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A Topological Switch in CFTR Modulates Channel Activity and Sensitivity to Unfolding

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) anion channel is essential to maintain fluid homeostasis in key organs. Functional impairment of CFTR due to mutations in the *cftr* gene leads to Cystic Fibrosis (CF). Here we show that the first nucleotide-binding domain (NBD1) of CFTR can spontaneously adopt an alternative conformation that departs from the canonical NBD fold previously observed for CFTR and related transporters. Crystallography studies reveal that this conformation involves a topological reorganization of NBD1. Single-molecule fluorescence resonance energy transfer microscopy shows that the equilibrium between the conformations is regulated by ATP binding. However, under destabilizing conditions, such as the prominent disease-causing mutation F508del, this conformational flexibility enables unfolding of the β -subdomain. Our data indicate that in wild-type CFTR this conformational transition of NBD1 regulates channel function, but, in the presence of the F508del mutation, it allows domain misfolding and subsequent protein degradation. Our work provides a framework to design conformation-specific therapeutics to prevent noxious transitions.

Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel that lead to impaired epithelial ion transport with dramatic consequences in multiple organs, particularly the lungs^{1,2}. CFTR belongs to the family of ATP-binding cassette (ABC) transporters, with a pseudo-dimeric architecture comprising 12 transmembrane helices connected to two nucleotide-binding domains (NBDs)³, as illustrated in Fig. 1A. In most CF patients, the disease is caused by deletion of the phenylalanine residue at position 508 (F508del) in NBD1, which leads to absence of the channel from the plasma membrane⁴. On the one hand, the mutation is known to perturb assembly of this multidomain protein⁵⁻⁸. On the other hand, studies have shown that F508del compromises CFTR by decreasing the thermal stability of NBD1 and subsequently of the entire protein^{6,9}. At physiological temperatures, the mutant channel is rapidly recognized as misfolded and is degraded by the cellular quality control system^{10,11} abolishing maturation and cell-surface expression. *In vitro* studies demonstrate that F508del has little effect on the NBD1 structure per se, but rather alters stability and dynamics of the domain^{6,12,13}. Yet, the molecular events that lead to NBD1 misfolding remain poorly understood. We can gain molecular insight from stabilizing mutations that compensate for the effects of F508del and lead to recovery of membrane expression and channel function¹⁴⁻¹⁶. For example, by studying CFTR from different species, it has been shown that replacing residues S492, A534 and I539 in the human channel by their avian counterparts largely suppresses the deleterious effects of F508del¹⁵. Another striking example where a sequence change rescues the mutant protein involves the regulatory insertion (RI), a 32-residue long segment found in NBD1 of all CFTR orthologs yet absent in related ABC transporters. The role of the RI has remained obscure. It is not resolved in crystal structures of NBD1^{12,17,18} nor cryo-EM structures^{3,19,20} of full-length CFTR and has been described as intrinsically disordered¹³. Remarkably, removal of the RI increases the stability of isolated NBD1 and, in the context of F508del-CFTR, largely recovers maturation, cell surface expression, activity and interdomain assembly of the mutant channel²¹. Another key factor is binding of ATP to NBD1, which is required for correct conformational maturation of CFTR²² and counterbalances destabilization by F508del to some degree^{7,23}. While the Walker B motif in NBD1 lacks a catalytic base¹⁷, making this ATP-binding site non-hydrolytic²⁴, ATP binding is proposed to drive opening of phosphorylated CFTR channels by promoting the formation of a NBD1:NBD2 dimer²⁵. We set out to examine how factors such as ATP, stabilizing mutations or removal of the RI impact the conformational landscape of NBD1 to understand the molecular origins of CF and inform therapeutic development.

Using single molecule Förster resonance energy transfer (smFRET) and nanobody-enabled structural studies, we captured and characterized a previously unknown conformation that exhibits significant structural rearrangements compared to the 'canonical' state of NBD1 originally described. We show that this non-canonical conformation confers enhanced channel activity on wild-type CFTR. Using smFRET and hydrogen deuterium exchange coupled to mass spectrometry, we characterized the conformational equilibrium between the two states of NBD1. We identify the molecular determinants of the equilibrium and show that the conformational transitions also favour local unfolding of NBD1 in the presence of the F508del mutation, elucidating the conformational pathway involved in the pathogenesis of CF.

70 Results

71 *The Regulatory Insertion Adopts Distinct Conformations*

72 We characterized the conformational landscape of the RI using smFRET which monitors the distance between
73 individual donor and acceptor dyes covalently attached to engineered cysteines in purified NBD1 (Extended
74 Data Figure 1).

75 To overcome the poor expression and stability of NBD1 from wild-type human CFTR, we used the 2PT-NBD1
76 variant containing three stabilizing mutations found in avian CFTR (S492P, A534P and I539T)¹⁵. Guided by
77 previous studies of a functional full-length cysteine-less CFTR variant^{26,27}, we replaced endogenous cysteines in
78 2PT-NBD1. Subsequently, for each FRET reporter, we introduced a pair of cysteines at desired locations and
79 labelled the purified variants with ATTO488 and Alexa647 as donor and acceptor fluorophores, respectively.

80 To monitor movements of the RI, we labelled position 426 within the RI (residues 405-436, red dashed line in
81 Fig. 1B) and residue 519 within a structured α -helix. The FRET efficiency between this 426-519 pair showed a
82 broad distribution of FRET states with a major population centred around $E_{FRET} = 0.4$ and two minor populations
83 at $E_{FRET} = 0.7$ and $E_{FRET} = 0.9$ when measured at room temperature (22 °C) in a buffer containing 2 mM ATP
84 (Fig. 1C). In the absence of ATP, we observed a dramatic change in the conformational profile as the entire
85 population was shifted to E_{FRET} values centred around 0.7 and 0.9 (Fig. 1D). To assess whether conformational
86 transitions occur on the submillisecond timescale, we generated two-dimensional (2D) plots of donor lifetime
87 versus FRET efficiency. The relationship between donor lifetime and FRET efficiency depends on the kinetics of
88 the conformational changes relative to the observation time²⁸. Specifically, if the signals fall on the *static FRET*
89 *line* (red line in Fig. 1E), there is no conformational exchange during the passage of the protein through the
90 confocal volume of the smFRET microscope (~1–5 ms), indicating that the protein exists in one conformation at
91 a time. By contrast, if the signals fall above the static FRET line structural changes occur, indicating dynamics on
92 the submillisecond timescale. For the 426-519 reporter pair, we observed that the population at $E_{FRET} = 0.4$ falls
93 on the static FRET line and thus is long-lived while the populations at 0.7 and 0.9 fall off the static FRET line and
94 appear to be dynamic (Fig. 1E-F). These two states can be better separated using 2D histograms of FRET
95 efficiency versus acceptor lifetime (Extended Data Figure 2).

96 To characterize the structural changes between the low and high FRET states, we screened a library of
97 nanobodies directed against 2PT-NBD1²⁹ to identify RI-conformation specific binders. We identified a nanobody
98 termed G11a which, upon binding to 2PT-NBD1, restricted the conformation of the RI to the high FRET state at
99 $E_{FRET} = 0.9$ (Fig. 1G). We observed similar high FRET (Figs. 1E-F) and acceptor lifetime (Extended Data Figure 2D)
100 values in the absence of the nanobody. This suggests that the nanobody does not induce new conformations of
101 2PT-NBD1, but rather stabilizes a protein state which is naturally present in the conformational ensemble.

102
103 To summarize, smFRET measurements demonstrated that the conformational space of the RI encompasses
104 several preferred states whose relative populations are regulated by ATP binding.

105 106 *Nanobody G11a Stabilizes an Alternative Conformation of NBD1*

107 We characterized the biophysical and biochemical properties of nanobody G11a and observed that it stabilizes
108 2PT-NBD1 upon binding, increasing its melting temperature by 10 °C in differential scanning fluorescence (DSF)
109 experiments (Extended Data Figure 3 and Supplementary Table 1). By contrast, G11a binding was lost with the
110 Δ RI-NBD1 variant where residues 405–436 have been removed, suggesting that G11a interacts directly with
111 part of the RI or binds a conformation adopted only in presence of the RI (Extended Data Figure 3B). We
112 determined the high-resolution crystal structure of the 2PT-NBD1:G11a complex (Fig. 2A) at 2.7 Å
113 (Supplementary Table 2) and found that the binding epitope includes several residues of the RI (residues 423–
114 436, red), as well as part of strand S4 (residues 476–487, blue). The conformation of NBD1 captured by G11a is
115 characterized by extensive structural changes in the β -subdomain resulting in an alternative topology
116 compared to the previously published crystal structures of NBD1 of CFTR^{12,17} and other ABC proteins (Fig. 2B-C).

117 All structures of human NBD1 published so far display a similar fold^{12,17,18}. In this canonical conformation
 118 (Fig. 2C), the β -subdomain is made up of three β -strands: the N-terminal S1 strand (389–404, purple), the S2
 119 strand (437–448, turquoise), and the S4 strand (473–487, blue). The RI (405–436, red dashed line) is
 120 unstructured and connects S1 and S2. In the G11a-bound structure, we observed an alternative topology
 121 (Fig. 2B) of this β -sheet where only the S2 strand is maintained in its original position. The S4 strand (blue) has
 122 unfolded to adopt a loop structure which no longer interacts with other secondary structures of NBD1. Part of
 123 the RI has become structured and interacts with the S2 strand in an antiparallel β -sheet (red) thus replacing the
 124 original S1 strand. Accordingly, the entire 389–422 segment including the canonical S1 strand, and the
 125 remainder of the RI (schematized as purple and red dashed lines in Fig. 2) is disordered in the crystal structure.
 126 As the S1 strand is replaced by a structured segment of the RI, we refer to this conformation as *β -strand-*
 127 *swapped* (β -SS).

