PI-(3,5)P_2-mediated oligomerization of the endosomal sodium/proton exchanger NHE9

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Abstract

Na^+/H^+ exchangers (NHE) are found in all cells to regulate intracellular pH, sodium levels and cell volume. The NHE isoform 9 (SLC9A9) fine-tunes endosomal pH, and its activity is linked to glioblastoma, epilepsy, autism spectrum and attention-deficit-hyperactivity disorders. Here, we report cryo-EM structures of *horse* NHE9 and a cysteine-variant at 3.6 and 3.1 Å resolution, respectively. We show how lysine residues, from a previously unresolved TM2-TM3 β-hairpin loop domain, are positioned above the dimerization interface and interact with the endosomal-specific PI-(3,5)P_2 lipid, together with residues located on dimer domain helices. Thermal-shift assays, solid-state membrane (SSM) electrophysiology and MD simulations, corroborates that NHE9 can specifically bind PI-(3,5)P_2, and that its addition stabilizes the homodimer and enhances NHE9 activity. We have further determined the cryo-EM structure of *E. coli* NhaA, confirming the expected coordination of cardiolipin at the dimerization interface, solidifying the concept that Na^+/H^+ exchanger dimerization and transporter activity can be regulated by specific lipids. Taken together, we propose that the activity of NHE9 is regulated by the PI-(3,5)P_2 lipid upon reaching endosomes, which we refer to as an lipid-activation-upon-arrival model.

Introduction

Na^+/H^+ exchangers (NHE) facilitate the electroneutral exchange of cations ($Na^+/Li^+/K^+$) and protons (H^+) across membranes to regulate intracellular pH, sodium levels and cell volume (Pedersen & Counillon, 2019). In mammals, there are 13 different isoforms belonging to the SLC9A (NHE1-9), SLC9B (NHA1-2) or sperm specific SLC9C families (Fuster & Alexander, 2014; Pedersen & Counillon, 2019). NHEs differ in their substrate preferences, kinetics, subcellular and tissue distribution (Pedersen & Counillon, 2019). Isoforms NHE1-5 are primarily expressed on the plasma membrane and have important physiological roles linked to cellular pH homeostasis (Pedersen & Counillon, 2019). Isoforms NHE6-9 are primary localized to intracellular compartments and work in concert with the V-type ATPase to fine-tune intracellular pH in their respective organelles.

NHEs are physiological homodimers (Brett *et al*, 2005; Fuster & Alexander, 2014; Pedersen & Counillon, 2019), with each monomer made up of a transporter unit and a C-terminal cytosolic tail between ~125-440 residues in length (Fuster & Alexander, 2014; Pedersen & Counillon, 2019). The mammalian transport modules share low sequence homology to bacterial Na^+/H^+ antiporters harbouring the "NhaA-fold", so-named after the first crystal structure obtained from *E. coli* (Brett *et al.*, 2005; Fliegel, 2019; Padan, 2008). The cryo-EM structures of NHE1 and NHE9 have been determined and nonetheless show a similar architecture to bacterial homologues with 13 transmembrane (TM) helices (Hunte *et al*, 2005; Lee *et al*, 2013; Paulino *et al*, 2014; Wohlert *et al*, 2014). The transporter unit consists of two distinct domains, a dimerization domain and an ion-transporting (core) domain. The 6-TM core domain undergoes global, elevator-like structural transitions to translocate ions across the membrane against the anchored dimerization domain (Coincon *et al*, 2016; Drew & Boudker, 2016). NHE1 is a house-keeping Na^+/H^+ exchanger, which is localised to the plasma membrane of most eukaryotic cells (Pedersen & Counillon, 2019). In contrast, NHE9 (SLC9A9) regulates the luminal pH of late- and recycling endosomes (Kondapalli *et al*, 2015; Kondapalli *et al*, 2014). Mutations in SLC9A9 have been associated with neurological disorders such as familial autism, ADHD and epilepsy (Kondapalli *et al.*, 2014; Zhang-James *et al*, 2019) and NHE9 expression is upregulated in glioblastoma, one of the most aggressive forms of brain cancers (Kondapalli *et al.*, 2015). NHE9 expression is also upregulated in response to viral infection (Slonchak *et*

al, 2019) and NHE9 variants are more frequent amongst patients who develop severe response to SARS-CoV-2 infection (Prasad, 2021).

A superimposition of the respective inward-facing structures of NHE1 and NHE9 confirms that the overall architecture is well-conserved (Fig. 1A). The main difference is that the transporter domain of NHE9 was predicted by AlphaFold 2 (AF2)(Jumper *et al*, 2021) to contain a ~ 60 residue extension of the TM2 -TM3 loop (Fig. 1B). In human NHEs, the extended loop domain sequence is present in organellar isoforms NHE7 and NHE9, as well as isoform-two of endosomal NHE6 (Supplementary Fig. 1A). Using native mass-spectrometry, we previously concluded that the NHE9 dimer co-purifies with lipid adducts of ~1KDa in size, whereas NHE9 monomers were essentially lipid-free (Winklemann *et al*, 2020). In the NHE1 nanodisc structure, aliphatic parts of fatty acids could be modelled at the dimerization interface (Fig. 1A) (Dong *et al*, 2021). The data quality limitation of the NHE9 structure in detergent, however, prevented the modelling of lipids at the dimer interface (Winklemann *et al.*, 2020). Nonetheless, we could detect map density for the TM2-TM3 loop, corresponding to a position above the aliphatic chains in NHE1 (Supplementary Fig. 1B). Native MS demonstrated that NHE9 co-purifies with negatively-charged phosphatidylinositol lipids matching the mass of PIP_2 (Winklemann *et al.*, 2020). Given the lack of positively-charged TM residues, it was hypothesized that the TM2-TM3 loop domain would help to coordinate the negatively-charged lipid head-groups. Consistently, the mutation of two lysine residues Lys105 and Lys107 in the unresolved TM2-TM3 loop domain, was sufficient to abolish PIP_2 stabilization and, under native MS conditions, the mutant was detected as a lipid-free monomer (Winklemann *et al.*, 2020). Here, we set out to resolve a cryo-EM structure of NHE9 with the TM2-TM3 loop domain to understand its role in lipid-mediated regulation.

Results

*Solid-supported membrane (SSM) electrophysiology of horse NHE9**

Previously, NHE9 from *horse* was selected for cryo-EM studies as it was found to be more detergent stable than *human* NHE9 (Winklemann *et al.*, 2020). Purified *horse* NHE9* (residues 8 to 574; out of 645) was reconstituted into liposomes together with F_0F_1-ATP synthase for proteoliposome studies and an apparent K_M of NHE9* for Na^+ of 20.5 ± 3 mM was determined (Winklemann *et al.*, 2020). However, a high

background from empty liposomes made studies of NHE9* activity challenging, as the signal-to-noise ratio was low ~ 3:1. More recently, we have used solid-supported membrane (SSM) electrophysiology to record Na^+ translocation of the mammalian Na^+/H^+ exchanger NHA2 (Matsuoka *et al*, 2022). In brief, proteoliposomes are adsorbed to an SSM and charge translocation is measured *via* capacitive coupling of the supporting membrane, following accumulation of transported ions (Bazzone *et al*, 2017). Here, to put the kinetic estimates for NHE9 on a firmer footing, we recorded SSM-data for NHE9* proteoliposomes upon increasing concentrations of Na^+ (Fig. 1D). Peak currents were ~6-fold higher than either the NHE9* variant for which ion-binding site residues Asn243 and Asp244 were substituted to alanine, or a mammalian transporter for fructose (GLUT5) (Fig. 1C). The estimated binding affinity (K_d) determined by SSM for Na^+ is 36.3 ± 4 mM, which is similar to the Michaelis–Menten K_M estimate for NHE9* in proteoliposomes (Supplementary Fig. 1D)(Winklemann *et al.*, 2020). Thus, we can confirm that NHE9* has binding affinity for sodium at a similar concentration range to the *in vivo* K_M estimates measured for other intracellular localized NHE6 and NHE8 isoforms (Pedersen & Counillon, 2019).

Cryo-EM structure of NHE9 with TM2-TM3 β-loop domain at low pH 6.5*

The previous *horse* NHE9* cryo-EM structure was determined from protein purified in the detergents LMNG and CHS and in buffer at pH 7.5 (Winklemann *et al.*, 2020). We attempted to improve the quality of the cryo-EM maps by incorporation of NHE9* into nanodiscs, but saw no significant improvement in the final map quality, when comparing to maps from the detergent samples (results not shown). As a next approach, we repeated the collection of NHE9* in detergent, but buffered to pH 6.5 where NHE9 is thought to be less active, and therefore presumably less dynamic. Overall, we collected ~4,300 movies and the final 3D-reconstruction contained data of 244,000 particles, from which an EM map could be reconstructed to 3.3 Å according to the gold-standard Fourier shell correlation (FSC) 0.143 criterion (Supplementary Fig. 2). Comparison of the NHE9* structures determined at pH 7.5 and pH 6.5 showed no apparent differences (Supplementary Fig. 1E). We speculated that the respective final 3D reconstructions may still contain heterogeneity and we therefore performed 3D-variability analysis in CryoSPARC (Punjani *et al*, 2017), followed by clustering (principal component) analysis to separate further classes. Selecting 40% of the

140 particles for heterogeneous refinement, enabled an improved cryo EM map
141 reconstruction of NHE9* at pH 6.5, with additional map features for the TM2-TM3
142 loop domain (Supplementary Fig. 3). Further homogenous refinement with C2
143 symmetry improved the cryo EM density for the TM2-TM3 loop domain with an
144 overall resolution of 3.6 Å (Supplementary Fig. 3A). As a last-step, a composite map
145 was generated for model building, combining the additional TM2-TM3 loop domain
146 features with the higher resolution EM maps (Supplementary Fig. 3B).
147
148 Previously, we modelled the TM2-TM3 loop domain of NHE9* based on several
149 structure prediction algorithms that detected the sequence homology to the β-hairpins
150 in *E. coli* NhaA (Supplementary Fig. 1C) (Lee *et al*, 2014; Winklemann *et al.*, 2020).
151 Consistently, the AF2 model of NHE9 predicts β-hairpins in the TM2-TM3 loop
152 domain with a high confidence score (Jumper *et al.*, 2021), but has a low confidence
153 score for the regions connecting the β-hairpins with extracellular ends of TM2 and TM3
154 (Fig. 1B). The AF2 prediction of the NHE9 monomer superimposes well with the
155 NHE9* structure (Supplementary Fig. 4A). The AF2 dimer model is also similar to the
156 AF2 monomer model, apart from the β-hairpin (Supplementary Fig. 4A), which is now
157 modelled to interact with the β-hairpin from its neighbouring protomer (Fig. 1E,
158 Methods). Subsequently, we refined the AF2 NHE9 dimer model into the NHE9* cryo-
159 EM maps with some further manual adjustment where needed (Methods and Fig. 1F).
160 At the extracellular end of TM2, a flexible loop, residues Pro72 to Asp82, were
161 predicted by AF2 as extending towards the centre of the dimerization interface,
162 followed by short-linker and a β-hairpin strand that is angled 60° away from the NHE9*
163 surface (Fig. 1B, E). Based on the cryo EM maps at ~ 4.0 Å resolution (Supplementary
164 Fig. 3A), we were able confidently refine the overall position of the short linker and β-
165 hairpin strand, but map density was insufficient to support some side-chains positioning
166 or the flexible loop (Fig. 1F). The β-hairpin strand loops back to the beginning of TM3
167 and contain a bent helix, annotated as extracellular helix (ECH1), which is well
168 supported by the cryo EM maps (Fig. 1F). The β-hairpins are predicted by AF2 to be
169 domain-swapped, and this arrangement refines well into the map density. In the middle
170 of the loop domain, a cluster of six positively-charged residues Lys105, Lys107 and
171 Arg108 from each of the two protomers extend towards the dimerization interface (Fig.
172 1F). Overall, the TM2-TM3 β-loop domain clasps the two protomers together on the

173 luminal side, and forms a highly positively-charged cluster that is now located above
174 the dimerization domain interface. As such, the re-modelled NHE9* structure is
175 consistent with its previously proposed requirement for binding negatively-charged
176 PIP_2 lipids (Winklemann *et al.*, 2020).

