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## AKAP18 $\delta$ Anchors and Regulates CaMKII Activity at Phospholamban-SERCA2 and RYR

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### Abstract

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#### DISCLOSURES

Carlson, Aronsen, Louch, Klussmann and Sejersted are partners in two Disclosure of inventions regarding the CaMKII activator and inhibitor peptides.

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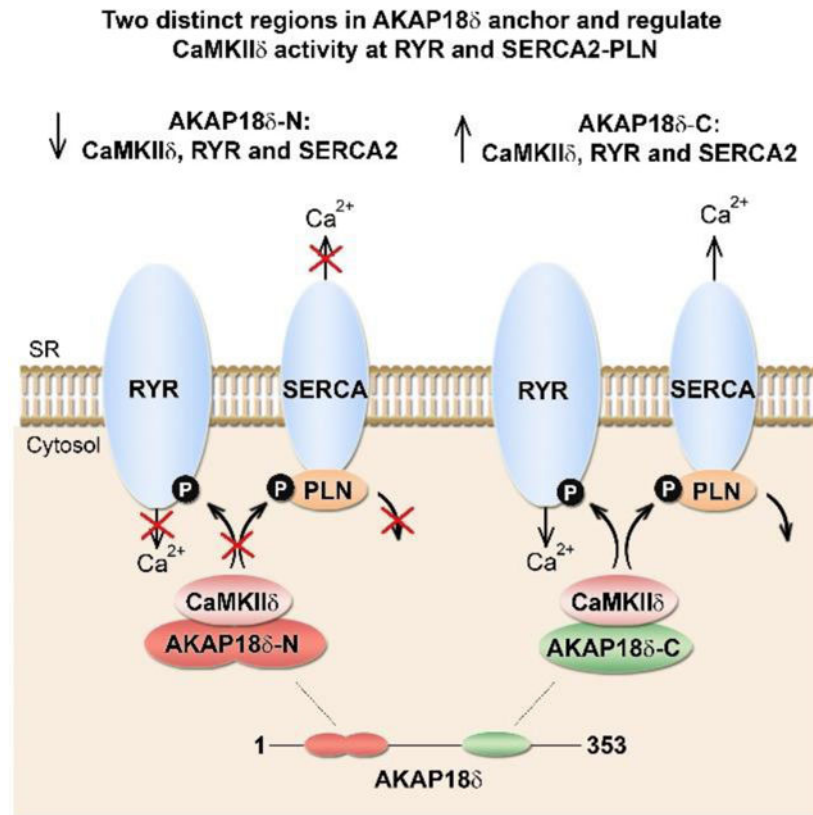
**Background:** The sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase 2 (SERCA2) mediates  $\text{Ca}^{2+}$  reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas the ryanodine receptor (RyR) mediates  $\text{Ca}^{2+}$  release from SR and triggers contraction.  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent protein kinase II (CaMKII) regulates activities of SERCA2 through phosphorylation of phospholamban (PLN) and RyR through direct phosphorylation. However, the mechanisms for CaMKII $\delta$  anchoring to SERCA2-PLN and RyR and its regulation by local  $\text{Ca}^{2+}$  signals remain elusive. The objective of this study was to investigate CaMKII $\delta$  anchoring and regulation at SERCA2-PLN and RyR.

**Methods:** A role for A-kinase anchoring protein 188 (AKAP188) in CaMKII $\delta$  anchoring and regulation was analyzed by bioinformatics, peptide arrays, cell-permeant peptide technology, immunoprecipitations, pull-downs, transfections, immunoblotting, proximity ligation, FRET-based CaMKII activity and ELISA-based assays, whole cell and SR vesicle fluorescence imaging, high-resolution microscopy, adenovirus transduction, adeno-associated virus injection, structural modeling, surface plasmon resonance and alpha screen technology.

**Results:** Our results show that AKAP188 anchors and directly regulates CaMKII $\delta$  activity at SERCA2-PLN and RyR, via two distinct AKAP188 regions. An N-terminal region (AKAP188-N) inhibited CaMKII $\delta$  through binding of a region homologous to natural CaMKII inhibitor peptide and Thr17-PLN region. AKAP188-N also bound CaM, introducing a second level of control. Conversely, AKAP188-C, which shares homology to neuronal CaMKII $\alpha$  activator peptide (N2B-s), activated CaMKII $\delta$  by lowering the apparent  $\text{Ca}^{2+}$  threshold for kinase activation and inducing CaM trapping. While AKAP188-C facilitated faster  $\text{Ca}^{2+}$  reuptake by SERCA2 and  $\text{Ca}^{2+}$  release through RyR, AKAP188-N had opposite effects. We propose a model where the two unique AKAP188 regions fine-tune  $\text{Ca}^{2+}$ -frequency-dependent activation of CaMKII $\delta$  at SERCA2-PLN and RyR.

**Conclusions:** AKAP188 anchors and functionally regulates CaMKII activity at PLN-SERCA2 and RyR, indicating a crucial role of AKAP188 in regulation of the heartbeat. To our knowledge this is the first protein shown to enhance CaMKII activity in heart and also the first AKAP reported to anchor a CaMKII isoform, defining AKAP188 also as a CaM-KAP.

## Graphical Abstract



## Keywords

Basic Science Research; Calcium Cycling/Excitation-Contraction Coupling; Cell Signaling/Signal Transduction; Contractile Function; Mechanisms

## INTRODUCTION

In cardiac myocytes, Ca<sup>2+</sup> cycling is centrally involved in excitation-contraction coupling (ECC) <sup>1</sup>. In this process, Ca<sup>2+</sup> enters the cell through L-type Ca<sup>2+</sup> channels leading to the opening of ryanodine receptors (RYR) in the sarcoplasmic reticulum (SR), and release of Ca<sup>2+</sup> (Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release). The resulting increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) causes Ca<sup>2+</sup> binding to troponin C and activation of the myofilaments leading to contraction. For diastolic relaxation to occur, Ca<sup>2+</sup> is removed from cytoplasm by the SR Ca<sup>2+</sup> ATPase 2 (SERCA2), and to a lesser extent by the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. Alterations in this Ca<sup>2+</sup> cycling are associated with decreased contractility and arrhythmia during heart failure <sup>2</sup>.

Ca<sup>2+</sup>/calmodulin (CaM)-dependent protein kinase  $\delta$  (CaMKII $\delta$ ), which is the predominant CaMKII isoform expressed in heart, regulates ECC by phosphorylating several Ca<sup>2+</sup> handling proteins, including RYR and phospholamban (PLN) <sup>3</sup>. PLN is a key modulator of SERCA2, and thus SR Ca<sup>2+</sup> reuptake, SR Ca<sup>2+</sup> load, and cardiomyocyte relaxation. Dephosphorylated PLN inhibits SERCA2 activity, whereas PLN phosphorylation at Thr17

by CaMKII $\delta$  (or Ser16 by protein kinase A (PKA)) reduces PLN interaction with SERCA2 and relieves this inhibition<sup>4</sup>. Likewise, phosphorylation at Ser2814-RYR by CaMKII $\delta$  increases RYR Ca<sup>2+</sup> sensitivity, leading to augmented SR Ca<sup>2+</sup> release and cardiomyocyte contraction. Inhibition of SR CaMKII $\delta$  activity results in decreased phosphorylation of RYR and PLN, and associated changes in Ca<sup>2+</sup> homeostasis and cardiac contractility<sup>5</sup>. These data support a pivotal role of CaMKII $\delta$  in fine-tuning ECC.

CaMKII forms a dodecamer that comprises two stacked 6-fold symmetric rings<sup>6</sup>. The CaMKII monomer contains an N-terminal ATP-binding pocket, catalytic and autoregulatory domains, and a C-terminal association domain mediating its oligomerization<sup>7</sup>. When CaMKII $\delta$  is in its inactive state, the Thr287 segment (Thr286 in CaMKII $\alpha$ ) in the autoregulatory domain binds to the so-called T-site in catalytic domain. This positions the adjacent sequence (pseudosubstrate) in the substrate-binding site (S-site) and ATP-binding pocket<sup>7</sup> (Suppl. Fig. 1a). Upon activation, Ca<sup>2+</sup>/CaM binds to the CaM binding site in the autoregulatory domain, displacing the Thr287 segment from the T-site (Suppl. Fig. 1b), and enabling the kinase to be phosphorylated by a neighboring active (open) CaMKII $\delta$  molecule. Thus, binding of at least two CaM molecules is required for CaMKII autophosphorylation. Autophosphorylation of Thr287 increases CaMKII affinity for CaM<sup>8</sup>, maintains the kinase in an autonomously active state<sup>9</sup> (Suppl. Fig. 1c), and permits the kinase to translate the frequency of Ca<sup>2+</sup> spikes into kinase activity *in vitro*,<sup>10</sup> a form of molecular memory or integration.

Although CaMKII $\delta$  regulates several aspects of ECC, it remains unclear how the kinase is functionally regulated within the distinct nanodomains where these Ca<sup>2+</sup> handling proteins are localized. For example, local [Ca<sup>2+</sup>] near the PLN-SERCA2 complex is expected to be too low to appreciably activate CaMKII, while CaMKII near RYRs in the dyadic cleft should be activated by the much higher local [Ca<sup>2+</sup>] levels<sup>11</sup>. Although activated CaMKII $\delta$  in myocytes is more mobile than traditionally thought<sup>12</sup>, another potential explanation for PLN phosphorylation by CaMKII $\delta$  is that as yet unidentified CaM-Kinase Anchoring Proteins (CaM-KAPs) could enable locally higher Ca<sup>2+</sup> sensitivity of the kinase.

We postulated that the A-kinase anchoring protein, AKAP18 $\delta$  (also known as AKAP7 $\delta$ ), could perform such a CaMKII $\delta$  anchoring and regulatory role at PLN-SERCA2 and RYR. Originally identified in rat kidney<sup>13</sup>, AKAP18 $\delta$  has been shown to interact directly with PKA and PLN, thereby enabling PKA-dependent phosphorylation at Ser16-PLN and augmentation of SERCA2 activity<sup>14</sup>. In human myocardium, the AKAP18 $\delta$  orthologue, AKAP18 $\gamma$ , similarly complexes with PKA, PLN-SERCA2 and phosphodiesterase 3A1 (PDE3A1) to control Ser16-PLN phosphorylation and SR Ca<sup>2+</sup> reuptake<sup>15</sup>. In the present work, we show that AKAP18 $\delta$  also anchors CaMKII $\delta$  to PLN-SERCA2 and RYR, defining AKAP18 $\delta$  as the first CaMKII anchoring protein. We further identify two unique regions in AKAP18 $\delta$  that inversely regulate CaMKII $\delta$  activity, CaMKII $\delta$ -catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and thus SERCA2 and RYR functional activities. This capability is enabled by one region of AKAP18 $\delta$  which binds CaM and inhibits CaMKII activation, and a second region which lowers the Ca<sup>2+</sup> threshold for CaMKII $\delta$  activation and induces CaM trapping. Based on our results we propose a model in which the two

AKAP188 regions fine-tune the Ca<sup>2+</sup>-frequency-dependent activation of CaMKII $\delta$  at PLN-SERCA2 and RYR.

## METHODS

### Data Availability.

The authors declare that all supporting data are available within the article and in its online supplementary files. Detailed methods are provided in the online Supplemental Materials. The data that support the findings of this study and analytical tools are available from the corresponding author upon reasonable request.

### Statistics.

All data sets with a small n (n<8) were tested for normal distribution using Kolmogorov-Smirnov, Shapiro-Wilk or D'Agostino & Pearson normality test (GraphPad Prism 8.0.1 or 9.1.0). Differences between groups with normally distributed data were analysed using ordinary one-way ANOVA with Dunnett's, Holm-Sidak's or Tukey's multiple comparisons test, or unpaired t-test for simple two-group comparison. Non-normal distributions were examined by Wilcoxon matched-pairs signed-rank test, Mann-Whitney test, or Kruskal-Wallis with Dunn's multiple comparisons test. When multiple measures were drawn from individual animals, nested t-test, nested one-way ANOVA with Tukey's or Dunnett's multiple comparisons test or the linear mixed effect model from the R nlme package (<https://CRAN.R-project.org/package=nlme>) with Tukey's post hoc correction was used. Only within-test corrections were made. *P* values <0.05 were considered statistically significant. Outliers were removed using the ROUT method (Q=1%) (GraphPad Prism) (Fig. 6a, Suppl. Fig. 3b–c and 6a–b). Power analysis was performed a priori to determine anticipated optimal sample size and number of AAV-injected animals. All experiments were performed in a randomized manner, and data analysis was performed blinded using name and allocation concealment. Representative immunoblots were selected to represent the means of the quantified data. Representative images were selected by eye and based on good signal/noise ratios.