128 Although this complex was crystallized in the presence of 2 mM ATP, we could only resolve the triphosphate of
 129 ATP with no clear density observed for the base. This observation is likely related to the fact that W401, which
 130 coordinates the adenosine base in the canonical conformation (Fig. 2C, purple), is disordered in the β -SS
 131 conformation.

132 In the β -SS conformation, residues 426 and 519, serving as anchor points for the FRET dyes, are separated by
 133 only 26.5 Å. Simulation of accessible dye positions using FRET-restrained positioning and screening (FPS)
 134 software³⁰ predicts an E_{FRET} value of 0.89 (Extended Data Figure 4 and Supplementary Table 3), which agrees
 135 remarkably well with the observed value of 0.9 (Fig. 1). We conclude that the high FRET state identified by
 136 smFRET corresponds to this G11a-bound β -SS conformation characterized by crystallography.

137 ***Functional wild type CFTR adopts the β -SS state***

138 The extensive and unexpected structural rearrangements observed in the β -SS conformation prompted us to
 139 question its biological relevance, especially in the context of the full-length protein. As G11a binding is not
 140 compatible with the canonical conformation of NBD1, we first established whether the nanobody binds a
 141 physiologically relevant form of CFTR, an indication that the β -SS state exists naturally. To this end, we
 142 performed flow cytometry experiments on permeabilized HEK293 cells stably expressing wild-type human CFTR
 143 incubated with either nanobody G11a, nanobody T2a (a nanobody that binds a conformationally invariant
 144 epitope on NBD1²⁹) or a negative control nanobody (that does not bind NBD1). We observed robust labelling of
 145 CFTR-expressing cells by nanobody G11a compared to the negative control (Fig. 3A), although signal intensity
 146 was reduced compared to T2a. This result indicates that the β -SS conformation is adopted by wild-type CFTR
 147 expressed in cells where it likely represents a subpopulation of the conformational ensemble. We also
 148 performed pull-down experiments with G11a on detergent solubilized membranes expressing wild-type CFTR.
 149 SDS-PAGE and Western blotting analysis demonstrated that G11a binds both immature and mature, fully
 150 glycosylated CFTR (Fig. 3B).

151 To investigate whether the β -SS conformation of NBD1 affects the function of wild-type CFTR, we studied the
 152 single-channel activity of wild-type CFTR. Figure 3D and Extended Data Figure 5 show representative recordings
 153 of an individual wild-type CFTR Cl⁻ channel in the absence and presence of saturating concentrations of G11a at
 154 37 °C. Nanobody G11a binding led to an altered gating pattern of CFTR, which increased the frequency but not
 155 the duration of channel openings, leading to a 50% increase in open probability (P_o ; a measure of the average
 156 fraction of time that a channel is open) (Fig. 3C). Analysis of prolonged recordings revealed that, in the
 157 presence of G11a, CFTR switched between two patterns of channel gating, one similar to wild-type and a
 158 second characterized by an elevated P_o (Extended Data Figure 5D-G). These data suggest that the alternative
 159 conformation represents an active channel state.

160 We conclude that nanobody G11a exclusively stabilizes a non-canonical conformation of the RI and X-ray
 161 crystallography shows that this conformation is associated with extensive structural changes in the β -
 162 subdomain of NBD1. This alternative, *β -strand-swapped* conformation is adopted by wild-type CFTR and
 163 constitutes a functional state of the channel.

164

165 ***The Conformational Equilibrium of NBD1 is Regulated by ATP***

166 To characterize the conformational equilibrium of NBD1, we performed smFRET on another distance reporter.
167 To follow the motions of the S4 strand, we labelled residues 479 and 519 which are separated by 41 Å in the
168 canonical conformation (C_{β} - C_{β} distance), versus 35 Å in the β -strand-swapped conformation (Fig. 4A-B).
169 Simulations of labelling these sites predict E_{FRET} values of 0.47 and 0.64, respectively (see Extended Data Figure
170 4B and Supplementary Table 3).

171 When we measured the smFRET signal of this pair (Fig. 4C), we observed two distinct states with a major
172 population centred on $E_{FRET} = 0.4$ in agreement with predictions for the canonical conformation and a smaller
173 population centred on 0.8 (larger than the value predicted from the crystal structure of the 2PT-NBD1:G11a
174 complex). This high FRET population increased sharply in the absence of ATP (Fig. 4D), confirming that it
175 corresponds to a non-canonical conformation. Furthermore, we observed that the data in the absence of ATP
176 lay off the static FRET, implying dynamic behaviour on the sub-millisecond timescale. Using photon distribution
177 analysis (PDA), we quantified the relative populations detected by FRET using 2 mM, 0.2 mM, and 0 mM ATP in
178 the buffer (Fig. 4E). We observed that, at 2 mM ATP, about 80% of the distribution sampled the canonical state
179 and 20% the putative β -SS conformation. The population of the canonical state was decreased to 58% and 25%
180 for 0.2 mM and 0 mM ATP, respectively, demonstrating that modulation by ATP is concentration dependent.

181 We then performed a kinetic experiment where, by using 0 mM ATP, the non-canonical state was initially
182 imposed, as evidenced by the 479-519 FRET signal at $E_{FRET} = 0.8$ (Fig. 4F). After 3000 s, we added ATP to a final
183 concentration of 2 mM and observed an immediate and stable change in the FRET signal to $E_{FRET} = 0.4$ indicating
184 transition to the canonical state. These data reveal that the non-canonical conformation is not an irreversible
185 misfolded end point of the conformation landscape, but an entirely reversible state.

186 Subsequently, we tested the effect of G11a binding on the 479-519 reporter pair using 2 mM ATP and detected
187 a shift of the population to $E_{FRET} = 0.8$ (Fig. 4G). We deduced that the high FRET state observed in the absence
188 of the nanobody matches the opening of the S4 strand in the β -SS state captured with the G11a-NBD1 crystal.
189 However, analysis of the FRET vs. donor lifetime 2D plot showed that this population lies on the static FRET line
190 (Fig. 4G), indicating that binding of G11a stabilizes the opening of the S4 and restricts its dynamics.

191 ***The RI Confers Structural Plasticity on the β -subdomain***

192 We performed the smFRET experiments with a Δ RI-2PT variant, where the regulatory insertion has been
193 removed (405-436, red in Fig. 4A-B), and observed a single population with $E_{FRET} = 0.4$, matching the expected
194 value for the canonical state (Fig. 4H).

195 To confirm that the RI increases plasticity of the S4 strand, we performed hydrogen deuterium exchange
196 coupled to mass spectrometry (HDX-MS), which identifies deuterium uptake of labile protons on backbone
197 amides to report solvent accessibility and conformational flexibility. In the presence of the RI, we observed a
198 rapid and marked exchange of backbone amide hydrogens of the peptide 475–490, containing the S4 strand
199 residues, indicative of increased structural flexibility (2PT-NBD1, blue; Extended Data Figure 6I). By contrast, for
200 the Δ RI-NBD1 variant, the uptake remained limited, even after 2 h of incubation (Δ RI-NBD1, black), thus
201 confirming that the RI enables flexibility of the S4 strand.

202 In the β -SS conformation, the N-terminal S1 strand is disordered and no longer interacts with the S2 and S4
203 strands of the β -subdomain (Fig. 2B). To probe the conformational equilibrium of the S1 strand, we used the
204 390-519 reporter pair and performed the same experiments as above (Extended Data Figure 6). We observed
205 two coexisting FRET states which can be reliably attributed to the canonical and β -SS conformations by using
206 either a Δ RI variant or the G11a nanobody, respectively. As observed with the 479-519 reporter pair, the
207 equilibrium is regulated by the presence of ATP and is reversible. Moreover, as the same conformational
208 profiles are observed with two different FRET pairs, we conclude that this represents the intrinsic dynamics of
209 the protein with little or no effect of the FRET labels.

210 HDX-MS also demonstrated that the dynamics of this N-terminal region of NBD1 is directly coupled to the
211 presence of the RI as we observed very limited exchange in the 392–399 peptide in Δ RI-NBD1 compared to

212 2PT-NBD1 (Extended Data Figure 6J). These findings are in agreement with previous HDX studies which
 213 identified the RI, the N-terminus and the S4 strand as fast-exchanging regions³¹.

214 We conclude that two conformations of the S4 and S1 segments can be distinguished by smFRET. One
 215 corresponds to the canonical conformation where both S1 and S4 segments are engaged in the canonical
 216 β -sheet. The other is promoted by depletion of ATP and matches predictions for the β -SS conformation
 217 observed by crystallography with an unstructured S4 and a disordered S1 segment.

