated in ice-cold lysis buffer (Cell Signaling) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science). Homogenates were centrifuged at 16,000 g and protein concentration in the supernatant determined using the DC™ protein assay kit (BioRad). Equal amounts of cell lysate were separated on 4-20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed with antibodies (Santa Cruz) against RARα, PLB, p-PLB (Ser16), p-PLB (Thr17), Akt, p-Akt (Ser473), SERCA2a, SOD1, SOD2, NOX2, NOX4 and antibodies against p-CaMKIIδ (Thr286) and CaMKIIδ (Cell Signaling Technology). Equal loading was confirmed by α-tubulin levels. The intensity of the bands was analyzed and quantified by densitometry.

Online Table 1. Cardiac function assessed by LV catheterization in WT and RARαKO mice

	WT (n=10)	RARαKO (n=9)
Heart rate (beat/min)	473±14	463±16
Blood pressure, mean (mmHg)	79.4±1.3	81.9±1.9
LVEDP (mmHg)	9.57±1.45	11.94±2.85
dP/dt_{max} (mmHg/sec)	8492±275	7869±261
dP/dt_{min} (mmHg/sec)	-8261±340	-6319±465*
dPR ratio	-0.94±0.014	-0.87±0.02*
Ejection fraction (%)	62.9±2.3	64.5±5.4
Stroke volume (μl)	22.9±1.7	18.8±2.1
Cardiac output (ml/min)	10.5±1.1	8.6±1.1
Tau-Glantz (ms)	9.79±0.96	15.31±3.07*
dP/dt_{max}/EDV (mmHg/ml)	244±19	272±13

WT: wild type; EDV: end diastolic volume; dPR ratio:dP/dt_{min} to dP/dt_{max} ratio.

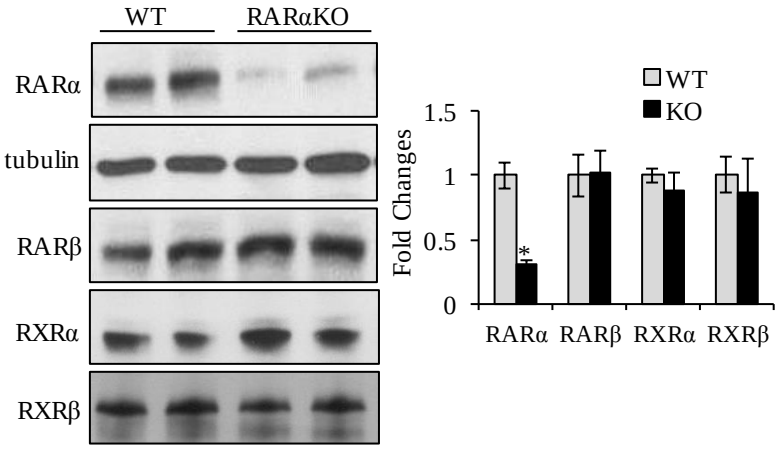
*P<0.05 vs WT.

Online Table 2. Biometric parameters in WT and RARαKO mice

	WT (n=8)	RARαKO (n=10)
BW (g)	41.5±3.03	45.1±2.32
HW (g)	0.165±0.005	0.185±0.005*
HW/TL ratio (g/cm)	0.072±0.002	0.084±0.002*
LV/TL ratio (g/cm)	0.053±0.003	0.067±0.002*
RV/TL ratio (g/cm)	0.0134±0.0014	0.0135±0.0013
LW/BW ratio (g/g)	0.072±0.002	0.084±0.003*
LW/TL ratio (g/cm)	0.090±0.008	0.105±0.006*

