Supplementary Information for

Cryo-EM structure of the human Kv3.1 channel reveals gating control by the cytoplasmic T1 domain

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			β1	β2	a1	α2	α3				
b. rz () (1 -	4										
hKv3.la	4	GESE	RIVINVGG	RHQTY	RSTLRTL	PGTRLAWLA	APP AHSH-		F1	DYDPRA	
nkv3.1D hKu3 2	4		RIVINVGG	RHQTT	RSTLRTL	PGTRLAWLF	A P ARSH-	ידייידי		DERA	SPOPOCOFFO
hKv3 3	81	GGGGGD	KTVTNVGGI	RHETY	RSTLRTI.	PGTRLAGUT	DPDAAAR-		FI	DECALLE MDECA	
hKv3.4	32	G ASE	KIIINVGG	RHETY	RSTLETL	PGTRLAWL	A P GGGR-			PE	TDGG
dShaw2	3	LINM	RVVLNVGG	RHETY	KATLKKI	PATRLSRII	ALA		1	JYDPIL	
hKv1.2	27	A-DH CC	RVVINISGI	RFETQ	LKTLAQF	PETLLEDPK	KKRMR			DPLR	
hKv2.1	30	S R	RVRLNVGGI	LA	WRTLDRL	PRTRLCKLF	CNTHDSL	LEVC	DI	SLDD	
hKv4.1	39	D	VLVVNV <mark>S</mark> GF	RFETW	K <mark>NTL</mark> DRY	PDTLLCSSE	EK	Е	FI	F <mark>YD</mark> ADS	
consensus	81		••••*	• • • • •	••**•••	* . * . *	•••		_	•••	-
						a 4			a 5)	a6
hKw3 1a	53			D DD	FFDBHDC	VFAHTLNYV					PCCWMTYBOH
hKv3.1b	53			D DD	FFDRHPG	VFAHILNYY	RT-GKLHC	PADVCGP	LYEEELAEV	IGIDEIDVE	PCCWMTYROH
hKv3.2	81	GAGNCSSR	GGRASDHPC	GGR	FFDRHPG	VFAYVLNYY	RT-GKLHC	PADVCGP	LFEEELAFV	GIDETDVE	PCCWMTYROH
hKv3.3	133			D DF	FFDRHPG	VFAYVLNYY	RT-GKLHC	PADVCGP	LFEEELGFV	GIDETDVE	ACCWMTYRQH
hKv3.4	80	GVGSSGSS	GG	-GGC	FFDRHPG	VFAYVLNYY	RT-GKLHC	PADVCGP	LFEEELTFV	VGIDETDVE	PCCWMTYRQH
dShaw2	51			N B Y	FFDRHPG	vfa <mark>q</mark> vlnyy	'RT-GKLHY	PTDVCGPI	LFEEELEFV	NGLDSNQVE	PCCWMTY <mark>T</mark> QH
hKv1.2	74			N D Y	FFDR <mark>NRP</mark>	SFDAILYY	QSGGRLRR	FVNVPLD	IFSEEIRF	(ELG <mark>D</mark> EAMD	M <u>F-</u>
hKv2.1	81			N D Y	FFDRHPG	AFTSILNFY	'RT-GRLHM	MEEMCALS	SESQELDY	VGIDEIYIE	S <mark>CC</mark> QAR Y HQK
hKv4.1	80			G B Y	FFDRDPD	MFRHVLNFY	RT-GRLHC	PRQECIQ	AFDEBIAF)	GUVPELNG	D <mark>CC</mark> LEE <mark>YR</mark> DR
consensus	101	α6		*•	****•••	• • • • • • • • •	••• •••••	• • • • • • • •	••••		• • • • • • • • • • •
		***	*							_	
hKv3.la	113	RDAEEA	LDSFGGAPI	JDNSAD	DADAD <mark>G</mark> P	GDS-		GD <mark>GED L</mark>	em <mark>tkrl</mark> als	SDS	PDCRPCG
hKv3.1b	113	RDAEEA	LDSFGGAPI	DNSAD	DADAD	GDS-		GDGEDEL	EMTKRLALS	SDS	PDCRP <mark>C</mark> G
hKv3.2	160	RDAEEA	LDIE	E	TPDLICG	UDCCL DDEA		GDDE-DLA	AA-KRIGIE	E AAGL-GG	PDCKSCR
HKV3.3	152	RDAEEA	IDSH APDE	AGAAN	SPDCC	CACDSD A-	GAGGGGLD	CAGG	AT ORTODI	2 - CC - ACC	HC CSC
dShaw2	111		AVLORI.D-				T.D	TEKPS E	T ARKEOFF	1 00 AO.	-DYYKCTI
hKv1.2	127	RDDDGY	KEE RPLE	PEN							
hKv2.1	141	KEQMNDDL	KREADT			LR RE	3	GEEFI	N		
hKv4.1	140	kken <mark>ae</mark> rl	ADDEDA			PQP	\	GDGP <i>I</i>	AI		_
consensus	241				•	•		•••••		•	
		SC)			S1			\	S1-S2 lii	nker
hKv3 la	168	-FWRRMOP	RTWALFEDE	VSSRV	ARVIAFA	SLEETLVST	TTTTCI.FTH	BRBNP	VNKTE F-	NV7	
