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Supplementary Information for

Single nucleotide polymorphisms alter kinase anchoring and the subcellular targeting of A-Kinase Anchoring Proteins

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Table S1

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SUPPORTING INFORMATION

SI Methods

Sequence analysis and variant identification.

The variants described here were captured at several different points in time as new SNPs were deposited into databases. Initially, NCBI dbSNP (https://www.ncbi.nlm.nih.gov/SNP/) was used to manually pick single nucleotide polymorphisms in the PKA binding helix of AKAP18 (AKAP7). Later, the NCBI Variation Viewer resource (https://www.ncbi.nlm.nih.gov/variation/view/) was used to scan other known A-kinase anchoring protein (AKAP)-PKA binding helices and pick missense variants that alter the protein sequence in this region.

Peptide array synthesis.

SPOT arrays were generated as described previously (1). Briefly, FMOC amino acids were purchased from Anaspec and cellulose membranes for SPOT synthesis were purchased from Intavis. Peptides were synthesized onto a membrane using the Intavis MultiPep solid-phase peptide synthesizer following the manufacturer's standard protocols.

RII overlay.

His-tagged mouse RIIα was expressed in BL21(DE3)pLysS *E. coli* and purified by Ni^{+}-affinity and size exclusion chromatography as described (2). Purified RII was then biotinylated using EZ-Link-Sulfo-NHS-biotin (Thermo) according to the product protocol. Biotinylated RII was incubated with peptide array membranes overnight at 4ºC in TBST containing 5% nonfat dry milk and 1% BSA. After extensive washing, the membrane was incubated with neutravidin-HRP (Thermo), washed again and imaged by ECL (Super-signal Pico, Thermo Pierce) on an AlphaInnotech Multiimager III. Quantification using densitometry of RII overlay signal was performed using Fiji/ImageJ and normalized to corresponding wildtype peptide signal (for each individual AKAP helix).

cDNA cloning and Mutagenesis.

Human AKAP18α-GFP was described previously (3). Human AKAP18γ-GFP was constructed as previously described (4). This cDNA was moved into pDONR221 (Thermo) via PCR and Gateway BP cloning and further transferred to a C-terminal mCherry destination vector via Gateway LR cloning. Rat AKAP18δ (5) was moved into pDONR221 via PCR and further transferred to a C-terminal YFP destination vector. Fragments of this AKAP were cloned similarly. Point mutations and deletions were introduced into full-length cDNAs in mammalian expression vectors via Phusion mutagenesis. Inverse PCR primers containing the desired mutations were used to amplify the entire plasmid. Template plasmid was digested with DpnI and the product was purified using the GeneJet PCR Purification kit (Thermo). Following phosphorylation of DNA ends with T4 polynucleotide kinase (NEB), plasmids were circularized with T4 ligase (NEB) and transformed into chemically competent GC10 E. coli (Genesee Scientific). Mutation was confirmed by Sanger sequencing (GeneWiz). Overlap

extension PCR was used to fuse cDNAs for AKAP18α variants and mCherry. This product was inserted into pDONR221 via Gateway BP reaction. Gateway LR cloning was used to transfer the fragment into pcDNA-DEST40 (Invitrogen). For cloning of the AKAP18 epsilon variant, reverse transcribed human cDNA (Universal cDNA, BioChain) was used as template for PCR with KAPA HotStart polymerase (Kapa Biosystems). The primer sequences used here are as follows:
p1F: 5'-ATGGAGCGCCCCGAAGCG
p2R: 5'-AGATTTCATAAGCTATGTTGTCACAATCGC
p3R: 5'-TTCCAATAGGTTTCTTGCCAATCACAATG
PCR products were gel purified, TOPO cloned into pCR-Blunt-II (Life Technologies) and sequenced from both ends. For further manipulations, attB sites were added by PCR and Gateway cloning was used to shuttle the cDNA into appropriate destination vectors as above.

