

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

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Abstract

Background - Ca^{2+} -dependent signaling through CaMKII and calcineurin was suggested to contribute to adverse cardiac remodeling. However, the relative importance of CaMKII versus calcineurin for adverse cardiac remodeling remained unclear.

Methods and Results - We generated double-knockout mice (DKO) lacking the two cardiac CaMKII genes δ and γ specifically in cardiomyocytes. We show that both CaMKII isoforms contribute redundantly to phosphorylation not only of phospholamban, ryanodine receptor 2, histone deacetylase 4 but also calcineurin. Under baseline conditions, DKO mice are viable and display neither abnormal Ca^{2+} handling nor functional and structural changes. Upon pathological pressure overload and beta-adrenergic stimulation, DKO mice are protected against cardiac dysfunction and interstitial fibrosis. But surprisingly and paradoxically, DKO mice develop cardiac hypertrophy driven by excessive activation of endogenous calcineurin, which is associated with a lack of phosphorylation at the auto-inhibitory calcineurin A site Ser411. Likewise, calcineurin inhibition prevents cardiac hypertrophy in DKO. Upon exercise performance, DKO mice show an exaggeration of cardiac hypertrophy with increased expression of the calcineurin target gene RCAN1-4 but no signs of adverse cardiac remodeling.

Conclusions - We established a mouse model in which CaMKII's activity is specifically and completely abolished. By the use of this model we show that CaMKII induces maladaptive cardiac remodeling while it inhibits calcineurin-dependent hypertrophy. These data suggest inhibition of CaMKII but not calcineurin as a promising approach to attenuate the progression of heart failure.

Key words: CaMKII, calcineurin, heart failure, signal transduction, cardiac hypertrophy

INTRODUCTION

Heart failure is the leading cause of death in developed countries.¹ It results from adverse cardiac remodeling upon pathological stress situations such as arterial hypertension, ischemic injuries or genetic causes. Adverse cardiac remodeling is usually described by a combined appearance of myocardial hypertrophy, activation of a fetal gene program, cell death and interstitial fibrosis.² Ca^{2+} -dependent signaling pathways including CaMKII and calcineurin were both proposed to play pivotal roles in adverse cardiac remodeling.³

The protein kinase CaMKII consists of four different isoforms with distinct expression patterns. CaMKII α and CaMKII β are enriched in the brain, but CaMKII δ and CaMKII γ are expressed ubiquitously. CaMKII δ is the predominant cardiac CaMKII isoform but CaMKII γ is also expressed in the heart.⁴ In human and experimental heart failure, CaMKII expression and activity is enhanced.^{4,5} Phosphorylation of the ryanodine receptor RyR2 by CaMKII has been reported to cause sarcoplasmic reticulum (SR) Ca^{2+} leak, which in turn seems to drive heart failure development.^{6,7} At the epigenetic level, CaMKII inactivates the negative regulator of adverse cardiac remodeling histone deacetylase 4 (HDAC4), leading to transcriptional activation of the myocyte enhancer factor 2 (MEF2), and phosphorylates histone 3.⁸⁻¹⁴ Transgenic overexpression of the splice variants CaMKII δ_B (localizes to the nucleus) and CaMKII δ_C (localizes to the cytosol) promote cardiac hypertrophy and dilated cardiomyopathy, respectively.^{15,16} CaMKII inhibitory peptides prevent structural heart disease.^{17,18} Mice with a global deletion of CaMKII δ were protected against adverse cardiac remodeling.^{7,8} However in all of these models, substantial phosphorylation of the target phospholamban (PLB) was still detectable, indicating an incomplete *loss-of-function*.

The protein phosphatase calcineurin consists of two subunits: calcineurin A (CnA), which contains the catalytic site and calcineurin B (CnB), the small regulatory Ca^{2+} -binding subunit.¹⁹ Transgenic CnA overexpression in mice induces massive cardiac hypertrophy by dephosphorylation-dependent translocation of the transcription factor NFAT from the cytosol to the nucleus.^{20,21} The CnA- α and CnA- β isoforms are expressed in the myocardium but only CnA β was demonstrated to be stress responsive.²² Moreover, mice lacking CnA β were

protected against cardiac hypertrophy.²³ Inhibition of CnA has been suggested as a treatment option for cardiac hypertrophy because studies with the calcineurin inhibitory agent cyclosporine A (CyA) have been successful in rodents.²⁴ On the other hand, the splicing variant CnA- β 1 seems to activate Akt-dependent cardioprotective pathways without inducing NFAT-dependent maladaptive hypertrophy.²⁵

We developed a cardiomyocyte-specific double knockout model, in which the two cardiac isoforms, CaMKII δ and CaMKII γ , were both deleted. This model identifies CaMKII as the critical regulator of maladaptive cardiac remodeling and reveals that CaMKII serves as a negative regulator of calcineurin activity *in vivo*. Unexpectedly, we provide evidence that cardiac hypertrophy due to over-activation of endogenous calcineurin does not cause systolic cardiac dysfunction.

METHODS

Mouse experiments. We generated conditional knockout mice containing cardiomyocyte-specific deletions of CaMKII δ (δ -CKO), CaMKII γ (γ -CKO) or both isoforms (DKO) by crossing previously described conditional knockout mice^{8, 26} to transgenic mice expressing Cre recombinase under the control of the α -MHC promoter (α MHC-Cre) or by using the mER-Cre-mER fusion protein under the control of a cardiomyocyte-specific α -MHC-promoter.^{27, 28} As controls, littermates homozygous for the conditional CaMKII δ and CaMKII γ alleles without α MHC-Cre transgene (δ -FF, γ -FF and FFFF) were used. For some experiments, C57BL/6 mice were obtained from Charles River (wild type mice). A detailed description of the surgical methods, the exercise protocol and *in vivo* imaging as well as hemodynamic measurement techniques can be found in *Supplemental Methods*. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Regierungspräsidium Karlsruhe, Germany.

Histology. The protocols for hematoxylin and eosin (H&E), Masson's trichrome staining and CD31 immunostaining can be found in the *Supplemental Methods*.

Western Blotting, GST-pulldown and immunocytochemistry. The immunoblotting, GST-pulldown and immunocytochemistry protocols as well as the antibodies used in this study can be found in the *Supplemental Methods*.

CaMKII kinase activity. CaMKII kinase activity was measured by detecting the amount of endogenous CaMKII that associates to GST-HDAC4 419-670 or by radioactive kinase assay using ³²P-ATP.¹⁰ A detailed description can be found in the *Supplemental Methods*.

Gene expression analysis. The RNA isolation and quantitative RT-PCR as well as the primer sequences used in this study can be found in the *Supplemental Methods*.

Culture of primary cardiomyocytes. Adenoviruses for gene transfer into cardiomyocytes were produced as described in the *Supplemental Methods*. Neonatal mouse ventricular myocytes (NMVMs) and adult mouse ventricular myocytes (AMVMs) were isolated from wild type and mutant mice, neonatal rat ventricular myocytes (NRVMs) were isolated from Wistar rats (Charles River). A detailed description is included in the *Supplemental Methods*.

Epifluorescence microscopy. Freshly isolated AMVMs were incubated with Fura-2 AM or Indo-1 AM and Ca^{2+} transients as well as SR Ca^{2+} load were measured. AMVMs were transilluminated by red light (>650 nm) to visualize sarcomeres, and fractional shortening was measured. A detailed description is included in the *Supplemental Methods*.

Statistical analysis. Data are summarized as mean $\pm$ SEM. Statistical analysis was performed with the Graph-Pad Prism Software Package Version 5.0 (GraphPad, Inc.). When two groups were compared, we used Wilcoxon-Mann-Whitney U tests. When more than two groups were compared, a Kruskal-Wallis test was applied. When we obtained a significant p-value we continued with pair-wise comparisons using Wilcoxon-Mann-Whitney U tests according to the closed testing principle. A value of $P<0.05$ was considered statistically significant.

RESULTS

CaMKII δ and CaMKII γ contribute redundantly to cardiac CaMKII activity. Gene-expression analysis of the four CaMKII genes (CaMKII α , CaMKII β , CaMKII δ and CaMKII γ) in RNA samples from healthy and diseased wild type mouse hearts was performed in relation to the expression levels in the brain. In accordance with others,⁴ we revealed that besides CaMKII δ , CaMKII γ is also expressed in the heart, whereas CaMKII α and CaMKII β are not (**Fig. 1A**). Moreover, both genes were up-regulated in diseased hypertrophic hearts after transverse aortic constriction (TAC)-induced pressure overload of the left ventricle. Thus, we took advantage of two conditional CaMKII knockout models that we developed previously,^{8, 26} and generated cardiomyocyte-specific CaMKII δ (δ -CKO), CaMKII γ (γ -CKO) and CaMKII δ /CaMKII γ double knockout mice (DKO) (**Fig. 1B**). As controls, α MHC-Cre negative littermates (δ -FF, γ -FF and FFFF) were used. Western blot analysis using an antibody, that was shown to recognize at least CaMKII δ and CaMKII γ ,^{8, 26} confirmed a marked loss of CaMKII in cardiac extracts of δ -CKO and DKO (**Fig. 1C**). With this antibody, CaMKII was almost not detectable in δ -CKO, suggesting (under the assumption that the antibody has the same affinity to CaMKII γ as to CaMKII δ) that CaMKII δ is largely more abundant than CaMKII γ in the heart (**Fig. 1C**, see also **Suppl. Fig. 1A**). A CaMKII γ -specific antibody could detect CaMKII γ in δ -CKO but not in γ -CKO and DKO mice, suggesting that CaMKII γ could compensate for the loss of CaMKII δ in δ -CKO mice. Notably, phosphorylation of the CaMKII target site of PLB Thr17 was only slightly reduced in δ -CKO mice, whereas it was completely abolished in DKO mice, confirming that CaMKII δ and CaMKII γ exert redundant roles and largely compensate for each other. As a control, phosphorylation at the protein kinase A (PKA) site PLB-Ser16 was not reduced (**Fig. 1C**). Thus, DKO represents a so far unique model for a complete loss of CaMKII activity in cardiomyocytes *in vivo*. We also analyzed the phosphorylation level of targets of interest. Consistent with PLB, phosphorylation of RyR2-Ser2814 and HDAC4-Ser632 was decreased moderately in δ -CKO but clearly in DKO animals. Again, phosphorylation at the PKA site RyR2-Ser2808 was not affected by CaMKII deletion (**Fig. 1C**).

CaMKII DKO mice are viable and do not display cardiac abnormalities. DKO mice developed normally and showed no increase in mortality or apparent morphological abnormalities. Cardiac size as indicated by heart weight/body weight ratios, ventricular chamber dimensions or fractional shortening, assessed by echocardiography were unchanged in mice at age of 12 weeks (**Suppl. Fig. 1B**). To study functional parameters in more detail we used a working heart preparation to standardize pre- and afterload (**Suppl. Fig. 1C**). We detected slight improvements in ESPVR (which was not significant in all experimental groups used in this study, see also Fig. 3A) as a measure for maximal pressure that can be developed by the ventricle at any given LV volume. Based on the observation that the CaMKII sites on PLB and RyR2 were hypo-phosphorylated, we measured global parameters of Ca^{2+} handling (**Suppl. Fig. 1D-E**). However, we did not detect any change in Ca^{2+} transients, SR Ca^{2+} content, diastolic Ca^{2+} concentration and single-cell fractional shortening, surprisingly indicating that – at least under basal conditions – CaMKII activity is dispensable for intracellular Ca^{2+} handling and cardiomyocyte contractility.

Calcineurin precedes CaMKII activation upon pathological pressure overload. We analyzed the time course of CaMKII and calcineurin activation after TAC (**Fig. 2A-B**). Starting at 24 hours after TAC, CaMKII expression ($\text{CaMKII}\delta_{\text{C}} > \text{CaMKII}\delta_{\text{B}}$) and CaMKII activity, as judged by its activity-dependent binding affinity to HDAC4,¹⁰ increased (~2-fold) (**Fig. 2A**). We did not use the usual antibodies recognizing CaMKII autophosphorylation to determine CaMKII activity because in DKO one of these antibodies still showed a signal although CaMKII protein was gone (**Suppl. Fig. 2A and Suppl. Fig. 1A**). As another indication for CaMKII activation, phosphorylation of HDAC4 at its CaMKII phosphorylation site Ser632 also started to increase three days after TAC. In contrast, expression of RCAN1-4, a specific target gene of the calcineurin-NFAT pathway, which is considered as an endogenous calcineurin reporter,²⁹ was dramatically overexpressed (~17-fold) one day after TAC (**Fig. 2B**). These data indicate that CaMKII activation and HDAC4 phosphorylation depend on upstream signals distinct from the upstream signals of calcineurin activation.

