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REVIEW

# The molecular evolution of function in the CFTR chloride channel

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**The ATP-binding cassette (ABC) transporter superfamily includes many proteins of clinical relevance, with genes expressed in all domains of life. Although most members use the energy of ATP binding and hydrolysis to accomplish the active import or export of various substrates across membranes, the cystic fibrosis transmembrane conductance regulator (CFTR) is the only known animal ABC transporter that functions primarily as an ion channel. Defects in CFTR, which is closely related to ABCC subfamily members that bear function as bona fide transporters, underlie the lethal genetic disease cystic fibrosis. This article seeks to integrate structural, functional, and genomic data to begin to answer the critical question of how the function of CFTR evolved to exhibit regulated channel activity. We highlight several examples wherein preexisting features in ABCC transporters were functionally leveraged as is, or altered by molecular evolution, to ultimately support channel function. This includes features that may underlie (1) construction of an anionic channel pore from an anionic substrate transport pathway, (2) establishment and tuning of phosphoregulation, and (3) optimization of channel function by specialized ligand–channel interactions. We also discuss how divergence and conservation may help elucidate the pharmacology of important CFTR modulators.**

## Introduction

The ATP-binding cassette (ABC) transporter superfamily includes many members of clinical relevance, such as the multidrug resistance proteins (MRPs) and other proteins involved in generation of antibiotic resistance, transport of a wide variety of substrates in pathogenic bacteria, and transport of bile acids, lipids, and lipopolysaccharides (Ford and Beis, 2019; Jetter and Kullak-Ublick, 2020). ABC transporter genes encode the largest family of transmembrane (TM) proteins among living organisms (Briz et al., 2019) and are expressed in all domains of life (Ford and Beis, 2019; Holland et al., 2003). Either function or dysfunction of ABC transporters is implicated in development or treatment of cancer (Briz et al., 2019; Nobili et al., 2020), neurological disorders (Jha et al., 2019; Sumirtanurdin et al., 2019), detoxification (Briz et al., 2019), visual function (Garces et al., 2018), and, among many other clinical presentations (Moitra and Dean, 2011), in cystic fibrosis (CF; Riordan et al., 1989). In CF, mutations in the gene encoding CFTR lead to loss of anion transport in a wide variety of epithelial tissues (Csanády et al., 2019). In this review, we use the data generated from >30 yr of intensive structure-function study of CFTR and related proteins to propose and evaluate a potential route by which CFTR may have evolved unique function as a phosphorylation-regulated chloride channel. New insights are made possible by the advent of high-resolution cryo-EM structures of CFTR and the recent cloning and characterization of the evolutionarily oldest known orthologue of CFTR, from sea lamprey (Lp-CFTR; see below), which exhibits many functional differences from the human CFTR orthologue (hCFTR; Cui et al., 2019a).

### *Overview of CFTR*

CFTR is a $Cl^-/HCO_3^-$ channel whose dysfunction directly leads to CF, the most common life-shortening genetic disease among Caucasians, affecting ~80,000 individuals worldwide (Riordan et al., 1989; https://cftr2.org/mutations_history). The role of CFTR has been well characterized in airway, intestine, and sweat gland epithelial cells (Buchwald et al., 1991; Gonska et al., 2009; Haq et al., 2016; Quinton et al., 2012; Quinton, 2007; Trezíse and Buchwald, 1991), where the anionic flux mediated by the protein contributes to water secretion and regulation of pH (Pezzulo

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et al., 2012; Rowe et al., 2014). CFTR also functions in several nonepithelial cell types (Cook et al., 2016; Edlund et al., 2014; Gao and Su, 2015; Guo et al., 2014; Norez et al., 2014; Pohl et al., 2014; Schulz and Tümmler, 2016; Su et al., 2011), including in the brain (Ballerini et al., 2002; Guo et al., 2009; Hincke et al., 1995; Johannesson et al., 1997; Mulberg et al., 1995; Mulberg et al., 1998; Parkerson and Sontheimer, 2004; Pfister et al., 2015; Plog et al., 2010; Weyler et al., 1999). Several hundred disease-causing mutations have been identified in the *CFTR* gene. For a subset of these mutations, four small-molecule modulator therapeutics from Vertex Pharmaceuticals, Inc. that increase the surface expression or activity of CFTR have been approved for clinical use. The first approved drug, VX-770 (ivacaftor), is a gating potentiator that increases function of certain CFTR mutants (Cui et al., 2019b; Sosnay et al., 2013; Van Goor et al., 2009; Van Goor et al., 2014; Yu et al., 2012). A better understanding of these drugs and their binding sites may aid in refining the next class of therapeutics.

ABC transporters use the energy of ATP binding and hydrolysis to accomplish the active import or export of various substrates across membranes (Rees et al., 2009). There are seven subfamilies of mammalian ABC transporters (ABCA, ABCB, ABCC,... ABCG), of which the E and F subfamilies do not bear actual transport function (Dean et al., 2001; Ford and Beis, 2019). A new classification of the ABC transporter superfamily that is based on the transmembrane domain (TMD) fold has recently been suggested (Thomas et al., 2020). CFTR is denoted ABCC7 and a member of type IV, respectively, according to these two classification schemes. CFTR bears ATPase activity like that of other ABCC subfamily members (Li et al., 1996; Stratford et al., 2007; Jordan et al., 2008), but biophysical methods have firmly established that CFTR functions as a phosphorylation-activated and ATP-gated ion channel (Anderson et al., 1991a; Anderson et al., 1991b; Bear et al., 1992; Berger et al., 1991; Sheppard et al., 1993), whereas its closest ABCC relatives function as multispecific exporters of organic anions (Jordan et al., 2008). CFTR may directly mediate the flux of glutathione (Gao et al., 1999; Kogan et al., 2003; Linsdell and Hanrahan, 1998), although CFTR-mediated active transport has not been shown, to our knowledge. Glutathione is transported by close ABCC relatives ABCC1/MRP1 (Mao et al., 1999) and ABCC4/MRP4 (Choi et al., 2001; Ko et al., 2002; Kogan et al., 2003; Ritter et al., 2005; Serrano et al., 2006); previous analysis has identified ABCC4 as CFTR's closest relative (Jordan et al., 2008; see also Cui et al., 2019a). The domain organization of CFTR is similar to that of its closest relatives, the "short transporters" of the ABCC subfamily (Jordan et al., 2008; Ford and Beis, 2019; Srikant and Gaudet, 2019), with two nucleotide-binding domains (NBDs) that function in ATP binding and hydrolysis, and two TMDs, each containing six TM helices that comprise the substrate transport pathway (Fig. 1). However, unique to CFTR is an intracellular regulatory (R) domain that contains multiple consensus sites for phosphorylation by PKA (Sebastian et al., 2013).

The opening of CFTR may be simplified to involve three sequential steps that have been uncovered via a combination of functional and structural data. First, PKA binds to (Mihályi et al., 2020) and phosphorylates (Rich et al., 1991) the aforementioned R domain, which results in loss of inhibitory interactions between that domain and the rest of the channel protein. Second, ATP binds to two sites at the interface of the cytoplasmic NBDs, which promotes a stable NBD dimer (Mense et al., 2006; Vergani et al., 2005). Finally, the wave of conformational changes associated with ATP-induced dimerization of the NBDs is transmitted to the pore domain, resulting in pore opening (Rahman et al., 2013; Simhaev et al., 2017; Sorum et al., 2015; Strickland et al., 2019). In related ABC exporters, ATP-dependent dimerization of the NBDs drives an overall transition from inward- to outward-facing conformation of the TMDs; this function was coopted by CFTR to drive ATP-induced channel opening (Fig. 2). At the level of individual residues, there is high conservation with transporters among amino acids in CFTR that are proposed to stabilize the inward-facing (closed) conformation in the absence of ATP (Wang et al., 2010; Wei et al., 2014; Wei et al., 2016), suggesting conservation of motifs integral to energetic signaling (Wang et al., 2014b; Wei et al., 2014; Wei et al., 2016). The close proximity of intracellular loops 2 and 4 (ICL2 and ICL4, respectively; Doshi et al., 2013; Wang et al., 2014b), constriction of the intracellular vestibule (Bai et al., 2011), and dilation of the extracellular vestibule, relative to the closed state, are all associated with channel opening (Beck et al., 2008; Infield et al., 2016; Norimatsu et al., 2012b; Rahman et al., 2013; Strickland et al., 2019). The CFTR pore opens in stages, requiring the sequential breaking and forming of intraprotein residue–residue interactions (Cui et al., 2013, 2014; Rahman et al., 2013), resulting in two subconductance states in addition to the full-conductance state (Gunderson and Kopito, 1995; Zhang et al., 2005a; Zhang et al., 2005b; Fig. 3). Using a particularly informative cysteine mutant at the outer vestibule, R334C-CFTR, the McCarty laboratory found that transitions between these subconductance states are highly dependent upon experimental conditions; for example, closing transitions almost always start from the s2 state in the presence of ATP, and transitions from s2 to f never occur in channels bound with the poorly hydrolyzable ATP analogue AMP-PNP (see also Langron et al., 2018), suggesting that this transition requires hydrolysis of nucleotide at the NBDs (Zhang et al., 2005a; Zhang et al., 2005b). Subconductance states are evident in recordings of WT CFTR from membrane patches and planar lipid bilayers, depending on experimental conditions, indicating that these represent inherent steps in gating of the channel pore (Gunderson and Kopito, 1995). In WT-hCFTR, this open pore is quite stable and does not close until ATP is hydrolyzed at the NBDs (Baukrowitz et al., 1994). Note that because CFTR displays three types of gating in one channel (phosphorylation-mediated, ligand-mediated, and pore-mediated gating), it serves as an exemplary target for studying the evolution of functional mechanisms within a single membrane protein.