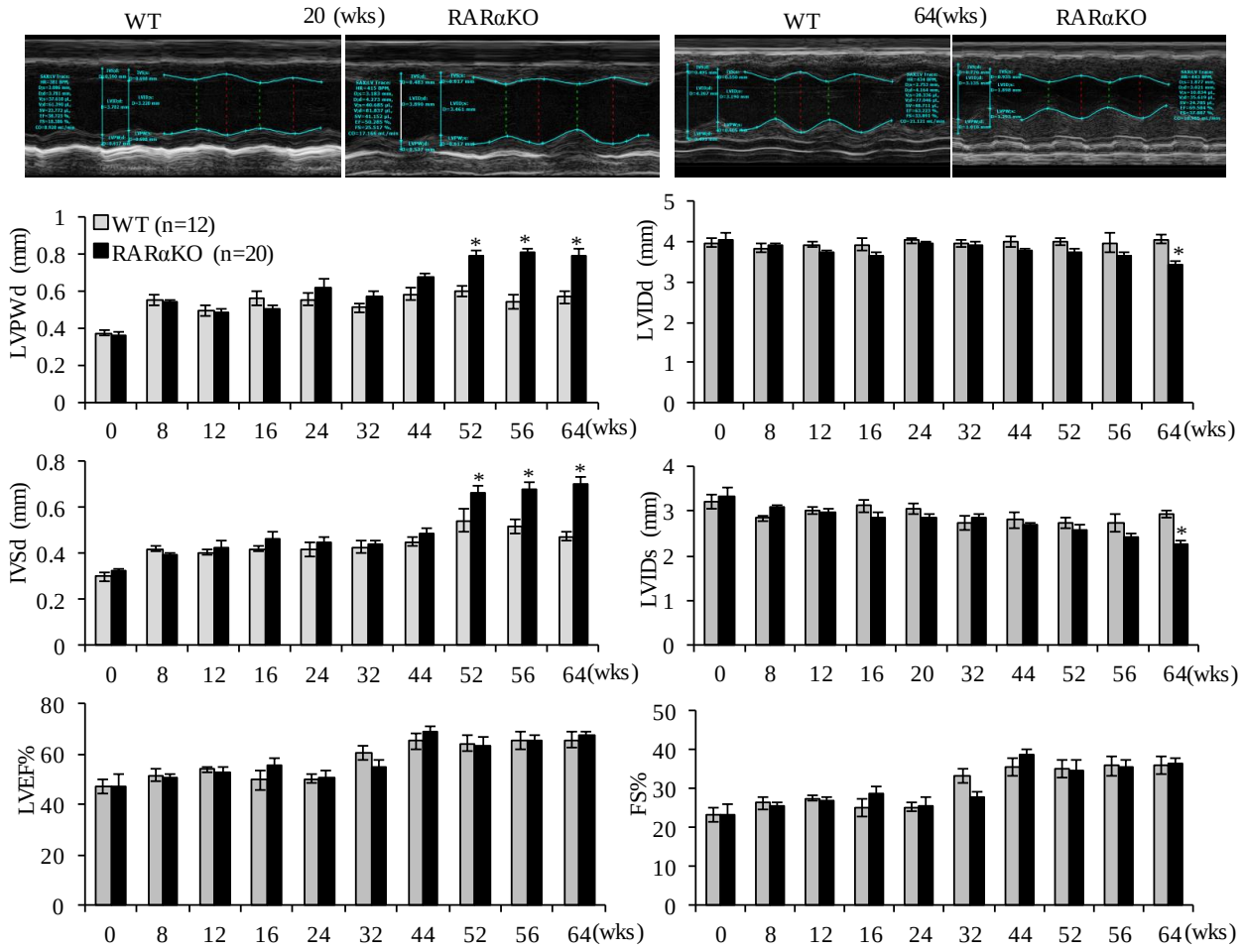
Hearts and lung were collected and weighed after 64 wks of gene deletion. BW: body weight; HW: heart weight; TL: tibia length; LW: lung weight. *p<0.05 vs WT.

