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Supplemental information

CaMKII binds both substrates and activators at the active site

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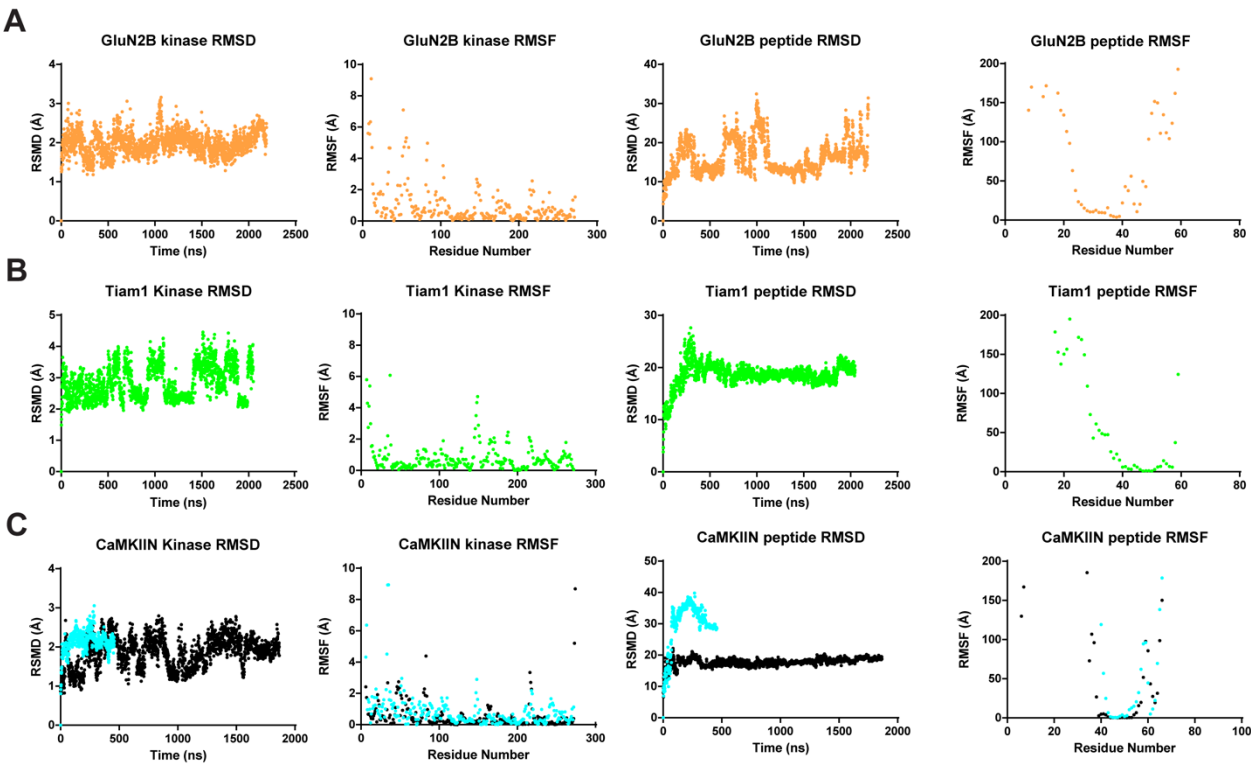

Figure S1. RMSD and RMSF calculations from MD trajectories. Related to Figure 5. RMSD and RMSF were calculated for both the kinase domain and the peptide for each of the trajectories. For CaMKIIN, the two trajectories are overlaid (cyan:0.46 μs and black:1.84 μs). For the kinase domains, RMSD and RMSF values are largely ~3 Å or less, with higher values at the termini, indicating high stability. For the peptides, RMSD values are higher which is not surprising considering this is a largely flexible polypeptide. Peptide RMSF plots are shown up to 200 Å. In the bound regions (residues ~23-40 in GluN2B, ~40-60 in Tiam1 and CaMKIIN) the RMSF values are quite low (<4 Å) indicating high stability. Whereas there is high mobility at the termini.

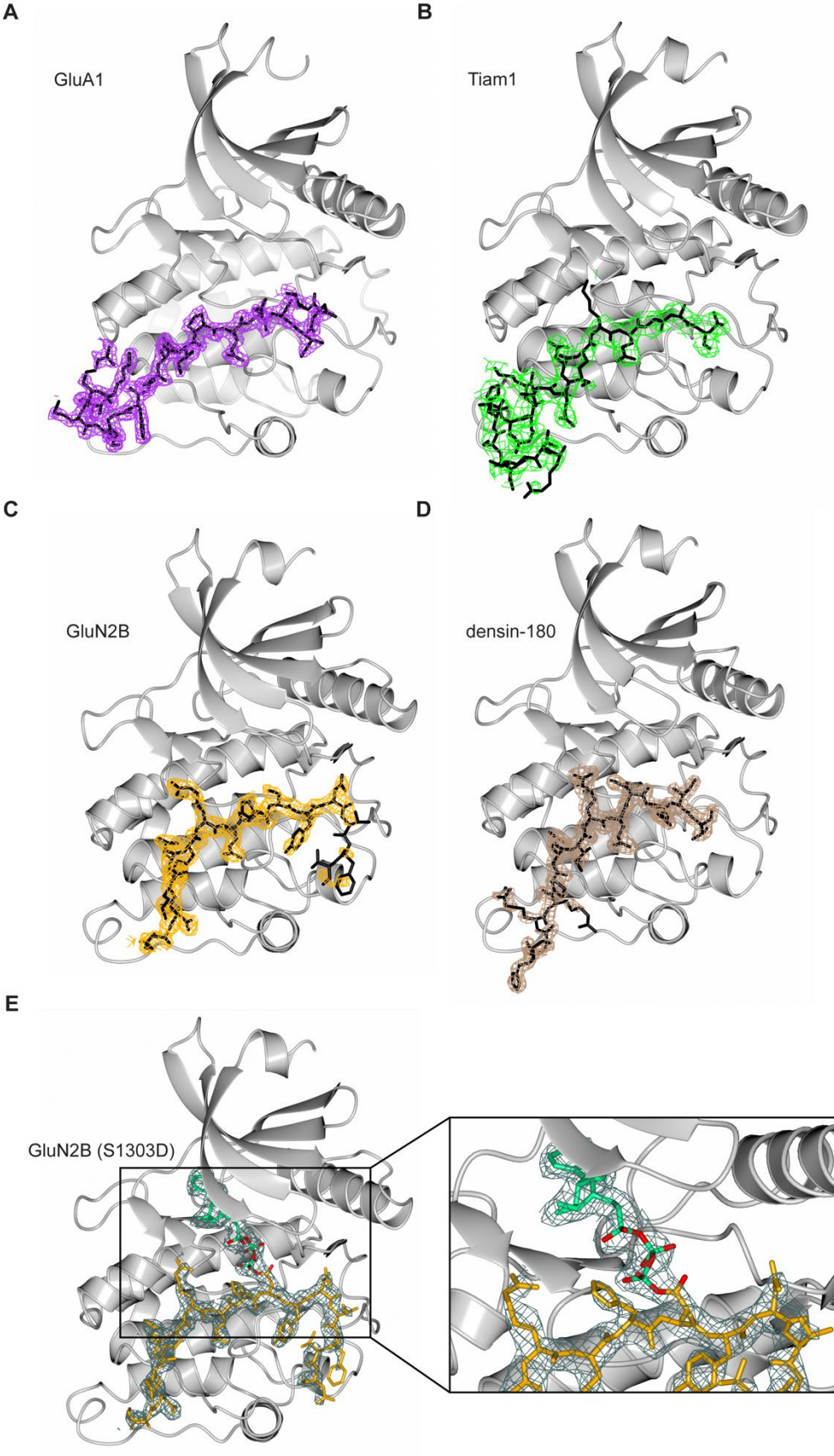
A
GluA1
B
Tiam1
C
GluN2B
D
densin-180
E
GluN2B (S1303D)

