SUPPLEMENTAL MATERIAL

The cardiac CaMKII genes δ and γ contribute redundantly to adverse remodeling but inhibit calcineurin-induced myocardial hypertrophy

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Supplemental Methods

Generation of DKO mice. All DKO animals (except the animals that were used for the endurance exercise experiment) were generated by crossing FFFF mice with transgenic mice expressing Cre-recombinase under the control of the α-MHC promoter (αMHC-Cre).[1] DKO mice that were used for the endurance exercise experiment were generated by crossing FFFF mice with transgenic mice expressing the mER-Cre-mER fusion protein under control of a cardiomyocyte-specific αMHC-promoter.[2] The latter animals were treated with tamoxifen to achieve CaMKII deletion. Therefore, 10 mg/ml tamoxifen (Sigma) was dissolved in a regular sunflower oil solution containing 6% ethanol. Tamoxifen was administered via gavage for 10 consecutive days once per day in a dose of 80 mg/Kg body weight per day. Efficient take-out of CaMKII was verified by Western blot. To avoid interferences with transient Cre expression, experiments were conducted at least 3 weeks after the last tamoxifen application.

Transverse aortic constriction. TAC to a 27 gauge stenosis was performed in 9-10 week-old male DKO, δ-KO, γ-KO, CaMKII $\delta^{-/-}/\gamma^{+/-}$, CaMKII$\gamma^{-/-}/\delta^{+/-}$, FFFF and wild type mice as described previously.[3, 4] A subgroup of the animals was treated twice daily by i.p. injection with cyclosporin A (CyA; Sandimmun, Novartis) with 24 mg, 12 mg or 4 mg/kg body weight per day for 7 days or 3 weeks, beginning at the day of surgery. CyA was diluted in 0.9% saline solution with 12% ethanol, and the vehicle alone served as control.

Isoproterenol administration. Isoproterenol (Iso; Sigma) diluted in 0.1% ascorbic acid was injected intraperitoneally (i.p.) once daily in 8-week-old male DKO mice and FFFF mice in a given dose of 10 mg/kg body weight per day over 14 days. 0.1% ascorbic acid alone (vehicle) served as control.

Swimming exercise protocol. Endurance exercise was carried out as described by others.[5, 6] In brief, a ramp protocol was used starting at 10 minutes two times daily, with 10 minutes increase each day until 90 minutes, two times per day was reached. The protocol ended after 14 days. The animals were closely observed at all times to avoid relative hypoxia. With this protocol, there were no events of mice submerging under the water surface.

Transthoracic echocardiography and working heart preparation. Echocardiography and studies on isolated hearts were carried out as previously described. [7, 8]

Histology. Hematoxylin and eosin (H&E), Masson´s trichrome and CD31 stainings were performed as previously described.[9, 10] Cardiomyocyte size was assessed on H&E-stained sections by using Image J software (http://rsb.info.nih.gov/ij/). More than 200 randomly chosen cardiomyocytes from each group were analyzed to measure cross-sectional cardiomyocyte area. To quantify cardiac fibrosis, 20 trichrome-stained sections (magnification 20x) from the left ventricle were randomly selected, and morphometric analysis by using Image J was performed. Capillary density was determined as the ratio of capillaries to 100 cardiomyocytes in transversely sectioned left ventricular tissue immunostained with CD31 and counterstained with DAPI. Photographs were acquired with an Olympus SZH zoom stereo dissection scope with an Optronics DEI-750 CCD digital camera. All data were analyzed by a single observer blinded to the mouse genotypes.

Western Blotting and GST-pulldown. Proteins from heart tissue and cultured cardiomyocytes were isolated, and Western blot analysis was performed according to protocols described previously.[11] GST-pulldown experiments were performed as described.[11] Besides GST-HDAC4 419-670 (which contains a CaMKII activity-dependent binding domain) we used GST-HDAC4 2-250 (which does not contain a CaMKII binding domain) as a negative control. Primary antibodies used were anti-CaMKII total (BD Bioscience), anti-CaMKIIγ (Santa Cruz), anti-p-CaMKII (Ser287) (Affinity Bioreagents), anti-PKD (Cell

signaling), anti-p-PKD (Ser744) (Cell signaling), anti-CnA (Abcam), anti-p-CnA (Ser411) (generated by Pineda antibodies), anti-GAPDH (Chemicon), anti-HDAC4 (Santa Cruz), anti-p-HDAC4 (Ser632) (Abcam), anti-phospholamban (PLB) (Upstate), anti-p-PLB (Thr17) (Santa Cruz), anti-p-PLB (Ser16) (Upstate), anti-RyR2 (Affinity BioReagents), anti-p-RyR2 (Ser2808) and anti-RyR2 (Ser2814) (kind gifts from Dr. Xander Wehrens, Houston, USA), anti-Akt (Cell signaling), anti-p-Akt (Ser473) (Cell signaling), anti-RCAN1-4 (kind gift from Dr. Timothy McKinsey, Denver, USA), and anti-α-actin (Sigma-Aldrich). Primary antibody incubation was followed by corresponding horseradish peroxidase (HRP)-conjugated secondary anti-mouse and anti-rabbit antibodies and ECL detection. Relative protein levels were detected by densitometry using the Image J program.

