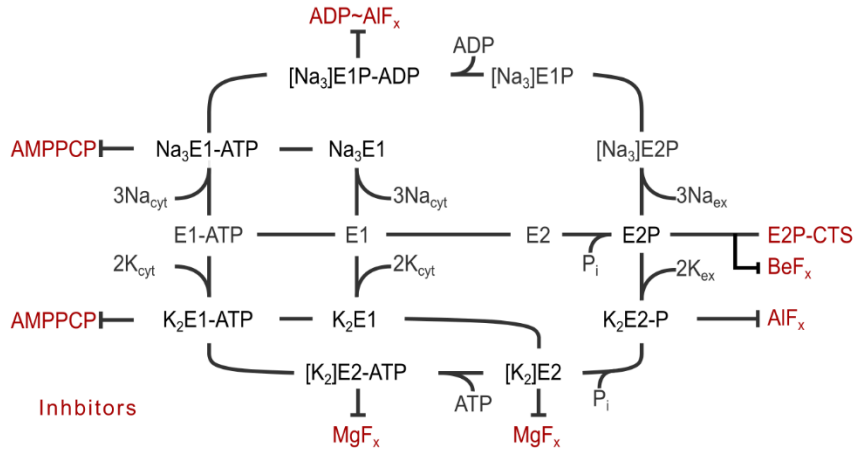
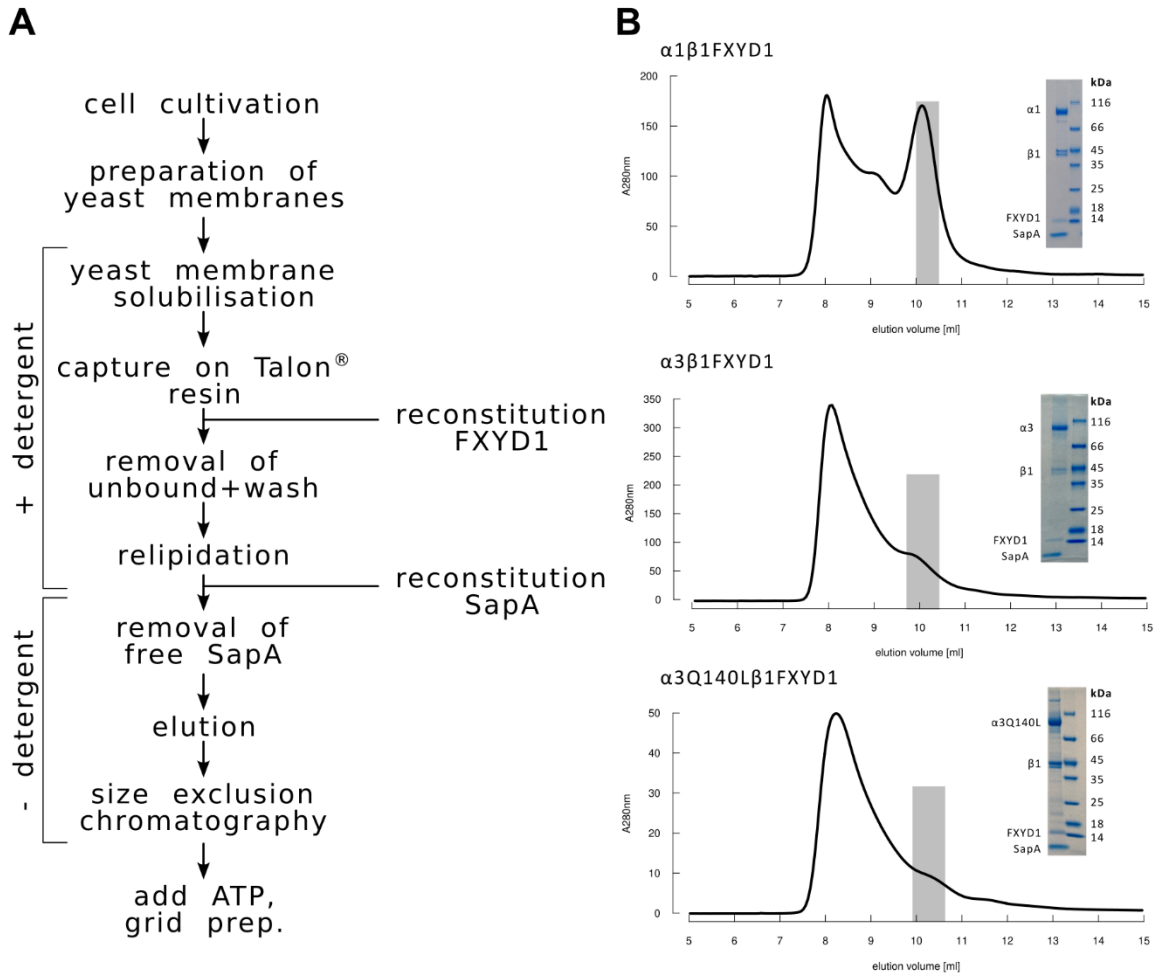
Supplementary Information

Supplementary Figure 1. Post Albers cycle of Na^+,K^+-ATPase



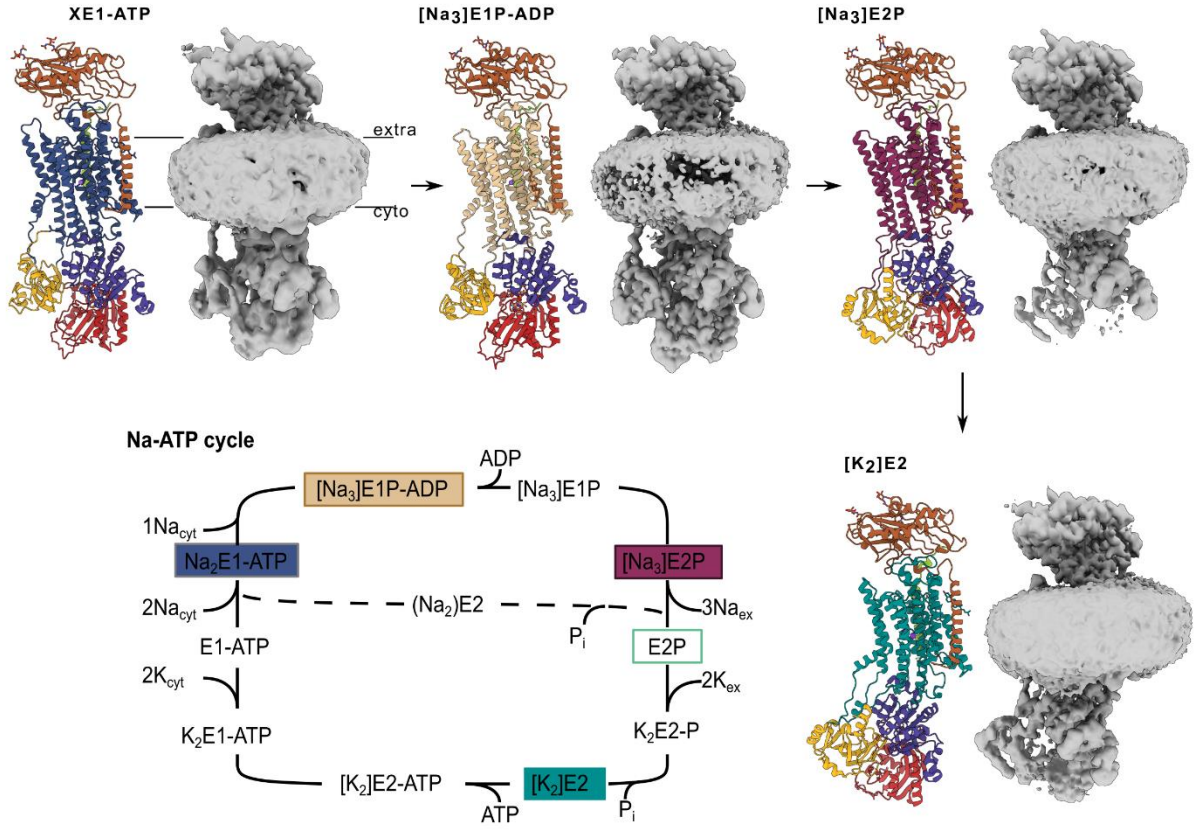
Post Albers scheme of the Na^+,K^+-ATPase. Conformations with prefix [Na3] or [K2] indicate occluded conformations. Inhibitors shown in red can be used to stabilise respective conformations when applied with appropriate ions and have been used for nanoDSF analysis shown in figure 3D.