hKv3.1b	168		RTWALFEDE	YSSRY	ARYVAFA	SLEFTLVST	TTECLETH	FRENP	IVNKTEIE-	NV	RNGTOVRYYR
hKv3.2	208	WRRLOP	RMWALFEDF	YSSRA	ARFIAFA	SLFFILVSI	TTFCLETH	EAFNI	/KNKTE	PV	INGTSWVLOY
hKv3.3	267	TWWRRWQP.	RVWALFEDE	YSSRA	ar <mark>y</mark> vafa	SLFFIL <mark>I</mark> SI	TTFCLETH	EGFIH	I SNKTVTQ <i>P</i>	SPIPGAPP	ENITNV
hKv3.4	205	CRGWQP	RMWALFEDF	YSSRA	ar <mark>v</mark> vafa	SLFFILVSI	TTFCLETH	EAFNII	DRNVTEIL-	RV0	GNITSVHFRR
dShaw2	151	SWWQEMKP	RIWSLFDEF	YSS <mark>N</mark> A	AKTIGVV	SVFFI <mark>CI</mark> SI	LSFCL <mark>K</mark> TH	PDMRVPI	/RNITVKTA	/	- <mark>N</mark> GSNGWFLD
hKv1.2	144	EFQR	QVWLLFEYE	ESSGP	ARIIAIV	SVMVILISI	VSFCLETL	PI B RD	ENEDMHO	SGVTFHTY:	SNSTIGYQQS
nKvZ.1	160	TCCAEKRK	KLWDLLPKF DIWDDAUNDNU	NSSVA	AKILALL	SIMFIVLS'I	TALSINIL	P		;L	DEOD
IIKV4.1	103 321	PAGSSLRQ	KLWRAFENE	HISIA : *	* *	IGFFIAVSV * *		PCRG5	ARRS		REQP
consensus	JZI			52 52		S2-S3 li	nker	 S3	· · · · · ·	2-C4 link	Š4
							0-0-			55-54 IIIK	
hKv3.la	238	FAFTOAFT	TYIEGVO	VVWFT	FEFLMRV	IFCPNKVEF	IKNSLNII	DFVAILPI	FYLEVGLSC	GLSS	KAAK-DV
hKv3.1b	238	DADIDAFI	TYIEGVO	VVWFT	FEFLMRV	IFCPNKVEF	IKNSLNII	DFVAILPI	FYLEVGLSC	LSS	KAAK-DV
hKv3.2	275	EIETDPAL	TYVEGVO		FEFLVRI	VFSPNKLEE	IKNLLNII	DFVAILPI	FYLEVGLSC	LSS	<mark>kaak-</mark> dv
hKv3.3	341	EVENDEFL	TYVEGVC	CVVWFT	FEFLMRI	TFCPDKVEF	IKSSLNII	DCVAILPI	FYGLSS	KAA	
hKv3.4	274		TY EGVO	VLWF'I	LEFLVRI	VCCPDTLDE	VKNLLNII TVOODITT	DEVALLPI	YLEVGLSC		KAAR-DV
uSHawz bKw1 2	222	TOTAL DEC		NAWET	PETLVRE	TACTORACT			ZTTTT ZTTTT		
hKv2.1	222	OSTDNPOL	AHVEAVC	LAWET	MEYLLRF	I SSPKKWKF	FKGPLNAI	DLUAILP	YNVTIFTTE	SNKSVLOF	2022 - 115 2NV -
hKv4.1	223	CGERFPQA	FF-CMDTA	VLIFT	GEYLLRL	FAAES CR	LRSVMSLI	DVVAILP	YIGLLVPF	NDDV	S
consensus	401			·*.	* * * *	**	*	* *			
			S4		S4-9	55L		S5		S5-PH II	nker (turret)
				<u> </u>	*	☆ ☆ ()-(
hKv3.1a	307	LGFLRVVF	FVRILRIF	KLTRHF	VGLRVLO	HTLRASTNE	EFLLLIIFI	ALGVLIF.	ATMIYYAEI	RI GA QPNDP	SASEHT <mark>HFKN</mark>
nKv3.lb	307	LGFLRVVF	FVRILRIF	KLTRHF	VGLRVLG	HTLRASTNI	SFLLLLIFI 797777777	ALGVLIF.	A1'MI YYAEI atmityyaei	KIGAQPNDP BUCAODMPD	SASEHTHEKN
11RVJ.2 hKv3 3	J44 ∆1∩	LGFLEWVE	T VRILRIFI FVRTI DI FI	ADI KHE XI.TDUU	VGLEVIC	HTLRASIN	ar an	ALGVLIF.	ATMITIAE ATMIYVAE	RIGA DE DE T	
hKv3.4	343	LGFLRVVF	FVRILRIF	(LTRHE	VGLRVLG	HTLRASTN	FLITTE	ALGVLIF	ATMTYYAEI	RIGARBSDP	RGNDHTDEKN
dShaw2	294	LEFSIE	IMRLFKLT	HSSGT	KITT	QTFRASAK	LTLLVFFT	VLGIVIF	ASLVYYAFI	RIQPNB	NDRNS
hKv1.2	290	LAILRVIF	LVRVFRIF	KLSRHS	K <mark>GI</mark> QILC	QTLKASMRI	ELGLLIFFI	FIGVILF	SAVYFAE	_ ~ u	ADERESQUEPS
hKv2.1	294	-RVVQIF	IMRILRIL	KLARHS	T <mark>GL</mark> QSLC	FTLRRSYN	LGLLIIFI	AMGIMIF	SLVFFAE	<	-DEDD TKFK S
hKv4.1	289	- C AFVT F	VFRVFRIFI	KFSRHS	QGLRILC	YTLKSCAS	LGFLI FSI	TMAIIIF.	ATVMFYAEI	K	-GINKINFTS
consensus	481	*	*		*.	•*•••••	* • • • * • • • *	· · · · · · *	••••		*