Dissociation of cardiac dysfunction and interstitial fibrosis from myocardial hypertrophy. Next, we performed TAC surgeries in DKO and FFFF mice. Strikingly, DKO were protected against cardiac fibrosis, increased caspase 3/7 activity as marker for apoptosis and systolic dysfunction and display an attenuated rarefication of capillarization (**Fig. 3A**, see also **Suppl. Fig. 3A**). This protective effect was not associated with changes in intracellular Ca^{2+} handling or cellular contractility (**Suppl. Fig. 2C+D**). Surprisingly, TAC still induced cardiac and cardiomyocyte hypertrophy in DKO. Moreover, the gene expression changes of typical fetal genes (ANP, BNP, βMHC) and remodeling marker (αMHC) were similar in DKO and FFFF (**Fig. 3B**). These data indicate a dissociation of cardiac hypertrophy and re-activation of the fetal gene program on one hand from maladaptive cardiac remodeling including interstitial fibrosis, apoptosis, reduced capillarization and cardiac dysfunction on the other hand. To confirm these data in an independent model, we applied neurohormonal stress to DKO. We injected isoproterenol (Iso) to DKO and FFFF mice. Iso led to cardiac hypertrophy (**Suppl. Fig. 4A**) and fetal gene activation in DKO as observed in FFFF mice (**Suppl. Fig. 4B**). But again and consistent with the TAC data, we observed less cardiac fibrosis, apoptotic markers and collagen expression in DKO (**Suppl. Fig. 4A+B**, see also **Suppl. Fig. 3A**). These observations were especially surprising in view of our previous finding that global deletion of one isoform, $\text{CaMKII}\delta$, attenuated cardiac hypertrophy and fetal gene activation.⁸ However, in our previous model we could still detect substantial phosphorylation of the typical CaMKII target PLB-Thr17 in the myocardium because $\text{CaMKII}\gamma$ was not deleted (see also **Fig. 1C**). Thus, we hypothesized that complete CaMKII inhibition might exert dual effects on anti- and pro-hypertrophic pathways. To substantiate this hypothesis we performed a gene dosage experiment (**Fig. 3C**). Consistent with our previous findings, the homozygous deletion of either $\text{CaMKII}\delta$ or $\text{CaMKII}\gamma$ resulted in a reduction of cardiac hypertrophy upon TAC. Paradoxically, the additional heterozygous deletion of the other CaMKII isoform resulted in a less pronounced reduction of cardiac hypertrophy upon TAC. Furthermore, the complete cardiac-specific deletion of all four $\text{CaMKII}\delta$ and $\text{CaMKII}\gamma$ gene copies (DKO) did not prevent cardiac hypertrophy. We also analyzed the effects of the

single deletion of CaMKII δ and CaMKII γ in isolated adult cardiomyocytes and could confirm the anti-hypertrophic effects of CaMKII δ and CaMKII γ (**Suppl. Fig. 3C**). We were also interested whether CaMKII δ or CaMKII γ play a specific role for apoptosis because our recent data suggested a specific role of CaMKII γ for apoptosis at least in macrophages.³⁰ However, TUNEL assays indicated that CaMKII δ deletion more than CaMKII γ deletion attenuates cardiomyocyte apoptosis and that the combined deletion is even more effective (**Suppl. Fig. 4B**).

CaMKII controls the calcineurin-NFAT pathway. Thus, we hypothesized that complete CaMKII deletion may result in an activation of a pro-hypertrophic pathway. Therefore, we searched for candidate signaling molecules that are activated by TAC or Iso. Whereas, protein kinase D (PKD) was not activated in DKO after TAC (**Suppl. Fig. 2A**), we found the calcineurin reporter gene RCAN1-4 to be strongly up-regulated in DKO mice after TAC and Iso (**Fig. 4A, Suppl. Fig. 2E**). This could not be observed after deletion of only one CaMKII gene, in particular CaMKII δ (**Suppl. Fig. 2E**), indicating that complete CaMKII deletion in the heart results in calcineurin activation. Consistently, Iso induced a dramatic increase in nuclear NFAT translocation and NFAT activity in NMVMs from DKO when compared to NMVMs from FFFF (**Fig. 4B+C**). In this regard, it was reported that CaMKII directly phosphorylates CnA-Ser411 *in vitro* but it remained unclear whether this mechanism was of biological significance *in vivo*.^{30, 31} Thus, using a newly synthesized specific antibody against CnA-Ser411 (**Fig. 4D**) we aimed to detect endogenous CnA phosphorylation. Indeed, overexpression of active CaMKII δ (T287D) in NRVMs resulted in hyper-phosphorylation of CnA-Ser411 (**Fig. 4E**). In accordance, we found that CnA was strongly hypo-phosphorylated in cardiac lysates from sham- and TAC-operated DKO hearts, which was associated with RCAN1-4 upregulation (**Fig. 4F**). Moreover, as compared to DKO CnA-Ser411 hypo-phosphorylation was less pronounced in cardiac extracts of δ -CKO, and not found in γ -CKO, explaining why calcineurin activity was only strongly increased in DKO but not in the single knockouts (**Fig. 4G**).

Cardiac hypertrophy, but not dysfunction is controlled by calcineurin during pathological and physiological hypertrophy in DKO. To test whether cardiac hypertrophy after TAC in DKO depended on calcineurin activation, we performed CnA knockdown experiments in isolated NMVMs of mutant mice. CnA knockdown, which resulted in approximately 50% decrease of CnA protein (**Suppl. Fig. 5A**), prevented Iso-induced cellular hypertrophy and activation of fetal genes in myocytes derived from DKO but not FFFF (**Fig. 5A-B**). We then repeated the TAC experiment in DKO with the calcineurin inhibitor cyclosporine A (CyA). CyA was shown to inhibit cardiac hypertrophy after TAC in dosages from 12.5 - 50 mg/kg body weight per day.³¹ Using a dosage of 4 mg/kg body weight per day, which did not affect TAC-induced cardiac hypertrophy in FFFF, we were able to inhibit RCAN1-4 expression as well as cardiac and cardiomyocyte hypertrophy in DKO but not FFFF (**Fig. 5C and Suppl. Fig. 5B**), indicating that TAC-dependent hypertrophy in DKO depends on calcineurin. As CnA knockdown, CyA attenuated also the expression of fetal genes in DKO (**Fig. 5B and Suppl. Fig. 5B**). In contrast to the anti-hypertrophic effects of CyA in DKO, CyA did not affect the protective effects of DKO on cardiac fibrosis or systolic function, providing evidence that - at least in this model - calcineurin-dependent cardiac hypertrophy is not maladaptive on the one hand but also not cardioprotective on the other hand. In line with the latter, we could not accumulate evidence that the potential cardioprotective calcineurin-dependent Akt pathway was activated (**Suppl. Fig. 5C**).

To clarify the role of the CaMKII-calcineurin pathway in physiological hypertrophy, we performed endurance exercise training by a swimming protocol in DKO and FFFF mice (**Fig. 5D-F**). Interestingly, whereas CaMKII protein levels were not altered after swimming in control mice (FFFF), CaMKII activity decreased about 30% (**Fig. 5D**). Remarkably, we found exaggerated heart weight/body weight (HW/BW) ratios and increased myocyte size in DKO mice after swimming, which was associated with increased expression of the calcineurin target gene RCAN1-4 (**Fig. 5E+F**). Again, despite calcineurin activation and increased cardiac hypertrophy no signs for systolic cardiac dysfunction were detected, indicating that activation of endogenous calcineurin is not maladaptive.

DISCUSSION

By generating mice lacking the two cardiac CaMKII isoforms δ and γ we introduce a new *loss-of-function* model with the deletion of both cardiac CaMKII isoforms in cardiomyocytes, and demonstrate that CaMKII - under unstressed conditions - is dispensable in cardiomyocytes with regard to integrity, growth, cardiomyocyte contractility and Ca^{2+} -handling. This is somewhat surprising in light of innumerable reports about CaMKII functions. But from the drug developer's point of view, this indicates that CaMKII inhibition does not exert major unwanted side effects on basal cardiac function. Furthermore, we show that - upon pathological stress conditions - CaMKII plays a dual role in the regulation of adverse cardiac remodeling (**Fig. 6**). On the one hand, CaMKII transduces maladaptive signaling. On the other hand, CaMKII negatively regulates calcineurin-dependent cardiac hypertrophy *in vivo*.

Redundancy of CaMKII δ and CaMKII γ

Because of a lack of *loss-of-function* models with the deletion of both cardiac CaMKII isoforms, it was so far not possible to study all essential roles of CaMKII in myocardial disease. Moreover, most of the reported CaMKII studies used non-genetic approaches such as overexpression of peptides or pharmacologic compounds such as KN-93. However, off-target effects, as e.g. described on protein kinase D or ion channels (e.g. L-type Ca^{2+} channels),^{8, 32, 33} could have complicated the conclusions. Therefore, we focused first on the development of a specific and complete genetic mouse model, in which the two CaMKII genes that are expressed in cardiomyocytes are deleted. We found that CaMKII δ and CaMKII γ compensate for each other and act redundantly on the phosphorylation of PLB-Thr17, RyR2-Ser2814, CnA-Ser411 and HDAC4-Ser632. In contrast to previously described approaches aiming at inhibiting CaMKII,^{7, 8, 17, 18, 34, 35} the new DKO model results in profound hypo-phosphorylation of these typical CaMKII phosphorylation targets.

CaMKII controls calcineurin signaling

Previous data from our lab showed that homozygous CaMKII δ knockout mice are protected against cardiac hypertrophy and interstitial fibrosis.⁸ Here we demonstrate that the loss of more than two cardiac CaMKII gene copies unmasks a dual role of CaMKII in regulating cardiac hypertrophy. DKO mice appear to develop a similar hypertrophic response as FFFF littermates after TAC and Iso, but histological, gene expression and biochemical analyses revealed that cardiac hypertrophy in DKO depends on activation of endogenous calcineurin. CaMKII phosphorylates CnA-Ser411, which was shown to lead to a decrease in its phosphatase activity.^{36, 37} Here, we show that this phosphorylation event occurs *in vivo* and in a CaMKII-dependent manner. Moreover, we found CnA-Ser411 hypo-phosphorylation to be associated with cardiac hypertrophy after pathological and physiological stress. Importantly and in contrast to current paradigms, the data of this study suggest that anti-hypertrophic effects are not required for an improved cardiac function. Moreover, a vast amount of literature suggested that calcineurin induces pathological cardiac hypertrophy, ultimately leading to heart failure.^{20, 21, 38, 39} However, the latter studies used forced overexpression of a truncated CnA construct lacking the regulatory domain that is phosphorylated by CaMKII, complicating the interpretation of these findings. A recent CnA β *loss-of-function* study seemed to prove the idea that calcineurin mediates pathological cardiac hypertrophy but this study focused mainly on cardiac hypertrophy but not on other aspects of pathological remodeling such as cardiac dysfunction.²³ In fact, histological analyses did not show a protection against cardiac fibrosis in CnA β null mice.²³ In the present study we identify DKO mice as a model with activation of endogenous calcineurin. We challenge current paradigms and conclude that - at least in the absence of CaMKII signals - activation of endogenous calcineurin is not maladaptive. Support comes from a recent study, demonstrating that calcineurin induces physiological hypertrophy in pregnant mice.⁴⁰ Moreover, Heineke et al. suggested that calcineurin exerts cardioprotective functions under certain conditions: hearts of transgenic mice with mild overexpression of CnA were protected in a murine model of dilated cardiomyopathy.⁴¹ Thus it is tempting to speculate that calcineurin activation in DKO

contributes to the observed cardioprotective effects upon TAC. Because it was suggested that the splice variant CnA β 1 exerts cardioprotection via Akt,²⁵ we measured Akt phosphorylation. But we found no changes. We cannot completely rule out that other calcineurin-dependent pathways contribute to the cardioprotective effects but our data show that CyA-treatment after TAC in DKO did not lead to cardiac dysfunction. Thus, we conclude that - in the setting of the current study - CaMKII-dependent but calcineurin-independent processes mediate pathological remodeling.