CaMKII kinase activity. CaMKII kinase activity was measured by radioactive kinase assays using GST-HDAC4 419-670 as a substrate and indirectly by detecting the amount of endogenous CaMKII that associates to GST-HDAC4 419-670 (see also above under GST pulldown). A detailed protocol has been described previously.[3, 11]

Caspase 3/7 activity measurements. The Caspase-Glo 3/7 Assay (Promega Corporation) was used to measure caspase-3 and -7 activities according to the manufacturer´s instructions. Heart lysates were mixed with reaction solution at equal volumes in a 96-well plate. Luminescence was detected after 60 minutes of incubation at room temperature.

RNA analysis. Total RNA was isolated from ventricular tissue or from cultured cardiomyocytes using TRIzol (Invitrogen). Total RNA was digested with DNase, and cDNA synthesis from 500 ng of RNA was carried out using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative real-time PCR (qPCR) was performed with Universal ProbeLibrary (Roche) by using TaqMan Universal PCR Mastermix (Applied Biosystems) and detection on a 7500 Fast Cycler (Applied Biosystems). Primers and probes were: For rat: *ANP* sense 5´-cccgacccagcatgg-3´ and antisense 5´-caactgctttctgaaaggggtg-

3′; For mouse: *CaMKIIα* sense 5′-gctgccaagattatcaacacc-3′ and antisense 5′-cacgctccagcttctggt-3′; *CaMKIIβ* sense 5′-gccatcctcaccactatgct-3′ and antisense 5′-ctccatctgctttcttgttgagt-3′; *CaMKIIγ* sense 5′-agttcacagggacctgaagc-3′ and antisense 5′-cgccttgaacttctatggcta-3′; *CaMKIIδ* sense 5′-gtgccatcctcacaaccat-3′ and antisense 5′-catctgacttcttgttcaataggc-3′; *GAPDH* sense 5′-gggttcctataaatacggactgc-3` and antisense 5′-ccatttttgtctacgggacga-3`; *Col5a1* sense 5`-ctacatccgtgccctggt-3` and antisense 5`-ccagcaccgtcttctggtag-3`; *Col16a1* sense 5`-gcattgcaggagaaaatggt-3` and antisense 5`-ccatcttgccataacctgga-3`; *ANP* sense 5`-cacagatctgatggatttcaaga-3` and antisense 5`-cctcatcttctaccggcatc-3`; *BNP* sense 5′-gtctggccggacactcag-3` and antisense 5`-tgcactggtgtcttcaacaac-3`; *βMHC* sense 5`-cgcatcaaggagctcacc-3` and antisense 5`-ctgcagccgcagtaggtt; *αMHC* sense 5`-cgcatcaaggagctcacc-3` and antisense 5`-cctgcagccgcattaagt-3`; *Rcan1-4* sense 5`-actggaaggtggtgtccttgt-3` and antisense 5′-tccagcttgggcttgactgag-3` .

Adenovirus production. Adenoviruses harboring CaMKII-T287D and GFP were generated according to the manufacturer's instructions (ViraPower Adenoviral Expression System; Invitrogen). Nuclear factor of activated T-cells (NFAT) luciferase, and NFAT-GFP adenoviruses were obtained from Seven Hills Bioreagents. After generation, the adenoviruses were amplified, purified with the Adeno-X Purification Kit (BD Biosciences) and its infectious units per µl were determined with the Adeno-X Rapid Titer Kit (BD Biosciences).

Cell culture of COS cells and transfection assays. COS cells were maintained in DMEM with 10% FBS, 2 mM l-glutamine, and penicillin-streptomycin. Transfection was performed with GeneJammer (Agilent Technologies) according to manufacturer's instructions. The expression constructs for CaMKII-T287D and CnA were described before.[11, 12]

Culture of neonatal rat ventricular cardiomyocytes (NRVMs). NRVMs were isolated from 1 to 2-day old Wistar rats as previously described.[11] After isolation, NRVMs were maintained

in DMEM/199 medium (4:1) with 10% FBS, 2 mM l-glutamine, and penicillinstreptomycin. NRVMs were infected 24 h after plating, grown 12 h later in serum-free media for another 4 h.

Culture of neonatal mouse ventricular cardiomyocytes (NMVMs). Neonatal cardiomyocytes were isolated from 1- to 2-day-old DKO or FFFF mice according to previously published methods.[13] Cells grew for 24 h in the fresh complete medium. Some of the cells were treated with CnA-siRNA or scrambled siRNA according to the manufacturer's instructions (Sigma-Aldrich). Medium was then replaced with serum-free medium 24 h prior to adding isoproterenol (Sigma-Aldrich, 0.1 μM) for another 24 h. Cells were then harvested for immunocytochemical staining or luciferase assay. For some of the immunostainings and for luciferase assay, adenovirus harboring NFAT-GFP or NFAT luciferase reporter were added, respectively.

Culture of adult mouse ventricular myocytes (AMVMs). For AMVM isolation, δ-CKO, γ-CKO and DKO mice and their Cre-negative littermates (control), respectively, were anesthetized with isoflurane and hearts were excised. Explanted hearts were retrogradely perfused and digested as described before.[14-16] Cells were plated onto regular cell culture plates or superfusion chambers, with the glass bottoms treated with laminin. Cells were immediately used for epifluorescence microscopy experiments or grew for 24 h in the fresh complete medium. Medium was then replaced with serum-free medium 24 h prior to adding isoproterenol (Sigma-Aldrich, 0.1 μM) for another 24 h.

