nature portfolio

Peer Review File

Integrative structural analysis of the human endosomal Na^+/H^+ exchanger NHE6 reveals a lipid-associated intracellular gate and a disordered C-terminus

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In general, the structural data are carefully done however, to support the various structure-function mechanistic hypotheses in the paper, various additional functional experiments should be performed as outlined below. In addition, the interpretation and specific details of how the functional experiments were performed need to be addressed.

1.Functional assay: The assay contained 150 mM RbCl. It is possible that ubidium can interact with the ion binding site and interfere with K interaction more than Na? In addition, in the presence of valinomycin in the functional assay, added K may not be as effective in driving NHE6 flux because of its permeation through valinomycin thereby collapsing and affecting concentration gradient.
In additional, before concluding that K and Na are equally as effective, it would be importance to provide full dose-response curves assessing the initial rates of H+ flux versus various concentrations of [Na] or [K] to determine their apparent affinities/K0.5 and Vmax for the exchanger. Moreover, it is also important to consider that the cytoplasm in vivo has a much higher concentration in mammalian cells of K than Na, therefore the chance of NHE6 binding K is significantly greater even were one to assume equal affinities and Vmax for both ions. Finally, the assay should be done also with an amiloride-like inhibitor to confirm that NHE-specific function is being assessed.

2.NHE6 ion binding site: It would be helpful to test the assigned residues using functional mutagenesis assays: A195, T262, D263, S288 and D292. The densities could correspond to Na+ or water molecules as mentioned. It would be useful do mention/discuss the putative water molecules in the context of H+ binding/transport.

3.Interaction of the C1 alpha helix L581/L582 residues with TM7 K350: Functional mutagenesis experiments should be performed to determine the biological importance of this proposed interaction. Deletion of the C1 alpha helix (W565–S585) could also potentially provide evidence for its functional importance.

4.Lipids interacting with IL2 connecting TM3 and TM4 and pushing the loop towards the entrance of the ion-binding site re-positioning R202 to act as a gate: This should be tested functionally by mutating R201, R202, Y194, E415 to de-stabilize the hypothesized hydrogen bond interactions. In addition, the location/residues of the lipid interactions is not experimentally shown/verified. In addition, a more detailed comparison between NHE6 and NHE9 lipid binding sites would be generally useful.

5.Patient missense mutations: The effect of the ΔE287-S288, G491D and L582P mutations on the function of the exchanger should be documented to confirm the hypothesized effects based on their structural localization.

6.N-terminal processing: SignalP 6.0 predicts a signal peptide and the most N-terminal peptide by MS is the semi-tryptic peptide beginning at L32. The cleavage should be validated by constructs with and without the signal peptide sequence.

Minor:

1.Modeling of the loops: LL1 was modeled from Alphafold/EcNHE9 to generate a composite model. The local resolution and

model confidence in these loops should be quantified and how any uncertainty impacts the gating model should be addressed. In the figures where appropriate, where loop positions impact the mechanistic hypotheses, loop residues that are built versus those that are grafted should be annotated/depicted clearly.

2.Elevator mechanism: The structure is in the IF open state. Previous NHE9 papers with some modelling and comparison to bacterial homologues (like NhaA) claim that the NHEs are elevator transporters. NHE1 has an OF and IF structure with elevator-like transport and a small vertical shift. This can be discussed and the NHE1 and NHE9 papers cited.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
This is a technically sound and timely integrative study of HsNHE6 that combines cryo-EM, functional reconstitution, lipidomics, and NMR/SAXS. The inward-open structure reveals the conserved NHE fold with 13 TMs per protomer, a plausible lipid-occupied cavity, and a C1 helix that contacts TM7. Functional assays show exchange of H^+ with both Na^+ and K^+, while NMR and SAXS confirm that the distal C-terminus is intrinsically disordered. Together, these findings build a framework for thinking about NHE6 regulation and disease mutations.
That said, several aspects need to be reinforced or clarified:

Major points
1. Ion selectivity and kinetics
The ACMA assay shows transport with both Na^+ and K^+, but the conclusion of “no preference” is too strong without kinetic data. Please provide quantification if possible and/or include mixed-ion competition and explore pH-dependence. A complementary assay (e.g., SSM-based electrophysiology or radiotracer flux) would strengthen the claim. Also discuss the discrepancy with yeast complementation data suggesting a K^+ preference, and the physiological context where cytosolic [K^+] far exceeds [Na^+].

2. Lipid-stabilized gate
The lipid densities near C1/IL2 and the arrangement of basic residues at the cytosolic entrance are intriguing, but at present the mechanism is largely inferential. It would strengthen the claim to:
o test transport in defined lipid mixtures (PC/PE variation, PIPs, ± cholesterol),
o mutate candidate gate residues (K200/R201/R202, Y194, E415, L581/L582) to see functional effects,
o and include map-only views/local resolution panels to show how confidently lipids can be modeled.

3. Ion-binding site density & assignments
Please provide local resolution maps, map–model FSC for the core, and clarify how alternative occupants (water vs cation) were treated in refinement. If the identity cannot be assigned unambiguously, the language should be tempered.

4. Functional link to disease variants
Mapping Christianson syndrome variants onto the model is useful, but the functional implications remain speculative. If feasible, it would be valuable to test one or two representative mutants (e.g., ΔE287–S288 near the catalytic site; L582P at the C1–TM7 interface) in transport assays or endosomal pH readouts.

5. Cellular localization: novelty and quantification
The IFM experiments essentially reproduce what is already known: NHE6 enrichment in early and recycling endosomes, with partial presence in lysosomes and Golgi. To clarify novelty, please quantify co-localization (Pearson/Manders coefficients), state how many cells/replicates were analyzed, and discuss the substantial plasma-membrane signal, which is often attributed to overexpression. Endogenous NHE6 is generally not considered a stable plasma-membrane protein.

6. Symmetry choice and modeling details
You note that LL1 is better resolved in the C1 map, yet you present a C2-symmetrized model. Please justify the choice explicitly (e.g., resolution gain, overall map quality, model validation metrics) and describe how LL1 was handled when preferring C2. Also consider highlighting LL1 in the relevant figure for clarity.

7. Entrance narrowing
Figure 3d would benefit from a side-by-side entrance view and a quantitative measure of the aperture (e.g., HOLE analysis or minimal cross-sectional distance) to support the “narrowing” claim. Cross-reference Figure 4a where the described interactions are depicted.

8. LL1 characterization
Given LL1’s modest size (~48 aa), have you considered NMR characterization (even 2D/3D of a peptide construct) to

strengthen its modeled features relative to AF predictions?

9. Comparative analysis with other NHEs
Please include a more quantitative comparison with published NHE structures (e.g., NHE9): RMSDs of TM cores, loop deviations, conservation of ion-site features). A small aligned-structures panel in Supplementary would help readers.

Minor points
• Methods:
o Ensure all buffers (pH/ionic strength), protein:lipid ratios, detergent/lipid identity and lots, and ACMA normalization steps (baseline, NH_4Cl de-quench) are specified.
o Define "biological replicate" vs "technical replicate."
• Figures:
o Label axes/units in transport plots.
o For lipid densities, show map-only panels at multiple thresholds, and state clearly how many lipids were modeled.
o Cross-reference figures where text describes specific panels (e.g., add "see Fig. 4a" to the sentence discussed).
• Text and style:
o Correct minor typos and wording listed below:

• Main text
o Pag. 2; line 56: harbor → harbors
o Pag. 3; line 73: "In the endosomal pathway, this..." → remove comma
o Pag. 3; line 79: "…disorders, such as autism, Christianson syndrome..." → disorders, such as autism or Christianson syndrome...
o Pag. 9; lines 230-231: "… (Fig. 2b). Here superscripts denote the TM α-helix to which each residue belongs) ..."
→ Option 1: remove "Here superscripts denote the TM α-helix to which each residue belongs)."
→ Option 2: … (Fig. 2b) (here superscripts denote the TM α-helix to which each residue belongs).
o Pag. 9; line 233: "Na+" → Na+ (superscript)
o Pag. 10; line 263: "The C-terminus has remained largely unresolved … likely due to their intrinsic disorder" → "The C-terminus has remained largely unresolved … likely due to its intrinsic disorder"
o Pag. 10; line 263: "Notably, 20 amino acids… (W565-S585)" → "Notably, 21 amino acids… (W565-S585)"
o Pag. 10; line 266-267: "…residues in C1 directly interacts with…" → "residues in C1 directly interact with"
o Pag. 11; line 290: "…This specific lipid-mediated positioning of a loop has not been observed in other, previously reported NHE structures, which lack defined cryo-EM densities corresponding to lipid molecules at this location and has their IL2 positioned differently than in HsNHE6…" → "This specific lipid-mediated positioning of a loop has not been observed in other previously reported NHE structures which lack defined cryo-EM densities corresponding to lipid molecules at this location and have their IL2 positioned differently than in HsNHE6"
o Pag. 12; line 306: Remove "As"
o Pag. 12; line 309: Remove "stable"
o Pag. 16; lines 390-391: "In addition, the ΔE287-S288TM6 mutations remove two residues…" → In addition, the ΔE287-S288TM6 mutation removes two residues…

• Figures & Supplementary
o Fig.1 caption, lines 189-191: "Transport activity is induced by the addition of 150 mM KCl (violet traces) or NaCl (green traces), respectively. d, De-quenching of ACMA (9-Amino-6-Chloro-2-Methoxyacridine) after the addition of 150 mM KCl (violet) or NaCl (green), after …" → KCL is blue (you write violet…) while NaCl is violet (there is no green…).
o Supplementary Fig. 2d: there is a typo: "CTF estiamtion (CTFFIND4)" → should be estimation.
o Supplementary Fig. 3: caption: "The resolution at … are indicated by arrows, respectively." → remove "respectively".

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #6

(Remarks to the Author)
In the manuscript "Integrative structural analysis of the human endosomal Na^+/H^+ exchanger NHE6 reveals a lipid-stabilized intracellular gate and a disordered C-terminus", Feilen et al. report a full-length structural model of the human endosomal Na+/H+ exchanger NHE6 obtained through a combination of techniques, including notably cryo electron microscopy, NMR spectroscopy and small-angle X-ray scattering. Their results show that NHE6 forms homodimers with a disordered C-terminus and a lipid-stabilized C-terminal helix with potential regulatory functions. The structural model allowed to map mutations of NHE6 that are associated with Christianson syndrome to functional regions of the protein.
Overall, the results provide valuable insight into how NHE6 operates on a molecular level. However, there are a few points that require further clarification:
The transport activity assay (Fig. 1c) shows that NHE6 exchanges Na+ and K+ against H+ without detectable preference.
1. The description of the transport assay setup is difficult to understand without prior knowledge. It should be mentioned that

the ATPase (and ATP added at the beginning) is required to establish an initial proton gradient before NHE6 activity can be measured. It would also be helpful to mention at which point of the assay the fluorophore was added, where it localizes and why NH4Cl is added at the end of the experiment.
2. The observed dequenching of ACMA upon addition of NaCl or KCl is ascribed to the activity of NHE6. However, a significant dequenching of ACMA was equally observed when NaCl and KCl were added in the absence of NHE6 (Fig. 1c). The authors should discuss this unexpected increase in ACMA fluorescence. Further, I do not agree with the statement "The dequenching signal was around two-fold higher than the signal from BrainPLEx liposomes containing only the E. coli F0F1-ATPase" (p. 8, l. 205). Based on the data shown in Fig. 1c, the signal is only about 50% higher than the background signal. Regarding this high background signal in the absence of NHE6 and the subtle differences measured here, I wonder if this assay is suitable to detect cation preferences of NHE6. As a control experiment, I would suggest adding a salt containing a monovalent cation that is not transported by NHE6.
The lipidomics analysis shows enrichment of PC and PE copurified with NHE6.
3. The authors state that PC and PE are the most enriched lipids in the NHE6 sample (p. 11, l. 276). However, looking at Supplementary Fig. 11, it is unclear whether this enrichment is statistically significant. Furthermore, in terms of fold-change, the enrichment of other lipids PI and sphingomyelin could also be important. Were these considered? I recommend to include p-values in the bar chart and discuss potential enrichment of less abundant membrane lipids.
4. In the discussion, the authors suggest that "The presence of a lipid-occupied cavity in HsNHE6 near C1 suggests a similar lipid-based mechanism may exist to restrict or tune activity based on lipid composition, ensuring compartment-specific function" (p. 18, l. 448). This statement is very intriguing and well-discussed. However, it does not fit with the assumption that the lipids occupying the NHE6 cavity are PC or PE. As bulk lipids present in the membranes of all organelles, they can hardly provide organelle-specific regulation of NHE6 depending on its localization. As mentioned by the authors a few lines above, HsNHE1 has a similar lipid-binding site that is occupied by PI(4,5)P2, a plasma membrane lipid marker. In the case of NHE6, an endosomal lipid marker such as PI(3)P would be a reasonable analogue (see Fig. 1 in https://www.nature.com/articles/s41580-022-00490-x for organelle-specific PIP distribution). Is it possible to derive any hints on the lipid identity from the shape of the cryo electron density?
In this context, the authors should mention whether or not PIPs can be detected using their lipidomics protocol. Could potentially enriched PIPs be picked up?