Other methods are given in details in the Online Supplement.

## RESULTS

### AKAP188-associated CaMKII $\delta$ controls Thr17-PLN phosphorylation.

First, we tested whether CaMKII $\delta$  and AKAP188 interact at SERCA2-PLN. Immunoprecipitation analysis revealed co-precipitation of CaMKII $\delta$  and SERCA2 with AKAP188 in adult cardiomyocyte lysate (Fig. 1a). High-resolution imaging of adult cardiomyocytes further demonstrated that AKAP188 and CaMKII $\delta$  (Fig. 1b, blue, upper and lower respectively) co-localized with SERCA2 (red) at Z-line. AKAP188 also co-localized with SERCA2 at M-line where little CaMKII $\delta$  was observed, indicating a role for AKAP188 also within the sarcomere center (Fig. 1b). WGA staining of t-tubules was used as marker for Z-lines, since these structures co-localize with  $\alpha$ -actinin (Suppl. Fig. 1d). Secondary

antibody controls and specificity of CaMKII $\delta$  and AKAP18 $\delta$  antibodies are shown in Suppl. Fig. 1e–g.

To analyze whether AKAP18 $\delta$ -associated CaMKII $\delta$  controls pThr17-PLN phosphorylation, AKAP18 $\delta$  was displaced from PLN using a cell-permeant AKAP18 $\delta$ -PLN competitor peptide<sup>14</sup>. Adult and neonatal cardiomyocytes treated with this peptide before isoproterenol (ISO)-stimulation, exhibited reduced pThr17-PLN (and pSer16-PLN as previously reported<sup>14</sup>) compared to control (Fig. 1c and Suppl. Fig. 1h, respectively), indicating that AKAP18 $\delta$ -associated CaMKII $\delta$  phosphorylates Thr17-PLN (illustrated in Fig. 1d). The peptides showed no changes at basal level or cytotoxicity (Suppl. Fig. 1i–j, respectively). Using biotin-labeled PLN peptides, we found that Thr17 phosphorylation reduced GST-AKAP18 $\delta$  binding (Fig. 1e), closely paralleling reported effects of Ser16 phosphorylation<sup>14</sup>. Thus, Thr17 phosphorylation seems to provide an on/off mechanism for the AKAP18 $\delta$ -PLN interaction.

Taken together, the data strongly support that AKAP18 $\delta$  anchors CaMKII $\delta$  to PLN-SERCA2, and thereby controls CaMKII $\delta$ -mediated Thr17-PLN phosphorylation.

### **CaMKII $\delta$ binds directly to two unique regions in AKAP18 $\delta$ .**

The CaMKII $\delta$ -AKAP18 $\delta$  interaction was further investigated using AlphaScreen™ technology. Only recombinant CaMKII $\delta$ -T287D (mimicking active kinase)<sup>16</sup> and not CaMKII $\delta$ -T287A (mimicking inactive kinase) (mutated proteins are validated in Suppl. Fig. 1k–l) was found to bind to AKAP18 $\delta$  (Fig. 1f), indicating that AKAP18 $\delta$  binds to autophosphorylated CaMKII $\delta$ .

To identify CaMKII $\delta$  binding sites, AKAP18 $\delta$ -YFP variants (Fig. 1g) were co-expressed with CaMKII $\delta$ -T287D. CaMKII $\delta$  precipitated all AKAP18 $\delta$  variants, except for GFP-AKAP18 $\delta$  (301–353) (Suppl. Fig. 1m), indicating that CaMKII $\delta$  binds between amino acids 201–301 (Fig. 1g, green region). This region is located C-terminally from the PLN binding domain (amino acids 124–220<sup>14</sup>) and N-terminally from the PKA binding domain (amino acids 301–314<sup>13</sup>). In addition, AKAP18 $\delta$ -YFP and GFP-AKAP18 $\delta$  (67–353) precipitated more strongly with CaMKII $\delta$  compared to other AKAP18 $\delta$  variants, consistent with a second CaMKII $\delta$  binding site towards the N-terminus of AKAP18 $\delta$  (Fig. 1g, red).

To more precisely identify CaMKII $\delta$  binding, rat AKAP18 $\delta$  was spot-synthesized as 20-mer overlapping peptides on membranes and incubated with active His-CaMKII $\delta$ . Immunoblotting identified CaMKII $\delta$  binding to two regions; amino acids 55–98 (AKAP18 $\delta$ -N, in red) and 238–266 (AKAP18 $\delta$ -C, in green) (Fig. 1h) and two homologous regions in human AKAP18 $\gamma$  (Suppl. Fig. 1n). CaMKII $\delta$ -T287D (coated in wells) binding was confirmed by an ELISA-based method using biotinylated peptides spanning the two AKAP18 $\delta$  regions (Suppl. Fig. 1o–p). Sequence alignments showed that the two CaMKII $\delta$ -binding regions were only present in AKAP18 $\delta$  and AKAP18 $\gamma$  and not in the shorter AKAP18 $\alpha$  and AKAP18 $\beta$  isoforms<sup>13</sup> (Fig. 1i–j).

### AKAP188 binds CaMKII $\delta$ through multiple sites.

We next sought to define AKAP188 binding in CaMKII $\delta_{C/2}$ , which is largely cytoplasmic and regulates ECC. CaMKII $\delta$  spot-synthesized as 20-mer overlapping peptides on membranes which were overlaid with GST-AKAP188 (Fig. 2a). AKAP188 bound to the ATP-binding region<sup>7</sup> (amino acids 19–68), S-site (amino acids 130–164) and T-site regions (amino acids 241–269) and a sequence within the autoregulatory domain (ARD) (amino acids 280–317)<sup>7</sup> (Fig. 2a, GST only as negative control is shown in Suppl. Fig. 2a, bottom). Notably, the S- and T-site regions within the catalytic domain have not been clearly defined, but are reported to contain at least residues Glu97 (S-site), Glu140 (S-site), Ile206 (T-site) and Trp238 (T-site)<sup>17</sup> (Fig. 2a, boxed regions in upper panel). Consistent with the above results, a biotinylated peptide covering the N-terminal region of AKAP188 (55–98; Suppl. Fig. 2b) showed an almost identical CaMKII $\delta$  binding pattern as GST-AKAP188.

CaMKII $\delta$  binding of biotin-ahx-AKAP188-C was too weak or dynamic to be detected by peptide arrays. However, overlaying a larger recombinant GST-AKAP188-C fragment (amino acids 201–301), revealed a binding site residing centrally within the T-site region (amino acids 205–233) (Fig. 2b). This binding site was not detected using GST-AKAP188 full length protein, suggesting that GST-AKAP188 (201–301) exhibits differential folding. These data are consistent with AKAP188-C and AKAP188-N binding to distinct T-site sequences. ELISA-based experiments confirmed AKAP188 binding to the autoregulatory domain, ATP binding region, S-site and two T-site regions of CaMKII $\delta$  (Suppl. Fig. 2c–e), supporting the interpretation of the overlay data. The five AKAP188 binding sites in CaMKII $\delta_{C/2}$  are indicated in Fig. 2c (19–68, 130–164, 205–233, 241–269 and 280–317, inhibitory ones in red and activating in green). The fact that AKAP188-N binds to several different regions in these critical CaMKII domains, may serve to stabilize (or rigidify) CaMKII in the closed inactivated state. Multiple CaMKII binding sites have also been identified in other proteins<sup>18–20</sup>, e.g. densin, which binds to several T-site sequences<sup>19</sup>.

### AKAP188-N inhibits CaMKII $\delta$ through sequences similar to the natural CaMKII inhibitor protein and Thr17-PLN region.

AKAP188-N (55–98) interacted only with activated CaMKII $\delta$  (Suppl. Fig. 2f). The effect of AKAP188-N on CaMKII $\delta$  activity was analyzed in an *in vitro* kinase assay. AKAP188-N (55–98) reduced CaMKII $\delta$ -catalyzed phosphorylation of syntide (a CaMKII substrate) by 50 % under high [Ca<sup>2+</sup>] and [CaM], conditions that should maximally activate CaMKII (Fig. 2d, left panel). Closer examination showed that amino acids 55–74 were less inhibitory (right panel). Bioinformatics revealed that this sequence exhibited similarities to CN27, a natural CaMKII inhibitory peptide<sup>21</sup> (Fig. 2e). In a crystal structure of CaMKII, a shorter variant of CN27 (CN21a in Fig. 2e) has been shown to bind to the T-site, while being sufficiently long to prevent access for substrate binding to the adjacent S-site<sup>21</sup>. In an ELISA-based assay, CN27 outcompeted the AKAP188-N (55–74)-CaMKII $\delta$  interaction, indicating that CN27 and AKAP188-N (55–74) may bind to the same site in CaMKII $\delta$  (Suppl. Fig. 2g). In addition, AKAP188-N (55–74) contains a proline (Pro64) in its central region (Fig. 2e, arrow); a feature also reported for the inhibitory T-site binding sequence in densin<sup>19</sup>, which might explain its weaker effect on CaMKII $\delta$  activity.



CaMKII $\delta$  binding of the more inhibitory amino acids 79–98 of AKAP18 $\delta$  (Fig. 2d) were not outcompeted by CN27 (Suppl. Fig. 2h). Bioinformatics revealed that these amino acids rather showed some sequence similarity to the Thr17-PLN region, with Ser95<sup>AKAP18 $\delta$</sup>  corresponding to Thr17<sup>PLN</sup> (Fig. 2f). Modeled placement of AKAP18 $\delta$  (89–99) into the peptide binding groove of CaMKII $\delta$  centered on Ser95 in AKAP18 $\delta$  (Fig. 2g), and suggested several favorable interactions between CaMKII $\delta$  and AKAP18 $\delta$ . Hydrophobic interactions between Pro90-Val103<sup>CaMKII $\delta$</sup> , Tyr92-Phe138<sup>CaMKII $\delta$</sup> , Phe93-Trp215<sup>CaMKII $\delta$</sup> , Ile96-Pro187<sup>CaMKII $\delta$</sup>  and Thr99-Phe174<sup>CaMKII $\delta$</sup>  were suggested (cyan dashed lines). The model indicated that Ser95 could interact with both Asp136<sup>CaMKII $\delta$</sup> , Lys138<sup>CaMKII $\delta$</sup>  and Thr177<sup>CaMKII $\delta$</sup>  (green dashed lines) via hydrogen bonds. A possible unfavorable repulsive interaction between Tyr92 and Glu97<sup>CaMKII $\delta$</sup>  was also indicated (red dashed line). Model predictions were validated by mutating key residues in AKAP18 $\delta$ , and subsequent ELISA analysis (Suppl. Fig. 2i, see legend for detailed description).

Kinetics of AKAP18 $\delta$ -N (55–98) binding to CaMKII $\delta$  were analyzed by surface plasmon resonance (SPR). A range of concentrations of recombinant CaMKII $\delta$ -T287D (47.6–500 nM) was injected over immobilized biotin-AKAP18 $\delta$ -N on a SA chip, and analyzed by fitting with a 1:1 interaction model (Langmuir). The dissociation equilibrium constant ( $K_D$ ) was  $23 \pm 2$  nM, with an association rate constant ( $k_a$ ) =  $(1.10 \pm 0.02) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  and a dissociation rate constant ( $k_d$ ) =  $(2.5 \pm 0.2) \times 10^{-4} \text{ s}^{-1}$  (Fig. 2h). These findings indicate a strong CaMKII $\delta$ -T287D interaction with AKAP18 $\delta$ -N with medium association and dissociation rates. We also performed SPR analyses of AKAP18 $\delta$ -N (55–74) and AKAP18 $\delta$ -N (79–98). The inhibitory AKAP18 $\delta$ -N (79–98) bound strongly to CaMKII $\delta$ -T287D with similar SPR values as identified above (Suppl. Fig. 2j). AKAP18 $\delta$ -N (55–74) which was less inhibitory and contained a proline in its central region, bound more weakly and exhibited higher association and dissociation rate constants (faster on/off) (Suppl. Fig. 2k).