Supplementary Figure 2. Purification of Na^+,K^+-ATPase in Saposin A nanodiscs



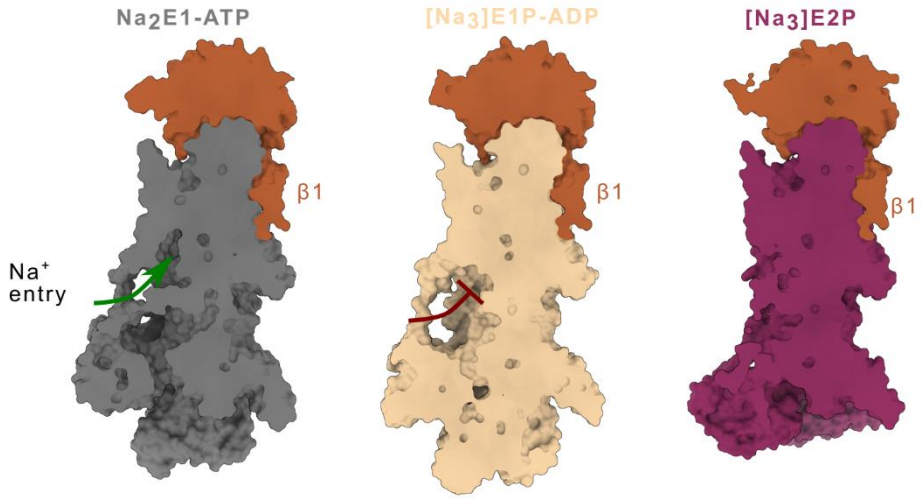
(A) Flowchart depicting the purification and reconstitution steps. (B) SDS-gels and size exclusion chromatograms of respective samples. Grey bars indicate fractions that were pooled and applied on grid. Please note that the minor size-exclusion peak at 10.5ml f used for cryoEM is predominantly monomeric while the majority of sample eluted at the void volume. Optimisation of the Saposin A reconstitution protocol did not increase the monomeric fraction, however ATPase activity measurements of the void fraction of α1β1-complexes showed activity of the void to be comparable to the monomeric fraction. Hence the void fraction is considered to represent functional but larger oligomeric assemblies that are not suitable for single particle cryo-EM.

Supplementary Figure 3. Cryo-EM of Na^+,K^+-ATPase under full turnover conditions in the presence of Na, K and ATP



Cryo-EM maps and structures of α3β1FXYD1 obtained in the presence of saturating concentrations of NaCl, KCl and ATP. Note that cations for the E1-ATP conformation have been donated X since it is unclear whether the state represents a Na+-, K+- or mixed Na+/K+- bound state. Under the given experimental conditions, a mix of all three is likely for this dynamic state. The Post-Albers scheme shows the conformation obtained under full turnover in filled coloured rectangles while conformations obtained under Na-only turnover are indicated in boxed rectangles.

Supplementary Figure 4. Comparison of Na+-bound conformations and the cytoplasmic access pathway

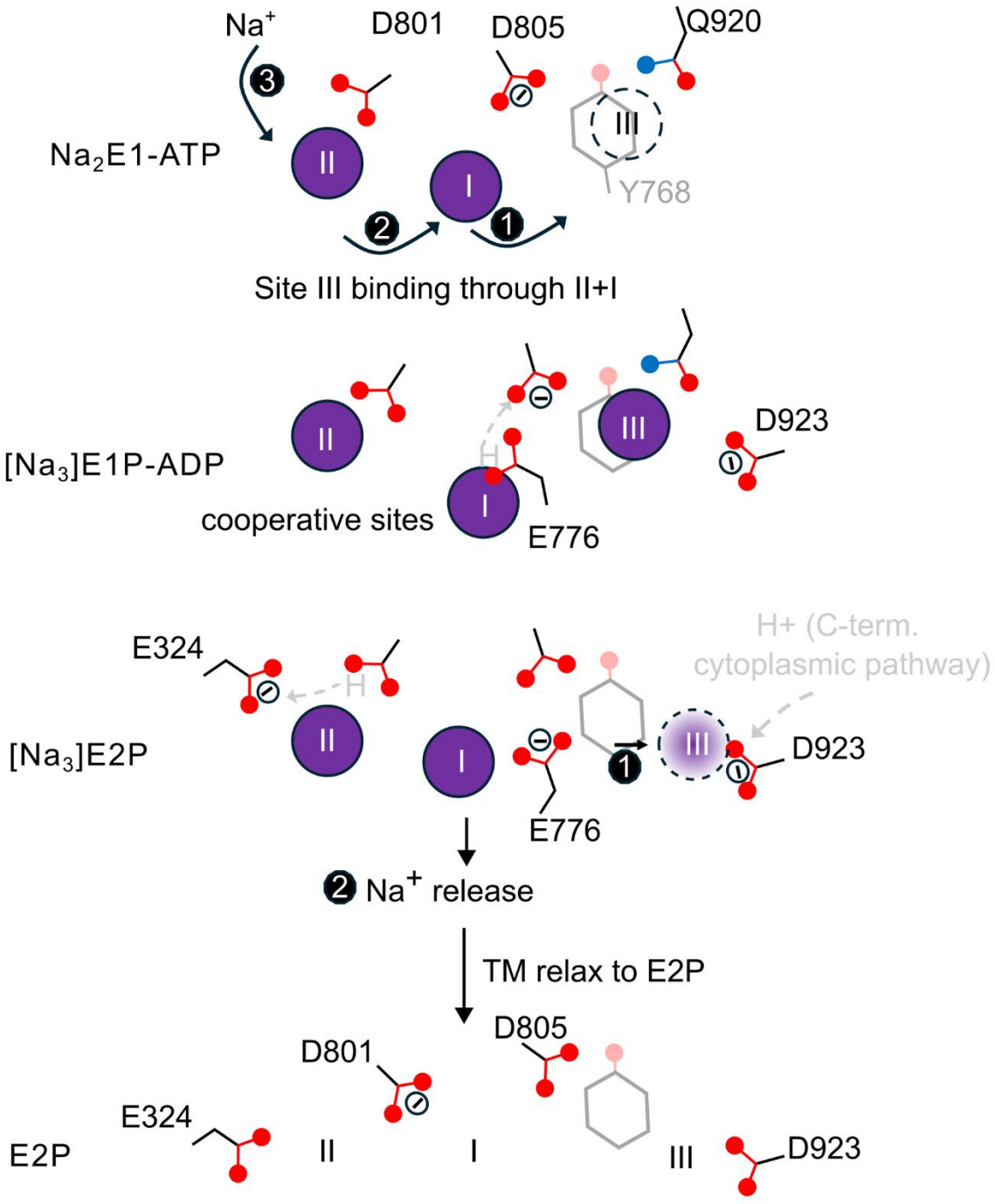


Clipped view of the Na+-bound states showing the opening towards the ion binding sites in E1 compared to the two occluded forms.

