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AKAP18δ 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) Ca^{2+}-ATPase 2 (SERCA2) mediates Ca^{2+} reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas the ryanodine receptor (RYR) mediates Ca^{2+} release from SR and triggers contraction. 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 CaMKIIδ anchoring to SERCA2-PLN and RYR and its regulation by local Ca^{2+} signals remain elusive. The objective of this study was to investigate CaMKIIδ anchoring and regulation at SERCA2-PLN and RYR.

Methods: A role for A-kinase anchoring protein 18δ (AKAP18δ) in CaMKIIδ 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 AKAP18δ anchors and directly regulates CaMKIIδ activity at SERCA2-PLN and RYR, via two distinct AKAP18δ regions. An N-terminal region (AKAP18δ-N) inhibited CaMKIIδ through binding of a region homologous to natural CaMKII inhibitor peptide and Thr17-PLN region. AKAP18δ-N also bound CaM, introducing a second level of control. Conversely, AKAP18δ-C, which shares homology to neuronal CaMKIIα activator peptide (N2B-s), activated CaMKIIδ by lowering the apparent Ca^{2+} threshold for kinase activation and inducing CaM trapping. While AKAP18δ-C facilitated faster Ca^{2+} reuptake by SERCA2 and Ca^{2+} release through RYR, AKAP18δ-N had opposite effects. We propose a model where the two unique AKAP18δ regions fine-tune Ca^{2+}-frequency-dependent activation of CaMKIIδ at SERCA2-PLN and RYR.

Conclusions: AKAP18δ anchors and functionally regulates CaMKII activity at PLN-SERCA2 and RYR, indicating a crucial role of AKAP18δ 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 AKAP18δ also as a CaM-KAP.

Graphical Abstract

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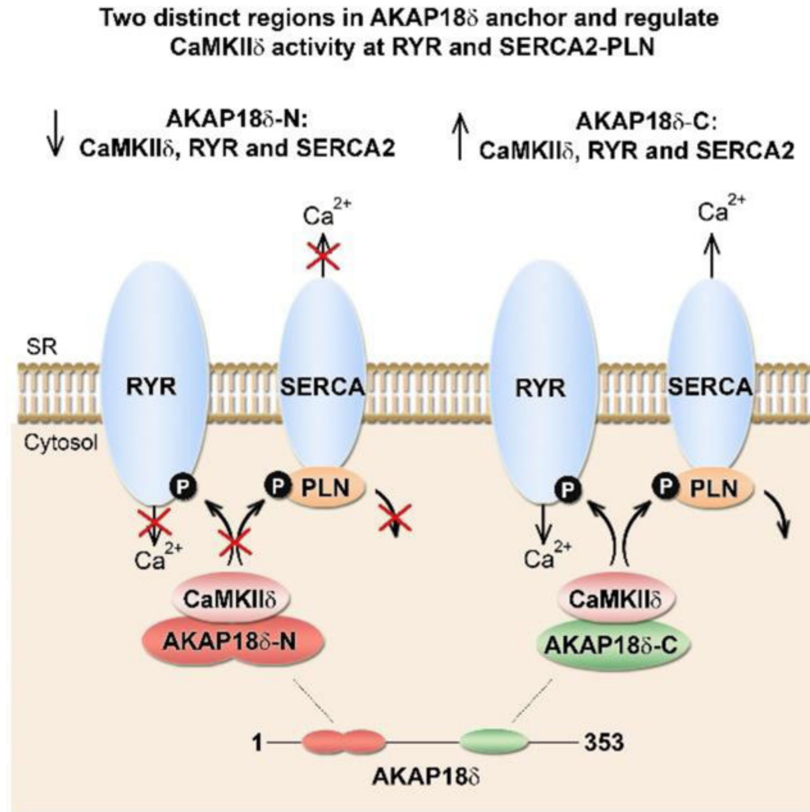

Keywords

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

INTRODUCTION

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

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

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

CaMKII forms a dodecamer that comprises two stacked 6-fold symmetric rings [6]. The CaMKII monomer contains an N-terminal ATP-binding pocket, catalytic and autoregulatory domains, and a C-terminal association domain mediating its oligomerization [7]. When CaMKIIδ is in its inactive state, the Thr287 segment (Thr286 in CaMKIIα) 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 [7] (Suppl. Fig. 1a). Upon activation, Ca^{2+}/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) CaMKIIδ molecule. Thus, binding of at least two CaM molecules is required for CaMKII autophosphorylation. Autophosphorylation of Thr287 increases CaMKII affinity for CaM [8], maintains the kinase in an autonomously active state [9] (Suppl. Fig. 1c), and permits the kinase to translate the frequency of Ca^{2+} spikes into kinase activity *in vitro,* [10] a form of molecular memory or integration.

Although CaMKIIδ regulates several aspects of ECC, it remains unclear how the kinase is functionally regulated within the distinct nanodomains where these Ca^{2+} handling proteins are localized. For example, local [Ca^{2+}] 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^{2+}] levels [11]. Although activated CaMKIIδ in myocytes is more mobile than traditionally thought [12], another potential explanation for PLN phosphorylation by CaMKIIδ is that as yet unidentified CaM-Kinase Anchoring Proteins (CaM-KAPs) could enable locally higher Ca^{2+} sensitivity of the kinase.

We postulated that the A-kinase anchoring protein, AKAP18δ (also known as AKAP7δ), could perform such a CaMKIIδ anchoring and regulatory role at PLN-SERCA2 and RYR. Originally identified in rat kidney [13], AKAP18δ has been shown to interact directly with PKA and PLN, thereby enabling PKA-dependent phosphorylation at Ser16-PLN and augmentation of SERCA2 activity [14]. In human myocardium, the AKAP18δ orthologue, AKAP18γ, similarly complexes with PKA, PLN-SERCA2 and phosphodiesterase 3A1 (PDE3A1) to control Ser16-PLN phosphorylation and SR Ca^{2+} reuptake [15]. In the present work, we show that AKAP18δ also anchors CaMKIIδ to PLN-SERCA2 and RYR, defining AKAP18δ as the first CaMKII anchoring protein. We further identify two unique regions in AKAP18δ that inversely regulate CaMKIIδ activity, CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and thus SERCA2 and RYR functional activities. This capability is enabled by one region of AKAP18δ which binds CaM and inhibits CaMKII activation, and a second region which lowers the Ca^{2+} threshold for CaMKIIδ activation and induces CaM trapping. Based on our results we propose a model in which the two

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AKAP18δ regions fine-tune the Ca^{2+}-frequency-dependent activation of CaMKIIδ 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

AKAP18δ-associated CaMKIIδ controls Thr17-PLN phosphorylation.

First, we tested whether CaMKIIδ and AKAP18δ interact at SERCA2-PLN. Immunoprecipitation analysis revealed co-precipitation of CaMKIIδ and SERCA2 with AKAP18δ in adult cardiomyocyte lysate (Fig. 1a). High-resolution imaging of adult cardiomyocytes further demonstrated that AKAP18δ and CaMKIIδ (Fig. 1b, blue, upper and lower respectively) co-localized with SERCA2 (red) at Z-line. AKAP18δ also co-localized with SERCA2 at M-line where little CaMKIIδ was observed, indicating a role for AKAP18δ 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 α-actinin (Suppl. Fig. 1d). Secondary

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antibody controls and specificity of CaMKIIδ and AKAP18δ antibodies are shown in Suppl. Fig. 1e–g.

To analyze whether AKAP18δ-associated CaMKIIδ controls pThr17-PLN phosphorylation, AKAP18δ was displaced from PLN using a cell-permeant AKAP18δ-PLN competitor peptide [14]. Adult and neonatal cardiomyocytes treated with this peptide before isoproterenol (ISO)-stimulation, exhibited reduced pThr17-PLN (and pSer16-PLN as previously reported [14]) compared to control (Fig. 1c and Suppl. Fig. 1h, respectively), indicating that AKAP18δ-associated CaMKIIδ 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-AKAP18δ binding (Fig. 1e), closely paralleling reported effects of Ser16 phosphorylation [14]. Thus, Thr17 phosphorylation seems to provide an on/off mechanism for the AKAP18δ-PLN interaction.

Taken together, the data strongly support that AKAP18δ anchors CaMKIIδ to PLN-SERCA2, and thereby controls CaMKIIδ-mediated Thr17-PLN phosphorylation.

CaMKIIδ binds directly to two unique regions in AKAP18δ.

The CaMKIIδ-AKAP18δ interaction was further investigated using AlphaScreen™ technology. Only recombinant CaMKIIδ-T287D (mimicking active kinase) [16] and not CaMKIIδ-T287A (mimicking inactive kinase) (mutated proteins are validated in Suppl. Fig. 1k–l) was found to bind to AKAP18δ (Fig. 1f), indicating that AKAP18δ binds to autophosphorylated CaMKIIδ.

To identify CaMKIIδ binding sites, AKAP18δ-YFP variants (Fig. 1g) were co-expressed with CaMKIIδ-T287D. CaMKIIδ precipitated all AKAP18δ variants, except for GFP-AKAP18δ (301–353) (Suppl. Fig. 1m), indicating that CaMKIIδ 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 [14]) and N-terminally from the PKA binding domain (amino acids 301–314 [13]). In addition, AKAP18δ-YFP and GFP-AKAP18δ (67–353) precipitated more strongly with CaMKIIδ compared to other AKAP18δ variants, consistent with a second CaMKIIδ binding site towards the N-terminus of AKAP18δ (Fig. 1g, red).

To more precisely identify CaMKIIδ binding, rat AKAP18δ was spot-synthesized as 20-mer overlapping peptides on membranes and incubated with active His-CaMKIIδ. Immunoblotting identified CaMKIIδ binding to two regions; amino acids 55–98 (AKAP18δ-N, in red) and 238–266 (AKAP18δ-C, in green) (Fig. 1h) and two homologous regions in human AKAP18γ (Suppl. Fig. 1n). CaMKIIδ-T287D (coated in wells) binding was confirmed by an ELISA-based method using biotinylated peptides spanning the two AKAP18δ regions (Suppl. Fig. 1o–p). Sequence alignments showed that the two CaMKIIδ-binding regions were only present in AKAP18δ and AKAP18γ and not in the shorter AKAP18α and AKAP18β isoforms [13] (Fig. 1i–j).

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AKAP18δ binds CaMKIIδ through multiple sites.

We next sought to define AKAP18δ binding in CaMKII$\delta_{C/2}$, which is largely cytoplasmic and regulates ECC. CaMKIIδ spot-synthesized as 20-mer overlapping peptides on membranes which were overlaid with GST-AKAP18δ (Fig. 2a). AKAP18δ bound to the ATP-binding region [7] (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) [7] (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) [17] (Fig. 2a, boxed regions in upper panel). Consistent with the above results, a biotinylated peptide covering the N-terminal region of AKAP18δ (55–98; Suppl. Fig. 2b) showed an almost identical CaMKIIδ binding pattern as GST-AKAP18δ.

CaMKIIδ binding of biotin-ahx-AKAP18δ-C was too weak or dynamic to be detected by peptide arrays. However, overlaying a larger recombinant GST-AKAP18δ-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-AKAP18δ full length protein, suggesting that GST-AKAP18δ (201–301) exhibits differential folding. These data are consistent with AKAP18δ-C and AKAP18δ-N binding to distinct T-site sequences. ELISA-based experiments confirmed AKAP18δ binding to the autoregulatory domain, ATP binding region, S-site and two T-site regions of CaMKIIδ (Suppl. Fig. 2c–e), supporting the interpretation of the overlay data. The five AKAP18δ 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 AKAP18δ-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 [18–20], e.g. densin, which binds to several T-site sequences [19].