177 *Cryo-EM structure of cysteine variant (NHE9*CC) shows binding of PI-(3,5)P_2 lipids*
178 In order to improve the resolution of the NHE9* structure, we substituted Leu139 and
179 Ile444 residues in an attempt to disulphide-trap the inward-facing state. In addition, we
180 added brain lipids during each step of the NHE9*CC purification (see Methods). We
181 subsequently collected a larger data-set of 13,780 movies and the final 3D-
182 reconstruction had 78,370 particles from which an EM map was reconstructed to 3.2 Å
183 according to the gold-standard Fourier shell correlation (FSC) 0.143 criterion
184 (Supplementary Fig. 4B). Despite the similar FSC resolution estimate, the new cryo-
185 EM maps were improved with some local regions extending to 2.5 Å resolution
186 (Supplementary Fig. 4B). In particular, the density was clearer for the TM2-TM3 β-
187 loop at lower contour levels (Supplementary Fig. 4B). In NHE9*, there was no cryo
188 EM map density for the 100-residue long C-terminal regulatory domain (CTD)
189 (Winklemann *et al.*, 2020), likely a result of its predicted dynamics and intrinsic
190 disorder (Hendus-Altenburger *et al*, 2014; Norholm *et al*, 2011; Pedersen & Counillon,
191 2019). In the improved NHE9* CC maps we could, however, model an additional 52
192 residues of the C-terminal regulatory domain (Fig. 2A and Supplementary Fig. 5A). A
193 structural superimposition of the refined NHE9* CC and NHE9* structures otherwise
194 show small differences (Supplementary Fig. 4C). Map density and sulphur atom
195 distances did not support any disulphide bond formation between the two introduced
196 cysteine residues (Supplementary Fig. 5B). SSM-based electrophysiology for
197 NHE9*CC mutant confirmed that the cysteine variant binds Na^+ with similar affinity
198 as NHE9* (Supplementary Fig. 1D). Currently, it's unclear whether the improved map
199 quality for NHE9*CC is due to the introduced cysteine mutations, or by the repeated
200 addition of lipids during purification.
201

202 At the dimerization interface we could now observe additional lipid-like features in the
203 cryo EM maps (Fig. 2A). Rather than the cylinder-like density of fatty acids as seen in
204 the NHE1 nanodisc structure in POPC lipids (Dong *et al.*, 2021), the cryo-EM maps
205 show larger, head-group lipid densities with distinct features (Fig. 2A, Supplementary

206 Fig. 5C). Given that NHE9* co-purified from yeast with several lipids corresponding
207 to the molecular mass of PIP_2 (Winklemann *et al.*, 2020), we attempted to model two
208 PIP_2 molecules into the NHE9* CC maps and found that the density was a better fit for
209 the lipid phosphatidylinositol-3,5-bisphosphate PI-(3,5)$P_{2,}$, rather than the more
210 abundant lipid phosphatidylinositol-4,5-bisphosphate PI-(4,5)P_2 (Fig. 2A,
211 Supplementary Fig. 5C). Although PI-(3,5)P_2 is a minor PIP_2 lipid, it is specific to late
212 endosomes and lysosomes (Hasegawa *et al*, 2017), which overlaps with the functional
213 localization of NHE9 (Hasegawa *et al.*, 2017). In contrast, the PIP_2 lipid PI-(4,5)P_2 is
214 principally found in the plasma membrane (Ho *et al*, 2012). In an electrostatic surface
215 potential map, the PI-(3,5)P_2 lipids could neutralise the positively-charged loop domain
216 residues Lys105, Lys105' Lys107, Lys107' previously predicted to interact with PIP_2
217 lipids (Fig. 2A,B) (Winklemann *et al.*, 2020).

218 Dimerization contacts are formed between TM1 and TM8' of the neighbouring
219 protomer. The glycerol backbone and connecting acyl chains of each PI-(3,5)P_2 lipid
220 forms hydrophobic stacking interactions to Trp321 (TM8, TM8') and its indole nitrogen
221 interacts with the phosphodiester (Fig. 2A). The glutamate residue Glu23 (TM1, TM1'),
222 further hydrogen bonds to the C5-OH of the *myo*-inositol sugar and also stabilises a
223 likely water-mediated sugar interaction. The position of Glu23 could partially explain
224 the preference for PI-(3,5)P_2 lipids, since a negative-charged PO_4^{2-} at the C5-OH
225 position would interact unfavourably with the carboxylate group of Glu23. Moreover,
226 a glutamine residue Gln21 (TM1, TM1') is also well positioned to interact with the
227 phosphomonoester in the C3-OH position, but map density was insufficient to confirm
228 this (Fig. 2A). Overall, the hydrophobic dimerization interface, together with polar and
229 aromatic residues located at its surface and positive-charged residues from the TM2-
230 TM3 β-hairpin loop, create an environment well-suited for binding PI-(3,5)P_2 lipids
231 (Fig. 2B).

232

233 *PI-(3,5)P_2 lipid binding by thermostability, electrophysiology, and MD simulations*
234 The cryo-EM maps of the TM2-TM3 β-hairpin loop domain in NHE9* CC were still
235 of insufficient quality for modelling side-chains in this region (Supplementary Fig. 4B),
236 likely due to inherent flexibility. We had previously detected interactions with PIP_2 and
237 PIP_3 lipids to NHE9* using FSEC-TS and GFP-based thermal stability assays (Nji *et*

238 *al*, 2018; Winklemann *et al.*, 2020), which we confirmed could monitor cardiolipin
239 binding to the *E. coli* Na^+/H^+ antiporter NhaA (Landreh *et al*, 2017; Nji *et al.*, 2018).
240 The average melting temperature (ΔT_m) of NHE9* increased by 8°C with PI-(4,5)P_2
241 addition, whereas other lipids POPC, POPE and POPA showed no stabilization
242 (Winklemann *et al.*, 2020). To validate that PI-(3,5)P_2 would also stabilise NHE9* in a
243 similar manner, NHE9* GFP-TS melting curves were recorded in the presence of either
244 PI-(4,5)P_2 or PI-(3,5)P_2 lipids (Methods and Fig. 2C). Unexpectedly, NHE9* show
245 greater thermostabilization in the presence of PI-(3,5)P_2, with a ΔT_m of 15°C, as
246 compared to ΔT_m of 8°C for PI-(4,5)P_2. Moreover, NHE9* starts to unfold around 30°C
247 with a shallow slope for the transition temperature, which is indicative of a mixed
248 protein population (Fig. 2C). In contrast, with PI-(3,5)P_2 added, NHE9* melts with a
249 sharp transition, consistent with the shift to a single oligomeric population (Fig. 2C).
250 Using FSEC we could confirm that a higher fraction of detergent-purified NHE9*
251 dimer is retained if PI-(3,5)P_2 lipid was added prior to heating at 50ºC for 10 mins, as
252 compared to either PI-(4,5)P_2 or PC lipid addition (Supplementary Fig. 6A). We had
253 previously shown that substitution of TM2-TM3 lysine residues to glutamine in NHE9*
254 (Lys85Gln-Lys105Gln-Lys107Gln) abolished PI-(4,5)P_2 stabilization and
255 oligomerization by native MS (Winklemann *et al.*, 2020). Indeed, the NHE9*
256 (Lys85Gln-Lys105Gln-Lys107Gln) variant purified as a monomer in detergent, and the
257 addition of either PI-(3,5)P_2, PI-(4,5)P_2 or PC lipids showed only minimal
258 thermostabilization (Supplementary Fig. 6B-C). Taken together, thermostability-shift
259 assays indicates that NHE9* has a preference for the lipid PI-(3,5)P_2 and its addition
260 likely stabilises the functional homodimer.
261

262 To assess the influence of PI-(3,5)P_2 lipid addition to NHE9* activity, we incubated the
263 purified NHE9* protein with either buffer, or buffer containing solubilised PI-(3,5)P_2
264 lipids, and reconstituted this mixture into liposomes made from yeast-polar lipids
265 (Methods). SSM-based electrophysiology of NHE9* incubated with buffer produces
266 similar peak currents upon Na^+ addition to those shown previously, whereas NHE9*
267 protein incubated with PI-(3,5)P_2 lipid produced a stronger response to Na^+ addition
268 (Fig. 2D, E and Supplementary Fig. 1D), and showed an four-fold increase in its binding
269 affinity for Na^+ (K_d = 9.6 mM) (Supplementary Fig. 6E). In contrast, the PI-(4,5)P_2 lipid
270 addition had a similar Na^+ binding affinity as the buffer only addition to NHE9*

271 (Supplementary Fig. 6E). To confirm that the response to PI-(3,5)P_2 was likely mediated
272 by the expected interaction with the lysine residues in the TM2-TM3 β-hairpin loop
273 domain, we recorded SSM-based currents for the purified lysine-to-glutamine NHE9*
274 (Lys85Gln-Lys105Gln-Lys107Gln) variant. The triple glutamine variant also showed a
275 weaker affinity for Na^+ (K_d = 55 mM) compared with NHE9* and, in fact, the addition
276 of the PI-(3,5)P_2 lipid decreased its apparent affinity for Na^+ (K_d = 88 mM)
277 (Supplementary Fig. 6E). The outside surface of the PI-(3,5)P_2-stabilised NHE9* TM2-
278 TM3 β-hairpin loop domain has a partially negatively-charged surface (Fig. 2B). It is
279 possible that stabilization of the TM2-TM3 β-hairpin loop domain by PI-(3,5)P_2 lipids
280 provides an electrostatic pathway that can better attract ions to the outward-facing
281 funnel.

282 To evaluate if that the lysine residues in the TM2-TM3 β-hairpin loop domain could
283 interact with PIP_2 lipids, we carried out molecular dynamics (MD) simulations of the
284 NHE9* structure and a *in silico* modelled NHE9* (Lys85Gln-Lys105Gln-Lys107Gln)
285 variant (see Methods). In the simulations using the NHE9* structure, the PI-(3,5)P_2
286 lipid stayed within less than 3 Å from its initial position for nearly the entire simulation
287 time (Fig. 3A,B). In contrast, a modelled PI-(4,5)P_2 lipid was less stably bound with a
288 higher fraction of frames showing > 3 Å movement from its initial position (Fig. 3B).
289 The distribution of salt-bridge interactions with Lys105 and Lys107 residues revealed
290 that Lys105 residues were nearly always in contact with the PI-(3,5)P_2 lipid, whereas
291 Lys107 residues remained in contact for around half of the simulation time (Fig. 3C,E).
292 The PI-(4,5)P_2 lipid had a much larger variation for interaction with the lysine residues
293 and the preferred interaction with Lys105 was less pronounced (Fig. 3D, E). In MD
294 simulations of the modelled NHE9*(Lys85Gln-Lys105Gln-Lys107Gln) variant, neither
295 of the PIP_2 lipids were stably bound and the lipids had no clear interaction with the
296 modelled glutamines (Fig. 3B-D). Overall, the MD simulations support that lysine
297 residues in the TM2-TM3 β-hairpin loop can form stable interactions with the modelled
298 PI-(3,5)P_2 lipids observed in the NHE9*CC variant.