		pore helix	SF		S6	hinge	S6T	ATM
	0.05							
hKv3.la	387	I PIGEWWAVVTMTTI	JGYGDMYPQTW	SGMLVGALC	CALAGVLTI	AMPVPVI	. VNNFGI	4YYSLAMAKQKLPKKKKKHIP
nKV3.1D	387	I PIGEWWAVVTMTTI	LGYGDMYPQTW	ISGMLVGALC	ALAGVLTI	AMPVPVI	. VNNFGI	4YYSLAMAKQKLPKKKKKHIP
nkv3.2	424	I PIGEWWAVVTMTTI	GYGDMY POTW	ISGMLVGALC	ALAGVLTI	AMPVPVI	. VNNFGI	4YYSLAMAKQKLPRKRKKHIP
NEVJ.J	490	IPIGEWWAVVIMITI	GIGDMIPKIW	ISGMLVGALC	ALAGVLII	AMPVPVI		ATTSLAMAKQKLPKKKNKHTP
debaw?	423 364		CYCDMARKTY	TCMEVGALC	ALAGVLII		VININE GI	ATTONDARY DEPARTARY P
bKul 2	261		GIGDMAPKII	CCKIVCSLO			VONFAL VONFAL	
hKw2 1	364		GIGDMVFIII CVCDIVPKTI	ICKIVGSLC		ALEVEVI	VONEN.	TYREORDOF A TRODE
hKrr4 1	250				SISCULVI		VINNESI	
	55				чило <mark>н</mark> онисти * *	*** **	10 1 N C V . * * * *	<pre>/ ** * / ******************************</pre>
conscisus	50	±	• ••			• •	• •	
					C-le	erminus		
hKv3.1a	462	RP			P <mark>Q</mark> L <mark>GS</mark>	PNYCKSV	VNS	PHHST
hKv3.1b	462	RP			PQL <mark>GS</mark>	PNYCKSV	VNS	PHHST
hKv3.2	499	PA			PQAS <mark>S</mark>	PTFCKTE	CLNM	ACN S T
hKv3.3	565	RP			PQPGS	PNYCKPE	PPPPPI	PPHPHSGGIS
hKv3.4	498	RP			AQLES	PMYCKSE	ETS	PRD S T
dShaw2	444	QP			RLP G A	FGGVSGC	GTPGS	GBSGPMGSGTG
hKvl.2	436	-B			KIPS	BDLKKSF	(SASTI)	SKSDYPEIQEGVN
nkvz.l	435		SIVSMNMKDA	FARSIEMML		NMGKKUK		SPNKWKWTKRTLSETS S SKSFETKE
nkv4.1	439 641	SGITNAFLQIKQNG	GLEDSGSGEE	QALCVRNRS	Arequinn		TTCHE	TDELTFSEALGAVSPGGRT
consensus	041				•	• • • • • • •	•	
hKv3.1a	484							QSDTC <mark>P</mark> LAQ <mark>E</mark> E-ILEI
hKv3.1b	484							QSDTC <mark>P</mark> LAQ <mark>EE-</mark> IL <mark>E</mark> I
hKv3.2	521							QSDICL-GKDNRLLEH
hKv3.3	596	PPPPITPPS	SMGVT-VAGAY	PAGPHTHPG	LLRGGAGG	LGIMGLE	PL	PAPGEPCPLAQEE-VIEI
hKv3.4	520							CSDTSPPAREEGMIER
dShaw2	476	PRRMNNKTKDLVSPK	(SDMA-FSFD-					
nKVI.Z	467	NSNEDFREENLK1	ANCT-LANTN	NEMPEROSO	DTINERE	A A OCKDR		
hKv4 1	513	SRSTSVSSOPVGPGS		PRRAKRRAT	RLANSTAS	VSRGSMC	DET.DML	GLERSHAPOSESSLNAKPHD-SLDL
consensus	721							
hV++2 1-	400				TDOMOT			
hKw3 1h	499	NRAGRA	TANEDCOUTC			COTOR		
hKw3 2	536	NRADSMENGEVARAA	U.SCD STCSE	PPLSP	PRIBTRR	SGIRE	IRRGET(TELLTICOVIC
hKv3.3	657	NRADPRPNGDPAAAA	LAHEDCPAIL	OPAMSP	EDKSPIT-	PGSRGRY	SRDRAG	CFNTDYAP
hKv3.4	536	KRADSKONGD-ANAV	USDE GAGLT		EBRRALRR	STTRDEN	IKKAAAG	CELLSTGDYAC
dShaw2				~				
hKv1.2								
hKv2.1	583	R-SMSSIDSFISCAT	DFPEAT	RFSHSP	LTSLPSK-	TGGSTAF	PEVGWR	GALGASGGRFVEANPSPDASQHSSFF
nKv4.1	588 001	N-CDSRDFVAAII	SIP1	PPAN	DesQess-	PGGGG	GSTLRI	SSEGEPCLEPETVKISSL
consensus	001 .	• •		•		•••••	•	
hKv3.la								
hKv3.1b	557							PPGGGM-RKDLCKE
hKv3.2	586							RKGYEKS
hKv3.3	716							SPDGSI-RKAT-GA
hKV3.4	600							A-DGSV-RKGTFVL
usliawz hKw1 2								
hKw2 1	654	TESPKSSMKTNNPLK	T.BAT.KVNFMF	COPSPLLPV	T.GMYHDPT	RNRGSAA		ECATLIDKAVI.SPESS VTTASAKT
hKv4 1	001							
consensus	881							
	-							
1 77 0 1								
nKv3.la	670					т ъ		
NKVJ.1D	5/0	SF						KYMP'I'-
111.VJ.2 hKv3 3	עענ 202	PP					D	GNALKT26A12510-
hKv3.4	612	RD						PLOHSPE
dShaw2								
hKv1.2								
hKv2.1	734	PPRSPEKHTAIAFNE	FEAGVHQYIDA	DTDDEGQLL	YSVDSSPF	KSIPGSI	S-PKFS	STGTRSEKNHFESSPLPTSPKFLRQN
hKv4.1	0.01							
consensus	Эрт							•