Immunocytochemistry. NMVMs or AMVMs were fixed in 4 % paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized with 0.3 % Triton X-100 (Sigma-Aldrich) for 10 min at room temperature and blocked for 30-60 min with 5 % goat serum (PAA) or BSA in PBS. Primary antibody for α-actinin (Sigma) was applied in PBS containing 5 % goat serum or BSA and 0.05 % Triton X-100 for 1 h. Cells were treated with secondary antibody (Texas Red-coupled

anti-mouse-antibody) in PBS containing 5 % goat serum and 0.1 % Triton X-100 for 1 h. Nuclei were labeled with DAPI (Invitrogen).

Apoptosis assay and cell size in AMVMs. TUNEL assays were performed using the in situ cell death detection kit (Roche) according to the manufacturer's protocol. To quantify the number of apoptotic AMVMs, AMVMs were counterstained with sarcomeric α-actinin, and the total numbers of AMVMs and TUNEL-positive nuclei were counted in 10 low power fields. For each experimental condition, contiguous visual fields were counted to accumulate data on 200 AMVMs per condition. Stained cells were normalized to total cell count as judged by DAPI staining. More than 80% of cells were sarcomeric α-actinin positive. Cardiomyocyte size was assessed by using Image J software (http://rsb.info.nih.gov/ij/). More than 100 randomly chosen cardiomyocytes from each group were analyzed to measure cross-sectional cardiomyocyte area.

Epifluorescence microscopy. A Nikon Eclipse TE2000-U inverted microscope which was provided with an IonOptix fluorescence detection system for assessment of Ca^{2+} handling properties was used (IonOptix). For the studies on unstressed mice (was performed in the Maier lab), AMVMs were loaded with Fura-2AM and analysed as described previously [14] For the studies on sham and TAC-operated mice (was performed in the Maack lab), AMVMs ere loaded with Indo-1AM and analysed as described previously. [15, 16]

Supplemental References:

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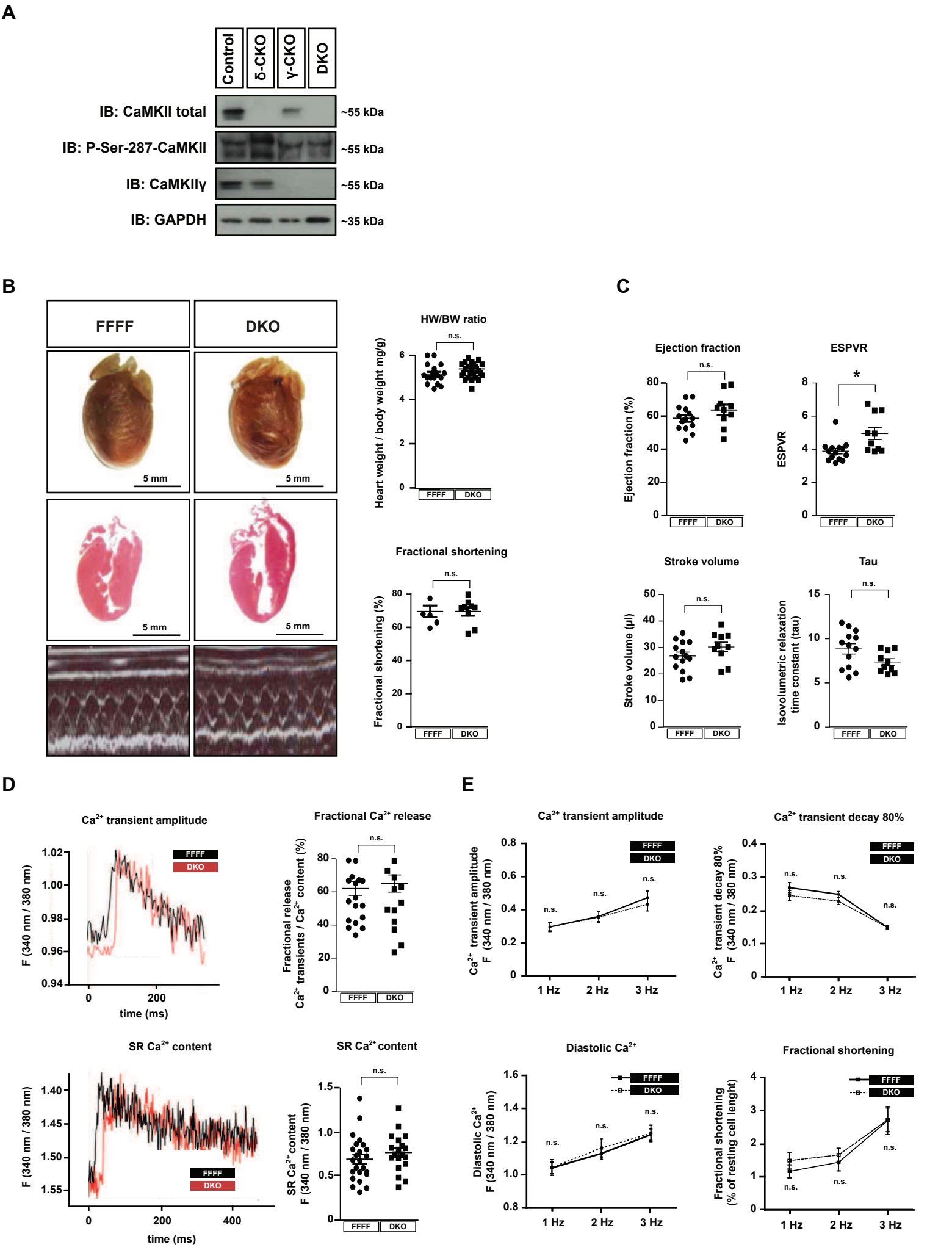
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Control
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γ-CKO
DKO
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IB: CaMKIIγ
IB: GAPDH
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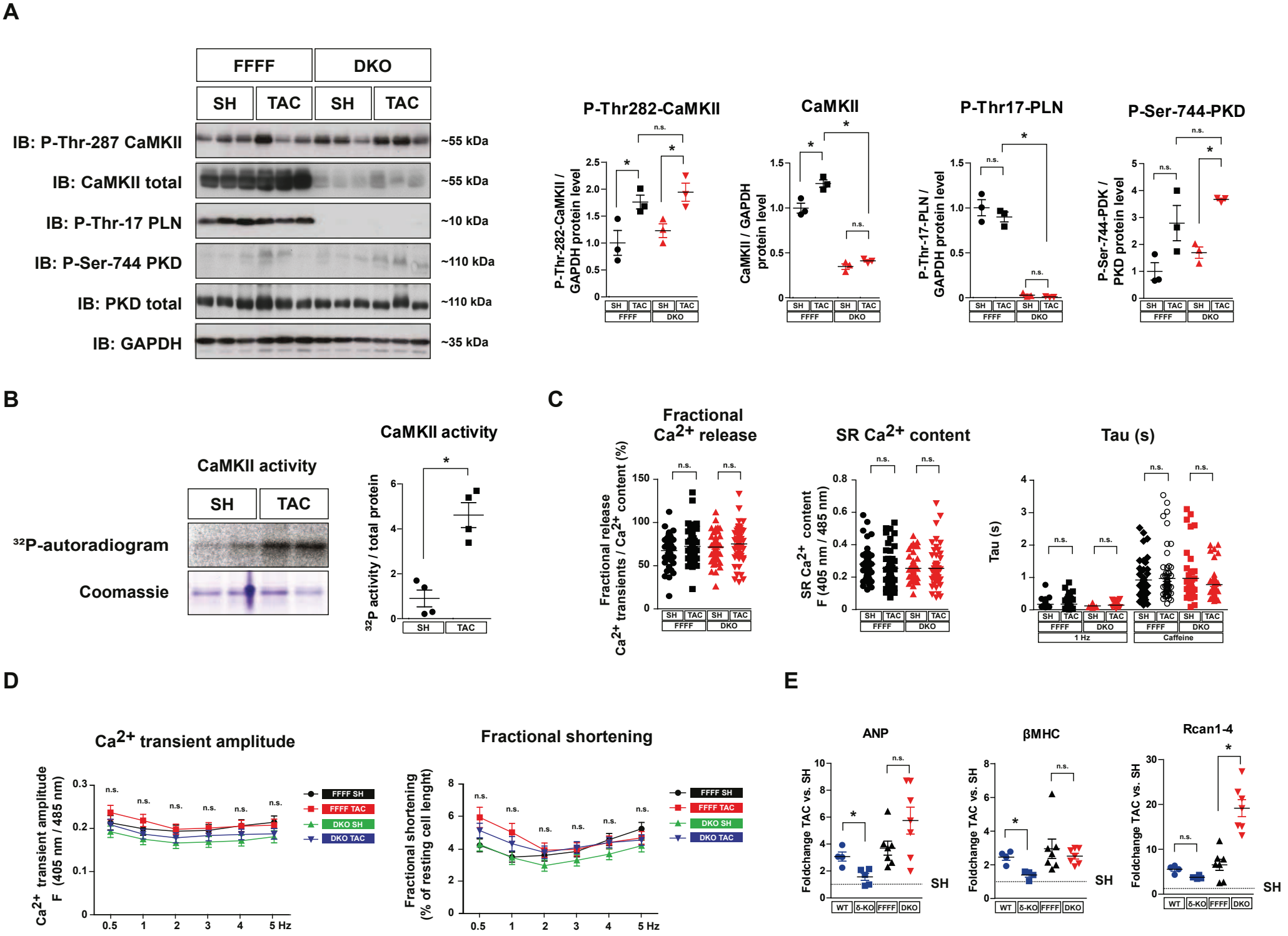
~55 kDa
~55 kDa
~35 kDa
B
FFFF
DKO
5 mm
HW/BW ratio
Heart weight / body weight mg/g)
Fractional shortening
Fractional shortening (%)
C
Ejection fraction
Ejection fraction (%)
ESPVR
Stroke volume
Stroke volume (µl)
Tau
Isovolumetric relaxation time constant (tau)
n.s.
*
D
Ca2+ transient amplitude
F (340 nm / 380 nm)
time (ms)
Fractional Ca2+ release
Fractional release Ca2+ transients / Ca2+ content (%)
SR Ca2+ content
SR Ca2+ content F (340 nm / 380 nm)
E
Ca2+ transient amplitude
Ca2+ transient amplitude F (340 nm / 380 nm)
Ca2+ transient decay 80%
Ca2+ transient decay 80% F (340 nm / 380 nm)
Diastolic Ca2+
Diastolic Ca2+ F (340 nm / 380 nm)
Fractional shortening
Fractional shortening (% of resting cell lenght)
1 Hz
2 Hz
3 Hz