299

300 *Cryo EM structure of NhaA confirms cardiolipin binding at the dimerization interface*
301 Whilst modulation of transporter activity, through means of lipid-dependent
302 oligomerization might seem unorthodox, the formation of the functional homodimer of

303 the bacterial Na^+/H^+ antiporter NhaA from *E. coli* has previously been shown to be
304 dependent on the negatively-charged lipid cardiolipin (Landreh *et al.*, 2017; Nji *et al.*,
305 2018; Quick *et al*, 2021; Rimon *et al*, 2019). Since cardiolipin synthesis in *E. coli* is
306 increased upon salt-stress and, because NhaA is required to alleviate salt-stress (Padan,
307 2008; Romantsov *et al*, 2009), there is a precedence for negatively-charged lipids
308 regulating the activity of Na^+/H^+ exchangers. Comparing structures of bacterial Na^+/H^+
309 exchangers that bind cardiolipin to those that do not, have shown that the sensitivity to
310 this lipid is matched with structural differences in their oligomeric interfaces (Gupta *et*
311 *al*, 2017; Nji *et al.*, 2018).
312

313 NhaA has become a model system for the lipid-dependent oligomerization of Na^+/H^+
314 exchangers and yet, we have no structural data confirming the coordination of
315 cardiolipin at the dimerization interface of NhaA. Hence, from grids of detergent-
316 purified NhaA, a data-set of 14,329 movies was collected and yielded an EM map
317 reconstructed to 3.37 Å according to the gold-standard Fourier shell correlation (FSC)
318 0.143 criterion (Supplementary Fig. 7). As expected from native MS and MD
319 simulations (Gupta *et al.*, 2017), thermal-shift assays (Nji *et al.*, 2018), and functional
320 activity analysis (Quick *et al.*, 2021; Rimon *et al.*, 2019) clear map density to support
321 the modelling of cardiolipin at the dimerization interface was observed (Fig. 4A, B).
322 One CDL was present in the middle of the positively-charged dimerization interface,
323 and two additional CDL lipids bind on either outside surface of the dimer interface (Fig.
324 4A, B). Although the dimerization interface is very positively-charged with four
325 arginine residues per protomer — Arg203, Arg204, Arg245 and Arg250 — only the
326 Arg204 residue directly interacts with the phosphate headgroups of CDL, and Thr205
327 hydrogen bonds to the oxygen atom of the distal phosphoester bonds (Fig. 4C, D).
328 Deeper in the pocket, the side chain of Trp258 interacts with both the central and the
329 flanking CDL molecules, where the side-chain is sandwiched between the distal
330 glycerol moieties of the CDL lipids, forming hydrophobic stacking interactions with
331 the acyl chains. This coordination of CDL is entirely consistent with computational
332 analysis of CDL binding sites from analysis of more than 40 different *E. coli* proteins
333 (Corey *et al*, 2021). Moreover, we have previously shown that an Arg203Ala and
334 Arg204Ala double-mutant abolished thermostabilization of NhaA by CDL
335 (Winkelmann *et al*, 2022). We have previously shown that between pH 4 and pH 6.5

336 the inward-facing cavity of NhaA opens up to allow Na^+ entry (Winkelmann *et al.*,
337 2022). The homodimer structure of NhaA at pH 7.5 also shows an open inward-facing
338 cavity like the NhaA monomeric crystal structure at pH 6.5 (Fig. 4E). Taken together,
339 we have been able to confirm the coordination of CDL binding to the NhaA homodimer,
340 representing its most native structural conformation seen to date.

341

342 *Further mechanistic insights from the improved cryo-EM NHE9 CC* structure*
343 A detailed mechanistic model for ion-exchange in NHE proteins is yet to be established,
344 as the coordination geometry of the transported Na^+ is unknown. Whilst molecular
345 dynamic simulations and biochemical and biophysical approaches have provided an
346 important framework in the bacterial exchangers (Alhadeff & Warshel, 2015; Arkin *et*
347 *al*, 2007; Huang *et al*, 2016; Kozachkov & Padan, 2013; Paulino & Kuhlbrandt, 2014),
348 a detailed understanding of the mammalian NHE's require an accurate model for the
349 position of the ion-binding sites. In the improved NHE9*CC maps, we were able to
350 confidently model all side-chains forming the ion-binding site, which are positioned
351 around the half-helical cross-over (Fig. 5A and B). We observed some minor differences
352 between the side-chain positions of Thr214, Asp215, Glu239 and Arg408 from the
353 previously reported NHE9* structure (Winklemann *et al.*, 2020), and also between
354 NHE9 and NHE1 (Fig. 5B, C). In MD simulations of NHE9 it was observed that Na^+
355 can be coordinated within the core domain forming interactions to Asp244, Asn243
356 Ser240 and Thr214 and several waters (Winklemann *et al.*, 2020). Based on phylogenic
357 analysis (Masrati *et al*, 2018), it was predicted that a salt-bridge would also form
358 between Glu239 in TM6 and Arg408 in TM11, but previous NHE9 and NHE1 models
359 were inconclusive, due to insufficient map density in this region.
360

361 Here, we can confirm a salt-bridge is indeed formed between Glu239 and Arg408
362 residues and given the position of Glu239 in the ion-binding site, we propose this salt-
363 bridge aids the stabilization of the residues required for coordinating Na^+ binding (Fig.
364 5C). Interestingly, Thr214 forms a parallel hydrogen bond to the ion-binding residue
365 Asn243 (Fig. 5C). This unexpected hydrogen bond establishes a more rigid ion-binding
366 site than that observed in NHE1, since the Thr214 residue is replaced by valine in NHE1
367 (Fig. 5B, C). Indeed, all the plasma membrane localized NHE isoforms have a
368 hydrophobic residue in this position, whereas the intracellular isoforms have a

threonine residue (Winklemann *et al.*, 2020). It seems likely that this structural difference might be the reason why intracellular isoforms are thought to be able to transport K^+ in addition to Na^+ (Donowitz *et al*, 2013; Pedersen & Counillon, 2019).

In NHE proteins, extrinsic factors bind to a large, intracellular C-terminal regulatory domain of ~125 – 440 amino acids, which is only found in the mammalian proteins (Brett *et al.*, 2005; Donowitz *et al*, 2009; Fuster & Alexander, 2014; Orlowski & Grinstein, 2004). The regulatory domain is partly structured and is predicted to be less ordered towards the distal end (Donowitz *et al.*, 2009; Norholm *et al.*, 2011). In NHE1, removal of the regulatory domain results in a constitutionally active transporter (Wakabayashi *et al*, 1992). The C-terminal domain has been referred to as an allosteric regulatory subunit, which influences ion-exchange activity *via* interaction with many effectors (Lacroix *et al*, 2004) e.g., calmodulin (CaM), calcineurin B-homologous protein (CHP) and others (Donowitz *et al.*, 2013; Donowitz *et al.*, 2009; Norholm *et al.*, 2011; Odunewu-Aderibigbe & Fliegel, 2014; Slepkov *et al*, 2007). The interactions of many of these effectors are dynamic, and can further modulate activity through multiple phosphorylation sites that leads to the recruitment of other factors leading to both up and down-stream signaling as well as trafficking (Donowitz *et al.*, 2009). Although, it is well-established that these interactions are important to NHE physiology, the molecular details are just emerging.

The recent human NHE1 structure in complex with CHP1 revealed an interaction with an interfacial α-helical stretch formed by residues 517 to 539 (Dong *et al.*, 2021). Unexpectedly, the CHP1 interacting with the interfacial helix was found to have no direct contacts with the transporter module itself, and is currently unclear how CHP1 binding increases NHE1 activity (Dong *et al.*, 2021). Key to developing an allosteric model for extrinsic regulation is to obtain an NHE structure without complex partners. In the improved NHE9*CC structure, we could model part of the C-terminal domain without protein complex partners and, despite low sequence identity to NHE1 (Winklemann *et al.*, 2020), we could model the interfacial helix aligning well with the position of the interfacial helix of NHE1 (Fig. 6). The interfacial helix in the CTD sits on the membrane interface and wraps around from the core domain to the linker helix TM7 (Fig. 6A and Supplementary Fig. 9). In particular, the highly-conserved Lys301 on the linker helix makes direct hydrogen bond interactions to Thr541 and backbone carbonyl oxygens, facilitated by a proceeding proline residue (Fig. 6A, B and

402 Supplementary Fig. 8).

403 Given that the NHE1 and NHE9 structures superimpose well, apart from the position
404 of the interfacial helix, it seems likely that the binding of CHP1 to the loop region,
405 proceeding the interfacial helix, has driven its dissociation from the linker helix (Fig.
406 6C). Consistently, the AF2 model of NHE1 is similar to the NHE9 with interactions
407 between TM7 and the CTD (Fig. 6D). The cytoplasmic surface is also more positively
408 charged with the CTD helix in the likely inhibited position, which could diminish the
409 attraction of positively-charged ions to the inward-facing cavity (Supplementary Fig.
410 9B-C).

411 We did not observe any map density for the 20-residue loop region proceeding the
412 interfacial helix in NHE9* CC and has been currently modelled by AF2 (Fig. 2A); this
413 loop is likely too dynamic in the absence of a binding partners, since half of the residues
414 are charged. Given the TM7 linker helix undergoes significant rearrangements between
415 outward and inward-facing states (Coincon *et al.*, 2016), we propose that the NHE9
416 activity is inhibited because the C-terminal helix restricts its mobility, thereby
417 restricting core domain movement. However, the binding of CHP1 likely removes this
418 physical constraint to enhance NHE9 activity (Fig. 6C). This autoinhibitory allosteric
419 model would be consistent with the fact the removal of the C-terminal tail in NHE1
420 results in a constitutional active transporter (Donowitz *et al.*, 2013; Pedersen &
421 Counillon, 2019; Wakabayashi *et al*, 1997). Notably, the surface of the interfacial helix
422 and proceeding loop is positively-charged and also harbors two histidine residues
423 His533 and His542 (Fig. 6A). It's possible that the mobility of the C-terminal tail would
424 further be influenced by pH and the binding of negatively-charged phosphatidylinositol
425 lipids to this region (Aharonovitz *et al*, 2000; Shimada-Shimizu *et al*, 2014), as
426 proposed for NHE1 and NHE3 proteins (Pedersen & Counillon, 2019).

427 **Discussion**

428 The mammalian NHE proteins are overall similar to the basic architecture defined by
429 the bacterial homologues with 13-TMs (Winklemann *et al.*, 2020). Cryo EM structure
430 of the plasma membrane localised isoform NHE1 and the endosomal isoform NHE9*
431 superimpose well (Dong *et al.*, 2021; Winklemann *et al.*, 2020), but with some
432 important structural differences revealed here. Although cell surface expression of
433 NHE9 is possible in mutant cell lines, NHE9 in the plasma membrane has shown to

434 have no detectable activity (Daniel Fuster, *personal communication*). Expression of the
435 intracellular NHE7 on the cell surface also results in a poor affinity for Na^+ (K_M at 240
436 mM) and no activity for K^+ (Milosavljevic *et al*, 2014; Pedersen & Counillon, 2019),
437 which is in contrast with the proposed activity of intracellular NHE7 and other members
438 for K^+ (Lin *et al*, 2005; Nakamura *et al*, 2005). Since intracellular NHEs recycle
439 through the plasma membrane they could, in principle, acidify vesicles upon exposure
440 to high Na^+ levels, and yet vesicular acidification in NHE7-expressing cells has not
441 been detectable (Milosavljevic *et al.*, 2014). The mechanistic basis for keeping them
442 inactive during plasma membrane recycling has been puzzling. Here, we have been able
443 to obtain cryo-EM maps of NHE9 that has enabled the refinement of a positively-
444 charged TM2-TM3 β-hairpin loop domain. The cryo-EM structure and functional
445 analysis, demonstrates that charged residues in the loop domain and polar residues in
446 TM1 and TM8 dimer domain helices, are well positioned to interact with the minor PI-
447 (3,5)P_2 lipid, rather than the more abundant PI-(4,5)P_2 lipid specific to the plasma
448 membrane. Although unexpected, PI-(3,5)P_2 is a minor lipid that is not found in the
449 plasma membrane, but only found in late endosomes and lysosomes (Hasegawa *et al.*,
450 2017; Ho *et al.*, 2012), which coincides with the localization of NHE9. Furthermore,
451 PI-(3,5)P_2 lipid is increased 20-fold upon osmoregulation and salt-stress (Dove *et al*,
452 1997; Hasegawa *et al.*, 2017), which are conditions that also increases expression of
453 yeast Na^+/H^+ exchangers (Pedersen & Counillon, 2019). Moreover, the V-type ATPase
454 is known to co-localise with NHE9, and work together to fine-tune organellular pH
455 (Kondapalli *et al.*, 2015). Consistent with the proposed model, the activity of the V-
456 type ATPase is also increased in the presence of PI-(3,5)P_2 lipids (Li *et al*, 2014). Lastly,
457 inhibition of enzymes required to produce PI-(3,5)P_2 lipids results in impaired
458 epidermal growth factor receptor (EGFR) trafficking (de Lartigue *et al*, 2009). Indeed,
459 NHE9 activity has been shown to be critical for EGFR sorting and turnover (Kondapalli
460 *et al.*, 2015).

