

Peer Review File

Manuscript Title: CFTR function, pathology and pharmacology at single-molecule resolution

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The Levring et al. paper seeks to determine how dimerization of CFTR NBDs is coupled to ATP binding and hydrolysis, and to pore opening, using a combination of smFRET and electrophysiology. CFTR cryo-EM studies revealed two conformations: one in absence of phosphorylation and ATP with the pore-closed and NBDs separated by and the R domain sterically blocking their dimerization and the other phosphorylated with ATP bound and NBDs dimerized with two ATP molecules bound at their interface. The goal here was to elucidate the relationship between NBD dimerization and gating, understand how ion permeation is coupled to ATP hydrolysis and NBD isomerization and gain insight into the effect of disease-causing mutations and pharmacological modulation for therapeutics. The manuscript presents a detailed molecular explanation for how conformational change and channel opening are coupled and provides insight into modes for potentiator action. The authors do a commendable job of integrating multiple types of biophysical and functional data. The writing is clear (in most places), the experiments are well designed and thoughtfully interpreted. The study contributes significantly to the field. Several issues of data presentation and interpretation should be addressed.

Major comments:

1) The conclusions hinge on quantitative comparisons between smFRET data and electrophysiology data. Some clarifications are needed.

The properties of CFTR are compared to the unlabeled CFTRFRET in E1b-g and the fluorophore-labelled version in Fig. E1i-k and E2. For Fig. 1 and 3 it is not clear whether this is the labeled or unlabeled version. The authors should clarify this in text, Methods and legends in a consistent manner. A clear nomenclature would help. If Figs. 1 and 3 use the unlabeled version, authors should justify why. Also, authors should note if the constructs being compared differ in possessing a C-terminal GFP.

In E1i-j the current–voltage relationships suggest that CFTRFRET has higher conductance (authors should indicate how many patches went into this comparison). In addition, missing is a comparison of open probability, which is critical for the interpretation.

2) Even when the reader knows that patch recordings and FRET come from different experiments, placing these side by side or one above the other could give the confusing impression that they were measured simultaneously. This confusion is aggravated by sentences like this in the text (line 206):

“The W401A variant, which is capable of binding and hydrolyzing ATP via the consensus site, underwent rapid transitions between NBD-separated and -dimerized states that correlated with channel gating (Figure 2c).” This sentence should be changed (e.g. to “more closely resembled,” not correlated). To further clarify this, authors should add to Fig 2 a panel showing dimerization probability and compare open and dimerized dwell times the way they did in WT channels in Fig 1. Authors should also define for panel f ratio between probabilities of opening and dimerization which is the numerator.

3) The conclusion of Fig. 3 is that NBD dimerization precedes channel opening. The average rise upon rapid ATP perfusion of the phosphorylated channel of high FRET occupancy (dimerization) in smFRET and of channel opening in patch is faster for dimerization. Quantification was by mono-exponential fits. These should be superimposed on the data. Tau of dimerization is shown as <100 ms. The precise value and SEM should be given.

Fig. E7 is supposed to demonstrate that differences in kinetic behavior seen in Fig 3 are not due to differences in solute exchange between the two sets of experiments, but this figure only displays e-phys data. What is the equivalent dataset for smFRET, and where is it displayed? The Tau of patch solution exchange in Fig. E7 is 150 ms, slower than the <100 ms value given for smFRET in Fig. 3a, so it is unclear how well the exchange rates actually match.

The authors state: “We conclude ... that the mean first passage time between NBD dimerization and channel opening in productive instances is approximately 500 ms.” However, their Tau of opening is 490 ms and they begin to see channels opening almost immediately. The delay they seek is the one between dimerization and opening and would be a smaller value.

Minor comments:

- 1) E1j is mistakenly referred to as E1k in the text (line 161)
- 2) All electrophysiology traces should have current amplitude scale bar (e.g. missing in Fig. 2c)
- 3) Figure 1. E1371Q should be shown without ATP as well.
- 4) No mass spec data to demonstrate that phosphorylation is consistent/present across all conditions
- 5) Figure 2. 2cd ATP concentration and phosphorylation status should be stated in legend for clarity. It is also not so easy to determine by eye the contribution of open probability and dimerization to the coupling ratio by looking at 2e and 2f. It would help to additionally show the dimerization probability for the five different variants.
- 6) Example traces in E1i look different at lower membrane potentials (-90 and -150)-- is there a better representative trace or what is the hypothesized reason for this difference?
- 7) Analogous examples of current from WT channels should be shown for comparison in E1b and E1k.
- 8) Line 140 NDB typo
- 9) A question regarding comparison of electrophysiology and FRET data-- are there any integral membrane phosphatases which could contribute to the observed differences between channel opening and NBD dimerization probability?
- 10) The authors should make clear that disease-causing CFTR variants have multiple modes/effects,

and should describe the direction in which a pharmacological agent should act (i.e. promote channel opening as opposed to promoting channel closure, etc). On p. 9, the effects of G551D and L927P on channel gating should be described first, before explaining effects on FRET-based readouts.

11) Presence of a native lipid membrane or detergent environment may affect the dynamics of the channel. To address this, the authors perform a set of FRET experiments in proteoliposomes, which should more accurately approximate a cell membrane. The FRET dynamics and dependence on ATP look similar to what they see in detergent, bolstering confidence in their experiments. One odd thing is that only a small fraction of channels are ATP sensitive. They attribute this to channel orientation within the bilayer. If the 10xHis tag is located on the NBD side of the protein, it is unclear why any immobilized molecules should have inward-facing NBDs which are inaccessible to ATP.

12) It is not clear if the kinetic model adds much to the discussion, but it also is presented with appropriate caveats. Does the model make a prediction in a way that could be used to test the alternative hypotheses presented in the introduction of the paper?

13) Figure 1i shows that a very large percentage of channel openings have a dwell time of at least 10 ms, which could be detected using smFRET. Though it may not be feasible for this study, it would be highly convincing if it were possible to design a FRET sensor of channel pore activity that approximates the electrophysiological readout? This would allow direct comparison within a single controlled system.

Referee #2 (Remarks to the Author):

The current manuscript by Levring et al. presents incredibly comprehensive studies of CFTR function as an ATP-gated ion channel; these studies include electrophysiological recordings of CFTR channel activity, real-time monitoring of the dimerization of CFTR's two nucleotide binding domains (NBDs) with smFRET imaging, biochemical measurements of ATP hydrolysis rate, and computer simulations based on a proposed gating model. Here are the main findings and their mechanistic implications:

1. The NBDs are readily dimerized by ATP binding to the two ATP-binding sites in fully phosphorylated CFTR, but the dimerized state (high FRET) is much more stable than the duration of either channel open state or the closed state at a saturating concentration of ATP. This uncoupling between NBD dimerization and channel gating suggests that multiple cycles of gate opening and closing can occur with NBDs remained dimerized (inferred from high FRET), contradicting previous idea of a strict coupling of NBD dimerization to gate opening and hydrolysis-triggered NBDs separation (low FRET) to gate closing (Csanady et al., 2010).

2. By manipulating ATP binding to each site (Y1219A for the consensus site and W401A for the degenerate site), they provided evidence for NBD dimerization by ATP occupancy at either site. While both NBD dimerization and gating were mostly preserved in W401A, Y1219A channels showed robust NBD dimerization but little activity, supporting previous reports that gating of CFTR is mainly through ATP binding to the consensus site (Vergani et al., 2003; Zhou et al., 2006).

3. By comparing the rate of NBD dimerization and the rate of channel activation upon sudden addition of ATP, they found that NBD dimerization takes place much faster than gate opening, contradicting previous proposition that NBD dimerization constitutes the rate-limiting step in CFTR

gating (Vergani et al., 2005). Although NBD dimerization precedes gate opening, NBD dimerization itself is not sufficient to open the gate because dimerization (high FRET) can be seen in conditions when there is little channel activity (e.g., upon ATP washout, or in the presence of inhibitory concentration of ADP).

4. Supporting the idea that ATP at the consensus site plays a critical role in opening the gate, the authors demonstrated that the disease-causing mutant G551D at the consensus site exhibits defective gating and NBD dimerization. Interestingly, the dimerized NBDs in G551D show FRET between high and low FRETs, suggesting the presence of an intermediate NBD dimer proposed previously (Tsai et al., 2010). Similar but not identical intermediate FRET was seen in another pathogenic mutation L927P (in TM8), which is 50 Å away from the consensus site. This latter observation suggests a long-distance effect of the L927P mutation on NBD dimerization, but the nature of this allosteric effect is unclear.

5. By testing the effects of CFTR potentiators ivacaftor and GLPG1837, they found a dramatic gating effect with minimal and moderate changes of NBD dimerization in G551D and L927P variants respectively. These are interesting observations, but it seems that not much additional insight can be gained (see below).

6. These results were put together to form the basis of a kinetic model, which was further examined through computer simulations. Although the proposed kinetic mechanism recapitulates many of the experimental data, some simulated results do deviate from actual data.

No doubt, these are important studies that reveal many unexpected phenomena, which lay a solid foundation for settling the debates over the coupling mechanism between NBD dimerization and gate opening, as well as for advancing our overall understanding of the structural-functional relationships of CFTR gating. This reviewer applauds the authors for such a remarkable attainment.

Major comments:

1. The two conformations of NBDs—separated versus dimerized—can be resolved by the shift of FRET from 0.25 to 0.49 (Fig. 1). In Fig. 1e, the FRET signal remains stable at 0.49 at 10 mM ATP while the electrophysiological recording shows repeated opening and closing of the WT-CFTR channel. The authors thus concluded that the NBDs remain dimerized throughout multiple rounds of ATP binding/hydrolysis (Fig. 5). Conceptually, the consensus site in the “dimerized NBDs” must open up a space wide enough to allow the exchange of hydrolytic products for the next ATP. Indeed, in ATP washout (and Mg addition) experiments (Fig. 3b, c), transition from high FRET to low FRET was observed immediately upon ATP removal (or Mg addition). As the waiting time is too short for ATP dissociation from the degenerate site, this result suggests that upon ATP hydrolysis at the consensus site, disengagement of the two NBDs does occur. The reason this high to low FRET transition is not seen in the continuous presence of millimolar ATP may be due to rapid rebinding and re-dimerization of the disengaged NBDs at millimolar ATP. This scenario supports the notion that the state “degenerate site ATP, pore closed” should assume a different dimeric state with a cracked consensus site to allow step 6 (Fig. 5) to take place. The canonical NBD dimer in the cryo-EM structure leaves no room for ATP to diffuse into (or out of) its binding pocket.

2. I wish the authors would have elaborated the rationale behind choosing positions 388 and 1435 for their FRET pair. T388 is at a position without a concrete secondary structure. Wouldn't this positioning result in some uncertainty in FRET measurements as the FRET efficiency is determined by not only the distance but also the relative orientation between the donor and the acceptor? It would be nice if another pair is chosen to replicate some of the key observations described above.

3. The W401A and Y1219A mutations were designed to weaken ATP binding. The authors observed that the NBDs of W401A and Y1219A mutants can still dimerize, and thus concluded that ATP binding at either site is sufficient for NBD dimerization (P7, line 215). Such conclusion is based on the assumption that the degenerate site in W401A (or the consensus site in Y1219A) must be vacant at 3 mM ATP. However, in electrophysiological study, the Y1219G mutation reduces the apparent affinity for ATP to a $K_{1/2}$ of ~ 5 mM (Zhou et al., 2006). If we assume that Y1219A affects the affinity similarly, at least a portion of the Y1219A channel population in Fig. 2 would still have ATP bound to the consensus site at 3 mM ATP. Given that the degenerate site has much higher affinity for ATP, it is unlikely that the degenerate site in W401A would be vacant at 3 mM ATP. Thus, the data in Fig. 2 only support that NBDs of W401A and Y1219A can dimerize. The occupancy of ATP at each site cannot be determined, and thus whether a single ATP is enough to trigger dimerization cannot be inferred conclusively. However, the conclusion may not be incorrect once data in Fig. 1j are taken into consideration. The ATP concentration dependence of high to low FRET transition rate suggests the existence of different states of NBD dimer with one or both sites occupied.

4. Some of the kinetic steps in Fig. 5 need justification/explanation:

a. Dissociation/binding of ATP in the degenerate site happens when the NBDs are separated (step 7, 8), but dissociation of hydrolytic products and binding of ATP to the consensus site happen when the NBDs are dimerized (step 4, 5, 6). Some intermediate conformations of NBDs need to exist.

b. Why step 3 is drawn differently in Fig. 5 and Fig. E11? Also, why did the authors propose only the open state hydrolyzes ATP, but not 2 ATP pore flicker-closed state or 2 ATP pore closed state?

c. Is the flicker-closed state the same as the cryo-EM structure of E1371Q-hCFTR? It's been known that many of the flickery events seen at negative membrane potentials are not likely real functional "state". Because of their high voltage dependence (see Fig. E1i.), many of the events are considered blocking of the pore by cytosolic large anions. To get the right kinetic parameter for the true flicker-closed state, one has to analyze data from recordings at positive membrane potentials.

d. There is a mistake both in the text and in the figure legend. Steps 6 – 8 represent transitions among closed states; therefore, it is not a new gating cycle, which by definition should include a closed event and an open event.

Minor comments:

1. I am not sure I truly understand the meaning of the term "hierarchical" gating mechanism.

2. P2, line 3: Do you mean electrophysiological properties?
3. P5, line 140: It should be "...to a stably NBD-dimerized state."
4. P6, line 157: Previous electrophysiological studies of R117H-CFTR and zebrafish CFTR, as well as the cryo-EM structure of E1371Q all indicate the presence of conductive and non-conductive states with dimerized NBDs. Please more explicitly state the unique insight from these data.
5. P6, line 161: ...with ATP concentration (Figure 1j), not 1k.
6. P6, line 172: The ADP experiments are interesting in that the low FRET value seems different from that without ATP. Is it reproducible? If it is, doesn't that suggest different degrees of disengagement of NBDs?
7. P7, line 191: The time constant for ligand exchange at the degenerate site is around 30 s (Tsai et al., 2010), which interestingly is very similar to the duration of high FRET upon ATP washout (Fig. 3b).
8. P7, line 226: It should be open probability, not opening probability. (In other parts of the text too.)
9. P8, line 237: The changes of FRET upon sudden addition of ATP show heterogeneity. This observation may be related to a recent paper by Yeh et al. (2021). The stated similarity to W401A and double exponential current rising upon addition of ATP (Fig. 1b and E1b) all remind this reviewer of the results published in this paper, which shows different closed channel conformations (perhaps different NBDs conformations) once ATP is removed.
10. P8, line 255: Of note, rapid removal of ATP caused a double exponential decay of WT-CFTR currents (Fig. 2B in Lin et al., 2014) with time constants ($\tau_1 < 1$ s and $\tau_2 = 29$ s) similar to these values reported for NBD dimerization relaxation. These shouldn't be just coincident.
11. P9, line 277: Not a correct reference and interpretation. The cited ligand exchange experiments lead to two different turn-over rates of ATP and the idea of a "partial dimer." On the other hand, by carrying out different ligand exchange experiments, Jih et al. (2012) propose a re-entry pathway featuring repetitive cycles of ATP turnover in a single opening burst. Both studies done by Hwang and colleagues invoke a partial dimer state where the consensus site is open up to allow replenish of ATP. They do not suggest that ATP turnover can occur in a single canonical NBD-dimerized conformation, where the ligand is occluded.
12. P10, line 312-314: I don't understand what the authors mean by the similar nature in "the similar nature of both mutations' impacts on NBD dimerization and channel opening". G551D and L927P actually exhibit different behaviors in their experiments. In Fig. 4b, the transitions between high and low FRET in G551D is different from those of L927P. Also, GLPG1837 drastically changes L927P's FRET pattern, but barely affects G551D. Moreover, electrophysiological properties of these two mutants and pharmacological effects of CFTR potentiators should be discussed in a more quantitative manner (see comment 18 below) before one can make more definitive conclusions.

