Supplemental Information

Table S1: Diffraction data and crystallographic refinement statistical values

Structure CaMKII kinase domain /dEAGsung/ Mg ²⁺ -AMPP CaMKII kinase domain /dEAG ² sung/ Mg ²⁺ -AMPP CaMKII pinase domain /dEAG ² sung/ Mg ²⁺ -AMPP PDB entry 5FG8 5H9B 5H9B SHU3 Diffraction data		<u> </u>	<u> </u>	
Diffraction data P12₁1 P12₁2₁1 P12₁1 P12₁1 P12₁1 P12₁2₁1 P12₁2₁1 P12₁2₁1 P12₁1 P1	Structure	/dEAG _{long} /	/dEAG _{long} /	domain /dEAG ^P long/
P12 ₁ 1	PDB entry	5FG8	5H9B	5HU3
Unit cell parameters a, b, c (Å), α=γ, β 36.50, 59.22, 70.76, 90.00, 97.23 35.82, 59.13, 70.34, 90.00, 99.02 36.91, 59.22, 70.30, 90.00, 96.68 Wavelength (Å) 0.98403 0.98406 0.97625 Resolution range (Å) 30.19-1.88 (1.95-1.88) 45.03-2.25 (2.33-2.25) 30.08-1.82 (1.88-1.82) No. of unique reflections 23,764 (2,099) 13,806 (1,342) 26,540 (2,412) No. of measured reflections 77,000 (5,111) 47,277 (4,686) 87,981 (5,854) Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) Vol 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Recas (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Record/Rece (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,288 2,271 <tr< td=""><td>Diffraction data</td><td></td><td></td><td></td></tr<>	Diffraction data			
a, b, c (Å), α=γ, β 90.00, 97.23 90.00, 99.02 96.68 Wavelength (Å) 0.98403 0.98406 0.97625 Resolution range (Å) 30.19-1.88 (1.95-1.88) 45.03-2.25 (2.33-2.25) 30.08-1.82 (1.88-1.82) No. of unique reflections 23,764 (2,099) 13,806 (1,342) 26,540 (2,412) No. of measured reflections 77,000 (5,111) 47,277 (4,686) 87,981 (5,854) Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) UGI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Remost (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24.034 Rework/Rirec (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22	Space group	P12 ₁ 1	P12 ₁ 1	P12 ₁ 1
Resolution range (Å) 30.19-1.88 (1.95-1.88) 45.03-2.25 (2.33-2.25) 30.08-1.82 (1.88-1.82) No. of unique reflections 23,764 (2,099) 13,806 (1,342) 26,540 (2,412) No. of measured reflections 77,000 (5,111) 47,277 (4,686) 87,981 (5,854) Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) I/oI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rework/Rirec (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): 76 49 Protein 58 76 49				
No. of unique reflections 23,764 (2,099) 13,806 (1,342) 26,540 (2,412) No. of measured reflections 77,000 (5,111) 47,277 (4,686) 87,981 (5,854) Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) I/σI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Rmess (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data 8 8 1.3780 24.034 Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rword/ Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): 76 49 Solvent	Wavelength (Å)	0.98403	0.98406	0.97625
reflections 77,000 (5,111) 47,277 (4,686) 87,981 (5,854) Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) I/σI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) R _{meas} (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 R _{work} /R _{free} (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39	Resolution range (Å)	30.19-1.88 (1.95-1.88)	45.03-2.25 (2.33-2.25)	30.08-1.82 (1.88-1.82)