Figure S2. Structures of CaMKII kinase domain bound to peptide binding partners. Related to Figure 2. Electron-density omit maps are shown as mesh for all peptide binding partners at σ=1. CaMKII kinase domain shown in light gray (A) GluA1 (PDB:6X5Q) in purple, (B) Tiam1(PDB:7UIQ) in green, (C) GluN2B (PDB:7UJQ) in orange, (D) densin-180 (PDB:6X5G) in brown, and (E) GluN2B (S1303D) in presence of ATP (PDB:7KL1). For (E), the peptide is shown in orange and omit map is shown in dark gray, ATP is colored green. D1303 sidechain is covalently linked to ATP molecule and is shown in red together with the phosphate oxygens.

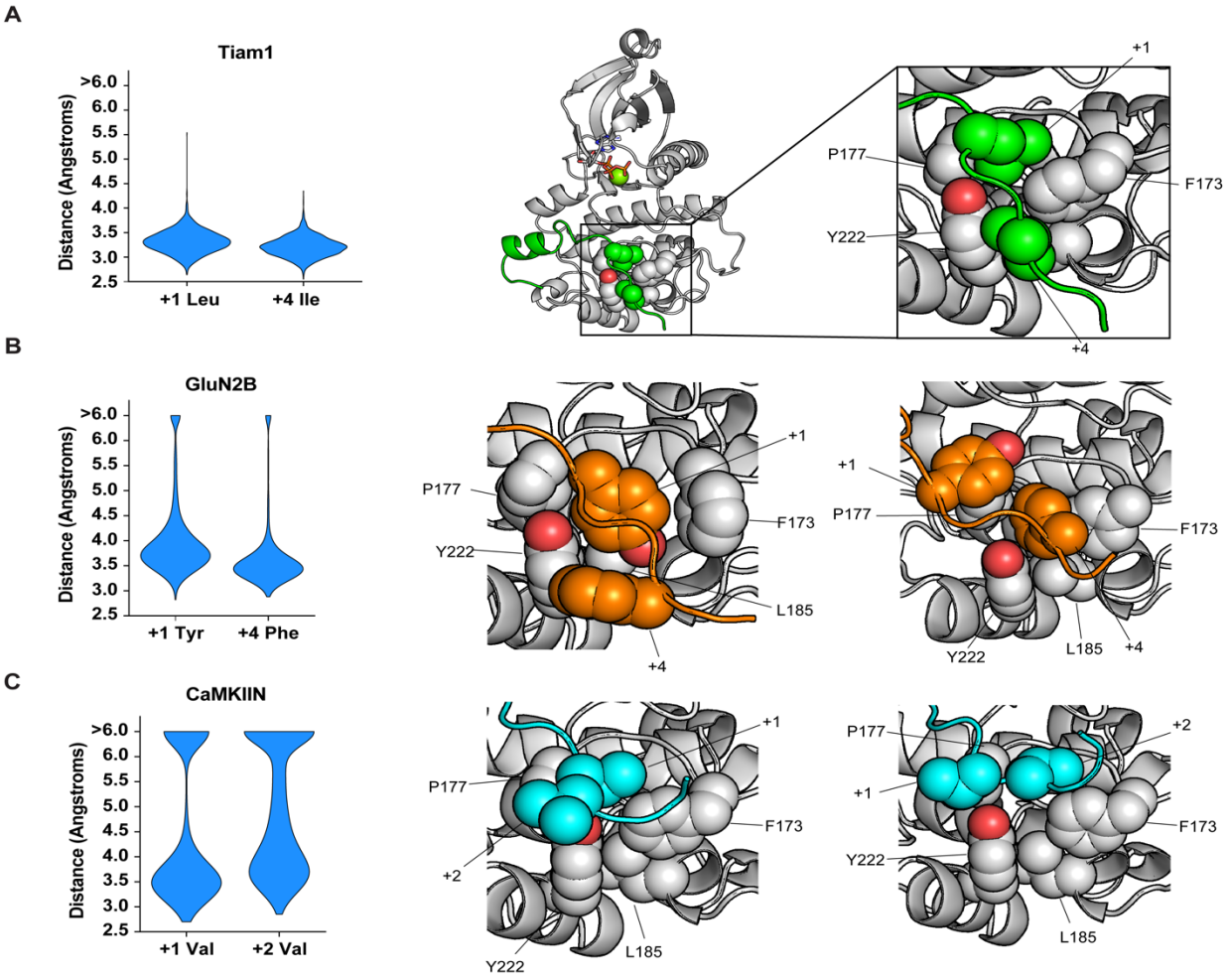

Figure S3. MD simulations highlight a hydrophobic interaction at the C-terminal end of peptides. Related to Figure 3 (A) Persistence of Tiam1 interaction with the F173, P177, L185, Y222 hydrophobic patch in MD simulations. Distance distributions for +1 leucine and +4 isoleucine of Tiam1 to patch (left), representative structure (middle). Zoomed view is labeled for clarity. (B) Persistence of GluN2B interaction with hydrophobic patch. Distance distributions for +1 tyrosine and +4 phenylalanine of GluN2B and two zoomed-in views of the interaction from different orientations. (C) Persistence of CaMKIIN1 interaction with hydrophobic patch from different orientations. Distance distributions for +1 and +2 valine residues of CaMKIIN and two zoomed-in views of the interaction from different orientations.