### *Natural history of the CFTR channel in vertebrates*

Given the structural conservation among CFTR and ABC exporters noted above, and functional conservation in terms of ATP dependence, how CFTR evolved to function as an anion channel regulating passive ionic diffusion has been an enduring question (Srikant, 2020; Srikant et al., 2020). Molecular

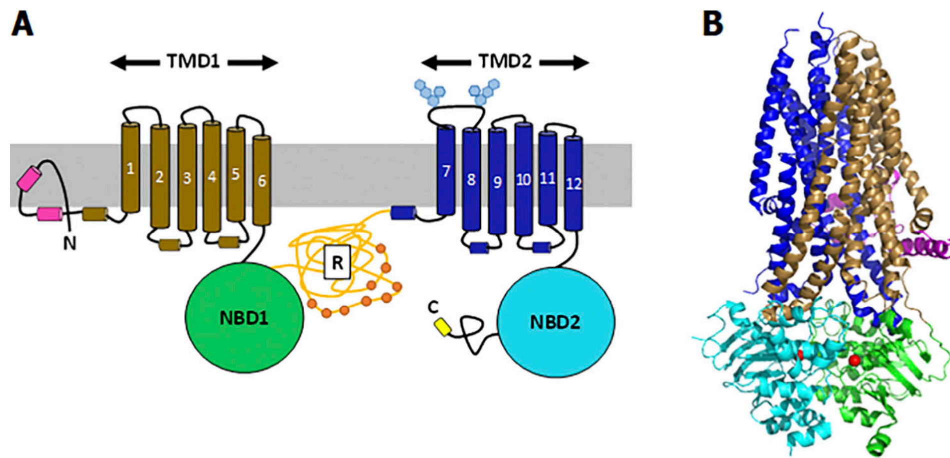


Figure 1. **Domain architecture of CFTR. (A)** Five functional domains: TMD1, NBD1, R domain with multiple phosphorylation sites, TMD2, and NBD2. Each TMD includes six transmembrane helices, numbered 1–12. The N-terminus includes the lasso motif (shown in pink), whereas the C-terminus includes a PDZ binding domain motif (yellow). **(B)** hCFTR from cryo-EM structure (PDB accession no. 6MSM). The R domain is not shown, because it is intrinsically unstructured.

evolution studies are facilitated by the availability of many orthologues for the protein/gene of interest, spanning as much of the evolutionary record as possible. Currently, ~300 CFTR orthologues are included in GenBank/UniProt, although not all of these are represented by expressible cDNA clones. Until very recently, the oldest CFTR orthologue known was from the dogfish shark, arising ~150 million yr ago (MYA; Fig. 4; Marshall et al., 1991); this orthologue bears functional characteristics similar to those of hCFTR. However, reasoning that the identification of an earlier CFTR orthologue with altered structure/function would provide novel insight into the evolution of epithelial anion transport, the Gaggar and McCarty laboratories recently led an effort to clone and characterize the Lp-CFTR (Cui et al., 2019a), which arose ~550 MYA (Smith et al., 2013). The identification of a CFTR orthologue in the jawless vertebrates establishes that CFTR exists across all vertebrates, predating the divergence of jawed and jawless vertebrates at the end of the Cambrian Period ~488 MYA. Sequence analysis indicates 46% sequence identity and 65% sequence similarity between Lp-CFTR and hCFTR, which is much lower

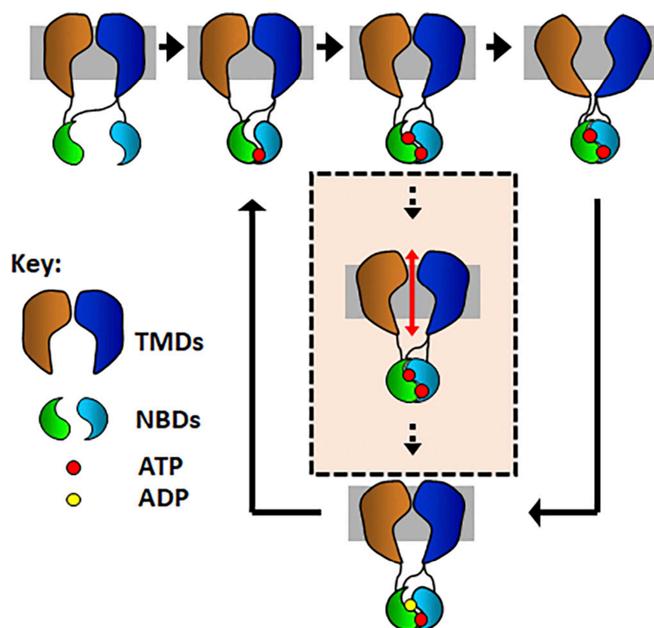


Figure 2. **Hypothesis for emergence of channel function in CFTR.** Modification of ATP-dependent transport activity in ABC transporters led to channel behavior, coopting the conformational changes necessary for unidirectional substrate transport in common ABC transporter systems. CFTR evolved features that break the alternating access cycle (solid-line arrows), enabling it to be open at both ends (box). Color scheme for major domains (again, lacking the R domain) is the same as in Fig. 1.

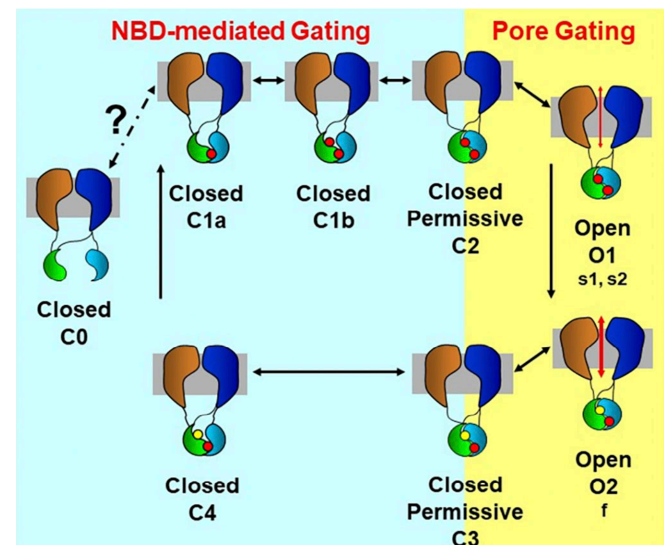


Figure 3. **Gating scheme for CFTR.** Prephosphorylated channels are shown in the membrane (gray slab) with two TMDs (brown and dark blue) and two NBDs (green and light blue), with ATP (red circle) and ADP (yellow circle). ATP-dependent gating is shown as including NBD-mediated gating steps leading to pore gating between conductance levels. Here, we do not distinguish between s1 and s2 subconductance levels, because s1→s2 occurs very rapidly in WT-hCFTR.

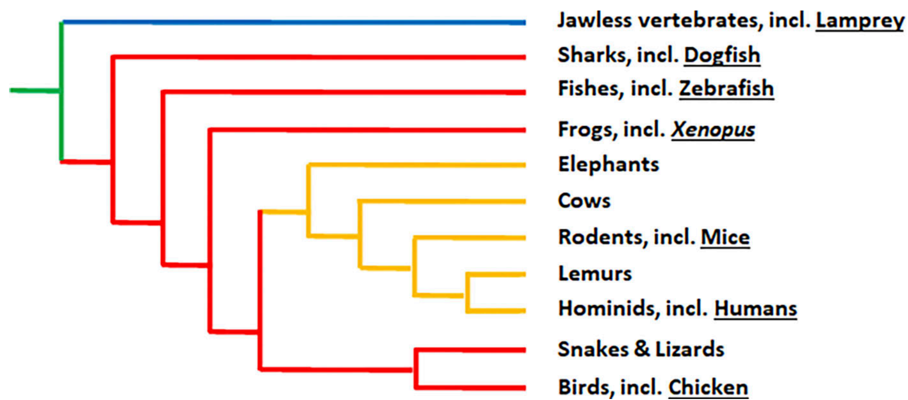


Figure 4. **Simplified and truncated evolutionary tree for vertebrates.** Green, common vertebrate ancestor; blue, jawless vertebrates; red and yellow, jawed vertebrates; yellow, mammals. CFTR orthologues studied in functional assays are shown underlined. (The time domain in this figure is not implied.)

than that among jawed vertebrate CFTRs (jv-CFTRs) and includes surprising divergence in functionally relevant motifs. Accordingly, Lp-CFTR differs from hCFTR in multiple functional characteristics (Table 1). The availability of this new orthologue thus provides the earliest evolutionary evidence of CFTR and lends insight into changes in gene and protein structure that underpin evolution of function from transporter to "optimized" anion channel. One important point to note in this respect is that, although the sea lamprey represents an evolutionary ancestor, it is also, of course, a currently living organism that may have undergone additional adaptation to its environment after the split with jawed vertebrates (Fig. 4). Thus, it cannot be automatically assumed that every position in CFTR that is unique in sea lamprey represents transitional change in the development of regulated channel activity. A good example in this regard is that of F508 in hCFTR, which is conserved across multiple ABC proteins but is leucine in lamprey (Cui et al., 2019a). Sorum et al. (2017) showed that replacing F508 with L in hCFTR significantly reduced its open probability. All known CFTRs other than Lp-CFTR and all known human ABCCs have F at this position, where the aromatic side chain is necessary for stabilizing the outward-facing state (Cui et al., 2006), so finding that this is substituted by a nonaromatic side chain in Lp-CFTR is mechanistically interesting and may represent a species-specific adaptation (Cui et al., 2019a).

