Supplementary Materials for

Structure of the voltage-gated K^+ channel Eag1 reveals an alternative voltage sensing mechanism

Authors: Jonathan R. Whicher[1], Roderick MacKinnon[1]*

Affiliations:

[1]Laboratory of Molecular Neurobiology and Biophysics, The Rockefeller University, Howard Hughes Medical Institute, 1230 York Avenue, New York, New York 10065, USA

*Correspondence to: mackinn@rockefeller.edu

Materials and Methods

Cloning of truncated rEag1 (rEag1Δ) and Calmodulin (CaM)

DNA encoding rat Eag1 (rEag1) was synthesized by Genewiz Inc. To improve the biochemical stability of rEag1, residues 773-886 from the C-terminus were removed with overlap PCR. First, N-terminal (residues 1-772) and C-terminal (residues 887-962) fragments with complementary ends were amplified with these primers (bold indicates complementary overhangs and italics indicates restriction sites used for cloning): rEag11-772_For – *TCTCGAGCCACC*ATGGCTACT, rEag11-772_Rev - **CTGCTCTGGAATAGGGTAGAA**GGAGACAGGAGTGGCTGG, rEag1887-962_For – **CCAGCCACTCCTGTCTCC**TTCTACCCTATTCCAGAGCAG, and rEag1887-962_Rev – *TTAAGAATTCGA*GGAAGCACCGAAGAT. Then the fragments were combined with primers rEag11-772_For and rEag1887-962_Rev to amplify the entire truncated construct. EcoRI and XhoI sites were used to clone rEag1Δ construct into a BacMam expression vector (*43*) with a C-terminal green fluorescent protein (GFP)-His_6 tag.

DNA encoding vertebrate CaM was cloned into the BacMam expression vector with a C-terminal stop codon directly after the coding sequence, which allowed for expression of CaM without a tag.

Electrophysiology of rEag1Δ

Chinese hamster ovary (CHO) cells, cultured in DMEM-F12 (Gibco) with 10% FBS, were transfected with the rEag1Δ expression plasmid with the FuGENE HD transfection reagent (Promega). 24hrs following transfection, the media was replaced with bath solution and experiments were performed at room temperature using either whole cell or inside-out patch clamp techniques with polished borosilicate glass pipettes with resistance between 2-4 MΩ. All recordings were measured with pClamp10.5 software (Molecular Devices), an Axopatch 200B amplifier (Molecular Devices), and an Axon digidata 1550 digitizer (Molecular Devices). Recordings were filtered at 1kHz and sampled at 10 kHz.

To determine the $V_{0.5}$ value for rEag1Δ, whole cell recordings were measured by holding the cells at -80mV, stepping to depolarized voltages up to 80mV in 10 mV steps, and then stepping back to -80mV. Normalized tail current vs. voltage was plotted for 4 separate recordings and fit with a Boltzmann function. The bath solution was 10mM HEPES pH 7.4, 60mM KCl, 95mM NaCl, 1mM $CaCl_2$ and the pipette solution was 10mM HEPES pH 7.4, 165mM KCl, 5mM EDTA.

To investigate the effect of holding potential on activation time for rEag1Δ, whole cell recordings were measured by holding cells for 500ms at increasing holding potentials from -150mV to -50mV in 20mV steps followed by a step to 40mV. The bath solution was 10mM HEPES pH 7.4, 15mM KCl, 140mM NaCl, 1mM $CaCl_2$ and the pipette solution was 10mM HEPES pH 7.4, 165mM KCl, 5mM EDTA.

To investigate CaM inhibition of rEag1Δ, the inside-out patch configuration was used. The bath and pipette solution was 10mM HEPES pH 7.4, 15mM KCl, 140mM NaCl, 5mM EDTA, 4.94mM $CaCl_2$ (10μM free Ca^{2+}). Patches were pulled in the presence of 10μM free Ca^{2+}, which promotes binding of endogenous CaM to rEag1 (*18*), then a 10mM HEPES pH 7.4, 165mM KCl, 5mM EDTA, 4.94mM $CaCl_2$ (10μM free Ca^{2+}) solution was locally perfused and current was measured by holding at -80mV and stepping to 80mV in 20 mV steps. Then a 10mM HEPES pH 7.4, 165mM KCl, 5mM EDTA solution was locally perfused to remove free Ca^{2+} and recordings were measured with the same protocol.

Expression and purification of rEag1Δ bound to CaM

Bacmids were generated for rEag1Δ and CaM with DH10Bac *E. coli* cells. SF9 cells in Grace's media supplemented with 10% FBS were transfected with bacmid DNA using the cellfectin II reagent (Invitrogen) to produce baculovirus. Then the baculovirus was amplified in 1L suspension cultures of SF9 cells at 27ºC.

1L cultures of HEK293S GnTI$^-$ at 3x10^6 cells/mL in Freestyle 293 media (Gibco) supplemented with 2% FBS were infected with both rEag1 and CaM amplified virus at a 4:1 rEag1:CaM ratio. Infected cells were incubated at 37ºC for 18hrs and expression was induced by adding 10μM sodium butyrate. After addition of sodium butyrate, the cells were incubated at 30ºC for 48hrs then harvested (*43*).

4L of cell pellet was resuspended in lysis buffer (20mM Tris pH 8, 5mM $CaCl_2$, 1μg/ml leupepetin, 1μg/ml pepstatin, 1mM benzamidine, 1μg/ml aprotonin, 0.01mg/ml DNase, 1mM PMSF), incubated at RT with stirring for 20min, and centrifuged for 40min at 35,000xg. Pellets were resuspended in extraction buffer (50mM Tris pH 8, 300mM KCl, 5mM $CaCl_2$, 2% n-Dodecyl-β-D-Maltopyranoside (DDM), 0.4% Cholesteryl hemisuccinate (CHS), 0.1mg/ml 3:1:1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC):1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE):1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1μg/ml leupepetin, 1μg/ml pepstatin, 1mM benzamidine, 1μg/ml aprotonin, 0.01mg/ml DNase, 1mM PMSF), incubated at 4ºC for 2hrs with stirring, and centrifuged for 90min at 35,000xg. The supernatant was added to CNBR-activated sepharose beads (GE healthcare) coupled to a nanobody with high affinity for GFP (GFP-NB) (*44*) and incubated for 3hrs at 4ºC. The beads were washed with wash buffer (20mM Tris pH 8, 300mM KCl, 5mM $CaCl_2$, 0.2% DDM, 0.04% CHS, 0.1mg/ml 3:1:1 POPC:POPE:POPS) first with and then without 10mM $MgCl_2$ and 5mM adenosine triphosphate (ATP) to remove bound heat shock proteins. The washed beads were incubated overnight at 4ºC with PreScission protease (10:1 w/w ration) to remove the GFP tag from rEag1Δ. The protein was eluted with wash buffer, concentrated, and

purified on a Superose 6 column (GE healthcare) equilibrated with 20mM Tris pH 8, 300mM KCl, 5mM $CaCl_2$, 5mM Dithiothreitol (DTT) 0.05% DDM, 0.01% CHS, 0.05mg/ml 3:1:1 POPC:POPE:POPS. Peak fractions of rEag1Δ bound to CaM (Figure S1) were pooled and concentrated to 4mg/ml for single particle cryo-EM structure determination.

EM sample preparation and imaging

Cryo grids were prepared with a Vitrobot Mark IV (FEI). Quantifoil R1.2/1.3 holey carbon grids (Quantifoil) were glow-discharged for 10s. Then 3.5µl of 4mg/ml rEag1Δ bound to CaM was pipetted onto the grids, which were blotted for 4s at 86% humidity and frozen in liquid nitrogen cooled liquid ethane. The grids were loaded onto a 300keV Titan Krios (FEI) with a Gatan K2 Summit direct electron detector (Gatan). Images were recorded with Serial EM (*45*) in super-resolution counting mode with a super resolution pixel size of 0.65Å and a defocus range of 1.5 to 3.0µm. Data were collected with a dose of 10 electrons per physical pixel per second (pixel size of 1.3Å at the specimen) and images were recorded with a 15s exposure and 300ms subframes (50 total frames) to give a total dose of 90 electrons per $Å^2$ (1.8 electrons per $Å^2$ per subframe).

Image processing and map generation

Dose fractionated subframes were 2 x 2 binned (giving a pixel size of 1.3 Å), aligned, and summed using Unblurr (*46*). The contrast transfer function was estimated for each summed image using CTFFIND4 (*47*). From the summed images, approximately 5,000 particles were manually picked and subjected to 2D classification in RELION (*48*)

specifying 20 classes. The three projection averages of the most populated classes were used as templates for automated picking in RELION. The automatically selected particles were manually inspected to remove false positives, resulting in a data set of 240,000 particles. The 240,000 particle images were subjected to 2D classification in RELION specifying 150 classes and the lowest populated classes were removed resulting in a data set of 215,000 particles. The best classes were used to generate an initial model with C4 symmetry using EMAN2 (*49*), which was subsequently used as a starting model for the 3D classification in RELION. The 215,000 particles were classified into 6 classes and each class had a similar number of particles and resolution. As a result, all 215,000 particles were combined for 3D refinement in RELION producing a map at 3.96Å resolution estimated by gold standard FSC at the 0.143 cutoff criteria. The refined particles were subjected to further rounds of 3D classification without image alignment, which produced a subset of 145,000 particles that generated a map at 3.86Å resolution estimated by gold standard FSC at the 0.143 cutoff criteria. The 145,000 particle data set was further refined in FREALIGN (*50*) with a resolution limit of 6.0Å to prevent overfitting of high-resolution terms. The gold standard FSC, calculated by comparing two independently determined half-maps from FREALIGN, estimated a nominal resolution of 3.78Å at the 0.143 cutoff criteria (Figure S1) (*51*). This map was used for model building and refinement (Figure S2, S3, S4).