reflections Multiplicity 3.2 (2.4) 3.4 (3.5) 3.3 (2.4) Completeness (%) 97.2 (88.0) 99.4 (99.5) 97.9 (91.0) I/σI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Remess (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rwork/Reree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²⁺ 1 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²⁺ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	-	23,764 (2,099)	13,806 (1,342)	26,540 (2,412)
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I/oI 12.4 (1.5) 10.4 (1.7) 19.3 (1.5) Remeas (%) 6.0 (86.2) 8.0 (93.9) 4.1 (73.6) Refinement data Esolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rwork/Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Multiplicity	3.2 (2.4)	3.4 (3.5)	3.3 (2.4)
Refinement data 8.0 (93.9) 4.1 (73.6) Refinement data Sesolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rwork/Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Completeness (%)	97.2 (88.0)	99.4 (99.5)	97.9 (91.0)
Refinement data 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rwork/Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Ι/σΙ	12.4 (1.5)	10.4 (1.7)	19.3 (1.5)
Resolution range (Å) 30.19-1.96 (2.04-1.96) 45.03-2.25 (2.42-2.25) 30.08-1.89 (1.96-1.89) No. of reflections 21,328 13,780 24,034 Rwork/Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	R _{meas} (%)	6.0 (86.2)	8.0 (93.9)	4.1 (73.6)
No. of reflections $21,328$ $13,780$ $24,034$ R_{work}/R_{free} (%) $19.1/22.1$ ($29.9/30.3$) $20.3/22.2$ ($32.2/37.8$) $19.2/22.8$ ($39.7/42.0$) No. of atoms in model: Protein $2,304$ $2,288$ $2,271$ Solvent 69 22 79 Nucleotide 27 27 27 Mg^{2+} 1 1 1 Average B value (Å ²): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg^{2+} 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Refinement data			
Rwork/Rfree (%) 19.1/22.1 (29.9/30.3) 20.3/22.2 (32.2/37.8) 19.2/22.8 (39.7/42.0) No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Resolution range (Å)	30.19-1.96 (2.04-1.96)	45.03-2.25 (2.42-2.25)	30.08-1.89 (1.96-1.89)
No. of atoms in model: Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų):	No. of reflections	21,328	13,780	24,034
Protein 2,304 2,288 2,271 Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų):	R _{work} /R _{free} (%)	19.1/22.1 (29.9/30.3)	20.3/22.2 (32.2/37.8)	19.2/22.8 (39.7/42.0)
Solvent 69 22 79 Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	No. of atoms in model:			
Nucleotide 27 27 27 Mg²+ 1 1 1 Average B value (Ų): Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Protein	2,304	2,288	2,271
Mg²+ 1 1 1 Average B value (Ų):	Solvent	69	22	79
Average B value (Ų): 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg²+ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Nucleotide	27	27	27
Protein 58 76 49 Solvent 44 49 41 Nucleotide 43 69 39 Mg ²⁺ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Mg ²⁺	1	1	1
Solvent 44 49 41 Nucleotide 43 69 39 Mg ²⁺ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Average B value (Å ²):			
Nucleotide 43 69 39 Mg ²⁺ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Protein	58	76	49
Mg ²⁺ 46 72 39 RMSD bond length (Å) 0.010 0.004 0.017	Solvent	44	49	41
RMSD bond length (Å) 0.010 0.004 0.017	Nucleotide	43	69	39
	Mg^{2+}	46	72	39
RMSD bond angle (°) 1.171 0.758 1.347	RMSD bond length (Å)	0.010	0.004	0.017
	RMSD bond angle (°)	1.171	0.758	1.347