PVP

Supplemental Figure S1. Sequence alignment of human Kv3 family members Kv3.1-Kv3.4, Shaw2 (a Kv3 orthologue from *d. melanogaster*) and other representative channels from human Kv1-4 subfamilies.

The sequences were aligned with MUSCLE and presented with BOXSHADE. Grey shaded regions indicate areas of high sequence conservation, invariant amino acids are shaded in black. Secondary structure elements are color-coded as in the main figures of the manuscript. Star symbols indicate residues mutated for electrophysiological characterization in this study. The uniprot IDs for the aligned sequences are: hKv3.1a: P48547, hKv3.1b: P48547-2, hKv3.1: Q96PR1, hKv3.3: Q14003, hKv3.4:Q03721, dShaw2: P17972, hKv1.2: P16389, hKv2.1: Q14721, hKv4.1: Q9NSA2.

	KCNC1 (Apo)	кс	NC1 with ED	KCNC1 with ZnCl ₂				
Data collection								
Microscope	Titan Krios (MRCEE, UK)	Tita	an Krios (CNC. L	Titan Krios (CNC LIK)				
Detector	K3		K3	,		(3		
Voltage (kV)	300		300		200			
Magnification	105.000		105.000	105.000				
Collection mode	Counting (superresolution)	Count	ing (superresol	ution)	Counting (su	perresolution)		
$\frac{1}{1} = \frac{1}{1} \sum_{i=1}^{n} \frac{1}{i} \sum_{j=1}^{n} \frac{1}{i} \sum_{i=1}^{n} \frac{1}{i} \sum_{j=1}^{n} \frac{1}{i} \sum_{j=1$		Count	17 6			7.6		
Electron exposure (e/A ⁻)	40		47.0		4	40		
	45		40		0 42			
	0.42		0.42	0.42				
Derocus range (µm; steps)	-1.0 to -2.6 (0.2)		$\frac{10.6 \text{ to } -2.4 (0.2)}{2.274}$	-0.6 t8 -2.4 (0.2)				
Number of movies	4,043		7,274		5,010			
Phase plate used	NO		NO	No				
Data processing		Consensus	Subclass 1	Subclass 2	Monomer	Dimer		
Initial Number of particles	1,539,146		3,177,434		2,672,854			
Number of particles after	58 244		217 788		263 026			
2D classification	33)211		217)700		200	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		
Symmetry	C4	C4	C1	C1	C4	C4		
Number of particles used	49,327	217,788	110,585	93,461	133,488	72,764		
for 3D refinement	,				,			
Map resolution	3.2	3.2	3.6	3.6	3.1	3.1		
(A; FSC threshold = 0.143)								
Resolution range (Å)	2.7 – 9.3	2.6 - 6.4	3.0 - 8.7	3.5 - 8.4	2.7 – 8.0	2.6 – 9.5		
Map sharpening B-factor	-134.4	-164.3	-135.0	-102	-153.2	-108.4		
(A²)								
Refinement								
Model resolution	25	2.4			2.4	2.2		
(Å; FSC threshold = 0.5)	5.5	5.4			5.4	5.5		
Model composition								
Non-hydrogon atoms	12 720	12 604			12 708	25 404		
Protein residues	1594	1564			1 572	23,404		
Ligands	1584	1504			16	32		
BMSD	10	10			10	02		
Bond lengths (Å)	0.007	0.007			0.003	0.004		
Bond angles (°)	0.300	0.672			0.494	0.530		
Validation	0.700	0.072			0.454	0.550		
Molprobity score	1 61	1 57			1 64	1 70		
	7.68	6.46			7 79	5.89		
Rotamer outliers	0.96	0.96			0.00	2 53		
Ramachandran nlot	0.50	0.50			0.00	2.33		
	0.00	0.00			0.00	0.00		
	3 09	3 22			3 3 2	2 3/		
Eavoured (%)	96.01	96.67			96.67	97.66		
	10.91	50.07			50.02	37.00		
EMDB Code	FMD-13416		FMD-13419		FMD-13417	FMD-13418		
PDB Code	7PHH		7PHL	7PHI	7PHK			

Supplemental Table 1. Cryo-EM data collection and processing parameters and model refinement statistics for Kv3.1a datasets collected under apo, Zn²⁺-free and Zn²⁺-containing conditions at the Midlands Regional cryo-EM Facility (MRCEF) or at the Cambridge Nanocentre (CNC), respectively.



3.5 Å (C1)

3.2 Å (C4)

Supplemental Figure S2. Purification and cryo-EM analysis of Kv3.1a under apo conditions.

(a) Flowchart for EM data processing and maps for color-coded local resolution estimation after processing with C1 and C4 symmetry, respectively.

(b) Representative size-exclusion chromatography profile on a Superose-6 column and SDS-PAGE of purified Kv3.1a. Multiple bands are due to complex N-linked glycosylation at N220 and N229.

(c) A representative electron micrograph (out of 4043), illustrating particle distribution of Kv3.1a. Scale bar: 50 nm.

(d) Representative 2-dimensional class averages from the electron micrographs. Scale bar: 10 nm.

- (e) Angular distribution of particles included in the final reconstructions.
- (f) FSC curves of the refined model for processing with different masks.



Supplemental Figure S3. Cryo-EM data processing of Kv3.1a in presence of EDTA or ZnCl₂.

(a) Flowchart for EM data processing including 3D variability analysis and maps for color-coded local resolution estimation after processing with C1 and C4 symmetry for a dataset obtained in presence of 1 mM EDTA.

(b) Flowchart for EM data processing and maps for color-coded local resolution estimation after processing with C4 symmetry for a dataset collected in presence of 400 µM ZnCl₂.



Supplemental Figure S4. EM density maps for representative segments in the Kv3.1a channel.

- (a) EM density map for the lipid at site II (near PH helix/turret)
- (b) EM density map for the lipid at site I (near S4 and S4/S5L)
- (c) EM density map for the S4 helix in the voltage-sensing domain (VSD)
- (d) EM density map for the selectivity filter with coordinated K+ ions
- (e) EM density maps for the α 6 helix in the cytoplasmic T1 domain
- (f) EM density maps for the turret domain
- (g) EM density maps for the Zn^{2+} binding motif in the T1 domain

(h-I) EM density maps for the axonal targeting motif (ATM) and C-terminal extension for a particle subclass showing extra densities for two chains of the tetramer.



Supplemental Figure S5. Detailed mapping of disease mutations in the human *KCNC1* gene linked to EPM7 and related epileptic encephalopathies.

Variants of the human *KCNC1* gene associated with the autosomal dominant disorder EPM7 (progressive myoclonic eplilepsy-7) listed at www.malacards.org were mapped onto the structure of the Kv3.1a tetramer, represented as grey cartoon. A single protomer is coloured in light green to highlight mutations located at intersubunit interfaces. Red star symbols indicate mutations which have been characterized extensively in the literature and the epileptic phenotype of the variant has been confirmed. Mutations labeled with orange star symbols are annotated to be "likely pathogenic". Yellow star symbols indicate amino acid positions of mutations with "uncertain significance" according to the variant database.



Supplemental Figure S6. Comparison of the T1 domain structure in Kv3.1a with T1 domains of representative members from the Kv1 and Kv4 subfamilies

(a) Superposition of the T1 domain from the Kv3.1a structure in the presence of $Z_n^{2^+}$ (dark blue cartoon) with the T1 domain from the Kv1.3/ β 2.1 structure (green, pdb: 7EJI, shown as surface and cartoon), highlighting a clash of the Kv3.1 α 3 helix with helix h6 of the β -subunit.

(b) Superposition of the T1 domain of Kv3.1a (dark blue) with the T1 domains of Kv1.2-2.1 (yellow, pdb: 6EBK) and Kv1.3 (green, pdb: 7EJ1) showing how S6T extensions in Kv1.3 would clash with an α 6 helical arrangement similar to Kv3.1a.

(c) Comparison of the T1 arrangement in Kv3.1a (dark blue), Kv1.2-2.1 (green, pdb: 6EBK) and Kv4.2 (orange, pdb: 7F0J) after superposing the S4/S5 linker regions from one protomer of the respective full-length structures.

(d) Close contact between N-terminal T1 domain and c-terminal S6T segment in Kv4.2 (pdb 7F01). An inter-subunit salt bridge connects S6T with the cytoplasmic T1 domain. Neighboring chains are shown in orange and yellow, respectively.



