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Lipid interactions enhance activation and potentiation of cystic fibrosis transmembrane conductance regulator (CFTR)

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Abstract: The recent cryo-electron microscopy structures of phosphorylated, ATP-bound CFTR in detergent micelles failed to reveal an open anion conduction pathway as expected on the basis of previous functional studies in biological membranes. We tested the hypothesis that interaction of CFTR with lipids is important for opening of its channel. Interestingly, molecular dynamics studies revealed that phospholipids associate with regions of CFTR proposed to contribute to its channel activity. More directly, we found that CFTR purified together with associated lipids using the amphipol: A8-35, exhibited higher rates of catalytic activity, channel activation and potentiation using ivacaftor, than did CFTR purified in detergent. Catalytic activity in CFTR detergent micelles was partially rescued by addition of phospholipids plus cholesterol, arguing that these lipids contribute directly to its modulation. In summary, these studies highlight the importance of lipids in regulated CFTR channel activation and potentiation.

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Introduction:

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44   Cystic Fibrosis is caused by mutations of the Cystic Fibrosis Transmembrane conductance
45   Regulator (*CFTR*) gene that codes for the ATP-binding cassette (ABC) anion channel, CFTR
46   (Tsui, Buchwald et al. 1985, Kerem, Rommens et al. 1989, Kartner, Hanrahan et al. 1991).
47   CFTR channel activity and anion conduction through its membrane pore is regulated by protein
48   kinase A (PKA) phosphorylation of its regulatory (R) domain as well as ATP binding and
49   hydrolysis by its nucleotide binding domains (NBDs) (Chang, Tabcharani et al. 1993, Winter and
50   Welsh 1997, Seibert, Chang et al. 1999, Ramjeesingh, Ugwu et al. 2008, Hwang and Sheppard
51   2009, Mihalyi, Torocsik et al. 2016). Biochemical and electrophysiological studies support a
52   model where phosphorylation promotes dissociation of the R domain from inhibitory locations at
53   the interface between the NBDs to facilitate their dimerization and ATPase activity (Kogan,
54   Ramjeesingh et al. 2001, Kogan, Ramjeesingh et al. 2002, Kidd, Ramjeesingh et al. 2004,
55   Vergani, Lockless et al. 2005, Aleksandrov, Aleksandrov et al. 2007, Baker, Hudson et al. 2007,
56   Stratford, Ramjeesingh et al. 2007, Ramjeesingh, Ugwu et al. 2008, Hwang and Sheppard 2009,
57   Kanelis, Hudson et al. 2010, Dawson, Farber et al. 2013). This model predicts that the NBDs of
58   the unphosphorylated and nonconductive form of CFTR may be dissociated, whereas the NBDs
59   will be dimerized in the phosphorylated and conductive form of the protein.

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61   Recently, four structures of CFTR have been determined using cryo-electron microscopy (EM)
62   at resolutions of 3.2 – 3.9 Å (Zhang and Chen 2016, Liu, Zhang et al. 2017, Zhang, Liu et al.
63   2017, Zhang, Liu et al. 2018). These structures include the unphosphorylated, ATP-free and
64   phosphorylated, ATP-bound forms and the unphosphorylated, ATP-free form of the zCFTR and
65   hCFTR proteins (Zhang and Chen 2016, Liu, Zhang et al. 2017, Zhang, Liu et al. 2017, Zhang,
66   Liu et al. 2018). Comparison of the structures show major conformational changes upon PKA
67   phosphorylation and ATP binding that are consistent with the molecular mechanism proposed for
68   opening of the conduction pore through CFTR. Such changes included the loss of stable R
69   domain interactions at the cleft between the two transmembrane domain (TMD)-NBD halves of
70   the CFTR molecule and tighter association of the NBDs (Zhang and Chen 2016, Zhang, Liu et
71   al. 2017, Zhang, Liu et al. 2018). There were also subtle conformational changes at the
72   transmembrane segments (TMs), notably at TM8 and TM12, upon PKA phosphorylation and
73   ATP binding (Zhang, Liu et al. 2017, Zhang, Liu et al. 2018). These structures suggest that both
74   TM8 and TM12 helices rotate to participate in novel interactions upon CFTR phosphorylation
75   (Zhang, Liu et al. 2017, Zhang, Liu et al. 2018). Unexpectedly, the structure of phosphorylated,
76   ATP-bound CFTR proteins did not reveal a continuous pathway for anion conduction (Zhang,
77   Liu et al. 2017, Zhang, Liu et al. 2018). Hence, factors required to stabilize the open conduction
78   pathway may be lacking in the CFTR proteins studied by cryo-EM.

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80   Recent studies have shown that ABC transporters specifically bind to lipids and these protein:
81   lipid interactions are structurally important (Taylor, Manolaridis et al. 2017, Jackson,
82   Manolaridis et al. 2018, Josts, Nitsche et al. 2018). Phospholipids and cholesterol are bound to
83   ABCG2 in a recent cryo-EM structure (Taylor, Manolaridis et al. 2017, Jackson, Manolaridis et
84   al. 2018) and conformers of MsbA solved in lipid containing nanodiscs differ from those
85   determined in detergent micelles (Josts, Nitsche et al. 2018). Furthermore, lipid interactions are
86   known to modify the activity of several ABC transporters including: P-glycoprotein (Ambudkar,
87   Lelong et al. 1998, Ritchie, Grinkova et al. 2009), ABCG1 (Hirayama, Kimura et al. 2013),
88   LmrA (Infed, Hanekop et al. 2011) and MsbA (Doerrler and Raetz 2002, Kawai, Caaveiro et al.
89   2011). In fact, the addition of lipids was found to stabilize CFTR after its purification in
90   detergent and modulate its ATPase activity (Yang, Wang et al. 2014, Hildebrandt, Khazanov et
91   al. 2017). Hence, we hypothesized that the conductance competent form of phosphorylated,
92   ATP-bound CFTR protein is stabilized through lipid interactions. To test this idea, we developed
93   a novel method to purify CFTR in a detergent-free environment. We confirmed that, relative to
94   detergent-purified protein, CFTR extracted from mammalian membranes using amphipol A8-35
95   retains associated lipids as well as exhibits enhanced ATPase activity, channel function and
96   response to the first drug targeting mutant CFTR.

97

98   **Results:**

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100   **Molecular dynamics (MD) simulation studies of "pre-open" state of CFTR reveal regions**
101   **with propensity for specific phospholipid interaction**

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103   We performed multiple microsecond-long MD simulations of a homology model of hCFTR in
104   the "pre-open" state (phosphorylated, ATP-bound) embedded in 1,2-palmitoyl-oleoyl-sn-
105   glycero-3-phosphocholine (POPC) or 1,2-palmitoyl-oleoyl-sn-glycero-3-phosphoserine (POPS)
106   bilayers, two lipids shown previously to support different levels of catalytic activity by CFTR
107   (Hildebrandt, Khazanov et al. 2017). By calculating the number density of POPC (Figure 1a,
108   green volumes) or POPS (Figure 1b, blue volumes), we observe an annular lipid shell
109   (transparent surface) wrapping around the TM region of hCFTR, revealing multiple sites
110   enriched for protein: lipid interactions (solid shapes). The number of lipid sites within the
111   annulus is 64 ± 3 for POPC (Figure 1a, transparent green surface) and 70 ± 4 for POPS (Figure
112   1b, transparent blue surface) bilayers, respectively (based on heavy-atom contacts within 4.5 Å).
113   Across multiple simulation repeats, we observe lipid binding sites that are enriched for
114   interactions with a particular lipid species. We show a differential association of POPC over
115   POPS with the outer vestibule of the CFTR protein (Figure 1c, green volumes) and POPS over
116   POPC with the inner vestibule of the channel (Figure 1d, blue volumes). POPC has a propensity
117   to associate with a groove between TM7 and TM9 in the outer vestibule. There are multiple
118   regions for which there is an increased propensity for POPS to associate with CFTR in the inner
119   membrane leaflet, including a cleft between TM4 and TM6 and another groove between TM10

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120 and TM11. As yet, the molecular basis for the positive effect of POPS on CFTR ATPase activity
121 remains unknown (Hildebrandt, Khazanov et al. 2017), but our analyses support the hypothesis
122 that its interaction with positively charged residues lining the cleft between TM4 and TM6 may
123 disrupt previously described interactions with organic anions known to be inhibitory to CFTR
124 ATPase activity (Kogan, Ramjeesingh et al. 2001, El Hiani and Linsdell 2015, El Hiani, Negoda
125 et al. 2016). Overall, these relative propensity maps of lipid interaction with the "pre-open" state
126 of CFTR correlate with regions that have been identified as important for channel opening
127 (Wang, El Hiani et al. 2014, El Hiani and Linsdell 2015, El Hiani, Negoda et al. 2016, Hwang,
128 Yeh et al. 2018).

129

130 **CFTR: lipid complexes are purified using amphipol A8-35**

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132 Full-length human Wt-CFTR, bearing its native sequence except for two affinity tags, a FLAG
133 tag (on the amino terminus) and a 10x histidine-tag (on the carboxy terminus) was expressed in
134 HEK293F cells. Amphipol A8-35 at 30 mg was effective in solubilizing CFTR from 1 ml of 2
135 mg/ml final concentration of resuspended membranes, which corresponds to an amphipol
136 concentration of 3% (w/v) (Figure 2a, lane 1). Finally, CFTR was purified to near homogeneity
137 in a single step by affinity to anti-FLAG® magnetic beads (Figure 2a, lane 3 and 4). In SDS-
138 PAGE, purified CFTR migrated as expected for the mature, complex glycosylated band C of the
139 protein (170-180 kDa, Figure 2a, lane 5). The immature, core glycosylated band B of CFTR was
140 also present in the eluted sample as a band at around 140-150 kDa (Figure 2a, lane 5).

141

142 Size exclusion chromatography (SEC) was used to characterize the purified CFTR protein on the
143 basis of differences in size using 280 nm to monitor the elution of CFTR (Figure 2b). The
144 fractions eluting between elution volume of 9-17 ml containing both core and complex
145 glycosylated CFTR, as confirmed by silver staining and immunoblotting, co-eluted with ATPase
146 activity (Figure 2b). Interestingly, the peak ATPase activity at elution volume of 14.5 ml was
147 slightly displaced from the peak CFTR protein abundance at elution volume of 13 ml, suggesting
148 that the population of purified CFTR molecules is heterogeneous with respect to specific enzyme
149 activity.

150

151 Previous studies found that amphipols do not compete with lipids, which remain associated to
152 membrane protein: amphipol complexes (Martinez, Gohon et al. 2002, Gohon, Dahmane et al.
153 2008, Bechara, Bolbach et al. 2012). Thus, we were interested in determining whether CFTR
154 purified using amphipol also retained lipids. We compared the amphipol-based purification to
155 that employed by the Chen group in their cryo-EM studies (Zhang and Chen 2016, Liu, Zhang et
156 al. 2017, Zhang, Liu et al. 2017). First, we confirmed that CFTR solubilized in Lauryl Maltose
157 Neopentyl Glycol (LMNG) detergent micelles (Zhang and Chen 2016, Liu, Zhang et al. 2017,
158 Zhang, Liu et al. 2017) could be purified using the same protocol that we developed for the
159 amphipol preparation (Figure 3a,b). Then, the relative degree of phospholipid association was

160   compared in the amphipol- and detergent-based purifications. Choline-containing lipids (i.e.
161   phosphatidylcholine or PC, lecithin, lysolecithin, and sphingomyelin) were quantified using a
162   fluorimetric assay after normalization for CFTR protein abundance (Refer to *CFTR*
163   *quantification* section in Materials and Methods for more details on the method). We detected a
164   higher number of approximately 56 to 76 choline-containing phospholipids associated per CFTR
165   molecule in amphipol compared to approximately 5 to 12 phospholipids associated per CFTR in
166   LMNG detergent micelles (Figure 3c). Interestingly, the number of measured lipid molecules
167   correlated with the number determined in our MD simulations of POPC or POPS. Notably, the
168   lipids that associated with CFTR in the amphipol purification include cholesterol, PC and
169   phosphatidylethanolamine (PE) as studied by lipid thin-layer chromatography (TLC) (Figure 3d).
170   We cannot exclude the presence of additional lipids, given the resolution of the solvent system
171   employed for TLC. In fact, we expect that phosphatidylserine (PS) is also associated given its
172   dominant presence in the inner leaflet of biological membranes.

173

174   **CFTR: lipid: amphipol complexes exhibit higher specific ATPase activity and higher**
175   **functional reconstitution as regulated anion channels than CFTR: detergent complexes**

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177   We compared the specific ATPase activities of CFTR purified using amphipol or LMNG after
178   PKA phosphorylation (P) to ensure that both preparations were maximally phosphorylated, a
179   modification known to be important for CFTR function as an enzyme and a channel (Chang,
180   Tabcharani et al. 1993, Li, Ramjeesingh et al. 1996, Winter and Welsh 1997, Seibert, Chang et
181   al. 1999, Szellas and Nagel 2003). We found that the K_m values of ATP were similar for the two
182   samples: K_m of 0.27 ± 0.08 mM ATP for the purified P-CFTR in amphipol and K_m of $0.32 \pm$
183   0.14 mM ATP for the purified P-CFTR in LMNG detergent micelles (Figure 4a). Interestingly,
184   the maximal ATPase activities, after normalization to protein amounts (Refer to *CFTR*
185   *quantification* section in Materials and Methods for more details on the method), were
186   significantly higher in the purified P-CFTR: amphipol complexes than in the purified P-CFTR:
187   LMNG micelles (V_{max} of 23.90 ± 1.91 nmol phosphate/mg protein/min versus V_{max} of $5.54 \pm$
188   0.66 nmol phosphate/mg protein/min respectively, Figure 4a).