Supplementary Figure 5 Alignment of published α3β1 structures with structures obtained in this study

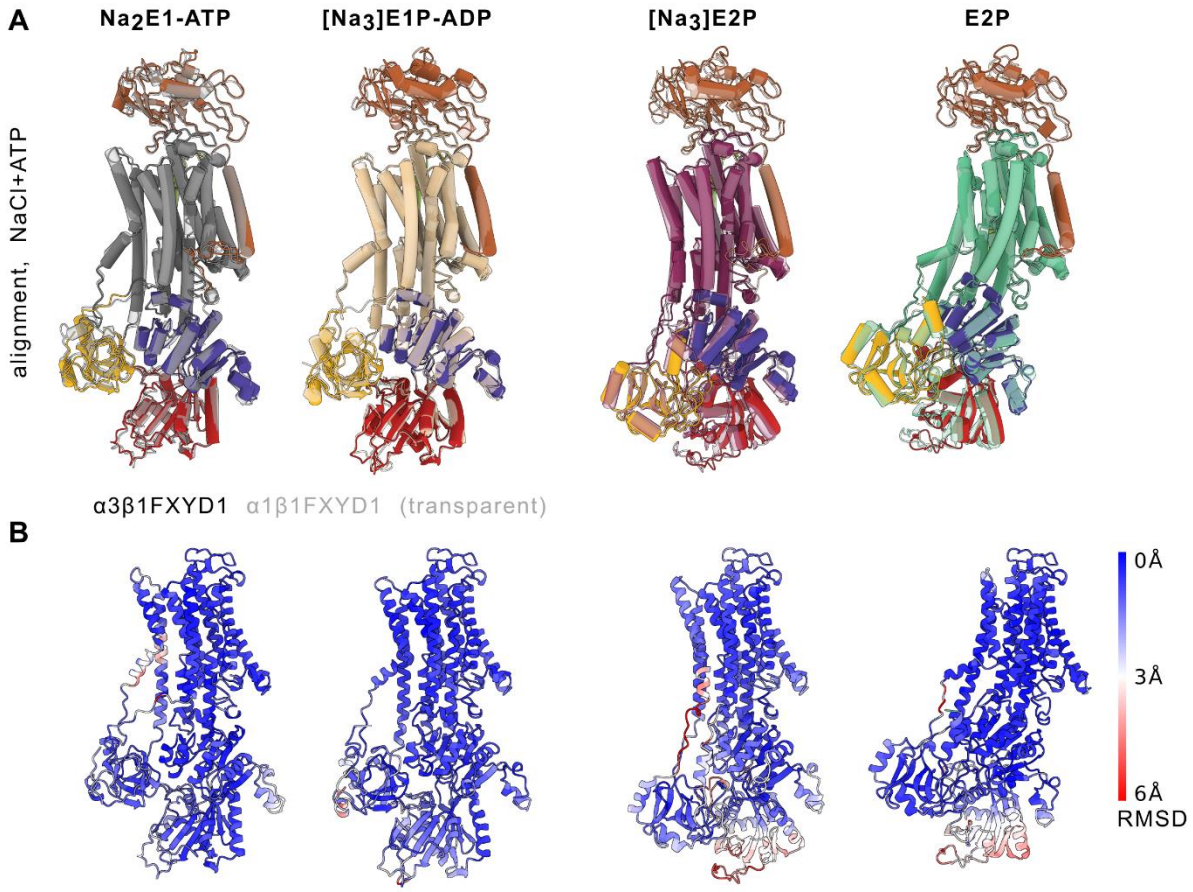
Models presented by Nguyen et al. are shown in semi-transparent view. Cα RMSD is plotted on the bottom graph. Please note the major difference for the Na_2E1-ATP state around TM1 (residue range 85-91. Also, the K^+-bound E2 conformation shows deviations around A and N-domain while the TM-domain is nearly identical.

Supplementary Figure 6. Sequence of Na^+-binding and release



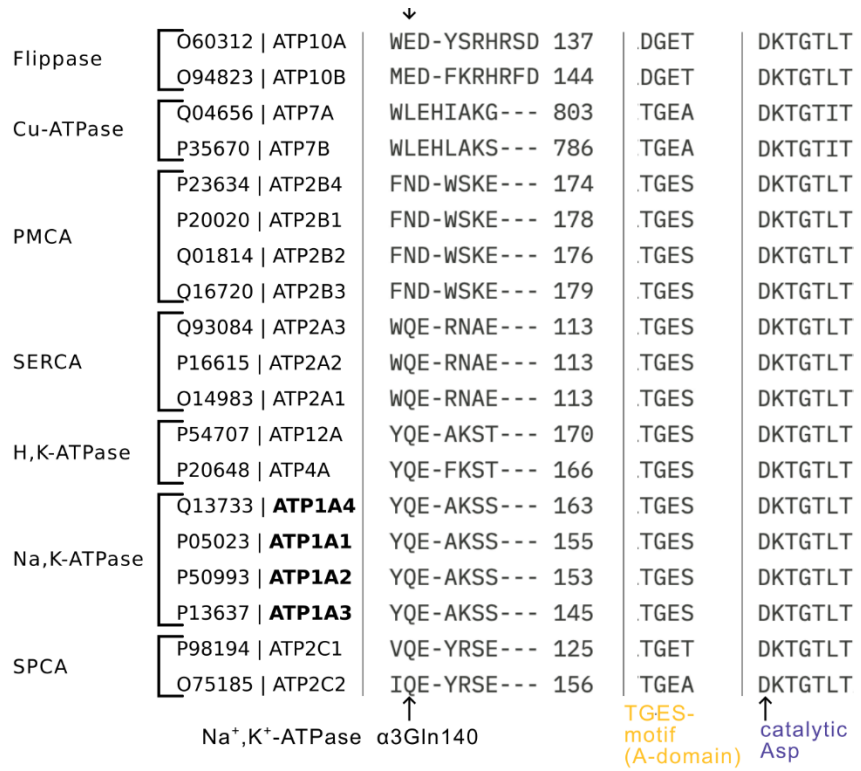
Black arrows indicate movements of sodium ions (purple), grey arrows of protons. Deprotonated residues are indicated by Θ. The model is based on structural findings in this study with information on protonation of residues gathered from references 44-47.