Live cell imaging.
HeLa cells grown on glass coverslips were transfected with vectors encoding AKAP18-GFP constructs using Lipofectamine 2000. Cells were incubated at 37 °C for 24 - 48 hours before imaging. Coverslips were washed in Hank's balanced salt solution, mounted in a Ludin chamber (Life Imaging Services) and imaged using a Leica AS-MDW workstation. Images were acquired using a Leica DM IRE2 microscope equipped with a CoolSNAP-HQ charge-coupled device camera (Roper Photometrics). Pre-treatment images were captured for 10 minutes prior to drug addition. For anchoring disruptor experiments, stearated-Ht31 or control st-Ht31P peptides (Promega) were added to 10 μM and images were captured every 5 minutes for 1 hour. For nuclear trapping experiments, Leptomycin B (Calbiochem) was added to 20 nM and images were captured every 5 minutes for at least 90 minutes. The rate of nuclear accumulation in these experiments was quantified by measuring fluorescence intensity in both a cytoplasmic and nuclear ROI at each time point in ImageJ. These values were expressed as the ratio between nuclear/cytoplasmic signal and graphed versus treatment time.

Fluorescence microscopy.
HeLa cells grown on glass coverslips were transfected with vectors encoding YFP-tagged regions of AKAP18δ using Lipofectamine 2000. Alternatively, cells were transfected with plasmids encoding AKAP18γ-mCherry or AKAP18γ–ΔPKA-mCherry along with RIIα-YFP. Cells were incubated at 37 °C for 24 - 48 hours and then fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. Coverslips were mounted in Prolong Gold antifade (Thermo) and imaged using a Leica AS-MDW workstation. Microscopy associated with proximity labeling experiments is described below.

Immunoprecipitation and western blotting.
HEK293A (Invitrogen) or HEK293T (GE) cells were transfected with 3 μg DNA per 10 cm dish using TransIt-LT1 (Mirus). After 40-48 hours cells were harvested in lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 2% glycerol, 1% Triton X-100) containing protease inhibitors. AKAP18γ/δ complexes were immunoprecipitated with rabbit anti-GFP IgG (Invitrogen) and protein A agarose for 2 hours at 4°C. Beads were washed 4 x 1 ml in lysis buffer. Proteins were separated on 4-12% gradient gels (Invitrogen) and transferred to nitrocellulose membranes. Primary antibodies (PKA catalytic subunit mAb (BD Biosciences clone 5B), 1:1000; RIIα mAb

(BD clone 40), 1:2000; GFP mAb (Santa Cruz Biotechnology sc-9996) were incubated with membranes overnight at 4°C in TBST/Blotto. The membranes were washed extensively in TBST, incubated with HRP-labeled secondary antibodies (Jackson Immunoresearch), washed as before and developed using ECL (ThermoFisher) on an Alpha Innotech MultiImage III with FluoroChem Q software, or with an iBright FL1000 imager (Thermo Scientific). For re-probing, membranes were stripped with 1X Re-Blot Plus Strong (Millipore) for 15 minutes and then re-blocked in Blotto before incubation with primary antibodies again. For experiments in figure 2B, 3T3-L1 cells (ATCC) grown in DMEM-H + 10% FBS were differentiated into adipocytes by switching to DMEM + 0.5 mM methyl-isobutylxanthine (IBMX), 1 µM dexamethasone and 10 µg/ml insulin for 3 days followed by a further 4 days in DMEM + 10 µg/ml insulin. Mouse hippocampal neurons were prepared as described (6). Both cell types were lysed in RIPA buffer and AKAP18 was immunoprecipitated from cleared lysates using a rabbit polyclonal antibody directed against the C-terminus harboring the anchoring helix (VO57, ref. (3)). AKAP18 isoforms were then detected by RII overlay as described above.

Structural modeling with AutoDock.
AutoDock calculations were carried out using AutoDock Tools 1.5.6, according to the methods described in (7). Briefly, mutant helix PDB files were prepared from PDB 4ZP3 using the mutagenesis wizard in PyMol (Schrodinger), and used as ligands in AutoDock, while the RIIα D/D structure from the same PDB files was used as the receptor. 50 docking simulations were performed, and the top-scoring simulation was chosen for further analysis. RMSD from the reference structure (PDB 4ZP3), residues in close contact, and predicted binding energies were obtained from AutoDock for AKAP18 wildtype, mutants and AKAP-*IS*. The calculated energy for AKAP-*IS* (5.49 kcal/mol) was compared to the previously experimentally determined energy (-12.8 kcal/mol; (8), and the discrepancy between the two values (-18.29 kcal/mol) was used to adjust AutoDock-obtained binding energies for all mutants. Conversions from ΔG to K_d were carried out using the formula $\Delta G = -RT \ln(1/K_d)$, where R=8.314 J/K*mol; T=298K.