Minor comments/spelling:
p.3, l. 79 such as autism and Christianson syndrome
p. 5, l. 158 "LC-MS/MS was also used to identify that the N-terminus is acetylated" – where is the data underlying this statement to be found?
Fig. 1 caption Brain Polar Lipid Extract
p. 10, l. 266 At this position, residues C1 directly interact
p. 11, l. 290 and have their IL2 positioned differently
Fig. 3 caption: The beginning of the caption does not seem quite right
p. 12, l. 306: Delete "As" at the beginning of the sentence to make it fit with the following sentence.

Reviewer #7

(Remarks to the Author)
Sodium/hydrogen exchangers (NHEs) are fundamental proteins essential for maintaining the most basic aspects of cellular homeostasis: pH, volume, and sodium levels. This manuscript performed integrative structural analysis of human NHE6, together with functional analysis. The authors solved the cryo-EM structure showing homodimer conformation of HsNHE6 with inward-open conformation. Furthermore, unresolved C-terminus was investigated by NMR and SAXS to be proved to be disordered. It is an excellent work to present the full-length model by integrative methods. However, there are some points need to be addressed for further consideration.

1. HsNHE6 present in an inward-open conformation dimeric architecture. No matter C1 or C2 was applied, the dimer shows high similarity. Means it is symmetric dimer. My first question would be, as you may agree, this symmetric dimer may not present the physiological conformation. Any tentative trials did the authors try to capture different conformation? Asymmetric dimer, and/or outward-open would provide more molecular mechanism of elevator transportation.
2. The radius of K+ and Na+ are definitely different, however HsNHE6 exchange them with H+ without preference and even without dequenching difference. It is a little confusing. Does this mean HsNHE6 is capable of transporting any monovalent cation? Although suspect Na+ density is observed, and unambiguous assignment is not possible. Any further detailed descriptions, embedded density or attached density, and so on would help for understanding.
3. Line 226-234, unassigned densities probably present the simultaneous occupancy of Na+. Thereby, the transport path could be analyzed by HOLE, to clarify the elevator mechanism.

Reviewer #8

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed most of the issues. One important wording issue remains: the title phrase “lipid-stabilized intracellular gate” still implies a degree of certainty without direct evidence, because the lipid densities were not chemically assigned and the gating model remains partly conjectural. I suggest a minor revision that softens the title to for example, lipid-associated or putative lipid-stabilized intracellular gate.

Reviewer #3

(Remarks to the Author)
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Reviewer #4

(Remarks to the Author)
The authors have thoroughly addressed the concerns raised during the review process. The revisions have significantly improved the clarity, rigor, and completeness of the manuscript. In particular, the additional analyses, expanded methodological descriptions, and careful clarification of interpretations strengthen both the technical soundness and the overall presentation of the work.
The manuscript now provides a coherent and well-supported integrative structural analysis, and the conclusions are appropriately framed within the limitations of the data.

Minor typographical and formatting issues can be corrected during the proof stage (e.g.: in the SI, Fig. 21: “vied” → “viewed”)
I have no further comments and recommend the manuscript for publication in its current form.

Reviewer #5

(Remarks to the Author)
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Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with an Early Career Researcher
We are both happy that our comments have been dealt with thoroughly and recommend this manuscript for publication .

Reviewer #8

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #9

(Remarks to the Author)
My assessment is limited to the modeling of the disordered C-terminal region of NHE6 and the reweighting against SAXS data of the conformational ensemble generated using simulations.

This work is well conducted and the strategy is appropriate for the question addressed. The authors show by NMR and CD that the C-terminal region is intrinsically disordered and does not have pronounced affinity for the neutral or negatively charged lipid bilayers tested. Thus, the generated ensemble for the isolated IDR can provide a reasonable estimate of the radius of reach of the IDR in the context of the membrane protein. As shown in Figure 5e, the Rg distributions for the isolated IDR before and after reweighting show considerable overlap. Moreover, Supplementary Figure 20b shows a high effective fraction of frames for the ensemble reweighted with theta = 200. Together, these results indicate that the initial ensemble generated from the CALVADOS 2 simulation is a suitable prior for BME reweighting.

Both the simulations and reweighting appear to have been carried out using a Colab notebook, with the URL reported in the Methods section. I only have minor comments aimed at improving the clarity of the legend of Figure 5e and ensuring the reproducibility of the results in case the notebook is modified or no longer available.

1. Figure 5e. The black curve is described as the Rg distribution from simulations before reweighting. However, the label reports the experimental Rg value, which may be confusing. I suggest replacing the label with the average Rg from the initial simulation ensemble. The experimental Rg could instead be shown as a vertical line.

2. Page 34, line 903. Although these details can currently be found in the Colab notebook, it would be useful to report in the Methods (i) how often conformations were saved during the simulation (every 70 ps in the Colab notebook) and (ii) the total number of conformations in the resulting ensemble. It would also be useful to specify that SAXS curves were calculated for each conformation using Pepsi-SAXS after backmapping from coarse-grained to all-atom structures using cg2all. The authors may consider citing the related work, i.e., PMID: 32006288 for BME, PMID: 28471369 for Pepsi-SAXS, and PMCID: PMC10872525 for cg2all.

3. Page 35, line 910. Since Supplementary Figure 20d shows the reduced chi-squared as a function of the effective fraction of frames, it may be clearer to refer to the "high effective fraction of frames, phi_eff=exp(S_rel)" rather than "high S_rel".

First, we would like to thank all the reviewers for the time they spent on our manuscript. We very much appreciate that they took the time to go through our work and for their kind suggestions on how to make our study stronger. We have revised our manuscript according to their highlighted comments as described in our point-by-point response below.

Reviewer #2 (Remarks to the Author)

In general, the structural data are carefully done however, to support the various structure-function mechanistic hypotheses in the paper, various additional functional experiments should be performed as outlined below. In addition, the interpretation and specific details of how the functional experiments were performed need to be addressed.

We would like to thank reviewer #2 for their comments on our manuscript and their recognition of our structural work as carefully executed. We appreciate their comments and suggestions to include additional functional experiments and believe that by revising our manuscript according to their suggestions, our study is significantly strengthened as described below.

1. Functional assay: The assay contained 150 mM RbCl. It is possible that ubidium can interact with the ion binding site and interfere with K interaction more than Na? In addition, in the presence of valinomycin in the functional assay, added K may not be as effective in driving NHE6 flux because of its permeation through valinomycin thereby collapsing and affecting concentration gradient.

We thank reviewer #2 for their comments regarding the transport of various monovalent cations by HsNHE6. The reviewer raises an important point that Rb^+ present in the assay buffer could potentially interact with the ion binding site and interfere with K^+ (and Na^+) transport. Similarly, the presence of valinomycin in the original assay may indeed have allowed K^+ permeation across the membrane, thereby collapsing and affecting the K^+ concentration gradient. We acknowledge that these confounding factors, together with the limited sensitivity of the original assay performed on a Flexstation plate reader, made it difficult to draw robust conclusions about cation preferences from our initial data. Motivated by these concerns and suggestions on the liposomal assay by all reviewers, we have significantly revised the functional assay and expanded upon the data analysis compared to our original submission.

To directly address these concerns and to investigate cation selectivity in more detail, we decided to replace NaCl with choline chloride (ChCl) in all purification buffers. Critically, by replacing RbCl with ChCl in the assay buffer, we eliminated the possibility of Rb^+ interfering with cation binding at the ion binding site. Furthermore, the omission of valinomycin from the assay removed the risk of K^+ permeation collapsing the driving force. These modifications, together with a change in the plate reader instrumentation from a Flexstation to a ClarioStar multi-mode microplate reader, substantially improved

assay sensitivity and enabled us to show that HsNHE6 reconstituted in liposomes transports Na^+, K^+, Li^+ and Rb^+ *in vitro*. Since we extended our functional analysis of HsNHE6 in liposomes to these four monovalent cations (and several different lipid environments), we have expanded the respective section in our manuscript, and dedicated an entire figure to the liposomal assay data in Fig. 3:

*"We next assessed whether detergent-purified HsNHE6 retains ion exchange activity and investigated its cation selectivity in vitro. Given the endosomal localization of HsNHE6 and the high cytosolic K^+ concentration (~140 mM K^+ vs. ~10–15 mM Na^+), we hypothesized that HsNHE6 could transport K^+ in addition to Na^+, as previously proposed based on the survival of salt-sensitive yeast overexpressing HsNHE6 in Na^+- and K^+-rich medium[26]. Moreover, it is well established that intracellular NHEs, including HsNHE6, can also transport Li^+[3,49], prompting us to additionally test Li^+ and Rb^+. To this end, we developed a coupled-proton transport assay in which HsNHE6 was co-reconstituted with the F_0F_1-ATPase from E. coli into liposomes composed of Brain Polar Lipid Extract (BrainPLEx), a physiologically relevant lipid composition, and CHS. As for the cell-derived membrane vesicle assay, ChCl was used throughout all purification and assay buffers to avoid interference from monovalent cations (see Methods). H^+ transport, initiated by addition of 150 mM of either cation salt, was monitored through the (de-)quenching of the proton-gradient-sensitive fluorophore ACMA[44] (**Fig. 3a**). Upon the addition of ATP, the F_0F_1-ATPase establishes a proton gradient resulting in the quenching of ACMA, followed by dequenching after the addition of either of the salts (**Fig. 3b**). Subsequent addition of NH_4Cl dissipates the pH gradient, causing complete dequenching of ACMA, and serves as confirmation that the observed quenching was driven by a proton gradient. HsNHE6 supported H^+ flux upon addition of Na^+, K^+, Li^+ and Rb^+, with no detectable preference among the four cations at 150 mM salt concentration (**Fig. 3b, c**)."*

Additionally, we have also extended our description of the experimental set-up in the methods section. The new section now reads as follows:

"*Coupled-proton transport assay*

The coupled-proton transport assay was carried out in a ClarioStar multi-mode microplate reader (BMG Labtech, Ortenberg, Germany) utilizing the pH-dependent quenching and dequenching of ACMA (9-Amino-6-chloro-2-methoxyacridine) fluorescence at 480 nm (excitation wavelength: 410 nm). For the functional assay, 10 µl of protein containing liposomes were diluted into 100 µl of assay buffer (30 mM HEPES (pH 7.4), 150 mM ChCl, 5 mM $MgCl_2$, 2.5 µM ACMA) in a 96-well plate. Upon the addition of 2.5 mM ATP, the F_0F_1-ATP synthetase establishes an outward-directed pH gradient by pumping protons into the liposomes, and the transport of HsNHE6 was initiated by the addition of 150 mM salt (NaCl, KCl, LiCl or RbCl) after equilibration (~3 min). After additional ~60 s, the proton gradient was dissipated by the addition of 20 mM NH_4Cl. The recorded fluorescence data were normalized: $F_{norm} = (F - F_{min})/(F_{max}-F_{min})$, where F

represents the fluorescence intensity at a given time point, F_{min} is the mean fluorescence after complete quenching (calculated over 161-175 s), and F_{max} the mean fluorescence before ATP injection (calculated over 0-14 s). Data are presented as the mean of three independent purifications and reconstitutions with the standard error of the mean (SEM)) after baseline subtraction. Biological replicates refer to experiments conducted using HsNHE6 from independent purifications and reconstitutions. In contrast, technical replicates involve repeating the experiment using HsNHE6 from the same purification and reconstitution batch."

