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Structural basis of CFTR folding, function and pharmacology

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Abstract

The root cause of cystic fibrosis (CF), the most common life-shortening genetic disease in the Caucasian population, is the loss of function of the CFTR protein, which serves as a phosphorylation-activated, ATP-gated anion channel in numerous epithelia-lining tissues. In the past decade, high-throughput drug screening has made a significant stride in developing highly effective CFTR modulators for the treatment of CF. Meanwhile, structural-biology studies have succeeded in solving the high-resolution structure of CFTR in different conformations. Here, we provide a brief overview of some striking features of CFTR folding, function and pharmacology, in light of its specific structural features within the ABC-transporter superfamily. A particular focus is given to CFTR's first nucleotide-binding domain (NBD1), because folding of NBD1 constitutes a bottleneck in the CFTR protein biogenesis pathway, and ATP binding to this domain plays a unique role in the functional stability of CFTR. Unraveling the molecular basis of CFTR folding, function, and pharmacology would inspire the development of next-generation mutation-specific CFTR modulators.

Keywords: CFTR, ABC transporter, modulator, corrector, potentiator

1. General introduction on CFTR 3D structures

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein is a member of the ATP-binding cassette (ABC) transporter family, forming a pseudo-dimeric architecture made of two units, each comprising a six-spanning transmembrane domain (TMD) and a nucleotide-binding domain (NBD) (**Figure 1**) [1-4]. CFTR stands apart in this large family however, as it possesses a unique, large cytosolic regulatory (R) domain linking the two TMD-NBD units. This R domain is largely disordered and contains multiple sites for PKA-dependent phosphorylation, which is required for CFTR function as an ATP-gated ion channel. The TMD-NBD units also contain specific structural elements that are associated with functional features (**Figure 1**) [3, 5-8]. These are:

(i) Asymmetric composite ATP-binding sites formed at the interface between NBD1 and NBD2 (i.e., catalysis-competent site 2 and non-canonical, degenerate site 1).

(ii) A regulatory insertion (RI) in NBD1 in the vicinity of the non-canonical ATP-binding site. Removal of RI (Δ RI) leads to rescue of F508del CFTR from retention and degradation and to improvement of its channel function [9]. Yet, the gating cycle of wild-type CFTR is only slightly affected by deletion of RI [10].

(iii) A partially broken transmembrane helix TM8 in TMD2, whose upper part is, together with TM7, in a non-canonical position. In all the CFTR 3D structures solved so far at high resolution (see [6, 8]), the channel is locked in a closed conformation, even in the phosphorylated, ATP-bound structure that would be expected to be an open state because of the introduced E1371Q mutation (at the Walker B motif of NBD2) that prolongs the CFTR opening-burst duration by abolishing ATP hydrolysis [3]. The bulky side chain of F337 in TM6 physically obstruct the pore, locked by the upper part of TM8, which together with TM1 and TM12 makes a significant contribution to the narrow region of the channel [11].

(iv) Lateral portals –or cytoplasmic pore entrances– at the level of the intracellular loops (ICLs) in the TMDs that connect to NBDs: one in TMD1 between TM4 and TM6 allowing anion flow towards the inner vestibule of the channel and the other one in TMD2 between TM10 and TM12, which in the cryo-EM 3D structures is not wide enough for ion entry due to the smaller distance between these two helices [7, 8, 12].

(v) An N-terminal extension called “lasso” motif common to proteins of the ABCC subfamily. The N-terminus is partially embedded in the lipid bilayer in contact with TM helices but likely adopts multiple conformations during folding or during the gating cycle, especially when interacting with potential partners, including the R domain [6, 13, 14].

