Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary Information for

Targeted disruption of PKC from AKAP Signaling Complexes

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METHODS

Peptide synthesis

Rink amide MBHA resin along with N-α-Fmoc protected amino acids were purchased from Novabiochem. (S)-NFmoc-2-(4'-pentenyl)alanine and Grubb's catalyst (1st Generation) were purchased from Sigma. All other reagents and organic solvents were purchased from Fisher Scientific unless specified. Solvents used in this synthesis were HPLC grade.

All peptides used in this study were synthesized using standard Fmoc (fluorenylmethoxycarbonyl) protected solid phase peptide synthesis. Rink amide MBHA resin was equilibrated in NMP (1-methyl-2-pyrrolidinone) for 15 minutes. Fmoc protection group was deprotected using 25% v/v solution of piperidine in NMP for 30 minutes. Deprotection step was followed by three washes with NMP. Amino acid coupling was carried out by adding 10 equivalents of Fmoc-protected amino acid (0.25 M final concentration) in NMP to the deprotected resin along with HCTU [O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] in NMP (0.24 M final concentration) followed by 8% v/v DIEA (N, N-Diisopropylethylamine). Amino acid coupling was carried out for 45 minutes followed by three washes with NMP. Deprotection and coupling steps were repeated for the addition of the remaining amino acid residues. Two residues of olefinic amino acid 'S5' [Fmoc-(S)-2-(4-pentenyl)alanine] were incorporated at suitable positions using standard coupling conditions. Olefin metathesis (ring closing metathesis) was performed on Fmoc-protected, resin bound peptide using 0.4 equivalents of Grubb's first-generation catalyst [Benzylidene-bis(tricyclohexylphosphine)dichlororuthenium] in 1,2-dichloroethane for 1 hour. This step

was carried out twice to ensure completion of staple formation. Addition of β-Alanine (Fmoc-β-Ala-OH) as a flexible N-terminal linker was performed under standard coupling conditions. Peptides were labeled with either fluorescein or biotin at the N-terminal end. For fluorescein labeling, deprotected, resin bound peptides were agitated overnight with 2 equivalents of 5(6)-carboxyfluorescein, 0.046 M HCTU and 2% v/v DIEA in DMF (N, N-Dimethylformamide). Biotin labeling was performed overnight with 10 equivalents of D-biotin, 0.14 M HCTU and 4% v/v DIEA in a 1:1 mixture of DMF and DMSO (dimethyl sulfoxide). Peptides were cleaved from resin in a cleavage cocktail of 95% TFA (trifluoroacetic acid), 2.5% water and 2.5% TIS (triisopropylsilane) for four hours. Products were then precipitated in ice cold MTBE (methyl-tert-butyl ether) and allowed to air dry. Crude products were dissolved in equal parts water and methanol and purified using high performance liquid chromatography. Product peptides were verified with ESI (electrospray ionization) mass spectroscopy. Fluorescein labeled peptides were quantified by measuring their absorbance at 495 nm. Biotin labeled peptides were quantified by measuring diminished absorbance of HABA [2-(4'-hydroxybenzeneazo)benzoic acid]-avidin complex at 500 nm. Pure, dry peptides were dissolved in DMSO to achieve 10 mM stocks and were stored at 4°C, protected from light.

Sequences for the peptides used in this study are as follows (red stars represent S_5 residues):

CSTAD1= (5/6 FAM) - β-Ala – K*S(Nle)L*FKRRKKAAKALK

CSTAD2= (5/6 FAM) - β-Ala – KA*(Nle)LC*KRRKKAAKALK

CSTAD3= (5/6 FAM) - β-Ala - KAS(Nle)LC*KRR*KAAKALK

CSTAD4= (5/6 FAM) - β-Ala - KAS(Nle)LCF*RRK*AAKALK

CSTAD5= (5/6 FAM) - β-Ala - KAS(Nle)LCFKRRK*AAK*LK

CSTAD5= Biotin - β-Ala - KAS(Nle)LCFKRRK*AAK*LK

CSTAD6= (5/6 FAM) - β-Ala - KAS(Nle)LCFKRRKK*AKA*K

CSTAD6= Biotin - β-Ala - KAS(Nle)LCFKRRKK*AKA*K

Scramble 5= (5/6 FAM) - β-Ala - LLSKAAFKAKK*RAR*(Nle)K

Scramble 5= Biotin - β-Ala - LLSKAAFKAKK*RAR*(Nle)K

Scramble 6= (5/6 FAM) - β-Ala - RLKAAFALKSKK*(Nle)KK*A

Scramble 6= Biotin - β-Ala - RLKAAFALKSKK*(Nle)KK*A

Molecular weights of purified peptides are as follows:

(5/6 FAM) - β-Ala - CSTAD1= 2564.4 (Expected mass= 2565.2)

(5/6 FAM) - β-Ala - CSTAD2= 2504.4 (Expected mass= 2505.1)

(5/6 FAM) - β-Ala - CSTAD3= 2464.2 (Expected mass= 2464.0)

(5/6 FAM) - β-Ala - CSTAD4= 2482.2 (Expected mass= 2483.0)

(5/6 FAM) - β-Ala - CSTAD5= 2539.2 (Expected mass= 2540.1)

Biotin - β-Ala - CSTAD5= 2407.5 (Expected mass= 2408.1)

(5/6 FAM) - β-Ala - CSTAD6= 2554.5 (Expected mass= 2555.1)

Biotin - β-Ala - CSTAD6= 2422.2 (Expected mass= 2423.1)

(5/6 FAM) - β-Ala - Scramble 5= 2508.0 (Expected mass= 2508.0)

Biotin - β-Ala - Scramble 5= 2376.0 (Expected mass= 2376.1)

(5/6 FAM) - β-Ala - Scramble 6= 2479.5 (Expected mass= 2480.1)

Biotin - β-Ala - Scramble 6= 2347.8 (Expected mass= 2348.0)

Cell culture

PC-3 and A549 cells were obtained from ATCC. Cell culture medium (RPMI-1640) and trypsin were purchased from Corning. Fetal bovine serum (FBS) was purchased from HyClone.

Cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C with 5% CO_2. Cells were passaged at least twice before being used in any experiment and all experiments were performed in at least triplicate at different passage numbers.

PMA induced cytoskeletal remodeling assay

$3x10^4$ cells were seeded per chamber on a chamber slide and allowed to adhere overnight. Cells were then briefly washed with PBS and serum starved for 24 hours. Following serum starvation, serum free media containing 5 μM CSTAD5, CSTAD6 or vehicle (DMSO) was introduced into respective chambers and slides were incubated for four hours. Following incubation with the treatment, media containing peptides (or vehicle) was aspirated and cells were briefly washed with serum free medium. Fresh serum free medium containing 500 nM PMA (Phorbol 12-myristate 13-acetate) was introduced to each chamber. Cells were imaged following 45 minutes of incubation with PMA containing

medium using Olympus IX71 inverted microscope. Cells were counted across three independent chambers for each treatment condition with at least 150 cells per image. Total quantification was performed with four independent experiments, statistical analysis was carried out with one-way ANOVA and Bonferroni's multiple comparisons test in GraphPad Prism.

Peptide uptake assay

$3x10^4$ cells were seeded per chamber on an 8-chamber slide (Nunc Lab-Tek II CC2) and allowed to adhere overnight. Cells were briefly washed with PBS and media containing 5 µM CSTAD5, CSTAD6 or vehicle (DMSO) was introduced to respective chambers. Cells were incubated with peptides (or vehicle) for four hours followed by three washes with PBS. Cells were then fixed using 2% paraformaldehyde solution for 10 minutes followed by three brief washes with PBS. Fixed cells were permeabilized using 0.1% Triton X-100 in PBS for 10 minutes. After permeabilization, cells were washed thrice with PBS. Nuclear staining was performed by adding 300 nM DAPI solution to each chamber followed by five minutes of incubation away from light. Cells were washed three times with PBS and slides were air dried. A coverslip was mounted overnight at 4°C using Permafluor aqueous mounting medium. Phase contrast and fluorescence imaging was performed using Olympus IX71 microscope equipped with Exfo X-Cite 120Q light source. ImageJ was used to generate channel overlays for acquired images.

Biotin-Streptavidin pulldown assay

PC-3 cells were lysed in ice cold NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40 supplemented with 1X Halt Protease Inhibitor Cocktail). Cell lysates

were treated with 5 μM biotin labeled peptides (CSTAD5 or CSTAD6), respective scrambled controls or vehicle (DMSO) and were agitated for four hours at 4°C. 25 μL of Streptavidin-Agarose resin (Millipore) was added to all samples and the samples were agitated overnight at 4°C. Resin was collected by centrifuging the lysate mixtures at 1000G for 5 minutes. Collected resin was washed at least three times with ice cold lysis buffer and boiled in Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) for 10 minutes at 95°C. SDS-PAGE was performed using 10% polyacrylamide gels (Tris-Glycine), followed by standard western blot on to PVDF (Immobilon-FL, Millipore) membranes. Pan-PKC antibody (1:1000, Rabbit mAb, Abcam) was used for primary immunodetection. Secondary antibody (1:15000, IRDye 800CW Goat anti-Rabbit, LI-COR) was used for fluorescence imaging. Membranes were imaged with Odyssey Fc imaging system (LI-COR). Densitometric quantifications were obtained using LI-LOR Image Studio. Statistical analysis was carried out with one-way ANOVA and Bonferroni's multiple comparisons test in GraphPad Prism.

Alternatively, A549 cells were lysed using Triton X-100, Tween 20 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.25 % Triton X-100, 0.05 % Tween-20 supplemented with 1X Halt Protease Inhibitor Cocktail). Pulldowns were performed as described previously followed by western blotting. Gravin antibody (1:1000, Mouse mAb, MilliporeSigma) and Pan-PKC antibody (1:1000, Rabbit mAb, Abcam) were used for primary immunodetection of gravin and PKC, respectively. Secondary antibodies (1:15000, IRDye 680RD Goat anti-Rabbit and IRDye 800CW Goat anti-Mouse, LI-COR)

were used for secondary immunodetection and fluorescence imaging. Membranes were imaged with Odyssey Fc imaging system (LI-COR).

Immunoprecipitation assay

A549 cells were seeded, allowed to attach overnight and serum starved for 24 hours. Serum starved cells were then treated with 5 μM CSTAD5, CSTAD6, respective scrambled controls and DMSO for four hours. Ice cold Triton X-100, Tween 20 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.25 % Triton X-100, 0.05 % Tween-20 supplemented with 1X Halt Protease Inhibitor Cocktail) was used to lyse the pretreated cells. Resulting lysates were agitated overnight with pre-conjugated gravin antibody (Mouse mAb, MilliporeSigma) and protein A/G-agarose resin beads (Protein A/G PLUS-Agarose, Santa Cruz Biotechnology) at 4ºC. Resin was collected by centrifuging the lysate mixtures at 1000 G for 1 minute. Resin was washed three times with ice cold lysis buffer and boiled in Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) for 10 minutes at 95°C followed by SDS-PAGE and western blotting. Blots were probed with pan-PKC antibody (1:1000, Rabbit mAb, Abcam) for primary immunodetection followed by secondary immunodetection (1:15000, IRDye 680RD Goat anti-Rabbit). Membranes were imaged and densitometric quantifications were obtained using Odyssey Fc imaging system (LI-COR). Statistical analysis was performed in GraphPad Prism with one-way ANOVA and Bonferroni's multiple comparisons test.

