Structural basis for gating mechanism of the human sodium-potassium pump

P2-type ATPase sodium-potassium pumps (Na’/K’-ATPases) are ion-transporting enzymes that use ATP to transport Na’ and K’ on opposite sides of the lipid bilayer against their electrochemical gradients to maintain ion concentration gradients across the membranes in all animal cells. Despite the available molecular architecture of the Na’/K’-ATPases, a complete molecular mechanism by which the Na’ and K’ ions access into and are released from the pump remains unknown. Here we report five cryo-electron microscopy (cryo-EM) structures of the human alpha3 Na’/K’-ATPase in its cytoplasmic side-open (E1), ATP-bound cytoplasmic side-open (E1•ATP), ADP•AlF4− trapped Na’-occluded (E1•P•ADP), BeF3− trapped exoplasmic side-open (E2P) and MgF4− trapped K’-occluded (E2•P) states. Our work reveals the atomically resolved structural detail of the cytoplasmic gating mechanism of the Na’/K’-ATPase.

The type-2 P-type sodium-potassium pump (Na’/K’-ATPase), first discovered in the 1950’s by Jens Christian Skou, plays significant roles in maintaining the electrochemical gradients for Na’ and K’ across the plasma membrane of animal cells. By utilizing energy from the hydrolysis of a single ATP, the Na’/K’-ATPase pumps three Na’ ions out and two K’ ions into the cell in each transport cycle. Structurally, the Na’/K’-ATPase is composed of three subunits assembled in a 1:1:1 stoichiometry: (1) alpha (α) – a catalytic unit (~110 kDa) that consists of three cytoplasmic domains (A, actuator; N, nucleotide binding; and P, phosphorylation) and a 10-transmembrane (TM)-helix domain (TMD); (2) beta (β) – a single TM extracellular unit (~35 kDa) required for Na’/K’-ATPase’s stability and membrane trafficking; (3) FXYD – a single TM regulatory unit (~10 kDa) that modulates the enzymatic activity by changing affinities of the Na’/K’-ATPase toward Na’, K’ or ATP (Fig. 1a)2,3,4. In humans, four α, three β, and seven FXYD isoforms have been identified, among which the expression of the α isoforms is tissue-specific. The α1 subunit is ubiquitously expressed in all tissues, whereas the α2, α3, and α4 are predominantly detected in muscle, brain and testis, respectively2,3. Currently, the only isoform structure determined by X-ray crystallography is the ubiquitously expressed α1 configuration5,6. It is proposed that the transport mechanism of the Na’/K’-ATPase follows the Post–Albers scheme—cycling between the E1 and E2 states. These states associate with Na’-dependent autophosphorylation and K’-dependent dephosphorylation, respectively. In the E1 state, the Na’/K’-ATPase adopts a cytoplasmic side-open conformation where the ion-binding sites open to the cytoplasm, allowing Na’ to access the transmembrane binding sites. Upon Mg-ATP and 3 Na’ binding, the Na’/K’-ATPase undergoes autophosphorylation at the conserved aspartate residue in the P domain of the α subunit, thereby switching to an α’-occluded E1•P•ADP state where the ion-binding sites are not accessible to the cytoplasm. Upon ADP release, the Na’/K’-ATPase switches to E2P state and the ion-binding sites open to the exoplasmic side for Na’ release and subsequent 2 K’ binding. The binding of K’ triggers dephosphorylation and drives the conformational change of the Na’/K’-ATPase to an K’-occluded state (E2•Pi). The Na’/K’-ATPase subsequently switches to the E2 state, and finally back to an E1 state releasing K’ into the cytoplasm allowing for the transport cycle to begin anew (Fig. 1b)12.

Previous X-ray crystallographic work revealed the architecture of the Na’/K’-ATPase in two distinct Na’-occluded (E1•P•ADP)5,6 and K’-occluded (E2•Pi)12 states. Recent reports of the exoplasmic side-open
(E2P) structure of the Na⁺/K⁺-ATPase indicates how the exoplasmic-gating mechanism works to release bound Na⁺ and facilitate extracellular K⁺ access into the pump’s ion-binding cavities. However, the cytoplasmic gating mechanism of the Na⁺/K⁺-ATPase still remains unclear due to the lack of structural information of the cytoplasmic side-open states (E1 and E1(Na⁺)-ATP). Here, we determined the cryo-EM structures of the human Na⁺/K⁺-ATPase in five intermediate states during its transport cycle, including the cytoplasmic side-open (E1), AMP-PCP-bound cytoplasmic side-open (E1(APC)), ADP-AlF₄⁻ trapped Na⁺-occluded (E1(P-ADP)), BeF₃⁻ trapped exoplasmic side-open (E2P) and MgF₄⁻ trapped K⁺-occluded (E2(P)-Pi) states. Structural comparison among these five states elucidated the conformational rearrangements in both cytoplasmic and transmembrane domains that are critical for the gating mechanism of the Na⁺/K⁺-ATPase. In brief, the opening cytoplasmic gate for Na⁺ access is triggered by a hinge-type rotation of the TMD’s M1 helix distal to M3 helix, while opening of the exoplasmic gate for K⁺ access is triggered by a twist of M4E away from M6. These structural insights and functional validation assays reveal structural mechanistic details of Na⁺/K⁺-ATPase gating.