CaMKII is required for pathological remodeling but not cardiac hypertrophy and fetal gene expression

The question arises of how CaMKII regulates pathological remodeling, especially cardiac fibrosis. We have shown that HDAC4 transmits CaMKII signals to the cardiac genome via MEF2.^{8, 10, 13} Because gene deletion and overexpression studies revealed that MEF2D primarily drives cardiac fibrosis and dysfunction rather than cardiac hypertrophy,⁴² the CaMKII-HDAC4-MEF2 pathway is a likely pathway leading to adverse cardiac remodeling (see model in **Fig. 6**). Likewise, HDAC4 hypo-phosphorylation was associated to the protective situation in DKO mice. But it was remarkable that DKO mice were not protected against re-activation of certain fetal genes including ANP, BNP and β MHC. It is important to note, that fetal gene activation is suggested to be driven not only by MEF2 but also NFAT.⁴³ Thus, we speculate that over-activation of the calcineurin-NFAT pathway in DKO compensates for the attenuation of the CaMKII-HDAC4-MEF2 pathway with regard to ANP, BNP and β MHC but not for attenuation of yet unidentified genes that cause cardiac fibrosis. Natriuretic peptides are actually known not to be causative but rather to be adaptive to combat the hemodynamic load and oxidative capacity of muscle cells.^{44, 45} On the other hand, MEF2-dependent genes that cause interstitial fibrosis are not clearly described yet. Taken together, our data indicate that fetal genes associated with calcineurin-dependent hypertrophy do not cause cardiac dysfunction. Likewise, in a model of physiological

hypertrophy we found no activation of CaMKII but activation of calcineurin-NFAT signaling without signs of pathological remodeling.

CaMKII is dispensable for intracellular Ca²⁺ handling under unstressed conditions

Another puzzling observation of this study was the lack of effect on Ca²⁺ handling although profound changes on phosphorylation on RyR2 and PLB were detected. We speculate that the lack of CaMKII phosphorylation on Ca²⁺ handling proteins might be compensated by other mechanisms such as PKA-mediated phosphorylation, although we could not detect significant increases in phosphorylation at the PKA sites PLB-Ser16 or RyR2-Ser2808. Knockin mouse models with a specific mutation of these sites would clarify the specific role of CaMKII phosphorylation at PLB-Thr17 and RyR2-Ser2814. Indeed, Respress et al. described RyR2-2814-S/A knockin mice.⁶ Consistent with our data, they also observed an unaltered SR Ca²⁺ content, Ca²⁺ transient decay and Ca²⁺ sparks at baseline, but they discovered that in the situation of heart failure, mutant mice were protected. Thus, further experiments are warranted to investigate DKO mice and Ca²⁺ handling in models of severe heart failure. However, it appears unlikely that an attenuation of disturbed Ca²⁺ handling contributes to the improved cardiac function as observed in DKO after TAC in the present study.

Summary

Taken together, we show that CaMKII is a key driver of maladaptive cardiac remodeling. This study describes maladaptive cardiac remodeling as a process of interstitial fibrosis and cardiac dysfunction but not cardiac hypertrophy and activation of commonly measured fetal genes such as ANP and BNP. Under certain conditions, CaMKII inhibits cardiac hypertrophy by a previously underestimated cross talk mechanism to calcineurin. In the absence of CaMKII signals, calcineurin does not seem to contribute to maladaptive cardiac remodeling, highlighting CaMKII and not calcineurin as a promising drug target to combat heart failure.

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DISCLOSURES

The authors of this manuscript have no conflicts to disclose.

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FIGURE LEGENDS

Fig. 1. CaMKII δ and CaMKII γ contribute redundantly to cardiac CaMKII activity. (A)

Real-time-PCR analysis of cardiac CaMKII isoforms three weeks after sham (SH) or transverse aortic constriction (TAC) surgery in wild type mice. The relative expression levels were normalized to CaMKII in brain tissue. All values are reported as mean $\pm$ SEM (n $\geq$ 3, *p<0.05). **(B)** To generate cardiomyocyte-specific conditional CaMKII δ and γ single knockout (δ -CKO and γ -CKO) and CaMKII δ /CaMKII γ double knockout mice (DKO), CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$ (δ -FF), CaMKII $\gamma^{\text{loxP-exon1/2-loxP}}$ (γ -FF) and CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$, CaMKII $\gamma^{\text{loxP-exon1/2-loxP}}$ (FFFF) mice were crossed to transgenic mice harboring Cre-recombinase under the control of the α MHC promoter. **(C)** Western blot analysis using antibodies directed against CaMKII, CaMKII γ , total PLB, phospho-PLB-Thr17, phospho-PLB-Ser16, total RyR2, phospho-RyR2-Ser2814, phospho-RyR2-Ser2808, total HDAC4, phospho-HDAC4-Ser632 and GAPDH (as loading control). Left ventricular extracts from δ -CKO, γ -CKO and DKO mice and their Cre-negative littermates (δ -FF, γ -FF and FFFF) were analyzed (n=4 per group). Quantitative analysis of the expression and phosphorylation of the proteins is shown at right. All values are presented as mean $\pm$ SEM. *p<0.05. n.s., not significant.

Fig. 2 Calcineurin precedes CaMKII activation upon pathological pressure overload.

Western blot analysis were performed in cardiac extracts from wild type mice. Organs were harvested at different time points after TAC surgery as indicated. **(A)** Western blot analysis after GST-HDAC4 pulldown using GST-HDAC4 419–670, which contains a CaMKII-activity dependent binding site. GST-HDAC4 2-250, which lacks a CaMKII binding domain, served as a negative control. As an additional negative control, protein lysates from global CaMKII δ mice versus wild type mice were used. GST-HDAC4 input was visualized by GST-immunoblotting, associated endogenous CaMKII is visualized by CaMKII-immunoblotting. The degree of CaMKII binding to GST-HDAC4 419-670 is a specific measure for CaMKII activity.¹⁰ A quantification of CaMKII binding normalized to the input of GST-HDAC4 is

shown. **(B)** Western blot analysis using antibodies directed against CaMKII, HDAC4, phospho-HDAC4-Ser632, RCAN1-4 and GAPDH. Quantitative analysis of the expression and phosphorylation of the proteins is shown. For all experiments $n \geq 3$ per group were used. All values are presented as mean $\pm$ SEM. * $p < 0.05$. n.s., not significant.

Fig. 3. Dissociation of cardiac dysfunction and interstitial fibrosis from myocardial hypertrophy. **(A)** DKO and FFFF mice were randomized to either TAC or SH surgery and sacrificed after 3 weeks. Representative images of the total hearts, H&E, Masson's trichrome and CD31 staining, echocardiographic M-modes and quantification of heart weight/body weight ratios, myocyte size, fibrosis area, capillary density and fractional shortening are shown ($n \geq 8$ per group). Values of left ventricular ejection fraction, isovolumetric relaxation time constant (Tau), end systolic pressure volume relation (ESPVR) and stroke volume measured in a working heart preparation are shown ($n \geq 7$ per group). **(B)** Fold-changes in mRNA levels of the hypertrophic markers ANP, BNP, α MHC and β MHC and fold-changes in collagen expression ($n \geq 5$ per group). **(C)** Heart weight/body weight ratios of SH- and TAC-operated mice with the indicated genotypes ($n \geq 4$ per group). All values are presented as mean $\pm$ SEM. * $p < 0.05$. n.s., not significant.

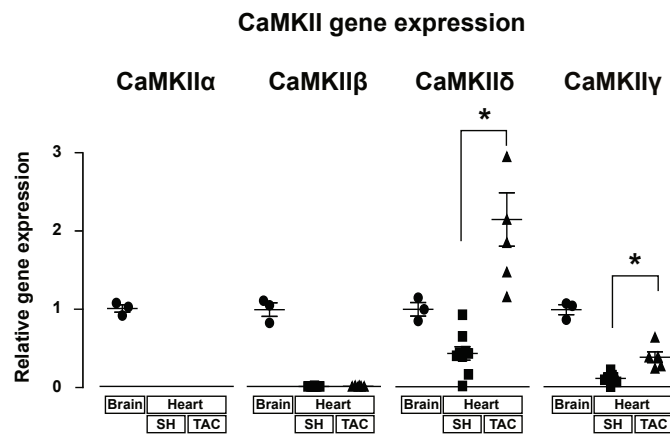
Fig. 4. CaMKII controls CnA activity. **(A)** Real-time-PCR analysis of RCAN1-4 in hearts from animals with genotypes and treatments as indicated (≥ 5 per group). **(B+C)** NMVMs from FFFF and DKO were used for cell-based experiments. **(B)** NFAT-GFP was adenovirally expressed in NMVMs. Cells were stained for α -actinin (shown in red) and with DAPI nuclear stain (shown in blue). The percentage of cells in which NFAT-GFP was localized to the nucleus is indicated (> 100 cardiomyocytes per well; $n \geq 3$ per condition). **(C)** NFAT-luciferase reporter assay in NMVMs ($n \geq 3$ per condition). **(D)** Western blot analysis was performed using lysates from COS-cells that were transfected with CnA in the absence and presence of constitutive active CaMKII δ (CaMKII-T287D). A schematic diagram of CnA indicates the position of the CaMKII phosphorylation site at Ser411, which is located within the calmodulin-

binding domain of CnA (CnB, calcineurin B-binding domain; CaM, calmodulin-binding domain; AID, autoinhibitory domain). **(E)** Adenoviral overexpression of CaMKII-T287D (adCaMKII-T287D) in NRVMs results in hyper-phosphorylation of CnA-Ser411 compared to non infected (control) NRVMs and NRVMs infected with GFP. **(F)** Western blot analysis of total CaMKII, RCAN1-4, total CnA and, CnA-Ser411 in cardiac extracts from FFFF and DKO mice 3 weeks after SH or TAC surgery ($n \geq 3$ per group). **(G)** Western blot analysis of total CnA and CnA-Ser411 in δ -CKO, γ -CKO or DKO versus their Cre-negative littermates ($n=4$ per group). For all Western blot experiments, GAPDH was used as loading control. Quantitative analysis of the expression and phosphorylation of the proteins is shown at right (F and G). All values are presented as mean $\pm$ SEM. * $p < 0.05$. n.s., not significant.

Fig. 5. Cardiac hypertrophy, but not dysfunction is controlled by calcineurin during pathological and physiological hypertrophy in DKO. (A-B) NMVMs from FFFF and DKO mice were transfected with two siRNAs directed against CnA or with scrambled siRNA (knockdown efficiency is shown in Suppl. Fig. 5A). NMVMs were then treated with Iso or vehicle. **(A)** ANP and BNP gene expression in NMVMs after Iso-stimulation ($n \geq 3$ per condition). **(B)** Cells were stained for α -actinin (shown in red) and cardiomyocyte size was determined after Iso-stimulation (>100 cardiomyocytes per well; $n \geq 3$ per condition). **(C)** FFFF and DKO mice underwent SH or TAC surgery and were treated with cyclosporine A (CyA; 4 mg/Kg/d, 3 weeks i.p.; $n \geq 7$) or vehicle ($n \geq 3$) as indicated. CyA treatment was started at the day of TAC or SH surgery. Three weeks later the mice were sacrificed. HW/BW ratios, myocyte cross sectional areas, quantification of cardiac fibrosis from Masson's trichrome-stained myocardial sections and fractional shortening assessed by echocardiography are shown. **(D-F)** FFFF and DKO mice were randomly assigned to a 14-day swimming or resting group, respectively. **(D)** Cardiac CaMKII activity was determined in heart lysates by GST-HDAC4 pulldown using GST-HDAC4 419–670 (GST-HDAC4 2-250 served as specificity control). A quantification of CaMKII-activity dependent binding as measure for CaMKII activity is given ($n \geq 11$ per group). **(E)** 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, fibrosis area and fractional shortening (n≥3 per group). **(F)** Western blot analysis using antibodies directed against CaMKII, total CnA, phospho-CnA-Ser411, RCAN1-4 and GAPDH. Quantitative analysis of the expression and phosphorylation of the proteins is shown (n≥7 per group). All values are presented as mean±SEM. *p<0.05. n.s., not significant.