Supplementary Figure 7. Comparison of Na^+,K^+-ATPase α1 and α3



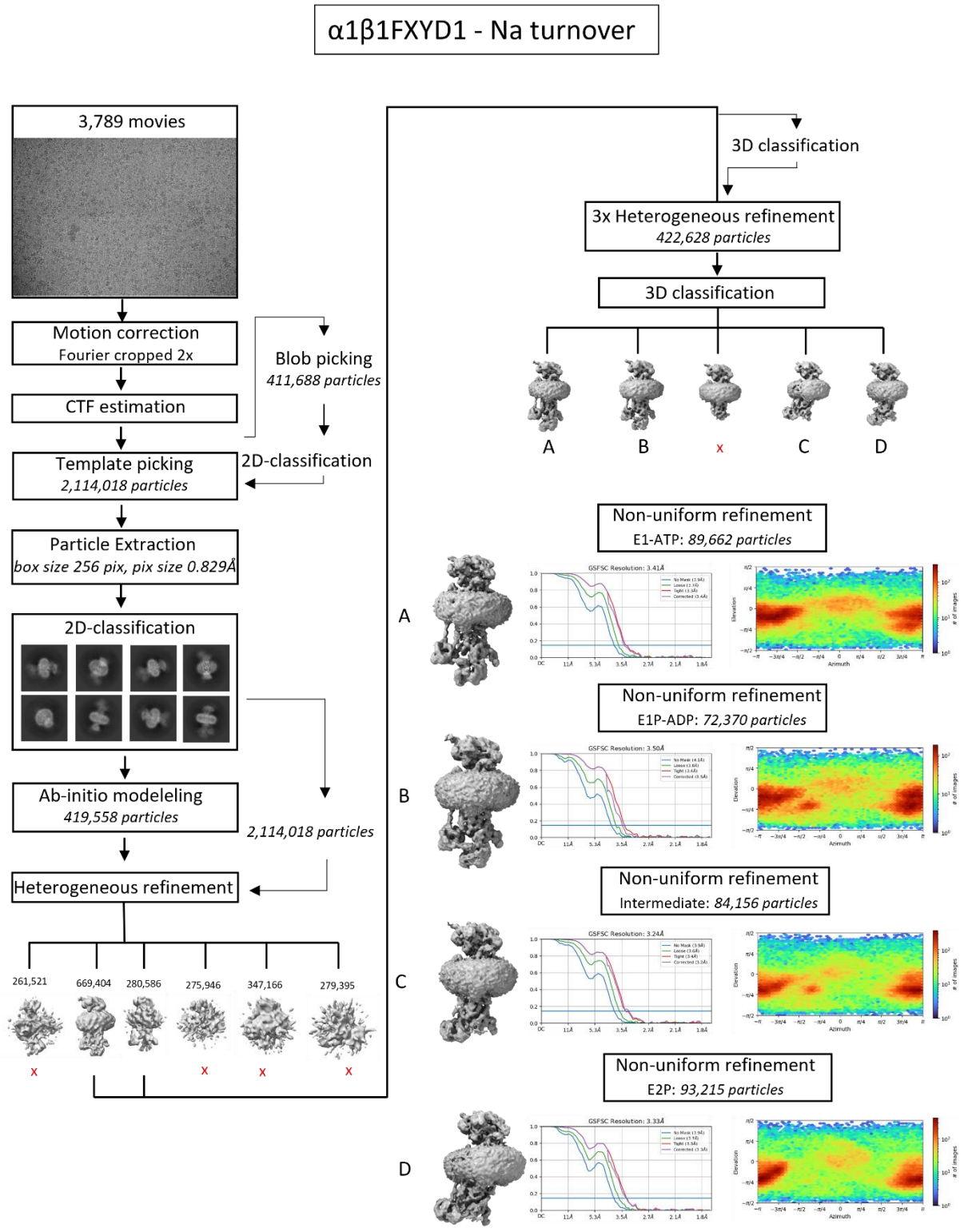
(A) Alignment of α1 (semi-transparent) and α3 structures on the β-subunit. (B) Cα-RMSDs for alignments shown in (A).

Supplementary Fig 8. Sequence alignment of human P-type ATPases



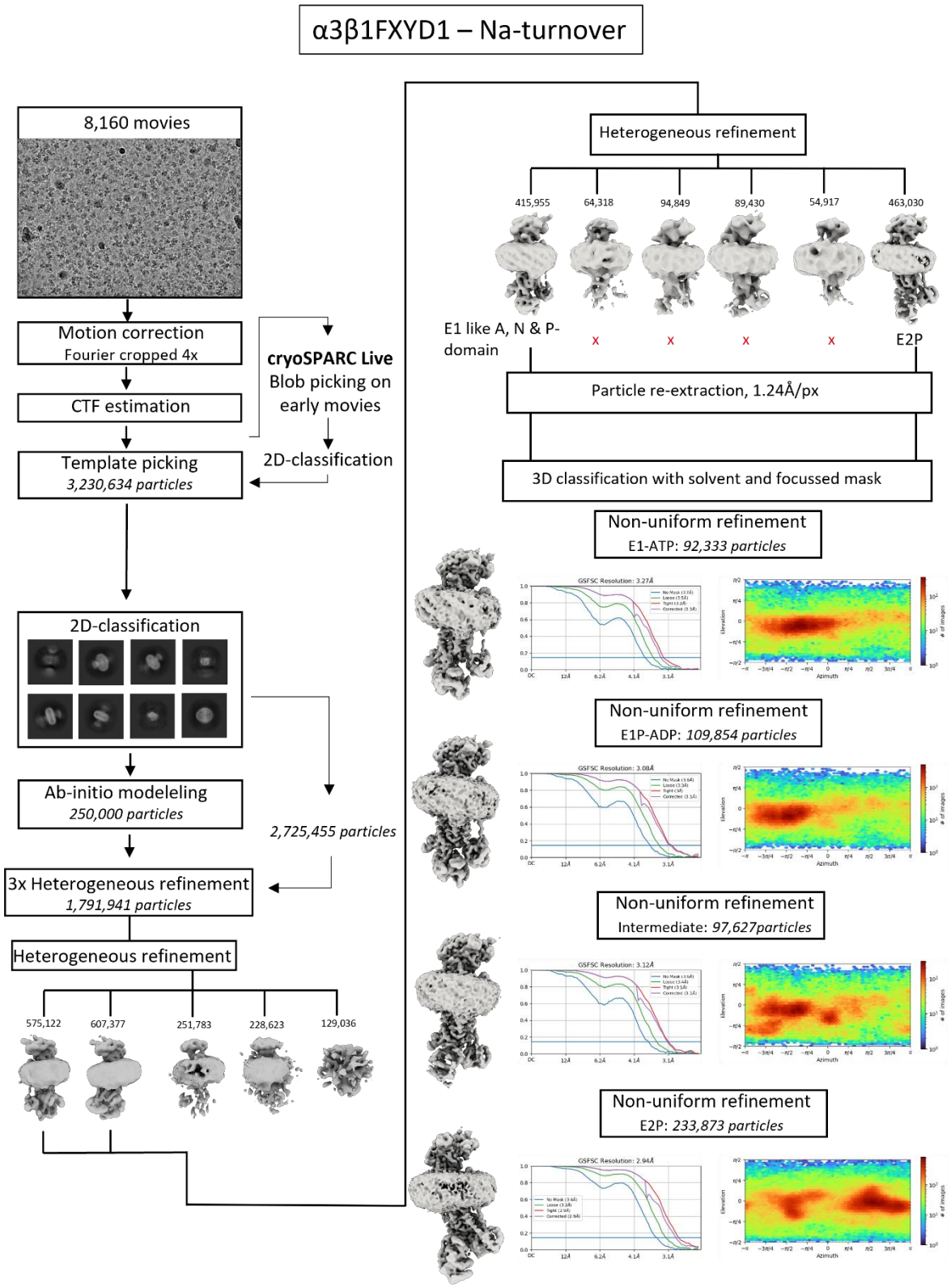
The alignment was performed using the EMBL-EBI MSA clustal Omega site using indicated uniport IDs.

