Structure, mechanism and lipid-mediated remodeling of the mammalian Na\(^+\)/H\(^+\) exchanger NHA2

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Supplementary Data Table 1: MD simulations.

| simulation name | state- A | state- B | run length (ns) | binding- A | binding- B |
|-----------------|----------|----------|-----------------|------------|------------|
| f-01-0          | 0        | 1        | 178522          | 716.6      | strong(1)  |
| f-01-1          | 0        | 1        | 178522          | 390.1      | —          |
| f-01-2          | 0        | 1        | 178522          | 386.9      | partial(2) |
| f-23-1          | 2        | 3        | 179068          | 374.1      | —          |
| f-23-2          | 2        | 3        | 179068          | 382.1      | —          |

Protomers A and B were prepared with different protonation states: state 0 (both Asp277 and Asp278 deprotonated), state 1 (Asp277 protonated, Asp278 deprotonated), state 2 (Asp277 deprotonated, Asp278 protonated), and state 3 (both Asp277 and Asp278 protonated).

(1) Spontaneous binding from 349.9 ns onwards until the end of the simulation.
(2) Spontaneous partial binding from 327.0 ns onwards until the end of the simulation.
**Supplementary Fig. 1**

a. Relative NHA2 expression level in salt-sensitive AB11c strain

b. FSEC traces of NHA2 constructs expressed in the salt-sensitive AB11c strain

c. Thermodynamic analysis of biNHA2

- **Supplementary Fig. 1a**: The graph shows the relative NHA2 expression level in a salt-sensitive AB11c strain. The constructs are labeled with their respective mutations, such as biNHA2 WT, hsNHA2 ΔN, and various single and double mutants.

- **Supplementary Fig. 1b**: The FSEC traces illustrate the fluorescence emission spectra of different NHA2 constructs expressed in the salt-sensitive AB11c strain. The constructs are color-coded for easy identification, and the traces highlight the differences in fluorescence intensities across various volumes.

- **Supplementary Fig. 1c**: This figure presents a thermodynamic analysis of biNHA2, with a peak at 57.4 ± 1.7 °C, indicating the optimal temperature range for the biNHA2 construct.
Supplementary Data Fig. 1. Expression and quality of NHA2-GFP fusions assessed by fluorescence-detection size exclusion chromatography (FSEC) in the ABc11 yeast strain. a. Bar represent normalized expression level compared to bison NHA2_{ΔN} for various NHA2 constructs and mutants as measured by GFP fluorescence in yeast whole-cells. Errors bars represent the: mean values ± s.d. of n = 4 biologically independent samples for biNHA2_{ΔN}, D277C-D278C, biNHA2 WT, ΔTM-1, E214A, R431A, T461E, E214R-R431E, hsNHA2_{ΔN}; mean values ± s.d. of n = 3 biologically independent samples for H169A, S244D, W456A, W456F, K459A, K459R; range of n = 2 biologically independent samples for R176A, E214R, R431E, T461A, D330A-Q331A; note only the full-length bison NHA2 construct is poorly expressed (10-fold less than bison NHA2_{ΔN}) and has a large fraction of aggregates (see b.), yet still shows significant complementation at 10 mM LiCl and some complementation at 25 mM LiCl (Supplementary Fig. 3b). b. FSEC traces of listed NHA2 constructs extracted by DDM/CHS from ABc11 yeast membranes. The peak at 11.5 ml corresponds to the NHA2 homodimer and all the constructs shown here have a main FSEC peak at 11.5ml; the only constructs with a shifted predominant peak at 12.2 ml for the monomer are NHA2_{TM-1} and NHA2_{ΔN} (Q330A, D331A) shown in Fig. 3b. All constructs showing a minor monomer peak at 12.2 ml show poor folding as apparent by the aggregation peak in the void. e.g., E214R, R431A, T461E, and bison NHA2 full-length (WT). c. Thermal shift of purified monomeric NHA2_{TM-1}-GFP in the presence of DDM/CHS. Data presented are normalized mean fluorescence as mean values ± s.e.m of n = 3 technical repeats; the apparent $T_M$ was calculated with a sigmoidal 4-parameter logistic regression function.
Supplementary Fig. 2

