

# Ca<sup>2+</sup>/calmodulin-dependent kinase II $\delta$ C-induced chronic heart failure does not depend on sarcoplasmic reticulum Ca<sup>2+</sup> leak

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## Abstract

**Aims** Hyperactivity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) has emerged as a central cause of pathologic remodelling in heart failure. It has been suggested that CaMKII-induced hyperphosphorylation of the ryanodine receptor 2 (RyR2) and consequently increased diastolic Ca<sup>2+</sup> leak from the sarcoplasmic reticulum (SR) is a crucial mechanism by which increased CaMKII activity leads to contractile dysfunction. We aim to evaluate the relevance of CaMKII-dependent RyR2 phosphorylation for CaMKII-induced heart failure development *in vivo*.

**Methods and results** We crossbred CaMKII $\delta$ C overexpressing [transgenic (TG)] mice with RyR2-S2814A knock-in mice that are resistant to CaMKII-dependent RyR2 phosphorylation. Ca<sup>2+</sup>-spark measurements on isolated ventricular myocytes confirmed the severe diastolic SR Ca<sup>2+</sup> leak previously reported in CaMKII $\delta$ C TG [4.65 ± 0.73 mF/F<sub>0</sub> vs. 1.88 ± 0.30 mF/F<sub>0</sub> in wild type (WT)]. Crossing in the S2814A mutation completely prevented SR Ca<sup>2+</sup>-leak induction in the CaMKII $\delta$ C TG, both regarding Ca<sup>2+</sup>-spark size and frequency, demonstrating that the CaMKII $\delta$ C-induced SR Ca<sup>2+</sup> leak entirely depends on the CaMKII-specific RyR2-S2814 phosphorylation. Yet, the RyR2-S2814A mutation did not affect the massive contractile dysfunction (ejection fraction = 12.17 ± 2.05% vs. 45.15 ± 3.46% in WT), cardiac hypertrophy (heart weight/tibia length = 24.84 ± 3.00 vs. 9.81 ± 0.50 mg/mm in WT), or severe premature mortality (median survival of 12 weeks) associated with cardiac CaMKII $\delta$ C overexpression. In the face of a prevented SR Ca<sup>2+</sup> leak, the phosphorylation status of other critical CaMKII downstream targets that can drive heart failure, including transcriptional regulator histone deacetylase 4, as well as markers of pathological gene expression including *Xirp2*, *Il6*, and *Col1a1*, was equally increased in hearts from CaMKII $\delta$ C TG on a RyR2 WT and S2814A background.

**Conclusions** S2814 phosphoresistance of RyR2 prevents the CaMKII-dependent SR Ca<sup>2+</sup> leak induction but does not prevent the cardiomyopathic phenotype caused by enhanced CaMKII $\delta$ C activity. Our data indicate that additional mechanisms—dependent of SR Ca<sup>2+</sup> leak—are critical for the maladaptive effects of chronically increased CaMKII $\delta$ C activity with respect to heart failure.

**Keywords** Ca<sup>2+</sup>/calmodulin-dependent kinase II; Ryanodine receptor 2; SR Ca<sup>2+</sup> leak; Heart failure

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## Introduction

The multifunctional serine/threonine kinase Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) has emerged as one of the most promising molecular targets for new pharmacological approaches in the therapy of heart failure (HF). CaMKII is overactive in experimental as well as human HF,<sup>1,2</sup> and growing evidence indicates its pivotal role in the pathophysiology of cardiac diseases.<sup>3,4</sup> The fundamental underlying CaMKII-dependent mechanisms with regard to HF pathophysiology, however, remain controversial, as CaMKII exhibits a broad impact on cardiomyocyte electrophysiology, excitation contraction coupling, inflammatory processes, apoptotic signalling, hypertrophic responses, and chronic remodelling processes.<sup>5,6</sup> In cardiomyocytes, CaMKII $\delta$  is the predominantly expressed CaMKII isoform, with three major splice variants identified in the heart. Splice variant CaMKII $\delta$ C localizes largely within the cytosol and is associated with the sarcoplasmic reticulum (SR) membrane and the nuclear envelope, whereas splice variant CaMKII $\delta$ B, which has a nuclear localization signal, is concentrated in the nucleus.<sup>7,8</sup> More recently, CaMKII $\delta$ 9 has been identified as a third substantially expressed splice variant, and like CaMKII $\delta$ C, it shows a cytosolic distribution pattern.<sup>9</sup> Transgenic (TG) mouse models revealed that overexpression of all three splice variants in cardiomyocytes causes cardiac hypertrophy and dilated cardiomyopathy in mice, which occurs more severely and is more rapidly progressing in the CaMKII $\delta$ C and CaMKII $\delta$ 9 TG.<sup>2,9,10</sup>