Supplementary Fig 9A CryoSparc processing workflows



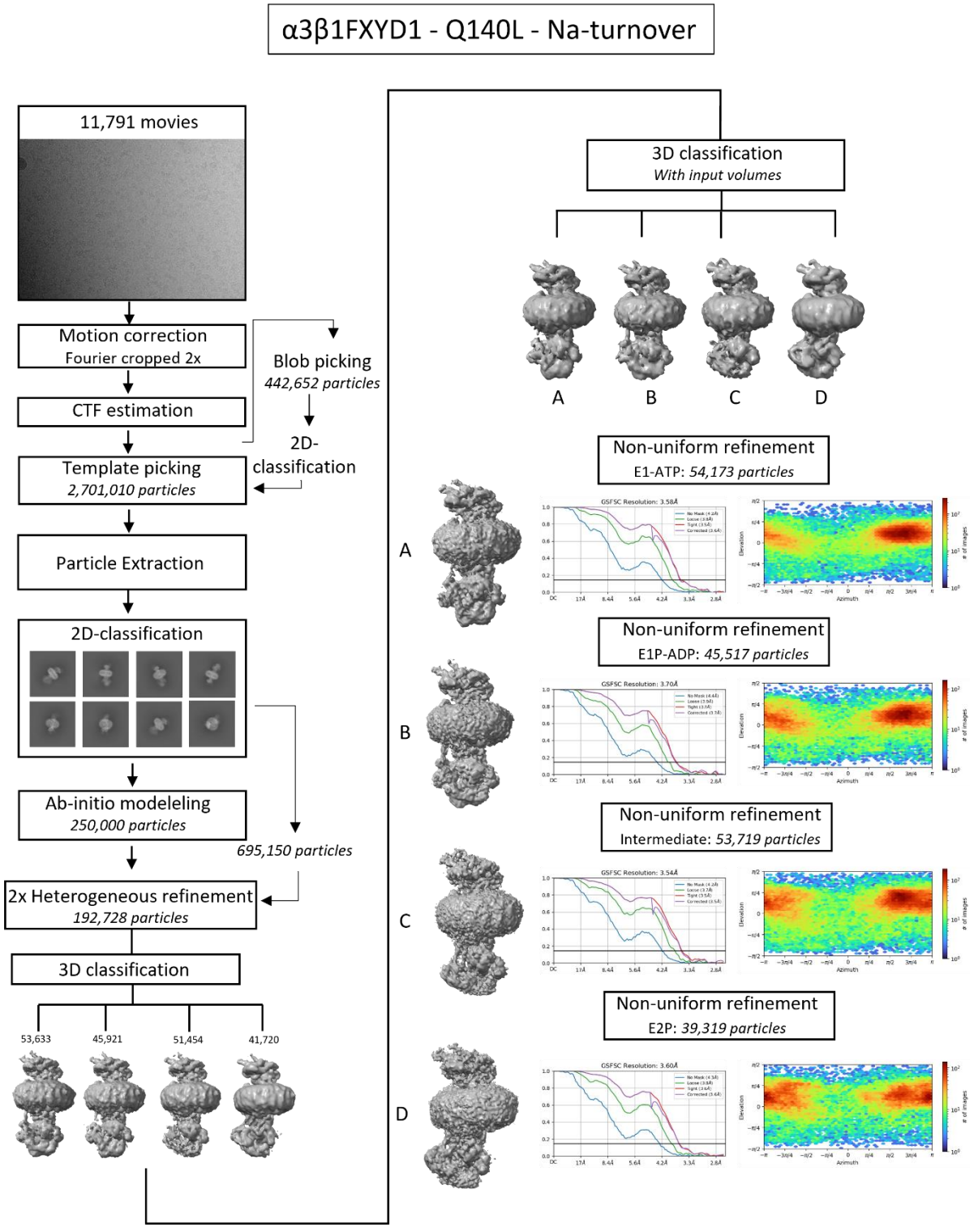
Resolution is determined at 0.143 FSC threshold. See supplementary figure 10B for local resolution maps.

Supplementary Fig 9B CryoSparc processing workflows



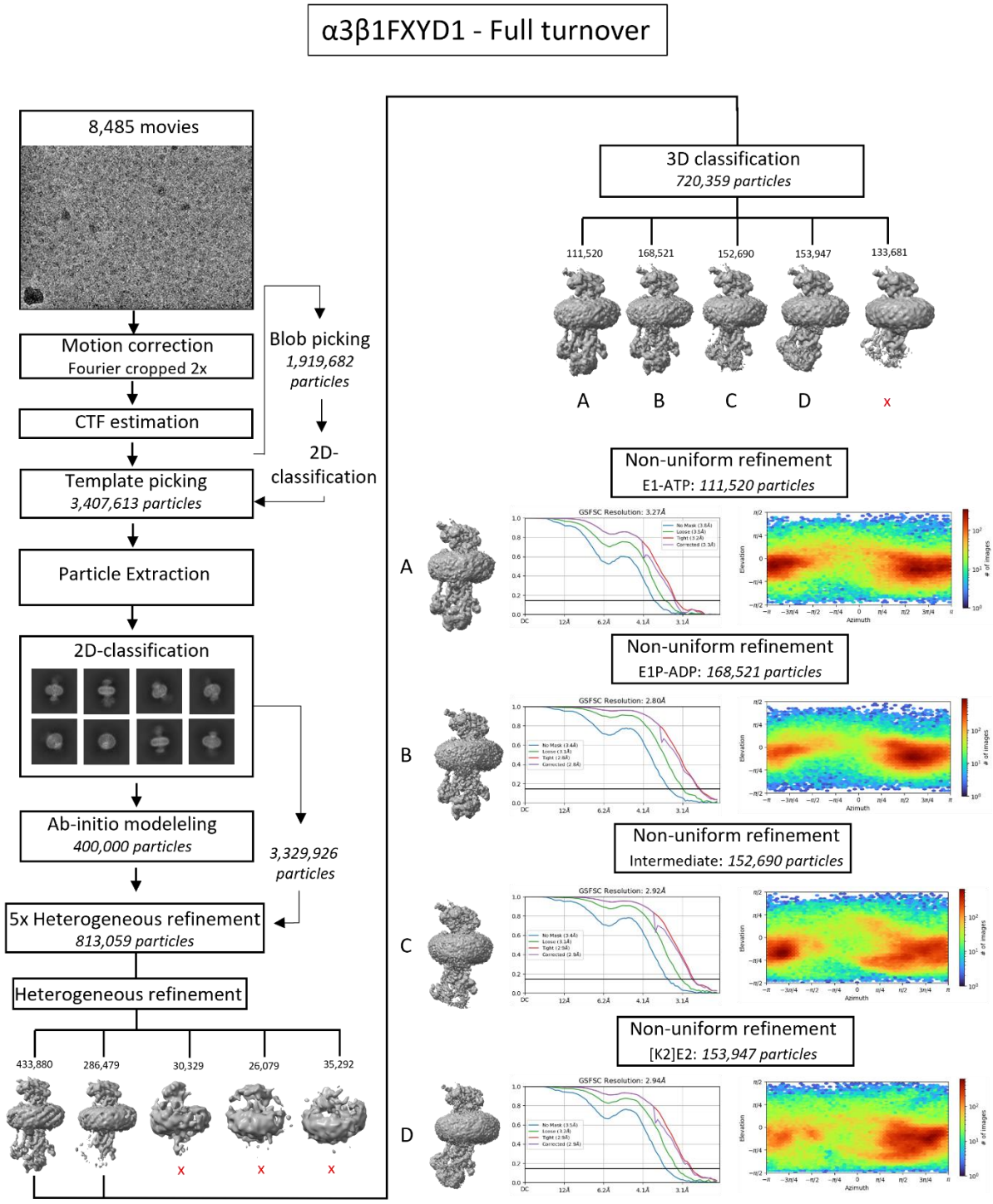
Resolution is determined at 0.143 FSC threshold. See supplementary figure 10A for map to model fit and local resolution maps.

