SUPPLEMENTAL MATERIALS

AKAP18δ anchors and regulates CaMKII activity at phospholamban-SERCA2 and RYR

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Expanded Materials & Metods

Please see the Major Resources Table in the Supplemental Materials.

Antibodies used for immunoblotting and immunoprecipitations

Immunoblotting (IB) and immunoprecipitations (IPs) were carried out using anti-AKAP18δ (1:1000 dilution for IB or 2 µl for IPs, obtained from [13]), anti-AKAP18δ (1:1000 dilution for IB, custom made by Genscript Corp.), anti-PLN (1:80000 dilution, MA3-922, Thermo Fischer Scientific), anti-pThr17-PLN (1:5000 dilution, A010-13, Badrilla), anti-pSer16-PLN (1:5000 dilution, A010-12, Badrilla), anti-CaMKIIδ (custom made from Genscript Corp. with PCIPNGKENF as antigen), anti-pThr286-CaMKII (corresponding to pThr287 in CaMKIIδ) (1:2000 dilution, ab2724, Abcam), anti-CaM (1:250 dilution, 4830, Cell Signaling), anti-GFP (1:5000 dilution, 632381, Clontech), anti-SERCA2 (1:2500 dilution, MA3-919, Thermo Fischer Scientific),

anti-RYR (1:1000 dilution, MA3-916, Thermo Fischer Scientific), anti-pSer2814-RYR (1:5000 dilution, A010-31, Badrilla), anti-pSer2808-RYR (1:5000 dilution, A010-30, Badrilla), anti-GAPDH (1:500 dilution, sc-20357, Santa Cruz), anti-HIS (A00186, Genscript Corp.), and monoclonal anti-biotin agarose conjugated beads (A-1559, Sigma). Horseradish peroxidase-(HRP) conjugated anti-mouse (1:3000 dilution, NA931V, GE Healthcare), anti-rabbit (1:3000 dilution, NA934V, GE Healthcare), monoclonal anti-rabbit light chain specific (1:10000, 211-002-171, Jackson ImmunoResearch), anti-goat (1:2500 dilution, HAF109, R&D Systems, Minneapolis, Minnesota), anti-GST (1:5000 dilution, RPN 1236, GE Healthcare), anti-HIS (1:5000 dilution, 46-0707, Thermo Fischer Scientific), and monoclonal anti-biotin (1:5000 dilution, A-0185, Sigma) were used as secondary antibodies.

Immunoprecipitation and pull down

Cardiomyocytes and HEK293 cells were first lysed in either IP-buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA**,** 1 % Triton X-100) or in a lysis buffer consisting of 1 x PBS, 1% Triton, and 0.1% Tween. Both lysis buffers were supplemented with phosphatase inhibitors (PhosSTOP tablets 49068370001, Roche Diagnostics, Indianapolis) and protease inhibitors (1183617001, Complete Mini EDTA-free tablets, Roche Diagnostics, Indianapolis). Cardiomyocyte lysate (in 3 mM $CaCl_2$) or lysates from HEK293 cells were further incubated with the specific antibodies and 50 µl protein PLUS A/G agarose beads (sc-2003, Santa Cruz) overnight at 4 °C. Immunocomplexes were washed three times in IP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA**,** 1 % Triton), centrifuged at 3000 x g for 1 min at 4 °C and boiled in 1x SDS loading buffer (4 x SDS loading buffer: 50 % sucrose, 7.5 % SDS, 62.5 mM Tris-HCl pH 6.8, 2 mM EDTA, 3.1% DTT, 0.01% Bromophenol Blue) before the immunoblot analyses. Rabbit IgG (sc-2027, AH Diagnostics) was used as negative control.

In the pull down assay, biotin-ahx-AKAP18δ-N (55-98) (10 µM) was coupled to 25 µl monoclonal anti-biotin conjugated beads (A1559, Sigma) in PBS for 2 hrs at 4 °C under gentle rotation. The beads with bound peptide were thereafter washed one time with PBS, incubated with 1 µg of His-CaMKIIδ-T287A in IP buffer with or without the presence of 1 µg recombinant CaM, and gently rotated for 2 hrs at 4 °C. The beads were then washed three times in 0.5 ml IP buffer and centrifuged at 3000 x *g* for 1 min at 4 °C before boiling in SDS loading buffer. The eluted complexes were subjected to immunoblot analysis.

Immunoblot analysis

Lysates (boiled in SDS loading buffer at 96 °C for 5 min or 10 min at 37 °C (RYR)) and immunoprecipitates were separated on 10 %, 12 %, 15 %, 4-15 % or a 4-20 % Criterion Tris-HCl or TGX precast gels, depending on protein sizes and samples, and blotted onto PVDF membranes. The PVDF membranes and peptide arrays were blocked in 2.5% or 5 % non-fat dry milk, 5 % BSA or 1 x casein, depending on the antibody used, in TBST for 60 min at room temperature. Following overnight incubation with primary antibodies at 4 °C, the membranes were washed five times 5 min or three times 10 min in TBST before subsequent incubation with primary or HRP-conjugated secondary antibody. Blots were developed using ECL Plus or ECL Prime (GE

Healthcare, RPN2132 and RPN2236). The chemiluminescence signals were detected by LAS1000 or LAS4000 (Fujifilm, Tokyo, Japan) or Azure Biosystems. Membranes were reprobed after stripping in Restore™ Western Blot Stripping Buffer (#21059, Thermo Scientific, United States) for 5 or 10 min at RT.

Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated as described previously [65], under approval number IV1-17U. The LVs of 1 to 3 day-old Wistar rats (Møllergaard Breeding and Research Center, Denmark) were excised, minced, and enzymatically digested with a collagenase solution (#LS004176, Worthington Biochemical). The cell suspension was transferred to uncoated culture flasks with serum-containing medium in a humidified incubator (5 % CO_2) for 20 min at 37 °C. Cardiomyocytes were isolated as non-adherent cells and seeded onto 6-well culture plates pre-coated with gelatin (G9391)/fibronectin (F1441) (Sigma), in plating medium consisting of DMEM (D1152, Sigma), M-199 (M2520, Sigma), penicillin/streptomycin, horse serum (14403E, BioWhittaker), and fetal bovine serum (14-701F, BioWhittaker). The cells were maintained in a humidified incubator with 5 % CO_2 at 37 °C for one or two days before treatment with cell-permeant peptides and isoproterenol stimulation.

Adeno-associated virus (AAV) vectors and in vivo gene transfer

An adeno-associated (AAV) serotype 9-based delivery system was used to overexpress AKAP18δ-N (55-74) *in vivo*. AKAP18δ-N (55-74) was linked to the fluorophore mCherry using a self-cleaving porcine teschovirus-1 2A (P2A) peptide by cloning the AKAP18δ-N (55-74) cDNA (sequence: cctcagggcaacgtgcctcagggcaaccctaagcggagcaaggagaaccggggcgaccgg) under control of the human cardiac troponin T promoter (TNNT2) into pSSV9-TnT-Cherry-2A using RsrII and AgeI restriction sites [66]. For AAV production, pSSV9-TnT-Cherry-2A with or without AKAP18δ-N (55-74) was co-transfected with the helper plasmid pDP9rs into low passage HEK293T cells and AAV vectors were purified and titrated as described before [67]. Then $1x10^{12}$ viral genomes/mouse of AAV-AKAP18δ-N (55-74) or control (AAV-TnT-Cherry-2A) were injected into tail vein (8-10 week old C57Bl6/J mice males and females randomized) and cardiomyocytes were isolated 4 weeks later as previously described [68]. Scientific personnel performing immunoblotting were blinded in terms of use of the tissue from AAV-AKAP18δ-N (55-74) versus control injected mice.

Isolation of adult rat cardiomyocytes

~300 g male Wistar rats (Janvier, France) were sedated in an anesthesia chamber and subsequently ventilated through an endotracheal tube with a combination of 64 % N_2O, 32 % O_2 and 3 % isoflurane. The heart was excised under deep surgical anesthesia, and perfused retrogradely through the aorta in a modified Langendorff setup with buffer containing (in mM) (pH adjusted to 7.4): NaCl 130, Hepes 25, D-glucose 22, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $CaCl_2$ 0. 67 and 0.8 g/L collagenase II (#LS004176, Worthington Biochemical Corporation, USA). Once cleared of blood, the perfusate was changed to one including collagenase (2 mg/mL, Worthington Biochemical Corp., Lakewood, NJ, USA) and low [Ca^{2+}] (0.05 mM). After 10 min of digestion, hearts were

cut into pieces, minced, and filtered, and isolated cardiomyocytes were allowed to sediment. Animal experiments were approved by the Norwegian Research Authority (FOTS ID: 3284), conforming to ETS no. 123.

Treating cardiomyocytes with cell-permeant peptides before immunoblot analyses

Isolated neonatal rat cardiomyocytes were cultured for one or two days in 6-well plates at a density of 4.4 x 10^6 cells /ml. A fraction of the cardiomyocytes was treated with arginine-coupled peptides (30 µM) for 45 min before isoproterenol stimulation (ISO, 0.1 µM) for 15 min. The cells were then washed twice with RNase-free PBS before harvesting in IP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA**,** 1 % Triton X-100) with phosphatase (PMSF, Na_3VO_4, NAF or PhosSTOP tablets, 49068370001, Roche Diagnostics, Indianapolis) and protease inhibitors (1183617001, Complete Mini EDTA-free tablets, Roche Diagnostics, Indianapolis) and centrifuged at 14,000 *g* for 10 min at 4 °C. Supernatants were collected and stored at -80 °C before SDS/PAGE and immunoblotting analysis.

Isolated adult cardiomyocytes were treated in 1.5 ml tubes (room temperature with gentle tilting) with Arg_9/TAT-coupled peptides for 45 min before isoproterenol stimulation (ISO) for 15 min. 55 min peptide treatment before isoproterenol stimulation (ISO) for 5 min was used in Fig. 6a. We used 0.2 µM ISO in Fig. 7e (RYR) and 0.5 µM ISO in the PLN competitor peptide experiments (Fig. 1c and Fig. 7h). Otherwise, 0.1 µM ISO was used. We used 30 µM of cell-permeant AKAP18δ-C, AKAP18δ-N (55-74), PLN competitor peptide or the respective control peptides, or 5 µM of AKAP18δ-N (79-98) or the respective scrambled control peptide. Only TFA free peptides were used in adult cardiomyocyte experiments. The cardiomyocytes were either lysed in the IP buffer described above or in a lysis buffer consisting of 1 x PBS, 1% Triton, 0.1% Tween supplemented with complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail.

