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Studies of the Structure, Regulation, and Pharmacology of the Cystic Fibrosis Transmembrane Conductance Regulator

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Studies of the Structure, Regulation, and Pharmacology of the Cystic Fibrosis Transmembrane Conductance Regulator

By

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B.S., Stetson University, 2009

Advisor: Nael A. McCarty, Ph.D

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science
Molecular and Systems Pharmacology

2016

Abstract

Studies of the Structure, Regulation, and Pharmacology of the Cystic Fibrosis Transmembrane Conductance Regulator

By Daniel T. Infield

Cystic Fibrosis (CF) is a devastating, life-shortening disease affecting approximately 70,000 patients worldwide. The research in this dissertation concerns the structure, function, and regulation of the product of the gene responsible for CF, an anion channel called the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The Introduction (Chapter 1) primarily serves to provide information relevant to our understanding of CF pathogenesis and of CFTR. The Methods (Chapter 2) detail the techniques I learned and modified to perform experiments testing CFTR function. The Results section (Chapter 3) is comprised of three related sections detailing discoveries on the conformational dynamics, regulation, and pharmacology of CFTR. Finally, the Discussion (Chapter 4) interprets the Results in light of existing knowledge on CFTR.

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ACKNOWLEDGMENTS

I would like to gratefully acknowledge several people who made this dissertation possible. First and foremost, thank you to my advisor, Nael A. McCarty PhD, for being a wonderful mentor and friend. Thank you for your patience and all of your advice pertaining to science and life. Thank you to everyone in the McCarty Lab, especially Guiying Cui PhD/MD, and Brandon Stauffer (with both of whom I have worked for 5+ years), for all of your encouragement and help in surmounting technical and conceptual hurdles. Thanks to my committee members for advice that has consistently proven timely and wise, and for the loan or use of equipment necessary for the completion of these studies. Thanks as well to several members of the ion channel and CFTR community, especially including David Dawson Ph.D, David Sheppard Ph.D, and Stephan Pless Ph.D, who were so forthcoming with advice to a trainee coming up in the field.

Thank you to my parents, siblings, and, most of all, my wife Kim. You have encouraged me all along the way, but probably more importantly, you reminded me (with and without words) that my scientific career is not the sum total of who I am.

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CHAPTER 1: INTRODUCTION

This chapter introduces the concepts relevant to the studies performed in this dissertation in five sections. First, I begin with a brief historical account of the fascinating history of the study of the pathophysiology of CF. Second, I discuss the seminal research that identified and ascertained the function and regulation of CFTR. I also explain the mechanisms of CFTR dysfunction caused by several types of CF-related mutations. Third, I discuss several indispensable roles of CFTR function in physiology, with a particular emphasis on those systems where dysfunction directly accounts for pathology in CF patients. Fourth, I discuss the currently FDA-approved functional modulator of CFTR, Ivacaftor (VX770), which is used in some of the studies in this dissertation. Fifth, I review relevant research pertaining to the structure-function relationship in CFTR. To tailor this section (of which an adapted version is in the process of being submitted as a freestanding publication) to this dissertation, I have focused on how the structure, function, and pharmacology of CFTR relates to structural and functional data from evolutionarily-related but functionally-divergent members of the ATP-binding cassette (ABC) superfamily of proteins. Finally, I introduce the three major interrelated research questions pertaining to CFTR structure and function that were asked in the course of this dissertation.

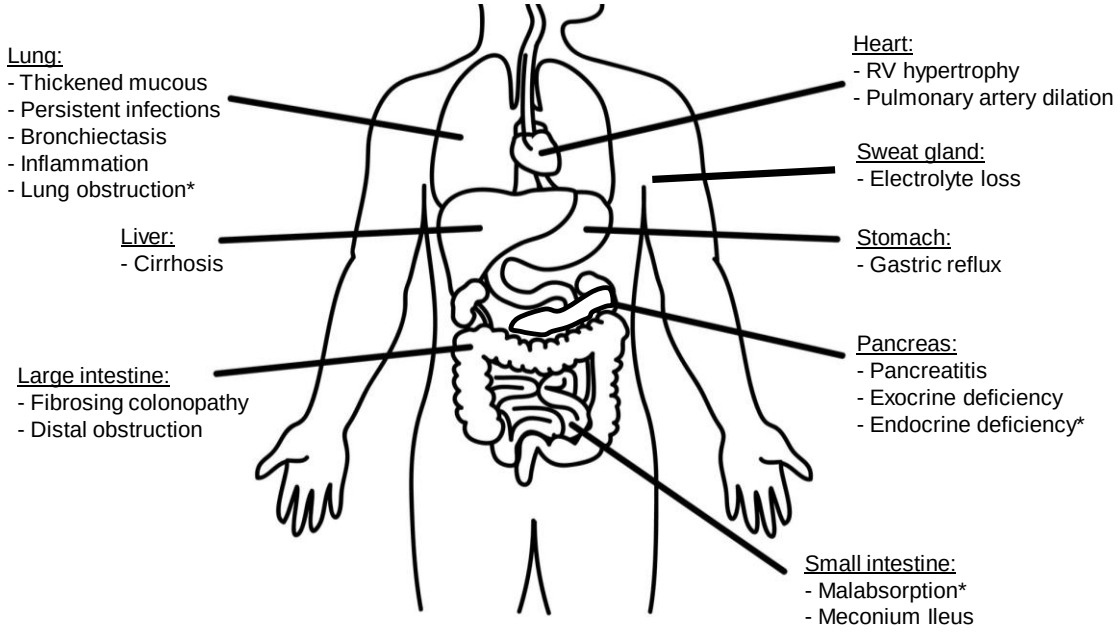
1.1: A brief history of research on CF pathophysiology

1.1.1 *Earliest indications.* Our history of "research" into CF actually begins as early as medieval times. In several countries of medieval Europe, there is evidence of a common (and dark) children's rhyme that "a child will die soon, whose forehead tastes salty when kissed."(Super, 1992) With the benefit of hindsight, we now know that this particular rhyme refers to the sweat gland electrolyte imbalance intrinsic to CF, a disease most common in ethnic Caucasians who claim these Europeans as ancestors (Conneally et al., 1973). Sadly, this pre-scientific society attributed the fact that this disease was often manifest in siblings as evidence of a cursed or "bewitched" family. As such, many of these CF children suffered perhaps the greatest condemnation of that time period—they were refused baptism (Super, 1992). In the early 20thcentury, Landsteiner (1905) and Garrod (1910) first recognized the existence of a disease involving concurrent meconium ileus (infantile intestinal blockage) and bronchopneumonia (Dodge, 2015). In addition, Garrod correctly hypothesized the autosomal recessive nature of the disease, based on patterns of inheritance in affected consanguineous families (Dodge, 2015).

1.1.2 Defining the disease. Major leaps forward occurred in the 1930s, when the French physician Guido Fanconi (1936) and American physician Dorothy Anderson (1938) independently described, in what even today is considered by physicians a high degree of accuracy (Super, 1992), the main pathological components of this disease (Dodge, 2015). Anderson in fact named the disease "Cystic Fibrosis," after the

pancreatic cysts that were characteristically observed in autopsy of CF patients. In her very first description, Dorothy Anderson even suggested treatment of using pancreatic enzymes, which would eventually be implemented as standard of care, but not until decades later (Somaraju and Solis-Moya, 2015). Fanconi and Anderson's work was followed up by myriad others and within 15 years, the majority of the presently known major systemic abnormalities in CF had been defined (Cutting, 2015). Figure 1.1 summarizes these effects, which will be revisited in the discussion of the clinical mechanism of a CFTR-directed therapeutic, Ivacaftor (Section 1.4). In addition, around this time, the historically hypothesized autosomal recessive inheritance of CF was firmly established via large scale genetic studies such as by Lowe *et al*. (Lowe et al., 1949).

1.1.3 Missing chloride conductance. Medieval nursery rhymes notwithstanding, the first solid evidence that CF was a disease of electrolytic imbalance came from a study by Paul Di Sant'Agnese et al., who noticed that CF patients were particularly susceptible to heat exhaustion during a heat wave in 1950s New York. They tested the sweat of CF patients and noted elevated sodium and chloride levels, indicating a defect in electrolyte reabsorption (Di Sant'Agnese et al., 1953). Approximately thirty years later, physiologist Paul Quinton (himself a CF patient) isolated CF sweat glands and observed an absence of physiological chloride permeability during microperfusion (Quinton, 1983; Quinton and Bijman, 1983). Around the same time, a lack of physiological chloride conductance (in these cases secretion) was reported in CF nasal epithelia (Knowles et al., 1983a) and airway epithelium (Frizzell et al., 1986; Knowles et al., 1983b; Welsh, 1986a; Welsh, 1986b; Welsh and Liedtke, 1986). The observation



Systemic effects of Cystic Fibrosis

Figure 1.1 Systemic Effects of Cystic Fibrosis. Author's own work, adapted from a purchased royalty-free stock photo of the internal organs of the human body. Effects taken from (Cutting, 2015). The effects that are asterisked have been specifically shown to be improved in G551D patients upon taking the FDA-approved CFTR potentiator Ivacaftor (Section 1.4).

that epithelial tissues from several different organs all featured loss of chloride conductance in led to the intuitive hypothesis that the autosomal recessive disease CF was caused by a genetic defect in a chloride channel.