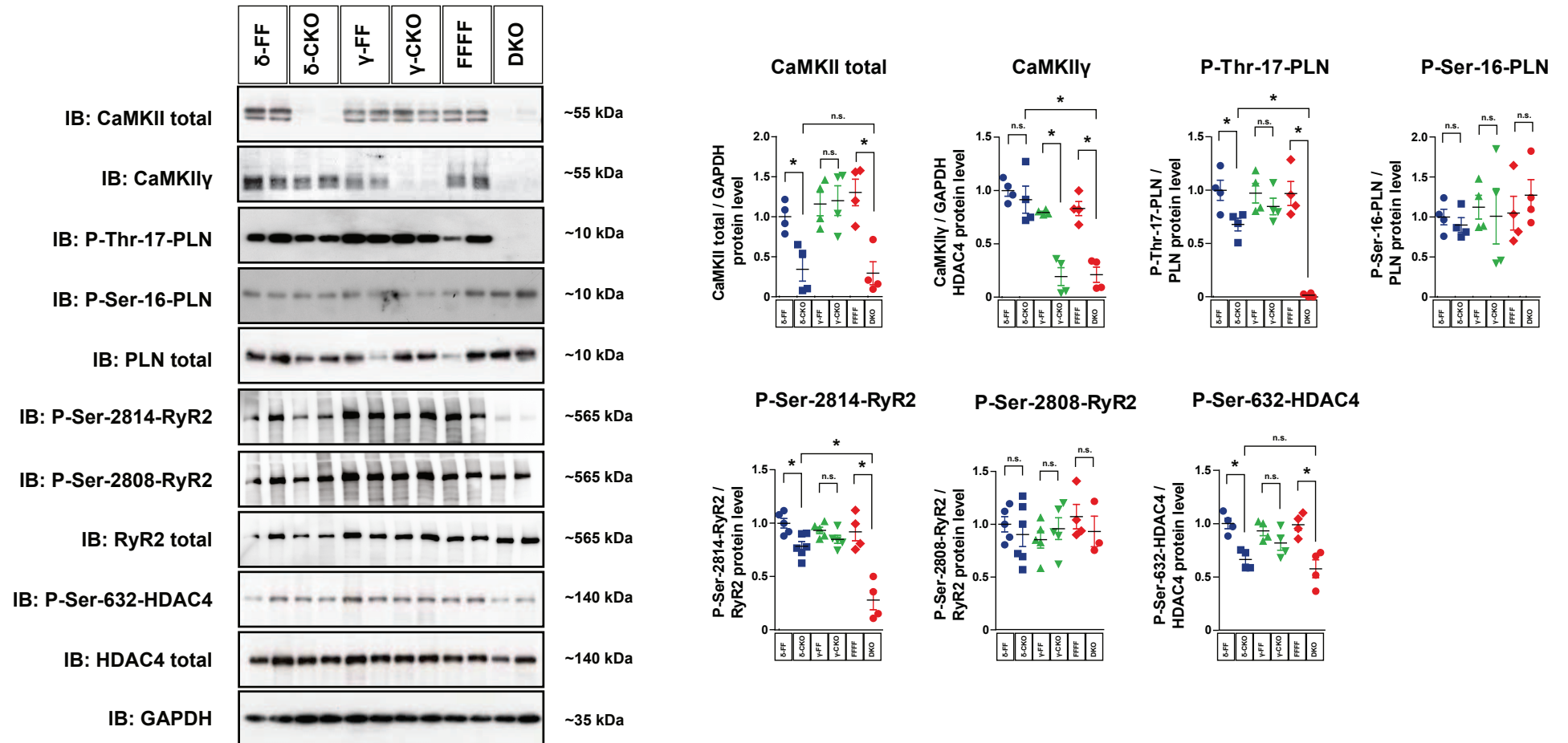
Fig. 6. Schematic model. Stimulation of β -adrenergic receptors by norepinephrine (NE) leads to an activation of the two Ca^{2+} -dependent pathways calcineurin and CaMKII (mediated by the isoforms CaMKII δ and CaMKII γ). Activated CaMKII phosphorylates HDAC4, which leads to a translocation of HDAC4 from the nucleus to the cytosol. Thereby, the repressive effect of HDAC4 on transcription factors as MEF2 is interrupted and a maladaptive remodeling gene program is initiated. As a second mechanism, CaMKII phosphorylates CnA, which leads to repression of the calcineurin-NFAT pathway and thereby acts anti-hypertrophic. Unexpectedly, the endogenous calcineurin-NFAT driven pro-hypertrophic pathway is not maladaptive.

A**B**

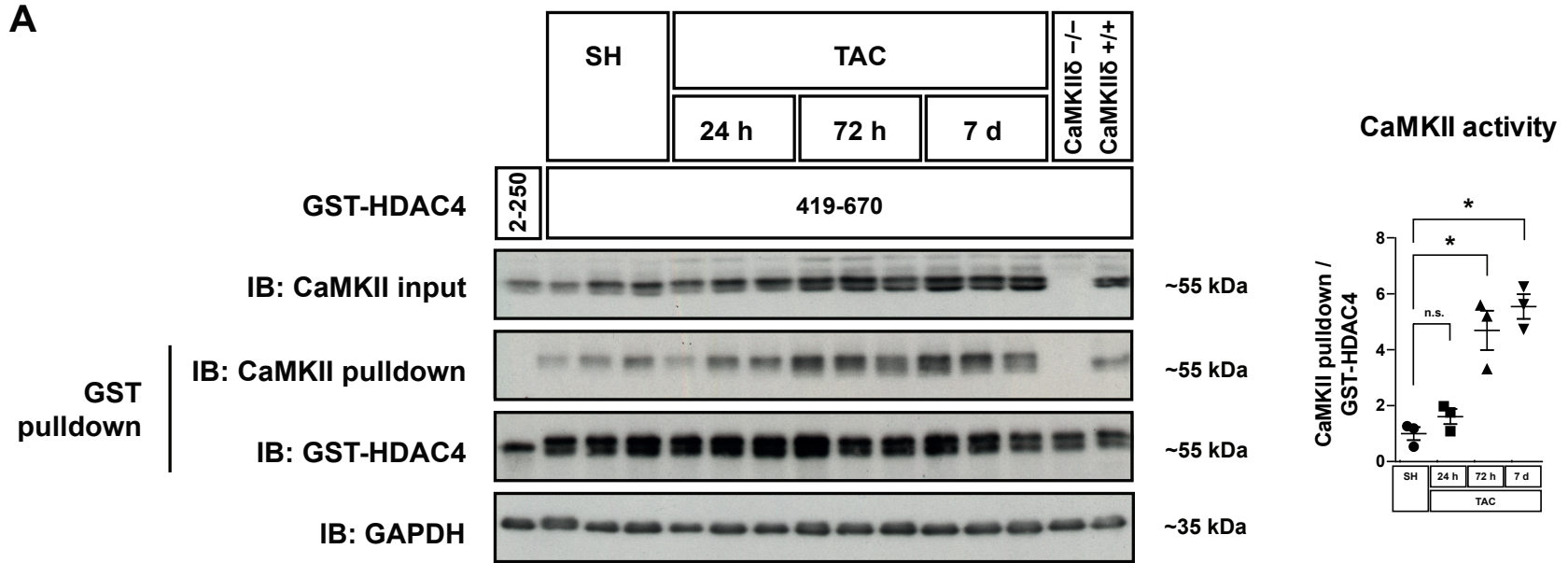
CaMKII knockout mice:

 δ -CKO (CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$; α MHC-Cre) γ -CKO (CaMKII $\gamma^{\text{loxP-exon1/2-loxP}}$; α MHC-Cre)DKO (CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$; CaMKII $\gamma^{\text{loxP-exon1/2-loxP}}$; α MHC-Cre)

Control Littermates:

 δ -FF (CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$) γ -FF (CaMKII $\gamma^{\text{loxP-exon1/2-loxP}}$)FFFF (CaMKII $\delta^{\text{loxP-exon1/2-loxP}}$)**C****Fig. 1**