Whole cell fluorescence imaging

Adult cardiomyocytes were loaded for 60 min with TAT-conjugated peptides and 5 min with 20 µM Fluo-4 AM (F14201, Molecular Probes, Eugene, USA) at room temperature. Whole cell Ca^{2+} transients were recorded with a photomultiplier (PTI model 4000, Photon Technology International, NJ, USA) during superfusion at 37 °C with a solution containing (mM): NaCl 140, D-glucose 5.5, KCl 5.4, Hepes 5, $CaCl_2$ 1, $MgCl_2$ 0.5, and NaH_2PO_4 0.4, pH=7.4. In a subset of the experiments, 20 nM ISO was added to the superfusate. Cells were field stimulated at 1, 2, 4 and 6 Hz (2.5 ms bipolar pulse) for 30s, with a 10s break between each stimulation frequency. Only cells observed to be stably stimulated without spontaneous Ca^{2+} waves were included. Background fluorescence was subtracted from each recording. Ca^{2+} extrusion time was analyzed as the time from the peak Ca^{2+} transient to 50% decline (t_{50}).

SR vesicles

Ca^{2+} handling was examined using crude homogenates from mice left ventricle, based on methods described by O'Brien and modified by Li et al [69, 70]. Fresh ventricular tissue was weighed and homogenized in ice-cold

buffer (1:10 wet weight/vol, pH 7.9) containing (in mM): 300 sucrose, 5 NaN_3, 1 EDTA, 40 L-histidine, 40 Tris HCl and protease inhibitors. Homogenization was performed with a Polytron 1200 (Kinematica AG, Luzern, Switzerland) at 25,000 rpm for 3 x 20 sec, with a 20 sec break between the bursts. Homogenates were then aliquoted, frozen in liquid N_2, and stored at -80 °C until use.

Ca^{2+} uptake was measured in 250 µl of assay buffer, containing (in mM): 165 KCl, 22 Hepes, 7.5 oxalate, 11 NaN_3, 0.0055 TPEN, 4.5 $MgCl_2$, 9 Tris HCl and 0.002 Fura-2 salt (pH = 7.0, 37 °C). Ca^{2+} fluxes were monitored with a Hidex Sense Multimodal Microplate Reader (Kem-En-Tec Nordic AS) after addition of 10-20 µl of freshly thawed and vortexed homogenate. The buffer with the homogenate was preincubated with 40 µM of TAT-AKAP18δ-N (79-98) or AKAP18δ-C (238-266) (or the scrambled control peptides) for 5 min at 37 °C before Ca^{2+} uptake by the vesicles was initiated by addition of Na_4ATP (2.2 mM), and then blocked by application of thapsigargin (1.5 mM) after 12 min. The fluorescence ratio was calibrated to $[Ca^{2+}]$ using the following equation: $[Ca^{2+}]=K_d *((R - R_{min})/(R_{max} - R))*(S_{f2}/S_{b2})$, where R is the 340 nm/390 nm fluorescence ratio, K_d is the dissociation constant of Fura-2 and Ca^{2+} and S_{f2}/S_{b2} is the ratio of measured fluorescence intensity at 390 nm when Fura-2 is Ca^{2+} free or saturated, respectively. R_{min} is the ratio at very low $[Ca^{2+}]$ and R_{max} is the ratio at saturating $[Ca^{2+}]$, obtained by adding 3.3 mM EGTA and 4.8 mM $CaCl_2$ respectively to the well at the end of each recording.

The maximal Ca^{2+} uptake rate was calculated as the minimal first derivative of the smoothed (TableCurve 2D, V 5.01, Systat Software Inc) Ca^{2+} uptake curve. The time constant (τ) of the Ca uptake were calculated by fitting the uptake curve to $Ca^{2+}=Ca^{2+}{}_0+ae^{-t*\tau}$ (Sigmaplot V14.0, Systat Software Inc).

Fluorescence resonance energy transfer (FRET)

An adenovirus encoding Camui FRET bio-sensor was transduced in isolated adult rabbit ventricular myocytes cultured for 2 - 4h (M.O.I 10–100) in PC-1 medium (5 % CO_2, 37 °C), as previously described [28]. Experiments were performed 24h after transduction. Transduced cardiomyocytes were incubated 90 min with 5 µM of TAT-AKAP18δ-C (238-266) or the scrambled control peptide. We recorded FRET images using confocal microscopy in frame scan mode (Zeiss LSM5 Pascal, ×40 water immersion objective). FRET signal was measured as an increase of the F_{CFP}/F_{YFP} ratio upon donor excitation. The λex was set at 458 nm (Ar laser) for CFP (donor) and at 510 nm for YFP (acceptor). Donor fluorescence emission was detected at 470–500 nm for CFP, and acceptor fluorescence was measured at ≥530 nm. Fluorescence images were analyzed using ImageJ software (NIH).

Immunostaining and airyscan microscopy

Freshly isolated rat cardiac myocytes were treated with consecutive steps for chemical fixation (4 % formaldehyde in 0.1 mol/L HEPES buffer, 10 min), quenching (PBS + 100 mM glycine, 10 min), permeabilization (PBS + 0.03 % Triton X-100, 10 min), and blocking (NaCl 150 mM, Na_3 citrate 17.5 mM, 5 % goat serum, 3 % BSA, and 0.02 % NaN_3, 2 hrs). Washing with PBS was performed in between each step. The sections were then incubated overnight with 1/100 dilutions of the following primary antibodies: anti-

AKAP18δ rabbit (custom made, Genscript), anti-SERCA2 mouse (MA3-919, Thermo Fischer Scientific), anti-CaMKIIδ rabbit (epitope against the unique 480-PCIPNGKENF-489 sequence in the extreme C-terminus of rat CaMKIIδ, custom made by Genscript), and anti-α-actinin mouse (ab9465, Abcam). The low blocking buffer employed for incubation contained 150 mM NaCl, 17.5 mM Na_3 citrate, 2 % goat serum, 1 % BSA, and 0.02 % NaN_3 at 4 °C. The following day, cells were washed with PBS and incubated with 1/200 dilutions of secondary antibodies in low blocking buffer for 2 hrs. Employed secondary antibodies were Alexa Fluor 680 conjugated goat-anti-mouse (A32729, Thermo Fisher Scientific) and Alexa Fluor 546 conjugated donkey anti-Rabbit (A10040, Thermo Fisher Scientific). In some experiments, t-tubules were labelled with 20 µg/mL Alexa Fluor 488 conjugated wheat germ agglutinin (W11261, Thermo Fischer Scientific).

For fluorescence microscopy, the sections were washed and sealed beneath a coverslip with SlowFade Diamond Antifade Mountant (S36963, Thermo Fisher Scientific). Imaging was performed on a ZEISS LSM 800 with Airyscan using the Airyscan super-resolution mode. Images were pre-processed with built-in software (Zeiss, Jena GmbH) using Airyscan processing and deconvolution algorithms, and then imported to FIJI (ImageJ). Images were subjected to background subtraction and contrast enhancement. Regions of interest were subsequently selected for profile plotting for each channel.

Proximity ligation assay

In situ protein-protein interactions between either AKAP18 and CaMKII or AKAP18 and RYR were assessed using Duolink® Proximity Ligation Assay (PLA) (DUO92101-1KT, Sigma-Aldrich). First, isolated rat ventricular cardiomyocytes were plated on laminin-coated glass bottom dishes (No 1.5, Ø 14 mm, γ-irradiated, Martek Corporation). The cells were subsequently fixed (4 % PFA, 10 min), quenched (150 mM Glycine, 10 min), permeabilized (Triton X-100, 0.5%, 10 min) and finally blocked using a blocking buffer containing 5% goat serum and 0.02% sodium azide in Dulbecco's PBS for 30 min at room temperature. Primary antibodies for rabbit RYR (1:100, HPA020028, Sigma-Aldrich), mouse RYR (1:100, MA3-916, ThermoFisher Scientific), pThr286-CaMKII (1:100, ab171095, Abcam) and AKAP18 (1:100, custom made by GenScript Corp.) were constituted in a mixture containing (2.5% goat serum, 0.02% sodium azide and 0,1% Triton X-100) and incubated overnight at 4 °C. Following primary antibody incubation, cells were prepared based on the online Duolink® PLA protocol (https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-fluorescence-user-manual.html#fluorescence). Briefly, cells were conjugated to complimentary oligonucleotides in a plus (rabbit) and minus (mouse) configuration and incubated for 1 hour at 37 °C. This was followed by ligation (30 min, 37 °C) and amplification (100 min, 37 °C) of fluorescent-labelled oligonucleotides. Finally, the signal of proximal proteins were visualized as distinct spots of fluorescence, using an LSM 800 confocal microscope (Carl Zeiss, Germany).

Ca^{2+} imaging and analysis

Isolated cardiomyocytes were first treated with 5 µM peptide in 0.2 mM Ca^{2+} cell isolation solution for 30 min, then loaded with fluo-4 AM (20 µmol/L, 15 min) with or without ISO and subsequently superfused with

Hepes-Tyrode solution containing (in mM): 140 NaCl, 0.5 $MgCl_2$, 5.0 HEPES, 5.5 glucose, 0.4 NaH_2PO_4, 5.4 KCl and 1.8 $CaCl_2$ (pH 7.4, 37°C). Spontaneous Ca^{2+} sparks were recorded in quiescent cardiomyocytes as confocal line scans using a Zeiss LSM 710 at a frame rate of 1.5 ms and pixel size of 0.1 μm for 6000 frames. Ca^{2+} spark analysis were performed using a custom program (CaSparks 1.01, D. Ursu, 2003) as previously described [71].

ELISA-based assay

An ELISA-based assay was performed as previously described [65]. Briefly, the ELISA plate was coated with 1 μg/well of recombinant GST-AKAP18δ, His-CaMKIIδ-T287D, His-CaMKIIδ (1-282), His-CaMKIIδ (1-75), CaM (C4874, Sigma) or plain CaMKIIδ peptides with the following amino acid sequence: 105-139, 130-164, 187-216, 215-246, or 236-269 in 1 x PBS and tilted gently overnight at 4 °C. The wells were thereafter washed in 1x PBS-T (0.1 %), blocked in 0.5 % gelatine (G-1890, Sigma) for 1 hour at room temperature, and incubated with 0.3, 1 or 5 μl of the given biotinylated peptide in 1 x PBS-T (0.1 %) for 2 hours at 37 °C with gently tilting. The wells were washed five times in 1 x PBS-T (0.1%), incubated with a monoclonal anti-biotin-HRP conjugated antibody (1:5000 dilution, A-0185, Sigma) for 30 min, washed again five times in 1x PBS-T (0.1%), before incubation with the Ultra TMB solution (34028, Thermo Fisher Scientific) for 15-30 min with gently tilting at room temperature. The reaction was stopped by adding 2 N HCl, and the signal was measured by a plate reader at 450 nm (Hidex Sense multimodal microplate reader, Finland).