Taken together, our data indicate that AKAP18 $\delta$ -N (55–98) is a potent inhibitor of the open CaMKII $\delta$  with strong affinity. Inhibition is effected by sequences with similarities to CN27 derived from natural CaMKII inhibitor protein (T-site binding, with associated S-site blockade) and Thr17-PLN region (S-site binding) (summarized in Fig. 2d, upper panel). CaMKII $\delta$  inhibition by AKAP18 $\delta$ -N is illustrated in Fig. 2i (open conformation). Since AKAP18 $\delta$ -N also bound to the autoregulatory domain, it is likely that AKAP18 $\delta$ -N also continues to bind and inhibit CaMKII $\delta$  after pThr287 dephosphorylation and CaM dissociation (Fig. 2j, closed conformation).

### **AKAP18 $\delta$ -N reduces pThr17-PLN and inhibits SR Ca<sup>2+</sup> reuptake.**

Analyses performed using biotin-ahx-PLN as a substrate showed that AKAP18 $\delta$ -N reduced CaMKII $\delta$ -T287D-catalyzed phosphorylation of Thr17 in PLN (Fig. 3a, full immunoblots in Suppl. Fig. 3a). We further examined whether AKAP18 $\delta$ -N also reduced Thr17-PLN phosphorylation in cardiomyocytes. Compared to the scrambled control peptide, cell-permeant AKAP18 $\delta$ -N (55–74) reduced the pThr17-PLN level in both ISO-treated (15 min) adult (Fig. 3b) and neonatal cardiomyocytes (Suppl. Fig. 3b), whereas pSer16, which is

a PKA substrate, was hardly affected. Neither AKAP188-N (55–74) nor control peptide showed any changes at basal level or cytotoxicity (Suppl. Fig. 3c–d, respectively).

Effects of AKAP188-N on  $\text{Ca}^{2+}$  fluxes in adult cardiomyocytes were also investigated. Rat adult cardiomyocytes were treated with the cell-permeant version of AKAP188-N (55–74) or a scrambled control peptide, with or without ISO-stimulation. Cell-permeant TAT-AKAP188-N (55–74) treatment prolonged decay time of  $\text{Ca}^{2+}$  transients at 4 Hz (Fig. 3c, representative tracings are shown in Suppl. Fig. 3e), where Thr17-PLN phosphorylation is increased<sup>22</sup> and SERCA2 plays a proportionally larger role in controlling  $\text{Ca}^{2+}$  removal from the cytosol<sup>23</sup>. In the presence of ISO, which augments SERCA2 activity via PLN phosphorylation, TAT-AKAP188-N (55–74) continued to slow  $\text{Ca}^{2+}$  transient decay at high stimulation frequencies (4 and 6 Hz) (Fig. 3d, representative tracings are shown in Suppl. Fig. 3f). Compared to the scrambled control peptide, AKAP188-N (79–98) also reduced SERCA2  $\text{Ca}^{2+}$  reuptake rate in isolated mouse SR vesicles (Fig. 3e). These findings are consistent with reduction of pThr17-PLN levels by AKAP188-N (Fig. 3a–b).

Finally, compared to control mice, adult mice injected with adeno associated virus (AAV) encoding AKAP188-N (55–74) exhibited reduced pThr17-PLN levels after ISO stimulation, suggesting that AKAP188-N (55–74) also inhibits CaMKII $\delta$ -catalyzed phosphorylation of Thr17 in PLN *in vivo* (Fig. 3f, upper panel). No effect of AAV-AKAP188-N (55–74) on pSer16-PLN was observed (Fig. 3f, middle panel).

### Calcified CaM binds to AKAP188-N in competition with CaMKII $\delta$ .

Rat AKAP188-N and human AKAP18 $\gamma$ -N contain stretches of alternating hydrophobic and basic residues, including valine (V), lysine (K), arginine (R), phenylalanine (F) and proline (P), which are characteristic for CaM binding motifs<sup>24</sup>. Closer inspection revealed that both AKAPs showed sequence similarities to the CaM binding motif in AKAP79<sup>25</sup> (Fig. 4a) and CaMKII $\delta$  (Suppl. Fig. 4a). We therefore analyzed whether AKAP188-N also bound calcified CaM. Immunoblotting demonstrated that the inhibitory AKAP188-N (79–98) peptide, the corresponding sequence in human AKAP18 $\gamma$ -N (52–71), and GFP-AKAP188 (67–353) precipitated with CaM-agarose in the presence of  $\text{Ca}^{2+}$  (Suppl. Fig. 4b–d, respectively). CaM binding to AKAP188-N (79–98) was also confirmed in an ELISA-based assay where CaM-coated wells were incubated with biotinylated AKAP188-N (79–98) or the scrambled control (Fig. 4b). Interestingly, in further experiments, the presence of CaM attenuated both the inhibitory AKAP188-N (79–98)-CaMKII $\delta$ -T287D (Fig. 4c) and AKAP188-N (79–98)-CaMKII $\delta$  (1–282) (Fig. 4d) interactions. Since CaMKII $\delta$  (1–282) lacks the CaM binding site, this finding indicates that CaM also outcompetes AKAP188-N (79–98) binding to CaMKII $\delta$  catalytic site.

Kinetics of the CaM-AKAP188-N (79–98) interaction were analyzed by SPR. In the presence of 1 mM  $\text{Ca}^{2+}$ , the dissociation equilibrium constant ( $K_D$ ) was  $4.7 \pm 0.7 \mu\text{M}$ , with an association rate constant ( $k_a$ ) =  $(1.3 \pm 0.3) \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  and a dissociation rate constant ( $k_d$ ) =  $(5.3 \pm 0.3) \times 10^{-4} \text{ s}^{-1}$  (Fig. 4e). These findings indicate that the CaM-AKAP188-N (79–98) interaction is quite slow and weak.

In summary, our data indicate that calcified CaM binds directly to AKAP186-N and outcompetes the inhibitory AKAP186-N-CaMKII $\delta$  interaction (illustrated in Fig. 4f). By this mechanism, rising Ca<sup>2+</sup> levels, and resulting calcification of CaM, relieve the inhibition of CaMKII.

### **AKAP186-C is homologous to the neuronal CaMKII $\alpha$ activator N2B-s and lowers the Ca<sup>2+</sup> threshold for CaMKII $\delta$ activation.**

As shown in Fig. 1j, both AKAP186 and AKAP18 $\gamma$  contain a second CaMKII $\delta$  interaction site positioned towards the C-terminus. Bioinformatics revealed that this region showed sequence similarity to the N2B-s sequence derived from the neuronal NMDA receptor NR2B subunit (Fig. 5a). N2B-s is homologous to the Thr286 autoinhibitory region in CaMKII $\alpha$ , and linked to autonomous CaMKII $\alpha$  activation in brain via T-site binding<sup>18, 26, 27</sup>. As demonstrated in Fig. 2b, AKAP186-C also bound to T site (amino acids 205–233).

In similarity to effects of N2B-s, we observed that AKAP186-C increased substrate phosphorylation by CaMKII $\delta$ -T287A in an *in vitro* kinase assay where CaM was omitted (Fig. 5b). This finding suggests that AKAP186-C augments CaMKII $\delta$  activity independently of Thr287 autophosphorylation and CaM binding. Consistently, cell-permeant TAT-AKAP186-C also increased Camui activity<sup>28</sup>, a FRET-based CaMKII activation state sensor, transduced into adult rabbit ventricular myocytes (Fig. 5c). As CaMKII requires relatively high intracellular Ca<sup>2+</sup> to achieve activation, we hypothesized that AKAP186-C was able to decrease the Ca<sup>2+</sup> threshold for CaMKII $\delta$  activation. Indeed, at 0.5  $\mu$ M free Ca<sup>2+</sup>, which does not fully activate CaMKII $\delta$ -T287A to phosphorylate Thr17-PLN, further increased Thr17-PLN phosphorylation in the presence of either AKAP186-C (tendency) or the N2B-s positive control peptide (Fig. 5d–e, respectively, complete immunoblots in Suppl. Fig. 5a–b). These observations suggest that AKAP186-C is able to potentiate CaMKII $\delta$  by keeping the inhibitory gate open as a wedge, thereby sensitizing CaMKII to Ca<sup>2+</sup>-dependent activation. Immunoprecipitations employing CaMKII $\delta$ -T287A in the presence of CaM showed that AKAP186-C also induced trapping of CaM to CaMKII $\delta$ -T287A (Fig. 5f); a feature which has been similarly described for N2B-s<sup>18</sup>.

Kinetics of the AKAP186-C (238–266)-CaMKII $\delta$  interaction analyzed by SPR revealed a dissociation equilibrium constant ( $K_D$ ) =  $340 \pm 46$  nM, association rate constant ( $k_a$ ) =  $(4.5 \pm 1.0) \times 10^3$  M<sup>-1</sup> s<sup>-1</sup> and dissociation rate constant ( $k_d$ ) =  $(1.4 \pm 0.1) \times 10^{-3}$  s<sup>-1</sup> (Fig. 5g). This indicated that the CaMKII $\delta$ -T287D-AKAP186-C interaction is weaker than the CaMKII $\delta$ -T287D-AKAP186-N interaction, has a slower on rate, and faster off rate.

Taken together, our data show that AKAP186-C is a CaMKII $\delta$  activator that lowers the Ca<sup>2+</sup> threshold for CaMKII $\delta$  activation (perhaps by keeping the inhibitory gate open) and also allows CaM trapping by the kinase (Fig. 5h–i).

### **AKAP186-C increases pThr17-PLN and facilitates faster SR Ca<sup>2+</sup> reuptake.**

When introduced into adult rat cardiomyocytes, TAT-AKAP186-C increased pThr17-PLN compared to both basal and ISO-stimulated cardiomyocytes, and when combined with ISO (5 min), the pThr17-PLN was further increased (tendency) (Fig. 6a). pThr17-PLN was also

augmented in neonatal rat cardiomyocytes treated with AKAP188-C or N2B-s<sup>18</sup> (Suppl. Fig. 6a–b). No cytotoxicity was observed for AKAP188-C or control peptide (Suppl. Fig. 3d).

Isolated adult rat cardiomyocytes were treated with cell-permeant TAT-AKAP188-C or scrambled control peptides, in the presence and absence of ISO. Ca<sup>2+</sup> transient recordings revealed findings opposite to those observed for AKAP188-N, as TAT-AKAP188-C accelerated Ca<sup>2+</sup> decline, both in the absence and presence of ISO (Fig. 6b–c, representative tracings are shown in Suppl. Fig. 6c–d). This effect was most marked at low frequencies (1 and 4 Hz), as rising resting Ca<sup>2+</sup> levels, CaMKII $\delta$  activation, and increasing pThr17-PLN at higher pacing rates likely overpowered the effects of AKAP188-C observed at baseline. Consistent with the data above, TAT-AKAP188-C also increased the SERCA2 Ca<sup>2+</sup>-uptake rate in isolated mouse SR vesicles (Fig. 6d).

In summary, these data support the notion that by activating CaMKII $\delta$ , AKAP188-C increases pThr17-PLN, and thereby accelerates Ca<sup>2+</sup> reuptake into the SR.