218 ***F508del Modulates the Conformational Equilibrium of NBD1***

219 We then investigated the effects of the F508del mutation and other destabilizing factors on the conformational
 220 equilibrium of NBD1 (Fig. 5A illustrates regions of interest). Using the 479-519 reporter as a proxy, we observed
 221 that removing F508 did not affect the equilibrium at 22 °C (Fig. 5B and Supplementary Table 4). It is known that
 222 the effects of F508del are strongly temperature-dependent as demonstrated by robust recovery of CFTR
 223 expression and function when cells expressing F508del-CFTR are grown below 30 °C³². To test whether
 224 increasing the temperature altered the relative equilibrium populations, we performed smFRET measurements
 225 at 37 °C and observed a decrease in the canonical state of 2PT-479-519 when compared to the same construct
 226 at 22 °C (Fig. 5B), indicating that the conformational equilibrium is temperature-dependent.

227 When we probed the conformational equilibrium at 37 °C, we observed that removal of F508del decreased the
 228 population of the canonical state (Fig. 5B), indicating that the CF-causing mutation modulates the equilibrium
 229 in favour of a non-canonical state at physiological temperatures.

230 The 2PT-NBD1 construct used here contains 3 stabilizing substitutions: S492P, A534P and I539T which reduce
 231 the impact of F508del on the maturation of the full-length protein¹⁵ (Fig. 5A, green). In human NBD1, Ser492 is
 232 a critical node in the allosteric network propagating thermal fluctuations between the β -subdomain and the
 233 F508 loop region³³. To better understand the consequences of deleting F508, we analysed the impact of
 234 F508del on the PT-NBD1 construct (carrying only two stabilizing mutations: A534P and I539T), bearing S492 as
 235 in wild-type human CFTR. Using the 479-519 reporter, we observed a modest decrease in the population of the
 236 canonical state from 80% (2PT-NBD1) to 76% (PT-NBD1) at room temperature but a stronger decrease at 37 °C,
 237 from 69% to 58% (Fig. 5B). Finally, when combining the destabilizing factors to probe the F508del-PT-NBD1
 238 variant at 37 °C, less than 40% of the population remained in the canonical state, despite the presence of 2 mM
 239 ATP (Fig. 5C). Extended Data Figure 7 details how each individual parameter gradually influences the
 240 conformational equilibrium.

241 We conclude that conditions that reduce the effects of F508del on the full-length CFTR protein (i.e. low
 242 temperature or stabilizing mutations) favour the canonical state in our smFRET experiments. By contrast,
 243 deletion of F508 decreases the canonical population, but only at permissive temperatures (i.e. 37 °C).

244 ***β -subdomain Unfolding Occurs in Non-Canonical States of NBD1***

245 We surmised that the conformational plasticity of the RI and the β -subdomain plays a key role in the molecular
 246 origin of NBD1 misfolding. As we observed that the presence of the RI allows the dissociation of the S1 and S4
 247 strands from the canonical β -sheet, we tracked domain unfolding by monitoring conformational changes in the
 248 rest of the β -sheet, specifically strand S2. Motion of this region is relevant to pathogenesis as residue K447
 249 located in the S2 strand (Fig. 5D, turquoise) is SUMOylated in F508del-NBD1, but not in WT-NBD1²³, suggestive
 250 of increased exposure in mutant CFTR. We monitored the conformations of the S2 strand in smFRET by
 251 labelling residues 442 and 519. Importantly, residue 447 (and 442) is not involved in the structural
 252 rearrangements that occur between the canonical and β -SS states (Fig. 2B-C). At 22 °C, the smFRET signal of the
 253 442-519 reporter pair in the context of 2PT-NBD1 showed a major static population with E_{FRET} centred on 0.4
 254 (Fig. 5E) which agrees with the expected value based on the crystal structures (Supplementary Table 3). In
 255 addition to this low FRET state, we observed a minor diffuse population with E_{FRET} values above 0.5. This
 256 indicates the coexistence of conformations where residue 442 approaches residue 519, a condition that
 257 requires local unfolding of the S2 strand. The diffuse population disappeared entirely when the RI was absent

258 (Fig. 5F), suggesting that it is linked to non-canonical states. We repeated the experiment at 37 °C where 2PT-
259 NBD1 showed a marked increase in this high E_{FRET} population (Fig. 5G).

260 When we measured the FRET signal of the 442-519 reporter pair at 37 °C in the PT-NBD1 variant, the
261 proportion of high FRET population further increased compared to 2PT-NBD1 (Fig. 5H). Thus, the presence of
262 S492, which decreased the population of canonical conformers (Fig. 5B), also favoured local unfolding of the β -
263 sheet.

264 Finally, upon deletion of F508, the high FRET population increased again with fluorescence signals deviating
265 from the static FRET line (Fig. 5I), indicative of highly dynamic behaviour. The broad distribution of E_{FRET} values
266 from 0.5 to 1 is suggestive of a disordered state. By contrast, preincubation of the F508del-PT variant with
267 G11a completely prevented local unfolding of the β -subdomain at 37 °C as the entire population became static
268 with $E_{FRET} = 0.4$ (Fig. 5J). This matches the expected values for the β -SS conformations and agrees with the
269 thermal stabilization of NBD1 upon binding of G11a. This suggests that binding of G11a traps NBD1 in the β -SS
270 state and protects the β -subdomain from unfolding.

271 Discussion

272 Our data demonstrate that CFTR alternates between the state observed by cryo-EM where NBD1 adopts the
273 canonical conformation (Fig. 6A), and one (or more) non-canonical states where NBD1 adopts a conformation
274 which has not been observed previously (Fig. 6B). Transitions towards the latter require the RI, a unique
275 32-residue intrinsically disordered segment^{13,34}. A β -strand-swapped state was trapped with nanobody G11a
276 and resolved by crystallography, revealing dramatic topological changes whereby the RI rearranges into a β -
277 strand with the S4 loop disengaging from the β -sheet, and the 389–422 segment becoming unstructured
278 (Figs. 2B and 6B).

279 Electrophysiological measurements showed that the G11a-stabilized β -SS state leads to enhanced CFTR
280 channel activity. Although more experiments are required to confirm that such an effect is solely due to
281 conformational restriction, this conclusion is supported by experiments showing that partial deletion of the RI
282 decreased the P_o of the channel³⁵.

283 If the β -SS state leads to enhanced CFTR channel activity, this alternative conformation might have evolved for
284 functional reasons. Indeed, the high conservation of the RI in all CFTR orthologs and its absence in other ABC
285 transporters endows a functional role specific to CFTR. Our study suggests that the RI might act as a
286 conformational switch (i.e. between low and high channel activities).

287 Intrinsically disordered regions have been proposed to achieve their functions through various processes such
288 as folding upon binding of a partner, known as “coupled folding and binding”³⁶, or forming dynamic, “fuzzy”
289 complexes. Our data offer a new mechanism where function (here increased channel activity) is achieved by
290 conferring structure on a disordered segment that replaces a structure which, in turn, needs to unfold. The
291 changes occur within a single domain, without requiring an external molecular partner and the two states are
292 reversible and in equilibrium. Our smFRET experiments show that ATP binding regulates this equilibrium and,
293 although both states co-exist at saturating concentration of ATP (2 mM), transient and/or local changes in ATP
294 concentration³⁷ could modulate the conformational equilibrium (and thus the activity) of CFTR. This could
295 explain why tight ATP binding is lost in F508del-CFTR and recovered upon removal of the RI as the mutation
296 decreases the population of the canonical conformation where W401 coordinates the adenine base of ATP.

297 Disordered regions are known to be hotspots for post-translational modifications which often trigger their
298 functional activation. Of note, the RI segment contains a phosphorylation site at position S422 and its
299 modification could therefore serve as a regulator of the conformational switch.

300 While the conformational plasticity conferred by the RI appears to provide functional gain, it may also bring
301 about increased vulnerability to misfolding, especially in the presence of destabilizing mutations such as
302 F508del. Our smFRET measurements show that conditions which are known to compensate for the effects of
303 the pathogenic mutation *in cellulo* (such as stabilizing mutations, decreased temperatures, or removal of the

304 RI) are associated with stabilization of the canonical population of NBD1. This is consistent with the observation
305 that F508del does not lead to structural changes in NBD1 constructs bearing stabilizing mutations¹². By
306 contrast, we observed that introducing F508del in a permissive context (37 °C, non-stabilized variant) leads to
307 loss of the canonical conformer, which was correlated with unfolding of the β -subdomain, measured by motion
308 of the S2 strand (Fig. 5). This state is thus distinct from the β -SS conformation where the S2 strand is well
309 folded (Fig 5J).

310 We propose that, in a structurally compromised context, the reorganization of the β -subdomain which
311 characterizes the β -SS conformation facilitates local unfolding (or prevents correct folding during biogenesis).
312 Specifically, destabilizing factors such as F508del would ease the transition from the β -SS state to an unfolding
313 intermediate (Fig. 6C) where the entire β -subdomain is unfolded, in agreement with the observed motion of
314 residue 442 (Fig. 5I). As the unfolding intermediates accumulate, the canonical population is decreased
315 (Fig. 6A-B) and transition to a fully misfolded state promoted (Fig. 6C). The idea that F508del leads to unfolding
316 in the β -subdomain is supported by previous HDX studies of NBD1 featuring only one stabilizing mutation³¹. In
317 these studies, removal of F508 led to substantially different exchange rates only in the region around F508
318 itself, the N-terminus (the S1 strand) and the S2 strand³¹.

319 Importantly, as this unfolding pathway strictly requires the presence of the RI, this model explains why its
320 removal is protective against F508del²¹. Likewise, it provides an explanation for protection by S492P¹⁵ (and
321 possibly other revertant mutations) as the mutation allosterically limits the conformational transitions
322 proposed above.