Fluorescence Polarization.
FP was performed as described previously (8). FITC-AKAP-*IS* (AMAQIEYLAKQIVDNAIQQAKA, 1 nM) was incubated with recombinant murine RIIα (20 nM) in 20 mM HEPES buffered saline + 0.1% Triton X-100 pH 7.5. Increasing concentrations (0.01 nM – 1 µM) of unlabeled AKAP18 (AMAELVRLSKRLVENAVLKAVQ) or V282M (AMAELVRLSKRL**M**ENAVLKAVQ) peptide were mixed with FITC-labeled AKAP-*IS*-RII complexes. Each sample was incubated for 10 min. Fluorescence polarization was measured on a Beacon 2000 (Panvera, Madison, WI), following the manufacturer's instructions. Saturation binding curves were generated with PRISM graphing software (GraphPad). IC_{50} values were calculated using the following equation: $y = 1-(m_0/(m_0+m_1*(1+([\text{FITC-}IS]/K_d))))$ where y equals the fraction bound (norm mA), m_0 equals ligand concentration, K_d is for FITC-*IS* (5 nM), and [FITC-*IS*] equals the concentration of FITC-*IS* (1 nM).

Electrophysiology.
HEK293 cells were transfected with cDNAs encoding the α1c and β2a subunits of cardiac L-type Ca^{2+} channels (3) and wildtype or V37M AKAP18α-GFP. Whole-cell recordings were performed using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Sunnyvale, CA). Signals were sampled at 2 kHz, filtered at 1 kHz, and acquired using pClamp software (version 7, Axon Instruments). Series resistance (90–

95%) and whole-cell capacitance compensation were used. Patch pipettes (2–4 MΩ) were filled with an intracellular solution containing 140 mM CsCl, 2 mM $MgCl_2$, 10 mM BAPTA, 10 mM HEPES, and 3 mM ATP. The external solution consisted of 15 mM $BaCl_2$, 145 mM Tetraethyl ammonium, 10 mM HEPES and 10 mM glucose. LTCC currents were evoked step depolarization (200 msec) from a holding potential of –80 mV to +10 mV. Rapid solution exchanges were accomplished through a two-barrel pipe controlled by a solution stimulus delivery device, SF-77B (Warner Instruments, Hamden, CT). CPT-cAMP (200 µM) was bath applied for 150 seconds before currents were evoked as above. Data are presented as mean ± SEM. Statistical analysis was performed using unpaired T-test or Mann-Whitney test in PRISM.

Calcium Imaging.