Whereas CaMKII $\delta$  splice variants CaMKII $\delta$ B and CaMKII $\delta$ C both regulate gene transcription, and overexpression of CaMKII $\delta$ 9 induces cardiomyocyte DNA damage (via phosphorylation of ubiquitin-conjugating enzyme E2T), CaMKII $\delta$ C was identified as the major regulator of SR Ca<sup>2+</sup> cycling.<sup>11,12</sup> Previous data consistently demonstrate that increased activity of CaMKII enhances ryanodine receptor 2 (RyR2) phosphorylation at S2814, which increases diastolic Ca<sup>2+</sup> leak from the SR.<sup>13–15</sup> Besides the pivotal role of SR Ca<sup>2+</sup> leakage for cardiac arrhythmia, including atrial fibrillation,<sup>16</sup> it has been hypothesized that this mechanism is also causative for the cardiomyopathic phenotype in CaMKII $\delta$ C TG mice (in which cardiac CaMKII activity and RyR2-S2814 phosphorylation are increased to similar levels as in human HF<sup>2,17,18</sup>) and for contractile dysfunction in HF of other origin.<sup>4,19–22</sup>

## Aims

In this proof-of-concept study, we set out to test the presumed central role of CaMKII-dependent RyR2-S2814 hyperphosphorylation and associated SR Ca<sup>2+</sup> leak for the development of CaMKII-dependent HF *in vivo*.

## Methods

### Mice

For generation of mice, see [2] and [23]. CaMKII $\delta$ C TG mice on a Black Swiss background were crossed to RyR2-S2814A knock-in (KI) mice on a C57/BL6N background. For all experiments, wild-type (WT), RyR2-S2814A KI, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A KI mice of the F2 generation were used. Groups were age-, sex-matched, and on the same background for all experiments. All animal experiments were performed according to the European Community guiding principles in the care and use of animals (2010/63/UE) and authorized by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Germany) or Regierung von Unterfranken (Veterinärwesen, Verbraucherschutz). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

### Echocardiography

Echocardiography in mice was performed under light isoflurane anaesthesia (1.5 vol% in 1 L O<sub>2</sub>/min) using a VisualSonics Vevo 2100 imaging set-up as previously described.<sup>24</sup>

### Biometry

Mice were euthanized at the age of 7–8 weeks. Hearts and lungs were quickly excised and weighed. Heart weight and lung weight were normalized to tibia length.

### Isolation of cardiomyocytes

The isolation of cardiomyocytes was performed as previously described.<sup>25</sup>

### Ca<sup>2+</sup>-spark measurements

Ca<sup>2+</sup> sparks were measured in cardiomyocytes loaded with Fluo-4AM (10  $\mu$ mol/L) with a laser (488 nm) scanning confocal microscope (LSM 5 Pascal, Zeiss, Germany) under superfusion with normal Tyrode's solution. Ca<sup>2+</sup> sparks were detected and quantified using the ImageJ plugin Sparkmaster,<sup>26</sup> with visual confirmation of sparks detected. Ca<sup>2+</sup>-spark frequency (CaSpF) was calculated from this and normalized to the scanned myocyte width and scanning interval. Ca<sup>2+</sup>-spark size (CaSpS; calculated as amplitude \* full width \* full duration) was added for all sparks within a cell

to calculate the SR Ca<sup>2+</sup> leak for this cell. The overall diastolic SR Ca<sup>2+</sup>-leak calculation was done both excluding and including non-sparking cells.