323 Previous models have proposed unfolding pathways for F508del-NBD1 in the absence of the RI⁶, suggesting
324 that the destabilizing effects of the deletion affects the integrity of the canonical conformation of NBD1. Our
325 data suggest that, in a physiological context (where the RI is present), another pathway must be considered. In
326 this pathway, F508del primarily topples CFTR from a topologically modified conformation that was not
327 observed previously and relies on the RI.

328 The discovery of a new NBD1 conformation with pathophysiological implications changes our understanding of
329 CFTR biology. It might also open conformation-specific therapeutic approaches for F508del and possibly other
330 related CF-causing mutations. Our study sheds new light on how structured and unstructured regions of a
331 protein can interchange for functional reasons and might constitute a new mechanism to modulate protein
332 function that could extend to other biological systems.

333 References

- 334 1. Riordan, J. R. *et al.* Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA.
335 *Science*. **245**, 1066–1073 (1989).
- 336 2. Ratjen, F. *et al.* Cystic fibrosis. *Nat. Rev. Dis. Prim.* **1**, 15010 (2015).
- 337 3. Zhang, Z., Liu, F. & Chen, J. Molecular structure of the ATP-bound, phosphorylated human CFTR. *Proc. Natl. Acad.*
338 *Sci. U. S. A.* **115**, 12757–12762 (2018).
- 339 4. Lukacs, G. L. & Verkman, A. S. CFTR: Folding, misfolding and correcting the Δ F508 conformational defect. *Trends*
340 *Mol. Med.* **18**, 81–91 (2012).
- 341 5. Serohijos, A. W. R. *et al.* Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D
342 structure crucial to assembly and channel function. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3256–3261 (2008).
- 343 6. Protasevich, I. *et al.* Thermal unfolding studies show the disease causing Δ F508 mutation in CFTR
344 thermodynamically destabilizes nucleotide-binding domain 1. *Protein Sci.* **19**, 1917–1931 (2010).
- 345 7. Wang, C. *et al.* Integrated biophysical studies implicate partial unfolding of NBD1 of CFTR in the molecular
346 pathogenesis of F508del cystic fibrosis. *Protein Sci.* **19**, 1932–1947 (2010).
- 347 8. He, L. *et al.* Restoration of NBD1 thermal stability is necessary and sufficient to correct Δ F508 CFTR folding and
348 assembly. *J. Mol. Biol.* **427**, 106–120 (2015).

- 349 9. Rabeh, W. M. *et al.* Correction of both NBD1 energetics and domain interface is required to restore $\Delta F508$ CFTR
350 folding and function. *Cell* **148**, 150–163 (2012).
- 351 10. Sharma, M., Benharouga, M., Hu, W. & Lukacs, G. L. Conformational and Temperature-sensitive Stability Defects of
352 the $\Delta F508$ Cystic Fibrosis Transmembrane Conductance Regulator in Post-endoplasmic Reticulum Compartments.
353 *J. Biol. Chem.* **276**, 8942–8950 (2001).
- 354 11. Okiyonedo, T. *et al.* Peripheral protein quality control removes unfolded CFTR from the plasma membrane.
355 *Science*. **329**, 805–810 (2010).
- 356 12. Lewis, H. A. *et al.* Impact of the $\Delta F508$ mutation in first nucleotide-binding domain of human cystic fibrosis
357 transmembrane conductance regulator on domain folding and structure. *J. Biol. Chem.* **280**, 1346–1353 (2005).
- 358 13. Lewis, H. A. *et al.* Structure and dynamics of NBD1 from CFTR characterized using crystallography and
359 hydrogen/deuterium exchange mass spectrometry. *J. Mol. Biol.* **396**, 406–430 (2010).
- 360 14. Yang, Z. *et al.* Structural stability of purified human CFTR is systematically improved by mutations in nucleotide
361 binding domain 1. *Biochim. Biophys. Acta - Biomembr.* **1860**, 1193–1204 (2018).
- 362 15. Aleksandrov, A. A. *et al.* Allosteric modulation balances thermodynamic stability and restores function of $\Delta F508$
363 CFTR. *J. Mol. Biol.* **419**, 41–60 (2012).
- 364 16. deCarvalho, A. C. V., Gansheroff, L. J. & Teem, J. L. Mutations in the nucleotide binding domain 1 signature motif
365 region rescue processing and functional defects of cystic fibrosis transmembrane conductance regulator $\Delta F508$. *J.*
366 *Biol. Chem.* **277**, 35896–35905 (2002).
- 367 17. Lewis, H. A. *et al.* Structure of nucleotide-binding domain 1 of the cystic fibrosis transmembrane conductance
368 regulator. *EMBO J.* **23**, 282–293 (2004).
- 369 18. Atwell, S. *et al.* Structures of a minimal human CFTR first nucleotide-binding domain as a monomer, head-to-tail
370 homodimer, and pathogenic mutant. *Protein Eng. Des. Sel.* **23**, 375–384 (2010).
- 371 19. Zhang, Z. & Chen, J. Atomic Structure of the Cystic Fibrosis Transmembrane Conductance Regulator. *Cell* **167**,
372 1586-1597.e9 (2016).
- 373 20. Liu, F., Zhang, Z., Csanády, L., Gadsby, D. C. & Chen, J. Molecular Structure of the Human CFTR Ion Channel. *Cell*
374 **169**, 85-95.e8 (2017).
- 375 21. Aleksandrov, A. A. *et al.* Regulatory insertion removal restores maturation, stability and function of $\Delta F508$ CFTR. *J.*
376 *Mol. Biol.* **401**, 194–210 (2010).
- 377 22. Lukacs, G. L. *et al.* Conformational maturation of CFTR but not its mutant counterpart ($\Delta F508$) occurs in the
378 endoplasmic reticulum and requires ATP. *EMBO J.* **13**, 6076–6086 (1994).
- 379 23. Gong, X. *et al.* Non-native conformers of cystic fibrosis transmembrane conductance regulator NBD1 are
380 recognized by Hsp27 and conjugated to SUMO-2 for degradation. *J. Biol. Chem.* **291**, 2004–2017 (2016).
- 381 24. Aleksandrov, L., Aleksandrov, A. A., Chang, X. B. & Riordan, J. R. The first nucleotide binding domain of cystic
382 fibrosis transmembrane conductance regulator is a site of stable nucleotide interaction, whereas the second is a
383 site of rapid turnover. *J. Biol. Chem.* **277**, 15419–15425 (2002).
- 384 25. Vergani, P., Lockless, S. W., Nairn, A. C. & Gadsby, D. C. CFTR channel opening by ATP-driven tight dimerization of
385 its nucleotide-binding domains. *Nature* **433**, 876–880 (2005).
- 386 26. Cui, L. *et al.* The role of cystic fibrosis transmembrane conductance regulator phenylalanine 508 side chain in ion
387 channel gating. *J. Physiol.* **572**, 347–358 (2006).
- 388 27. Mense, M. *et al.* In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain
389 heterodimer. *EMBO J.* **25**, 4728–4739 (2006).
- 390 28. Kalinin, S., Valeri, A., Antonik, M., Felekyan, S. & Seidel, C. A. M. Detection of Structural Dynamics by FRET: A
391 Photon Distribution and Fluorescence Lifetime Analysis of Systems with Multiple States. *J. Phys. Chem. B* **114**,
392 7983–7995 (2010).

- 393 29. Sigoillot, M. *et al.* Domain-interface dynamics of CFTR revealed by stabilizing nanobodies. *Nat. Commun.* **10**, 2636
394 (2019).
- 395 30. Kalinin, S. *et al.* A toolkit and benchmark study for FRET-restrained high-precision structural modeling. *Nat.*
396 *Methods* **9**, 1218–1225 (2012).
- 397 31. Premchandrar, A. *et al.* New insights into interactions between the nucleotide-binding domain of CFTR and keratin
398 8. *Protein Sci.* **26**, 343–354 (2017).
- 399 32. Denning, G. M. *et al.* Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-
400 sensitive. *Nature* **358**, 761–4 (1992).
- 401 33. Proctor, E. A. *et al.* Rational coupled dynamics network manipulation rescues disease-relevant mutant cystic
402 fibrosis transmembrane conductance regulator. *Chem. Sci.* **6**, 1237–1246 (2015).
- 403 34. Kanelis, V., Hudson, R. P., Thibodeau, P. H., Thomas, P. J. & Forman-Kay, J. D. NMR evidence for differential
404 phosphorylation-dependent interactions in WT and $\Delta F508$ CFTR. *EMBO J.* **29**, 263–77 (2010).
- 405 35. Csanády, L., Chan, K. W., Nairn, A. C. & Gadsby, D. C. Functional roles of nonconserved structural segments in
406 CFTR's NH₂-terminal nucleotide binding domain. *J. Gen. Physiol.* **125**, 43–55 (2005).
- 407 36. Wright, P. E. & Dyson, H. J. Intrinsically disordered proteins in cellular signalling and regulation. *Nature Reviews*
408 *Molecular Cell Biology* vol. 16 18–29 (2015).
- 409 37. Ainscow, E. K., Mirshamsi, S., Tang, T., Ashford, M. L. J. & Rutter, G. A. Dynamic imaging of free cytosolic ATP
410 concentration during fuel sensing by rat hypothalamic neurones: Evidence for ATP-independent control of ATP-
411 sensitive K⁺ channels. *J. Physiol.* **544**, 429–445 (2002).
- 412 38. Cui, L. *et al.* Domain Interdependence in the Biosynthetic Assembly of CFTR. *J. Mol. Biol.* **365**, 981–994 (2007).
- 413 39. Goddard, T. D. *et al.* UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Sci.* **27**, 14–
414 25 (2018).