Supplemental Figure S7. C-terminal extension beyond the axonal targeting motif (ATM) interacts with two neighboring chains of the T1 domain.

(a) Close-up view of the T1 domain from the Kv3.1a with chains from different subunits shown as green, yellow, blue and grey cartoon. Residues 452-472 of the C-terminus from chain A are shown in stick representation. Residues 452-463 shown in bright red and residues 464-472 shown in dark red.

(b) Inset from A, showing intra-subunit interactions between residues of the C-terminal extension (residues 464-472, shown in dark red) and residues in the $\alpha 2$ and $\alpha 3$ helices (yellow cartoon) of the T1 domain.



Supplemental Figure S8. Comparison of the Kv3.1a cryo-EM structure to model predicted by AlphaFold2 shows differences in the arrangement of S4 and the S4/S5 linker.

(a) Superposition of the protomer structure of human Kv3.1a predicted by AlphaFold2 (blue cartoon, taken from: alphafold.ebi.ac.uk/entry/P48547) onto 1 chain from the cryo-EM structure of Kv3.1a (yellow cartoon). The rest of the Kv3.1a tetramer is represented as grey cartoon.

(b) AlphaFold2 model for one protomer of Kv3.1a colored by level of confidence.

(c) Close-up showing the arrangement of the voltage-sensing S4 helix with positions of gating charges R2-R6 with respect to F356 of the CTC predicted by AlphaFold2. Compared to the experimentally determined cryo-EM structure, S4 is in a more upward-shifted position, with R4 located above F256 in the S1 segment.

(d) Close-up view of the S4/S5 linker region in the AlphaFold2 model, highlighting interactions to residues in S4 and a6 of the T1 domain. The model predicts a helical extension of two amino acids (S121 and F122) beyond the experimentally determined model. In this arrangement, the side chain of H336 interacts with the hydroxyl and backbone carbonyl groups of S121. K449 in S6T also interacts with a different set of residues (backbone carbonyls of V119 and G123). This moves the closest point of contact between S4/S5L and a6 in T1 closer to S4 (between R332 and H336), whereas this interdomain interaction is closer to S5 according to the cryo-EM structure (between H336 and R339, see main Figure 4 c). The R326/R332 H-bond between S4 and S4/S5 linker is present in the AI-predicted and the experimentally determined model.



Supplemental Figure S9. Comparison of electrostatic surface potential distribution for human Kv channel structures of Kv3.1a, Kv1.2-2.1 and Kv1.3. (a, b) Surface representation of human Kv3.1a in front view (A) and cross-sectional view (B). (c, d) Surface representation of human Kv1.2-2.1 (pdb:6EBK) in front view (C) and cross-sectional view (D). (e, f) Surface representation of human Kv1.3 in front view (E) and cross-sectional view (F). Surfaces are coloured by electrostatic potential (red, -5 kT e^{-1} ; blue, $+5 \text{ kT e}^{-1}$).



Kv4.3 T1 (2l2R)

Kv4.2 T1 (7F3F)

Supplemental Figure S10. Comparison of electrostatic surface charges near d4 in T1 of Kv3.1a and T1 domains from other representative channels of the Kv1 and Kv4 subfamilies.

(a) Slab view of the full length Kv3.1a structure in electrostatic surface representation. Left inset: Close-up view of the upper vestibule near the selectivity filter (SF). Densities for K^+ ions (purple spheres) in the SF and densities for unidentified small molecule(s) are shown as blue mesh.

Right inset: close-up view of the lower vestibule in T1 near helix a4, highlighting position of D81 in Kv3.1a.

(b) Close-up view of the lower vestibule in T1 near helix α 4 in the full-length rat Kv1.2-2.1 structure (pdb 6EBK).

(c) Close-up view of the lower vestibule in T1 near helix α 4 in the full-length human Kv1.3 structure (pdb 7EJ1).

(d) Close-up view of the lower vestibule near a4 for the rat Kv4.3 T1 structure (pdb 212R).

(e) Close-up view of the lower vestibule near $\alpha 4$ for the T1 domain in the full-length human Kv4.2 structure (pdb 7F3F).

Surfaces are coloured by electrostatic potential (red, -5 kT e^{-1} ; blue, $+5 \text{ kT e}^{-1}$). Residues in corresponding position to D81 in Kv3.1 are labeled in navy.



Supplemental Figure S11. Pore hydration properties and characterisation of the D81K mutant by MD simulations and TEVC recordings.

(a) Water density within the Kv3.1a channel calculated from unbiased MD simulations. The constriction site in the T1 domain at D81 is indicated by the red arrow symbol.

(b, c) Evolution of the pore radius in nm (B) and water density in nm³ within the Kv3.1a pore (D) over simulation time (in nsec), showing that the pore region near the PVP hinge remains hydrated throughout the simulation.

(d, e) K^{+} occupancy densities in simulations of wild-type (E) and D81K (F) mutant, illustrated at an isosurface value of 0.2 molecules/nm³. The interstitial void occupancy by K^{+} ions (indicated by the red arrow) is disrupted by the charge-inverting D81K mutation. The difference in abundance is seen in MD simulations at 0 mV and -300 mV.

(f, g) Current responses for wildtype and D81K evoked by 400-ms step depolarizations from a holding voltage of -100 mV. The steps are delivered in increments of 10 mV from -60 mV to 50 mV and the start-to-start interval is 5 s. The horizontal and vertical scale bars indicate 100 ms and 1 μ A, respectively.

(h) Representative current responses from D81K and WT Kv3.1a expressing oocytes (at + 50 mV, circles) and mean value (bars) for WT (black) and D81K mutant (red). P value for one-sided ANOVA test is indicated on the graph. Data are averages from 21 (WT) and 18 (D81K) oocytes, respectively.

(I) G-V curves for wildtype Kv3.1a (black circles) and mutant D81K (red circles). Data are averages from 9 oocytes, respectively. Solid lines represent the best first-order Boltzmann fits.





Supplemental Figure S12. Comparison of extra- and intracellular linker arrangement in the voltage-sensing domains of Kv3.1a and Kv1.2-2.1 and VSD arrangement in Kv4.2.

(a) Cartoon representation of the extracellular half of the Kv3.1a VSD (S1-S4) with S1/S2 and S3/S4 linkers shown in green and blue cartoon representation.