To improve the portion of the map corresponding to CaM, the 145,000 particle data set was subjected to 3D classification in FREALIGN with a resolution limit of 8.0Å. Of the 5 resulting classes, 1 of the classes, corresponding to 42,000 particles, displayed

improved CaM density. This subset of particles was refined in FREALIGN with a 6.0Å resolution limit, producing a map with a nominal resolution of 3.92Å estimated by gold standard FSC at the 0.143 cutoff criteria. This map, which showed the linker between the CaM N- and C-lobes (Figure S9), was used to build the CaM region of the structure.

Model building

The model for rEag1 bound to CaM was built in Coot (*52*). For model building, BFACTOR.EXE (written by Nikolaus Grigorieff) was used to sharpen the maps from FREALIGN to different b-factor values. For figure generation and deposition, the EM density map amplitudes were scaled using a map calculated from the refined atomic model of rEag1 in DIFFMAP.EXE (written by Nikolaus Grigorieff) and low-pass filtered to the nominal resolution indicated above or to the resolution explicitly stated in the figure legend.

For the PAS and CNBHD domains, the crystal structure of the PAS/CNBHD complex from mouse Eag1 (PDB ID 4LL0) (*16*) was fit into the map and regions that did not agree with the map were rebuilt. Regions of the PAS and CNBHD not observed in the crystal structure, which included residues 10-15, 136-205, 693-696, and 704-722, were built de-novo (Figure S7). The S1-S6 segments (residues 206-484) and the C-linker (residues 485-521) were built de-novo. For CaM, the crystal structure of vertebrate CaM/Ca^{2+} in an extended conformation (PDB ID 1CLL) (*53*) was split into N-lobe (residues 5-75) and C-lobe (residues 81-147) fragments and individually fit into the map as a rigid body. The linker between the N- and C-lobes (residues 76-81) was built using the map with

improved CaM density. The model is mostly complete except for residues 1-9, 407-411, 697-703, and 723-849 (unstructured C-terminus) of rEag1 and residues 1-5 and 147 of CaM. The majority of the side chains were removed from lower resolution regions, including residues 136-205 (C-terminus of the PAS domain), 305-325 (S3b and the top of S4), 704-722 (C-terminus of CNBHD), and the CaM.

Model refinement and validation

Real and reciprocal space refinement of the model in *p*4 crystallographic symmetry with one monomer per "asymmetric unit" was conducted using phases and amplitudes extracted from one of the independently calculated half maps generated by FREALIGN. At the end of refinement, Fourier shell correlations (FSC) were calculated between the refined model and the half map used for refinement (work), the other half map (free), and the full map to assess over fitting (Figure S4).

To minimize computation during refinement of the model, both the model and the half map were translated into a box that extended 5Å away from the model and map in each direction (*54*). Then the model was refined against the working half map in real space using PHENIX real space refine (*55*) with secondary structure restraints. Following real space refinement, the working half map was solvent flattened by creating a 3Å mask around the model and setting the regions of the map outside of the mask to 0 (*54*). Structure factors were calculated for the solvent flattened working half map (*54*) and the model was refined against the working half map in reciprocal space using Refmac (*56, 57*) (Figure S4). ProSMART was used to generate secondary structure restraints used

during reciprocal space refinement (*58*). Figures were generated with Chimera (*59*), Pymol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.), HOLE (*60*), APBS (*61, 62*), and the Bsoft package (*63*) and structure calculations were performed with the SBgrid suite of programs (*64*).

Figs. S1-S9

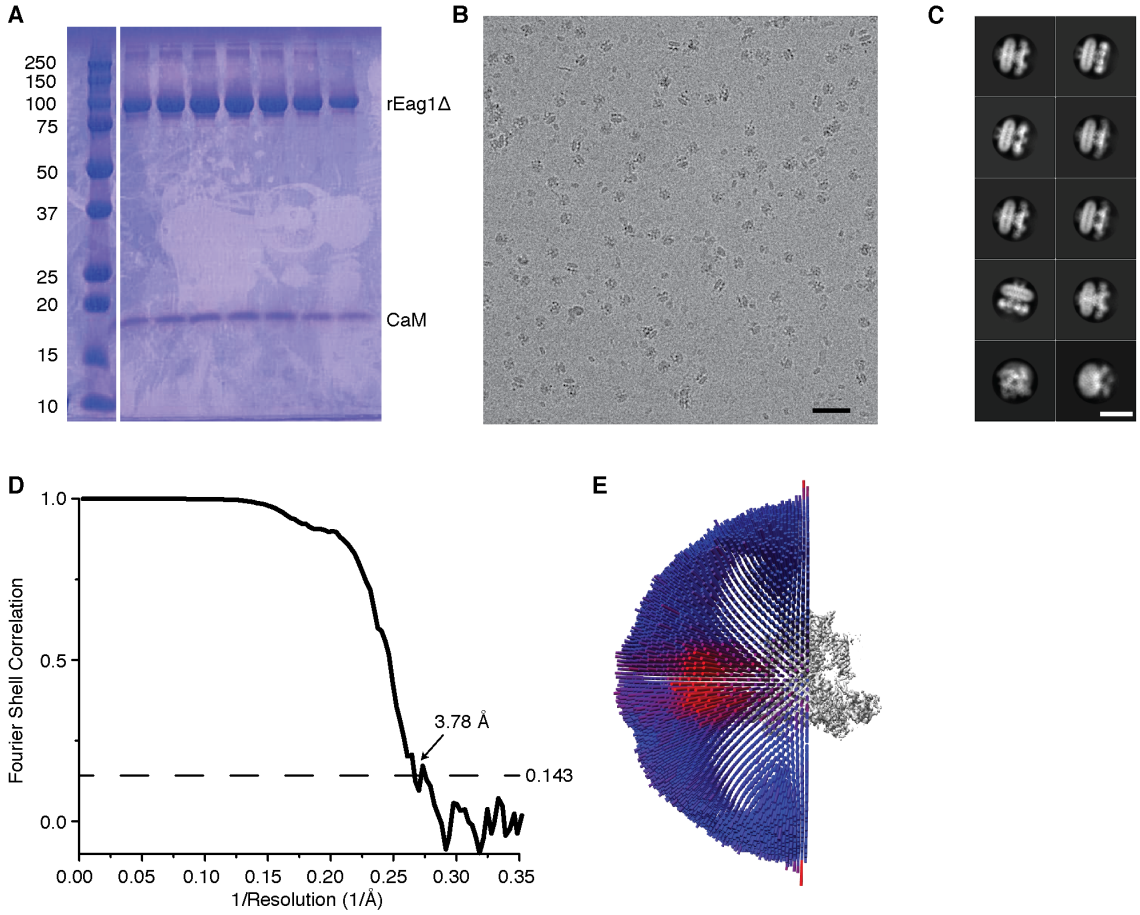


Fig. S1. Single-particle cryo-EM structure determination of rEag1Δ bound to CaM. (**A**) SDS-PAGE gel of rEag1Δ fractions from the final gel filtration column (molecular weight standards are in kDa). rEag1Δ and CaM co-elute demonstrating a strong binding interaction. (**B**) Representative micrograph of rEag1Δ bound to CaM in vitreous ice (scale bar = 500 Å). (**C**) 10-highest populated classes from 2D classification (scale bar = 100 Å). (**D**) Gold standard FSC of rEag1Δ bound to CaM channel. The gold standard FSC was calculated by comparing the two independently determined half-maps from FREALIGN. The dotted line indicates the 0.143 FSC cutoff which corresponds to a nominal resolution of 3.78 Å. (**E**) Orientation distribution of particles in the final reconstruction. Each column indicates one view and the number of particles in each view is indicated by the size and color of the column (larger columns colored red contain a higher number of particles).

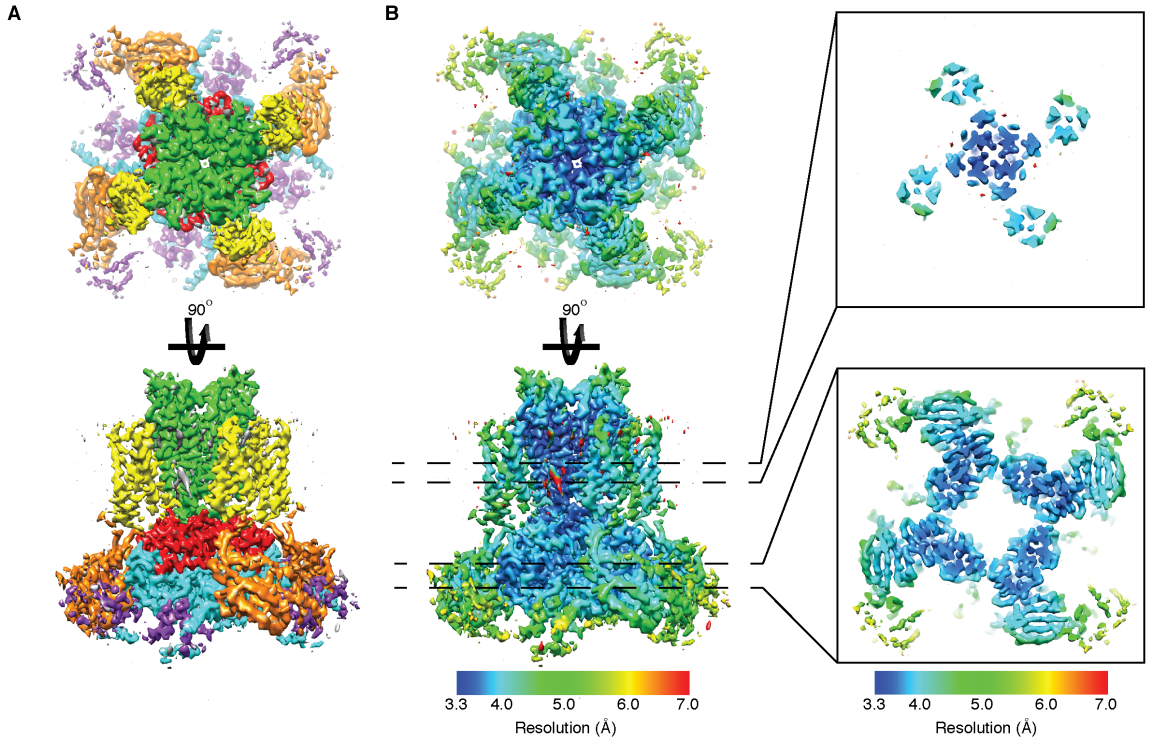
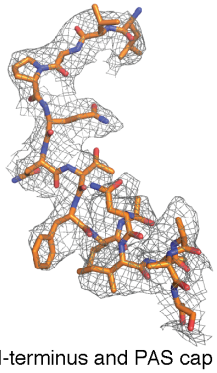
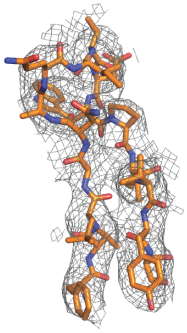