1.2 Cloning and characterization of the CF gene.

1.2.1 Cloning of CFTR. With both the genetic and physiological bases of CF reasonably well established by the late 1980s, the "race was on" to identify the gene harboring mutations in CF. This effort predated the mapping of the human genome, and as such was facilitated by intensive linkage studies that had mapped the CF locus to a particular region of chromosome 7 (Knowlton et al., 1985; Tsui et al., 1985; White et al., 1985). In 1989, a group led by Francis Collins (now director of the National Institutes of Health), John Riordan, and Lap-Chee Tsui ultimately used gene jumping (concentrating on GC-rich segments of the region that were likely to harbor genes (Rommens et al., 1989)) to discover and publish the sequence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Riordan et al., 1989), as well as localize the most common CF-associated gene mutation, the deletion of phenylalanine 508 (F508del) (Kerem et al., 1989). Based on hydropathy plots and sequence similarity with exporters of the ATP binding cassette (ABC) protein superfamily, this group proposed that CFTR was a transmembrane protein with 2 major transmembrane domains (each having 6 transmembrane helices) and three major cytoplasmic domains, including two ABC-like nucleotide binding domains, NBD1 and NBD2 (Riordan et al., 1989). This topology is rendered in Figure 1.2 as a cartoon layout, which is how CFTR has been

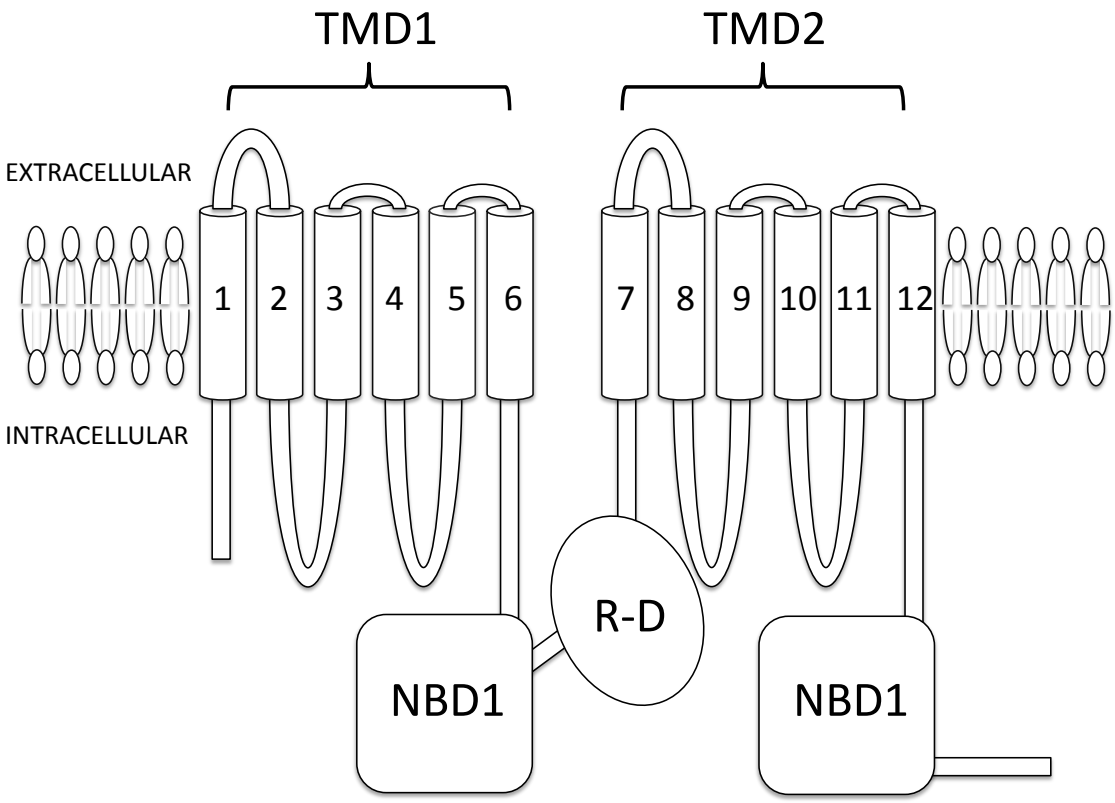


Figure 1.2 Cartoon depiction showing the overall organization and topology of CFTR. TMD = transmembrane domain, NBD1 = first nucleotide binding domain, NBD2 = second nucleotide binding domain, R-D = Regulatory "R" domain. Transmembrane helices are numbered.

depicted essentially since the gene was cloned. Though CFTR's kinship to other ABC transporters has ultimately proven useful in elucidating details about its structure and conformational dynamics (see Section 1.5), it led to initial confusion in the understanding the function of the protein. After all, why would an ion channel, which regulates the passive flow of ions down their electrochemical gradients, need to hydrolyze ATP, as necessary for active transport? The initial uncertainty as to the function of CFTR is why it was named a "transmembrane conductance regulator" rather than an ion channel at the time of cloning—a name that, however awkward, has stood until the present day.

1.2.2 CFTR is an anion channel. Earlier studies done on normal and CF epithelia had shown that CF caused a loss of a phosphorylation-regulated chloride conductance (Schoumacher et al., 1987). Congruent with this, it was shown that expression of CFTR in CF airway cells (Rich et al., 1990) and in CFTR-less cell lines (Berger et al., 1991) imparted a new PKA-dependent chloride conductance, strongly implying that CFTR functions as a chloride channel. Ultimately, definitive demonstration that CFTR itself directly mediated the chloride conductance was accomplished using two independent approaches. The Welsh group showed that point mutations in the putative pore domain of CFTR altered the halide selectivity of the channel (Anderson et al., 1991b) while the Riordan group purified CFTR, reconstituted the protein in a cell-free system, and demonstrated that CFTR alone was sufficient to mediate an ATP and cAMP-dependent chloride conductance (Bear et al., 1992).

It bears mentioning at this juncture that although the vast number of studies of CFTR have concentrated on its physiologically relevant chloride channel activity exclusively, chloride channels are in general notoriously nonselective, so it is not surprising that CFTR has been found to conduct many different (physiological and non-physiological) anions (Linsdell et al., 1997; Yeh et al., 2015). In part prompted by very early clinical research reports demonstrating that pancreatic bicarbonate ($HC0_3^-$) secretion is impaired in CF patients (Gaskin et al., 1982), and by the fact that current measurements across airway epithelia could not be fully accounted by chloride as a sole anion (Smith and Welsh, 1992), several groups tested and ultimately confirmed that airway epithelial cells exhibit a cAMP-stimulated, CFTR-dependent $HC0_3^-$ conductance (Choi et al., 2001; Ko et al., 2002; Smith and Welsh, 1992). The list of physiological anionic solutes able to permeate through CFTR has in fact even been reported to include the reducing tripeptide glutathione (GSH) (Linsdell and Hanrahan, 1998). However, while this promiscuous permeability is interesting and often useful in mechanistic studies of the channel pore (Serrano et al., 2006; Yeh et al., 2015), thus far only CFTR chloride and bicarbonate conductances have been strongly implicated in the pathophysiology of CF, by the mechanisms described in Section 1.3.

1.2.3 CFTR is activated by phosphorylation. Its identity as a *bona fide* chloride channel established, intensive electrophysiological and biochemical studies were done to elucidate the molecular mechanisms of CFTR regulation. It was found that deleting the intracellular R domain eliminated dependence on cAMP, suggesting that CFTR is activated by direct PKA-mediated phosphorylation of this region (Rich et al., 1991).

Specific consensus sites were identified as targets of PKA phosphorylation; it was found that mutation of these sites either to alanine (to eliminate the side chain) (Cheng et al., 1991) or to aspartic acid (to mimic phosphorylation (Rich et al., 1993)) altered the dependence of the channel on cAMP or PKA. The fact that both phosphorylation, and phospho-mimicking at these positions reduced phospho-dependence strongly implied that the unphosphorylated R-domain overall served as an inhibitory domain and that phosphorylation relieved this inhibition. The above data conjured hypotheses relating the R-domain to that of the inactivation "ball" responsible for inactivation of voltage-gated ion channels (Armstrong and Bezanilla, 1977). This idea was indirectly supported by the fact that there was redundancy observed in terms of the ability of four main serine residues to inhibit the channel when unphosphorylated (Cheng et al., 1991).

However, subsequent studies have complicated this model significantly. First, it was shown that an exogenous unphosphorylated R domain peptide failed to inhibit R domain-less (ΔR-CFTR) channels, while adding a phosphorylated R domain peptide actually stimulated them (Winter and Welsh, 1997). Second, it was shown via a combination of approaches (electrophysiology, mass spectroscopy, and phospho-western blotting) that specific sites are phosphorylated at different rates (Csanady et al., 2005b) and mediate opposite functions; this is to say, some sites inhibit CFTR when unphosphorylated, while some sites inhibit CFTR when phosphorylated (Csanady et al., 2005b; Wilkinson et al., 1997). Finally, despite the observed redundancy in terms of functionally validated PKA consensus sites to confer phospho-regulation on CFTR *per se*, it also has been shown that the *degree* of phosphorylation (above that necessary to

activate the channel at all) affects the activity of CFTR in a graded (rather than binary) fashion (Hwang et al., 1993). Particularly, partially de-phosphorylated *vs.* strongly phosphorylated CFTR channels differ at least in terms of their sensitivity to ATP (Csanady et al., 2000) (the gating ligand of CFTR, see below), gating kinetics (Csanady et al., 2010; Vergani et al., 2003), and response to pharmacological modulators (Wang et al., 2005). Though as-yet poorly understood in terms of its mechanistic or structural basis, this phenomenon is likely highly relevant in the context of epithelial tissues, wherein PKA signaling is highly dynamic (Mutlu and Factor, 2008).

The complex nature of CFTR regulation by phosphorylation is still not well understood, particularly since it is now thought that in addition to (and in combination with) PKA, CFTR is modulated by PKC (Chappe et al., 2004; Seavilleklein et al., 2008) and tyrosine kinases (Billet et al., 2013) through distinct sites. However, of these modes, activation of CFTR by PKA alone is the best understood at the molecular level. Intersubunit cysteine cross-linking demonstrated that PKA-mediated phosphorylation of the R domain of CFTR promotes formation of the NBD heterodimer necessary for channel opening *in vivo* (Mense et al., 2006). Similar results were found in experiments using purified NBD-R domain fragments (Howell et al., 2004). More recently, extensive nuclear magnetic resonance studies have mapped the majority of the residues of the R domain and demonstrated that PKA phosphorylation causes the loss of specific secondary helical structures within the domain (Baker et al., 2007) and the loss of inhibitory interactions between the R domain and the rest of the CFTR protein, including at the interface between the two NBDs (Bozoky et al., 2013a; Bozoky et al., 2013b).

Thus, at least one mechanism by which the R domain regulates CFTR activity is to function as an inhibitory "gear" that gets in the way of the conformational changes described below that are necessary for channel opening in the presence of ATP. However, other mechanisms must also exist, at least to account for the stimulation of ΔR-CFTR channel activity by the fully phosphorylated R domain(Winter and Welsh, 1997) and for the finding that the R-domain still regulates the activity of a truncation mutant of CFTR that is missing NBD2 (Wang et al., 2010).