(b) Same region as in A but for the Kv1.2-2.1 VSD (pdb: 2R9R).

(c) Superposition of the VSDs from Kv3.1a (blue) and Kv1.2 (orange), using S1-S3 as a reference point. S4 (shown in a bolder colour than S1-S3) is upward shifted for Kv3.1 by one helical turn, compared to the position of S4 in Kv1.2.

(d) Cartoon representation of the intracellular half of the VSD (S1-S4) in KV1.3a, with the S2/S3 linkers shown in purple cartoon representation.

(e) Same as in D, but for the respective S2/S3 linker in Kv1.2-2.1 (pdb: 2R9R).

(f) Extracellular view of the VSD superposition of Kv3.1 and Kv1.2 in C.

(g) VSD arrangement in Kv4.2 (pdb: 7F0J).S3 is ommited for clarity. A salt bridge between R6 and R311 in S4/S5L is indicated by dashed lines.



Supplemental Figure S13. TEVC recordings of Kv3.1a channels in Xenopus oocytes.

(a-b) Representative whole-oocyte currents of wild type Kv3.1a (WT) with (+) and without (-) C-terminal FLAG tag. Currents are evoked by 400-ms step depolarizations from a holding voltage of -100 mV. The steps are delivered in increments of 10 mV from -60 mV to 50 mV, and the start-to-start interval is 5 s. The horizontal and vertical scale bars indicate 100 ms and 1 μ A, respectively.

(**c-k**) Representative whole-oocyte currents of several alanine replacement mutants introduced into the wild type Kv3.1a construct. The voltage protocol and scale bars are as described for panel a. For R332A and K449A, steps are extended to + 60 mV.

(I) G-V curves for wildtype Kv3.1a with (+) and without (-) the C-terminal FLAG tag. Data are scatter plots from 11 and 10 oocytes, respectively.



Supplemental Figure S14. Activation and deactivation time constants for mutant Kv3.1a channels in comparison to wildtype Kv3.1a.

(a) The voltage dependence of the time constants of activation (hollow symbols) and deactivation (filled symbols) from wildtype Kv3.1a (black circles) and mutant D114A (blue circles). Symbols and error bars represent the mean ± SEM. Error bars are smaller than the symbol size.

(b-h) same as (a), but data for mutants E116A, E117A, D120A, R332A, R339A, K449A and D81K respectively.

In each group, n=8-10 oocytes. To evaluate differences relative to WT, we use the one-way (or Kruskal-Wallis if necessary) ANOVA test. ^ P<0.05 # P<0.01 * P<0.001.

Exact values for n and P for the time constant comparisons at each voltage are listed in the source file included with this study.



Supplemental Figure S15. Z-position of the S4 gating charges R1-R4 relative to the CTC in Kv3.1a for subunits A-D in response to activating and deactivating potentials.

(a) Z-position of the S4 gating charges R1-R4 within the Kv3.1a subunits relative to the CTC (cyan dashed line) in response to application of activating (+300 mV) potentials.

(b-c) Z-position of the S4 gating charges R1-R4 within the Kv3.1a subunits relative to the CTC (cyan dashed line) in response to deactivating (-650 mV) potentials.



Supplemental Figure S16. Analysis of lipid/protein interactions in the human Kv3.1a channel.

(a) Lipid-protein interactions at Site I: a phospholipid (PC, green sticks) is bound near the S4/S5 linker (purple) and voltage-sensing helix S4 (blue) of Kv3.1a. The binding site exhibits similarity to the known PIP_2 (wheat-coloured sticks) binding site in the structure of Kv7.1 (pdb: 6V01).

(b) Lipid-protein interactions at Site II: a phospholipid (PC, green sticks) is bound at the interface between S6 (yellow) and PH (green) in Kv3.1a compared to phosphatidylcholine (PC, pink sticks) in the Kv1.2/Kv2.1 structure (pdb: 4JTC).

(c, d) MD simulations demonstrate that anionic lipids predominate interactions at lipid site I. In contrast, no lipid preferentially localizes to site II.

(c) Normalized number of contacts between Kv3.1a residues and headgroups of PS/PIP_2 . A contact was assumed if a residue's bead was within 5.5 Å of the lipid headgroup's bead and contacts were subsequently averaged across the four subunits. Residues with a contact frequency > 17.5% (half the ratio of anionic lipids in the inner leaflet) are coloured red.

(d) Densities of the anionic lipids calculated from CG simulations illustrated at one of the subunit interfaces at an isosurface value of 4.0 molecules/nm³.



Supplemental Figure S17. Illustration of dimer interface interactions for a subset of particles forming Kv3.1a dimers in the dataset with 400 μ M ZnCl₂.

Cartoon representation of the Kv3.1a dimer arrangement observed for a subset of particles present in the $ZnCl_2$ -containing EM dataset.

Inset: Close-up showing that the dimer interaction is facilitated by stabilizing salt bridges between the side chains of D51 and R52 in the cytoplasmic T1 domain.

	Region	n	V _{0.5} (mV)	Р	z (e ₀)	Р
WT FLAG(+)		29	8.5 ± 0.6		3.7 ± 0.1	
WT FLAG(-)		11	8.9 ± 0.7		3.8 ± 0.2	
D81K	Alpha 4/alpha5 linker	9	12.7 ± 0.6	0.014	3.1 ± 0.1	0.493
D114A	Alpha 6	9	-1.9 ± 0.9	3.0x10 ⁻⁶	3.8 ± 0.1	0.640
E116A	Alpha 6	10	4.4 ± 0.8	6.0x10 ⁻³	3.2 ± 0.1	0.068
E117A	Alpha 6	10	6.4 ± 0.9	0.274	3.6 ± 0.2	0.437
D120A	Alpha 6	11	11.0 ± 1.6	0.090	3.6 ± 0.3	0.833
E116A/D120A	Alpha 6	10	5.1 ± 0.8	0.027	3.0 ± 0.2	8.3x10 ⁻³
R332A	S4-S5	10	27.9 ± 1.7	1.1x10 ⁻¹⁰	2.9 ± 0.2	2.7x10 ⁻³
H336A	S4-S5	9	10.3 ± 1.1	0.260	3.8 ± 0.1	0.941
R339A	S4-S5	11	12.6 ± 1.2	0.017	3.7 ± 0.1	0.378
K449A	S6	10	21.4 ± 1.0	2.7x10 ⁻⁸	3.6 ± 0.1	0.409

Supplemental Table 2. Summary of best-fit Boltzmann function parameters of G-V curves obtained for wildtype or mutant Kv3.1a channels.