13. P11, line 347: It should be noted that both VX-770 and GLPG1837 work on a CFTR variant with its NBD2 completely removed (Yeh et al., 2017; Yeh et al., 2015). These drugs also exert dramatical gating effects on G551D whose NBDs may not assume a canonical NBD dimer state (Lin et al., 2014; Yeh et al., 2017) (Fig. 4 in current study).

14. P11, line 375-377: Previous studies suggest that reagents such as NPPB and high-affinity ATP analogs, act synergistically with Ivacaftor and GLPG1837 on G551D channels by promoting NBD dimerization (Lin et al., 2016; Yeh et al., 2017). The authors may consider incorporating these studies in the Discussion.

15. Figure 1: I suggest the authors unify the spelling of "dimerization." It is dimerization in the text but dimerisation in Fig 1 and Fig 4.

16. Figure 1e: If I understand correctly, the electrophysiological recordings are from WT-CFTR, not from the CFTRFRET protein. Wouldn't it be a better comparison to use the electrophysiological recording of CFTRFRET protein here? Of note, the CFTRFRET construct altered the majority of the endogenous cysteine residues; this alteration could affect CFTR gating as seen in the literature. In Figure E1, the authors demonstrated their ability to use CFTRFRET proteins in electrophysiological experiments and concluded that its gating kinetics are similar to WT-CFTR (but see comment 19 below).

17. Figure 1j: I don't understand the data points at 0 ATP. Do the authors still observe FRET transitions even without ATP, meaning that the NBDs dimerize without ATP? In 1e, FRET always stays at low FRET.

18. Figure 4b: In response to GLPG1837, the current trace of G551D barely changes as if GLPG1837 has little effect. This potentiation effect is far from the reported ~30-fold stimulation shown in Fig. 4g. Please comment on the discrepancy and consider replacing the recording with a more representative one. Similar concerns are raised for the effect of GLPG1837 on L927P. The single-channel activity in 4b does not change 20-fold as indicated in Fig. 4g.

19. Figure 4f: It is shown that the P_o of E1371Q is ~0.7 and increases to ~0.8 with GLPG1837. It is puzzling how E1371Q has a P_o of only 0.7. Numerous studies showed that with hydrolysis eliminated, the closing rate of E1371Q (or E1371S) is >100-fold slower than WT, resulting in a P_o close to unity (Bompadre et al., 2005; Csanady et al., 2013; Vergani et al., 2003; Yu et al., 2016). Indeed, the E1371Q single channel in Figure 1f seems to be almost always open, though it is hard to determine whether those brief downward deflections are flicker closures or interburst closures with this time scale. It would be helpful to expand an E1371Q single channel trace to allow a better assessment of channel behavior in a quantitative manner.

20. Figure E1i: From the raw trace, the P_o of the FRET construct is apparently higher than the WT. The open burst time of FRET variant is visibly longer than WT. Is this a consistent finding? Please comment on the discrepancy.

21. Figure E8g: What is the second arrow below the ADP+Pi for?

22. Figure E11b, d, and e: In b, the simulated single-channel trace shows a much longer open time than the experiment, while the closed time seems not affected much (or slightly shorter). However, in d and e, the experimental and simulated dwell times match perfectly but the two closed times deviate significantly. Please comment on this discrepancy.

23. Figure E11h: The experimental ATP turnover rate is 15/min (or 0.25/s), corresponding to one ATP hydrolyzed every four seconds, a much longer time than a gating cycle time of wildtype CFTR (~1 s). If, as proposed, multiple ATP hydrolysis cycles occur, the theoretical ATP hydrolysis rate must be even higher than 1/s. As stated, there is a likelihood that this biochemical measurement is not very accurate because of imperfect protein preparation. Alternatively, the proposed gating model is overly simplified. Either way, I wonder whether the simulation really provides more insights into the gating mechanism of CFTR. Sometimes it may be better to be vaguely right than precisely wrong. I leave this to the authors to decide.

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Referee #3 (Remarks to the Author):

The manuscript by Levring et al describes characterization of molecular cycles involved in activation of the CFTR channels. Specifically, single molecule FRET studies done in parallel with single channel electrophysiology enable to characterize the relationship between ATP binding, hydrolysis and channel opening. Furthermore, how disease-causing mutations and therapeutic correctors modulates these events are studied and discussed.

The study is very nicely crafted, the experiments are very well performed, and the conclusions are convincing.

This work brings a clear view on a long-standing issue in the CF field and deserves publication.

Nevertheless, a number of points require clarification.

1/ FRET values

CryoEM structures of human CFTR have -so far- captured 2 conformations with the NBDs either separated or dimerized. Structural data on ABC structures suggest more extended conformational diversity with several different substates. The differences in FRET values observed in the present manuscript may be indicative of such diversity.

Therefore, the authors need to clearly assign -when possible- the link between the FRET value and the identified conformer .

The ATP-free CFTR shows a low FRET state at 0.25. Does this match the cryo-EM structures of the NBD separated state ? Similarly does the 0.49 FRET value match the NBD-dimerized state for the used probes?

The authors need to run careful simulations (for examples with FPS, see Kalinin et al) with the appropriate probe models to evaluate the calculated FRET value for each conformation.

ATP-bound dephosphorylated CFTR shows a 0.28 FRET value. How does that translate in terms of

distance change given the R0 for these probes ?

The corresponding cryo-EM structure showed in Figure E4 does not address these issues, in great part due to the limited resolution. In fact, the authors only vaguely describe “proximal changes in local structure”. What does that mean? In my opinion in its current state this structure does not bring relevant information and should be either improved or removed from the manuscript.

2/ The E1371Q mutant appear to be locked in the high FRET state during the time course of the experiments, at least in the trace shown (100s, Fig1f). However, the distribution on Fig1c seems to show a significant low FRET population (~5-10%), pretty much identical to that seen for the “wt” CFTR (which can hydrolyze ATP and thus should spend more time in the NBD separated). In that line, it is unclear to me why the authors see no NBD separation in this mutant after phosphatase treatment (Fig E3). One may expect to see some separation over the course of the measurement.

In the same Figure, can the author comment on the difference in ATP concentration dependence of channel gating vs NBD dimerization? At 3mM ADP 80% of the channels are still in dimers but gating is almost gone.

3/ Very little is shown regarding the membrane-embedded measurements. What does the sentence “While the fraction of the molecules responsive to ATP was significantly reduced” refer to ? Only single traces are shown. No statistics (“significantly” ?).

4/ ATP binding sites

The authors use the W401A and Y1219A mutants originally described by Hwang and colleagues (although mutated into Gly in that study). What do the authors mean by “destabilize ATP binding” ?

It should be noted that the mutations decrease affinity but may not prevent it. For example, W401A is still able to bind ATP (albeit with lower affinity) and 3mM is quite a large concentration.

Perturbing the ATP binding at the degenerate site may also alter the conformational equilibrium of NBD1 that we recently described (Scholl et al, 2021)

On P8 the authors state that for mutant Y1219A “ATP binds only at the degenerate site”. This is not proven.

On P7, the authors describe that “the Y1219A variant, which binds ATP mainly at the degenerate site, transitioned between NBD-dimerized and separated states slowly, and very rarely exhibited channel opening” but they only show single traces. They should show distributions.

5/ Figure E3K is unclear to me. Is it expected to see such a significant effect at 0.1 and 0.3 mM ADP while 3mM ATP is maintained (and while Fig E3I show predominantly NBD in dimer form)? I assume that the last part of the graph has 0mM ADP (correct?). Why is the rebound much faster than what is observed during the first 2 mins?

6/ The coupling ratio seems unclear to me. What if the two would anticorrelate ? The coupling ratio would still be 1 ?

7/ P7 the authors state “We infer from these observations that channel opening probability is enhanced by interactions of CFTR with the terminal phosphate moiety of the ATP molecule bound within the consensus site.”. This should be readily testable as the terminal phosphate at the consensus site is coordinated by K1250 and T1246. Point mutations should support this statement.

8/ Mutation G551D

The authors show an intermediate FRET value of 0.37. The authors suggest that the mutation introduce a “steric and electrostatic repulsion at the NBD interface” which is very vague.

An Asp at position 551 will likely prevent binding of ATP at the consensus site (at least in a NBD dimerize state) because the acidic side chain would directly and closely face the terminal phosphate. This suggests that the NBD1-NBD2 dimer is not formed at the consensus site and that the protein is adopting an intermediate/different conformation. This also suggest that the ATP “stimulation” is due to dimerization at the degenerate site.

Therefore the “dimerized dwell time” on Fig E9E may actually not correspond to dimerized state as “expected”, ie. where both sites are closed, as observed by Cryo EM.

In addition, the statement on P10 that “Importantly, both the G551D and L927P variants exhibited ATP-dependent FRET response indicative of wild-type ATP binding affinities” is hard to reconcile with the position of D551 in the mutant

Furthermore, the effect of potentiators on G551D are not clear, and not discussed.

Ivacaftor was approved to treat G551D patients, we would expect to see an effect. The authors show some individual traces with GLPG1837, but not the approved drug Ivacaftor (why?) from which there seem to be no significant effect. The authors should explore and or discuss this.

In contrast, stimulation of the L927P mutation does lead to fully dimerized state which does seem to correlate with increase channel opening (although Fig 4f shows quite modest improvement).

Author Rebuttals to Initial Comments:

Point-by-point response

We sincerely thank the Reviewers for their time and constructive comments, which have helped to significantly improve the clarity and scholarship of the manuscript.

Reviewer #1 (Remarks to the Author):

The Levring et al. paper seeks to determine how dimerization of CFTR NBDs is coupled to ATP binding and hydrolysis, and to pore opening, using a combination of smFRET and electrophysiology. CFTR cryo-EM studies revealed two conformations: one in absence of phosphorylation and ATP with the pore-closed and NBDs separated by and the R domain sterically blocking their dimerization and the other phosphorylated with ATP bound and NBDs dimerized with two ATP molecules bound at their interface. The goal here was to elucidate the relationship between NBD dimerization and gating, understand how ion permeation is coupled to ATP hydrolysis and NBD isomerization and gain insight into the effect of disease-causing mutations and pharmacological modulation for therapeutics. The manuscript presents a detailed molecular explanation for how conformational change and channel opening are coupled and provides insight into modes for potentiator action. The authors do a commendable job of integrating multiple types of biophysical and functional data. The writing is clear (in most places), the experiments are well designed and thoughtfully interpreted. The study contributes significantly to the field. Several issues of data presentation and interpretation should be addressed.

Major comments:

1) The conclusions hinge on quantitative comparisons between smFRET data and electrophysiology data. Some clarifications are needed. The properties of CFTR are compared to the unlabeled CFTR_{FRET} in E1b-g and the fluorophore-labelled version in Fig. E1i-k and E2. For Fig. 1 and 3 it is not clear whether this is the labeled or unlabeled version. The authors should clarify this in text, Methods and legends in a consistent manner. A clear nomenclature would help.

We revised the text, figure legends, and methods to clearly indicate which CFTR construct was used in every experiment.

If Figs. 1 and 3 use the unlabeled version, authors should justify why. Also, authors should note if the constructs being compared differ in possessing a C-terminal GFP.

Macroscopic current measurements using inside-out patches (e.g. **Extended Data Figure 1b-g**) were not labeled with fluorophores because efficient and specific labeling of CFTR cannot be achieved in the heterogeneous cellular membrane. C-terminally GFP-fused CFTR were used in these experiments as it has been shown previously that C-terminal fusion with GFP does not affect CFTR gating (https://bpsbioscience.com/media/wysiwyg/60506_3.pdf). In our revised manuscript, we now provide further evaluations of fluorophore-labelled CFTR_{FRET} that support our conclusion that it recapitulates wild-type CFTR behaviour (see **Extended Data Figure 1**). Site-specifically labeled CFTR_{FRET} solubilized in detergent (**Figure E2a-b**) hydrolyzed ATP with

41 a rate nearly identical to that of wild-type CFTR (**Figure E1h**). Fluorophore-labelled CFTR_{FRET}
42 and wild-type CFTR (without fluorophore labels) reconstituted into synthetic planar lipid bilayers
43 also exhibited similar current–voltage relationships, open probabilities, and responses to
44 GLPG1837 (**Figure E1i-m**).

45
46 In E1i-j the current–voltage relationships suggest that CFTR_{FRET} has higher conductance
47 (authors should indicate how many patches went into this comparison). In addition, missing is a
48 comparison of open probability, which is critical for the interpretation.

49 We have now discussed the higher conductance on page 4 “*Single-channel conductance of*
50 *fluorophore-labelled CFTR_{FRET} was slightly higher (Figure E1i-j), possibly due to the C343S*
51 *substitution, a residue bordering the pore.*” This difference does not affect interpretation of single-
52 molecule FRET measurements, as it does not relate to the dynamics of the nucleotide-binding
53 domains. We also noted that the data points in **Extended Data Figure 1j** reflect means and
54 standard errors of 3-18 channels reconstituted in synthetic planar lipid bilayers.

55 Per the Reviewer’s suggestion, we determined the open probability of the fluorophore-conjugated
56 CFTR_{FRET} variant channels and show that it is essentially identical to that of the wild-type,
57 unlabeled CFTR (**Extended Data Figure 1l**).