189

190   In order to determine the role of lipids in enhancing the ATPase activity of the amphipol
191   preparation, we tested the effect of re-introducing PC, PS or a mix of PE:PS:PC:cholesterol
192   (5:2:1:1 ratio by weight) in the LMNG preparation (Figure 4b). We measured a significant
193   increase in ATPase activity of purified P-CFTR in LMNG detergent micelles upon pre-treatment
194   with PE:PS:PC:cholesterol (5:2:1:1 ratio by weight) albeit only reaching half of the relative
195   ATPase activity of purified P-CFTR in amphipol (Figure 4b). These results suggest that these
196   lipids, including cholesterol, enhance catalytic activity of the functional CFTR molecules in the
197   LMNG preparation and that the number of functional CFTR molecules is greater in the amphipol
198   preparation.

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200 We were then prompted to determine if the amphipol preparation enhanced the functional
201 reconstitution of CFTR channel activity relative to the LMNG preparation. Each preparation was
202 separately reconstituted into pre-formed POPC liposomes. We measured anion electrodiffusion
203 from liposomes normalized for the amount of CFTR inserted into liposomes (Refer to *CFTR*
204 *quantification* section in Materials and Methods for more details on the method) in order to
205 determine the relative proportion of channel competent CFTR molecules from the two
206 preparations (Eckford, Li et al. 2012, Eckford, Li et al. 2015). Briefly, in this assay, CFTR
207 protein in amphipols or LMNG micelles was reconstituted into POPC proteoliposomes with
208 equal concentrations of potassium iodide inside and potassium glutamate outside of the
209 liposomes to provide a driving force for iodide efflux and to maintain the osmolarity (Eckford, Li
210 et al. 2012, Eckford, Li et al. 2015). Addition of valinomycin, an ionophore that selectively
211 facilitates potassium ion flux out of the proteoliposomes, alleviates the charge build up in the
212 proteoliposomes and allows for iodide efflux through activated CFTR (Eckford, Li et al. 2012,
213 Eckford, Li et al. 2015). A schematic of this assay is shown in Figure 5a.
214
215 We measured iodide efflux after valinomycin addition to proteoliposomes containing either
216 amphipol or LMNG preparations of CFTR (Figure 5a). As expected for CFTR-mediated flux,
217 electrodiffusion of iodide was minimal for unphosphorylated CFTR or phosphorylated CFTR in
218 the absence of Mg-ATP (Figure 5b) (Eckford, Li et al. 2012). In addition, the CFTR inhibitor,
219 CFTR$_{inh}$-172, reduced flux for the phosphorylated protein in the presence of Mg-ATP, pointing
220 to the specificity of this function (Figure 5b) (Eckford, Li et al. 2012). Together, the
221 proteoliposomal flux assay reports on the phosphorylation and ATP-dependent channel activity
222 of CFTR. Importantly, we observed a significantly higher rate of iodide efflux from
223 proteoliposomes containing P-CFTR purified using amphipol relative to the proteoliposomes
224 containing P-CFTR purified using LMNG (Figure 5b). Given that these proteoliposomal flux
225 studies were controlled for the abundance of total CFTR reconstituted in proteoliposomes and
226 the number of proteoliposomes (estimated by total trapped iodide, Supplementary Figure 5),
227 these findings show that the proportional functional reconstitution is greater in the amphipol
228 preparation. These findings can also be interpreted to suggest that the lipids including cholesterol
229 retained in the CFTR: amphipol complexes exert an activating effect on the CFTR channel.
230
231 Ivacaftor (or VX-770) potentiates the regulated channel activity of Wt-CFTR and is approved as
232 a therapeutic intervention for a number of disease-causing mutations (Van Goor, Hadida et al.
233 2009, Eckford, Li et al. 2012, Jih and Hwang 2013, Van Goor, Yu et al. 2014). To date, its
234 binding site is unknown, although we previously showed using reconstituted CFTR purified
235 using the detergent, *fos*-choline 14, that VX-770 directly modulates channel opening of PKA
236 phosphorylated CFTR (Eckford, Li et al. 2012). Previous studies have suggested that the
237 lipophilic compound, VX-770, interacts with the lipid bilayer and may bind at a CFTR: lipid
238 interface (Jih and Hwang 2013, Baroni, Zegarra-Moran et al. 2014, Hwang, Yeh et al. 2018).
239 Thus, we reasoned that CFTR extracted with its associated lipids using amphipol would exhibit a

240   greater response to VX-770 than CFTR extracted with detergents. Now, we show in paired
241   studies, that the fold increase in iodide electrodiffusion caused by VX-770 (1 μM) from
242   proteoliposomes containing amphipol extracted CFTR was approximately twice that measured
243   for proteoliposomes containing LMNG extracted protein (Figure 5c). We interpret these findings
244   to suggest that the lipids present in the CFTR: amphipol complexes facilitate VX-770 binding
245   and/or the conformational changes required for its potentiation.
246
247

248   **Discussion:**

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250   We provide direct evidence that lipid interaction with purified CFTR enhances its catalytic
251   function, an activity that is coupled to its ion channel activity. Furthermore, the lipids associated
252   with CFTR participate in VX-770 mediated potentiation, supporting the hypothesis that VX-770
253   mediates its effects by binding at a protein: lipid interface (Baroni, Zegarra-Moran et al. 2014,
254   Hwang, Yeh et al. 2018).

255

256   We also report several methodological advances in the study of CFTR. Our studies suggest that
257   purification of membrane proteins that are relatively intractable, such as CFTR, can be achieved
258   using amphipol A8-35 with yields that are comparable to those achieved by detergents. There are
259   several other examples of membrane proteins that have been directly extracted from membranes
260   by amphipol A8-35 (Popot, Berry et al. 2003, Popot 2018, Marconnet 2019). Further, affinity
261   chromatography can be used to purify membrane proteins either extracted from membranes with
262   amphipol A8-35 (Popot 2018) or after exchanging detergent for amphipol A8-35 (Giusti, Kessler
263   et al. 2015) since the affinity tag is accessible to solution and not embedded in the amphipol belt
264   (Etzkorn, Zoonens et al. 2014). Once purified together with native lipids interacting in a complex
265   with amphipols, these proteins will exhibit enhanced stability and improved suitability for
266   biophysical studies (Marconnet 2019).

267

268   This is not the first study to show that CFTR purified in detergents can be functionally
269   reconstituted as a phosphorylated and ATP-regulated anion channel in phospholipid liposomes
270   (Bear, Li et al. 1992, Li, Ramjeesingh et al. 1996, Ramjeesingh, Li et al. 1997, Kogan,
271   Ramjeesingh et al. 2002, Ramjeesingh, Ugwu et al. 2008). In the very first purification papers,
272   we used relatively harsh detergents to extract and purify CFTR prior to reconstitution (Bear, Li et
273   al. 1992, Ramjeesingh, Li et al. 1997, Eckford, Li et al. 2012, Eckford, Li et al. 2015). We
274   estimated that the proportion of functionally reconstituted proteins after detergent purification
275   was low, less than 20% of the total number of CFTR molecules incorporated into liposomes
276   (Bear, Li et al. 1992). Unfortunately, the proportion of purified CFTR that can be functionally
277   reconstituted as an anion channel was not routinely defined in other published purification
278   protocols so a systematic comparison using values in the literature is not possible. However, our

279   proteoliposomal flux studies in the current work show that we can at least double the proportion
280   of channel competent molecules using the amphipol A8-35 relative to the detergent LMNG.
281
282   MD simulations carried out on phosphorylated, ATP-bound hCFTR revealed potential
283   phospholipid binding sites. Importantly, this model was built based on a template structure
284   (zCFTR), which shares 55% of sequence identity with hCFTR and shows a nonconductive state.
285   As previously mentioned, preferential binding of POPC was found at the external cleft between
286   TM7 and TM9 and preferential binding of POPS was found at the inward facing gaps between
287   TM4-TM6 and TM10-TM11 (El Hiani and Linsdell 2015, El Hiani, Negoda et al. 2016). It has
288   been speculated that TM8 plays a major role in gating of the conductance path (Zhang and Chen
289   2016, Liu, Zhang et al. 2017, Zhang, Liu et al. 2017, Hwang, Yeh et al. 2018), hence the
290   localization of a putative POPC interaction site near TM8 (the groove between TM7 and TM9)
291   implicates a role for this lipid in modulation of the gate. Similarly, POPS association with a
292   region in the "pre-open" state of the CFTR channel, that was previously shown to comprise an
293   aqueous portal in cysteine accessibility and electrophysiology studies (El Hiani and Linsdell
294   2015, El Hiani, Negoda et al. 2016, Hwang, Yeh et al. 2018, Li, Cowley et al. 2018), points to its
295   possible modulation of the channel opening transition. Altogether, the simulations suggest that
296   lipids interact with regions that participate in conformational changes leading to pore opening
297   and suggest that delipidation during purification in detergents may disrupt this mechanism. This
298   interpretation aligns with our functional studies that revealed the importance of lipids in
299   maintaining the conduction competent configuration of CFTR.
300
301   We reason that in addition to an important role for the phospholipid interactions modeled in our
302   simulations, the cholesterol identified in the CFTR: amphipol complexes also contributes to the
303   enhanced function mediated by this complex. Cholesterol is present at the plasma membrane and
304   interacts with a population of CFTR in microdomains known as lipid rafts (Cholon, O'Neal et al.
305   2010, Abu-Arish, Pandzic et al. 2015). The interaction of CFTR and cholesterol regulates the
306   confinement and dynamics of CFTR at the cell surface (Cholon, O'Neal et al. 2010, Abu-Arish,
307   Pandzic et al. 2015). However, not much is known on the effect of the interaction of CFTR and
308   cholesterol on CFTR channel activity. This is the first study to show a direct role for cholesterol
309   in augmenting the intrinsic catalytic and channel activity of CFTR.
310
311   We showed previously that the channel activity of detergent purified CFTR was potentiated by
312   ivacaftor (VX-770) following its reconstitution into liposomes, supporting the claim that VX-770
313   acts via direct binding to CFTR (Eckford, Li et al. 2012). Another group recently studied the
314   interaction of VX-770 with detergent solubilized CFTR using hydrogen/deuterium exchange
315   (Byrnes, Xu et al. 2018). This method showed that there were multiple regions in CFTR that
316   underwent changes in conformation after VX-770 interaction; however, the specific drug binding
317   site has yet to be defined. We and others hypothesize that VX-770 binds at a lipid: CFTR protein
318   interface given its lipophilicity (log$P$ score of 5.76) (Baroni, Zegarra-Moran et al. 2014, Chin,

319   Hung et al. 2018) and that VX-770 has been found to insert into the lipid bilayer (Baroni,
320   Zegarra-Moran et al. 2014). In this study, we provide direct evidence that the potentiating effect
321   of VX-770 is facilitated in reconstitutions of CFTR where native lipid interactions are preserved.
322
323   In summary, it is clear from these studies of purified and reconstituted CFTR, that the intrinsic
324   catalytic and channel activities of CFTR are modulated by associated lipids. This work points to
325   the need to define the molecular basis for these interactions in order to advance our
326   understanding of channel gating, conduction and the mechanism of action of clinically approved
327   drugs such as ivacaftor (VX-770).
328
329

330   **Materials and Methods:**
331

332   **Homology modeling of human CFTR:**
333   A homology model of the NBD-dimerized hCFTR was constructed using MODELLER (Fiser
334   and Sali 2003) with the cryo-EM structure of phosphorylated, ATP-bound *Danio rerio* CFTR
335   channel (zCFTR, PDB: 5W81) as template (Zhang, Liu et al. 2017). The sequence of zCFTR
336   (5W81), denoted as ΔzCFTR below, does not contain segments connecting each TMD-NBD pair
337   (406-436, 1182-1202), the R-domain (642-843), the glycosylated extracellular loop connecting
338   TM7 and TM8 (887-917), and the C-terminal stretch after NBD2 (1459-1485). The full-length
339   sequence of hCFTR and the sequence of PDB 5W81 were aligned with *align2d* program of
340   MODELLER (Zhang, Liu et al. 2017). To avoid modelling long *de novo* segments of zCFTR and
341   prevent short dangling fragments in the final homology model, the protein sequence alignment
342   between hCFTR and ΔzCFTR generated by *align2d* was edited to exclude segments that are not
343   aligned, are too short, or have no corresponding structural information from cryo-EM. The final
344   sequence used for homology modelling consists of five discontinuous segments (hCFTR; 1-403,
345   438-593, 846-886, 910-1172, and 1204-1457). Fifty homology models of human CFTR were
346   generated using the *model-single* program, and the model with the lowest global DOPE score
347   was chosen as the final model. The resulting structural model of human CFTR was superimposed
348   onto PDB 5W81 with respect to the Cα's of aligned residues through a translation-rotation
349   operation that minimizes RMSD.
350

351   **Preparation of simulation systems:**
352   Two Mg-ATP moieties from the zCFTR cryo-EM structure were inserted at the NBD interface
353   of hCFTR by superimposing the hCFTR homology model onto zCFTR using VMD (Humphrey,
354   Dalke et al. 1996). All five proteogenic segments of hCFTR were acetylated at the N-termini and
355   amidated at the C-termini. This hCFTR and Mg-ATP construct was embedded in either POPC or
356   POPS bilayers in hexagonal unit cells. hCFTR was embedded in a POPC bilayer using two
357   InflateGRO methods with slight modification catering to hexagonal geometry (Kandt, Ash et al.
358   2007, Schmidt and Kandt 2012). This procedure resulted in a bilayer consisting of 306 and 265