### Measurement of sarcoplasmic reticulum Ca<sup>2+</sup> load

Cardiomyocytes were loaded with Fura-2AM (10  $\mu$ mol/L) and were alternately excited at 340 and 380 nm. The amplitude (Fura-2 F340/380 ratio) of the Ca<sup>2+</sup> transient induced by rapid application of caffeine (10 mmol/L) was used to assess the SR Ca<sup>2+</sup> load as previously described.<sup>22</sup>

### Immunoblot analysis

Left ventricular tissue was lysed in buffer containing 30 mmol/L Tris/HCl pH 8.8; 5 mmol/L EDTA; 30 mmol/L NaF; 3% SDS; and 10% glycerol + protease inhibitor cocktail + phosphatase inhibitor cocktail (Sigma-Aldrich). Protein samples were separated by SDS-PAGE and subsequently transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat dry milk in TBST for 1 h and afterward incubated with the primary antibodies overnight at 4°C (all 1:1000 in TBST). After incubation with appropriate secondary antibodies (1:5000 in TBST) for 1 h, chemiluminescence was detected using a Fusion FX (Vilber Lourmat) imaging system and quantified with Fusion-Capt™ (Vilber Lourmat) software. Details on the antibodies used are provided in the supporting information.

### RT-qPCR

Left ventricular tissue was dissolved in TRIzol™ reagent (Thermo Fisher), and RNA extraction was performed according to the manufacturer's instructions. cDNA was generated from 1  $\mu$ g of RNA using the primaREVERSE BASIC Reverse-Transcription Kit (Steinbrenner). RT-qPCR was performed with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) on a LightCycler 480 II system (Roche). The data were analysed using LinRegPCR Version 2018.0. Primer sequences are provided in the supporting information.

### Statistics

Statistical analyses were performed using ordinary one-way ANOVA, Brown-Forsythe and Welch ANOVA, or Kruskal–Wallis test as specified in the figure legends. Survival data were analysed by log-rank (Mantel–Cox) test. *P* values are presented within each figure.

An extended methods section can be found in the supporting information.

## Results

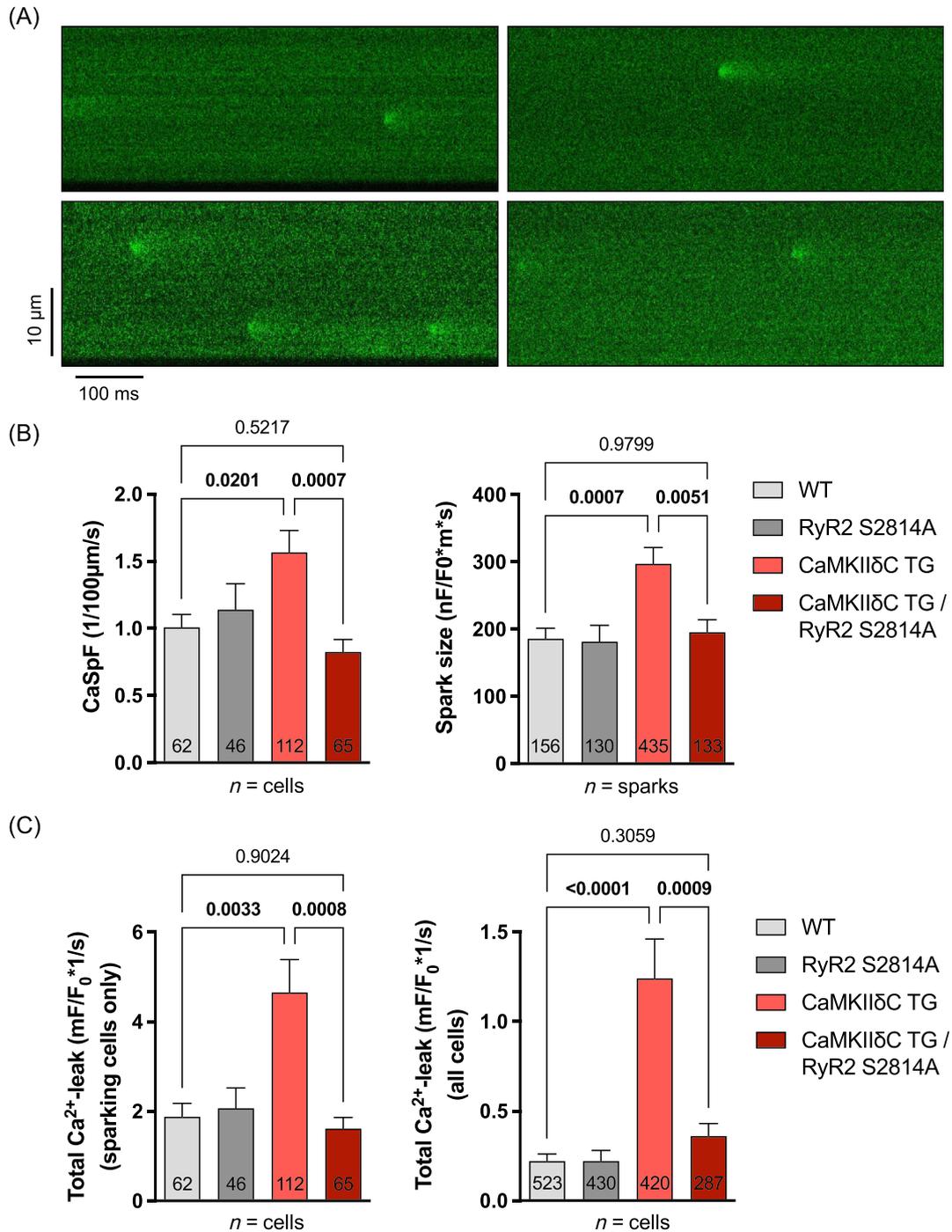
### The Ryanodine receptor 2-S2814A mutation prevents sarcoplasmic reticulum Ca<sup>2+</sup> leak in Ca<sup>2+</sup>/calmodulin-dependent kinase II $\delta$ C transgenic mice

