Supplementary Note 1

Titration experiments defining competitive interactions between Na^+ and Sr^{2+} or Ca^{2+} – Two sets of titration experiments were performed to define the mode of Sr^{2+} binding and its competition with Na^+. In the first set of experiments, the NCX_Mj crystals were titrated in solutions containing 10, 1 or 0.1 mM Sr^{2+} in the presence of 2.5 mM Na^+, which is necessary for crystal stabilization (**Methods**). To examine the Sr^{2+} occupancy as a function of concentration, F_o-F_c ion-omit maps of soaked crystals were calculated to the same resolution (2.9 Å for this set) and contoured at 7σ, using data that had been scaled against the same reference data. At 10 mM Sr^{2+}, a strong electron density peak was observed at S_{Ca}, indicating binding of a single Sr^{2+} ion (**Supplementary Fig. 2a**). The data shows that Sr^{2+} binding displaces Na^+, as no density was observed in any the other three sites, even at lower contour levels. Reducing Sr^{2+} from 10 to 1 mM resulted in a significant decrease of Sr^{2+} occupancy, as demonstrated by the weakened density at S_{Ca} site, indicating low Sr^{2+} affinity in outward facing NCX_Mj. At 0.1 mM, there is no measurable Sr^{2+} binding, as no density is observed at the S_{Ca} site at an equivalent map contour level. However, by recalculating the map obtained at low Sr^{2+} concentration to a higher resolution (2.55 Å) and lowering the map contour (4σ), it is possible to discern that the electron density distribution within the ion binding sites is virtually identical to that obtained with 2.5 mM Na^+ only, indicating the displacement of Sr^{2+} by Na^+ and water (**Supplementary Fig. 2a**, inset). This change in Sr^{2+} occupancy did not introduce any significant conformational change in the protein, as this Sr^{2+}-loaded structure is in a partially open state highly similar to that observed in the structure obtained at a low Na^+ concentration.

The low affinity of Sr^{2+} binding to NCX_Mj was further demonstrated in a second set of titration experiments, in which we maintained a high Sr^{2+} concentration of 10 mM while increasing Na^+ from 2.5 mM to 10 and 100 mM. At 10 mM, Na^+ can already competitively displace a large portion of bound Sr^{2+} as indicated by the weakened S_{Ca} density (**Supplementary Fig. 2b**). Concurrently, density signals at S_{int} and S_{mid} emerge from the partially bound Na^+ ion and water, respectively, as revealed by the map at lower contour levels (**Supplementary Fig. 2b**). Further increase of Na^+ amounts to 100 mM results in no observable Sr^{2+} binding, and the electron density distribution within all four sites is virtually identical to that in NCX_Mj wih 100 mM Na^+ only (**Supplementary Fig. 2b**), indicating a complete displacement of Sr^{2+} by Na^+. In addition, high Na^+ concentrations also induced the NCX_Mj structure to adopt the occluded state.

Ca^{2+} binding was defined similarly in a set of titration experiments in which the NCX_Mj crystals are soaked in solutions containing various concentrations of Ca^{2+} in the presence of 2.5 mM Na^+. At 10 mM Ca^{2+}, the Ca^{2+} density was observed at S_{mid} and S_{Ca} sites, indicating that Ca^{2+} can bind to either site, though not simultaneously owing to their close proximity (**Supplementary Fig. 2c**). At least one water molecule co-coordinates the bound Ca^{2+} ion (**Fig. 3b**). Similar to Sr^{2+}, Ca^{2+} binding excludes Na^+ as no density is observed at the Na^+ specific S_{int} site, and this Ca^{2+}-loaded structure is also in a partially open state. Reducing Ca^{2+} to 1 and 0.1 mM led to a sequential decrease in the Ca^{2+} density; Ca^{2+} thus binds with low affinity to outward-facing NCX_Mj. At 0.1 mM Ca^{2+}, there is no obvious Ca^{2+} binding and the density distribution is the same as that in the structure at low Na^+ concentration. The low affinity of Ca^{2+} binding also can be demonstrated by increasing Na^+. In 10 mM Ca^{2+}, 10 mM of Na^+ can replace Ca^{2+} to significant extent. The electron density distribution in this condition appears to reflect a mixture of partially bound Ca^{2+} (at sites S_{Ca} or S_{mid}) and partially bound Na^+ (at sites S_{Ca} and S_{int}) (**Supplementary Fig. 2c**).