In additional, before concluding that K and Na are equally as effective, it would be importance to provide full dose-response curves assessing the initial rates of H+ flux versus various concentrations of [Na] or [K] to determine their apparent affinities/K0.5 and Vmax for the exchanger. Moreover, it is also important to consider that the cytoplasm in vivo has a much higher concentration in mammalian cells of K than Na, therefore the chance of NHE6 binding K is significantly greater even were one to assume equal affinities and Vmax for both ions. Finally, the assay should be done also with an amiloride-like inhibitor to confirm that NHE-specific function is being assessed.

We thank reviewer #2 for their suggestions regarding dose-response characterization and the important consideration of physiological cation concentrations. We agree that it is important to provide full dose-response curves to draw clear conclusions regarding the cation preferences. Therefore, we have performed full experiments titrating Na^+ and K^+ from 0 to 80 mM while maintaining a constant total ionic strength of 150 mM using ChCl as an osmotic substitute. These experiments were performed in POPC:POPE:POPS liposomes, which gave us the best signal-to-noise of all the lipid compositions we tested and therefore allowed us to resolve the small differences in initial transport rates required for kinetic analysis. Fitting the resulting transport activity curves to the Michaelis-Menten equation revealed an apparent K_m of 6.75 mM for K^+ and 25.13 mM for Na^+, representing an approximately 4-fold higher affinity for K^+. This is further supported by the relative transport efficiency (V_{max}/K_m) of 0.007 mM^{-1} for K^+ compared to 0.005 mM^{-1} for Na^+, confirming a kinetic preference for K^+ over Na^+. We note that this preference was not apparent from our original endpoint measurements at saturating concentrations (150 mM salt), which explains the initial conclusion of equal selectivity. We have revised the manuscript accordingly to reflect these new kinetic data and included the titration curve in the figure dedicated to the ion transport (*Fig. 3*). The corresponding text in the manuscript reads as follows:

"The endpoint measurements at saturating salt concentrations did not reveal a preference between Na^+ and K^+, but the endosomal localization of HsNHE6 facing a K^+-rich cytosol suggests that preference for K^+ would be physiologically meaningful. To resolve potential differences in the apparent affinities of HsNHE6 for these two physiologically most relevant cations, we performed kinetic experiments in

*POPC:POPE:POPS (50:30:20 mol/mol) liposomes, which provided the most favorable signal-to-noise ratio of all conditions tested, and determined the apparent K_m for Na^+ and K^+ (**Fig. 3f**). Na^+ or K^+ concentrations were titrated from 0 to 80 mM while maintaining a constant total ionic strength of 150 mM by supplementation with ChCl. From these experiments, we determined an apparent K_m of 25.13 mM for Na^+ and 6.75 mM for K^+ (**Fig. 3f**), comparable to values reported for EcNHE9[6], and for HsNHE6 in cells[3], and revealing a ~4-fold higher apparent affinity for K^+. The corresponding relative transport efficiencies (V_{max}/K_m) of 0.005 mM^{-1} for Na^+ and 0.007 mM^{-1} for K^+, further indicate that HsNHE6 operates more efficiently at physiologically relevant K^+ concentrations."*

We further agree that the substantially higher cytosolic K^+ concentration (~140 mM) compared to Na^+ (~10–15 mM) means that *in vivo*, the chance of HsNHE6 binding K^+ at the endosomal membrane is significantly greater, even assuming equal affinities for both ions. We have now addressed this point explicitly in the revised manuscript, where we discuss the physiological implications of our *in vitro* findings in the context of the cellular ion environment. The relevant passage in the discussion now reads:

"Functional reconstitution of HsNHE6 into liposomes confirmed its activity as a monovalent cation/H^+ exchanger, capable of transporting Na^+, K^+, Li^+ and Rb^+. While transport activity at saturating cation concentrations (150 mM) showed no marked selectivity between the tested cations, kinetic analysis revealed a clear preference for K^+ over Na^+, with an approximately 4-fold lower K_m and a higher relative transport efficiency, in line with previous yeast complementation assays[26]. Notably, the ability of HsNHE6 to transport Rb^+ further supports its capacity to accommodate K^+. This contrasts with NHE1[64], which does not transport K^+ or Rb^+, underscoring functional differences between these isoforms. In the physiological context, the substantially higher cytosolic K^+ concentration (~140 mM) compared to Na^+ (~10-15 mM) would strongly favor K^+ as the transported substrate at the endosomal membrane, even assuming equal affinities. Conversely, if HsNHE6 is transiently active at the plasma membrane, or immediately after invagination, where Na^+ concentration remains high and K^+ concentration low, such conditions would instead favor Na^+ transport. Together, the structural and functional data underscore the versatility of the HsNHE6 ion-binding site and its capacity to accommodate multiple physiological cations."

Regarding the suggestion to use an amiloride-like inhibitor to confirm NHE-specific function: we have tested several amiloride-like inhibitors in both functional (liposome) and binding (Flow Induced Dispersion Analysis) assays but were unable to confirm inhibition of or binding to HsNHE6. The pharmacological profile of organellar NHEs (NHE6, NHE7, NHE9) has not been systematically characterized in the literature (Pedersen and & Counillon 2019), and direct comparisons between plasma membrane and organellar isoforms with the classical amiloride derivatives are scarce. Looking at our model of HsNHE6 and the model of HsNHE1 with bound cariporide (Dong *et al.*, 2021;

PDB 7DSX) in addition to sequence alignment between all NHEs suggests that some of the key interacting residues in the HsNHE1 cariporide binding site are altered in the intracellular transporters. Our negative result is therefore difficult to interpret unambiguously: it may reflect a genuine difference in pharmacology, technical limitations of our assay, or a combination of both. Nevertheless, we want to point out that liposomes containing only the ATPase serve as robust negative control, as subtraction of the 0 mM condition eliminates any background signal and no dose-dependent transport is observed across the full concentration range tested (Fig. 3f).

2.NHE6 ion binding site: It would be helpful to test the assigned residues using functional mutagenesis assays: A195, T262, D263, S288 and D292. The densities could correspond to Na+ or water molecules as mentioned. It would be useful do mention/discuss the putative water molecules in the context of H+ binding/transport.

We thank reviewer #2 for their suggestion to functionally test the assigned ion binding site residues. To assess the functional importance of the ion binding site, we generated the mutants S288A and D292A, which target two of the key ion-coordinating residues identified in our structure. S288 is directly involved in ion coordination, while D292 is the most highly conserved residue in the NHE ion binding site. Both mutants showed reduced transport activity compared to wild-type HsNHE6 (Supplementary Fig. 11), supporting their critical role in the transport mechanism. We note that due to limited time and resources, we focused on a representative subset of mutants at functionally critical positions rather than mutating all five residues of the ion binding site.

Regarding the reviewer's suggestion to discuss putative water molecules in the context of H^+ binding and transport: we have now extended the discussion and describe the potential role of water molecules for the cation coordination, as well as H^+ transport. We again emphasize that our current resolution is not sufficient to unambiguously assign these densities. The corresponding section in the discussion reads as follows:

"Water molecules are commonly found within the coordination shell of cation transporters and may also facilitate H^+ translocation via a Grotthuss-like mechanism[63], whereby protons are relayed along a chain of ordered water molecules. The observed densities could therefore have implications for both cation coordination and H^+ transport. However, at the current resolution of 3.4 Å, unambiguous assignment is not possible."

3.Interaction of the C1 alpha helix L581/L582 residues with TM7 K350: Functional mutagenesis experiments should be performed to determine the biological importance of this proposed interaction. Deletion of the C1 alpha helix (W565–S585) could also potentially provide evidence for its functional importance.

We thank reviewer #2 for their suggestion to functionally test the C1-TM7 interaction. To probe the biological importance of this interaction, we chose to mutate K350 to alanine

(on the TM7 side), as the side chain of this residue forms hydrogen bonds with the backbone carbonyls of both L581 and L582 in C1 and is therefore central to the proposed interaction. The K350A mutation is expected to abolish both hydrogen bonds simultaneously, providing a direct readout of the functional relevance of the C1-TM7 interface. Additionally, the Christianson syndrome-linked mutation L582P, which disrupts the interaction from the C1 side, serves as a complementary probe for this interface (see also our response to point 5 below). Together, these two mutants test the C1-TM7 interaction from both sides. The K350A mutation resulted in a slight increase in transport activity compared to wild-type HsNHE6 in a cell-derived membrane vesicle assay (Supplementary Fig. 11), suggesting that the C1-TM7 interaction modulates HsNHE6 activity by stabilizing the inward-open conformation. It is worth noting that this modulatory effect may be further tuned by specific lipids, which cannot be probed in the current assay setup.

Regarding the suggestion to delete the entire C1 helix (W565-S585): we considered this approach but reasoned that a complete deletion would likely cause substantial structural perturbation beyond the specific C1-TM7 interaction, complicating the interpretation. We think that the combination of K350A and L582P allowed us to assess the importance of this interaction in a more targeted manner.

4.Lipids interacting with IL2 connecting TM3 and TM4 and pushing the loop towards the entrance of the ion-binding site re-positioning R202 to act as a gate: This should be tested functionally by mutating R201, R202, Y194, E415 to de-stabilize the hypothesized hydrogen bond interactions. In addition, the location/residues of the lipid interactions is not experimentally shown/verified. In addition, a more detailed comparison between NHE6 and NHE9 lipid binding sites would be generally useful.

We thank reviewer #2 for their suggestion to functionally test the proposed gate residues. To assess the role of the positively charged residues at the cytoplasmic entrance, we generated the R202A variant, targeting the conserved basic residue positioned at the entrance to the negatively charged funnel. Compared to WT HsNHE6, the R202A mutant showed a slightly increased transport activity in a cell-derived membrane vesicle assay (Supplementary Fig. 11), consistent with a role for R202 in restricting ion access to the binding site.

5.Patient missense mutations: The effect of the ΔE287-S288, G491D and L582P mutations on the function of the exchanger should be documented to confirm the hypothesized effects based on their structural localization.

To directly test the functional impact of CS-linked mutations, we generated the ΔE287-S288 and L582P mutants. ΔE287-S288 removes two residues near the ion binding site, including S288 which is directly involved in ion coordination. L582P disrupts the hydrogen bond between the C1 backbone and K350 at the C1–TM7 interface. Strikingly, the two

mutations showed opposing effects: while ΔE287-S288 abolished transport activity, L582P slightly increased activity compared to WT HsNHE6 (Supplementary Fig. 11), suggesting that these mutations cause Christianson syndrome through distinct mechanisms. ΔE287-S288 thus represents a loss-of-function mutation *in vitro* leading to reduced endosomal alkalization, whereas L582P acts as a gain-of-function mutation *in vitro* resulting in an increased endosomal alkalization. Both ultimately disrupt the fine-tuning of endosomal pH and endosomal-lysosomal trafficking. Notably, the gain-of-function effect of L582P mirrors that of the K350A mutation at the same interface, as discussed above (see response to point 3).

6.N-terminal processing: SignalP 6.0 predicts a signal peptide and the most N-terminal peptide by MS is the semi-tryptic peptide beginning at L32. The cleavage should be validated by constructs with and without the signal peptide sequence.

We thank reviewer #2 for highlighting the importance of defining the N-terminal processing of HsNHE6. Our data provides converging evidence for proteolytic removal of an endogenous signal peptide during biogenesis: SignalP 6.0 predicts a cleavable signal peptide with a cleavage site just before S43, and our LC-MS/MS analysis independently identifies a semi-tryptic peptide starting at L32 as the most N-terminal peptide in the mature protein (Supplementary Fig. 6). While the two approaches do not pinpoint an identical cleavage site, they are broadly consistent and both support processing in this region, in agreement with a previous study proposing that residues P27–F37 form a short α-helix that targets HsNHE6 to the ER and is subsequently cleaved.