Online Fig. 1



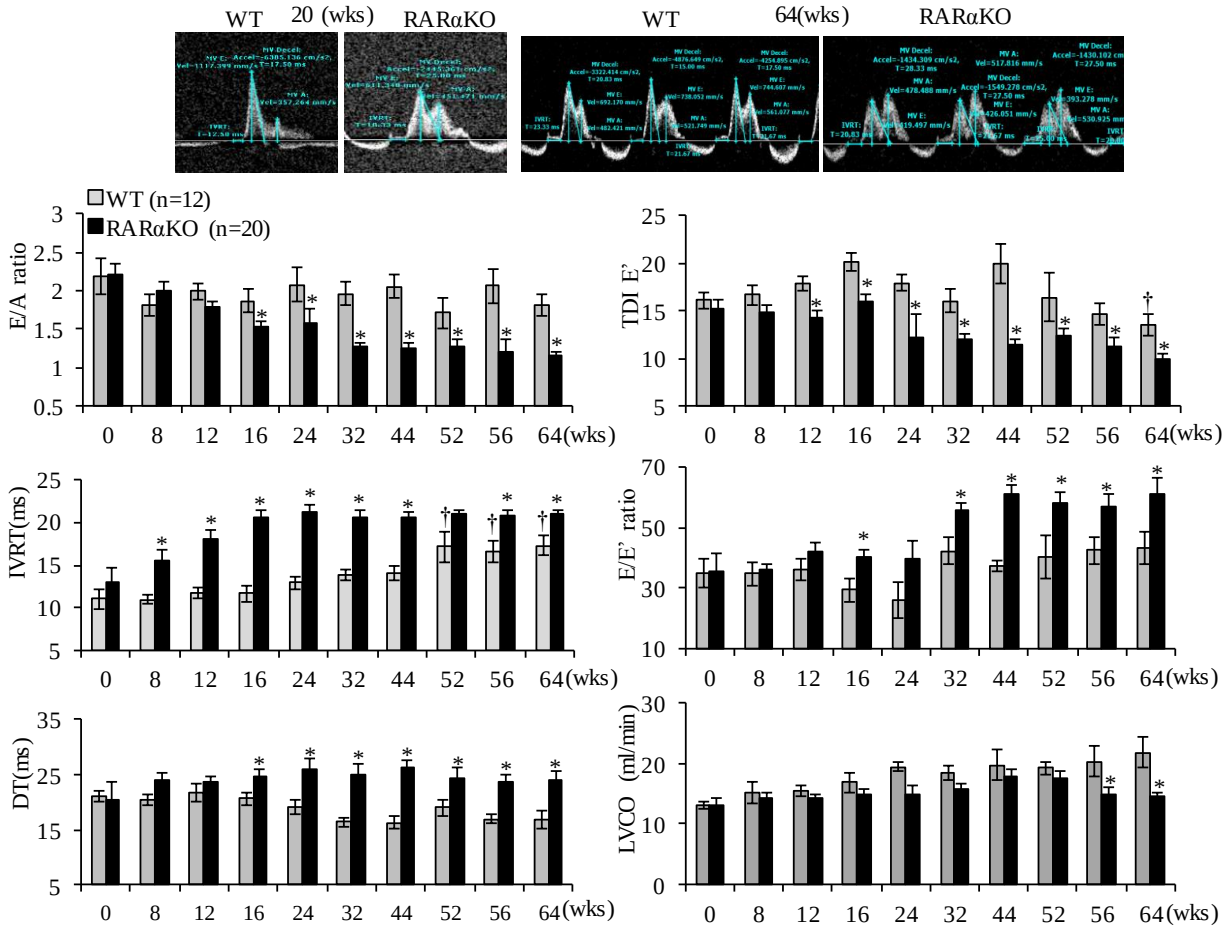
Online Fig. 1. Cardiac specific gene deletion of RARα. Alpha-MHC-Cre-RARα$^{fl/fl}$ mice were treated with tamoxifen (0.5 mg/day) or vehicle for 4 days, at 6 wks of age, hearts collected after 20 wks of gene deletion. Protein expression of RARα, RARβ, RXRα and RXRβ were determined by Western blot and quantification analysis performed. Equal loading was verified by α-tubulin expression. *p<0.05 vs WT.