Supplementary Fig 9C CryoSparc processing workflows



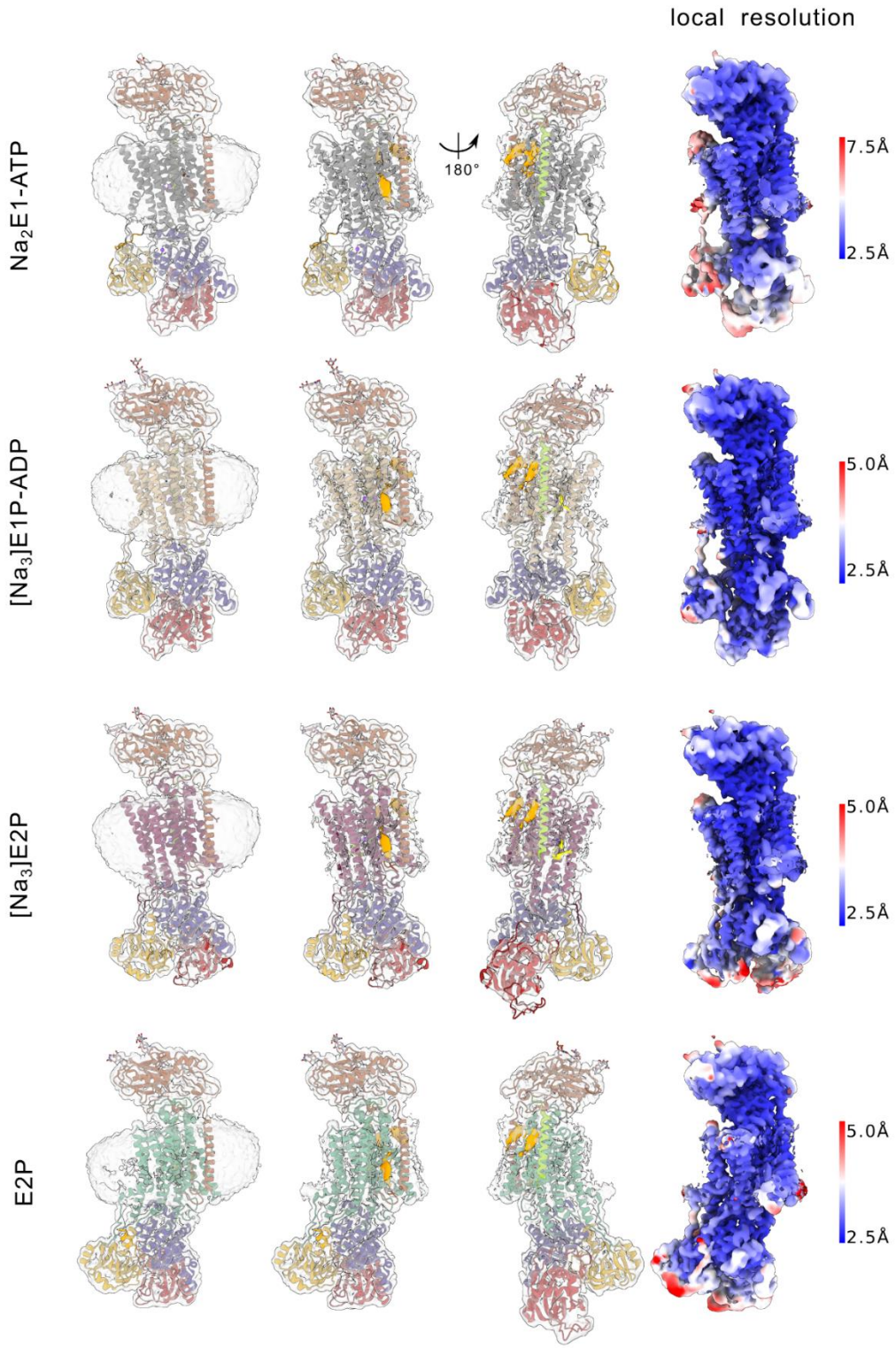
Resolution is determined at 0.143 FSC threshold. See supplementary figure 10B for local resolution maps.

Supplementary Fig 9D CryoSparc processing workflows



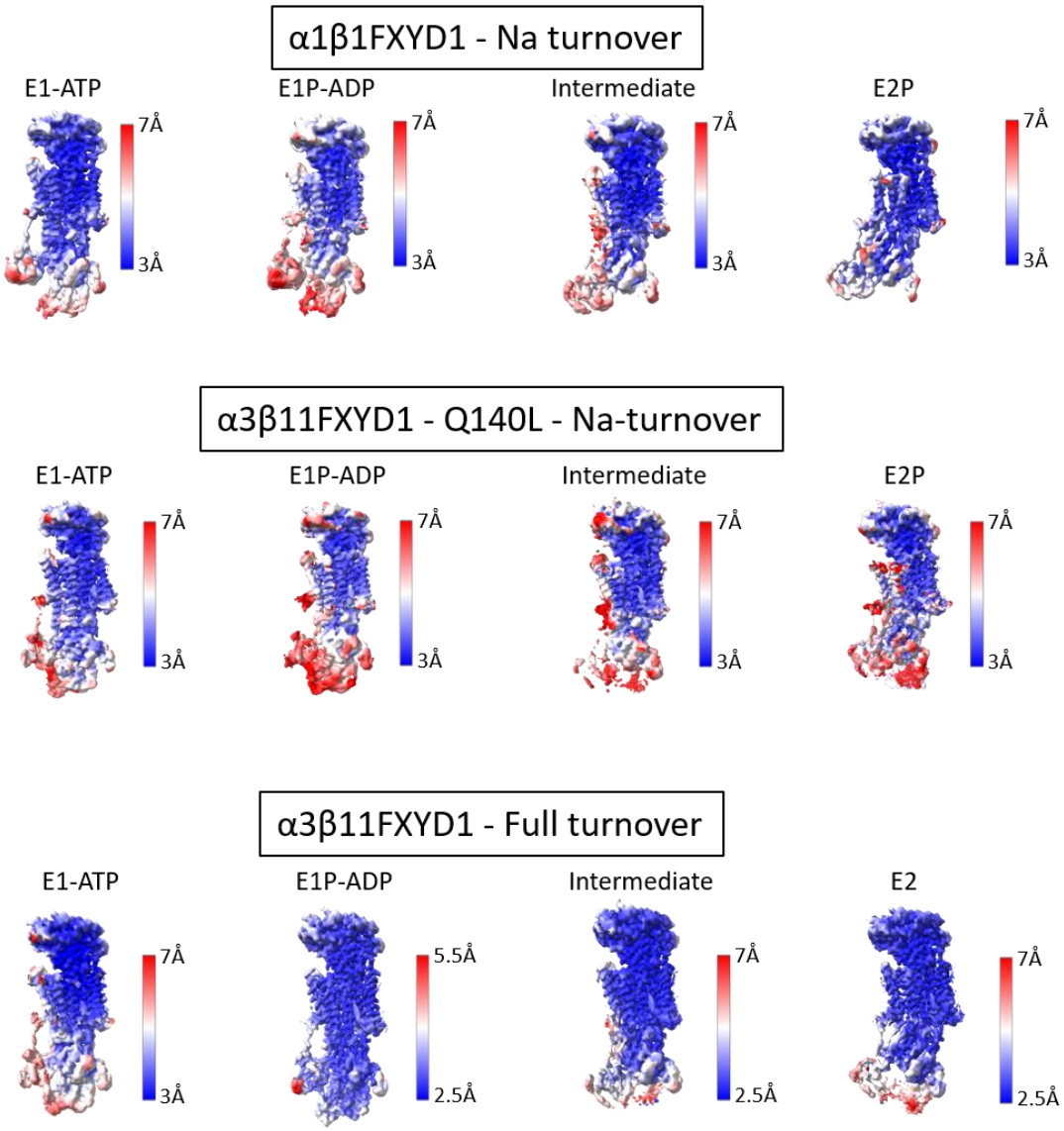
Resolution is determined at 0.143 FSC threshold. See supplementary figure 10B for local resolution maps.