Supplementary Note 2

Prediction of the mode of Ca^{2+} coordination in outward-facing occluded NCX_Mj – At the time this computational study was initiated, no crystal structure had been resolved for any Ca^{2+} bound state of NCX_Mj. As mentioned, our previous simulation study (Marinelli et al. *Proc Natl Acad Sci USA* **111**, E5354-5362, 2014) showed that the functional state of the crystal structure of NCX_Mj then available (PDB 3V5U) (Liao et al. *Science* **335**, 686-690, 2012) is that of the fully occupied Na^+ state, i.e. with Na^+ bound at S_{ext}, S_{Ca} and S_{int}, a water molecule at S_{mid}, and D240 protonated (and E54 and E213 ionized) – an interpretation now confirmed by the new high-resolution crystal structures of NCX_Mj obtained at high and low Na^+ concentrations. This notwithstanding, a weak anomalous-scattering signal (2.0 Å wavelength) had been detected at the S_{Ca} site in that Na^+-bound structure (Liao et al. *Science* **335**, 686-690, 2012), indicating that a minority subpopulation of NCX_Mj co-existed in the crystal lattice with a Ca^{2+} ion bound at this position. Albeit the detailed structure of this state could not be resolved, it was hypothesized that Ca^{2+} would be coordinated by E54 and E213 in this site, with both side-chains interacting in a bi-dentate configuration (Liao et al. *Science* **335**, 686-690, 2012).

To assess this hypothesis further (and independently from the crystallographic studies reported here), we first carried out a survey of observed Ca^{2+}-coordination geometries in protein structures the Protein Data Bank. This statistical analysis revealed that over 90% of the Ca^{2+}-binding sites that include two coordinating carboxyl groups in a bi-dentate mode also feature a total ion-coordination number of 8 (**Supplementary Fig. 3a**). This finding is consistent with a previous analysis of the Cambridge Structural Database, which reported an increased propensity for bi-dentate carboxyl-Ca^{2+} interactions in binding sites with greater coordination numbers (Katz et al. *J. Am. Chem. Soc.* **118**, 5752-5763, 1996). (Similarly for Sr^{2+}, which is transported by NCX_Mj, the most frequently observed coordination number is 8 – data not shown.) In addition, most of the two-carboxyl Ca^{2+}-binding sites in the PDB show a pseudo-octahedral coordination geometry, with each carboxylate moiety lying on an octahedron vertex. This octahedral geometry is also possible when only one carboxyl group forms a bi-dentate interaction, which likely implies a coordination number of 7 (**Supplementary Fig. 3a**). However, when there are two opposing bi-dentate contacts and the coordination-number is 8, the two carboxyl groups are most often nearly orthogonal relative to each other (**Supplementary Fig. 3b**); this configuration closely resembles the arrangement of E54 and E213 around the S_{Ca} site in the 3V5U NCX_Mj structure (Liao et al. *Science* **335**, 686-690, 2012). Conversely, the less frequent 7-coordination mode typically implies a more in-plane arrangement of the opposing carboxyl groups, which are also at a shorter distance from each other (**Supplementary Fig. 3b**).

Based on this analysis, therefore, we hypothesized that in NCX_Mj the bound Ca^{2+} ion would also have a coordination number of 8. To produce an initial model of this state, we replaced the Na^{+} ion bound to S_{Ca} in the published X-ray structure (PDB 3V5U) of the outward-facing, occluded state, and removed the Na^{+} ions from the S_{ext} and S_{int} sites. Then, to reach coordination 8 we placed two additional water molecules according to the consensus geometry of known Ca^{2+}-binding site with two opposing coordinating carboxyl groups (**Supplementary Fig. 3b**). Specifically, the first water molecule lies between S51 and D240 while the second water molecule is located between S210 and N81. This initial model was then equilibrated using extensive conventional MD simulations (**Methods**). The equilibrated structure of the Ca^{2+}-bound state is shown in **Supplementary Fig. 4b**. It is worth noting that in the proposed structure, the binding site features the same C_2 pseudo-symmetry axis observed in the 3×Na^{+} state (**Supplementary Fig. 4a**), running parallel to the membrane mid-plane. This symmetry implies that the structure of the ion-binding region, both for Na^{+} and Ca^{2+}, is likely be largely unchanged as the transporter alternates between outward- and inward-facing occluded states. Importantly, this model is consistent with the crystal structures of the Ca^{2+}- and Sr^{2+}-bound states now available (**Fig. 3**), in that the ion occupies S_{Ca}, coordinated by the carboxyl groups of E54 and E213, each in a bi-dentate mode, in addition to the carbonyl groups of T50 and T209. One of the predicted water molecules is also resolved in the new crystal structure, while the other probably contributes to the second putative Ca^{2+} signal in S_{mid} (**Fig. 3**).