Table 1. **Comparison of features between human and lamprey orthologues, focusing on three major domains of function: channel behavior, regulation, and modulation**

| | Lp-CFTR | hCFTR |
|---|---|---|
| **Functional domain: channel behavior** | | |
| Open channel stability (open burst duration) | Low | High |
| Frequency of subconductance states | High | Low |
| Single-channel open conductance | Low | High |
| Shape of I-V relationship | Rectified | Linear |
| Sensitivity to (affinity for) ATP for channel opening | Very low | High |
| **Functional domain: regulation by phosphorylation** | | |
| Rate of activation by PKA-mediated phosphorylation | Low | High |
| Number of predicted PKA sites in the R domain | 4 | 8 |
| **Functional domain: pharmacological modulation** | | |
| Effect of VX-770/ivacaftor (inhibition versus potentiation) | Small inhibition | Potentiation |
| Inhibition by CFTRinh172 | Low | High |
| Sensitivity to pore block by GlyH-101 | None | High |
| Sensitivity to pore block by NPPB | Low | High |
| Sensitivity to pore block by glibenclamide | Equal | Equal |

NPPB, 5-nitro-2-(3-phenyl-propylamino) benzoic acid. Related to Cui et al., 2019a.

Below, we identify several potential routes by which CFTR evolved regulated channel behavior. We propose that many features shared among bona fide ABCC proteins and present in recent ABCC ancestors of CFTR provided a unique opportunity for emergence of novel channel function by incremental evolutionary changes.

## Molecular evolution of channel function

### *Construction of an anionic pore from an anionic substrate pathway*

Both the passive conduction of anions by CFTR and the unidirectional transport of highly structurally diverse organic anions by its ABCC relatives (Sauna et al., 2004) is accomplished by pathways through the TMDs. Therefore, divergence in these pathways would be expected to most closely reflect the principal difference between channels and transporters: channels contain a pore that allows uninterrupted permeation across the plasma membrane, a violation of the "alternating access" mechanism of transporters (Fig. 2; Bai et al., 2011; Gadsby, 2009). This divergence would be accomplished by evolutionary changes distributed broadly through the TMDs, as suggested by a recent study of mutations that alter substrate specificity in a fungal pheromone transporter (Srikant and Gaudet, 2019; Srikant et al., 2020). In formation of the CFTR chloride channel, this would require both degradation of the "gates" seen in ABC transporters and stabilization of an open pore conformation (Bai et al., 2011). The relationship between substrate binding and opening/closure of these gates, relevant to establishing the occluded state in transporters, may remain in CFTR in a vestigial state, as evidenced by reports that permeating anions may affect gating transitions (Sorum et al., 2015; Yeh et al., 2015; Zhang et al., 2000; Zhang et al., 2002).

Understanding how the CFTR pore evolved requires the integration of functional and structural information. Early 2-D electron crystallography of hCFTR at low resolution (Rosenberg et al., 2004; Rosenberg et al., 2011) confirmed the general ABC-like architecture of CFTR predicted in the initial gene discovery study (Riordan et al., 1989). In addition, several homology models of CFTR were developed using structures of related ABC transporters as a template. These studies contributed to the understanding of the molecular interface encompassing the most common CF-causing mutation (ΔF508; Mornon et al., 2008; Serohijos et al., 2008), as well as several details relating to the conformational transitions underlying CFTR gating (Corradi et al., 2015; Dalton et al., 2012; Furukawa-Hagiya et al., 2013; Mornon et al., 2015; Mornon et al., 2009; Rahman et al., 2013; Strickland et al., 2019). However, the disparity between the wide variety of substrates of nonchannel ABC transporters and the chloride channel function of CFTR resulted in intrinsically limited confidence in these homology models, at least with respect to the TMDs.

In the last 5 yr, eight structures of detergent-solubilized CFTR from three orthologues have been released from two laboratories in a large range of resolutions, all solved by single-particle cryo-EM (Table 2 and Fig. 5).

The first structures were of the ATP-free, dephosphorylated zebrafish CFTR (zfCFTR) in inward-facing conformation at a reported resolution of 3.7 Å and, under the same conditions, hCFTR at a reported resolution of 3.9 Å. In both structures, the NBDs were of significantly lower resolution than the rest of the protein, and thus crystal structures of exogenous NBDs were used to construct the final models (Liu et al., 2017; Zhang and Chen, 2016). Subsequently, the structures of phosphorylated, ATP-bound, hydrolysis-deficient mutants of zfCFTR and hCFTR in the outward-facing state were resolved at reported resolutions of 3.4 Å and 3.2 Å, respectively (Zhang et al., 2017; Zhang et al., 2018). In addition to revealing a structural motif unsuspected for CFTR—the lasso motif found in other ABCC transporters (e.g., SUR1, SUR2, MRP1) in which the N-terminus loops into the lipid bilayer (Fig. 1 A)—these CFTR structures exhibited TM helix positioning and secondary structure that may be unique to CFTR among the ABCs. Of note, TM7 and TM8 are rearranged such that the top-down TM helix symmetry of most ABC transporters is broken. There are also kinks in TM8 and TM5 helices in approximately the same vertical position. We note that two structures from recombinant thermostabilized chicken CFTR (chCFTR), one in dephosphorylated conditions with ATP present (resolution, 4.3 Å) and one in phosphorylated conditions with ATP present (resolution, 6.6 Å), show TM8 as fully helical and lack the rearrangement of TM7 and TM8, instead positioning TM7 nearly orthogonal to the fatty acid tails of the lipid bilayer (see Fig. 5; Fay et al., 2018).

The positioning of TM8 in the Chen structures has been supported by functional evidence suggesting that some residues of TM8 line the CFTR channel pore (Negoda et al., 2019). The unwound portion of TM8 has been proposed by the Chen laboratory to underlie CFTR's unique channel function (Liu et al., 2017), and molecular dynamics studies suggest that this unwinding would be maintained in a lipid bilayer (Corradi et al.,

Table 2. **High-resolution CFTR structures to date**

| **Protein** | **zfCFTR** | **hCFTR** | **zfCFTR** | **hCFTR** | **hCFTR** | **hCFTR** | **chCFTR** | **chCFTR** |
|---|---|---|---|---|---|---|---|---|
| Orthologue | Zebrafish | Human | Zebrafish | Human | Human | Human | Chicken | Chicken |
| Resolution | 3.7 Å | 3.9 Å | 3.4 Å | 3.2 Å | 3.3 Å | 3.2 Å | 4.3 Å | 6.6 Å |
| Detergent | Detergent (LMNG, digitonin, CHS) | Detergent (LMNG, digitonin, CHS) | Detergent (LMNG, digitonin, CHS) | Detergent (LMNG, digitonin, CHS) | Detergent (LMNG, digitonin, CHS) | Detergent (LMNG, digitonin, CHS) | Detergent (DMNG, digitonin) | Detergent (DMNG, digitonin) |
| Mutation | - | - | E1372Q | E1371Q | E1371Q | E1371Q | ΔRI/1404S/1441X | ΔRI/1404S/1441X |
| State | Closed, inward facing, dephosphorylated, apo-ATP | Closed, inward facing, dephosphorylated, apo-ATP | Closed, outward facing, phosphorylated, ATP-bound | Closed, outward facing, Phosphorylated, ATP-bound | Closed, outward facing, Phosphorylated, ATP-bound, VX-770-bound | Closed, outward facing, Phosphorylated, ATP-bound, GLPG1837-bound | Closed, inward facing, dephosphorylated, ATP-present | Closed, inward facing, phosphorylated, ATP-present |
| PDB accession no. | 5UAR | 5UAK | 5W81 | 6MSM | 6O2P | 6O1V | 6D3R | 6D3S |
| Year | 2016 | 2017 | 2017 | 2018 | 2019 | 2019 | 2018 | 2018 |

ch, chicken; CHS, cholesteryl hemisuccinate; DMNG, decyl maltose neopentyl glycol; LMNG, lauryl maltose neopentyl glycol; zf, zebrafish.


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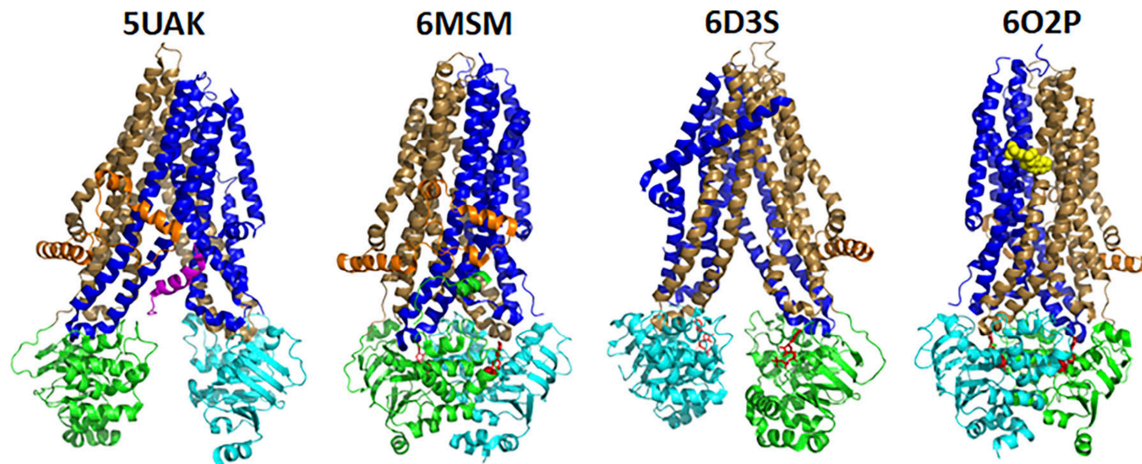



Figure 5. **High-resolution structures of CFTR.** See Table 2 for conditions for each PDB accession number shown here. Color coding is as follows: orange, lasso domain; brown, TMD1; blue, TMD2; green, NBD1; cyan, NBD2; magenta, R domain; red sphere, $Mg^{2+}$; red sticks, ATP; yellow spheres in 6O2P, VX-770.