The image contains a diagram illustrating the alignment of protein sequences for various species, including human, chimpanzee, bison, rat, mouse, and Xenopus. The diagram highlights conserved ion-binding sites (PI binding sites) and flexible regions enabling TM-1 mobility. Each species is represented with a set of aligned amino acid sequences, with specific regions marked for TM1 to TM13, showing the extent of sequence conservation and divergence across different species.
Supplementary Fig. 2. Multiple sequence alignment of bison NHA2 and eukaryotic homologues. NHA2 selected sequences of mammalian vertebrates, Xenopus laevis (African clawed frog) and Human (SLC9B2) were aligned. Residues with over 70% sequence identity are indicated by purple background. Conserved ion-binding site residues are highlighted with a red border. Positions which have been identified as part regions for lipid binding are highlighted with yellow border, and proline and glycine clusters associated with TM–1 mobility with orange border. Helix breakpoints (zigzag-line), and helices of the core 6-TM transport domain TMs (pink), dimerization domain (green) and domain-swapped helix TM –1 (blue) are shown and enumerated.
**Supplementary Fig. 3**

**a.** Salt-stress (Li⁺ or Na⁺) -> NHA2 active (vacuoles) -> Cell growth (A₆₀₀)

**b.** Normalised lithium sensitivity

**c.** Oligomerization

**d.** Ion-binding site

**e.** Normalised sodium sensitivity

**f.** Normalised sodium sensitivity

- 0 mM NaCl
- 300 mM NaCl
- 400 mM NaCl
Supplementary Fig. 3. Functional complementation of Li⁺ sensitivity in the yeast strain AB11c by heterologous expression of NHA2 constructs. a. Schematic showing the yeast salt-stress assay used for screening for NHA2 functional activity. b. AB11c strain was transformed with bison NHA2 WT, bison NHA2 ΔN, human NHA2 ΔN or bison NHA2 ΔN with both Asp277 and Asp278 have been substituted with cysteine (D277C-D278C). Yeast were grown as outlined in Methods in -URA media supplemented with 2% galactose and LiCl at listed concentrations and growth was determined by optical density of the culture at 600 nm (OD₆₀₀) after 48 at 30°C and normalised against growth of bison NHA2 ΔN. Errors bars represent the mean values ± s.d. of n = 4 biologically independent samples. c. As in a. for all bison NHA2 ΔN structural and oligomerization mutants. d. As in a. for bison NHA2 ΔN ion-binding site mutants. Errors bars represent the mean values ± s.d. of n = 4 biologically independent samples. e. In most cases Li⁺ was used to assess NHA2 complementation as Li⁺ is more toxic to yeast cells and NHA2 ΔN has a higher apparent affinity (K_D) for Li⁺ vs. Na⁺. Nevertheless, for those ion-binding site mutants that retained some Li⁺-complementation, they were further assessed for Na⁺ sensitivity. above: Yeast were grown as outlined in Methods in -URA media supplemented with 2% galactose and NaCl and growth was determined by optical density of the culture at 600 nm (OD₆₀₀) after 72 at 30°C and normalised against growth of bison NHA2 ΔN. below: To facilitate comparison the above data is shown as bar graphs for 300 and 400 mM NaCl. Errors bars represent the mean values ± s.d. of n = 3 biologically independent samples for biNHA2 ΔN, D277C-D278C, W456F, T461E and E214R-R431E; range of n = 2 biologically independent samples for E214R, R431E, T461A.
**Supplementary Fig. 4.** Cryo-EM density of *bison* NHA2\textsubscript{DN} in detergent before masked refinement.  

**a.** Cryo-EM density map of NHA2\textsubscript{DN} prior to masked refinement with the 6-TM core transport domains (coloured in pink), the dimer domain (coloured in green) and the N-terminal domain detergent with additional density that swapped helix TM –1 from the neighbouring protomer (blue).  

