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Byrne et al. Supplementary Information

Supplementary Methods

Tryptic digestion

Protein samples were reduced with 3 mM DTT in 50 mM ammonium bicarbonate (AmBic) and heated at 60 °C for 10 minutes. Free cysteine residues were subsequently alkylated with 14 mM iodoacetamide (dark, at room temperature, 30 minutes). Excess iodoacetamide was quenched upon addition of DTT to a final concentration of 7 mM. Proteins were digested overnight with trypsin (2% w/w) at 37 °C.

Titanium dioxide enrichment

Titansphere Phos-TiO_2 spin tips (GLSciences) were conditioned with solution A (2 % TFA, 80 % MeCN) by centrifugation at 3000 *x g* for 2 minutes and then equilibrated with solution B (25 % lactic acid, 1.5 % TFA, 60 % MeCN), centrifuging as before. Peptides were diluted with solution B, loaded on to the tip and centrifuged at 1000 *x g* for 10 minutes. Unbound material (flow through) was re-loaded on to the tip and centrifuged again to increase phosphopeptide recovery. Spin columns were washed using solution B followed by solution A. Phosphopeptides were eluted from the tips with 5% ammonium hydroxide (1000 *x g*, 5 minutes) followed by 5% (v/v) pyrrolidine solution (1000 *x g*, 5 minutes). Samples were dried to completion by vacuum centrifugation and reconstituted with water: acetonitrile (97:3) containing 0.1% (v/v) TFA.

LC-MS/MS and data analysis

Tryptic peptides were analysed by LC-MS/MS before and after titanium dioxide-based phosphopeptide enrichment. nLC-ESI-MS/MS analysis was performed using either an Orbitrap Fusion Tribrid mass spectrometer (ThermoScientific) attached to a Ultimate 3000 nano system (Dionex), or an AmaZon ETD ion trap (Bruker Daltonics) arranged in-line with nanoAcquity n-UHPLC system (Waters). The tandem mass spectra for all identified phosphopeptides were interrogated manually.

Orbitrap Fusion LC-MS/MS method Peptides were loaded onto the trapping column (Thermo Scientific, PepMap100, C18, 300 µm X 5 mm), using partial loop injection, for seven minutes at a flow rate of 9 µL/min with 2% (v/v) MeCN 0.1% (v/v) TFA and then resolved on an analytical column (Easy-Spray C18 75 µm x 500 mm 2 µm bead diameter column) using a gradient of 96.2% A (0.1% FA) 3.8% B (80% MeCN 19.9% H_2O 0.1% FA) to 50% B over 30 minutes at a flow rate of 300 nL min^{-1}. A full scan mass spectrum was acquired over *m/z* 400-1500 in the Orbitrap (60K resolution at *m/z* 200) and data-dependent MS/MS analysis performed using a top speed approach (cycle time of 3 s), using either HCD and/or ETcaD fragmentation modes, with product ions being detected in the ion trap (rapid mode).

AmaZon ETD LC-MS/MS method Peptides were loaded onto a trapping column (Waters Symmetry C_{18}, 5 µm packing material, 180 µm x 20 mm) for three minutes with a flow rate of 5 µL/min with solvent A (0.1% (v/v) FA), and then resolved on a Waters nanoACQUITY C_{18} analytical column (1.8 µm packing material, 75 µm x 150 mm) using a gradient of 97% A, 3% B (0.1% (v/v) formic acid in MeCN) to 40% B over 60 min at 300 nL/min. A full scan mass spectrum was acquired over *m/z* 150-2000 and the three most abundant ions selected for isolation and fragmentation by CID and ETD. Dynamic exclusion was set to 1 minute. CID was performed with helium as the target gas, with the MS/MS fragmentation amplitude set at 1.20 V, and ramped from 30 to 300% of the set value.

ThermoScientific .raw files were converted to .mgf files in Proteome Discoverer. HCD and ETcaD spectra were separated according to ETD reaction time (<39 ms selects HCD spectra) generating two separate .mgf files which could be assigned as HCD or ETcaD spectra; they were then merged using an in-house Perl script. Bruker data files were converted to .mgf files using CompassXport software (Bruker Daltonic).