Online Fig. 2



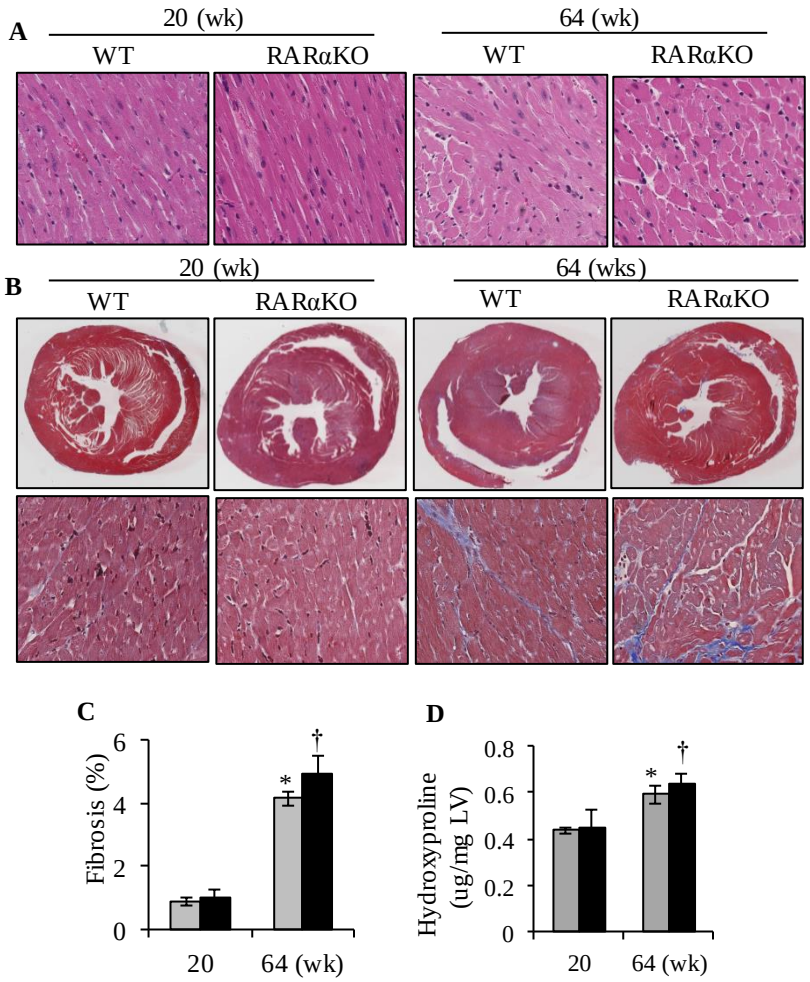
Online Fig. 2. Cardiac hypertrophy and systolic function in RARαKO mice. Echocardiography was performed in WT and RARαKO mice at indicated times, before (0) and after gene deletion. Upper panel: A representative M-mode tracing of LV showing internal diameter and posterior wall. Structural and functional parameters derived from M-mode tracings (n=12/group) were presented as mean ± SEM._LVPWd: LV posterior wall end diastole; IVSd: interventricular septum end diastole; LVIDs: LV internal diameter end diastole; LVIDs: LV internal diameter end systole; LVEF%: LV ejection fraction and FS%: fraction shortening. *p<0.05 vs age matched WT.