359   lipid molecules for InflateGRO and InflateGRO2 methods, respectively. hCFTR embedded in a
360   POPS bilayer was prepared and solvated using CHARMM-GUI (Jo, Kim et al. 2008), resulting
361   in a bilayer consisting of 287 lipid molecules. To add water and ions to the POPC-embedded
362   systems, tools from GROMACS package were used (Abraham MJ 2015). For POPS-embedded
363   systems, CHARMM-GUI automatically adds water and ions after adding the lipid bilayer. Both
364   POPC-embedded and POPS-embedded systems were solvated in an aqueous solution of 150 mM
365   NaCl in a hexagonal unit cell. No water molecules or ions were placed within the channel pore or
366   within the lipid bilayer in both initial systems. The number of atoms in the simulation cells were
367   181,000 to 208,000 and unit cell dimensions were: $a = b = 105 \sim 111$ Å, $c = 165 \sim 191$ Å, $\alpha = \beta =$
368    $90°$, $\gamma = 120°$.
369

370   **MD simulations:**
371   The CHARMM36 force field was used for protein, ions, lipids and ATP along with the TIP3P
372   water model (Jorgensen 1983, MacKerell, Bashford et al. 1998, Klauda, Venable et al. 2010,
373   Huang and MacKerell 2013). All systems (POPC-embedded by InflateGRO, POPC-embedded
374   by InflateGRO2, POPS-embedded) were subject to steepest descent energy minimization until
375   the maximum computed forces were below 1000 kJ/mol/nm. All simulations were conducted in
376   the NpT ensemble with T=300 K, p=1 atm, and with a timestep of 2 fs. Constant temperature and
377   pressure were maintained via Nosé–Hoover thermostat (Nosé 1984, Hoover 1985) (temperature
378   coupling of 0.5 ps) and Parrinello-Rahman barostat (Parrinello 1980, Nosé 1983) (time constant
379   of 2.0 ps), respectively. Lennard-Jones interactions were cut off at 1.2 nm and a force-based
380   switching function with a range of 1.0 nm was used. Electrostatic interactions were calculated
381   using particle-mesh Ewald (Darden T 1993, Essmann U 1995) with a real-space cut-off distance
382   of 1.2 nm. Nonbonded interactions were calculated using Verlet neighbor lists (Verlet 1967, Páll
383   2013). All covalent bonds involving hydrogen atoms were constrained using the LINCS
384   algorithm (Hess 2008). Simulations were performed using GROMACS 2018.1 (Abraham MJ
385   2015). All systems were NpT-equilibrated in three 10 ns-stages before production runs,
386   successively with protein heavy atoms, protein backbone atoms, and protein Cα atoms restrained.
387   All positional restraints used a force constant of 1000 kJ/mol/nm^2. Random initial velocities were
388   generated for twenty replicas of POPC-embedded system (embedded using either InflateGRO or
389   InflateGRO2 procedures), followed by 1 μs production runs of each replica, for an aggregate
390   total sampling time of 20 μs for POPC-embedded hCFTR. Random initial velocities were
391   generated for ten replicas of the POPS-embedded system (embedded using CHARMM-GUI),
392   followed by 1 μs production runs of each replica, for an aggregate total sampling time of 10 μs
393   for POPS-embedded hCFTR.
394

395   To obtain values of bulk lipid densities for normalizing lipid spatial distribution functions of
396   protein-embedded systems, a pure POPC bilayer and a pure POPS bilayer in rectangular cells
397   were constructed and surrounded by explicit water and 150 mM NaCl using CHARMM-GUI
398   membrane builder (cell dimensions: $50 \times 50 \times 69$ Å^3). The neat bilayer systems contained a total

399   of 84 and 74 lipid molecules for POPS and POPC, respectively. Each system was simulated for
400   50 ns using GROMACS 2018.1 (Abraham MJ 2015) using identical simulation parameters as
401   those used for protein-embedded bilayer simulations.
402
403   **Model validation:**
404   The alignment carried out by *align2d* program of the MODELLER gave a different sequence
405   alignment from that reported by Zhang and colleagues in the C-terminal region of NBD1. For
406   example, residues 593-597 (protein sequence: LMASK) of zebrafish CFTR was incorrectly
407   aligned to residues 602-606 (protein sequence: LVTSK) of human CFTR instead of residues
408   594-598 (protein sequence: LMANK). The differently aligned sequences were deleted from our
409   model because they were regarded as short dangling sequences; as a result, the homology model
410   did not include structure for a small region of NBD1 for which structural information exists in
411   the template structure. To verify whether this truncation alters the conformational and dynamic
412   properties of the transmembrane region of hCFTR, we reproduced all molecular simulations
413   using the original zCFTR model. Molecular models and simulations of zCFTR were conducted
414   using an identical protocol to the methods described above for hCFTR using the structure of
415   zCFTR (PDB 5W81). These simulations were performed for POPS and POPC lipids (the former
416   was embedded using CHARMM-GUI; the latter was embedded using both the InflateGRO and
417   InflateGRO2 protocols), for identical simulation lengths.
418
419   To examine structural fluctuations, we computed the RMSD of three subsets of Cα atoms ("All
420   Residues", "All Without C-terminus", and "TM Helices") over simulation time for both the
421   hCFTR homology model and zCFTR, as shown in Supplementary Figure 1. For the "All
422   Residues" subset, all CFTR residues are included except the segments not modeled by cryo-EM
423   (zCFTR) or MODELLER (hCFTR), as explained in the detailed methods of homology modeling.
424   The "All Without C-terminus" subset is the "all" subset minus residues 1438-1485 in zCFTR or
425   1437-1480 in hCFTR. The C-terminal stretches of both zCFTR and hCFTR are found to be
426   highly dynamic during simulation and account for considerable variation in RMSD amongst the
427   replicas. For the "TM Helices" subset, TM1-TM12 helices and the Lasso motif helices are
428   included. This subset includes all helices that form contacts with lipids. Overall, the RMSDs of
429   zCFTR and hCFTR converged to similar values, especially when only TM helices were
430   considered. The results indicate that the structure of the TM region was not significantly affected
431   by the exclusion of the segment during our simulations. To examine the convergence of average
432   protein-lipid interactions, we computed the average number of lipids within 4.5 Å of protein
433   heavy atoms over simulation time for zCFTR and hCFTR (Supplementary Figure 2). The
434   average number of protein-lipid interactions are stable throughout simulations and zCFTR and
435   hCFTR models are within one standard deviation for the majority of simulation time.
436
437
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11

Analysis of MD simulations:

Prior to analysis, all simulation frames were aligned using the Cα atoms of all TM segments of the cryo-EM structure. Analysis was performed on all simulation frames spaced by 5.0 ns after removing the first 100 ns of data from each simulation repeat. For time-averaged analyses, only POPC-embedded by InflateGRO2 and POPS-embedded simulations were utilized based on the agreement we observed between zCFTR and hCFTR models (Supplementary Figure 1). Protein-lipid contact propensities were measured for each amino acid of hCFTR by computing a binary contact (yes/no) whenever any protein-lipid heavy-atom pair was less than 4.5 Å using MDTraj (McGibbon, Beauchamp et al. 2015). These data were calculated for each simulation repeat and averaged over all repeats.

Spatial distribution functions for all lipids were calculated as a number density on a grid with 1 Å spacing using MDAnalysis (Michaud-Agrawal, Denning et al. 2011). POPC and POPS spatial distribution functions ($g_{PC(r)}$ and $g_{PS(r)}$) were normalized with respect bulk lipid number density in a neat bilayer system. PC and PS spatial distribution functions were computed using all simulation frames and the average number density of the bilayer was computed (by truncating all six edges of the box along X, Y, and Z by 10 Å, and taking a mean of the entire lipid region, both lipid headgroups and tails). The number average lipid density was computed to be 0.0956 ± 0.0152 and 0.1015 ± 0.0138 Å^{-3} for PC and PS, respectively. POPC and POPS spatial distribution functions were normalized by dividing by the bulk values PC and PS in the neat bilayer systems. Volume maps were rendered at an isovalue cutoff of 1.6 for POPC and POPS; showing regions with > 60% increased density relative to the bulk bilayer concentration. Difference maps were computed by computing the difference between spatial distribution functions ($g_{PC(r)}$ - $g_{PS(r)}$, and $g_{PS(r)}$ - $g_{PC(r)}$). Volumes of the difference map were rendered at an isovalue cutoff of 0.8; showing regions with an absolute difference > 80% between PC and PS, and vice versa. Manipulation of spatial distribution functions was performed using the volutil functionality of VMD (Humphrey, Dalke et al. 1996).

Generation of FLAG-CFTR-His construct:

N-terminal DYKDDDDK (FLAG) tag was introduced on the CFTR-His construct (with a C-terminal 10x His-tag) on the plasmid DNA containing WT-CFTR cDNA (in pcDNA3.1) as the template using the KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA), primers (Forward: 5'- GAG ATG GAT TAT AAA GAT GAT GAT G -3', Reverse: 5'- CAT CAT CAT CTT TAT AAT CCA TCT C -3') and polymerase chain reaction. The construct was validated by DNA sequencing (TCAG, Toronto, Ontario, Canada) and amplified by transformation and plasmid maxi-prep (Qiagen, Hilden, Germany). High-quality (260/280 nm ratio of 1.8 and higher) plasmid DNA was concentrated to a final concentration of 1 μg/μl.

478   **Expression of FLAG-CFTR-His construct in HEK293F cells and generation of crude**
479   **membranes:**
480   The FLAG-CFTR-His construct was transiently transfected in HEK293F (Thermo Fisher
481   Scientific) suspension cell line capable of complex N-glycosylation. DNA at 50 μg was mixed in
482   a 1:1 ratio with transfection reagent FectoPRO® (Polyplus, Berkeley, CA) and added to each 200
483   ml suspension culture at 0.8×10^6 cells per ml, as previously described (Ereno-Orbea, Sicard et
484   al. 2017, Ereno-Orbea, Sicard et al. 2018). Cells were grown at 37°C shaking at 180 rpm with
485   8% CO_2 for 24 h. The next day, cells were treated with 10 mM sodium butyrate (Sigma-Aldrich,
486   St. Louis, MO) at 37°C shaking for 24 h. HEK293F cells transfected with FLAG-CFTR-His
487   were collected and spun down at 6000 rpm at 4°C for 30 min. Fresh or frozen cell pellet
488   generated from a 600 ml cell suspension was resuspended in 40 ml of phosphate-buffered saline
489   (PBS, Wisent Inc., Saint-Jean Baptiste, Quebec, Canada) containing one tablet of cOmplete,
490   EDTA free protease inhibitor (Roche, Mannheim, Germany). The cells were lysed using
491   Emulsiflex C3 high pressure homogenizer (Avestin, Ottawa, Ontario, Canada) with at least 5
492   passages during the lysing process. Cell debris were spun down at 1000g at 4°C for 15 min. The
493   supernatant from this spin was further spun down at 100,000g at 4°C for 2 h. The pelleted crude
494   membrane was resuspended in 20 ml of PBS containing 0.1 mM DDM (BioShop, Burlington,
495   ON, Canada) and protease inhibitor (Roche). Syringes with 21G and 27G needles (BD, Franklin
496   Lakes, NJ) were used to disaggregate the pellet. The DDM treated membranes were incubated on
497   ice for 10 min. Any heavy particulate matter that came down were discarded before spinning the
498   membrane suspension at 100,000g at 4°C for 2 h. The extracted crude membrane pellet was
499   again resuspended with the aid of the syringe and previously described needles in 20 ml of 25
500   mM HEPES, 100 mM NaCl buffer with protease inhibitor (Roche) and then aliquoted. Total
501   protein concentration of the crude membranes was determined by Bradford assay (Bradford
502   1976) and presence of CFTR was confirmed by immunoblotting.
503
504   **Purification of FLAG-CFTR-His in amphipol or detergent:**
505   Crude membranes were solubilized to a final concentration of 2 mg/ml with 1% LMNG
506   (Anatrace, Maumee, OH) and 0.2% cholesterol hemisuccinate (Anatrace) in buffer A (20 mM
507   Tris-HCl pH 7.5, 2 mM $MgCl_2$, 200 mM NaCl, 20% glycerol, and 2 mM DTT, with protease
508   inhibitor, Roche) as previously described (Zhang and Chen 2016, Liu, Zhang et al. 2017, Zhang,
509   Liu et al. 2017) or with 3% (w/v) amphipol A8-35 (Anatrace) in buffer B (25 mM HEPES pH 8,
510   100 mM NaCl with protease inhibitor, Roche) at 4°C for 2 h with shaking. Insoluble fractions
511   were removed by ultracentrifugation at 45,000 rpm at 4°C for 1 h. Anti-FLAG® M2 magnetic
512   beads (Sigma-Aldrich) were washed with corresponding buffers (buffer A with 0.025% LMNG
513   or buffer B with 0.025% amphipol) using a magnetic rack (New England Biolabs, Ipswich, MA)
514   for a total of 3 times. Soluble fractions were incubated with anti-FLAG® M2 magnetic beads at
515   4°C overnight with shaking. Unbound proteins were removed the next day with a magnetic rack
516   (New England Biolabs) and anti-FLAG® M2 magnetic beads (Sigma-Aldrich) were washed 5
517   times with corresponding buffers. If phosphorylation is required, the anti-FLAG® M2 magnetic