**Results**

**Construct optimization and functional characterization of the recombinant human α3-isoform Na⁺/K⁺-ATPase**

To exogenously reconstitute the human α3 Na⁺/K⁺-ATPase, we screened different combinations of βs and FXYDs with α3, and found out that a combination of the α3, β1 and FXYD6 yielded the highest expression level. The three genes encoding for the α3, β1 and FXYD6 were cloned into a single vector containing the CMV promoter and the P2A ribosomal skipping sequences in between the three genes (see Methods for more details). The baculovirus-mammalian (bac-mam) expression system was utilized to express the human α3 Na⁺/K⁺-ATPase. The Flag-tagged α3 Na⁺/K⁺-ATPase was purified by anti-Flag antibody-conjugated resin (FlagM2). The purified Na⁺/K⁺-ATPase was shown as a single peak on gel filtration (Supplementary Fig. 1a) and fully assembled with the three subunits (α3, β1 and FXYD6) (>95% purity) as shown by SDS-PAGE analysis (Supplementary Fig. 1b). To validate the functionality of this recombinant Na⁺/K⁺-ATPase, we performed whole-cell patch-clamp using HEK 293 F cells that over-express α3/β1/FXYD6. Indeed, the transduced HEK 293 F cells show ~4-fold enhanced pump activity, 2.17 ± 0.90 pA/pF as compared with background pump-activity in mock-transduced cells, 0.57 ± 0.06 pA/pF (Fig. 1c, Supplementary Fig. 1c). For this recombinant Na⁺/K⁺-ATPase, the number of phosphorylation sites measured is 252 ± 14 nmol Pi/mg protein (Fig. 1d) and the ATP turnover rate measured is 252 ± 14 μmol Pi/mg protein/hr (Fig. 1e), which are comparable to those of the tissue-prepared endogenous Na⁺/K⁺-ATPases.

**Structural determination of the human Na⁺/K⁺-ATPase in its Na⁺- and K⁺-occluded states**

We trapped the human α3 Na⁺/K⁺-ATPase in its Na⁺-occluded state using a buffer containing Na⁺ and ADP in conjunction of AlF₄⁻, and its
K⁺-occluded state using K⁺ and MgF₂ (see Methods for more details). The single-particle cryo-EM analysis yielded 3.7-Å-resolution Na⁺-occluded (Fig. 2a) and 4.1-Å-resolution K⁺-occluded (Fig. 2b) maps. The cryo-EM densities of the ADP-ALF₃ (Fig. 2c) and MgF₂ (Fig. 2d) in the cytoplasmic domains in both maps are well-defined. The cryo-EM densities of the two maps are sufficient to build reliable models for the structures of the human α3 Na⁺/K⁺-ATPase in the Na⁺-occluded (E1-P-ADP) (Fig. 2e) and K⁺-occluded (E2-Pi) (Fig. 2f) states, with the help of structures of the pig Na⁺/K⁺-ATPase (PDBS 3WGU and 3BSE).

Architecturally, the structure of the human sodium-potassium pump is virtually identical to that of other Na⁺/K⁺-ATPases, which were previously determined by X-ray crystallography. α3, β1 and FXD6 form a stable complex assembly through the interactions of their TMDs. In particular, the 45°-tilted single TM of the FXYD6 form a stable complex assembly through the interactions of occluded-state (E2) due to radiation damage. Nevertheless, we can model the binding structures of the pig Na⁺/K⁺-ATPase (PDBs 3WGU and 3BSE).

As anticipated, in the presence of Na⁺ and ADP in conjunction of ALF₃, the human α3 Na⁺/K⁺-ATPase is trapped in the Na⁺-occluded state (E1-P-ADP). Superposition of the Na⁺-occluded structures of the human α3 (this study) and the pig α1 Na⁺/K⁺-ATPase (PDB: 3WGU) by aligning their alpha subunit showed no major structural difference with a root-mean-square deviation (r.m.s.d) - 0.8Å (Supplementary Fig. 1d). The transmembrane helices M6 adopt a typical occluded conformation as seen in the pig ortholog. Like the Na⁺-occluded state, the K⁺-occluded-state (E2-P) structure of the human α3 (this study) is nearly identical to that of the pig α1 ortholog (PDB: 3BSE) (r.m.s.d - 0.8Å) (Supplementary Fig. 1e). Despite the strong densities of all the helices in the TMDs, except Q920, other highly conserved cation-binding residues, including E324 (M4), N773 and E776 (M5), D801 and D805 (M6) are poorly resolved in the occluded-state cryo-EM maps (Fig. 2g, h) due to radiation damage. Nevertheless, we can model the binding cavity and bound cations reliably using the coordinates from the previously determined occluded structures of the pig Na⁺/K⁺-ATPase (Supplementary Fig. If, g).

Stabilization and structural determination of the fully exoplasmic side-open and cytoplasmic side-open states

We speculated that the reason why Na⁺/K⁺-ATPase favors adopt an Na⁺-occluded or K⁺-occluded ligand-bound conformations is due to the co-purified Na⁺ or K⁺ ions. To trap Na⁺/K⁺-ATPase in its non-occluded states, we substituted the four cation-binding residues (E324, E776, D801 and D805) of the α3 (Supplementary Fig. If, g) into alanine to completely diminish the affinities of Na⁺ and K⁺ binding. This cation-binding deficient Na⁺/K⁺-ATPase, termed “4A mutant” has a relatively high surface expression (Supplementary Fig. 1h), but exhibits significantly reduced pump activity (0.88 ± 0.19 pA/pF) (Fig. 1c, Supplementary Fig. 1c). In consistency with the reduction in transport activity, the 4A mutant reduces both its phosphorylation activity to 0.29 ± 0.07 nmol sites/mg protein (Fig. 1d) and its ATP turnover rate to 17 ± 5 µmol P/mg protein/hour (Fig. 1e). These observations suggested that the 4A mutant has correct protein folding; however, its enzymatic activities, including Na⁺-induced phosphorylation and K⁺-induced ATP hydrolysis become less sensitive to Na⁺ and K⁺, respectively. Using this cation-binding deficient 4A mutant Na⁺/K⁺-ATPase, we determined three cryo-EM structures of the Na⁺/K⁺-ATPase in its exoplasmic side-open state at 3.9Å resolution, as mimicked the E2 state, and its cytoplasmic side-open states at 3.4Å and 3.5Å resolutions, as mimicked the E1 and E1-ATP, respectively.