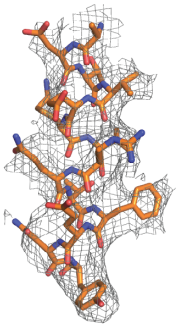
Fig. S2. Cryo-EM density map of rEag1Δ bound to CaM. Cryo-EM maps filtered at 3.78 Å are colored based on the corresponding rEag1 domains (**A**) (PAS is orange, VS is yellow, S5-S6 is green, C-linker is red, CNBHD is cyan, and CaM is purple) or local resolution (**B**). Slices of the transmembrane domain (top panel) and intracellular domains (bottom panel) colored by local resolution are shown on the right.



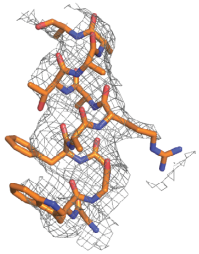
N-terminus and PAS cap
Residues 10-25



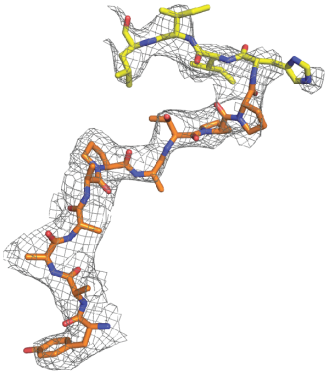
PAS β-sheet
Residues 30-45



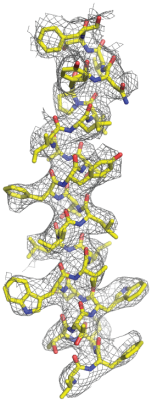
PAS α-helix
Residues 77-90



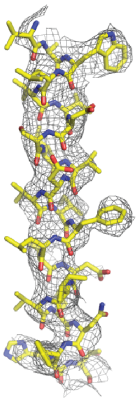
CaM binding α-helix
Residues 147-158



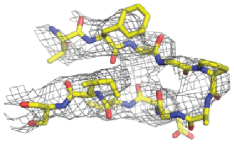
PAS C-terminal linker
Residues 198-211



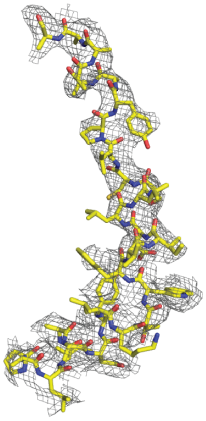
S1
Residues 215-241



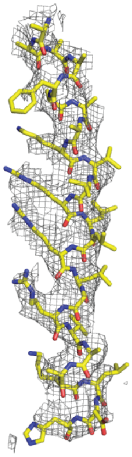
S2
Residues 248-271



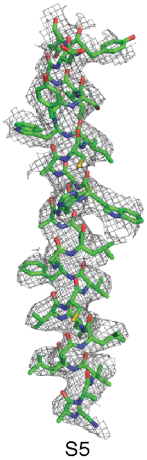
S2-S3 linker
Residues 272-282



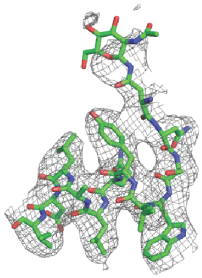
S3
Residues 284-312



S4
Residues 319-343



S5
Residues 348-377



Turret helix
Residues 388-399

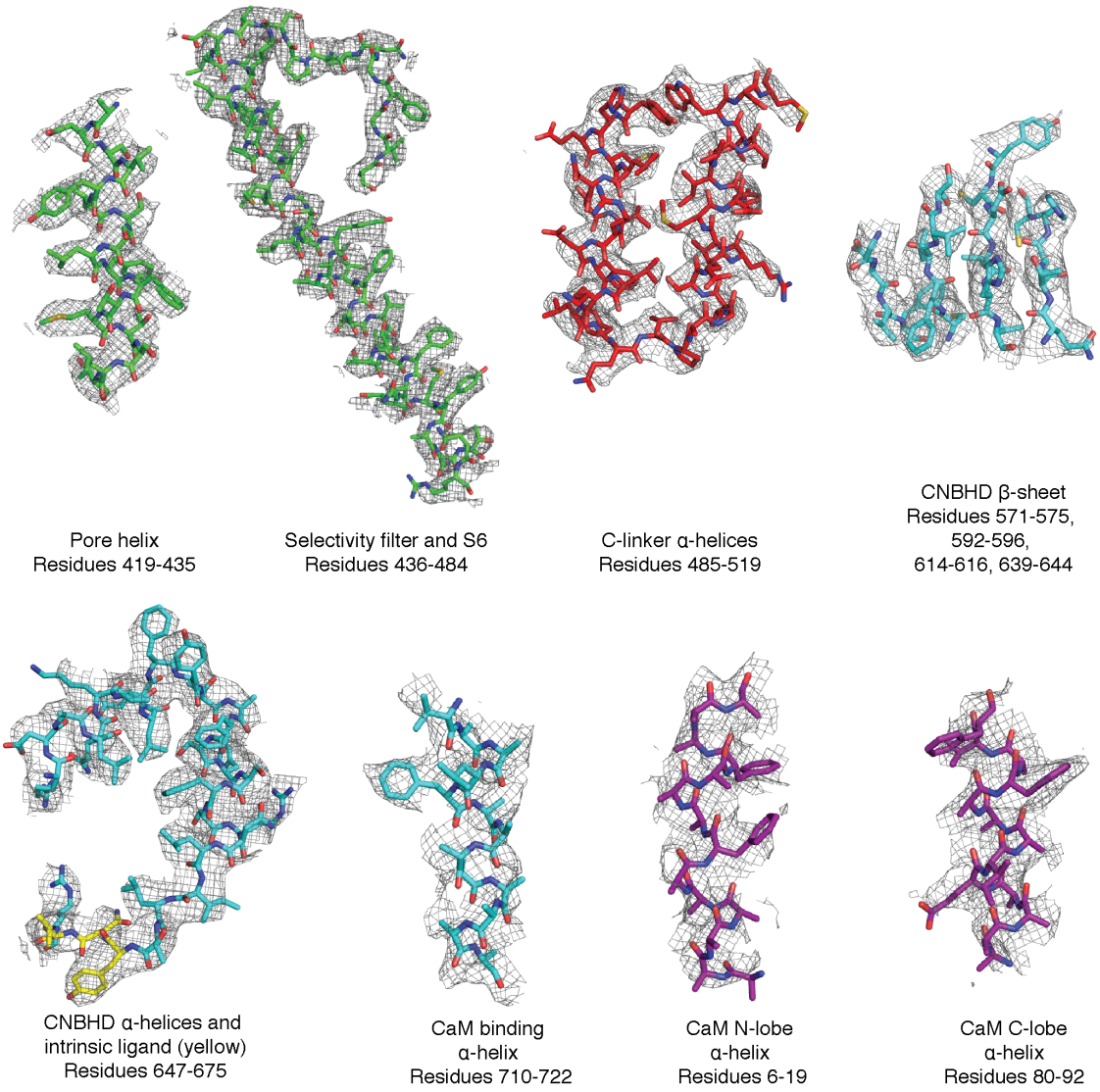
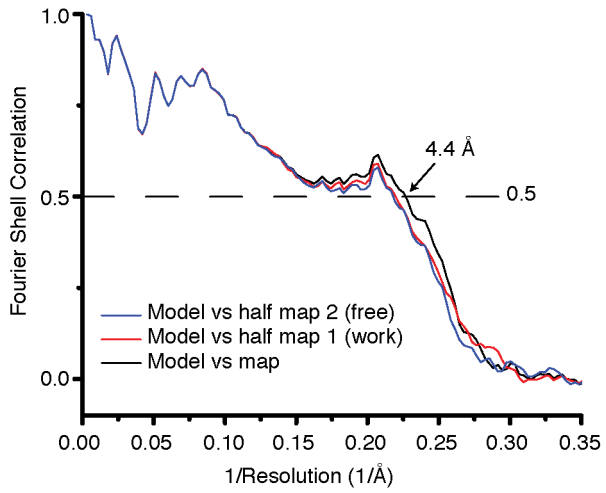


Fig. S3. Regional cryo-EM density of rEag1Δ bound to CaM. Representative density (filtered at 3.78 Å) for all regions of the rEag1 structure. PAS domain is orange, VS is yellow, S5-S6 is green, C-linker is red, CNBHD is cyan (the intrinsic ligand or the portion of the Eag1 sequence that occupies the cyclic nucleotide binding site shown as yellow), and CaM is purple.