Parameters are expressed as mean \pm SEM from n oocytes. WT values are global.

P values are compared to WT from the same batch or consecutive combined batches. One-way ANOVA or Kruskall-Wallis ANOVA.

Supplementary Results & Discussion

Kv3.1a/Zn²⁺- structure does not support evidence for secondary Zn²⁺-binding sites

Gu *et al.* report that zinc reversibly binds to Kv3.1 at several sites other than T1 domain's zinc finger and this has various effects on ion channel activity, depending on where the sites are located [1]. Additionally, our structures suggest another pocket resembling zinc finger motif formed by H60, H66 and D81. Therefore, we collected datasets of Kv3.1 in the presence of 400 μ M ZnCl₂ or 1 mM EDTA to resolve any extra zinc-binding sites which may exist in Kv3.1a. However, no conformational change of Kv3.1a was observed between the two maps and no inorganic ion was seen in the ZnCl₂ sample in the four suspected sites (H60/D81, C208/H212, H381/H383 and H459). The first hypothesis to explain this is that zinc ions do not bind to Kv3.1 other than the zinc finger motif in the near-open state. There is also the possibility that zinc ions do not bind tightly to the suspected sites.

While we did not find evidence for additional zinc-binding sites with our structural biology efforts, we serendipitously discovered a potential cause of higher-order dimerisation seen with some tetrameric ion channels (Figure S3 b). Ion channels with T1 domain often show dimerisation at the distal end of T1 domain, for example a plant hyperpolarisation-activated K⁺ channel KAT1 [2]. On visual examination of electron micrographs, we found that Kv3.1a shows increased dimerisation in the presence of ZnCl₂ but does not form a dimer with 1 mM EDTA. Reconstruction of the dimer showed that this oligomer was stabilised by R51 and D53 of one subunit interacting with D53 and R51 with the other subunit, respectively (Figure S17). It is beyond the scope of this study to investigate the mechanism of such oligomerisation and its physiological significance, however our observation provides basis for further exploration of such feature.

Subclass map of Kv3.1a structure in EDTA shows C-terminus encircling T1 domain

Kv3.1a cryo-EM maps show protein density on the lateral side of T1 domain at low contour levels. In order to better resolve this region, we performed 3D variability analysis on Cryosparc with mask around the cytoplasmic domain. One of the modes showed this feature more clearly for two subunits diagonal to each other, and it was identified as further C-terminal extension of axonal targeting motif (ATM).

In this extended state, ATM makes contact with two T1 domains, the one of neighbouring subunit on its N-terminal side and T1 of its own subunit on the C-terminal side. If extrapolated to all four subunits, this would create a "ring" of ATM strand around the lateral side of T1 domain. Such positioning of ATM is similar to that of KChIPs, which bind to the sides of T1 domains of Kv4 channels [3, 4]. This suggests ATM's involvement in coordinating Kv3.1a gating between T1 domain and pore domain. This is supported by H459A mutation leading to shift in Kv3.1 activation threshold without affecting the channel's sensitivity to zinc [1], as well as the channel-inactivating K457Δ truncation associated with a rare form of epilepsy.

Small molecules occupy the aqueous cavity below Kv3.1 selectivity filter

We observe non-peptide density in the aqueous cavity on the cytoplasmic side of selectivity filter of Kv3.1a. Although the shape of the density is probably distorted due to its location on the four-fold symmetry axis, the generally tubular shape of this density parallel to the symmetry axis appears to be a genuine feature of this small molecule. This density fits well with the shape and size of polyamines such as spermidine coordinating with water (Figure. S10 a), as well as short-chain phospholipids and free fatty acids. Similar densities are also present in the cytoplasmic cavities of Kv1.2/2.1 chimera [5], NavAb [6], TRAAK [7], KirBac3.1 [8] and Kv4.2 [9] channels, all of them in the open state, suggesting that the presence of small molecules at this site is a common feature for ion channels.

Some features of this aqueous cavity suggests this molecule being a polyamine. First, the surface of this cavity is strongly negatively charged (Figure S9 b), an ideal environment for occupation by positively charged molecules. Although there is no report of Kv3.1 (or any Shaker superfamily channel) being modulated by polyamines, they are known open-pore blockers of inward-rectifying potassium channels (such as KirBac3.1) by occupying analogous site [8]. Interestingly, all known small molecule pore-blockers of Kv3.1 such as tetraethylammonium (TEA), 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP), and dimethylguanidine (DMG) feature amine group and are thought to occupy this aqueous cavity. These provide circumstantial evidence for this molecule being an amine derivative.

There is also the possibility of this molecule being a phospholipid or free fatty acid. While there is little structural evidence of this unlike the polyamine hypothesis, functional data exist for the modulation of Shaker superfamily channels by free fatty acids. For example, arachidonic acid is known to inhibit Kv4.2 and Kv1.4 [10]. It is also a rationally most sensible explanation as the aqueous cavity is still located within the transmembrane domain and phospholipids have been observed partially occupying this cavity in Nav1.4, albeit with a significantly different binding mode [11]. It is also possible that a detergent is trapped in the open pore, similar to the configuration observed in

the Nav1.4 structure from electric eel [12]. In contrast to the Nav1.4 structure where the pseudo 4fold symmetry is broken due to the complex formation with the β 1-subunit and the shape of the density matches well with a digitonin molecule, the 4-fold symmetrical appearance of the densities below the selectivity filter in our Kv3.1a structure could be an EM processing artefact. Because of its location on the 4-fold axis of symmetry, the density from the bound molecule is possibly distorted and may not faithfully resemble the shape of a detergent/lipid/free fatty acid, even if the data is processed in C1.

We performed metabolomics mass spectrometry in an effort to determine the identity of this molecule (data not shown), however the result was inconclusive. We did not detect presence of polyamines or other unique small molecules, however this might have been due to the molecules still being trapped in the protein as it collapsed in the denaturation process. We detected a number of fatty acids, most notably eicosanoic acid, however they might have been products of the fragmentation of annular phospholipids. Therefore, we could not ascertain the identity of this molecule and therefore excluded it from structural model despite its potential importance in ion channel function.