A



B

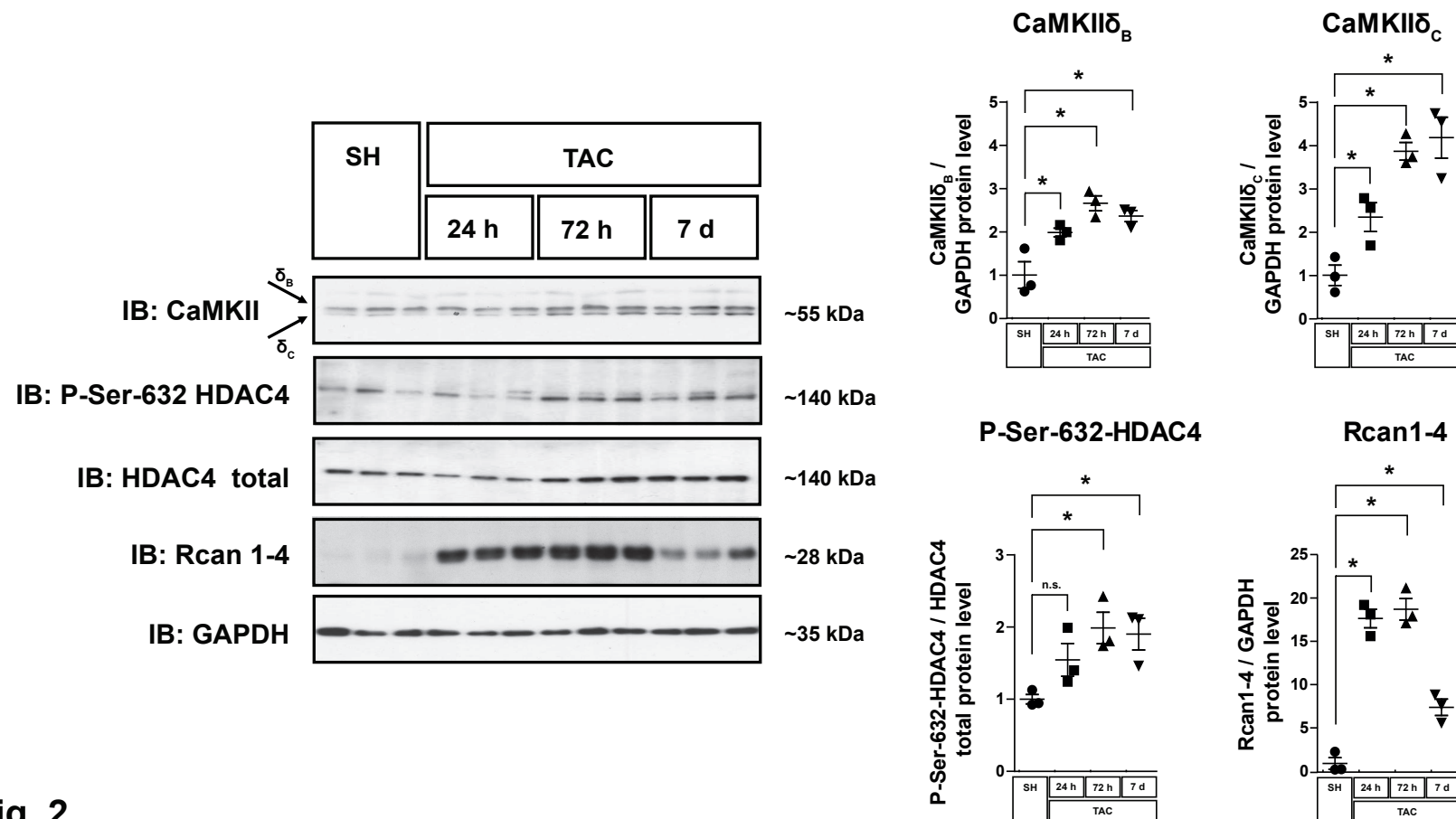
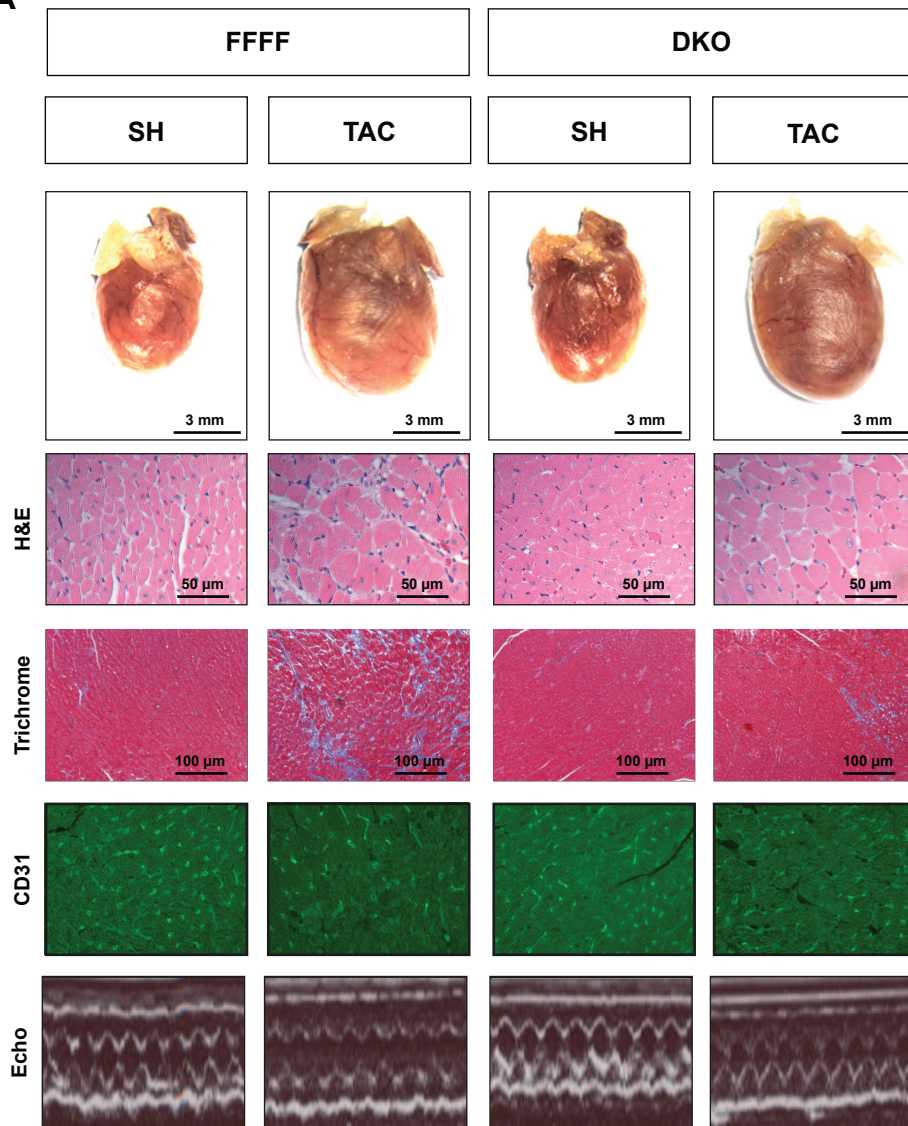
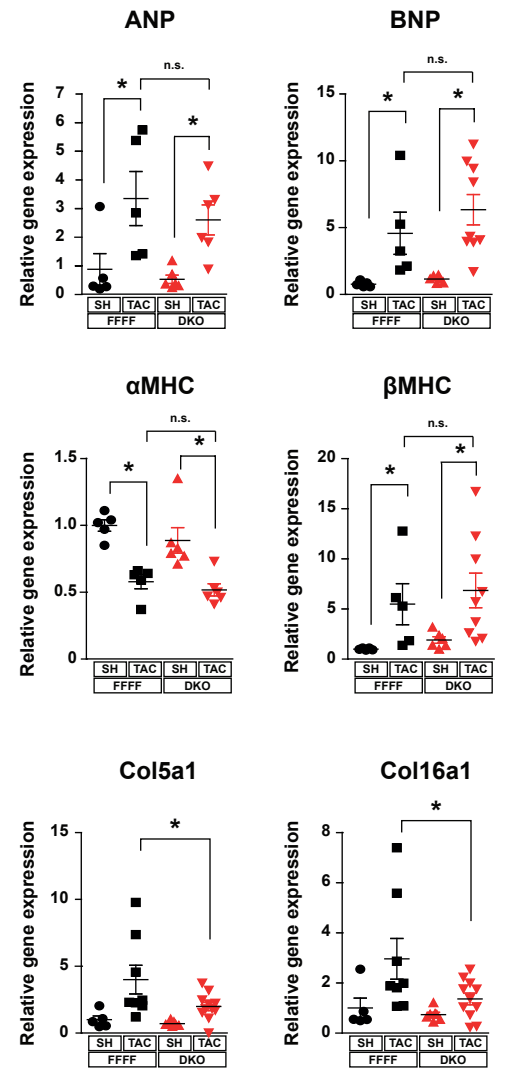


Fig. 2

A



B



C

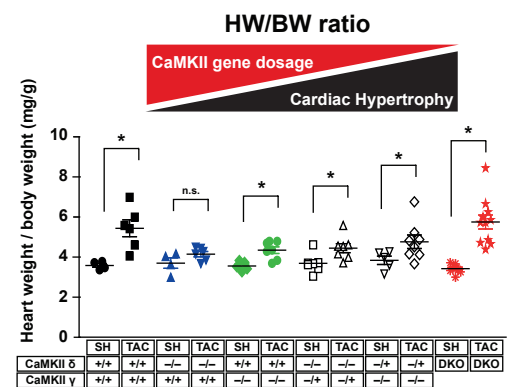


Fig. 3

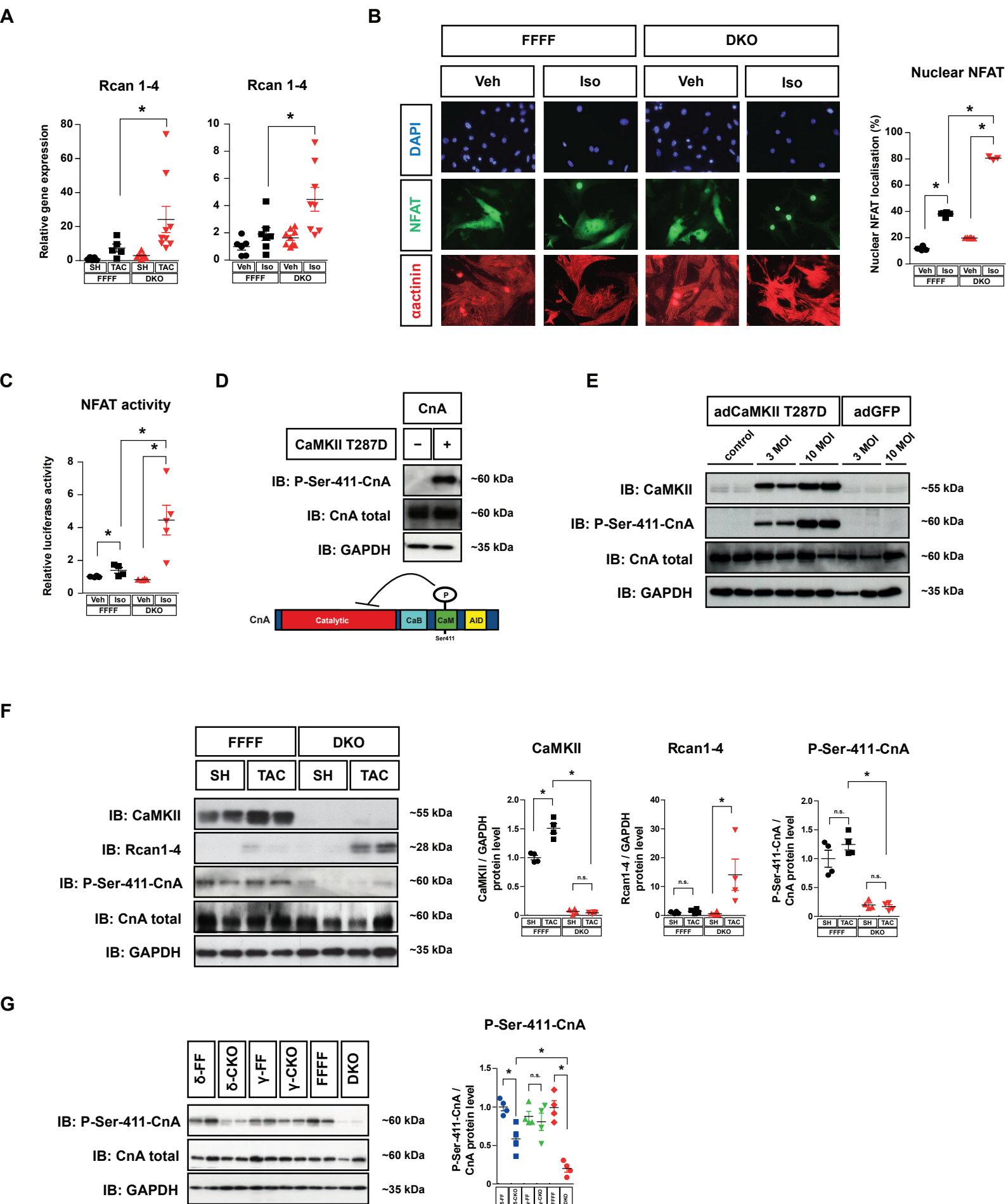


Fig. 4

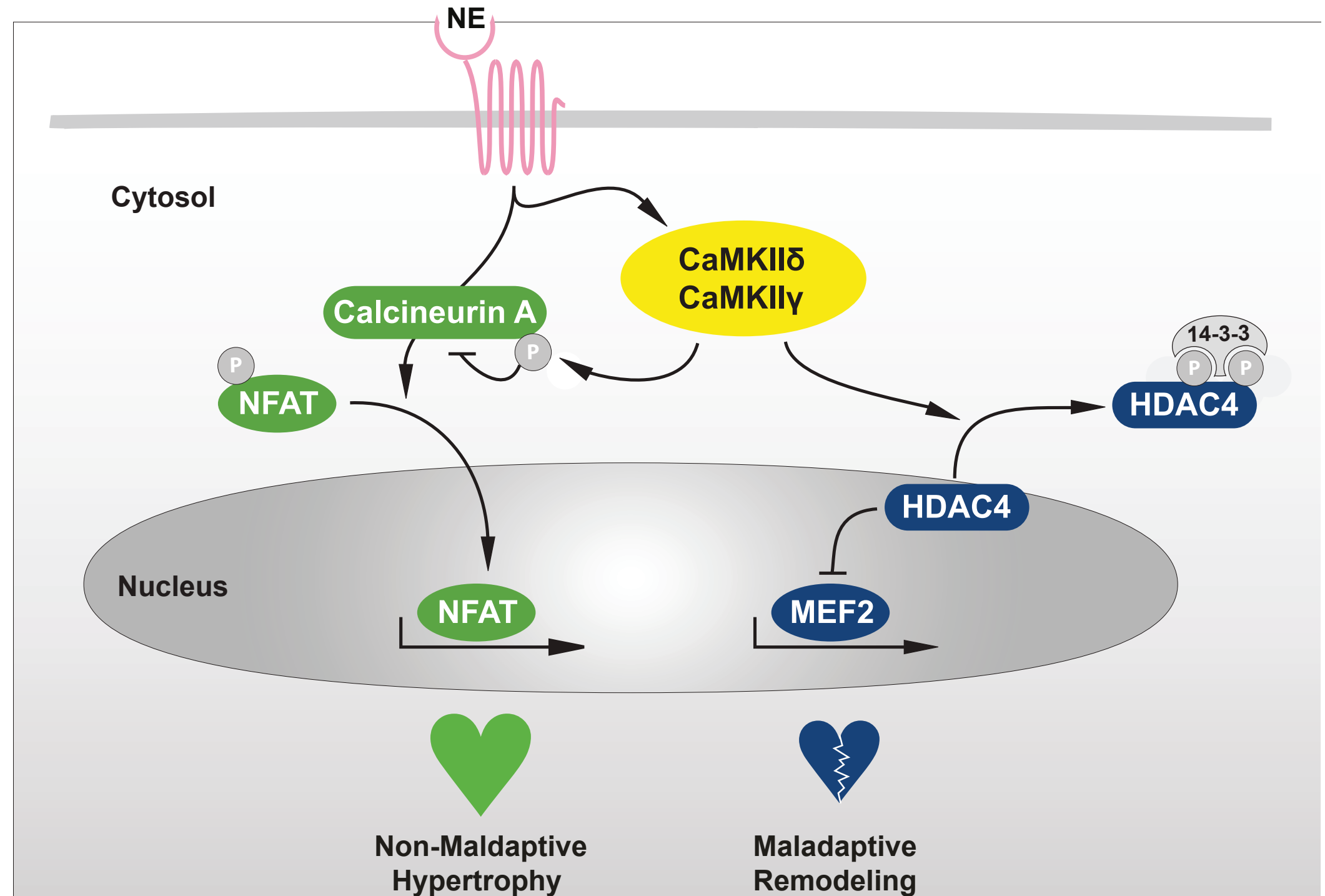


Fig. 6

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 (α MHC-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 α MHC-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-CaMKII γ (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'-cccgaccagcatgg-3' and antisense 5'-caactgcttctgaaaggggtg-3'; For mouse: *CaMKII α* sense 5'-gctgccaagattatcaacacc-3' and antisense 5'-cacgctccagctctggt-3'; *CaMKII β* sense 5'-gccatcctcaccactatgct-3' and antisense 5'-ctccatctgcttctgttgagt-3';