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518   beads (Sigma-Aldrich) were treated with a phosphorylation cocktail (10,000 U of PKA (New
519   England Biolabs) and 5 mM Mg-ATP (Sigma-Aldrich) in buffer A with 0.025% LMNG or
520   buffer B with 0.025% amphipol) at room temperature for 1 h with shaking. The phosphorylation
521   cocktail was removed by magnetic rack and the anti-FLAG® M2 magnetic beads were washed
522   again for 5 times with the corresponding buffers. FLAG-CFTR-His protein was eluted with 200
523   μg/ml FLAG® peptide (DYKDDDDK, Sigma-Aldrich) in buffer A with 0.025% LMNG or
524   buffer B with 0.025% amphipol using the magnetic rack after 45 min incubation with shaking at
525   4°C. Purified protein at 500 μl was loaded in Superose 6 Increase 10/300 column (GE
526   Healthcare, Chicago, IL) to further characterize the protein. Purity of purified CFTR was
527   determined by densitometry of the CFTR bands compared to other bands present on the 4-12%
528   silver stained gels by Image Studio™ Lite software.
529
530   **CFTR quantification:**
531   Crude membranes at 2.6 mg were solubilized in amphipol and CFTR was purified in amphipol as
532   previously described to generate 400 μl of eluted standard in the presence of FLAG® peptide. An
533   aliquot of standard (100 μl) was subjected to amino acid analysis with the Waters Pico-Tag
534   System (Waters ACQUITY UPLC, Milford, MA). Refer to Supplementary Figure 3 and
535   Supplementary Table 1 for complete results of amino acid analysis of the purified CFTR
536   standard in amphipol. Alanine (1015.112 pmoles was present in the 100 μl standard) was chosen
537   to determine the concentration of CFTR standard as amino acid hydrolysis with various solvents
538   resulted in 100% recovery of alanine (Adebiyi, Jin et al. 2005) and there are no alanine residues
539   in the FLAG® peptide that was also present in the standard. The calculation of the concentration
540   of the CFTR standard is as follows:

541    $$\frac{weight\ Ala}{weight\ CFTR} = \frac{number\ of\ Ala*(MW\ Ala)}{MW\ CFTR}$$

542

543    $$\frac{72065\ pg}{weight\ CFTR} = \frac{83*71\ pg/pmol}{170507.57\ pg/pmol}$$

544
545   Weight of CFTR = 208512269 pg or 2085.12269 ng
546   Volume used for amino acid analysis = 100 μl
547   Concentration of CFTR standard = 2085.12269 ng / 100 μl = 20.85 ng/μl
548
549   The remaining standard was aliquoted and stored at -80°C. When required, various nanogram
550   amounts of the standard were run with purified protein samples of unknown amounts on SDS-
551   PAGE and subjected to immunoblotting. Immunoblot bands were analyzed with Image Studio™
552   Lite software and densitometry of unknowns were interpolated on the standard curve on
553   GraphPad Prism. Refer to Supplementary Figure 4 for example of standard curve for
554   concentration determination.
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Phospholipid assay:

Purified CFTR in amphipol, purified CFTR in LMNG and the appropriate controls were subjected to a 96 well phospholipid assay kit according to the manufacturer's protocol (Cat. no. MAK122, Sigma-Aldrich). Fluorescence was read at excitation of 530 nm and emission of 585 nm on the SpectraMax i3X plate reader (Molecular Devices, San Jose, CA).

Lipid TLC:

Lipid containing samples were extracted as previously described (Dorr, Koorengevel et al. 2014). The lipid containing organic phase was first dried with sodium sulfate for 1 h, filtered and concentrated under a stream of argon to a volume of 50 μl. Silica gel plates (Analtech Inc., Newark, DE) were first activated at 160°C for 1 h before sample application. Samples (10 μl) and polar lipid standard (2 μl, Matreya LLC, State College, PA) were spotted and developed using chloroform: methanol: water (65:25:4) as the solvent system. Visualization of the lipids was conducted as previously described (Dorr, Koorengevel et al. 2014).

ATPase assay - ATP dose response:

Purified protein (approximately 50-200 ng in 25 μl) was incubated with 50 μl of various concentrations of Na-ATP (Sigma-Aldrich) solubilized in 10 mM HEPES, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM DDM, pH 7.4 buffer at 37°C for 2 h. A colorimetric assay was applied as previously described (Chifflet, Torriglia et al. 1988) to detect phosphate at absorbance 800 nm on the SpectraMax i3X plate reader (Molecular Devices). Values were subtracted from controls (amphipol or LMNG buffer with various concentrations of ATP), converted to nmol phosphate by interpolation of phosphate standard curve on GraphPad Prism and then to nmol phosphate/mg protein/min after CFTR quantification as previously described. Values were fit on Michaelis-Menten curves with GraphPad Prism to determine the K_m and V_{max} values of ATPase activity.

ATPase assay - Re-introducing lipids to purified CFTR in LMNG detergent micelles:

Egg PC, porcine brain PS, or a mix of PE/brain PS/egg PC/cholesterol in a 5:2:1:1 (w/v) ratio (all lipids in chloroform from Avanti® Polar Lipids, Inc., Alabaster, AL) were dried in a glass tube under argon gas. Dried lipids were resuspended in 25 mM HEPES, 100 mM NaCl, pH 8.0 buffer with sonication to a stock concentration of 10 mg/ml. Resuspended lipid (100 μg in 10 μl) was added to purified protein (approximately 50-200 ng in 25 μl) on a 96 well plate along with controls: 1) 10 μl of 25 mM HEPES, 100 mM NaCl, pH 8.0 buffer added to 25 μl of purified protein; 2) 10 μl of resuspended lipid added to 25 μl of buffer A with 0.025% LMNG; 3) 10 μl of 25 mM HEPES, 100 mM NaCl, pH 8.0 buffer added to 25 μl buffer A with 0.025% LMNG. Protein with lipids and controls were incubated at 1 h at room temperature. Na-ATP (40 μl of 0.9375 mM stock concentration to a final concentration of 0.5 mM Na-ATP) solubilized in 10 mM HEPES, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM DDM, pH 7.4 buffer was added to the mix and the reaction was incubated at 37°C for 2 h. Colorimetric assay was applied as previously described above.

597

Iodide efflux:

CFTR protein in amphipol was incubated with 5 mM DDM during the elution step with FLAG®

peptide prior to reconstitution whereas protein in LMNG remained as is. Purified protein

(approximately 0.4 μg) was reconstituted in 5 mg of POPC (Avanti® Polar Lipids, Inc.) and

subjected to iodide efflux as previously described (Eckford, Li et al. 2012, Eckford, Li et al.

2015).

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Author contributions

Study was designed by S.C. and C.E.B. based on discussions with the other authors. S.C. generated and analyzed the SEC, CFTR quantification, phospholipid, ATPase and iodide efflux data with the help from M.R., J.E.-O., C.E.B. and J-P.J. M.R. assisted in optimization of the novel purification method, ATPase assay, lipid TLC, and amino acid quantification of CFTR standard. M.H. assisted in optimization of the novel purification method. J.E.-O. conducted transfections of CFTR in HEK-293F cells in suspension and conducted SEC of purified protein. H.C. conducted transfections of CFTR in HEK-293F cells. C.I. and Z.W.Z. generated homology model of hCFTR, and conducted and analyzed MD simulations. R.P. provided suggestions on presentation and interpretation of MD simulation data. J-P.J assisted with optimization of novel purification method and suggestions for improvement of assays. Manuscript was written by S.C. and C.E.B. with input from all the authors.

References:

Abraham MJ, M. T., Schulz R, Páll S, Smith JC, Hess B & Lindahl E. (2015). GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 1-7(2015): 19-25.

Abu-Arish, A., E. Pandzic, J. Goepp, E. Matthes, J. W. Hanrahan and P. W. Wiseman (2015). Cholesterol modulates CFTR confinement in the plasma membrane of primary epithelial cells. *Biophys J* 109(1): 85-94.

Adebiyi, A. P., D. H. Jin, T. Ogawa and K. Muramoto (2005). Acid hydrolysis of protein in a microcapillary tube for the recovery of tryptophan. *Biosci Biotechnol Biochem* 69(1): 255-257.

Aleksandrov, A. A., L. A. Aleksandrov and J. R. Riordan (2007). CFTR (ABCC7) is a hydrolyzable-ligand-gated channel. *Pflugers Arch* 453(5): 693-702.

Ambudkar, S. V., I. H. Lelong, J. Zhang and C. Cardarelli (1998). Purification and reconstitution of human P-glycoprotein. *Methods Enzymol* 292: 492-504.

Baker, J. M., R. P. Hudson, V. Kanelis, W. Y. Choy, P. H. Thibodeau, P. J. Thomas and J. D. Forman-Kay (2007). CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices. *Nat Struct Mol Biol* 14(8): 738-745.

Baroni, D., O. Zegarra-Moran, A. Svensson and O. Moran (2014). Direct interaction of a CFTR potentiator and a CFTR corrector with phospholipid bilayers. *Eur Biophys J* 43(6-7): 341-346.

Bear, C. E., C. H. Li, N. Kartner, R. J. Bridges, T. J. Jensen, M. Ramjeesingh and J. R. Riordan (1992). Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell* 68(4): 809-818.

Bechara, C., G. Bolbach, P. Bazzaco, K. S. Sharma, G. Durand, J. L. Popot, F. Zito and S. Sagan (2012). MALDI-TOF mass spectrometry analysis of amphipol-trapped membrane proteins. *Anal Chem* 84(14): 6128-6135.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254.

Byrnes, L. J., Y. Xu, X. Qiu, J. D. Hall and G. M. West (2018). Sites associated with Kalydeco binding on human Cystic Fibrosis Transmembrane Conductance Regulator revealed by Hydrogen/Deuterium Exchange. *Sci Rep* 8(1): 4664.

Chang, X. B., J. A. Tabcharani, Y. X. Hou, T. J. Jensen, N. Kartner, N. Alon, J. W. Hanrahan and J. R. Riordan (1993). Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J Biol Chem* 268(15): 11304-11311.

Chifflet, S., A. Torriglia, R. Chiesa and S. Tolosa (1988). A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. *Anal Biochem* 168(1): 1-4.

Chin, S., M. Hung, A. Won, Y. S. Wu, S. Ahmadi, D. Yang, S. Elmallah, K. Toutah, C. M. Hamilton, R. N. Young, R. D. Viirre, C. M. Yip and C. E. Bear (2018). Lipophilicity of the Cystic Fibrosis Drug, Ivacaftor (VX-770), and Its Destabilizing Effect on the Major CF-causing Mutation: F508del. *Mol Pharmacol* 94(2): 917-925.

Cholon, D. M., W. K. O'Neal, S. H. Randell, J. R. Riordan and M. Gentzsch (2010). Modulation of endocytic trafficking and apical stability of CFTR in primary human airway epithelial cultures. *Am J Physiol Lung Cell Mol Physiol* 298(3): L304-314.

Darden T, Y. D. P. L. (1993). Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. *Journal of chemical physics* 98(12): 10089–10092

Dawson, J. E., P. J. Farber and J. D. Forman-Kay (2013). Allosteric coupling between the intracellular coupling helix 4 and regulatory sites of the first nucleotide-binding domain of CFTR. *PLoS One* 8(9): e74347.

680   Doerrler, W. T. and C. R. Raetz (2002). ATPase activity of the MsbA lipid flippase of Escherichia
681   coli. *J Biol Chem* 277(39): 36697-36705.
682   Dorr, J. M., M. C. Koorengevel, M. Schafer, A. V. Prokofyev, S. Scheidelaar, E. A. van der
683   Cruijsen, T. R. Dafforn, M. Baldus and J. A. Killian (2014). Detergent-free isolation,
684   characterization, and functional reconstitution of a tetrameric K+ channel: the power of native
685   nanodiscs. *Proc Natl Acad Sci U S A* 111(52): 18607-18612.
686   Eckford, P. D., C. Li and C. E. Bear (2015). Functional reconstitution and channel activity
687   measurements of purified wildtype and mutant CFTR protein. *J Vis Exp*(97).
688   Eckford, P. D., C. Li, M. Ramjeesingh and C. E. Bear (2012). Cystic fibrosis transmembrane
689   conductance regulator (CFTR) potentiator VX-770 (ivacaftor) opens the defective channel gate
690   of mutant CFTR in a phosphorylation-dependent but ATP-independent manner. *J Biol Chem*
691   287(44): 36639-36649.
692   El Hiani, Y. and P. Linsdell (2015). Functional Architecture of the Cytoplasmic Entrance to the
693   Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore. *J Biol Chem*
694   290(25): 15855-15865.
695   El Hiani, Y., A. Negoda and P. Linsdell (2016). Cytoplasmic pathway followed by chloride ions to
696   enter the CFTR channel pore. *Cell Mol Life Sci* 73(9): 1917-1925.
697   Ereno-Orbea, J., T. Sicard, H. Cui, I. Akula and J. P. Julien (2018). Characterization of
698   Glycoproteins with the Immunoglobulin Fold by X-Ray Crystallography and Biophysical
699   Techniques. *J Vis Exp*(137).
700   Ereno-Orbea, J., T. Sicard, H. Cui, M. T. Mazhab-Jafari, S. Benlekbir, A. Guarne, J. L.
701   Rubinstein and J. P. Julien (2017). Molecular basis of human CD22 function and therapeutic
702   targeting. *Nat Commun* 8(1): 764.
703   Essmann U, P. L., Berkowitz ML, Darden T, Lee H, Pedersen LG. (1995). A smooth particle
704   mesh Ewald method. *Journal of chemical physics* 103(19): 8577–8593
705   Etzkorn, M., M. Zoonens, L. J. Catoire, J. L. Popot and S. Hiller (2014). How amphipols embed
706   membrane proteins: global solvent accessibility and interaction with a flexible protein terminus. *J
707   Membr Biol* 247(9-10): 965-970.
708   Fiser, A. and A. Sali (2003). Modeller: generation and refinement of homology-based protein
709   structure models. *Methods Enzymol* 374: 461-491.
710   Giusti, F., P. Kessler, R. W. Hansen, E. A. Della Pia, C. Le Bon, G. Mourier, J. L. Popot, K. L.
711   Martinez and M. Zoonens (2015). Synthesis of a Polyhistidine-bearing Amphipol and its Use for
712   Immobilizing Membrane Proteins. *Biomacromolecules* 16(12): 3751-3761.
713   Gohon, Y., T. Dahmane, R. W. Ruigrok, P. Schuck, D. Charvolin, F. Rappaport, P. Timmins, D.
714   M. Engelman, C. Tribet, J. L. Popot and C. Ebel (2008). Bacteriorhodopsin/amphipol
715   complexes: structural and functional properties. *Biophys J* 94(9): 3523-3537.
716   Hess, B. (2008). P-LINCS: A Parallel Linear Constraint Solver for Molecular Simulation. *J Chem
717   Theory Comput* 4(1): 116-122.
718   Hildebrandt, E., N. Khazanov, J. C. Kappes, Q. Dai, H. Senderowitz and I. L. Urbatsch (2017).
719   Specific stabilization of CFTR by phosphatidylserine. *Biochim Biophys Acta* 1859(2): 289-293.
720   Hirayama, H., Y. Kimura, N. Kioka, M. Matsuo and K. Ueda (2013). ATPase activity of human
721   ABCG1 is stimulated by cholesterol and sphingomyelin. *J Lipid Res* 54(2): 496-502.
722   Hoover, W. G. (1985). Canonical dynamics: Equilibrium phase-space distributions. *Phys Rev A
723   Gen Phys* 31(3): 1695-1697.
724   Huang, J. and A. D. MacKerell, Jr. (2013). CHARMM36 all-atom additive protein force field:
725   validation based on comparison to NMR data. *J Comput Chem* 34(25): 2135-2145.
726   Humphrey, W., A. Dalke and K. Schulten (1996). VMD: visual molecular dynamics. *J Mol Graph*
727   14(1): 33-38, 27-38.
728   Hwang, T. C. and D. N. Sheppard (2009). Gating of the CFTR Cl- channel by ATP-driven
729   nucleotide-binding domain dimerisation. *J Physiol* 587(Pt 10): 2151-2161.