AKAP18δ-N inhibits CaMKIIδ through sequences similar to the natural CaMKII inhibitor protein and Thr17-PLN region.

AKAP18δ-N (55–98) interacted only with activated CaMKIIδ (Suppl. Fig. 2f). The effect of AKAP18δ-N on CaMKIIδ activity was analyzed in an *in vitro* kinase assay. AKAP18δ-N (55–98) reduced CaMKIIδ-catalyzed phosphorylation of syntide (a CaMKII substrate) by 50 % under high [Ca^{2+}] 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 [21] (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 [21]. In an ELISA-based assay, CN27 outcompeted the AKAP18δ-N (55–74)-CaMKIIδ interaction, indicating that CN27 and AKAP18δ-N (55–74) may bind to the same site in CaMKIIδ (Suppl. Fig. 2g). In addition, AKAP18δ-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 [19], which might explain its weaker effect on CaMKIIδ activity.

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CaMKIIδ binding of the more inhibitory amino acids 79–98 of AKAP18δ (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 $Ser95^{AKAP18\delta}$ corresponding to $Thr17^{PLN}$ (Fig. 2f). Modeled placement of AKAP18δ (89–99) into the peptide binding groove of CaMKIIδ centered on Ser95 in AKAP18δ (Fig. 2g), and suggested several favorable interactions between CaMKIIδ and AKAP18δ. Hydrophobic interactions between $Pro90\text{-}Val103^{CaMKII\delta}$, $Tyr92\text{-}Phe138^{CaMKII\delta}$, $Phe93\text{-}Trp215^{CaMKII\delta}$, $Ile96\text{-}Pro187^{CaMKII\delta}$ and $Thr99\text{-}Phe174^{CaMKII\delta}$ were suggested (cyan dashed lines). The model indicated that Ser95 could interact with both $Asp136^{CaMKII\delta}$, $Lys138^{CaMKII\delta}$ and $Thr177^{CaMKII\delta}$ (green dashed lines) via hydrogen bonds. A possible unfavorable repulsive interaction between Tyr92 and $Glu97^{CaMKII\delta}$ was also indicated (red dashed line). Model predictions were validated by mutating key residues in AKAP18δ, and subsequent ELISA analysis (Suppl. Fig. 2i, see legend for detailed description).

Kinetics of AKAP18δ-N (55–98) binding to CaMKIIδ were analyzed by surface plasmon resonance (SPR). A range of concentrations of recombinant CaMKIIδ-T287D (47.6–500 nM) was injected over immobilized biotin-AKAP18δ-N on a SA chip, and analyzed by fitting with a 1:1 interaction model (Langmuir). The dissociation equilibrium constant (K_D) was 23 ± 2 nM, with an association rate constant (k_a) = $(1.10 \pm 0.02) \times 10^4\ M^{-1}\ s^{-1}$ and a dissociation rate constant (k_d) = $(2.5 \pm 0.2) \times 10^{-4}\ s^{-1}$ (Fig. 2h). These findings indicate a strong CaMKIIδ-T287D interaction with AKAP18δ-N with medium association and dissociation rates. We also performed SPR analyses of AKAP18δ-N (55–74) and AKAP18δ-N (79–98). The inhibitory AKAP18δ-N (79–98) bound strongly to CaMKIIδ-T287D with similar SPR values as identified above (Suppl. Fig. 2j). AKAP18δ-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 AKAP18δ-N (55–98) is a potent inhibitor of the open CaMKIIδ 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). CaMKIIδ inhibition by AKAP18δ-N is illustrated in Fig. 2i (open conformation). Since AKAP18δ-N also bound to the autoregulatory domain, it is likely that AKAP18δ-N also continues to bind and inhibit CaMKIIδ after pThr287 dephosphorylation and CaM dissociation (Fig. 2j, closed conformation).

AKAP18δ-N reduces pThr17-PLN and inhibits SR Ca^{2+} reuptake.

Analyses performed using biotin-ahx-PLN as a substrate showed that AKAP18δ-N reduced CaMKIIδ-T287D-catalyzed phosphorylation of Thr17 in PLN (Fig. 3a, full immunoblots in Suppl. Fig. 3a). We further examined whether AKAP18δ-N also reduced Thr17-PLN phosphorylation in cardiomyocytes. Compared to the scrambled control peptide, cell-permeant AKAP18δ-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

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a PKA substrate, was hardly affected. Neither AKAP18δ-N (55–74) nor control peptide showed any changes at basal level or cytotoxicity (Suppl. Fig. 3c–d, respectively).

Effects of AKAP18δ-N on Ca^{2+} fluxes in adult cardiomyocytes were also investigated. Rat adult cardiomyocytes were treated with the cell-permeant version of AKAP18δ-N (55–74) or a scrambled control peptide, with or without ISO-stimulation. Cell-permeant TAT-AKAP18δ-N (55–74) treatment prolonged decay time of Ca^{2+} transients at 4 Hz (Fig. 3c, representative tracings are shown in Suppl. Fig. 3e), where Thr17-PLN phosphorylation is increased [22] and SERCA2 plays a proportionally larger role in controlling Ca^{2+} removal from the cytosol [23]. In the presence of ISO, which augments SERCA2 activity via PLN phosphorylation, TAT-AKAP18δ-N (55–74) continued to slow 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, AKAP18δ-N (79–98) also reduced SERCA2 Ca^{2+} reuptake rate in isolated mouse SR vesicles (Fig. 3e). These findings are consistent with reduction of pThr17-PLN levels by AKAP18δ-N (Fig. 3a–b).

Finally, compared to control mice, adult mice injected with adeno associated virus (AAV) encoding AKAP18δ-N (55–74) exhibited reduced pThr17-PLN levels after ISO stimulation, suggesting that AKAP18δ-N (55–74) also inhibits CaMKIIδ-catalyzed phosphorylation of Thr17 in PLN *in vivo* (Fig. 3f, upper panel). No effect of AAV-AKAP18δ-N (55–74) on pSer16-PLN was observed (Fig. 3f, middle panel).

Calcified CaM binds to AKAP18δ-N in competition with CaMKIIδ.

Rat AKAP18δ-N and human AKAP18γ-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 [24]. Closer inspection revealed that both AKAPs showed sequence similarities to the CaM binding motif in AKAP79 [25] (Fig. 4a) and CaMKIIδ (Suppl. Fig. 4a). We therefore analyzed whether AKAP18δ-N also bound calcified CaM. Immunoblotting demonstrated that the inhibitory AKAP18δ-N (79–98) peptide, the corresponding sequence in human AKAP18γ-N (52–71), and GFP-AKAP18δ (67–353) precipitated with CaM-agarose in the presence of Ca^{2+} (Suppl. Fig. 4b–d, respectively). CaM binding to AKAP18δ-N (79–98) was also confirmed in an ELISA-based assay where CaM-coated wells were incubated with biotinylated AKAP18δ-N (79–98) or the scrambled control (Fig. 4b). Interestingly, in further experiments, the presence of CaM attenuated both the inhibitory AKAP18δ-N (79–98)-CaMKIIδ-T287D (Fig. 4c) and AKAP18δ-N (79–98)-CaMKIIδ (1–282) (Fig. 4d) interactions. Since CaMKIIδ (1–282) lacks the CaM binding site, this finding indicates that CaM also outcompetes AKAP18δ-N (79–98) binding to CaMKIIδ catalytic site.

Kinetics of the CaM-AKAP18δ-N (79–98) interaction were analyzed by SPR. In the presence of 1 mM Ca^{2+}, the dissociation equilibrium constant (K_D) was 4.7 ± 0.7 μM, with an association rate constant (k_a) = $(1.3 \pm 0.3) \times 10^2$ M^{-1} s^{-1} and a dissociation rate constant (k_d) = $(5.3 \pm 0.3) \times 10^{-4}$ s^{-1} (Fig. 4e). These findings indicate that the CaM-AKAP18δ-N (79–98) interaction is quite slow and weak.

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In summary, our data indicate that calcified CaM binds directly to AKAP18δ-N and outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction (illustrated in Fig. 4f). By this mechanism, rising Ca^{2+} levels, and resulting calcification of CaM, relieve the inhibition of CaMKII.

AKAP18δ-C is homologous to the neuronal CaMKIIα activator N2B-s and lowers the Ca^{2+} threshold for CaMKIIδ activation.

As shown in Fig. 1j, both AKAP18δ and AKAP18γ contain a second CaMKIIδ 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 CaMKIIα, and linked to autonomous CaMKIIα activation in brain via T-site binding [18, 26, 27]. As demonstrated in Fig. 2b, AKAP18δ-C also bound to T site (amino acids 205–233).

In similarity to effects of N2B-s, we observed that AKAP18δ-C increased substrate phosphorylation by CaMKIIδ-T287A in an *in vitro* kinase assay where CaM was omitted (Fig. 5b). This finding suggests that AKAP18δ-C augments CaMKIIδ activity independently of Thr287 autophosphorylation and CaM binding. Consistently, cell-permeant TAT-AKAP18δ-C also increased Camui activity [28], a FRET-based CaMKII activation state sensor, transduced into adult rabbit ventricular myocytes (Fig. 5c). As CaMKII requires relatively high intracellular Ca^{2+} to achieve activation, we hypothesized that AKAP18δ-C was able to decrease the Ca^{2+} threshold for CaMKIIδ activation. Indeed, at 0.5 μM free Ca^{2+}, which does not fully activate CaMKIIδ-T287A to phosphorylate Thr17-PLN, further increased Thr17-PLN phosphorylation in the presence of either AKAP18δ-C (tendency) or the N2B-s positive control peptide (Fig. 5d–e, respectively, complete immunoblots in Suppl. Fig. 5a–b). These observations suggest that AKAP18δ-C is able to potentiate CaMKIIδ by keeping the inhibitory gate open as a wedge, thereby sensitizing CaMKII to Ca^{2+}-dependent activation. Immunoprecipitations employing CaMKIIδ-T287A in the presence of CaM showed that AKAP18δ-C also induced trapping of CaM to CaMKIIδ-T287A (Fig. 5f); a feature which has been similarly described for N2B-s [18].

Kinetics of the AKAP18δ-C (238–266)-CaMKIIδ interaction analyzed by SPR revealed a dissociation equilibrium constant (K_D) = 340 ± 46 nM, association rate constant (k_a) = $(4.5 \pm 1.0) \times 10^3$ M^{-1} s^{-1} and dissociation rate constant (k_d) = $(1.4 \pm 0.1) \times 10^{-3}$ s^{-1} (Fig. 5g). This indicated that the CaMKIIδ-T287D-AKAP18δ-C interaction is weaker than the CaMKIIδ-T287D-AKAP18δ-N interaction, has a slower on rate, and faster off rate.

Taken together, our data show that AKAP18δ-C is a CaMKIIδ activator that lowers the Ca^{2+} threshold for CaMKIIδ activation (perhaps by keeping the inhibitory gate open) and also allows CaM trapping by the kinase (Fig. 5h–i).

AKAP18δ-C increases pThr17-PLN and facilitates faster SR Ca^{2+} reuptake.

When introduced into adult rat cardiomyocytes, TAT-AKAP18δ-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

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augmented in neonatal rat cardiomyocytes treated with AKAP18δ-C or N2B-s [18] (Suppl. Fig. 6a–b). No cytotoxicity was observed for AKAP18δ-C or control peptide (Suppl. Fig. 3d).

Isolated adult rat cardiomyocytes were treated with cell-permeant TAT-AKAP18δ-C or scrambled control peptides, in the presence and absence of ISO. Ca^{2+} transient recordings revealed findings opposite to those observed for AKAP18δ-N, as TAT-AKAP18δ-C accelerated Ca^{2+} 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^{2+} levels, CaMKIIδ activation, and increasing pThr17-PLN at higher pacing rates likely overpowered the effects of AKAP18δ-C observed at baseline. Consistent with the data above, TAT-AKAP18δ-C also increased the SERCA2 Ca^{2+}-uptake rate in isolated mouse SR vesicles (Fig. 6d).