AlphaScreen assay

In vitro interaction of GST-AKAP18δ with His-CaMKIIδ-T287A or His-CaMKIIδ-T287D was analyzed by AlphaScreen™ technology (Perkin Elmer). GSH-donor beads and Ni-acceptor beads (each 20 μg/ml) were incubated with recombinant GST-AKAP18δ (100 nM) and recombinant His-CaMKIIδ-T287A or His-CaMKIIδ-T287D (10, 30, or 100 nM) in PBS buffer (1 x PBS pH 7.4, 0.1 % Tween 20, 0.1 % BSA) for 2 hours at room temperature in the dark. Alpha-signal was detected using an EnSpire® Multimode Plate Reader.

Transfection of HE293 cells

HEK293 cells were grown in DMEM (41965-039, Thermo Fischer Scientific) supplemented with 10 % FBS (14-701F, BioWhittaker), 1 % non-essential amino acids (10370-021, Thermo Fischer Scientific), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (penicillin/streptomycin, P4333, Sigma) in a 5 % CO_2 humidified incubator at 37 °C. Lipofectamine 2000 (11668-019, Thermo Fischer Scientific) was used for transient transfection as instructed by the manufacturer. After 18-20 hours, the cells were harvested in immunoprecipitation (IP)-buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA**,** 1 % Triton) with complete protease inhibitor mixture tablets (11836170001, Roche Diagnostics, Indianapolis).

Plasmids and fusion proteins

Full length AKAP18δ-YFP and AKAP18δ-YFP deletion mutants were as previously described [72]. His-CaMKIIδ-T287D (NM_012519), the mutated active form of the kinase, was cloned into pcDNA3.1 (Genscript, Piscataway, NJ).

Peptide arrays and peptide synthesis

Rat AKAP18δ (NP_001001801), human AKAP18γ (Q9P0M2) and rat CaMKIIδ (NP_036651) were synthesized as 20-mer overlapping peptides on cellulose paper using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Köln, Germany).

Peptides were synthesized at 80-98 % purity by Genscript (Corp, Piscataway, New Jersey, USA). For cell experiments the peptides were synthesized either with an Arg_9 (RRRRRRRRR) (Fig. 1c, Fig. 7h, Suppl. Fig. 3b, and Suppl. Fig. 6a-b) or a TAT (RKKRRQRRR) (Fig. 3b-d, Fig. 5c, Fig. 6a-c, and Fig. 7e-g) sequence at the N-terminus to make them cell-permeant. All TAT-conjugated peptides were made with formic salt (TFA free). For the ELISA-based assay and overlay experiments the peptides were synthesized with an N-terminal biotin-tag attached via an ahx-linker to avoid potential steric hindrance. The CN21a and N2B-s (kinase assays in Fig. 2d and Fig. 5b), CN27 (competition assay in Suppl. Fig. 2g-h), S-site and T-site peptides (to coat the wells in Suppl. Fig. 2d-e) were synthesized without any tags.

PLN_comp pep: RRASTIEMPQQ

PLN_ctrl pep: RRApSTIEMPQQ

PLN (1-30): MEKVQYLTRSAIRRASTIEMPQQARQNLQN

pThr16-PLN (1-30): MEKVQYLTRSAIRRApSTIEMPQQARQNLQN

pThr17-PLN (1-30): MEKVQYLTRSAIRRASpTIEMPQQARQNLQN

RYR (2797-2827): MALYNRTRRISQTSQVSVDAAHGYSPRAID

AKAP18δ-N (55-98): PQGNVPQGNPKRSKENRGDRNDHVKKRKKAKKDYQPNYFLSIPI

Scram pep (55-98): QFDQNERKVVKKAKSSRYGIPDPLPYKGDRNHGIRNKQPPNKKN

AKAP18δ-N (55-74): PQGNVPQGNPKRSKENRGDR

Scram pep (55-74): PSVQQNNRGRRDEGGNKKPP

AKAP18δ-N (70-89): NRGDRNDHVKKRKKAKKDYQ

AKAP18δ-N (79-98): KKRKKAKKDYQPNYFLSIPI

Scram pep (79-98): PIKKYNKRYFIQDAKSLKPK

AKAP18δ-C (238-266): KKGVRKIEPGLYEQFIDHRFGEEILYQID

Scram pep (238-266): QPQGRRGEGIIEDDFFLLHVIIKEKEKYY (adult cardiomyocytes) or IDKVEGRFEQQLLGEYGHFKDIEPKRYII (ELISA assay)

AKAP18δ-C_ctrl pep: KKGVRKIEPGLpYEQFIDHRFGEEILYQID (neonatal cardiomyocytes)

AKAP18δ-N (79-103): KKRKKAKKDYQPNYFLSIPITNKKI

AKAP18δ-N (F93A, S95A): KKRKKAKKDYQPNYALAIPITNKKI

AKAP18δ-N (F93A, S95V): KKRKKAKKDYQPNYALVIPITNKKI

AKAP18δ-N (Y92D, F93A, S95A): KKRKKAKKDYQPNDALAIPITNKKI

AKAP18δ-N (L94A, P97A, T99A): KKRKKAKKDYQPNYFASIAIANKKI

AKAP18δ-N (I96D, P97A): KKRKKAKKDYQPNYFLSDAITNKKI

Syntide: PLARTLSVAGLPGKK

N2B-s: KAQKKNRNKLRRQHSYDTFVDL

CN21a: KRPPKLGQIGRSKRVVIEDDR

AIP: RKKALRRQGAVDAL

CN27: KRPPKLGQIGRAKRVVIEDDRIDDVLK

CaMKIIδ (105-139): REYYSEADASHCIQQILESVNHCHLNGIVHRDLKP

CaMKIIδ (130-164): NGIVHRDLKPENLLLASKSKGAAVKLADFGLAIEV

CaMKIIδ (187-216): RKDPYGKPVDMWACGVILYILLVGYPPFWDED

CaMKIIδ (215-246): WDEDQHRLYQQIKAGAYDFPSPEWDTVTPEAK

CaMKIIδ (236-269): WDTVTPEAKDLINKMLTINPAKRITASEALKH

Recombinant proteins

GST-AKAP18δ was expressed in *E. coli* BL21 by IPTG induction and purified as previously described [73]. Recombinant His-CaMKIIδ-T287D, His-CaMKIIδ-T287A, His-CaMKIIδ-T287D (1-74), His-CaMKIIδ (1-282), GST-AKAP18δ (201-301) and GST were generated by Genscript (Corp, Piscataway, New Jersey, USA). Active His-CaMKIIδ (PV3373) was obtained from Thermo Fisher Scientific. Recombinant bovine CaM (C4874) was purchased from Sigma.

Peptide array overlays and epitope mapping

Peptide arrays were overlaid with 1 µg/ml of recombinant active His-CaMKIIδ in CaMKII binding buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 15 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 % triton), 2 µg/ml of GST-AKAP18δ, 2 µg/ml GST-AKAP18δ (201-301), 2 µg/ml GST (control) or 5 µM of the biotinylated AKAP18δ-N (55-98) peptide in 1 x casein overnight at 4 °C. Arrays were then washed three times 10 min in TBS-T before incubation with HRP-conjugated anti-GST, anti-HIS or anti-biotin secondary antibody for 1 hour (described in the antibody section). The washing procedure was repeated before the signal was developed and detected as described in the immunoblot section.

For epitope mapping, the peptide arrays were blocked in 1 x casein and thereafter overlaid with the antibody of interest overnight at 4 °C with gentle tilting. The peptide arrays were further washed before incubation with the secondary antibody and washed again before binding was detected as described above.

CaMKII activity assay using syntide as substrate

In Fig. 2d and Fig. 5b we performed a CaMKII assay as described [74]. In brief, 70 nM of recombinant His-CaMKIIδ-T287D was incubated together with 5-40 µM of different test peptides in 10 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5 mM $CaCl_2$, 10 µg/ml CaM (14-368, Merck Millipore), 5 mM DTT, 0.1 mM ATP and 0.1 µCi/µl [γ-^{33}P]ATP (Hartmann Analytic GmbH, Germany) for 30 min at 30 °C. For assays

performed with recombinant His-CaMKIIδ-T287A no CaM was included in the kinase buffer (Fig. 5b). Syntide-2 was used as substrate [75]. The reactions were terminated with 250 mM EDTA before being spotted onto Whatman P81 phosphocellulose chromatography paper (cat. no 3698-915, Fisher Scientific) [76,77] and subsequently washed 3 x 10 min in 1 % phosphoric acid, rinsed in 100 % methanol, and air dried. The membranes were exposed to Fujifilm phosphor imaging plates and measured with a BAS-1800 phosphor imager (Fuji Medical Systems). CN21a [21] and N2B-s [18] were used as control peptides.

CaMKII activity assay using PLN or RYR as a substrate

In Fig. 3a, Fig. 5d-e and Fig. 7d, we performed a CaMKII activity assay using PLN or RYR as a substrate. Briefly, in Fig. 3a or Fig. 7d, recombinant His-CaMKIIδ-T287D and biotin-ahx-PLN (1-30) or biotin-axh-RYR (2797-2827) were incubated with 50 µM of AKAP18δ-N (55-74) or the corresponding scrambled control peptide in CaMKII kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 % Triton X-100, 1 % BSA, 0.1 mM ATP, 10 µg/ml CaM) for 30 min at 37 °C. The assay was also performed in the absence of recombinant His-CaMKIIδ-T287D and/or AKAP18δ-N (55-74) (additional controls in Suppl. Fig. 3a). The reactions were stopped by boiling in SDS loading buffer for 5 min at 95 °C. In Fig. 5d-e, 50 µM of AKAP18δ-C (238-266) or the N2B-s control peptide with CaMKIIδ-T287A were tested in CaMKII kinase buffer with different concentrations of $CaCl_2$ (0 – 1.0 µM). The level of CaMKIIδ phosphorylation of Thr17-PLN was analyzed by immunoblotting.