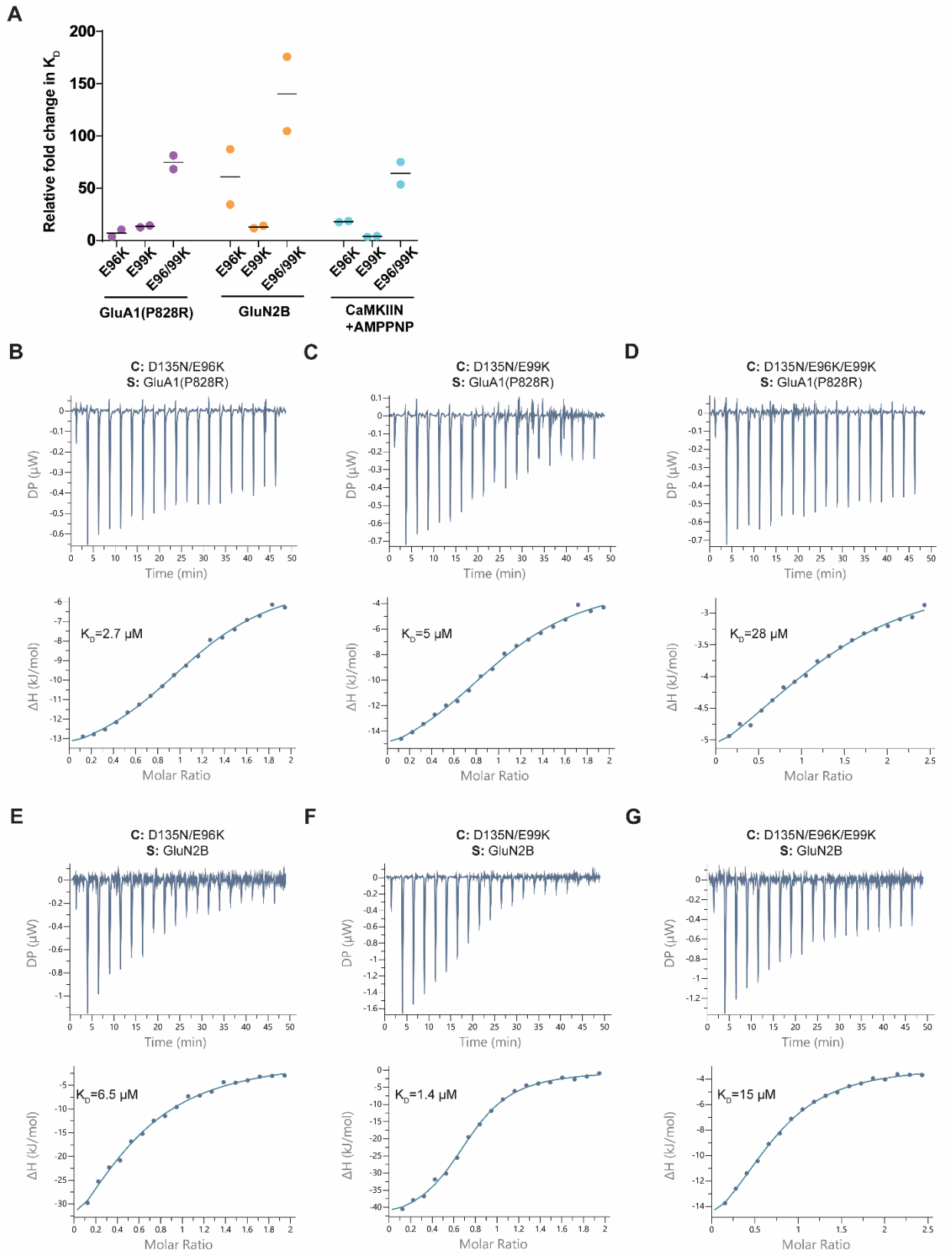

Figure S4. Effect of charge reversal mutations at the -3 position. Related to Figure 4. (A)K_d values were extracted from ITC data and relative fold changes were calculated by dividing the observed K_d from the mutant by the D135N kinase domain. Individual data points are shown, the line indicates the average. ITC measurements of

D135N kinase domain mutants and interaction partners. All measurements were performed in duplicate, here one representative dataset is shown. (B) E96K and GluA1 (P828R), (C) E99K and GluA1 (P828R), (D) E96K/E99K and GluA1 (P828R), (E) E96K and GluN2B, (F) E99K and GluN2B, and (G) E96K/E99K and GluN2B. The mean K_d value from two independent measurements is labeled in the figure.