2018). The stability of this segment may be enhanced by interactions between R933, located at the intracellular boundary of the unwound portion of TM8, and E873, in TM7. In both the structures of closed hCFTR (Protein Data Bank [PDB] accession no. 5UAK) and nearly open hCFTR with ATP bound (PDB accession no. 6MSM), the oppositely charged ends of these residues essentially overlap. It is very interesting to note that R933 is conserved within CFTR and ABCC4 orthologues among both jawed and jawless vertebrates. However, E873 is conserved within jawed vertebrates but is Q in both Lp-CFTR and all ABCC4s, although this assignment must remain tentative due to the poor alignment between CFTR and ABCC4 sequences in TM7. Within the unwound stretch of TM8 itself, sequences are poorly conserved even within the CFTR and ABCC4 branches.

Importantly, an open structure of CFTR with a fully conducting ion pore has yet to be published. Currently, all structures have been determined with CFTR in detergent; additional structures of CFTR in a lipidic environment may be needed to elucidate the fully conducting ion pathway as well as to understand the complex conformational transitions between open and closed states. Regardless of these considerations, these structures can certainly be used to spatially locate amino acids that have been implicated in CFTR channel function. In aid of this, significant effort has been expended to functionally map the chloride conduction pathway through CFTR. Many studies have mutated putative pore residues and characterized channel behavior and modulation (Linsdell et al., 1997; McCarty et al., 1993; McDonough et al., 1994; Tabcharani et al., 1997). To identify explicitly "pore-lining" residues, several groups have employed the substituted cysteine accessibility method. This approach probes the environment of specific residues by mutating them to cysteine and characterizing their reaction to sulfhydryl-specific chemicals (Karlin and Akabas, 1998).

In the process of going through the channel to exit the cell, the chloride ion first encounters highly conserved basic residues in the ICLs, including K190, R248, R303, K370, R1030, K1041, and R1048. These residues are proposed to play roles in attracting chloride ions into the pore because charge-eliminating mutations reduce single-channel conductance (Aubin and Linsdell, 2006; El Hiani and Linsdell, 2015; Zhou et al., 2008). Considering that they mediate anion conduction, it is initially surprising that this group of residues is very highly conserved in transporter ABCCs: all seven residues analogous to those listed above are basic in ABCC4 and most (five of seven) are basic in ABCC5. To our knowledge, the effect of mutations at these positions on the function of ABCC4 or ABCC5 has not been directly tested. However, functional studies of MRP1 (ABCC1) have specifically implicated several basic residues in analogous regions in the binding of organic anionic substrates (Conseil et al., 2006; Haimeur et al., 2004) that are transported by the majority of ABCCs, including ABCC4 and ABCC5 (Jansen et al., 2015; Ritter et al., 2005). These data are intriguing because they suggest that one way in which CFTR evolved chloride channel activity was to use residues already functionally important in the transport of organic anionic substrates and repurpose them toward the novel function of conducting inorganic anions through the channel pore. In further support of this, several substrates of ABCC transporters inhibit CFTR by blocking the pore from the intracellular side (Linsdell and Hanrahan, 1999). Hence, these residues may contribute to a vestigial binding site for these substrates within CFTR. Another intriguing possibility is that ABCC4 and ABCC5 may allow the conductance of chloride along with their traditional substrates during transport, in a manner akin to the leak current associated with the function of neurotransmitter transporters (Fairman et al., 1995; Sonders and Amara, 1996; Wadiche et al., 1995). Such a substrate-induced current has not yet been measured from cells expressing ABCC4 or ABCC5, although this would be expected to be of very low amplitude (due to the slower nature of transporter function) and would likely be challenging to measure because substrate binds intracellularly in these proteins.

As the chloride ion travels further up the CFTR pore toward the extracellular space, it encounters pore-lining residues contributed by TM helices 1, 5, 6, 8, 9, 11, and 12 (Alexander et al., 2009; Bai et al., 2010; Bai et al., 2011; Gao et al., 2013; McDonough et al., 1994; Wang et al., 2014a; Zhang and Hwang, 2015; Zhang

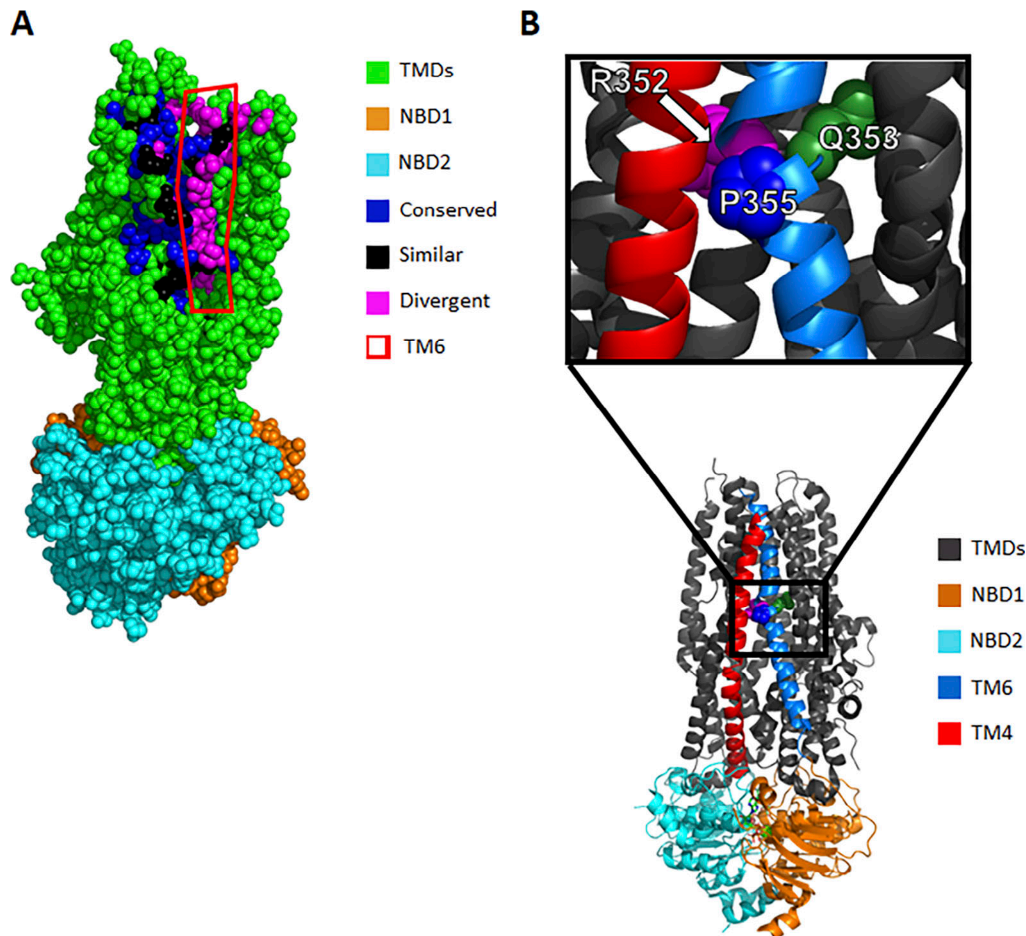



Figure 6. **Conservation with ABCC4 in residues lining the CFTR channel pore. (A)** hCFTR structure (PDB accession no. 6MSM) in nearly open state, showing major domains, with sections of non–pore-lining helices removed in order to visualize the chloride ion permeation pathway. Dark blue residues, identical between jawed vertebrate consensus CFTR and ABCC4; black residues, biochemically similar; magenta, biochemically divergent. The highly divergent pore-lining TM6 is bounded in red. **(B)** hCFTR (PDB accession no. 6MSM) is again shown, highlighting a lateral portal proposed to enable unique chloride channel activity among ABCCs. Inset is a closeup view of a kink in TM6. P355 is conserved with ABCC4, whereas R352 and Q353 are divergent.

et al., 2005b; Zhang et al., 2002). Fig. 6 A shows the nearly open structure of hCFTR, wherein we have highlighted residues shown by the substituted cysteine accessibility method to line the pore (Akabas, 1998; Alexander et al., 2009; Aubin and Linsdell, 2006; Bai et al., 2010; Bai et al., 2011; El Hiani and Linsdell, 2015; El Hiani et al., 2016; Fatehi and Linsdell, 2009; Gao et al., 2013; Liu et al., 2004; Negoda et al., 2019; Norimatsu et al., 2012a; Norimatsu et al., 2012b; Qian et al., 2011; Rubaiy and Linsdell, 2015; Serrano et al., 2006; Wang et al., 2011; Wang et al., 2014a; Zhang and Hwang, 2015; Zhou et al., 2008). Residues are colored according to conservation between CFTR and ABCC4 (Jordan et al., 2008; dark blue, conserved; black, similar; magenta, divergent).