Exoplasmic-gating mechanism

The structure of the exoplasmic side-open human α3 (Fig. 3a, b) is nearly identical to that of the pig kidney α1 Na⁺/K⁺-ATPase (r.m.s.d - 0.9Å) stabilized in the E2P conformation by BeF₃ bound to the Aspartate 366 residues of the P domain (Supplementary Fig. 2a, b). All the subunits and ten transmembrane helices of the human α3 subunit overlap perfectly with those of the pig kidney α1 subunit (Supplementary Fig. 2b, c) confirming that the cation-binding deficient 4A mutant is functionally and structurally relevant.

To study the exoplasmic-gating mechanism of the Na⁺/K⁺-ATPase, we superimposed the structures of the exoplasmic side-open and K⁺-occluded states by aligning the M7-M10 helices. This comparison revealed the M1-M4 helices undergo major conformational rearrangements between the K⁺-occluded and the exoplasmic side-open state while minor changes were observed in the M5-M10 (Supplementary Fig. 2d-f). In the exoplasmic side-open state, the A domain tilts ~7° along the vertical axis in the presence of BeF₃ relative to that of the K⁺-occluded state (Fig. 3c). Along with the conformational change of the A domain, the M1-M2 and M3-M4 helices rotate -15° and 14°, respectively horizontally as a whole rigid body (Fig. 3c). In the K⁺-occluded compared to those in the exoplasmic side-open states. The M2 is unwound in the K⁺-occluded state, but forms a well-folded α-helix in the exoplasmic side-open state. Such conformational change of M2, in turn, drives its downward movement (Supplementary Fig. 2e). Most of the key K⁺-binding residues, including N773 and E776 (M5), D801 and D805 (M6) undergo minor rearrangements (Supplementary Fig. 2g) between the two states.

The exoplasmic gate of the Na⁺/K⁺-ATPases is defined by the arrangement of the M4E relative to the M6 helix (Fig. 3d). In the K⁺-occluded state, M4E and M6 helices are in a proximal distance. Particularly, V319 of M4E and L798 of M6 (7.7 Å Ca-Ca distance) are situated closely with each other (Fig. 3d) and localized on top of the potassium binding cavity, serving as the exoplasmic-gating latch closing the K⁺ entry pathway (Fig. 3e). In the exoplasmic side-open state, the rotation of M4E distances V319 from L798 of M6 helix (12.6 Å Ca-Ca distance) (Fig. 3d) and thereby creates an open K⁺ entry pathway (Fig. 3f) along M4E and M6 helices. To study the role of the exoplasmic-gating latch residue V319 (M4E), we mutated it to alanine. This mutation has minor effect on the phosphorylation activity, 0.84 ± 0.15 nmol sites/mg protein compared to the WT, 0.95 ± 0.09 nmol sites/mg protein (Fig. 3g). Interestingly, V319A mutation significantly increases the maximal ATPase activity of the Na⁺/K⁺-ATPase (Figs. 3h, 4b). However, while the V319A mutant remains its apparent affinity toward K⁺, 0.5 ± 0.1 mM, compared to that of the WT, 0.4 ± 0.1 mM (Fig. 3h), its apparent affinity toward Na⁺ significantly reduces, 24.0 ± 3.3 mM compared to that of the WT, 9.2 ± 1.3 mM (Fig. 4d). These data indicated that V319A mutation likely shifts its E1-E2 equilibrium toward E2 state that prefers K⁺ ions to Na⁺ ion binding, thus increases the dephosphorylation rate of the Na⁺/K⁺-ATPase.

Cytoplasmic-gating mechanism

The well-defined 3.4Å and 3.5Å maps of the human α3 4A mutant stabilized in its cytoplasmic side-open state in the absence or presence of AMPPNP enabled us to build reliably the atomic models of the human α3 Na⁺/K⁺-ATPase in its cytoplasmic-side open (E1) (Fig. 4a, b) and AMPPNP-bound cytoplasmic-side open (E1-ATP) (Fig. 4c, d). Superposition of the Na⁺/K⁺-ATPase in the cytoplasmic side-open and Na⁺-occluded states by the TM7-10 helices revealed that major conformational changes occur at the α3 subunit while the β1s and FXYD6’s conformations remain unchanged (Supplementary Fig. 3a). Within the TMD of the α3, M1-M4 helices undergo major rearrangements while minor conformational changes occur at M5-M10 helices.
Fig. 2 | Cryo-EM maps and structures of the human α3 Na+/K+ ATPase in its Na+- and K+-occluded states. a 3.7 Å resolution cryo-EM map of the human α3 Na+-occluded structure. exo: exoplasm, cyto: cytoplasm. b 4.1 Å resolution cryo-EM map of the human α3 K+-occluded structure. exo: exoplasm, cyto: cytoplasm. c ADP-AlF4− trapped cytoplasmic domains of the Na+-occluded structure. The mesh represents electron density of ADP-AlF4−. d MgF42− trapped cytoplasmic domains of the K+-occluded-state structure. e Structural model of the human α3 Na+/K+ ATPase in its Na+-occluded state. The black dashed line represents missing density of amino acid residues 261-270 in the structural model. f Structural model of the human α3 Na'/K'-ATPase in its K'-occluded state. g, h Densities of the cation-binding residues and their respective helices in the Na+-occluded (g) and K+-occluded structure (h).
Except E324 in M4, most of the Na⁺ binding residues situated in the unmoving part of the TMD, including helices M5 (N773 and E776), M6 (D801 and D805), and M8 (Q920). Hence, these cation-binding residues only move slightly within the Na⁺ binding pockets between the cytoplasmic side-open and the Na⁺-occluded state to accommodate for Na⁺ binding (Supplementary Fig. 3d).