Supplementary Fig 10A. Map to model fit and local resolution plots for α3β1 structures obtained under Na^+-turnover condition



Cryo-EM densities are shown either uncropped or with the SaposinA nanodisc removed and density contoured at 4 s.d. within 3Å radius of the model. Cholesterol densities are shown in orange, and phospholipid density is marked in yellow.

Supplementary Fig 10B. Local resolution plots of the other three datasets. (1) α1β1 structures obtained under Na^+-turnover condition, (2) α3Q140Lβ1 structures obtained under Na^+-turnover condition and (3) α3β1 structures obtained under full-turnover condition



Supplementary Table 1A

Refinement statistic α1β1FXYD1 – Na^+- turnover

	9RON EMD-54125 $Na_2E1 \cdot ATP$	9ROO EMD-54126 $[Na_3]E1P \cdot ADP$	9ROP EMD-54127 $[Na_3]E2P$	9ROQ EMD-54128 E2P
Data collection and processing				
Microscope		Titan Krios I (eBIC)		
Magnification		130,000		
Voltage (kV)		300		
Pixel size (Å)		0.414		
Electron dose ($e^-/Å^2$)		58.8		
Defocus range (μm)		-0.6 to -1.8		
Nr. of collected micrographs		3,789		
Initial particle picks		2,114,018		
Final particle picks	89,662	72,370	84,156	93,215
Refinement & validation				
Map resolution (Å)				
FSC 0.143	3.41	3.50	3.24	3.33
Map sharpening B-factor ($Å^{-2}$)	-102.7	-107.2	-103.0	-102.5
Initial model used (PDB)	3WGU	3WGU	3WGU/4HYT	4HYT
Model composition				
non-hydrogen atoms	10,272	10,161	10,269	10,306
Protein residues	1306	1296	1309	1313
Ligands	1	0	0	0
R.m.s. deviations				
Bond length (Å)	0.003	0.003	0.004	0.003
Bond angles (°)	0.583	0.607	0.551	0.614
Validation				
MolProbity score	2.11	1.92	1.96	2.04
Clash score	16.44	12.48	11.42	12.69
Rotamer outliers (%)	0.36	0.09	0.27	0.18
Ramachandran (%)				
Favored	94.15	95.58	94.24	93.57
Allowed	5.69	4.42	5.76	6.35
Outliers	0.15	0	0	0.08
Rama-Z				
whole	0.01	-0.97	-0.80	-1.5
helix	1.13	-0.1.	0.48	-0.35
sheet	-0.76	-1.20	-0.88	-1.22
loop	-0.92	-0.91	-1.45	-1.48

Supplementary Table 1B

Refinement statistic α3β1FXYD1 – Na^+-turnover

	9RO9 EMD-54113 $Na_2E1 \cdot ATP$	9ROA EMD-54114 $[Na_3]E1P \cdot ADP$	9ROD EMD-54115 $[Na_3]E2P$	9ROE EMD-54116 E2P
Data collection and processing				
Microscope		Titan Krios II (EMBION)		
Magnification		130,000		
Voltage (kV)		300		
Pixel size (Å)		0.647		
Electron dose ($e^-/Å^2$)		59.3		
Defocus range (μm)		-0.6 to -1.8		
Nr. of collected micrographs		8,160		
Initial particle picks		3,230,634		
Final particle picks	92,333	109,854	97,627	233,873
Refinement & validation				
Map resolution (Å)				
FSC 0.143	3.27	3.08	3.12	2.94
Map sharpening B-factor ($Å^{-2}$)	-86.7	-85.9	-90.5	-96.3
Initial model used (PDB)	9RON	This study 9ROO	9ROP	9ROM
Model composition				
non-hydrogen atoms	10,301	10,212	10,331	10,429
Protein residues	1297	1277	1300	1312
Ligands	11	16	11	9
R.m.s. deviations				
Bond length (Å)	0.003	0.004	0.003	0.003
Bond angles (°)	0.530	0.525	0.512	0.474
Validation				
MolProbity score	1.92	1.71	1.90	2.29
Clash score	10.80	7.33	9.32	15.34
Rotamer outliers (%)	0.18	0.36	0.54	1.87
Ramachandran (%)				
Favored	94.58	95.65	93.88	94.17
Allowed	5.19	4.35	6.12	5.68
Outliers	0.23	0	0	0.15
Rama-Z				
whole	0.40	0.74	0.55	0.73
helix	1.84	1.26	1.85	1.72
sheet	-1.05	0.07	-0.86	0.11
loop	-0.94	-0.17	-0.94	-0.67

Supplementary Table 1C

Refinement statistic α3Q140Lβ1FXYD1 - Na^+-turnover

	9ROJ EMD-54121 $Na_2E1 \cdot ATP$	9ROK EMD-54122 $[Na_3]E1P \cdot ADP$	9ROL EMD-54123 $[Na_3]E2P$	9ROM EMD-54124 E2P
Data collection and processing				
Microscope		Titan Krios II (eBIC)		
Magnification		130,000		
Voltage (kV)		300		
Pixel size (Å)		0.653		
Electron dose ($e^-/Å^2$)		59.8		
Defocus range (μm)		-0.6 to -1.8		
Nr. of collected micrographs		11,791		
Initial particle picks		2,701,010		
Final particle picks	54,173	45,517	53,719	39,319
Refinement & validation				
Map resolution (Å)				
FSC 0.143	3.58	3.70	3.54	3.60
Map sharpening B-factor ($Å^{-2}$)	-65.4	-72.7	-70.4	-66.5
Initial model used (PDB)	9RO9	This study 9ROA	9ROD	9ROE
Model composition				
non-hydrogen atoms	10,266	10,127	10,299	10,400
Protein residues	1297	1277	1300	1315
Ligands	7	7	6	6
R.m.s. deviations				
Bond length (Å)	0.002	0.003	0.003	0.004
Bond angles (°)	0.486	0.530	0.525	0.584
Validation				
MolProbity score	1.81	1.99	1.83	2.01
Clash score	8.50	11.56	8.13	12.07
Rotamer outliers (%)	0.18	0.36	0.09	0.08
Ramachandran (%)				
Favored	94.89	93.75	94.19	93.78
Allowed	5.11	6.17	5.81	5.14
Outliers	0	0.08	0	0.08
Rama-Z				
whole	0.47	-0.15	0.00	-0.20
helix	2.28	0.76	1.88	1.04
sheet	-1.18	-0.37	-1.25	-0.73
loop	-1.19	-0.86	-1.56	-1.14