### **AKAP188 also anchors and functionally regulates CaMKII $\delta$ activity at RYR.**

RYR has been identified together with PLN-SERCA2 in SR nanodomains<sup>29</sup>. We therefore analysed whether AKAP188 also regulates CaMKII $\delta$  activity at RYR. Co-localization and immunoprecipitation of CaMKII $\delta$  and RYR with AKAP188 in adult rat cardiomyocytes was shown using proximity ligation assay (Fig. 7a–b, yellow spots) and western blotting (Fig. 7c). In similarity to effects on pThr17-PLN (Fig. 3a), AKAP188-N (55–98) also reduced CaMKII $\delta$  phosphorylation of Ser2814-RYR using biotin-ahx-RYR (2797-2827) as substrate (Fig. 7d). When introduced into adult cardiomyocytes, cell-permeant TAT-AKAP188-N (79–98) and TAT-AKAP188-N (55–74) reduced Ser2814-RYR phosphorylation (Fig. 7e) and RYR functional activity after ISO stimulation (Fig. 7g), whereas TAT-AKAP188-C in the absence of ISO exhibited opposite effects (Fig. 7f–g). TAT-AKAP188-N (79–98) exhibited no effect on pSer2814-RYR at basal level (Suppl. Fig. 7b) or at pSer2808-RYR after ISO stimulation (Suppl. Fig. 7c), consistent with Ser2814 being the main CaMKII phosphorylation site in RYR<sup>30</sup>. Finally, we tested the effect of the AKAP188-PLN competitor peptide on Ser2814-RYR phosphorylation. Contrary to pThr17-PLN (Fig. 1c), no significant changes in the pSer2814-RYR level in cardiomyocytes treated with the AKAP188-PLN competitor peptide were observed (Fig. 7h). This finding indicates that RYR and PLN-SERCA2 might not compete for the same AKAP188-CaMKII $\delta$  pool.

Taken together, our data indicate that in addition to effects at PLN-SERCA2, AKAP188 also anchors and regulates CaMKII $\delta$  activity at RYR.

## **DISCUSSION**

Here, we have provided mechanistic insight into the anchoring and regulation of CaMKII $\delta$  activity by AKAP188 at PLN-SERCA2 and RYR. We identified two unique regions in AKAP188 that inversely regulate CaMKII $\delta$  activity, CaMKII $\delta$ -catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and SERCA2 and RYR functional activities. We specifically showed that an inhibitory domain (AKAP188-N) also binds calcified CaM, while an

activating domain (AKAP188-C) wedges CaMKII $\delta$  open, trapping CaM within the kinase, and lowering the Ca<sup>2+</sup> threshold for its activation.

### Working model to explain AKAP188 effects on CaMKII $\delta$ in myocytes.

CaMKII activity has been shown to be sensitive to the frequency of Ca<sup>2+</sup> oscillations *in vitro*<sup>10</sup> and in intact adult cardiomyocytes<sup>28</sup>. Based on our data, we propose a working hypothesis whereby AKAP188 fine-tunes this frequency-dependent activation of CaMKII $\delta$ . At low stimulation frequency (Fig. 8a), AKAP188-N (in red) binds the CaMKII $\delta$  autoregulatory domain (pink), the ATP binding pocket, T- and S-site in the catalytic domain (green). This multi-point contact may stabilize the closed, inactive CaMKII conformation. Inhibition of the kinase persists. However, when the frequency of the Ca<sup>2+</sup> transients increases (Fig. 8b), accumulated calcified CaM (beige) may outcompete the inhibitory AKAP188-N-CaMKII $\delta$  interaction by binding to the CaMKII $\delta$  autoregulatory domain (pink) and AKAP188-N (red). These events lead to displacement of the S-site from AKAP188-N. CaM binding to AKAP188-N may alter its conformation, leading to its release also from the ATP binding region and T-site (Fig. 8b), augmented access of Thr17-PLN to the S-site, and greater CaMKII $\delta$  potency. Concomitantly, AKAP188-C (in green) binds to the released T-site, keeping the inhibitory gate open as a wedge. This potentiates CaMKII $\delta$  by lowering the Ca<sup>2+</sup> threshold for its activation and by trapping CaM, leading to substrate phosphorylation (e.g. Thr287 in the neighboring CaMKII $\delta$  subunit, Thr17-PLN or Ser2814-RYR) during subsequent Ca<sup>2+</sup> transients in a feed-forward manner: molecular memory of CaMKII $\delta$  results. Thereafter, when the frequency of Ca<sup>2+</sup> transients declines, CaM dissociates from CaMKII $\delta$  and AKAP188-N, making the latter accessible to bind the CaMKII $\delta$  autoregulatory domain, ATP binding region, and S- and T-sites, leading to re-inhibition of CaMKII $\delta$  (Fig. 8a). While plausible, it is unknown whether in the intact continuously beating heart the activating effect of AKAP188-C is dominant over the inhibitory stabilization caused by AKAP188-N.

In contrast to the CaMKII $\delta$  autoregulatory domain, AKAP188-N does not contain the cluster of Phe293, Asn294 and Arg296 which is critical for CaM trapping by CaMKII $\delta$ <sup>31</sup>, suggesting that AKAP188-N does not trap calcified CaM as the Ca<sup>2+</sup> transient frequency declines. This may allow AKAP188-N to inhibit CaMKII $\delta$  prior to complete dissociation of CaM from CaMKII $\delta$ . In fact, since AKAP188-C and AKAP188-N (55–74) bind differently within the T-site region, there might not be any need for AKAP188-C to dissociate from CaMKII $\delta$  when the frequency declines. With this positioning, AKAP188-C might rapidly potentiate kinase activity during a subsequent rise in Ca<sup>2+</sup> transient frequency since this could occur as soon as AKAP188-N dissociates, and independently of the Thr287 autophosphorylation state or other posttranslational modifications reported to promote autonomous activation of CaMKII<sup>32–35</sup>. In the presence of posttranslational modifications, AKAP188-C may increase CaMKII $\delta$  activation to an even higher level, whereas AKAP188-N is expected to still be able to inhibit CaMKII $\delta$  (illustrated in Fig. 2i), even if its binding to the autoregulatory domain is abolished by oxidation (Met281/282)<sup>32</sup> or S-nitrosylation (Cys290)<sup>35</sup>. The stoichiometry of the AKAP188-CaMKII $\delta$  interaction is not known, but since AKAP188 has been shown to oligomerize<sup>36</sup>, it is plausible that several AKAP188 molecules anchor and regulate different CaMKII $\delta$  monomers within the same CaMKII

oligomer. Bioinformatics revealed that the AKAP18 $\delta$  binding regions are well conserved across CaMKII isoforms (Suppl. Fig. 8), suggesting that AKAP18 $\delta$  may be able to anchor and regulate different CaMKII isoforms, and might provide an explanation for some Thr17-PLN phosphorylation observed in the CaMKII $\delta$  knock out mouse model <sup>37</sup>.

Additional studies are needed to further interrogate the proposed AKAP18 $\delta$ -CaMKII $\delta$ -SERCA2-PLN/RYR model (Fig. 8). Both PLN and RYR are thought to be important targets for CaMKII $\delta$  effects during the force frequency relationship (FFR) <sup>30, 38–40</sup>. While the effects of CaMKII and PLN on frequency-dependent acceleration of relaxation (FDAR) have been more controversial <sup>41–43</sup>, recent works employing transgenic CaMKII inhibition in PLN deficient mice have indicated that CaMKII-dependent regulation of PLN is critical to achieve FDAR, but that as yet unidentified CaMKII targets may also contribute <sup>40</sup>. Our data support an important role of CaMKII during FDAR, as we also observed that AKAP18 $\delta$ -C-dependent CaMKII stimulation accelerated Ca<sup>2+</sup> transient decline at low stimulation frequency (Fig. 6b).

### **AKAP18 $\delta$ is a novel CaMKII Anchoring Protein.**

To our knowledge, this is the first AKAP reported to anchor a CaMKII isoform, defining AKAP18 $\delta$  also as a CaM-KAP. From the crystal structures of fragments of AKAP18 $\delta$  <sup>44</sup> and AKAP18 $\gamma$  <sup>45</sup> it appears as though the two CaMKII $\delta$  binding sites are mostly accessible and do not overlap with the PLN <sup>14</sup> or PKA <sup>13</sup> binding sites. Thus, both CaMKII $\delta$  and PKA may bind to AKAP18 $\delta$  and phosphorylate PLN at the same time. AKAP18 $\delta$  also coordinates phosphorylation of inhibitor-1 <sup>46</sup>, which in turn inhibits protein phosphatase 1, the major phosphatase responsible for dephosphorylating PLN <sup>47–49</sup>.

It remains to be determined whether the opposing effects of the two AKAP18 $\delta$  regions on CaMKII $\delta$  activity are unique to AKAP18 $\delta$ , or whether a similar arrangement exists in other proteins. CaMKII $\alpha$  interacts with two sites in the NMDA (N-methyl-D-aspartate) receptor subunit NR2B <sup>18</sup>. One site (N2B-s) is similar to AKAP18 $\delta$ -C, both at sequence level, as well as functionally, as it generates autonomous activation of CaMKII $\alpha$  <sup>18</sup>. The second binding site is reported to be CaMKII $\alpha$ -Thr286 dependent <sup>18</sup>. Densin, located in neuronal postsynaptic densities, also inhibits CaMKII-mediated phosphorylation through T-site binding <sup>19</sup>, but binds to the CaMKII association domain through a second site <sup>50–52</sup>. Furthermore, GTPase Rem2, a critical regulator of dendritic branching and homeostatic plasticity, binds also to the association domain, but inhibits CaMKII rather through the S-site <sup>20</sup>. Interestingly, Rem2 also interacts with CaM <sup>53, 54</sup>.

### **Pathophysiological relevance.**

Understanding the molecular mechanisms of CaMKII regulation is important in the context of various heart diseases and the development of new treatment strategies. Sustained CaMKII activation is linked to impaired cardiomyocyte Ca<sup>2+</sup> homeostasis, cardiac dysfunction and arrhythmias in diseases spanning atrial fibrillation, heart failure and diabetic cardiomyopathy. Although CaMKII activity and RYR functional activity are often increased in these conditions <sup>4</sup>, decreased SERCA2 activity is often reported. During heart failure, reduction in SERCA2 activity is likely a result of both lowered protein expression <sup>55</sup> and

hypo-phosphorylation of PLN following increased phosphatase activity<sup>56,57</sup>. Restoration of SR Ca<sup>2+</sup> re-uptake through increasing PLN phosphorylation levels and/or SERCA2 activity is therefore considered to be a potential therapeutic strategy<sup>58,59</sup>. Future studies are needed to test whether the CaMKII activator peptide identified in this study (AKAP188-C) can be used to increase pThr17-PLN levels and thus SERCA2 activity *in vivo*. One strategy may be to target AKAP188-C to longitudinal SR (LSR), using a strategy similar to that described for the AIP<sub>4</sub>-LSR transgenic mice<sup>60,61</sup>. To this end, the peptide sequences presently derived from the two AKAP188 regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte Ca<sup>2+</sup> cycling.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms:

<b>AAV</b>	adeno associated virus
<b>AKAP</b>	A-kinase anchoring protein
<b>Arg9</b>	RRRRRRRRR
<b>CaM</b>	calmodulin
<b>CaM-KAP</b>	CaM-kinase anchoring protein
<b>CaMKII</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II
<b>CN27</b>	a CaMKII inhibitor peptide
<b>ECC</b>	excitation-contraction coupling
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>FDAR</b>	frequency-dependent acceleration of relaxation
<b>FFR</b>	force frequency relationship
<b>FRET</b>	fluorescence resonance energy transfer

<b>GST</b>	glutathione S-transferase
<b>IB</b>	immunoblotting
<b>IP</b>	immunoprecipitation
<b>ISO</b>	isoproterenol
<b>LDH</b>	lactate dehydrogenase
<b>LSR</b>	longitudinal SR
<b>LV</b>	left ventricle
<b>NMDA</b>	N-methyl-D-aspartate
<b>N2B-s</b>	a CaMKII activator peptide
<b>PDE</b>	phosphodiesterase
<b>PKA</b>	protein kinase A
<b>PLA</b>	proximity ligation assay
<b>PLN</b>	phospholamban
<b>PVDF</b>	polyvinylidene fluoride
<b>RYR</b>	ryanodine receptor
<b>SA</b>	streptavidin affinity
<b>Scram pep</b>	scrambled peptide
<b>SERCA2</b>	sarco/endoplasmic reticulum Ca <sup>2+</sup> -ATPase 2
<b>SPR</b>	surface plasmon resonance
<b>SR</b>	sarcoplasmic reticulum
<b>TAT</b>	RKKRRQRRR
<b>TFA</b>	trifluoroacetic acid
<b>TMB</b>	3,3',5,5'-tetramethylbenzidine
<b>WGA</b>	wheat germ agglutinin