Supplementary Note 3

Optimization of the simulation parameters describing Ca^{2+}-carboxylate interactions – In the CHARMM27 force-field used in the simulations in this study, atoms are represented as spheres carrying a fixed electronic charge that does not change depending on their environment. That is, CHARMM27 does not account explicitly for electronic polarization and charge transfer effects, which can be very significant particularly for divalent cations (Ngo et al. *J. Chem. Theor. Comp.* **11**, 4992-5001, 2015). This notwithstanding, CHARMM27 describes Ca^{2+} in solution fairly accurately. The charge of the ion is set to +2, thus neglecting any charge-transfer to the solvent, but the van der Waals interactions between the ion and the surrounding water molecules (i.e. the Lennard-Jones (LJ) parameters ε and σ) are adjusted so as to reproduce the experimental Ca^{2+} hydration free energy, as well as Ca^{2+}-water interaction energies derived from quantum chemical calculations in the gas phase (Marchand et al. *Proteins* **33**, 265-284, 1998). As shown in **Supplementary Fig. 5a**, this model results in Ca^{2+}-water structural parameters that are in good agreement with neutron diffraction and EXAFS experiments (Hewish et al. *Nature* **297**, 138-139, 1982; Jalilehvand et al. *J. Am. Chem. Soc.* **123**, 431-441, 2001; Fulton et al. *J. Phys. Chem. A* **107**, 4688-4696, 2003). In particular, the calculated water-oxygen coordination number in solution, N, is 7.25; this value is within the experimental range, and also in very good agreement with simulations based on the AMOEBA polarizable force field (N = 7.3) (Jiao et al. *J. Phys. Chem. B* **110**, 18553-18559, 2006) or a quantum-mechanical (DFT) energy function (N ~ 7) (Bogatko et al. *Chem. Eur. J.* **19**, 3047-3060, 2013; Kahlen et al. *J. Phys. Chem. B* **118**, 3960-3972, 2014). The success of the CHARMM27 model seems to result from the fact that the peak of the Ca^{2+}-water-oxygen radial distribution function is at a slightly shorter distance (2.3 Å) than what is observed experimentally (2.4 - 2.46 Å); thus, ion-water electrostatics seem to partially compensate for the missing polarization and charge transfer effects.

We sought to examine whether CHARMM27 is similarly accurate in its description of the interaction between Ca^{2+} and carboxyl groups, which are often key for Ca^{2+} binding to proteins. We and others have previously shown that in the case of Na^+, this interaction is too strong and need be corrected (Marinelli et al. *Proc Natl Acad Sci USA* **111**, E5354-5362, 2014). Following a similar approach, we calculated the dissociation constant of the Ca^{2+}-carboxylate pair in water, and compared with experimental values. To calculate this dissociation constant, a series of simulation systems were prepared containing 1,470 water molecules, an acetate anion and a Ca^{2+} ion; a Cl^- ion was also included to neutralize the total charge of the system. 86 configurations were prepared in which the distance between the Ca^{2+} and the acetate molecule (defined by the central carbon atom) was set at values ranging from 2 to 5 Å in intervals of 0.05 Å, and from 5 to 12 Å in intervals of 0.2 Å. To avoid artifacts the Cl^- ion was kept at a distance larger than 8 Å from either Ca^{2+} or acetate, using a flat-bottom harmonic potential of force constant k = 100 kcal/Å^2. The potential-of-mean-force (PMF) as a function of the cation-acetate distance, *r*, was then computed at constant temperature (298 K) and pressure (1 atm), using the Thermodynamic Integration method. Specifically, for each configuration we carried out a 2-ns simulation in which the cation-acetate distance is restrained to the initial reference value (using a harmonic potential of force constant k = 4,000 kcal/mol Å^2). From each of these simulations we computed the mean value of the projection of all interatomic forces (i.e. excluding those from the restraint) on the distance vector, using the implementation of the Adaptive-Biasing-Force method in NAMD (Henin et al. *J. Chem. Theor. Comp.* **6**, 35-47, 2010). The PMF was then obtained by integrating the mean-force profile along *r*. The dissociation constant of the ion pair was then derived from the PMF using the following equation (Gilson et al. *Biophys. J.* **72**, 1047-1069, 1997):

$$K_d = \left[4\pi \int_0^{R_{\mathrm{off}}} \exp\left\{ -\frac{\mathrm{PMF}(r)}{k_B T} \right\} r^2 \, dr \right]^{-1}$$