461 The sequence for the β-hairpin TM2-TM3 loop domain is absent in the plasma
462 membrane NHE isoforms, which adds support this structural-feature has evolved as an
463 additional, regulatory element. Interestingly, the closely-related NHE6 isoform has the
464 TM2-TM3 loop domain present in most organisms, including the great apes, but in
465 human its only present in isoform two (Supplementary Fig.1A). A lipid-activated model
466 by dimerization through an endosomal lipid could further explain the poor and/or absent

467 activity for NHE7 and NHE9 proteins measured at the plasma membrane in mutant cell
468 lines. Indeed, the increased Na^+ binding (K_d) affinity from 36 to 9 mM in the presence
469 of PI-(3,5)P_2 would be more consistent with NHE9 utilizing cytoplasmic Na^+ in
470 neurons, which is reported to be around 15 mM (Kondapalli *et al*, 2013). To provide
471 additional support for lipid-dependent oligomerization in Na^+/H^+ exchangers we
472 determined the cryo-EM structure of *E. coli* NhaA homodimer, confirming the expected
473 coordination of lipids at the dimerization interface. In an analogous manner to PI-
474 (3,5)P_2, the negatively-charged cardiolipin lipid head-groups bind between positively
475 charged residues located at the dimerization interface (Nji *et al.*, 2018).

476 More recently, the cryo EM structure of *bison* Na^+/H^+ exchanger NHA2 was determined
477 and it was shown that it has an additional TM helix at the N-terminus, as compared to
478 the NHE9 and related 13-TM members (Matsuoka *et al.*, 2022). The N-terminal helix
479 (TM –1) is repeat-swapped, and makes all of the dimerization contacts and, in doing
480 so, establishes a very large, cytoplasmic opening between the two protomers. The
481 dimerization interface buries a total surface area of only 1,000 $Å^2$, making it one of the
482 smallest oligomeric interfaces seen for any elevator protein. Removal of the additional
483 TM –1 helix, or the mutation of just two polar residues at the dimer interface, was
484 enough to disrupt dimerization of NHA2 (Matsuoka *et al.*, 2022). NHA2 is well-folded
485 as a monomer, yet mutations resulting in only the monomer were non-functional when
486 assessed for their ability to rescue cell growth in a salt-sensitive yeast strain (Matsuoka
487 *et al.*, 2022). This example with NHA2 clearly shows that oligomerization is essential
488 for activity in Na^+/H^+ exchangers. By combining native MS with thermal-shift assays,
489 PI lipid stabilization of the NHA2 homodimer was concluded and a cryo EM structure
490 in nanodiscs with PI lipids showed that the cytoplasmic opening was now closed, with
491 map density matching PI lipids at the dimerization interface (Matsuoka *et al.*, 2022).
492 Taken together, lipid-mediated oligomerization of Na^+/H^+ exchanger's is becoming
493 clear, yet the physiological basis for such regulation is just emerging. For NHE9, the
494 binding of PI-(3,5)P_2 is consistent with its subcellular location, and the lipid could either
495 enhance or completely turn on NHE9 activity by stabilizing the homodimer once it
496 reaches late-endosomes (Fig. 7). To the best of our knowledge, such a lipid-activation-
497 upon-arrival model would be a novel regulatory mechanism for ion-transporters and
498 SLCs in general.

499 In addition to the lipid-dependent oligomerization, mammalian NHEs have a long C-
500 terminal regulatory domain that regulates their activity (Pedersen & Counillon, 2019).
501 The C-terminal domain is poorly conserved across the different NHE members and, so
502 far, only part of the C-terminal tail could be modelled for *human* NHE1 in complex
503 with Calcineurin B homologous protein 1 (CHP1)(Dong *et al.*, 2021). Structures of
504 human NHE1-CHP1 in outward and inward-facing states show some differences in the
505 position of the C-terminal tail (Dong *et al.*, 2021). Based on these structural differences,
506 it was proposed that CHP1 may increase activity by favouring an outward-facing state.
507 In the improved the NHE9*CC structure we could model the corresponding region of
508 the C-terminal tail, but in the absence of any interacting regulatory proteins.
509 Surprisingly, we find that NHE9*CC has a similar interfacial helix between the core
510 and dimer domains to that seen in NHE1. However, in the absence of a regulatory
511 protein, the C-terminal interfacial helix in NHE9*CC is making a number of direct
512 contacts with the linker helix TM7, likely restricting its mobility and that of the core
513 transport domains. An autoinhibitory role of the C-terminal tail has been previously
514 proposed for NHE1 (Wakabayashi *et al.*, 1997), which is removed upon Ca^{2+}-
515 calmodulin binding to a site distal to the peripheral helix (Sjogaard-Frich *et al*, 2021).
516 Our structure of NHE9*CC implies that the C-terminal tail may act in an autoinhibitory
517 manner in all the NHEs in the absence of binding partners. It, thus, seems likely that
518 the relocation of an auto-inhibitory C-terminal tail by binding partners could represent
519 a general mode for positive allosteric regulation in all NHEs. Nevertheless, it's possible
520 positive regulators like Ca^{2+}-calmodulin, can both displace this C-terminal helix and
521 promote homodimerization by itself forming oligomers (Sjogaard-Frich *et al.*, 2021).
522 Furthermore, PIP_2 lipids interacting with the C-terminal tail and its degree of
523 phosphorylation may all contribute to the mobility of the CTD, and the ultimate level
524 of autoinhibition apparent under resting conditions. Indeed, the complexity of auto-
525 regulation was seen in a more recent structure of *human* NHE3, which was still in an
526 auto-inhibited state with CHP1 bound, as distal loop downstream of the CTD helix was
527 found to have protruded into the inward-facing cavity (Dong *et al*, 2022).

528 **Summary**

529 Our work provides a molecular framework for the allosteric regulation of NHE9 by
530 modulating oligomerization in a specific lipid-dependent manner. The structure of the
531 extracellular TM2-TM3 β-loop domain expands the possibilities for pharmacological

532 control of NHE9 and provides a conceptual framework for how the cell might control
533 transporters by regulating their oligomerization with organelle-specific cues. Recently,
534 it was shown that plant hormone (auxin) PIN-formed transporters are functional
535 homodimers sharing the same fold as the Na^+/H^+ exchangers (Su *et al*, 2022; Ung *et al*,
536 2022; Yang *et al*, 2022). PIN transporters likewise harbour a C-terminal regulatory
537 domain of varying length (Krecek *et al*, 2009), and also have both organellar and plasma
538 membrane isoforms (Mravec *et al*, 2009), which must be regulated individually.
539 Moreover, it has been shown that the plasma membrane localized PIN1 is in a dynamic
540 equilibrium between monomers and dimers, and that the dimeric form can be regulated
541 in the plant cell by endogenous flavonols (Teale *et al*, 2021). Thus, the allosteric
542 regulatory mechanisms shown here could reveal themes relevant to other transporters
543 in general.

544

545 **Materials and Methods**

546 **Expression and purification of NHE9* and its variants**

547 The *horse* NHE9* structural construct (UniProt accession: F7B133) was identified
548 previously (Winklemann *et al.*, 2020), and is partially truncated on the C-terminal tail
549 consisting of residues 8 to 575 out of a total of 644. The constructs NHE9*CC,
550 NHE9*(N243A-D244A), NHE9*(K85Q-K105Q-K107Q) were synthesized and cloned
551 into the GAL1 inducible TEV-site containing GFP-TwinStrep-His_8 vector $pDDGFP_3$.
552 The cloned *horse* NHE9* and its variants were transformed into the *S. cerevisiae* strain
553 FGY217 and cultivated in 24-L cultures of minus URA media with 0.2% of glucose at
554 30°C at 150 RPM Tuner shaker flasks using Innova 44R incubators (New Brunswick).
555 Upon reaching an OD_{600} of 0.6 AU galactose was added to a final concentration of 2%
556 (w/v) to induce protein overexpression. Following incubation at the same conditions
557 the cells were harvested 22h after induction by centrifugation (5,000 × *g*, 4°C, 10 min).
558 The cells were resuspended in cell resuspension buffer (CRB, 50 mM Tris-HCl pH 7.6,
559 1 mM EDTA, 0.6 M sorbitol) and subsequently lysed by mechanical disruption as
560 previously described(Drew *et al*, 2008). Centrifugation (10,000 × *g*, 4°C, 10 min) was
561 used to remove cell debris. Membranes were subsequently isolated from the supernatant
562 by ultracentrifugation (195,000 × *g*, 4°C, 2 h), resuspended and homogenized in

563 membrane resuspension buffer (MRB 20 mM Tris-HCl pH 7.5, 0.3 M sucrose, 0.1 mM
564 $CaCl_2$).

565 For structural studies of NHE9* at pH 6.5, the membranes were extracted and purified
566 as described previously (Winklemann *et al.*, 2020). In short, the Streptag-purified
567 protein after removal of the C-terminal affinity-GFP-tag was collected and concentrated
568 using 100 kDa MW cut-off spin concentrator (Amicon Merck-Millipore) further
569 purified by size-exclusion chromatography (SEC), using a Superose 6 increase 10/300
570 column (GE Healthcare) and an Agilent LC-1220 system in 20 mM Mes-Tris pH 6.5,
571 150 mM NaCl, 0.003% (w/v) LMNG, 0.0006% (w/v) CHS. For NHE9*CC, the isolated
572 membranes were solubilized as mentioned before (Winklemann *et al.*, 2020) with
573 addition of brain extract from bovine brain type VII (Sigma-Aldrich, cat. nr. B3635) to
574 a total concentration of 0.003mg/ml, in the solublization, wash and elution buffers
575 (Winklemann *et al.*, 2020). The cleaved protein was collected and concentrated using
576 100 kDa MW cut-off spin concentrators (Amicon Merck-Millipore) separated by size-
577 exclusion chromatography (SEC), using a Superose 6 increase 10/300 column (GE
578 Healthcare) and an Agilent LC-1220 system in 20 mM Tris-HCl pH 7.5, 150 mM NaCl,
579 0.003% (w/v) LMNG, 0.0006% (w/v) CHS.

580

581 *Expression and purification of EcNhaA-mut2*

582 *Ec*NhaA WT-like triple mutant (A109T, Q277G, L296M), with a TEV-cleavable C-
583 terminal GFP-His_8 tag was overexpressed in the *E. coli* strain Lemo21 (DE3) and
584 purified as previously described (Lee *et al.*, 2014). Briefly, the *Ec*NhaA triple mutant
585 was extracted from membranes with *n*-Dodecyl β-D-maltoside (DDM; Glycon) and
586 purified by Ni-nitrilotriacetic acid (Ni-NTA; Qiagen) affinity chromatography. To
587 purified NhaA-triple-mutant-GFP fusion, a final concentration of 3 mM cardiolipin
588 (18:1) in 0.05% DDM was added, and then dialyzed overnight against buffer consisting
589 of 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.03% DDM. The dialysed NhaA-triple-
590 mutant-GFP fusion was subjected to size-exclusion chromatography and the peak
591 concentrated to 3.5 $mg.ml^{-1}$.