The resulting .mgf files were searched using MASCOT (version 2.1) against the *E. coli* IPI database (downloaded 24th March 2015) with the sequence of His-tagged PKA-Cα included. Parameters were set as follows: MS1 tolerance of 10 ppm (Fusions) or 0.6 Da (AmaZon); MS/MS mass tolerance of 0.6 Da; carbamidomethylation of cysteine was set as a fixed modification; phosphorylation of serine and threonine, oxidation of methionine and deamidation of asparagine and glutamine were set as variable modifications.

Supplementary Table 1. Sites of auto-phosphorylation identified on recombinant wild-type PKA (catalytic domain), PKA-Cα WT, by reverse-phase chromatography of tryptic peptides and tandem mass spectrometry using either collision-mediated dissociation (CID/HCD) or electron-transfer dissociation (ETD). Mass, observed charge states, fragmentation mode, maximum MASCOT ion score and maximum delta score are indicated for each phosphopeptide/phosphosite. Mascot delta score gives an indication of degree of confidence in phosphosite localisation within a given peptide sequence. Where phosphosite localisation is potentially ambiguous, the alternate site with respect to the residues within the peptide sequence (alt. site) is also indicated. Novel sites of phosphorylation are marked with an asterisk. Tag indicates a phosphosites identified in the purification tag.

Peptide	Site	Mass	Charge States	HCD/CID/ ETD	Max. Ion Score (Delta: alt. site)
MHHHHHHpSpSGLVPR	Tag	1827.73	2+, 3+, 4+	HCD	66
QHMDpSPDLGTDDDDK	Tag	1767.64	2+, 3+	HCD	36 (24: Thr10)
KGpSEQESVK	Ser10	1070.47	2+	HCD/ETD	42 (30: Ser7)
KGpSEQESVKEFLAK	Ser10	1658.77	3+	ETD	44 (32: Ser7)
WETPpSQNTAQLDQFDR	Ser34	2014.84	2+, 3+	CID	52 (21: Thr3)
KWETPpSQNTAQLDQFDR	Ser34	2142.94	2+, 3+	HCD	91 (20:Thr4)
WETPSQNpTAQLDQFDR	*Thr37	2014.83	3+	CID	40 (2: Ser5)
KWETPSQNpTAQLDQFDR	*Thr37	2142.94	3+	HCD	77 (0: Ser6)
IKpTLGTGSFGR	Thr48	1215.60	2+	HCD	26 (13: Thr6)
IKpTLGTGpSFGR	Thr48, Ser53	1295.57	2+	HCD	27 (7: Thr3, Thr6)
TLGpTGSFGR	Thr51	974.42	2+	HCD	30 (1:Thr1, Ser6)
IKTLGpTGSFGR	Thr51	1215.60	3+	HCD	53 (12: Thr3)
TLGTGpSFGR	Ser53	974.42	2+	CID	43 (23: Thr4)
HKEpSGNHYAMK	*Ser65	1380.56	2+, 3+	HCD/ETD	45
IGRFpSEPHAR	Ser139	1248.58	2+, 3+	HCD/ETD	33
TWpTLCGTPEYLAPEIILSK	Thr197	2271.09	2+, 3+	HCD	79 (2: Thr1)
FPpSHFSSDLK	Ser259	1243.53	2+, 3+	HCD/ETD	54 (24: Ser6)
VpSINEK	Ser338	768.34	2+	HCD/ETD	27