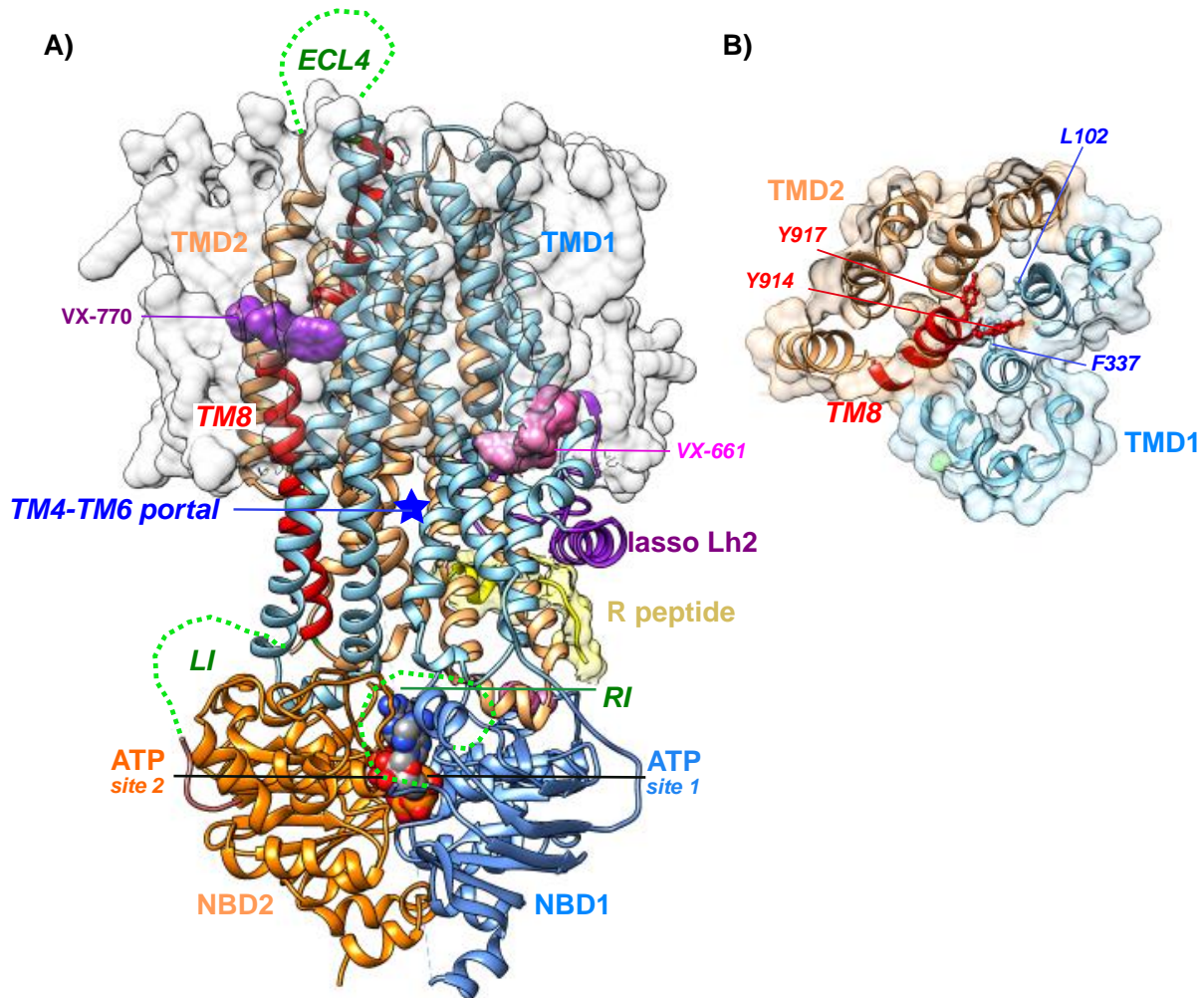


Figure 1. CFTR 3D structure

A) Ribbon representation of the experimental 3D structure of human CFTR (phosphorylated, ATP-bound, pdb 6MSM), inserted in a membrane bilayer model (grey surfaces). The polypeptide chain is colored according to the different domains, with the TM8 helix highlighted in red. VX-770 (pdb 6O2P [15]) and VX-661 (pdb 7SV7 [16], also see [17]) are shown in their respective binding sites. The regulatory insertion (RI) in NBD1, the linker insertion (LI) between TMD2 and NBD2 and the extracellular loop ECL4 (disordered and thus absent from the cryo-EM data) are depicted with green, dashed lines. The regulatory domain (R) was also not solved in the cryo-EM 3D structure, except for a small peptide segment assuming an alpha-helical structure (yellow); however, precise amino acids were not assigned for this segment because of the poor resolution. **B)** Top view of the external portions of the TMDs, showing the unusual position of TM8 coming down onto the constrictive part of the channel pore and F337 in TM6 physically obstructing the pore.