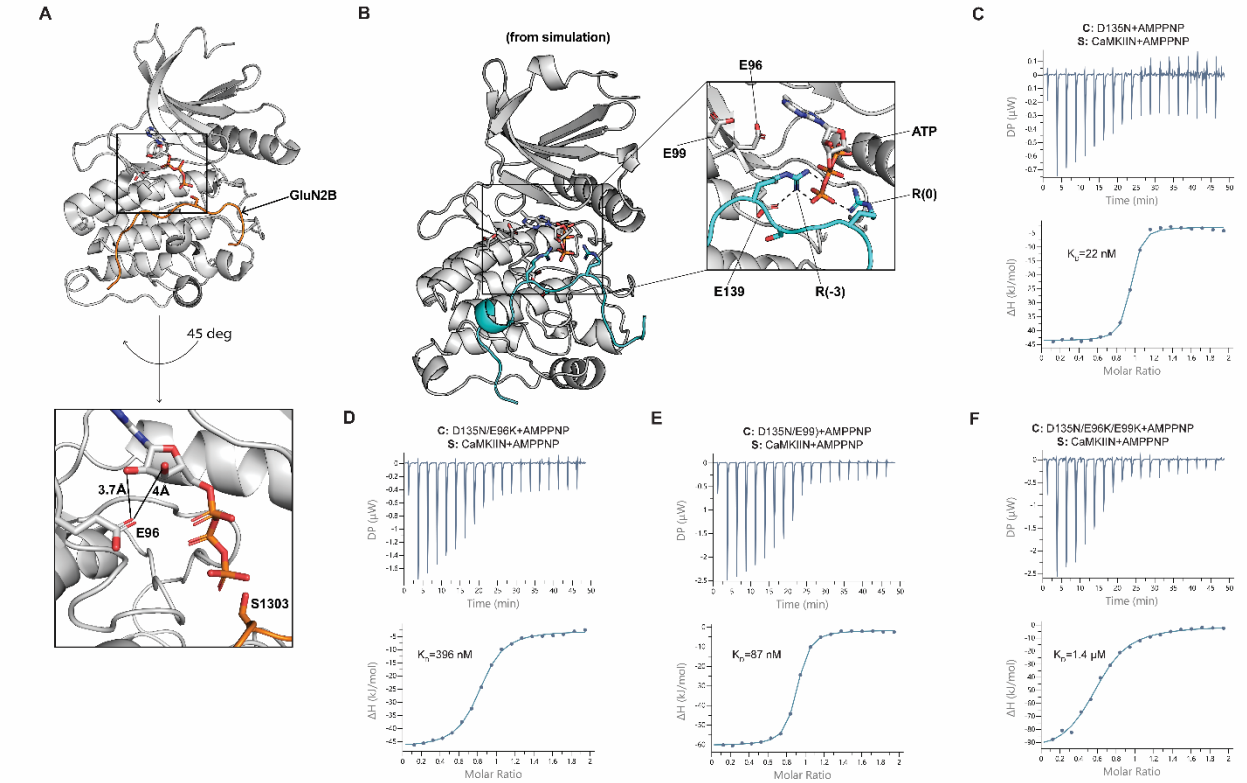

Figure S5. CaMKIIN has unique interactions with ATP. Related to Figure 4. (A) Co-crystal structure of CaMKII kinase domain and GluN2B in complex with ATP (PDB 7UJP). Bottom image highlights the interaction between E96 sidechain and ribose hydroxyl groups of ATP. The gamma phosphate group faces S1303. (B) Snapshot from an MD trajectory with CaMKIIN peptide and ATP bound. The arginine at the -3 position of CaMKIIN mostly interacts with E139, minimally with E96, and not at all with E99. All interactions depicted with dashed lines are below 3 Å. This conformational change precludes the ATP adenosine from forming ionic interactions with the backbones of D90 and V92, as observed in the crystal structure. As a result, the ATP adenosine group exits the binding pocket in the CaMKIIN trajectories. ITC measurements of CaMKIIN in the presence of AMPPNP are shown in C-F. Contents of the cell (C) and syringe (S) used in the ITC measurements are listed in the figure panels. The mean K_d value from two independent measurements is labeled in the figure.