Online Fig. 3



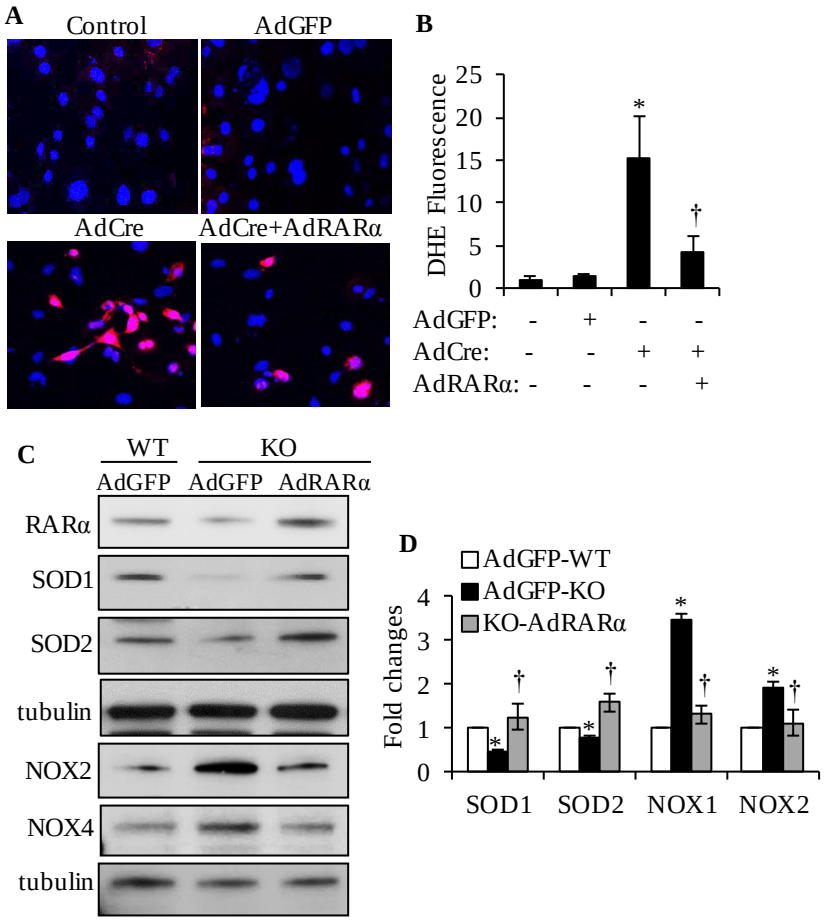
Online Fig. 3. Diastolic dysfunction in RARαKO mice. Diastolic heart function was determined by echocardiography, in WT and RARαKO mice. Upper panel: Pulsed-wave Doppler echocardiography images of transmitral flow patterns. Measurements of peak E and A velocity, IVRT, DT are shown. E/A: ratio of mitral valve flow velocities; IVRT: isovolumic relaxation time; DT: deceleration time of the E-wave; TDI E': tissue Doppler early diastolic mitral annular velocity; E/E' ratio: ratio of transmitral Doppler early filling velocity to tissue Doppler early diastolic mitral annular velocity; LVCO: cardiac output. *p<0.05 vs age matched WT; †p<0.05 vs WT at 0 point.

Online Fig. 4



Online Fig. 4. Cardiac morphological changes in RARαKO mice. (A) Paraffin sections from WT and RARαKO mouse hearts were stained with hematoxylin-eosin (HE, x40) after 20 and 64 wks of gene deletion. **(B)** Paraffin sections from WT and RARαKO mouse hearts were stained with Masson's Trichrome **(**upper panel: x1.3; bottom panel: x40), after 20 and 64 wks of gene deletion. **(C)** Quantification of fibrotic areas in WT and RARαKO mice, after 20 and 64 wks of gene deletion. Data were presented as mean ± SEM (n=6). * $p<0.05$ vs WT at 20 wks; †, $p<0.05$ vs RARαKO at 20 wks. (D) Hydroxyproline levels in LVs of WT and RARαKO mice (n=6). * $p<0.05$ vs WT at 20 wks; †, $p<0.05$ vs RARαKO at 20 wks.

Online Fig. 5



Online Fig. 5. Gene deletion of RARα increases intracellular ROS generation in cardiomyocytes. (A) Neonatal cardiomyocytes isolated from RARα$^{fl/fl}$ mice were transfected with adenovirus-mediated overexpression of Cre recombinase (AdCre), then transfected with or without wild type RARα (AdRARα). DHE staining (red) was performed and quantified **(B)**. **(C)** Adult cardiomyocytes isolated from WT and RARαKO mice at 20 wks were transfected with AdGFP or AdRARα for 24 h. Protein expression of SOD1, SOD2, Nox2 and Nox4 were determined by Western blot and quantified **(D)**. *p<0.05 vs AdGFP-WT; †p<0.05 vs AdGFP-KO.