2. Folding mechanisms and mode of action of correctors.

CFTR has attracted much attention due to its intrinsically complex folding pathway, which is impaired by multiple disease-causing mutations. Understanding the folding mechanisms of wild-type CFTR uncovers the folding defects in disease-causing mutants. The converse is true as well. Structural data facilitate prediction of the impact of mutations, while biomarkers are globally used to evaluate correlation of CFTR folding or function with clinical data. Through innovative computational methods and machine learning, the Balch lab recently used functional phenotypes and ER-Golgi trafficking of a limited dataset of 64 CFTR2 mutants to predict how each residue in CFTR contributes to function and folding [18, 19]. Interestingly, these novel methods may also contribute to predicting personalized medication of CF [18].

Grasping CFTR folding is also important from a more general perspective in that it may help unravel commonalities of mammalian ABC-transporter folding. This pathway, monitored by a cellular quality-control machinery, includes co- and post-translational steps and involves insertion of 12 TMs into the lipid bilayer, individual folding of each domain and their assembly into a mature 3D structure [20-24]. As the CFTR molecule spans three topological spaces, folding of its domains will depend on the help of chaperones localized within the endoplasmic reticulum (ER) lumen, the ER bilayer, and the cytoplasm [25-27]. Failure to meet quality-control checkpoints will lead to retention in the ER and degradation. Precisely which pathways are involved in degradation is not known, but proteasome inhibitors are known to stabilize certain mutants while for instance N1303K is removed by autophagy [28, 29]. Class III and class IV mutations bypass ER quality control at least to some extent, as they are dysfunctional and hence cannot be properly folded but nevertheless reach the cell surface [30, 31].

An important factor in the triage process is the CFTR mutation itself. In fact recent top down proteomic studies have begun to uncover differential interactions of distinct mutants with specific degradative machineries [26] and more generally have revealed mutant-specific interactomes [25, 27, 32].

The folding of NBD1 represents the Achilles' heel in CFTR biosynthesis [24], a weakness that is accentuated by the F508 deletion in NBD1, the most common CF-causing mutation. F508del has little effect on the NBD1 3D structure but does decrease its folding efficiency and thermal stability. As a consequence, assembly of all domains is affected, in particular the interface of NBD1 with ICL4 in TMD2 [33-35], and hence the folding and stability of the entire protein [35-37].

An advantage of such a clear 2-phase folding defect is that rescue of defective CFTR is achievable through different means, by rescuing folding defects in NBD1 and/or by rescuing domain assembly. Here, we focus on recent advances made in the field of CFTR basic science research that provide insights into the role of ligands on the folding and function of CFTR: the folding and activity of the critical NBD1 domain and the domain assembly of CFTR.

Stability of F508del NBD1 can be improved in many ways: specific second-site suppressor mutations in NBD1 (for example I539T, R553M), removal of the regulatory insertion (RI), or binding of ATP or nucleotide analogs [9, 10, 33-35]. As a consequence, both folding and function of F508del CFTR improve, implying that rescue at the defective mutated domain improves the whole protein.

Isolated NBD1 needs ATP to be stable on its own [38]. This stabilizing effect of ATP binding on NBD1 has been observed in the nascent polypeptide chain upon *in vitro* translation, and may hence facilitate subdomain folding in the ER [39]. The intrinsically disordered RI is a special feature in CFTR's NBD1, but even more special is that it can adopt an alternative conformation when studied in isolation. This beta-strand-swapped conformation essentially leads to a reorganization of the beta-subdomain and a shift of the disordered part to the linker between TMD1 and NBD1, increasing its length [40]. The equilibrium is pulled towards the topologically alternative conformation in the absence of ATP or by a specific nanobody. As gating of CFTR is not much changed by deletion of RI [9], this disordered region may well be more important for CFTR folding than for ATP-dependent CFTR gating.

The potential roles of RI in CFTR biogenesis and channel function have been investigated quite a while ago. As described above, Csanády et al. [10] reported a slight decrease of the open probability with shorter open time in a construct lacking amino-acid residues 415-432 in RI. On the other hand, removal of RI in the F508del background significantly improves biogenesis and channel function [9]. Of particular interest is the observation that Δ RI-F508del channels assume a much-prolonged lifetime in the plasma membrane with a longer ATP resident time in the degenerate site 1 (see below for more details).