730 Hwang, T. C., J. T. Yeh, J. Zhang, Y. C. Yu, H. I. Yeh and S. Destefano (2018). Structural
731 mechanisms of CFTR function and dysfunction. *J Gen Physiol* 150(4): 539-570.
732 Infed, N., N. Hanekop, A. J. Driessen, S. H. Smits and L. Schmitt (2011). Influence of detergents
733 on the activity of the ABC transporter LmrA. *Biochim Biophys Acta* 1808(9): 2313-2321.
734 Jackson, S. M., I. Manolaridis, J. Kowal, M. Zechner, N. M. I. Taylor, M. Bause, S. Bauer, R.
735 Bartholomaeus, G. Bernhardt, B. Koenig, A. Buschauer, H. Stahlberg, K. H. Altmann and K. P.
736 Locher (2018). Structural basis of small-molecule inhibition of human multidrug transporter
737 ABCG2. *Nat Struct Mol Biol* 25(4): 333-340.
738 Jih, K. Y. and T. C. Hwang (2013). Vx-770 potentiates CFTR function by promoting decoupling
739 between the gating cycle and ATP hydrolysis cycle. *Proc Natl Acad Sci U S A* 110(11): 4404-
740 4409.
741 Jo, S., T. Kim, V. G. Iyer and W. Im (2008). CHARMM-GUI: a web-based graphical user
742 interface for CHARMM. *J Comput Chem* 29(11): 1859-1865.
743 Jorgensen, W., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. (1983). Comparison
744 of Simple Potential Functions for Simulating Liquid Water. . *Journal of chemical physics* 79: 926-
745 935.
746 Josts, I., J. Nitsche, S. Maric, H. D. Mertens, M. Moulin, M. Haertlein, S. Prevost, D. I. Svergun,
747 S. Busch, V. T. Forsyth and H. Tidow (2018). Conformational States of ABC Transporter MsbA
748 in a Lipid Environment Investigated by Small-Angle Scattering Using Stealth Carrier Nanodiscs.
749 *Structure*.
750 Kandt, C., W. L. Ash and D. P. Tieleman (2007). Setting up and running molecular dynamics
751 simulations of membrane proteins. *Methods* 41(4): 475-488.
752 Kanelis, V., R. P. Hudson, P. H. Thibodeau, P. J. Thomas and J. D. Forman-Kay (2010). NMR
753 evidence for differential phosphorylation-dependent interactions in WT and DeltaF508 CFTR.
754 *Embo j* 29(1): 263-277.
755 Kartner, N., J. W. Hanrahan, T. J. Jensen, A. L. Naismith, S. Z. Sun, C. A. Ackerley, E. F.
756 Reyes, L. C. Tsui, J. M. Rommens, C. E. Bear and et al. (1991). Expression of the cystic fibrosis
757 gene in non-epithelial invertebrate cells produces a regulated anion conductance. *Cell* 64(4):
758 681-691.
759 Kawai, T., J. M. Caaveiro, R. Abe, T. Katagiri and K. Tsumoto (2011). Catalytic activity of MsbA
760 reconstituted in nanodisc particles is modulated by remote interactions with the bilayer. *FEBS
761 Lett* 585(22): 3533-3537.
762 Kerem, B., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M.
763 Buchwald and L. C. Tsui (1989). Identification of the cystic fibrosis gene: genetic analysis.
764 *Science* 245(4922): 1073-1080.
765 Kidd, J. F., M. Ramjeesingh, F. Stratford, L. J. Huan and C. E. Bear (2004). A heteromeric
766 complex of the two nucleotide binding domains of cystic fibrosis transmembrane conductance
767 regulator (CFTR) mediates ATPase activity. *J Biol Chem* 279(40): 41664-41669.
768 Klauda, J. B., R. M. Venable, J. A. Freites, J. W. O'Connor, D. J. Tobias, C. Mondragon-
769 Ramirez, I. Vorobyov, A. D. MacKerell, Jr. and R. W. Pastor (2010). Update of the CHARMM all-
770 atom additive force field for lipids: validation on six lipid types. *J Phys Chem B* 114(23): 7830-
771 7843.
772 Kogan, I., M. Ramjeesingh, L. J. Huan, Y. Wang and C. E. Bear (2001). Perturbation of the pore
773 of the cystic fibrosis transmembrane conductance regulator (CFTR) inhibits its atpase activity. *J
774 Biol Chem* 276(15): 11575-11581.
775 Kogan, I., M. Ramjeesingh, C. Li and C. E. Bear (2002). Studies of the molecular basis for cystic
776 fibrosis using purified reconstituted CFTR protein. *Methods Mol Med* 70: 143-157.
777 Li, C., M. Ramjeesingh, W. Wang, E. Garami, M. Hewryk, D. Lee, J. M. Rommens, K. Galley
778 and C. E. Bear (1996). ATPase activity of the cystic fibrosis transmembrane conductance
779 regulator. *J Biol Chem* 271(45): 28463-28468.

20

780   Li, M. S., E. A. Cowley, Y. El Hiani and P. Linsdell (2018). Functional organization of
781   cytoplasmic portals controlling access to the cystic fibrosis transmembrane conductance
782   regulator (CFTR) chloride channel pore. *J Biol Chem* 293(15): 5649-5658.
783   Liu, F., Z. Zhang, L. Csanady, D. C. Gadsby and J. Chen (2017). Molecular Structure of the
784   Human CFTR Ion Channel. *Cell* 169(1): 85-95.e88.
785   MacKerell, A. D., D. Bashford, M. Bellott, R. L. Dunbrack, J. D. Evanseck, M. J. Field, S.
786   Fischer, J. Gao, H. Guo, S. Ha, D. Joseph-McCarthy, L. Kuchnir, K. Kuczera, F. T. Lau, C.
787   Mattos, S. Michnick, T. Ngo, D. T. Nguyen, B. Prodhom, W. E. Reiher, B. Roux, M. Schlenkrich,
788   J. C. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorkiewicz-Kuczera, D. Yin and M. Karplus
789   (1998). All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J*
790   *Phys Chem B* 102(18): 3586-3616.
791   Marconnet, A., Pozza, A., Ilioaia, O., Giusti, F. & Zoonens, M. (2019). Efficiency of amphipathic
792   polymers in solubilizing and stabilizing membrane proteins: a comparative study (in
793   preparation).
794   Martinez, K. L., Y. Gohon, P. J. Corringer, C. Tribet, F. Merola, J. P. Changeux and J. L. Popot
795   (2002). Allosteric transitions of Torpedo acetylcholine receptor in lipids, detergent and
796   amphipols: molecular interactions vs. physical constraints. *FEBS Lett* 528(1-3): 251-256.
797   McGibbon, R. T., K. A. Beauchamp, M. P. Harrigan, C. Klein, J. M. Swails, C. X. Hernandez, C.
798   R. Schwantes, L. P. Wang, T. J. Lane and V. S. Pande (2015). MDTraj: A Modern Open Library
799   for the Analysis of Molecular Dynamics Trajectories. *Biophys J* 109(8): 1528-1532.
800   Michaud-Agrawal, N., E. J. Denning, T. B. Woolf and O. Beckstein (2011). MDAnalysis: a toolkit
801   for the analysis of molecular dynamics simulations. *J Comput Chem* 32(10): 2319-2327.
802   Mihalyi, C., B. Torocsik and L. Csanady (2016). Obligate coupling of CFTR pore opening to tight
803   nucleotide-binding domain dimerization. *Elife* 5.
804   Nosé, S. (1984). A molecular dynamics method for simulations in the canonical ensemble.
805   *Molecular Physics* 52: 255-268.
806   Nosé, S. a. K., M.L. (1983). Constant pressure molecular dynamics for molecular systems.
807   *Molecular Physics* 50: 1055-1076.
808   Páll, S. a. H., B. (2013). A flexible algorithm for calculating pair interactions on SIMD
809   architectures. *Computer Physics Communications* 184: 2641-2650.
810   Parrinello, M. a. R., A. (1980). Crystal structure and pair potentials: A molecular-dynamics
811   study. *Physical Review Letters* 45: 1196-1199.
812   Popot, J. L. (2018). Membrane Proteins in Aqueous Solutions, Springer International Publishing.
813   Popot, J. L., E. A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D. M. Engelman, M. Flotenmeyer,
814   F. Giusti, Y. Gohon, Q. Hong, J. H. Lakey, K. Leonard, H. A. Shuman, P. Timmins, D. E.
815   Warschawski, F. Zito, M. Zoonens, B. Pucci and C. Tribet (2003). Amphipols: polymeric
816   surfactants for membrane biology research. *Cell Mol Life Sci* 60(8): 1559-1574.
817   Ramjeesingh, M., C. Li, E. Garami, L. J. Huan, M. Hewryk, Y. Wang, K. Galley and C. E. Bear
818   (1997). A novel procedure for the efficient purification of the cystic fibrosis transmembrane
819   conductance regulator (CFTR). *Biochem J* 327 (Pt 1): 17-21.
820   Ramjeesingh, M., F. Ugwu, F. L. Stratford, L. J. Huan, C. Li and C. E. Bear (2008). The intact
821   CFTR protein mediates ATPase rather than adenylate kinase activity. *Biochem J* 412(2): 315-
822   321.
823   Ritchie, T. K., Y. V. Grinkova, T. H. Bayburt, I. G. Denisov, J. K. Zolnerciks, W. M. Atkins and S.
824   G. Sligar (2009). Chapter 11 - Reconstitution of membrane proteins in phospholipid bilayer
825   nanodiscs. *Methods Enzymol* 464: 211-231.
826   Schmidt, T. H. and C. Kandt (2012). LAMBADA and InflateGRO2: efficient membrane alignment
827   and insertion of membrane proteins for molecular dynamics simulations. *J Chem Inf Model*
828   52(10): 2657-2669.