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429 Author contributions

430 D.S., D.N.S. and C.G. conceived and designed the experiments. D.S., M.S., M.O., and R.C. performed NBD1 and
431 nanobody expression, purification and biochemical characterization. D.S., M.S. and M.O. performed nanobody
432 discovery, cloning and purification which E.P. and T.L. supervised. M.S. designed and conducted crystallography
433 experiments and collected the X-ray data. M.S., C.G., A.G.P. and D.S. processed and refined the X-ray data.
434 M.O. performed and analysed flow cytometry and CFTR pull-down experiments. Y.W. performed patch-clamp
435 electrophysiological experiments which D.N.S. supervised. C.M. conducted the HDX-MS data collection and
436 analysis. D.S. performed modelling, construction and labelling of FRET reporters, and smFRET experiments.

437 D.S., J.H. and R.C. recorded and analysed the smFRET data. D.S., D.N.S., J.S., J.H. and C.G. co-wrote the
438 manuscript. D.N.S., J.S., J.H., and C.G. directed the study. All authors approved the final version of the
439 manuscript.

440 **Competing financial interests**

441 The authors declare no competing financial interests.

442 **Figure Legends**

443 **Fig. 1 | Single molecule FRET analysis for the 426-519 reporter pair of NBD1.**

444 **A**, Depiction of the cryo-EM structure of the full-length CFTR protein (PDB: 5UAK) with its transmembrane domains (TMDs)
445 and cytoplasmic nucleotide-binding domains (NBDs). TMD1 is coloured grey and TMD2 green. The regulatory domain and
446 RI are unresolved. Phenylalanine-508 (F508) is shown in orange. **B**, Ribbon representation of NBD1 showing the location of
447 the smFRET reporters in the canonical conformation (based on the published crystal structure of human NBD1,
448 PDB: 2BBO). The positions of the labelled cysteines are shown as yellow spheres. The RI, which is not resolved in the
449 structure, is depicted as a red dashed line. ATP and F508, located in the α -subdomain, are shown. **C-D**, FRET efficiency
450 histograms of the 426-519 reporter in the presence and absence of 2 mM ATP and 3 mM MgCl₂. **E-G**, Two-dimensional
451 plots of donor fluorescence lifetime in presence of acceptor ($\tau_{D(A)}$) versus FRET efficiency of the 426-519 reporter pair for
452 the indicated conditions. For panel **G**, in the presence of 5 μ M of nanobody G11a.

453 **Fig. 2 | The G11a-bound structure reveals a new conformation of NBD1 with a different topology.**

454 **A**, High-resolution structure of 2PT-NBD1 in complex with nanobody G11a. No density is observed for residues 389–422. **B**,
455 and **C**, comparative representations highlighting the topological changes in the β -subdomain observed between the
456 G11a-bound structure (**B**, the nanobody is omitted for clarity) and the canonical conformation as previously published (**C**,
457 here PDB: 2BBO). Unresolved segments are illustrated as random coils with dashed lines in red for the RI (404–436) and in
458 magenta for the N-terminus (389–403). The segment including the S4 strand (473–487) is coloured in blue. A topological
459 representation of the β -subdomain is shown to the right of each conformation. In the new conformation, residues from
460 the RI adopt a β -sheet structure and replace the S1 segment which becomes unstructured. We therefore refer to it as β -
461 *strand-swapped*.

462 **Fig. 3 | The G11a nanobody binds to full-length CFTR in cells and increases channel activity.**

463 **A**, Flow cytometry analysis of CFTR recognition by nanobodies in CFTR overexpressing HEK293 cells. Data were normalized
464 to the number of events acquired in each condition. Graph depicts one representative of six independent experiments.
465 The inset shows the average median fluorescence $\pm$ SEM (fold against negative control, dashed line=1). **B**, Immunoblot of
466 CFTR pulled down with TwinStrep-tagged nanobodies from solubilized HEK293 cells. Eluted nanobody-CFTR complexes
467 were separated by SDS-PAGE and presence of CFTR was detected with a mouse anti-CFTR monoclonal antibody (596,
468 recognizing residues 1204–1211 in NBD2³⁸) after immunoblotting. Arrows indicate the mature (band C) and immature
469 (band B) forms of CFTR. The blot shown is representative of 3 independent experiments. **C**, Open probabilities of wild-type
470 CFTR in the absence and presence of G11a (1 μ M). Symbols represent individual values and columns are means $\pm$ SEM (n =
471 6); *, P = 0.000157 vs control; Student's t-test; two-sided. **D**, Representative recordings of a wild-type CFTR Cl⁻ channel in
472 an excised inside-out membrane patch from a C127 cell heterologously expressing wild-type human CFTR. The recordings
473 were acquired at 37 °C in the presence of ATP (0.3 mM) and PKA (75 nM) in the intracellular solution. After the channel
474 was fully activated, G11a (1 μ M) was directly added to the intracellular solution bathing the membrane patch. Dotted lines
475 indicate where the channel is closed, and downward deflections correspond to channel openings.

476 **Fig. 4 | Single molecule FRET analysis for the 479-519 reporter pair of NBD1.**

477 **A-B**, Positions of the labelled cysteines 479 and 519 for the canonical and β -SS conformations. **C-D**, Two-dimensional plots
478 of donor fluorescence lifetime ($\tau_{D(A)}$) versus FRET efficiency for the indicated constructs and conditions. In the absence of
479 conformational dynamics, the data clouds fall on the static FRET line (red continuous line). **E**, Overlay of FRET efficiency
480 histograms of 2PT-479-519 for the indicated ATP concentrations. Raw data (filled) were fitted using the PDAFit software
481 (thick lines, see Methods). Inset: Centre of the error bars shows the obtained fit for the population of the canonical
482 conformation as calculated by PDAFit. Error bars indicate 95% confidence interval. **F**, Reversibility of the conformational
483 change. **G-H**, Two-dimensional plot of 2PT-479-519 in presence of G11a (**G**) and of the Δ RI-2PT-NBD1 variant with 2 mM
484 ATP (**H**).

485

486 **Fig. 5 | The effects of F508del, temperature and S492P on the β -subdomain**

487 **A**, Structure of the canonical conformation highlighting the positions of the 3 stabilizing mutations and F508. **B**,
488 Histograms of the obtained fit for the population of the canonical states of the 479-519 reporter pair as calculated by
489 PDAFit. Values are shown for 2PT-NBD1 and PT-NBD1 (with S492) variants at 22 °C and 37 °C in the presence ('F') or
490 absence (' Δ F') of F508. Error bars indicate 95% confidence interval. **C**, Overlay of FRET efficiency histograms of
491 2PT-479-519 at 22 °C and F508del-PT-479-519 at 37 °C. **D**, Structure of NBD1 in the β -strand swapped conformation
492 illustrating the positions of residues 442 and 519 (yellow spheres) along with K447 in the S2 strand (turquoise). **E-J**, Two-
493 dimensional plots of donor fluorescence lifetime ($\tau_{D(A)}$) vs FRET efficiency of the 442-519 reporter pair for the indicated
494 constructs and conditions.

495 **Fig. 6 | Model of an unfolding pathway through the β -strand-swapped conformation.**

496 Cartoon representations of the different conformational states of NBD1 superimposed onto the dephosphorylated and
497 ATP-free full-length CFTR structure (PDB: 5UAK). **A**, The canonical conformation of human NBD1 (PDB: 2BBO) is mostly
498 structured, only the RI (red) is unstructured. **B**, In the β -strand-swapped conformation, the N-terminus and the first half of
499 the RI are unstructured (purple and red dashed, respectively). The second part of the RI is structured (red thick) and the S4
500 strand unfolds to become a loop (blue). **C**, In the unfolding intermediate, the RI and all strands of the β -sheet are
501 unstructured (S1 in purple, RI in red, S2 in turquoise and S4 in blue). Figures of full-length CFTR structures were created
502 using ChimeraX³⁹.

503 **Methods**

504 ***Human NBD1 expression and purification***

505 Human 2PT-NBD1 (residues 387-646 containing the mutations S492P, A534P, I539T) and Δ RI-NBD1 (residues
506 387-646, Δ 405-436) were expressed as N-terminal, His₆-SUMO fusion proteins in *Escherichia coli* (BL21 (DE3)
507 pLysS cells) as previously described^{6,17}. Cells were lysed using a French press and recombinant proteins were
508 purified by nickel ion affinity chromatography (HisTrap HP, 1ml - GE Healthcare). The His₆-SUMO tag was
509 removed using Ulp1 protease⁴⁰ followed by a second nickel ion chromatography. The samples were then
510 passed through a gel filtration column (Superdex 200 Increase 10/300 GL - GE Healthcare) previously
511 equilibrated in 20 mM Hepes pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 10% (w/v) ethylene glycol, 2 mM ATP, 3
512 mM MgCl₂, 1 mM Tris (2-carboxyethyl)phosphine (TCEP). Protein concentration was determined using
513 Coomassie Plus (Bradford) Assay Kit (ThermoScientific).