A



B

Space Group	P4
Cell Dimensions for structure determination	
a,b,c (Å)	332.8, 332.8, 332.8
α,β,γ (°)	90, 90, 90
Cell Dimensions for refinement	
a,b,c (Å)	165, 165, 143
α,β,γ (°)	90, 90, 90
Resolution (Å)	165-3.78
No. Reflection	38,517
R_{work}	27.2
No. protein atoms	6,178
Mean B-factor	301.0
RMSD	
Bond lengths (Å)	0.007
Bond angles (°)	1.188
Ramachandran	
Favored (%)	92.57
Allowed (%)	7.31
Outliers (%)	0.12
Molprobity	
Clash score	3.23
Rotomer oultliers (%)	0.35
Overall score	1.58

Fig. S4. Structure validation of the rEag1 atomic model. (**A**) FSC cross validation between the rEag1 model and the half map used during refinement (working set, red), the other half map (free set, blue), and the full map (black). (**B**) Refinement statistics for the rEag1 model.

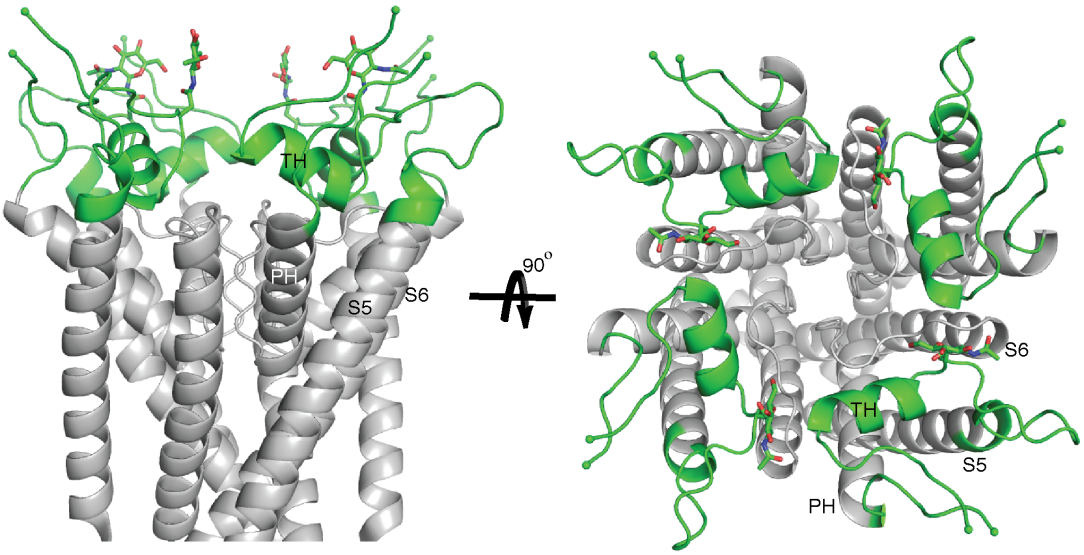


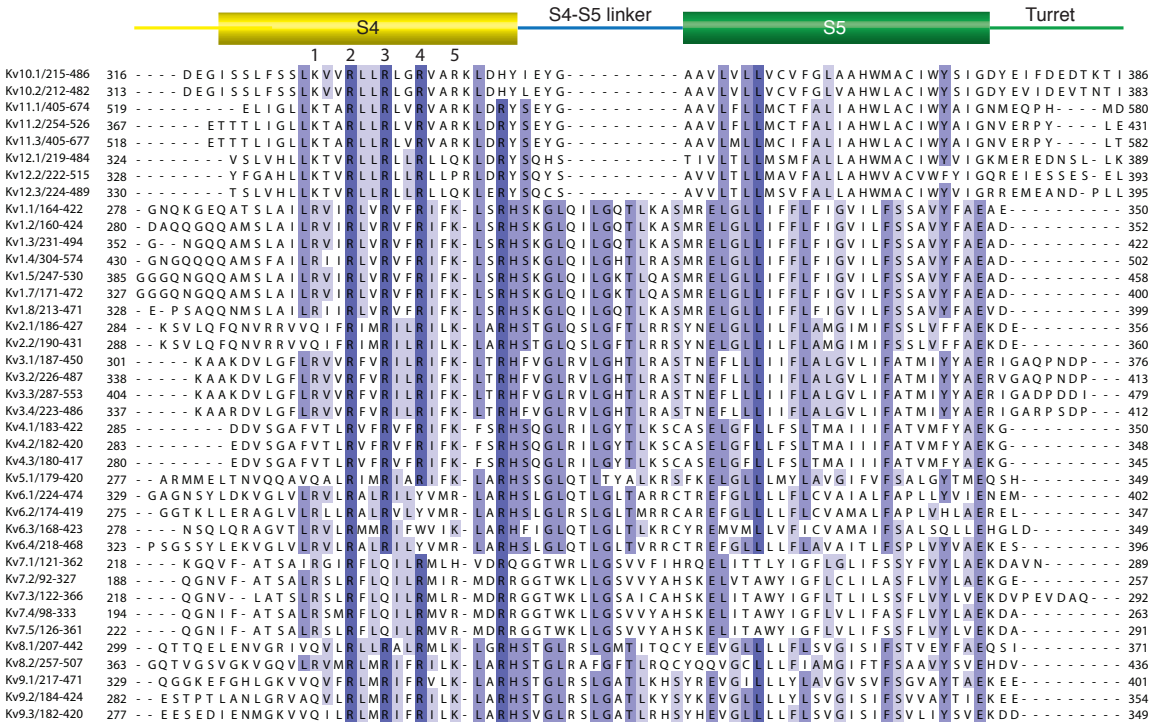
Fig. S5. Structure of the extracellular turret. The turret is colored green (TH-turret helix) and S5, S6 and pore helix (PH) are colored grey. N388 and the attached N-acetyl glucosamine are represented as sticks with green C, blue N, and red O.

S1 | S1-S2 linker

```
Kv10.1/215-486  215 VFKTTWDWIILILTFYTAILVPYNVSFKTR---------------------------------------------------------- 244
Kv10.2/212-482  212 AFKTTWDWVILILTFYTAIMVPYNVSFKTK---------------------------------------------------------- 241
Kv11.1/405-674  405 PFKAVWDWLILLLVIYTAVFTPYSAAFLLKETEE-GPPAT------------------------------------------------ 443
Kv11.2/254-526  254 PFKAVWDWLILLLVIYTAVFTPYSAAFLLSDQDE--SRRG------------------------------------------------ 291
Kv11.3/405-677  405 PFKAVWDWLILLLVIYTAIFTPYSAAFLLNDREE--QKRR------------------------------------------------ 442
Kv12.1/219-484  219 TFKAGWDWLILLATFYVAVTVPYNVCFIGNDDLS---------------------------------------------------------- 252
Kv12.2/222-515  222 ALRATWDGFILLATLYVAVTVPYSVCVSTAREPS---------------------------------------------------------- 255
Kv12.3/224-489  224 VSKAIWDGLILLATFYVAVTVPYNVCFSGDDDTP---------------------------------------------------------- 257
Kv1.1/164-422   164 GPARVIAIVSVMVILISIV-IFCLETLPELKDDKDFTGTVH-------------------------------------RI-DNTTV-I 211
Kv1.2/160-424   160 GPARIIAIVSVMVILISIV-SFCLETLPIFRDENEDMHGSGV----------------------TF-----H----TY-SNSTIGY 212
Kv1.3/231-494   231 GPARGIAIVSVLVILISIV-IFCLETLPEFRDEKDYPASTSQ-----D---------------SF-----E----AAGNSTSGSR 285
Kv1.4/304-574   304 SPARGIAIVSVLVILISIV-IFCLETLPEFRDDRDLVMALSA-----G----------------GH-----G-GLLNDTSAPHLEN 361
Kv1.5/247-530   247 GSARAIAIVSVLVILISII-TFCLETLPEFRDERELLRHPPAPHQPPAPAPGANGS----------------GVMAPP-SGPTVAP 314
Kv1.7/171-472   171 GPARGIAIVSVLVILISIV-IFCLETLPQFRVDGRGGNNGGVSRVSPVSRGSQEEEEDEDDSYTFHHGITPGEMGTG-GSSSLST 253
Kv1.8/213-471   213 SAARAVAVVSVLVVVISIT-IFCLETLPEFREDRELKVVRD-----------------------------------PN-LNMSKTV 261
Kv2.1/186-427   186 VAAKILAIISIMFIVLSTI-ALSLNTLPELQSLDEFG------------------------------------------------- 221
Kv2.2/190-431   190 VAAKILAIVSILFIVLSTI-ALSLNTLPELQETDEFG------------------------------------------------- 225
Kv3.1/187-450   187 RYARYVAFASLFFILVSIT-TFCLETHERFNPIVNKT-------EIENVRNGT------------------Q--V----------RY 235
Kv3.2/226-487   226 RAARFIAFASLFFILVSIT-TFCLETHEAFNIVKNKT-------E--PVINGT------------------S--V----------VL 272
Kv3.3/287-553   287 RAARYVAFASLFFILISIT-TFCLETHEGFIHISNKTV----TQASPIPGAP-P----------------E--N----------IT 338
Kv3.4/223-486   223 RAARVVAFASLFFILVSIT-TFCLETHEAFNIDRNVT-------EILRVGNIT------------------S--V----------HF 271
Kv4.1/183-422   183 TAALVFYYVTGFFIAVSVI-ANVVETIPCRGSARRSS-------R-----------------------------E----------Q 221
Kv4.2/182-420   182 TMALVFYYVTGFFIAVSVI-ANVVETVPCGSSPG-HI-------K-----------------------------E----------L 219
Kv4.3/180-417   180 TLALVFYYVTGFFIAVSVI-TNVVETVPCGTVPG-S-------K-----------------------------E----------L 216
Kv5.1/179-420   179 CPARVVAVLSFLLILVSSV-VMCMGTIPELQVLDAEG------------------------------------------------- 214
Kv6.1/224-474   224 LPGKVFACLSVLFVTVTAV-NLSVSTLPSLREEEEQG------------------------------------------------- 259
Kv6.2/174-419   174 LAGKLFACVSVSFVAVTAV-GLCLSTMPDIRAEEERG------------------------------------------------- 209
Kv6.3/168-423   168 LAAQILASVSVVFVIVSMV-VLCASTLPDWRNAAADNRSLD------D----------------------------------RSR 211
Kv6.4/218-468   218 LPGKVFACLSILFVATTAV-SLCVSTMPDLRAEEDQG------------------------------------------------- 253
Kv7.1/121-362   121 KC-FVYHFAVFLIVLVCLI-FSVLSTIEQYAA------------------------------------------------------ 150
Kv7.2/92-327     92 A--FIYHAYVFLLVFSCLV-LSVFSTIKEYEK------------------------------------------------------ 120
Kv7.3/122-366   122 A--LLYHALVFLIVLGCLI-LAVLTTFKEYET------------------------------------------------------ 150
Kv7.4/98-333     98 A--FVYHVFIFLLVFSCLV-LSVLSTIQEHQE------------------------------------------------------ 126
Kv7.5/126-361   126 A--FIYHAFVFLLVFGCLI-LSVFSTIPEHTK------------------------------------------------------ 154
Kv8.1/207-442   207 TAARIFGVISIIFVVVSII-NMALMSAEL---------------------------------------------------------- 234
Kv8.2/257-507   257 VAAKAIGVASSTFVLVSVV-ALALNTVEEMQQHSGQG------------------------------------------------- 292
Kv9.1/217-471   217 LPSKLFSCVSISVVLASIA-AMCIHSLPEYQAREAAAAVAA------VA-----------------------------------AGRS 262
Kv9.2/184-424   184 VLSRVFSILSILVVMGSII-TMCLNSLPDFQIPDSQG------------------------------------------------- 219
Kv9.3/182-420   182 LSAKLIAISSLSVVLASIV-AMCVHSMSEFQNEDG------------------------------------------------- 215
```