The functional consequence of the phosphoresistant RyR2-S2814A mutation on CaMKII-induced SR Ca<sup>2+</sup> leak was investigated by measuring Ca<sup>2+</sup> sparks. As illustrated by the original line scans of Ca<sup>2+</sup> sparks shown in *Figure 1A*, ventricular cardiomyocytes from CaMKII $\delta$ C TG mice show a much higher frequency and size of individual Ca<sup>2+</sup> sparks (CaSpF, CaSpS) (*Figure 1B*), resulting in a massively increased SR Ca<sup>2+</sup> leak (*Figure 1C*), as previously reported.<sup>19</sup> In CaMKII-TG mice carrying the RyR2-S2814A mutation, however, this induction of SR Ca<sup>2+</sup> leak due to CaMKII overactivity was completely prevented both regarding CaSpF and CaSpS (*Figure 1B*), resulting in a total leak per cell that was unaltered compared with WT cells (*Figure 1C*). These data suggest that the SR Ca<sup>2+</sup> leak induced by CaMKII $\delta$ C overexpression entirely depends on CaMKII-specific RyR2 phosphorylation at the S2814 site. Noteworthy, SR Ca<sup>2+</sup> load was not different between CaMKII $\delta$ C TG vs. CaMKII $\delta$ C TG/RyR2-S2814A cardiomyocytes (Supporting Information, *Figure S3*).