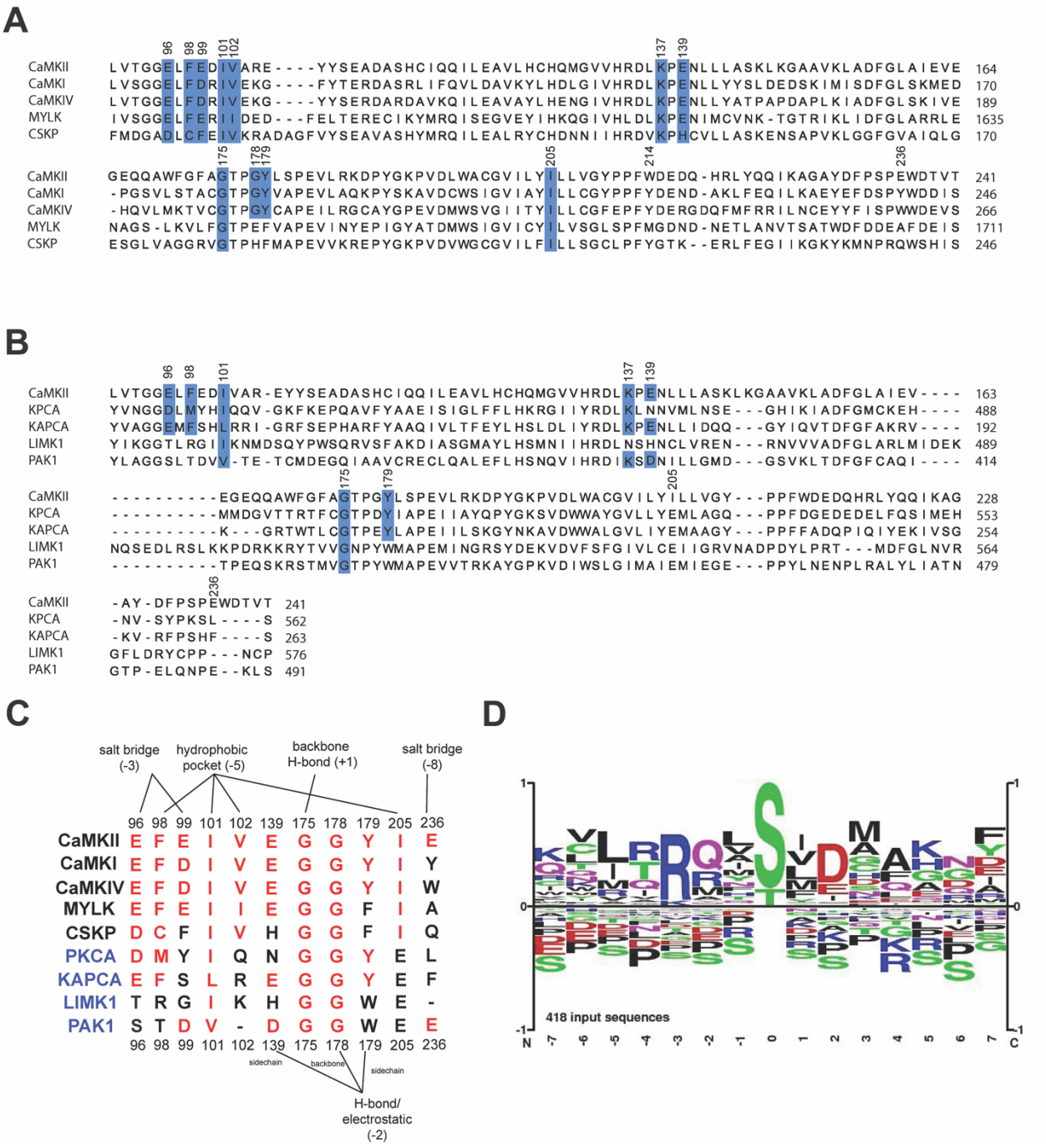

Figure S6. Multi-sequence alignment of kinase domains. Related to Figures 3, 4, 5 and 6.
Alignment of CaMKII from residue 91-241 (according to CaMKIIα numbering) is shown (A) across Ca^{2+}/CaM (CaMK) family and (B) across different kinase families. Conserved residues are highlighted blue. (C) Alignment of key residues involved in binding substrates across kinases from the Ca^{2+}/CaM family (black text) and four kinases from different families (blue text). PKCA and KAPCA (cAMP dependent kinase) belong to the AGC Ser/Thr family. LimK1 and Pak1 belong to TKL and STE Ser/Thr family. (D) The sequence logo for all previously identified substrates of CaMKII from the PhosphoSitePlus database with the phosphorylation site at the 0 position.

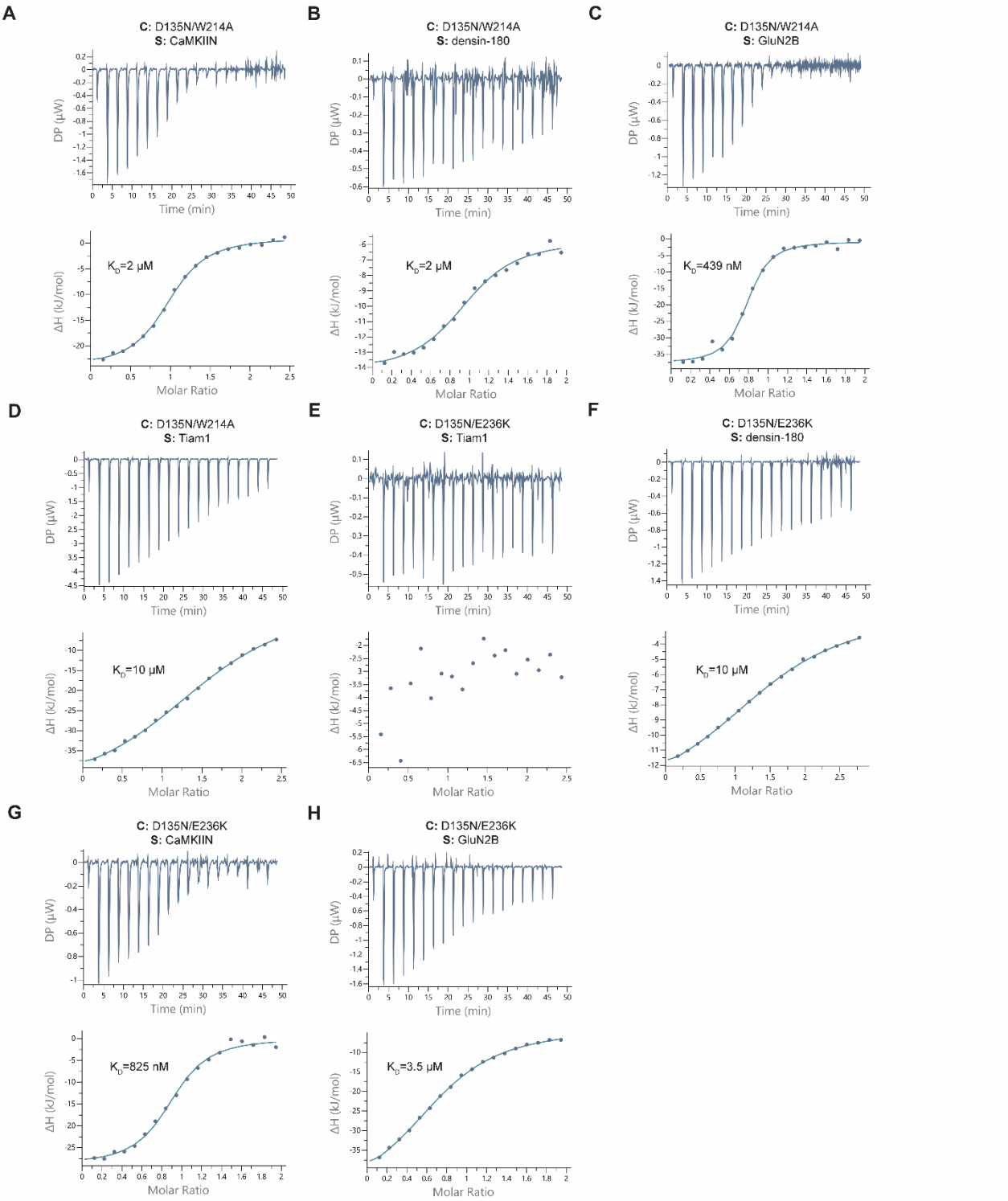

Figure S7. Effect of W214A and E236K mutations on binding affinity. Related to Figures 5 and 6. ITC measurements between D135N kinase domain mutants and interaction partners. Contents of the cell (C) and syringe (S) used in the ITC measurements are listed in the figure panels. The mean K_d value from two independent measurements is labeled in the figure.