58
59 2) Even when the reader knows that patch recordings and FRET come from different experiments,
60 placing these side by side or one above the other could give the confusing impression that they
61 were measured simultaneously. This confusion is aggravated by sentences like this in the text (line
62 206): “The W401A variant, which is capable of binding and hydrolyzing ATP via the consensus
63 site, underwent rapid transitions between NBD-separated and -dimerized states that correlated with
64 channel gating (Figure 2c).” This sentence should be changed (e.g. to “more closely resembled,”
65 not correlated). To further clarify this, authors should add to Fig 2 a panel showing dimerization
66 probability and compare open and dimerized dwell times the way they did in WT channels in Fig
67 1. Authors should also define for panel f ratio between probabilities of opening and dimerization
68 which is the numerator.

69 We thank the Reviewer for pointing out this confusion and we have now revised the text to clearly
70 indicate that channel recordings and FRET were two different experiments. For example, on page
71 7: “*Relative to wild-type CFTR, the W401A variant, which is capable of binding and hydrolyzing*
72 *ATP at the consensus site, underwent comparatively rapid transitions between NBD-separated*
73 *and -dimerized states that **more closely resembled** the dynamics of pore opening **measured in***
74 *electrophysiological recordings*”. And: “*By contrast, the Y1219A variant, which binds ATP*
75 *principally at the degenerate site, slowly transitioned between NBD-dimerized and separated*
76 *states ... **single-channel measurements** of the Y1219A variant exhibited only sporadic opening*
77 *events*”. On page 8: “*Here, we **separately tracked** the time-courses of NBD dimerization and*
78 *macroscopic current increase upon application of saturating ATP (3 mM)*”.

79 As requested, we also added a panel quantifying dimerization probability of the variants (**Figure**
80 **2f**), and a panel comparing the open and NBD-dimerized dwell times for the W401A CFTR variant
81 (**Figure 2d**). The Y1219A variant has an open probably of nearly zero and gates slowly, preventing
82 us to quantify its open dwell time.

83 The definition of coupling ratio is now clarified in the **Figure 2** legend as: “open probability
84 divided by dimerization probability.”

85

86 3) The conclusion of Fig. 3 is that NBD dimerization precedes channel opening. The average rise
87 upon rapid ATP perfusion of the phosphorylated channel of high FRET occupancy (dimerization)
88 in smFRET and of channel opening in patch is faster for dimerization. Quantification was by
89 mono-exponential fits. These should be superimposed on the data. Tau of dimerization is shown
90 as <100 ms. The precise value and SEM should be given.

91 As requested, we now superimposed mono-exponential fits on the individual time-courses as
92 **Extended Data Figure 7a-b** in the revision. In **Figure 3a** we reported the means and standard
93 errors of $\tau_{opening}$ from 42 inside-out membrane patches. We did not specify the relaxation time
94 constant for dimerization as the measured rate may be limited by mixing time and therefore not
95 inform on the biologically relevant transition. The fitted values for $\tau_{Dimerization}$ (~100 ms) are on the
96 same scale as measured rates of mixing ($\tau_{Exchange} = 115$ ms, see below), indicating the measured
97 dimerization response may be limited by the rate of solvent mixing in the fluorescence microscope.
98 For these reasons, we believe it is appropriate to report the upper bound on the rate of dimerization
99 instead of a precise value.

100

101 Fig. E7 is supposed to demonstrate that differences in kinetic behavior seen in Fig 3 are not due to
102 differences in solute exchange between the two sets of experiments, but this figure only displays
103 e-phys data. What is the equivalent dataset for smFRET, and where is it displayed? The Tau of
104 patch solution exchange in Fig. E7 is 150 ms, slower than the <100 ms value given for smFRET
105 in Fig. 3a, so it is unclear how well the exchange rates actually match.

106 We performed additional experiments to measure the solute exchange within the microfluidics of
107 our single-molecule imaging microscope (**Extended Data Figure 7e**). The estimated exchange
108 rate is $\tau_{Exchange} = 115$ ms. We revised the text on pages 8-9 to include this new data: “The rate of
109 channel opening ($\tau_{opening} = 490 \pm 40$ ms) (**Figure 3a, E7a**), was approximately 3-fold slower than
110 the solvent exchange rate of the perfusion system ($\tau_{exchange} \sim 150$ ms) (**Figure E7c,d**). By contrast,
111 the fitted rates for NBD dimerization from FRET measurements ($\tau_{Dimerization} \sim 100$ ms) were on the
112 same scale as the solvent exchange rate in the fluorescence microscope ($\tau_{exchange} = 115$ ms) (**Figure**
113 **E7b,e**). Thus, the observed delay in current activation could not be ascribed to differences in rates
114 of mixing of the two experimental methods. We therefore conclude that the observed delay reflects
115 conformational changes within the NBD-dimerized state that precede channel opening...”

116 The authors state: “We conclude ... that the mean first passage time between NBD dimerization
117 and channel opening in productive instances is approximately 500 ms.” However, their Tau of
118 opening is 490 ms and they begin to see channels opening almost immediately. The delay they
119 seek is the one between dimerization and opening and would be a smaller value.

120 Given the imprecision of our estimate of the rate of the initial dimerization step ($\tau_{Dimerization} < 100$
121 ms), we cannot confidently deconvolve the individual sequential rates that lead to the pore open
122 state. However, the Reviewer is correct that the delay time must be corrected relative to $\tau_{Opening} =$
123 490 ms. We modify the text in the revision to include this consideration (page 9): “... the mean
124 first passage time of this process is approximately 400-500 ms.”

125

126 Minor comments:

127

128 1) E1j is mistakenly referred to as E1k in the text (line 161)

129 Corrected.

130

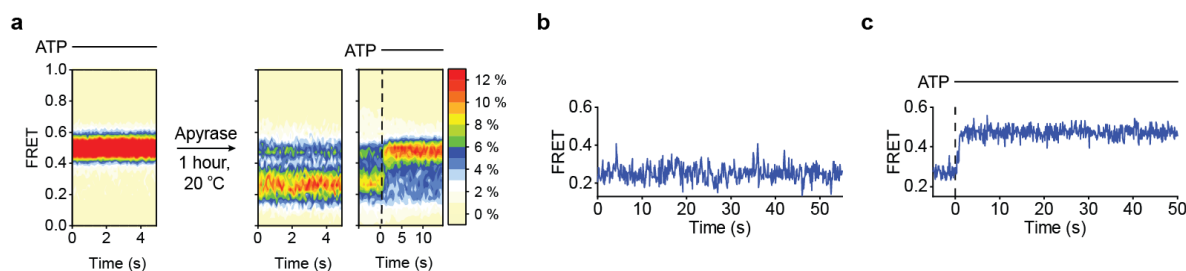
131 2) All electrophysiology traces should have current amplitude scale bar (e.g. missing in Fig. 2c)

132 Corrected.

133

134 3) Figure 1. E1371Q should be shown without ATP as well.

135 We agree that addition of panels reflecting ATP-free E1371Q CFTR would nicely demonstrate
136 retention of ATP-dependence for this variant. However, due to the slow rate of non-hydrolytic
137 ATP dissociation we have found it difficult to isolate a homogenous population of ATP-free
138 E1371Q CFTR molecules. Incubation with Apyrase for 1 hour at 20 °C was insufficient to produce
139 a uniformly low-FRET (NBD-separated) population (**Figure R1**).



140

141 **Figure R1 | Nucleotide-free E1371Q CFTR**

142 **a.** Contour plots of phosphorylated E1371Q CFTR. CFTR was incubated with 3 mM ATP (left), treated with Apyrase for 1 hour
143 at 20 °C in nucleotide-free buffer (middle), and 3 mM ATP was then reintroduced (right). **b-c.** Example single-molecule traces for
144 E1371Q CFTR in ATP-free buffer (**b**) and upon rapid delivery of 3 mM ATP (**c**).

145 Nonetheless, these experiments confirm that dimerization of E1371Q is ATP-dependent and
146 reversible, and are consistent with electrophysiological recordings. However, as we have been
147 unable to perform ‘ATP-free’ measurements of E1371Q CFTR at steady state, we believe that
148 addition of such panels to **Figure 1** may confuse the reader.

149 4) No mass spec data to demonstrate that phosphorylation is consistent/present across all
150 conditions.

151 In electrophysiological measurements protein kinase A (PKA) was applied until current amplitude
152 saturated (see e.g. **Figure 1b**). This state should be consistently phosphorylated.

153 In cases where a real-time read-out is not available such as for ATP hydrolysis or single-molecule
154 FRET measurements, CFTR was incubated with PKA and ATP for 30-60 minutes at room
155 temperature. We have experimentally determined that under these conditions the response to PKA-
156 phosphorylation saturated within 1-2 minutes (**Figure 1d**).

157 Similarly, CFTR was treated with Lambda protein phosphatase for 30 minutes at room temperature
158 to dephosphorylate the R-domain. Under these conditions the response to phosphatase also
159 saturated within 1-2 minutes (**Extended Data Figure 3r**).

160 While a minor population of molecules may be refractory to kinase or phosphatase treatment, we
161 would expect for this to be consistent across experiments. Mass spec quantification of the
162 phosphorylation status in each experiment would be a significant undertaking and beyond the
163 scope of the present study.

164

165 5) Figure 2. 2cd ATP concentration and phosphorylation status should be stated in legend for
166 clarity. It is also not so easy to determine by eye the contribution of open probability and
167 dimerization to the coupling ratio by looking at 2e and 2f. It would help to additionally show the
168 dimerization probability for the five different variants.

169 We have now specified ATP concentration to be 3 mM in the legend of **Figure 2**. We also add a
170 panel that shows the dimerization probability for the nucleotide binding site variants (**Figure 2f**).

171 6) Example traces in E1i look different at lower membrane potentials (-90 and -150)-- is there a
172 better representative trace or what is the hypothesized reason for this difference?

173 We have updated the single-channel traces of the current-voltage relationship in **Extended Data**
174 **Figure 1i** to better reflect open probabilities of the CFTR_{FRET} variant.

175 7) Analogous examples of current from WT channels should be shown for comparison in E1b
176 and E1k.

177 Example current traces of wild-type CFTR have been added to **Extended Data Figure 1b and 1k**.

178 8) Line 140 NDB typo

179 Corrected.

180 9) A question regarding comparison of electrophysiology and FRET data-- are there any integral
181 membrane phosphatases which could contribute to the observed differences between channel
182 opening and NBD dimerization probability?

183 Channel open probabilities were determined using purified CFTR reconstituted into synthetic lipid
184 bilayers. NBD dimerization probability was determined by FRET measurements performed with
185 purified protein. Neither system contains cellular phosphatases.

186 10) The authors should make clear that disease-causing CFTR variants have multiple
187 modes/effects, and should describe the direction in which a pharmacological agent should act (i.e.
188 promote channel opening as opposed to promoting channel closure, etc). On p. 9, the effects of
189 G551D and L927P on channel gating should be described first, before explaining effects on FRET-
190 based readouts.

191 As suggested, the channel gating and ATPase activities of the G551D and L927P mutants are now
192 described before the FRET data. The effects of the potentiators are now specified as to “*promote*
193 *channel opening*” (pages 10-11).

194
195 11) Presence of a native lipid membrane or detergent environment may affect the dynamics of the
196 channel. To address this, the authors perform a set of FRET experiments in proteoliposomes, which
197 should more accurately approximate a cell membrane. The FRET dynamics and dependence on
198 ATP look similar to what they see in detergent, bolstering confidence in their experiments. One
199 odd thing is that only a small fraction of channels are ATP sensitive. They attribute this to channel
200 orientation within the bilayer. If the 10xHis tag is located on the NBD side of the protein, it is
201 unclear why any immobilized molecules should have inward-facing NBDs which are inaccessible
202 to ATP.

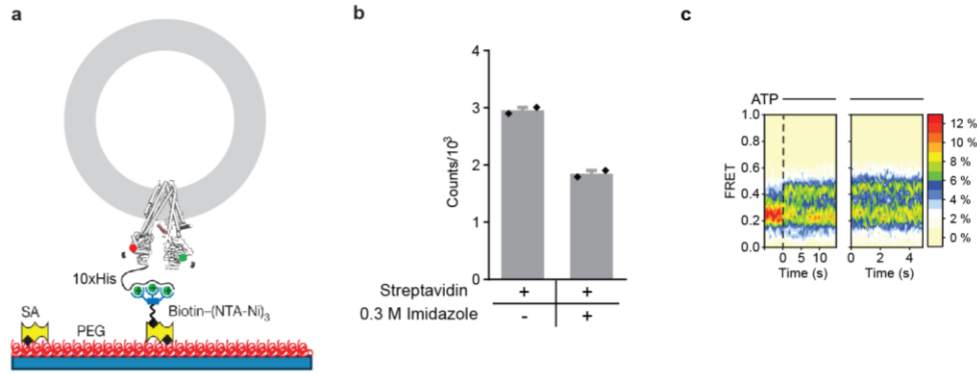
203 Immobilization of proteoliposome-reconstituted CFTR through the C-terminal His-tag (**Figure**
204 **R2a**) was designed to select CFTR molecules with the ‘NBDs-out’ orientation, as the Reviewer
205 correctly specifies. This orientation is necessary for sensitivity to externally applied membrane
206 impermeable reagents.

207 In practice, proteoliposome experiments with CFTR proved challenging for a number of reasons.
208 First, although CFTR was reconstituted at a low protein-to-lipid ratio, aiming to produce vesicles
209 with zero to one CFTR molecules incorporated, the sample may also contain vesicles containing
210 more than one CFTR molecule. In these cases vesicles may be immobilized via a non-fluorophore
211 conjugated CFTR molecule with the ‘NBDs-out’ orientation, while FRET is being measured by a
212 passenger molecule with the ‘NBDs-in’ orientation. The proteoliposome reconstitution procedure
213 may also negatively impact CFTR folding and function so as to produce inactive protein within
214 the bilayer.

215 We also found that nearly half of our proteoliposomes tethered within microfluidic chambers were
216 unable to be released by imidazole treatment (**Figure R2b**). By contrast, near-quantitative release
217 from the surface with imidazole was demonstrated with the detergent-solubilized sample
218 (**Extended Data Figure 2g**). We have previously observed that anionic lipids (e.g. POPS) may
219 associate with Ni²⁺-coated surfaces and the POPS content of our vesicles may contribute to this
220 non-specific association issue. The extent to which such issues relate to losses in CFTR activity in
221 this context is not presently known.

222 Approximately half of the immobilized and phosphorylated CFTR channels displayed sensitivity
223 to externally applied ATP (**Figure R2c**). Consistent with a permanent loss of CFTR activity
224 associated with the proteoliposome reconstitution or immobilization process, the responsive and
225 non-responsive molecules did not dynamically interconvert within the time-scale of imaging.

226 These complications were a component of decision to focus our quantitative analysis on digitonin-
227 solubilized CFTR.



228

229 **Figure R2 | Proteoliposome-reconstituted CFTR**

230 **a.** Schematic drawing of the immobilization strategy for proteoliposome-reconstituted CFTR. **b.** Quantification of the specificity
 231 of His-tag-dependent immobilization. **c.** Contour plots showing the response of proteoliposome-reconstituted CFTR molecules to
 232 rapid application of 3 mM ATP. Proteoliposomes were kinase-treated before the experiments.