RMSD: root-mean-square deviation. High-resolution bin statistics are shown in parentheses.

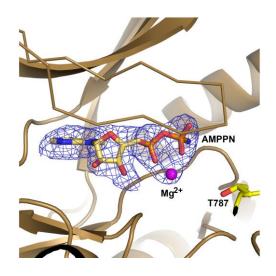


Figure S1: CaMKII kinase domain bound with AMPPN

View of active site of native CaMKII kinase domain (residues 1-283) crystallized in the presence of $dEAG_{long}$ and AMPPNP. Mesh shows omit density map revealing the presence of AMPPN. Kinase shown as dark-wheat cartoon and T787 in channel fragment as yellow stick. AMPPN and Mg^{2+} are indicated.

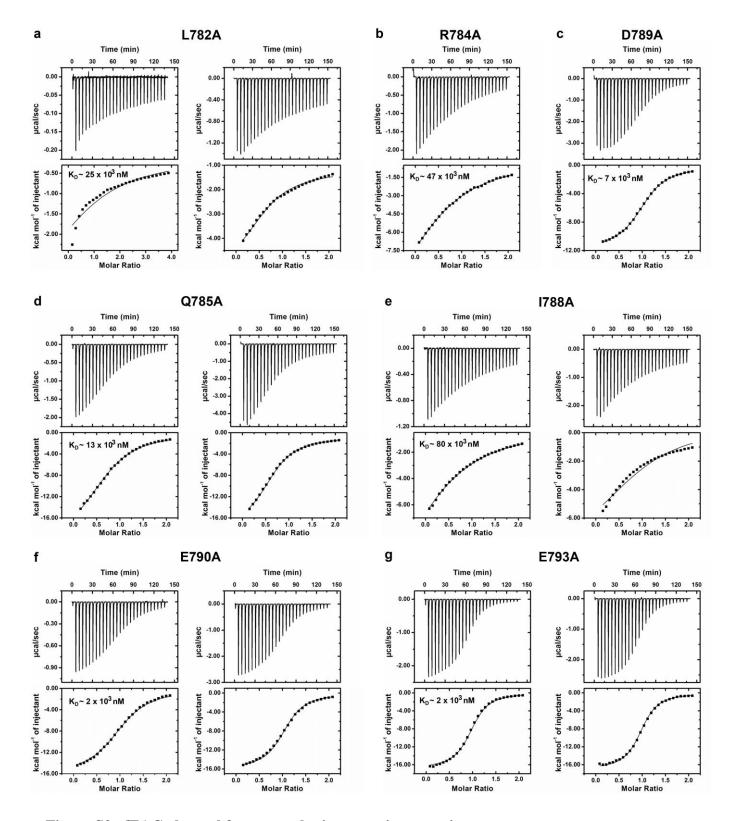


Figure S2: dEAG channel fragment alanine scanning experiments.

ITC experiments of wild type CaMKII kinase domain and mutants of $dEAG_{long}$ channel fragment in the presence of Mg^{2+} -AMPPNP. Mutants as indicated. Titration injection heats are shown in the upper panel and binding isotherm of integrated binding enthalpies in the lower panel. K_D values are shown for each mutant in lower panels. The large decrease in affinity and/or decrease in protein expression levels for some mutants led us to perform just 2 independent calorimetric assays for L782A, Q785A, I788A, E790A and E793A. In these cases we executed assays at two different

concentrations and performed a global fit of the data using the Affinimeter package. Data and fit for both these experiments is shown. For R784A and D789A one of the three replicas performed is shown.

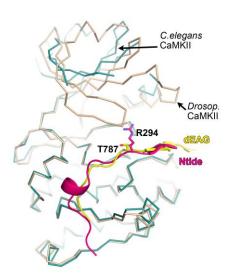


Figure S3: Structural similarity between kinase domain bound to dEAG and bound to the CaMKII inhibitor Ntide.

CaMKII kinase domain/dEAG_{long} complex (in wheat color) with bound Mg²⁺-ADP superposed on CaMKII kinase domain/Ntide complex (shown in cyan; PDB entry: 3KL8) from *C. elegans*. Channel fragment (dEAG) in yellow and Ntide (Ntide) in magenta. Residues T787 in dEAG and R294 in Ntide shown in stick. Superposition matched position of C α -atoms in the C-lobe of the two kinase domains (residues 97-275).

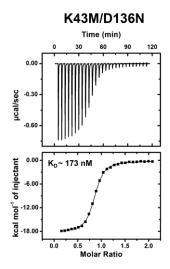


Figure S4: Determination of affinity of channel fragment for CaMKII kinase domain mutant.

ITC experiment of kinase domain K43M/D136N double mutant titrated with $dEAG_{short}$ channel fragment in the presence of Mg^{2+} -AMPPNP. Titration injection heats are shown in the upper panel and binding isotherm of integrated binding enthalpies in the lower panel. K_D value is shown in lower panel.