The rearrangement of M1–M4 helices couples with the conformational change of the cytoplasmic A domain. Specifically, the A domain rotates ~14° horizontally along the axis perpendicular to the plasma membranes in the cytoplasmic side-open state relative to that in the Na⁺-occluded state (Fig. 4e). As the cytoplasmic A domain is connected to M1–M3 through three rigid linkers, this rotation in the A domain can be visualized by the movement of the rigid M3 helix.
Fig. 3 | 3.9 Å resolution structure of the human α3 Na’/K’-ATPase in its exoplasmic side-open state. a, b Cryo-EM map (a) and structural model (b) of the human α3 Na’/K’-ATPase in its exoplasmic side-open state (E2P), exo: exoplasm, cyto: cytoplasm. c Superimpose the M1–M2 helices (brown), M3–M4 helices (slate) and A domain (yellow) of the human α3 exoplasmic side-open (colored) and K’-occluded (gray) structures by aligning their M7–M10 helices. d Superimpose K’ entry pathway in the exoplasmic side-open (colored) and K'-occluded (gray) structures of the human α3. Back view: FXDYD6 was shown in the front of the structural model. e Slab view of the K’-occluded structure shows no K’ entry pathway (red trace). f Slab view of the human exoplasmic side-open structure shows the K’ entry pathway (red trace). g, h the numbers of phosphorylation sites (g) and K’-dependent ATPase activities (h) of the wild-type (WT) Na’/K’-ATPase and its mutants, including F90A, L93A and V319A. N = 6 independent experiments. Data were presented as mean ± standard deviation (SD). One-way ANOVA test was used to compare mean values of multiple different variants. P-values were shown as numerical numbers in the subtitle 3g. Source data are provided as a Source Data file.

Discussion
In this work, we focused on structural and functional characterization of the human α3 Na’/K’-ATPase that comprises α3, β1 and FXDYD6. We reported the structures of the human α3 Na’/K’-ATPase in five distinct intermediate states, of which the cytoplasmic side-open-state structures fill a gap in the structural understanding of the Na’/K’-ATPase's transport cycle and gating mechanism of the Na’/K’-ATPase. To capture the cytoplasmic side-open states, we introduced a quadruple alanine mutation variant (4A mutant) at the key ion binding residues to diminish the ion-binding capacity of the Na’/K’-ATPase. We anticipated that our approach may lead to unforeseen changes in the structure of the Na’/K’-ATPase. Therefore, we performed functional and structural characterization on the 4A mutant. Our functional data suggests that the 4A mutant still retained a certain level of its phosphorylation and ATPase activities (Fig. 1d, e). In consistency with the functional data, our structural data also showed a virtually identical structure of the human α3 4A mutant and the pig α1 WT ATPase in the exoplasmic side-open state (Supplementary Fig. 2b). These evidences confirmed that the 4A mutant was appropriate to use for solving the cytoplasmic side-open states of the Na’/K’-ATPase.

Superposition of the M1–M6 helices between the cytoplasmic side-open and the exoplasmic side-open states revealed the gating mechanism of the Na’/K’-ATPase exists in each state of the Na’/K’-ATPase during its transport cycle; for instance, in the cytoplasmic side-open state, the cytoplasmic gate is open, whereas in the exoplasmic side-open state, the exoplasmic gate is open while the cytoplasmic gate is closed (Supplementary Fig. 4). These results together suggest that both F90 and L93 residues are critical for the functionality of Na’, K’-ATPase, which strongly supports our structural model.

In addition, the M1 and M2 helices also undergo a one-helix-turn screw-like movement along their axis toward the membranes while the M3 vertically shifts upward by ~3 Å (Supplementary Fig. 3b). The M4 helix is directly connected to the P domain, so its movement is dictated by the P domain’s rearrangement. The cytoplasmic side-open M4E (M4’s residues 309–320) is slightly tilted compared to that in the Na’-occluded state (Supplementary Fig. 3b). The movements described above potentially expand the Na’ entry pathway and facilitate Na’ access into the binding pockets of the cytoplasmic side-open Na’/K’-ATPase.

In the E1+ATP structure, the cytoplasmic domains are stabilized by the AMP/PPCP bound to the interface of N and P domains of the α3 subunit (Supplementary Fig. 3e). Superposition of the cytoplasmic domains in the E1+ATP and E1+P-ADP states by the P domain showed that a helix formed by residues 708-717 of the P domain rotates about ~14° closer to the N domain in the E1+P-ADP compared to that in E1+ATP state (Supplementary Fig. 3f). This rotation creates a hydrogen bond between residue N710 and the gamma phosphate group of the ATP molecule which may facilitate the autophosphorylation reaction of the human α3.

Comparison of gating mechanisms of P2 ATPases
Our structural insights of the human α3 Na’/K’-ATPase in its exoplasmic-side open state re-emphasizes the rearrangement of the M4E relative to the M6 helix to open the ion pathway toward the exoplasm. Interestingly, the M1–M4 rearrangement in the exoplasmic side-open state is similar to that of the Na’/K’-ATPase in different cardiac glycoside-bound structures8,23,24 (Supplementary Fig. 5a) and the gastric proton pump type-2 H+/K+-ATPases in its blocker-bound conformation23. A similar rotation of the M3–M4 was also observed in sarcoendoplasmic reticulum Ca2+-ATPase (SERCA) in its luminal side-open state compared to its Ca2’-occluded state23,24 (Supplementary Fig. 5b). These similar conformational changes suggest that the exoplasmic-gating mechanism is conserved among P2 ATPase family.