233 12) It is not clear if the kinetic model adds much to the discussion, but it also is presented with
 234 appropriate caveats. Does the model make a prediction in a way that could be used to test the
 235 alternative hypotheses presented in the introduction of the paper?

236 We acknowledge that the model is simplified and therefore cannot recapitulate all experimental
 237 observables. We believe having a kinetic model is useful to tie all experimental observables
 238 together and to guide future experiments for the following reasons:

239
 240 Establishing the basic topology of the CFTR gating mechanism is a fundamental goal as it provides
 241 an essential framework for kinetic modeling, the context for understanding the impact of mutations
 242 and drug action and a physical guide for establishing and testing hypotheses about function.

243
 244 The prevailing kinetic frameworks available to the field only included states that could be directly
 245 discerned from electrophysiological data. Parallel assessment of smFRET dynamics and
 246 electrophysiology demanded additional considerations and constraints to the available kinetic
 247 information and thus a new kinetic model had to be established that parsimoniously recapitulates
 248 the major kinetic and structure features of CFTR function revealed by both methodologies as well
 249 as the estimated ATP hydrolysis rate based on ensemble measurements. Its inclusion ties all
 250 available information together into a single kinetic framework with a defined topology and is thus
 251 an essential message of our investigation.

252
 253 Kinetic models of function are also critical to scientific advancement because they provide a
 254 critical framework for others to challenge and test through new experiments. For instance, our
 255 findings suggest that the kinetic model presented explains the majority of the data obtained, but
 256 that additional complexities associated with ATP binding and hydrolysis in the consensus site alone
 257 should likely be considered in future work. The kinetic framework presented also points to the
 258 need for mechanistic clarifications regarding “flicker” open/closed states that have been evidenced
 259 in the field for decades. Prior investigations have suggested that such processes reflect “flicker
 260 closing” events from otherwise open channel states that reflect clogging of the pore by solutes. In
 261 other words, these events are kinetically “off-pathway”. While we cannot yet be conclusive in this
 262 regard, the kinetic framework proposed leaves open the possibility that “flicker” events may

263 instead reflect “flicker open” events that are “on-pathway” barrier crossing attempts prior to ATP
264 hydrolysis.

265
266 Both sets of considerations, together with the identification of rate-limiting intramolecular
267 conformational changes within CFTR that govern gating kinetics that is defined by the kinetic
268 framework, will be vital for the field to consider in future investigations.

269
270 13) Figure 1i shows that a very large percentage of channel openings have a dwell time of at least
271 10 ms, which could be detected using smFRET. Though it may not be feasible for this study, it
272 would be highly convincing if it were possible to design a FRET sensor of channel pore activity
273 that approximates the electrophysiological readout? This would allow direct comparison within a
274 single controlled system.

275 We share the Reviewer’s ambition to directly correlate the conformational transition of CFTR with
276 a functional readout of pore opening. Making such experiments feasible is certainly an exciting
277 direction to pursue in the future.

278

279 **Reviewer #2 (Remarks to the Author):**

280

281 The current manuscript by Levring et al. presents incredibly comprehensive studies of CFTR
282 function as an ATP-gated ion channel; these studies include electrophysiological recordings of
283 CFTR channel activity, real-time monitoring of the dimerization of CFTR's two nucleotide
284 binding domains (NBDs) with smFRET imaging, biochemical measurements of ATP hydrolysis
285 rate, and computer simulations based on a proposed gating model. Here are the main findings and
286 their mechanistic implications:

287

288 1. The NBDs are readily dimerized by ATP binding to the two ATP-binding sites in fully
289 phosphorylated CFTR, but the dimerized state (high FRET) is much more stable than the duration
290 of either channel open state or the closed state at a saturating concentration of ATP. This
291 uncoupling between NBD dimerization and channel gating suggests that multiple cycles of gate
292 opening and closing can occur with NBDs remained dimerized (inferred from high FRET),
293 contradicting previous idea of a strict coupling of NBD dimerization to gate opening and
294 hydrolysis-triggered NBDs separation (low FRET) to gate closing (Csanady et al., 2010).

295

296 2. By manipulating ATP binding to each site (Y1219A for the consensus site and W401A for the
297 degenerate site), they provided evidence for NBD dimerization by ATP occupancy at either site.
298 While both NBD dimerization and gating were mostly preserved in W401A, Y1219A channels
299 showed robust NBD dimerization but little activity, supporting previous reports that gating of
300 CFTR is mainly through ATP binding to the consensus site (Vergani et al., 2003; Zhou et al.,
301 2006).

302

303 3. By comparing the rate of NBD dimerization and the rate of channel activation upon sudden
304 addition of ATP, they found that NBD dimerization takes place much faster than gate opening,
305 contradicting previous proposition that NBD dimerization constitutes the rate-limiting step in
306 CFTR gating (Vergani et al., 2005). Although NBD dimerization precedes gate opening, NBD
307 dimerization itself is not sufficient to open the gate because dimerization (high FRET) can be seen
308 in conditions when there is little channel activity (e.g., upon ATP washout, or in the presence of
309 inhibitory concentration of ADP).

310

311 4. Supporting the idea that ATP at the consensus site plays a critical role in opening the gate, the
312 authors demonstrated that the disease-causing mutant G551D at the consensus site exhibits
313 defective gating and NBD dimerization. Interestingly, the dimerized NBDs in G551D show FRET
314 between high and low FRETs, suggesting the presence of an intermediate NBD dimer proposed
315 previously (Tsai et al., 2010). Similar but not identical intermediate FRET was seen in another
316 pathogenic mutation L927P (in TM8), which is 50 Å away from the consensus site. This latter
317 observation suggests a long-distance effect of the L927P mutation on NBD dimerization, but the
318 nature of this allosteric effect is unclear.

319

320 5. By testing the effects of CFTR potentiators ivacaftor and GLPG1837, they found a dramatic
321 gating effect with minimal and moderate changes of NBD dimerization in G551D and L927P
322 variants respectively. These are interesting observations, but it seems that not much additional
323 insight can be gained (see below).

324
325 6. These results were put together to form the basis of a kinetic model, which was further examined
326 through computer simulations. Although the proposed kinetic mechanism recapitulates many of
327 the experimental data, some simulated results do deviate from actual data.

328
329 No doubt, these are important studies that reveal many unexpected phenomena, which lay a solid
330 foundation for settling the debates over the coupling mechanism between NBD dimerization and
331 gate opening, as well as for advancing our overall understanding of the structural-functional
332 relationships of CFTR gating. This reviewer applauds the authors for such a remarkable
333 attainment.

334
335 We thank this Reviewer for their concise, thorough, and positive summary of our investigations.

336
337 Major comments:

338
339 1. The two conformations of NBDs—separated versus dimerized—can be resolved by the shift of
340 FRET from 0.25 to 0.49 (Fig. 1). In Fig. 1e, the FRET signal remains stable at 0.49 at 10 mM ATP
341 while the electrophysiological recording shows repeated opening and closing of the WT-CFTR
342 channel. The authors thus concluded that the NBDs remain dimerized throughout multiple rounds
343 of ATP binding/hydrolysis (Fig. 5). Conceptually, the consensus site in the “dimerized NBDs”
344 must open up a space wide enough to allow the exchange of hydrolytic products for the next ATP.
345 Indeed, in ATP washout (and Mg addition) experiments (Fig. 3b, c), transition from high FRET to
346 low FRET was observed immediately upon ATP removal (or Mg addition). As the waiting time is
347 too short for ATP dissociation from the degenerate site, this result suggests that upon ATP
348 hydrolysis at the consensus site, disengagement of the two NBDs does occur. The reason this high
349 to low FRET transition is not seen in the continuous presence of millimolar ATP may be due to
350 rapid rebinding and re-dimerization of the disengaged NBDs at millimolar ATP. This scenario
351 supports the notion that the state “degenerate site ATP, pore closed” should assume a different
352 dimeric state with a cracked consensus site to allow step 6 (Fig. 5) to take place. The canonical
353 NBD dimer in the cryo-EM structure leaves no room for ATP to diffuse into (or out of) its binding
354 pocket.

355
356 The CFTR gating cycle involves hydrolytic turnover and nucleotide exchange in the consensus
357 ATP binding site. We agree with the Reviewer that nucleotide exchange cannot occur from the
358 conformation adopted by phosphorylated and ATP-bound E1371Q CFTR (PDB 6MSM). In this
359 state the ATP is occluded from solvent. The absence of observable FRET dynamics in the ATP
360 saturated state and the monotonic increase of the dwell time of the high FRET state with ATP
361 concentration supports that ATP may rebind without complete separation of the NBDs. We posit
362 that small conformational changes occur after ATP hydrolysis that are sufficient to allow inorganic
363 phosphate and ADP release while the NBD’s remain ostensibly dimerized. As noted in our
364 discussion (page 12): “*The structure of the NBD-dimerized CFTR¹⁴ suggests that only small*
365 *changes at the consensus site, such as disrupting the hydrogen bond between R555 and T1246¹⁵,*
366 *would be sufficient for inorganic phosphate, ADP release and ATP rebinding.” Given the absence*
367 *of additional structural information on such processes, we may only conclude that the magnitude*
368 *and rate of such changes are not detectable with the specific structural perspective that CFTR*
369 *folding and biochemistry enabled us to achieve.*

370 To clarify this point we revised the text on page 6: “*These findings suggest that FRET-silent*
371 *processes occur within the NBD-dimerized conformation that trigger channel opening and closure*
372 *and that only subtle rearrangements at the dimer interface are required for nucleotide exchange.*”

373 The legend for **Figure 5** was also revised to clarify the need for conformational change to allow
374 for nucleotide exchange. See also our response for point 4a.

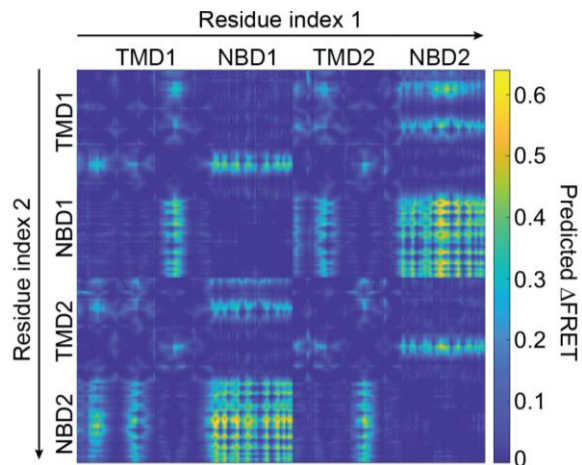
375
376 2. I wish the authors would have elaborated the rationale behind choosing positions 388 and 1435
377 for their FRET pair. T388 is at a position without a concrete secondary structure. Wouldn't this
378 positioning result in some uncertainty in FRET measurements as the FRET efficiency is
379 determined by not only the distance but also the relative orientation between the donor and the
380 acceptor? It would be nice if another pair is chosen to replicate some of the key observations
381 described above.

382
383 As CFTR has intrinsic folding/stability issues, the identification of structurally informative and
384 functionally tolerable sites of labelling was a substantial undertaking. These efforts were not
385 elaborated in the present manuscript due to space constraints and because it was noncritical for the
386 conclusions drawn in the manuscript. We briefly summarize the selection of labelling sites below:

387 Based on extant structures of dephosphorylated and ATP-free wild-type CFTR (PDB 5UAK) and
388 phosphorylated and ATP-bound E1371Q CFTR (PDB 6MSM) we measured the separation of
389 alpha-carbons for all structurally resolved residue pairs in CFTR. Using the Förster radius of the
390 LD555/LD655 FRET pair, we predicted FRET efficiencies in both conformational states, as well
391 as Δ FRET upon conformational isomerization (**Figure R3**). From residue pairs with favourable
392 predicted FRET efficiencies, we selected a subset of residues with solvent-exposed sidechains to
393 ensure efficient labelling with maleimide-conjugated fluorophores. Residues mutated in cystic
394 fibrosis patients or reported to affect folding or gating in the literature were excluded.

395 A panel (40) of cysteine-substituted CFTR variants were tested for whether they expressed, folded,
396 site-specifically labelled with maleimide-conjugated fluorophores, and retained key functional
397 properties of the wild-type channel. Several (5) pairs were experimentally tested for interpretable
398 FRET responses. This led to the final choice of labelling positions 388 and 1435. While T388 is
399 not involved in secondary structure, the density corresponding to the backbone of this residue is
400 well-defined in maps of both the ATP-free and dephosphorylated CFTR, as well as the ATP-bound
401 and phosphorylated E1371Q CFTR. Consistent with this, the 388/1435 pair exhibited tight
402 distributions of FRET efficiencies in conditions corresponding to those used for structure
403 determination.

404



405
 406 **Figure R3 | Residue selection.** Heat map of predicted changes in FRET efficiencies upon conformational isomerization for all
 407 possible pairs of labelling in CFTR.

408
 409 3. The W401A and Y1219A mutations were designed to weaken ATP binding. The authors
 410 observed that the NBDs of W401A and Y1219A mutants can still dimerize, and thus concluded
 411 that ATP binding at either site is sufficient for NBD dimerization (P7, line 215). Such conclusion
 412 is based on the assumption that the degenerate site in W401A (or the consensus site in Y1219A)
 413 must be vacant at 3 mM ATP. However, in electrophysiological study, the Y1219G mutation
 414 reduces the apparent affinity for ATP to a $K_{1/2}$ of ~5 mM (Zhou et al., 2006). If we assume that
 415 Y1219A affects the affinity similarly, at least a portion of the Y1219A channel population in Fig.
 416 2 would still have ATP bound to the consensus site at 3 mM ATP. Given that the degenerate site
 417 has much higher affinity for ATP, it is unlikely that the degenerate site in W401A would be vacant
 418 at 3 mM ATP. Thus, the data in Fig. 2 only support that NBDs of W401A and Y1219A can
 419 dimerize. The occupancy of ATP at each site cannot be determined, and thus whether a single ATP
 420 is enough to trigger dimerization cannot be inferred conclusively. However, the conclusion may
 421 not be incorrect once data in Fig. 1j are taken into consideration. The ATP concentration
 422 dependence of high to low FRET transition rate suggests the existence of different states of NBD
 423 dimer with one or both sites occupied.