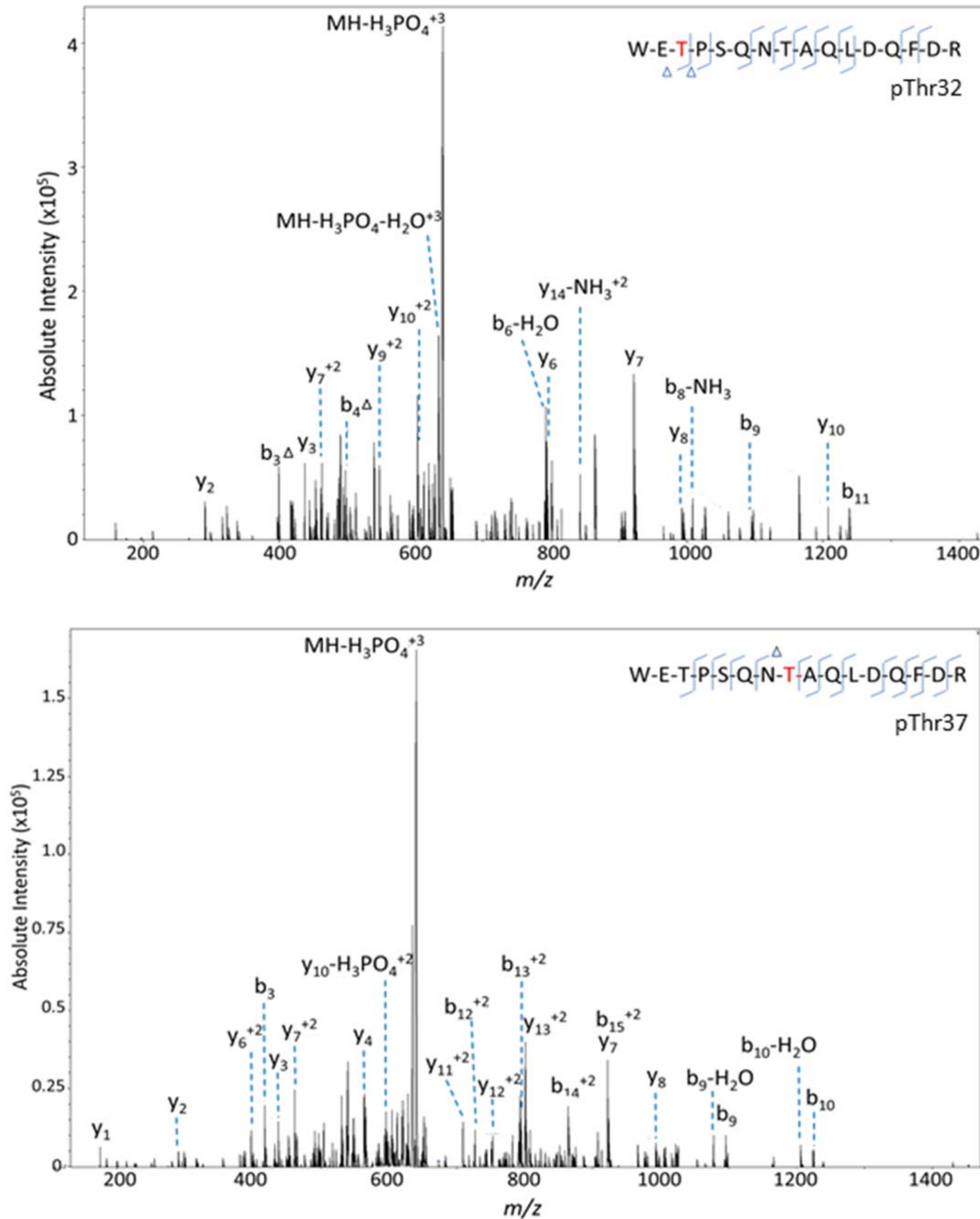
Supplementary Table 2. Sites of auto-phosphorylation identified on recombinant PKA R133A, by reverse-phase chromatography of tryptic peptides and tandem mass spectrometry using either collision-induced dissociation (CID) or electron-transfer dissociation (ETD). Mass, observed charge states, fragmentation mode, maximum MASCOT ion score and maximum delta score are indicated for each phosphopeptide/phosphosite. Mascot delta score gives an indication of degree of confidence in phosphosite localisation within a given peptide sequence. Where phosphosite localisation is potentially ambiguous, the alternate site with respect to the residues within the peptide sequence (alt. site) is also indicated. Novel sites of phosphorylation are marked with an asterisk.