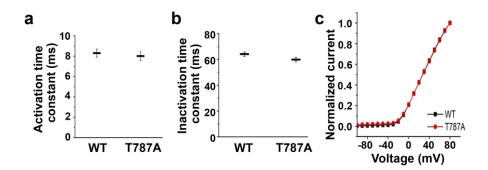


Figure S5: Current characteristics for WT and T787A dEAG channels

Comparison of **a**) activation time constant and **b**) inactivation time constant of WT and T787A dEAG channel current evoked at 60 mV. **c**) Comparison of the WT and T787A dEAG channel current-voltage relationship. Data are mean \pm SEM and n=7-8.

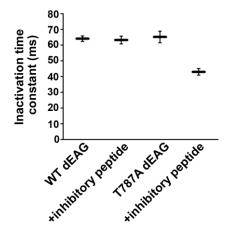


Figure S6: Inactivation time constant for dEAG channel

Inactivation time constant of current at 60 mV mediated by WT dEAG channel alone and exposed to the inhibitory peptide, T787A dEAG channel alone and exposed to the inhibitory peptide. Data are mean \pm SEM and n=7-10.

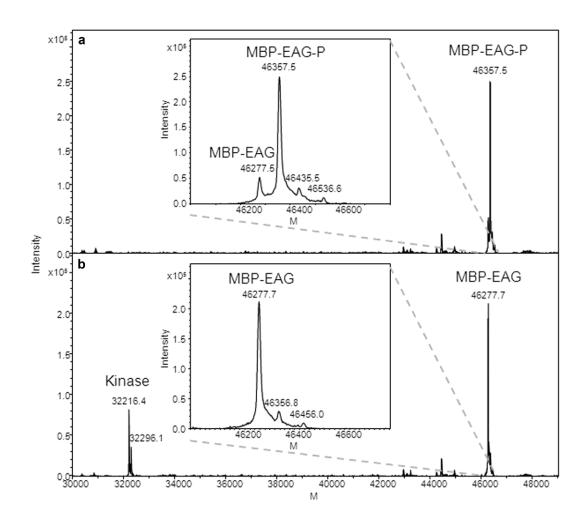


Figure S7: Mass spectrometry spectra of samples before and after ITC experiments showing dephosphorylation.

Deconvoluted UHR-QTOF mass spectra of phosphorylated MBP-dEAG_{short} before **a**) and after **b**) ITC experiment in the presence of wild type CaMKII kinase domain and Mg²⁺-AMPCP.

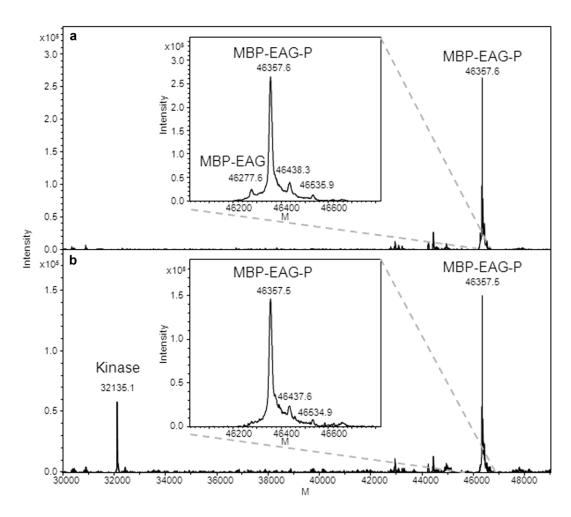


Figure S8: Mass spectrometry spectra of samples before and after ITC experiments showing no change in phosphorylation.

Deconvoluted UHR-QTOF mass spectra of phosphorylated MBP-dEAG before **a**) and after **b**) ITC experiment in the presence of D136N CaMKII kinase domain and Mg²⁺-ADP.