CaM trapping

Recombinant CaMKIIδ-T287A (2 µg) in 100 µl kinase buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 0.1 % Triton X-100, 1 % BSA, 0.1 mM ATP, 10 µg/ml CaM) containing 0.5 µM $CaCl_2$ was incubated with 50 µM of AKAP18δ-C (238-266) or the scrambled control peptide for 30 min at 37 °C. Thereafter HIS antibody (2 µg, A00186, Genscript Corp.) in 100 µl ice cold IP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA**,** 1.0 % Triton) and 50 µl protein PLUS A/G agarose beads (sc-2003, Santa Cruz) were added and gently rotated overnight at 4 °C. Immunocomplexes were washed three times in IP buffer, centrifuged at 3000 x *g* for 1 min at 4 °C, and boiled in SDS loading buffer before immunoblot analyses were performed. 100 ng of recombinant bovine CaM and 200 ng of CaMKIIδ-T287A were used as input controls.

Surface plasmon resonance (SPR) analysis

The SPR analyses were performed using Biacore X100 (Biacore Inc, Uppsala, Sweden). The biotinylated peptide (ligand) was immobilized on SA chips (BR100032, Cytiva) in the range of 300-350 resonance units (RU) after conditioning the chip with three consecutive 1 min injections of 1 x Biacore running buffer (BR100826, Cytiva). Recombinant His-CaMKIIδ-T287D (analyte, Genscript) or CaM (C4874, Sigma) were dialysed into the running buffer and diluted over a range of concentrations (47.6-500, 95.2-1000, 952.6-10000 nM), and injected over the sensor surface at a flow rate of 30 µl/min for 180s. The dissociation time was set at

600 s. The obtained sensorgrams were analyzed using Biacore X100 evaluation software and curve fitting was performed with the assumption of one-to-one binding (Langmuir).

CaM agarose pull down

CaM agarose (A6112, Sigma Aldrich) pull down with biotin-AKAP18δ (79-98), biotin-AKAP18γ (52-71), or GFP-AKAP18δ (67-353) was performed in either 2.5 mM $CaCl_2$ or 5 mM EGTA (control) as previously described [74]. Binding was detected by immunoblotting.

Cytotoxicity detection kit (LDH)

Cytotoxicity was measured according to the manufacturer's protocol (cat. no. 11-644-793-001, Roche Diagnostics).

Densitometric analysis

Densitometric analysis was performed using Scion Image (Scion Corp., Frederick, Maryland) or ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

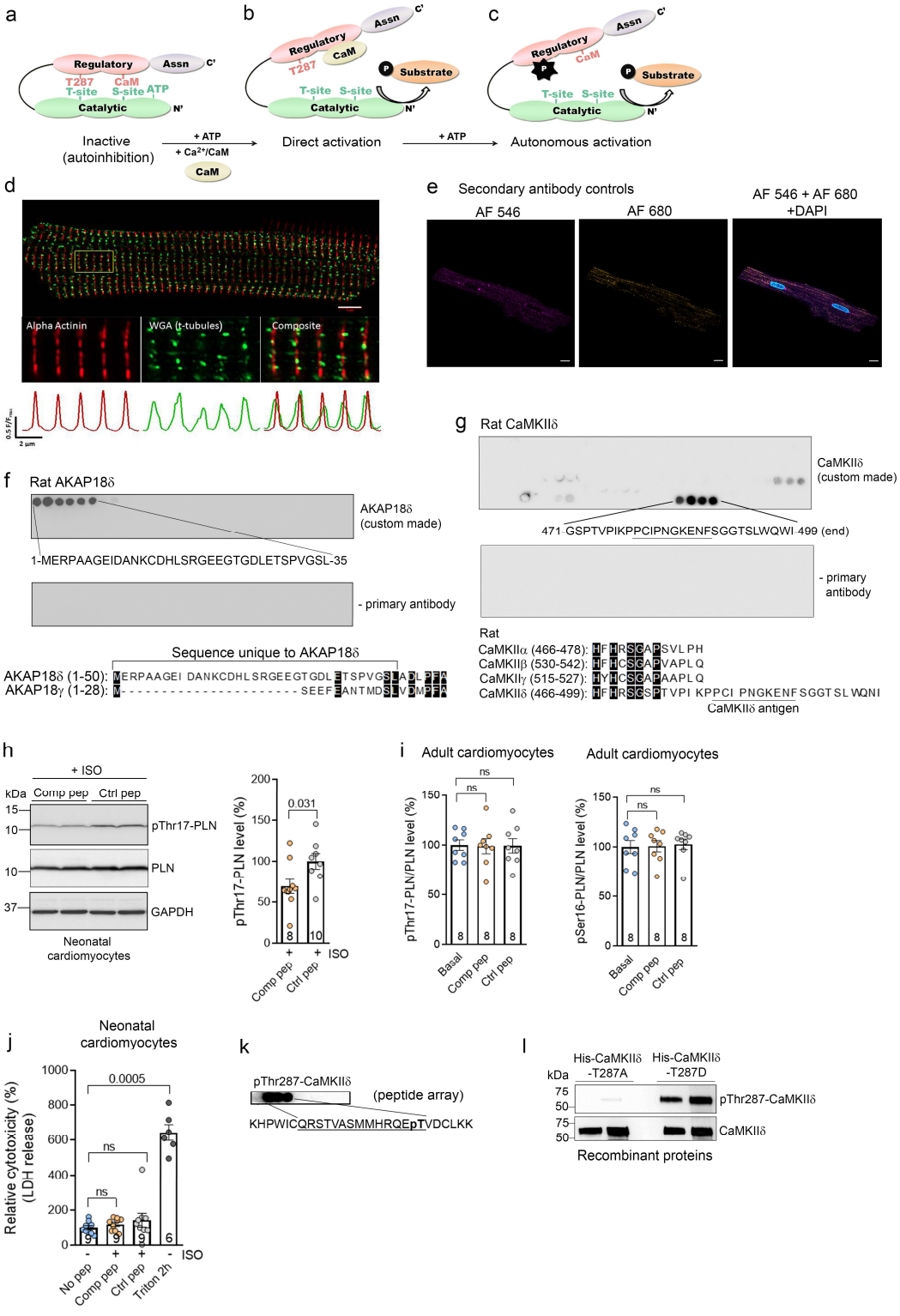
Modeling

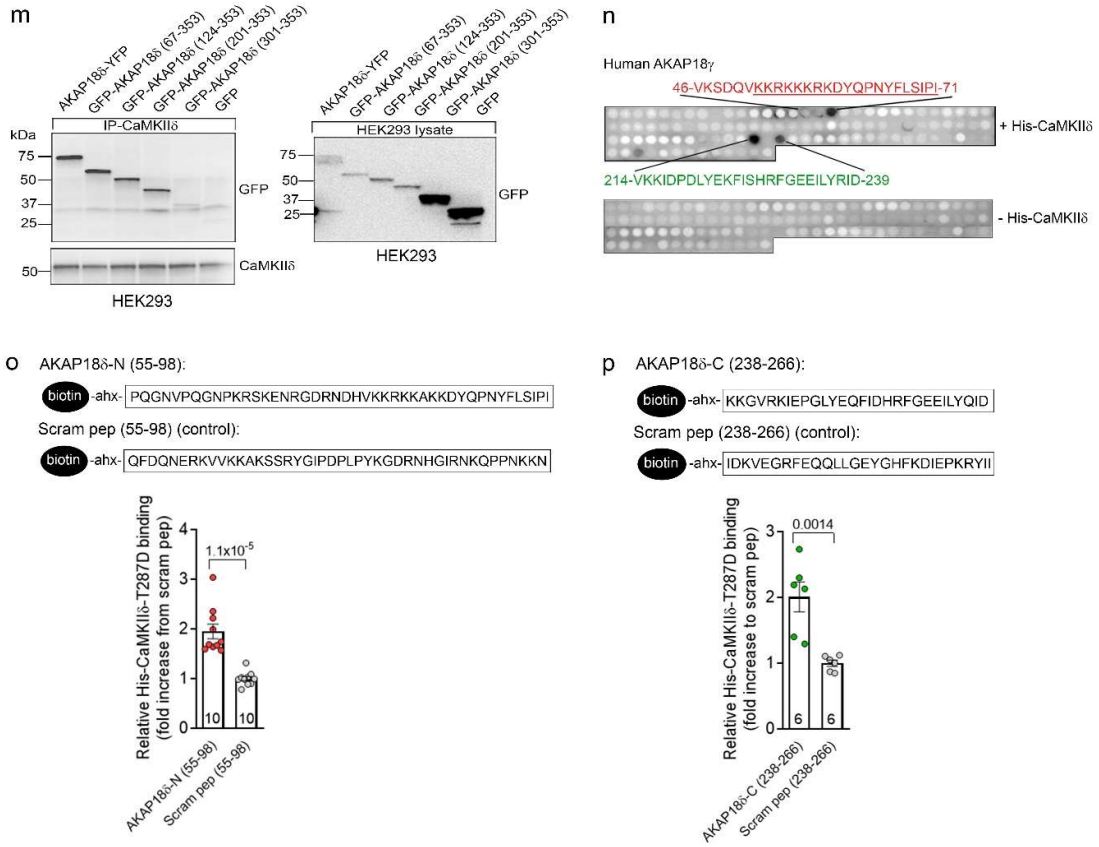
The published structure of human CaMKIIδ (PDB code 2WEL) [78] was used as a template for homology modeling of the AKAP18δ peptide 89-QPNYFLSIPIT-99 in the peptide binding groove of CaMKIIδ, with Ser95 corresponding to Thr287 in the autophosphorylation site of CaMKIIδ. The side chain conformations for each residue in the modeled AKAP18δ peptide were selected based on a combination of the orientation of the corresponding residues in CaMKIIδ, combined with an assessment of optimal interaction/least steric occlusion with CaMKIIδ. The structural model was analyzed and visualized by PyMOL (Schrodinger LLC).

Bioinformatic analyses

We used DNA Star, Lasergene (Madison, WI) to align the following sequences: Rat AKAP18δ (NP_001001801), human and mouse AKAP18γ (Q9P0M2 and AAP55205, respectively), AKAP18α (O43687), AKAP18β (NP_619539), rat CaMKIIδ (NP_036651), rat CaMKIIα (P11275), rat CaMKIIβ (P08413), rat CaMKIIγ (P11730), rat CaM-KIIN (NP_067710) rat PLN (NP_073198), human neuronal NR2B (NP_000825) and human AKAP79 (P24588).