Peptide	Site	Mass	Charge States	CID/ETD	Max. Ion Score (Delta: alt. site)
MHHHHHHpSpSGLVPR	Tag	1827.57	4+	CID	34
QHMDpSPDLGTDDDDKMGNAAAAK	Tag	2481.89	3+	CID	62 (46: Thr10)
GpSGMKETAAAK	Tag	1129.45	2+, 3+	ETD	80 (64)
GpSGMKETAAAKFER	Tag	1561.58	2+, 3+	ETD	63 (23)
GpSEQESVKEFLAK	Ser10	1530.51	3+	ETD	32 (19: Ser6)
KGpSEQESVK	Ser10	1070.33	2+	CID	48 (39: Ser7)
KGpSEQESVKEFLAK	Ser10	1658.64	2+, 3+	ETD	57 (28: Ser7)
MGNAAAAKKGpSEQESVK	Ser10	1784.67	2+, 3+	ETD	66 (41: Ser15)
WEpTPSQNTAQLDQFDR	*Thr32	2014.75	3+	CID	28 (2: Ser5)
WETPpSQNTAQLDQFDR	Ser34	2014.79	2+, 3+	CID	72 (26: Thr3)
KWETPpSQNTAQLDQFDR	Ser34	2142.96	3+	CID	38 (6: Thr9)
KWETPSQNpTAQLDQFDR	*Thr37	2142.76	3+	CID	42 (0: Ser6)
IKpTLGTGpSFGR	Thr48, Ser53	1295.53	2+	CID	42 (0: Thr3, Thr6)
IKTLGpTGSFGR	Thr51	1215.54	2+	CID	59 (2: Ser8)
TLGTGpSFGR	Ser53	974.37	2+	CID	60 (31: Thr4)
IKTLGTGpSFGR	Ser53	1215.47	2+, 3+	ETD/CID	53 (19: Thr6)
HKEpSGNHYAMK	*Ser65	1380.60	3+	ETD	46
VMLVKHKEpSGNHYAMK	*Ser65	1950.94	2+, 3+	ETD	54
IGRFpSEPHAR	Ser139	1248.48	3+	ETD	29
TWpTLCGTPEYLAPEIILSK	Thr197	2271.10	2+, 3+	CID	72 (12: Thr1)
GRTWpTLCGTPEYLAPEIILSK	Thr197	2484.19	3+	CID	32 (0: Thr3)
GPGDTSNFDDYEEEEIRVpSINEK	Ser338	2721.97	3+	CID	36 (30: Ser6)
FKGPGDTSNFDDYEEEEIRVpSINEK	Ser338	2997.21	3+, 4+	ETD/CID	54