In summary, these data support the notion that by activating CaMKIIδ, AKAP18δ-C increases pThr17-PLN, and thereby accelerates Ca^{2+} reuptake into the SR.

AKAP18δ also anchors and functionally regulates CaMKIIδ activity at RYR.

RYR has been identified together with PLN-SERCA2 in SR nanodomains [29]. We therefore analysed whether AKAP18δ also regulates CaMKIIδ activity at RYR. Co-localization and immunoprecipitation of CaMKIIδ and RYR with AKAP18δ 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), AKAP18δ-N (55–98) also reduced CaMKIIδ phosphorylation of Ser2814-RYR using biotin-ahx-RYR (2797-2827) as substrate (Fig. 7d). When introduced into adult cardiomyocytes, cell-permeant TAT-AKAP18δ-N (79–98) and TAT-AKAP18δ-N (55–74) reduced Ser2814-RYR phosphorylation (Fig. 7e) and RYR functional activity after ISO stimulation (Fig. 7g), whereas TAT-AKAP18δ-C in the absence of ISO exhibited opposite effects (Fig. 7f–g). TAT-AKAP18δ-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 [30]. Finally, we tested the effect of the AKAP18δ-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 AKAP18δ-PLN competitor peptide were observed (Fig. 7h). This finding indicate that RYR and PLN-SERCA2 might not compete for the same AKAP18δ-CaMKIIδ pool.

Taken together, our data indicate that in addition to effects at PLN-SERCA2, AKAP18δ also anchors and regulates CaMKIIδ activity at RYR.

DISCUSSION

Here, we have provided mechanistic insight into the anchoring and regulation of CaMKIIδ activity by AKAP18δ at PLN-SERCA2 and RYR. We identified two unique regions in AKAP18δ that inversely regulate CaMKIIδ activity, CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and SERCA2 and RYR functional activities. We specifically showed that an inhibitory domain (AKAP18δ-N) also binds calcified CaM, while an

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activating domain (AKAP18δ-C) wedges CaMKIIδ open, trapping CaM within the kinase, and lowering the Ca^{2+} threshold for its activation.

Working model to explain AKAP18δ effects on CaMKIIδ in myocytes.

CaMKII activity has been shown to be sensitive to the frequency of Ca^{2+} oscillations *in vitro* [10] and in intact adult cardiomyocytes [28]. Based on our data, we propose a working hypothesis whereby AKAP18δ fine-tunes this frequency-dependent activation of CaMKIIδ. At low stimulation frequency (Fig. 8a), AKAP18δ-N (in red) binds the CaMKIIδ 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^{2+} transients increases (Fig. 8b), accumulated calcified CaM (beige) may outcompete the inhibitory AKAP18δ-N-CaMKIIδ interaction by binding to the CaMKIIδ autoregulatory domain (pink) and AKAP18δ-N (red). These events lead to displacement of the S-site from AKAP18δ-N. CaM binding to AKAP18δ-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 CaMKIIδ potency. Concomitantly, AKAP18δ-C (in green) binds to the released T-site, keeping the inhibitory gate open as a wedge. This potentiates CaMKIIδ by lowering the Ca^{2+} threshold for its activation and by trapping CaM, leading to substrate phosphorylation (e.g. Thr287 in the neighboring CaMKIIδ subunit, Thr17-PLN or Ser2814-RYR) during subsequent Ca^{2+} transients in a feed-forward manner: molecular memory of CaMKIIδ results. Thereafter, when the frequency of Ca^{2+} transients declines, CaM dissociates from CaMKIIδ and AKAP18δ-N, making the latter accessible to bind the CaMKIIδ autoregulatory domain, ATP binding region, and S- and T-sites, leading to re-inhibition of CaMKIIδ (Fig. 8a). While plausible, it is unknown whether in the intact continuously beating heart the activating effect of AKAP18δ-C is dominant over the inhibitory stabilization caused by AKAP18δ-N.

In contrast to the CaMKIIδ autoregulatory domain, AKAP18δ-N does not contain the cluster of Phe293, Asn294 and Arg296 which is critical for CaM trapping by CaMKIIδ [31], suggesting that AKAP18δ-N does not trap calcified CaM as the Ca^{2+} transient frequency declines. This may allow AKAP18δ-N to inhibit CaMKIIδ prior to complete dissociation of CaM from CaMKIIδ. In fact, since AKAP18δ-C and AKAP18δ-N (55–74) bind differently within the T-site region, there might not be any need for AKAP18δ-C to dissociate from CaMKIIδ when the frequency declines. With this positioning, AKAP18δ-C might rapidly potentiate kinase activity during a subsequent rise in Ca^{2+} transient frequency since this could occur as soon as AKAP18δ-N dissociates, and independently of the Thr287 autophosphorylation state or other posttranslational modifications reported to promote autonomous activation of CaMKII [32–35]. In the presence of posttranslational modifications, AKAP18δ-C may increase CaMKIIδ activation to an even higher level, whereas AKAP18δ-N is expected to still be able to inhibit CaMKIIδ (illustrated in Fig. 2i), even if its binding to the autoregulatory domain is abolished by oxidation (Met281/282) [32] or S-nitrosylation (Cys290) [35]. The stoichiometry of the AKAP18δ-CaMKIIδ interaction is not known, but since AKAP18δ has been shown to oligomerize [36], it is plausible that several AKAP18δ molecules anchor and regulate different CaMKIIδ monomers within the same CaMKII

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oligomer. Bioinformatics revealed that the AKAP18δ binding regions are well conserved across CaMKII isoforms (Suppl. Fig. 8), suggesting that AKAP18δ may be able to anchor and regulate different CaMKII isoforms, and might provide an explanation for some Thr17-PLN phosphorylation observed in the CaMKIIδ knock out mouse model [37].

Additional studies are needed to further interrogate the proposed AKAP18δ-CaMKIIδ-SERCA2-PLN/RYR model (Fig. 8). Both PLN and RYR are thought to be important targets for CaMKIIδ effects during the force frequency relationship (FFR) [30, 38–40]. While the effects of CaMKII and PLN on frequency-dependent acceleration of relaxation (FDAR) have been more controversial [41–43], 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 [40]. Our data support an important role of CaMKII during FDAR, as we also observed that AKAP18δ-C-dependent CaMKII stimulation accelerated Ca^{2+} transient decline at low stimulation frequency (Fig. 6b).

AKAP18δ is a novel CaMKII Anchoring Protein.

To our knowledge, this is the first AKAP reported to anchor a CaMKII isoform, defining AKAP18δ also as a CaM-KAP. From the crystal structures of fragments of AKAP18δ [44] and AKAP18γ [45] it appears as though the two CaMKIIδ binding sites are mostly accessible and do not overlap with the PLN [14] or PKA [13] binding sites. Thus, both CaMKIIδ and PKA may bind to AKAP18δ and phosphorylate PLN at the same time. AKAP18δ also coordinates phosphorylation of inhibitor-1 [46], which in turn inhibits protein phosphatase 1, the major phosphatase responsible for dephosphorylating PLN [47–49].

It remains to be determined whether the opposing effects of the two AKAP18δ regions on CaMKIIδ activity are unique to AKAP18δ, or whether a similar arrangement exists in other proteins. CaMKIIα interacts with two sites in the NMDA (N-methyl-D-aspartate) receptor subunit NR2B [18]. One site (N2B-s) is similar to AKAP18δ-C, both at sequence level, as well as functionally, as it generates autonomous activation of CaMKIIα [18]. The second binding site is reported to be CaMKIIα-Thr286 dependent [18]. Densin, located in neuronal postsynaptic densities, also inhibits CaMKII-mediated phosphorylation through T-site binding [19], but binds to the CaMKII association domain through a second site [50–52]. 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 [20]. Interestingly, Rem2 also interacts with CaM [53, 54].

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^{2+} 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 [4], decreased SERCA2 activity is often reported. During heart failure, reduction in SERCA2 activity is likely a result of both lowered protein expression [55] and

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hypo-phosphorylation of PLN following increased phosphatase activity [56, 57]. Restoration of SR Ca^{2+} re-uptake through increasing PLN phosphorylation levels and/or SERCA2 activity is therefore considered to be a potential therapeutic strategy [58, 59]. Future studies are needed to test whether the CaMKII activator peptide identified in this study (AKAP18δ-C) can be used to increase pThr17-PLN levels and thus SERCA2 activity *in vivo*. One strategy may be to target AKAP18δ-C to longitudinal SR (LSR), using a strategy similar to that described for the AIP_4-LSR transgenic mice [60, 61]. To this end, the peptide sequences presently derived from the two AKAP18δ regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte Ca^{2+} cycling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

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

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

REFERENCES

1. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002;415:198–205. [PubMed: 11805843]
2. Bers DM. Altered cardiac myocyte Ca regulation in heart failure. Physiology (Bethesda). 2006;21:380–7. [PubMed: 17119150]
3. Maier LS and Bers DM. Role of Ca2+/calmodulin-dependent protein kinase (CaMK) in excitation-contraction coupling in the heart. Cardiovasc Res. 2007;73:631–640. [PubMed: 17157285]

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Author Manuscript
Author Manuscript

4. Mattiazzi A and Kranias EG. The role of CaMKII regulation of phospholamban activity in heart disease. Front Pharmacol. 2014;5.

5. Ji Y, Zhao W, Li BL, Desantiago J, Picht E, Kaetzel MA, Schultz JE, Kranias EG, Bers DM and Dedman JR. Targeted inhibition of sarcoplasmic reticulum CaMKII activity results in alterations of Ca2+ homeostasis and cardiac contractility. Am J Physiol Heart Circ Physiol. 2006;290:H599–H606. [PubMed: 16143658]

6. Rosenberg OS, Deindl S, Sung RJ, Nairn AC and Kuriyan J. Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. Cell. 2005;123:849–860. [PubMed: 16325579]

7. Hudmon A and Schulman H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J. 2002;364:593–611. [PubMed: 11931644]

8. Meyer T, Hanson PI, Stryer L and Schulman H. Calmodulin Trapping by Calcium-Calmodulin Dependent Protein-Kinase. Science. 1992;256:1199–1202. [PubMed: 1317063]

9. Miller SG, Patton BL and Kennedy MB. Sequences of Autophosphorylation Sites in Neuronal Type II Cam Kinase That Control Ca-2+-Independent Activity. Neuron. 1988;1:593–604. [PubMed: 2856100]

10. De Koninck P and Schulman H. Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. Science. 1998;279:227–230. [PubMed: 9422695]

11. Saucerman JJ and Bers DM. Calmodulin mediates differential sensitivity of CaMKII and calcineurin to local Ca2+ in cardiac myocytes. Biophys J. 2008;95:4597–612. [PubMed: 18689454]

12. Wood BM, Simon M, Galice S, Alim CC, Ferrero M, Pinna NN, Bers DM and Bossuyt J. Cardiac CaMKII activation promotes rapid translocation to its extra-dyadic targets. J Mol Cell Cardiol. 2018;125:18–28. [PubMed: 30321537]

13. Henn V, Edemir B, Stefan E, Wiesner B, Lorenz D, Theilig F, Schmitt R, Vossebein L, Tamma G, Beyermann M, Krause E, Herberg FW, Valenti G, Bachmann S, Rosenthal W and Klussmann E. Identification of a novel A-kinase anchoring protein 18 isoform and evidence for its role in the vasopressin-induced aquaporin-2 shuttle in renal principal cells. J Biol Chem. 2004;279:26654–65.