Aorta from 5 mice were dissected and placed in ice-cold DMEM containing 1X glutamate, 1X pyruvate, 1X penicillin/streptavidin and fungizone (0.25 g/ml). Aorta segments were transferred and incubated in a DMEM solution containing 2.2 mg/mL of collagenase Type 2 (Worthington) at 37° C for 15 minutes to remove the adventitia. To disperse and culture unpassaged arterial myocytes, the tissue was cut into 2-5 mm segments and incubated at 37° C with constant shaking in a buffer containing 134 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 7 mM D-glucose and 2.2 mg/mL of collagenase. The digestion was stopped by adding an equal volume of DMEM containing 5% fetal bovine serum. The digested tissue was then centrifuged for 5 minutes at 14,000 rpm. The pellet containing the digested tissue was resuspended in DMEM containing 1X glutamate, 1X pyruvate, 5% serum and 5 mM D-glucose with gentle resuspension, which resulted in dispersion of individual arterial myocytes. Cells were then seeded on glass coverslips coated with laminin and kept in an incubator at 37° C with 5% CO_2 for 7-10 days before transfection. Transfection of unpassaged arterial myocytes with cDNAs encoding either AKAP18α-mCherry or AKAP18α-V37M-mCherry was performed using the TransfeX reagent (ATCC) following the manufacturer's protocol. Cells were used for calcium imaging experiments 24 hours after transfection. For global intracellular calcium ($[Ca^{2+}]_i$) imaging experiments, transfected arterial myocytes were loaded with the membrane-permeable acetoxymethyl-ester (AM) Fluo-4 for 20 minutes at room temperature. Cells exhibiting mCherry fluorescence were selected for imaging. Cells were bathed in a HEPES-buffered saline solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose and 10 mM HEPES, pH 7.4. Images were collected using an Andor spinning disk confocal system coupled to an Olympus iX-81 inverted microscope equipped with an Olympus 40X oil immersion lens (NA=0.75). 60 mM K^+ was added to depolarize the membrane potential of the cells and activate L-type calcium channels. After a response was observed, cells were washed with control solution for 10 minutes. For the second round of stimulation, cells were incubated with 1 µM forskolin for 3 minutes, and then challenged again with 60 mM K^+. Images were normalized by dividing the fluorescence intensity of each pixel (F) by the average resting fluorescence intensity (F_o) of a confocal image to determine the F/F_o-time relationship.

Proximity labeling and mass spectrometry.

The cDNA for miniTurbo (9) was fused to 3' end of AKAP18γ cDNAs and tested for expression in HEK293T cells. Cells were transfected using calcium phosphate precipitation. After 24 hours, biotin labeling was performed by incubating cells in media containing 50 µM biotin for 1 hour. Biotinylation of proteins was verified by western blot using NeutrAvidin-HRP (1:5000, Thermo Scientific). For immunostaining, cells were washed in PBS and fixed in 4% PFA. Biotinylated proteins were detected using

NeutrAvidin-Rhodamine Red X (1:1000, Thermo Scientific). AKAP18-miniTurbo constructs were detected using a mouse antibody to a V5 epitope (1:1000, Invitrogen MA5-15253). Actin was visualized with ActinGreen 488 (Molecular Probes). Cells were imaged using a Keyence BZX-700 workstation. For biotin labeling/MS experiments, transfections were scaled up and cells were labeled with biotin as above. Cells were then lysed in 800 µl RIPA buffer (20mM Tris pH 7.5, 130mM NaCl, 2mM EDTA, 20mM NaF, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS and protease inhibitors), rotated for 10 min at 4ºC, and clarified at 15000xg for 8 min. Lysates were incubated with 30 µl of magnetic streptavidin beads (Tritech/Solulink) rotating at RT for 1.5 hrs. Beads were washed 2x in RIPA, 1x in 1M KCl, 1x in 0.1M $NaCO_3$, 2x in 2M Urea, and 2x in 20mM Tris pH 7.5/150mM NaCl, and resuspended in 50 µl 6M guanidinium HCl. Proteins were reduced and alkylated by the addition of 2 mM TCEP and 4 mM chloroacetamide, and incubated at 70°C for 15 min. Samples were then diluted with 50 µL of 50 mM ammonium bicarbonate digested using 2 µg of Lys-C (Wako) for 2 hours. After Lys-C digestion, samples were further diluted by 100 µL of ammonium bicarbonate and digested using 1 µg of trypsin (Thermo Scientific) overnight at 37°C. Peptides were desalted and eluted from StageTips using elution buffer (50% acetonitrile, 0.1% TFA) and then loaded on a self-pulled 360 µm OD x 100 µm ID 20 cm column with a 7 µm tip packed with 3 µm Reprosil C18 resin (Dr. Maisch, Germany). Peptides were analyzed by nanoLC-MS in a 120 minutes, 10% to 24% acetonitrile gradient in 0.1% acetic acid at 300 nL/min (Thermo EASY nLC 1200) on an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer. Orbitrap FTMS spectra (R = 60 000 at 200 m/z; m/z 350–1600; 7e5 target; max 20ms ion injection time) and Top Speed data dependent acquisition with 2 s cycle time; HCD MS/MS spectra (5e3 target; max 50 ms injection time); CID MS/MS spectra (2e3 target; max 50 ms injection time) were collected with dynamic exclusion for 60 s; The normalized collision energy applied for HCD was 25 % and CID was 28%.Mass spectra were searched against the UniProt human reference proteome (July 2016) using MaxQuant v1.6.1.0. Samples were grouped into separate fractions: “LFQ mode” with “Fast LFQ.” “Match between runs” mode was enabled. Other settings were kept as default. Data analysis was carried out in the R environment. Briefly, LFQ intensities calculated by MaxQuant were log2 and Z-score transformed; only proteins quantified in at least 60% of the replicates were selected for further analysis; missing values were replaced by random numbers drawn from a distribution with mean of -2 and a standard deviation of 0.1 to represent protein abundance below the detection limit; a two-sided Student’s t-test, assuming equal variances was used to determine the *p*-values and adjusted for multiple hypothesis testing using “fdr” method. Protein network prediction and GO analysis were performed using STRING database version 10.5 (10). Networks were visualized using Cytoscape v3.6.1 software. Default settings were adjusted to disregard genomic proximity and gene fusion data when assigning confidence scores. A confidence level of 0.5 was used as a cutoff. Darkness of edges scales with confidence score.