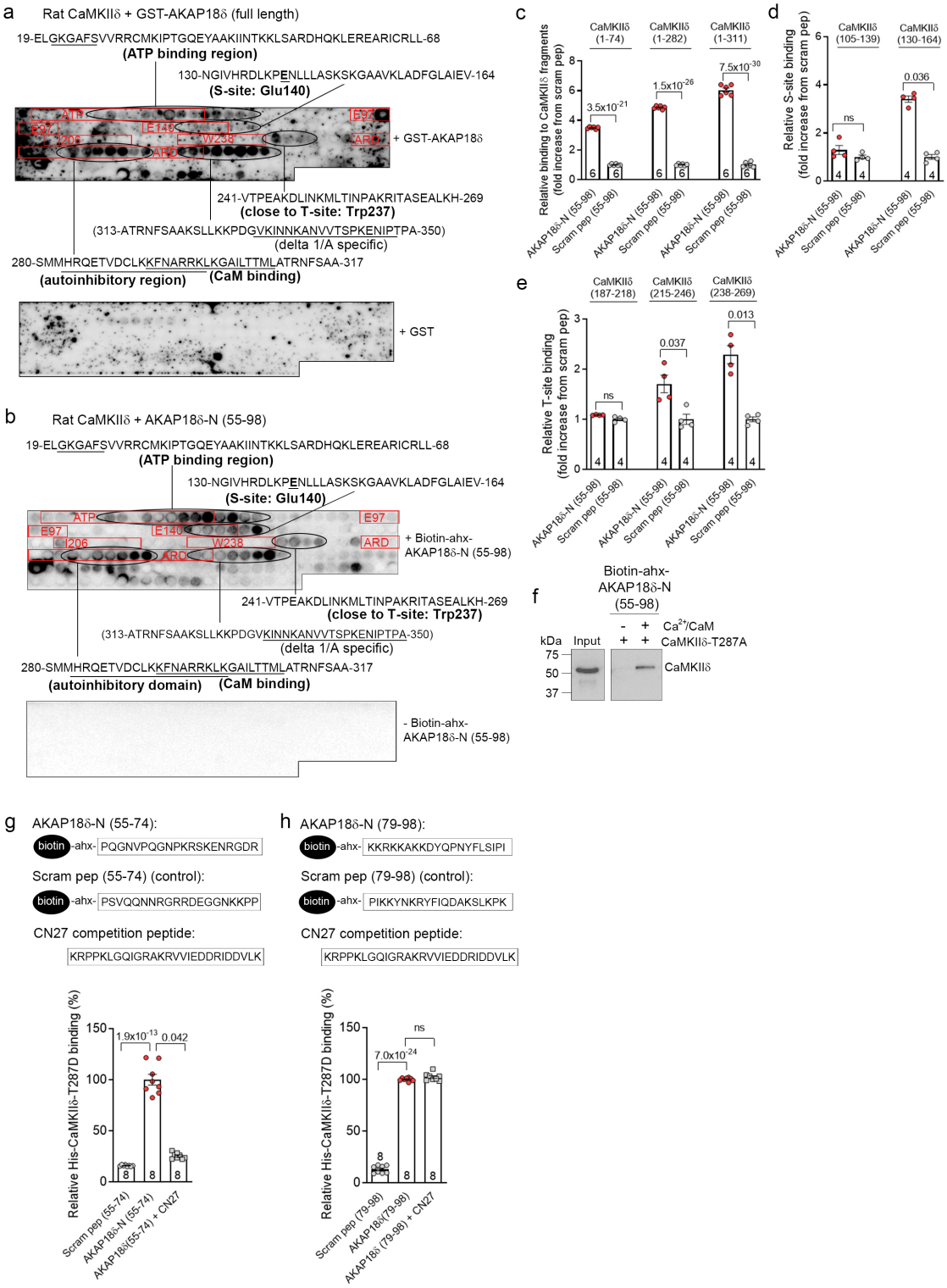
SUPPLEMENTAL FIGURES

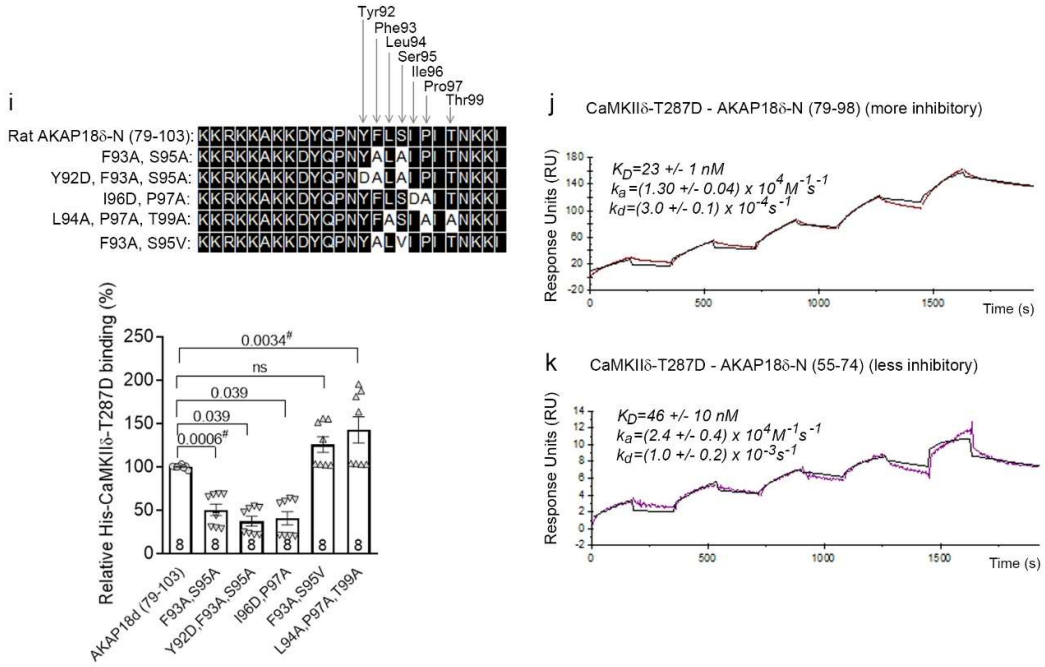




Supplemental figure 1. Illustration of CaMKII activation, validation of anti-CaMKIIδ, anti-AKAP18δ, CaMKIIδ-T287A and CaMKIIδ-T287D mutations, CaMKIIδ-AKAP18δ binding, Z-line staining, cytotoxicity analysis of PLN-derived peptides and pThr17-PLN levels in neonatal and adult cardiomyocytes treated with cell-permeant PLN competitor or control peptide. (**a-c**) Illustration of CaMKIIδ activation (modified from [79]). (**a**) Each CaMKII monomer is composed of an N-terminal catalytic domain (light green), an autoregulatory domain (pink), and a C-terminal association domain (Assn, lilac) which mediates oligomerization [7]. The catalytic domain contains a T-site (Thr287 binding segment), S-site (substrate binding) and an ATP binding pocket. The regulatory domain contains an autoinhibitory region, where Thr287 and the CaM binding motif are located. When CaMKIIδ is in its inactive state, the Thr287 segment (Thr286 in CaMKIIα) in the regulatory domain binds to the T-site in the catalytic domain and positions the adjacent sequence in the S-site and ATP-binding pocket. (**b**) Upon activation, Ca^{2+}/CaM (beige) binds to the regulatory domain and displaces it from the ATP binding pocket, S-and T-sites. This opens the kinase and enables it to be phosphorylated at Thr287 by a neighboring active CaMKIIδ molecule (also open). (**c**) Thr287 autophosphorylation maintains the kinase in an autonomously active state. (**d**) Co-localization of WGA (t-tubules) with α-actinin (Z-line). Single images and traces are shown in the lower panels. Scale bar=5 µm. (**e**) Secondary antibody controls using Alexa Fluor (AF) 546 (left panel) and AF 680 (middle panel) showed no specific staining. DAPI was used to stain the nucleus (blue in composite image at right). Scale bars=10 µm. The entire amino acid sequences of rat (**f**) AKAP18δ and (**g**) CaMKIIδ were spot-synthesized as 20-mer overlapping peptides on cellulose membranes and overlaid with polyclonal antibodies directed against AKAP18δ and CaMKIIδ, respectively. The