14. Lygren B, Carlson CR, Santamaria K, Lissandron V, McSorley T, Litzenberg J, Lorenz D, Wiesner B, Rosenthal W, Zaccolo M, Tasken K and Klussmann E. AKAP complex regulates Ca2+ re-uptake into heart sarcoplasmic reticulum. Embo Rep. 2007;8:1061–1067. [PubMed: 17901878]

15. Ahmad F, Shen WX, Vandeput F, Szabo-Fresnais N, Krall J, Degerman E, Goetz F, Klussmann E, Movsesian M and Manganiello V. Regulation of Sarcoplasmic Reticulum Ca2+ ATPase 2 (SERCA2) Activity by Phosphodiesterase 3A (PDE3A) in Human Myocardium PHOSPHORYLATION-DEPENDENT INTERACTION OF PDE3A1 WITH SERCA2. J Biol Chem. 2015;290:6763–6776. [PubMed: 25593322]

16. Fong YL, Taylor WL, Means AR and Soderling TR. Studies of the Regulatory Mechanism of Ca-2+-Calmodulin-Dependent Protein Kinase II - Mutation of Threonine-286 to Alanine and Aspartate. J Biol Chem. 1989;264:16759–16763.

17. Yang EY and Schulman H. Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. J Biol Chem. 1999;274:26199–26208.

18. Bayer KU, De Koninck P, Leonard AS, Hell JW and Schulman H. Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature. 2001;411:801–805. [PubMed: 11459059]

19. Jiao YX, Jalan-Sakrikar N, Robison AJ, Baucum AJ, Bass MA and Colbran RJ. Characterization of a Central Ca2+/Calmodulin-dependent Protein Kinase II alpha/beta Binding Domain in Densin That Selectively Modulates Glutamate Receptor Subunit Phosphorylation. J Biol Chem. 2011;286:24806–24818.

20. Royer L, Herzog JJ, Kenny K, Tzvetkova B, Cochrane JC, Marr MT and Paradis S. The Ras-like GTPase Rem2 is a potent inhibitor of calcium/calmodulin-dependent kinase II activity. J Biol Chem. 2018;293:14798–14811.

21. Vest RS, Davies KD, O'Leary H, Port JD and Bayer KU. Dual mechanism of a natural CaMKII inhibitor. Mol Biol Cell. 2007;18:5024–5033. [PubMed: 17942605]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

22. Zhao W, Uehara Y, Chu G, Song Q, Qian J, Young K and Kranias EG. Threonine-17 phosphorylation of phospholamban: a key determinant of frequency-dependent increase of cardiac contractility. J Mol Cell Cardiol. 2004;37:607–12. [PubMed: 15276030]
23. Louch WE, Stokke MK, Sjaastad I, Christensen G and Sejersted OM. No rest for the weary: diastolic calcium homeostasis in the normal and failing myocardium. Physiology (Bethesda). 2012;27:308–23. [PubMed: 23026754]
24. James P, Vorherr T and Carafoli E. Calmodulin-Binding Domains - Just 2-Faced or Multifaceted. Trends Biochem Sci. 1995;20:38–42. [PubMed: 7878743]
25. Faux MC and Scott JD. Regulation of the AKAP79-protein kinase C interaction by Ca2+/ calmodulin. J Biol Chem. 1997;272:17038–17044.
26. Strack S and Colbran RJ. Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem. 1998;273:20689–20692.
27. Strack S, McNeill RB and Colbran RJ. Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem. 2000;275:23798–23806.
28. Erickson JR, Patel R, Ferguson A, Bossuyt J and Bers DM. Fluorescence resonance energy transfer-based sensor Camui provides new insight into mechanisms of calcium/calmodulin-dependent protein kinase II activation in intact cardiomyocytes. Circ Res. 2011;109:729–38. [PubMed: 21835909]
29. Alsina KM, Hulsurkar M, Brandenburg S, Kownatzki-Danger D, Lenz C, Urlaub H, Abu-Taha I, Kamler M, Chiang DY, Lahiri SK, Reynolds JO, Quick AP, Scott L Jr., Word TA, Gelves MD, Heck AJR, Li N, Dobrev D, Lehnart SE and Wehrens XHT. Loss of Protein Phosphatase 1 Regulatory Subunit PPP1R3A Promotes Atrial Fibrillation. Circulation. 2019;140:681–693. [PubMed: 31185731]
30. Wehrens XH, Lehnart SE, Reiken SR and Marks AR. Ca2+/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. Circ Res. 2004;94:e61–70. [PubMed: 15016728]
31. Singla SI, Hudmon A, Goldberg JM, Smith JL and Schulman H. Molecular characterization of calmodulin trapping by calcium/calmodulin-dependent protein kinase II. J Biol Chem. 2001;276:29353–60.
32. Erickson JR, Joiner MLA, Guan X, Kutschke W, Yang JY, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJL, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ and Anderson ME. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell. 2008;133:462–474. [PubMed: 18455987]
33. Erickson JR, Pereira L, Wang LG, Han GH, Ferguson A, Dao K, Copeland RJ, Despa F, Hart GW, Ripplinger CM and Bers DM. Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. Nature. 2013;502:372–376. [PubMed: 24077098]
34. Coultrap SJ and Bayer KU. Nitric Oxide Induces Ca2+-independent Activity of the Ca2+/ Calmodulin-dependent Protein Kinase II (CaMKII). J Biol Chem. 2014;289:19458–19465. [PubMed: 24855644]
35. Erickson JR, Nichols CB, Uchinoumi H, Stein ML, Bossuyt J and Bers DM. S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II delta. J Biol Chem. 2015;290:25646–25656.
36. Singh A, Rigatti M, Le AV, Carlson CR, Moraru, II and Dodge-Kafka KL. Analysis of AKAP7gamma Dimerization. J Signal Transduct. 2015;2015:371626.
37. Grimm M, Ling H and Brown JH. Crossing signals: relationships between beta-adrenergic stimulation and CaMKII activation. Heart Rhythm. 2011;8:1296–8. [PubMed: 21354330]
38. Bluhm WF, Kranias EG, Dillmann WH and Meyer M. Phospholamban: a major determinant of the cardiac force-frequency relationship. Am J Physiol Heart Circ Physiol. 2000;278:H249–55. [PubMed: 10644605]
39. Kushnir A, Shan J, Betzenhauser MJ, Reiken S and Marks AR. Role of CaMKIIdelta phosphorylation of the cardiac ryanodine receptor in the force frequency relationship and heart failure. Proc Natl Acad Sci U S A. 2010;107:10274–9.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

40. Wu Y, Luczak ED, Lee EJ, Hidalgo C, Yang J, Gao Z, Li J, Wehrens XH, Granzier H and Anderson ME. CaMKII effects on inotropic but not lusitropic force frequency responses require phospholamban. J Mol Cell Cardiol. 2012;53:429–36. [PubMed: 22796260]
41. DeSantiago J, Maier LS and Bers DM. Frequency-dependent acceleration of relaxation in the heart depends on CaMKII, but not phospholamban. J Mol Cell Cardiol. 2002;34:975–84. [PubMed: 12234767]
42. Valverde CA, Mundiña-Weilenmann C, Said M, Ferrero P, Vittone L, Salas M, Palomeque J, Petroff MV and Mattiazzi A. Frequency-dependent acceleration of relaxation in mammalian heart: a property not relying on phospholamban and SERCA2a phosphorylation. J Physiol. 2005;562:801–13. [PubMed: 15528241]
43. Picht E, DeSantiago J, Huke S, Kaetzel MA, Dedman JR and Bers DM. CaMKII inhibition targeted to the sarcoplasmic reticulum inhibits frequency-dependent acceleration of relaxation and Ca2+ current facilitation. J Mol Cell Cardiol. 2007;42:196–205. [PubMed: 17052727]
44. Gold MG, Smith FD, Scott JD and Barford D. AKAP18 contains a phosphoesterase domain that binds AMP. J Mol Biol. 2008;375:1329–43. [PubMed: 18082768]
45. Bjerregaard-Andersen K, Ostensen E, Scott JD, Tasken K and Morth JP. Malonate in the nucleotide-binding site traps human AKAP18 gamma/delta in a novel conformational state. Acta Crystallogr F. 2016;72:591–597.
46. Singh A, Redden JM, Kapiloff MS and Dodge-Kafka KL. The Large Isoforms of A-Kinase Anchoring Protein 18 Mediate the Phosphorylation of Inhibitor-1 by Protein Kinase A and the Inhibition of Protein Phosphatase 1 Activity. Mol Pharmacol. 2011;79:533–540. [PubMed: 21149637]
47. Carr AN, Schmidt AG, Suzuki Y, del Monte F, Sato Y, Lanner C, Breeden K, Jing SL, Allen PB, Greengard P, Yatani A, Hoit BD, Grupp IL, Hajjar RJ, DePaoli-Roach AA and Kranias EG. Type 1 phosphatase, a negative regulator of cardiac function. Mol Cell Biol. 2002;22:4124–4135. [PubMed: 12024026]
48. Nicolaou P, Hajjar RJ and Kranias EG. Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology. J Mol Cell Cardiol. 2009;47:365–371. [PubMed: 19481088]
49. Pathak A, del Monte F, Zhao W, Schultz JE, Lorenz JN, Bodi I, Weiser D, Hahn H, Carr AN, Syed F, Mavila N, Jha L, Qian J, Marreez Y, Chen GL, McGraw DW, Heist EK, Guerrero JL, DePaoli-Roach AA, Hajjar RJ and Kranias EG. Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1. Circ Res. 2005;96:756–766. [PubMed: 15746443]
50. Strack S, Robison AJ, Bass MA and Colbran RJ. Association of calcium/calmodulin-dependent kinase II with developmentally regulated splice variants of the postsynaptic density protein densin-180. J Biol Chem. 2000;275:25061–25064.
51. Mcneill RB and Colbran RJ. Interaction of Autophosphorylated Ca2+ Calmodulin-Dependent Protein-Kinase-Ii with Neuronal Cytoskeletal Proteins - Characterization of Binding to a 190-Kda Postsynaptic Density Protein. J Biol Chem. 1995;270:10043–10049.
52. Walikonis RS, Oguni A, Khorosheva EM, Jeng CJ, Asuncion FJ and Kennedy MB. Densin-180 forms a ternary complex with the alpha-subunit of Ca2+/calmodulin-dependent protein kinase II and alpha-actinin. J Neurosci. 2001;21:423–433. [PubMed: 11160423]
53. Beguin P, Mahalakshmi RN, Nagashima K, Cher DHK, Kuwamura N, Yamada Y, Seino Y and Hunziker W. Roles of 14–3-3 and calmodulin binding in subcellular localization and function of the small G-protein Rem2. Biochem J. 2005;390:67–75. [PubMed: 15862114]
54. Flynn R, Labrie-Dion E, Bernier N, Colicos MA, De Koninck P and Zamponi GW. Activity-Dependent Subcellular Cotrafficking of the Small GTPase Rem2 and Ca2+/CaM-Dependent Protein Kinase II alpha. Plos One. 2012;7.
55. Studeli R, Jung S, Mohacsi P, Perruchoud S, Castiglioni P, Wenaweser P, Heimbeck G, Feller M and Hullin R. Diastolic dysfunction in human cardiac allografts is related with reduced SERCA2a gene expression. Am J Transplant. 2006;6:775–82. [PubMed: 16539635]
56. Schwinger RH, Munch G, Bolck B, Karczewski P, Krause EG and Erdmann E. Reduced Ca(2+)-sensitivity of SERCA 2a in failing human myocardium due to reduced serin-16 phospholamban phosphorylation. J Mol Cell Cardiol. 1999;31:479–91. [PubMed: 10198180]