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## NOVELTY AND SIGNIFICANCE

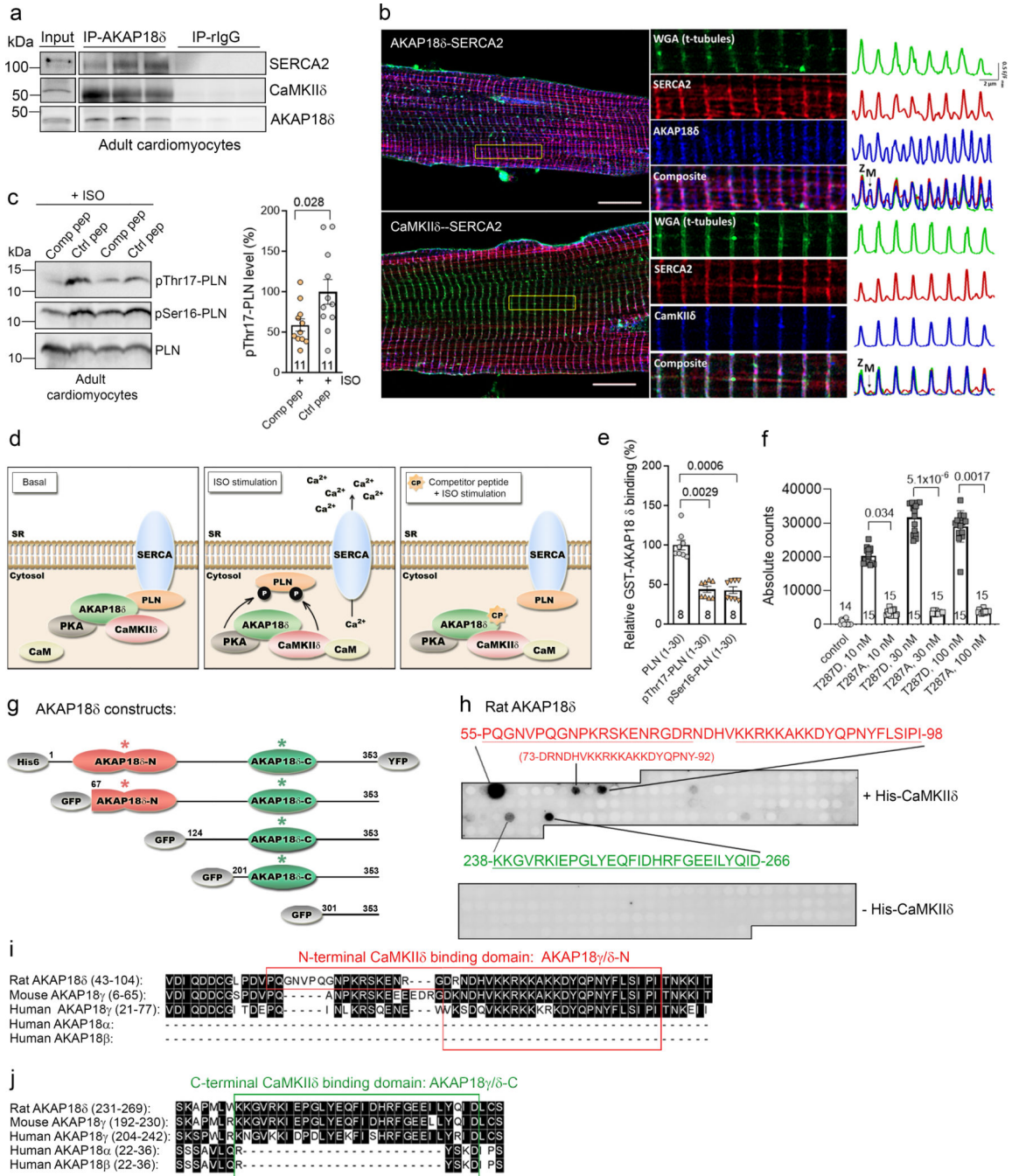
### What Is Known?

- Sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase 2 (SERCA2) and ryanodine receptor (RZR) are essential for cardiac excitation-contraction coupling.
- $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent protein kinase II (CaMKII) modulates SERCA2 and RZR activities through indirect and direct phosphorylation events, respectively, but CaMKII anchoring and local regulation mechanisms remain elusive.
- A-Kinase Anchoring Protein 18 delta (AKAP18 $\delta$ ) anchors protein kinase A to SERCA2-phospholamban (PLN).

### What New Information Does This Article Contribute?

- AKAP18 $\delta$  anchors and functionally regulates CaMKII activity at SERCA2-PLN and RZR, indicating a crucial role of AKAP18 $\delta$  in heartbeat regulation.
- AKAP18 $\delta$  also anchors calmodulin (CaM), inducing a second level of control.
- AKAP18 $\delta$  is the first AKAP reported to anchor a CaMKII isoform, defining AKAP18 $\delta$  as a CaM-Kinase Anchoring Proteins (CaM-KAP).

SERCA2 mediates  $\text{Ca}^{2+}$  reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas RZR mediates  $\text{Ca}^{2+}$  release from SR and triggers contraction. CaMKII $\delta$  regulates activities of SERCA2, through phosphorylation of PLN, and RZR by direct phosphorylation. However, the mechanisms for CaMKII $\delta$  anchoring to SERCA2-PLN and RZR and its regulation by local  $\text{Ca}^{2+}$  signals remain unclear. Here, we provide mechanistic insight into the anchoring and regulation of CaMKII $\delta$  activity by AKAP18 $\delta$  at SERCA2-PLN and RZR. We identified two unique regions in AKAP18 $\delta$  that inversely regulate CaMKII $\delta$  activity (CaMKII $\delta$ -catalyzed phosphorylation of Thr17-PLN and Ser2814-RZR) and SERCA2 and RZR function. We specifically showed that an inhibitory domain (AKAP18 $\delta$ -N) also binds calcified CaM, while an activating domain (AKAP18 $\delta$ -C) wedges CaMKII $\delta$  open, trapping CaM within the kinase, and lowering the  $\text{Ca}^{2+}$  threshold for its activation. Based on our data we propose a working model where the two unique AKAP18 $\delta$  regions fine-tune  $\text{Ca}^{2+}$ -frequency-dependent activation of CaMKII $\delta$  at SERCA2-PLN and RZR. The peptide sequences derived from the two AKAP18 $\delta$  regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte  $\text{Ca}^{2+}$  cycling.

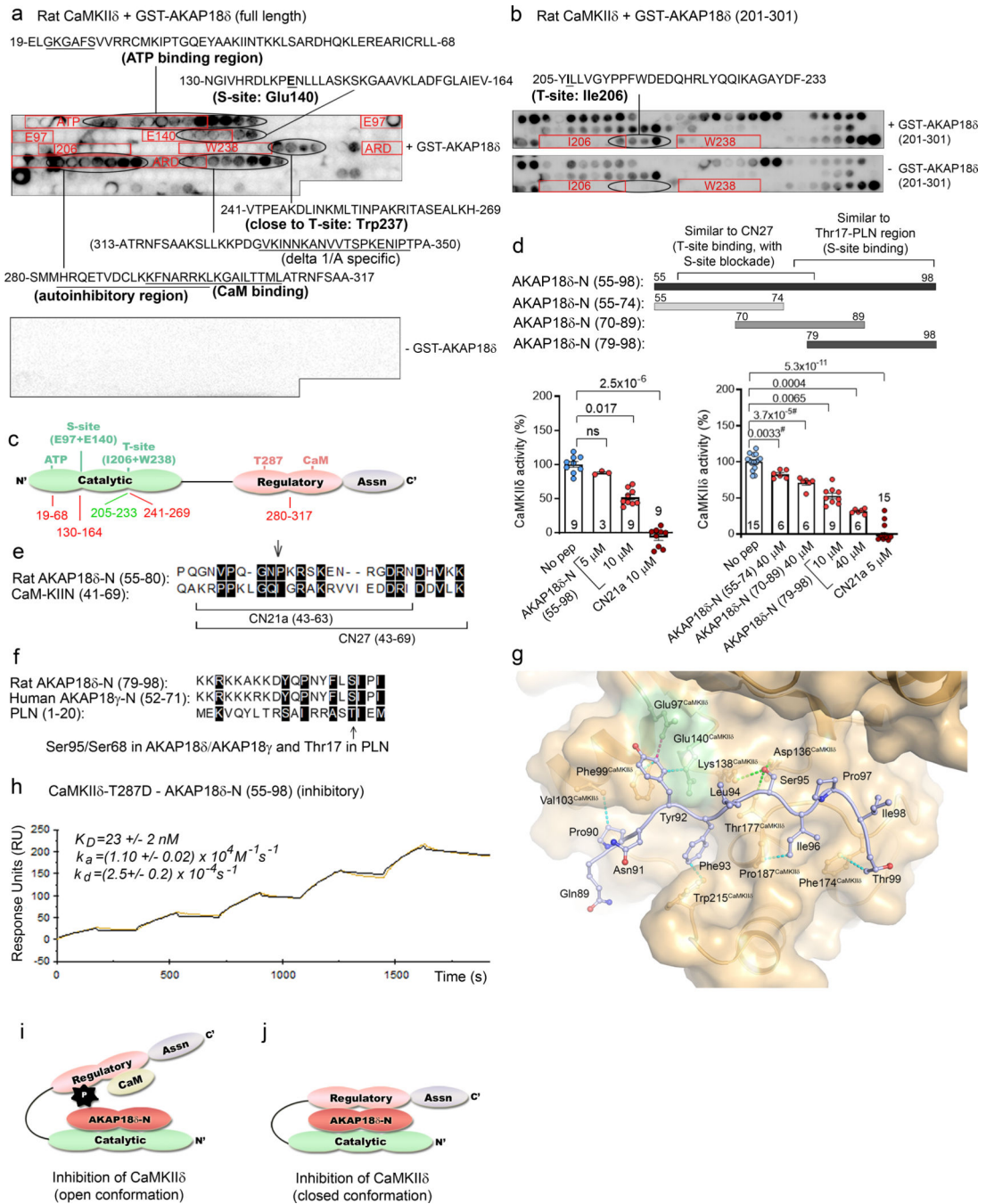


**Figure 1. AKAP18δ anchors CaMKIIδ to the PLN-SERCA2 complex and controls Thr17-PLN phosphorylation.**

(a) Immunoprecipitation of CaMKIIδ-AKAP18δ from adult cardiomyocyte lysate detected by immunoblotting. Rabbit IgG was used as control. (b) High-resolution imaging of SERCA2-AKAP18δ (upper) and SERCA2-CaMKIIδ (lower) in adult mouse cardiomyocytes using anti-SERCA2, anti-AKAP18δ and anti-CaMKIIδ. Single images and corresponding traces are shown in middle and at right. Z/ M-lines are indicated. Scale bars=10 μm. (c) pThr17-PLN, pSer16-PLN and PLN levels in ISO-stimulated