592

593

594

595 **Cryo-EM sample preparation and data acquisition and processing**
596 3 µg of purified NHE9*CC sample was applied to freshly glow-discharged Quantifoil
597 R2/1 Cu300 mesh grids (Electron Microscopy Sciences) and blotted for 3.0s with a 20s
598 waiting time prior, under 100% humidity and subsequently plunge frozen in liquid
599 ethane using a Vitrobot Mark IV (Thermo Fisher Scientific). Cryo-EM datasets were
600 collected on a Titan Krios G3i electron microscope operated at 300 kV equipped with
601 a GIF (Gatan) and a K3 Bioquantum direct electron detector (Gatan) in counting mode.
602 The movie stacks were collected at 130,000× magnification corresponding to a pixel
603 size of 0.66 Å in a counted super-resolution mode. All movies were recorded with a
604 defocus range of -0.4 to -2.5 µm. Similarly, 2.4 µg of purified NHE9* at pH 6.5 was
605 blotted and the movie stacks were collected at 165,000x magnification, with a pixel size
606 of 0.82 Å on Titan Krios G2 electron microscope operated at 300 kV equipped with a
607 GIF (Gatan) and a K2 summit direct electron detector (Gatan) in counting mode The
608 movies were recorded with a defocus range of -0.9 to -2.3 µm. 3µl of *Ec*NhaA-mut2
609 with concentration of 3.5mg/ml was blotted and the movie stack was collected at
610 130,000x magnification, with a pixel size of 0.6645 Å. The movies were recorded with
611 a defocus range of -0.6 to -2.0 µm. The statistics of all cryo-EM data acquisition are
612 summarized in Supplementary Table 1.
613

614 *Image processing NHE9* at pH 6.5:*
615 The dose-fractioned movies were corrected by using MotionCorr2 (Zheng *et al*, 2017).
616 The dose-weighted micrographs were used for contrast-transfer-function estimation by
617 CTFFIND-4.1.13 (Rohou & Grigorieff, 2015). The dose-weighted images were used
618 for auto-picking, classification and 3D reconstruction. Approximately 1,000 particles
619 were manually picked, followed by a round of 2D classification to generate templates
620 for a subsequent round of auto-picking using RELION-3.0 beta (Zivanov *et al*, 2018).
621 The auto-picked particles were subjected to multiple rounds of 2D classification using
622 RELION-3.0 beta to remove bad particles and "junk". The particles belonging to
623 "good" 2D classes were extracted and used for initial model generation using RELION-
624 3.0 beta (Zivanov *et al.*, 2018).
625

626 To visualize the extracellular loop domain of NHE9, the aligned 244,279 particles from
627 RELION was imported into CryoSPARC (Punjani *et al.*, 2017). *UCSF pyem (Daniel*

628 *Asarnow, 2019)* was used for file format conversion from RELION to CryoSPARC. 3D
629 Variability Analysis (3DVA) in CryoSPARC (Punjani *et al.*, 2017) was performed and
630 set up 6 variable components with the filter resolution 4 Å and high-pass filter 20 Å,
631 respectively. After cluster analysis, the remaining 103,815 particles in 1 of 3 cluster
632 was selected and subsequently, homogeneous refinement in CryoSPARC was
633 performed after applying C2 symmetry and the reconstructed map reached to 3.1 Å
634 resoltuion at the gold standard FSC (0.143).

635 *Image processing NHE9*CC:*
636 The dataset was processed using CryoSPARC (Punjani *et al.*, 2017). Dose fractionated
637 movie frames were aligned using "patch motion correction," and contrast transfer
638 function (CTF) were estimated using "Patch CTF estimation". The particles were
639 picked using automated blob picker. Particles with good 2D classes were used for
640 template based particle picking and extracted using a box size of 300 pixels. 268,054
641 particles were further used for *ab initio* model building and hetero refinements. Final
642 round of non-uniform refinement with C2 symmetry and masked local refinement did
643 result in 3D reconstruction with a gold standard FSC resolution estimation of 3.15Å.
644

645 *Image processing EcNhaA-mut2:*
646 14,329 dose fractionated movie frames were aligned using "patch motion correction,"
647 and contrast transfer function (CTF) were estimated using "Patch CTF estimation" in
648 CryoSPARC (Punjani *et al.*, 2017). 4,401,429 particles were extracted and cleaned up
649 using multiple rounds of 2D classification. 542,404 particles were used for *ab initio*
650 model building and cleaned using multiple rounds of hetero refinement. 78,917
651 particles were further selected for a final round of non-uniform refinement and masked
652 local refinement which resulted in 3D reconstruction with a gold standard FSC
653 resolution estimation of 3.37Å.
654

655 **Cryo EM model building and refinement**
656 Previously determined NHE9 structure (PDB id: 6Z3Z) was fitted into the cryoEM
657 density map of NHE9* CC mutant. Iterative model building and real space refinement
658 was performed using COOT (Emsley *et al*, 2010) and PHENIX.refine (Afonine *et al*,
659 2018). Prior to model building of the extracellular loop domain, AlphaFold2 (AF2) with
660 poly-glycine linker was performed to predict the NHE9 dimer structure assembly. After

automatically fitting the initial model into the cryo-EM map, iterative model building and real space refinement was performed using COOT (Emsley *et al.*, 2010) and PHENIX.refine (Afonine *et al.*, 2018). The refinement statistics are summarized in Supplementary Table 1. For generating structural figures PyMOL was used, and for figures of cryo-EM maps either Chimera (Pettersen *et al*, 2004) or ChimeraX (Pettersen *et al*, 2021) was used.

GFP-based thermal shift assay

To characterize lipid binding for NHE9* we used the GFP-Thermal Shift assay (Chatzikyriakidou *et al*, 2021). In brief, purified NHE9*-GFP fusions was isolated as described previously (Winklemann *et al.*, 2020). A buffer containing 20mM Tris, 150mM NaCl and 0.03% (w/v) DDM 0.006% (w/v) CHS was used to dilute samples to a final concentration of 0.05–0.075 mg/ml with DDM added to a final concentration of 1% (w/v), and incubated for 30 min at 4°C. Stock solutions of the respective lipids, phosphatidylinositol-bis-4,5-phosphate (PI-(4,5)P_2 dioctanoyl Echelon Biosciences cat no. P-4508), and phosphatidylinositol-bis-3,5-phosphate (PI-(3,5)P_2 dipalmitoyl, Echelon Biosciences cat no. P-3516) were prepared with final concentration of 1 mg/ml in buffer mentioned above. Respective lipids were added to purified NHE9* sample to a final concentration of 0.1 mg/ml and incubated for 10 mins on ice. Subsequently, β-D-Octyl glucoside (Anatrace) was added to a final concentration of 1% (w/v) in 300 µl and the sample aliquots of 100 µl were heated at individual temperatures ranging from 20-80°C for 10 min using a PCR thermocycler (Veriti, Applied Biosystems). Heat-denatured material was pelleted at 5000 × g for 30 min at 4°C. The resulting supernatants were collected and fluorescence values recorded (Excitation: 488, Emission 512 nm) using 96-well plate (Thermo Fisher Scientific) measured with Fluoroskan microplate fluorometer (Thermo Scientific) reader. The apparent *T*m was calculated by plotting the average GFP fluorescence intensity from three technical repeats per temperature and fitting a resulting curve using a sigmoidal 4-parameter logistic regression using GraphPad Prism software (GraphPad Software Inc.). The ΔT_M was calculated by subtracting the apparent T_M of the lipid free sample, from the apparent T_M of the sample with the respective lipid added.

695 **Solid Supported Membrane-based electrophysiology**

696 For SSM-based electrophysiology measurements, protein was reconstituted in yeast
697 polar lipids (Avanti). The lipids were prepared by solubilization in chloroform and dried
698 using a rotary evaporator (Hei-Vap Core, Heidolph Instruments). Dry lipids were
699 thoroughly resuspended in 10 mM MES-Tris pH 8.5, 10 mM $MgCl_2$ buffer at a final
700 concentration of 10 mg ml^{-1}. Unilamellar vesicles were prepared by extruding the
701 resuspended lipids using an extruder (Avestin) with 400-nm polycarbonate filters
702 (Whatman). The vesicles were destabilized by the addition of Na-cholate (0.65% w/v
703 final concentration). SEC-purified protein was added to the destabilized liposomes at a
704 lipid-to-protein ratio (LPR) of 5:1 and incubated for 5 min at room temperature. The
705 sample was added to a PD SpinTrap G-25 desalting column for removing detergent and
706 the reconstituted proteoliposomes were collected in a final volume of 100µl. The
707 sample was diluted to final lipid concentration of 5 mg ml^{-1} in 10mM MES-Tris pH 7.5,
708 10 mM $MgCl_2$ buffer, flash frozen I liquid nitrogen and stored at −80 °C.
709 Proteoliposomes were diluted 1:1 (vol/vol) with non-activating buffer (10 mM MES-
710 Tris pH 7.5, 300 mM Choline chloride, 10 mM $MgCl_2$) and sonicated using a bath
711 sonicator. 10µl of sample was loaded on 1mm sensor. For sample measured with lipid
712 PI-(3,5)P_2, 20µM of lipid was added to the protein and incubated for 15 min. The sample
713 was again reconstituted in yeast polar lipids for making proteoliposomes using the
714 protocol mentioned above.

715

716 Sensor preparation for SSM-based electrophysiology using the SURFE^{2}R N1(Nanion
717 Technologies) system was performed as described previously (Bazzone *et al.*, 2017).
718 During the experiments, NHE9 was activated by solution exchange from non-activating
719 buffer to an activating buffer containing the substrate i.e NaCl. For binding kinetics
720 measurements, *x* mM Choline chloride was replaced by (300-x) mM NaCl in the
721 activating buffer to get peaks corresponding to increase in substrate concentration. For
722 kinetic analysis, the response to increasing concentrations of NaCl was fitted from
723 triplicate measurements to nonlinear regression curve-fit to one site specific binding
724 using GraphPad Prism software. The peak current values were normalized with respect
725 to the average of maximum value obtained for all measurements. The final K_d values
726 reported are the mean ± s.d. of n = 3 independent sensors.

727 **All-atom molecular dynamics simulations**

728 The NHE9* CC structure and NHE9* TM2-TM3 loop variant (K58Q, K105Q, K107Q)
729 were embedded into the POPC bilayer and solvated in 0.15 M NaCl using CHARMM-
730 GUI (Qi *et al*, 2015). Either PI-(3,5)P_2 and PI-(4,5)P_2 were placed in the PIP_2 binding
731 site identified from the cryo-EM structure. All simulations were simulated with a 2 fs
732 timestep using the CHARMM36m forcefield in GROMACS 2022.1(Abraham, 2015).
733 The system was then energy minimised and equilibrated using the standard CHARMM-
734 GUI protocol (Abraham, 2015), where the last step of the protocol was extended to 5
735 ns. The production runs were conducted for 250 ns under 303.15 K using the v-rescale
736 thermostat. The pressure of all systems was maintained at 1 bar using a C-rescale
737 barostat. All simulations were carried out in triplicates, where simulation frames were
738 saved every 0.1 ns.

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Author contributions

D.D. designed the project. Cloning, expression screening and sample preparation for cryo-EM was carried out by P.M. and S.K. Cryo-EM data collection and map reconstruction was carried out by P.M, S.K, R.M, A.G an D.D. Model building was carried out by R.M, A.G and D.D. MD simulations were carried out by T.D and L.D. All authors discussed the results and commented on the manuscript. The authors declare no competing financial interests. Correspondence and request for materials should be addressed to D.D. (d.drew@dbb.su.se).