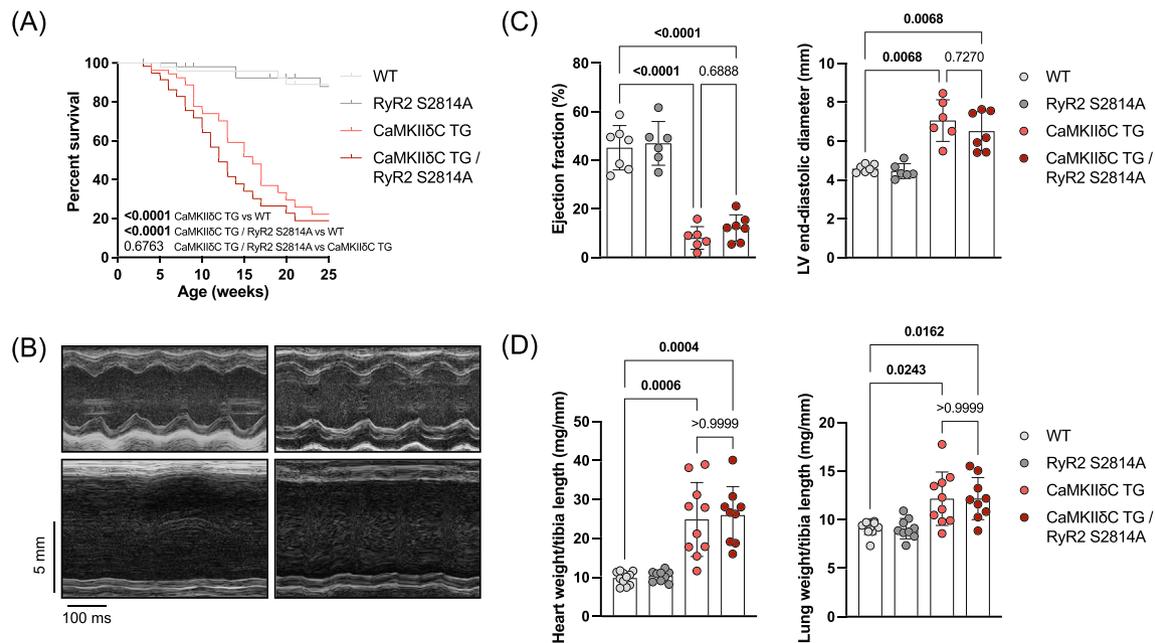
### The Ryanodine receptor 2-S2814A mutation does not prevent heart failure in Ca<sup>2+</sup>/calmodulin-dependent kinase II $\delta$ C transgenic mice

To determine the relevance of RyR2-S2814 phosphorylation for the CaMKII $\delta$ C-induced cardiomyopathic phenotype, we performed survival analysis, assessed cardiac function using echocardiography, and quantified cardiac hypertrophy and pulmonary congestion. Surprisingly, despite the beneficial effect on SR Ca<sup>2+</sup> leak, the RyR2-S2814A mutation did not improve mortality in the CaMKII $\delta$ C TG, which displayed premature death with a median survival of <20 weeks (*Figure 2A*). Echocardiographic analysis revealed that the decline of cardiac function in the CaMKII $\delta$ C TG was only slightly attenuated by RyR2-S2814 phosphoresistance at 7 weeks of age (Supporting Information, *Figure S1A,B*), and this effect diminished over time. At 12 weeks of age, the CaMKII $\delta$ C TG carrying the RyR2-S2814A had developed the same degree of severe contractile dysfunction and left ventricular dilation as the TG on a RyR2 WT background (*Figure 2B,C*). Similar findings were made with crosses of CaMKII $\delta$ C TG with -RyR2-S2814A mutant mice generated in the Heller Brown lab:

**Figure 1** Effect of the RyR2-S2814A mutation on SR  $\text{Ca}^{2+}$  leak in CaMKII $\delta$ C TG cardiomyocytes. (A) Original line scans of  $\text{Ca}^{2+}$  sparks in isolated ventricular cardiomyocytes from wild-type (WT), RyR2-S2814A knock-in, CaMKII $\delta$ C transgenic (TG) mice, and CaMKII $\delta$ C TG on a RyR2-S2814A knock-in background. (B)  $\text{Ca}^{2+}$ -spark frequency and  $\text{Ca}^{2+}$ -spark size, and (C) total SR  $\text{Ca}^{2+}$  leak calculated both excluding (left panel) and including (right panel) non-sparking cardiomyocytes from WT, RyR2-S2814A, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A mice. Cell numbers or spark numbers are indicated in the bar graphs. Data are presented as mean  $\pm$  SEM. *P* values were determined by Brown-Forsythe and Welch ANOVA with Games–Howell multiple comparisons test.



**Figure 2** Effect of the RyR2-S2814A mutation on heart failure development in CaMKII $\delta$ C TG mice. (A) Kaplan–Meier curves ( $n \geq 27$ ) from WT, RyR2-S2814A, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A mice. (B) Representative echocardiographic M-mode images; (C) cardiac ejection fraction and left ventricular end-diastolic diameter measured by echocardiography ( $n \geq 6$ ; 12 weeks); (D) heart weight and lung weight normalized to tibia length ( $n \geq 9$ ; 7–8 weeks) in WT, RyR2-S2814A, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A mice.  $P$  values were determined by log-rank (Mantel–Cox) test (survival data), ordinary one-way ANOVA with Sidak multiple comparisons test (ejection fraction), Brown–Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons test (LV end-diastolic diameter), and Kruskal–Wallis with Dunn’s multiple comparisons test (heart weight and lung weight).