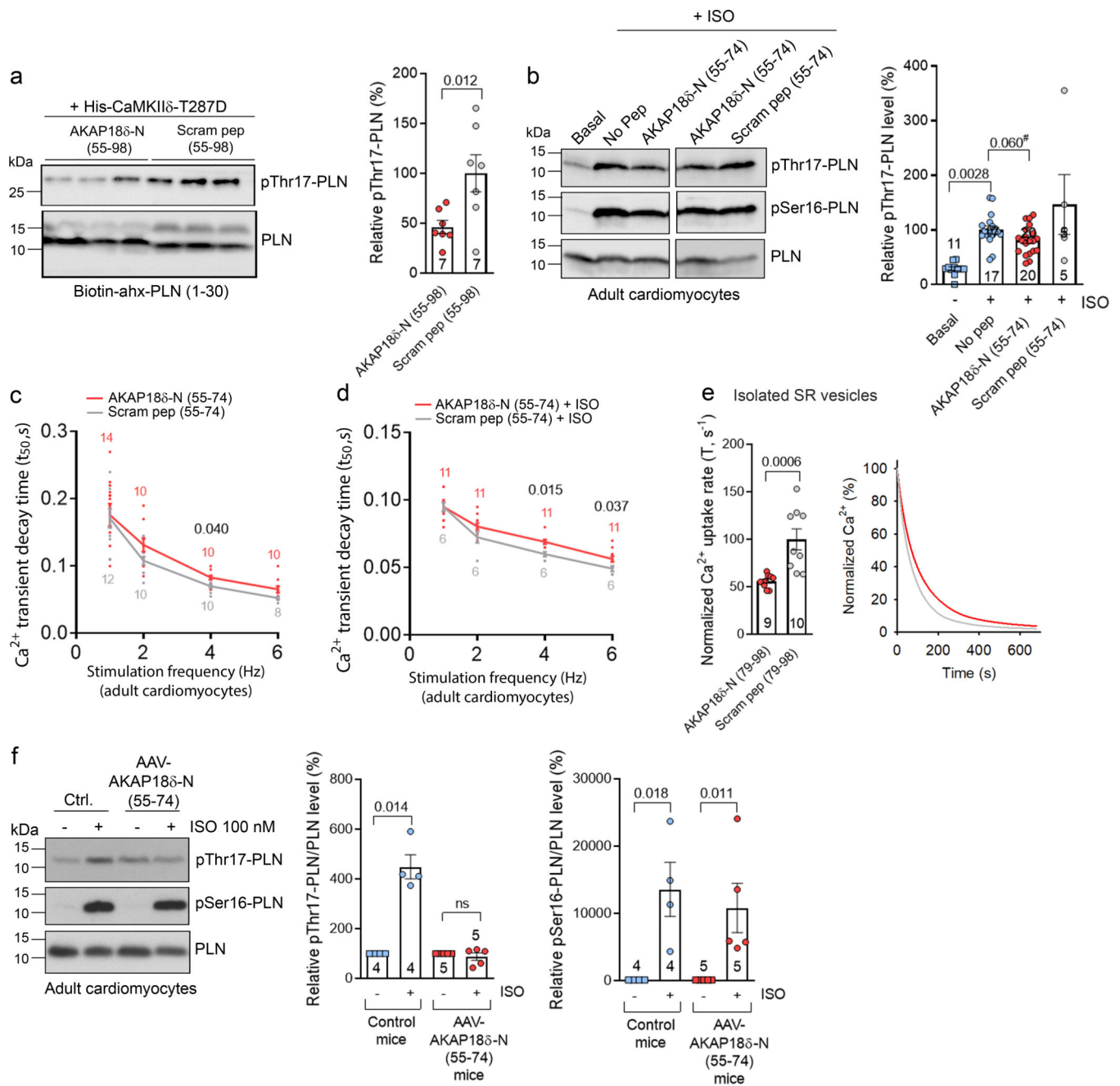
adult cardiomyocytes pre-treated with cell-permeant AKAP18 $\delta$ -PLN competitor or control peptide. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by the linear mixed effect model from the R nlme package with Tukey's post hoc correction (n=11, 4 rats). **(d)** Illustration of the AKAP18 $\delta$ -PLN competitor experiment. Left: Without ISO, PLN is dephosphorylated and inhibits SERCA2 activity. Middle: During ISO-stimulation, AKAP18 $\delta$ -associated CaMKII $\delta$  (and PKA) phosphorylates PLN, leading to SERCA2 activation and Ca<sup>2+</sup> uptake into SR. Right: In the presence of the cell-permeant AKAP18 $\delta$ -PLN competitor peptide (CP)<sup>14</sup>, AKAP18 $\delta$  displaces from PLN-SERCA2. AKAP18 $\delta$ -associated CaMKII $\delta$  is no longer able to phosphorylate PLN. Both AKAP18 $\delta$ <sup>62</sup> and PLN locate to membrane, but this is not shown for simplicity. **(e)** Analyses of biotin-ahx-PLN (1–30), biotin-ahx-pSer16-PLN (1–30) and biotin-ahx-pThr17-PLN (1–30) binding to GST-AKAP18 $\delta$  (coated in wells) by an ELISA-based assay. Binding was detected with a biotin-HRP conjugated antibody and incubation with Ultra TMB. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=8). **(f)** Analysis of AKAP18 $\delta$ -CaMKII $\delta$  interaction by AlphaScreen<sup>TM</sup>. GST-AKAP18 $\delta$  was incubated with increasing concentrations of recombinant CaMKII $\delta$ -T287D or CaMKII $\delta$ -T287A. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=14–15). **(g)** The two CaMKII $\delta$  binding regions in AKAP18 $\delta$  are illustrated in red and green. **(h)** Residues important for CaMKII $\delta$  binding were identified by overlaying 20-mer overlapping AKAP18 $\delta$  peptides spot-synthesized on membranes with active His-CaMKII $\delta$  and immunoblotting. Immunoblotting without His-CaMKII $\delta$  was used as control (lower panel). Underlined sequences were synthesized as soluble peptides for further experiments. **(i-j)** The two CaMKII $\delta$  binding regions (red and green) are indicated in the alignment of rat AKAP18 $\delta$ , human and mouse AKAP18 $\gamma$ , and the smaller AKAP18 $\alpha$  and AKAP18 $\beta$ . Black boxes indicate identical amino acids (DNA Star).





**Figure 2. AKAP18 $\delta$  binds CaMKII $\delta$  through multiple sites and inhibits CaMKII $\delta$  through sequences similar to the natural CaMKII inhibitor protein and Thr17-PLN region.** AKAP18 $\delta$  binding was identified by overlaying 20-mer overlapping CaMKII $\delta$  peptides with (a) GST-AKAP18 $\delta$  or (b) GST-AKAP18 $\delta$  (201–301) and immunoblotting with anti-GST-HRP. Immunoblotting without recombinant protein (lower panel) or GST (Suppl. Fig. 2a, lower panel) was used as control. Peptides containing ATP binding region<sup>7</sup> (ATP binding motif underlined), S-site (E97 and E140-containing sequences), T-site (I206 and W238-containing sequences) or autoregulatory domain (ARD) are boxed. Stretches of spots that

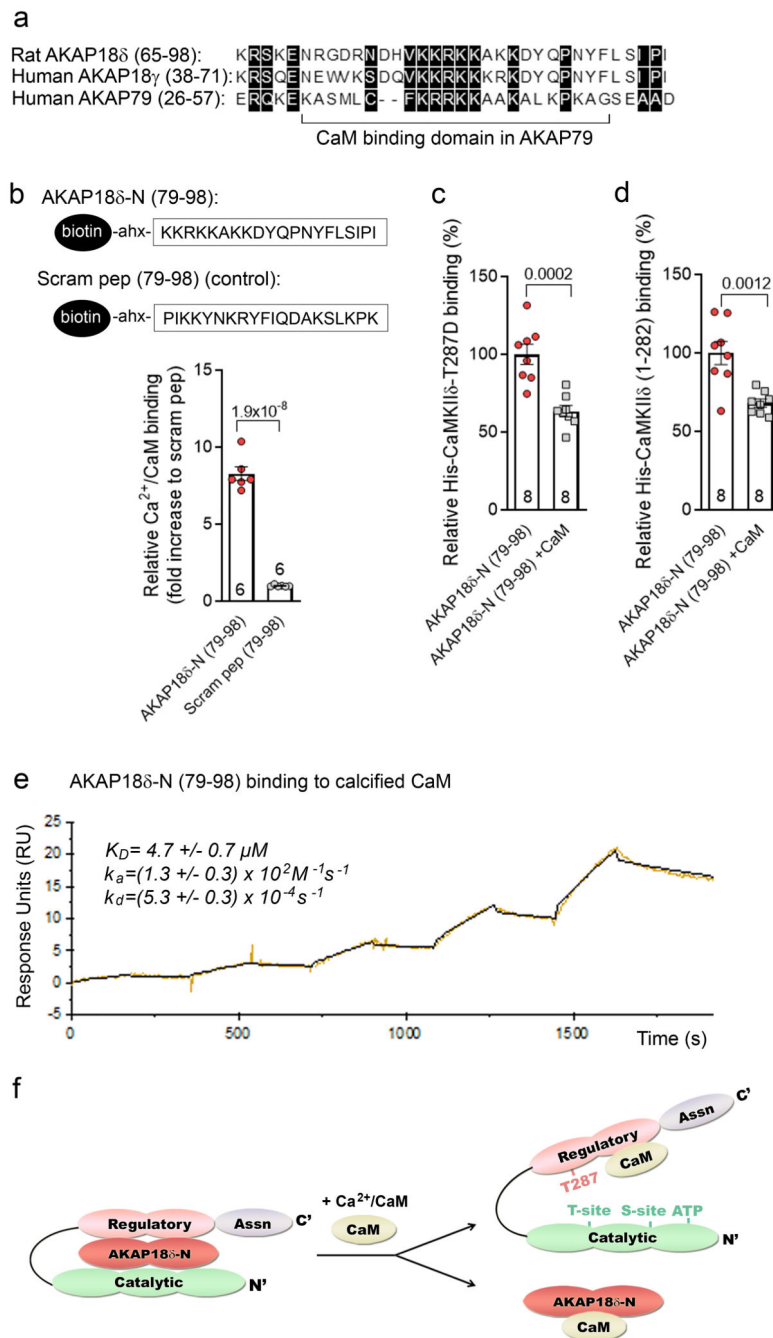
were not apparent in negative controls were regarded as potential binding sites. Sequences for spots with strongest signal are given. Autoinhibitory region and CaM binding site are underlined <sup>7</sup>. The CaMKII $\delta_{1/A}$ -specific sequence is only present in neonatal mouse hearts <sup>63</sup>. Amino acids 205–233 (upper panel in **b**) was regarded as potential binding site, since it was absent in negative control (lower panel). (**c**) The five AKAP18 $\delta$  binding regions identified by peptide arrays (in **a-b**) are indicated in CaMKII $\delta_{2/C}$  (inhibitory ones in red and activating in green). (**d**) Effect of different AKAP18 $\delta$ -N sequences on CaMKII $\delta$ -T287D activity (<sup>32</sup>P incorporation into syntide). CN21a derived from natural CaMKII inhibitor protein (CaM-KIIN) <sup>21</sup> was used as control. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=3–9 in left panel, n=6–15 in right panel). # Significant differences were detected by Mann-Whitney test. Upper panel illustrates the different AKAP18 $\delta$ -N peptides. (**e**) Alignment of rat AKAP18 $\delta$  (55–80) with the natural CaMKII inhibitor protein (amino acids 41–69). CN27 and CN21a are indicated <sup>21</sup>. Arrow denotes a proline in AKAP18 $\delta$ . (**f**) Alignment of rat AKAP18 $\delta$  (79–98) and human AKAP18 $\gamma$  (52–71) with PLN (1–20). Arrow indicates Ser68/Ser95 in AKAP18 $\gamma$ /AKAP18 $\delta$  and Thr17 in PLN. Black boxes indicate identical or functionally similar amino acids (DNA Star). (**g**) Structural model of the AKAP18 $\delta$  (89-QPNYFLSIPIT-99) binding to CaMKII $\delta$  centered at Ser95 (corresponds to Thr17 in PLN). AKAP18 $\delta$  is shown as a ball-and-stick model, while CaMKII $\delta$  peptide backbone is shown as a cartoon with central residues as a ball-and-stick motif. The negatively charged patch around S-site residues Glu97 and Glu140 is colored light green. Rest of the transparent CaMKII $\delta$  surface is shown in yellow. (**h**) SPR analysis of immobilized biotin-ahx-AKAP18 $\delta$  (55–98) on an SA chip and recombinant CaMKII $\delta$ -T287D injected at a range of concentrations (47.6–500 nM) (n=3). (**i-j**) Illustration of CaMKII $\delta$  inhibition by AKAP18 $\delta$ -N. AKAP18 $\delta$ -N (red) binds to catalytic region (light green) and inhibits CaMKII $\delta$  in both an (**i**) open and (**j**) closed conformation after pThr287 dephosphorylation and CaM dissociation.