S2 | S2-S3 linker | S3 | S3-S4 linker

```
Kv10.1/215-486  245 ----QNNVAWLVVDSIVDVIFLVDIVLNFHTTFVGPAGEVISDPKLIRMNYLKTWFVIDLLSCLPYDVINAFENV---------- 315
Kv10.2/212-482  242 ----QNNIAWLVLDSVVDVIFLVDIVLNFHTTFVGPGGEVISDPKLIRMNYLKTWFVIDLLSCLPYDIINAFENV---------- 312
Kv11.1/405-674  444 -ECGYACQPLAVVDLIVDIMFIVDILINFRTTYVNANEEVVSHPGRIAVHYFKGWFLIDMVAAIPFDLLIFGSGSE--------- 518
Kv11.2/254-526  292 -ACSYTCSPLTVVDLIVDIMFVVDIVINFRTTYVNTNDEVVSHPRRIAVHYFKGWFLIDMVAAIPFDLLIFRTGSD--------- 366
Kv11.3/405-677  443 -ECGYSCSPLNVVDLIVDIMFIIDILINFRTTYVNQNEEVVSDPAKIAIHYFKGWFLIDMVAAIPFDLLIFGSGSD--------- 517
Kv12.1/219-484  253 -----TTRSTTVSDIAVEILFIIDIILNFRTTYVSKSGQVIFEARSICIHYVTTWFIIDLIAALPFDLLYAFNVTV--------- 323
Kv12.2/222-515  256 ----AARGPPSVCDLAVEVLFILDIVLNFRTTFVSKSGQVVFAPKSICLHYVTTWFLLDVIAALPFDLLHAFKVNV--------- 327
Kv12.3/224-489  258 ----ITSRHTLVSDIAVEMLFILDIILNFRTTYVSQSGQVISAPRSIGLHYLATWFFIDLIAALPFDLLYIFNITV--------- 329
Kv1.1/164-422   212 YNSNIFTDPFFIVETLCIIWFSFELVVRFFAC-----------PSKT-DFFKNIMNFIDIVAIIPYFITLGTEIAEQE-------- 277
Kv1.2/160-424   213 QQSTSFTDPFFIVETLCIIWFSFEFLVRFFAC-----------PSKA-GFFTNIMNIIDIVAIIPYFITLGTELAEKPE------- 279
Kv1.3/231-494   286 AGASSFSDPFFVVETLCIIWFSFELLVRFFAC-----------PSKA-TFSRNIMNLIDIVAIIPYFITLGTELAERQ-------- 351
Kv1.4/304-574   362 SGHTIFNDPFFIVETVCIVWFSFEFVVRCFAC-----------PSQA-LFFKNIMNIIDIVSILPYFITLGTDLAQQQGG----- 429
Kv1.5/247-530   315 LLPRTLADPFFIVETTCVIWFTFELLVRFFAC-----------PSKA-GFSRNIMNIIDVVAIFPYFITLGTELAEQQPG---GG 384
Kv1.7/171-472   254 LGGSFFTDPFFLVETLCIVWFTFELLVRFSAC-----------PSKP-AFFRNIMNIIDLVAIFPYFITLGTELVQQQEQQPASG 326
Kv1.8/213-471   262 LSQTMFTDPFFMVESTCIVWFTFELVLRFVVC-----------PSKT-DFFRNIMNIIDIISIIPYFATLITELVQET-------- 327
Kv2.1/186-427   222 --QSTDNPQLAHVEAVCIAWFTMEYLLRFLSS-----------PKKW-KFFKGPLNAIDLLAILPYYVTIFLTESN--------- 283
Kv2.2/190-431   226 --QLNDNRQLAHVEAVCIAWFTMEYLLRFLSS-----------PNKW-KFFKGPLNVIDLLAILPYYVTIFLTESN--------- 287
Kv3.1/187-450   236 YREAETEAFLTYIEGVCVVWFTFEFLMRVIFC-----------PNKV-EFIKNSLNIIDFVAILPFYLEVGLSGLSS-------- 300
Kv3.2/226-487   273 QYEIETDPALTYVEGVCVVWFTFEFLVRIVFS-----------PNKL-EFIKNLLNIIDFVAILPFYLEVGLSGLSS-------- 337
Kv3.3/287-553   339 NVEVETEPFLTYVEGVCVVWFTFEFLMRITFC-----------PDKV-EFLKSSLNIIDCVAILPFYLEVGLSGLSS-------- 403
Kv3.4/223-486   272 RREVETEPILTYIEGVCVLWFTLEFLVRIVCC-----------PDTL-DFVKNLLNIIDFVAILPFYLEVGLSGLSS-------- 336
Kv4.1/183-422   222 PCGERFPQAFFCMDTACVLIFTGEYLLRLFAA-----------PSRC-RFLRSVMSLIDVVAILPYYIGLLVPKN---------- 284
Kv4.2/182-420   220 PCGERYAVAFFCLDTACVMIFTVEYLLRLAAA-----------PSRY-RFVRSVMSIIDVVAILPYYIGLVMTDN---------- 282
Kv4.3/180-417   217 PCGERYSVAFFCLDTACVMIFTVEYLLRLFAA-----------PSRY-RFIRSVMSIIDVVAIMPYYIGLVMTNN---------- 279
Kv5.1/179-420   215 --NRVEHPTLENVETACIGWFTLEYLLRLFSS-----------PNKL-HFALSFMNIVDVLAILPFYVSLTLTHLG--------- 276
Kv6.1/224-474   260 -HCSQMCHNVFIVESVCVGWFSLEFLLRLIQA-----------PSKF-AFLRSPLTLIDLVAILPYYITLLVDGAAAGRRKP--- 328
Kv6.2/174-419   210 -ECSPKCRSLFVLETVCVAWFSFEFLLRSLQA-----------ESKC-AFLRAPLNIIDILALLPFYVSLLLGLAAGP-------- 274
Kv6.3/168-423   212 YSAGPGREPSGIIEAICIGWFTAECIVRFIVS-----------KNKC-EFVKRPLNIIDLLAITPYYISVLMTVFTGE-------- 277
Kv6.4/218-468   254 -ECSRKCYYIFIVETICVAWFSLEFCLRFVQA-----------QDKC-QFFQGPLNIIDILAISPYYVSLAVSEEPPEDGER--- 322
Kv7.1/121-362   151 ----LATGTLFWMEIVLVVFFGTEYVVRLWSAGCRSKYVGLW--GRL-RFARKPISIIDLIVVVASMVVLCVG--S---------- 217
Kv7.2/92-327    121 ----SSEGALYILEIVTIVVFGVEYFVRIWAAGCCCRYRGWR--GRL-KFARKPFCVIDIMVLIASIAVLAAG--S---------- 187
Kv7.3/122-366   151 ----VSGDWLLLLETFAIFIFGAEFALRIWAAGCCCRYKGWR--GRL-KFARKPLCMLDIFVLIASVPVVAVG--N---------- 217
Kv7.4/98-333    127 ----LANECLILEFVMIVVFGLEYIVRVWSAGCCCRYRGWQ--GRF-RFARKPFCVIDFIVFVASVAVIAAG--T---------- 193
Kv7.5/126-361   155 ----LASSCLLILEFVMIVVFGLEFIIRIWSAGCCCRYRGWQ--GRL-RFARKPFCVIDTIVLIASIAVVSAK--T---------- 221
Kv8.1/207-442   235 --SWLDLQLLEILEYVCISWFTGEFVLRFLCV-----------RDRC-RFLRKVPNIIDLLAILPFYITLLVESLSGS-------- 298
Kv8.2/257-507   293 EGGPDLRPILEHVEMLCMGFFTLEYLLRLAST-----------PDLR-RFARSALNLVDLVAILPLYLQLLLECFTGEGHQR--- 362
Kv9.1/217-471   263 PEGVRDDPVLRRLEYFCIAWFSFEVSSRLLLA-----------PSTR-NFFCHPLNLIDIVSVLPFYLTLLAGVALGD-------- 328
Kv9.2/184-424   220 --NPGEDPRFEIVEHFGIAWFTFELVARFAVA-----------PDFL-KFFKNALNLIDLMSIVPFYITLVVNLVV---------- 281
Kv9.3/182-420   216 ---EVDDPVLEGVEIACIAWFTGELAVRLAAA-----------PCQK-KFWKNPLNIIDFVSIIPFYATLAVDTKE---------- 276
```