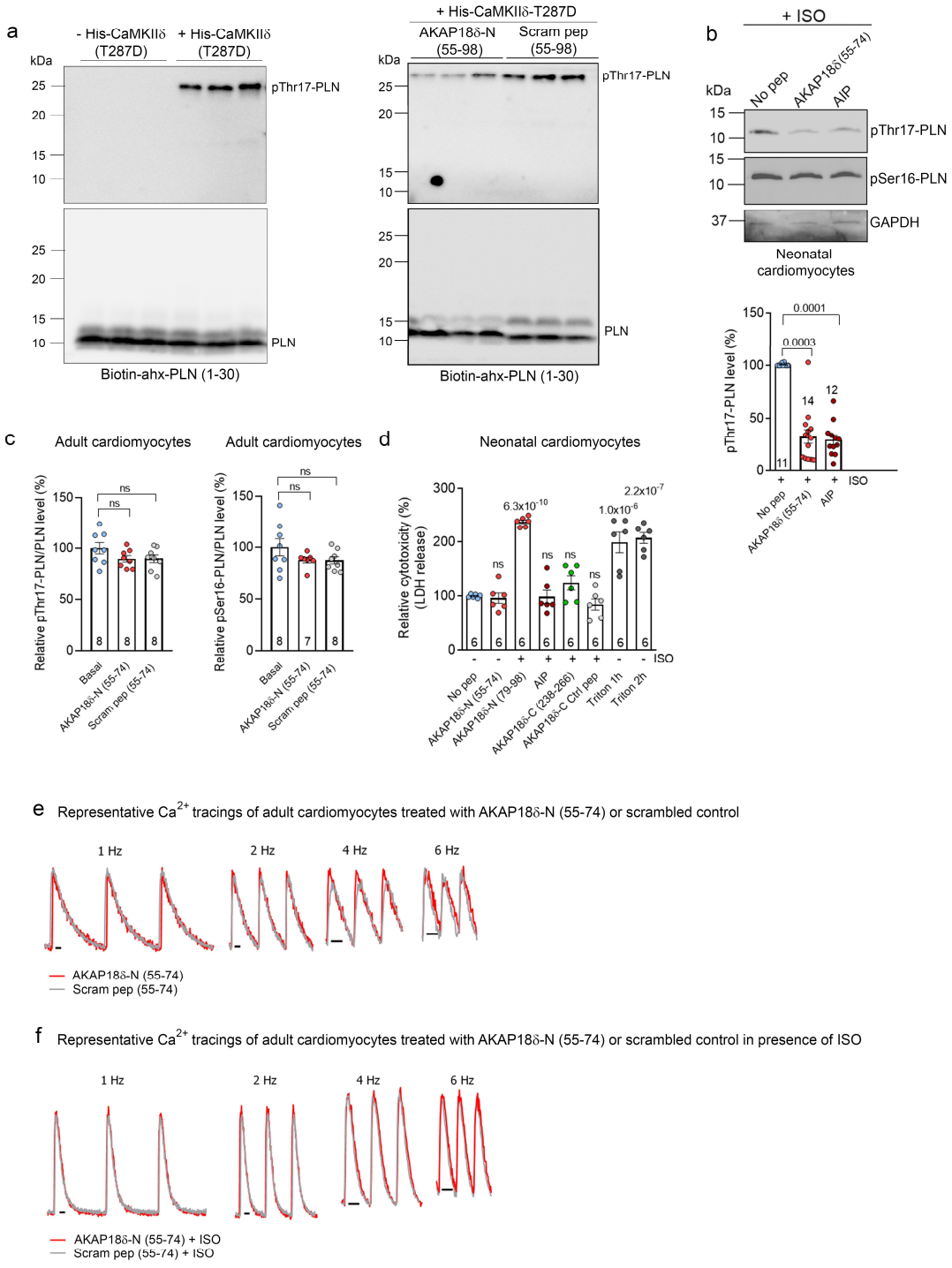
antibodies were highly specific against their respective epitope sequences and binding to other sequences was not observed. Peptide membranes without primary antibody were used as negative controls (lower panels). Alignment of AKAP18δ and AKAP18γ shows that the anti-AKAP18δ epitope is only present in AKAP18δ (lower panel in **f**). Alignment of CaMKII isoforms shows that the anti-CaMKIIδ epitope is only present in the unique C-terminus of CaMKIIδ (underlined sequence in the peptide array and in the alignment in **g**). (**h**) ISO-stimulated neonatal rat cardiomyocytes were pre-treated with a cell-permeant AKAP18δ-PLN disruptor or control peptide, as previously described [14]. pThr17-PLN (upper) and PLN (middle) levels were analyzed by immunoblotting. GAPDH was used as loading control. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired t-test (n=8-10). (**i**) The graphs show the pThr17-PLN/PLN (left panel) and pSer16-PLN/PLN (right panel) levels in adult cardiomyocytes treated with or without the cell-permeant AKAP18δ-PLN disruptor or control peptide at basal level. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by nested one-way ANOVA with Dunnett's multiple comparisons test (n=8, 3 rats). Ns: non-significant. (**j**) LDH release from neonatal rat cardiomyocytes treated with or without the cell permeant PLN competitor peptide or the control peptide (30 µM). Incubation with 2 % Triton for 2 hours was used as a positive control for cytotoxicity. The LDH level without any peptide treatment was set to 100 %. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=6-9). (**k**) Anti-pThr287-CaMKIIα was overlaid onto membranes with 20-mer overlapping pre-phosphorylated Thr287-CaMKIIδ peptides. The common core sequence in all three spots is underlined. (**l**) Recombinant full length CaMKIIδ-T287D and CaMKIIδ-T287A were immunoblotted with pThr287-CaMKIIδ antibodies. As expected, only CaMKIIδ-T287D were recognized by anti-pThr287-CaMKIIδ. Immunoblotting with CaMKIIδ antibodies showed similar levels of CaMKIIδ-T287D and CaMKIIδ-T287A (loading control). (**m**) Lysates from HEK293 cells co-transfected with CaMKIIδ-T287D and full length AKAP18δ-YFP, AKAP18δ-GFP deletions [72] or GFP subjected to immunoprecipitation using CaMKIIδ antibodies (n=3). Co-precipitation of AKAP18δ was detected by immunoblotting with GFP antibodies. (**n**) Residues important for CaMKIIδ binding were identified by overlaying human AKAP18γ full length protein spot-synthesized as 20-mer peptides with 3 amino acid offsets on membranes with active His-CaMKIIδ. His-CaMKIIδ binding was detected by immunoblotting. Immunoblotting without His-CaMKIIδ was used as negative control (lower panel). Underlined sequence was synthesized as a soluble peptide for further experiment. Binding of (**o**) biotin-ahx-AKAP18δ-N (55-98) and (**p**) biotin-ahx-AKAP18δ-C (238-266) to recombinant CaMKIIδ-T287D (coated in wells) were confirmed in ELISA-based assays. Binding was detected by a monoclonal anti-biotin-HRP conjugated antibody followed by incubation with an Ultra TMB solution. CaMKIIδ-T287D binding to the corresponding scrambled control peptides was set to 1. Significant differences were tested by Mann Whitney test (n=10 in **o**) or unpaired t-test (n=6 in **p**). Normal distribution was confirmed by Shapiro-Wilk test in **p**. Sequences of the different peptides are given in the upper panels in **o** and **p**. An ahx-linker was included between the biotin tag and peptide sequence to avoid potential steric hindrance.


a
Rat CaMKIIδ + GST-AKAP18δ (full length)
19-ELGKGAFSVVRRCMKIPTGQEYAAKIINTKKLSARDHQKLEREARICRLL-68
(ATP binding region)
130-NGIVHRDLKPENLLLASKSKGAAVKLADFGLAIEV-164
(S-site: Glu140)
+ GST-AKAP18δ
241-VTPEAKDLINKMLTINPAKRITASEALKH-269
(close to T-site: Trp237)
(313-ATRNFSAAKSLLKKPDGVKINNKANVVTSPKENIPTPA-350)
(delta 1/A specific)
280-SMMHRQETVDCLKKFNARRKLKGAILTTMLATRNFSAA-317
(autoinhibitory region)
(CaM binding)
+ GST
b
Rat CaMKIIδ + AKAP18δ-N (55-98)
19-ELGKGAFSVVRRCMKIPTGQEYAAKIINTKKLSARDHQKLEREARICRLL-68
(ATP binding region)
130-NGIVHRDLKPENLLLASKSKGAAVKLADFGLAIEV-164
(S-site: Glu140)
+ Biotin-ahx-AKAP18δ-N (55-98)
241-VTPEAKDLINKMLTINPAKRITASEALKH-269
(close to T-site: Trp237)
(313-ATRNFSAAKSLLKKPDGVKINNKANVVTSPKENIPTPA-350)
(delta 1/A specific)
280-SMMHRQETVDCLKKFNARRKLKGAILTTMLATRNFSAA-317
(autoinhibitory domain)
(CaM binding)
- Biotin-ahx-AKAP18δ-N (55-98)
c
Relative binding to CaMKIIδ fragments (fold increase from scram pep)
CaMKIIδ (1-74)
CaMKIIδ (1-282)
CaMKIIδ (1-311)
3.5x10-21
1.5x10-26
7.5x10-30
AKAP18δ-N (55-98)
Scram pep (55-98)
d
Relative S-site binding (fold increase from scram pep)
CaMKIIδ (105-139)
CaMKIIδ (130-164)
ns
0.036
e
Relative T-site binding (fold increase from scram pep)
CaMKIIδ (187-218)
CaMKIIδ (215-246)
CaMKIIδ (238-269)
ns
0.037
0.013
f
Biotin-ahx-AKAP18δ-N (55-98)
- + Ca2+/CaM
+ + CaMKIIδ-T287A
kDa
Input
75
50
37
CaMKIIδ
g
AKAP18δ-N (55-74):
biotin -ahx- PQGNVPQGNPKRSKENRGDR
Scram pep (55-74) (control):
biotin -ahx- PSVQQNNRGRRDEGGNKKPP
CN27 competition peptide:
KRPPKLGQIGRAKRVVIEDDRIDDVLK
Relative His-CaMKIIδ-T287D binding (%)
1.9x10-13
0.042
Scram pep (55-74)
AKAP18δ-N (55-74)
AKAP18δ(55-74) + CN27
h
AKAP18δ-N (79-98):
biotin -ahx- KKRKKAKKDYQPNYFLSIPI
Scram pep (79-98) (control):
biotin -ahx- PIKKYNKRYFIQDAKSLKPK
CN27 competition peptide:
KRPPKLGQIGRAKRVVIEDDRIDDVLK
Relative His-CaMKIIδ-T287D binding (%)
7.0x10-24
ns
Scram pep (79-98)
AKAP18δ(79-98)
AKAP18δ (79-98) + CN27



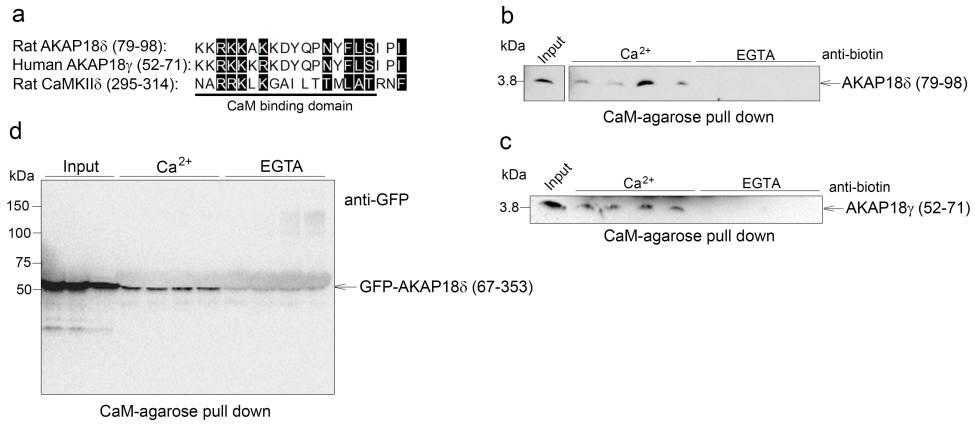
Supplemental figure 2. Validation of AKAP18δ binding to the ATP-binding region, T-site, S-site and autoregulatory domain (ARD) in CaMKIIδ, CN27 competition, mutation and SPR analyses. (**a**) AKAP18δ binding was identified by overlaying 20-mer overlapping CaMKIIδ peptides with either GST-AKAP18δ (upper panel) or GST (negative control, lower panel). Binding was detected by immunoblotting with anti-GST-HRP. The ATP binding region [7] (consensus ATP binding motif is underlined), S-site (E97 and E140-containing sequences), T-site (I206 and W238 containing sequences), the autoregulatory domain (ARD), the autoinhibitory region, and CaM binding site are indicated. The CaMKII$\delta_{1/A}$ specific sequence is only present in neonatal mouse hearts [63] (in parenthesis). (**b**) Biotin-ahx-AKAP18δ-N (55-98) was overlaid with 20-mer overlapping CaMKIIδ peptides spot-synthesized on cellulose membrane. Binding was identified by immunoblotting with anti-biotin-HRP conjugated antibodies. Immunoblotting without incubation of biotin-ahx-AKAP18δ (55-98) was used as negative control (lower panel). See figure legend in **a** for detailed description. Biotin-ahx-AKAP18δ-N (55-98) binding to the (**c**) ATP binding region, catalytic domain (1-282), catalytic + autoregulatory domains (1-311) (n=6), (**d**) S-site and (**e**) T-site (coated as either recombinant proteins or synthetic peptides in wells) confirmed using ELISA-based assays. Binding was detected with a monoclonal anti-biotin-HRP conjugated antibody followed by incubation with an Ultra TMB solution. Normal distribution was confirmed by Kolmogorov-Smirnov test, and significant differences were examined by ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test (in **c**). Significant differences were examined by Kruskal-Wallis test with Dunn's multiple comparisons test (in **d-e**). Binding to the biotinylated corresponding scrambled control peptide was set to 1 (**c-e**) (n=6 in **c**, and n=4 in **d-e**). (**f**) Binding of biotin-ahx-AKAP18δ-N (55-98) to recombinant CaMKIIδ-T287A with or without the presence of Ca^{2+}/CaM was analysed in a pull down experiment. Precipitated CaMKIIδ-T287A was detected by immunoblotting with CaMKIIδ antibodies. No binding was observed when Ca^{2+}/CaM was omitted. The T-site binding CN27 peptide competed with CaMKIIδ-T287D binding to (**g**) biotin-ahx-AKAP18δ-N (55-74), but not to (**h**) biotin-