Strikingly, the pore-lining residues of several TMs are highly conserved between CFTR and ABCC4; for example, in TM1, six of seven pore-lining residues in CFTR are identical in ABCC4. Regarding this conservation, TM6 (see region bounded in red in Fig. 6) is an outlier, both in terms of the number of biochemically divergent pore-lining residues and as calculated as a sum of the Grantham scores (incorporating differences in composition, polarity, and molecular volume; Grantham, 1974) to gauge evolutionary distance between consensus amino acids of CFTR and ABCC4 sequences from jawed vertebrates (Table 3). In addition, the substituted cysteine reactivity pattern for the extracellular end of TM6 is anomalous for an α-helix in a membrane protein; the stretch of residues from L331 to V345 is nearly uninterrupted in terms of accessibility to membrane-impermeant thiol-directed reagents applied extracellularly (Alexander et al., 2009; Bai et al., 2010; Norimatsu et al., 2012a), whereas residues F337 through V345 exhibit a helical pattern of modification by MTS reagents applied intracellularly (Bai et al., 2010; El Hiani and Linsdell, 2010). This also contrasts with better-conserved helices such as TM1 and TM11, wherein reactivity follows a helical periodicity (Table 3).

Divergence in TM6, a highly discriminatory region of the CFTR pore (McCarty and Zhang, 2001), may play important roles in neofunctionalization toward channel activity while retaining glutathione transport capacity (Kogan et al., 2003). Divergent residues such as R334 in TM6 also play important enough roles in the electrostatic attraction of $Cl^-$ and in pore stability (Zhang et al., 2005b) that their mutation causes CF (Sheppard et al., 1993).

Table 3. **Pore-lining residues in hCFTR predicted by experiment**

| Region | Residue numbers | Aggregate Grantham score[a] |
|---|---|---|
| TM1 | *92, 95, 98, 102, 106,* 107, *109* | 111 |
| ICL1 | *186,* 188, *189, 190,* | 32 |
| TM3 | *191, 192,* 193, 194, 195, 196, 197, *199, 200,* 203, *205, 207,* 211, 213, *215* | 532 |
| ICL2 | *241,* 243, 244, *248, 252,* 299, *303,* | 142 |
| TM5 | *306,* 307, *310, 311,* 326 | 209 |
| TM6 | 331, 333, 334, 335, 336, *337,* 338, 339, 340, 341, 342, 344, 345, 348, 349, 352, 353, 355, 356, 360, 367, 370 | 1,389 |
| TM8 | 913, 914, 917 | 327 |
| ICL3 | *986, 988, 989, 990* | 0 |
| TM9 | *993,* 1000, *1003,* 1008, 1009, 1010 | 361 |
| ICL4 | *1030, 1041, 1048* | 0 |
| TM11 | *1112,* 1115, *1118* | 58 |
| TM12 | *1127, 1129,* 1131, 1132, 1134, 1135, 1137, 1138, 1139, *1140,* 1141, 1142, *1144, 1145, 1147,* 1148, *1150,* 1152, 1156 | 561 |

Italics = identical; underlined = divergent; unformatted = similar.
[a]A higher Grantham score indicates less conservation.

How may this divergence be responsible for the structural changes necessary for the development of ion channel activity? First, divergence in TM6 may play a central role in the degradation of an intracellular transporter gate. In the human and zebrafish ATP-bound CFTR cryo-EM structures (PDB accession nos. 6M2M and 5W81), the intracellular region of TM6 is subtly kinked outward (Fig. 6 B), as opposed to being curved but tightly packed in ABCC1, the closest relative to CFTR for which a structure exists. It has been proposed that this change may have created an aqueous "portal" that contributes to the ion permeation pathway (Zhang et al., 2017). Both functional and structural studies support the importance of these changes (El Hiani and Linsdell, 2015; El Hiani et al., 2016; Li et al., 2018; Zhang et al., 2017). Sequence comparisons in this region reveal that a proline was already present in this region in an ancestral ABCC. In the place of conserved hydrophobic residues in ABCC4, CFTR has hydrophilic residues in this region, including R352 and Q353. These residue changes may be responsible for fundamentally altering the interaction of TM6 with surrounding helices, ultimately contributing to the degradation of the intracellular gate. Notably, the Lp-CFTR sequence uniquely contains a serine residue analogous to position 353.

Second, divergence in the TMDs also apparently enabled the formation of several intraprotein interactions that stabilize the open CFTR pore, which would be antithetical to the rapid transitions in conformation of the substrate binding pocket in a transporter undergoing alternating access. Previously, to identify important loci of divergence between CFTR and transporters of the ABCC subfamily, the McCarty laboratory performed type II divergence analysis between CFTR and ABCC4 sequences (Jordan et al., 2008). This approach identified residues maximally conserved within groups and biochemically divergent between groups. Type II divergence is exemplified by residue positions within an alignment that (1) are completely conserved within paralogous groups and (2) have amino acids with biochemically different properties between paralogous groups (e.g., acidic charge versus basic charge; Gu, 1999; Gu, 2001). The concept as applied here is that use of type II divergence analysis would identify the specific domains and residues most likely to be involved in the evolutionary transition from transporter activity (ABCC4) to channel activity (CFTR). In this study, we found that two salt bridges (Fig. 7) that stabilize the open pore architecture of CFTR (R347-D924 [Cotten and Welsh, 1999] and R352-D993 [Cui et al., 2008]) consist of one residue that is highly conserved between CFTR and ABCC4 (R347 in TM6 and D993 in TM9) and one that is type II divergent (D924 in TM8 and R352 in TM6). Interestingly, both interactions include residues mutated in CF disease (Jordan et al., 2008). Here we note that in both of these salt bridge interactions, the residue

| hCFTR Pair: | R347-D924 | | R352-D993 | |
|---|---|---|---|---|
| | R347 | D924 | R352 | D993 |
| jv-CFTR | R | D | R | D |
| Lp-CFTR | R | D 2 | R 2 | D |
| hABCC4 | R 1 | V | L | D 1 |
| hABCC5 | T | A | V | M |

Figure 7. **Evolution of pore-stabilizing salt bridges absolutely conserved in CFTRs from jawed vertebrates, including hCFTR.** For the two intraprotein salt bridges included here, as examples, one can trace the appearance of residue–residue interactions, and their fixation as conserved features, in the evolutionary lineage from ABCC5 and ABCC4 transporters to Lp-CFTR and jv-CFTR.

biochemically conserved between CFTR and ABCC4 is divergent in ABCC5. Thus, in each pair, the first residue likely emerged in a common ancestor of CFTR and ABCC4 after divergence from ABCC5, thereby providing the basis of a salt bridge when the other residue subsequently emerged in CFTR (Fig. 7). For the R352-D993 pair, the evolution of R352 from divergent hydrophobic residues in the ancestors was highly adventitious because it appears to have simultaneously contributed to the formation of a pore-stabilizing salt bridge and the destabilization of the secondary structure of TM6 that potentially contributed to a cytoplasmic gate (see above). Similar evolutionary pathways may have been at play with interactions involving charged residues in extracellular loop 1, such as R117 (Cui et al., 2014). Of these, it is notable that R117 is not found in Lp-CFTR, where it is instead a hydrophobic residue as in ABCC4 and ABCC5. Thus, it is likely that additional residues, such as R117, emerged late in evolution to stabilize the pore in jv-CFTR. The existence of high-resolution structures for hCFTR in closed and nearly open states will facilitate the identification of other intraprotein interactions and allow us to ask whether these residues exhibit evolutionary patterns across species. Testing of the above will require structural and functional interrogation of CFTR transporter chimeras.

### *Evolution of CFTR regulation by phosphorylation of its R domain*

CFTR is activated by PKA-mediated phosphorylation at consensus sites in the R domain representing a functional linker encoded between NBD1 and TMD2 (Fig. 1; Ford et al., 2020; Hunt et al., 2013). The structural mechanism for the phosphorylation-mediated regulation of CFTR by this intrinsically disordered domain is poorly understood but evidently involves dynamic, phosphosensitive interactions between R domain helices and nearby domains of CFTR, including NBD1 and NBD2 (Baker et al., 2007; Bozoky et al., 2013a; Bozoky et al., 2013b; Chappe et al., 2005). The R domain also has been suggested to plug the channel pore in a phosphorylation-dependent manner (Meng et al., 2019). Interestingly, although the fully dephosphorylated R domain precludes ATP-induced channel opening (Rich et al., 1991), biophysical studies strongly suggest that channel activity depends on the degree of PKA-mediated phosphorylation, in a rheostat-like manner, and that these sites play specific roles in "graded" activation of the channel (Csanády et al., 2005a; Csanády et al., 2000; Csanady et al., 2005b; Wilkinson et al., 1997). The phosphorylation of ABC proteins other than CFTR has not been extensively studied; however, there is some evidence that several members of the superfamily, including P-glycoprotein (ABCB1; Mellado and Horwitz, 1987), are phosphorylated in cells (see Stolarczyk et al., 2011 for a comprehensive review on this subject). There is evidence that several ABCB and ABCC proteins are phosphorylated in a region connecting NBD1 and TMD2 (Ford et al., 2020; Mellado and Horwitz, 1987; Stolarczyk et al., 2011). However, there is no clear evidence that mutation or phosphorylation of this region significantly affects the function of these transporters, as it profoundly does in CFTR (Stolarczyk et al., 2011). Moreover, the relevant PKA consensus sites in CFTR's R domain are located in an ~200-aa region that is absent in other ABC transporters (including other ABCCs; Sebastian et al., 2013). Based on data available at the time, the McCarty and Jordan laboratory suggested that this region arose in CFTR specifically as the result of the loss of an RNA splice site at the end of exon 14 in the lineage between jawless and jawed vertebrates (Sebastian et al., 2013). However, revised sea lamprey gene assemblies (see https://genomes.stowers.org/organism/Petromyzon/marinus and Smith et al., 2018) no longer indicate this splice junction, which explains the presence of an R domain in the cloned sea lamprey sequence (Cui et al., 2019a).