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57. Munch G, Bolck B, Karczewski P and Schwinger RH. Evidence for calcineurin-mediated regulation of SERCA 2a activity in human myocardium. J Mol Cell Cardiol. 2002;34:321–34. [PubMed: 11945024]
58. del Monte F and Hajjar RJ. Targeting calcium cycling proteins in heart failure through gene transfer. J Physiol. 2003;546:49–61. [PubMed: 12509478]
59. Lipskaia L, Chemaly ER, Hadri L, Lompre AM and Hajjar RJ. Sarcoplasmic reticulum Ca(2+) ATPase as a therapeutic target for heart failure. Expert Opin Biol Ther. 2010;10:29–41. [PubMed: 20078230]
60. Ji Y, Li B, Reed TD, Lorenz JN, Kaetzel MA and Dedman JR. Targeted inhibition of Ca2+/calmodulin-dependent protein kinase II in cardiac longitudinal sarcoplasmic reticulum results in decreased phospholamban phosphorylation at threonine 17. J Biol Chem. 2003;278:25063–71.
61. Ji Y, Zhao W, Li B, Desantiago J, Picht E, Kaetzel MA, Schultz Jel J, Kranias EG, Bers DM and Dedman JR. Targeted inhibition of sarcoplasmic reticulum CaMKII activity results in alterations of Ca2+ homeostasis and cardiac contractility. Am J Physiol Heart Circ Physiol. 2006;290:H599–606. [PubMed: 16143658]
62. Horner A, Goetz F, Tampe R, Klussmann E and Pohl P. Mechanism for targeting the A-kinase anchoring protein AKAP18delta to the membrane. J Biol Chem. 2012;287:42495–501.
63. Gray CBB and Brown JH. CaMKIIdelta subtypes: localization and function. Front Pharmacol. 2014;5.
64. Hou Z, Kelly EM and Robia SL. Phosphomimetic mutations increase phospholamban oligomerization and alter the structure of its regulatory complex. J Biol Chem. 2008;283:28996–9003.
65. Mathiesen SB, Lunde M, Aronsen JM, Romaine A, Kaupang A, Martinsen M, de Souza GA, Nyman TA, Sjaastad I, Christensen G and Carlson CR. The cardiac syndecan-4 interactome reveals a role for syndecan-4 in nuclear translocation of muscle LIM protein (MLP). J Biol Chem. 2019;294:8717–8731. [PubMed: 30967474]
66. Werfel S, Jungmann A, Lehmann L, Ksienzyk J, Bekeredjian R, Kaya Z, Leuchs B, Nordheim A, Backs J, Engelhardt S, Katus HA and Müller OJ. Rapid and highly efficient inducible cardiac gene knockout in adult mice using AAV-mediated expression of Cre recombinase. Cardiovasc Res. 2014;104:15–23. [PubMed: 25082846]
67. Jungmann A, Leuchs B, Rommelaere J, Katus HA and Müller OJ. Protocol for Efficient Generation and Characterization of Adeno-Associated Viral Vectors. Hum Gene Ther Methods. 2017;28:235–246. [PubMed: 29048971]
68. Börner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ and Nikolaev VO. FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. Nat Protoc. 2011;6:427–38. [PubMed: 21412271]
69. Li JL, Wang XN, Fraser SF, Carey MF, Wrigley TV and McKenna MJ. Effects of fatigue and training on sarcoplasmic reticulum Ca(2+) regulation in human skeletal muscle. J Appl Physiol (1985). 2002;92:912–22. [PubMed: 11842021]
70. O'Brien PJ. Calcium sequestration by isolated sarcoplasmic reticulum: real-time monitoring using ratiometric dual-emission spectrofluorometry and the fluorescent calcium-binding dye indo-1. Mol Cell Biochem. 1990;94:113–9. [PubMed: 2374546]
71. Kolstad TR, van den Brink J, MacQuaide N, Lunde PK, Frisk M, Aronsen JM, Norden ES, Cataliotti A, Sjaastad I, Sejersted OM, Edwards AG, Lines GT and Louch WE. Ryanodine receptor dispersion disrupts Ca(2+) release in failing cardiac myocytes. Elife. 2018;7.
72. Stefan E, Wiesner B, Baillie GS, Mollajew R, Henn V, Lorenz D, Furkert J, Santamaria K, Nedvetsky P, Hundsrucker C, Beyermann M, Krause E, Pohl P, Gall I, MacIntyre AN, Bachmann S, Houslay MD, Rosenthal W and Klussmann E. Compartmentalization of cAMP-dependent signaling by phosphodiesterase-4D is involved in the regulation of vasopressin-mediated water reabsorption in renal principal cells. J Am Soc Nephrol. 2007;18:199–212. [PubMed: 17135396]
73. Schafer G, Milic J, Eldahshan A, Gotz F, Zuhlke K, Schillinger C, Kreuchwig A, Elkins JM, Abdul Azeez KR, Oder A, Moutty MC, Masada N, Beerbaum M, Schlegel B, Niquet S, Schmieder P, Krause G, von Kries JP, Cooper DM, Knapp S, Rademann J, Rosenthal W and Klussmann E.

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Highly functionalized terpyridines as competitive inhibitors of AKAP-PKA interactions. Angew Chem Int Ed Engl. 2013;52:12187–91.

74. Ottesen AH, Louch WE, Carlson CR, Landsverk OJB, Kurola J, Johansen RF, Moe MK, Aronsen JM, Hoiseth AD, Jarstadmarken H, Nygard S, Bjoras M, Sjaastad I, Pettila V, Stridsberg M, Omland T, Christensen G and Rosjo H. Secretoneurin is a novel prognostic cardiovascular biomarker associated with cardiomyocyte calcium handling. J Am Coll Cardiol. 2015;65:339–351. [PubMed: 25634832]
75. Chang BH, Mukherji S and Soderling TR. Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A. 1998;95:10890–5.
76. Asensio CJ and Garcia RC. Determination of a large number of kinase activities using peptide substrates, P81 phosphocellulose paper arrays and phosphor imaging. Anal Biochem. 2003;319:21–33. [PubMed: 12842103]
77. Witt JJ and Roskoski R, Jr. Rapid protein kinase assay using phosphocellulose-paper absorption. Anal Biochem. 1975;66:253–8. [PubMed: 1147218]
78. Rellos P, Pike AC, Niesen FH, Salah E, Lee WH, von Delft F and Knapp S. Structure of the CaMKIIdelta/calmodulin complex reveals the molecular mechanism of CaMKII kinase activation. PLoS Biol. 2010;8:e1000426.
79. Erickson JR. Mechanisms of CaMKII Activation in the Heart. Front Pharmacol. 2014;5:59. [PubMed: 24765077]

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) and ryanodine receptor (RYR) are essential for cardiac excitation-contraction coupling.
- Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) modulates SERCA2 and RYR activities through indirect and direct phosphorylation events, respectively, but CaMKII anchoring and local regulation mechanisms remain elusive.
- A-Kinase Anchoring Protein 18 delta (AKAP18δ) anchors protein kinase A to SERCA2-phospholamban (PLN).

What New Information Does This Article Contribute?

- AKAP18δ anchors and functionally regulates CaMKII activity at SERCA2-PLN and RYR, indicating a crucial role of AKAP18δ in heartbeat regulation.
- AKAP18δ also anchors calmodulin (CaM), inducing a second level of control.
- AKAP18δ is the first AKAP reported to anchor a CaMKII isoform, defining AKAP18δ as a CaM-Kinase Anchoring Proteins (CaM-KAP).

SERCA2 mediates Ca^{2+} reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas RYR mediates Ca^{2+} release from SR and triggers contraction. CaMKIIδ regulates activities of SERCA2, through phosphorylation of PLN, and RYR by direct phosphorylation. However, the mechanisms for CaMKIIδ anchoring to SERCA2-PLN and RYR and its regulation by local Ca^{2+} signals remain unclear. Here, we provide mechanistic insight into the anchoring and regulation of CaMKIIδ activity by AKAP18δ at SERCA2-PLN and RYR. We identified two unique regions in AKAP18δ that inversely regulate CaMKIIδ activity (CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR) and SERCA2 and RYR function. We specifically showed that an inhibitory domain (AKAP18δ-N) also binds calcified CaM, while an activating domain (AKAP18δ-C) wedges CaMKIIδ open, trapping CaM within the kinase, and lowering the Ca^{2+} threshold for its activation. Based on our data we propose a working model where the two unique AKAP18δ regions fine-tune Ca^{2+}-frequency-dependent activation of CaMKIIδ at SERCA2-PLN and RYR. The peptide sequences derived from the two AKAP18δ regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte Ca^{2+} cycling.

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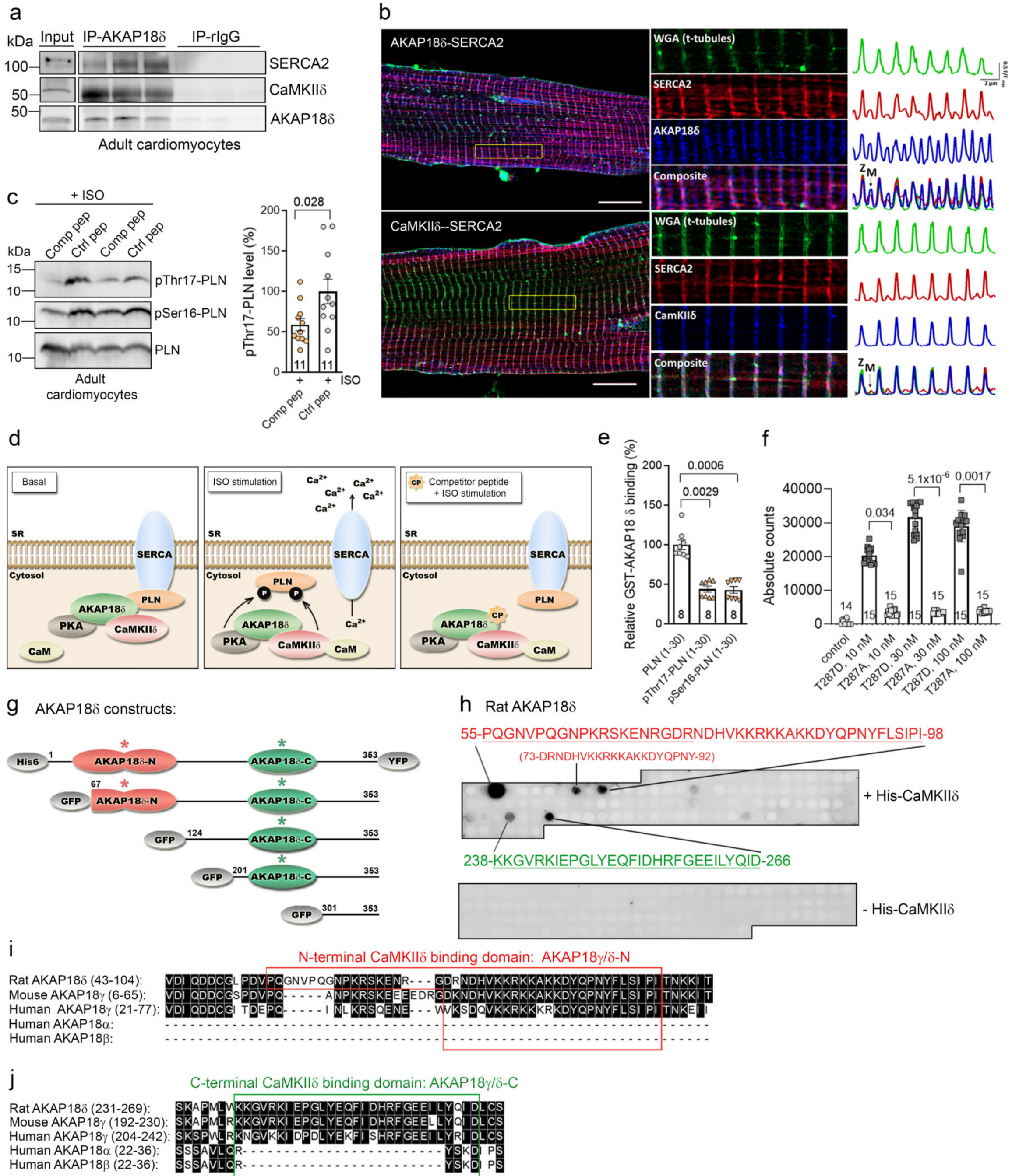

Figure 1. AKAP18δ anchors CaMKIIδ to the PLN-SERCA2 complex and controls Thr17-PLN phosphorylation.