1.2.4 CFTR is opened by ATP binding and closed by ATP hydrolysis at the NBDs. Not long after CFTR was shown to function as a chloride channel, excised patch clamp studies demonstrated that nucleoside triphosphates (NTPs, with a strong preference for ATP) are required to open the phosphorylated CFTR channel (Anderson et al., 1991a). This was consistent with the initial proposal that the cystosolic regions of CFTR conserved with ABC transporters were nucleotide binding domains (Riordan et al., 1989). In addition, the dose dependence of channel opening as a function of ATP concentration showed negative cooperativity, suggesting that there were two ATP binding sites (ABSs) in the channel, analogous to the two sites in the NBDs of transporters (Anderson et al., 1991a; Gunderson and Kopito, 1994). The finding that non-hydrolyzable ATP analogs such as AMP-PNP were unable to open CFTR (Anderson et al., 1991a; Carson and Welsh, 1993) initially caused some confusion, because it seemed to implicate not just binding of an ATP ligand but also hydrolysis (consumption) of that ligand to channel opening. However, it was subsequently demonstrated that while AMP-PNP was unable to open CFTR on its own, it could bind and open the channel if

applied in combination with ATP (Baukrowitz et al., 1994; Gunderson and Kopito, 1994) or immediately after washout of ATP (Hwang et al., 1994) . In these contexts, AMP-PNP has the effect of "locking" the channel open, presumably due to its binding to and disabling one ATP-hydrolyzing pocket of CFTR along with ATP binding to another, structurally distinct site (Gunderson and Kopito, 1994; Hwang et al., 1994).

Strong evidence was attained that ATP hydrolysis was likely not required to open CFTR through mutation of residues in the NBDs that are analogous to ones demonstrated to be important for ATP hydrolysis in other ABC proteins (Gunderson and Kopito, 1995; Vergani et al., 2005). Far from precluding channel opening, these mutations instead "locked" the channel into open bursts orders of magnitude longer than observed for Wild-type (WT) CFTR (Gunderson and Kopito, 1994; Vergani et al., 2005). These studies also provided additional evidence that the two predicted ABSs in CFTR are non-equivalent, since mutations of analogous lysine residues abolished channel closure when mutated in NBD2 (K1250A) but not when mutated in NBD1 (K464A) (Berger et al., 2005; Gunderson and Kopito, 1995). Subsequent biochemical experiments would demonstrate that this non-equivalence was in part due to the fact that the ABS incorporating K464 was already enzymatically "dead" (likely due to the absence of other canonical catalytic residues in this site), while the ABS incorporating K1250 was enzymatically functional (Aleksandrov et al., 2002). (See section 1.5 for a more detailed discussion of the structural and evolutionary basis for this aspect of CFTR function.) Overall, these results suggested that WT-CFTR opening is dependent on ATP binding *to* the channel, while channel closure is dependent on ATP hydrolysis *by* the

channel. This so-called "non-equilibrium" gating model has been more or less supported by subsequent studies wherein other catalytic residues in the hydrolytic ABS near K1250 have been mutated (Csanady et al., 2010; Kloch et al., 2010; Vergani et al., 2005), although it is important to note that the strictness of dependence of CFTR opening on ATP binding and CFTR closing on ATP hydrolysis is not absolute: highly phosphorylated CFTR channels can open and close (vary rarely) in the absence of free ATP, and even in the absence of the NBD2 domain (Wang et al., 2010).

1.2.5 Mechanisms of dysfunction in CF-related CFTR mutants. As discussed above, CF is directly caused by mutations in CFTR. Consequently, as the field has worked to understand the the WT-CFTR channel, concurrent study has aimed to elucidate the mechanisms by which mutations in CFTR cause channel dysfunction. To date, approximately 1900 different mutations in the CFTR gene have been reported (www.genet.sickkids.on.ca). Of these, at least 159 occur with enough frequency that they are known to be reliably CF-causing, and they are very diverse in terms of proposed mechanism of dysfunction (Sosnay et al., 2013). In order to make sense of this diversity, these mutations have been grouped into classes (Cutting, 2015) (Figure 1.3): Class I mutations disrupt synthesis of the full length protein; Class II affect processing (including trafficking); Class III cause dysfunction in the channel's responsiveness to cellular

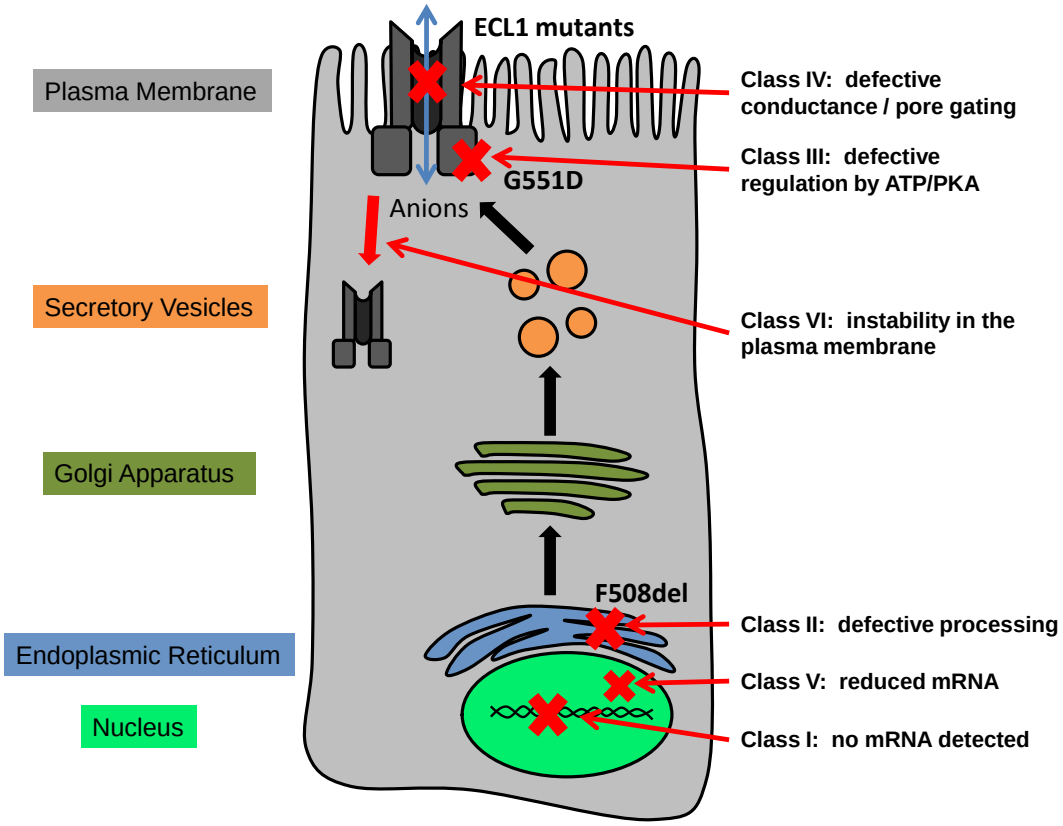


Figure 1.3 Mechanistic classes of Cystic Fibrosis-related mutations in CFTR in a schematized epithelial cell. Author's own work. Labeled with archetype mutants of Class II, III, and IV discussed in this section. The CFTR molecule (gray) is purposely drawn drastically out of scale.

regulation (ATP and PKA); Class IV cause dysfunction in open pore stability and/or anion conduction; and Class V mutations cause transcription of mRNA to be reduced, and Class VI mutations cause defects in plasma membrane stability leading to enhanced endocytosis (Cutting, 2015). Several different missense, deletion, and nonsense mutations in CFTR genes are found in substantial numbers of CF patients (Sosnay et al., 2013) (Table 1.1). Since our group is most interested in the structure and function of the channel, we concentrate on mutation classes wherein full-length CFTR protein is made but is dysfunctional (particularly Classes II, III, IV). Below, I use archetype mutations to explain these three classes. As will become clear, these classes are not mutually exclusive; this is to say, it is possible for one mutation to cause defects of several class types.

Class II: F508del-CFTR. The CF-causing mutation F508del-CFTR was identified when the gene was cloned and at that time it was estimated to be present in approximately 70% of all CF patients (Kerem et al., 1989). It has been shown subsequently that the F508del mutation is present on at least one allele in approximately 90% of American CF patients, making it by far the most common CF-causing CFTR mutation (Sosnay et al., 2013). The first evidence that this mutation alters CFTR processing was generated by comparing glycosylation patterns between WT- and F508del-CFTR (Cheng et al., 1990). Specifically, glycanase treatment of transfected cells showed that WT-CFTR is both “core” and “complex” glycosylated, whereas F508del-CFTR is only “core” glycosylated (Cheng et al., 1990). Since proteins are core glycosylated in the endoplasmic reticulum (ER) and complex glycosylated in the

TABLE 1.1 Prevalence and class overlap in CF-related CFTR mutations

Mutation	Domain	Mutation Class[1]	Number of Patients[5]
P67L	N-terminus	IV	238
G85E	N-terminus	II	580
D110H[2,4]	TMD1	IV	57
R117H[2,3]	TMD1	**IV**	**2042**
R117C[3]	TMD1	IV	141
Y122X	TMD1	I	75
L206W	TMD1	II	330
R334W	TMD1	IV	404
T338I	TMD1	IV	51
R347P	TMD1	IV	512
R347H	TMD1	IV	191
R352Q	TMD1	IV	99
A455E	NBD1	II, V	495
I507del	NBD1	II	629
F508del[2]	**NBD1**	**II, II, IV**	**64868**
V520F	NBD1	II	155
G542X	NBD1	I	3474
S549R	NBD1	III	61
S549N	NBD1	III	184
G551D[2]	NBD1	**III**	**2915**
R553X	NBD1	I	1298
A559T	NBD1	II	85
R560T	NBD1	II	340
R1066C	TMD2	II	212
L1077P	TMD2	II,III	93
M1101K	TMD2	II	176
D1152H	TMD2	IV	555
R1162X	TMD2	I	611
D1270N	NBD2	III	52
W1282X	NBD2	I	1552

Data culled from CFTR2 database: www.genet.sickkids.on.ca

1. Mutation class for a given mutant is that ascertained *thus far* from the functional experiments or genetics. This categorization will likely expand over time: for example, many class II mutants (like F508del) may also have functional defects observable after correction of trafficking.
2. Mutations discussed as archetypes of a class
3. Most common mutations in a region of CFTR (ECL1) whose structure was investigated as part of this dissertation (Section 3.2).
4. This mutation was specifically investigated as a part of this dissertation (Section 3.1)
5. This is the number of patients ever recorded to have a given mutation.

Golgi apparatus, this discrepancy indicated that the absence of F508 causes F508del-CFTR to fail to exit the ER. Immunofluorescence (Cheng et al., 1990) and biotinylation (Denning et al., 1992c) data showed that the consequence of this trapping is that most if not all of F508del-CFTR fails to localize to the plasma membrane and is eventually targeted for proteasomal degradation (Jensen et al., 1995; Ward et al., 1995).