We appreciate the suggestion to validate this cleavage using constructs with and without the predicted signal peptide sequence. However, signal peptides function as targeting determinants rather than isolated processing motifs, and their removal or alteration would fundamentally redirect HsNHE6 within the secretory pathway, typically causing mislocalization, misfolding, or ER retention of membrane proteins. Such effects would confound rather than clarify the physiological cleavage event. Importantly, with the native signal peptide intact, our IFM analysis confirms that HsNHE6 correctly localizes to early and recycling endosomes as well as the plasma membrane (Supplementary Fig. 7), consistent with previous reports (Pescosolido et al., 2021, Xinhan et al., 2011) and supporting that our construct undergoes normal biogenesis and trafficking.

Minor:

1.Modeling of the loops: LL1 was modeled from Alphafold/EcNHE9 to generate a composite model. The local resolution and model confidence in these loops should be quantified and how any uncertainty impacts the gating model should be addressed. In the figures where appropriate, where loop positions impact the mechanistic hypotheses, loop residues that are built versus those that are grafted should be annotated/depicted clearly.

We thank reviewer #2 for raising this important point. We would like to clarify that the AlphaFold model of LL1 from HsNHE6 was used to construct our full-length composite model; the LL1 from EcNHE9 only served as a template to guide the positioning of this loop onto our model.

Regarding local resolution: in neither the C1 nor C2 cryo-EM map was the local resolution of LL1 sufficient to support confident *de novo* model building. This is also why it is marked as a dotted line in Fig. 1b. We have made this explicit in the revised manuscript, where we now state:

*"The luminal loop 1 (LL1) between TM2 and TM3 appeared slightly better resolved in the C1 map (**Supplementary Fig. 5b**), likely because LL1 lies on the C2 symmetry axis at the dimer interface, but in neither case was the local resolution sufficient to support confident de novo model building of the loop. We therefore refrained from building LL1 directly from the cryo-EM maps and limited model interpretation accordingly."*

Importantly, LL1 was grafted into the composite full-length model presented in Fig. 6, where it is depicted in a distinct color (light blue) and clearly annotated in the figure legend. LL1 does not appear in any of the figures supporting our mechanistic hypotheses. We want to emphasize that all our mechanistic interpretations, including the proposed lipid-stabilized gating by R202 in IL2, are based solely on the experimental cryo-EM structure and model. IL2 connecting TM3 and TM4, which is central to the gating hypothesis, is well-resolved in the cryo-EM map and was built *de novo*, unlike LL1. The uncertainty in the LL1 region therefore does not impact on the proposed gating mechanism. Q-scores (Pintilie et al., 2025) for the IL2 region, including residues R201, R202, and Y194, are in the range of 0.03-0.56 (Supplementary Fig. 17). While the Q-scores for R201 and R202 fall below the expected value for a structure at 3.4 Å resolution, the local density was sufficient to support confident placement of these residues, which are central to the proposed gating mechanism.

We agree that our original description of the LL1 modeling may have created confusion when we refer to the full-length composite model versus the experimental cryo-EM model. To avoid this, we have removed the following sentence from the revised manuscript:

"*While neither the C1 nor the C2 cryo-EM maps allowed complete model building of LL1, we incorporated structural guidance from the EcNHE9 structural model[39] and AlphaFold3[40] predictions for the composite model (see later).*"

See also our response to Reviewer #4, point 6 below.

2.Elevator mechanism: The structure is in the IF open state. Previous NHE9 papers with some modelling and comparison to bacterial homologues (like NhaA) claim that the NHEs are elevator transporters. NHE1 has an OF and IF structure with elevator-like

transport and a small vertical shift. This can be discussed and the NHE1 and NHE9 papers cited.

We thank reviewer #2 for this suggestion. We have expanded our manuscript to include a more thorough analysis and discussion of the elevator mechanism beyond a mention in the introduction as in the original version. In the results section entitled "*HsNHE6 adopts an inward-facing conformation with the ion binding site occupied by additional cryo-EM density*", we now also compare our inward-open HsNHE6 structure with the outward-open, cariporide-bound HsNHE1 structure (Dong et al., 2021; PDB 7DSX), aligned on the dimerization domain, in addition to the comparison with the inward-open HsNHE1, HsNHE3 and EcNHE9. This comparison reveals a vertical shift of the core domain between the two conformational states, consistent with the proposed elevator mechanism. We present this analysis in a new supplementary figure (Supplementary Fig. 9) and have revised the manuscript accordingly:

*"Comparison of the HsNHE6 model with the published outward-open structure of HsNHE1 revealed a larger difference than when comparing to the inward-open HsNHE1 structure, with an RMSD of C^{α} atoms of 2.7 Å (**Supplementary Fig. 9**). Notably, the main differences were observed in the core domain TM helices, which undergo a vertical displacement within the membrane during the transition from the inward-open to the outward-open conformation."*

Additionally, we have expanded our discussion to address the implications for the transport cycle, including references to relevant publications (Okazaki et al. 2019, Winkelmann et al., 2020, Dong et al., 2021), and note that capturing HsNHE6 in an outward-open conformation remains an important future goal:

*"NHEs are proposed to transport substrates via an elevator mechanism, in which the core domain undergoes a vertical displacement relative to the static dimerization domain, alternately exposing the ion binding site to either side of the membrane as it has been previously described for HsNHE1, EcNHE9, and PaNhaP[6,7,14,59]. Consistent with this, alignment of our inward-open HsNHE6 model with the outward-open, inhibitor-bound HsNHE1 structure on the dimerization domain revealed a vertical shift of the core domain (**Supplementary Fig. 9**) with the largest per-residue C^{α} RMSD values concentrated in the core domain TM helices, further supporting an elevator-like transport mechanism. Capturing HsNHE6 in an outward-open conformation remains an important goal for fully untangling the conformational cycle of NHEs."*

Reviewer #3 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We appreciate Reviewer #3 for participating in the co-review of this manuscript and thank them for their contributions to the reviewing process.

Reviewer #4 (Remarks to the Author)

This is a technically sound and timely integrative study of HsNHE6 that combines cryo-EM, functional reconstitution, lipidomics, and NMR/SAXS. The inward-open structure reveals the conserved NHE fold with 13 TMs per protomer, a plausible lipid-occupied cavity, and a C1 helix that contacts TM7. Functional assays show exchange of H^+ with both Na^+ and K^+, while NMR and SAXS confirm that the distal C-terminus is intrinsically disordered. Together, these findings build a framework for thinking about NHE6 regulation and disease mutations.

That said, several aspects need to be reinforced or clarified:

Major points

1. Ion selectivity and kinetics

The ACMA assay shows transport with both Na^+ and K^+, but the conclusion of "no preference" is too strong without kinetic data. Please provide quantification if possible and/or include mixed-ion competition and explore pH-dependence. A complementary assay (e.g., SSM-based electrophysiology or radiotracer flux) would strengthen the claim. Also discuss the discrepancy with yeast complementation data suggesting a K^+ preference, and the physiological context where cytosolic [K^+] far exceeds [Na^+].

We thank reviewer #4 for their thorough suggestions regarding ion selectivity and kinetics.

In response to concerns about the original assay, we have fundamentally redesigned our functional characterization (see detailed description in our response to Reviewer #2, point 1). Briefly, we replaced all Na^+- and Rb^+-containing buffers with ChCl, omitted valinomycin, and changed the plate reader instrumentation, substantially improving assay sensitivity. With this optimized assay, we demonstrate that HsNHE6 transports Na^+, K^+, Li^+ and Rb^+ against H^+ without detectable preference under saturating conditions (Fig. 3). However, full dose-response experiments performed in POPC:POPE:POPS liposomes (which provided the best signal-to-noise of the lipid compositions tested), titrating Na^+ and K^+ from 0 to 80 mM while maintaining a constant total ionic strength of 150 mM using ChCl as an osmotic substitute revealed a clear kinetic preference for K^+ over Na^+. Fitting the transport activity curves to the Michaelis-Menten equation yielded approximately 4-fold higher affinity for K^+. This is further supported by a higher relative transport efficiency for K^+ compared to Na^+. We note that this kinetic preference was not apparent from our original endpoint measurements at saturating concentrations, which explains the initial conclusion of no marked selectivity. The manuscript has been revised accordingly.

Regarding the suggestion for a complementary assay such as SSM-based electrophysiology: while SSM-based electrophysiology using specialized equipment such as the SURFE^{2}R system (Nanion Technologies) has been successfully applied to

characterize other NHEs, this equipment was not available to us. Instead, we invested substantial effort in optimizing our liposomal coupled-proton transport assay, including redesigning the buffer system (replacing Na+ and Rb+ with ChCl, omitting valinomycin), changing the plate reader instrumentation, and systematically testing multiple lipid environments. We believe that the resulting assay, combined with the cellular functional assays on key mutants, provides a robust and comprehensive characterization of HsNHE6 transport activity.

Regarding the discrepancy with yeast complementation data and the physiological context of cytosolic K^+ and Na^+ concentrations: we have addressed both points in the discussion (see our response to Reviewer #2, point 1, second part). In brief, we now discuss how the substantially higher cytosolic K^+ concentration (~140 mM vs. ~10–15 mM Na^+) would strongly favor K^+ as the transported substrate at the endosomal membrane in vivo, and how the kinetic preference observed here, reflected in the ~4-fold lower K_m for K^+, is consistent with previous yeast complementation data (Hill et al., 2006). Cation selectivity *in vivo* may thus arise from a combination of intrinsic affinity differences and the prevailing ion gradients at the membrane where HsNHE6 resides.

2. Lipid-stabilized gate

The lipid densities near C1/IL2 and the arrangement of basic residues at the cytosolic entrance are intriguing, but at present the mechanism is largely inferential. It would strengthen the claim to:

- test transport in defined lipid mixtures (PC/PE variation, PIPs, ± cholesterol),

We have expanded our functional characterization of HsNHE6 in liposomes, which now includes five different synthetic lipid mixtures in addition to Brain polar lipid extract (BrainPLEx) included in our original submission. In liposomes composed of POPC alone, POPC:POPE (80:20 mol/mol) or POPC:SoyPI (95:5 mol/mol), we could not detect transport activity for HsNHE6 (Supplementary Fig. 14), indicating that these lipid compositions are not sufficient to support HsNHE6 activity *in vitro*. In contrast, liposomes composed of POPC:POPE:POPS (50:30:20 mol/mol) supported transport of all four tested monovalent cations against H^+ (Fig. 3d), suggesting that negatively charged lipids such as PS are required for HsNHE6 activity. Interestingly, the activity in POPC:POPE:POPS liposomes was ~2-fold higher than in BrainPLEx liposomes (Fig. 3c), suggesting that additional lipid species present in BrainPLEx may modulate HsNHE6 activity. To investigate this further, we tested the effect of PI, which has previously been shown to be important for the stability of the NHE dimer. Addition of SoyPI to POPC:POPE:POPS liposomes abolished HsNHE6 transport activity (Fig. 3e), indicating a modulatory, potentially inhibitory, role of PI. While we appreciate the suggestion of testing the presence of cholesterol in the liposomes, this was not feasible with our current setup, which relies on the presence of cholesteryl hemisuccinate (CHS) in the

liposome preparation to reduce membrane leakiness. We have added the description of the results above to the results section in the text which reads:

*"We next sought to investigate how the transport of the four different cations might depend on the lipid environment. To this end, we co-reconstituted HsNHE6 with the E. coli F_0F_1-ATPase in liposomes composed of five different synthetic lipid mixtures each supplemented with CHS (**Fig. 3**; **Supplementary Fig. 14a**). HsNHE6 supported transport of all four cations when reconstituted in liposomes composed of POPC:POPE:POPS (50:30:20 mol/mol). In this composition there was no cation preference at saturating salt concentrations, however the activity was ~2-fold higher than in BrainPLEx liposomes (**Fig. 3d**). In contrast, no transport activity was detected in liposomes composed of POPC alone, POPC:POPE (80:20 mol/mol) or POPC:SoyPI (95:5 mol/mol) (**Supplementary Fig. 14b**), even though the F_0F_1-ATPase was active in these liposomes, as evidenced by formation of the proton gradient. Together, these results indicate that PS-containing lipid mixtures support HsNHE6 activity in vitro, whereas mixtures lacking PS do not, suggesting that negatively charged lipids such as PS are required for HsNHE6 activity. The higher activity in POPC:POPE:POPS compared to BrainPLEx further suggested that some lipid species present in BrainPLEx may negatively modulate HsNHE6. To test this directly, and given that PI has previously been proposed to be important for NHE dimer stability[6,50], we asked whether PI itself might affect transport. Addition of SoyPI to POPC:POPE:POPS liposomes (POPC:POPE:POPS:SoyPI, 45:30:20:5 mol/mol) abolished transport activity (**Fig. 3e**), suggesting that phosphoinositide species, may exert an inhibitory or modulatory effect on HsNHE6 transport activity."*

- mutate candidate gate residues (K200/R201/R202, Y194, E415, L581/L582) to see functional effects,

To assess the functional importance of the proposed gate and C1-TM7 interface, we generated mutants targeting key residues from both sides of this structural network. The R202A mutant targets one of the conserved basic residues at the cytoplasmic entrance to the ion-binding funnel which restricts the access to the negatively charged funnel. From the C1-TM7 side, K350A disrupts the hydrogen bonds between TM7 and the backbone carbonyls of L581 and L582 in the C1 helix, while the CS-linked mutation L582P probes this interaction from the C1 side (see also our response to Reviewer #2, points 3 and 5).