Fig. S1

Fig. S1. Baseline characterization of DKO mice. (A) Western blot analysis of total CaMKII, CaMKIIγ, phospho-CaMKII-Thr287, and GAPDH as loading control in adult ventricular myocytes (AMVMs) from δ-CKO, γ-CKO and DKO mice and Cre-negative littermates (Control). **(B)** Representative images of whole hearts, sections and M-mode echocardiograms from DKO mice and floxed Cre-negative littermates (FFFF). Heart weight/body weight (HW/BW) ratios and fractional shortening for both groups are also indicated. Values are presented as mean±SEM (n≥18). **(C)** Hemodynamic measurements in isolated Langendorff-perfused hearts (n≥10). Ejection fraction, end systolic pressure volume relations (ESPVR), stroke volume and isovolumetric relaxation time constant (tau) are shown. **(D)** Representative single cell measurements for Ca^{2+} transient amplitude, fractional Ca^{2+} release and sarcoplasmatic reticulum (SR) Ca^{2+} content in isolated ventricular cardiomyocytes from DKO and FFFF mice, loaded with fluo-4. **(E)** Ca^{2+} transient decay after field stimulation with 1, 2 and 3 Hz. Measurements for intracellular diastolic Ca^{2+} and fractional shortening (FS) given as % of resting cell length after stimulation with 1, 2 and 3 Hz. All measurements were conducted in n≥30 single cardiomyocytes from n≥3 mice. Values are reported as mean±SEM. *p<0.05. n.s., not significant.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FFFF
DKO
SH
TAC
SH
TAC
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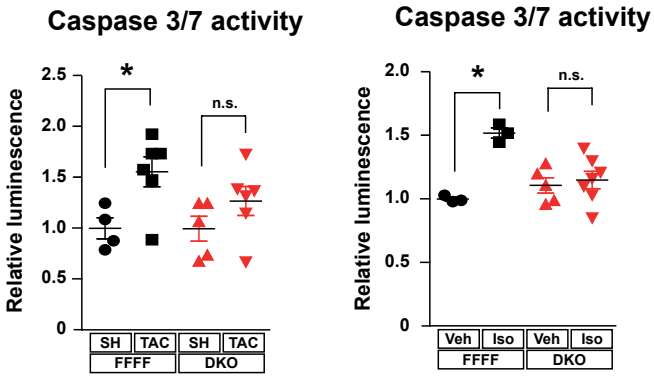
IB: PKD total
IB: GAPDH
~55 kDa
~55 kDa
~10 kDa
~110 kDa
~110 kDa
~35 kDa
P-Thr282-CaMKII
P-Thr-282-CaMKII / GAPDH protein level
CaMKII
CaMKII / GAPDH protein level
P-Thr17-PLN
P-Thr-17-PLN / GAPDH protein level
P-Ser-744-PKD
P-Ser-744-PDK / PKD protein level
n.s.
*
SH
TAC
FFFF
DKO
B
CaMKII activity
SH
TAC
32P-autoradiogram
Coomassie
CaMKII activity
32P activity / total protein
C
Fractional Ca2+ release
Fractional release Ca2+ transients / Ca2+ content (%)
SR Ca2+ content
SR Ca2+ content F (405 nm / 485 nm)
Tau (s)
1 Hz
Caffeine
D
Ca2+ transient amplitude
Ca2+ transient amplitude F (405 nm / 485 nm)
FFFF SH
FFFF TAC
DKO SH
DKO TAC
0.5
1
2
3
4
5 Hz
Fractional shortening
Fractional shortening (% of resting cell lenght)
E
ANP
βMHC
Rcan1-4
Foldchange TAC vs. SH
SH
WT
δ-KO
FFFF
DKO

Fig. S2

Fig. S2. Ca^{2+}-handling after TAC in DKO. (A) Western blot analysis of total CaMKII, phospho-CaMKII-Thr287, phospho-PLB-Thr17, phospho-PKD-Ser744, total PKD and GAPDH as loading control in cardiac extracts from FFFF and DKO mice three weeks after SH or TAC surgery. Quantitative analysis is shown (n≥3 per group). **(B)** Radioactive CaMKII kinase activity measured by *in vitro* kinase assay using GST-HDAC4 419-670 as a substrate. Coomassie staining was used to demonstrate equivalent GST-HDAC4 input. Representative blots and quantification (n=4) are shown. **(C+D)** Intracellular Ca^{2+} handling and cell shortening after TAC. Measurements were conducted in adult mouse ventricular myocytes (AMVMs) from FFFF and DKO mice three weeks after SH or TAC surgery. AMVMs were loaded with indo-1. Averaged data for intracellular Ca^{2+} transient amplitudes and fractional shortening given as % of resting cell length after field stimulation with 0.5, 1, 2, 3, 4 and 5 Hz. Fractional Ca^{2+} release and averaged sarcoplasmatic (SR) Ca^{2+} content were estimated from the caffeine-induced peak in Ca^{2+} transients. Time constant (tau) of intracellular Ca^{2+} decline after field stimulation and under caffeine. All measurements were conducted in n≥25 single AMVMs from n≥3 mice. **(E)** Gene expression in wild type and δ-KO as well as DKO and FFFF mice after TAC or SH surgery. Animals were sacrificed after 3 weeks. Fold-changes in mRNA levels of ANP, βMHC and Rcan1-4 (n≥3 per group). All values are reported as mean±SEM. *$p<0.05$. n.s., not significant.