modest rescue of contractile dysfunction in mice of  $<9$  weeks that was lost in mice older than 9 weeks of age (Supporting Information, Figure S2A,B). Moreover, RyR2-S2814A phosphoresistance had no effect on the CaMKII $\delta$ C TG-induced massive cardiac hypertrophy or on pulmonary congestion as a sign of left ventricular backward failure (Figure 2D). Thus, despite the prevention of SR  $\text{Ca}^{2+}$  leak, phosphoresistance of the RyR2 at the CaMKII-specific S2814 site did not prevent the progression to HF induced by CaMKII $\delta$ C overexpression.

### Phosphorylation of other Ca<sup>2+</sup>/calmodulin-dependent kinase II targets and maladaptive gene expression are not affected by the Ryanodine receptor 2-S2814A mutation in Ca<sup>2+</sup>/calmodulin-dependent kinase II $\delta$ C transgenic hearts

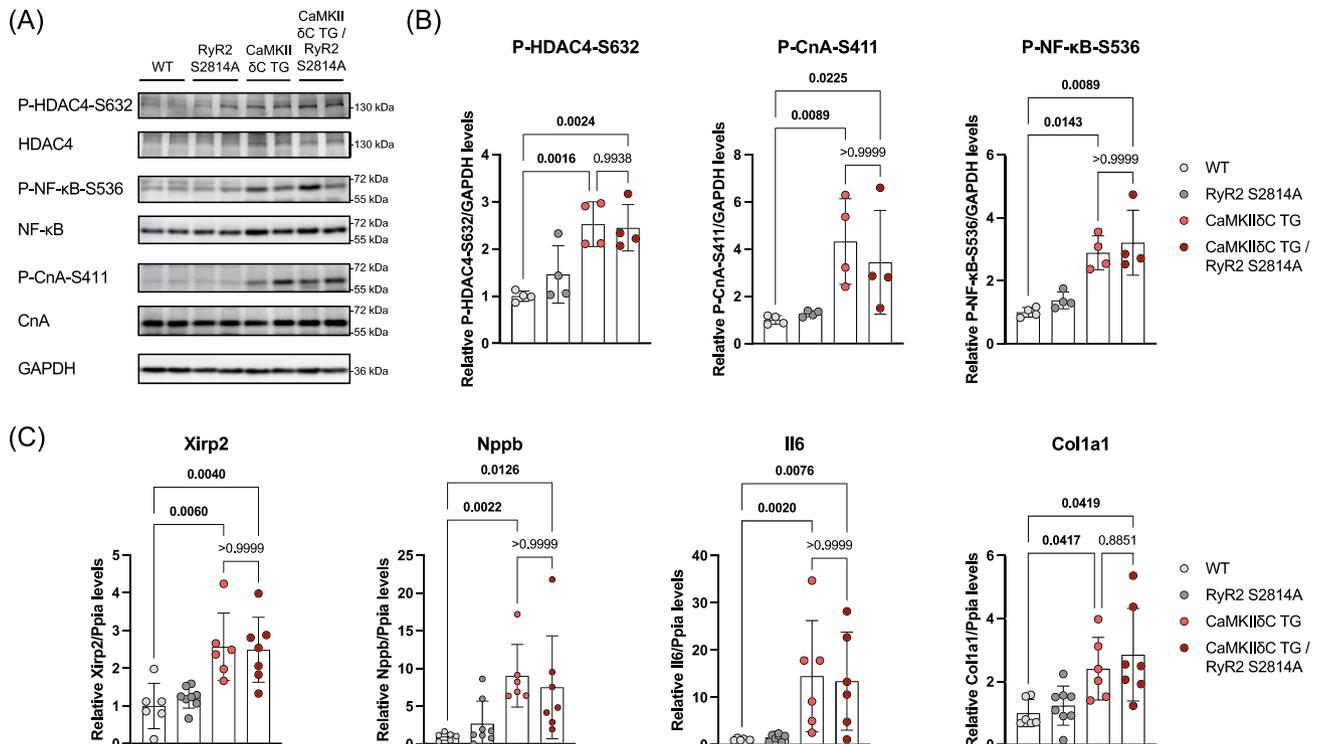
To investigate the effect of the RyR2-S2814A mutation on the activity of other CaMKII-dependent critical pathways in the heart, we analysed the phosphorylation status of transcriptional regulators histone deacetylase 4 (HDAC4) and calcineurin A (CnA) at their respective CaMKII-specific phosphosites.<sup>27</sup> We could not detect any RyR2-S2814A phosphoresistance-dependent difference in the CaMKII $\delta$ C