CaMKII γ sense 5'-agttcacagggacctgaagc-3' and antisense 5'-cgccttgaacttctatggcta-3'; *CaMKII δ* sense 5'-gtgccatcctcacaacccat-3' and antisense 5'-catctgacttctgttcaataggc-3'; *GAPDH* sense 5'-gggttcctataaatacggactgc-3' and antisense 5'-ccattttgtctacgggacga-3'; *Col5a1* sense 5'-ctacatccgtgccctggt-3' and antisense 5'-ccagcaccgtcttctggtag-3'; *Col16a1* sense 5'-gcattgcaggagaaaaatggt-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'; *β MHC* sense 5'-cgcatcaaggagctcacc-3' and antisense 5'-ctgcagccgcagtaggt; *α MHC* sense 5'-cgcatcaaggagctcacc-3' and antisense 5'-cctgcagccgcattaagt-3'; *Rcan1-4* sense 5'-actggaaggtggtgtccttgt-3' and antisense 5'-tccagctgggcttgactgag-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 μ l 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 penicillin-streptomycin. 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 μ M) 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 μ M) for another 24 h.

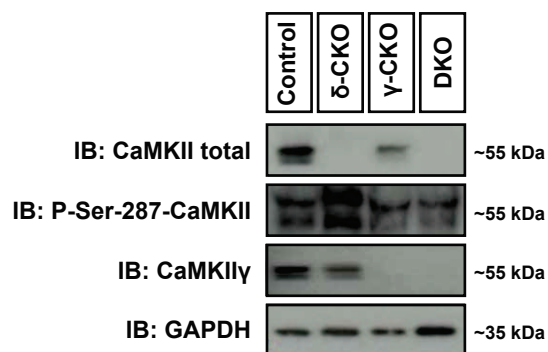
Immunocytochemistry. NMVMs or AMVMs were fixed in 4 % paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized with 0.3 % Triton X-100 (Sigma-Aldrich) for 10 min at room temperature and blocked for 30-60 min with 5 % goat serum (PAA) or BSA in PBS. Primary antibody for α -actinin (Sigma) was applied in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

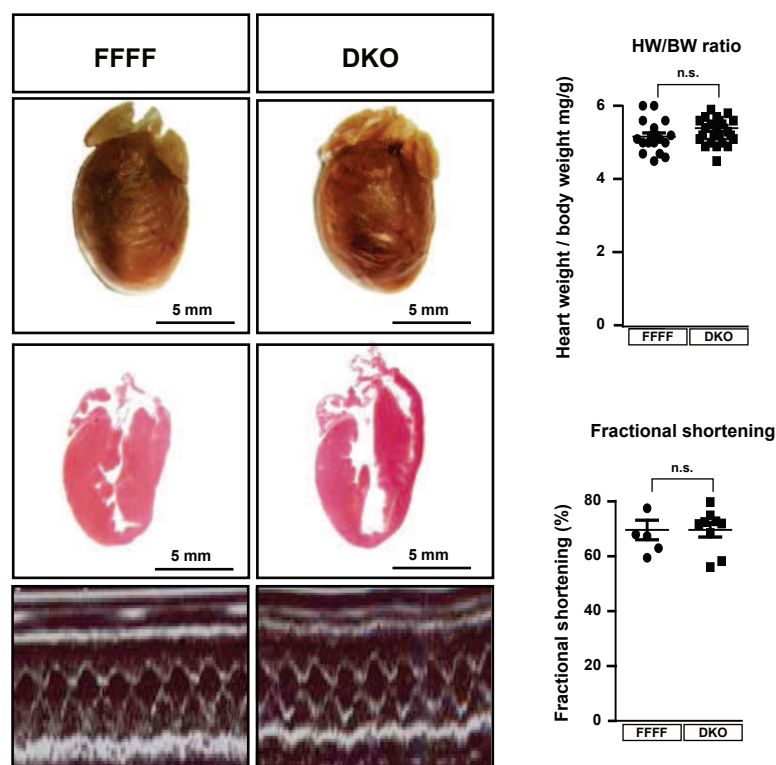
Epifluorescence microscopy. A Nikon Eclipse TE2000-U inverted microscope which was provided with an IonOptix fluorescence detection system for assessment of Ca^{2+} handling properties was used (IonOptix). For the studies on unstressed mice (was performed in the Maier lab), AMVMs were loaded with Fura-2AM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

SUPPLEMENTAL FIGURES

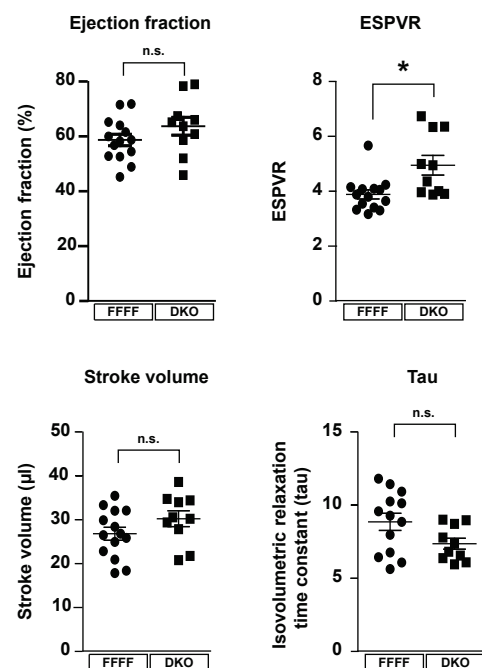
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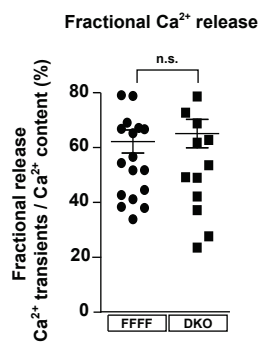
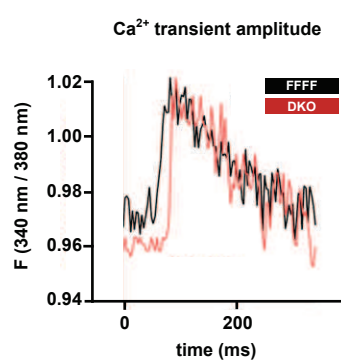
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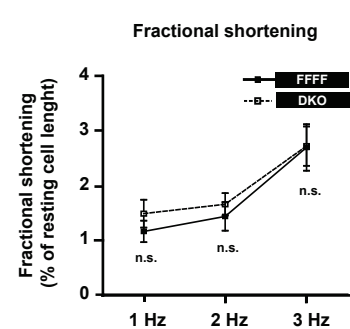
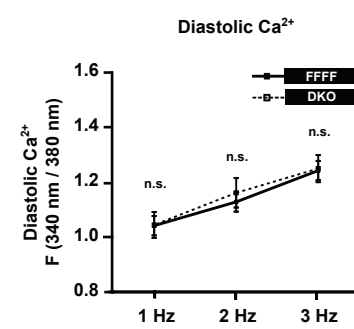
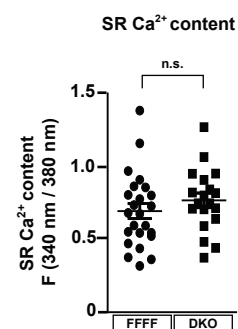
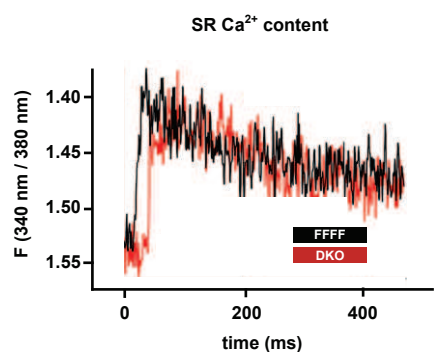
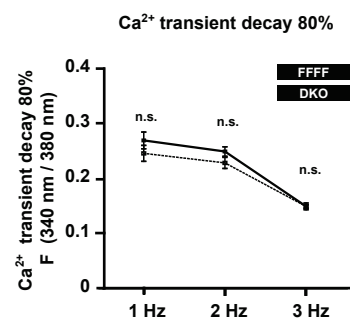
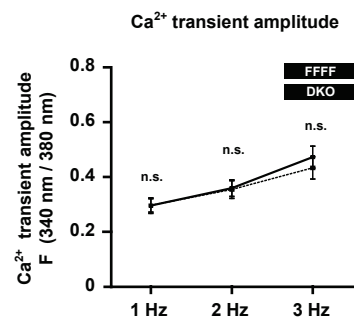


Figure I

Fig. 1. Baseline characterization of DKO mice. **(A)** Western blot analysis of total CaMKII, CaMKII γ , 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 $\pm$ SEM (n $\geq$ 18). **(C)** Hemodynamic measurements in isolated Langendorff-perfused hearts (n $\geq$ 10). Ejection fraction, end systolic pressure volume relations (ESPVR), stroke volume and isovolumetric relaxation time constant (τ) are shown. **(D)** Representative single cell measurements for Ca²⁺ transient amplitude, fractional Ca²⁺ release and sarcoplasmic reticulum (SR) Ca²⁺ content in isolated ventricular cardiomyocytes from DKO and FFFF mice, loaded with fluo-4. **(E)** Ca²⁺ transient decay after field stimulation with 1, 2 and 3 Hz. Measurements for intracellular diastolic Ca²⁺ and fractional shortening (FS) given as % of resting cell length after stimulation with 1, 2 and 3 Hz. All measurements were conducted in n $\geq$ 30 single cardiomyocytes from n $\geq$ 3 mice. Values are reported as mean $\pm$ SEM. *p<0.05. n.s., not significant.