424
 425 We agree with the Reviewer that the mutations may not completely abolish ATP binding and we
 426 revised the text as that the mutations were designed “to *reduce the affinity for ATP*” and “*the*
 427 *Y1219A variant, which binds ATP principally at the degenerate site*” (page 7). However, the
 428 fraction of such a dual ATP-occupied mutants is small and its presence will not alter the
 429 conclusions we draw, for the following reasons:

- 430
 431 1. The bulk ATPase activity of the Y1219A variant at 3 mM ATP is reduced to only 15 % of
 432 the wild-type CFTR (**Figure 2b**). This indicates that the Y1219A substitution nearly
 433 abolished ATP binding at the consensus site.
 434 2. The open probability of the Y1219A variant is reduced to nearly zero, also suggesting that
 435 the substitution nearly abolished ATP binding (**Figure 2f**).
 436 3. The NBD-dimerization probability of the Y1219A variant is approximately 50 % of the
 437 wild-type level, consistent with ATP binding being perturbed. Yet the relative reduction in
 438 dimerization probability is much less than the effects on ATP hydrolysis and pore opening.
 439 These observations are consistent with the degenerate site ATP supporting dimerization

440 events, that in the absence ATP occupancy in the consensus site do not lead to pore opening
441 or ATP hydrolysis (**Figure 2e**).

442 4. Most importantly, the FRET and gating dynamics of the mutants are completely different
443 from that of wild-type CFTR, indicating the mutated site is mostly vacant under the
444 experimental conditions (**Figure 2c**).

445 5. As the Reviewer points out, data in **Figure 1i** lends further support that NBD may dimerize
446 with one or both sites occupied.

447

448 4. Some of the kinetic steps in Fig. 5 need justification/explanation:

449

450 a. Dissociation/binding of ATP in the degenerate site happens when the NBDs are separated (step
451 7, 8), but dissociation of hydrolytic products and binding of ATP to the consensus site happen
452 when the NBDs are dimerized (step 4, 5, 6). Some intermediate conformations of NBDs need to
453 exist.

454

455 We agree with the Reviewer that intermediate conformations of NBD separation must exist.
456 Because we cannot explicitly measure transitions of CFTR to intermediate states or estimate the
457 lifetimes of inferred intermediates we have not included them in our simplified kinetic scheme in
458 **Figure 5**.

459 To clarify this point we revised the legend: “ATP rebinding may occur with subtle rearrangement
460 at the dimer interface (step 6) or with complete NBD-separation (step 8) to initiate a new gating
461 cycle.”

462

463 b. Why step 3 is drawn differently in Fig. 5 and Fig. E11? Also, why did the authors propose only
464 the open state hydrolyzes ATP, but not 2 ATP pore flicker-closed state or 2 ATP pore closed state?

465

466 In **Figure 5** we deliberately draw step 3 from bracketed channel conformations as we do not have
467 evidence allowing us to determine which of ‘2 ATP pore flicker-closed’ and ‘2 ATP pore open’
468 states are catalytically competent. We are also unaware of literature that might distinguish between
469 models allowing hydrolysis from both or either states. One might speculate that flicker closures
470 are local to the pore and therefore do not affect hydrolysis. As the rates of exchange between these
471 states is much greater than the rates into or out of these states, this ambiguity has negligible impact
472 on our kinetic simulations as direct connectivity to either state yielded indistinguishable results.

473 For the simulations presented, we explicitly specified the connectivity of the underlying model by
474 allowing only hydrolysis from the on-pathway, ‘2 ATP pore open’ state. We altered the depiction
475 of the kinetic scheme in **Extended Data Figure 11** accordingly. [REDACTED]

476

477 To our best knowledge, there is no evidence to support that ATP hydrolysis occurs without pore
478 opening. In our kinetic scheme, we aim only to outline the dominant gating path, deliberately
479 ignoring rare events, even those that we know to occur.

480

481

[REDACTED]

490 c. Is the flicker-closed state the same as the cryo-EM structure of E1371Q-hCFTR? It's been
491 known that many of the flickery events seen at negative membrane potentials are not likely real
492 functional "state". Because of their high voltage dependence (see Fig. E1i.), many of the events
493 are considered blocking of the pore by cytosolic large anions. To get the right kinetic parameter
494 for the true flicker-closed state, one has to analyze data from recordings at positive membrane
495 potentials.

496
497 The molecular nature of flicker-closure events, the origin of their voltage-dependence, and whether
498 the structure of human E1371Q CFTR (PDB 6MSM) reflects a flicker-closed state has yet to be
499 elucidated to our knowledge. As our kinetic scheme allows ambiguity in this regard, we believe
500 that detailed discussions on this specific point lie beyond the scope of the current study. For the

501 purpose of our gating model and simulations, we aim only to recapitulate the empirical observation
502 of flicker-closure events during the pre-hydrolytic period of the open burst.

503
504 d. There is a mistake both in the text and in the figure legend. Steps 6 – 8 represent transitions
505 among closed states; therefore, it is not a new gating cycle, which by definition should include a
506 closed event and an open event.

507
508 We thank the Reviewer for pointing this out. We mean to state that ADP dissociation (step 5) leads
509 to a dynamic intermediate that may isomerize (step 7) and to which ATP may bind to NBD-
510 separated (step 8) or NBD-dimerized states (step 6). A new gating cycle is initiated after ATP
511 binding (steps 1 and 2). We do not consider steps 6-8 a separate gating cycle. We have revised the
512 text as (page 13): “*Dissociation of ADP (Step 5) results in a dynamic intermediate to which ATP*
513 *can rebind (steps 6-8) thereby initiating another gating cycle.*” and the legend of **Figure 5** as “*ADP*
514 *dissociation (step 5) leads to a dynamically isomerizing intermediate (step 7). ATP rebinding may*
515 *occur with subtle rearrangement at the dimer interface (step 6) or with complete NBD-separation*
516 *(step 8) to initiate a new gating cycle.*”

517
518 Minor comments:

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520 1. I am not sure I truly understand the meaning of the term “hierarchical” gating mechanism.

521
522 We use this word to emphasize our finding that NBD dimerization does not necessarily lead to
523 pore opening. Pore opening requires conformational changes within the NBD-dimerized channel,
524 governed by ATP hydrolysis.

525
526 2. P2, line 3: Do you mean electrophysiological properties?

527
528 Corrected.

529
530 3. P5, line 140: It should be “...to a stably NBD-dimerized state.”

531
532 Corrected.

533
534 4. P6, line 157: Previous electrophysiological studies of R117H-CFTR and zebrafish CFTR, as
535 well as the cryo-EM structure of E1371Q all indicate the presence of conductive and non-
536 conductive states with dimerized NBDs. Please more explicitly state the unique insight from these
537 data.

538
539 As the Reviewer has pointed out earlier, whether NBD dimerization is strictly coupled to gate
540 opening has been a long-debated topic. There are reports in the literature supporting both
541 arguments. Here we address this question by directly comparing the kinetics of NBD dimerization
542 with channel gating. Furthermore, our finding that CFTR spends most of the time in the NBD-
543 dimerized state is original.

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545 5. P6, line 161: ...with ATP concentration (Figure 1j), not 1k.

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Corrected.

6. P6, line 172: The ADP experiments are interesting in that the low FRET value seems different from that without ATP. Is it reproducible? If it is, doesn't that suggest different degrees of disengagement of NBDs?

The data is reproducible from three independent experiments. To investigate if this reflects global or local conformational changes of the NBDs would require cryo-EM analyses that are beyond the scope of this study.

7. P7, line 191: The time constant for ligand exchange at the degenerate site is around 30 s (Tsai et al., 2010), which interestingly is very similar to the duration of high FRET upon ATP washout (Fig. 3b).

Please see our response to point 10 below.

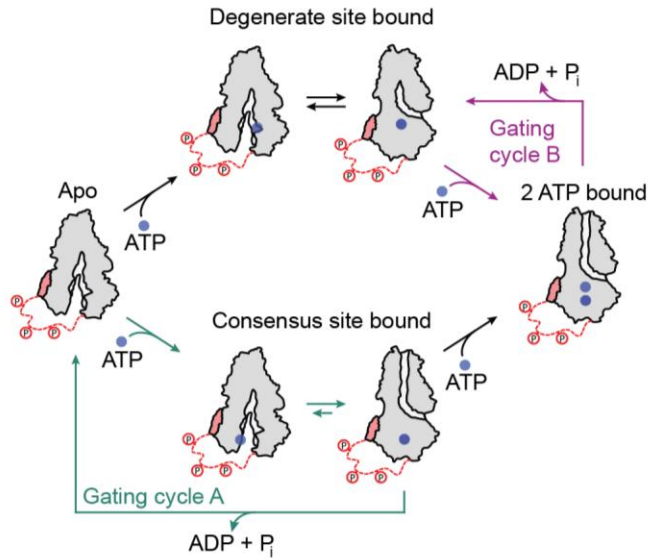
8. P7, line 226: It should be open probability, not opening probability. (In other parts of the text too.)

Corrected here and throughout the text.

9. P8, line 237: The changes of FRET upon sudden addition of ATP show heterogeneity. This observation may be related to a recent paper by Yeh et al. (2021). The stated similarity to W401A and double exponential current rising upon addition of ATP (Fig. 1b and E1b) all remind this reviewer of the results published in this paper, which shows different closed channel conformations (perhaps different NBDs conformations) once ATP is removed.

We thank the Reviewer for pointing this out. The different configurations of closed states described by Yeh et al. (2021) are clearly evident in our ATP washout experiments (**Figure 3b**) where molecules first release nucleotide from the consensus site, but retain ATP in the degenerate site which supports dimerization (**Extended Data Figure 8g**). Reversible rundown may correspond to release of degenerate site ATP leading to stably separated NBDs. We have now added the following sentence on page 9: "*Dissociation of ATP from both sites likely leads to the reversible rundown of CFTR currents that occurs after prolonged exposure to nucleotide-free solutions*³⁰"

The observed heterogeneity in the ATP injection experiment (**Figure 3a**) may have a different origin. At time of ATP injection CFTR molecules had been in ATP-free buffer for a prolonged period and both ATP binding sites should be vacant. To us, the simplest origin of heterogeneity arises from stochastic and sequential ATP binding to the individual binding sites (**Figure R5**). That is, the population is homogenous at time of injection. CFTR molecules that bind ATP in the consensus site first, may dimerize, hydrolyze ATP, release products, and relax back to the starting point. Repeated cycles of this may occur. Upon ATP binding in the degenerate site the NBDs dimerize stably. Eventually, every molecule reaches steady state with both binding sites simultaneously occupied.



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Figure R5 | Sequential ATP binding to consensus and degenerate binding sites in the pre-steady state. Schematic of the events occurring after rapid ATP application to PKA-phosphorylated CFTR. If ATP is bound at the consensus site first, CFTR enters Gating cycle A. The NBDs of CFTR dimerize, the pore opens, ATP is hydrolyzed, and catalytic products are released. Repeated cycles may occur. Every passage through Gating cycle A involves complete NBD dimerization and separation. Eventually, CFTR molecules will bind ATP in both sites simultaneously, and gate at steady state (Gating cycle B). Gating cycle B involves only subtle rearrangements at the NBD interface.

10. P8, line 255: Of note, rapid removal of ATP caused a double exponential decay of WT-CFTR currents (Fig. 2B in Lin et al., 2014) with time constants ($\tau_1 < 1$ s and $\tau_2 = 29$ s) similar to these values reported for NBD dimerization relaxation. These shouldn't be just coincident.

We agree. The apparent correlation between the biexponential time constants for relaxation of pore opening and dimerization suggests a common underlying molecular mechanism determining both transitions.

To us, the simplest explanation is that CFTR relaxes in two steps associated with release of ATP from either site. Hydrolysis-catalyzed release from the consensus site is fast. Dissociation from the degenerate site is slow. The intermediate with ATP in the degenerate site alone behaves like the Y1219A CFTR variant, exhibiting high dimerization probability but low channel open probability. This interpretation is in line with conclusions made by Lin et al (2014).

The biphasic current relaxation upon nucleotide exchange reported by Tsai et al., 2010 (Reviewer comment 7) is also consistent with this model. Further, we note that the reduced rate of the slow exchange component reported by Tsai et al ($\tau_2 = 51$ s) compared to the slow decay component measured in washout experiments ($\tau_2 \approx 20$ -30 s) is consistent with our observations. During the second phase of washout, CFTR dynamically isomerizes between dimerized and separated conformations (**Figure 3b**). When NBDs separate, both the consensus and degenerate site are able to readily release nucleotide. In the ligand exchange experiment continuous presence of nucleotide stabilizes the dimerized conformation thereby reducing the rate of exchange at the degenerate site.

We revised the text to reference the correlation with the cited manuscripts (page 9): “*Parallel FRET experiments showed that the time course of NBD-separation is biphasic, with time constants of 1.6 seconds and 20 seconds, respectively (Figure 3b). These rates correlate with the double*

619 *exponential time constants reported for CFTR current decay and ligand exchange*^{18,29}. *This*
620 *apparent correlation suggests a common underlying molecular mechanism determining both*
621 *transitions.”*

622
623 11. P9, line 277: Not a correct reference and interpretation. The cited ligand exchange experiments
624 lead to two different turn-over rates of ATP and the idea of a “partial dimer.” On the other hand,
625 by carrying out different ligand exchange experiments, Jih et al. (2012) propose a re-entry pathway
626 featuring repetitive cycles of ATP turnover in a single opening burst. Both studies done by Hwang
627 and colleagues invoke a partial dimer state where the consensus site is open up to allow replenish
628 of ATP. They do not suggest that ATP turnover can occur in a single canonical NBD-dimerized
629 conformation, where the ligand is occluded.

630
631 We thank the Reviewer for pointing out the differences in the models. The shared concept of the
632 present inferences and the cited manuscript by Tsai et al., is that during wild-type CFTR gating at
633 steady state, the NBDs do not fully separate for each hydrolysis event and open burst. Rather they
634 remain associated through continuous ATP binding in the degenerate site. Nonetheless, nucleotide
635 exchange at the consensus site necessarily requires some degree of separation (see also discussion
636 above). Tsai et al. propose the formation of a partial dimer. By contrast, we do not detect changes
637 in FRET efficiency between repeated gating cycles and therefore infer that the changes required
638 for nucleotide exchange must be subtle in magnitude and/or transient in time. We revise the text
639 to reflect this (pages 9-10): *“These findings suggest that the probability of ATP rebinding exceeds*
640 *that of complete NBD separation when ATP concentration is greater than 100 μM. This concept,*
641 *consistent with ligand exchange experiments*¹⁸, *suggests that repetitive cycles of ATP turnover can*
642 *occur in an ostensibly NBD-dimerized conformation with only subtle changes at the consensus site*
643 *required for nucleotide exchange. In cellular settings, repetitive gating cycles are therefore*
644 *expected to persist until the finite rate of NBD separation at cellular ATP concentrations allows*
645 *the dephosphorylated R-domain to reinsert, terminating CFTR gating.”*

646
647 12. P10, line 312-314: I don’t understand what the authors mean by the similar nature in “the
648 similar nature of both mutations’ impacts on NBD dimerization and channel opening”. G551D and
649 L927P actually exhibit different behaviors in their experiments. In Fig. 4b, the transitions between
650 high and low FRET in G551D is different from those of L927P. Also, GLPG1837 drastically
651 changes L927P’s FRET pattern, but barely affects G551D. Moreover, electrophysiological
652 properties of these two mutants and pharmacological effects of CFTR potentiators should be
653 discussed in a more quantitative manner (see comment 18 below) before one can make more
654 definitive conclusions.