Online Fig. 6

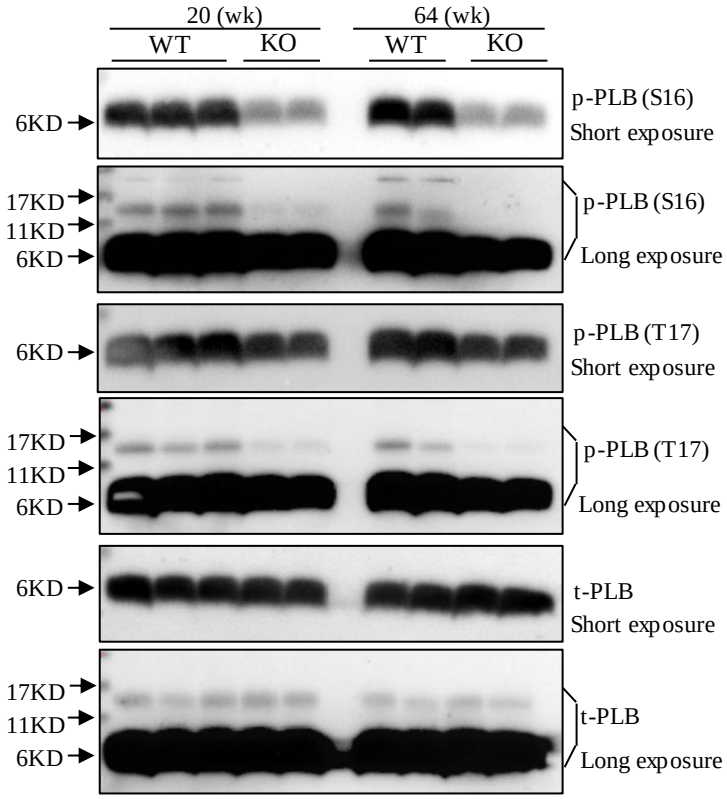


Fig. 6. RARα deletion inhibits the phosphorylation of PLB. Cardiac total and phosphorylated PLB (S16 and T17) in WT and RARαKO mice, after 20 and 64 wks of gene deletion, were determined by Western blot. The pentamer or monomer of PLB (5-24KD) was visualized with short and long exposure.