S4
S4-S5 linker
S5
Turret
1 2 3 4 5
Kv10.1/215-486 316 ----DEGISSLFSSLKVVRLLRLGRVARKLDHYIEYG----------AAVLVLLVCVFGLAAHWMACIWYSIGDYEIFDEDTKTI 386
Kv10.2/212-482 313 ----DEGISSLFSSLKVVRLLRLGRVARKLDHYLEYG----------AAVLVLLVCVFGLVAHWLACIWYSIGDYEVIDEVTNTI 383
Kv11.1/405-674 519 ---------ELIGLLKTARLLRLVRVARKLDRYSEYG----------AAVLFLLMCTFALIAHWLACIWYAIGNMEQPH----MD 580
Kv11.2/254-526 367 ------ETTTLIGLLKTARLLRLVRVARKLDRYSEYG----------AAVLFLLMCTFALIAHWLACIWYAIGNVERPY----LE 431
Kv11.3/405-677 518 ------ETTTLIGLLKTARLLRLVRVARKLDRYSEYG----------AAVLMLLMCIFALIAHWLACIWYAIGNVERPY----LT 582
Kv12.1/219-484 324 --------VSLVHLLKTVRLLRLLRLLQKLDRYSQHS----------TIVLTLLMSMFALLAHWMACIWYVIGKMEREDNSL-LK 389
Kv12.2/222-515 328 --------YFGAHLLKTVRLLRLLRLLPRLDRYSQYS----------AVVLTLLMAVFALLAHWVACVWFYIGQREIESSES-EL 393
Kv12.3/224-489 330 --------TSLVHLLKTVRLLRLLRLLQKLERYSQCS----------AVVLTLLMSVFALLAHWMACIWYVIGRREMEAND-PLL 395
Kv1.1/164-422 278 -GNQKGEQATSLAILRVIRLVRVFRIFK-LSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAE---------- 350
Kv1.2/160-424 280 -DAQQGQQAMSLAILRVIRLVRVFRIFK-LSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAD---------- 352
Kv1.3/231-494 352 -G--NGQQAMSLAILRVIRLVRVFRIFK-LSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAD---------- 422
Kv1.4/304-574 430 -GNGQQQQAMSFAILRIIRLVRVFRIFK-LSRHSKGLQILGHTLRASMRELGLLIFFLFIGVILFSSAVYFAEAD---------- 502
Kv1.5/247-530 385 GGGQNGQQAMSLAILRVIRLVRVFRIFK-LSRHSKGLQILGKTLQASMRELGLLIFFLFIGVILFSSAVYFAEAD---------- 458
Kv1.7/171-472 327 GGGQNGQQAMSLAILRVIRLVRVFRIFK-LSRHSKGLQILGKTLQASMRELGLLIFFLFIGVILFSSAVYFAEAD---------- 400
Kv1.8/213-471 328 -E-PSAQQNMSLAILRIIRLVRVFRIFK-LSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEVD---------- 399
Kv2.1/186-427 284 --KSVLQFQNVRRVVQIFRIMRILRILK-LARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMIFSSLVFFAEKDE---------- 356
Kv2.2/190-431 288 --KSVLQFQNVRRVVQIFRIMRILRILK-LARHSTGLQSLGFTLRRSYNELGLLILFLAMGIMIFSSLVFFAEKDE---------- 360
Kv3.1/187-450 301 -----KAAKDVLGFLRVVRFVRILRIFK-LTRHFVGLRVLGHTLRASTNEFLLLIIFLALGVLIFATMIYYAERIGAQPNDP--- 376
Kv3.2/226-487 338 -----KAAKDVLGFLRVVRFVRILRIFK-LTRHFVGLRVLGHTLRASTNEFLLLIIFLALGVLIFATMIYYAERVGAQPNDP--- 413
Kv3.3/287-553 404 -----KAAKDVLGFLRVVRFVRILRIFK-LTRHFVGLRVLGHTLRASTNEFLLLIIFLALGVLIFATMIYYAERIGADPDDI--- 479
Kv3.4/223-486 337 -----KAARDVLGFLRVVRFVRILRIFK-LTRHFVGLRVLGHTLRASTNEFLLLIIFLALGVLIFATMIYYAERIGARPSDP--- 412
Kv4.1/183-422 285 --------DDVSGAFVTLRVFRVFRIFK-FSRHSQGLRILGYTLKSCASELGFLLFSLTMAIIIFATVMFYAEKG---------- 350
Kv4.2/182-420 283 --------EDVSGAFVTLRVFRVFRIFK-FSRHSQGLRILGYTLKSCASELGFLLFSLTMAIIIFATVMFYAEKG---------- 348
Kv4.3/180-417 280 --------EDVSGAFVTLRVFRVFRIFK-FSRHSQGLRILGYTLKSCASELGFLLFSLTMAIIIFATVMFYAEKG---------- 345
Kv5.1/179-420 277 --ARMMELTNVQQAVQALRIMRIARIFK-LARHSSGLQTLTYALKRSFKELGLLLMYLAVGIFVFSALGYTMEQSH---------- 349
Kv6.1/224-474 329 -GAGNSYLDKVGLVLRVLRALRILYVMR-LARHSLGLQTLGLTARRCTREFGLLLLFLCVAIALFAPLLYVIENEM---------- 402
Kv6.2/174-419 275 --GGTKLLERAGLVLRLLRALRVLYVMR-LARHSLGLRSLGLTMRRCAREFGLLLLFLCVAMALFAPLVHLAEREL---------- 347
Kv6.3/168-423 278 ----NSQLQRAGVTLRVLRMMRIFWVIK-LARHFIGLQTLGLTLKRCYREMVMLLVFICVAMAIFSALSQLLEHGLD-------- 349
Kv6.4/218-468 323 -PSGSSYLEKVGLVLRVLRALRILYVMR-LARHSLGLQTLGLTVRRCTREFGLLLLFLAVAITLFSPLVYVAEKES--------- 396
Kv7.1/121-362 218 ----KGQVF-ATSAIRGIRFLQILRMLH-VDRQGGTWRLLGSVVFIHRQELITTLYIGFLGLIFSSYFVYLAEKDAVN------- 289
Kv7.2/92-327 188 ----QGNVF-ATSALRSLRFLQILRMIR-MDRRGGTWKLLGSVVYAHSKELVTAWYIGFLCLILASFLVYLAEKGE--------- 257
Kv7.3/122-366 218 ----QGNV--LATSLRSLRFLQILRMLR-MDRRGGTWKLLGSAICAHSKELITAWYIGFLTLILSSFLVYLVEKDVPEVDAQ--- 292
Kv7.4/98-333 194 ----QGNIF-ATSALRSMRFLQILRMVR-MDRRGGTWKLLGSVVYAHSKELITAWYIGFLVLIFASFLVYLAEKDA--------- 263
Kv7.5/126-361 222 ----QGNIF-ATSALRSLRFLQILRMVR-MDRRGGTWKLLGSVVYAHSKELITAWYIGFLVLIFSSFLVYLVEKDA--------- 291
Kv8.1/207-442 299 --QTTQELENVGRIVQVLRLLRALRMLK-LGRHSTGLRSLGMTITQCYEEVGLLLLFLSVGISIFSTVEYFAEQSI--------- 371
Kv8.2/257-507 363 -GQTVGSVGKVGQVLRVMRLMRIFRILK-LARHSTGLRAFGFTLRQCYQQVGCLLLFIAMGIFTFSAAVYSVEHDV--------- 436
Kv9.1/217-471 329 --QGGKEFGHLGKVVQVFRLMRIFRVLK-LARHSTGLRSLGATLKHSYREVGILLLYLAVGVSVFSGVAYTAEKEE--------- 401
Kv9.2/184-424 282 --ESTPTLANLGRVAQVLRLMRIFRILK-LARHSTGLRSLGATLKYSYKEVGLLLLYLSVGISIFSVVAYTIEKEE--------- 354
Kv9.3/182-420 277 --EESEDIENMGKVVQILRLMRIFRILK-LARHSVGLRSLGATLRHSYHEVGLLLLFLSVGISIFSVLIYSVEKDD--------- 349
Selectivity Filter
Turret Helix
Pore Helix
S6
Kv10.1/215-486 387 RNNSWLYQLAMDIGTPYQFN----------GSGSGKWEGGPSKNSVYISSLYFTMTSLTSVGFGNIAPSTDIE 449
Kv10.2/212-482 384 QIDSWLYQLALSIGTPYRYN----------TSAGIWEGGPSKDSLYVSSLYFTMTSLTTIGFGNIAPTTDVE 445
Kv11.1/405-674 581 SRIGWLHNLGDQIGKPY-------N----------S---SGLGGPSIKDKYVTALYFTFSSLTSVGFGNVSPNTNSE 637
Kv11.2/254-526 432 HKIGWLDSLGVQLGKRY-------N----------GS--DPASGPSVQDKYVTALYFTFSSLTSVGFGNVSPNTNSE 489
Kv11.3/405-677 583 DKIGWLDSLGQQIGKRY-------N----------DS--DSSSGPSIKDKYVTALYFTFSSLTSVGFGNVSPNTNSE 640
Kv12.1/219-484 390 WEVGWLHELGKRLESPYYG----------NNTLGGPSIRSAYIAALYFTLSSLTSVGFGNVSANTDAE 447
Kv12.2/222-515 394 PEIGWLQELARRLETPYYLVGRRPAGGNSSGQSDNCSSSSEANGTGLELLGGPSLRSAYITSLYFALSSLTSVGFGNVSANTDTE 478
Kv12.3/224-489 396 WDIGWLHELGKRLEVPY-V----------NGSVGGPSRRSAYIAALYFTLSSLTSVGFGNVCANTDAE 452
Kv1.1/164-422 351 ----------EAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGG 385
Kv1.2/160-424 353 ----------ERESQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGG 387
Kv1.3/231-494 423 ----------DPTSGFSSIPDAFWWAVVTMTTVGYGDMHPVTIGG 457
Kv1.4/304-574 503 ----------EPTTHFQSIPDAFWWAVVTMTTVGYGDMKPITVGG 537
Kv1.5/247-530 459 ----------NQGTHFSSIPDAFWWAVVTMTTVGYGDMRPITVGG 493
Kv1.7/171-472 401 ----------DDDSLFPSIPDAFWWAVVTMTTVGYGDMYPMTVGG 435
Kv1.8/213-471 400 ----------EPESHFSSIPDGFWWAVVTMTTVGYGDMCPTTPGG 434
Kv2.1/186-427 357 ----------DDTKFKSIPASFWWATITMTTVGYGDIYPKTLLG 390
Kv2.2/190-431 361 ----------DATKFTSIPASFWWATITMTTVGYGDIYPKTLLG 394
Kv3.1/187-450 377 ----------SASEHTHFKNIPIGFWWAVVTMTTLGYGDMYPQTWSG 413
Kv3.2/226-487 414 ----------SASEHTQFKNIPIGFWWAVVTMTTLGYGDMYPQTWSG 450
Kv3.3/287-553 480 ----------LGSNHTYFKNIPIGFWWAVVTMTTLGYGDMYPKTWSG 516
Kv3.4/223-486 413 ----------RGNDHTDFKNIPIGFWWAVVTMTTLGYGDMYPKTWSG 449
Kv4.1/183-422 351 ----------TNKTNFTSIPAAFWYTIVTMTTLGYGDMVPSTIAG 385
Kv4.2/182-420 349 ----------SSASKFTSIPAAFWYTIVTMTTLGYGDMVPKTIAG 383
Kv4.3/180-417 346 ----------SSASKFTSIPASFWYTIVTMTTLGYGDMVPKTIAG 380
Kv5.1/179-420 350 ----------PETLFKSIPQSFWWAIITMTTVGYGDIYPKTLLG 383
Kv6.1/224-474 403 ----------ADSPEFTSIPACYWWAVITMTTVGYGDMYPRSTPG 437
Kv6.2/174-419 348 ----------GARRDFSSVPASYWWAVISMTTVGYGDMVPRSLPG 382
Kv6.3/168-423 350 ----------LETSNKDFTSIPAACWWVIISMTTVGYGDMYPITVPG 386
Kv6.4/218-468 397 ----------GRVLEFTSIPASYWWAIISMTTVGYGDMVPRSVPG 431
Kv7.1/121-362 290 ----------ESGRVEFGSYADALWWGVVTVTTIGYGDKVPQTWVG 325
Kv7.2/92-327 258 ----------NDHFDTYADALWWGLITLTTIGYGDKYPQTWNG 290
Kv7.3/122-366 293 ----------GEEMKEEFETYADALWWGLITLATIGYGDKTPKTWEG 329
Kv7.4/98-333 264 ----------NSDFSSYADSLWWGTITLTTIGYGDKTPHTWLG 296
Kv7.5/126-361 292 ----------NKEFSTYADALWWGTITLTTIGYGDKTPLTWLG 324
Kv8.1/207-442 372 ----------PDTTFTSVPCAWWWATTSMTTVGYGDIRPDTTTG 405
Kv8.2/257-507 437 ----------PSTNFTTIPHSWWAAVSISTVGYGDMYPETHLG 470
Kv9.1/217-471 402 ----------DVGFNTIPACWWWGTVSMTTVGYGDVVPVTVAG 434
Kv9.2/184-424 355 ----------NEGLATIPACWWWATVSMTTVGYGDVVPGTTAG 387
Kv9.3/182-420 350 ----------HTSSLTSIPICWWWATISMTTVGYGDTHPVTLAG 383