Prior to our work, the SERCA was the only member of the P2 ATPase family whose cytoplasmic gating mechanism was functionally and structurally well-characterized23,30. Our cytoplasmic side-open structures of the human α3 Na’/K’-ATPase reveal a different operation of the cytoplasmic gate compared to that of the SERCA. In particular, to expose the Ca2’ binding sites in the transmembrane domain, the SERCA’s M1 (L61) moves vertically along its own axis by 12 Å (two-helix turns) into the luminal membranes as a “sliding-door” mechanism (Supplementary Fig. 5c). On the other hand, the Na’/K’-ATPase uses a “hinged-door” 19 Å outward rotation of M1 (F90 and L93) distal from M3 (T286) to create a wide-open cytoplasmic gate for Na’ access in the human α3 Na’/K’-ATPase (Fig. 4f). We speculate these different operations of the two M1 helices are due to the different movements of the A domains that directly link to the M1 helices of the two ATPases. In particular, while the SERCA’s A domain moves up ~12 Å along a vertical axis (perpendicular to the lipid bilayers) (Supplementary Fig. 5c), pushing the M1 toward the lumen membrane, the Na’/K’-ATPase’s A domain rotates outward relative to the P domain in the E1 and E1+ATP
structures. In addition, while the SERCA comprises a single alpha subunit, the Na⁺/K⁺-ATPases compose of multiple subunits, including alpha, beta and FXYD. We can’t rule out the possibility that some structurally unresolved N- and C-terminal regions of the beta and FXYD subunits may play a certain roles in regulating the cytoplasmic gate of the Na⁺/K⁺-ATPases. Hence, further functional studies need to be done to understand any potential role(s) of the beta and FXYD subunits in the cytoplasmic gating mechanism of the Na⁺/K⁺-ATPases.

A structural model for gating mechanism of the Na⁺/K⁺-ATPase

Our work demonstrates a complete transport cycle for transporting Na⁺/K⁺ with different intermediate states of the human α3
**Methods**

**Con structs and cell growth**

Full-length human N-terminal Flag-tagged ATP3A, ATP3B1, and FXYD6 cDNAs were sub-cloned into pE3T-BM vector\(^{25}\) (Addgene) with ribosome skipping sequences (P2A) (pEZT-NKA construct). Mutations, including substrate-binding-deficient 4 A (E327A, E776A, D801A, and D805A), cytoplasmic-gating deficient F90A and L93A, and exoplasmic-gating deficient V319A were introduced by site-directed mutagenesis. The Na\(^+\)/K\(^+\)-ATPase in its Na\(^+\)-occluded state (E1). exo: exoplasm, cyt: cytoplasm.

**Sample preparation and EM data acquisition**

To trap the human \(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase in its Na\(^+\)-occluded state (E1-P-ADP), the WT Na\(^+\)/K\(^+\)-ATPase was incubated in a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 1% LMNG, 10% (v/v) glycerol, 1% (v/v) LMNG and incubated at 4 °C for 1 h. The unlysed cells, debris and excess membranes were removed by centrifuging at 48,000 × g for 30 min at 4 °C. The Na\(^+\)/K\(^+\)-ATPase protein was captured by affinity purification using Flag M2 resin (Sigma), eluted by buffer containing 200 μg/ml Flag peptide (Fisher Scientific) and further purified by size exclusion chromatography equilibrated in a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 1% LMNG or 20 mM HEPES-K pH 7.4, 150 mM KCl, 1% (v/v) LMNG. The LMNG-solubilized Na\(^+\)/K\(^+\)-ATPase was used for ATPase activity assays. Proteins used for cryo-EM sample preparation were purified in the same manner with a minor modification. Detergent was exchanged from LMNG to digitonin during the affinity purification and size exclusion chromatography using a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 0.06% (w/v) Digitonin; or 20 mM HEPES-K pH 7.4, 150 mM KCl, 0.06% (w/v) Digitonin.

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\(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase (Supplementary Fig. 6). With an emphasis on revealing the structural insights for gating mechanism of the Na\(^+\)/K\(^+\)-ATPase, we generated a model focusing on the M1-M6 helices, with M7-M10 helices removed for better illustration (Fig. 5). The gating regulation of the Na\(^+\)/K\(^+\)-ATPase is done at two distinct cytoplasmic and exoplasmic gates controlling directionality of ion trafficking into and out of Na\(^+\)/K\(^+\)-ATPase at specific intermediate states. The cytoplasmic gate is regulated by M1 and M3, while the exoplasmic gate is regulated by M4E and M6. The transport begins with an open cytoplasmic gate created by the distal arrangement of M1 and M3 allowing Na\(^+\) ions to enter the cytoplasmic gate, while the M4E and M6 form a closed exoplasmic gate preventing K\(^+\) or non-specific ions to enter from the exoplasm. Both gates are closed to secure Na\(^+\) in the ion-binding pockets in the Na\(^+\)-occluded state where autophosphorylation occurs. ADP release triggers a large rotation of the cytoplasmic A domain, inserting its TGES motif into the nucleotide binding cavity, previously formed by the N and P domains in the Ei-P-ADP state (Supplementary Fig. 7a, b). This rotation induces conformational changes in the M1 and M2, and subsequently M4E and M6 (Supplementary Fig. 7c) to open the exoplasmic gate, releasing Na\(^+\) ions and allowing K\(^+\) binding in the exoplasmic side-open state. In this state, the M1 and M3 remain proximal, closing the cytoplasmic gate to prevent Na\(^+\) or other ions to pass through, while the K\(^+\) ions are selected at the K\(^+\) binding sites. Upon K\(^+\) binding, M4E and M6 close their K\(^+\) entry pathway and K\(^+\) ions are secured inside the K\(^+\) binding pockets. Dephosphorylation triggers the opening of the cytoplasmic gate to release K\(^+\) into the cytoplasm and the cycle restarts (Fig. 5).

In summary, we report five structures representing different intermediate states of the human \(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase during its transport cycle. Our finding suggests the structural insights for gating mechanism of the Na\(^+\)/K\(^+\)-ATPase (Fig. 5) and describes a complete transport cycle of the Na\(^+\)/K\(^+\)-ATPase (Supplementary Fig. 6). Our work provides opportunities to understand both the structure-function relationship and pathophysiology of the human \(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase.

4 °C for 20 min. Cell pellets were homogenized in hypotonic buffer containing 10 mM HEPES-Na pH 7.4, 10 mM NaCl, 10% glycerol, 0.2 mM PMSF and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benazamidine HCl, 1 μg/ml aprotinin, 100 μg/ml trypsin inhibitor from soybean (Sigma)), flash-frozen by liquid nitrogen and stored at −80 °C.