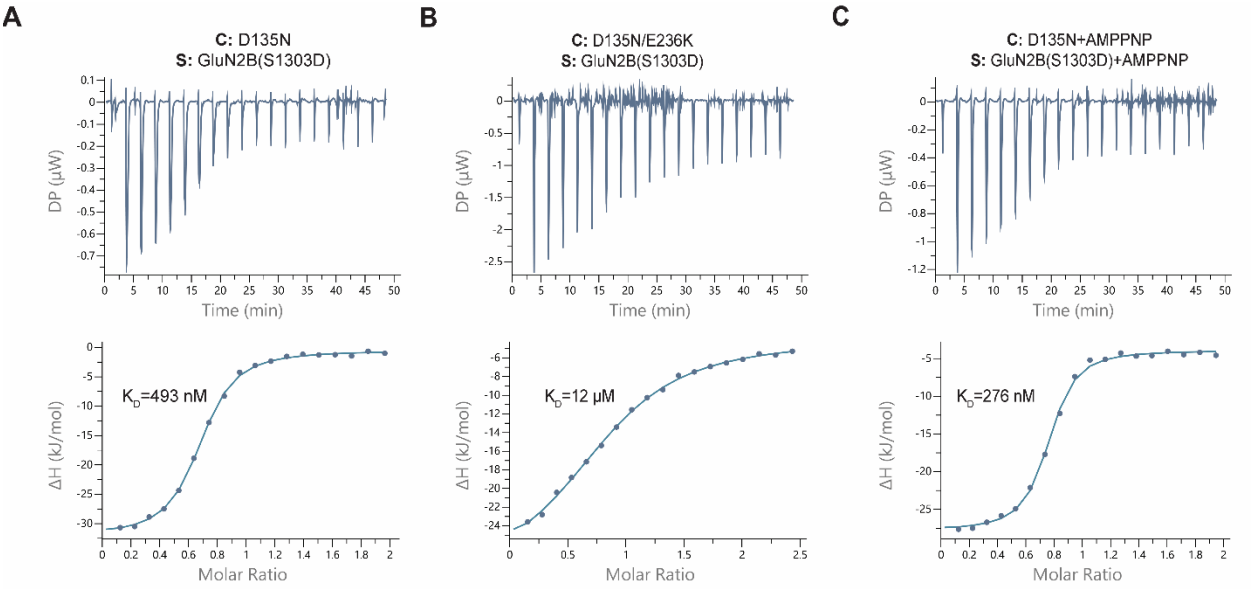

Figure S8. Effect of the phosphomimetic mutation on GluN2B binding affinity. Related to Figures 6 and 7. ITC measurements between D135N kinase domain mutants and interaction partners. Contents of the cell (C) and syringe (S) used in the ITC measurements are listed in the figure panels. The mean K_d value from two independent measurements is labeled in the figure.

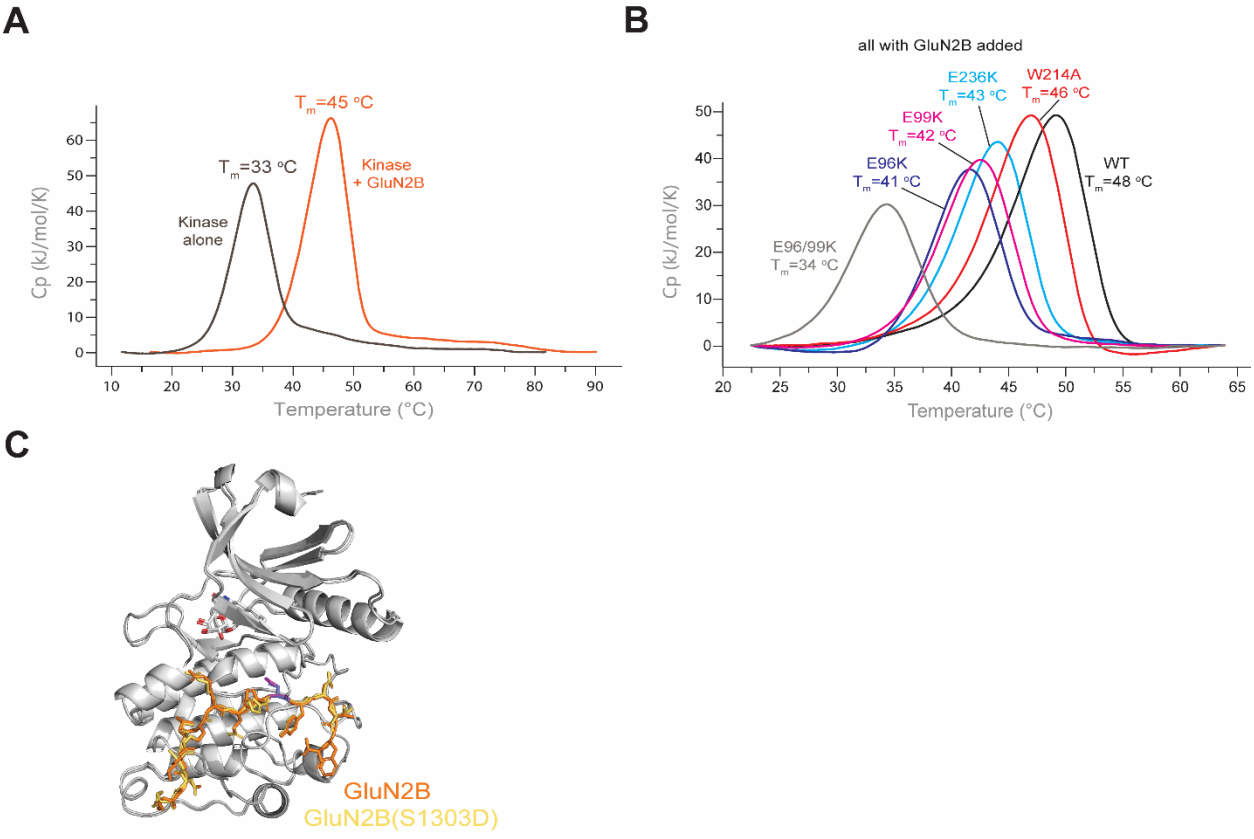

Figure S9. DSC for GluN2B binding and alignment of WT and S1303D GluN2B bound to the kinase domain. Related to Figure 4, 5, 6 and 7.