PKC substrate phosphorylation assay

PC-3 cells were seeded in 12 well plates and allowed to attach overnight. Cells were washed briefly with PBS and serum starved for 24 hours. Following serum starvation, cells were treated with various concentrations (1, 2.5, 5 μM) of peptides (CSTAD5 and CSTAD6), 500 nM Sotrastaurin or vehicle (DMSO) for four hours. Post treatment, cells were briefly washed with PBS and incubated with serum free cell medium containing 100 nM PMA for 5 minutes. Following stimulation, cell medium was aspirated and cells were harvested using 2X Laemmli sample buffer. Harvested samples were boiled at 95°C for 10 minutes and briefly centrifuged. SDS-PAGE was performed using 8% polyacrylamide gels, followed by standard western blot on to PVDF (Immobilon-FL, Millipore) membranes. Phospho-(Ser) PKC Substrate antibody (1:1000, Rabbit mAb, Cell Signaling Technology) was used to detect phosphorylated PKC substrates along with α-Tubulin (1:1000, Mouse mAb, DSHB, 12G10) antibody as loading control. Secondary antibodies (1:15000, IRDye 800CW Goat anti-Rabbit and IRDye 680RD Goat anti-Mouse, LI-COR) were used for secondary immunodetection and fluorescence imaging. All membranes were imaged with LI-COR Odyssey Fc imaging system.

Cell motility assay

PC-3 cells were seeded on to 24 well plates and allowed to form confluent monolayer. A vertical wound was introduced into each well using a pipette tip. Cells were then briefly washed with PBS and serum free medium to remove any detached cells and cell debris. Media supplemented with 5% FBS along with 5 μM peptides (CSTAD5, CSTAD6) or vehicle (DMSO) was carefully introduced into respective wells. Cells were imaged for ‘0 hour’ time point using Olympus IX71 inverted microscope before being incubated. Following 24 hours of incubation, cells were imaged once again at the 24-hour time point.

Wound area calculation for all captured images was performed using MRI wound healing tool for ImageJ. Zero hour and 24-hour time point wound areas were used to calculate percentage wound healing for each individual well across three independent assays. Statistical analysis was carried out in GraphPad Prism with one-way ANOVA and Bonferroni's multiple comparisons test.

Proteomic mass spectrometry

A549 cells were lysed using Triton X-100, Tween 20 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.25 % Triton X-100, 0.05 % Tween-20 supplemented with 1X Halt Protease Inhibitor Cocktail). Resulting lysate was treated with 5 μM biotin labeled peptides (CSTAD5 or CSTAD6) or their respective scrambled controls and were agitated for four hours at 4°C. 25 μL of Streptavidin-Agarose resin (Millipore) was added to all samples and the samples were agitated overnight at 4°C. Resin was collected by centrifuging the lysate mixtures at 1000 G for 5 minutes. Collected resin was washed at least four times with ice cold lysis buffer and boiled in Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) for 10 minutes at 95°C. SDS-PAGE was performed using 8% polyacrylamide gels (Tris-Glycine). Electrophoresis was stopped after the proteins had travelled a nominal distance into the separating gel. Gel was thoroughly washed with deionized water followed by staining with colloidal Coomassie blue to visualize protein bands. Bands for each treatment were then excised. Trapped proteins were digested using mass spectrometry grade trypsin and the resultant peptides were extracted in 50% acetonitrile and 0.1% TFA. 1 μL of sample was injected and analyzed via LC-MS/MS on a ThermoScientific Orbitrap Velo Elite. Proteome Discoverer Version 1.4 was used to

identify interacting proteins. A cut-off score of 125 was set for the Proteome Discoverer score and proteins above the cut-off were compared between the lead peptides and their respective scrambled controls. A list of proteins enriched by the lead peptides but not by their scrambled controls along with the common proteins enriched in both was compiled and sorted according to the scores.

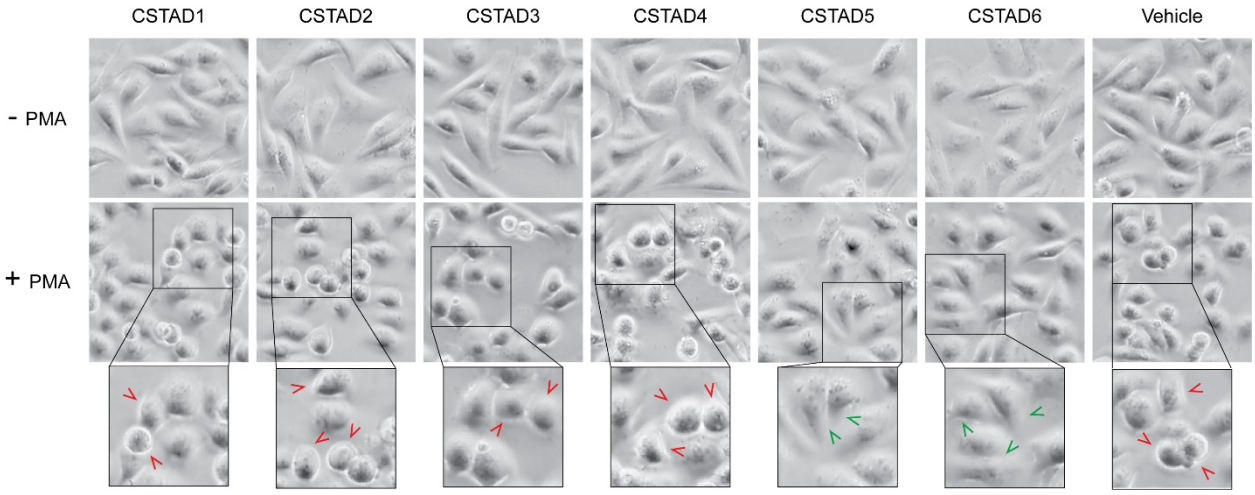

Figure S1. CSTAD5 and CSTAD6 peptides downregulate PMA induced cytoskeletal remodeling in PC-3 cells.

Serum starved PC-3 cells were treated with 5 μM CSTAD peptides or vehicle (DMSO) for four hours, followed by 500 nM PMA for 45 minutes. Phase contrast images pre- and post-PMA stimulation displaying altered in morphology (cell rounding) for cells treated with CSTAD 1-4 whereas CSTAD 5 and CSTAD 6 treated cells preserve a more flattened morphology.