**Figure 3. AKAP188-N reduces pThr17-PLN and inhibits Ca<sup>2+</sup> reuptake into SR.**

(a) CaMKII $\delta$  phosphorylation of biotin-ahx-PLN (1–30), in the presence of AKAP188-N (55–98) or a scrambled control peptide. Phosphorylated Thr17-PLN was observed at 25 kDa consistent with induced oligomerization<sup>64</sup> (Suppl. Fig. 3a, complete immunoblots). Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired t-test (n=7). (b) Adult cardiomyocytes were treated with TAT-AKAP188-N (55–74) or the scrambled control for 45 min before ISO-stimulation (15 min) and immunoblotted with pThr17-PLN, pSer16-PLN and PLN antibodies. pThr17-PLN was quantified against PLN or GAPDH. Normal distribution was confirmed by Shapiro-Wilk test (not tested for

scram pep with  $n < 6$ ). Significant differences were examined by nested one-way ANOVA with Tukey's multiple comparisons test ( $n = 5-20$ , 2-6 rats). #Significant differences were examined by nested t-test. The effect of TAT-AKAP188-N (55-74) on decay time of  $Ca^{2+}$  transients at different stimulation frequencies of adult cardiomyocytes in the (c) absence or (d) presence of ISO. Scrambled TAT-AKAP188-N (55-74) peptide was used as control. Normal distribution was confirmed by Shapiro-Wilk test (in c) and Kolmogorov-Smirnov or D'Agostino & Pearson test (in d, except for the scrambled peptide at 1 Hz). Significant difference was examined by nested t-test,  $n = 8-14$  in c (2-4 rats) and  $n = 6-11$  in d (3-4 rats). Representative  $Ca^{2+}$  tracings are shown in Suppl. Fig. 3e-f. (e) The effect of TAT-AKAP188-N (79-98) or a scrambled control on SERCA2  $Ca^{2+}$  reuptake rate in isolated mouse SR vesicles (left ventricle crude homogenate). Normal distribution was confirmed by D'Agostino & Pearson test. Significant differences were examined by unpaired t-test,  $n = 9-10$ . (f) Levels of pThr17-PLN, pSer16-PLN and PLN in adult cardiomyocytes isolated from control (WT) and AAV-AKAP188-N (79-98) mice, treated with or without ISO. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test ( $n = 4-5$ , 3-4 mice in each group).



**Figure 4. Calcified CaM binds to AKAP18 $\delta$ -N and outcompetes the inhibitory AKAP18 $\delta$ -N-CaMKII $\delta$  interaction.**

(a) Alignment of rat AKAP18 $\delta$ -N (65–98) and human AKAP18 $\gamma$  (38–71) with the CaM-binding domain in AKAP79<sup>25</sup>. Black boxes indicate identical or functional similar amino acids (DNA Star). (b) Binding of biotin-ahx-AKAP18 $\delta$ -N (79–98) or a scrambled control to Ca<sup>2+</sup>/CaM (coated in wells) analyzed by an ELISA-based method. Normal distribution was confirmed by Kolmogorov-Smirnov test. Significant differences were examined by unpaired t-test (n=6). Biotinylated AKAP18 $\delta$ -N (79–98) peptide was incubated with or

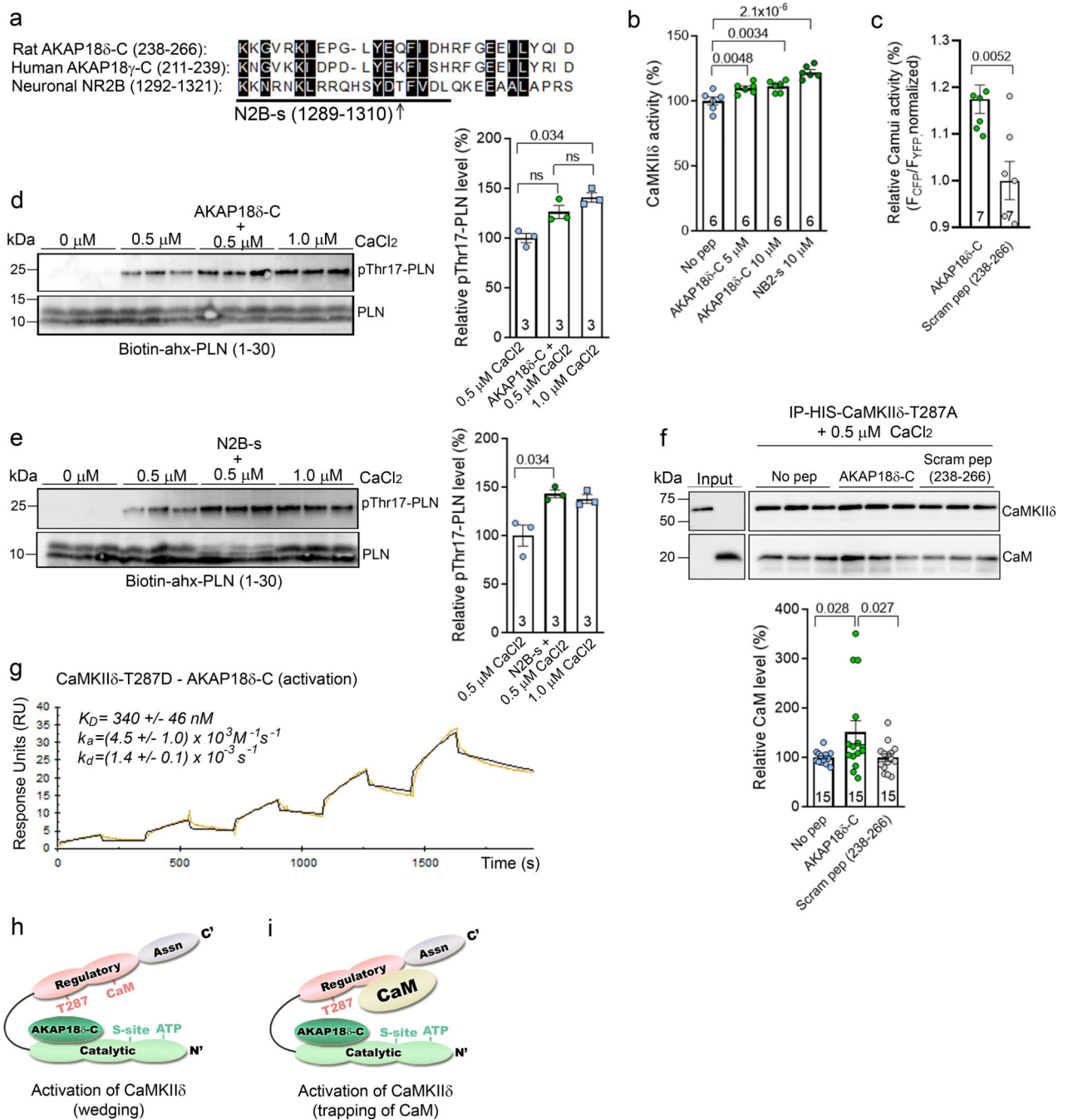
without CaM in wells coated with recombinant **(c)** CaMKII $\delta$ -T287D or **(d)** CaMKII $\delta$  (1–282). Binding was detected with anti-biotin-HRP. Normal distribution was confirmed by Shapiro-Wilk test **(c-d)**. Significant differences were examined by unpaired t-test (n=8 in **c-d**). **(e)** SPR analysis of immobilized biotin-ahx-AKAP18 $\delta$  (79–98) and recombinant CaM injected (952.6–10000 nM) (n=3). **(f)** CaM (beige) outcompetes the inhibitory AKAP18 $\delta$ -N-CaMKII $\delta$  interaction upon binding to the CaMKII $\delta$  regulatory region (pink) and AKAP18 $\delta$ -N (red).

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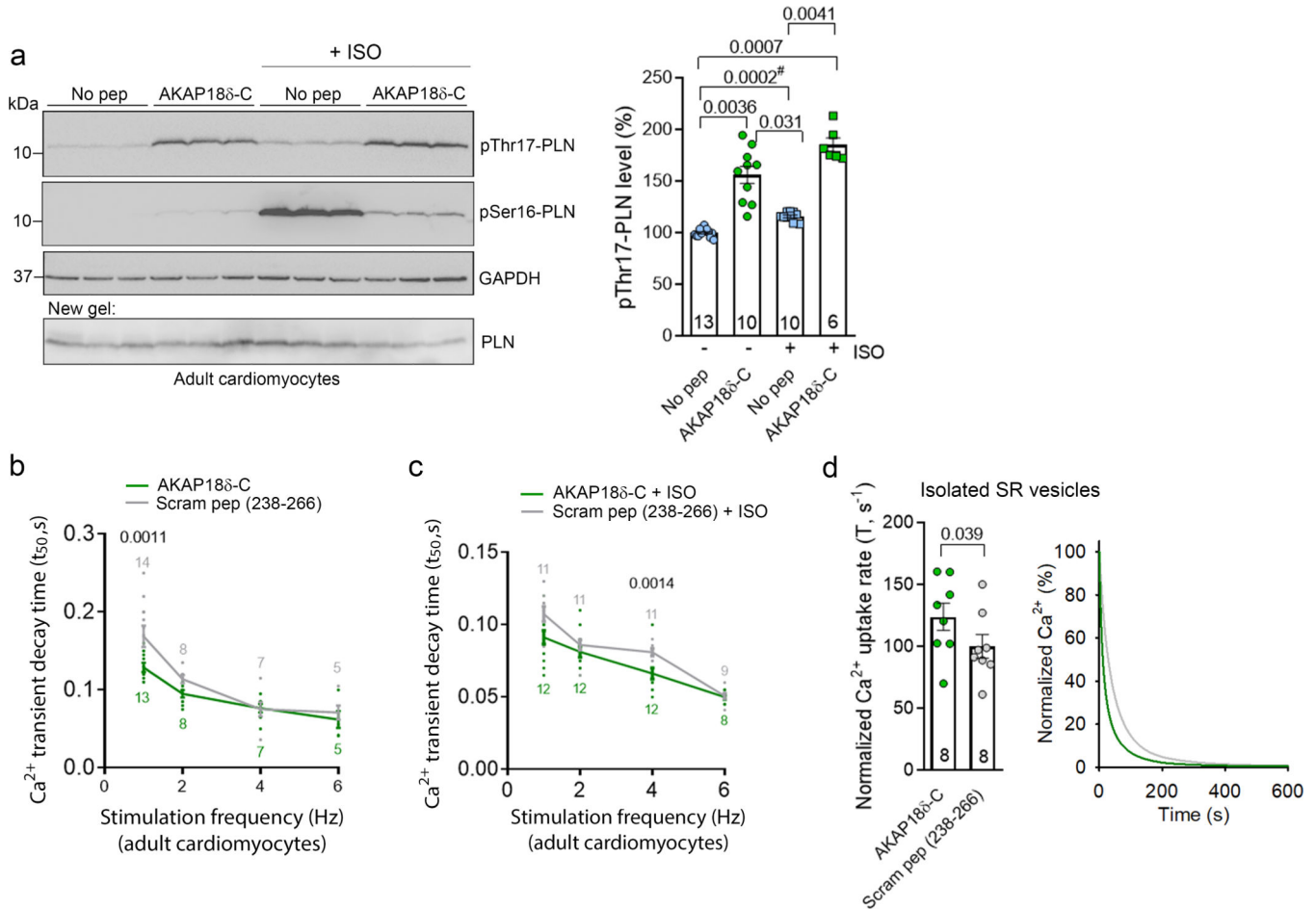


**Figure 5. AKAP18 $\delta$ -C is homologous to the neuronal CaMKII $\alpha$  activator N2B-s and lowers the Ca<sup>2+</sup> threshold for CaMKII $\delta$  activation.**