where R_{off} is the distance that defines the binding region. Note that if *r* is in Å, this equation implies the K_d is in units of 1 molecule/Å^3 (to convert to moles/liter, multiply by 1,660.54). As shown in **Supplementary Fig. 5b**, this analysis demonstrates CHARMM27 severely overestimates the interaction between Ca^{2+} and carboxyl groups; the measured and calculated dissociation constants for the Ca^{2+}-acetate ion-pair in solution is off by several orders of magnitude. Similarly to Na^+, however, this deficiency can be corrected by shifting the distance at which the LJ potential that specifically describes the ion-carboxyl-oxygen interaction reaches its minimum, or R_{min} ($R_{\mathrm{min}} = 2^{1/6}\sigma$) (Marinelli et al. *Proc Natl Acad Sci USA* **111**, E5354-5362, 2014). Here, we find that for Ca^{2+}, a value of 3.23 Å (compared with the original 3.067 Å) results in calculated dissociation constants that are in excellent agreement with experimental values (Luo et al. *J. Phys. Chem. Lett.* **1**, 183-189, 2010; Kahlen et al. *J. Phys. Chem. B* **118**, 3960-3972, 2014), with a minor alteration in the geometry of the cation-anion pair (**Supplementary Fig. 5b**). Specifically, when the two ions are in contact, the Ca^{2+} to carboxylate- oxygen distances increase from 2.2 Å for the original CHARMM27 to 2.34 Å for the corrected force-field. Consistently, the Ca^{2+}-water coordination number (in the context of the ion-pair) does not vary significantly when introducing this correction (**Supplementary Fig. 5c**), matching the value obtained with *ab initio* MD simulations (N ~ 5) (Kahlen et al. *J. Phys. Chem. B* **118**, 3960-3972, 2014).

Supplementary Note 4

Correcting the free-energy landscape of Ca^{2+}-bound NCX_Mj for charge-transfer effects – From a mechanistic standpoint, a key feature of the free-energy landscape of the Ca^{2+}-bound state calculated with the fixed-charge CHARMM27 force-field (**Fig. 6a**, 'uncorrected') is that it reflects a coexistence of the open, semi-open and occluded states. It is however intriguing that the open, maximally hydrated state appears to be the most energetically preferred, relative to the semi-open and occluded states, in contrast to what we observe for Na^{+} (**Fig. 5a**). Although the magnitude of this preference (~2 kcal/mol) is sufficiently small to be entirely compatible with a plausible transport mechanism, the current and previous structural studies (Liao et al. *Science* **335**, 686-690, 2012) do indicate that the Ca^{2+}-bound transporter tends to adopt occluded or semi-open conformations, at least in the context of the crystal lattice. Therefore we questioned whether the computational result might require further examination.

Specifically, we reasoned that the preference for the open, hydrated state might stem from the fact that the fixed-charge CHARMM27 force-field does not account for variations in charge transfer effects in different environments. That is, in the fixed-charge model, the electronic charge carried by the isolated Ca^{2+} in solution is unchanged when the ion is bound to a protein site. Although as discussed above this model can be reasonably corrected, for example, in the case of a single carboxyl group interacting with Ca^{2+} in solution, the fixed-charge approximation is likely to be less suitable when Ca^{2+} interacts with multiple electronegative groups concurrently, as in NCX_Mj (**Supplementary Fig. 4b**). Charge-transfer effects are indeed known to be particularly significant for divalent cations (Kohagen et al. *J. Phys. Chem. Lett.* **5**, 3964-3969, 2014; Ngo et al. *J. Chem. Theor. Comp.* **11**, 4992-5001, 2015), and imply a reduction in the electronic charge of the atoms involved (in absolute value); thus, we sought to examine whether such a change in the charge distribution of the ion-binding sites would diminish the cost of dehydration of the protein interior.

To assess the magnitude of these hypothetical charge-transfer effects in NCX_Mj, we examined the ion-binding region (in the outward-occluded conformation) occupied by either 3 or 2 Na^{+} ions, and either by Ca^{2+} or Sr^{2+}, through quantum-mechanical (QM) calculations; analogous calculations were carried out for clusters of a cation and water and of a cation-carboxyl pair and water.