Supplementary Table 1D

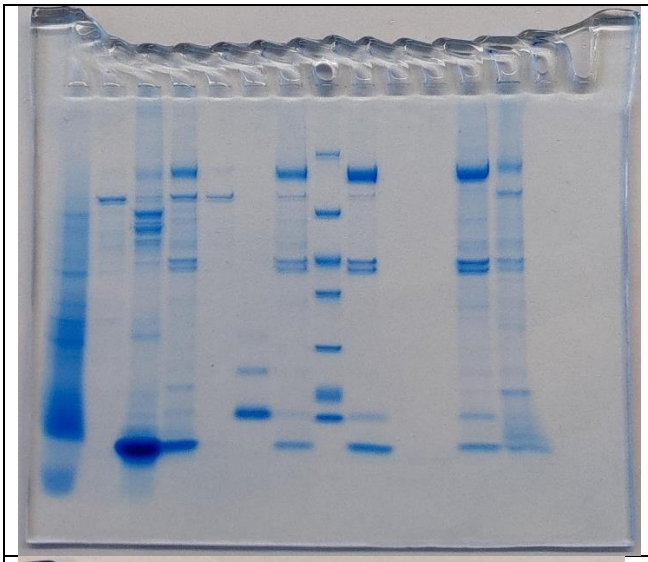
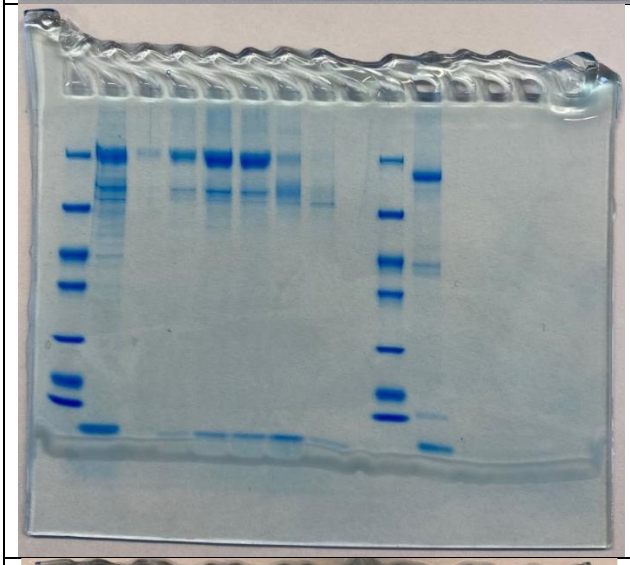
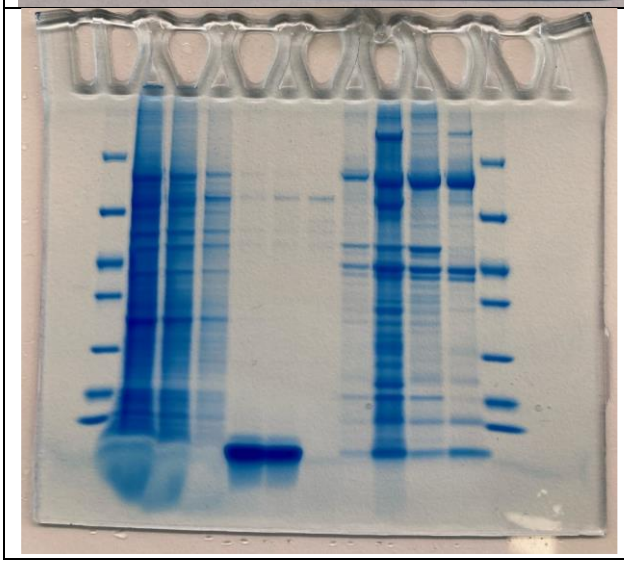
Refinement statistic α3β1FXYD1 - full turnover

	9ROF EMD-54117 X_2E1 · ATP	9ROG EMD-54118 [Na_3]E1P · ADP	9ROH EMD-54119 [Na_3]E2P	9ROI EMD-54120 [K_2]E2P
Data collection and processing				
Microscope		Titan Krios II (EMBION)		
Magnification		130,000		
Voltage (kV)		300		
Pixel size (Å)		0.647		
Electron dose (e^-/Å^2)		59.3		
Defocus range (μm)		-0.6 to -1.8		
Nr. of collected micrographs		8,485		
Initial particle picks		3,407,613		
Final particle picks	111,520	168,521	152,690	153,947
Refinement & validation				
Map resolution (Å)				
FSC 0.143	3.27	2.80	2.92	2.94
Map sharpening B-factor (Å^{-2})	-91.9	-83.8	-88.8	-102.3
Initial model used (PDB)	9RO9	This study 9ROA	9ROD	3KDP
Model composition				
non-hydrogen atoms	10,299	10,156	10,304	10,355
Protein residues	1297	1277	1300	1306
Ligands	9	10	8	8
R.m.s. deviations				
Bond length (Å)	0.002	0.003	0.003	0.003
Bond angles (°)	0.565	0.561	0.580	0.550
Validation				
MolProbity score	2.00	1.76	1.84	1.90
Clash score	12.45	6.34	8.33	9.39
Rotamer outliers (%)	0.36	1.55	1.17	1.34
Ramachandran (%)				
Favored	94.11	96.13	95.12	96.07
Allowed	5.89	3.87	4.80	3.93
Outliers	0	0	0.08	0
Rama-Z				
whole	-0.15	1.30	0.41	-0.56
helix	0.98	1.87	1.68	0.04
sheet	-0.19	-0.73	-0.34	-0.90
loop	-1.17	0.25	-1.02	-0.54

Supplementary Table 2 Ouabain binding, Na^+,K^+-ATPase and turnover numbers determined in yeast membranes. Each value ±SEM represents the average of 3-5 determinations.

Membranes	**Ouabain binding (pmol/mg)**	**Na,K-ATPase activity (µmol/mg/min)**	**Turnover rate (min^{-1})**
α1β1 (wt)	15.5±0.6	0.131±0.002	8452±456
α1Q150Lβ1	10.2±0.3	0.019±0.002	1863±251 (22% of wt)
α1Q150Hβ1	15.9±0.7	0.073±0.003	4591±391 (54% of wt)
α3β1 (wt)	8.1±0.2	0.044±0.004	5432±628
α3Q140Lβ1	7.4±0.3	0.004±0.0003	541±63 (10% of wt)

Uncropped gel files from supplementary figure 2

	**α1β1FXYD1**, lanes from right): 1-7.) samples from different purification steps 8.) Marker 9.) collected monomer fraction SEC (EM sample) 10-11.) - 12-13.) samples from different purification steps
	**α3β1FXYD1**, lanes from right: 1.) Marker 2-8.) undisclosed sample 10.) Marker 11.) collected monomer fraction SEC (EM sample)
	**α3Q140Lβ1FXYD1**, lanes from right): 1.) Marker 2-10.) samples from different purification steps 11.) collected monomer fraction SEC (EM sample) 12.) Marker