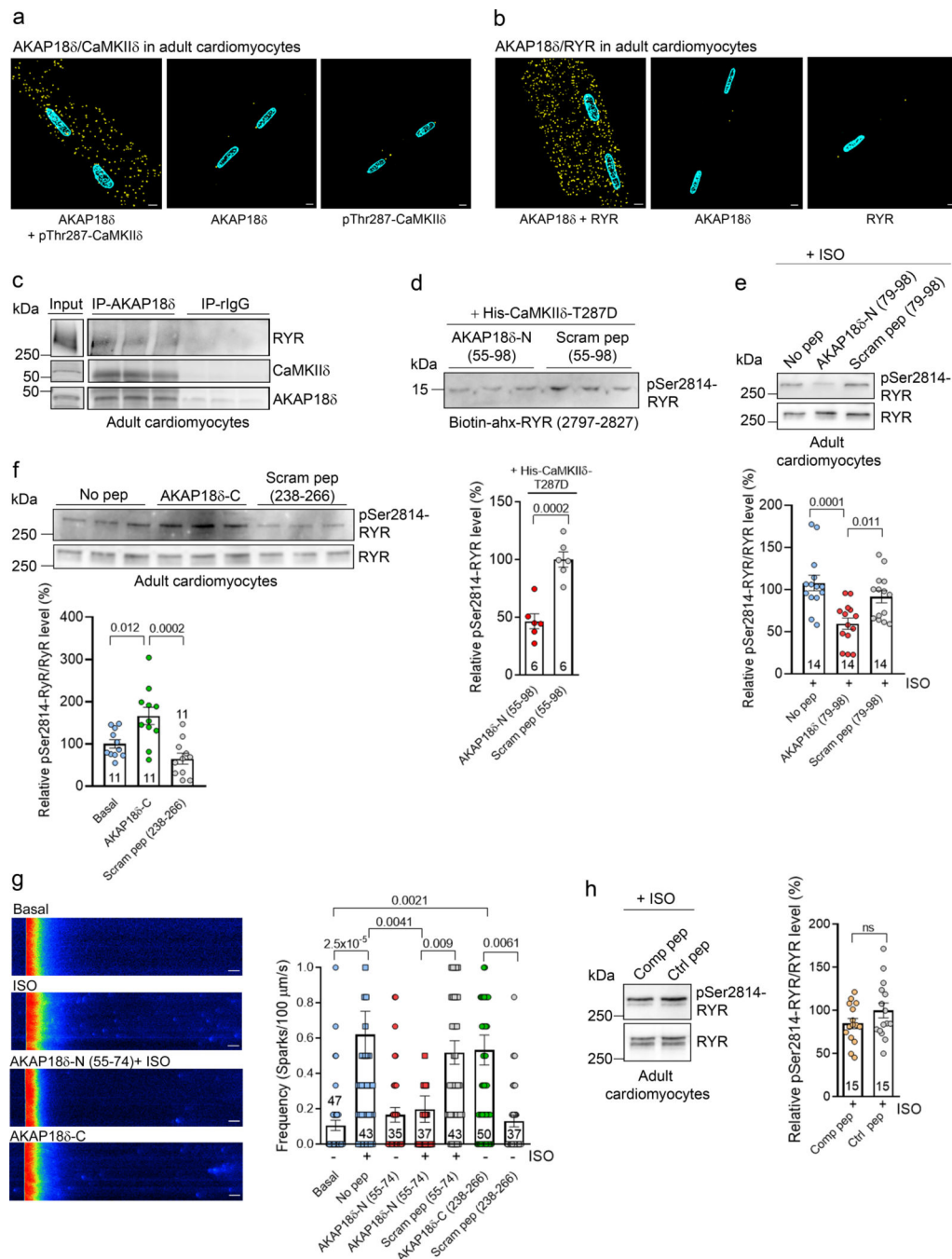
(a) Alignment of AKAP18 $\delta$ -C (238–266) and AKAP18 $\gamma$ -C (211–239) with CaMKII $\alpha$  binding site in neuronal NMDA receptor NR2B subunit (amino acids 1289–1321)<sup>18</sup>. N2B-s (1292–1310) is underlined. Arrow denotes CaMKII $\alpha$  phosphorylation site in NR2B (Thr1306). Black boxes indicate identical or functionally similar amino acids. (b) Effect of AKAP18 $\delta$ -C on CaMKII $\delta$ -T287A activity analyzed in a CaMKII kinase assay in the absence of CaM (<sup>32</sup>P incorporation into syntide). N2B-s<sup>18</sup> and CN21a<sup>21</sup> were used as controls. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences

were examined by ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test (n=6). (c) Effect of TAT-AKAP188-C on a Camui FRET-based bio-sensor<sup>28</sup>, transduced into adult rabbit ventricular myocytes. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired t-test (n=7). Immunoblot analysis of CaMKII $\delta$ -catalyzed phosphorylation of Thr17-PLN at 0.5  $\mu$ M CaCl<sub>2</sub> with or without the presence of (d) AKAP188-C or (e) N2B-s. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (d-e). (f) Immunoblot analyses of immunoprecipitations of CaM with CaMKII $\delta$ -T287A in the absence or presence of AKAP188-C (n=15). Normal distribution was confirmed by D'Agostino & Pearson test, and significant differences were examined by ordinary one-way ANOVA with Dunnett's multiple comparisons test. (g) SPR analysis of immobilized biotin-ahx-AKAP188-C and recombinant CaMKII $\delta$ -T287D injected (95.2–1000 nM) (n=3). (h-i) AKAP188-C (dark green) binds to catalytic region of CaMKII $\delta$  (light green) and activates CaMKII $\delta$  by (h) lowering the Ca<sup>2+</sup> threshold for activation by keeping the inhibitory gate open and (i) trapping CaM (beige) within the kinase.



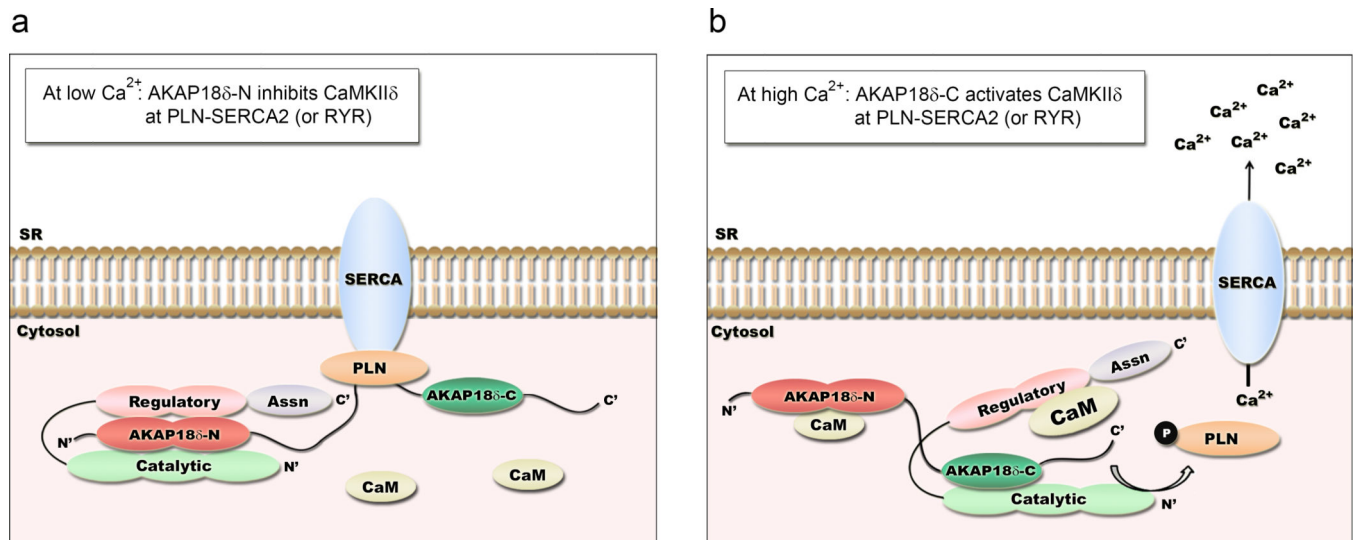


**Figure 6. AKAP188-C increases pThr17-PLN and facilitates faster  $\text{Ca}^{2+}$  reuptake into SR.** (a) Effect of TAT-AKAP188-C on pThr17-PLN in basal and during ISO-stimulation (5 min) in adult cardiomyocytes. pThr17-PLN, pSer16-PLN and PLN levels were detected by immunoblotting. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by nested one-way ANOVA with Tukey's multiple comparisons test ( $n=6-13$ , 4 rats). Effect of TAT-AKAP188-C or a scramble control on decay time of  $\text{Ca}^{2+}$  transients across a range of stimulation frequencies in adult cardiomyocytes in (b) absence or (c) presence of ISO. Normal distribution was confirmed by Shapiro-Wilk test (not tested for the 6 Hz data set with  $n<6$ ). Significant difference was examined by nested t-test,  $n=5-14$  in b (3-4 rats) and  $n=8-12$  in c (5-6 rats). Representative tracings are shown in Suppl. Fig. 6c-d. (d) Effect of TAT-AKAP188-C or a scrambled control on  $\text{Ca}^{2+}$  reuptake rate in isolated mouse SR vesicles (left ventricle crude homogenate). Significant differences were examined by Wilcoxon matched-pairs signed rank test.



**Figure 7. AKAP186 anchors and functionally regulates CaMKII $\delta$  activity also at RYR.** *In situ* proximity ligation assay of (a) AKAP186-pThr287-CaMKII $\delta$  and (b) AKAP186-RYR (yellow dots in left panels) in adult cardiomyocytes (see method section for detailed description). Incubations with only anti-AKAP186, anti-pThr287-CaMKII $\delta$  or anti-RYR were used as negative controls (middle and right panels). Positive control for the assay is shown in Suppl. Fig. 7a. Scale bars=5  $\mu$ m. (c) Immunoprecipitation of AKAP186-CaMKII $\delta$ -RYR in adult cardiomyocyte lysate detected by immunoblotting. (d) CaMKII $\delta$  phosphorylation of biotin-ahx-RYR (2797–2827), with or without presence of

AKAP188-N (55–98) or the scrambled control. Biotin-ahx-pSer2814-RYR was detected by immunoblotting. Normal distribution was confirmed by Shapiro-Wilk test, and significant differences were examined by unpaired t-test  $n=6$ . pSer2814-RYR and RYR levels in adult cardiomyocytes treated with **(e)** TAT-AKAP188-N (79–98) in presence of ISO or **(f)** AKAP188-C (238–266) in absence of ISO. The respective scrambled peptides were used as controls. Normal distribution was confirmed by Shapiro-Wilk test (in **e-f**). Significant differences examined by the linear mixed effect model from the R nlme package with Tukey's post hoc correction ( $n=14$ , 5 rats in **e**, and  $n=11$ , 4 rats in **f**). **(g)** Line scans and  $Ca^{2+}$  sparks of cardiomyocytes treated with or without ISO, TAT-AKAP188-N (55–74), AKAP188-C (238–266) or the respective scrambled control peptides. Significant differences examined by the linear mixed effect model from the R nlme package with Tukey post hoc correction ( $n=35-50$ , 5 rats). Scale bars=150 ms. **(h)** Immunoblotting of pSer2814-RYR and RYR in ISO-stimulated cardiomyocytes pre-treated with the cell-permeant AKAP188-PLN competitor or control peptide. Normal distribution was confirmed by Shapiro-Wilk test  $N_s$ ; not significant, examined by nested t-test ( $n=12$ , 5 rats).



**Figure 8. A model where the two unique regions in AKAP186 fine-tune CaMKII $\delta$  activation at PLN-SERCA2 (or RYR).**

(a) At low  $\text{Ca}^{2+}$  transient frequency, AKAP186-N (red) inhibits CaMKII $\delta$  by binding to the regulatory domain (pink), and ATP-binding pocket, T- and S-sites in the catalytic domain (green). No Thr17-PLN (or Ser2814-RYR) phosphorylation results. (b) When the  $\text{Ca}^{2+}$  transient frequency increases, accumulated calcified CaM (beige) outcompetes the inhibitory AKAP186-CaMKII $\delta$  interaction by binding to the regulatory domain (pink) and AKAP186-N (red). AKAP186-C (dark green) binds to the released T-site in CaMKII $\delta$  and lowers the  $\text{Ca}^{2+}$  threshold for kinase activation, by keeping the inhibitory gate open and trapping CaM within CaMKII $\delta$ . CaMKII $\delta$  catalyzes further Thr17-PLN (or Ser2814-RYR) phosphorylation, leading to reduced PLN association and faster SR  $\text{Ca}^{2+}$  reuptake by SERCA2 (or  $\text{Ca}^{2+}$  release by RYR). It is also possible that AKAP186-N and AKAP186-C regulate two different CaMKII molecules. Assn; association domain.