829   Seibert, F. S., X. B. Chang, A. A. Aleksandrov, D. M. Clarke, J. W. Hanrahan and J. R. Riordan
830   (1999). Influence of phosphorylation by protein kinase A on CFTR at the cell surface and
831   endoplasmic reticulum. *Biochim Biophys Acta* 1461(2): 275-283.
832   Stratford, F. L., M. Ramjeesingh, J. C. Cheung, L. J. Huan and C. E. Bear (2007). The Walker B
833   motif of the second nucleotide-binding domain (NBD2) of CFTR plays a key role in ATPase
834   activity by the NBD1-NBD2 heterodimer. *Biochem J* 401(2): 581-586.
835   Szellas, T. and G. Nagel (2003). Apparent affinity of CFTR for ATP is increased by continuous
836   kinase activity. *FEBS Lett* 535(1-3): 141-146.
837   Taylor, N. M. I., I. Manolaridis, S. M. Jackson, J. Kowal, H. Stahlberg and K. P. Locher (2017).
838   Structure of the human multidrug transporter ABCG2. *Nature* 546(7659): 504-509.
839   Tsui, L. C., M. Buchwald, D. Barker, J. C. Braman, R. Knowlton, J. W. Schumm, H. Eiberg, J.
840   Mohr, D. Kennedy, N. Plavsic and et al. (1985). Cystic fibrosis locus defined by a genetically
841   linked polymorphic DNA marker. *Science* 230(4729): 1054-1057.
842   Van Goor, F., S. Hadida, P. D. Grootenhuis, B. Burton, D. Cao, T. Neuberger, A. Turnbull, A.
843   Singh, J. Joubran, A. Hazlewood, J. Zhou, J. McCartney, V. Arumugam, C. Decker, J. Yang, C.
844   Young, E. R. Olson, J. J. Wine, R. A. Frizzell, M. Ashlock and P. Negulescu (2009). Rescue of
845   CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S*
846   *A* 106(44): 18825-18830.
847   Van Goor, F., H. Yu, B. Burton and B. J. Hoffman (2014). Effect of ivacaftor on CFTR forms with
848   missense mutations associated with defects in protein processing or function. *J Cyst Fibros*
849   13(1): 29-36.
850   Vergani, P., S. W. Lockless, A. C. Nairn and D. C. Gadsby (2005). CFTR channel opening by
851   ATP-driven tight dimerization of its nucleotide-binding domains. *Nature* 433(7028): 876-880.
852   Verlet, L. (1967). Computer 'Experiments' on Classical Fluids. I. Thermodynamical Properties of
853   Lennard-Jones Molecules. *Physical Review* 159: 98-103.
854   Wang, W., Y. El Hiani, H. N. Rubaiy and P. Linsdell (2014). Relative contribution of different
855   transmembrane segments to the CFTR chloride channel pore. *Pflugers Arch* 466(3): 477-490.
856   Winter, M. C. and M. J. Welsh (1997). Stimulation of CFTR activity by its phosphorylated R
857   domain. *Nature* 389(6648): 294-296.
858   Yang, Z., C. Wang, Q. Zhou, J. An, E. Hildebrandt, L. A. Aleksandrov, J. C. Kappes, L. J.
859   DeLucas, J. R. Riordan, I. L. Urbatsch, J. F. Hunt and C. G. Brouillette (2014). Membrane
860   protein stability can be compromised by detergent interactions with the extramembranous
861   soluble domains. *Protein Sci* 23(6): 769-789.
862   Zhang, Z. and J. Chen (2016). Atomic Structure of the Cystic Fibrosis Transmembrane
863   Conductance Regulator. *Cell* 167(6): 1586-1597.e1589.
864   Zhang, Z., F. Liu and J. Chen (2017). Conformational Changes of CFTR upon Phosphorylation
865   and ATP Binding. *Cell* 170(3): 483-491.e488.
866   Zhang, Z., F. Liu and J. Chen (2018). Molecular structure of the ATP-bound, phosphorylated
867   human CFTR. *Proc Natl Acad Sci U S A*.
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869 **Figure Legends:**

870
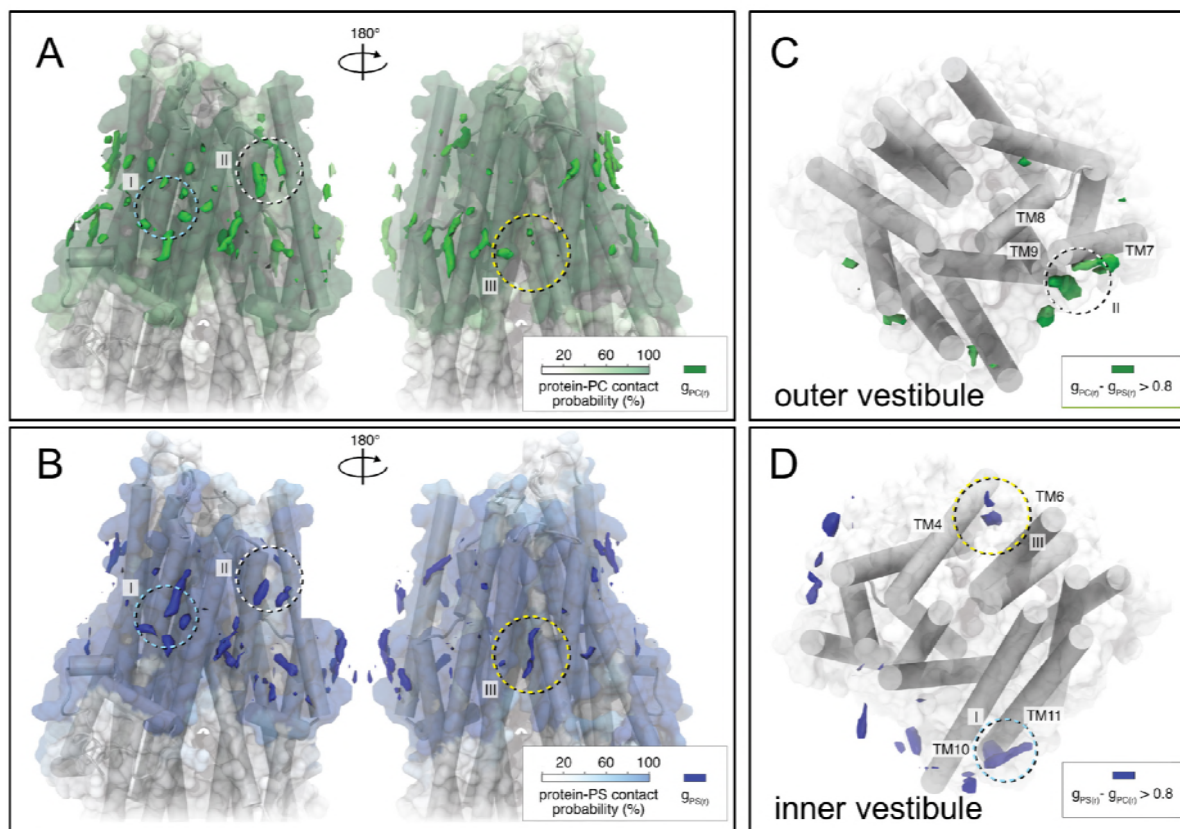
871 **Figure 1:** Phospholipids interact with CFTR. (a and b) Molecular renderings of hCFTR models
872 viewed from a side orientation. The spatial distribution function of lipid heavy atoms over all
873 MD simulations is depicted as colored volumes for (a) POPC ($g_{PC(r)}$, green) and (b) POPS ($g_{PS(r)}$,
874 blue). Each map is normalized by the average number density of the lipids in a neat bilayer and
875 only regions with density > 160% of this value are shown. A transparent surface is colored by the
876 percentage of simulation data where heavy-atom contacts are made between protein and lipids
877 atoms. A difference map shows regions with increased binding for (c) POPC ($g_{PC(r)}$ - $g_{PS(r)}$ > 0.8)
878 in the outer leaflet and (d) POPS ($g_{PS(r)}$ - $g_{PC(r)}$ > 0.8) in the inner leaflet. Labels I, II, and III
879 highlight regions where differences can be observed between these spatial distribution functions.
880 Preferential binding of POPC was found at the external cleft between TM7 and TM9 (region II).
881 Preferential binding of POPS was found at the internal clefts between TM10 and TM11 (region
882 I) and between TM4 and TM6 (region III).

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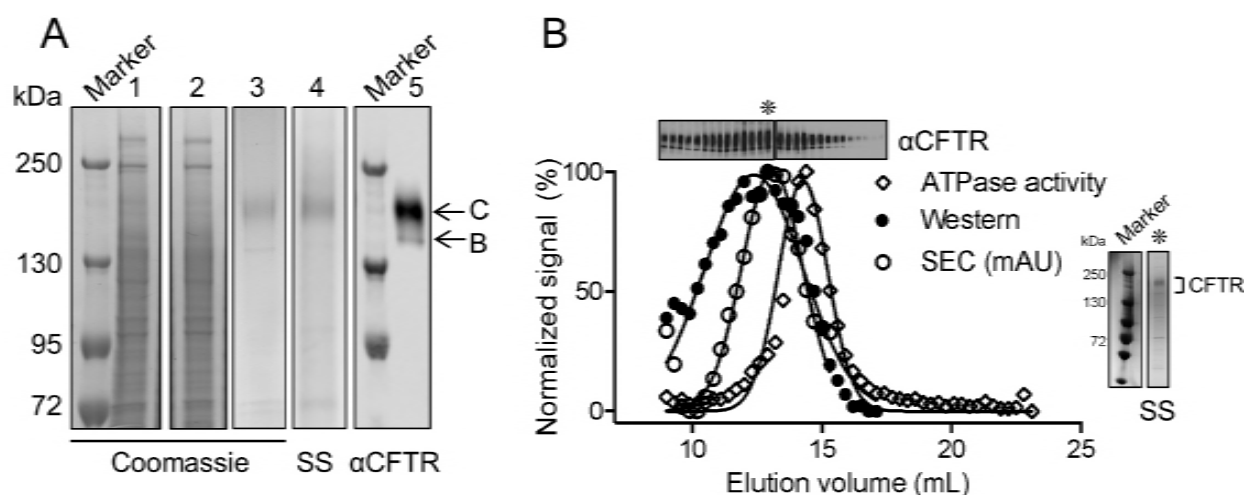
Figure 2: Functional CFTR can be purified using amphipol. (a) Protein gels stained by Coomassie blue of lysate solubilized by amphipol (1), unbound fraction (2) and eluate from anti-FLAG® M2 magnetic beads (3). Eluate from anti-FLAG® M2 magnetic beads was also stained by silver stain (SS) (4) and immunoblotted (5) showing that CFTR was effectively purified as a relatively pure population. CFTR appeared as two bands that include a band at approximately 170-180 kDa that represents the mature, complex glycosylated form of the protein (band C) and a band at approximately 140-150 kDa that represents the immature, core glycosylated form of the protein (band B). (b) Overlay of normalized signal of CFTR ATPase activity (open diamonds) and CFTR protein detected by immunoblot (closed circles) to SEC trace on a Superose 6 10/300 GL (GE Healthcare) (open circles) showing that the SEC peak at (asterisk) corresponds to CFTR protein separation and the shoulder of that peak corresponds to functional CFTR. SS of the SEC peak (asterisk) shows the purified CFTR protein is relatively pure.
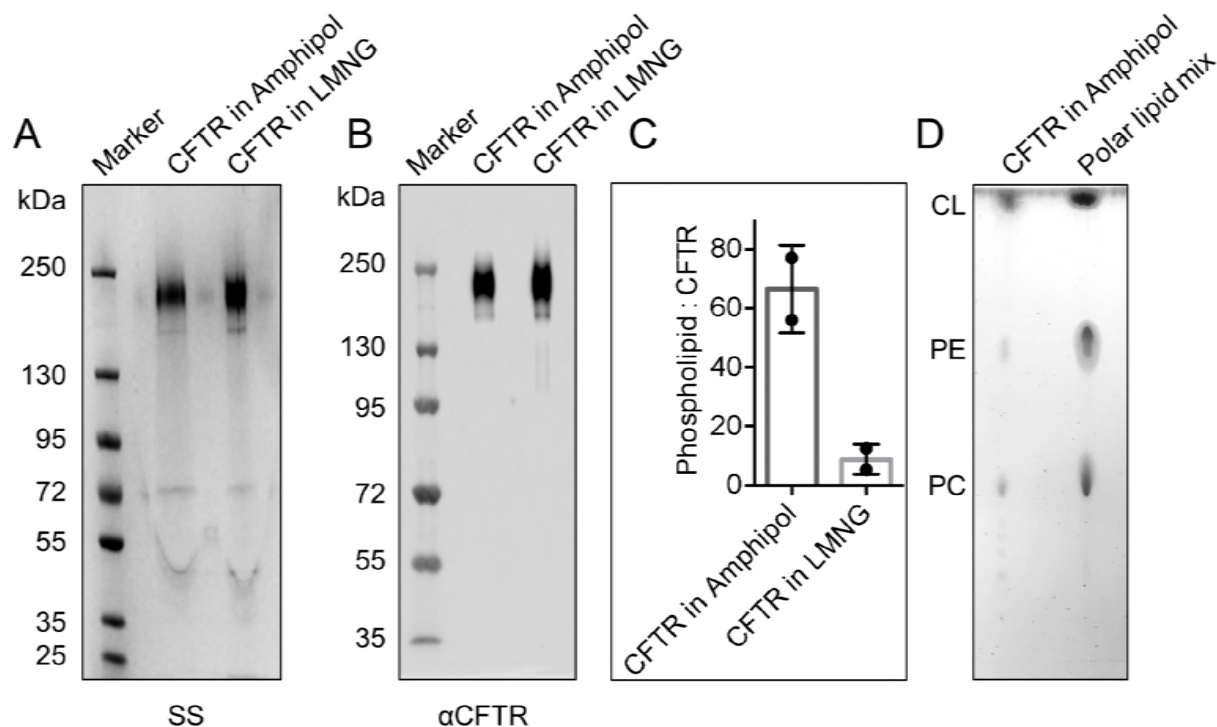
Figure 3: Yield of CFTR is similar for amphipol and LMNG purifications and amphipol purified CFTR retains an annulus of phospholipids and cholesterol. (a) SS and (b) immunoblot of purified CFTR from amphipol and LMNG purifications showing that both purifications yielded high purity of CFTR protein at similar yields. (c) Phospholipid analysis showing that purified CFTR solubilized in amphipol contained more phospholipids (56 to 76 phospholipids per CFTR molecule) than CFTR solubilized in LMNG (5 to 12 phospholipids per CFTR molecule) after normalizing to protein amounts. The data is presented as a range of n = 2 technical replicates. (d) Lipid TLC showing that extracted lipids from purified CFTR in amphipol include cholesterol, PE and PC as confirmed by similar migration of known lipids from polar lipid mix standard. These lipids were not present in amphipol by itself.