Encouragingly, studies contemporary to these demonstrated that F508del-CFTR does generate current when expressed in non-mammalian expression systems, such as *Xenopus* oocytes (Drumm et al., 1991). It was hypothesized these currents were enabled by the lower culturing temperature for these cell types, and in agreement with this, subsequent studies demonstrated that F508del-CFTR trafficking could be "corrected" in mammalian cells by lowering the culturing temperature below 37° C (Denning et al., 1992a). It is, however, also well established that the function of temperature-corrected F508del-CFTR is impaired (regardless of expression system); therefore, F508del must be thought of as both a Class II and a Class III mutant. In particular, at room temperature, a given level of activation requires higher levels of PKA for F508del-CFTR than for WT-CFTR (Drumm et al., 1991; Wang et al., 2000), and even when fully phosphorylated, the mutant has an approximately 40-fold lower opening frequency in response to ATP (Miki et al., 2010). More recently, it has been shown that activity is essentially eliminated when temperature-corrected F508del-CFTR recorded in excised patches is raised from room temperature to 37°C (Liu et al., 2012; Wang et al., 2011b).

Nevertheless, the demonstration that trafficked F508del-CFTR retains marginal function has led to efforts to develop pharmacological "correctors" capable of increasing the pool of plasma membrane-localized F508del-CFTR in patients. These efforts have been supported by intensive mechanistic work on the structural defects caused by deletion of F508. These studies have essentially all converged on two main structural phenotypes caused by this mutation. First, deletion of this residue renders the domain in which it is found (NBD1) structurally distorted and biochemically unstable (Ko et al., 1993; Mendoza et al., 2012; Rabeh et al., 2012). Second, loss of F508 disrupts an interaction between NBD1 and intracellular loop 4 (ICL4) that is critical to both the folding and subsequent gating of the channel (He et al., 2008; Rabeh et al., 2012; Serohijos et al., 2008). In the context of the whole protein, these defects can be distinguished via second-site suppressor mutations that repair either one defect or the other (Mendoza et al., 2012). Similarly, it is thought that some pharmacological correctors act on the NBD1 stability defect, while others (including the most promising pharmacological corrector of F508del, Lumacaftor (VX809)) act by helping to repair the NBD1-ICL4 interaction defect (Okiyoneda et al., 2013).

Class III: G551D-CFTR. The relatively common G551D mutation is associated with severe disease (Cutting et al., 1990). As opposed to mutations like F508del, the G551D mutation does not apparently affect protein trafficking or stability. Rather, this mutant has a profound defect in its opening rate in response to activating stimuli—approximately 100-fold lower than that of WT-CFTR (Bompadre et al., 2005; Miki et al., 2010). Since G551D is one of several disease-related mutations localized to one of the

previously-described ATP binding pockets of the NBDs of CFTR (Cutting et al., 1990; Smit et al., 1993), it has been hypothesized that the mutation alters ATP binding to these domains. In support of this, biochemical assays with purified NBD proteins demonstrated that G551D NBD1 binds ATP with an approximately four-fold lower affinity as compared to WT-NBD1 (or F508del NBD1) (Logan et al., 1994). However, in excised patches, the activity of G551D-CFTR is dependent on PKA phosphorylation at the R domain, but essentially independent of ATP concentration (Bompadre et al., 2007). Therefore, either the G551D mutation more profoundly impairs ATP binding in the context of the whole protein than in isolated NBD1, or a more complex, as yet undescribed mechanism accounts for the anomalous behavior of G551D-CFTR.

Class IV: ECL1 mutants. A group of missense mutations found in the pore domains of CFTR have been found to affect CFTR function through defects in gating of or conductance through the pore (Table 1.1). Many of these mutations affect pore stability, as evidenced by diminished open burst durations and subconductances in their single channel behavior (Cotten and Welsh, 1999). At the structural level, one hypothesized mechanism by which some Class IV mutants cause dysfunction in the CFTR channel is by disrupting an electrostatic “salt-bridge” interaction necessary for maintenance of pore architecture and stability.

For example, in the first extracellular loop (ECL1) of CFTR, mutations at six different positions have been associated with CF disease, including D110H and D110E (Van Goor et al., 2014). Characterization of some CF-causing mutations in this region,

including R117H (Hammerle et al., 2001; Sheppard et al., 1993), as well as studies using multi-species CFTR chimeras (Price et al., 1996), had ascribed to ECL1 an important role in maintaining the stability of the open pore of the channel. We recently investigated the functional role of three charged residues (D110, E116, and R117), wherein mutations cause mild CF disease (Cui et al., 2014). Charge-reversed (D110R- and E116R-CFTR) or neutralized (R117A-CFTR) mutants displayed very brief mean burst durations compared to WT-CFTR (~ 700 ms) and significant subconductance behavior. Interestingly, though, when the full conductance was observed in these mutants, it was similar in amplitude to WT-CFTR (Cui et al., 2014). For R117A-CFTR, this result was suggestive that this basic residue was likely not involved in the electrostatic attraction of anions into or through the pore, as had been suggested by another group (Zhou et al., 2008). As discussed above, poorly-hydrolyzable ATP analogs such as AMP-PNP "lock" WT-CFTR open (when applied concurrently with ATP) by disallowing ATP hydrolysis necessary for channel closure (Gunderson and Kopito, 1994). However, the mean burst durations of D110R-, E116R, and R117A-CFTR were not increased by ATP + AMP-PNP, further suggesting that their defects are in "pore gating" – that is, regulation of the CFTR gate kinetically downstream of ATP binding and NBD dimerization. The failure of these ECl1 mutants to be locked open by AMP-PNP, combined with their diminished burst duration, strongly suggests that channel closure is primarily the result of instability of the channel pore, as opposed to ATP hydrolysis (as in WT-CFTR) (Section 1.2.4).

Mutations of these three residues that spared native charge partially rescued mean open burst durations, perhaps pointing to a role of residue charge at these

positions in maintaining open pore stability (Cui et al., 2014). Electrophysiological evidence from single channel recording of charge-swapped mutants suggested that E116 may interact with R104 of TM1, while R117 may interact with E1126 of ECL6 (Cui et al., 2014). Outside of ECl1, at least two other electrostatic interactions involving positions of Class IV mutation have been identified, one involving R347 (Cotten and Welsh, 1999) and the other involving R352 (Cui et al., 2008) (Table 1.1).

1.3 Physiological roles of CFTR

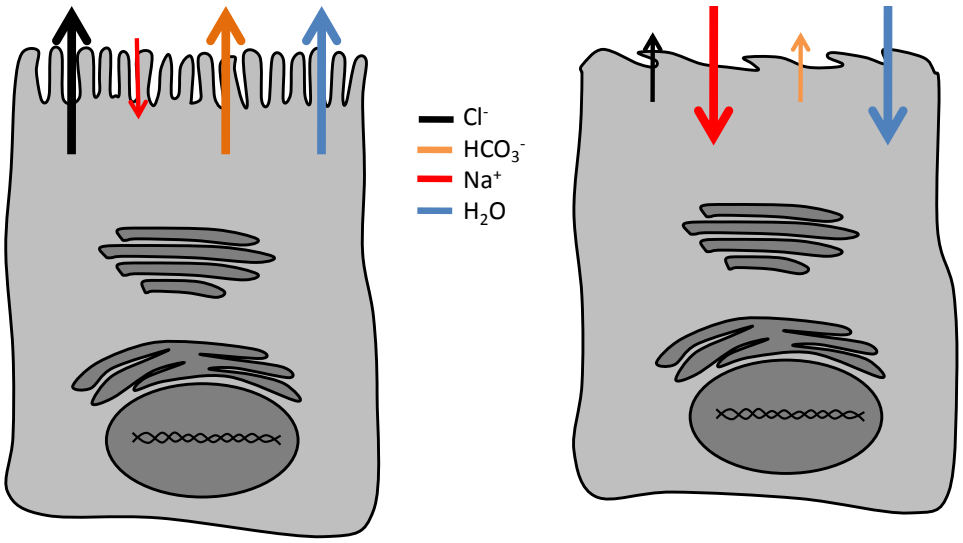
1.3.1 Epithelial and exocrine physiology: roles of chloride and bicarbonate permeation through CFTR. As discussed above, even before the discovery of CFTR, the pathophysiology of the multisystem disease CF had already been somewhat consolidated around the idea of secretory defects in epithelia (Knowles et al., 1983a; Knowles et al., 1983b; Quinton, 1983; Quinton and Bijman, 1983; Welsh, 1986a; Welsh, 1986b; Welsh and Liedtke, 1986). This pathophysiological characterization therefore provided retroactive mechanistic insight into the physiological roles of CFTR in those tissues after CFTR was cloned and functionally characterized as a chloride and bicarbonate channel.

CFTR is expressed on the luminal sides of polarized epithelial cells (Denning et al., 1992b). Both the function of apical CFTR and that of important basolateral anion transporters are regulated by PKA (McCann and Welsh, 1990), which allows for the vectorial transport of anions to occur in response to adrenergic signaling elicited by physiological secretagogues such as epinephrine (Bossard et al., 2011; Frizzell et al.,

1986; Quinton et al., 2012). Ionic transport in turn directly modulates the physiological secretion and reabsorption of other ions and water due to electrogenic and osmotic forces (Figure 1.4). In the airway, CFTR dysfunction causes loss of chloride efflux into the airway surface liquid (Rich et al., 1990). This loss of chloride efflux is accompanied by increased sodium absorption through the Epithelial Sodium Channel, or ENaC, as evidenced by increased amiloride-sensitive current in CF epithelia (Knowles et al., 1983a; Knowles et al., 1983b). In order to maintain isotonicity in the airway surface liquid (ASL) (Knowles et al., 1997), these alterations in ionic flux to the lumen would be expected to impair the physiological secretion of water and therefore dehydrate the ASL. In support of this, it was found that when airway epithelial cells from CF patients are cultured to form polarized epithelia *in vitro,* the depth of liquid maintained at the air-surface interface is only half of that of cells from normal controls, or less, but it is isotonic (Matsui et al., 1998; Tarran et al., 2001). This dehydration has been traditionally proposed to underlie the basis for the mucovicidosis (thickened mucous) in CF, which impairs mucocilliary clearance (Matsui et al., 1998) and ultimately contributes to the vicious pathophysiological cycle of persistent bacterial and viral lung infection, inflammation from a futile immune response, and ultimate airway remodeling and destruction (Rowe et al., 2005).