**Protein purification**

Homogenized cells were thawed and centrifuged at 4000 × g at 4 °C for 20 min. Supernatants containing cytosolic lysate were discarded. Pellets were resuspended into lysis buffer containing 20 mM HEPES-Na pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 1% (v/v) LMNG and incubated at 4 °C for 1 h. The unlysed cells, debris and excess membranes were removed by centrifuging at 48,000 × g for 30 min at 4 °C. The Na\(^+\)/K\(^+\)-ATPase protein was captured by affinity purification using Flag M2 resin (Sigma), eluted by buffer containing 200 μg/ml Flag peptide (Fisher Scientific) and further purified by size exclusion chromatography equilibrated in a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 1% LMNG or 20 mM HEPES-K pH 7.4, 150 mM KCl, 1% (v/v) LMNG. The LMNG-solubilized Na\(^+\)/K\(^+\)-ATPase was used for ATPase activity assays. Proteins used for cryo-EM sample preparation were purified in the same manner with a minor modification. Detergent was exchanged from LMNG to digitonin during the affinity purification and size exclusion chromatography using a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 0.06% (w/v) Digitonin; or 20 mM HEPES-K pH 7.4, 150 mM KCl, 0.06% (w/v) Digitonin.

**Sample preparation and EM data acquisition**

To trap the human \(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase in its Na\(^+\)-occluded state (E1-P-ADP), the WT Na\(^+\)/K\(^+\)-ATPase was incubated in a buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 1 mM MgCl\(_2\), 8 mM NaF, 2 mM AlCl\(_3\) and 0.06% (w/v) digitonin at room temperature for 1 h. Similarly, a buffer containing 20 mM HEPES-K pH 7.4, 150 mM KCl, 1 mM MgCl\(_2\), 8 mM NaF and 0.06% (w/v) digitonin was used to trap the protein in its K\(^+\)-occluded state (E2-Pi).

The 4A mutant \(\alpha\)3 Na\(^+\)/K\(^+\)-ATPase in its purification buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl and 0.06% (w/v) digitonin was used to capture its cytoplasmic side-open state (E1). A supplement of 1 mM AMPPCP pH 7.4 and 4 mM MgCl\(_2\) was used to trap the protein in its ATP-bound cytoplasmic side-open state (E1-ATP). The mixture was incubated on ice for 1 h to trap the exoplasmic side-open state (E2P), a supplement of 1 mM BeSO\(_4\), 4 mM NaF and 1 mM MgCl\(_2\) was added to the 4A mutant Na\(^+\)/K\(^+\)-ATPase. The mixture was incubated at room temperature for 3 h.

All the proteins samples above were spun down at 18,000 × g at 4 °C for 30 min before subjected to the cryo-EM grids. The cryo-EM grids were prepared by applying 3 μl of protein samples (4 mg/ml) to glow discharged Quantifoil R2/1.3 300-mesh gold holey carbon grids (Quantifoil, Micro Tools GmbH, Germany). Grids were blotted for 40 s under 100% humidity at 4 °C before being plunged into liquid ethane using Mark IV Vitrobot (FEI).

Micrographs were acquired on a Titan Krios microscope (FEI) operated at 300 kV with a K3 Summit direct electron detector.
Na+/K+-ATPase complex. The resolution for the resulting cryo-EM map selected particles was performed with a soft mask around the whole density for the entire complex. The angular sampling (3.7°), resulting one new good class with improved resolution into the original pixel size. Subsequently, we performed showing good secondary structural features were selected and re-classified by using local search in combination with small filter.

Image processing

The cryo-EM datasets of all 5 different Na+/K+-ATPase samples were processed in identical manners. The detailed image processing workflows were shown in Supplementary Figs. 8–12. The cryo-EM statistics for five different datasets were summarized in Table 1. Briefly, movie frames were motion-corrected and binned two-fold, and dose-weighted using MotionCor2. The CTF parameters were estimated using Gctf. RELION-3 was used for the following processing. Particles were first roughly picked by using the Laplacian-of-Gaussian blob method, and then subjected to 2D classification. Class averages representing projections of Na+/K+-ATPase in different orientations were used as templates for reference-based particle picking. Extracted particles were binned three times and subjected to 2D classification. Particles from the classes with fine structural feature were selected for 3D classification using an initial model generated from a subset of the particles in RELION. Particles from one of the 3D classes showing good secondary structural features were selected and re-extracted into the original pixel size. Subsequently, we performed finer 3D classification by using local search in combination with small angular sampling (3.7°), resulting one new good class with improved density for the entire complex. The final 3D refinement with the selected particles was performed with a soft mask around the whole Na+/K+-ATPase complex. The resolution for the resulting cryo-EM map was further improved by CTF refinement and particle polishing. Local resolution was calculated in RELION3. Resolution was estimated by applying a soft mask around the protein density with the Fourier Shell Correlation (FSC) 0.143 criterion.

Model building, refinement and validation

Overall, of the 5 cryo-EM maps, the α3’s TMD’s helices, especially M1–M4 had clearly well-defined densities (Supplementary Fig. 13) that enabled de novo building, while α3’s cytoplasmic domains, β1 and FXYD6 were built based on their homologous models. In brief, the 3.7-Å-resolution Na+–occluded (E1-P-ADP) and 4.1-Å-resolution K+–occluded (E2-Pi) human α3 Na+/K+-ATPase structures were built based on the homologous structures of the pig Na+/K+-ATPase (PDBs 3WGU and 3B8E). The models were manually adjusted in Coot. The 3.5-Å resolution cryo-EM map of E1-Pi state is resolved in the highest quality with many well-defined side chains density for all of the α3, β1 and FXYD6, which enabled de novo building of an accurate model for the human α3 Na+/K+-ATPase, with the help of the Na+-occluded structure described above. The TMD was well resolved in the 3.4-Å resolution cryo-EM map of E1 state; however, it showed poor densities for the P and N domains presumably due to the high flexibility of these domains in the absence of ATP molecule. We rigid-body fitted the P and N domains of the E1-Pi into the E1 map guided by a few well-resolved helices. The cryo-EM structure of the BeF3-–bound exoplasmic side-open (E2P) state was built in a similar manner as that used for cytoplasmic side-open structures. Briefly, the TMD of α3 was de novo built, whereas other domains were modeled mainly by rigid-body fitting. Phenix’s real space refinements were used to refine coordinates of all the models. MolProbity in Phenix was used to validate the models. The FSCs between models and maps were computed using the “Comprehensive validation” function in Phenix.