These QM calculations were carried out using Gaussian 09 (Frisch et al., 2009). The protein region that was analyzed was defined using a truncation radius of ~8 Å around each of the binding sites; this region therefore includes most of the conserved residues in the so-called α-repeats, for a total of ~260 atoms. Truncated residues in this region were capped with acetyl and methyl groups. The starting structures for the QM calculations were those obtained from the classical MD simulations described above; specifically we analyzed the occluded-state structures of outward facing NCX_Mj with either 3 Na^{+} or 1 Ca^{2+} bound (**Fig. 5c, 6c**), as well as the semi-open structure with 2 Na^{+} ions (**Fig. 5c**). A structure with bound Sr^{2+} was also studied, which we generated from the Ca^{2+}-bound state by substituting Ca^{2+} with Sr^{2+}. For all these systems we carried out DFT-B3LYP calculations (Lee et al. *Phys. Rev. B* **37**, 785-789, 1988; Becke. *J. Chem. Phys.* **98**, 5648-5652, 1993). The geometry of the 1×Ca^{2+}, 3×Na^{+} and 2×Na^{+} systems was energy-optimized using a double-zeta atomic basis set with polarization functions, i.e., cc-pv-dz (Dunning. *J. Chem. Phys.* **90**, 1007-1023, 1989). During this optimization the positions of the Cα atoms were fixed (in addition to the nitrogen atoms of the two amide moieties in residues N73 and N232). A single-point energy calculation using the triple-zeta basis set cc-pv-tz (Dunning. *J. Chem. Phys.* **90**, 1007-1023, 1989) was then carried out on the optimized geometry for further analysis of the electronic properties of the ion-protein complex. For the Sr^{2+} system, we used the SDD basis set, using the Stuttgart-Dresden quasi-relativistic effective core potential (Kaupp et al. *J. Chem. Phys.* **94**, 1360-1366, 1991) for Sr^{2+} and the Dunning-Huzinaga full double-zeta basis set (D95) (Dunning et al. **3**, 1977) for the protein atoms. (To directly compare the Ca^{2+} and Sr^{2+} states, we also optimized the Ca^{2+} state using the SDD basis set.) Finally, for the smaller clusters comprising a hydrated ion or an ion-ligand pair in solution, we used the cc-pv-tz basis set both for the geometry optimization (cc-pv-dz yielded near identical results) and the single-point electronic property analysis. In all systems, Natural Bonding Orbital (NBO) and Natural Population analyses (Reed et al. *J. Chem. Phys.* **83**, 1736-1740, 1985; Reed et al. *J. Chem. Phys.* **83**, 735-746, 1985; Reed et al. *Chem. Rev.* **88**, 899-926, 1988) were used to quantify the degree of charge transfer between the ions and their environment, and to also assess the magnitude of associated donor-acceptor interaction energies.

The results of these QM calculations demonstrate, first, that the ion coordination geometries predicted by the classical MD simulations closely correspond to minima of the QM energy function (**Supplementary Table 2**). In all cases examined, the DFT-optimized geometry deviates from the input structures derived from the MD simulations by less than 0.5 Å. Interestingly, the optimized geometries for the Ca^{2+}- and Sr^{2+}-bound states are almost identical to each other (RMSD ~ 0.14 Å), consistent with the fact that NCX_Mj transports both ions similarly (as opposed to e.g. Cd^{2+}). The second clear conclusion from this analysis is that the extent of charge transfer from Ca^{2+} or Sr^{2+} to its coordinating residues in the S_{Ca} site of NCX_Mj is greater than when the ion is in solution, either solvated only by water, or interacting with a single carboxyl ligand (**Supplementary Fig. 6**). Specifically, the excess amount of charge transferred by either of the divalent cations, $\Delta\Delta q$, is approximately $0.2e$ (**Supplementary Fig. 6a, 6c**). NBO analysis indicates that 58% of this charge is transferred to the carboxyl groups of E54 and E213, 30% to the carbonyl groups of T50 and T209 and the backbone and side-chains of S51 and S210, and the rest to the two water molecules coordinating Ca^{2+} (**Supplementary Fig. 4b**). By contrast, the amount of charge transferred by Na^{+} to its ligands at the equivalent S_{Ca} site (both in the 3×Na^{+} and 2×Na^{+} states) is similar to that observed for the hydrated ion, or for ion interacting with a single carboxyl group in solution, i.e. $\Delta\Delta q \sim 0$ (**Supplementary Fig. 6b**).

To account for the excess charge-transfer effect observed in the Ca^{2+}-bound state, we introduced a correction in the derivation of the free-energy landscape from the Bias-Exchange Metadynamics simulations. In essence, this correction assigns greater statistical weight to the configurations of the system sampled during the simulation that are energetically more consistent with the adjusted charge distribution (**Methods**). As shown in **Fig. 6a**, the corrected landscape indeed favors the occluded and semi-open conformations to a greater degree than the uncorrected landscape, relative to the open, maximally hydrated state. Therefore, the charge-transfer correction yields better agreement with the previous and current crystallographic studies.