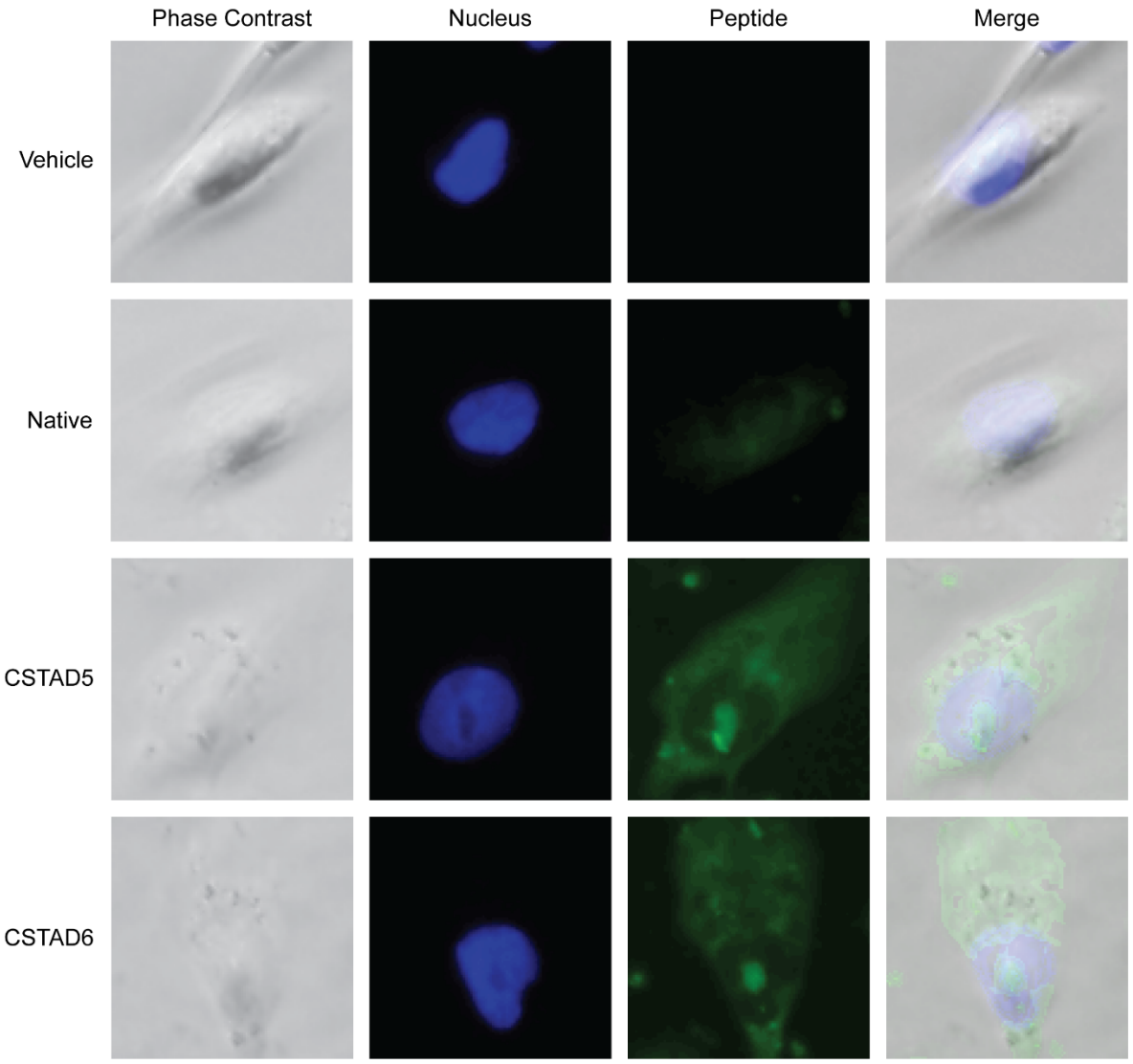

Figure S2. CSTAD peptides permeate cell membranes and display cytoplasmic localization.

PC-3 cells were treated with 5 μM vehicle (DMSO), CSTAD5, CSTAD6 or native peptide for four hours. Cells were washed, fixed with paraformaldehyde, permeabilized with Triton X-100 followed by nuclear staining with DAPI. Fluorescence images along with their overlays demonstrate the primarily cytoplasmic localization of CSTAD peptides with some degree of presence within the nucleus.

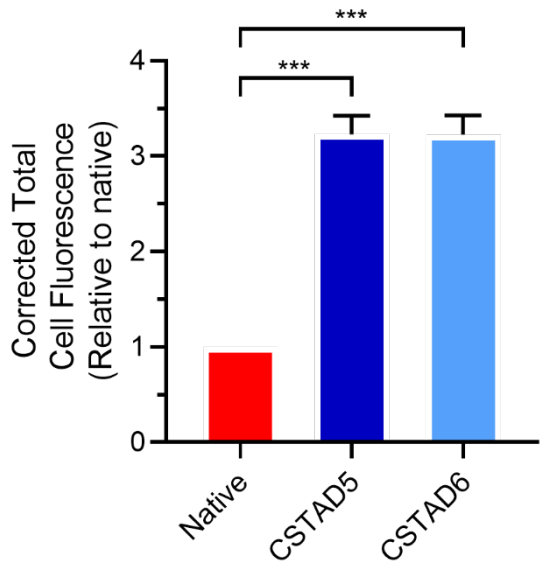

Figure S3. Addition of an all-hydrocarbon staple improves cellular uptake.

PC-3 cells were treated with 5 μM CSTAD5, CSTAD6 or vehicle (DMSO) for four hours. Cells were washed, fixed with paraformaldehyde, permeabilized with Triton X-100 followed by nuclear staining with DAPI. Peptide uptake was quantified as Corrected Total Cell Fluorescence (CTCF) using ImageJ. *** $p<0.01$ by one-way ANOVA and Bonferroni's multiple comparisons test. Error bars SEM from n=100 cells.