514 ***Nanobodies discovery, cloning, expression and purification***

515 Nanobodies against 2PT-NBD1 were obtained as previously described²⁹. Briefly, a llama was immunized 6 times
516 with purified 2PT-NBD1. Peripheral blood lymphocytes were collected after the final boost and RNA was
517 purified to generate a phage library. Nanobodies were obtained after phage display selection, using established
518 protocols⁴¹. After two rounds of selection on biotinylated 2PT-NBD1, 12 different families, classified according
519 to the sequences of the third complementarity determining region (CDR3), were discovered. Nanobodies were
520 cloned in pXAP100 vector encoding His₆-tag and Myc-tag at the C-terminal end of the protein. This vector was
521 also modified to encode Twin-Strep-tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys repeated twice with an internal linker
522 region) instead of the His₆-tag, which was used for CFTR pull-down experiments. The expression and
523 purification of nanobodies was performed as previously described⁴¹. Briefly, nanobodies were produced in
524 *Escherichia coli* (BL21 (DE3) pLysS cells), purified from the periplasmic extract via either HisPur Ni-NTA resin
525 (ThermoScientific) or Strep-Tactin XT Superflow resin (Iba) followed by a size exclusion chromatography (SEC)
526 (Superdex 200 Increase 10/300 GL -GE Healthcare) into 20 mM HEPES pH 7.5, 150 mM NaCl, and 10% (w/v)
527 glycerol.

528
529 ***NBD1-ELISA assay***

530 For dose-response assays, Nunc MaxiSorp 96-well plates (ThermoScientific), were coated with 5 μ g/ml
531 NeutrAvidin Biotin-Binding protein (ThermoScientific) overnight at 4 °C and blocked for 2 h at room
532 temperature (RT) with 4% milk in phosphate-buffered saline. Each new reagent addition was preceded by three

533 washes with 200 μ l of NBD1 buffer (20 mM HEPES pH 7.5, 150 mM NaCl, and 10% (w/v) glycerol, 10% (w/v)
534 ethylene glycol, 2 mM ATP, 3 mM $MgCl_2$). Then, biotinylated purified NBD1 at 5 μ g/ml was immobilized for 45
535 min at RT followed by 1 h RT incubation with 100 μ l of purified nanobody at varying concentrations (0-100
536 μ g/ml). Signal detection was followed using His₆-tag specific antibody (0.5 μ g/ml - Invitrogen) to detect the
537 nanobodies and secondary antibody anti-mouse coupled to horse radish peroxidase (HRP) (0.5 μ g/ml –
538 Millipore). 50 μ l of 1-Step UltraTMB-ELISA (ThermoScientific) served as substrate for the peroxidase and
539 intensity of the reaction was proportional to absorbance measured at 450 nm with SynergyMx (BioTek) after
540 addition of 50 μ l H_2SO_4 at 1 M.

541 ***Thermal shift assay (DSF)***

542 Solutions of NBD1 constructs (10 μ M final concentration), nanobodies (30 μ M final concentration) and 2.5x
543 concentrated SYPRO Orange Protein Stain (Molecular Probes) diluted in NBD1 buffer, were added to the wells
544 of 96-well PCR plates (VWR) in a final volume of 25 μ l. Plates were sealed with EasySeal sheets (Molecular
545 Dimensions) and spun for 2 min at 900 x g. SYPRO orange fluorescence was monitored in CFX96 Touch Real-
546 Time PCR Detection System (Bio-Rad) using plate type BR white and scan mode FRET from 10 to 80 °C in
547 increments of 1 °C.

548 ***Crystallization trials***

549 For 2PT-NBD1:G11a complex formation, G11a nanobody was SEC purified the day before in 20 mM Hepes
550 pH 7.5, 150 mM NaCl, 10% (w/v) glycerol and mixed with freshly SEC purified NBD1 with 1.2 molar excess of
551 G11a, and keeping 2 mM ATP, 3 mM $MgCl_2$ and 1 mM TCEP final concentrations. Protein complex was
552 incubated overnight on ice and then concentrated onto 30 kDa MWCO Amicon concentrator (Millipore) until
553 protein concentration reached 11 mg/ml. Crystallization was performed in sitting drops at room temperature,
554 adding 100 nl of protein to 100 nl of the precipitant (0.2 M $CaCl_2$, 0.1 M Tris pH 8.5, 25% (w/v) PEG 4000 and
555 were set up immediately using a Mosquito robot (Art Robbins). Crystallization plates were incubated at 20 °C.
556 Single crystals were mounted in CryoLoops (Molecular Dimensions Ltd) and flash-frozen in liquid nitrogen.

557 ***Crystal structure determination***

558 Diffraction data were collected at 100 K at Proxima1 beamline of Soleil (Gif-sur-Yvette, France) synchrotron as
559 indicated in Table S2. Data were processed using the Autoproc program⁴². The dataset was solved by molecular
560 replacement using Molrep⁴³. Subsequently, several cycles of model building, using COOT⁴⁴, combined with
561 refinement using BUSTER 2.10.1⁴⁵ were conducted. Finally, structure validation was performed with
562 MOLPROBITY⁴⁶. Figures and structural comparisons of the 2PT-NBD1:G11a complex with the human NBD1
563 structure previously published (PDB: 2BBO¹³) were prepared using Chimera⁴⁷.

564 ***Flow cytometry***

565 HEK293 cells stably overexpressing human wt-CFTR⁴⁸ were permeabilized with 0.01%
566 n-Dodecyl- β -D-Maltopyranoside (β -DDM - Inalco) at least for 2 h on ice. Cells were incubated with 50 μ g/ml
567 nanobodies and DAPI (2.5 μ M – Invitrogen) to monitor the permeabilization state. Nanobody binding was
568 detected using Myc-tag specific antibody (2 μ g/ml – Life Technologies) and then anti-mouse-Alexa Fluor 700
569 (1.3 μ g/ml – Invitrogen) for 30 min on ice. We used the T2a nanobody as positive control for CFTR binding²⁹ and
570 a nanobody directed against *Lactococcus lactis* multidrug resistance protein as negative control. Cells were
571 washed once between each step by centrifugation (200 x g for 5 min at 4 °C). All incubations (100 μ l) and
572 washes (1.5 ml) were performed in PBS with 6% fetal bovine serum (FBS) and 0.01% β -DDM on ice. Cell
573 fluorescence was measured with Gallios Flow Cytometer (Beckman Coulter). Alexa-Fluor signal (laser : 633 nm
574 – detector : 725/20 nm) was recorded after gating on cells positive for CFTR-EGFP (laser : 488 nm – detector :
575 525/50 nm) and permeabilized/positive for DAPI (laser : 405 nm – detector : 450/50 nm). Gating graphs are
576 shown in Fig S8. Data was analyzed with Kaluza software.

577 **CFTR pull-down**

578 Human wt-CFTR was extracted from HEK293 cell pellets by solubilization with 0.1% DMNG in PBS with protease
579 inhibitors for 1 h at 4 °C. The cell debris was removed by centrifugation (16,000 x g for 30 min at 4 °C).
580 Supernatant was diluted 10 times in PBS with protease inhibitors and incubated for 30 min on 10 µl Strep-
581 Tactin XT Superflow resin (Iba) pre-loaded with 200 µg nanobodies. Resin was washed and eluted with Buffer
582 BXT (Iba). The presence of CFTR in each sample was detected by SDS-PAGE and immuno-blotting.

583 **SDS-PAGE and immunoblotting**

584 Cell extracts were separated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to nitrocellulose
585 membranes (Bio-Rad) for immunodetection. After blocking for 1 h with 5% bovine serum albumin (BSA) in Tris-
586 buffered saline added Tween-20 (TBST), CFTR was detected using monoclonal antibody 596³⁸ for 1 h in blocking
587 buffer. Blots were washed 3 times for 5 min and incubated with anti-mouse-HRP antibody (0.2 µg/ml –
588 Millipore) for 1 h in TBST. Membranes were washed 3 times for 5 min. CFTR was visualized by
589 chemiluminescence using Luminata Forte Western HRP Substrate (Millipore) and detected with ImageQuant
590 400 (GE Healthcare).

591 **Single-channel studies**

592 For patch-clamp experiments, we used mouse mammary epithelial (C127) cells stably expressing wild-type
593 human CFTR (gift of C. R. O’Riordan (Sanofi Genzyme)). They were cultured and used as described previously⁴⁹.

594 CFTR Cl⁻ channels were recorded in excised inside-out membrane patches using an Axopatch 200B patch-clamp
595 amplifier and pCLAMP software (version 10.4) both from Molecular Devices (San Jose, CA, USA) as described
596 previously⁴⁹. The pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine, 140 aspartic acid,
597 5 CaCl₂, 2 MgSO₄ and 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), adjusted to pH 7.3
598 with Tris ([Cl⁻], 10 mM). The bath (intracellular) solution contained (mM): 140 NMDG, 3 MgCl₂, 1 CsEGTA and 10
599 TES, adjusted to pH 7.3 with HCl ([Cl⁻], 147 mM; free [Ca²⁺], < 10⁻⁸ M) and was maintained at 37 °C.