Phosphorylation activity

Phosphorylation activity was determined in the absence of K’ as described elsewhere. In brief, LMNG-solubilized Na+/K’-ATPase wild-type (wt) and mutant proteins were reacted with excessive amount of [γ-32P]ATP in a reaction buffer containing 20 mM HEPES-Na pH 7.4, 150 mM NaCl, 0.125 mM EDTA, 0.02% (w/v) LMNG and 10 mM MgCl2. The reaction was incubated at 0 °C for 20 s, then terminated by adding a stop solution containing 10% w/v trichloroacetic acid and 2 mM HCl.
sodium pyrophosphate. The protein samples were washed twice with a wash solution containing 0.1% w/v trichloroacetic acid and 10 mM KH2PO4 and then resuspended in 1 M NaOH at 55 °C. The radioactivity was determined by liquid scintillation counting and compared with the specific radioactivity of the ATP to compute the amount of 32P incorporated in the protein samples. The protein concentration was determined by BCA assay kit (Thermo Fisher Scientific, cat# 23227).

ATPase activity

The K+ dependence of ATPase activity was determined in the presence of 120 mM NaCl and varied concentrations of KCl (0–20 mM). The Na+ dependence of ATPase activity was determined in the presence of 20 mM KCl and varied concentrations of NaCl (0–120 mM). The amount of inorganic phosphate released from the reactions was measured by Enzchek™ phosphate assay kit (Thermo Fisher Scientific, cat# E6646). LMNG-solubilized wild-type (wt) and mutant Na+/K+-ATPase proteins were premixed with 1 mM ATP, 0.1 units of purine nucleoside phosphorylase (PNP) enzyme, 200 µM 2-amino-6-mercapto-7-methylpurine riboside (MESG) substrate and KCl or NaCl at different concentrations shown above. The reaction was initiated by adding 10 mM MgCl2 and incubated at room temperature for 5 min. A negative control reaction was set up in the presence of 100 mM Oucabain. The absorbance at 360 nm was measured by Omega CLARIOstar Plus instrument (BMG Labtech). The protein concentration was determined by BCA assay kit (Thermo Fisher Scientific, cat# 23227).

Electrophysiology

Patch-clamp methods were described previously.37,38 All recordings were performed at a holding potential of 0 mV. The extracellular solution contained 120 mM NaOH, 4 mM MgCl2, 0.5 mM EGTA, 15 mM TEA-OH, 7 mM NaCl or KCl, and 10 mM HEPES, set to pH 7.4 with aspartate. The cytoplasmic solution contained 20 mM NaCl, 125 mM aspartate, 0.5 mM EGTA, 0.5 mM MgCl2, 0.2 mM CaCl2, 20 mM HEPES, 1 mM KH2PO4, 6 mM Mg-ATP, 0.2 mM GTP, set to pH 7.4 with KOH.

### Table 1 | Cryo-EM data collection and model statistics

| PDB  | EMDB  | Na+-occluded | K+-occluded | Exoplasmic side-open | Cytoplasmic side-open | AMPPCP-bound cytoplasmic side-open |
|------|-------|---------------|-------------|----------------------|-----------------------|-----------------------------------|
| 8D3U | EMD-2164 | 300 | 300 | 300 | 300 | 300 |
| 8D3X | EMD-27167 | 60 | 60 | 60 | 60 | 60 |
| 8D3Y | EMD-27168 | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 |
| 8D3V | EMD-27165 | 0.83 | 0.83 | 1.08 | 1.08 | 1.08 |
| 8D3W | EMD-27166 | C1 | C1 | C1 | C1 | C1 |
| Initial particle images (no.) | 1,889,299 | 2,017,712 | 1,559,941 | 1,970,027 | 2,926,310 |
| Final particle images (no.) | 108,246 | 91,096 | 84,639 | 227,349 | 98,088 |
| Map resolution (Å) | 3.7 | 4.1 | 3.9 | 3.4 | 3.5 |
| FSC threshold | 0.143 | 0.143 | 0.143 | 0.143 | 0.143 |
| Map resolution range (Å) | 3.7–5.2 | 3.9–5.3 | 3.7–5.6 | 3.2–4.6 | 3.3–4.6 |

### Data collection and processing

| Voltage (kV) | 300 | 300 | 300 | 300 | 300 |
| Electron exposure (e−/Å²) | 6 | 0 | 6 | 0 | 6 |
| Defocus range (µm) | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 | 1.6–2.6 |
| Pixel size (Å) | 0.83 | 0.83 | 1.08 | 1.08 | 1.08 |
| Symmetry imposed | C1 | C1 | C1 | C1 | C1 |
| Initial particle images (no.) | 1,889,299 | 2,017,712 | 1,559,941 | 1,970,027 | 2,926,310 |
| Final particle images (no.) | 108,246 | 91,096 | 84,639 | 227,349 | 98,088 |
| Map resolution (Å) | 3.7 | 4.1 | 3.9 | 3.4 | 3.5 |
| FSC threshold | 0.143 | 0.143 | 0.143 | 0.143 | 0.143 |
| Map resolution range (Å) | 3.7–5.2 | 3.9–5.3 | 3.7–5.6 | 3.2–4.6 | 3.3–4.6 |