A



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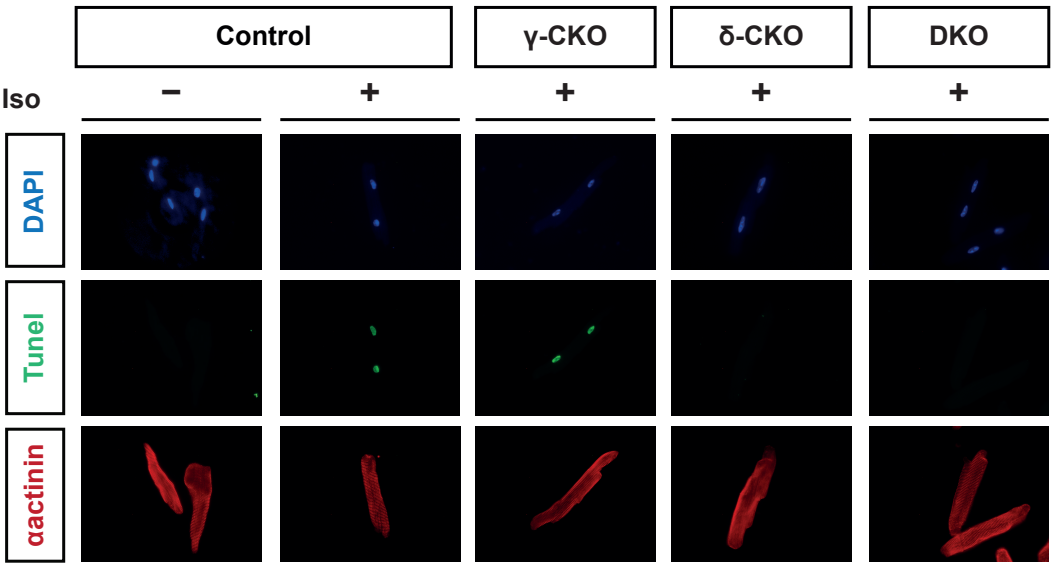

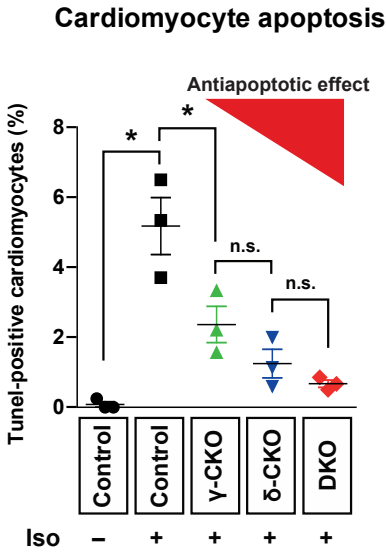

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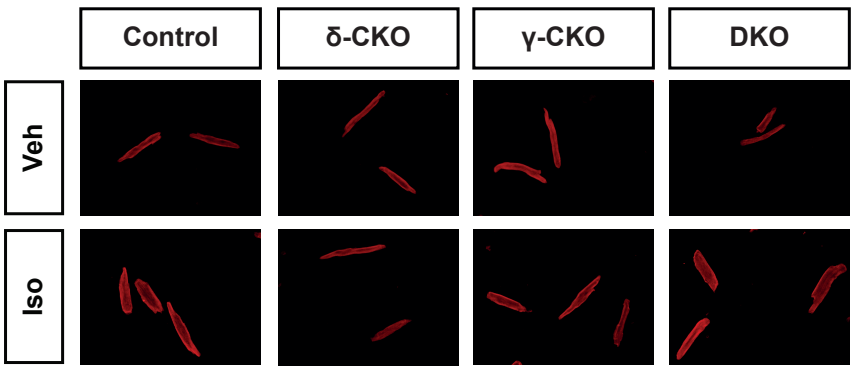