The unique functional phosphoregulation of CFTR by the R domain may directly relate to its identity as the sole ion channel in the ABC superfamily. In the case of many bona fide ABC transporters, the activity of the protein, including hydrolysis of ATP (Senior et al., 1998), is highly dependent on the availability of substrates. These substrates, which include xenobiotics (Chen and Tiwari, 2011), are typically present at low concentrations in the cell, resulting in low transporter-associated ATPase activity. By contrast, CFTR always has access to chloride, and binding of chloride is not required for ATPase activity in the same way that binding of substrate is required for ATPase activity in other ABC superfamily members. Because ATP is present in the cell at concentrations well above the half-maximal effective concentration for channel opening (Csanády et al., 2000), without some other means of regulation, CFTR would allow unproductive high ATPase rates and the uninterrupted flow of chloride down the electrochemical gradient—in either direction with respect to the cell. By coupling the R domain–mediated regulation of the channel to PKA-mediated phosphorylation, the CFTR-expressing epithelial cell ensures that chloride is brought to the appropriate electrochemical potential by the coordinated action of basolateral chloride transporters, which are also regulated by PKA (McCann and Welsh, 1990), and CFTR-mediated permeability in the apical membrane.

The overall sequence of the R domain is poorly conserved across CFTR orthologues, but the PKA consensus sites shown to be functionally relevant in hCFTR are highly conserved across jv-CFTRs (Sebastian et al., 2013). However, half of the consensus dibasic PKA sites are missing in Lp-CFTR (Fig. 8); furthermore, some of those that are found in both human and lamprey orthologues exhibit substantial divergence in the context surrounding the phosphorylated serine, which may contribute to differences in the rate of phosphorylation or to changes in conformation after phosphorylation. This is consistent with the observation that Lp-CFTR exhibits a greatly slowed response to PKA-induced activation (Cui et al., 2019a). The additional sites may have evolved in jv-CFTRs, after the split from jawless vertebrates, as a means of fine-tuning the graded activation intrinsic to hCFTR. Future work may explore the functional effects of transplantation of PKA recognition motifs and surrounding primary sequence from hCFTR into Lp-CFTR.

### *An inherited ATPase defect intrinsic to CFTR NBD-mediated gating kinetics*

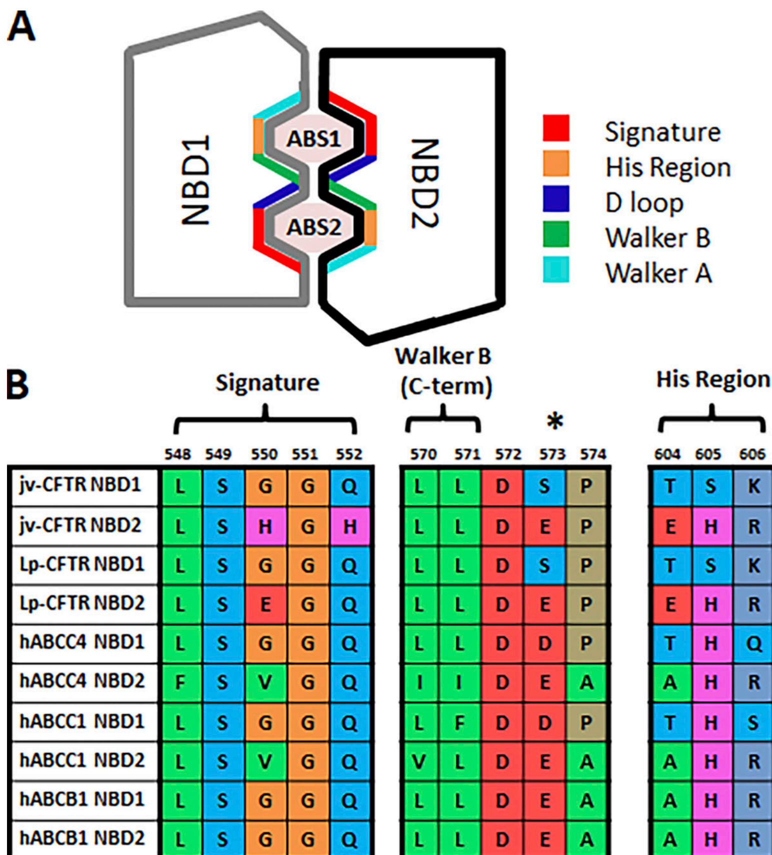
In ABC transporters, ATP binds at two composite sites (ABS1 and ABS2) formed by conserved motifs from NBDs positioned in a head-to-tail arrangement (Smith et al., 2002). Fig. 9 A depicts a

| PKA Consensus Site: | S660 | S670 | S700 | S712 | S737 | S768 | S795 | S813 |
|---|---|---|---|---|---|---|---|---|
| Human CFTR | RRNSI | **H**RFSL | RKNSI | RKFSI | RRLSL | RRQSV | RKVSL | RRLSQ |
| Mouse CFTR | RRSSI | RRFSV | RKNSI | RKISI | KRLSL | RRQSV | RKISL | RRLSQ |
| Chicken CFTR | RRNSI | RRFSF | RKNSI | RKLSI | RRISL | RRQSV | RKMSV | RRLSR |
| *Xenopus* CFTR | RRNSI | RRCSI | RKNSV | RKFSI | RKLSL | RRQSV | RKMSV | RRLSQ |
| Dogfish CFTR | RRNSI | RRCSV | RKSSL | KKFSL | RHFSL | RRQSV | RKMSM | RRLSE |
| Lamprey CFTR | RRGSM | RRGST | **PQGTQ** | **Q**RFSV | RRLSL | RRKSV | **HAPHG** | **P**RKSL |

Figure 8. **Conservation among CFTR orthologues in PKA consensus sites in the R domain.** Primary sequences equivalent to each of the eight consensus sites for PKA-mediated phosphorylation found in hCFTR are shown for mouse, chicken, frog, shark, and lamprey. Numbering for consensus sites at the top of the table refers to the hCFTR orthologue. Residues bearing divergence from the consensus dibasic sequence are shown in bold and underlined. Other variability in the primary sequence surrounding the target serine also is evident, which may contribute to altered response to phosphorylation.

simplified model of these sites, wherein each ABS is shown to consist of the so-termed Walker A, Walker B, and H loop regions from one NBD and the ABC signature and D loops from the other NBD. ATP binding to an ABS promotes NBD dimerization, which "powers" active transport by driving conformational changes in the TMDs (Rahman et al., 2013; Strickland et al., 2019); in ABC exporters, this flips the TMD conformation from inward to outward facing (Rees et al., 2009). ATP hydrolysis at these sites leads to dissociation of the NBD dimer, which allows the re-adoption of the inward-facing conformation to bind new intracellular substrates, although there is significant disagreement regarding the degree of dissociation undergone at the NBDs to accomplish this (George and Jones, 2012; Hohl et al., 2014; Puljung, 2015; Zoghbi et al., 2012). Structural (Zhang et al., 2017) and functional (Chaves and Gadsby, 2015) studies support the idea that CFTR uses the same overall scheme, wherein opening involves binding of ATP to both ABSs and dimerization of the NBDs, whereas closing results from ATP hydrolysis, which promotes the subsequent dedimerization of the NBDs.