(**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

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adult cardiomyocytes pre-treated with cell-permeant AKAP18δ-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 AKAP18δ-PLN competitor experiment. Left: Without ISO, PLN is dephosphorylated and inhibits SERCA2 activity. Middle: During ISO-stimulation, AKAP18δ-associated CaMKIIδ (and PKA) phosphorylates PLN, leading to SERCA2 activation and Ca^{2+} uptake into SR. Right: In the presence of the cell-permeant AKAP18δ-PLN competitor peptide (CP) [14], AKAP18δ displaces from PLN-SERCA2. AKAP18δ-associated CaMKIIδ is no longer able to phosphorylate PLN. Both AKAP18δ [62] 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-AKAP18δ (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 AKAP18δ-CaMKIIδ interaction by AlphaScreen™. GST-AKAP18δ was incubated with increasing concentrations of recombinant CaMKIIδ-T287D or CaMKIIδ-T287A. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=14–15). (**g**) The two CaMKIIδ binding regions in AKAP18δ are illustrated in red and green. (**h**) Residues important for CaMKIIδ binding were identified by overlaying 20-mer overlapping AKAP18δ peptides spot-synthesized on membranes with active His-CaMKIIδ and immunoblotting. Immunoblotting without His-CaMKIIδ was used as control (lower panel). Underlined sequences were synthesized as soluble peptides for further experiments. (**i-j**) The two CaMKIIδ binding regions (red and green) are indicated in the alignment of rat AKAP18δ, human and mouse AKAP18γ, and the smaller AKAP18α and AKAP18β. Black boxes indicate identical amino acids (DNA Star).

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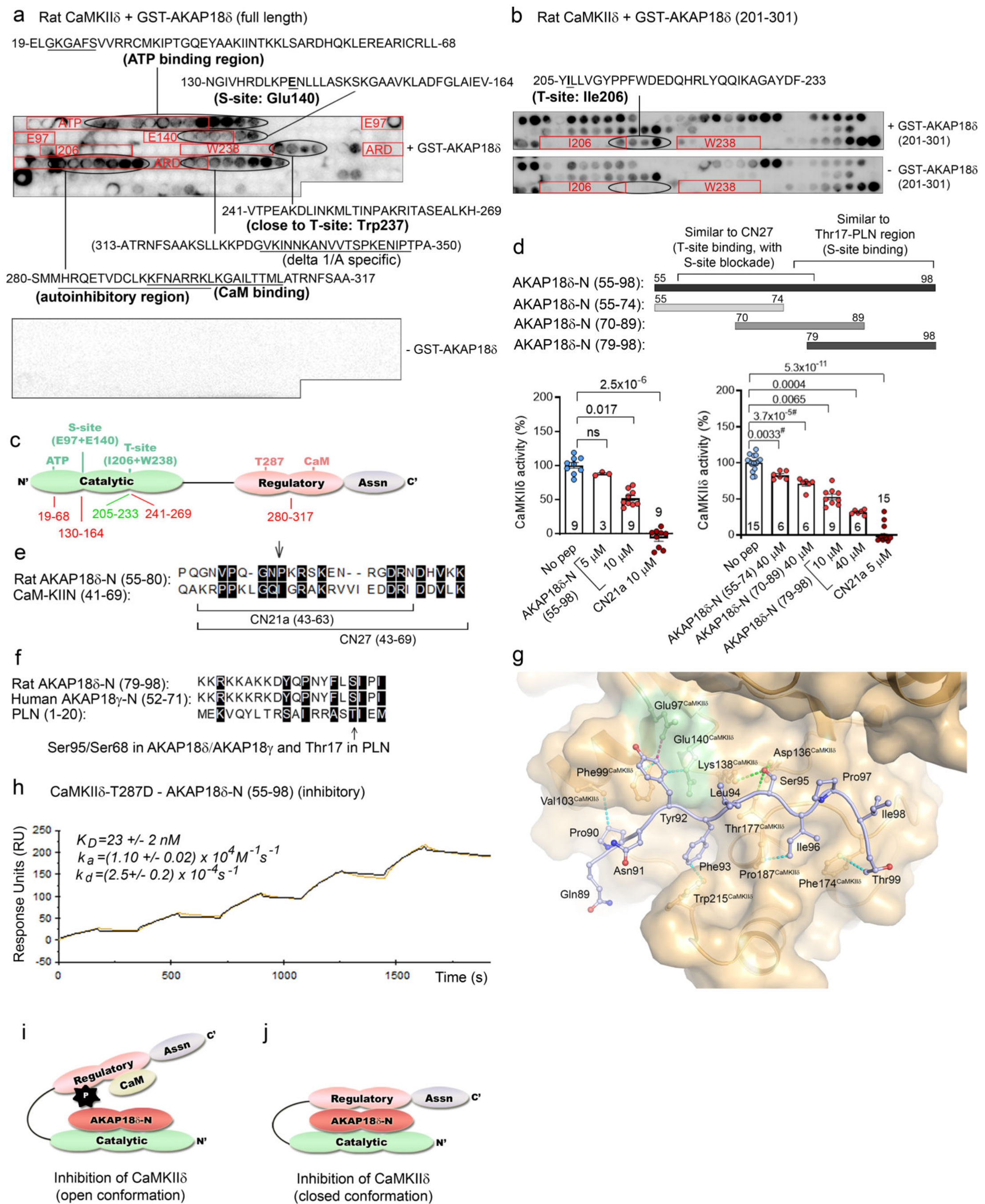

Figure 2. AKAP18δ binds CaMKIIδ through multiple sites and inhibits CaMKIIδ through sequences similar to the natural CaMKII inhibitor protein and Thr17-PLN region.

AKAP18δ binding was identified by overlaying 20-mer overlapping CaMKIIδ peptides with (**a**) GST-AKAP18δ or (**b**) GST-AKAP18δ (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 [7] (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 [7]. The CaMKIIδ$_{1/A}$-specific sequence is only present in neonatal mouse hearts [63]. 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 AKAP18δ binding regions identified by peptide arrays (in **a**-**b)** are indicated in CaMKIIδ$_{2/C}$ (inhibitory ones in red and activating in green). (**d**) Effect of different AKAP18δ-N sequences on CaMKIIδ-T287D activity (^{32}P incorporation into syntide). CN21a derived from natural CaMKII inhibitor protein (CaM-KIIN) [21] 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 AKAP18δ-N peptides. (**e**) Alignment of rat AKAP18δ (55–80) with the natural CaMKII inhibitor protein (amino acids 41–69). CN27 and CN21a are indicated [21]. Arrow denotes a proline in AKAP18δ. (**f**) Alignment of rat AKAP18δ (79–98) and human AKAP18**γ** (52–71) with PLN (1–20). Arrow indicates Ser68/Ser95 in AKAP18**γ**/AKAP18δ and Thr17 in PLN. Black boxes indicate identical or functionally similar amino acids (DNA Star). (**g**) Structural model of the AKAP18δ (89-QPNYFLSIPIT-99) binding to CaMKIIδ centered at Ser95 (corresponds to Thr17 in PLN). AKAP18δ is shown as a ball-and-stick model, while CaMKIIδ 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 CaMKIIδ surface is shown in yellow. (**h**) SPR analysis of immobilized biotin-ahx-AKAP18δ (55–98) on an SA chip and recombinant CaMKIIδ-T287D injected at a range of concentrations (47.6–500 nM) (n=3). (**i-j**) Illustration of CaMKIIδ inhibition by AKAP18δ-N. AKAP18δ-N (red) binds to catalytic region (light green) and inhibits CaMKIIδ in both an (**i**) open and (**j**) closed conformation after pThr287 dephosphorylation and CaM dissociation.

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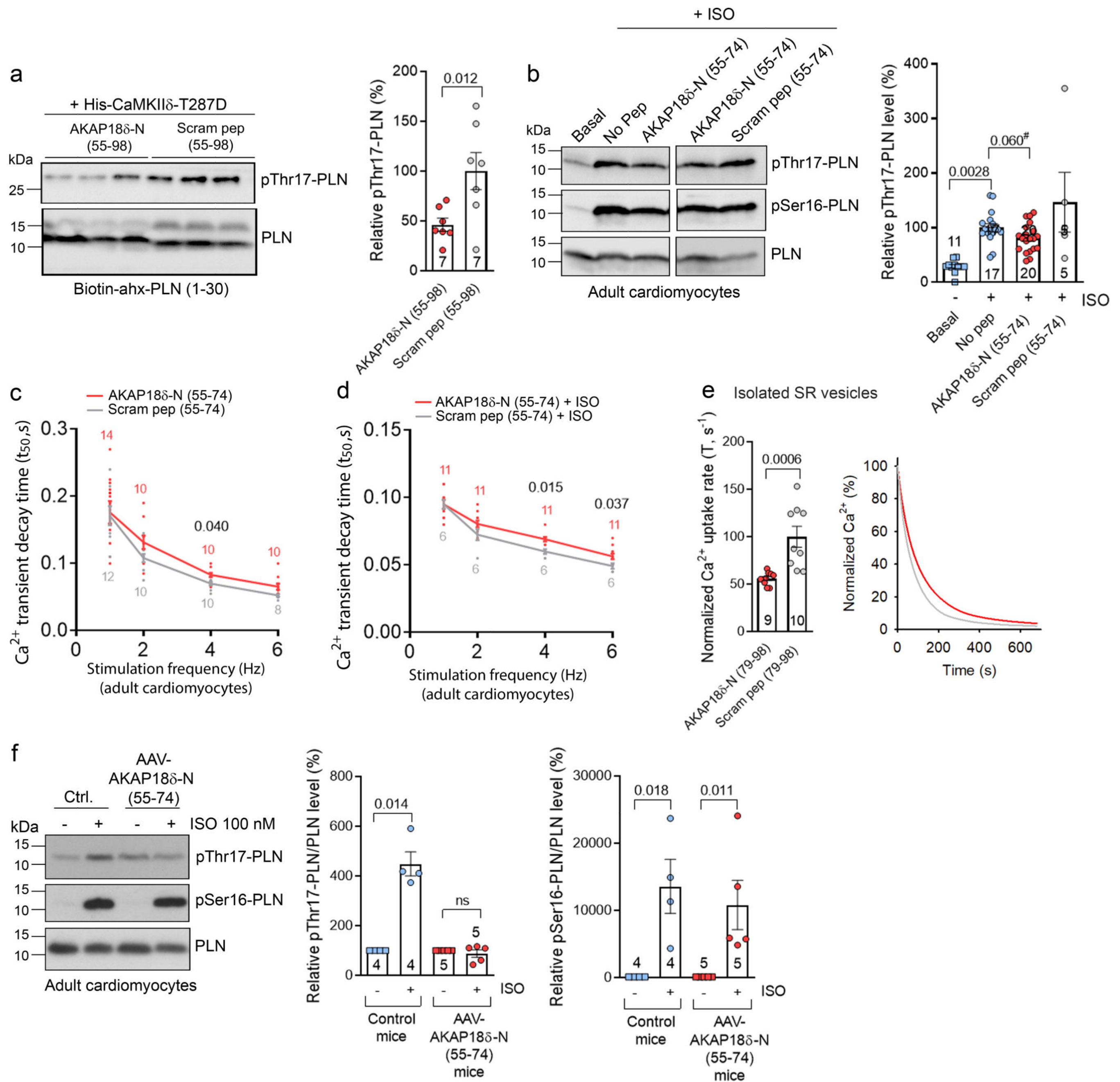

Figure 3. AKAP18δ-N reduces pThr17-PLN and inhibits Ca^{2+} reuptake into SR.