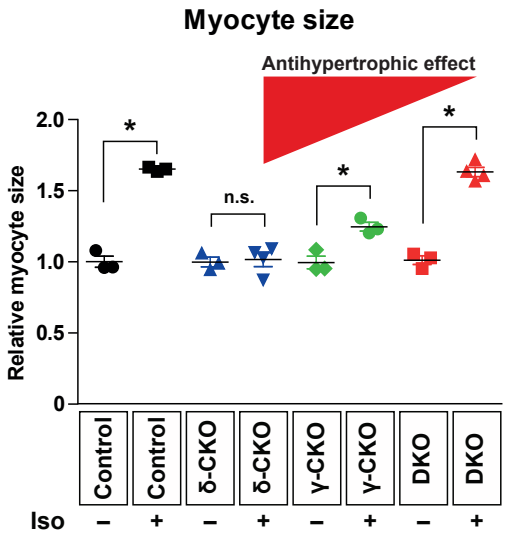
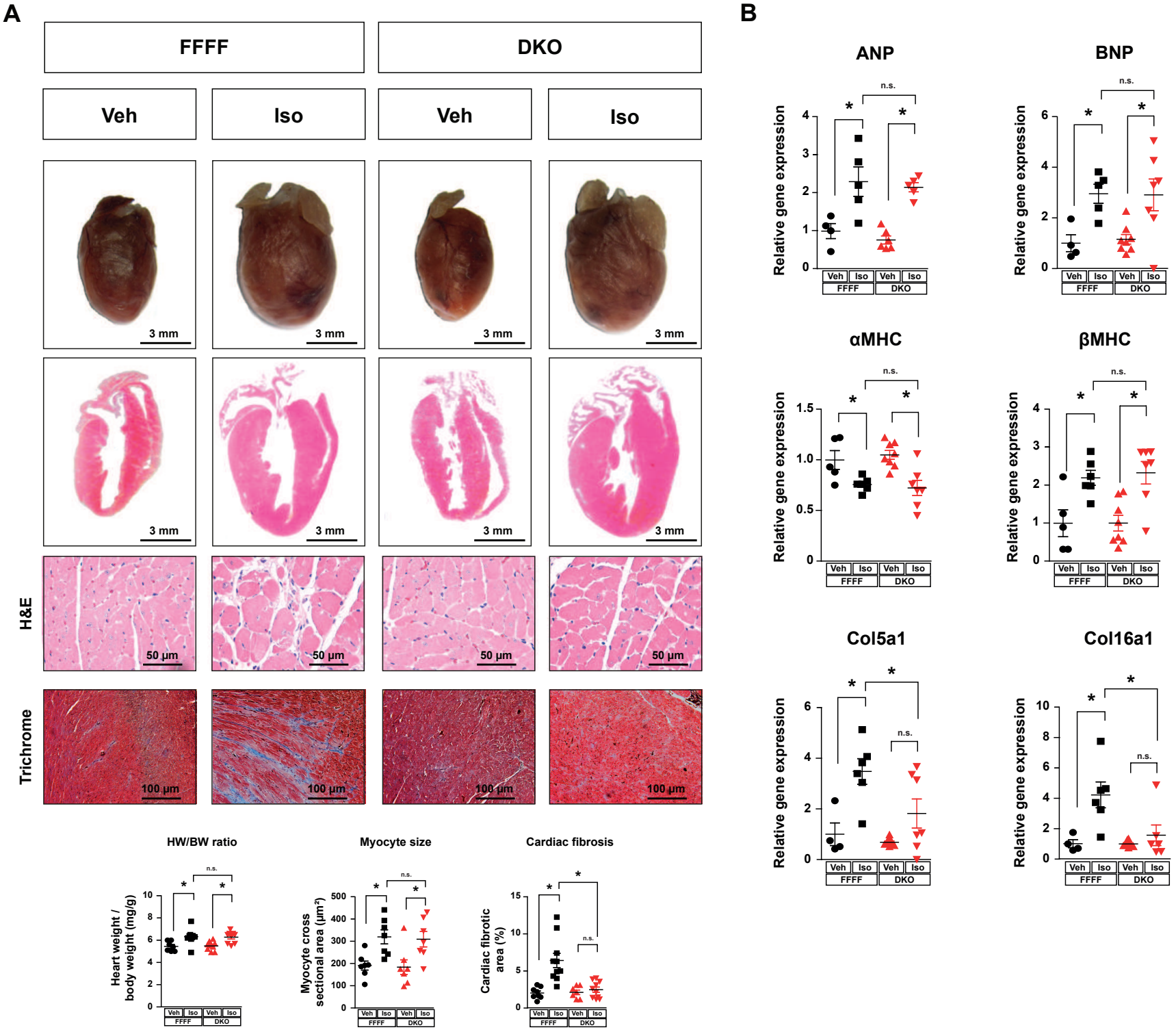


Figure S3

Fig. S3. Redundant roles for CaMKIIδ and γ for cardiomyocyte apoptosis and hypertrophy. (A) Quantification of cardiac caspase 3/7 activity in DKO and FFFF mice as measure for apoptosis induced by 3 weeks after TAC or 14 days of isoproterenol (Iso) treatment (n≥3 per group). **(B-C)** Experiments were conducted in adult ventricular myocytes (AMVMs) from δ-CKO, γ-CKO and DKO mice and their Cre-negative littermates (Control). Cells were stimulated with Iso or treated with vehicle. **(B)** Cells were stained for α-actinin (shown in red) and with DAPI nuclear stain (shown in blue). TUNEL-positive cells display green nuclei (more than 200 myocytes were analyzed per well). Shown are selected myocytes with TUNEL-positive and negative staining. The percentage TUNEL-positive cells is indicated (n=4 per condition). **(C)** Myocyte size was determined by measuring >100 cardiomyocytes per well (n=4 per condition). All values are presented as mean±SEM. *p<0.05. n.s., not significant.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FFFF
DKO
Veh
Iso
Veh
Iso
3 mm
H&E
50 μm
Trichrome
100 μm
HW/BW ratio
Heart weight / body weight (mg/g)
Myocyte size
Myocyte cross sectional area (μm²)
Cardiac fibrosis
Cardiac fibrotic area (%)
n.s.
B
ANP
BNP
αMHC
βMHC
Col5a1
Col16a1
Relative gene expression
Veh
Iso
FFFF
DKO

Fig. S4

Fig. S4. CaMKII-dependent dissociation of cardiac fibrosis and dysfunction from cardiac hypertrophy and fetal gene activation after isoproterenol. (A) Representative images of whole hearts as well as H&E and Masson´s trichrome staining of left ventricular wall sections. Quantification of HW/BW ratios, myocyte size and fibrosis area (n>6 per group). FFFF and DKO mice were treated i.p. with isoproterenol (Iso) 10 mg/Kg/d or vehicle for 14 days, respectively **(B)** Fold-changes in mRNA levels of the hypertrophic markers ANP, BNP, α-MHC and β-MHC and fold-changes in collagen expression (n≥4 per group). All values are presented as mean±SEM. *p<0.05. n.s., not significant.