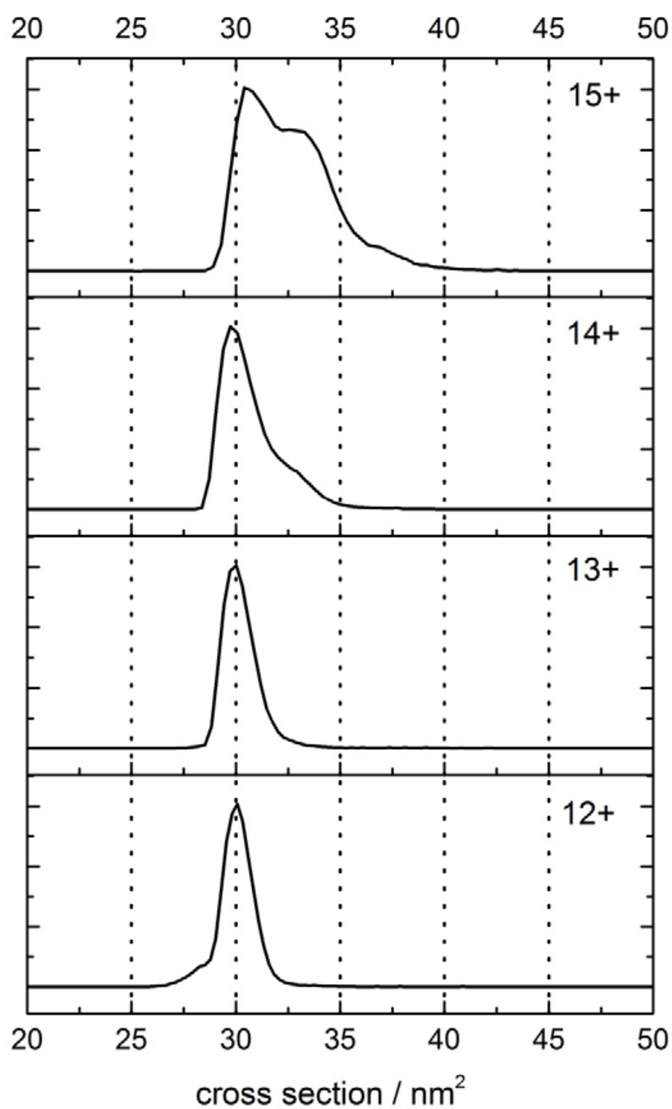
Supplementary Table 3. Sites of auto-phosphorylation identified on Mn^{2+}-lambda protein phosphatase treated recombinant PKA WT, by reverse-phase chromatography of tryptic peptides and tandem mass spectrometry using either collision-induced dissociation (CID) or electron-transfer dissociation (ETD). Mass, observed charge states, fragmentation mode, maximum MASCOT ion score and maximum delta score are indicated for each phosphopeptide/phosphosite. Mascot delta score gives an indication of degree of confidence in phosphosite localisation within a given peptide sequence. Where phosphosite localisation is potentially ambiguous, the alternate site with respect to the residues within the peptide sequence (alt. site) is also indicated. Novel sites of phosphorylation are marked with an asterisk.

Peptide	Site	Mass	Charge States	CID/ETD	Max. Ion Score (Delta: alt. site)
KpSEQESVKEFLAK	Ser10	1658.79	3+	ETD	58 (45: S7)
TLGTGpSFGR	Ser53	974.42	2+	HCD	33 (12: T4)
FpSEPHAR	Ser139	922.37	2+	HCD	24
TWpTLCGTPEYLAPEIILSK	Thr197	2271.09	3+	HCD	52 (1: T1)
FPpSHFSSDLK	Ser259	1243.53	3+	ETD	40 (22: S6)
GPGDTSNFDDYEEEEIRVpSINEK	Ser338	2722.13	3+	HCD	45 (25: S6)