However, there is also evidence that loss of CFTR's physiological bicarbonate permeation (Smith and Welsh, 1992) is a major contributor to pathophysiology in the CF lung. The ASL of patients (and CF animal models) has been shown to be approximately 0.6 pH units more acidic than normal controls (even in the absence of overt bacterial

A.



B.

Physiological parameter	**Change in CF epithelia**
Chloride luminal efflux	Decreased
Bicarbonate luminal efflux	Decreased
Sodium influx	Increased
H_20 secretion to airway	Lower
ASL pH	More acidic
ASL depth *in vitro*	Shallower
Mucous quality	More viscous
Ciliary function	Severely impaired

Figure 1.4 Consequences of CFTR dysfunction on physiology of an idealized epithelial cell. A. Author's own work, depicting the changes in ionic fluxes. Note that Chloride and Bicarbonate efflux are depicted as significantly impaired (smaller arrows) rather than absent in the CF cell because many epithelial tissues (including in the airway) also contain Ca^{2+} - dependent chloride and bicarbonate conductances that are independent of CFTR (Smith and Welsh, 1992).

infection (Pezzulo et al., 2012; Song et al., 2006)), presumably as a result of the loss of the flux of this important base into the lumen. This acidity is proposed to not only underlie the viscous nature of CF mucous (Tang et al., 2016; Yang et al., 2013) but also directly render several proteins of the innate immune system in the ASL less effective in killing bacteria (Pezzulo et al., 2012; Shah et al., 2016; Tang et al., 2016).

These mechanisms are closely paralleled in the intestinal and pancreatic epithelium. As in the lung, these organs are affected with mucovicidosis, although in these internal organs, the effect of the sticky mucous is not to incur infection but rather to block pancreatic exocrine function (ultimately leading to pancreatic remodeling) and nutrient absorption through the ileum (Quinton, 2010). As with the airway, acidity in these organ systems suggests that bicarbonate transport is also impaired (Gelfond et al., 2013). The pancreas is the main bicarbonate-secreting organ in the body, and, as discussed previously, it has been long known that bicarbonate levels are abnormally low in pancreatic secretions from CF patients (Gaskin et al., 1982; Quinton, 2010). It is therefore highly likely that defects in CFTR-mediated bicarbonate transport are important (if not predominant) causes of dysfunction in these internal organs, particularly in light of recent studies demonstrating that mutations that predominantly affect bicarbonate permeation through CFTR are more highly associated with pancreatitis than with Cystic Fibrosis (LaRusch et al., 2014). Finally, note that an important exception to the directionality of ion transport as discussed in the epithelial tissues above is found in the unique electrochemical environment of the sweat gland, wherein CFTR channels expressed on both the apical and basolateral sides of cells lining

the reabsorptive duct (Kartner et al., 1992) primarily mediate transcellular chloride absorption (Reddy and Quinton, 1992). Impairment by CFTR dysfunction therefore leads directly to hypertonicity of the sweat, as initially observed by Di Sant'Agnese and colleagues so many decades ago (Di Sant'Agnese et al., 1953).

1.3.2 Pancreatic beta cell (endocrine) function. Diabetes mellitus is the most common co-morbidity with CF, occurring in approximately 50% of CF patients over the age of 30 (Brennan and Beynon, 2015). This co-morbidity is tied both to acceleration in the rate of lung function decline and to increased mortality in patients (Marshall et al., 2005); as such, it has become increasingly important to address as the lifespan of CF patients has improved. So-called Cystic Fibrosis Related Diabetes (CFRD) shares similarity with both type I and type II diabetes—there is evidence of impairment of both insulin secretion in nearly all patients with CFRD (Cano Megias et al., 2015; Cucinotta et al., 1994; De Schepper et al., 1992) and of insulin resistance in some patients with CFRD (Cano Megias et al., 2015; Elder et al., 2007; Hardin et al., 2001; Tofe et al., 2005) and in CF mice (Fontes et al., 2015). The cellular mechanism of insulin resistance in CFRD is poorly understood (see Appendix A for data that I collected as a part of a study on possible defects in insulin responsiveness in CF airway cells).

Abnormalities in insulin secretion have conventionally been attributed to the pancreatic destruction resulting from exocrine pathway blockage, as discussed above (Gibson-Corley et al., 2016). However, recent clinical data have established that CF patients have impaired insulin secretion even if they are pancreatic exocrine sufficient

(Cano Megias et al., 2015; Wooldridge et al., 2015). Moreover, the CF pigs have been shown to have impaired insulin secretion from the pancreas in the absence of detectable pathology in the islet cells (Uc et al., 2015). These data suggest that at least some degree of defect of insulin secretion may be due to pancreatic beta cell dysfunction. To this end, several very recent studies have suggested that CFTR is functionally expressed in pancreatic beta cells. CFTR (Guo et al., 2014; Ntimbane et al., 2016) (or possibly a calcium-activated chloride channel regulated by the activity of CFTR (Edlund et al., 2014)) appears to play a role in maintaining the physiological membrane potential of the beta cell. When absent or inhibited, intracellular chloride (Cl_i) is raised, which in turn hyperpolarizes the resting membrane potential for the cell relative to beta cells with functional WT-CFTR (Guo et al., 2014). This counteracts the effect of inhibition of K_{ATP} channels by ATP, which normally depolarizes the membrane potential of the beta cell and ultimately leads to insulin secretion (Edlund et al., 2014; Guo et al., 2014). This novel and direct role for CFTR channel activity in endocrine physiology has opened the possibility that fixing CFTR in CF patients may repair physiological defects including and beyond those related to epithelial cells.

Relevant to this, it is notable that CFTR has been found to be transcribed in a wide variety of cell types (Ott and Harris, 2011). However, in many of these tissues, the question of whether CFTR protein is actually made is highly controversial, and/or the proposed roles of CFTR are poorly understood (Cook et al., 2015; Divangahi et al., 2009; Haggie and Verkman, 2007; Lamhonwah et al., 2010; Michoud et al., 2009; Norez et al., 2014; Pohl et al., 2014). In fact, quantitative studies of mRNA levels in these tissues

indicate that CFTR is transcribed at a rate approximately 400-fold lower than that of epithelial tissues—near the limit of reliable detection by reverse transcription polymerase chain reaction (RT-PCR) in many cases (Yoshimura et al., 1991). Nevertheless, although I decided in the preceding section to focus on systems where convincing evidence has been generated implicating CFTR function to the mechanism of a life-threatening aspect of CF pathophysiology, in so doing, I do not intend to exclude the possibility of roles of CFTR in the physiology of other systems.

1.4 *Discovery and characterization of a clinically efficacious potentiator of CFTR.*

As discussed above, although CF is caused by mutations in a single chloride channel, it is a multisystem disease. As a result, in the absence of a silver bullet therapeutic that could completely replace or repair mutant CFTR, physicians have historically treated the symptoms of the disease in isolation. For example, the nutritional aspects of the disease have been substantially addressed through the administration of pancreatic enzymes overcome the pancreatic insufficiency of the digestive system, as well as through vitamin supplementation and customization of the diet (Solomon et al., 2016). The persistent infections in CF have been treated with antibiotics (oral, intravenous, and inhaled (Stephens et al., 1983)); however, it is important to note that by and large, these antibiotics do not prevent the eventual stable colonization of the lungs by opportunistic bacteria such as *Pseudomonas Aeuriginosa* (Bianconi et al., 2015). More recently, treatments were introduced that more directly targeted pathophysiological features of CF, most specifically the mucovicidosis in the

lung. For example, it was found that a major and exceptionally large constituent of CF mucous is DNA from dead human and bacterial cells (Eisenberg et al., 1997; Fuchs et al., 1994). Nebulized recombinant DNAse has therefore proved an effective mucolytic agent and is currently prescribed for most patients over the age of 6 (Eisenberg et al., 1997). In addition, the administration of nebulized hypertonic saline was been found to improve mucous clearance and lung function in CF patients (Donaldson et al., 2006), presumably by modulating the osmotic gradient in the ASL described in Section 1.3.1.

All this being said, significant research effort also has been expended to identify positive modulators of CF-related mutants of CFTR, with the hope of treating the underlying cause of the disease. In this regard, the first undeniable success has been achieved by Vertex Pharmaceuticals, who in 2009 reported the discovery of VX770 (Ivacaftor), a small molecule potentiator of CFTR (Van Goor et al., 2009). The lead compound was discovered through a cell-based high throughput screen using NIH 3T3 cells stably expressing mutant CFTR and a halide-sensitive variant of yellow fluorescent protein (YFP) that functions as an indicator of anionic channel function (Van Goor et al., 2009). While displaying unimpressive potency ($EC_{50} \approx 2$ μM), this lead was attractive because it showed higher *in vitro* efficacy than a benchmark investigational CFTR potentiator (genistein), and it was able to potentiate both G551D- and temperature-corrected F508del-CFTR currents from human bronchial epithelial cells (Hadida et al., 2014). A 48-step optimization process significantly improved the potency (EC_{50} to 20 nM) and the pharmacokinetic properties of the drug (Hadida et al., 2014). In phase 3 clinical trials, the drug met its primary endpoint goals, improving lung function >10% on

average in G551D patients (Accurso et al., 2010), leading to its initial approval for G551D patients over the age of 6. Subsequent studies have demonstrated that, at least in G551D patients, the increase in lung function upon taking Ivacaftor appears to be stable over time (> 3 years) (McKone et al., 2014).

Follow-up clinical studies of patients receiving Ivacaftor have shown, perhaps more powerfully than ever before, the broad physiological consequences of CFTR function and dysfunction. For example, in addition to increases in lung function and decreases in bacterial infection (Accurso et al., 2010), treatment of G551D patients with Ivacaftor thus far has been associated with increased nutrient absorption and weight gain (Accurso et al., 2010; Borowitz et al., 2016), improved insulin secretion and glucose tolerance (i.e. improved pancreatic beta cell function (Bellin et al., 2013; Tsabari et al., 2015)), normalization of gastrointestinal pH (Rowe et al., 2014), and reversal of chronic sinusitis (a symptom resulting from CFTR dysfunction in the nasal epithelia) (Chang et al., 2015) (Figure 1.1, asterisked effects). To the ion channel investigator, it is inspiring to see the many ways in which one drug has validated years of effort into understanding the molecular basis for CF disease.

How does Ivacaftor work? The drug potentiates, rather than activates, CFTR (Van Goor et al., 2009); *i.e.,* the drug does not increase the concentration of cAMP in cells to activate PKA and phosphorylate the channel (Pyle et al., 2011). At the single channel level, the drug increases the frequency and duration of channel openings, rather than increasing the unitary conductance of the channel (Van Goor et al., 2009).