655
656 This section has been extensively revised based on this comment and Reviewer 1’s comments.
657 Thank you.

658
659 13. P11, line 347: It should be noted that both VX-770 and GLPG1837 work on a CFTR variant
660 with its NBD2 completely removed (Yeh et al., 2017; Yeh et al., 2015). These drugs also exert
661 dramatical gating effects on G551D whose NBDs may not assume a canonical NBD dimer state
662 (Lin et al., 2014; Yeh et al., 2017) (Fig. 4 in current study).
663

664 Thank you for pointing this out. We have revised the text to include this information. On page 12:
665 *“These data lead to the conclusion that the major effect of Ivacaftor or GLPG1837 is not to support*
666 *transition from NBD-separated to dimerized conformations. Rather, these potentiators principally*
667 *operate by promoting pore opening in CFTR when NBDs are already dimerized. In other words,*
668 *potentiators affect the coupling efficiency between NBD dimerization and channel opening,*
669 *possibly by stabilizing the TMDs in the pore open configuration⁴³. This effect also manifests in*
670 *variants unable to form a canonical NBD dimer, such as G551D and a variant devoid of the entire*
671 *NBD⁴⁰.”*

672
673 14. P11, line 375-377: Previous studies suggest that reagents such as NPPB and high-affinity ATP
674 analogs, act synergistically with Ivacaftor and GLPG1837 on G551D channels by promoting NBD
675 dimerization (Lin et al., 2016; Yeh et al., 2017). The authors may consider incorporating these
676 studies in the Discussion.

677 We included a discussion of the investigational compound NPPB in the revision (page 12): *“The*
678 *investigational compound 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB) was proposed to*
679 *stimulate pore opening by such a mechanism⁴⁴.”*

680
681 15. Figure 1: I suggest the authors unify the spelling of “dimerization.” It is dimerization in the
682 text but dimerisation in Fig 1 and Fig 4.

683
684 Spelling has been unified as “dimerization”.

685
686 16. Figure 1e: If I understand correctly, the electrophysiological recordings are from WT-CFTR,
687 not from the CFTRFRET protein. Wouldn't it be a better comparison to use the
688 electrophysiological recording of CFTRFRET protein here? Of note, the CFTRFRET construct
689 altered the majority of the endogenous cysteine residues; this alteration could affect CFTR gating
690 as seen in the literature. In Figure E1, the authors demonstrated their ability to use CFTRFRET
691 proteins in electrophysiological experiments and concluded that its gating kinetics are similar to
692 WT-CFTR (but see comment 19 below).

693
694 Yes, **Figure 1e** displays electrophysiological recordings of wild-type CFTR. The comparison is
695 justified by measurements made in **Extended Data Figure 1**, which demonstrate that the
696 CFTR_{FRET} and wild-type variants have similar time-courses of current relaxation in response to
697 rapid ATP application or withdrawal. Dependences on ATP and PKA, and sensitivity to
698 GLPG1837 are also similar. Finally, ATP hydrolysis, which reflects flux through the gating cycle
699 is also comparable between the two variants.

700 Consistent with literature reports (e.g. Cui et al. 2006 and Mense et al. 2006), we found that
701 substituting all cysteines in CFTR to serine or alanine caused expression defects that precluded
702 purification. We therefore conducted an extensive screen of cysteine variants to isolate residues
703 causing this unwanted effect. Residues C524 and C590 are buried within NBD1 and could be
704 retained without a great extent of non-specific fluorophore labelling. C76L and C592M
705 substitutions were found to not affect expression. Collectively, these changes to the cysteine-less
706 CFTR variant allowed purification.

707 While cysteine substitutions may cause subtle changes to gating, our observations seem
708 incompatible with large gating defects that would alter the major conclusions drawn in this
709 manuscript.

710 To further substantiate that key gating properties are retained by the CFTR_{FRET} variant we add a
711 comparison of open probabilities and open dwell times for the wild-type CFTR and the
712 fluorophore-conjugated CFTR_{FRET} to the revision (**Extended Data Figure 11,m**).

713 Cui, L., et al. The role of cystic fibrosis transmembrane conductance regulator phenylalanine 508
714 side chain in ion channel gating. *J Physiol* **572**, 347-358 (2006).

715 Mense, M., et al. In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding
716 domain heterodimer. *EMBO J* **25**, 4728-4739 (2006).

717
718 17. Figure 1j: I don't understand the data points at 0 ATP. Do the authors still observe FRET
719 transitions even without ATP, meaning that the NBDs dimerize without ATP? In 1e, FRET always
720 stays at low FRET.

721 NBD-dimerization in the absence of ATP does occur, albeit infrequently (see e.g. **Figure 1e** - end
722 of the no ATP trace and **Extended Data Figure 10**). We also observe infrequent pore opening in
723 the absence of ATP (**Extended Data Figure 10**) consistent with literature reports (e.g. Wang et
724 al. 2010 and Mihályi et al. 2016). Both dimerization and pore opening also occur with Apyrase,
725 which was added to remove potential ATP contamination.

726 Our working model is that CFTR gating, like other stochastic processes in biology, is facilitated
727 by ATP binding and NBD dimerization, but can occur independently with very low efficiency and
728 duration with finite probability.

729 Wang, W., et al. ATP-independent CFTR channel gating and allosteric modulation by
730 phosphorylation. *Proc Natl Acad Sci USA* **107**, 3888–3893 (2010).

731 Mihályi, C., Töröcsik, B., & Csanády, L. Obligate coupling of CFTR pore opening to tight
732 nucleotide-binding domain dimerization. *eLife* **5**, e18164 (2016).

733
734 18. Figure 4b: In response to GLPG1837, the current trace of G551D barely changes as if
735 GLPG1837 has little effect. This potentiation effect is far from the reported ~30-fold stimulation
736 shown in Fig. 4g. Please comment on the discrepancy and consider replacing the recording with a
737 more representative one. Similar concerns are raised for the effect of GLPG1837 on L927P. The
738 single-channel activity in 4b does not change 20-fold as indicated in Fig. 4g.

739
740 The open probability of G551D is very low and gating is slow. Therefore, in any given 60 second
741 window we are likely to see zero or one open events. Neither is truly representative. We have
742 updated the current trace in **Figure 4b** to better represent the relative current stimulation with
743 GLPG1837.

744 The window of time presented for the L927P trace is also updated in **Figure 4b** of the revision.
745 The traces reflect the relative potentiation presented in **Figure 4g**. Because of the very short open
746 dwell time and only modestly affected closed dwell time of unpotentiated L927P CFTR, the open
747 probability may be perceived greater in sample traces than it is.

748
749 19. Figure 4f: It is shown that the Po of E1371Q is ~0.7 and increases to ~0.8 with GLPG1837. It
750 is puzzling how E1371Q has a Po of only 0.7. Numerous studies showed that with hydrolysis
751 eliminated, the closing rate of E1371Q (or E1371S) is >100-fold slower than WT, resulting in a
752 Po close to unity (Bompadre et al., 2005; Csanady et al., 2013; Vergani et al., 2003; Yu et al.,
753 2016). Indeed, the E1371Q single channel in Figure 1f seems to be almost always open, though it
754 is hard to determine whether those brief downward deflections are flicker closures or interburst
755 closures with this time scale. It would be helpful to expand an E1371Q single channel trace to
756 allow a better assessment of channel behavior in a quantitative manner.

757
758 The deflections are flicker-closures – however, the duration of flickers make them hard to
759 distinguish from inter-burst closures. Consistent with literature, we determine very slow current
760 relaxation for E1371Q upon ATP withdrawal in excised inside-out patches: $\tau_{closure} = 427 \pm 91$ s
761 (mean and standard error, n = 6 patches) (**Extended Data Figure 8a**). Thus, the vast majority of
762 the observed closures do not involve release of ATP from the consensus site.

763 We add an expanded view of the E1371Q single-channel trace to **Figure 1e** of the revision.

764
765 20. Figure E1i: From the raw trace, the Po of the FRET construct is apparently higher than the
766 WT. The open burst time of FRET variant is visibly longer than WT. Is this a consistent finding?
767 Please comment on the discrepancy.

768
769 We have updated the single-channel traces of the current-voltage relationship in **Extended Data**
770 **Figure 1i** to better reflect the open probability of the CFTR_{FRET} variant. We also add a comparison
771 of open probabilities and open dwell times for the phosphorylated wild-type and fluorophore-
772 conjugated CFTR_{FRET} variant channels (**Extended Data Figure 11-m**). We do not find a
773 statistically significant increase in open probability for the CFTR_{FRET} variant.

774
775 21. Figure E8g: What is the second arrow below the ADP+Pi for?

776
777 The multiple arrows aimed to illustrate that several molecular events occur within this step. The
778 second arrow is removed in the revision to avoid confusion.

779
780 22. Figure E11b, d, and e: In b, the simulated single-channel trace shows a much longer open time
781 than the experiment, while the closed time seems not affected much (or slightly shorter). However,
782 in d and e, the experimental and simulated dwell times match perfectly but the two closed times
783 deviate significantly. Please comment on this discrepancy.

784
785 The open burst dwell times of the experimental and simulated traces in **Extended Data Figure**
786 **11b** match, as is also reflected in the quantification in **Extended Data Figure 11d**. However, as
787 we do not have an accurate estimate of flicker-closure rates, this may give rise to the different
788 appearances of the open burst in experimental and simulated traces. Due to the limited bandwidth
789 of our experiments, we ignore flicker-closure events in idealization of experimental single-channel
790 recordings. The dwell time histogram therefore reflect the life-time of the open burst. To best
791 mimic the experimental analysis, the dwell times of the open burst were also extracted from
792 simulated traces, again ignoring flicker-closure events. In our revised manuscript we clarify these
793 points in the legend of **Extended Data Figure 11**.

794
795 23. Figure E11h: The experimental ATP turnover rate is 15/min (or 0.25/s), corresponding to one
796 ATP hydrolyzed every four seconds, a much longer time than a gating cycle time of wildtype
797 CFTR (~1 s). If, as proposed, multiple ATP hydrolysis cycles occur, the theoretical ATP hydrolysis
798 rate must be even higher than 1/s. As stated, there is a likelihood that this biochemical measurement
799 is not very accurate because of imperfect protein preparation. Alternatively, the proposed gating
800 model is overly simplified. Either way, I wonder whether the simulation really provides more
801 insights into the gating mechanism of CFTR. Sometimes it may be better to be vaguely right than
802 precisely wrong. I leave this to the authors to decide.
803

804 [As we have elaborated in the response to Reviewer 1 point 12, we believe having a kinetic model](#)
805 [is useful to tie all experimental observables together and to guide future experiments.](#)

806

807

808 References:

809

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811 CFTR gating II: Effects of nucleotide binding on the stability of open states. *J Gen Physiol*,
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832 ATP-driven tight dimerization of its nucleotide-binding domains. *Nature*, 433(7028), 876-880.
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834 Vergani, P., Nairn, A. C., & Gadsby, D. C. (2003). On the mechanism of MgATP-dependent
835 gating of CFTR Cl⁻ channels. *J Gen Physiol*, 121(1), 17-36.

836 [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12508051)
837 [uids=12508051](#)

838 Yeh, H. I., Sohma, Y., Conrath, K., & Hwang, T. C. (2017). A common mechanism for CFTR
839 potentiators. *J Gen Physiol*, 149(12), 1105-1118. <https://doi.org/10.1085/jgp.201711886>

840 Yeh, H. I., Yeh, J. T., & Hwang, T. C. (2015). Modulation of CFTR gating by permeant ions. *J*
841 *Gen Physiol*, 145(1), 47-60. <https://doi.org/10.1085/jgp.201411272>
842 Yeh, H. I., Yu, Y. C., Kuo, P. L., Tsai, C. K., Huang, H. T., & Hwang, T. C. (2021). Functional
843 stability of CFTR depends on tight binding of ATP at its degenerate ATP-binding site. *J Physiol*,
844 599(20), 4625-4642. <https://doi.org/10.1113/JP281933>
845 Yu, Y. C., Sohma, Y., & Hwang, T. C. (2016). On the mechanism of gating defects caused by
846 the R117H mutation in cystic fibrosis transmembrane conductance regulator. *J Physiol*, 594(12),
847 3227-3244. <https://doi.org/10.1113/jp271723>
848 Zhou, Z., Wang, X., Liu, H. Y., Zou, X., Li, M., & Hwang, T. C. (2006). The two ATP binding
849 sites of cystic fibrosis transmembrane conductance regulator (CFTR) play distinct roles in gating
850 kinetics and energetics. *J Gen Physiol*, 128(4), 413-422. <https://doi.org/10.1085/jgp.200609622>
851

852 **Reviewer #3 (Remarks to the Author):**

853

854 The manuscript by Levring et al describes characterization of molecular cycles involved in
855 activation of the CFTR channels. Specifically, single molecule FRET studies done in parallel with
856 single channel electrophysiology enable to characterize the relationship between ATP binding,
857 hydrolysis and channel opening. Furthermore, how disease-causing mutations and therapeutic
858 correctors modulates these events are studied and discussed.

859

860 The study is very nicely crafted, the experiments are very well performed, and the conclusions are
861 convincing.

862

863 This work brings a clear view on a long-standing issue in the CF field and deserves publication.

864

865 We thank this Reviewer for these supportive summary comments.

866

867 Nevertheless, a number of points require clarification.

868

869 1/ FRET values

870 CryoEM structures of human CFTR have -so far- captured 2 conformations with the NBDs either
871 separated or dimerized. Structural data on ABC structures suggest more extended conformational
872 diversity with several different substates. The differences in FRET values observed in the present
873 manuscript may be indicative of such diversity. Therefore, the authors need to clearly assign -when
874 possible- the link between the FRET valued and the identified conformer.

875

876 The ATP-free CFTR shows a low FRET state at 0.25. Does this match the cryo-EM structures of
877 the NBD separated state ? Similarly does the 0.49 FRET value match the NBD-dimerized state for
878 the used probes? The authors need to run careful simulations (for examples with FPS, see Kalinin
879 et al) with the appropriate probe models to evaluate the calculated FRET value for each
880 conformation.