Online Fig. 7

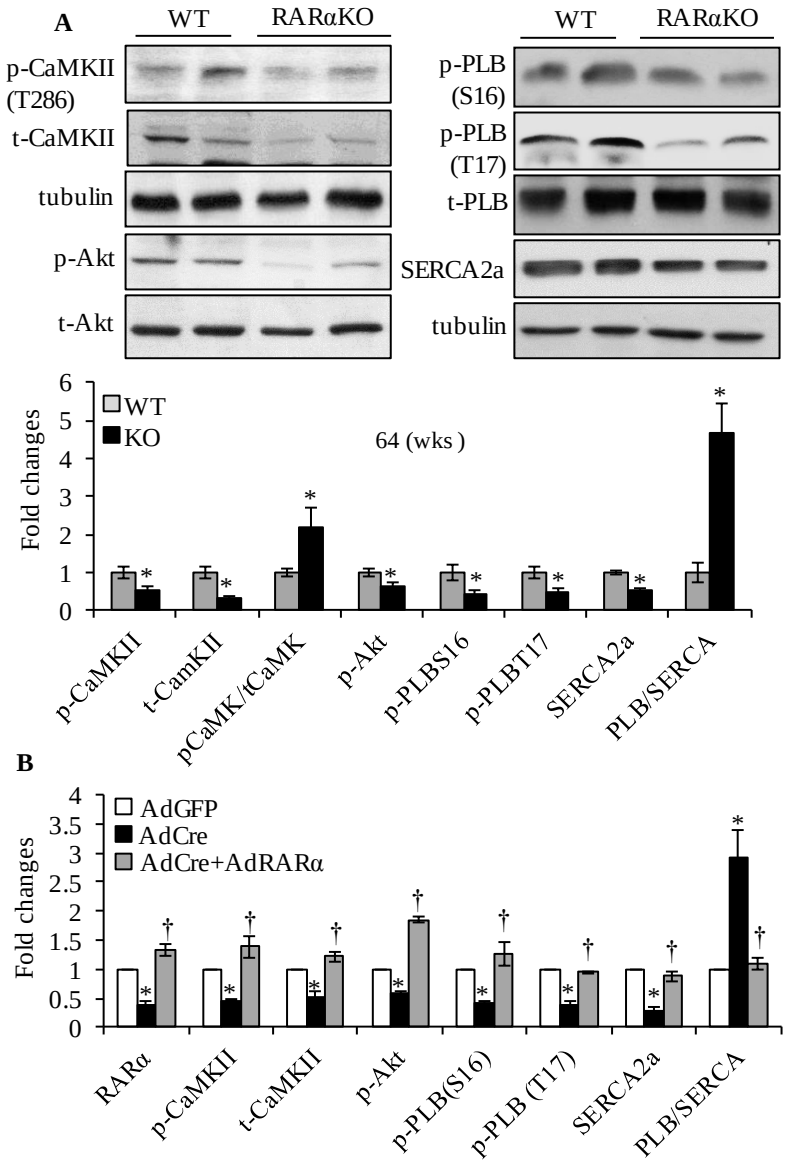


Fig. 7. RARα deletion impairs calcium handling signaling. (A) Cardiac total and phosphorylated CaMKIIδ, Akt, PLB and SERCA2a in WT and RARαKO mice, at 64 wks, were determined by Western blot and quantified. Loading control was determined by α-tubulin expression. ***** P<0.05 vs WT. **(B)** Neonatal cardiomyocytes isolated from RARα$^{fl/fl}$ mice were transfected with AdCre, then transfected with or without AdRARα for 24 h. Protein expression and phosphorylation of CaMKIIδ, Akt, PLB, SERCA2a and RARα were determined by Western blot and quantified. *****p<0.05 vs AdGFP control; † p<0.05 vs AdCre group.

Online Fig. 8

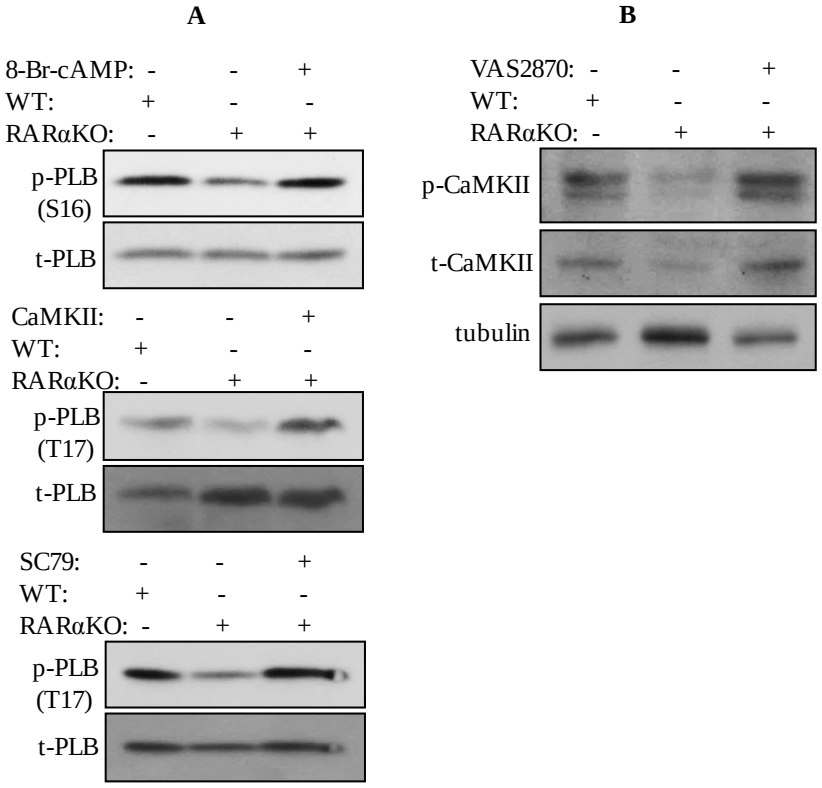


Fig. 8. PKA, CaMKII and Akt are involved in regulation of the phosphorylation of PLB. (A) Adult cardiomyocytes isolated from WT and RARαKO mice were treated with 8-Br-cAMP (PKA activator, 25 μmol/L), activated CaMKII (0.6 μg/ml) and SC79 (Akt activator, 4 μg/ml) for 30 min, protein expression/phosphorylation of PLB determined by Western blot (n=3). **(B) Role of NOX in regulation of the expression/phosphorylation of CaMKIIδ.** Adult cardiomyocytes isolated from WT and RARαKO mice were treated with or without VAS2870 (5 μmol/L, NOX inhibitor) for 12 h, and protein expression and phosphorylation of CaMKIIδ determined by Western blot.

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