600 CFTR Cl⁻ channels were activated promptly following membrane patch excision and clamping voltage at -50 mV
601 using the catalytic subunit of protein kinase A (PKA (purified from bovine heart); 75 nM) and ATP (1 mM), both
602 from Merck Life Science UK Ltd.. To test the effects of nanobodies on wild-type CFTR, we added them to the
603 intracellular solution and acquiring 5 – 10 minutes of single-channel data in the continuous presence of ATP
604 (0.3 mM) and PKA (75 nM). Because of the difficulty of removing nanobodies from the recording chamber,
605 specific interventions were compared with the pre-intervention control period made with the same
606 concentration of ATP and PKA, but without nanobodies. To minimise rundown, PKA and ATP were added to all
607 intracellular solutions. On completion of experiments, the recording chamber was thoroughly cleaned before
608 re-use.

609 In this study, we used excised inside-out membrane patches containing ≤ 5 active channels, determined using
610 the maximum number of simultaneous channel openings observed during an experiment⁵⁰. After recording,
611 filtering and digitizing data⁵¹, single-channel current amplitude (*i*), open probability (*P*_o), mean burst duration
612 (MBD) and interburst interval (IBI) were determined as described previously^{50,51}. Results are expressed as
613 means ± SEM of *n* observations, where *n* represents the number of individual membrane patches obtained
614 from different cells. Using SigmaPlot™ (version 13.0, Systat Software Inc., San Jose, CA, USA), we tested for
615 differences between two groups of data acquired within the same experiment with Student’s *t*-test.
616 Differences were considered statistically significant when *P* < 0.05.

617 **Hydrogen deuterium exchange mass spectrometry**

618 *Experiments*

619 Hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments were performed on a Synapt G2Si
620 HDMS coupled to an Acquity UPLC M-Class system with HDX and automation (Waters Corporation, Manchester

UK). Protein samples were used at a concentration of 50 μ M. Isotope labelling was initiated by diluting 5 μ l of each protein sample into 95 μ l of buffer L (HEPES 20 mM pD 7.5, NaCl 100 mM) complemented with 2 mM ATP, 3 mM $MgCl_2$ when specified. The protein was incubated for 1, 5, 30, and 120 min to capture a range of exchange times and then quenched in buffer Q (100 mM potassium phosphate, brought to pH 2.3 with formic acid) at 4 °C before being digested on-line with a Waters Enzymate BEH pepsin column at 20 °C, for ~30 s, at a pressure of ~2000 psi. The same procedure was used for undeuterated controls, with the labelling buffer being replaced by buffer E (HEPES 20 mM pH 7.5, NaCl 100 mM). The peptides were trapped on a Waters BEH C18 VanGuard pre-column for 3 min at a flow rate of 200 μ l/min in buffer A (0.1% formic acid ~ pH 2.5) for desalting before being applied to a Waters BEH C-18 analytical column. Peptides were eluted with a linear gradient of buffer B (8-40% gradient of 0.1% formic acid in acetonitrile) at a flow rate of 40 μ l / min. All trapping and chromatography steps were performed at 1 °C to minimize back exchange. The electrospray ionization source was operated in the positive ion mode and ion mobility was enabled for the MS instrument. HDMS^E data were acquired with a 20 to 30 V trap collision energy ramp for high-energy acquisition of product ions. Optimized peptide identification and peptide coverage for all samples was performed from undeuterated controls (five replicates). All deuterium time points were performed in triplicate. Leucine Enkephalin (LeuEnk - Sigma) was used as a lock mass for mass accuracy correction and the MS was calibrated with sodium iodide. The on-line Enzymate pepsin column was washed between each injection with pepsin wash (1.5 M Gu-HCl, 4% MeOH, 0.8% formic acid) recommended by the manufacturer to prevent significant peptide carry-over from the pepsin column and a blank run was performed between each technical triplicate. Measurements were performed on three biological replicates.

641 *Data evaluation and statistical analysis*

642 Sequence identification was made from MS^E data from the undeuterated samples using ProteinLynx Global
643 Server 2.5.1 (PLGS Waters Corp. Manchester UK). The output peptides were filtered using DynamX (v. 3.0)
644 using the following filtering parameters: minimum intensity of 1000, minimum and maximum peptide
645 sequence length of 5 and 25 respectively, minimum MS/MS products of 2, minimum products per amino acid
646 of 0.28, minimum score of 5, and a maximum MH^+ error threshold of 15 ppm. The peptides had to be identified
647 in 3 out of 5 replicates. Additionally, all the spectra were visually examined and only those with high signal to
648 noise ratios were used for HDX-MS analysis. The amount of relative deuterium uptake for each peptide was
649 determined using DynamX (v. 3.0) and were not corrected for back exchange since only relative differences
650 were used for analysis and interpretation and there was no benefit from normalizing the data⁵². The relative
651 fractional uptake (RFU) was calculated from $RFU_a = [Y_{a,t} / (MaxUptake_a \times D)]$, where Y is the deuterium uptake
652 for peptide a at incubation time t , and D is the percentage of deuterium in the sample after mixing the protein
653 with the labeling solution. 99% Confidence intervals were calculated using Deuterios⁵³ and were found to be of
654 0.7 Da or smaller for the sum of RFU over all time points.

655 *Modeling of dye distributions using the FPS simulation program*

656 We used the FRET-restrained positioning and screening (FPS) software³⁰ to calculate accessible volumes and
657 theoretical approximations of distances and FRET efficiencies between fluorophores fixed onto cysteines in key
658 regions of NBD1.

659 *Site-directed mutagenesis for the generation of single molecule PIE-FRET constructs*

660 Guided by previous studies of functional full-length cysteine-less CFTR variants^{26,27}, we replaced endogenous
661 cysteines of 2PT-NBD1. Cysteines 491 and 524 were converted to alanine and cysteines 590 and 592 to valine.
662 Substitutions were done by the QuikChange™ site-directed mutagenesis procedure using a modified protocol
663 for primer design⁵⁴.

664 *NBD1 fluorophore labeling*

665 For smFRET experiments engineered cysteines of NBD1 constructs were labeled with ATTO488 and Alexa647
666 maleimide dyes (ATTO-TEC GmbH AD 488-41 and ThermoFisher A20347, respectively). Affinity-purified NBD1
667 at 70 μM in labeling buffer (20 mM HEPES pH 7.20-7.40, 150 mM NaCl, 10% (w/v) glycerol, 10% (w/v) ethylene
668 glycol 2 mM ATP, 3 mM MgCl_2 and 1 mM TCEP) was incubated with a 3 times molar excess of each dye to NBD1
669 for a total of 3 h. Labeled NBD1 was spun at 4 $^\circ\text{C}$ and 16,000 g for 30 minutes and passed through a gel
670 filtration column (Superdex 200 Increase 10/300 GL - GE Healthcare) previously equilibrated in 20 mM HEPES
671 pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 10% (w/v) ethylene glycol, 2 mM ATP, 3 mM MgCl_2 .

672 ***Single-molecule FRET data recording***

673 Protein at 5-15 μM was diluted 1000-fold into the buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) prior to mixing
674 with other reagents. Then the protein was diluted 100-fold in the final measurement sample containing also
675 0.1 mg/mL BSA, MgATP and/or nanobody, reaching a final concentration of 50-150 pM. The BSA-coated
676 (1 mg/ml BSA) coverslip (Nunc Lab-Tek Chambered Coverglass, Thermo Fisher Scientific) was rinsed two times
677 with the sample solution (30 μl), prior to depositing a 30 μl drop of the same solution. Background or scatter
678 samples were prepared similarly but without protein.

679 Per sample, 1-3 hour datasets were recorded at 22 $^\circ\text{C}$ on a homebuilt multiparameter fluorescence detection
680 microscope with pulsed interleaved excitation (MFD-PIE) as established⁵⁵, with minor modifications. Emission
681 from a pulsed 483-nm laser diode (LDH-P-C-470, PicoQuant) was cleaned up (Chroma ET485/20x, F49-482; AHF
682 analysentechnik AG), emission from a 635-nm laser diode (LDH-P-C-635B, PicoQuant) was cleaned up (Chroma
683 z635/10x, PicoQuant), and both lasers were alternated at 26.67 MHz (PDL 828 Sepia II, PicoQuant), delayed ~ 18
684 ns with respect to each other, and combined with a 483-nm reflecting dichroic mirror in a single-mode optical
685 fiber (coupler, 60FC-4- RGBV11-47; fiber, PMC-400Si-2.6-NA012-3-APC-150-P, Schäfter + Kirchhoff GmbH).
686 After collimation (60FC-L-4-RGBV11-47, SuK GmbH), the linear polarization was cleaned up (CODIX VIS-600-
687 BC-W01, F22-601; AHF analysentechnik AG), and the light (75 μW of 483-nm light and 50 μW of 635-nm light)
688 was reflected into the back port of the microscope (IX70, Olympus Belgium NV) and upward [3-mm-thick full-
689 reflective Ag mirror, F21-005 (AHF) mounted in a total internal reflection fluorescence filter cube for BX2/IX2,
690 F91-960; AHF analysentechnik AG] to the objective (UPLSAPO-60XW, Olympus). Sample emission was
691 transmitted through a 3-mm-thick excitation polychroic mirror (Chroma zt470-488/640rpc, F58-PQ08; AHF
692 analysentechnik AG), focused through a 75-mm pinhole (P75S, Thorlabs) with an achromatic lens (AC254-200-
693 A-ML, Thorlabs), collimated again (AC254-50-A-ML, Thorlabs), and spectrally split (Chroma T560lpxr, F48-559;
694 AHF analysentechnik AG). The blue range was filtered (Chroma ET525/50m, F47-525, AHF analysentechnik AG),
695 and polarization was split (PBS251, Thorlabs). The red range was also filtered (Chroma ET705/100m, AHF
696 analysentechnik AG), and polarization was split (PBS252, Thorlabs). Photons were detected on four avalanche
697 photodiodes (PerkinElmer or EG&G SPCM-AQR12/14, or Laser Components COUNT BLUE), which were
698 connected to a time-correlated single-photon counting (TCSPC) device (SPC-630, Becker & Hickl GmbH) over a
699 router (HRT-82, Becker & Hickl) and power supply (DSN 102, PicoQuant). Signals were stored in 12-bit first-in-
700 first-out (FIFO) files. Microscope alignment was carried out using fluorescence correlation spectroscopy (FCS)
701 on freely diffusing ATTO 488-CA and ATTO 655-CA (ATTO-TEC) and by connecting the detectors to a hardware
702 correlator (ALV-5000/EPP) over a power splitter (PSM50/51, PicoQuant) for alignment by real-time FCS.
703 Instrument response functions (IRFs) were recorded one detector at-a-time in a solution of ATTO 488-CA or
704 ATTO 655-CA in near-saturated centrifuged potassium iodide at a 25-kHz average count rate for a total of
705 25×10^6 photons. Macrotime-dependent microtime shifting was corrected for two (blue/parallel and
706 red/perpendicular) of four avalanche photodiodes (APDs) using the IRF data as input.