881

882 As requested, we performed all-atom molecular dynamics simulations of CFTR (PDB codes
883 5UAK and 6MSM) with the LD555 and LD655 dyes attached at their sites of labeling for
884 comparison with the FRET experiments. As previously described, we utilized a simplified
885 potential that maintains native contacts in the original structure while allowing the fluorophores to
886 explore all possible positions and conformations (Noel et al., 2016 and Girodat et al., 2020). Using
887 this approach, we can confirm that our FRET efficiencies are consistent with the existing CryoEM
888 structures of the two conformations (**Table R1**). For reference, we also included the distances
889 obtained from FPS as suggested by the Reviewer (Kalinin et al., 2012 and Sindbert et al., 2011),
890 which show larger differences, which we attribute to the simplified nature of the ball-and-stick
891 model used by the FPS program.

892

893 ATP-bound dephosphorylated CFTR shows a 0.28 FRET value. How does that translate in terms
894 of distance change given the R0 for these probes ?

895

896 Regarding the ATP-bound dephosphorylated state (0.28 FRET efficiency), this corresponds to an
897 inter-dye distance of 72.6 Å, which is a ~2 Å decrease from the apo state.

State	PDB	FRET Efficiency	FRET Distance	SMOG	FPS (R_{mp})	C_{α} - C_{α}
Deph. Apo	5UAK	0.25	74.5	72.1	65.9	69.9
Deph. ATP		0.28	72.6			
Phos. ATP	6MSM	0.49	62.4	66.0	55.4	46.4

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Table R1. Comparison of inter-dye distances estimated from FRET and CryoEM structures.

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Noel, J. K., Levi, M., Raghunathan, M., Lammert, H., Hayes, R. L., Onuchic, J. N., & Whitford, P. C. (2016). SMOG 2: a versatile software package for generating structure-based models. *PLoS computational biology*, 12(3), e1004794.

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Girodat, D., Pati, A. K., Terry, D. S., Blanchard, S. C., & Sanbonmatsu, K. Y. (2020). Quantitative comparison between sub-millisecond time resolution single-molecule FRET measurements and 10-second molecular simulations of a biosensor protein. *PLoS computational biology*, 16(11), e1008293.

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Kalinin, S., Peulen, T., Sindbert, S., Rothwell, P. J., Berger, S., Restle, T., ... & Seidel, C. A. (2012). A toolkit and benchmark study for FRET-restrained high-precision structural modeling. *Nature methods*, 9(12), 1218-1225.

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Sindbert, S., Kalinin, S., Nguyen, H., Kienzler, A., Clima, L., Bannwarth, W., ... & Seidel, C. A. (2011). Accurate distance determination of nucleic acids via Forster resonance energy transfer: implications of dye linker length and rigidity. *Journal of the American Chemical Society*, 133(8), 2463-2480.

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The corresponding cryo-EM structure showed in Figure E4 does not address these issues, in great part due to the limited resolution. In fact, the authors only vaguely describe “proximal changes in local structure”. What does that mean? In my opinion in its current state this structure does not bring relevant information and should be either improved or removed from the manuscript.

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We have performed additional cryo-EM analysis and improved the structure to 4.3 Å, which enables us to position the TMDs and NBDs with confidence. Furthermore, the presence of ATP is unambiguous (**Figure E4**). Comparing this structure with the two published conformations show that the dephosphorylated CFTR retains the NBD-separated conformation even with ATP bound at both NBDs. In addition to correlating the FRET value with structure, this structure also provides an important missing piece in understanding CFTR regulation. It shows that ATP-binding to dephosphorylated CFTR does not induce large-scale conformational changes, thus explaining why R-domain phosphorylation is required for channel opening even at physiological ATP concentrations (1-10 mM).

938
939 2/ The E1371Q mutant appear to be locked in the high FRET state during the time course of the
940 experiments, at least in the trace shown (100s, Fig1f). However, the distribution on Fig1c seems
941 to show a significant low FRET population (~5-10%), pretty much identical to that seen for the
942 “wt” CFTR (which can hydrolyze ATP and thus should spend more time in the NBD separated).
943

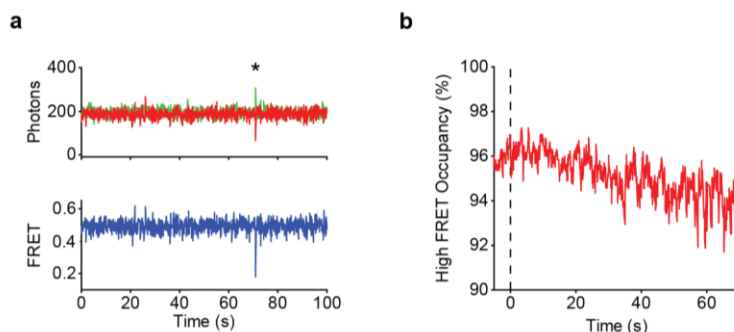
944 The fraction of E1371Q molecules that does not occupy the high FRET state reflects two
945 populations: 1) Infrequent NBD-separation events from the high FRET state (**Figure R6a**); 2) A
946 small fraction of nonresponsive molecules. The latter population does not respond to stimuli like
947 ATP addition and might reflect molecules that have denatured during handling, surface tethering,
948 and/or the small fraction of non-specifically fluorophore-labelled molecules (**Extended Data**
949 **Figure 2a**).

950 In that line, it is unclear to me why the authors see no NBD separation in this mutant after
951 phosphatase treatment (Fig E3). One may expect to see some separation over the course of the
952 measurement.
953

954 The NBD separation of E1371Q in response to phosphatase does occur, but exceedingly slow. The
955 zoomed-in view of **Extended Data Figure 3r** reveals a subtle inflection in the dimerized state
956 occupancy of E1371Q CFTR upon phosphatase injection (**Figure R6b**). The response is too slow
957 to accurately estimate a time-constant in our smFRET setup.

958 Our experiments suggest that autoinhibitory re-engagement of the R domain requires prior NBD-
959 separation. NBD-separation is limited by the rate of hydrolytic or non-hydrolytic ATP dissociation
960 from wild-type and E1371Q CFTR variants, respectively. Our best estimate of non-hydrolytic ATP
961 dissociation is from E1371Q current relaxation upon ATP withdrawal measured in inside-out
962 excised patches (**Extended Data Figure 8a**). We have measured the time constant of this reaction
963 to be $\tau_{closure} = 427 \pm 91$ s (mean and standard error, $n = 6$). A similar value was reported by David
964 Gadsby and colleagues (Vergani et al. 2005): $\tau_{closure} = 476$ s.

965 In the continued presence of ATP, non-hydrolytic ATP dissociation from dephosphorylated
966 E1371Q CFTR may lead to either R domain autoinhibition or ATP rebinding. Thus, 450 seconds
967 should be a lower bound on the predicted time-constant of relaxation. Altogether, this is consistent
968 with the slow rate of NBD separation measured in **Extended Data Figure 3r**.



969
970 **Figure R6 | E1371Q CFTR NBD-separation.**
971 **a.** Example single-molecule trace showing a rare NBD separation event (indicated by an asterisk) for E1371Q CFTR. CFTR was
972 phosphorylated and in the presence of 3 mM ATP. Top panel shows donor (green) and acceptor (red) fluorescence intensities.
973 Bottom panel shows FRET. **b.** Depopulation from the High FRET state after λ phosphatase injection (at the dashed line) for E1371Q
974 CFTR. The plot is a zoomed-in view of **Extended Data Figure 3r** in the manuscript.

975

976 Vergani, P., Lockless, S., Nairn, A. et al. CFTR channel opening by ATP-driven tight dimerization
977 of its nucleotide-binding domains. *Nature* **433**, 876–880 (2005).

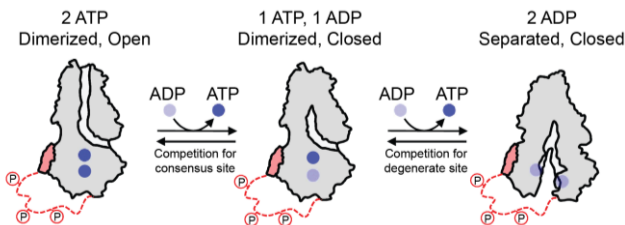
978

979 In the same Figure, can the author comment on the difference in ATP concentration dependence
980 of channel gating vs NBD dimerization? At 3mM ADP 80% of the channels are still in dimers but
981 gating is almost gone.

982

983 Due to space-constraints we did not describe the origin of the asymmetry between ADP-mediated
984 inhibition of pore opening and NBD-dimerization. Our working model (**Figure R7**) posits that
985 ADP competition for the consensus site is sufficient to prevent opening of the pore, but is
986 insufficient to cause separation of NBDs. Thus, at intermediate ADP concentrations CFTR
987 channels are trapped in a configuration equivalent to the post-hydrolytic state which is also
988 transited during normal gating (**Figure 5**). At higher concentration, ADP competes for both
989 nucleotide-binding sites to inhibit both NBD-dimerization and pore opening.

990



991

992 **Figure R7 | Competitive inhibition by ADP.** At lower concentrations, ADP competes for ATP binding in the consensus site to
993 inhibit pore opening but not NBD dimerization. Higher ADP concentrations compete for ATP binding in the degenerate site to
994 inhibit NBD dimerization.

995

996 3/ Very little is shown regarding the membrane-embedded measurements.

997 What does the sentence “While the fraction of the molecules responsive to ATP was significantly
998 reduced” refer to ? Only single traces are shown. No statistics (“significantly” ?).

999 We refer to our response to Reviewer 1 point 11.

1000 The text was revised to remove the word “*significantly*”.

1001

1002 4/ ATP binding sites

1003 The authors use the W401A and Y1219A mutants originally described by Hwang and colleagues
1004 (although mutated into Gly in that study). What do the authors mean by “destabilize ATP
1005 binding” ?

1006 It should be noted that the mutations decrease affinity but may not prevent it. For example, W401A
1007 is still able to bind ATP (albeit with lower affinity) and 3mM is quite a large concentration.
1008 Perturbating the ATP binding at the degenerate site may also alter the conformational equilibrium
1009 of NBD1 that we recently described (Scholl et al, 2021). On P8 the authors state that for mutant
1010 Y1219A “ATP binds only at the degenerate site”. This is not proven.

1011

1012 We agree with the Reviewer that the mutations may not completely abolish ATP binding and we
1013 revised the text as the mutations used were designed “to *reduce the affinity for ATP*” and “*the*

1014 *Y1219A variant, which binds ATP principally at the degenerate site*” (page 7). However, the
1015 fraction of such a dual ATP-occupied mutants is small and its presence will not alter the
1016 conclusions we draw. Please see our response to Reviewer 2, point 3.

1017
1018 On P7, the authors describe that “the Y1219A variant, which binds ATP mainly at the degenerate
1019 site, transitioned between NBD-dimerized and separated states slowly, and very rarely exhibited
1020 channel opening” but they only show single traces. They should show distributions.

1021
1022 As suggested, the distributions of NBD dimerization and channel opening are now included as
1023 **Figure 2e** and **2f**.

1024
1025 5/ Figure E3K is unclear to me. Is it expected to see such a significant effect at 0.1 and 0.3 mM
1026 ADP while 3mM ATP is maintained (and while Fig E3I show predominantly NBD in dimer form)?

1027
1028 Based on the literature, we do expect to observe ADP inhibition under those experimental
1029 conditions. The dose-dependence of competitive ADP inhibition described in **Figure E3K** is
1030 consistent with measurements by Welsh and colleagues (Randak and Welsh 2005).

1031 Randak, C. O. & Welsh, M. J. ADP inhibits function of the ABC transporter cystic fibrosis
1032 transmembrane conductance regulator via its adenylate kinase activity. *Proc Natl Acad Sci USA*
1033 **102**, 2216–2220 (2005).

1034 I assume that the last part of the graph has 0mM ADP (correct?).

1035 Yes, at the end of the current trace in **Extended Data Figure 3k** there is no ADP.

1036 Why is the rebound much faster than what is observed during the first 2 mins?

1037 We think that this is related to the ‘reversible rundown’ effect described by Hwang and colleagues
1038 (Yeh et al. 2021). Hwang and colleagues describe a slow component for the current activation after
1039 ATP application, that appears after extended intervals without ATP. The effect is ascribed to
1040 release of ATP from the degenerate ATP binding site. Our model for competitive inhibition
1041 described in **Figure R7** predicts that the ADP-dependent current inhibition observed in **Extended**
1042 **Data Figure 3k** would not involve ATP dissociation from the degenerate site, and thus that no
1043 reversible rundown should occur. Therefore, a slow component of activation is evident upon initial
1044 ATP application, but not upon ADP withdrawal.

1045 Yeh, H.-I., Yu, Y.-C., Kuo, P.-L., Tsai, C.-K., Huang, H.-T. and Hwang, T.-C. Functional stability
1046 of CFTR depends on tight binding of ATP at its degenerate ATP-binding site. *J Physiol*, **599**:
1047 4625-4642 (2021).

1048
1049 6/ The coupling ratio seems unclear to me. What if the two would anticorrelate ? The coupling
1050 ratio would still be 1 ?

1051 The definition of coupling ratio is clarified in the revised legend of **Figure 2** as: “*open probability*
1052 *divided by dimerization probability.*” In interpreting the coupling ratio it is important to note that
1053 NBD dimerization and pore opening correlate – at least for wild-type CFTR. In **Figures 1d,g** we
1054 demonstrate correlation between current amplitude and NBD-dimerization probability in the time-
1055 courses of activation and in the ATP dose-dependences for wild-type CFTR. The coupling ratio is
1056 then the scalar that relates open probability and dimerization probability.

1057 Whilst not essential for the interpretations made in the manuscript, the ratio may be most easily
1058 understood by making a simplifying assumption: NBD-dimerization is required for pore opening.
1059 In support of this assumption, we note that across all variants and conditions that we have tested,
1060 the probability of dimerization exceeds the probability of opening. Under this assumption, the
1061 coupling ratio reflects: What is the probability of pore opening, given that the NBDs have
1062 dimerized? We find that ATP occupancy in the consensus site is crucial for the coupling, disease
1063 mutations weaken coupling, and potentiators partially rescue coupling.

1064

1065 7/ P7 the authors state “We infer from these observations that channel opening probability is
1066 enhanced by interactions of CFTR with the terminal phosphate moiety of the ATP molecule bound
1067 within the consensus site.”. This should be readily testable as the terminal phosphate at the
1068 consensus site is coordinated by K1250 and T1246. Point mutations should support this statement.

1069 Effects of substituting the Walker A lysine of the consensus site (K1250) have been reported in
1070 the literature. Consistent with our inferences and analogous to the E1371Q variant studied in the
1071 present manuscript, substitution of K1250 results in CFTR variants that bind ATP but lose
1072 hydrolysis activity, thereby trapping CFTR in dramatically prolonged open bursts (Gunderson and
1073 Kopito, 1995 and Zeltwanger et al. 1999). Like E1371Q, these variants likely mimic the pre-
1074 hydrolytic state of wild-type CFTR bound to two ATP molecules.