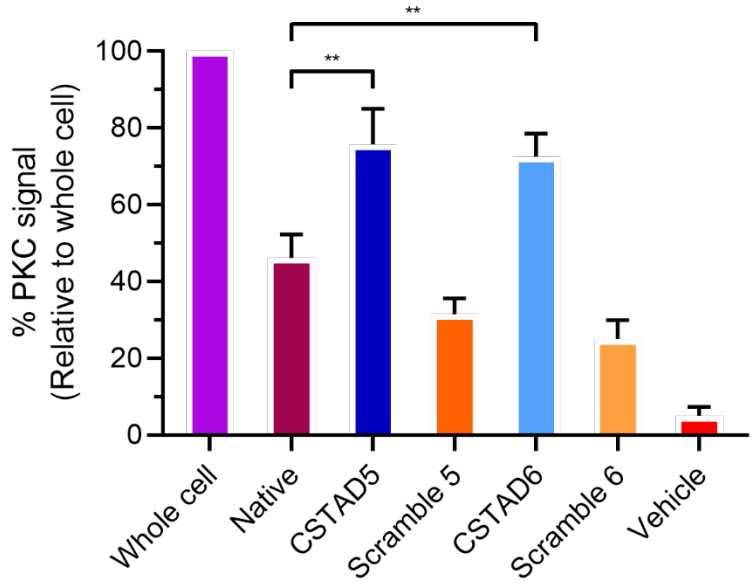

Figure S4: Addition of an all-hydrocarbon staple improves PKC binding.

PC-3 cell lysates were treated with biotin-labeled peptides for four hours. Pulldowns were performed using streptavidin-agarose resin. PKC was detected by immunoblotting. Densitometric quantifications were performed using LI-LOR Image Studio. * $p<0.05$; ** $p<0.01$ by one-way ANOVA and Bonferroni's multiple comparisons test. Error bars represent standard deviation from n=5 experiments.

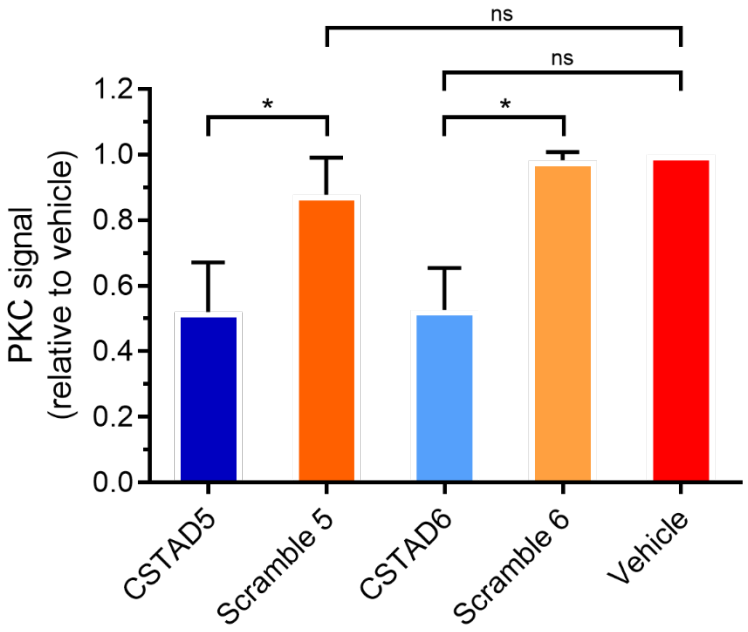

Figure S5. CSTAD5 and CSTAD6 reduce PKC scaffolding by gravin.

Serum starved A549 cells were treated with 5 μM CSTAD5 or CSTAD6, their respective scrambled controls or DMSO for four hours. Treated cells were lysed and gravin was immunoprecipitated followed by SDS-PAGE and immunoblotting for PKC. Densitometric quantifications show reduced PKC coprecipitation with gravin in lysates from cells pretreated with CSTAD5 or CSTAD6 as compared to their scrambled controls. * $p<0.05$; ns, not significant by one-way ANOVA and Bonferroni's multiple comparisons test. Error bars represent standard deviation from n=4 experiments.