Type-I correctors (lumacaftor/VX-809, tezacaftor/VX-661, C18) and the novel corrector elexacaftor/VX-445 in contrast do not rescue NBD1 folding but do salvage mutant CFTR from retention and degradation [22, 41]. These compounds enhance overall transmembrane chloride flux primarily because of an increase in the number of CFTR molecules (N) on the cell surface due to their positive effects on CFTR folding and stability [22, 41-46]. The mechanisms of CFTR function, the role of potentiators, and importance of NBD1 will be discussed below.

High-throughput compound screenings in many industrial and academic labs [45, 47, 48] have yielded a series of modulators that together rescue F508del CFTR to wild-type levels and function, despite the misfolded NBD1 [22]. Rescue by rectification of the primary defect – F508del NBD1 misfolding– must be more difficult than rescue by enhancing domain assembly. This rather indirect improvement of CFTR mutants is a blessing in disguise as most mutants respond well to these correctors. FDA approval for Trikafta (VX770 + VX661 + VX445) has been made to >150 distinct CFTR mutations up to now.

Enhanced domain assembly is effected by type-I correctors through their stabilization of TMD1, apparently irrespective of NBD1 stability [33, 34, 49]. The binding site for the type-I correctors VX-809 and VX-661 has recently been identified by two independent studies [16, 17]. The drugs insert their common 1,3-benzodioxol-5-yl-cyclopropane carboxamide group in a hydrophobic pocket in TMD1, while their variable parts are in contact with lipids (Figure 1). Type I correctors were proposed to stabilize a fragile region of the protein corresponding to a hot spot of class II mutations, which includes P67L, G85E, E92K, L206W, P205S, and W361R. The stabilization of this area allows allosteric rescue of mutations in other parts of the protein. In particular, an

allosteric pathway towards NBD1 was highlighted, involving the lasso helix 2 (Lh2 in the N-terminus of TMD1), ICL1 and ICL4 [17]. This may explain how drug binding acts allosterically on the ICL4:NBD1 interface. The allosteric pathway through CFTR explains as well why disease-causing class-II mutation P67L, just downstream of Lh2, [44] and the rare mutation E92K in the elbow helix 1 (Eh1) [50] respond so strongly to VX-809. The G85E mutant, in contrast, lacks any response to type-I correctors likely due to a misalignment of the G85E-containing TM1 in the ER membrane [51, 52], as enhanced packing of the TMD1 transmembrane helices by type-I correctors is only possible when the helices are properly aligned. Fascinating then is the effect of ribosomal-protein Rpl12 depletion, which effects local changes in the translation rate to improve folding (domain assembly) of G85E and its export from the ER [53].

Another recent advance was the report describing the mode of action of VX-445 and defining this as a different type of corrector. A first study reported its binding and activity *in vitro* at concentrations of 50-100 μ M to purified F508del-NBD1 and a rescue effect on F508del function *in vivo* [46]. More recent folding experiments in cells however show that 3 μ M VX-445 does not rescue NBD1 folding, nor that of TMD1 or TMD1-NBD1 [41]. Molecular docking followed by molecular dynamics simulations suggested a potential binding site for VX-445 in NBD1, corresponding to the region that is targeted by the dual-acting potentiator-corrector CFFT-01 [54] and that was also predicted to accommodate VX-809 or VX-661 as well [17]. The same study also revealed a separate binding site for VX-445 in TMD1, distinct from that of VX-809 and VX-661 [17]. Whether VX-445 binding to NBD1 or to another domain is related to its potentiator activity, which is additive to ivacaftor [55-57], remains to be explored. It is very likely that a binary classification of CFTR modulators into correctors and potentiators is too simple, given results of ongoing medicinal-chemistry research yielding new therapeutic compounds with unanticipated modes of action on CFTR. After all, a lack of functionality is always caused by a conformation/structure defect, whether recognized by the cellular quality-control system or not, and whether due to retention and degradation or channel dysfunction.