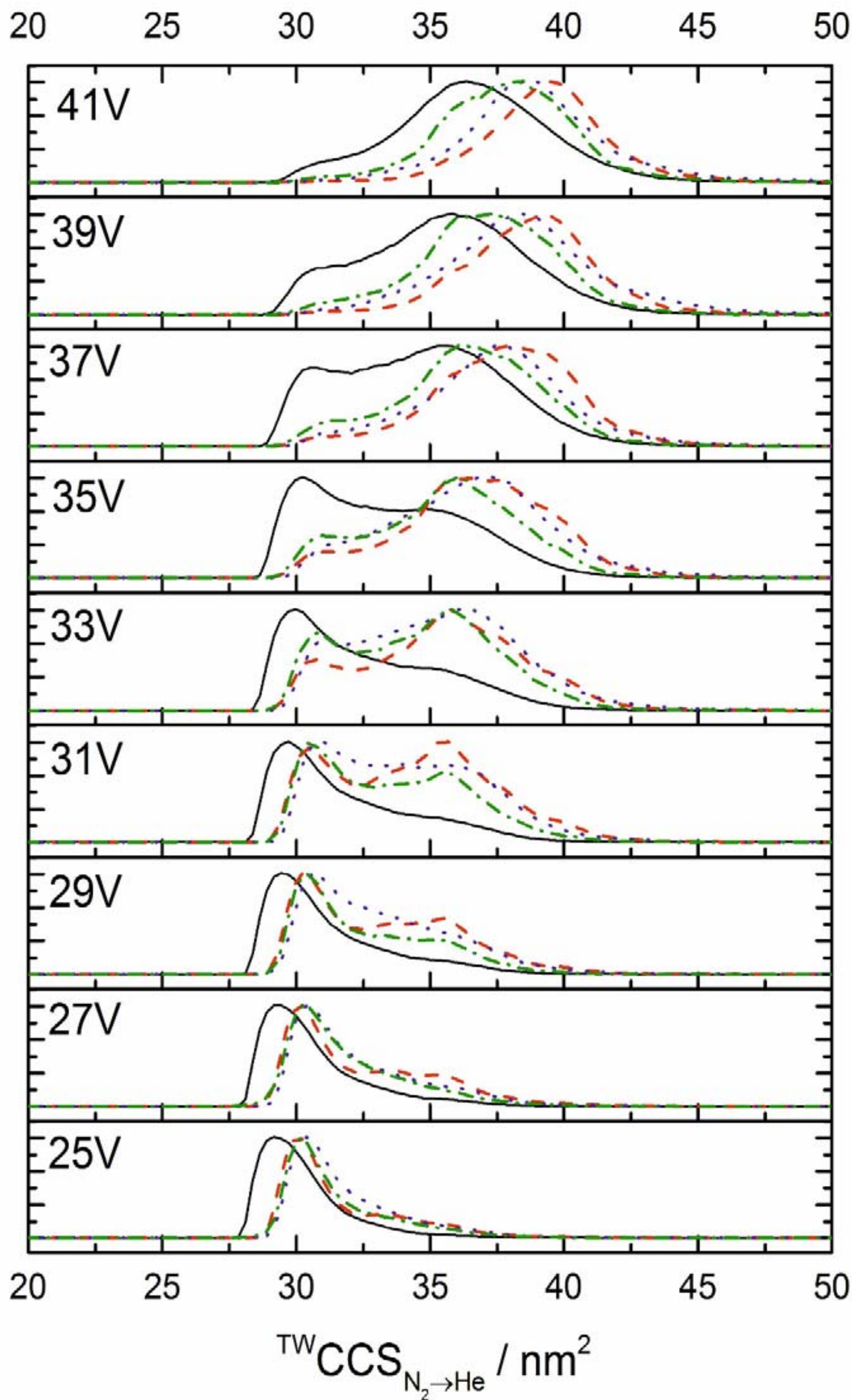
Supplementary Figure 1. Collision dissociation product ion tandem mass spectra of triply charged ion (top) identifying phosphorylation of Thr32 and (bottom) identifying phosphorylation at Thr37. Matched product ions are indicated.



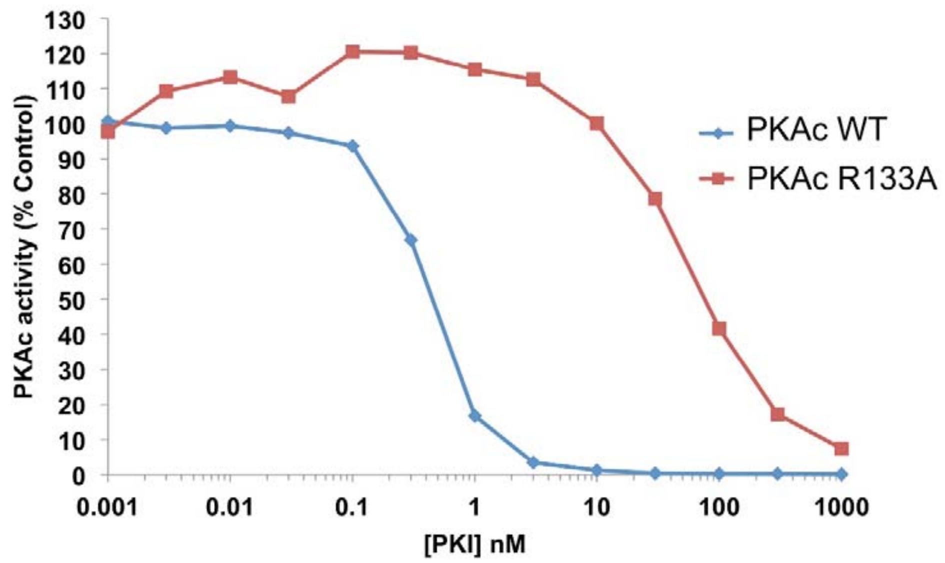
Supplementary Figure 2. ${}^{TW}CCS_{N2\rightarrow He}$ for each of the four charges states observed following ESI-IM-MS of wild-type PKA-Cα under non-denaturing conditions.