Many ABC proteins feature homodimeric NBDs that together form two ABS sites with equivalent functions, but the monomeric ABCCs contain significant divergence in ABS1 (Gadsby et al., 2006). A sequence alignment of the relevant motifs (Fig. 9 B) demonstrates major points of divergence as compared with P-glycoprotein (ABCB1), which has essentially homodimeric NBDs. Note that the ABCC family shows divergence




| | 548 | 549 | 550 | 551 | 552 | 570 | 571 | 572 | 573 | 574 | 604 | 605 | 606 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| jv-CFTR NBD1 | L | S | G | G | Q | L | L | D | S | P | T | S | K |
| jv-CFTR NBD2 | L | S | H | G | H | L | L | D | E | P | E | H | R |
| Lp-CFTR NBD1 | L | S | G | G | Q | L | L | D | S | P | T | S | K |
| Lp-CFTR NBD2 | L | S | E | G | Q | L | L | D | E | P | E | H | R |
| hABCC4 NBD1 | L | S | G | G | Q | L | L | D | D | P | T | H | Q |
| hABCC4 NBD2 | F | S | V | G | Q | I | I | D | E | A | A | H | R |
| hABCC1 NBD1 | L | S | G | G | Q | L | F | D | D | P | T | H | S |
| hABCC1 NBD2 | L | S | V | G | Q | V | L | D | E | A | A | H | R |
| hABCB1 NBD1 | L | S | G | G | Q | L | L | D | E | A | A | H | R |
| hABCB1 NBD2 | L | S | G | G | Q | L | L | D | E | A | A | H | R |

Figure 9. **Evolutionary divergence within the NBD1–NBD2 interface. (A)** Schematic representation of a prototypical head-to-tail NBD dimer sandwich and the interfacial regions that interact with ATP. **(B)** Alignment of several relevant regions of the NBDs from CFTR and more distant homologues. Numbering is of hCFTR NBD1. Note that jv-CFTR represents the consensus sequence from CFTR from jawed vertebrates, whereas Lp-CFTR specifically refers to the sequence of Lp-CFTR. Significant ABCC- and CFTR-specific divergence is seen in ABS1, particularly in the NBD2 signature sequence, the NBD1 Walker B motif, and the NBD1 His region. To facilitate identification of differences, amino acids in the table are colored according to common chemical properties (charge, polarity, etc.). Note that the ABCC family shows divergence adjacent to the NBD1 Walker B loop that is integral to ABS1 at the position indicated by an asterisk.

adjacent to the NBD1 Walker B loop that is integral to ABS1 at the position indicated by an asterisk in Fig. 9 B. Here, a critical catalytic glutamate conserved in canonical ABS sites (Orelle et al., 2003) is substituted in most ABCCs with an aspartate or serine in NBD1, and the following alanine is substituted with a proline (Payen et al., 2003). In ABCC1, these two substitutions may be responsible for increased affinity for ATP and significantly slowed ATP hydrolysis at ABS1 (the so-called incompetent site) as compared with the canonical ABS2 site (the "competent" site; Gao et al., 2000; Hagmann et al., 1999; Hou et al., 2000; Payen et al., 2003; Qin et al., 2008). In addition, the NBD2 signature sequence contributing to ABS1 is F/LS**V**GQ in most ABCCs, as opposed to the canonical LS**G**GQ as in ABCB1; this also may impact affinity for ATP (Smith et al., 2002). In CFTR, where ATP hydrolysis at ABS1 is essentially absent (Aleksandrov et al., 2002; Basso et al., 2003), there is additional, lineage-specific divergence evident in these alignments. In NBD1, instead of the conservative ABCC aspartate substitution for the catalytic glutamate adjacent to the Walker B region (asterisked position noted above), all CFTRs have a serine residue (e.g., **S**573 in hCFTR). Additionally, the NBD2 signature sequence integral to ABS1 of CFTR is also unique among ABCCs.

What purpose in CFTR may degeneration/divergence in the NBD dimer interface serve? As explained previously, the ABC transporter duty cycle requires the consumption of ATP. Adaptation of the cycle for optimal chloride channel activity would ideally allow a maximal amount of chloride to be diffused per ATP consumed. In this regard, it is highly advantageous that members of the ABCC subfamily of proteins harbor a degenerate ABS1, because any ion channel built on this scaffold would only consume one ATP molecule per gating cycle rather than two. This potential is generally borne out by biochemical studies. Recently developed spectroscopic methods for measuring ATP hydrolysis from model ABC transporters support the general inference that homodimeric transporters catalyze ATP at a significantly higher overall rate than heterodimeric transporters (Collauto et al., 2017). Specific to mammalian transporters, the absolute ATP turnover rate for hCFTR as calculated from channel closing rate is ∼0.5/s (Li et al., 1996), which correlates well with published rates from purified, detergent-solubilized protein (∼130 nmol/mg/min; Liu et al., 2017). This rate is roughly half that of the homodimeric P-glycoprotein expressed and purified similarly (∼230 nmol/mg/min in the presence of substrate; Kim and Chen, 2018).

It is not yet well understood how additional divergence found in CFTR orthologues may contribute to any unique behavior(s). In all jv-CFTRs, the signature sequence in NBD2 is LS**H**G**H**—more divergent from consensus than ABCC homologues in its substitution of histidine for the C-terminal glutamine found in canonical ABSs (Fig. 9 B; Smith et al., 2002). Interestingly, uniquely among CFTRs, the NBD2 signature sequence from the Lp-CFTR orthologue retains this canonical glutamine (LSEG**Q**). Whether the unique composition of the CFTR ABS1 is necessary for normal gating or ATP hydrolysis is a question that needs further study using rigorous biochemical and electrophysiological methods. One intriguing explanation has been proposed on the basis of recent FRET experiments on ABCC1/MRP1 demonstrating important differences in its NBD dynamics as compared with CFTR. Electrophysiological data from CFTR suggest that ATP hydrolysis is quickly followed by dedimerization of the NBD heterodimer (Csanády et al., 2010). However, in MRP1, the post-hydrolytic NBD dimer is apparently much longer lived (Wang et al., 2020). Could CFTR-specific divergence in the NBD interface play a role in tuning CFTR gating, making it highly responsive to ATP hydrolysis at ABS2? Support for this possibility is found in a study demonstrating that mutating certain amino acids in the CFTR NBD interface to ABC transporter consensus results in a highly stable ATP-dependent dimer and prolonged open channel burst durations (Tsai et al., 2010).

### Hypothesized route for the evolution of regulated channel activity in CFTR

How did CFTR evolve its indispensable channel function? Our analyses demonstrate that many of the amino acid residues and motifs that bestow on hCFTR its function and regulation were already present to different degrees in closely related but functionally divergent ancestors. Hence, it is possible to compare the sequence of CFTR with that of increasingly distant homologues, infer what features are common, and propose a chronology for the molecular evolution of CFTR function and its optimization (Fig. 10). From such analysis, we suggest that residues underpinning interdomain energetic signaling, degeneration of the ATPase activity in ABS1, and intracellular basic residues critical to future CFTR $Cl^-$ channel activity were present in a common ancestor of the ABCC family (Fig. 10, point 1). Following divergence from ABCC5, an ancestor of ABCC4 and CFTR retained these features and added to them; at this point, many residues that would eventually line and stabilize the $Cl^-$ channel pore of CFTR emerged, possibly in use to bind and transport anionic substrates (Fig. 10, point 2). A common CFTR ancestor accumulated critical channel-specific residues in TM6 and elsewhere, which led to secondary structure changes around a conserved proline (P355 in CFTR) and pore-stabilizing salt bridges. Some degree of phosphoregulation was present as well (Fig. 10, point 3). Finally, fine-tuning of channel regulation and pore architecture continued after the split between jawless vertebrate CFTRs and jv-CFTRs (Fig. 10, point 4), but was largely consolidated before significant additional speciation in jv-CFTRs. This timeline is ripe for exploration in functional experiments with mutagenesis guided by structural and bioinformatics analysis.

### Translational relevance: Toward therapeutic development across ABC transporters

As discussed above, CFTR is clinically relevant to the pathogenesis of CF, an impactful genetic disease. The continued development of efficacious CFTR modulators requires a better understanding of the function of this channel. The modulators from Vertex, although highly efficacious, do not impact all patients with eligible CFTR genotypes, nor do they solve all of the problems in this multiple organ system disease or lead to long-term stabilization of lung function (Flume et al., 2018; Gauthier et al., 2020; Guimbellot et al., 2017; Konstan et al., 2017; Li et al., 2019; McKinzie et al., 2017; Moheet et al., 2021; Patel et al., 2020;

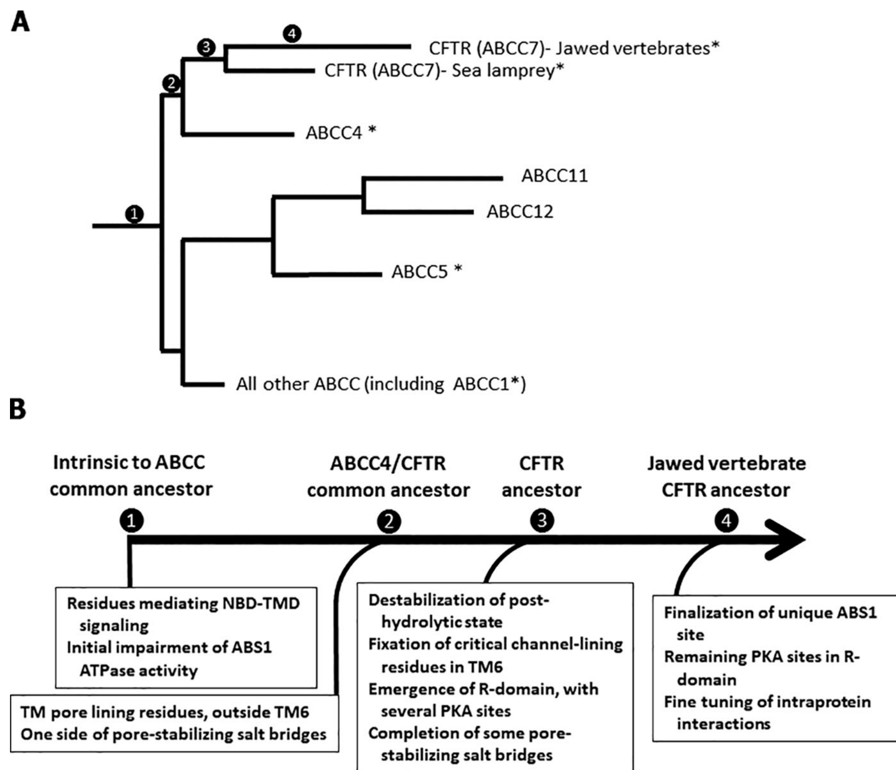



Figure 10. **ABCC subfamily dendrogram and proposed chronology of molecular evolution of CFTR function. (A)** Dendrogram adapted from two previous studies on CFTR evolution (Jordan et al., 2008; Sebastian et al., 2013). Proteins discussed in this review are indicated with *. **(B)** Chronology of emergence of functional features of jv-CFTR, as supported by the analyses in this review. Ancestors labeled with circled numbers correspond to the dendrogram points in A.