### Refinement

| Model composition | 10135 | 10217 | 9896 | 10019 | 10048 |
| Non-hydrogen atoms | 1289 | 1305 | 1298 | 1280 | 1280 |
| Protein residues | 2 (ALF, ADP) | 1 (MF4) | 0 | 0 | 1 (ACP) |
| Ligands | | | | | |
| Bonds (RMSD) | 0.003 (0) | 0.003 (0) | 0.003 (0) | 0.003 (0) | 0.003 (0) |
| Lengths (Å) (# > 4σ) | 0.652 (6) | 0.587 (3) | 0.570 (0) | 0.495 (0) | 0.571 (5) |
| Angles () (# > 4σ) | 1.93 | 1.90 | 1.96 | 1.68 | 1.86 |
| Validation | 11.11 | 12.13 | 10.11 | 6.63 | 9.56 |
| MolProbity score | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Clashscore | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Ramachandran plot | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Outliers (%) | 5.39 | 4.39 | 6.75 | 4.57 | 5.20 |
| Allowed (%) | 94.61 | 95.61 | 93.25 | 95.43 | 94.80 |
| Favored (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| B factors (Å) | 76.90 | 64.28 | 73.26 | 45.39 | 40.85 |
| Protein (mean) | 57.14 | 37.07 | – | – | 33.72 |
| Ligand (mean) | 0.81 | 0.78 | 0.81 | 0.79 | 0.80 |
| CC (mask) | 0.64 | 0.57 | 0.60 | 0.58 | 0.61 |
| CC (box) | 0.52 | 0.44 | 0.52 | 0.53 | 0.56 |
| CC (peaks) | 0.79 | 0.76 | 0.77 | 0.74 | 0.76 |
| CC (volume) | 0.83 | 0.88 | – | – | 0.83 |

Mean CC for ligands
Free Ca²⁺ of cytoplasmic solutions was calculated to be 0.05 μM, and free Mg²⁺ was calculated to be 0.4 mM using WEBMAX EXTENDED (http://www.stanford.edu/~cpatton/webmax/webmaxE.htm). Highly polished dental wax coated pipette tips with inner diameters of 4-6 μm were employed generating a typical access resistance during recordings ranging from 1.2 to 4 MΩ. To activate Na⁺/K⁺-ATPase activity, solutions were maintained at 37 °C, a giga seal was established and the membrane was ruptured with mild suction. Cells were held at a holding potential of 0 mV and stabilized for greater than 30 s to allow for cytoplasmic diffusion of ATP other ionic constituents. After 30 s, the cells were rapidly switched among up to four parallel solution streams. The base extracellular solution containing an additional 7 mM NaCl and 0 mM KCl was rapidly exchanged with a solution containing 7 mM kCl and 0 additional NaCl. The presence of extracellular K⁺ ions and cytoplasmic Na⁺ with ATP allowed for the electrogenic cycle of pump and 0 mM KCl was rapidly exchanged with a solution containing 7 mM NaCl and 0 mM KCl. The cells were stimulated to half-maximal current levels for 15 s, followed by a 1 s application of 0 mM NaCl, 70 mM KCl, 25 mM HEPES-Na pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 1% (w/v) LMNG. The biotinylated proteins were pulled down using Neutavidin agarose (Thermo Fisher, cat#29202). The biotinylated α3, β1, and FXYD6 were detected by SDS-PAGE and Western blot techniques using polyclonal antibodies against α3 (Abclonal, cat# A16036) (1:1000 dilution), FXYD6 (Abclonal, cat# AP182P) (1:10,000 dilution) was used as a secondary antibody. Actin served not only as a loading control (Supplementary Fig. 1h, WCL panel) but also as a cell surface labeling control (Supplementary Fig. 1g, h, i) to make sure only the cell surface proteins were labeled. HRP-conjugated anti-actin antibody (Santa Cruz, cat# sc-47778-HRP) (1:2000 dilution) was used. Experiment was done in triplicate. Uncropped blots were included in the Source Data file.

Statistical analysis and graph plotting

Unpaired t-tests (for 2-group comparison) and one-way ANOVA tests (for multiple-group comparison) were performed and graphs were plotted using Prism software (version 9.3.1, GraphPad). Data were represented as mean ± standard deviation (if applied).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support this study are available from the corresponding authors upon reasonable request. The cryo-EM maps of the human α3 Na⁺/K⁺-ATPase have been deposited in the Electron Microscope Data Bank (EMDB) under accession codes: EMD-27164 (Na⁺-occluded state), EMD-27167 (K⁺-occluded state), EMD-27168 (exoplasmic side-open state), EMD-27165 (cytoplasmic side-open state) and EMD-27166 (AMPPCP-bound cytoplasmic side-open state). The AMPPCP-bound cytoplasmic side-open state. The AMPPCP-bound cytoplasmic side-open state. The atomic coordinates for the human α3 Na⁺/K⁺-ATPase have been deposited to the RCSB Protein Data Bank (PDB) under accession codes: 8D3U (Na⁺-occluded state), 8D3X (K⁺-occluded state), 8D3Y (exoplasmic side-open state), 8D3Z (cytoplasmic side-open state) and 8D3W (AMPPCP-bound cytoplasmic side-open state).

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Author contributions
P.T.N, X.-C.B., and B.L. conceived the project. P.T.N. optimized the protein expression and purification procedures and prepared samples for cryo-EM analyses. P.T.N. performed cell-surface protein expression, ATPase assays. T.T.S. and P.T.N. performed phosphorylation assays. C.D. and M.F. conducted patch-clamp whole-cell recordings. X.-C.B. and E.U. collected the cryo-EM data. X.-C.B. and P.T.N. carried out cryo-EM reconstruction and model building. P.T.N and X.-C.B. wrote the paper with inputs from other authors.

Competing interests
The authors declare no competing interests.

Additional information
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