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Table S1: Links to NCBI Gene and Variation Information

AKAP2

https://www.ncbi.nlm.nih.gov/gene/11217

https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=11217

mRNA	Protein	Contig	Missense Variant	SNP id
NM_001198656.1	NP_001185585.1	NT_008470.20	Y657C	rs772943520
			Q658H	rs762558016
			A659S	rs113341282
			A659T	rs113341282
			G660S	rs751539414
			G660D	rs367980096
			Q664P	rs767483282
			A666P	rs752491691
			I667F	rs755845466
			I667V	rs755845466
			Q668K	rs746046130
			Q669K	rs758492339

AKAP5

https://www.ncbi.nlm.nih.gov/gene/9495

https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=9495

mRNA	Protein	Contig	Missense Variant	SNP id
NM_004857.3	NP_004848.3	NT_026437.13	T395P	rs79474776
			S398T	rs764236886
			I408V	rs753889145
*after this work was completed 2 new SNPs were annotated:			T395I	rs906590680
			L391F	rs377585854

AKAP7

https://www.ncbi.nlm.nih.gov/gene/9465

https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=9465

mRNA	Protein	Contig	Missense Variant	SNP id
NM_016377.3	NP_057461.2	NT_025741.16	R280S (R302S)	rs376123315
			V282M (V304M)	rs11538901
			V282G (V304G)	rs757764447
			E283G (E305G)	rs746428158
			V286A (V308A)	rs141169465

AKAP11

https://www.ncbi.nlm.nih.gov/gene/11215

https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=11215&ctg=NT_024524.15&mrna=NM_016248.3&prot=NP_057332.1&orien=forward

mRNA	Protein	Contig		Missense Variant	SNP id
NM_016248.3	NP_057332.1	NT_024524.15	AKAP11 (site 1)	N617H	rs761516240
				V620L	rs766993879
				S621N	rs749993203
				E622G	rs377262755
				E622A	rs377262755
	*after this work was completed another SNP was annotated:			L628F	rs899983160
			AKAP11 (site 2)	A1648S	rs147330536
				A1648V	rs140983171
				L1650F	rs776327237
				A1651V	rs759046890
				E1652Q	rs764768179
				I1654V	rs752657729
				A1656V	rs202188943
				I1659M	rs751315176
	*after this work was completed 2 new SNPs were annotated:			R1664P/Q	rs536746094
				E1665K	rs755073794

AKAP13

https://www.ncbi.nlm.nih.gov/gene/11214

https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=11214&ctg=NT_010194.18&mrna=NM_006738.5&prot=NP_006729.4&orien=forward

mRNA	Protein	Contig	Missense Variant	SNP id
NM_006738.5	NP_006729.4	NT_010194.18	I1248V	rs114556336
			S1253R	rs773395257
			R1254C	rs759396524
			R1254H	rs138629757
			V1256M	rs752435400
			D1257V	rs758332238
			E1261K	rs114446918
			E1261V	rs200595324
			K1264E	rs546556841