(A) Differential Scanning Calorimetry data from CaMKII kinase domain alone (brown) and CaMKII kinase domain bound to GluN2B peptide (orange). (B) Differential Scanning Calorimetry data from CaMKII kinase domain mutants bound to GluN2B. (C) Overlay of WT and S1303D GluN2B bound kinase domain structures with hecameg bound (detergent present in crystallization condition) (PDB: 7UJQ and 7KL0). The residue at 1303 is highlighted in blue (serine) and magenta (aspartate).

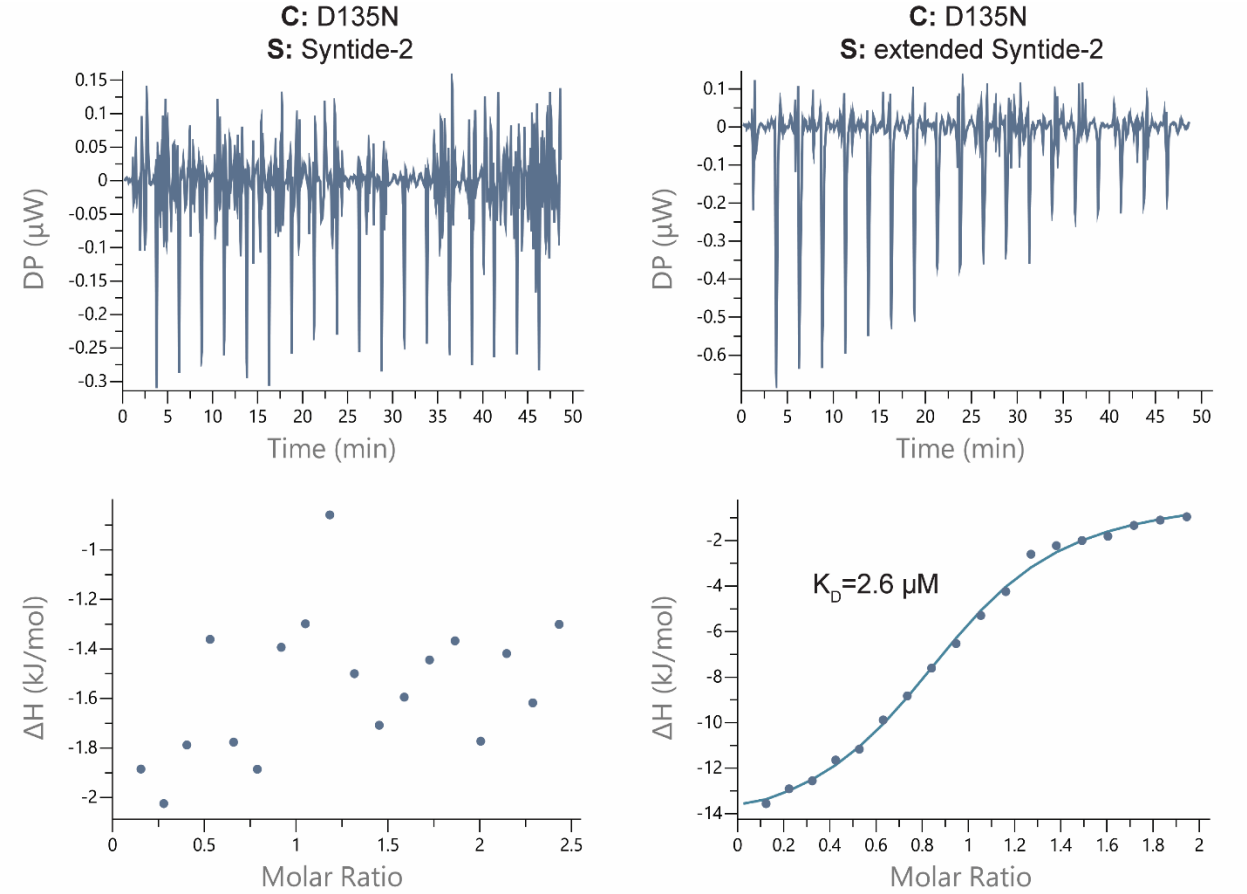

Figure S10. Far salt bridge interaction facilitates low micromolar affinity. Related to Figure 7.
Comparison of Syntide-2 to extended Syntide-2. Contents of the cell (C) and syringe (S) used in the ITC measurements are listed in the figure panels. The mean K_d value from two independent measurements is labeled.

Table S1. GluN2B WT cocrystal structures. Related to Figure 2.

	GluN2B/WT kinase/ADP (7UJS)	GluN2B/WT kinase (7UJR)	GluN2B/D135N kinase/ATP (7UJP)	GluN2B/D135N kinase/Hecameg (7UJQ)
Data collection				
Space group	$P12_11$	$P12_11$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, b, c (Å)	45.01, 65.23, 53.71	45.09, 66, 45	73.14, 91.42, 91.92	73.25, 91.78, 91.77
α, β, γ (°)	90, 95.26, 90	90, 97.61, 90	90, 90, 90	90, 90, 90
Resolution (Å)	50 – 2.75 (2.85 – 2.8)	50 – 1.95 (1.99 – 1.96)	50 – 2.56 (2.60 – 2.56)	50 – 2.25 (2.29 – 2.25)
R_{merge}	0.102 (0.374)	0.13 (0.485)	0.232 (0.812)	0.116 (0.374)
Mean $I/\sigma I$	5 (1.57)	9.5 (2)	5 (1.86)	9.3 (4.36)
Completeness (%)	93.8 (90.9)	93.8 (82.7)	100 (99.8)	96.9 (90.1)
Redundancy	2.6 (2.5)	2.7 (2.5)	6.3 (5.4)	6.3 (5.7)
$CC_{1/2}$	0.985 (0.711)	0.956 (0.678)	0.976 (0.572)	0.99 (0.868)
CC*	0.996 (0.912)	0.989 (0.899)	0.994 (0.853)	0.997 (0.964)
Refinement				
Resolution (Å)	44.86 – 2.75 (2.82 – 2.75)	33.92 – 1.95 (2.00 – 1.95)	48.56 – 2.56 (2.62 – 2.56)	45.93 – 2.25 (2.31 – 2.25)
Unique reflections	7103 (428)	16929 (1027)	19465 (1411)	27648 (1847)
R_{work} / R_{free} (%)	22.28/25.56	20.93/25.16	23.18/28.08	21.99/27.11
No. atoms				
Protein	2253	2234	4479	4507
Water	0	93	45	129
Ligand	27	17	67	52
Ramachadran plot				
In preferred regions (%)	93.57	96.03	96.93	95.85
In allowed regions (%)	5.71	3.97	3.07	3.79
Outliers (%)	0.71	0.0	0.0	0.36
B-factors				
Protein	38.04	30.62	32.74	27.15
Water	0	33.63	18.61	21.49
Ligand	71.96	45.15	48.55	23.01
R.m.s. deviations				
Bond lengths (Å)	0.0033	0.0021	0.0037	0.0028
Bond angles (°)	1.2665	1.11809	1.2648	1.2374

Table S2. GluN2B S1303D cocrystal structures. Related to Figure 2.