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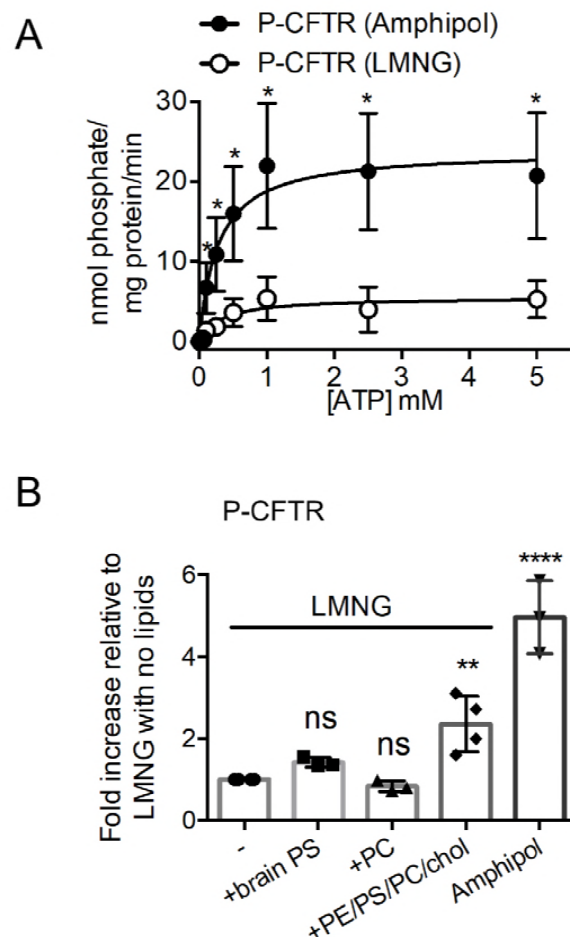
911 **Figure 4:** Specific ATPase activity of phosphorylated CFTR is higher in amphipol preparation
912 than in detergent preparation and this difference is related to lipid associations of CFTR. (a)
913 ATPase activity of P-CFTR in amphipol (closed circles) and in LMNG detergent (open circles)
914 across ATP concentrations (mM) expressed as nmol phosphate/mg protein/min. The data is fitted
915 to Michaelis-Menten curves and is presented as mean $\pm$ SD (n = 3 biological replicates and n = 6
916 technical replicates). *P = 0.0026 at 0.1 mM ATP; *P = 0.0008 at 0.25 mM ATP; *P = 0.0006 at
917 0.5 mM ATP; *P = 0.0006 at 1.0 mM ATP; *P = 0.0003 at 2.5 mM ATP; *P = 0.0009 at 5.0
918 mM ATP; multiple t-tests. (b) Fold change of ATPase activity at 0.5 mM ATP relative to protein
919 amounts of P-CFTR in LMNG detergent pre-treated with PC, brain PS or a mix of lipids
920 PE/brain PS/egg PC/cholesterol (PE/PS/PC/chol) at 5:2:1:1 weight ratio for 1 h relative to P-
921 CFTR in LMNG detergent. ATPase activity at 0.5 mM ATP of P-CFTR in amphipol was also
922 compared as a reference. The data is presented as mean $\pm$ SD (n = 4 biological replicates, n = 4
923 technical replicates for P-CFTR in LMNG and P-CFTR in LMNG pre-treated with
924 PE/PS/PC/chol; n = 3 biological replicates, n = 3 technical replicates for P-CFTR in LMNG pre-
925 treated with brain PS, PC and P-CFTR in amphipol). ns, not significant; **P = 0.0012; ****P <

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926 0.0001; One-way ANOVA with Dunnett's multiple comparisons test comparing each condition
927 to P-CFTR in LMNG detergent.
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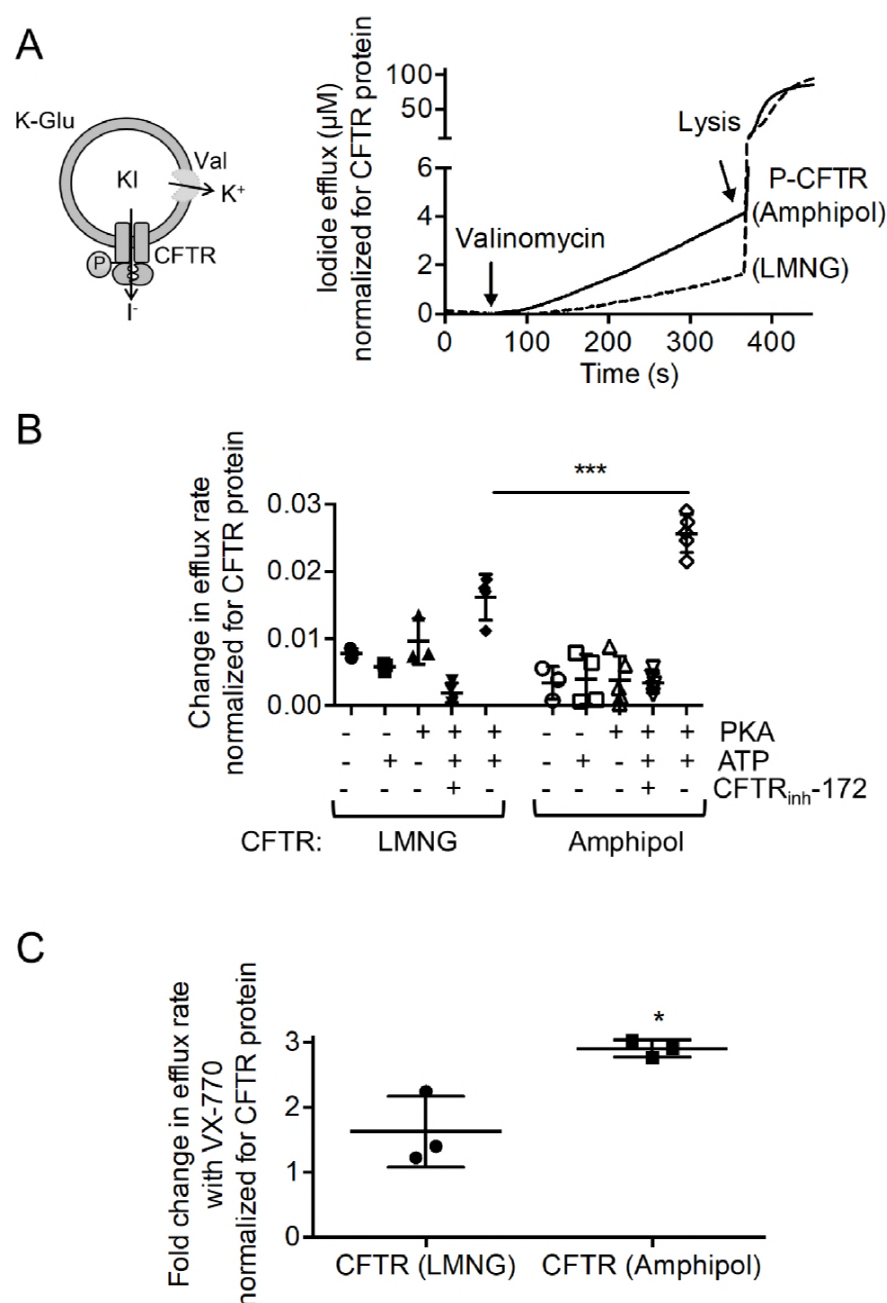
929
930 **Figure 5:** Amphipol protects channel-active conformation of CFTR. (a) Cartoon showing that
931 proteoliposomes are loaded with potassium iodide (KI) on the inside with an equal concentration
932 of potassium glutamate (K-Glu) on the outside. Valinomycin (Val), a potassium ionophore,
933 provides a counterion pathway thereby facilitating iodide (I⁻) electrodiffusion via activated
934 CFTR. The amount of I⁻ effluxed can be detected by an iodide-selective electrode. Representative
935 iodide efflux traces showing iodide effluxed (µM) normalized for CFTR protein (ng)

27

936   reconstituted in liposomes. Iodide efflux traces of proteoliposomes reconstituted with P-CFTR
937   pre-treated with Mg-ATP in amphipol and of proteoliposomes reconstituted with P-CFTR pre-
938   treated with Mg-ATP in LMNG that were both treated with valinomycin and then lysed with
939   Triton. (b) Change in iodide efflux rate normalized to CFTR amounts (ng) before and after
940   valinomycin treatment of reconstituted P-CFTR in LMNG detergent and in amphipol with Mg-
941   ATP along with negative controls: unphosphorylated CFTR with and without Mg-ATP, P-CFTR
942   without Mg-ATP and P-CFTR with Mg-ATP treated with $CFTR_{inh}$-172. Reconstituted P-CFTR
943   in LMNG detergent and amphipol with Mg-ATP resulted in significant increases in change of
944   slope of iodide efflux compared to negative controls. Reconstituted P-CFTR in amphipol with
945   Mg-ATP resulted in a significantly higher change in slope of iodide efflux compared to
946   reconstituted P-CFTR in LMNG detergent with Mg-ATP. The data is presented as mean ± SD (n
947   = 3 biological replicates, n > 3 technical replicates). ***P < 0.0001; One-way ANOVA with
948   Tukey's multiple comparisons test. (c) Fold change in iodide efflux rate normalized for CFTR
949   protein with 1 μM VX-770 compared to vehicle shows that the potentiation effect of VX-770
950   was significantly higher in proteoliposomes reconstituted with P-CFTR in amphipol compared to
951   proteoliposomes reconstituted with P-CFTR in LMNG detergent micelles. The data is presented
952   as mean ± SD (n = 3 biological replicates, n = 3 technical replicates). *P = 0.0355; paired t-test.
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955 **Supplementary Figures and Tables Legends:**