TG-induced hyperphosphorylation of either HDAC4-S632 or CnA-S411, indicating overall unaffected CaMKII activity regarding transcriptional regulators (Figure 3A, B). Moreover, we observed the same increase in NF- $\kappa$ B-S536 phosphorylation, indicating CaMKII $\delta$ C TG-induced activation of inflammation (Figure 3A, B).<sup>28,29</sup> Analysis of markers of MEF2-dependent gene expression (*Xirp2*), foetal gene expression (*Nppb*), inflammation (*Il6*), and structural remodelling (*Col1a1*)<sup>30</sup> confirmed the same degree of pathological gene expression in the CaMKII $\delta$ C TG, regardless of RyR2-S2814A phosphoresistance, indicating that multiple CaMKII-dependent maladaptive signalling pathways are still activated despite SR  $\text{Ca}^{2+}$ -leak prevention (Figure 3C).

## Discussion

Our data confirm that the increased SR  $\text{Ca}^{2+}$  leak upon CaMKII $\delta$ C overexpression completely depends on RyR2-S2814A phosphorylation. However, preventing the induction of SR  $\text{Ca}^{2+}$  leak does not rescue the CaMKII $\delta$ C TG-induced cardiomyopathic phenotype. Consequently, additional CaMKII-dependent mechanisms must be important for the deleterious effects of CaMKII overactivity with respect to the development of HF in the applied model of CaMKII $\delta$ C

**Figure 3** Effect of the RyR2-S2814A mutation on the phosphorylation of selected CaMKII targets and marker genes in CaMKII $\delta$ C TG hearts. (A) Representative immunoblots of histone deacetylase 4 (HDAC4) Ser632 phosphorylation, HDAC4, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) Ser536 phosphorylation, NF- $\kappa$ B, calcineurin A (CnA) Ser411 phosphorylation, CnA, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from WT, RyR2-S2814A, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A hearts and (B) quantifications of HDAC4-Ser632, CnA-Ser411, and NF- $\kappa$ B-Ser536 phosphorylation ( $n = 4$ ; 7–8 weeks). (C) Quantification of Xin actin-binding repeat-containing protein 2 (*Xirp2*), natriuretic peptide B (*Nppb*), interleukin 6 (*Il6*), and collagen type I alpha 1 (*Col1a1*) expression normalized to peptidylprolyl isomerase A (*Ppia*) in WT, RyR2-S2814A, CaMKII $\delta$ C TG, and CaMKII $\delta$ C TG/RyR2-S2814A hearts ( $n \geq 6$ ; 7–8 weeks). *P* values were determined by ordinary one-way ANOVA with Sidak multiple comparisons test (P-HDAC4-Ser632), Brown–Kruskal–Wallis with Dunn’s multiple comparisons test (P-CnA-Ser411, P-NF- $\kappa$ B-Ser536, *Xirp2*, *Nppb*, *Il6* expression), and Brown-Forsythe and Welch ANOVA with Dunnett T3 multiple comparisons test (*Col1a1* expression).



overexpression. We show that maladaptive transcriptional and inflammatory processes, which are essential downstream pathways of pathological CaMKII activation,<sup>6,29</sup> persist despite SR Ca<sup>2+</sup> leak prevention and are likely to drive structural remodelling and contractile impairment in the setting of chronic CaMKII overactivity. Our conclusions are in line with previous observations that SR-targeted inhibition of CaMKII and phospholamban ablation could not rescue the cardiac phenotype of CaMKII $\delta$ C TG mice,<sup>31,32</sup> indicating that the maladaptive effects of increased CaMKII $\delta$ C activity appear to be indeed SR independent.