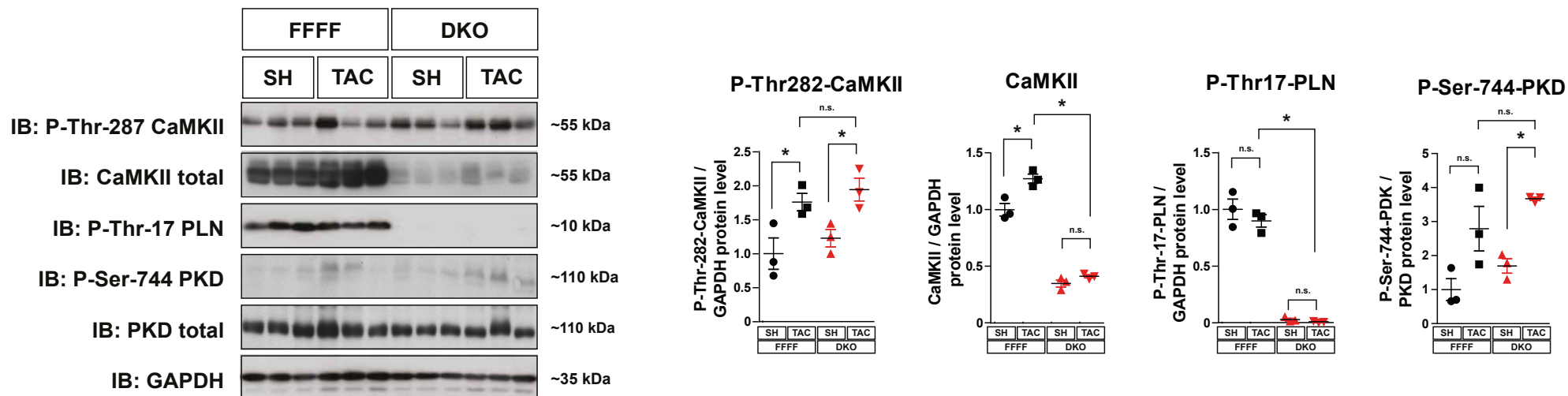
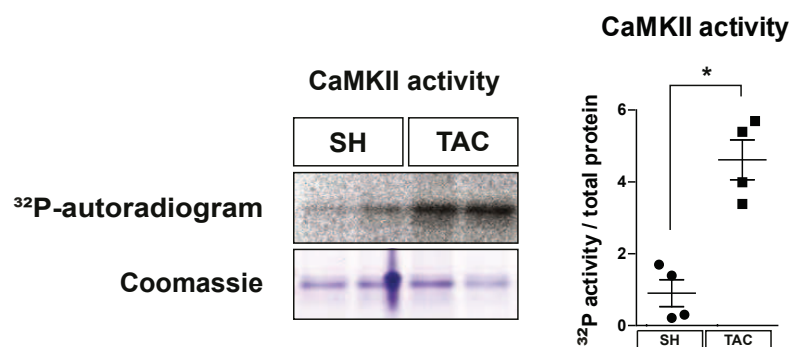
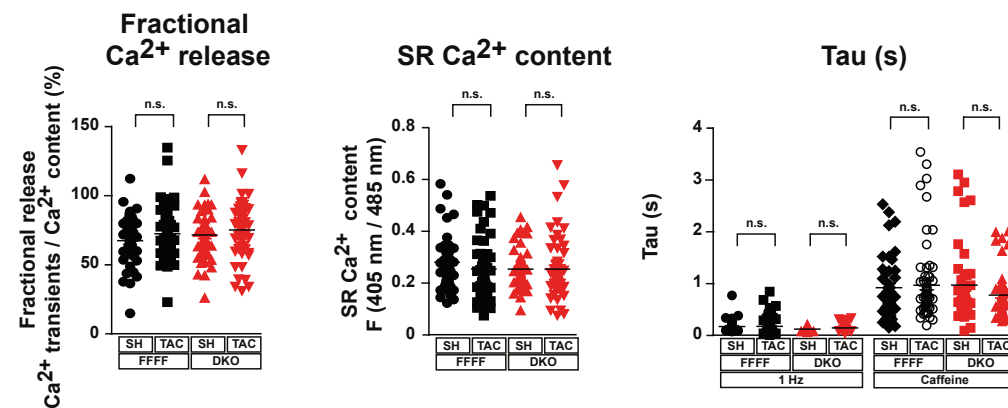
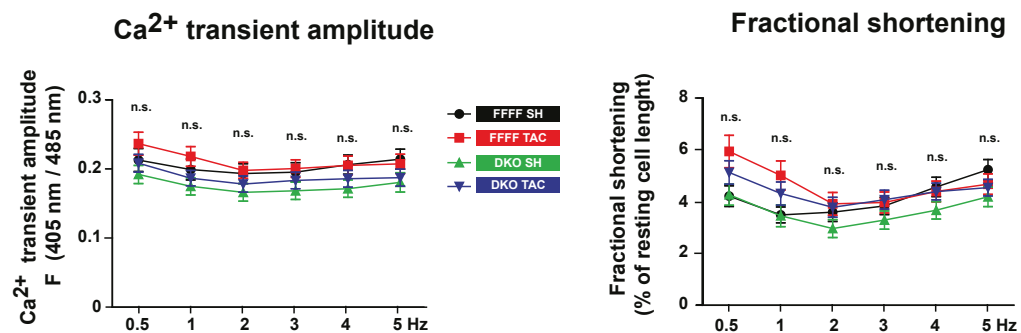
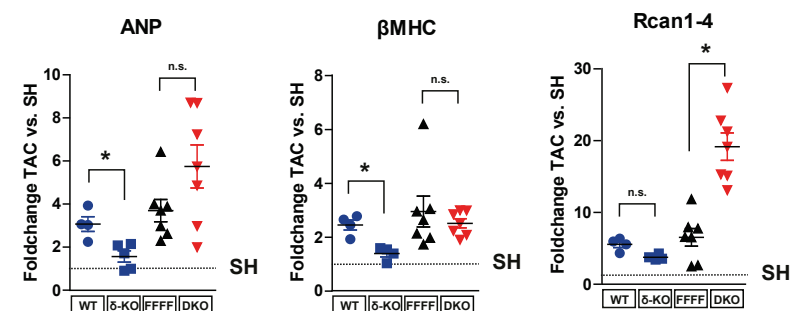
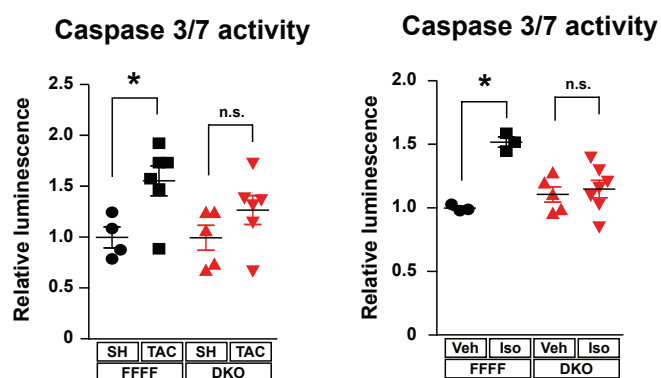
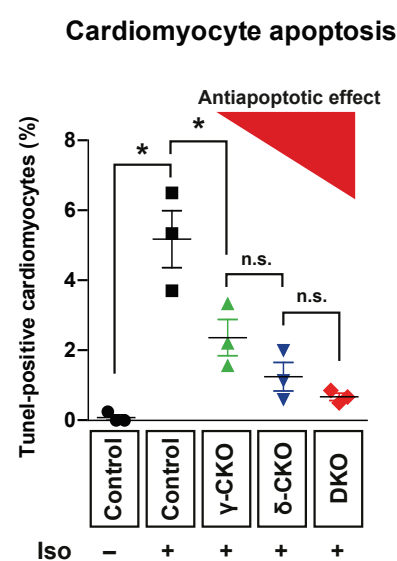
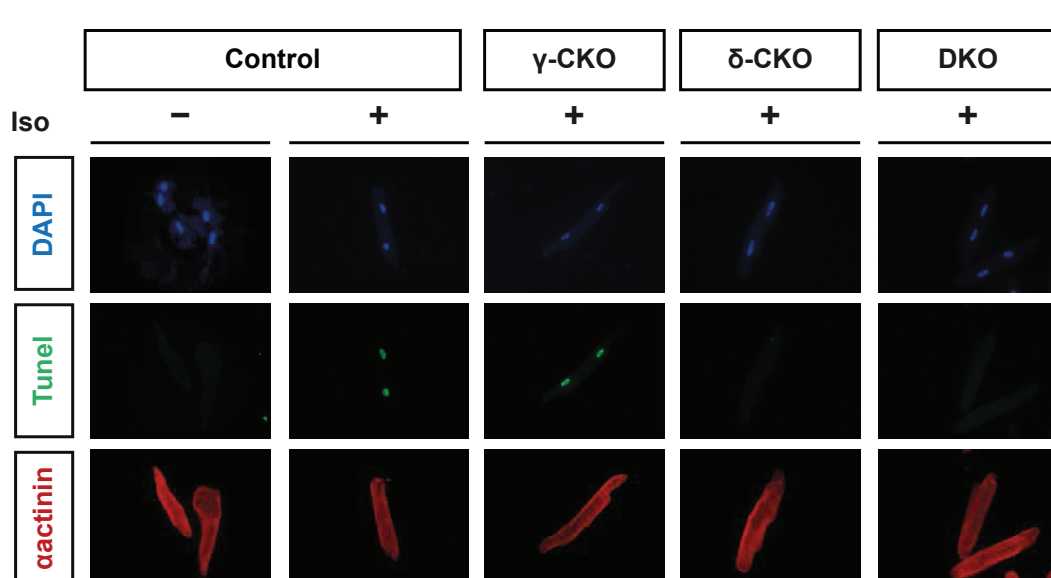
A**B****C****D****E****Figure II**

Fig. II. Ca²⁺-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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ release and averaged sarcoplasmatic (SR) Ca²⁺ content were estimated from the caffeine-induced peak in Ca²⁺ transients. Time constant (tau) of intracellular Ca²⁺ 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.

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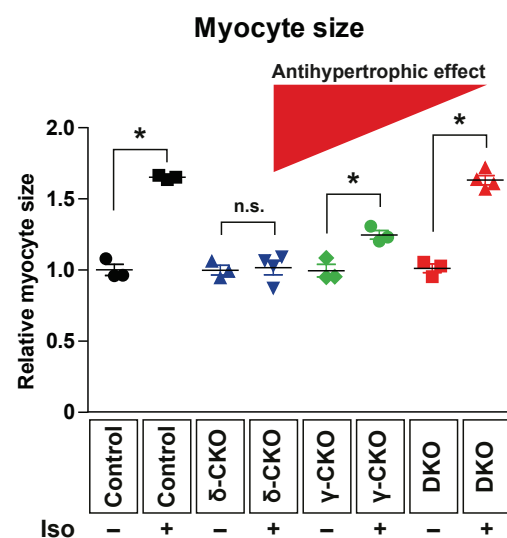
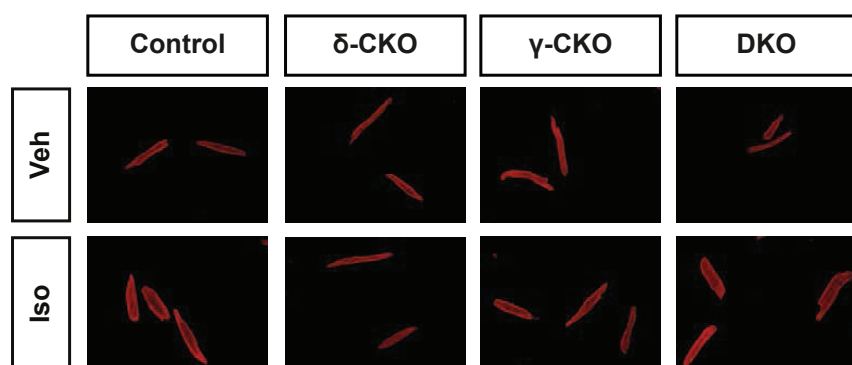
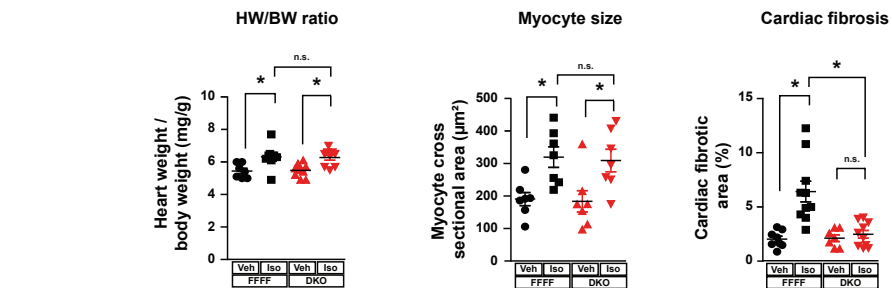
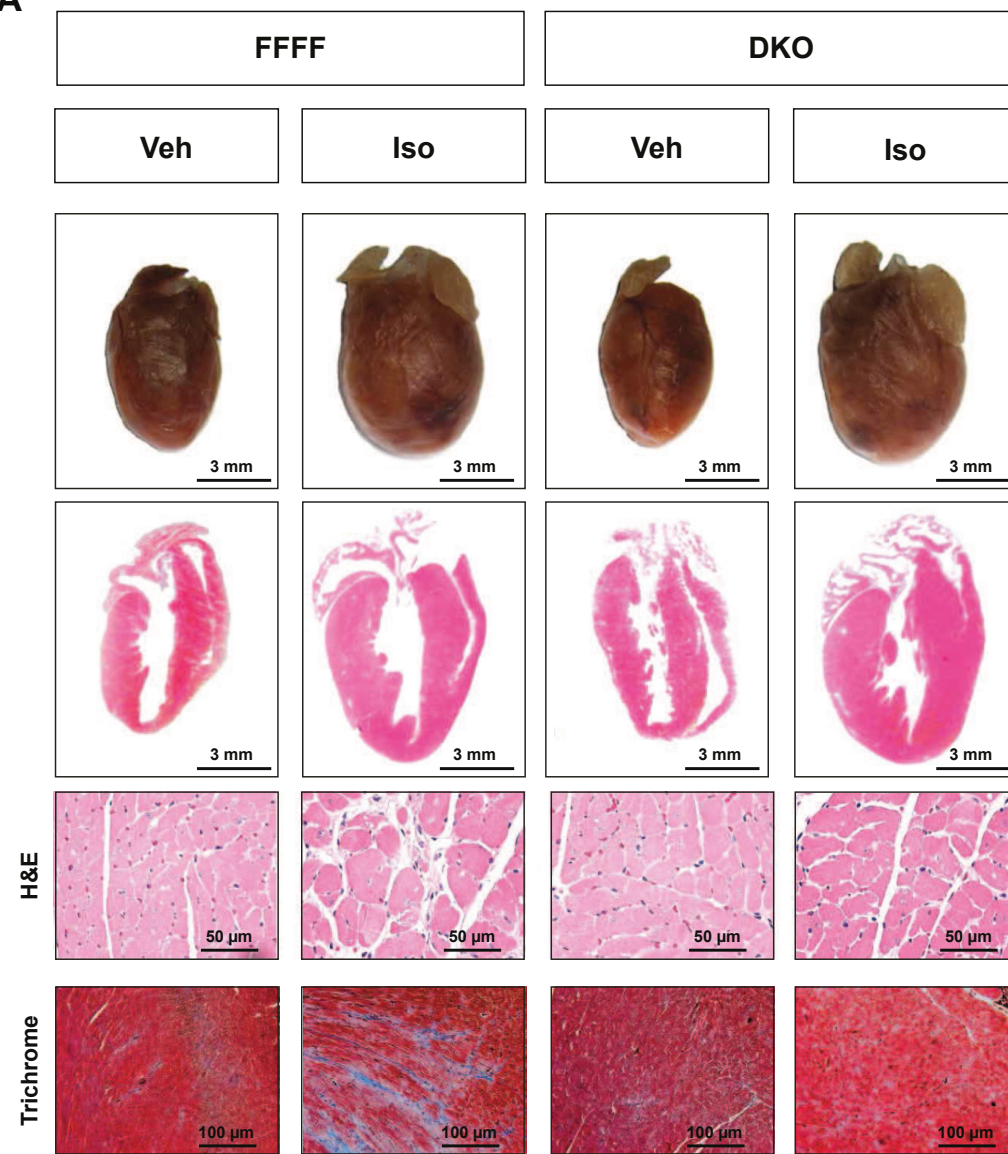