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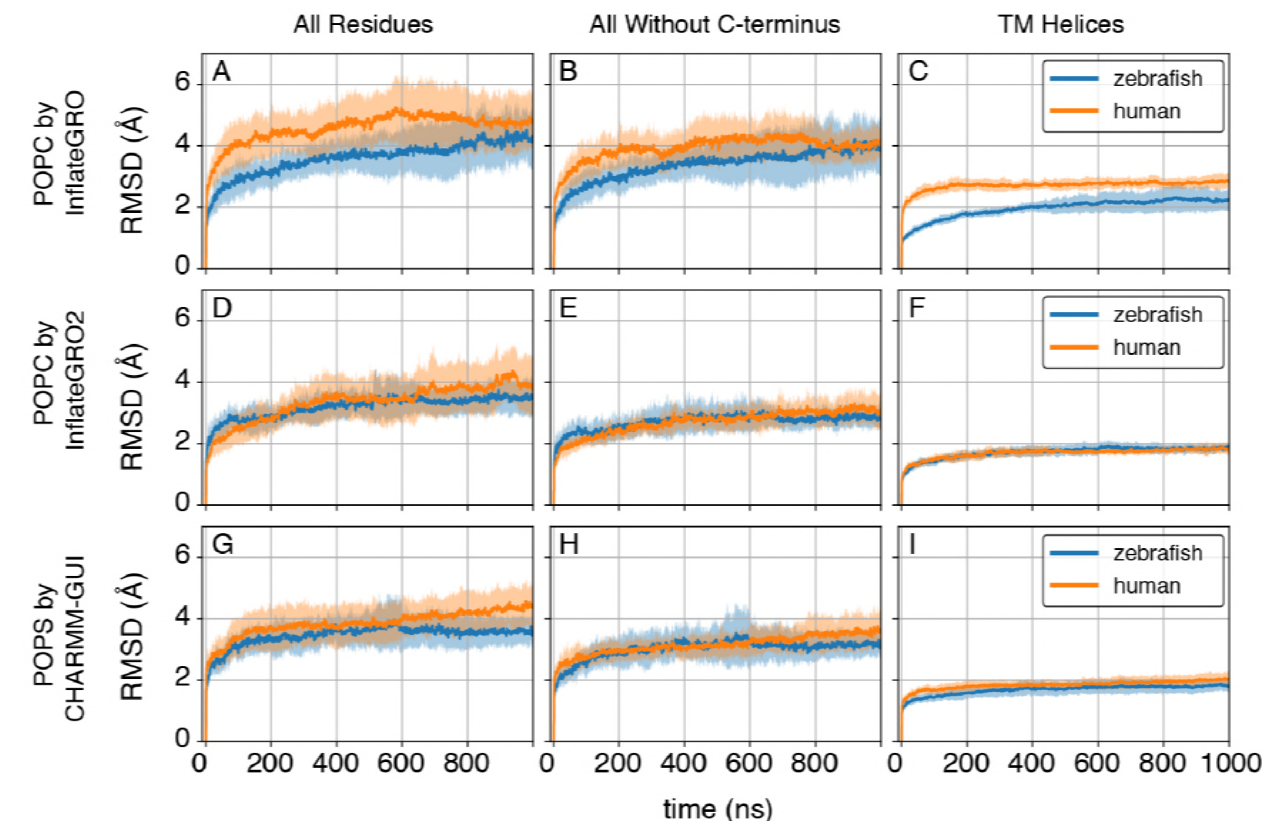
958 **Supplementary Figure 1:** Cross-validation of hCFTR and zCFTR models. RMSD of Cα atoms
959 versus time, averaged over simulation replicas. Regions within one standard deviation from the
960 mean RMSD are shaded. Each row identifies a bilayer type and embedding method; (a-b-c)
961 POPC-embedded using InflateGRO, (d-e-f) POPC-embedded using InflateGRO2, (g-h-i) POPS-
962 embedded using CHARMM-GUI. The three columns show RMSD values with respect to the
963 initial structures for different subsets of protein Cα atoms for hCFTR (orange) and zCFTR (blue)
964 models. Both zCFTR and hCFTR RMSD values are within one standard deviation of the mean
965 for systems POPC-embedded using InflateGRO2 and POPS-embedded using CHARMM-GUI.
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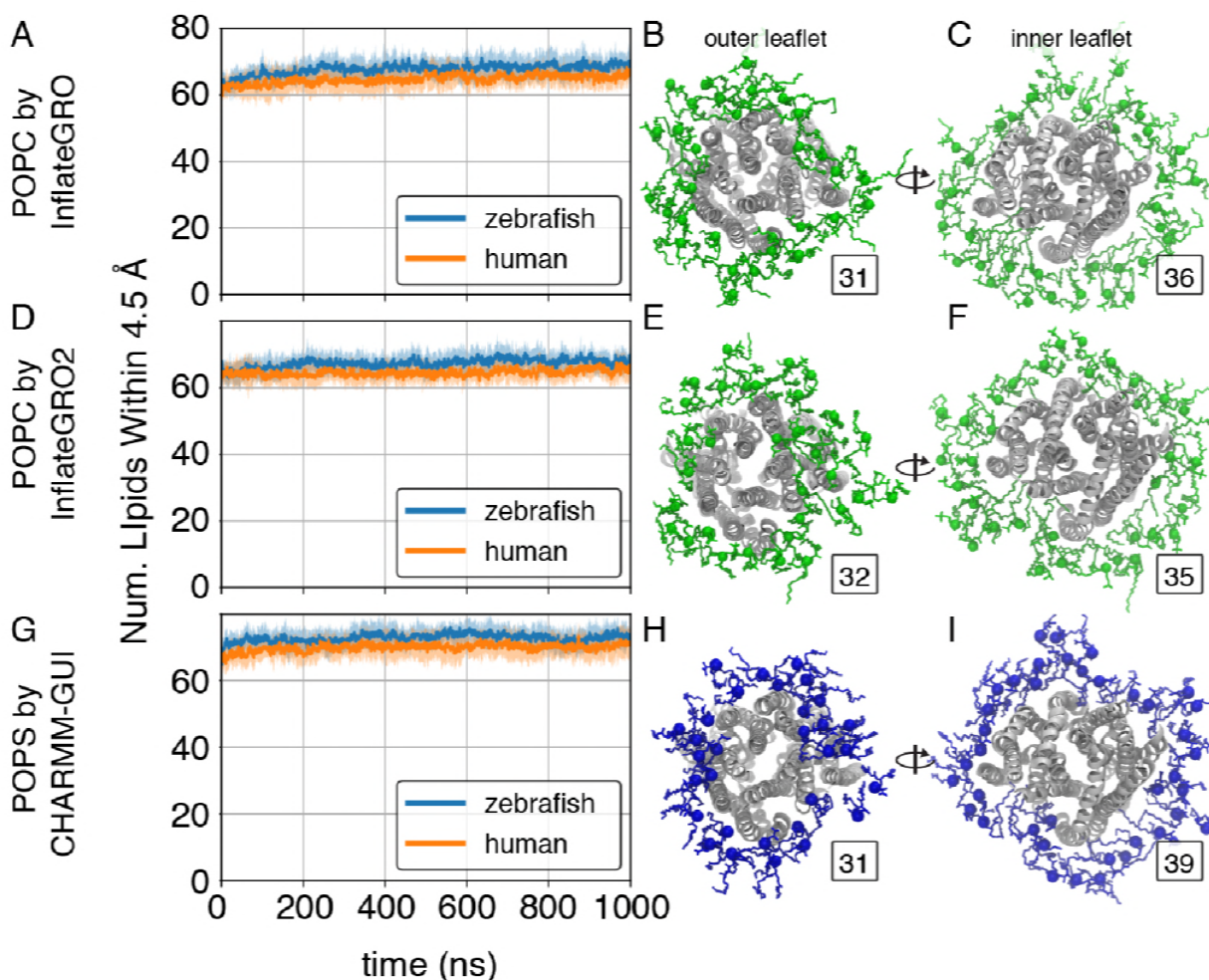
968   **Supplementary Figure 2:** Protein-lipid interactions in hCFTR and zCFTR models. Average
969   number of lipids interacting with protein heavy atoms versus time. All lipid molecules with a
970   heavy-atom pair distance less 4.5 Å are counted. Regions within one standard deviation from the
971   mean are shaded. Each row corresponds to a bilayer type and embedding method; (a) POPC-
972   embedded using InflateGRO, (d) POPC-embedded using InflateGRO2, (g) POPS-embedded
973   using CHARMM-GUI. In these panels, average lipid counts from simulations conducted with the
974   hCFTR (orange) and zCFTR (blue) models are shown. For each bilayer type and embedding
975   method, molecular renderings of individual snapshots depicting all lipid molecules within 4.5 Å
976   of protein heavy-atoms are shown from the extracellular and intracellular orientation (b-c, e-f, h-
977   i). POPC (green) and POPS (blue) are shown in licorice representation with phosphorous
978   headgroup atoms depicted as spheres. The total number of lipids in the outer and inner leaflet
979   within 4.5 Å are shown as an inset number.

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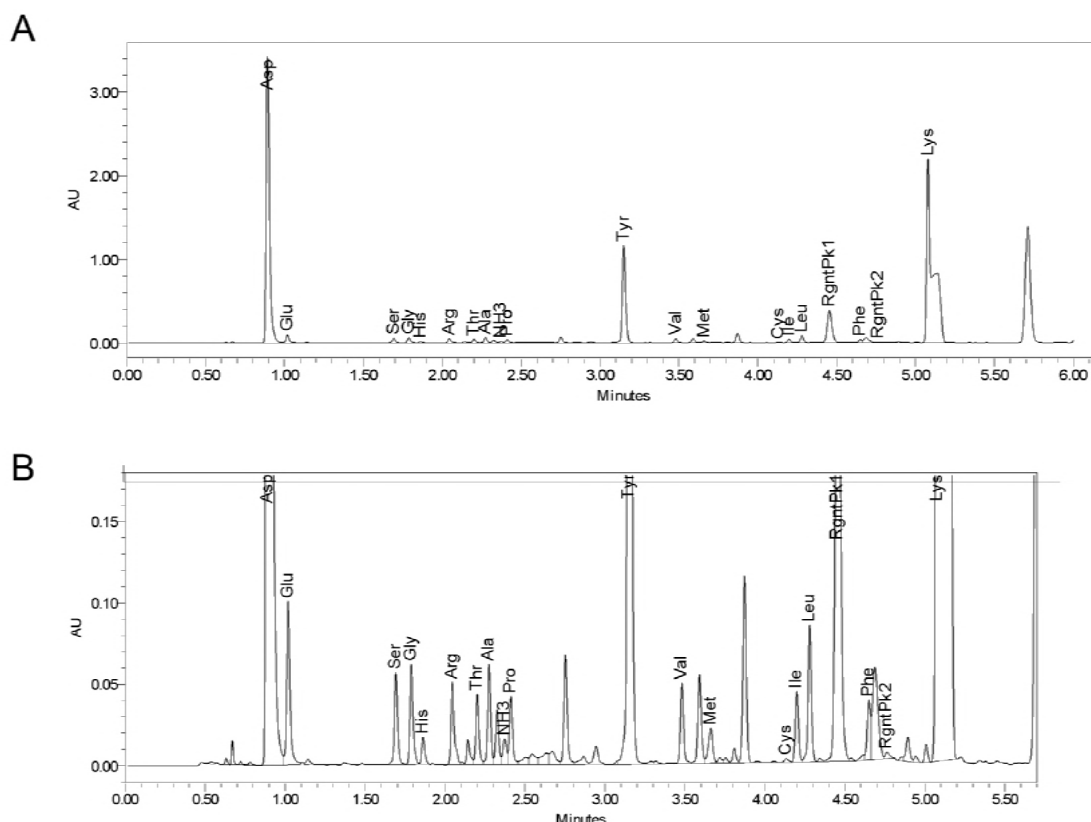
Supplementary Figure 3: Results of amino acid analysis of CFTR standard. (a) Chromatogram of all the amino acids present in CFTR standard. (b) Scaled chromatogram to magnify the smaller peaks.
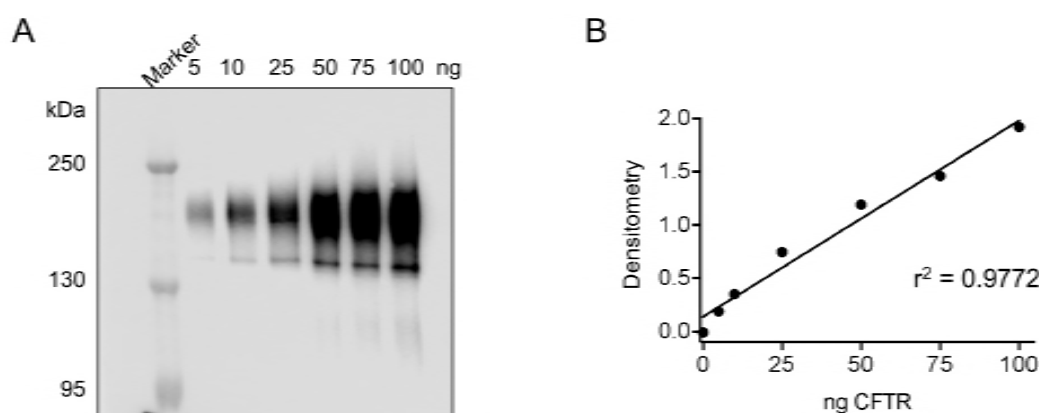
Supplementary Figure 4: Representative immunoblot and standard curve applied for CFTR quantification. (a) Immunoblot of CFTR standard at increasing amounts (nanograms). (b) Standard curve of (a) that is used for interpolation of unknown purified protein.

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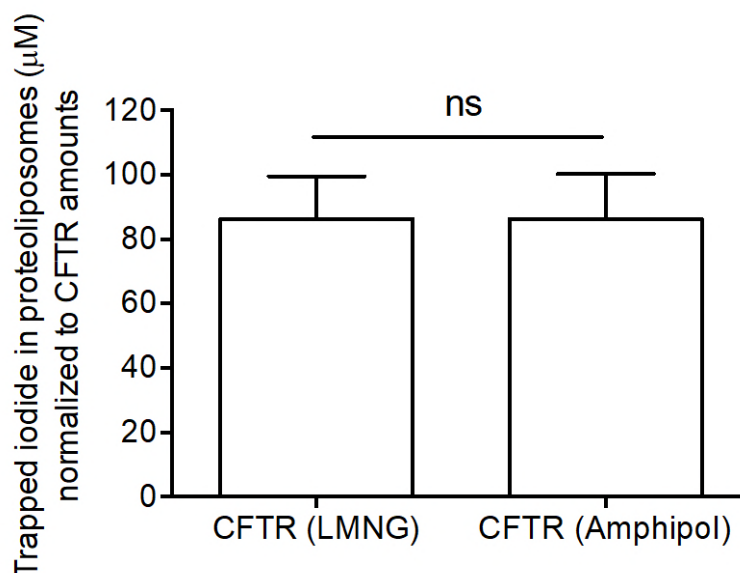
992 **Supplementary Figure 5:** Trapped iodide is similar for the two reconstitutions, reconstitution of
993 LMNG purified CFTR and amphipol purified CFTR. Bars correspond to iodide measurements
994 before valinomycin addition and after detergent lysis normalized to CFTR amounts. The data is
995 presented as mean ± SD (n > 3 biological replicates and n > 3 technical replicates). $P = 0.9974$;
996 paired t-test.

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1002 **Supplementary Table 1:** Summary table of amino acid analysis results of CFTR standard. Table
1003 shows the amino acid (3-letter code), retention time (RT) in minutes of when the amino acid
1004 shows up, the area under the peak and the corresponding amount of the amino acid in picomoles
1005 (pmoles) in a 100 µl purified CFTR standard.

Amino acid	RT	Area	Amount (pmoles)
Asp	0.892	5099166	66398.567
Glu	1.019	147780	2021.338
Ser	1.692	79642	1035.873
Gly	1.788	86381	1139.691
His	1.862	22241	289.816
Arg	2.045	74065	947.141
Thr	2.201	59033	747.984
Ala	2.275	83745	1015.112
Pro	2.412	59190	713.162
Tyr	3.150	1680122	19755.248
Val	3.480	70278	882.894
Met	3.661	41282	496.935
Cys	4.132	4485	37.648
Ile	4.198	60431	686.995
Leu	4.279	124398	1497.008
Phe	4.650	53427	618.165
Lys	5.079	5901164	38071.770

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1007

1008 **Supplementary Table 2:** Variables for determining concentration of CFTR standard.

Variable	Value
MW Ala	89.0935 – 18 (for peptide bond) = 71 g/mol or pg/pmole
weight Ala	1015.12 pmoles (from chromatogram) 1015.12 pmoles x 71.0935 pg/pmole = 72065 pg
number of Ala in CFTR	83

1009

A

| kDa | Marker | 1 | 2 | 3 | 4 | Marker | 5 |

Coomassie   SS   αCFTR

C
B

B

* αCFTR

◇ ATPase activity
● Western
○ SEC (mAU)

Normalized signal (%)

Elution volume (mL)

kDa
250
130
72

Marker   *

] CFTR

SS

SS   αCFTR

A POPC by InflateGRO

B outer leaflet
C inner leaflet

D POPC by InflateGRO2

E
F

G POPS by CHARMM-GUI

H
I

Num. Lipids Within 4.5 Å

time (ns)

zebrafish
human

A

Marker 5 10 25 50 75 100 ng

kDa

250

130

95

B

r² = 0.9772