The story would not be complete without mentioning the importance of lipids in the biogenesis of the CFTR protein, which not only drive clustering of CFTR into nanoscale membrane

domains – or lipid rafts – [58, 59], but also likely play a role in its folding and activity [60]. The clustering of CFTR is dependent on cholesterol, but is decreased in CF, despite an increase of cholesterol in CF epithelia; correctors restore the clustering [59]. How allosteric interactions with lipids participate in the modulation of stability and/or activity of CFTR has yet to be explored. The large pocket occupied by type-I correctors in TMD1 may well be a primary candidate, as this spot can stably accommodate lipid acyl chains [61]. Membrane lipids may also play an important role in maintaining a patent pore structure, as all the structures so far solved at atomic resolution in detergents are in a closed conformation despite the use of strategies to promote the open state.

3. Gating mechanisms and mode of action of potentiators

The operation of CFTR as an ion channel can be simplified into two distinct steps: the phosphorylation-activation step and the ATP-dependent gating step [1, 3, 5]. In the first step, slow spontaneous dislodgement of the R domain exposes its consensus serine residues to protein kinase A (PKA)-dependent phosphorylation [62, 63]. The phosphorylated R domain is sequestered at the periphery of CFTR, permitting free access of ATP to the two ATP-binding sites at the interface between the two TMD-NBD complexes [8, 64, 65]. In the second step, the phosphorylated channel opens upon ATP binding-induced NBD dimerization [2, 66, 67].

Despite this clear regulation, phosphorylated CFTR assumes a noticeable spontaneous opening rate in the absence of ATP [68]; in the presence of a maximally effective concentration of ATP, the opening rate is increased by ~100 - 200 fold. ATP-dependent gating kinetics is slow with open (burst) time as well as closed (interburst) time constants in the range of hundreds of milliseconds for wild-type CFTR channels. It is the catalysis-competent site 2 that is mostly responsible for the gating effect of ATP on phosphorylated CFTR, although it is *not* ATP binding *per se*, but ATP binding-induced NBD dimerization that is coupled to gate opening [66, 69]. Closing of the channel is facilitated by ATP hydrolysis at site 2 [2, 70, 71].

Comparison of the unphosphorylated and phosphorylated, ATP-bound CFTR structures, reveals not only a canonical NBD dimer with two ATP molecules sandwiched at the dimer interface, but

also rearrangements of the TMDs so that the above-mentioned, two cytoplasmic portals narrow, and the extracellular segments of TM8 and TM12 tilt towards TM1 and TM2 [65]. While these conformational changes induced by phosphorylation and ATP binding constitute parts of the channel-opening process, the fact that all the structures of phosphorylated ATP-bound CFTR do not possess an open pore suggests that additional conformational changes are needed to “open” the channel.

Two kinetic mechanisms have been proposed to explain ATP-dependent gating of CFTR. 1) The strict coupling-gating model, an idea borrowed from the proposed molecular motions of ABC transporters. It depicts a synchronized movement of the CFTR TMDs and NBDs as a rigid body so that each opening of the gate is tightly coupled to NBD dimerization and each closing to a complete separation of NBDs triggered by ATP hydrolysis at site 2 [6, 72]. 2) In contrast, the energetic-coupling model for ATP-dependent gating of CFTR follows the principle of classical allosteric modulation: a probabilistic relationship between opening/closing of the gate in TMDs and association/dissociation of the two NBDs [73-75](cf. [76, 77]). The structural implications of these kinetic models have been discussed extensively [3].

Regardless of the exact mechanism of CFTR gating, one puzzle has lingered for quite some time: As site 1 does not hydrolyze ATP, and hence is called a “degenerate site”, what is the functional role of tightly-bound ATP in this site [78-80]? Of note, there is a large gap between the lifetime of site 1 ATP in biochemical data (tens of minutes) and the lifetime estimated from functional studies (~30 s). Recent biochemical experiments however show that this slow release of ATP at site 1 is greatly accelerated by phosphorylation of the R domain or phosphorylation-mimetic mutations at the consensus serines in the R domain [81]. No matter which lifetime is taken into consideration, the duration of ATP trapping at site 1 is way longer than the duration of a gating cycle, suggesting that site-1 ATP turnover is not associated with opening and closing of CFTR. Indeed, earlier studies have not assigned a definite role of the degenerate site 1 in controlling channel opening by ATP [67, 82-84]. A recent report by Yeh et al. [85] offered some clues however. It showed that opening of CFTR by ATP becomes sluggish upon prolonged deprivation of ATP, suggesting that a state with completely separated and emptied NBDs is dysfunctional (**Figure 2**). The study also showed experimental evidence linking this functional deterioration