(**a**) CaMKIIδ phosphorylation of biotin-ahx-PLN (1–30), in the presence of AKAP18δ-N (55–98) or a scrambled control peptide. Phosphorylated Thr17-PLN was observed at 25 kDa consistent with induced oligomerization [64] (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-AKAP18δ-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-AKAP18δ-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-AKAP18δ-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-AKAP18δ-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-AKAP18δ-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).

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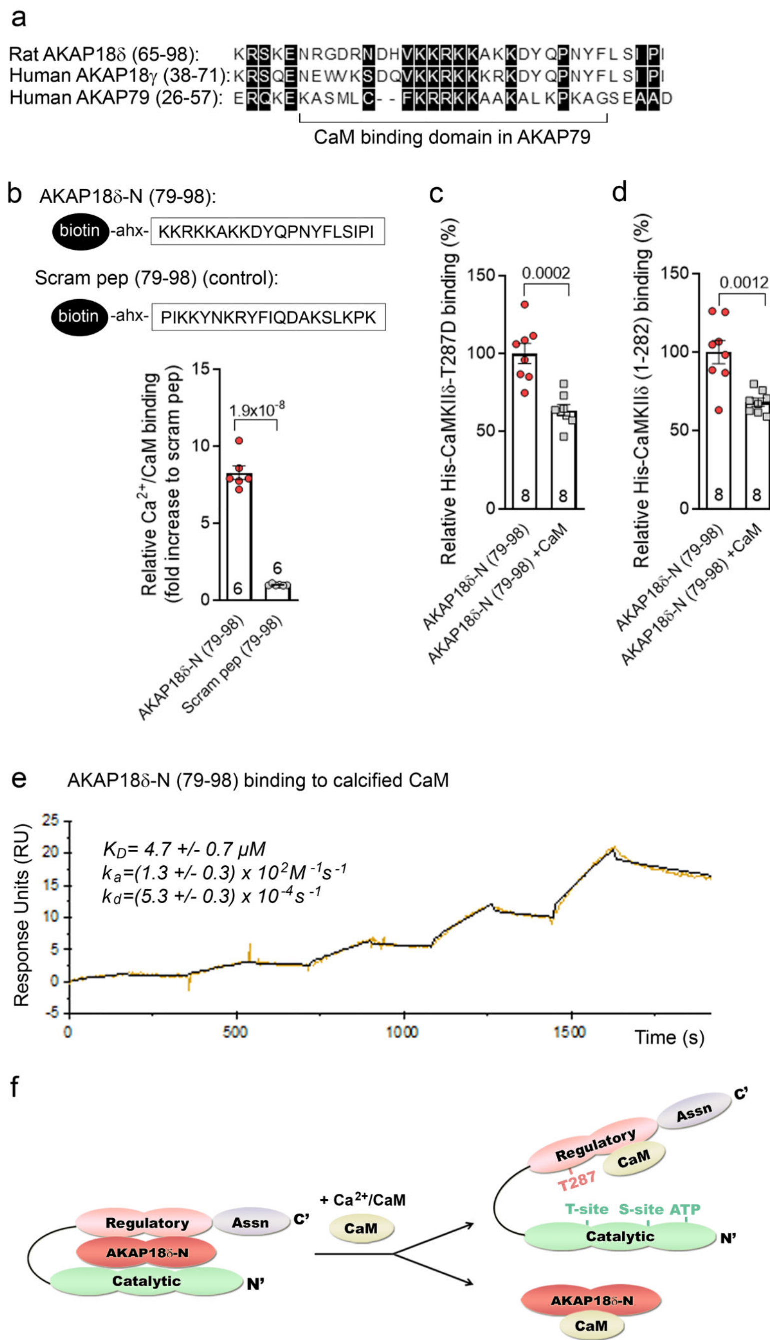

Figure 4. Calcified CaM binds to AKAP18δ-N and outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction.

(**a**) Alignment of rat AKAP18δ-N (65–98) and human AKAP18γ (38–71) with the CaM-binding domain in AKAP79 [25]. Black boxes indicate identical or functional similar amino acids (DNA Star). (**b**) Binding of biotin-ahx-AKAP18δ-N (79–98) or a scrambled control to Ca^{2+}/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 AKAP18δ-N (79–98) peptide was incubated with or

without CaM in wells coated with recombinant (**c**) CaMKIIδ-T287D or (**d**) CaMKIIδ (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-AKAP18δ (79–98) and recombinant CaM injected (952.6–10000 nM) (n=3). (**f**) CaM (beige) outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction upon binding to the CaMKIIδ regulatory region (pink) and AKAP18δ-N (red).

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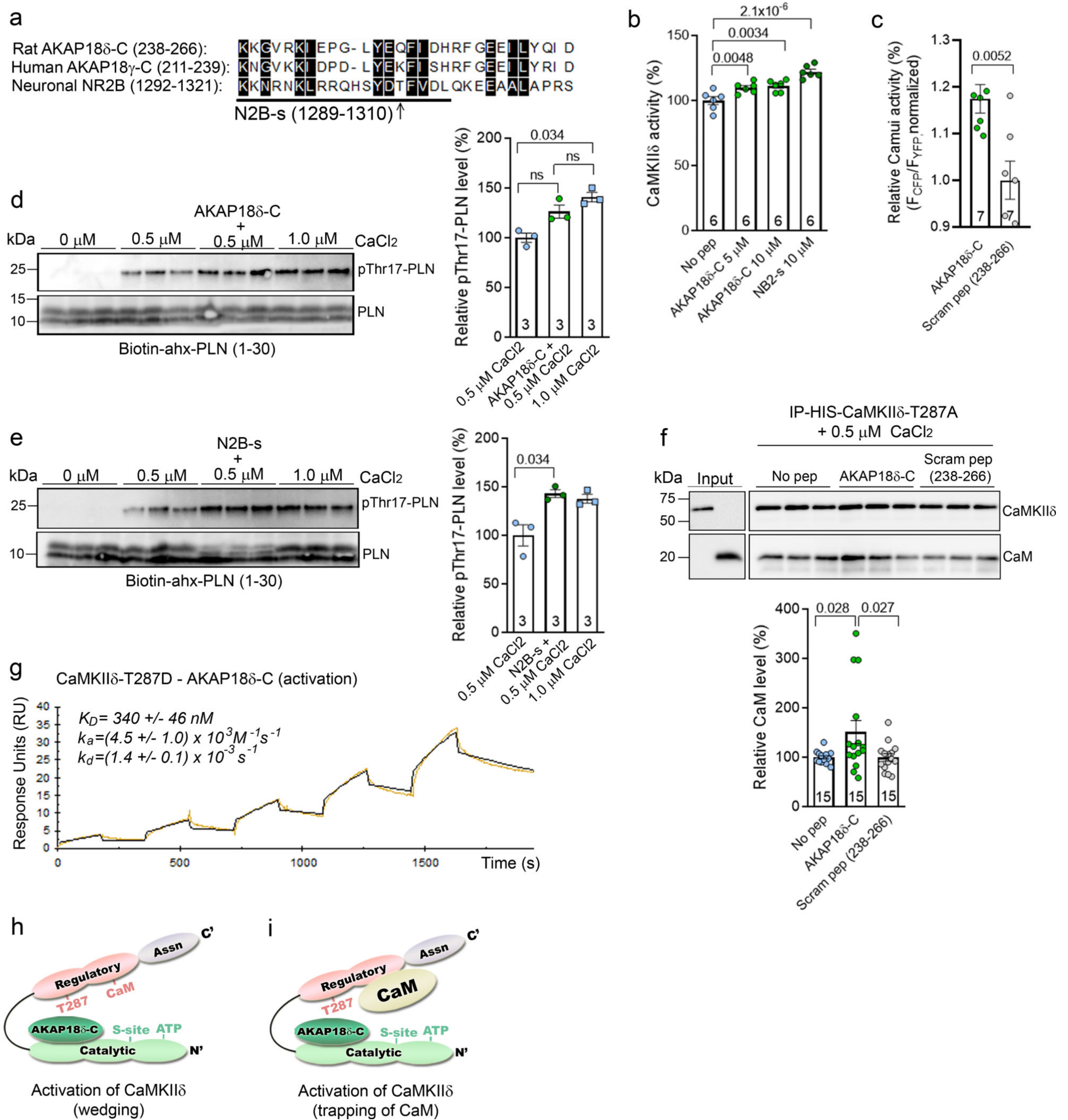

Figure 5. AKAP18δ-C is homologous to the neuronal CaMKIIα activator N2B-s and lowers the Ca^{2+} threshold for CaMKIIδ activation.

(**a**) Alignment of AKAP18δ-C (238–266) and AKAP18γ-C (211–239) with CaMKIIα binding site in neuronal NMDA receptor NR2B subunit (amino acids 1289–1321) [18]. N2B-s (1292–1310) is underlined. Arrow denotes CaMKIIα phosphorylation site in NR2B (Thr1306). Black boxes indicate identical or functionally similar amino acids. (**b**) Effect of AKAP18δ-C on CaMKIIδ-T287A activity analyzed in a CaMKII kinase assay in the absence of CaM (^{32}P incorporation into syntide). N2B-s [18] and CN21a [21] were used as controls. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences

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were examined by ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test (n=6). (**c**) Effect of TAT-AKAP18δ-C on a Camui FRET-based bio-sensor [28], 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 CaMKIIδ-catalyzed phosphorylation of Thr17-PLN at 0.5 μM $CaCl_2$ with or without the presence of (**d**) AKAP18δ-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 CaMKIIδ-T287A in the absence or presence of AKAP18δ-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-AKAP18δ-C and recombinant CaMKIIδ-T287D injected (95.2–1000 nM) (n=3). (**h-i**) AKAP18δ-C (dark green) binds to catalytic region of CaMKIIδ (light green) and activates CaMKIIδ by (**h**) lowering the Ca^{2+} threshold for activation by keeping the inhibitory gate open and (**i**) trapping CaM (beige) within the kinase.