707 ***Single-molecule MFD-PIE-FRET data analysis***

708 Data were analyzed with the PAM software⁵⁶ via standard procedures for MFD-PIE smFRET burst analysis^{57,58}.
709 Signals from each TCSPC routing channel (corresponding to the individual detectors) were divided in time gates
710 to discern 483-nm excited FRET photons from 635-nm excited acceptor photons. A two-color MFD all-photon

burst search algorithm using a 500- μ s sliding time window (minimum of 100 photons per burst, minimum of 5 photons per time window) was used to identify single donor- and/or acceptor-labeled molecules in the fluorescence traces. Double labelled single molecules were selected from the raw burst data using a kernel density estimator (ALEX-2CDE < 12)⁵⁹, that also excluded other artefacts. Sparse slow-diffusing aggregates were removed from the data by excluding bursts exhibiting a burst duration > 20 ms. By generating histograms of E versus measurement time, we corroborated that the distribution of E was invariant over the duration of the measurement. Data was corrected in this order to obtain the absolute stoichiometry parameter S and absolute FRET efficiency E : background subtraction, donor emission crosstalk correction, acceptor direct excitation correction and relative detection efficiency correction. The last value depends on the used dye combination, but also on the used detectors. By making histograms of E versus measurement time, we corroborated that the distribution of E was invariant over the duration of the measurement.

Photon distribution analysis (PDA)

Static PDA was carried out to obtain the absolute interdye distance distribution assuming two Gaussian distributed states⁶⁰. For each FRET data set, raw bursts were re-binned in 1 ms time bins, and histograms were constructed and analyzed. Binned data was plotted in a raw (uncorrected) FRET efficiency (E_{PR}) versus uncorrected stoichiometry (S_{PR}) plot and only bins with $0.2 < S_{PR} < 0.6$ were included in the analysis to remove burst sections containing complex acceptor photophysics or photobleaching. Furthermore, only bins with at least 20 and maximally 200 photons (to reduce calculation time) were used for PDA analysis. A two-state model for a Gaussian distance distribution was used to generate a library of simulated E_{FRET} values, which was fitted to the experimental E_{FRET} histogram using a reduced χ^2 -guided simplex search algorithm to obtain the amplitude, mean distance R and width σ of all Gaussian distributed substates and, in the case of multiple states, their area fraction A (%). A probability density function (PDF) was calculated per state using the R and σ parameters that describe the underlying Gaussian distributed states. The summed PDF was scaled to a total area of unity, with the PDF area of each state scaled to the corresponding fraction of molecules. A Jacobian was used to estimate confidence intervals (95%) of the fit parameters. Criteria for a good fit were a low reduced χ^2 value, as well as a weighted-residual plot free of trends.

Data availability

Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. The atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank (PDB). The accession numbers for the structure reported in this paper is PDB: 6ZE1. The source data underlying Figs. 1-5 and Extended Data Figures 2, 3, 6 and 7 will be provided as Source Data files.

References

40. Li, S. J. & Hochstrasser, M. A new protease required for cell-cycle progression in yeast. *Nature* **398**, 246–251 (1999).
41. Pardon, E. *et al.* A general protocol for the generation of Nanobodies for structural biology. *Nat. Protoc.* **9**, 674–693 (2014).
42. Vonrhein, C. *et al.* Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **67**, 293–302 (2011).
43. Vagin, A. & Teplyakov, A. *MOLREP*: an Automated Program for Molecular Replacement. *J. Appl. Crystallogr.* **30**, 1022–1025 (1997).
44. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **66**, 486–501 (2010).
45. Bricogne, G. *et al.* Bricogne, BUSTER version 2.10.1., Cambridge, United Kingdom: Global Phasing Ltd. (2017).
46. Chen, V. B. *et al.* MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr.*

755 *Sect. D Biol. Crystallogr.* **66**, 12–21 (2010).

756 47. Pettersen, E. F. *et al.* UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem.*
757 **25**, 1605–1612 (2004).

758 48. Hildebrandt, E. *et al.* A Stable Human-Cell System Overexpressing Cystic Fibrosis Transmembrane Conductance
759 Regulator Recombinant Protein at the Cell Surface. *Mol. Biotechnol.* **57**, 391–405 (2015).

760 49. Sheppard, D. N. & Robinson, K. A. Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane
761 conductance regulator Cl⁻ channels expressed in a murine cell line. *J. Physiol.* **503**, 333–346 (1997).

762 50. Cai, Z., Taddei, A. & Sheppard, D. N. Differential sensitivity of the Cystic Fibrosis (CF)-associated mutants G551D
763 and G1349D to potentiators of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel. *J. Biol.*
764 *Chem.* **281**, 1970–1977 (2006).

765 51. Rodrat, M. *et al.* Carbon monoxide-releasing molecules inhibit the cystic fibrosis transmembrane
766 conductance regulator Cl⁻ channel. *Am J Physiol Lung Cell Mol Physiol.* **319**, L997-L1009 (2020).

767 52. Wales, T. E., Eggertson, M. J. & Engen, J. R. Considerations in the Analysis of Hydrogen Exchange Mass
768 Spectrometry Data. in *Mass Spectrometry Data Analysis in Proteomics* (ed. Matthiesen, R.) 263–288 (Humana
769 Press, 2013). doi:10.1007/978-1-62703-392-3_11.

770 53. Lau, A. M. C., Ahdash, Z., Martens, C. & Politis, A. Deuterios: Software for rapid analysis and visualization of data
771 from differential hydrogen deuterium exchange-mass spectrometry. *Bioinformatics* **35**, 3171–3173 (2019).

772 54. Liu, H. & Naismith, J. H. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid
773 mutagenesis protocol. *BMC Biotechnol.* **8**, 91 (2008).

774 55. Talavera, A. *et al.* Phosphorylation decelerates conformational dynamics in bacterial translation elongation factors.
775 *Sci. Adv.* **4**, (2018).

776 56. Schrimpf, W., Barth, A., Hendrix, J. & Lamb, D. C. PAM: A Framework for Integrated Analysis of Imaging, Single-
777 Molecule, and Ensemble Fluorescence Data. *Biophys. J.* **114**, 1518–1528 (2018).

778 57. Hellenkamp, B. *et al.* Precision and accuracy of single-molecule FRET measurements—a multi-laboratory
779 benchmark study. *Nat. Methods* **15**, 669–676 (2018).

780 58. Kudryavtsev, V. *et al.* Combining MFD and PIE for accurate single-pair förster resonance energy transfer
781 measurements. *ChemPhysChem* **13**, 1060–1078 (2012).

782 59. Tomov, T. E. *et al.* Disentangling subpopulations in single-molecule FRET and ALEX experiments with photon
783 distribution analysis. *Biophys. J.* **102**, 1163–1173 (2012).

784 60. Antonik, M., Felekyan, S., Gaiduk, A. & Seidel, C. A. M. Separating structural heterogeneities from stochastic
785 variations in fluorescence resonance energy transfer distributions via photon distribution analysis. *J. Phys. Chem. B*
786 **110**, 6970–6978 (2006).

787

A **B β -strand-swapped conformation** **C Canonical conformation**

A Canonical state**B β -strand-swapped state****C Unfolding intermediate**

RI unstructured
S4-S1-S2 sheet
active channel

RI partially structured
389-422 unfolding
S4 opening
hyperactive channel

β -sheet unfolding

B **2 mM ATP**

C **0 mM ATP**

D **2 mM ATP + G11a**

A ATTO488 and Alexa647 on 426-519
(β -SS conformation)

predicted $\langle R_{DA} \rangle = 35.0 \pm 11 \text{ \AA}$
predicted $E_{FRET} = 0.89$

B ATTO488 and Alexa647 on 479-519 (2BBO)

predicted $\langle R_{DA} \rangle = 58.4 \pm 9 \text{ \AA}$
predicted $E_{FRET} = 0.47$