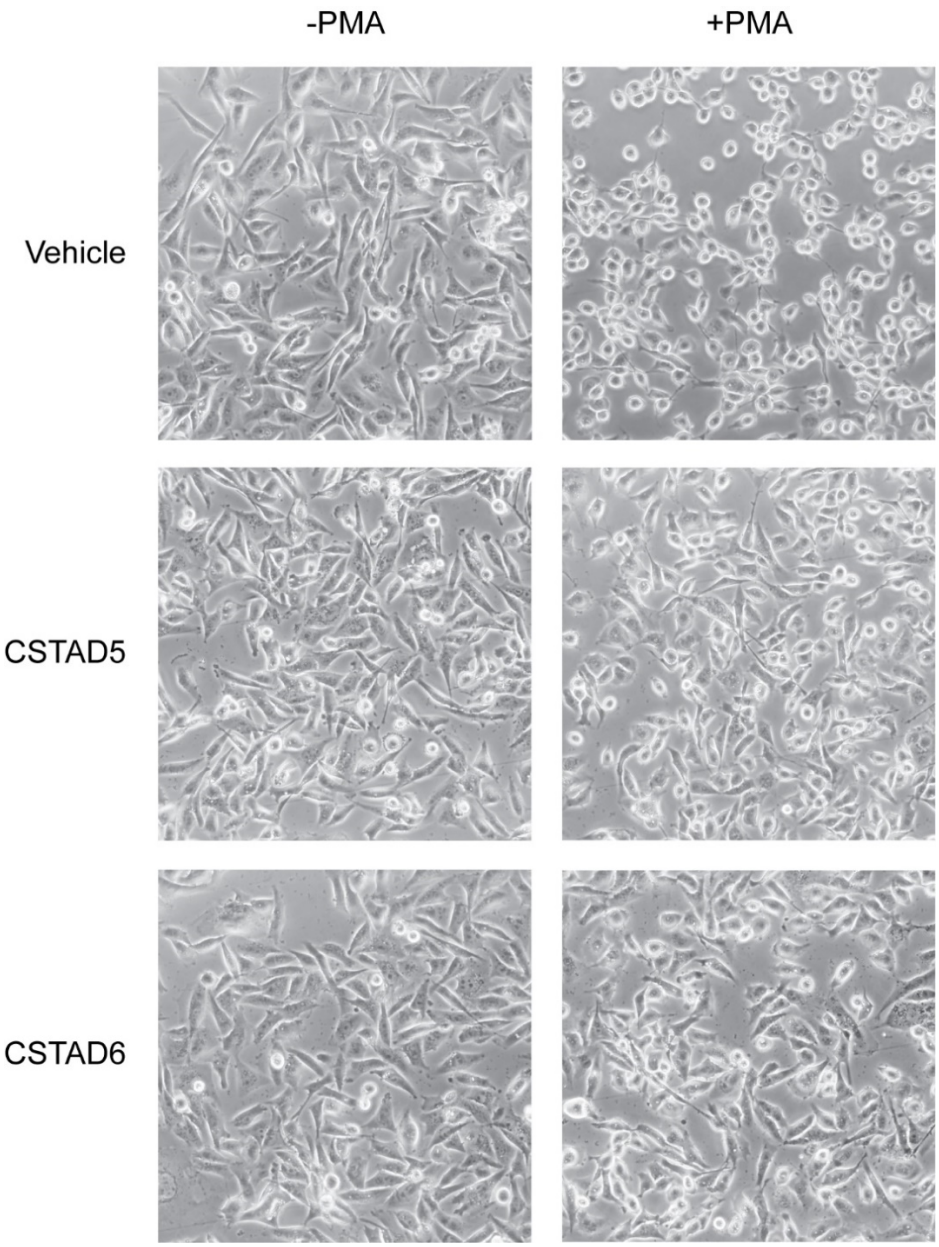

Figure S6. CSTAD peptides reduce PMA induced cytoskeletal remodeling in PC-3 cells. Serum starved (24 hours) PC-3 cells were treated with 5 μM CSTAD peptides or vehicle (DMSO) for four hours followed by 45 minutes with 500 nM PMA. Phase contrast images pre- and post-PMA stimulation show the preservation of non-rounded cell morphology in CSTAD5- or CSTAD6-treated cells as compared to vehicle-treated cells.

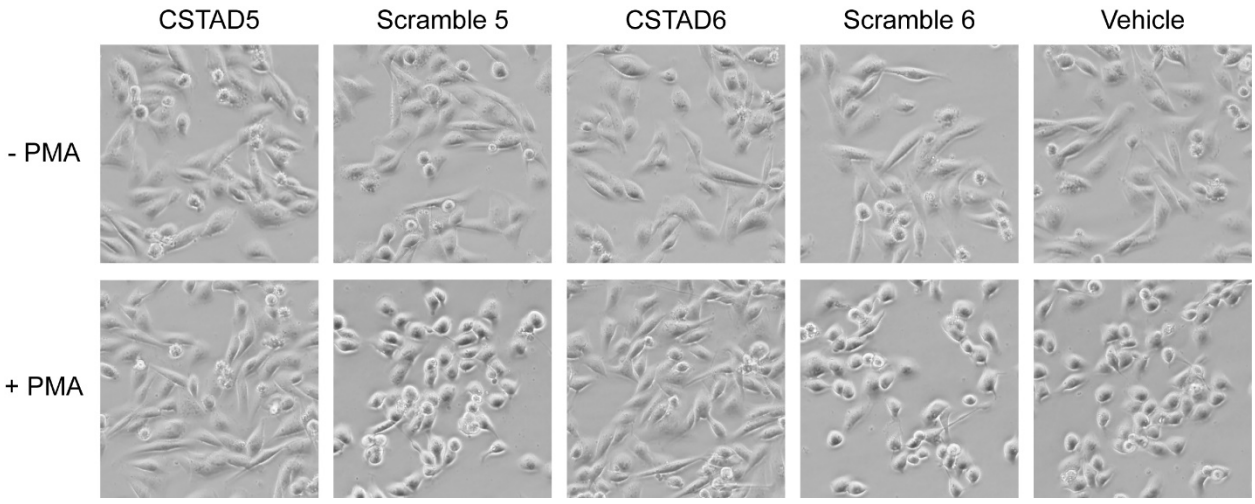

Figure S7. Scramble controls do not downregulate PMA-induced cytoskeletal remodeling.

Serum starved (24 hours) PC-3 cells were treated with 5 μM CSTAD5, CSTAD6, scramble controls or vehicle (DMSO) for four hours followed by a 45-minute treatment with 500 nM PMA. Phase contrast images pre- and post-PMA stimulation show the preservation of normal cell morphology in CSTAD5- or 6-treated cells as compared to their respective scramble controls or vehicle treated cells.