Data availability

The coordinates and the maps for cryo-EM structures of *horse* NHE9* with TM2-TM3 loop at pH 6.5, *hor*se NHE9* CC bound to PI-(3,5)P_2, and the *E. coli* NhaA dimer with cardiolipin, have been deposited in the Protein Data Bank (PDB) and Electron Microscopy Data Bank (EMD) with entries PDB ID: 8PVR, 8PXB, 8PS0, respectively.

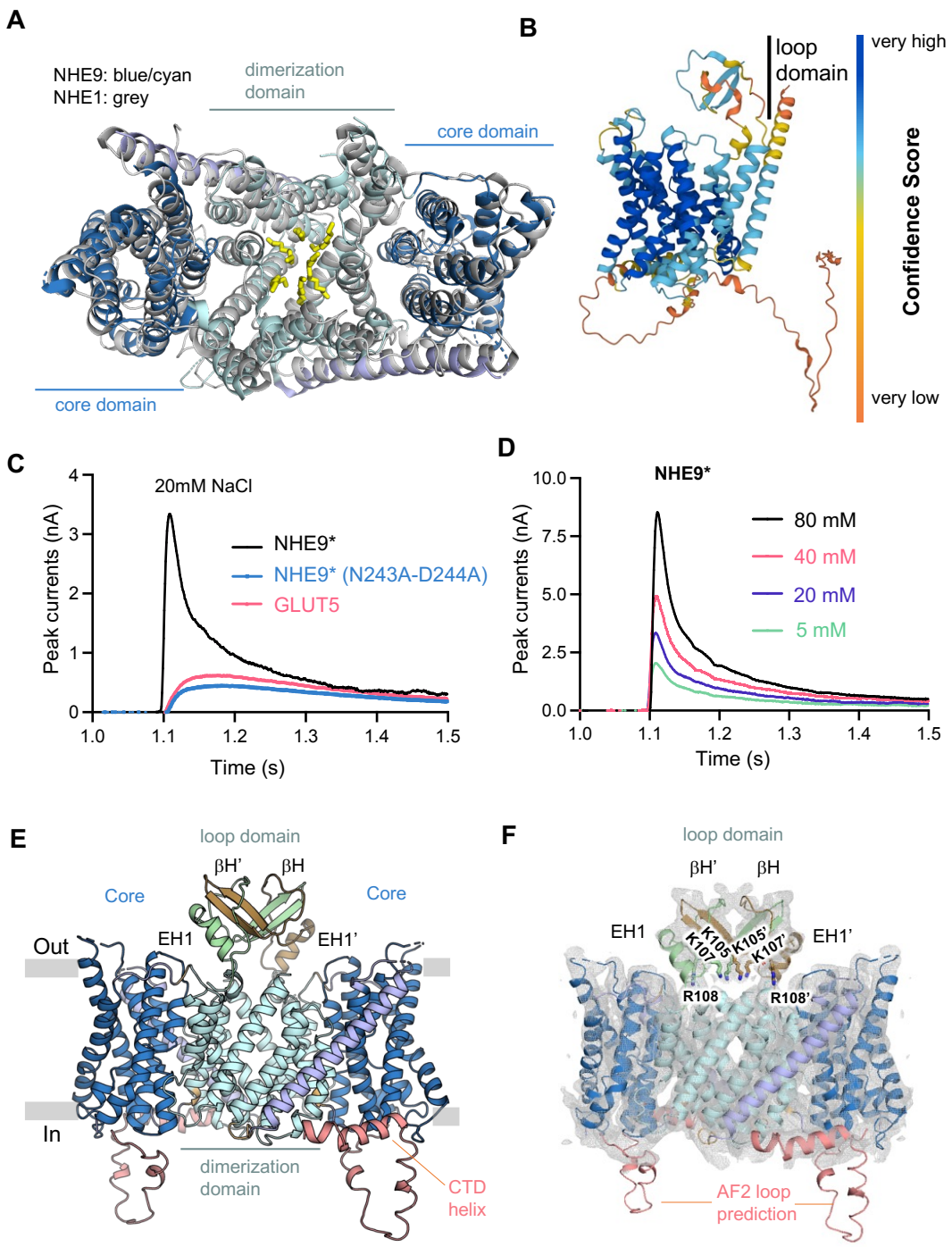
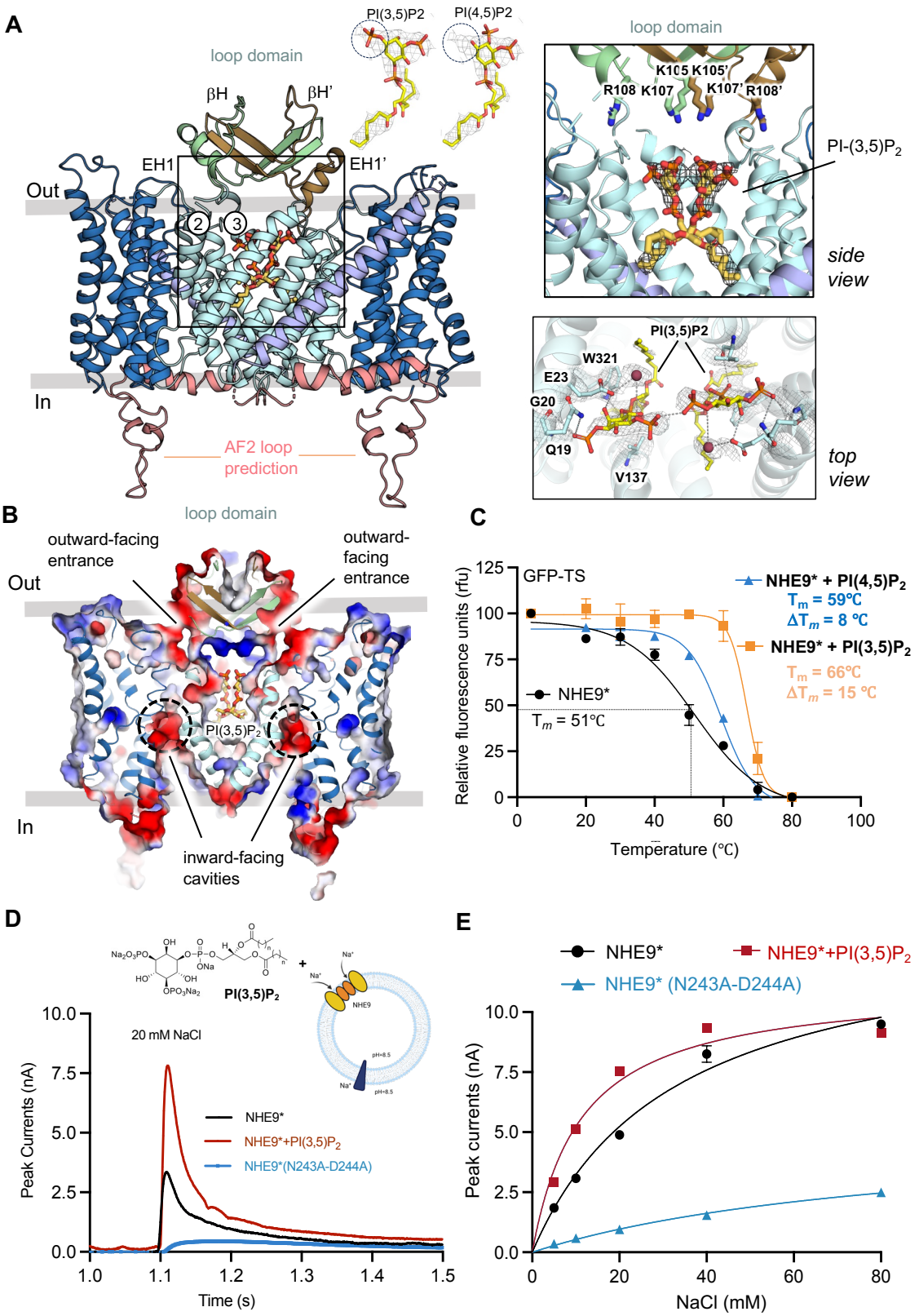


Figure 1. SSM-based electrophysiology and cryo EM NHE9* structure with TM2-TM3 β-loop domain modelled. (**A**) Structural superimposition of NHE9 with inward-facing NHE1 (C_α RMSD = 2.91 Å) shows the overall structural conservation. Inward-facing NHE9 ΔCTD (PDB: 6Z3Z) with dimerization domain (cyan), core domain (blue) and TM7 linking helix (light-purple) all shown as cartoon. Inward-facing NHE1 (PDB: 7DSW) (grey, cartoon) with its lipids (yellow, sticks) inside the cavity between the

991 dimerization domains. (**B**) AF2 model of the human NHE9 monomer that predicts an
992 additional TM2-TM3 loop domain containing a β-hairpin with reasonable confidence.
993 (**C**) SSM-based electrophysiology. Transient currents were recorded for NHE9*
994 proteoliposomes (black trace) at symmetrical pH 7.5 after the addition of 20 mM NaCl.
995 Peak currents for an ion-binding site NHE9* variant N243A-D244A (blue trace) and
996 fructose transporter GLUT5 (Suades *et al*, 2023) (red trace) after the addition of 20 mM
997 NaCl are also shown (**D**) SSM-based electrophysiology measurements of NHE9*
998 proteoliposomes with transient currents recorded after Na^+ concentration jumps at pH
999 7.5 on both sides. (**E**) AF2 model of the *horse* NHE9 dimer with an TM2-TM3 loop
1000 domain containing an β-hairpin (βH1, βH1') and extracellular helix (EH1, EH1') from
1001 each of the two protomers coloured in brown and green, respectively. The core domain
1002 (dark-blue), dimerization domain (light-blue) and CTD domain (salmon) is further
1003 highlighted. (**F**) The AF2 dimer model for *horse* NHE9 after its refinement into cryo
1004 EM maps, as shown in E with charged residues in loop domain highlighted in sticks.
1005 An additional C-terminal helix in the CTD could be modelled (salmon, cartoon), but
1006 there is no density to support the 20 residue AF2 loop model that is located between the
1007 end of the core domain and the beginning of the interfacial helix**.**



1008 **Figure 2. TM2-TM3 β-loop domain structure of NHE9* CC with negatively-**
1009 **charged PI-(3,5)P_2 lipids bound at the dimer interface. (A)** *left:* Structure of the
1010 *horse* NHE9*CC determined by cryo-EM and guided by the refined NHE9* model for
1011 the β-hairpin TM2-TM3 loop domain. Domain-swapped βH, βH' and EH1, EH1' in

1012 green and brown respectively, and NHE9 core and dimer domains coloured as in Fig.
1013 1E. Two PIP_2 lipids (yellow sticks) are located in the middle at the dimerization
1014 interface between the two protomers and, in the insert above, the fitting of PI-(3,5)P_2
1015 vs PI-(4,5)P_2 lipids into the cryo EM maps are compared. *right above:* Magnification
1016 of the two PIP_2 lipids, their interdomain binding groove and part of the loop-domain
1017 residues at the dimer interface. TM segments TM1 and TM8 are not shown for better
1018 visualization of the bound lipids. In total six positively-charged amino acids (K105,
1019 K107, R108 of each monomer) in the loop domains are positioned above the two
1020 negatively-charged PI-(3,5)P_2 lipids (yellow-sticks). *right below:* Inclusion of TM1 and
1021 TM8 helices and viewed from the extracellular side, shows the coordination of PI-
1022 (3,5)P_2 lipids (yellow sticks and grey mesh) by aromatic and polar residues **(B)**
1023 Electrostatic surface potential map cross-section with two negatively-charged PI-
1024 (3,5)P_2 modelled in the hydrophobic and positively-charged dimerization interface
1025 (blue). The interface between the loop domains and core domains (indicated by the
1026 black-line) are negatively-charged and provide an electrostatic pathway for cations in
1027 the outward-facing state. Protein shown as cartoon colored as in Fig. 1E, and PI-(3,5)P_2
1028 as yellow-sticks and the ion-binding site is highlighted (dotted-circle). (**C**) Thermal
1029 shift stabilization of purified dimeric NHE9*-GFP in the presence of PI-(4,5)P_2 (blue)
1030 and PI-(3,5)P_2 (mustard) compared to lipid-free (black). Data presented are normalized
1031 fluorescence of mean values ± data range of n = 3 technical repeats; the apparent T_M
1032 was calculated from datapoints fitted according to a sigmoidal 4-parameter logistic
1033 regression function. (**D**) *top*: Structure of the lipid PI(3,5)P_2 and its addition to NHE9
1034 prior to incorporation into proteoliposomes and an illustration of the experimental
1035 setup. *Bottom:* resulting peak transient currents following addition of 20 mM NaCl
1036 under symmetrical pH conditions. (**E**) Fit of the amplitude of the transient currents as
1037 a function of Na^+ concentrations at pH 7.5 for horse NHE9* proteoliposomes pre-
1038 incubated with either buffer (black-trace) or synthetic PI-(3,5)P_2 lipids (red trace) and
1039 the corresponding binding affinity (K_D). Error bars are the mean values ± s.e.m. of:
1040 n = 3 individual repeats.