Thus, our data reflect the ongoing debate regarding the relevance of the RyR2-S2814 phosphorylation site in cardiac pathology. Whereas consistent findings point to a pivotal role of RyR2-S2814 phosphorylation in arrhythmias,<sup>23,33,34</sup> the relevance of this specific phosphorylation site in the (clinical) manifestation of HF is less clear. Notably, while mice with the phosphoresistant S2814A mutation were relatively protected from cardiac dysfunction induced by pressure overload, this mutation did not protect from cardiac failure after

myocardial infarction.<sup>21</sup> In addition, the phosphomimetic S2814D mutation in mice induces only a mild decrease in cardiac contractility with aging under unstressed conditions.<sup>34</sup> Also, RyR stabilization via rycal S36 treatment was shown to reduce arrhythmias but not contractile impairment and structural remodelling after pressure overload and myocardial infarction *in vivo*, further highlighting the pivotal role of RyR2 leakiness in cardiac arrhythmogenesis but not chronic HF development.<sup>35</sup> Consistent with these findings, our investigation of SR Ca<sup>2+</sup> load in CaMKII $\delta$ C TG vs. CaMKII $\delta$ C TG/RyR2-S2814A cardiomyocytes revealed unaltered SR Ca<sup>2+</sup> content (Supporting Information, *Figure S3*). This observation further indicates that in conditions of chronic CaMKII overactivity, SR Ca<sup>2+</sup> leak normalization does not primarily improve the progression of contractile dysfunction.

In conclusion, our data challenge the view of SR Ca<sup>2+</sup> leakage as a major factor in CaMKII-driven HF. We previously showed that *acute* pharmacologic inhibition of CaMKII reduces SR Ca<sup>2+</sup> leak and can to some extent improve cardiomyocyte function in human HF as well as in CaMKII $\delta$ C TG

cells.<sup>20,36</sup> However, other CaMKII-dependent mechanisms besides RyR2 phosphorylation and SR Ca<sup>2+</sup> leak must be of predominant relevance for the *chronic* development of HF. Yet, there are specific cases, such as acute ischaemia/reperfusion injury and atrial fibrillation, where inhibition of SR Ca<sup>2+</sup> leak has been shown to prevent CaMKII-dependent cell death and atrial remodelling in preclinical models and thus may have beneficial effects on disease progression.<sup>37,38</sup>

From a translational perspective, it will be key to discern whether there is actually a single, central mediator mainly responsible for the detrimental CaMKII effects or whether CaMKII-dependent pathology is rather the result of multiple complex target interactions. In a scenario of long-term chronic inhibition of CaMKII, it is essential to also consider its many physiological functions.<sup>39</sup> Designing specific approaches to selectively inhibit CaMKII-dependent detrimental downstream mechanisms may hold a major therapeutic promise to circumvent compromising the many physiological functions of CaMKII.

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## Conflict of interest

None declared.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1:** Effect of RyR2-S2814A mutation on cardiac performance of CaMKII $\delta$ C TG mice at 7 weeks of age. (A) Ejection fraction (EF) and (B) left ventricular end-diastolic diameter (LVEDD) measured by echocardiography in WT, RyR2 S2814A, CaMKII $\delta$ C TG and CaMKII $\delta$ C TG/RyR2 S2814A mice ( $n \geq 8$ ). \* $P < 0.05$ ; n.s.: not significant.

**Figure S2:** Effect of RyR2-S2814A mutation on cardiac contractility of CaMKII $\delta$ C TG mice at different timepoints. Fractional shortening measured by echocardiography in WT, RyR2 S2814A, CaMKII $\delta$ C TG and CaMKII $\delta$ C TG/RyR2 S2814A mice (A) younger than 9 weeks and (B) older than 9 weeks of age. \* $P < 0.05$ ; n.s.: not significant.

**Figure S3:** Effect of RyR2-S2814A mutation on SR Ca<sup>2+</sup>-load in CaMKII $\delta$ C TG cardiomyocytes. SR Ca<sup>2+</sup>-load in cardiomyocytes from CaMKII $\delta$ C TG and CaMKII $\delta$ C TG/RyR2 S2814A mice ( $n \geq 12$ ).

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