**b.** Cryo-EM density map for that fitted a short helix at the N-terminus.  

**c.** The full-length *bison* NHA2 N- sequence was subjected to secondary structure prediction by Jpred ([https://www.compbio.dundee.ac.uk/jpred/](https://www.compbio.dundee.ac.uk/jpred/)) with the predicted N-terminal helix that was modelled shown as a red-bar.
\[ \text{Bison NHA2}_{\Delta N}\-\mathrm{GFP} \]
\[ \text{Human NHA2}_{\Delta N}\-\mathrm{GFP} \]
\[ \text{Human KDEL3}\-\mathrm{GFP} \]
\[ \text{Bison NHA2}_{\Delta N}\-\mathrm{TM-1}\-\mathrm{GFP} \]
\[ \text{Bison NHA2}_{\Delta N}\-\mathrm{(D277C-D278C)}\-\mathrm{GFP} \]
\[ \text{Bison NHA2}_{\Delta N}\-\mathrm{(E214A)}\-\mathrm{GFP} \]
Supplementary Fig 5. Confocal localization of human and bison NHA2ΔN-GFP fusions and derived mutants. a. Confocal and widefield images of Bison NHA2ΔN-GFP, Human NHA2ΔN-GFP, Human KDEL3-GFP (ER-marker protein), and non-active ion-binding site mutants Bison NHA2ΔN (D277C-D78C)-GFP, and Bison NHA2ΔN (E214A)-GFP in the salt-sensitive strain Ab11c. Expression of NHA2 in either plasma membrane and/or vacuoles will rescue salt-stress similar to the vacuolar Na+/H+ exchanger NHX1 (J. Biol. Chem. 272, 26145-26152; Microbiology 145, 3221-3228; PNAS, 2007 104 (47) 18677-18681). b. As in a., for Bison NHA2ΔN1-GFP and the TM7-TM8 loop mutant Bison NHA2ΔN (D330A-Q331A)-GFP that disrupt homodimerization and yet show similar localization to Bison and Human NHA2ΔN-GFP and clearly different from the ER marker (KDEL3).
Supplementary Fig 6. Native MS of pure yeast PI lipids. Mass spectra of commercially available PI lipids from yeast reported to be >98% pure (product no: 37-0132, Laradon). Mass spectrum were recorded in negative ionization mode and because of negative mode, the mass label in the PI spectrum is the mass minus one proton. The two most abundant PI lipids (32:1 and 34:1) in this mixture are consistent with the reportedly most abundant PI lipids from lipidomics of *S. cerevisiae*, which in yeast are as abundant as PC lipids (PLoS ONE 8(9): e73936; Genome Res. 2003 Feb;13(2):244-53.).
a. Intrinsic TM-1 dynamics of NHA2: lowest to mid energy modes

b. Intrinsic elevator-like motions of NHA2: mid-energy modes

Supplementary Fig. 7. Elastic Network Modelling of NHA2 dimers and monomers. a. Intrinsic TM –1 dynamics of both the monomeric (left) and the dimeric (right) forms of NHA2_{AN}. The lowest frequency modes of bison NHA2_{AN} are dominated by intrinsic TM–1 dynamics that spontaneously interconvert between the two observed structural conformations (detergent and nanodisc). b. Elevator-like motions of both monomeric and dimeric forms of NHA2. Mid-range modes (NM12-18) encode displacements of the dimer versus the transport domain that can drive transitions to inward-like states resembling the inward conformation of NapA.
Supplementary Fig. 8

a. CA RMSD (Å) vs. time t (ns)

b. RMSF (Å)

c. Na+ distance (Å)

d. Residue distances

| residue         | n1   |
|-----------------|------|
| Gly243:O        | 0.361|
| Ser243:O        | 0.035|
| Asp274-OD*      | 1.343|
| Asp275-OD*      | 0.956|
| Gly360:O        | 0.001|
| Water           | 3.491|
| **Total**       | **6.187**|

e. Molecular model

f. Molecular model
Supplementary Fig. 8 Average structural variability of the NHA2 nanodisc structure in MD simulations and ion-binding events. Conformational drift from the initial experimental structure and local flexibility was assessed with root mean square quantities. Protomers A and B, which were simulated in different protonation states were structurally superimposed on themselves and analyzed independently. Left: state 0 (D277 and D278 deprotonated). Right: state 1 (D277 protonated, D278 deprotonated). a. Cα root mean square distances (RMSD) from the first trajectory frame after energy minimization and short equilibration MD. b. Cα root mean square fluctuations (RMSF). Locations of key residues are highlighted as coloured circles. c. In simulation f-01-2, a sodium ion partially bound to the ion binding residues in protomer A (protonation state 0, i.e., D277 and D278 negatively charged). Shortest distance of any sodium ion to either carboxylate oxygen in D277 or D278: timeseries (left) and histogram (right). d. Coordination of the partially bound sodium ion, quantified by the average contributions of oxygen atoms from different residues to the first hydration shell of the bound sodium ion, $n_1$. $n_1<0.001$ are not shown. Only backbone carbonyl oxygen atoms (“O”) or the delta oxygen atoms of the carboxylate groups participated in binding. e. Top view (from the lysosolic side), with dimer domain in light green and core domain in light purple at time 327.0 ns. Water molecules within 3 Å of the sodium ion (yellow) are included. f. Top view at the end of the simulation at 386.9 ns.
Supplementary Fig. 9 Estimation of bison NHA2ΔN orientation into liposomes. *Left:* Cartoon showing the possible orientations of NHA2ΔN-GFP upon insertion into liposomes with the cleavage site inaccessible (right-side out) or accessible (inside-out). *Right:* NHA2-GFP proteoliposomes were incubated o/n with a 1:1 ratio of TEV protease or with an equal volume of buffer. TEV Cleavage efficiency was estimated at an average of 75.3 ± 1.6 (%) by band intensities of SDS-page by in-gel fluorescence (left) and Coomassie staining (right). n = 2 biologically independent experiments.
Uncropped gel for Supplementary Fig. 9