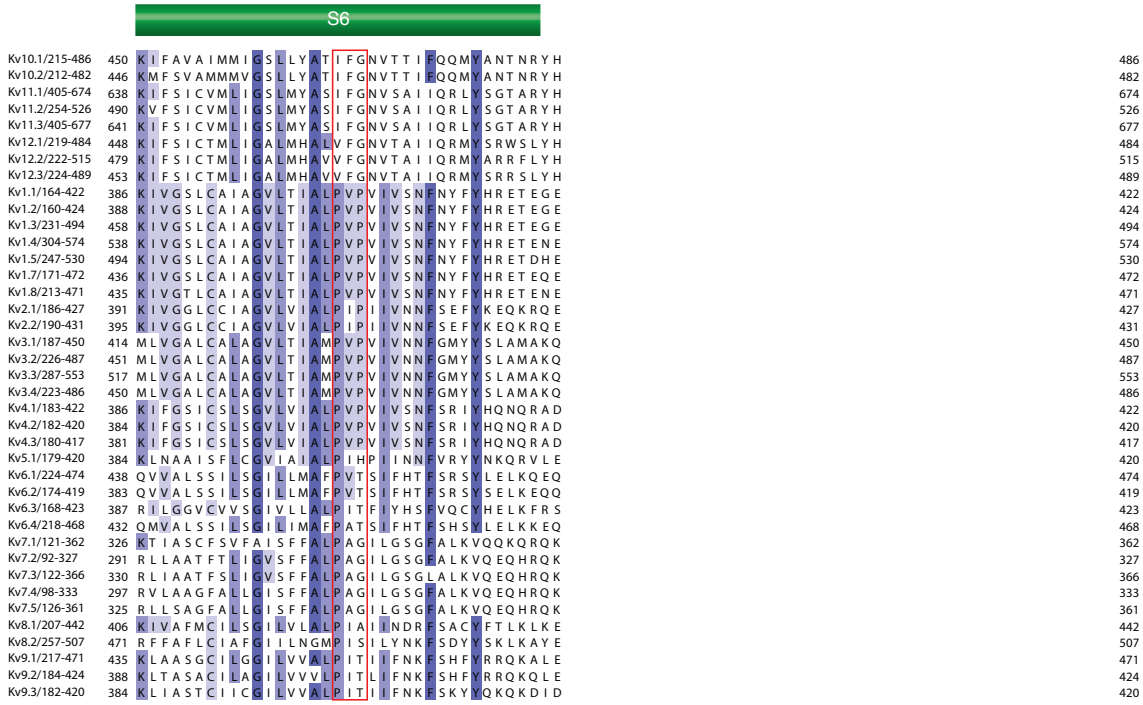


Fig. S6. Sequence alignment for the transmembrane region (S1-S6) of human voltage-gated potassium channels. S1-S6 of human Eag1 are identical to rat Eag1. Helix and loop annotations are based on the rEag1 structure. The 5 positive charges on S4 and the PVP hinge are indicated.