ahx-AKAP18δ-N (79-98) in ELISA based assays. Briefly, recombinant CaMKIIδ-T287D was coated in the wells and binding of the biotinylated AKAP18δ-N peptides was detected with a monoclonal anti-biotin-HRP. Sequences of the different peptides are shown in the upper panels in **g** and **h**. An ahx linker was included between the biotin tag and the AKAP18δ-N sequences to avoid potential steric hindrance. Binding of biotin-ahx-AKAP18δ-N (55-74) (in **g**) and biotin-ahx-AKAP18δ-N (79-98) (in **h**) to CaMKIIδ-T287D without the presence of CN27 was set to 100 %. Normal distribution was confirmed by Shapiro-Wilk test (in **g**) or Kolmogorov-Smirnov test (in **h**). Significant differences were examined by ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test (n=8, in **g-h**). (**i**) Analysis of CaMKIIδ-T287D binding (coated in wells) to different AKAP18δ (79-103) mutated biotinylated peptides using an ELISA based assay. Binding was detected with a monoclonal anti-biotin-HRP conjugated antibody and thereafter incubation with an Ultra TMB solution. The Phe93Ala-Ser95Ala (F93A, S95A) double mutation reduced the CaMKIIδ binding by 50 % compared to the wild-type AKAP18δ peptide. This reduction in binding was likely due to the loss of the aromatic interaction between Phe93 and $Trp215^{CaMKII\delta}$ combined with loss of hydrogen bond interactions between Ser95 and residues $Asp136^{CaMKII\delta}$, $Lys138^{CaMKII\delta}$ and/or $Thr177^{CaMKII\delta}$. The Tyr92Asp-Phe93Ala-Ser95Ala (Y92D, F93A, S95A) triple mutant bound even more weakly to CaMKIIδ, which likely reflected an additional repulsion between $Asp92^{AKAP18\delta}$ and the negatively charged S-site patch in CaMKIIδ. The mutated peptide Ile96Asp-Pro97Ala (I96D, P97A) also showed a low degree of binding to CaMKIIδ, suggesting that $Ile96^{AKAP18\delta}$ too plays an important role in the interaction. The stronger binding of Leu94Ala-Pro97Ala-Thr99Ala (L94A, P97A, T99A) to CaMKIIδ is less easily explained by the current model (Fig. 2g). A plausible explanation could be that an increased flexibility due to the three alanine residues in the triple-mutant peptide is able to induce conformations of the peptides with stronger binding to CaMKIIδ. CaMKIIδ binding to non-mutated AKAP18δ (79-103) was set to 100 %. Significant differences were examined by Kruskal-Wallis test with Dunn's multiple comparisons test (n=8). # Significant differences for these three groups were detected with one-way ANOVA with Sidak's multiple comparisons test (normal distribution confirmed for the three groups by by Shapiro-Wilk test). Ns: non-significant. Analyses of the affinity and kinetics of the (**j**) AKAP18δ (79-98)-CaMKIIδ-T287D and (**k**) AKAP18δ (55-74)-CaMKIIδ-T287D interactions. SPR analysis of immobilized biotin-ahx-AKAP18δ (79-98) (in **j**) and biotin-ahx-AKAP18δ (55-74) (in **k**) on SA chips and recombinant CaMKIIδ-T287D injected at a range of concentrations (47.6-500 nM in **j** and 95.2-1000 nM in **k**) (n=3).

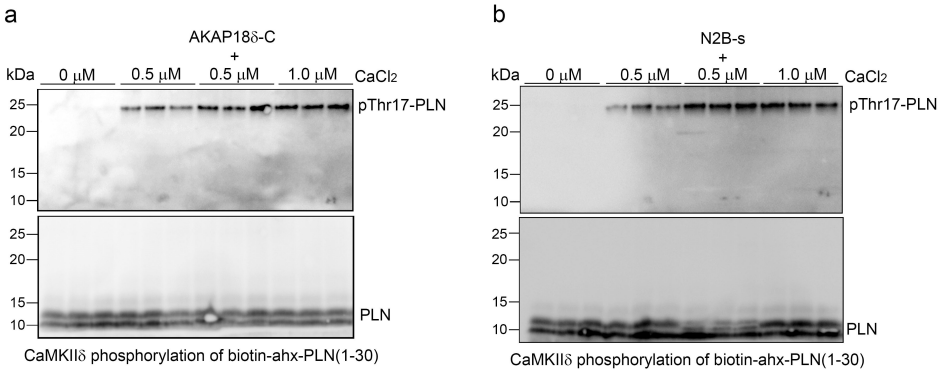


Supplemental figure 3. Complete immunoblots of Thr17-PLN phosphorylation in the presence of the inhibitory AKAP18δ-N sequence, pThr17-PLN levels in AKAP18δ-N (55-74) treated neonatal and adult cardiomyocytes, cytotoxicity analyses of AKAP18δ derived peptides and representative Ca^{2+} tracings. (**a**) Right panel: Complete immunoblot of CaMKIIδ phosphorylation of the biotin-ahx-PLN (1-30) peptide treated with the inhibitory AKAP18δ-N (55-98) peptide or the corresponding

scrambled peptide (negative control). pThr17-PLN was detected at 25 kDa due to PLN oligomerization [64]. Left panel: The 25 kDa band was not detected when CaMKIIδ was omitted. (**b**) Rat neonatal cardiomyocytes were untreated or pre-treated with cell-permeant AKAP18δ-N (55-74) or AIP for 45 min before ISO-stimulation (15 min) and analyzed for pThr17-PLN or pSer16-PLN by immunoblotting. GAPDH was used as loading control. Significant differences were examined by Kruskal-Wallis test with Dunn's multiple comparisons test (n=11-14). (**c**) The graphs show pThr17-PLN/PLN (left) and pSer16-PLN/PLN (right) levels in adult cardiomyocytes treated with or without the cell-permeant AKAP18δ-N (55-74) or scrambled control peptide at basal level. Normal distribution was confirmed by Shapiro-Wilk test. Ns: non-significant, examined by nested one-way ANOVA with Dunnett's multiple comparisons test (n=7-8, 3 rats). (**d**) LDH release analysis of cell permeant AKAP18δ peptides and AIP. The cell-permeant AKAP18δ-N (79-98) peptide was cytotoxic to neonatal rat cardiomyocytes at 30 µM, whereas no cytotoxicity was observed for the cell-permeant versions of AKAP18δ-N (55-74), AKAP18δ-C (238-266), AKAP18δ-C (238-266) control peptide and AIP (all 30 µM). Incubation with 2 % Triton for 2 hours was used as a positive control. LDH release from cells without any peptide treatment was set to 1. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences versus "No pep" were examined by ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test (n=6). Representative tracings of Ca^{2+} transients after stimulation frequency and treatment with AKAP18δ-N (55-74) (red) or the scrambled control (grey) in (**e**) absence or (**f**) presence of ISO. Width of black line under first transient at each frequency indicates 0.1 seconds. Amplitudes of tracings are normalized.



Supplemental figure 4. Validation of AKAP18δ and AKAP18γ binding to calcified CaM. (**a**) Alignment of rat AKAP18δ (79-98) and the corresponding sequence of human AKAP18γ (52-71) with the CaM binding region in CaMKII [7]. The CaM binding domain is underlined. Black boxes indicate identical or functionally similar amino acids (DNA Star, Lasergene). (**b**) Biotin-AKAP18δ-N (79-98) and (**c**) biotin-AKAP18γ-N (52-71) were incubated with CaM-agarose in the presence of either 1 mM Ca^{2+} or 1 mM EGTA (control). Input and precipitates were immunoblotted with anti-biotin-HRP. (**d**) GFP-AKAP18δ (67-353) expressed in HEK293 was incubated with CaM-agarose in the presence of either 1 mM Ca^{2+} or 1 mM EGTA (control). Input and precipitates were examined by immunoblotting with anti-GFP.



Supplemental figure 5. Complete immunoblots of Thr17-PLN phosphorylation in presence of AKAP18δ-C or N2B-s. Complete immunoblots of CaMKIIδ-T287A phosphorylation of the biotin-ahx-PLN (1-30) peptide treated with or without the activator peptides (**a**) AKAP18δ-C (238-266) or (**b**) N2B-s, in presence of different $CaCl_2$ concentrations. pThr17-PLN detected by immunoblotting was observed at 25 kDa due to PLN oligomerization [64]. No pThr17-PLN was detected when $CaCl_2$ was omitted (three first lanes in **a** and **b**).