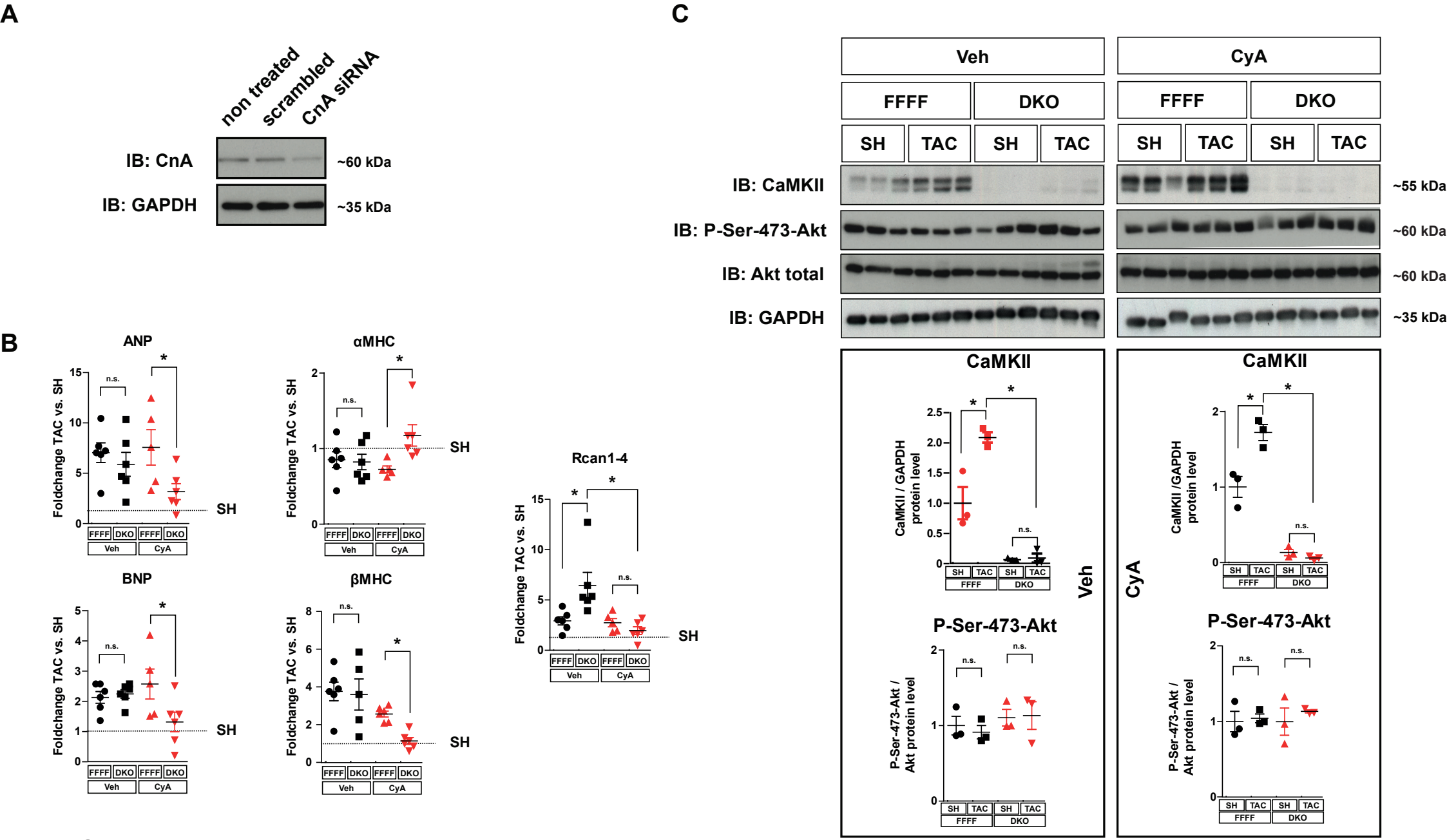
A
non treated
scrambled
CnA siRNA
IB: CnA
~60 kDa
IB: GAPDH
~35 kDa
B
ANP
αMHC
BNP
βMHC
Rcan1-4
Foldchange TAC vs. SH
n.s.
*
SH
FFFF
DKO
Veh
CyA
C
Veh
CyA
FFFF
DKO
SH
TAC
IB: CaMKII
~55 kDa
IB: P-Ser-473-Akt
~60 kDa
IB: Akt total
~60 kDa
IB: GAPDH
~35 kDa
CaMKII
CaMKII / GAPDH protein level
P-Ser-473-Akt
P-Ser-473-Akt / Akt protein level

Figure S5

Fig. S5. Normalization of fetal gene program and unaltered Akt phosphorylation after CnA inhibition in DKO mice. (A) For data presented in Fig. 5A+B, neonatal mouse ventricular myocytes (NMVMs) were transfected with CnA-siRNA or scrambled siRNAs, or they were not transfected (non treated). Western blot analysis demonstrates efficient CnA knockdown. GAPDH shows equal loading. **(B+C)** Analog to the experiment shown in Fig. 5C, DKO and FFFF mice were randomized to either TAC or SH surgery and were treated with cyclosporine A (CyA; 4 mg/Kg/d i.p.) or vehicle (n≥3). Mice were sacrificed 7 days after TAC surgery. CyA-treated DKO mice were protected from TAC-induced hypertrophy (data not shown) as described in Fig. 5C (3 weeks after TAC). **(B)** Fold-changes in cardiac mRNA levels of the hypertrophic markers ANP, BNP, αMHC and βMHC and fold-changes of RCAN 1-4 expression (n≥3 per group). **(C)** Western blots for CaMKII, Akt total, phospho-Akt-Ser473, and GAPDH as loading control were performed in lysates from left ventricle. Quantitative analysis of the expression and phosphorylation of the proteins is shown (n=3). All values are presented as mean±SEM. *p<0.05. n.s., not significant.

Cardiac CaM Kinase II Genes δ and γ Contribute to Adverse Remodeling but Redundantly Inhibit Calcineurin-Induced Myocardial Hypertrophy

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