(or rundown) to ATP dissociation from site 1. Interestingly, eliminating ATP hydrolysis at site 2 minimizes this ATP depletion-caused channel rundown. The latter observation raises the possibility that some evolutionary advantages emerge for those ABC transporters in which one of the ATP-binding sites is disabled.

Moreover, this role of site-1 ATP in stabilizing CFTR function is defective in F508del [85], an observation consistent with a previous report that showed decreased site-1 ATP binding in F508del [86]. As described above, removal of RI stabilizes the channel function of F508del [9], and Δ RI-CFTR is more resistant to prolonged ATP deprivation (Yeh and Hwang, unpublished observation). These data all point to a yet-to-be-defined role of RI and site-1 ATP in determining the structural—and hence functional—stability of CFTR.

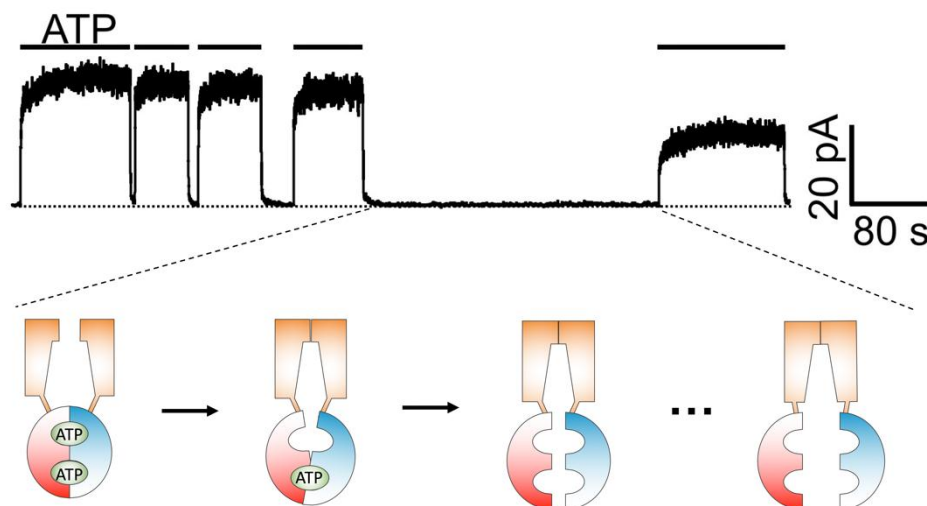


Figure 2. Role of tight ATP binding in maintaining proper CFTR gating. Real-time recording of wild-type CFTR in response to removal of ATP for various intervals and subsequent addition of ATP. The longer the duration of ATP removal, the larger the fraction of CFTR currents that rise more slowly and the lower the final steady-state currents observed. The cartoon depicts a hypothetical structural alteration of the NBDs upon prolonged deprivation of ATP: Forced depletion of ATP results in channel dysfunction due to disengagement of the two NBDs. (Adapted from [85])

Gating abnormalities account for at least 5% of all disease-associated mutations; even the most common F508del exhibits a gating defect nearly as severe as the well-established defect in biogenesis [87]. Indeed, it takes a combination of correctors and potentiator to significantly improve the clinical manifestations of CF patients carrying the F508del mutation [88, 89]. A wide spectrum of compounds with potentiator activities has been reported over the past two

decades (see [90] for review). The discovery of VX-770 [48] and subsequent success in clinical trials [91, 92] no doubt ushered in a new era of precision medicine, targeting directly the root cause of CF.