An *in vitro* study using purified and reconstituted G551D-CFTR protein generated data suggesting that Ivacaftor is able to open CFTR in the absence of ATP (Eckford et al., 2012), which has led to the hypothesis that the drug opens CFTR via an ATP-independent mechanism. However, the plausibility of this mechanism to explain the *physiological* action of this drug has been challenged by a recent study from our group showing that the drug is unable to potentiate phosphorylated CFTR in excised patches wherein ATP has been thoroughly washed out of the recording chamber (Cui and McCarty, 2015).

And while it has been reasonably well established that Ivacaftor can bind CFTR directly (Eckford et al., 2012), the mechanism of this drug is not well understood at the structural level—its binding site on the protein is not known. In this regard, we know certain useful things; for example, *in vitro,* Ivacaftor potentiates WT-CFTR and CFTR variants harboring mutations in diverse locations (Van Goor et al., 2014). Therefore, while the drug was discovered via screening of one CFTR particular mutant (dF508-CFTR), this mutation does not confer upon CFTR a binding site for Ivacaftor. We have also recently observed that VX770 potentiates non-human CFTR orthologs including murine CFTR (Cui and McCarty, 2015) and *Xenopus* CFTR (*Cui, et al., Submitted 2016*). In the work described herein, I take another step towards understanding the structural determinants of the binding and activity of Ivacaftor by testing the effect of the drug on a version of the protein lacking the CFTR-specific R-domain important for channel regulation (Section 3.3), and in the discussion I relate these and other results from our group in the context of a hypotheses of the drug's mechanism of action.

1.5 *CFTR: the evolution of structure and function*

CFTR, the protein genetically linked to the devastating disease Cystic Fibrosis, is the only member of the ATP-Binding Cassette Transporter superfamily so far demonstrated to bear ion channel activity. Thanks to 25 years of intensive biophysical study, many structural features important to CFTR's channel function have been identified, but the mechanisms by which CFTR uniquely evolved this function remain poorly understood. To lay groundwork to understanding these mechanisms, here I compare the sequence of CFTR to related ABC transporters at different evolutionary distances in the context of recently published functional data, to identify determinants of both conservation and divergence in the structure and function of these proteins. The analyses demonstrate several ways in which the ABCC subfamily of ABC transporters provided a unique opportunity for emergence of a chloride channel. They also allow me to propose the molecular mechanisms and chronological order by which CFTR evolved the structural features necessary for regulated channel activity.

1.5.1 CFTR evolved from a family of efflux transporters. CFTR is located in the C subfamily of the ABC transporter superfamily of proteins (ABCC7) (Jordan et al., 2008; Stratford et al., 2007). ABC transporters are transmembrane proteins that use the energy of ATP binding and hydrolysis to accomplish the active import or export of various substrates across membranes (Rees et al., 2009). Indeed, CFTR also bears ATPase activity (Li et al., 1996; Stratford et al., 2007); but despite this similarity, biophysical methods have firmly demonstrated that CFTR uniquely 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 ABCC relatives described here function as multi-specific exporters. There is some evidence that CFTR may directly mediate flux of glutathione (Kogan et al., 2003; Linsdell and Hanrahan, 1998), a transport substrate of close ABCC relatives MRP1 (ABCC1) (Mao et al., 1999) and MRP4 (ABCC4) (Ritter et al., 2005). However, in CFTR, the continuous flux of glutathione is not dependent ATP hydrolysis (Kogan et al., 2003), so it does not appear that this permeability mirrors ATP hydrolysis-powered transport function *per se*. Instead, glutathione may be conducted through the promiscuous channel pore of CFTR, which conducts not only halides but also bicarbonate (Choi et al., 2001; Ko et al., 2002) and gold dicyanate (Serrano et al., 2006).

In general, ABC exporters have proven resistant to crystallization. However, several structures of efflux transporters have been solved in recent years, including several representatives of the nucleotide-free "inward-facing" conformation (Hohl et al., 2012; Lee et al., 2014; Li et al., 2014; Ward et al., 2007) and the nucleotide-bound "outward-facing" conformation (Choudhury et al., 2014; Dawson and Locher, 2006; Dawson and Locher, 2007; Ward et al., 2007). The modular subunit layout found in these ABC-protein structures matches that predicted for CFTR, and experimental studies have demonstrated that CFTR contains inter-subunit connections analogous to those in other ABC proteins that are required for folding and function (He et al., 2008; Serohijos et al., 2008). Helical packing also appears analogous, in as much as the helices lining the

pore of CFTR also appear to line the substrate pathway in a crystal structure of the bacterial transporter Sav1866 (Norimatsu et al., 2012a; Norimatsu et al., 2012b).

A high resolution structure of CFTR (or of any other ABCC) remains elusive. Therefore, many groups have applied homology modeling and molecular dynamics simulations to generate structural hypotheses (Belmonte and Moran, 2015; Corradi et al., 2015; Dalton et al., 2012; Norimatsu et al., 2012a; Rahman et al., 2013; Serohijos et al., 2008). But homology models are only as relevant as the templates used are similar to the target molecule; this is especially complicated for CFTR, which functions as a channel, whereas even its closest relatives function as transporters. Moreover, CFTR contains entire stretches of amino acid sequence important for channel phospho-regulation (Cheng et al., 1991; Csanady et al., 2005a; Lewis et al., 2004; Rich et al., 1993) that are not found in other ABC proteins (Sebastian et al., 2013) (see further on this topic below).

Nevertheless, several structural features of outward-facing ABC transporter crystal structures (Dawson and Locher, 2006; Dawson and Locher, 2007; Ward et al., 2007) have been verified experimentally in the extensively-studied open state of CFTR (Figure 1.5). First (Figure 1.5, magenta boxes), it has been shown via cysteine cross-linking experiments that the open state of CFTR entails the tight, head-to-tail dimerization between NBD1 and NBD2 (Mense et al., 2006), perhaps involving an inter-subunit hydrogen bond between R555 and T1246 (Vergani et al., 2005). Second (Figure 1.5, orange boxes), the open pore has been associated with the close proximity of ICL2

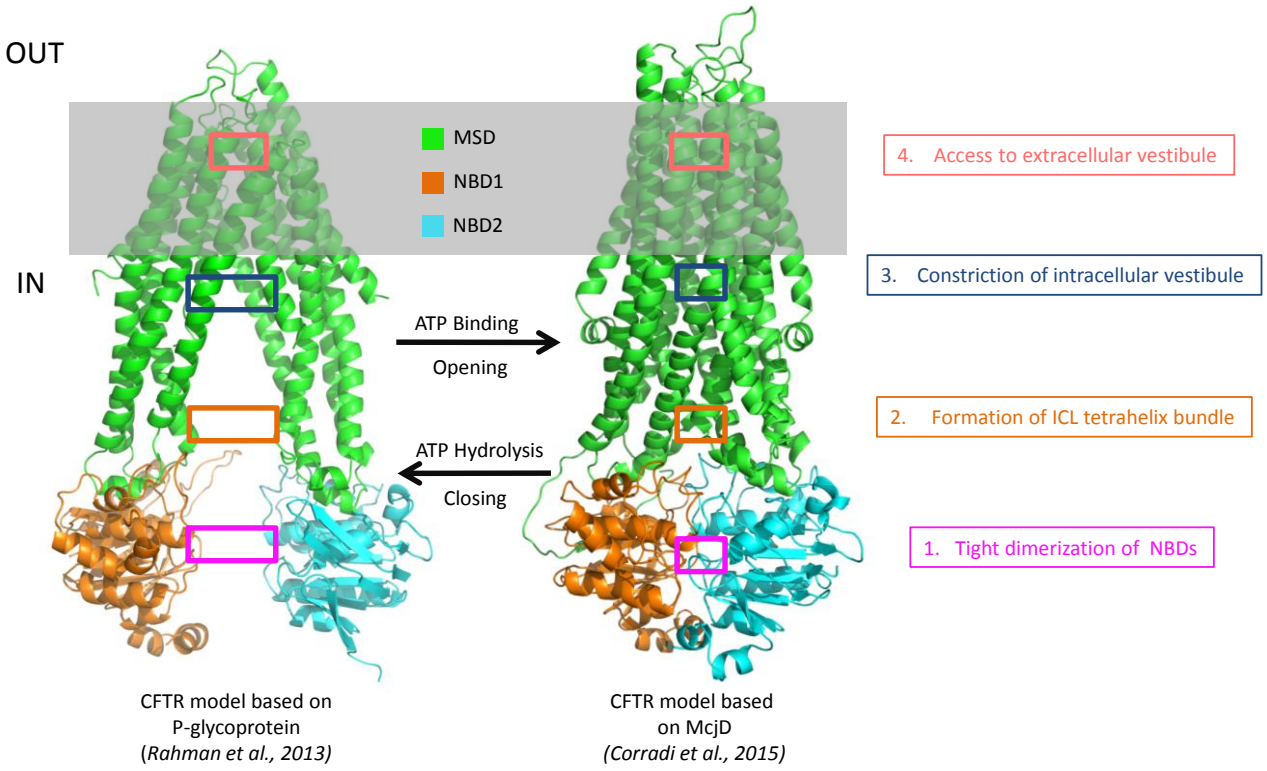


Figure 1.5 Conformational changes shared between gating in CFTR and transport in ABC Transporters. The inward-facing (closed) CFTR model is based on the ATP-free mouse P-glycoprotein structure (Li et al., 2014; Rahman et al., 2013), and the outward-facing (open) CFTR model is based on the substrate-occluded structure of McjD (Choudhury et al., 2014; Corradi et al., 2015). Four loci of conformational change are boxed, with relevant studies cited in the text. Images generated with PyMol 0.99.

and ICL4 (Wang et al., 2014b), at a region that forms an intracellular tetrahelix bundle critical to function of ABC transporters (Doshi et al., 2013). Third (Figure 1.5, dark blue boxes), it has been shown that access to the intracellular vestibule of CFTR by large (> 13 Å diameter) probes is slowed in the open state relative to the closed state (Bai et al., 2011), suggesting that channel opening entails constriction of the intracellular vestibule. Conversely, it has been shown that access to pore-lining residues in the extracellular vestibule (such as T338) is faster in the open state than in the closed state, suggesting that channel opening involves at least some degree of dilation in this region (Figure 1.5, salmon boxes) (Beck et al., 2008; Norimatsu et al., 2012b). Crystal structures from related transporters have therefore proven to be valuable guides to understanding the structure and function of CFTR.