1075 While T1246 coordinates the terminal phosphate moiety of the consensus site ATP, the side-chain
1076 is also engaged in an important hydrogen bond with R555 across the NBD interface. Disruption
1077 of the NBD dimer interface was demonstrated in the literature by T1246N substitution (Vergani et
1078 al. 2005). Deconvolving the dual effects of substitution at this site is likely to be difficult.

1079 Gunderson KL, Kopito RR. Conformational states of CFTR associated with channel gating: the
1080 role ATP binding and hydrolysis. *Cell* **82**: 231-9 (1995).

1081 Zeltwanger S, Wang F, Wang GT, Gillis KD, Hwang TC. Gating of cystic fibrosis transmembrane
1082 conductance regulator chloride channels by adenosine triphosphate hydrolysis. Quantitative
1083 analysis of a cyclic gating scheme. *J Gen Physiol* **113**:541-54 (1999).

1084 Vergani P, Lockless SW, Nairn AC, Gadsby DC. CFTR channel opening by ATP-driven tight
1085 dimerization of its nucleotide-binding domains. *Nature* **433**: 876–880 (2005).

1086

1087 8/ Mutation G551D

1088 The authors show an intermediate FRET value of 0.37. The authors suggest that the mutation
1089 introduce a “steric and electrostatic repulsion at the NBD interface” which is very vague. An Asp
1090 at position 551 will likely prevent binding of ATP at the consensus site (at least in a NBD dimerize
1091 state) because the acidic side chain would directly and closely face the terminal phosphate. This
1092 suggests that the NBD1-NBD2 dimer is not formed at the consensus site and that the protein is
1093 adopting an intermediate/different conformation. This also suggest that the ATP “stimulation” is
1094 due to dimerization at the degenerate site. Therefore the “dimerized dwell time” on Fig E9E may
1095 actually not correspond to dimerized state as “expected”, ie. where both sites are closed, as
1096 observed by Cryo EM.

1097

1098 We agree and revised the manuscript. Page 10: “The high FRET, NBD-dimerized conformation is

1099 *likely to be different from that of E1371Q CFTR previously observed by cryo-EM, evident by a*
1100 *lower coupling ratio (Figure 4e) and a shorter life-time (Extended Data Figure 9e,f). In*
1101 *agreement with these data, a recent cryo-EM study showed that the G551D variant adopts*
1102 *conformations in between the extremes observed for fully NBD-separated (PDB 5UAK) and -*
1103 *dimerized (PDB 6MSM) states³⁵.”*

1104 Wang C., et al. Mechanism of dual pharmacological correction and potentiation of human CFTR.
1105 *bioRxiv* 2022.10.10.510913; doi: <https://doi.org/10.1101/2022.10.10.510913> (2022).

1106

1107 In addition, the statement on P10 that “Importantly, both the G551D and L927P variants exhibited
1108 ATP-dependent FRET response indicative of wild-type ATP binding affinities” is hard to
1109 reconcile with the position of D551 in the mutant

1110 The FRET response of G551D refers to formation of the intermediate conformation, in which ATP
1111 binds in the NBD partially-separated conformation, where residue 551 likely does not contribute
1112 to binding. We revised the manuscript (pages 10-11): “*For both G551D and L927P variants, FRET*
1113 *transitions exhibited ATP-dependence indicative of wild-type ATP binding affinities (Figure E9i-*
1114 *k). Their functional defects are caused by deficits in ATP effecting formation of a tight NBD dimer*
1115 *and in the coupling of the allosteric processes within NBD-dimerized CFTR that give rise to*
1116 *channel opening (Figure 4e).”*

1117

1118 Furthermore, the effect of potentiators on G551D are not clear, and not discussed.

1119 Ivacaftor was approved to treat G551D patients, we would expect to see an effect. The authors
1120 show some individual traces with GLPG1837, but not the approved drug Ivacaftor (why?) from
1121 which there seem to be no significant effect. The authors should explore and or discuss this. In
1122 contrast, stimulation of the L927P mutation does lead to fully dimerized state which does seem to
1123 correlate with increase channel opening (although Fig 4f shows quite modest improvement).

1124 GLPG1837 and Ivacaftor both have large effects on G551D pore opening and share a common
1125 binding site (Liu et al. 2019), and mechanism of action (Yeh, et al. 2017). While Ivacaftor is
1126 clinically approved, electrophysiologists (including us) favor GLPG1837 because Ivacaftor is very
1127 hydrophobic, making it difficult to determine its effective concentration, which has led to some
1128 inconsistent reporting for this compound (Csanády and Töröcsik, 2019). As Ivacaftor and
1129 GLPG1837 share the same mechanism of action, lessons learned from one compound can be
1130 applied to the other.

1131 Our smFRET measurements show that GLPG1837 does not rescue the defect of G551D
1132 dimerization. Consistently, Hunt and colleagues (Wang et al. 2022) described a lack of an effect
1133 with Ivacaftor in their structural studies of a CFTR variant harboring the G551D substitution.

1134 While GLPG1837 does promote formation of the dimer for the L927P CFTR variant, the key
1135 observation is that the relative stimulation of open probability greatly exceeds the relative
1136 stimulation of dimerization probability (Figure 4f,g). We conclude that the major effect of
1137 potentiators is not to support transition from the separated to dimerized conformation. Rather,
1138 potentiator binding acts allosterically on molecules that have already formed an NBD dimer by
1139 favoring conformational changes that couple dimerization to pore opening.

1140 As suggested, we have revised the text to incorporate these discussions (page 11): “*Consistent*
1141 *with previous reports*⁴⁰⁻⁴³, we observed that both potentiators induced marked increases of channel
1142 *open probabilities (Figures 4b,f, E10a). By comparison their effects on NBD dimerization were*
1143 *much smaller for all CFTR variants tested (Figures 4b,f, E10a). For example, GLPG1837*
1144 *increased the open probability of the G551D variant by more than 30-fold, while the change in*
1145 *NBD dimerization was marginal (Figure 4b). This observation, together with the recent cryo-EM*
1146 *study of the G551D CFTR variant in the presence of Ivacaftor*³⁵, *demonstrates that neither*
1147 *Ivacaftor nor GLPG1837 promotes NBD dimerization. Similarly, for the L927P variant, the*
1148 *relative stimulation of open probability greatly exceeded the relative stimulation of dimerization*
1149 *probability (Figure 4f,g).”*

1150 Liu, F., et al. Structural identification of a hotspot on CFTR for potentiation. *Science*, **364**, 1184–
1151 1188 (2019).

1152 Yeh, H. I., Sohma, Y., Conrath, K., & Hwang, T. C. A common mechanism for CFTR potentiators.
1153 *J Gen Physiol* **149**, 1105–1118 (2017).

1154 Csanády L, Töröcsik B. Cystic fibrosis drug ivacaftor stimulates CFTR channels at picomolar
1155 concentrations. *Elife*. Jun 17;8:e46450 (2019).

1156 Wang C., et al. Mechanism of dual pharmacological correction and potentiation of human CFTR.
1157 *bioRxiv* 2022.10.10.510913; doi: <https://doi.org/10.1101/2022.10.10.510913> (2022).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have made a strong effort and well answered the questions raised in the review. The paper is worthy of publication in its current form.

Referee #2 (Remarks to the Author):

This revised manuscript by Levring et al. has been improved significantly. I only have the following comments for the authors to consider before they finalize their paper:

1. It remains vague to me what the word “hierarchical” means when is used to describe the gating mechanism of CFTR (in Abstract and discussion). The key issue under debate is whether NBD dimerization leads to obligatory gate opening (strict coupling model), or NBD dimerization does not guarantee gate opening (allosteric mechanism with a probabilistic relationship between NBD dimerization and gate opening). The data presented in the current paper apparently support the latter idea; thus, a simple allosteric gating mechanism should be sufficient.
2. On page 8, line 164: It is not appropriate to conclude that “NBD-dimerization is insufficient for channel opening” at this point (I don’t mean this conclusion is wrong), because the steady state data (open probability and probability of dimerized state) are used to draw conclusion for a kinetic step, channel opening. Here one can certainly conclude that the NBD-dimerized state is not equal to the open channel conformation. In fact, if one puts Figures 1i (red) and 3e together, NBD dimerization and gate opening seem very much coupled. The most important data for the conclusion that “NBD-dimerization is insufficient for channel opening” are those in Figure 3b and EFigure 3i: robust NBD-dimerization in conditions where negligible channel opening is expected.
3. Figure 3: The x-axis scale of upper panel in a is different from that in the lower panel. Is this correct? In b, the x-axis is not labeled in the upper panel. Is it the same as that in the lower panel?
4. Page 12, line 387: “nearly quantitatively”?

Referee #3 (Remarks to the Author):

In the revised version of the manuscript and in the rebuttal, Levring et al at thoroughly answering all comments and questions from the 3 reviewers (while not performing new experiments...).

Regarding my specific questions, I am overall satisfied with the answers, I would still make note of the following :

1/ Line 896. What is the author’s interpretation of a 2A change ?

2/ Line 944: the ~0.2 FRET population is present in phosphorylated WT and 1371Q, but not (to my

eye at least) in phosphorylated APO wt. How is that compatible with denatured subpopulation ?

3/ Line 1022. I was expecting a distance distribution.

4/Line 1069: After reading the authors reply and going back to the two references describing mutation of K1250 (which effectively prevents hydrolysis and not binding, like E1371Q), I'm still puzzled by the sentence "We infer from these observations that channel opening probability is enhanced by interactions of CFTR with the terminal phosphate moiety of the ATP molecule bound within the consensus site." These interactions are established from the structure: K1250, T1246, Q1291 and the backbone nitrogen of G551. They almost perfectly conserved in the degenerate site (K464, T460, Q493 and G1349), suggesting that their assumption may not be correct and that something else must be at play.

1 **Author Rebuttals to First Revision:**

2 **Point-by-point response**

3

4 We sincerely thank the Reviewers for their added time and comments.

5 **Referee #1 (Remarks to the Author):**

6 The authors have made a strong effort and well answered the questions raised in the review. The
7 paper is worthy of publication in its current form.

8

9 **Referee #2 (Remarks to the Author):**

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13 mechanism of CFTR (in Abstract and discussion). The key issue under debate is whether NBD
14 dimerization leads to obligatory gate opening (strict coupling model), or NBD dimerization does
15 not guarantee gate opening (allosteric mechanism with a probabilistic relationship between NBD
16 dimerization and gate opening). The data presented in the current paper apparently support the
17 latter idea; thus, a simple allosteric gating mechanism should be sufficient.

18 We have changed the title to “*CFTR function, pathology and pharmacology at single-molecule*
19 *resolution*”.

20 The wordings in the abstract, introduction, and discussion have been adjusted. Page 2: “*CFTR*
21 *exhibits an **allosteric** gating mechanism in which conformational changes within the NBD-*
22 *dimerized channel, governed by ATP hydrolysis, regulate chloride conductance*”. Page 3: “*The*
23 *information obtained reveals an **allosteric** gating mechanism...*”. Page 11: “*Strikingly, the*
24 ***allosteric** relationship evidenced between NBD dimerization and pore opening held true across*
25 *diverse conditions and CFTR variants...*”.

26 2. On page 8, line 164: It is not appropriate to conclude that “NBD-dimerization is insufficient for
27 channel opening” at this point (I don’t mean this conclusion is wrong), because the steady state
28 data (open probability and probability of dimerized state) are used to draw conclusion for a kinetic
29 step, channel opening. Here one can certainly conclude that the NBD-dimerized state is not equal
30 to the open channel conformation. In fact, if one puts Figures 1i (red) and 3e together, NBD
31 dimerization and gate opening seem very much coupled. The most important data for the
32 conclusion that “NBD-dimerization is insufficient for channel opening” are those in Figure 3b and
33 EFigure 3i: robust NBD-dimerization in conditions where negligible channel opening is expected.

34 As suggested, we simplified the statement on page 6 to “*We thus conclude that both conductive*
35 *and non-conductive NBD-dimerized states must exist.*”.

36 3. Figure 3: The x-axis scale of upper panel in a is different from that in the lower panel. Is this
37 correct? In b, the x-axis is not labeled in the upper panel. Is it the same as that in the lower panel?

38 The interpretation of the Reviewer is correct. We have now labelled the x-axes of all the panels to
39 avoid confusion.

40 4. Page 12, line 387: “nearly quantitatively”?

41 We deleted “nearly quantitatively”, it now reads “*At physiological ATP concentrations, fully*
42 *phosphorylated CFTR remains NBD dimerized for many cycles of ATP turnover and pore*
43 *opening.*”

44

45 **Referee #3 (Remarks to the Author):**

46 In the revised version of the manuscript and in the rebuttal, Levring et al at thoroughly answering
47 all comments and questions from the 3 reviewers (while not performing new experiments...).
48 Regarding my specific questions, I am overall satisfied with the answers, I would still make note
49 of the following:

50 1/ Line 896. What is the author’s interpretation of a 2Å change?

51 Our structure clearly demonstrated that ATP binding to the dephosphorylated CFTR does not lead
52 to large-scale conformational change. Therefore the 2 Å change likely reflects local
53 conformational changes of the sites of labelling as we stated on page 5: “*The small shift in FRET*
54 *efficiency is likely due to local changes that affect either the position and/or dynamics of the sites*
55 *of labelling*”.

56 2/ Line 944: the ~0.2 FRET population is present in phosphorylated WT and 1371Q, but not (to
57 my eye at least) in phosphorylated APO wt. How is that compatible with denatured subpopulation?

58 Given that the 0.2 FRET population does not resolve from the dominant 0.25 population for the
59 phosphorylated APO wt condition, it may simply be hidden in the left shoulder of the 0.25 peak.
60 The same is true for dephosphorylated APO wt and dephosphorylated ATP wt conditions.

61 3/ Line 1022. I was expecting a distance distribution.

62 We believe it is more appropriate to present the distributions of NBD dimerization and channel
63 opening, thus we can directly compare the Y1219A variant with the WT CFTR and other variants.

64 4/Line 1069: After reading the authors reply and going back to the two references describing
65 mutation of K1250 (which effectively prevents hydrolysis and not binding, like E1371Q), I’m still
66 puzzled by the sentence “We infer from these observations that channel opening probability is
67 enhanced by interactions of CFTR with the terminal phosphate moiety of the ATP molecule bound
68 within the consensus site.” These interactions are established from the structure: K1250, T1246,
69 Q1291 and the backbone nitrogen of G551. They almost perfectly conserved in the degenerate site
70 (K464, T460, Q493 and G1349), suggesting that their assumption may not be correct and that
71 something else must be at play.

72 For clarity we removed this sentence from the manuscript.