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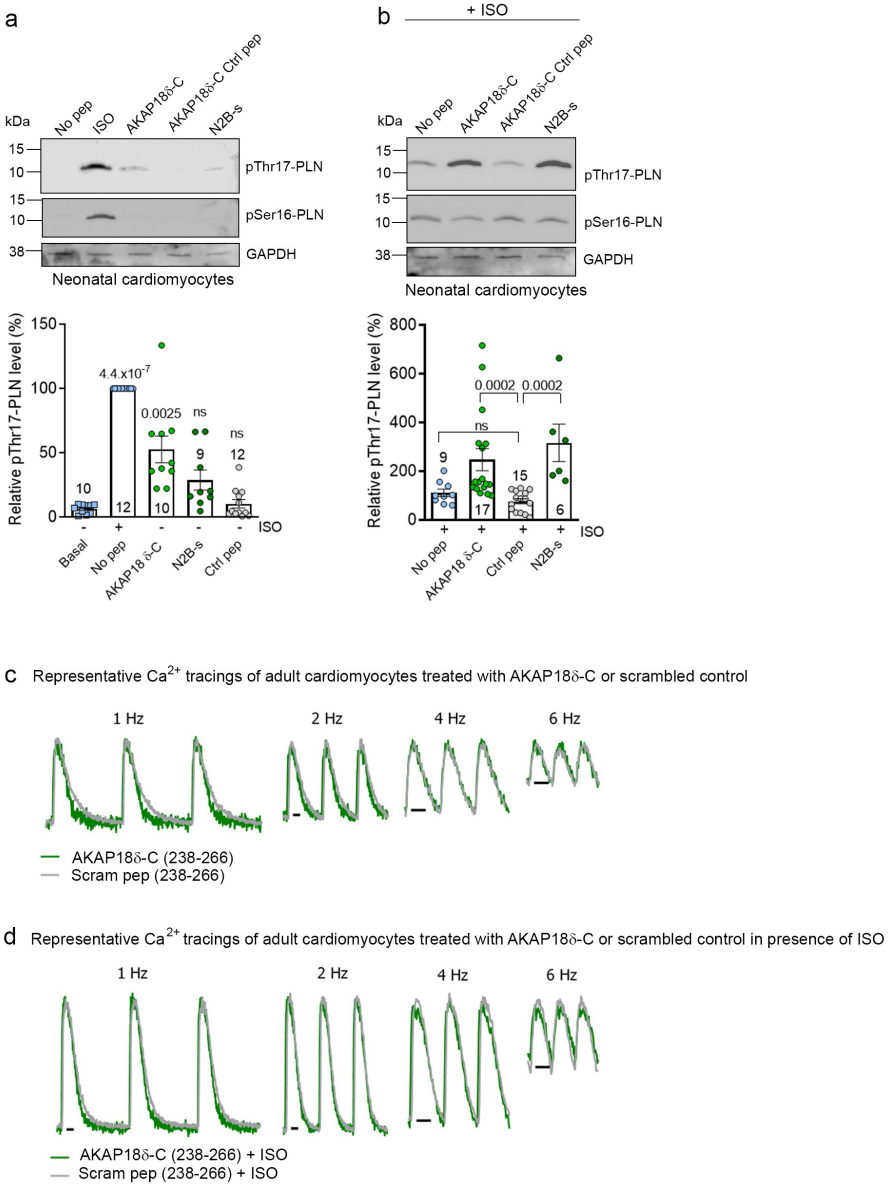

Supplementary Figure 6
Carlson et al.

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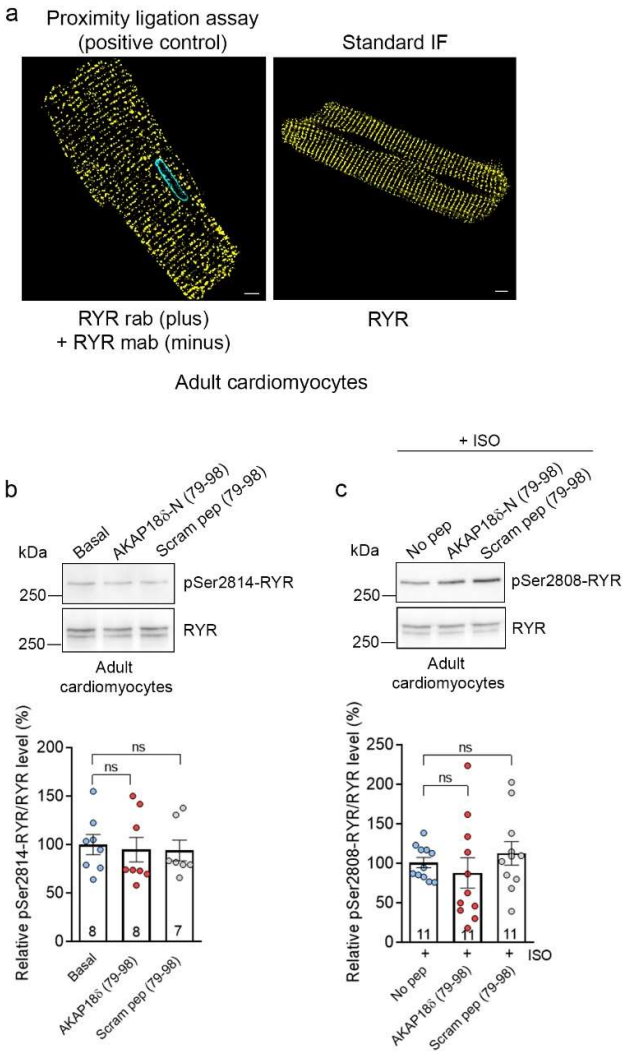
575 **Supplemental figure 6. Validation of AKAP18δ-C as a CaMKIIδ activator in neonatal rat**
576 **cardiomyocytes and representative Ca^{2+} tracings.** Rat neonatal cardiomyocytes were treated with
577 or without 30 µM of cell-permeant Arg_9-AKAP18δ-C (238-266), Arg_9-N2B-s or the control peptide for
578 45 min in (**a**) absence or (**b**) presence of ISO (15 min). The pThr17-PLN (upper panels) or pSer16-PLN
579 (middle panels) levels were analyzed by immunoblotting. GAPDH was used as a loading control
580 (lower panels in **a** and **b**). The relative pThr17-PLN level at basal and in ISO stimulated cells was set
581 to 100 % (**a** and **b**, respectively). Significant differences were examined by Kruskal-Wallis test with
582 Dunn's multiple comparisons test (versus "Basal", n=9-12 in **a**, and n=6-17 in **b**). Representative

583 tracings of Ca^{2+} transients after stimulation frequency and treatment with AKAP18δ-C (238-266)
584 (green) or the scrambled control (grey) in (**c**) absence or (**d**) presence of ISO. Width of black line
585 under first transient at each frequency indicates 0.1 seconds. Amplitudes of tracings are normalized.



Supplemental Figure 7. Validation of the *in situ* proximity ligation assay and levels of pSer2808-RYR and pSer2814-RYR in adult cardiomyocytes treated with cell-permeant AKAP18δ-N (79-98). (**a**) Positive control using RYR antibodies from two different species (rab; rabbit = PLUS probe and mab; mouse = MINUS probe) is shown in the left panel. Standard immunofluorescence using RYR antibody is shown to the right. Nuclei were stained with DAPI. Scale bars=5 µm (**b**) Adult cardiomyocytes were treated with or without cell-permeant AKAP18δ-N (79-98) or a scrambled control peptide for 60 min. Levels of pSer2814-RYR and total RYR were detected by immunoblotting. Ns, not significant, examined by either nested one-way ANOVA with Dunnett's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test (n=7-8, 2 rats). (**c**) Adult cardiomyocytes were treated with or without cell-permeant AKAP18δ-N (79-98) or a scrambled control peptide for 45 min before ISO-stimulation (15 min). Levels of pSer2808-RYR and total RYR were detected by immunoblotting. Normal distribution was confirmed by Shapiro-Wilk test. Ns (not significant), examined by nested one-way ANOVA with Dunnett's multiple comparisons test (n=11, 5 rats).

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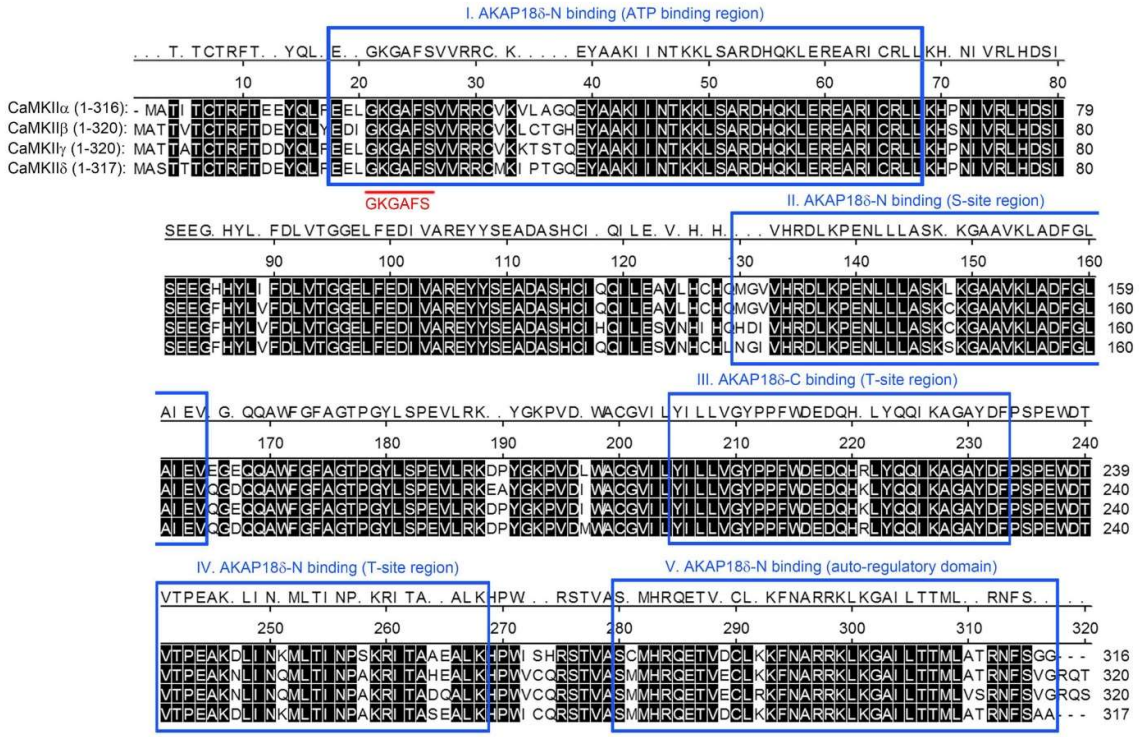

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604 **Supplemental figure 8. Alignment of different CaMKII isoforms.** The five AKAP18δ binding sites
605 identified in the different CaMKIIδ regions are indicated in the alignment of the rat CaMKIIα, β and
606 γ isoforms (DNA Star, Lasergene). The association domains of the CaMKII isoforms are not shown
607 for simplicity. Black boxes indicate identical amino acids. The consensus ATP binding motif (GKGAFS)
608 [7] is indicated in red.

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