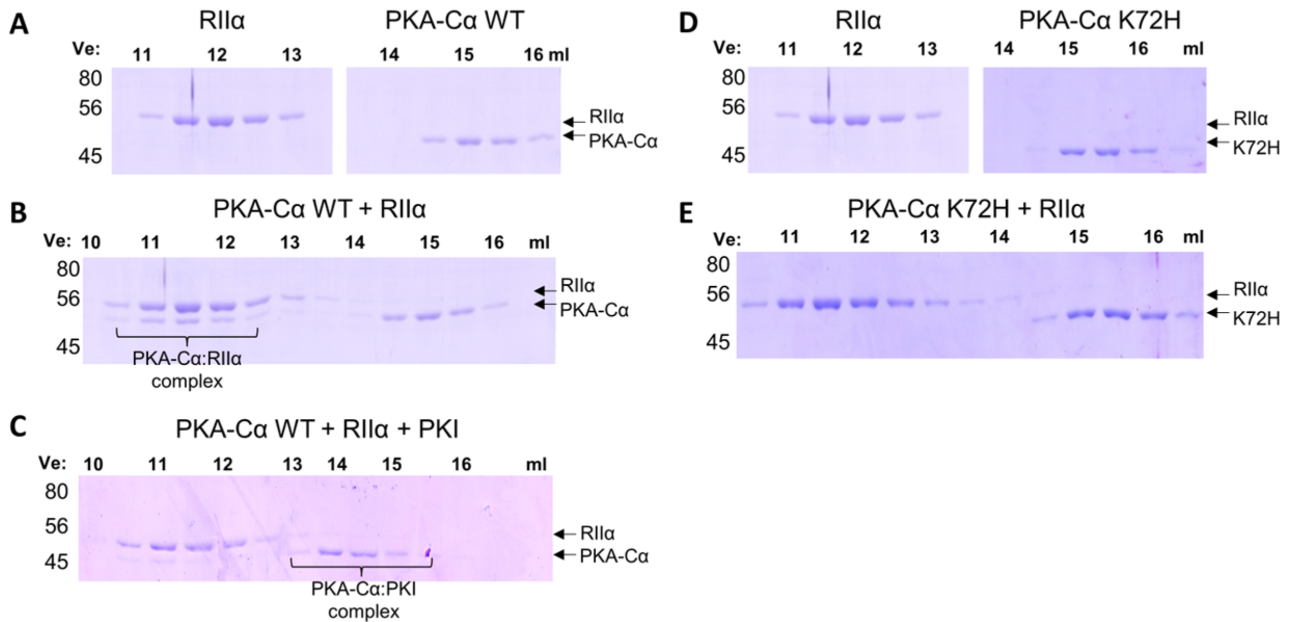
Supplementary Figure 3. Collision-induced unfolding profiles of PKA wild type (black line), λPP treated wild type (red dashed line), R133A (blued dotted line) and K72H (green dashed/dotted line). $^{TW}CCS_{N2\rightarrow He}$ is shown for each step of increased trap collision energy (V).