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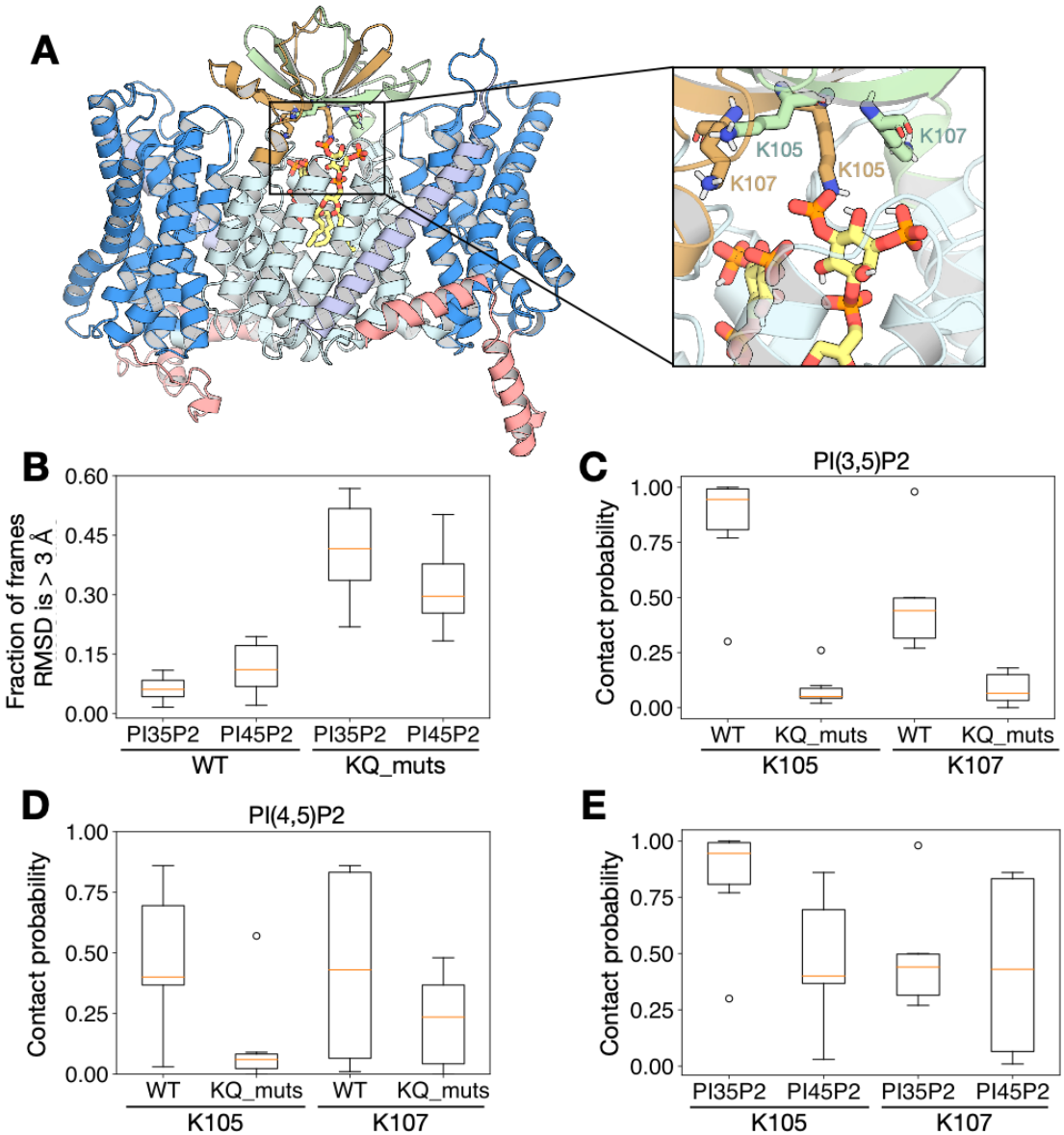

Figure 3. MD simulations of PIP_2 interaction to NHE9* CC. (**A**) A representative snapshot of NHE9 with PI-(3,5)P_2 after a 250 ns simulation. The structure of PI-(3,5)P_2 is shown in yellow. K105 and K107 on different chains are shown in green and brown, respectively. (**B**) Box plot representation of the distribution of the fractions of frames where the root mean square deviation (RMSD) of PIP_2 is > 3 Å with respect to its previous 10 ns structure. (**C**) Box plot representation of the distribution of frames where PI-(3,5)P_2 are within 4 Å (defined as contact probability) with either K105, K107 or its mutants. (**D**) Box plot representation of the distribution of frames where PI-(4,5)P_2 are within 4 Å (defined as contact probability) with either K105, K107 or its mutants. (**E**) Box plot representation of the distribution of the contact probability between K105 and K107 with either PI-(3,5)P_2 or PI-(4,5)P_2.

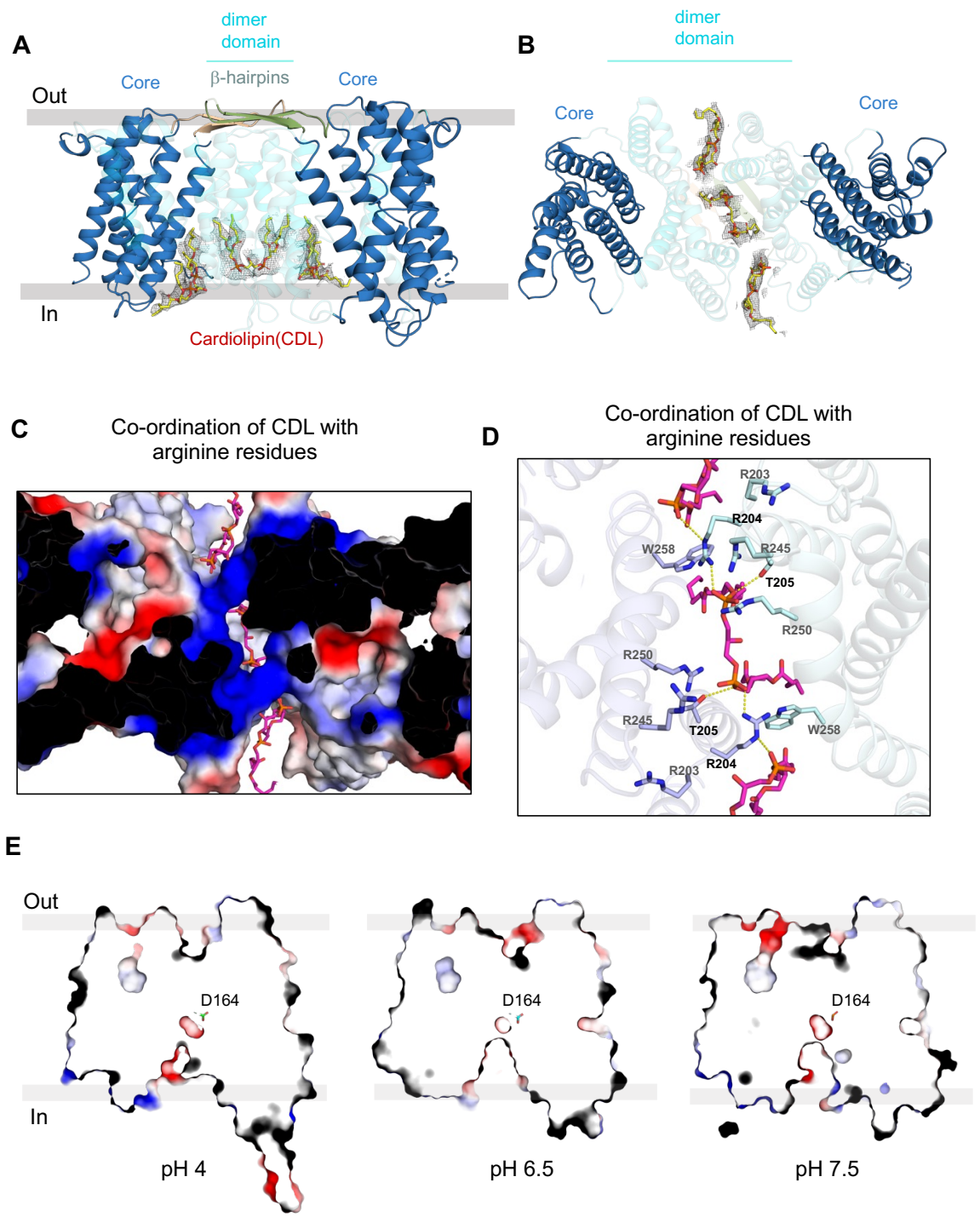

Figure 4. Structure of cardiolipin (CDL) bound to *Ec*NhaA homodimer. (**A**) Model of *Ec*NhaA homodimer obtained by cryo-EM and with β-hairpin loop domains (palegreen, sand, cartoon). Large densities between the dimerization domains indicate the presence of three CDL (yellow, sticks) fitted in the model. (**B**) Cytoplasmic view of *Ec*NhaA model with the three CDL fitted between the dimer domains. (**C**) Cytoplasmic view of slice through an electrostatic surface potential map with three cardiolipins (mangenta, sticks) positioned in the hydrophobic and positively-charged dimerization

1060 interface (blue). (**D**) Cytoplasmic view of *Ec*NhaA model (cartoon) with CDL
1061 (magenta, sticks) coordinating residues. Positively charged arginine residues are
1062 labelled and shown in cyan and purple sticks. (**E**) Slice through an electrostatic surface
1063 representation of *Ec*NhaA structures, perpendicular to the membrane plane, at active
1064 pH 6.5 (left, PDB ID: 7S24), inactive pH 4.0 (right, PDB ID: 4AU5) and pH 7.5-CDL
1065 bound. The ion-binding aspartate is indicated and shown as cyan sticks. The ion-binding
1066 funnel at the cytoplasmic side is much more open at active pH 6.5 and pH 7.5-CDL
1067 bound structure than at pH 4.