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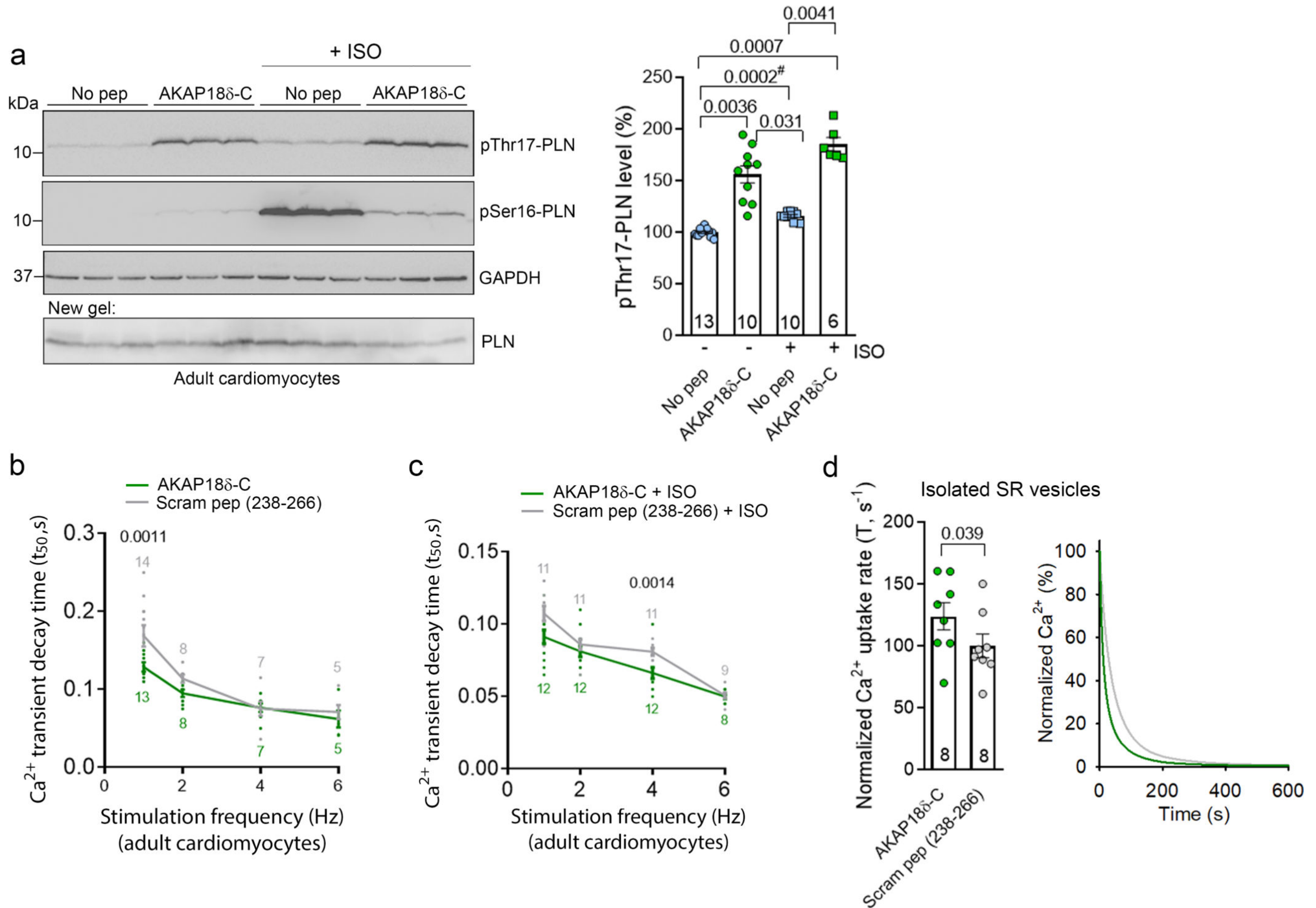

Figure 6. AKAP18δ-C increases pThr17-PLN and facilitates faster Ca^{2+} reuptake into SR.
(**a**) Effect of TAT-AKAP18δ-C on pThr17-PLN in basal and during ISO-stimulation (5 min) in adult cardiomyocytes. pThr17-PLN, pSer16-PLN and PLN levels were detected by immunoblotting. Normal distribution was confirmed 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-AKAP18δ-C or a scramble control on decay time of 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-AKAP18δ-C or a scrambled control on Ca^{2+} reuptake rate in isolated mouse SR vesicles (left ventricle crude homogenate). Significant differences were examined by Wilcoxon matched-pairs signed rank test.

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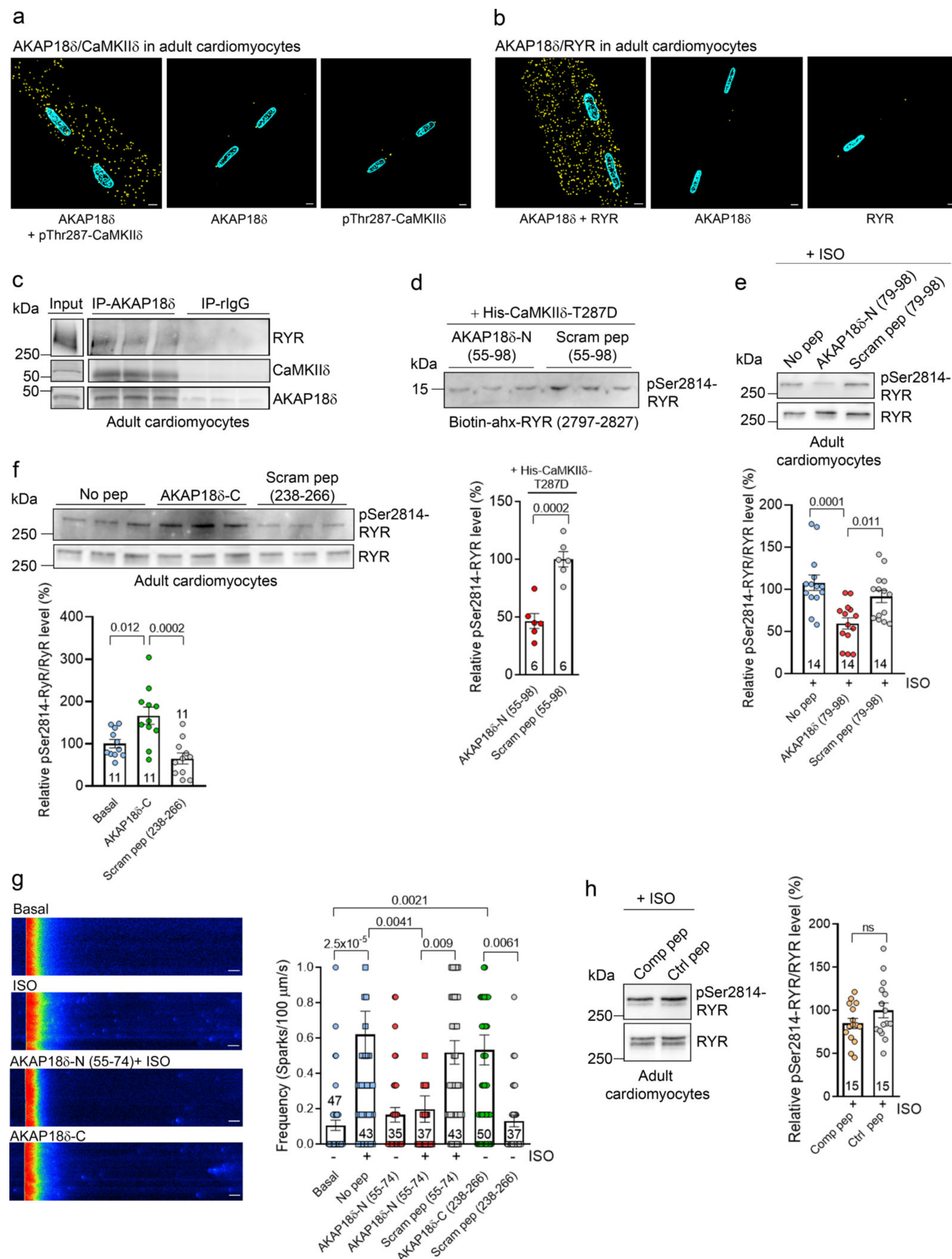

Figure 7. AKAP18δ anchors and functionally regulates CaMKIIδ activity also at RYR.

In situ proximity ligation assay of (**a**) AKAP18δ-pThr287-CaMKIIδ and (**b**) AKAP18δ-RYR (yellow dots in left panels) in adult cardiomyocytes (see method section for detailed description). Incubations with only anti-AKAP18δ, anti-pThr287-CaMKIIδ 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 μm. (**c**) Immunoprecipitation of AKAP18δ-CaMKIIδ-RYR in adult cardiomyocyte lysate detected by immunoblotting. (**d**) CaMKIIδ phosphorylation of biotin-ahx-RYR (2797–2827), with or without presence of

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AKAP18δ-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-AKAP18δ-N (79–98) in presence of ISO or (**f**) AKAP18δ-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-AKAP18δ-N (55–74), AKAP18δ-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 AKAP18δ-PLN competitor or control peptide. Normal distribution was confirmed by Shapiro-Wilk test Ns; not significant, examined by nested t-test (n=12, 5 rats).

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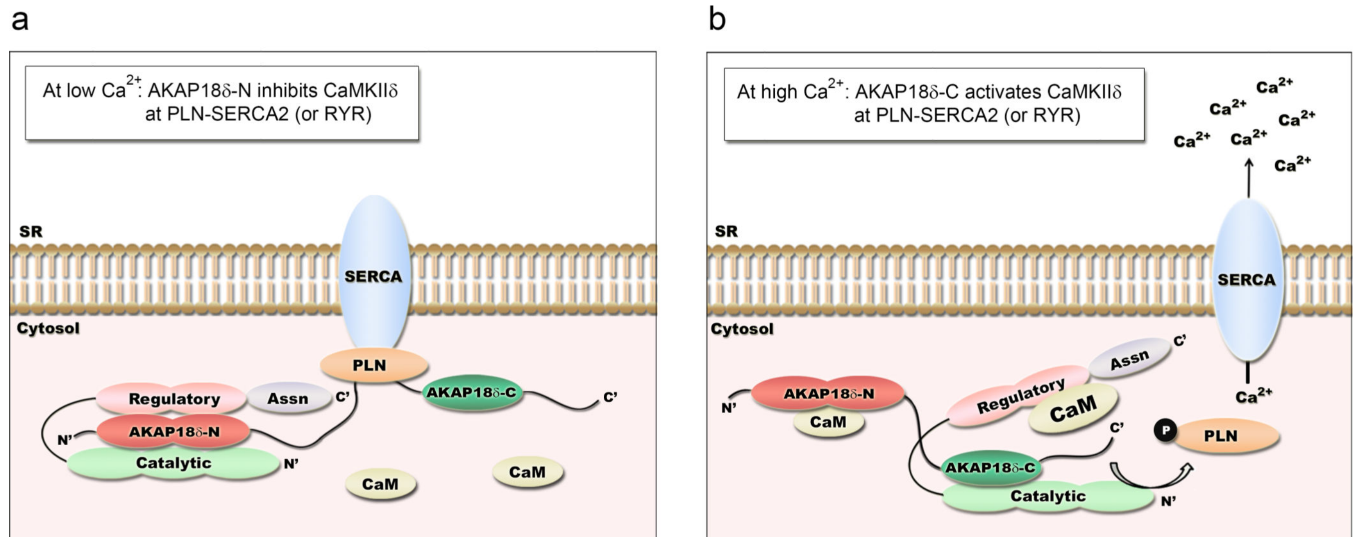

Figure 8. A model where the two unique regions in AKAP18δ fine-tune CaMKIIδ activation at PLN-SERCA2 (or RYR).

(**a**) At low Ca^{2+} transient frequency, AKAP18δ-N (red) inhibits CaMKIIδ 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 Ca^{2+} transient frequency increases, accumulated calcified CaM (beige) outcompetes the inhibitory AKAP18δ-CaMKIIδ interaction by binding to the regulatory domain (pink) and AKAP18δ-N (red). AKAP18δ-C (dark green) binds to the released T-site in CaMKIIδ and lowers the Ca^{2+} threshold for kinase activation, by keeping the inhibitory gate open and trapping CaM within CaMKIIδ. CaMKIIδ catalyzes further Thr17-PLN (or Ser2814-RYR) phosphorylation, leading to reduced PLN association and faster SR Ca^{2+} reuptake by SERCA2 (or Ca^{2+} release by RYR). It is also possible that AKAP18δ-N and AKAP18δ-C regulate two different CaMKII molecules. Assn; association domain.