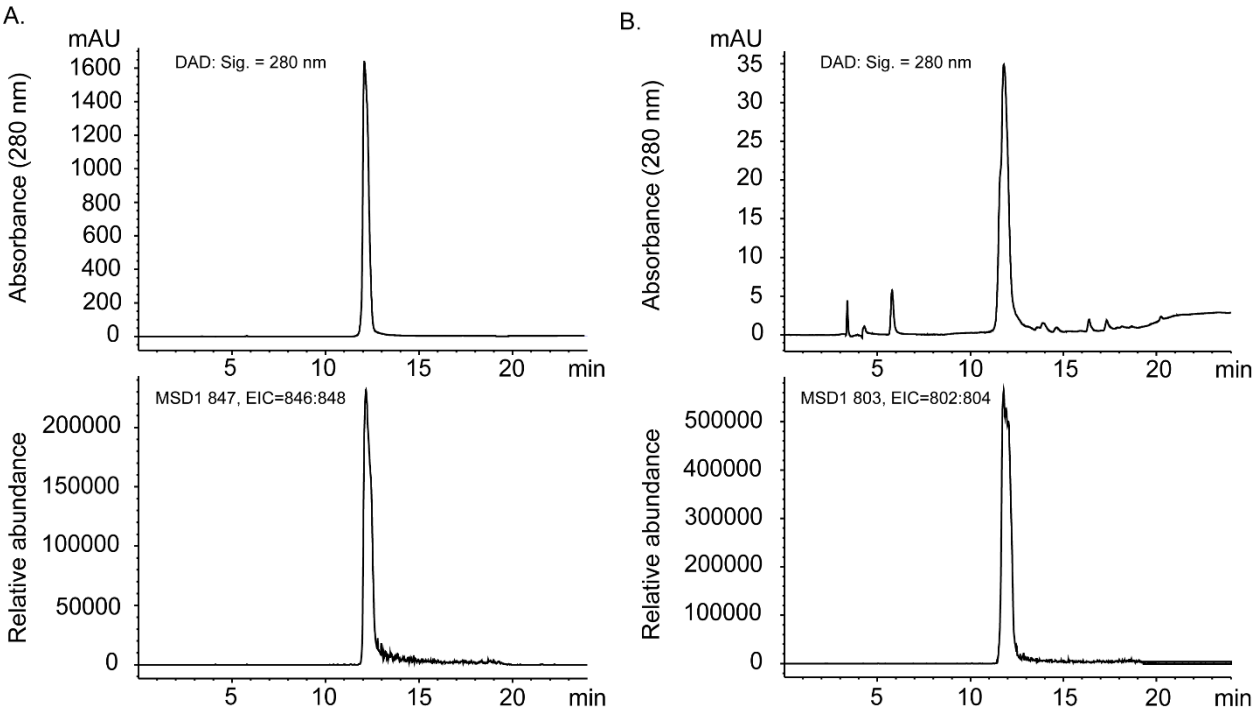

Figure S8. LC/MS spectra of (5/6 FAM)- and biotin-labeled CSTAD5 peptide.

(A) Spectra of purified (5/6 FAM) labeled CSTAD5 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 847.7. **(B)** Spectra of purified biotin labeled CSTAD5 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 803.7.

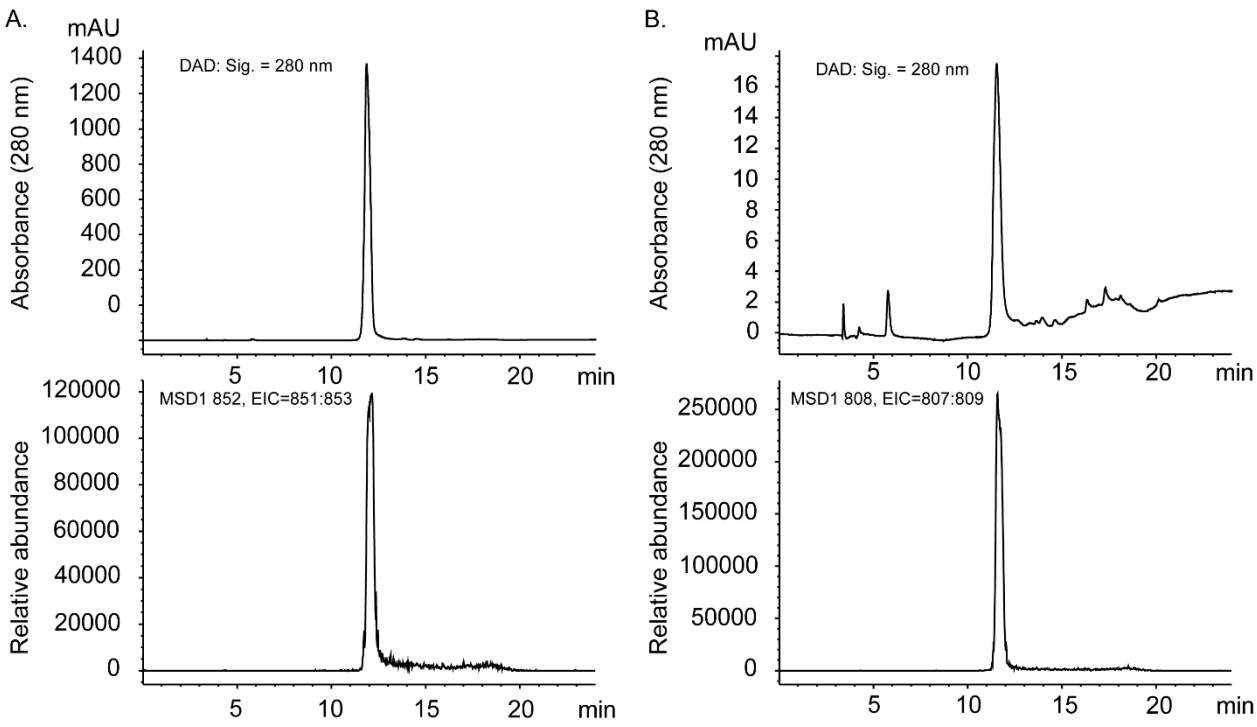

Figure S9. LC/MS spectra of (5/6 FAM)- and biotin-labeled CSTAD6 peptide.

(A) Spectra of purified (5/6 FAM) labeled CSTAD6 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 852.7. **(B)** Spectra of purified biotin labeled CSTAD6 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 808.7.