Figure III

Fig. III. Redundant roles for CaMKII δ 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 $\geq$ 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 $\pm$ SEM. *p<0.05. n.s., not significant.

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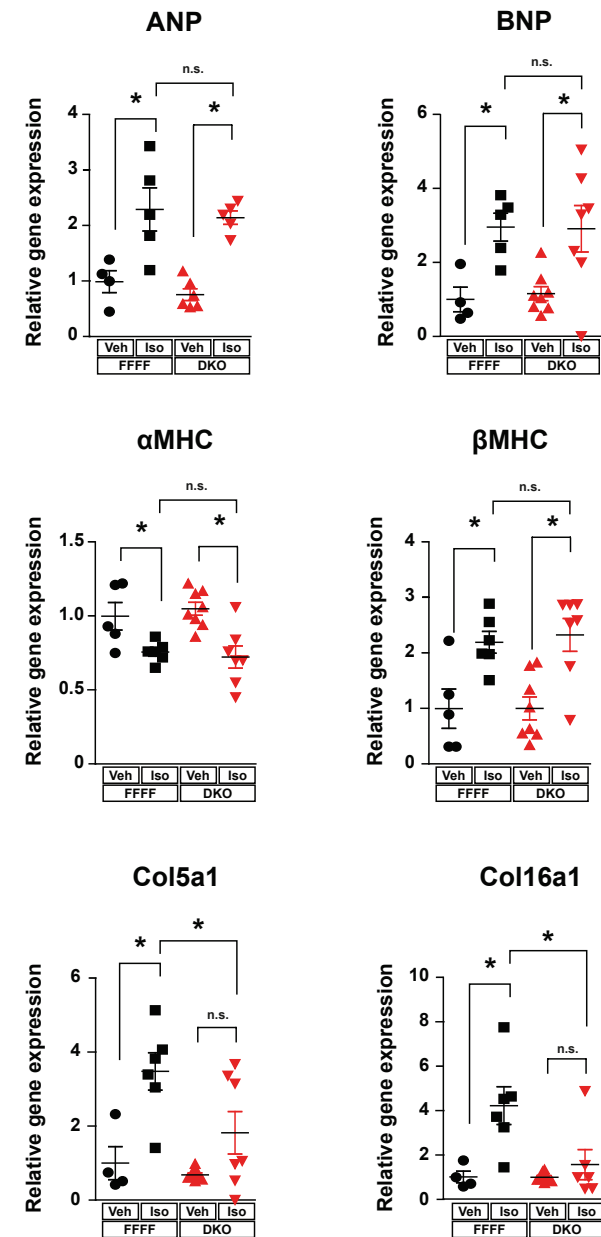
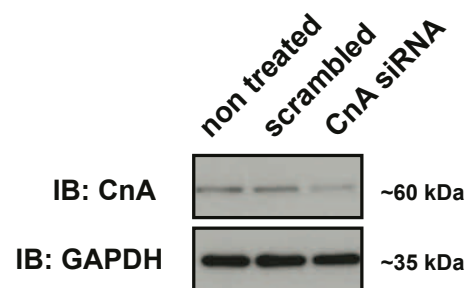


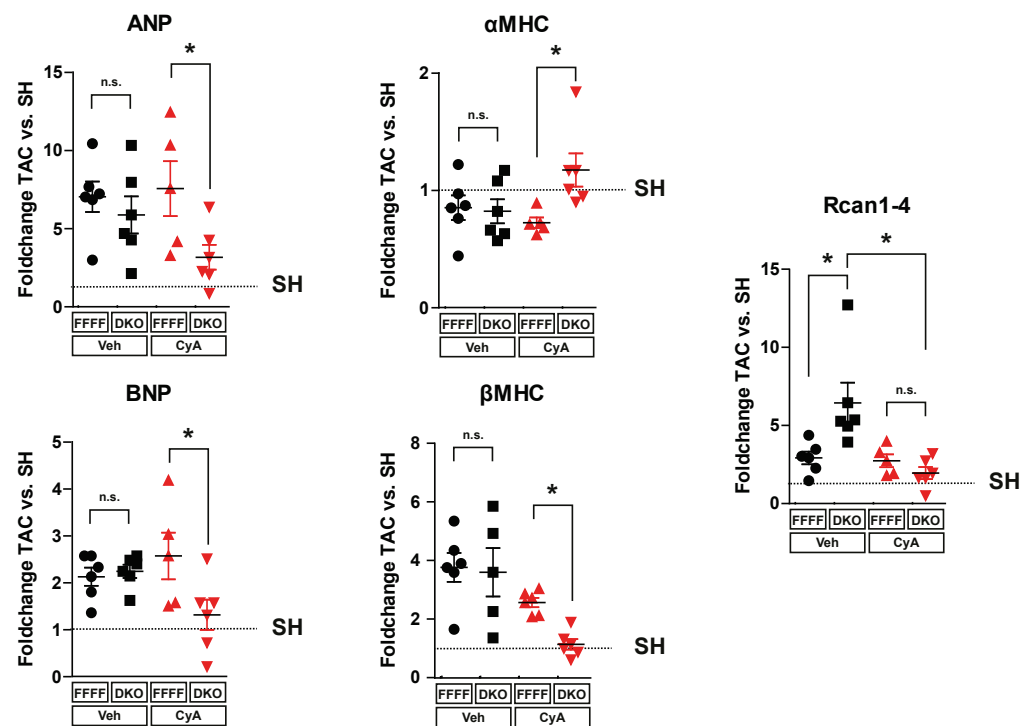
Figure IV

Fig. IV. 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 ($n \geq 4$ per group). All values are presented as mean $\pm$ SEM. * $p < 0.05$. n.s., not significant.

A



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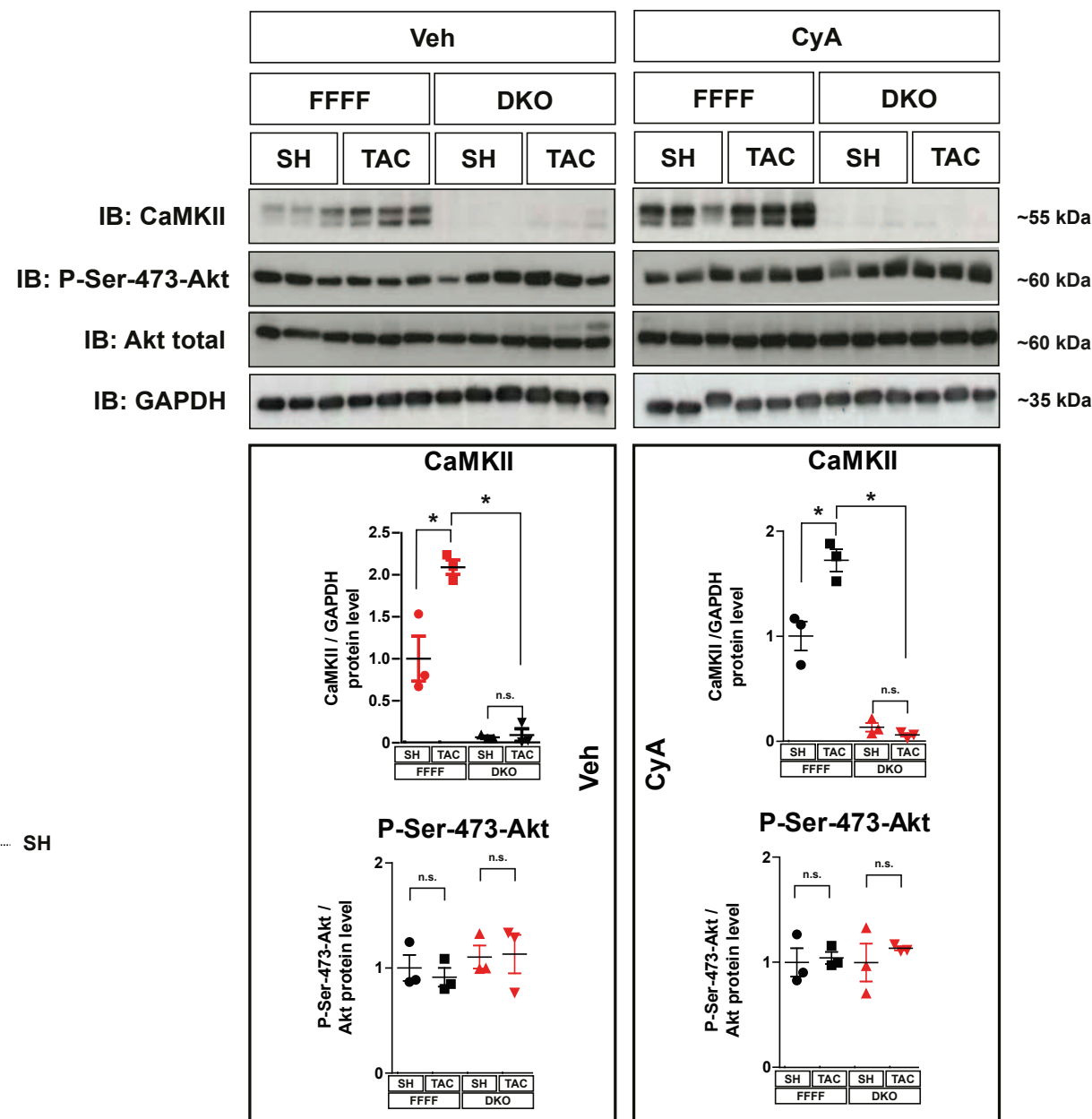


Figure V

Fig. V. 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.

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