All three mutations resulted in a slight increase in transport activity compared to wild-type HsNHE6 in a cell-derived membrane vesicle assay (Supplementary Fig. 11). The increased activity upon R202A substitution is consistent with a role for this residue in restricting ion access to the binding site, while the equivalent effect observed for K350A and L582P suggests that the C1-TM7 interaction modulates transport activity by

stabilizing the inward-open conformation of HsNHE6 (see also responses to reviewer #2 points 3, 4, and 5).

We note that due to limited time and resources, we focused on a representative subset of mutants at structurally informative positions rather than mutating all proposed residues. The selected mutants probe the gating mechanism (R202A) and the C1-TM7 interaction (K350A and L582P) that together form the structural basis for the proposed lipid-stabilized regulation of ion access.

- and include map-only views/local resolution panels to show how confidently lipids can be modeled.

We have now also looked at the local resolution estimation at the putative lipid binding site at the C-terminal helix (Supplementary Fig. 15). Based on the local resolution estimation for the putative lipid densities, the observed densities are at a resolution between 3.2 to 3.5 Å. This resolution is not sufficient to confidently model lipid molecules into the map, which we also have not done.

3. Ion-binding site density & assignments

Please provide local resolution maps, map–model FSC for the core, and clarify how alternative occupants (water vs cation) were treated in refinement. If the identity cannot be assigned unambiguously, the language should be tempered.

We thank reviewer #4 for these suggestions. We now provide the Q-score (Pintilie et al., 2025) a local/per-atom measure of how well an amino acid is resolved in a cryo-EM map for the putative ion binding site residues (A195^{TM3}, D263^{TM5}, S288^{TM6}, D292^{TM6}, and T262^{TM5}) in **Supplementary Fig. 10b**. For these residues, Q-scores ranging from 0.46 to 0.55 were observed, which is above the average Q-score expected for a structure at 3.4 Å resolution (0.45). Additionally, the figure also contains a panel (**Supplementary Fig. 10a**) showing the local resolution estimation for the unassigned densities at the putative ion binding sites, which we hypothesize may represent bound ions or water molecules. The observed densities are at a resolution of 3.2–3.4 Å, which is insufficient to confidently model cation(s) and/or water molecules.

Regarding the treatment of the unassigned densities during refinement: given that the resolution at the ion binding site is insufficient to unambiguously distinguish between Na^+ ions (which was added during purification) and water molecules, we chose not to model any occupants at these positions in the final refined model. Since this site corresponds to the well-characterized ion binding site in other NHEs as referenced to in the text, we interpret the densities as likely reflecting bound cation(s) and/or water molecules, but we have been careful not to assign a specific identity.

We believe the language in the manuscript is appropriately cautious but have made it clear that we did not model the densities. We explicitly state that "*the resolution of the HsNHE6 cryo-EM structure is insufficient for unambiguous density assignment and model building*" and use terms such as "*hypothesize*" and "*putative*" when referring to these densities.

4. Functional link to disease variants

Mapping Christianson syndrome variants onto the model is useful, but the functional implications remain speculative. If feasible, it would be valuable to test one or two representative mutants (e.g., ΔE287–S288 near the catalytic site; L582P at the C1–TM7 interface) in transport assays or endosomal pH readouts.

We thank reviewer #4 for this suggestion. We have generated two representative CS-linked mutants for functional characterization: ΔE287-S288, which is located near the catalytic site and removes the ion-coordinating residue S288, and L582P, which disrupts the C1–TM7 interface. Both mutations showed opposing effects on the transport activity of HsNHE6 (see also responds to reviewer #2 point 5). ΔE287-S288 abolishes HsNHE6 transport activity and L582P slightly increased activity compared to WT HsNHE6 (Supplementary Fig. 11).

5. Cellular localization: novelty and quantification

The IFM experiments essentially reproduce what is already known: NHE6 enrichment in early and recycling endosomes, with partial presence in lysosomes and Golgi. To clarify novelty, please quantify co-localization (Pearson/Manders coefficients), state how many cells/replicates were analyzed, and discuss the substantial plasma-membrane signal, which is often attributed to overexpression. Endogenous NHE6 is generally not considered a stable plasma-membrane protein.

We thank the reviewer for their suggestion. We agree that the IFM experiments confirm what is already known about NHE6, however, this was precisely their purpose. To ensure that the construct used for structural analysis behaves similarly to the native protein in mammalian cells, it was essential to verify its localization. We acknowledge that this rationale was not clearly described in the original manuscript, and we have now clarified it in the revised version.

We also agree that the observed substantial non-endosomal localization, including at the plasma membrane and Golgi, is likely due to overexpression, as noted by the reviewer. Importantly, however, the experiment demonstrates that the Flag-tagged construct does localize to early and recycling endosomes, supporting the conclusion that the construct remains structurally intact.

The revised section in the manuscript now reads as follows:

*"To validate that HsNHE6 exhibited the expected subcellular localization, HEK293F cells were transiently transfected with a pcDNA3.1(+) expression vector encoding HsNHE6-FLAG. Following fixation, cells were processed for IFM analysis to assess co-localization of the FLAG epitope with markers for the plasma membrane and specific cellular compartments (**Supplementary Fig. 7**). Confocal imaging revealed prominent localization of HsNHE6-FLAG with Rab5-positive early endosomes and transferrin receptor (TfR)-positive early/recycling endosomes. Substantial localization of HsNHE6-FLAG at the plasma membrane was also observed, likely reflecting over-expression. HsNHE6-FLAG co-localized to some extent with LAMP-1-positive lysosomes and partially with Golgin97-positive Golgi structures. These observations are consistent with reported localization patterns of HsNHE6 in cultured cells in other studies[29,42]."*

Additionally, Supplementary Fig. 7 now also contains a quantification (Pearson coefficients) of the co-localization.

6. Symmetry choice and modeling details

You note that LL1 is better resolved in the C1 map, yet you present a C2-symmetrized model. Please justify the choice explicitly (e.g., resolution gain, overall map quality, model validation metrics) and describe how LL1 was handled when preferring C2. Also consider highlighting LL1 in the relevant figure for clarity.

We thank reviewer #4 for this important point. We processed our data with both C1 and C2 symmetry. Consistent with the approach taken for other published mammalian NHE structures (Dong et al. 2021, Kokane et al. 2025), we applied C2 symmetry given the homodimeric nature of HsNHE6. Both reconstructions are of comparable quality as shown in Supplementary Fig. 3. The C1 reconstruction confirmed that the dimer is symmetric, with the two resulting models overlaying closely (RMSD of ~0.3 Å across Cα atoms, Supplementary Fig. 5a), and no meaningful structural differences were observed between the two protomers in the C1 reconstruction. We therefore chose to focus our analysis on the C2-symmetrized map, which simplifies model building and interpretation by treating both protomers equivalently. We acknowledge that this choice precludes detection of potential asymmetries between protomers, which may exist in a functional context; however, no such asymmetry was evident in our C1 data. We have added an explanation of our choice in the results section:

*"Given the high similarity between the C1 and C2 maps, the absence of detectable asymmetry between protomers (**Supplementary Fig. 5a**), and the dimeric nature of HsNHE6, we focused the structural analysis on the model derived from the C2-symmetrized reconstruction."*

Regarding LL1: We note that LL1 lies on the C2 symmetry axis at the dimer interface, which may explain why this region appears slightly better resolved in the C1 map as symmetry averaging across the axis can reduce density for features that are not perfectly

symmetric. Nevertheless, in neither map was the local resolution of LL1 sufficient for confident *de novo* model building. LL1 has therefore only been incorporated into the composite full-length model (deposited on Zenodo *(10.5281/zenodo.18628699)*) seen in Fig. 6 based on a high-confidence AlphaFold3 prediction and guided by the positioning of the homologous loop in EcNHE9. In this figure, LL1 is depicted in a distinct color (light blue) and annotated with a legend added directly to the figure. Importantly, LL1 does not appear in any figures supporting our mechanistic hypotheses, which are based solely on the experimental cryo-EM model (see also our response to Reviewer #2, Minor point 1).

To avoid confusion between the composite full-length model and the model derived from the experimental cryo-EM maps, we have removed the following sentence from the revised manuscript:

"*While neither the C1 nor the C2 cryo-EM maps allowed complete model building of LL1, we incorporated structural guidance from the EcNHE9 structural model*[39] *and AlphaFold*[3,40] *predictions for the composite model (see later).*"

7. Entrance narrowing

Figure 3d would benefit from a side-by-side entrance view and a quantitative measure of the aperture (e.g., HOLE analysis or minimal cross-sectional distance) to support the "narrowing" claim. Cross-reference Figure 4a where the described interactions are depicted.

The manuscript contains a comparison of the entrance with other published NHE models, showing the entrance in Supplementary Fig. 19. Here we show the electrostatic surface of HsNHE1 (inward and outward open conformation PDB ID 7DSV, 7DSX), HsNHE9 (PDB ID 8PXB) and NhaP (PDB ID 4CZA) as well as a cartoon representation comparing each of the published models with our HsNHE6 model.

Additionally, we have now performed a MOLE (Raček et al., 2025) analysis of our HsNHE6 structural model and compared it to HsNHE1 (PDB ID 7DSV) and EcNHE9 (PDB ID 8PXB). In Supplementary Fig. 18, we compare the radius of the calculated tunnels as well as represent the tunnel within the respective structural models. This analysis revealed that the tunnel of HsNHE6 is indeed narrow at the position where it passes R202, which could not be observed for HsNHE1 and EcNHE9.

We have now included the interaction between K350 and L581 and L582 in Fig. 4a (previously Fig. 3a) and added the appropriate cross-reference to provide a clear overview of this interaction.

8. LL1 characterization

Given LL1's modest size (~48 aa), have you considered NMR characterization (even 2D/3D of a peptide construct) to strengthen its modeled features relative to AF predictions?

We appreciate the reviewer's suggestion to characterize LL1 by NMR. We have carefully considered this and believe that, while technically feasible given the modest size of LL1 (~48 aa), NMR characterization of an isolated LL1 peptide construct would be of limited value in this context. In the full-length protein, LL1 is positioned at the dimer interface where it interacts with the opposing protomer and is in proximity to putative lipid densities. These interactions, which are critical for the positioning of the loop, would not be recapitulated in an isolated peptide, which would likely adopt a different conformation. Furthermore, LL1 contains a predicted N-linked glycosylation site at N128, which would be absent in a recombinantly expressed peptide and may contribute to the structure of the loop in vivo. We note that the AlphaFold3 prediction for LL1 shows high confidence and predicts a β-hairpin structure and disorder propensity if low for LL1, consistent with the structure of the homologous loop in EcNHE9. Importantly, LL1 is only included in the composite full-length model (Fig. 6) and does not contribute to any of the mechanistic hypotheses presented in this study.