Phuan et al., 2018), revealing a need to continue to study CFTR to develop new therapies (Davies et al., 2019; Grand et al., 2021; Veit et al., 2018). Understanding the nature of the stable open state may aid in the rational design of drugs that can lock mutant CFTR channels open, leading to increased $Cl^-$ secretion and amelioration of CF disease and potentially some forms of chronic obstructive pulmonary disease and other lung disorders (Raju et al., 2016; Solomon et al., 2016a; Solomon et al., 2016b). Conversely, overactivity of CFTR may contribute to polycystic kidney disease (Hanaoka et al., 1996) and secretory diarrhea, including cholera (Thiagarajah and Verkman, 2003). A better understanding of CFTR may lead to the design of clinically useful inhibitors to treat these secretory disorders. Comparative pharmacology is conceptually tangential to evolution of function, particularly for synthetic drugs that are not mimics of natural ligands that CFTR could have "evolved" to bind. That being said, an improved understanding of the structural relationships between groups of ABC transporters may be relevant to the investigation of the mechanisms of action of CFTR-targeted drugs discovered through high-throughput screening. In fact, distant CFTR orthologues and transporter homologues may assist in the elucidation of mechanisms and binding sites of the Food and Drug Administration–approved CFTR-directed therapeutic compounds using approaches similar to those used to understand the action of CFTR inhibitors (Stahl et al., 2012). While data suggest that many pharmacological agents correct the folding of trafficking mutants of both CFTR (ABCC7) and P-glycoprotein (ABCB1; Loo et al., 2012), lumacaftor, which may bind MSD1 of CFTR (Loo et al., 2013), is unable to correct trafficking mutants of P-glycoprotein (Loo et al., 2012). The drug is, however, able to correct trafficking mutants of ABCA4 associated with macular degeneration (Sabirzhanova et al., 2015).

VX-770/ivacaftor has been shown in some studies to potentiate (and therefore likely directly bind) CFTR from multiple species, including human, murine (i.e., in Cui et al., 2016; Cui and McCarty, 2015; but not in Van Goor et al., 2009; Bose et al., 2019), and *Xenopus* (Cui et al., 2016) orthologues. Surprisingly, Lp-CFTR is not potentiated by VX-770 (Cui et al., 2019a); in fact, a small degree of inhibition was observed. Recently, the Chen laboratory solved a cryo-EM structure of CFTR in the presence of VX-770 at 3.3 Å resolution (PDB accession no. 6O2P) and identified residues contributing to the binding energy (Liu et al., 2019). This study revealed that VX-770 binds at a cleft formed by TMs 4, 5, and 8 deep inside the membrane core (see Fig. 5) at the interface between protein and the membrane lipid. Whether this structure demonstrates the binding site responsible for therapeutic potentiation is currently unclear (Csanády and Töröcsik, 2019; Yeh et al., 2019), although the same site also coordinated another potentiator, GLPG1837 (Liu et al., 2019). The conservation in this binding site is mixed; of the amino acids whose mutation strongly affect affinity, some are highly conserved across CFTRs and ABCC4s (e.g., R933 in hCFTR, but not S308), whereas others are conserved among CFTRs but not with ABCC4s (e.g., Y304), and some sites are uniquely divergent in Lp-CFTR (e.g., F931, a proline in lamprey).

A very recent study from the Bear laboratory (Laselva et al., 2021) explored VX-770 binding sites using photo-induced crosslinking. This study confirmed a position proximal to the site identified by the Chen laboratory, noted above, but also identified a site within the ICLs linking the TMDs to the NBDs. This second location, formed by residues in ICL4, was previously

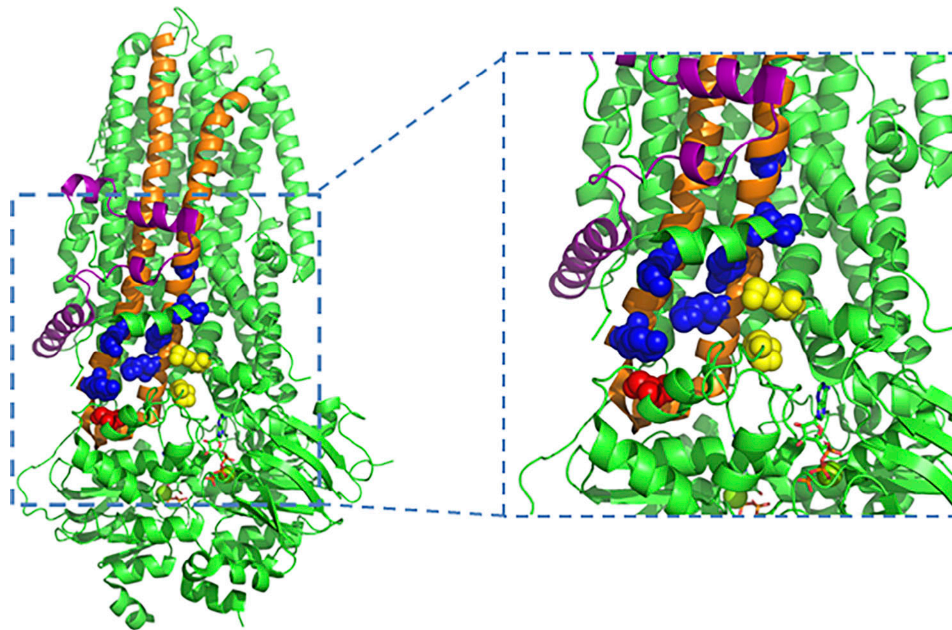


Figure 11. **Residues contributing to second potential binding site for VX-770 are located in a domain tightly linked to channel opening and to the most common mutation causing CF disease.** Residues from Laselva et al. (2021) are mapped onto the 6MSM structure from the Chen laboratory. Purple, lasso domain; orange, TM10 and TM11, whose cytoplasmic tails comprise ICL4; blue, sites contributing to VX-770 binding site; yellow, E543 and K968, identified by Strickland et al. (2019) as responsive to the occupancy of the NBDs by ATP; red, F508.

nominated as a VX-770 binding site by the observation that ICL4 was protected from hydrogen/deuterium exchange in the presence of drug (Byrnes et al., 2018). Note that ICL4 also is the portion of the TMDs that most closely approaches position F508, which is deleted in most CF alleles in North America (Mornon et al., 2008; Serohijos et al., 2008). Residues making the strongest contribution to binding energy at this second site include K1041, E1046, P1050, F1052, H1054, Y1073, and K1080. In their hands, mutation F1052A at the second site had a significantly (approximately fivefold) larger effect on VX-770 affinity than alanine mutations of aromatics within the first site. This site also is much closer to the NBDs and interestingly is adjacent to residues E543 and K968 (Fig. 11), which were previously identified as involved in signaling the state of NBD occupancy by ATP to the TMDs (Strickland et al., 2019; of note, K968 is type II divergent between CFTRs and ABCC4s, with the exception of Lp-CFTR, where the equivalent position bears a glutamine). Hence, this newly identified pocket may contribute to the mechanism by which VX-770 stabilizes the channel open state (Cui et al., 2019b; Langron et al., 2018). We note that all of the residues listed above that contribute to this second site are conserved in Lp-CFTR, which is not potentiated by VX-770 (Cui et al., 2019a), other than K1080 (a glutamine, in lamprey). A lack of functional potentiation is, however, at most indirect evidence of loss of binding. In fact, because a small degree of inhibition was observed, it is possible that the drug binds to a site or sites on Lp-CFTR similar to that on hCFTR but that the nature of the interaction is subtly altered by divergence in the site such that potentiation does not occur. Conceptual precedence for such a scenario may be found in the pharmacology of closely structurally related drugs that bind to similar sites on receptors but induce opposing functional outcomes, such as the dihydropyridine class of voltage-sensitive $Ca^{2+}$ channel modulators (Zhao et al., 2019). The emergence of a biotinylated, photo-cross-linkable ivacaftor analogue (Laselva et al., 2021) is expected to significantly aid in the dissection of the effect of a given mutation on binding versus potentiation or inhibition.

## Conclusion

There are many questions that have yet to be answered with respect to the structure–function relationship in CFTR and related transporters. Many of these questions now can be answered through the study of revertant mutants between groups, retracing a possible evolutionary path. The results of these studies have the potential to shed light on the structures of both channel and nonchannel ABC proteins and may reveal channel-specific features in CFTR that serve as levers for the pharmacological repair of mutant channels in patients with CF. Although this article focuses on only one member of the ABC transporter superfamily, CFTR (ABCC7), many others have been implicated in disease, including close relatives, such as P-glycoprotein (ABCB1) and MRPs 1, 4, and 5 (ABCC1, 4, and 5), which confer life-threatening resistance to therapeutics when overexpressed (Chen and Tiwari, 2011). The extent to which structural and functional information gained about one ABCC can be mapped to another is an important consideration in both the discovery and mechanistic understanding of therapeutics directed against these proteins. Looking forward, the study of the molecular evolution of function in ABC proteins may therefore lead to exciting advances in the pharmacological and structural understanding of these highly medically relevant proteins.

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