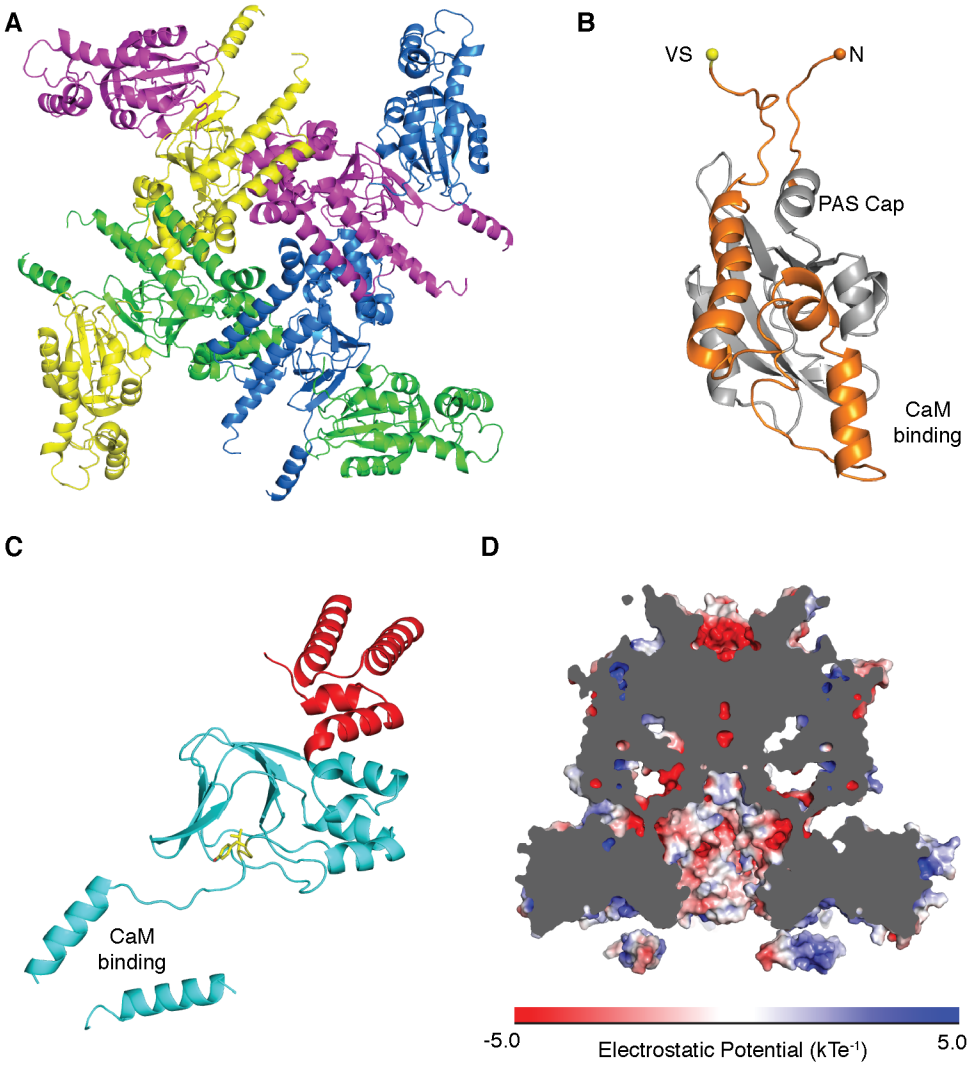


Fig. S7. Intracellular domains. (**A**) Subunit organization of the intracellular domains. Extracellular view of the intracellular domains with each subunit colored differently. (**B**) Structure of rEag1 PAS domain. Structural elements not observed in previous crystal structures are shown in orange. The orange sphere indicates the N-terminus and the yellow sphere indicates the connection to the VS. (**C**) Structure of rEag1 C-linker (red) and CNBHD (cyan). The top two helices of the C-linker are from the neighboring subunit. The portion of the rEag1 sequence that occupies the cyclic nucleotide-binding site (Y672 and L674) is shown as sticks with yellow C and red O. (**D**) Electrostatic surface potential of the rEag1 intracellular vestibule.

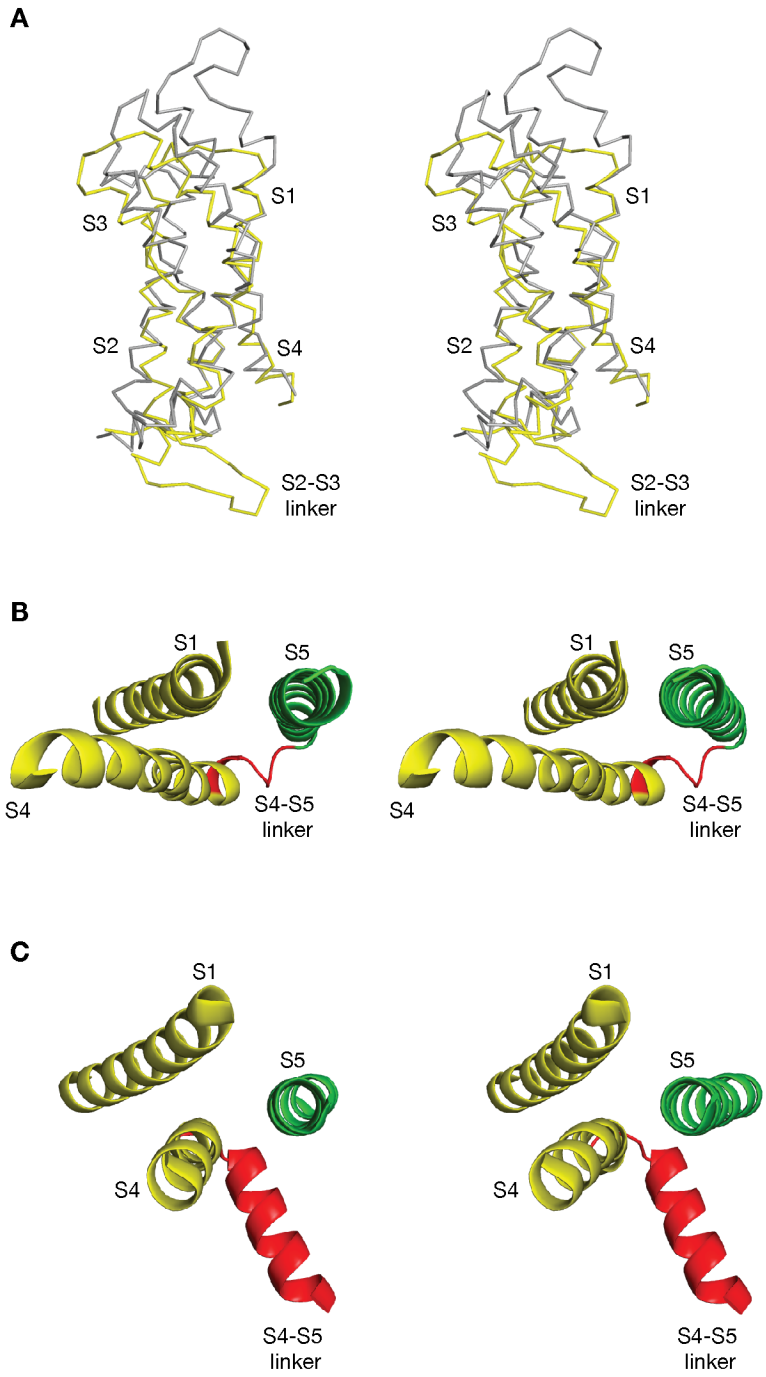


Fig S8. Comparison of Eag1 and K_v1.2-2.1 VS. (**A**) Superposition of the Eag1 VS (yellow) and the K_v1.2-2.1 VS (grey) shown in stereoview. The S1 helix of the VS was used for superposition. Interaction between S1 (yellow), S4 (yellow), and S5 (green) in Eag1 (**B**) and K_v1.2-2.1 (**C**). Images are shown in stereoview and the S4-S5 linker is red.

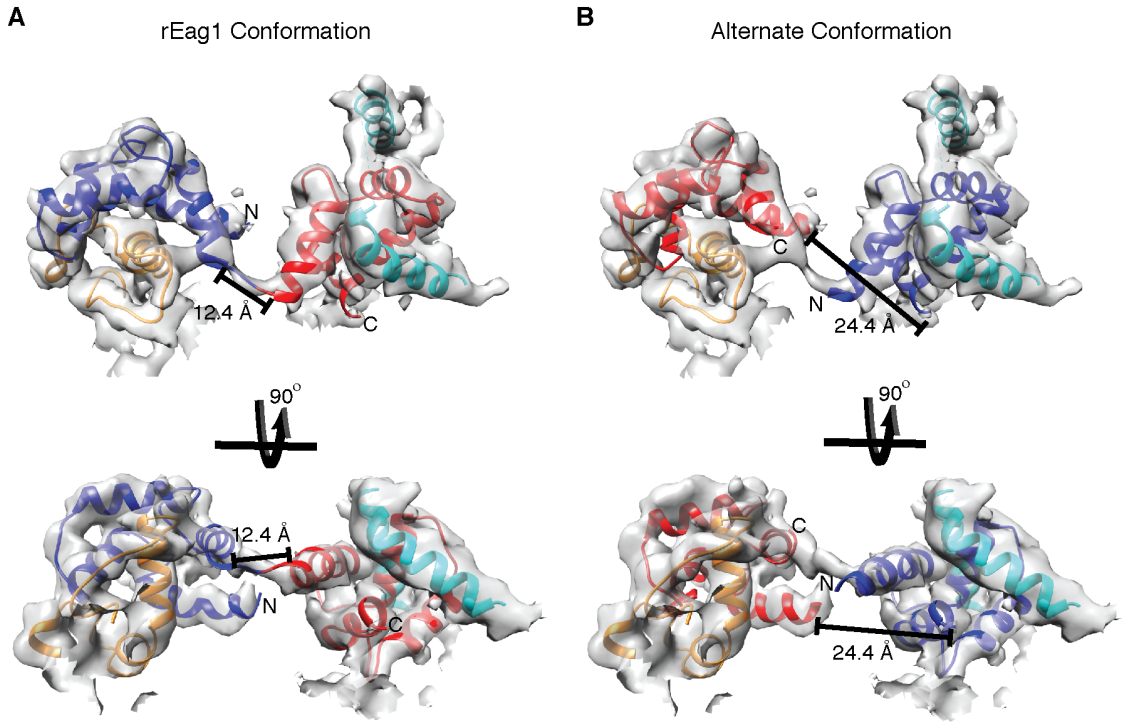


Fig. S9. Evidence for the proposed orientation of CaM binding. (**A**) The proposed conformation of CaM binding to rEag1, in which the CaM N-lobe (blue) binds to the PAS domain (orange) and the CaM C-lobe (red) binds to the CNBHD (cyan). In this orientation the distance between the N and C-lobes of CaM is approximately 12.4 Å, a reasonable distance for the 5-residue linker between the lobes (residues 76-80). In addition, through further 3D classification density for the 5-residue linker was observed in a subset of particles (the cryo-EM map is filtered at 5 Å). **B.** In the alternate conformation, in which the CaM N-lobe binds to the CNBHD and the CaM C-lobe binds to the PAS domain, it would be impossible for the 5-residue linker to span the 24.4 Å distance. Furthermore, we did not observe density for such a linker.