Supplementary Methods

Coarse Grain Simulations

Unresolved residues in the cryo-EM structure within the S1/S2, S3/S4 and T1/S1 loops were built using Modeller [13]. The long unresolved loop between the T1 α 6 and S1 helices was modelled as a combination of two unstructured regions $_{121}$ SFGGAP $_{126}$ and $_{159}$ DSPDGRPGGF $_{168}$. Coarse-grained simulations were performed using the Martini 2.2 force field [14] with the protein embedded in an asymmetric model membrane [15] using the Martini Bilayer Maker [16] of CHARMM-GUI [17]. After energy minimization and equilibration for 20 ns, three replicates of the system were simulated for 20 μ s each using Gromacs 2020 [18] with the protein backbone beads restrained to allow convergence of lipid interactions. The final 7.5 μ s of the simulation trajectories were used for analysis using the PyLipID [19] and MDTraj [20] libraries . A contact was assumed if a residue's bead was within 5.5 Å of a lipid head-group bead and occupancy probability density calculations were performed using a grid of 2 Å resolution.

Free-Energy perturbation calculations following the methodology of Corey et al. [21] were performed to ascertain the identity of lipid site I without preferential interactions in the CG

simulations. The PC lipid in CG resolution was alchemically transformed into an anionic PA lipid previously identified to occupy the analogous site in prokaryotic KcsA channels [22, 23]. Coulomb and van-der-Waals interactions were perturbed separately using 10 windows each along the λ chemical space. Each λ window was energy-minimized, equilibrated and then simulated for 300 ns using a leapfrog stochastic dynamics integrator. The *alchemlyb* library [24] was used to calculate energies from the individual windows through the BAR method [25]. Results were calculated as a mean of three independent perturbation simulations.

References

- 1. Gu, Y., J. Barry, and C. Gu, *Kv3 channel assembly, trafficking and activity are regulated by zinc through different binding sites.* J Physiol, 2013. **591**(10): p. 2491-507.
- 2. Clark, M.D., et al., *Electromechanical coupling in the hyperpolarization-activated K.* Nature, 2020. **583**(7814): p. 145-149.
- 3. Wang, H., et al., Structural basis for modulation of Kv4 K+ channels by auxiliary KChIP subunits. Nat Neurosci, 2007. **10**(1): p. 32-9.
- 4. Pioletti, M., et al., *Three-dimensional structure of the KChIP1-Kv4.3 T1 complex reveals a cross-shaped octamer.* Nat Struct Mol Biol, 2006. **13**(11): p. 987-95.
- 5. Matthies, D., et al., *Single-particle cryo-EM structure of a voltage-activated potassium channel in lipid nanodiscs.* Elife, 2018. **7**.
- 6. Wisedchaisri, G., et al., *Resting-State Structure and Gating Mechanism of a Voltage-Gated Sodium Channel.* Cell, 2019. **178**(4): p. 993-1003 e12.
- 7. Brohawn, S.G., E.B. Campbell, and R. MacKinnon, *Physical mechanism for gating and mechanosensitivity of the human TRAAK K+ channel.* Nature, 2014. **516**(7529): p. 126-30.
- 8. Clarke, O.B., et al., *Domain reorientation and rotation of an intracellular assembly regulate conduction in Kir potassium channels.* Cell, 2010. **141**(6): p. 1018-29.
- 9. Kise, Y., et al., *Structural basis of gating modulation of Kv4 channel complexes*. Nature, 2021. **599**(7883): p. 158-164.
- 10. Angelova, P.R. and W.S. Müller, *Arachidonic acid potently inhibits both postsynaptic-type Kv4.2 and presynaptic-type Kv1.4 IA potassium channels.* Eur J Neurosci, 2009. **29**(10): p. 1943-50.
- 11. Pan, X., et al., *Structure of the human voltage-gated sodium channel Na*. Science, 2018. **362**(6412).
- 12. Yan, Z., et al., *Structure of the Nav1.4-beta1 Complex from Electric Eel*. Cell, 2017. **170**(3): p. 470-482 e11.
- Sali, A. and T.L. Blundell, *Comparative protein modelling by satisfaction of spatial restraints*. J Mol Biol, 1993. 234(3): p. 779-815.
- 14. de Jong, D.H., et al., *Improved Parameters for the Martini Coarse-Grained Protein Force Field*. J Chem Theory Comput, 2013. **9**(1): p. 687-97.

- Duncan, A.L., R.A. Corey, and M.S.P. Sansom, *Defining how multiple lipid species interact with inward rectifier potassium (Kir2) channels*. Proc Natl Acad Sci U S A, 2020. **117**(14): p. 7803-7813.
- 16. Qi, Y., et al., *CHARMM-GUI Martini Maker for Coarse-Grained Simulations with the Martini Force Field.* J Chem Theory Comput, 2015. **11**(9): p. 4486-94.
- 17. Jo, S., et al., *CHARMM-GUI: a web-based graphical user interface for CHARMM.* J Comput Chem, 2008. **29**(11): p. 1859-65.
- 18. Abraham, M., et al., *High performance molecular simulations through multi-level parallelism from laptops to supercomputers*. 2015: SoftwareX.
- 19. Song, W., et al., *PyLipID: A Python Package for Analysis of Protein-Lipid Interactions from Molecular Dynamics Simulations*. J Chem Theory Comput, 2022. **18**(2): p. 1188-1201.
- 20. McGibbon, R.T., et al., *MDTraj: A Modern Open Library for the Analysis of Molecular Dynamics Trajectories.* Biophys J, 2015. **109**(8): p. 1528-32.
- 21. Corey, R.A., et al., *Insights into Membrane Protein-Lipid Interactions from Free Energy Calculations.* J Chem Theory Comput, 2019. **15**(10): p. 5727-5736.
- 22. Oakes, V., S. Furini, and C. Domene, *Effect of anionic lipids on ion permeation through the KcsA K.* Biochim Biophys Acta Biomembr, 2020. **1862**(11): p. 183406.
- Poveda, J.A., et al., Modulation of the potassium channel KcsA by anionic phospholipids: Role of arginines at the non-annular lipid binding sites. Biochim Biophys Acta Biomembr, 2019.
 1861(10): p. 183029.
- 24. Chodera, J.D., *A Simple Method for Automated Equilibration Detection in Molecular Simulations.* J Chem Theory Comput, 2016. **12**(4): p. 1799-805.
- 25. Bennett, C., *Efficient estimation of free energy differences from Monte Carlo data.* 1976: Journal of Computational Physics. p. 245-268.