9. Comparative analysis with other NHEs

Please include a more quantitative comparison with published NHE structures (e.g., NHE9): RMSDs of TM cores, loop deviations, conservation of ion-site features). A small aligned-structures panel in Supplementary would help readers.

We appreciate the interest in enhancing the clarity of our manuscript by reviewer #4. We would kindly like to point out that a comparison with published NHE structural models is included in Supplementary Fig. 8 of the original submission. This figure presents an overlay of our HsNHE6 structural model other inward-open mammalian NHEs, HsNHE1, HsNHE3, and EcNHE9. The figure includes both the overall Cα atom RMSD values and the per-residue RMSD analysis. In the main text this reads as:

*"The RMSDs of C^{α} atoms between HsNHE6 and inward-open HsNHE1 or HsNHE3 are approximately 2 Å, with significant variations observed in the loop regions and between TM1 and TM2 (**Supplementary Fig. 8a, b**). In contrast, the RMSD between HsNHE6 and EcNHE9 is around 1 Å, with differences primarily observed in the loops (**Supplementary Fig. 8c**)."*

Additionally, we now also compare our inward-open HsNHE6 structural model with the outward-open HsNHE1 structural model (**Supplementary Fig. 9**) and added a short description of this comparison in the main text:

*"Comparison of the HsNHE6 model with the published outward-open structure of HsNHE1 revealed a larger difference than when comparing to the inward-open HsNHE1 structure, with an RMSD of C^{α} atoms of 2.7 Å (**Supplementary Fig. 9**). Notably, the main differences were observed in the core domain TM helices, which undergo a vertical displacement within the membrane during the transition from the inward-open to the outward-open conformation."*

We hope that both the figures and the accompanying text address the concern of the reviewer.

Minor points

Methods:

Ensure all buffers (pH/ionic strength), protein:lipid ratios, detergent/lipid identity and lots, and ACMA normalization steps (baseline, NH_4Cl de-quench) are specified.

We thank the reviewer for highlighting the lack of clarity in our original manuscript regarding the buffer composition, protein:lipid ratio and normalization steps. To improve reproducibility, we now include a more thorough description of the protein:lipid ratios in the method section:

"Briefly, liposomes were destabilized with 0.65 % (v/v) sodium cholate before addition of 100 µg of HsNHE6 and E. coli F0F1 ATP synthetase (2:1 molar ratio) resulting in a 1:25 protein:lipid w/w ratio."

Furthermore, we decided to extend upon the lipid compositions addressed in our study, adding five synthetic mixtures and have therefore added a new section, describing which liposomes were formed and how HsNHE6 together with the F0F1 ATP synthetase was reconstituted into these liposomes:

"Protein reconstitution into liposomes

For functional characterization, HsNHE6 was reconstituted in liposomes formed of either POPC (Avanti Research, Alabaster, Alabama, US), POPC:POPE (80:20 mol/mol, Avanti Research), POPC:SoyPI (95:5 mol/mol, Avanti Research), POPC:POPE:POPS (50:30:20 mol/mol), POPC:POPE:POPS:SoyPI (45:30:20:5 mol/mol , Avanti Research) or Brain Polar Lipid Extract (BrainPLEx, Avanti Research). Lipids solubilized in chloroform were dried under a constant stream of N_2, CHS powder was added in a 5:1 w/w ratio and lipids and CHS were resuspended in 30 mM HEPES (pH 7.4), 150 mM ChCl, 5 mM $MgCl_2$ to a final lipid concentration of 10 mg/ml. The lipid solution underwent four rounds of sonication in a bath sonicator before the liposomes were subjected to extrusion using a polycarbonate filter with a pore size of 100 nm. The reconstitution of protein into the lipid/CHS liposomes was performed based on the previous published protocol[44]. Briefly, liposomes were destabilized with 0.65 % (v/v) sodium cholate before addition of 100 µg of HsNHE6 and E. coli F0F1 ATP synthetase (2:1 molar ratio) resulting in a 1:25 protein:lipid w/w ratio. After incubation for 30 min at room temperature, detergent was removed using PD-10 desalting columns."

Additionally, we now also describe, how the ACMA data were normalized:

"The recorded fluorescence data were normalized: $F_{norm} = (F - F_{min})/(F_{max}-F_{min})$, where F represents the fluorescence intensity at a given time point, F_{min} is the mean fluorescence

after complete quenching (calculated over 161-175 s), and F_{max} the mean fluorescence before ATP injection (calculated over 0-14 s).”

Define “biological replicate” vs “technical replicate.”

We thank the reviewer for highlighting the lack of clarity in our original manuscript regarding the definition of biological and technical. To improve understanding, we included now a more explicit definition of biological and technical replicates to the corresponding method section:

“Data are presented as the mean of three independent purifications and reconstitutions, with the standard error of the mean (SEM) after baseline subtraction. Biological replicates refer to experiments conducted using HsNHE6 from independent purifications and reconstitutions. In contrast, technical replicates involve repeating the experiment using HsNHE6 from the same purification and reconstitution batch.”

Figures:

- Label axes/units in transport plots.
- For lipid densities, show map-only panels at multiple thresholds, and state clearly how many lipids were modeled.
- Cross-reference figures where text describes specific panels (e.g., add “see Fig. 4a” to the sentence discussed).

Transport plots: We would like to thank reviewer #4 for their attention to details. We have ensured that all axes are labeled with the correct units and text in the transport plots. In the revised manuscript, the transport data is displayed in its own main text figure (Fig. 3) with supplementary data plots of various lipid compositions in Supplementary Fig. 14.

Lipid densities: We appreciate the suggestion by reviewer #4 and for highlighting that our text on the lipid densities was unclear. In our cryo-EM maps we observe several non-protein densities that we interpret as likely lipids. This is further supported by our lipidomics analysis (Supplementary Fig. 16), which demonstrates the presence of several phospholipid classes in the purified HsNHE6 sample. However, because these densities cannot be assigned unambiguously to a specific lipid, we intentionally chose not to model any lipids in the final model. We have now made it more explicit in the manuscript text that we did not include lipid molecules in the final models the first time we mention non-protein densities:

*“LL1 lies across the dimerization interface, in proximity to several non-protein densities that we interpret as putative lipids in both the C1 and C2 cryo-EM reconstructions (**Supplementary Fig. 5c**), although no lipid molecules were modeled.”*

We fully understand the rationale behind showing map-only panels at different thresholds. In this case, however, we found that varying global thresholds does not substantially improve interpretability, because the appearance of the entire map changes uniformly and does not provide additional information that could guide confident lipid assignment. For this reason, and to avoid overinterpreting the data, we believe the current set of annotated figure panels in Fig. 4 offers the most accurate and transparent presentation. Instead, to provide a clearer visualization of the two lipid densities, we have added a new figure (Supplementary Fig. 15) to show the putative lipid densities colored according to the local resolution in the context of the HsNHE6 model (panel a) and structure (panel b). With this, we hope that readers can assess the strength and quality of the underlying densities.

We hope the expanded explanations and revised supplementary figure fully address the reviewer's concerns regarding lipid densities.

Text and style:
Correct minor typos and wording listed below:

- Main text
  - Pag. 2; line 56: harbor → harbors
  - Pag. 3; line 73: "In the endosomal pathway, this…" → remove comma
  - Pag. 3; line 79: "…disorders, such as autism, Christianson syndrome…" → disorders, such as autism or Christianson syndrome…
  - Pag. 9; lines 230-231: "… (Fig. 2b). Here superscripts denote the TM α-helix to which each residue belongs) …"
    → Option 1: remove "Here superscripts denote the TM α-helix to which each residue belongs)."
    → Option 2: … (Fig. 2b) (here superscripts denote the TM α-helix to which each residue belongs).
  - Pag. 9; line 233: "Na+" → Na+ (superscript)
  - Pag. 10; line 263: "The C-terminus has remained largely unresolved … likely due to their intrinsic disorder" → "The C-terminus has remained largely unresolved … likely due to its intrinsic disorder"
  - Pag. 10; line 263: "Notably, 20 amino acids… (W565-S585)" → "Notably, 21 amino acids… (W565-S585)"
  - Pag. 10; line 266-267: "…residues in C1 directly interacts with…" → "residues in C1 directly interact with"
  - Pag. 11; line 290: "…This specific lipid-mediated positioning of a loop has not been observed in other, previously reported NHE structures, which lack defined cryo-EM densities corresponding to lipid molecules at this location and has their IL2 positioned differently than in HsNHE6…" → "This specific

lipid-mediated positioning of a loop has not been observed in other previously reported NHE structures which lack defined cryo-EM densities corresponding to lipid molecules at this location and have their IL2 positioned differently than in HsNHE6"

- Pag. 12; line 306: Remove "As"
- Pag. 12; line 309: Remove "stable"
- Pag. 16; lines 390-391: "In addition, the ΔE287-S288TM6 mutations remove two residues…" → In addition, the ΔE287-S288TM6 mutation removes two residues…

- Figures & Supplementary
  - Fig.1 caption, lines 189-191: "Transport activity is induced by the addition of 150 mM KCl (violet traces) or NaCl (green traces), respectively. d, De-quenching of ACMA (9-Amino-6-Chloro-2-Methoxyacridine) after the addition of 150 mM KCl (violet) or NaCl (green), after …" → KCL is blue (you write violet…) while NaCl is violet (there is no green…).
  - Supplementary Fig. 2d: there is a typo: "CTF estiamtion (CTFFIND4)" → should be estimation.
  - Supplementary Fig. 3: caption: "The resolution at … are indicated by arrows, respectively." → remove "respectively".

Once again, we thank reviewer #4 for their attention to details. We have corrected the minor typos mentioned above as well as revised the text according to their kind suggestions. Additions to the text are highlighted in light blue throughout.

Reviewer #5 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank Reviewer #5 for co-reviewing this manuscript and for their time and contribution to the review process.

Reviewer #6 (Remarks to the Author)

In the manuscript “Integrative structural analysis of the human endosomal Na^+/H^+ exchanger NHE6 reveals a lipid-stabilized intracellular gate and a disordered C-terminus”, Feilen et al. report a full-length structural model of the human endosomal Na+/H+ exchanger NHE6 obtained through a combination of techniques, including notably cryo electron microscopy, NMR spectroscopy and small-angle X-ray scattering. Their results show that NHE6 forms homodimers with a disordered C-terminus and a lipid-stabilized C-terminal helix with potential regulatory functions. The structural model allowed to map mutations of NHE6 that are associated with Christianson syndrome to functional regions of the protein.

Overall, the results provide valuable insight into how NHE6 operates on a molecular level.

We thank reviewer #6 for their comments on our manuscript and their recognition of our study of HsNHE as valuable.

However, there are a few points that require further clarification:

The transport activity assay (Fig. 1c) shows that NHE6 exchanges Na+ and K+ against H+ without detectable preference.

1. The description of the transport assay setup is difficult to understand without prior knowledge. It should be mentioned that the ATPase (and ATP added at the beginning) is required to establish an initial proton gradient before NHE6 activity can be measured. It would also be helpful to mention at which point of the assay the fluorophore was added, where it localizes and why NH4Cl is added at the end of the experiment.

We agree with the important point raised by the reviewer. To address this, we have expanded the description of our transport assay to provide additional context and detail, particularly for readers who may not be familiar with this type of experiment. In the results section, we now describe the assay principle step by step:

*“To this end, we developed a coupled-proton transport assay in which HsNHE6 was co-reconstituted with the F_0F_1-ATPase from E. coli into liposomes […]. H^+ transport, initiated by addition of 150 mM of either cation salt, was monitored through the (de-)quenching of the proton-gradient-sensitive fluorophore ACMA[44] (**Fig. 3a**). Upon the addition of ATP, the F_0F_1-ATPase establishes a proton gradient resulting in the quenching of ACMA, followed by de-quenching after the addition of either of the salts (**Fig. 3b**).”*

We have also included a schematic of the assay set-up in Fig. 3a to aid understanding.