	GluN2B(S1303D) /D135N kinase/ATP (7KL1)	GluN2B(S1303D) /D135N kinase/ATP (7UJT)	GluN2B(S1303D) /D135N kinase (PDB 7UIS)	GluN2B(S1303D) /D135N kinase/Hecameg (7KL0)
Data collection				
Space group	$P2_12_12_1$	$P12_11$	$P12_11$	$P2_12_12_1$
Cell dimensions				
a, b, c (Å)	72.89, 92.13, 91.34	47.31, 67.25, 45.72	43.41, 71.43, 45.28	72.99, 91.29, 92.08
α, β, γ (°)	90, 90, 90	90, 94.43, 90	90, 97.51, 90	90, 90, 90
Resolution (Å)	50 – 2.4 (2.44 – 2.4)	50 – 2.1 (2.14 – 2.1)	50 – 2.58 (2.64 – 2.6)	50-2.4 (2.44-2.4)
R_{merge}	0.183 (0.527)	0.098 (0.297)	0.162 (0.678)	0.188 (0.627)
Mean $I/\sigma I$	5.9 (2.65)	9.9 (3.51)	5.8 (1.52)	4.9 (2.04)
Completeness (%)	99.9 (98.8)	99.6 (97.3)	96.9 (92.5)	99.9 (99.5)
Redundancy	5.7 (5)	3.3 (2.8)	3.3 (3.4)	5.6 (4.9)
$CC_{1/2}$	0.976 (0.740)	0.993 (0.824)	0.977 (0.743)	0.984 (0.652)
CC*	0.994 (0.922)	0.998 (0.951)	0.994 (0.658)	0.996 (0.888)
Refinement				
Resolution (Å)	48.5 – 2.4 (2.46 – 2.4)	38.65 – 2.1 (2.15 – 2.1)	24.37 – 2.58 (2.65 – 2.58)	48.52 – 2.4 (2.46 – 2.4)
Unique reflections	23497 (1650)	15852 (1096)	7962 (502)	23396 (1610)
R_{work} / R_{free} (%)	20.60/25.75	20.34/24.65	21.73/ 26.75	20.71/25.84
No. atoms				
Protein	4524	2268	2207	4497
Water	118	96	0	131
Ligand	76	38	0	54
Ramachadran plot				
In preferred regions (%)	97.12	95.71	95.64	96.74
In allowed regions (%)	2.88	3.93	4.36	3.26
Outliers (%)	0.0	0.36	0.0	0.0
B-factors				
Protein	28.11	28.27	43.53	25.95
Water	20.96	28.26	0	20.24
Ligand	32.40	41.33	0	26.08
R.m.s. deviations				
Bond lengths (Å)	0.0074	0.0021	0.0030	0.0073
Bond angles (°)	1.4853	1.1765	1.2691	1.4717

Table S3. Tiam1, Densin-180 and GluA1 cocrystals structures structures. Related to Figure 2.

	Tiam1/D135N kinase/ATP (7UIR)	Tiam1/D135N kinase (7UIQ)	Densin-180/D135N kinase (PDB 6X5G)	GluA1/D135N kinase (6X5Q)
Data collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P12_11$	$P2_12_12_1$
Cell dimensions				
a, b, c (Å)	43.49, 138.84, 154.97	43.43, 137.42, 156.47	36.29, 66.17, 61.71	42.63, 57.46, 107.71
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 99.78, 90	90, 90, 90
Resolution (Å)	50 – 3.1 (3.18 – 3.13)	50 – 3.11 (3.17 – 3.12)	50 - 1.85 (1.88-1.85)	50 – 2.1 (2.18 - 2.14)
R_{merge}	0.287 (0.87)	0.266 (0.67)	0.065 (0.325)	0.179 (0.377)
Mean $I/\sigma I$	3.9 (1.47)	3.9 (1.53)	9.6 (3.14)	3.2 (2.21)
Completeness (%)	98 (96.5)	93.4 (91.9)	93.6 (86.5)	96.9 (92.7)
Redundancy	5.6 (4.6)	4.6 (3.5)	3.4 (3.3)	4.9 (4.4)
$CC_{1/2}$	0.975 (0.618)	0.992 (0.565)	0.99 (0.829)	0.992 (0.82)
CC*	0.994 (0.831)	0.969 (0.831)	0.997 (0.952)	0.998 (0.949)
Refinement				
Resolution (Å)	33.31 – 3.1 (3.183 – 3.103)	31.54 – 3.11 (3.20 – 3.12)	33.11 - 1.85 (1.895-1.85)	39.32 – 2.14 (2.159 – 2.104)
Unique reflections	16512 (1130)	15535 (1042)	22043 (1457)	14475 (798)
R_{work} / $R_{free\ (\%)}$	23.21/27.44	25.85/30.92	18.71/22.65	18.93/23.5
No. atoms				
Protein	4438	4430	2259	2241
Water	0	0	107	96
Ligand	79	0	23	18
Ramachadran plot				
In preferred regions (%)	93.48	91.11	97.48	95.71
In allowed regions (%)	5.62	8.17	2.52	4.29
Outliers (%)	0.91	0.73	0.0	
B-factors				
Protein	45.73	33.76	24.09	17.35
Water	0	0	25.19	14.48
Ligand	62.04	0	53.79	30.61
R.m.s. deviations				
Bond lengths (Å)	0.0052	0.0068	0.0096	0.0080
Bond angles (°)	1.4030	1.5793	1.6299	1.5184