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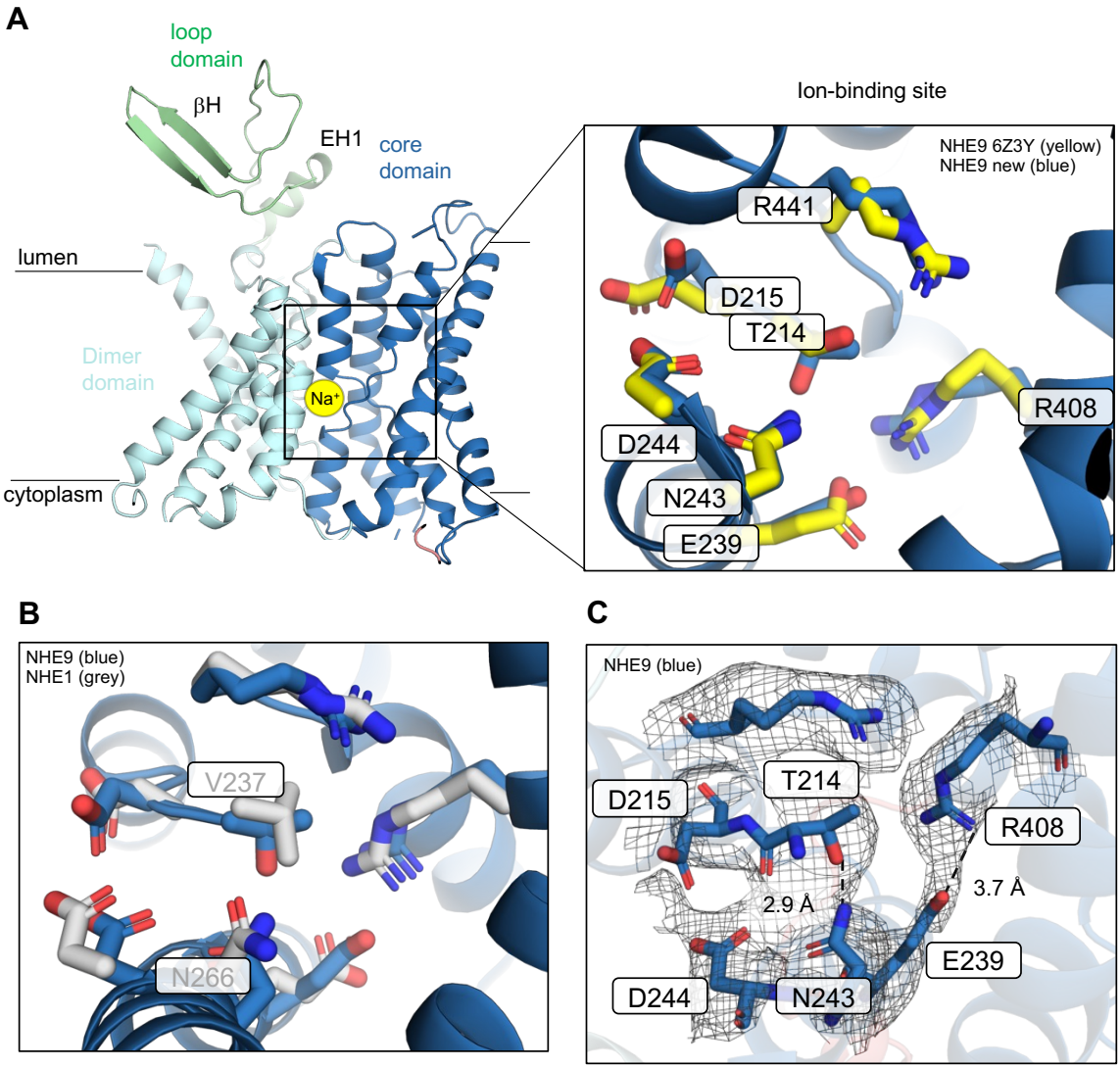

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1081 **Figure 5. Ion-binding site of the NHE9*CC (A)** *left:* cartoon representation of the
1082 NHE9 ion-binding site, located in the 6-TM core transporter domain, which is made up
1083 of two broken helices. The sodium ion (yellow sphere) is located and coordinated at the
1084 ion-binding site. *right:* ion-binding residues of the NHE9*CC structure are shown as
1085 blue sticks and labelled, with the identical residues of NHE9* shown as yellow sticks
1086 (PDB id: 6Z3Y). **(B)** Ion-binding residues of NHE9*CC are shown as blue sticks and
1087 NHE1 residues shown as grey sticks with Val237 labelled**. (C)** Hydrogen-bonding
1088 between T214-N243 and salt-bridge interaction between E239-R408 in the NHE9*CC
1089 structure are illustrated by dashed lines and the cryo EM maps shown as grey mesh..

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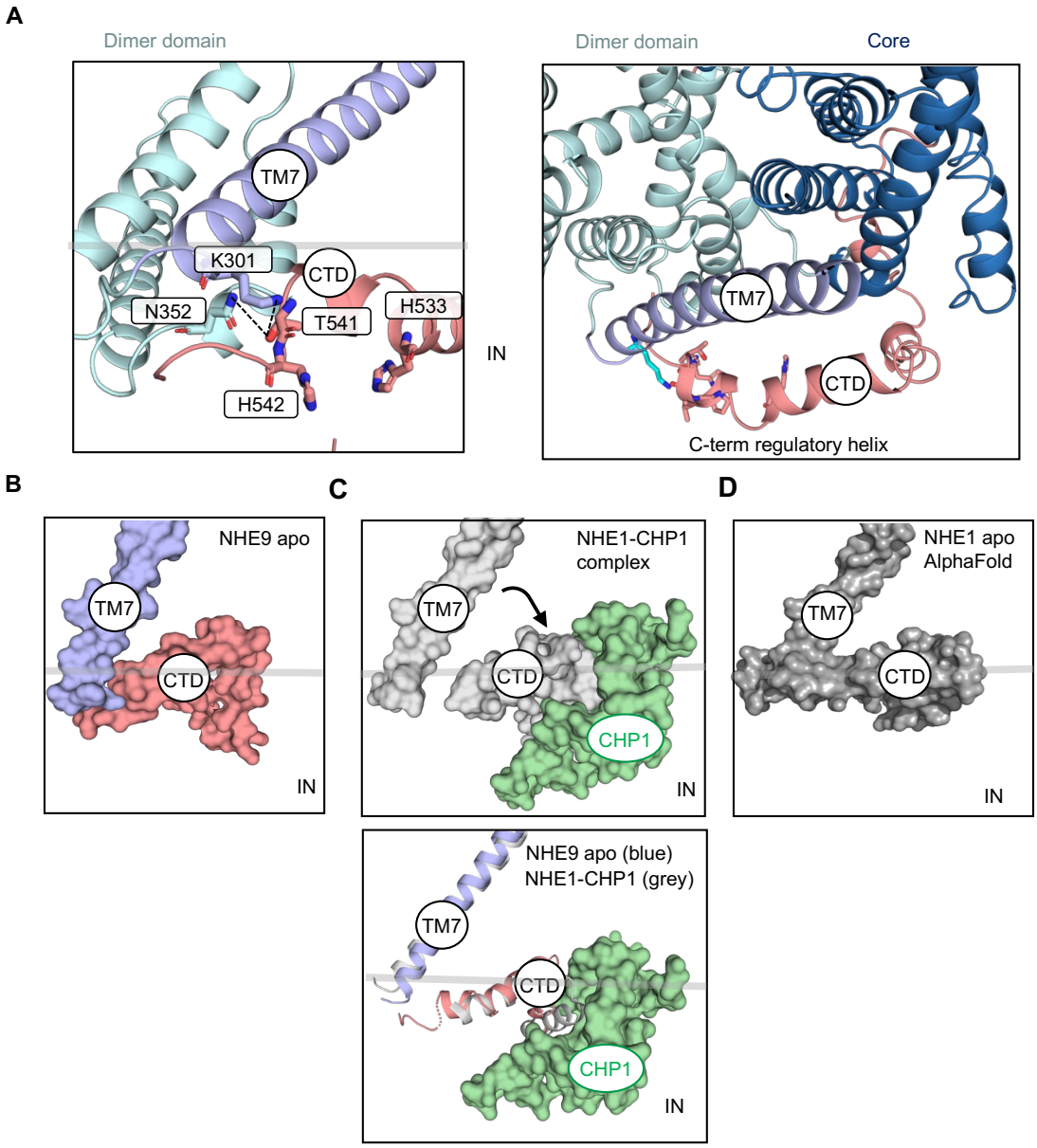
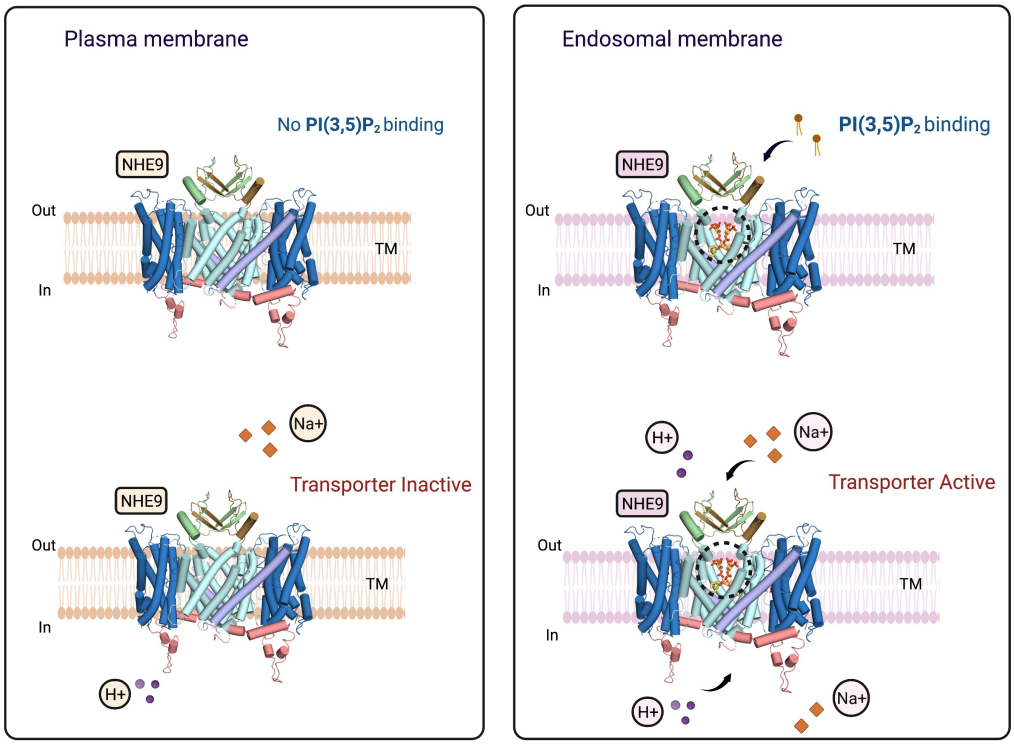


Figure 6 The autoinhibition and regulation of NHE9. (A) Numerous contacts are formed between the CTD and the linker helix TM7 (depicted as side view right; depicted as top view left (shown in cartoon, colored as in Figure 1A). **(B)** In the horse NHE9* CC structure the CTD stays connected to the linker helix TM7 (protein shown as surface, colored as in Fig1A). **(C)** *above:* AF2 model of NHE1 predicts CTD interacts to TM7 as seen in the NHE9* CC cryo EM structure. *below:*Aligning the NHE9*CC structure (blue-salmon) with inward-facing human NHE1 CTD (grey) and CHP1 (green) **(D)** CHP1 (green, surface) binding to the CTD of NHE1 (grey, surface) moves it away from the linker helix TM7.



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1103 **Figure 7. The PI-(3,5)P_2-dependent activation of NHE9 in late endosomes.**
1104 Schematic representation of NHE9 ion translocation, dimer stabilisation and transport
1105 activation in the endosomes vs. the cell membrane. NHE9 is inactive in the plasma
1106 membrane. Upon relocation into late endosomes, a concomittant bindning of PI-(3,5)P_2
1107 at the dimerization interface improves the stability of homodimer and activates the
1108 transporter. The figure was created using Biorender.com