Motivated by these recent studies, here I have performed sequence comparisons among CFTR and well-aligning ABC proteins of increasing phylogenetic distance in light of relevant biophysical data. For the purpose of this analysis, I have grouped together several orthologs of CFTR which have been functionally characterized (human, mouse, chicken, frog, and shark) as “jawed vertebrate consensus” CFTR. This consensus CFTR is aligned and compared to a recently published sequence of the distant CFTR ortholog from Sea lamprey (Ren et al., 2015) and to sequences of transporter homologs including ABCC4 (Ritter et al., 2005), ABCC5 (Jansen et al., 2015), ABCC1 (Cole, 2014), and ABCB1 (P-glycoprotein) (Kartner et al., 1983) (Figure 1.6). To view these alignments in their entirety, please refer to Appendix B. Analysis is divided into four sections that

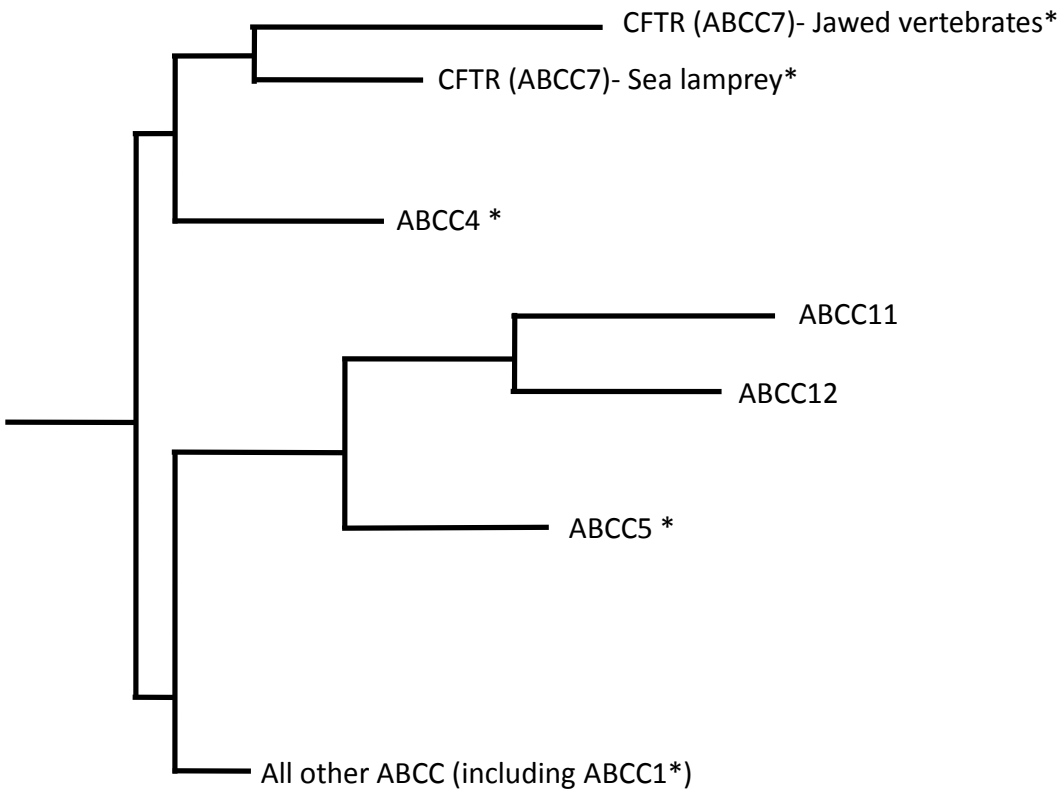


Figure 1.6: ABCC subfamily dendrogram. Adapted from two previous studies on CFTR evolution (Jordan et al., 2008; Sebastian et al., 2013). Proteins discussed in this section are indicated with an asterisk.

correspond to regions of CFTR that mediate the protein's catalytic activity, inter-domain energetic signaling, actuation of function, and phospho-regulation.

1.5.2 A degenerate ATP binding site in the NBDs of CFTR shows evidence of unique functional divergence. In ABC transporters, ATP binds at two sites (ABS 1 and 2) that incorporate several conserved motifs from nucleotide-binding domains (NBDs) positioned in a head-to-tail arrangement (Smith et al., 2002). The top of Figure 1.7 depicts a simplified model of these sites wherein each ABS is shown to consist of the so-termed Walker A, Walker B, and Histidine 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 transmembrane domains (TMDs); in ABC exporters this flips the TMD conformation from inward- to outward-facing (Rees et al., 2009). Conversely, ATP hydrolysis at these sites is proposed to lead to dissociation of the NBD dimer, which allows re-adoption of the inward-facing conformation to bind new intracellular substrates (Zoghbi et al., 2012), although it is important to note that there is significant disagreement as to the *degree* of dissociation undergone at the NBDs to accomplish this (George and Jones, 2012; Hohl et al., 2014; Puljung, 2015).

Many ABC transporters feature homodimeric NBDs that together form two ABS sites with equivalent functions. However, the ABCCs contain significant divergence in one site (ABS1) in the functional motifs introduced above (Gadsby et al., 2006). A sequence alignment of these motifs demonstrates major points of divergence in ABCCs as compared to P-glycoprotein, an ABCB subfamily member (ABCB1) with homodimeric

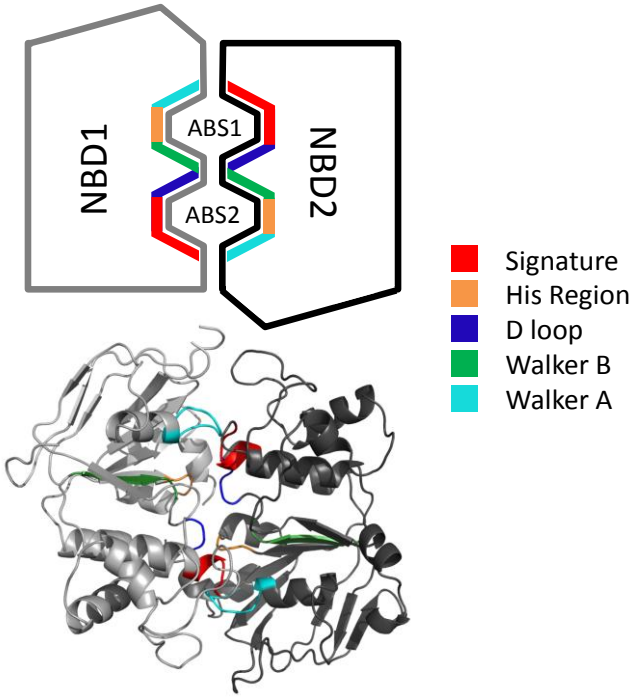


Figure 1.7: Schematic (top) and homology model (below)(Corradi et al., 2015) of the CFTR NBD dimer with regions of NBDs that comprise ATP binding sites (ABS 1 and 2) highlighted. Homology model image generated via PyMol 0.99.

NBDs (Figure 1.8). Note that the ABCC family shows divergence adjacent to the NBD1 Walker B loop that is integral to ABS1. At the asterisked position in the alignment in Figure 1.8, a critical catalytic glutamate conserved in canonical ABS sites (Orelle et al., 2003) is substituted in most ABCCs with an aspartate, and the following alanine is substituted with a proline (Payen et al., 2003). In ABCC1, these two substitutions may be responsible for increased ATP affinity to and significantly slowed ATP hydrolysis at ABS1 (the so-called "incompetent" site) as compared to 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 seen in ABCB1 (Figure 1.8). The functional consequence of this valine substitution is not known, but it would be expected to distort the ATP binding pocket since the canonical glycine at this position is predicted to directly interact with the γ-phosphate of ATP (Smith et al., 2002).

In human CFTR, ATP hydrolysis at ABS1 is essentially absent (Aleksandrov et al., 2002; Basso et al., 2003). Interestingly, additional, lineage-specific divergence exists in this region. In NBD1, instead of the conservative ABCC aspartate substitution for the catalytic glutamate adjacent to the Walker B region (Figure 1.8, asterisked position), all CFTRs have a serine residue (**S**573). In addition, a critical catalytic histidine (Kloch et al., 2010) in the nearby His region is uniquely substituted with a serine (**S**605) in all CFTRs. The NBD2 Signature sequence integral to ABS1 of CFTR is also unique among ABCCs. In CFTR from jawed vertebrates, the sequence of this motif is LS**H**G**H**—more divergent from consensus than ABCC homologs in its substitution of the C-terminal glutamine that

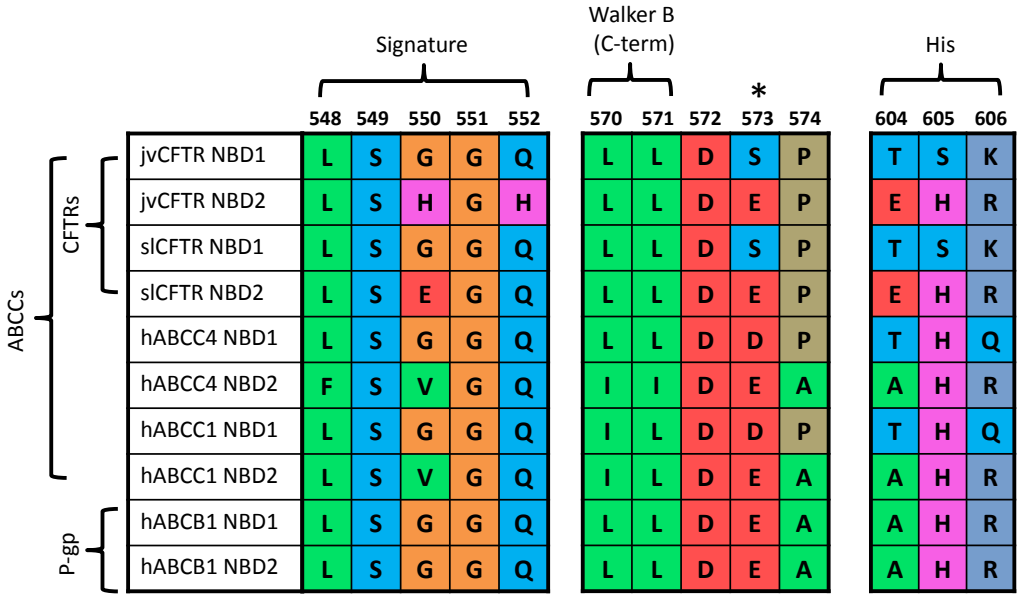


Figure 1.8 Multiple sequence alignment of catalytic regions of ABC transporter NBDs. Residue numbering along the top corresponds to NBD1 of human CFTR. JvCFTR is consensus jawed vertebrate CFTR. slCFTR is sea lamprey CFTR.

interacts directly with the ribose moiety of ATP bound in canonical ABSs (Smith et al., 2002). Interestingly, uniquely among CFTRs, the NBD2 Signature sequence from the sea lamprey ortholog retains this canonical glutamine (LSEG**Q**). Considering the early divergence of this ortholog (Figure 1.6), this discrepancy gives insight into the timing and order of evolutionary changes in ABS1 that ultimately led to the composition found in the human channel.