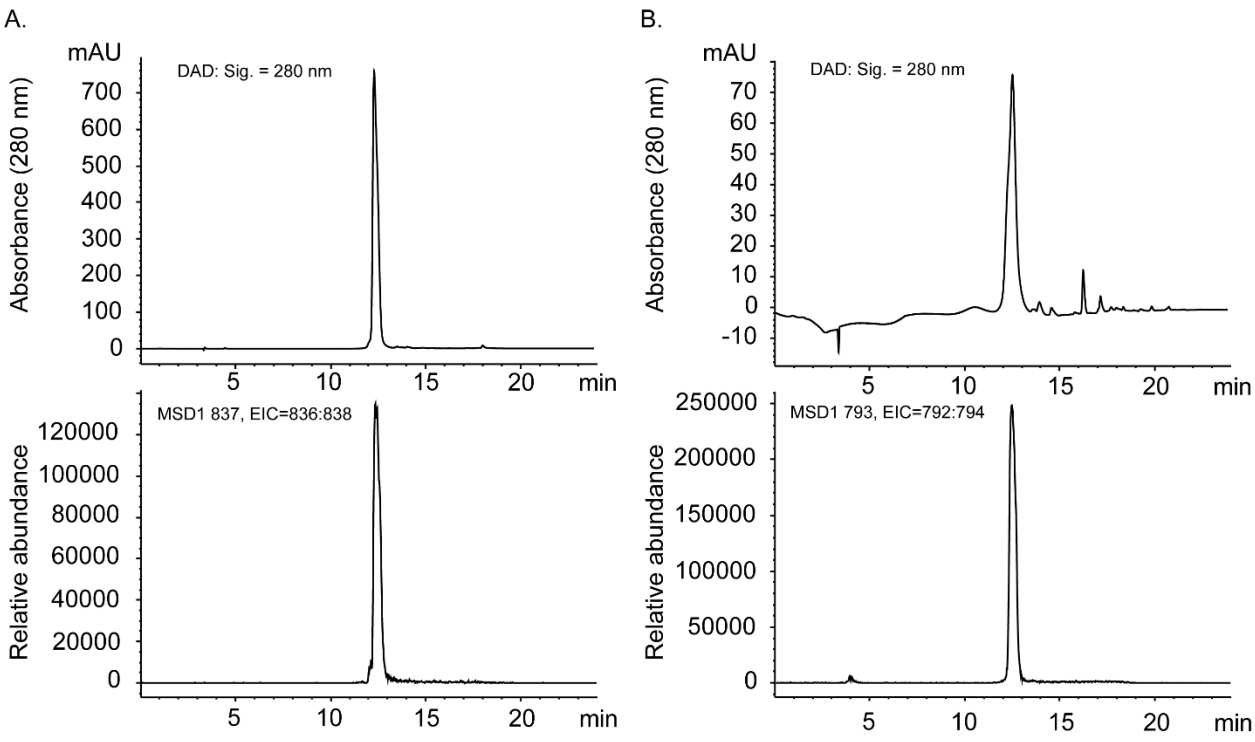

Figure S10. LC/MS spectra of (5/6 FAM)- and biotin-labeled Scramble 5 peptide.

(A) Spectra of purified (5/6 FAM) labeled Scramble 5 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 837.0. **(B)** Spectra of purified biotin labeled Scramble 5 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 793.0.

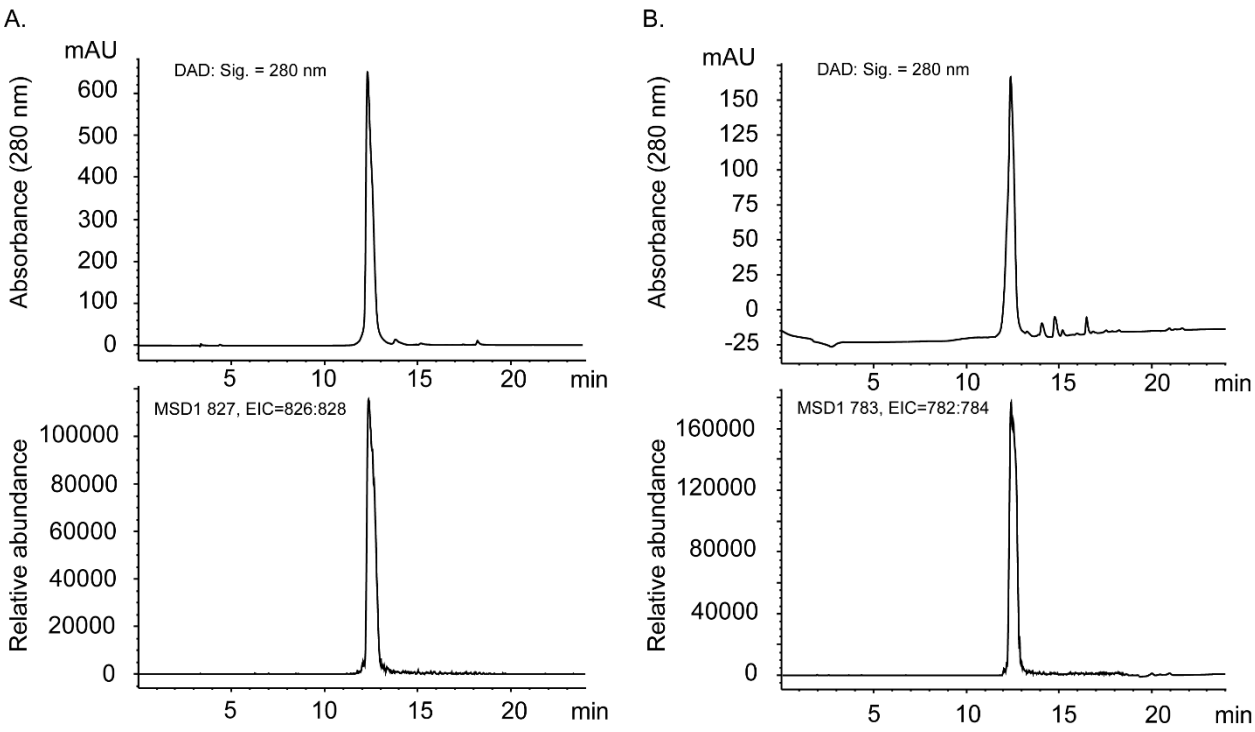

Figure S11. LC/MS spectra of (5/6 FAM)- and biotin-labeled Scramble 6 peptide.

(A) Spectra of purified (5/6 FAM) labeled Scramble 6 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 827.7. **(B)** Spectra of purified biotin labeled Scramble 6 peptide. Absorbance at 280 nm along with extracted ion current (EIC) mass spectrometric detection (MSD) are shown. Expected mass (M+3)/3 = 783.7.

Table S1. Proteins enriched in streptavidin-biotin pulldowns performed using CSTAD5 and CSTAD6 along with their respective scrambled controls. Proteins above a Proteome Discoverer score of 125 are listed serially.