In the methods section, we now provide a detailed description of the assay procedure, including the buffer composition, the timing of each addition (ATP, salt, NH4Cl), and the normalization of the fluorescence data (see "Coupled-proton transport assay" in Methods).

Regarding the localization of ACMA: the fluorophore is present in the assay buffer from the beginning. Since the liposomes are added to the assay buffer after protein reconstitution, it is assumed that ACMA primarily resides on the outside of the liposomes. However, it was shown that ACMA can cross hydrophobic membranes and its membrane permeability strongly depends on the protonation state of ACMA. Protonated ACMA becomes quenched and membrane impermeable. In our assay, the protonation state of ACMA will change, making it difficult to definitively determine how ACMA is distributed during the assay (Islam et al., 2021).

NH4Cl is added as a control step at the end of the experiment where it dissipates any remaining proton gradient. NH3 (in equilibrium with $NH4^+$) freely permeates the liposome membrane and captures luminal protons, thereby collapsing the pH difference and causing complete dequenching of ACMA. This serves as an internal control to confirm that the observed quenching was driven by a proton gradient. We have added an explanation to the results section of the text:

"Subsequent addition of NH_4Cl dissipates the pH gradient, causing complete dequenching of ACMA, and serves as confirmation that the observed quenching was driven by a proton gradient."

We hope that this more in-depth explanation enhances the clarity of the experimental set-up and improves the understanding of both the methodology and the results.

2. The observed dequenching of ACMA upon addition of NaCl or KCl is ascribed to the activity of NHE6. However, a significant dequenching of ACMA was equally observed when NaCl and KCl were added in the absence of NHE6 (Fig. 1c). The authors should discuss this unexpected increase in ACMA fluorescence. Further, I do not agree with the statement "The dequenching signal was around two-fold higher than the signal from BrainPLEx liposomes containing only the E. coli F0F1-ATPase" (p. 8, l. 205). Based on the data shown in Fig. 1c, the signal is only about 50% higher than the background signal. Regarding this high background signal in the absence of NHE6 and the subtle differences measured here, I wonder if this assay is suitable to detect cation preferences of NHE6. As a control experiment, I would suggest adding a salt containing a monovalent cation that is not transported by NHE6.

We thank reviewer #6 for their critical assessment of the original transport assay. We fully agree with the concerns raised regarding the high background signal and the overstatement of the two-fold difference. In response to these and related comments from other reviewers, we have fundamentally redesigned the functional. As outlined in our response to the two reviewers above, we replaced NaCl and RbCl in all purification and assay buffers with choline chloride, which is not transported by NHEs and therefore serves as the non-transported cation control as suggested by reviewer 2. We also omitted valinomycin and changed the plate reader instrumentation from a Flexstation to a

ClarioStar multi-mode microplate reader. Together, these modifications substantially reduced the background dequenching signal and improved the sensitivity of the assay (Fig. 3). The statement about the two-fold difference has been removed from the revised manuscript. With the optimized assay, we are now able to clearly distinguish HsNHE6-specific transport from background across four different monovalent cations (Na+, K+, Li+, Rb+) and multiple lipid environments (Fig. 3, Supplementary Fig. 14). Despite our optimization of the assay, some background dequenching in the ATPase-only liposomes upon salt addition is still there. We assume that this dequenching is a result of osmotic perturbations or effects of ionic strength on ACMA fluorescence after the addition of salt. This has been also already previously observed for ACMA-based liposomal assays for other NHEs (Uzdavinys et al., 2017, Winkelmann et al. 2020).

The lipidomics analysis shows enrichment of PC and PE copurified with NHE6.

3. The authors state that PC and PE are the most enriched lipids in the NHE6 sample (p. 11, l. 276). However, looking at Supplementary Fig. 11, it is unclear whether this enrichment is statistically significant. Furthermore, in terms of fold-change, the enrichment of other lipids PI and sphingomyelin could also be important. Were these considered? I recommend to include p-values in the bar chart and discuss potential enrichment of less abundant membrane lipids.

We thank reviewer #6 for this important point. In response, we repeated the lipidomics analysis with an optimized protocol, now comprising two independent biological replicates, each with technical quadruplicates. The updated analysis (Fig. 4e, f; Supplementary Fig. 16) shows that the most enriched lipid species in the purified HsNHE6 sample compared to HEK293F cells expressing HsNHE6 are PS, PE, LysoPE and sphingomyelin (SM). We note that the enrichment profile differs from our original submission, which reported PC and PE as the most enriched species; this reflects the improved consistency of the optimized lipidomics protocol.

Regarding the addition of p-values, we want to note that our lipidomics dataset comprises only two biological replicates, which precludes meaningful statistical testing. Therefore, variance cannot be reliably estimated, and p-values would be statistically uninformative and potentially misleading. However, we have chosen to present the data additionally as fold-changes with the individual data points shown (Fig. 4f; Supplementary Fig. 16b), which we believe provides the most transparent representation of the data given the sample size.

As the reviewer suggested, we have now also considered the enrichment of less abundant lipid species. SM is indeed enriched and is now included in our revised analysis, as is LysoPE. PI was detected in both samples but was not enriched in the purified HsNHE6 sample relative to cells. Interestingly, the enrichment of PS in the purified sample is consistent with our functional data showing that PS-containing

liposomes (POPC:POPE:POPS) support HsNHE6 transport activity, whereas liposomes lacking PS (POPC, POPC:POPE) do not (Fig. 3, Supplementary Fig. 14). We have updated the manuscript text, figures, and discussion accordingly.

4. In the discussion, the authors suggest that "The presence of a lipid-occupied cavity in HsNHE6 near C1 suggests a similar lipid-based mechanism may exist to restrict or tune activity based on lipid composition, ensuring compartment-specific function" (p. 18, l. 448). This statement is very intriguing and well-discussed. However, it does not fit with the assumption that the lipids occupying the NHE6 cavity are PC or PE. As bulk lipids present in the membranes of all organelles, they can hardly provide organelle-specific regulation of NHE6 depending on its localization. As mentioned by the authors a few lines above, HsNHE1 has a similar lipid-binding site that is occupied by PI(4,5)P2, a plasma membrane lipid marker. In the case of NHE6, an endosomal lipid marker such as PI(3)P would be a reasonable analogue (see Fig. 1 in https://www.nature.com/articles/s41580-022-00490-x for organelle-specific PIP distribution). Is it possible to derive any hints on the lipid identity from the shape of the cryo electron density?

In this context, the authors should mention whether or not PIPs can be detected using their lipidomics protocol. Could potentially enriched PIPs be picked up?

We appreciate the reviewer's insightful suggestion regarding organelle-specific lipid markers such as PI(3)P and for the suggested paper on the topic. We agree that our original suggestion of compartment-specific regulation was difficult to reconcile with the observation that the bulk lipids PC and PE were the most abundant species detected. With the updated lipidomics data now showing enrichment of PS, PE, LysoPE and SM, the picture has changed, though the fundamental question of whether lipid identity at this site confers organelle-specific regulation remains open.

Regarding the cryo-EM density: the resolution of the putative lipid densities (estimated at 3.2–3.5 Å, Supplementary Fig. 15) is not sufficient to confidently distinguish between lipid headgroups, and we therefore chose not to model specific lipid species into the map.

Regarding PIP detection: our lipidomics protocol detects phosphatidylinositol (PI) but does not have sufficient sensitivity to reliably detect and quantify individual phosphoinositide (PIP) species, which are present at very low abundances in cellular membranes. Therefore, we cannot rule out that specific PIPs co-purify with HsNHE6 below our detection limit.

We have added a passage to the discussion that ties together the lipidomics, functional and structural data, and explicitly addresses the possibility that PIPs may occupy the lipid binding site *in vivo*:

"We therefore cannot rule out that PIPs such as PI(3)P, an endosomal lipid marker, may occupy the lipid binding site in vivo and contribute to compartment-specific regulation of

HsNHE6 activity, analogous to what has been suggested for PI(4,5)P_2 at the homologous site in HsNHE1[66].”

Minor comments/spelling:

- p.3, l. 79 such as autism and Christianson syndrome
- p. 5, l. 158 “LC-MS/MS was also used to identify that the N-terminus is acetylated“ – where is the data underlying this statement to be found?
- Fig. 1 caption Brain Polar Lipid Extract
- p. 10, l. 266 At this position, residues C1 directly interact
- p. 11, l. 290 and have their IL2 positioned differently
- Fig. 3 caption: The beginning of the caption does not seem quite right
- p. 12, l. 306: Delete “As” at the beginning of the sentence to make it fit with the following sentence.

We thank reviewer #6 for their attention to details. We have corrected the minor typos in the text, figure legends and figure according to their kind suggestions. Additions to the text are highlighted in light blue throughout.

As for the LC-MS/MS, we have now included an overview about the peptides with N-terminal acetylation from the LC-MS/MS experiment as Source Data to support the identification of these peptides. Additionally, we have deposited the complete Mascot Search Results from the LC-MS/MS experiment on Zenodo. In the text, we have added a reference to this data and revised the Data availability section accordingly:

“*The three full-length HsNHE6 models depicted in Fig. 6 are deposited on Zenodo (10.5281/zenodo.18628699), as well as the Mascot Search Results from the LC-MS/MS experiment and all source data. Source Data are provided with this paper.”*

Reviewer #7 (Remarks to the Author):

Sodium/hydrogen exchangers (NHEs) are fundamental proteins essential for maintaining the most basic aspects of cellular homeostasis: pH, volume, and sodium levels. This manuscript performed integrative structural analysis of human NHE6, together with functional analysis. The authors solved the cryo-EM structure showing homodimer conformation of HsNHE6 with inward-open conformation. Furthermore, unresolved C-terminus was investigated by NMR and SAXS to be proved to be disordered. It is an excellent work to present the full-length model by integrative methods. However, there are some points need to be addressed for further consideration.

We thank reviewer #7 for their comments on our manuscript and their recognition of our integrative approaches to study the structure of HsNHE.

1. HsNHE6 present in an inward-open conformation dimeric architecture. No matter C1 or C2 was applied, the dimer shows high similarity. Means it is symmetric dimer. My first question would be, as you may agree, this symmetric dimer may not present the physiological conformation. Any tentative trials did the authors try to capture different conformation? Asymmetric dimer, and/or outward-open would provide more molecular mechanism of elevator transportation.

   We fully agree with the reviewer that an asymmetric dimer and/or an outward-open conformation would provide more details regarding the transport mechanism, and we agree that a symmetric dimer may not represent the physiological conformation. Therefore, we also processed our data in C1 symmetry but could not observe an asymmetric dimer. We approached this through different means, including symmetry expansion, symmetry relaxation and focused 3D classification with/without image alignment. None of these approaches resulted in a noticeably asymmetric dimer nor a dimer with a different conformation than an inward-open conformation. This is in line with most of the published structures of mammalian NHEs, which were all solved as an inward-open symmetric dimer. The only exception to this is the HsNHE1 structure (Dong et al., 2021), which had both the inhibitor cariporide and the Calcineurin B-homologous protein 1 (CHP1) bound and was solved in a symmetric outward-open conformation. From the literature, it seems like the inward-open conformation might be an energetically favored conformation for purified (mammalian) NHEs. Additionally, it should be mentioned that NHEs in their natural environment/localization are facing a monovalent cation/H^+ gradient, which is not recapitulated in the current available structures of NHEs in either detergent micelles or nanodiscs.

In the revised manuscript, we now compare our inward-open HsNHE6 structure with the outward-open HsNHE1 structure, aligned on the TMs of the dimerization domain, which reveals a vertical shift of the core domain consistent with the proposed elevator mechanism (Supplementary Fig. 9). We highlight this in the results section and have added a point to the discussion and note that capturing HsNHE6 in an outward-open conformation remains an important future goal (see also our response to Reviewer #2, Minor point 2).

2. The radius of K+ and Na+ are definitely different, however HsNHE6 exchange them with H+ without preference and even without dequenching difference. It is a little confusing. Does this mean HsNHE6 is capable of transporting any monovalent cation? Although suspect Na+ density is observed, and unambiguous assignment is not possible. Any further detailed descriptions, embedded density or attached density, and so on would help for understanding.