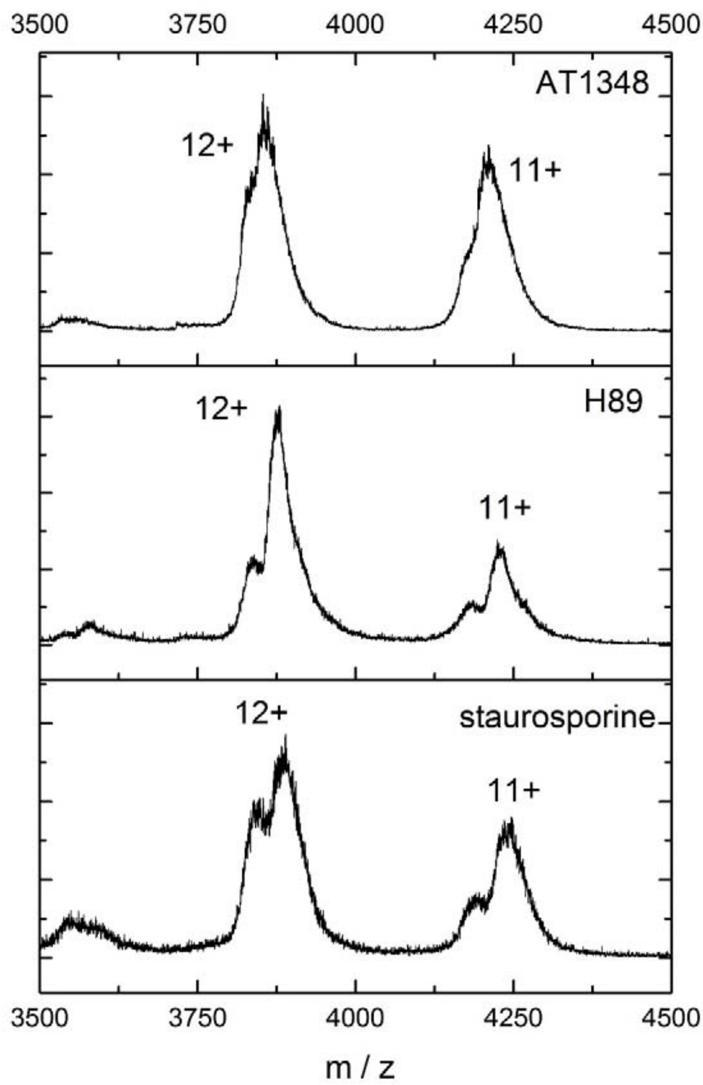
Supplementary Figure 4. Activity of purified WT (blue) or R133A (red) PKAc in the presence of increasing concentrations of PKI as assessed by kinetic assay using fluorescent Kemptide as substrate.



Supplementary Figure 5. Size exclusion chromatography (SEC) investigation of holoenzyme complex formation between catalytic (PKA-Cα) and regulatory (RIIα) PKA subunits. PKA-Cα WT and RIIα were analysed by SEC separately (A) or following pre-incubation at 4°C for 2 h in the absence (B) or presence of PKI (C). Eluted fractions (0.5 ml) were collected and protein detected following SDS-PAGE and Coomassie staining. At the concentrations used, PKI could not be detected by Coomassie and is therefore not shown. PKA-Cα K72H was also analysed (D) and formation of a RIIα: K72H PKA-Cα complex assessed (E). Evidence for co-elution of catalytic and regulatory subunit, which is indicative of holoenzyme complex formation, was only detected for WT PKA-Cα, and was blocked upon inclusion of PKI. Holoenzyme formation was completely abolished for K72H (E).



Supplementary Figure 6. Native ESI-MS demonstrating partial dissociation of non-covalently bound small molecule ligands to WT PKAc arising from elevated collision energy applied in the transfer region of the Synapt G2-Si.



Supplementary Figure 7. Native ESI mass spectra acquired with increasing (bottom to top) transfer collision energy for PKA WT (blue circle) in the presence of staurosporine (yellow star) and PKI (red triangle). Collision energy applied (V) is shown on the left.