VX-770 increases CFTR activity by stabilizing the open channel conformation [48, 93, 94]. As its action is independent of ATP hydrolysis or NBD dimerization, VX-770 was proposed to bind to CFTR's TMDs [95]. Experimental data support the proposition that the CFTR's TMDs have a common spot for VX-770 and a structurally different CFTR potentiator GLPG1837 [96]. This latter idea was indeed verified when the cryo-EM structure of CFTR/VX-770 (or GLPG1837) was solved [15]. This hot spot for CFTR potentiators is formed mostly by hydrophobic amino acids from TM4, 5, and 8. Of particular interest in this binding site is Y304, which is a phenylalanine in mouse or rat CFTR. Although mouse CFTR does not respond to VX-770 (or GLPG1837), converting F304 in mouse CFTR to tyrosine restores the effects of VX-770 and GLPG1837 [48, 97, 98]. The observation that residues in TM8 contribute to binding of these CFTR potentiators supports the notion that gating of CFTR involves the molecular motion of TM8 discussed above. It is surprising however that the CFTR structures with potentiators bound still assume a closed conformation in the pore (**Figure 1**), suggesting that cryo-EM experimental conditions somehow favor a closed pore and that other strategies are needed to obtain the open-channel structure. Alternatively, multiple binding sites may exist for CFTR potentiators [97, 99], or membrane lipids play an essential role in conformational changes of CFTR [100]. Regardless, solving the open state of CFTR is expected to help future structure-based drug design as the allosteric modulation mechanism proposed for CFTR potentiators dictates that the drug binds to the open state with higher affinity than to the closed state [96].

CF-causing CFTR mutants that have functional defects and reach the cell surface sufficiently (if not to wild-type levels) are grouped into classes III and IV. Although these mutants are licensed to leave the ER to some extent, they should not be considered properly folded. Functionally, and therefore structurally/conformationally, their gating (class III) and/or conductance (class IV) are defective. For instance, R347P leaves the ER for only ~15%, R117C for ~50% and R117H even much better than wild-type [30, 101]; all have been FDA-approved for Trikafta. These three variants and R334W all respond well to corrector VX-809, while R347P does not respond at all

(biochemically nor functionally) to potentiator VX-770 [30]. On the other hand, G551D, a prototypical class-III mutation that responds to VX-770 extremely well, exhibits severe gating defects presumably due to an electrostatic repulsion between the negatively charged side chain of aspartate 551 and gamma phosphate of the bound ATP at site 2 [74].

Unlike G551D, most other pathogenic mutations in CF cannot be explained mechanistically in a straightforward manner, but the atomic structures of CFTR now afford a great opportunity to understand possible structural basis for their pathophysiological and pharmacological properties. For example, R347 in TM6 forms a salt bridge with D924 in TM8 [102]. The role of this salt bridge is unclear in CFTR gating as it is present in both unphosphorylated and phosphorylated, ATP-bound CFTR structures. Nonetheless, it is interesting to note that this salt bridge lies just behind the binding pocket for VX-770. Thus, one can speculate that the salt bridge between R347 and D924 may be important for the structural integrity of CFTR at this local area, and that disruption of the salt bridge changes the conformation of the VX-770 binding site and thereby renders the mutant unresponsive to VX-770.

Similar structural insights may be attained for other pathogenic mutations such as R117H, a mutation at the first extracellular loop. This mild-CF mutation causes both conductance and gating defects [103, 104]. Some experimental data suggest that disruption of the interaction between R117 and E1124 is responsible for the gating defect of R117H [105], but more studies are required to address the exact role of this residue in gating and how a mutation so far away from the NBDs causes gating defects.

Conclusion

The last couple of years have witnessed enormous progress in the field of CF, with in particular the development of remarkably effective combinatorial therapy for a large number of mutations and the successes in solving the structure of CFTR at an atomic resolution. Nevertheless, the mechanisms underlying the action of CFTR modulators on the mutated proteins, and more broadly the molecular basis of the wild-type CFTR folding and function, remain not fully understood. Future work will contribute to improved fundamental knowledge that will eventually

help guide the development of novel therapies. In particular, the roles of lipids and of the lasso motif in the mechanisms of folding and activation of the channel need clarification (see for instance [106]), we will need to understand the full conformational landscape of CFTR. This ongoing basic research is indeed still relevant insofar as some rare mutations can escape current treatments, or current medications may induce intolerances or unbearable side effects. Finally, this in-depth knowledge on CFTR structure/function will also be beneficial in general to our understanding of diseases that affect other ABC transporters.

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Conflict of interest statement: no competing interest associated with the manuscript.

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