We have now included a more thorough investigation of the transport capabilities of HsNHE6 comparing Na^+, K^+, Li^+, Rb^+ (Fig. 3, Supplementary Fig. 14). For a detailed description of how the assay was redesigned, we refer to our response to Reviewer #2, point 1. Under the conditions investigated, all four monovalent cations are transported, and we conclude that HsNHE6 can transport different monovalent cations *in vitro*. The actual cation specificity *in vivo* would then arise from its cellular localization. As an endosomal transporter, HsNHE6 experiences a K+ gradient due to the high cytosolic K+ concentration (~140 mM vs. ~10-15 mM Na+) and therefore would transport K+ over Na+ when located in endosomes. If HsNHE6 is active at the plasma membrane, we would expect Na^+ transport to dominate due to the high extracellular Na^+ concentration (~145 mM). We have included a deeper discussion of this in the revised manuscript, which now reads:

"Functional reconstitution of HsNHE6 into liposomes confirmed its activity as a monovalent cation/H^+ exchanger, capable of transporting Na^+, K^+, Li^+ and Rb^+. While transport activity at saturating cation concentrations (150 mM) showed no marked selectivity between the tested cations, kinetic analysis revealed a clear preference for K^+ over Na^+, with an approximately 4-fold lower K_m and a higher relative transport efficiency, in line with previous yeast complementation assays[26]. Notably, the ability of HsNHE6 to transport Rb^+ further supports its capacity to accommodate K^+. This contrasts with NHE1[64], which does not transport K^+ or Rb^+, underscoring functional differences between these isoforms. In the physiological context, the substantially higher cytosolic K^+ concentration (~140 mM) compared to Na^+ (~10-15 mM) would strongly favor K^+ as the transported substrate at the endosomal membrane, even assuming equal affinities. Conversely, if HsNHE6 is transiently active at the plasma membrane, or immediately after invagination, where Na^+ concentration remains high

and K^+ concentration low, such conditions would instead favor Na^+ transport. Together, the structural and functional data underscore the versatility of the HsNHE6 ion-binding site and its capacity to accommodate multiple physiological cations."

In Fig. 2b we are providing a view into the structural model of the ion binding site, overlayed with unassigned densities which we speculate represent bound Na+ and/or water. Importantly, we have not built any ions in the final model. In line with point 3 from Reviewer #4 on the ion binding site residues and the additional density in the ion binding site, we now provide the Q-score a local/per-atom measure of how well an amino acid is resolved in a cryo-EM map for the putative ion binding site residues ($A195^{TM3}$, $D263^{TM5}$, $S288^{TM6}$, and $D292^{TM6}$, and $T262^{TM5}$) in Supplementary Fig. 10.

3. Line 226-234, unassigned densities probably present the simultaneous occupancy of Na+. Thereby, the transport path could be analyzed by HOLE, to clarify the elevator mechanism.

We have now included a MOLE (Raček et al., 2025) analysis of the ion transport tunnel in the inward-open conformation (Supplementary Fig. 18), comparing it to other mammalian NHEs in an inward-open conformation. We have added this analysis to the results section that describes the C-terminal α-helix:

*"This narrowing by $R202^{IL2}$ was also shown by MOLE analysis[52]. Here, a tunnel formed from the putative ion binding site towards the cytoplasmic interface is narrowed around $R202^{IL2}$, compared to HsNHE1 and EcNHE9 (**Supplementary Fig. 18**)."*

Since we were not able to resolve the structure of HsNHE6 in a different conformation (asymmetric dimer, outward-open conformation), we cannot analyze the full path. Since the elevator mechanism is the assumed transport mechanism for NHEs, we use this term throughout our manuscript to describe the transport mechanism. But we would agree with the reviewer that further work needs to be done to fully untangle the elevator mechanism of NHEs, which is currently beyond the scope of this project.

Reviewer #8 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are grateful to Reviewer #8 for their contribution as a co-reviewer and for taking their time to evaluate our manuscript.

First, we would like to thank all the reviewers for their time spent on our manuscript. We are grateful to reviewer #9 for stepping in for reviewer #7 and for their useful comments regarding the modeling of the C-terminal region of NHE6. We have revised our manuscript according to the raised points by all reviewers and provide an overview of the changes in our point-by-point response below.

Reviewer #2 (Remarks to the Author)

The authors have addressed most of the issues. One important wording issue remains: the title phrase "lipid-stabilized intracellular gate" still implies a degree of certainty without direct evidence, because the lipid densities were not chemically assigned and the gating model remains partly conjectural. I suggest a minor revision that softens the title to for example, lipid-associated or putative lipid-stabilized intracellular gate.

We thank reviewer #2 again for their constructive feedback regarding our manuscript. We are glad that we could address their concerns. We have adapted our title as they suggested, which now reads: "Integrative structural analysis of the human endosomal Na^+/H^+ exchanger NHE6 reveals a lipid-associated intracellular gate and a disordered C-terminus".

Reviewer #3 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We would like to acknowledge reviewer #3 for participating in the co-review of this manuscript and thank them for their helpful input during the review.

Reviewer #4 (Remarks to the Author)

The authors have thoroughly addressed the concerns raised during the review process. The revisions have significantly improved the clarity, rigor, and completeness of the manuscript. In particular, the additional analyses, expanded methodological descriptions, and careful clarification of interpretations strengthen both the technical soundness and the overall presentation of the work.

The manuscript now provides a coherent and well-supported integrative structural analysis, and the conclusions are appropriately framed within the limitations of the data.

Minor typographical and formatting issues can be corrected during the proof stage (e.g.: in the SI, Fig. 21: “vied” → “viewed”)

I have no further comments and recommend the manuscript for publication in its current form.

Once again, we are grateful for the time reviewer #4 spent on our manuscript. We are pleased that we could address their concerns, leading to such a positive assessment and for recognizing that the revisions have improved the clarity and completeness of our manuscript.

We have corrected various typos in the manuscript in addition to the 'vied' → 'viewed' typo in SI Fig. 21.

Reviewer #5 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Once again, we sincerely thank reviewer #5 for their involvement in the co-review of this manuscript and for the valuable contributions they made throughout the reviewing process.

Reviewer #6 (Remarks to the Author)

I co-reviewed this manuscript with an Early Career Researcher We are both happy that our comments have been dealt with thoroughly and recommend this manuscript for publication .

Again, thank you to reviewer #6 for taking their time to review our manuscript and for their useful feedback. We are glad that we could address all concerns raised by the reviewer.

Reviewer #8 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are grateful to reviewer #8 for taking part in the co-review of this manuscript and appreciate the time and effort they devoted to the review process.

Reviewer #9 (Remarks to the Author)

My assessment is limited to the modeling of the disordered C-terminal region of NHE6 and the reweighting against SAXS data of the conformational ensemble generated using simulations.

This work is well conducted and the strategy is appropriate for the question addressed. The authors show by NMR and CD that the C-terminal region is intrinsically disordered and does not have pronounced affinity for the neutral or negatively charged lipid bilayers tested. Thus, the generated ensemble for the isolated IDR can provide a reasonable estimate of the radius of reach of the IDR in the context of the membrane protein. As shown in Figure 5e, the Rg distributions for the isolated IDR before and after reweighting show considerable overlap. Moreover, Supplementary Figure 20b shows a high effective fraction of frames for the ensemble reweighted with theta = 200. Together, these results indicate that the initial ensemble generated from the CALVADOS 2 simulation is a suitable prior for BME reweighting.

We thank reviewer #9 for the very useful comments which helped increase the clarity of our analysis.

Both the simulations and reweighting appear to have been carried out using a Colab notebook, with the URL reported in the Methods section. I only have minor comments aimed at improving the clarity of the legend of Figure 5e and ensuring the reproducibility of the results in case the notebook is modified or no longer available.

1. Figure 5e. The black curve is described as the Rg distribution from simulations before reweighting. However, the label reports the experimental Rg value, which may be confusing. I suggest replacing the label with the average Rg from the initial simulation ensemble. The experimental Rg could instead be shown as a vertical line.

We have modified figure 5e according to the suggestion by reviewer #9, changing the label and indicating the experimental Rg by a purple vertical line. We have updated the figure legend accordingly.

2. Page 34, line 903. Although these details can currently be found in the Colab notebook, it would be useful to report in the Methods (i) how often conformations were saved during the simulation (every 70 ps in the Colab notebook) and (ii) the total number of conformations in the resulting ensemble. It would also be useful to specify that SAXS curves were calculated for each conformation using Pepsi-SAXS after backmapping from coarse-grained to all-atom structures using cg2all. The authors may consider citing the

related work, i.e., PMID: 32006288 for BME, PMID: 28471369 for Pepsi-SAXS, and PMCID: PMC10872525 for cg2all.

3. Page 35, line 910. Since Supplementary Figure 20d shows the reduced chi-squared as a function of the effective fraction of frames, it may be clearer to refer to the "high effective fraction of frames, phi_eff=exp(S_rel)" rather than "high S_rel".

We thank reviewer #9 for their kind suggestions. We have expanded the methods section describing the Molecular Dynamics simulations of HsNHE6$_{G586\text{-}A701}$, adding more details and clarity. As suggested, we have (i) included how often conformations were saved and (ii) the total number of conformations in the resulting ensemble. We now also specify that the SAXS curves were calculated for each conformation using Pepsi-SAXS after backmapping from coarse-grained to all-atom structures using cg2all, and cite the suggested papers throughout. Finally, we refer to the "high effective fraction of frames, phi_eff=exp(S_rel)" rather than "high S_rel". Our updated text now reads:

"To obtain an ensemble of the HsNHE6$_{G586\text{-}A701}$ protein, CALVADOS 2[53] was used. We reweighted the ensembles of HsNHE6$_{G586\text{-}A701}$ against the SAXS data using Bayesian maximum entropy (BME) reweighting (https://colab.research.google.com/github/KULL-Centre/ColabCALVADOS/blob/main/simulate_and_reweight/CALVADOS_simulate_and_reweight.ipynb#scrollTo=Oy-YHFWr_w5n)[97]. θ is a scaling parameter in BME reweighting that is set to balance the experimental and simulations data, enduring a good agreement with the experimental data (low $\chi 2$) and retaining as much information as possible from the starting simulation (high effective fraction of frames, phi_eff=exp($Srel$))[97]. We selected θ= 200 (**Supplementary Fig. 20d**). Conformations were saved every 70 ps during the simulations with the total number of conformations in the resulting ensemble reaching 1000. The input parameters were the amino acid sequence, pH 7.4, 0.15 M NaCl, and 20 °C, as well as the SAXS scattering curve. The SAXS curve was then calculated for each conformation using Pepsi-SAXS[98] after back mapping from coarse-grained to all-atom structures using cg2all[99]. From these curves, an average SAXS curve was generated and the *Rg* distribution extracted."

Fig. 5. Order and disorder in the C-terminus of HsNHE6. a, Cartoon representation of one HsNHE6 monomer with a zoom into the C-terminal C1 helix next to TM4, 7 and 9, with the two hydrogen bonds between K350^{TM7} and the C1 backbone carbonyls. Different domains are colored as in Fig. 1. **b,** NMR secondary chemical shifts (SCS) of C$^{\alpha}$ nuclei for HsNHE6$^{G586\text{-}A701}$. The cylinders indicate the positions of transient helices and arrows transient extended structure. **c,** Experimental SAXS scattering data of HsNHE6$^{G586\text{-}A701}$ (pink) and back-calculated SAXS scattering curve from the reweighted ensemble from the simulations (gray line). **d,** Dimensionless Kratky plot of the SAXS data. Error bars in c and d represent propagated statistical uncertainties from detector counting statistics, radial averaging, and buffer subtraction. **e,** Probability distribution of R_g before weighting to the experimental SAXS data (black) and from the reweighted ensemble from the simulations (gray). The experimentally determined Rg is indicated by a vertical line (pink). Three structures from the ensemble from the reweighted simulations with different R_gs

are represented in a bead-model in gray.