As of this writing, we know very little about the role that degeneration in ABS1 plays in the context of the overall function of ABCC proteins, and still less how additional divergence that is found to be conserved within CFTR orthologs may contribute to any unique function(s). The absolute ATP turnover rate for human CFTR is 0.5-1/s, as measured by both biochemistry and electrophysiology (Li et al., 1996). This hydrolysis rate, which governs channel closure (Gunderson and Kopito, 1995), is 10 to 20-fold slower than the substrate-induced ATP turnover achieved by ABC exporters with two competent ATPase sites, such as P-glycoprotein (Senior et al., 1998; Sharom et al., 1995; Urbatsch et al., 1994). Comparing the above ATPase rates to those of the *transporter* ABCCs is more difficult: wide ranges are reported in the literature, but they generally fall between those of CFTR and P-glycoprotein (Bakos et al., 2000; Hagmann et al., 1999; Malofeeva et al., 2012; Sauna et al., 2004). Overall, it seems reasonable to implicate the degeneration in ABS1 and the overall slow ATP hydrolysis of CFTR in the long (~0.6 s), stable openings that characterize the gating behavior of the channel. Whether the unique composition of the CFTR ABS1 is necessary for this gating behavior (either

through direct effects on ABS1 or cross talk to the competent ABS2) is a question that necessitates future study.

There is also some evidence that the conformational changes driven by ATP binding differ between transporters bearing heterodimeric *vs.* homodimeric NBDs. One recent spectroscopic study observed that binding of a non-hydrolyzable ATP analog AMP-PNP to the heterodimeric exporter BmrCD elicited partial NBD closure and minimal movement in the TMDs. ADP + vanadate (which models post-hydrolytic ATP) elicited tighter dimerization of the NBDs and conformational changes in the TMDs consistent with an outward-facing state (Mishra et al., 2014). Similar results were reported in a study of the heterodimeric protein complex TM287-288 (Hohl et al., 2014). This is in contrast to transporters with homodimeric NBDs, wherein both the formation of a tight NBD dimer and the transition to outward-facing TMDs are accomplished by AMP-PNP and thus without ATP hydrolysis (Dawson and Locher, 2007; Ward et al., 2007).

However, these results are somewhat difficult to relate to CFTR, considering that ATP hydrolysis is not required to reach the open state of the channel, which structurally resembles AMP-PNP-bound, outward-facing transporter structures (Figure 1.5). In fact, abolishing ATP hydrolysis (for example through mutation of catalytic residues in the competent ABS2) leads to channels "trapped" open in long bursts (Gunderson and Kopito, 1995; Vergani et al., 2005). In this regard, it is worth mentioning that AMP-PNP, used to mimic ATP in many structural studies, is unable to open CFTR on its own (Gunderson and Kopito, 1994), but instead must be applied concurrently with ATP. This nucleotide mixture locks CFTR open (Baukrowitz et al., 1994; Gunderson and Kopito,

1994) presumably as a result of AMP-PNP binding to the competent ABS2 site and ATP binding to the incompetent ABS1 site (Hwang et al., 1994). The simplest explanation of this observation is that AMP-PNP does not bind to the degenerate ABS1 site in CFTR in a manner native enough to fully replicate the binding and tight NBD dimerization accomplished by physiological ATP. If so, the spectroscopic and structural studies using nucleotide analogs as ATP mimics should be interpreted with caution, at least with respect to CFTR. Moreover, the difference in the effect of ATP *vs.* AMP-PNP in CFTR but not in homodimeric transporters supports the idea that divergence in ABS1 may have an important structural consequence, even if neither modeling (Huang et al., 2009) nor functional studies (Chaves and Gadsby, 2015; Mense et al., 2006) have yet to note a large difference in the arrangement of the ATP-bound NBD1/2 heterodimer of CFTR as compared to that of other ABC proteins.

This being said, it is quite possible that the TMDs of the pre-hydrolytic and post-hydrolytic states of ABC transporters are in fact conformationally distinct. Indeed, when recorded in lipid bilayers, WT-CFTR displays a slight increase in single-channel conductance in parts of the open burst associated with a post-hydrolyzed state of ATP (Gunderson and Kopito, 1995). This behavior can also be observed in certain CFTR mutants wherein transitions between conformational substates are slowed (Cui et al., 2008; Zhang et al., 2005).

In terms of the conformational changes induced by ATP hydrolysis, data from a recent study strongly suggests that the NBDs of CFTR fully dissociate upon ATP hydrolysis (Chaves and Gadsby, 2015), as proposed for homodimeric transporters (Zou

et al., 2009). This dissociation may be initiated by hydrolysis at the hydrolysis competent site, as suggested by spectroscopic studies done with nucleotide binding domain fragments from other heterodimeric ABC proteins (Zoghbi et al., 2012). Future structural studies directly probing NBD dynamics in whole heterodimeric transporters (ideally using several different means of controlling state) may therefore identify interesting differences in conformational dynamics intrinsic to the function of homodimeric transporters, heterodimeric transporters, and CFTR.

1.5.3 Regions mediating inter-subunit energetic signaling are conserved in ABCCs. Both CFTR channel gating and ABC transport activity involve harnessing the conformational energy of ATP-driven NBD dimerization to actuate a function in the TMDs. Therefore, CFTR and related transporters have a similar need to energetically (and therefore physically) connect these domains by way of inter-subunit interactions. Hence, it is not surprising that, despite their functional divergence, CFTR and its closest transporter homolog ABCC4 share high identity and similarity in most of the intracellular loops (ICLs) understood to mediate this connection (Table 1.2). Intuitively, when NBD-ICL interactions are disrupted in CFTR through mutation (including through the common CF-disease mutation F508del), folding abnormalities result that adversely affect protein trafficking (Cheng et al., 1990; Cotten et al., 1996; Serohijos et al., 2008). Functionally, disruption of NBD-ICL interactions via interfering peptides or mutations predominantly affects the opening rate of CFTR; *i.e.*, the frequency of the conformational transition in the TMDs that gates the pore (Cotten et al., 1996; Ehrhardt et al., 2015; He et al., 2008; Serohijos et al., 2008). Considering the structural evidence closely relating CFTR channel

Table 1.2: Sequence comparison in intracellular loops (ICLs) of CFTR and ABCC4 (residue numbers correspond to hCFTR)

	ICL1 (148-192)	ICL2 (241-306)	ICL3 (931-990)	ICL4 (1030-1099)
Identity	50%	24%	42%	50%
Similarity	86%	67%	76%	76%

opening to the inward-to-outward transition undergone in ABC exporter function (Figure 1.5), one might regard the opening rate of CFTR as a transporter-analogous process: one dependent on relaying the movement from NBD dimerization to the actuation domain located in the TMDs. As such, it is perhaps not surprising that these evolutionarily conserved regions mediate transporter-analogous roles, as opposed to channel-specific roles in anion conduction or pore stability.

Strikingly, high conservation in energetic signaling exists between CFTR and other ABC transporters in several residues along the longitudinal axis of the TMDs where mutations increase the unliganded (ATP-independent) opening rate of CFTR. These residues (F337, P355, K190, and K978 (Wang et al., 2010; Wei et al., 2014; Wei et al., 2015)) are proposed to contribute to setting the energy barrier to channel opening, and are all biochemically conserved with ABCC4 and ABCC5. Although these residues have not yet been studied in mammalian ABCCs, mutations of conserved equipositional residues have been shown to rescue function of ATP-insensitive mutants of the yeast multidrug resistance protein Yor1p, suggesting that residues integral to energetic signaling in CFTR may play similar roles in *bona fide* transporters (Wang et al., 2014b; Wei et al., 2014; Wei et al., 2015). However, not all residues and motifs involved in this process are so tidily conserved between CFTR and related transporters. A notable exception is the recently described ICL2-ICL4 electrostatic pair E267-K1060 in human CFTR (Wang et al., 2014b). There is compelling evidence that breakage of this electrostatic interaction increases the energetic input required to open the channel, yet this pair (which is conserved in all CFTRs) is absent in other ABCCs. Interestingly

enough, the E267-K1060 interaction was identified on the basis of an equipositional electrostatic pair that mediates intracellular tetrahelix bundle formation in the bacterial ABC exporter MsbA (Doshi et al., 2013). Neutral, polar residues compatible with hydrogen bonding are found in the same positions in other ABCCs, and these residues may interact, as observed in the crystal structure of the ABC exporter Sav1866 (Dawson and Locher, 2007)). But even if they do, one wonders why CFTR evolved an important interaction to be chemically different from its closest relatives, but concordant with that found in distantly related ABC exporters such as MsbA.

1.5.4 Transmembrane pathways in ABCCs employ conserved and divergent residues towards distinct functions. The transmembrane pathways mediating functions in CFTR and its relatives mediate unmistakably different functional roles. Conceptually, the divergence in these pathways most directly targets the principal difference between channels and transporters: only channels contain a pore that allows uninterrupted permeation across the plasma membrane (Gadsby, 2009). With this in mind, what degree of conservation is found between CFTR and *bona fide* transporter relatives in residues in the TMDs confirmed to be relevant to channel function in CFTR?

Significant effort has been expended to 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). Recently, several groups have employed the substituted cysteine accessibility method (SCAM) to this end. 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). Figure 1.9 shows a model of CFTR (Corradi et al., 2015) based on the ABC transporter McjD (Choudhury et al., 2014), wherein I colored residues shown by this 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., 2015; Fatehi and Linsdell, 2009; Gao et al., 2013; Liu et al., 2004; Norimatsu et al., 2012a; Norimatsu et al., 2012b; Qian et al., 2011; Rubaiy and Linsdell, 2015; Serrano et al., 2006; Wang et al., 2011a; Wang et al., 2014a; Zhang and Hwang, 2015; Zhou et al., 2008) according to conservation between CFTR and the organic anion transporter ABCC4, the closest transporter relative to CFTR (dark blue = conserved, gray = similar, magenta = divergent). To allow visualization of the permeation pathway, I have removed some helical regions which have not been shown thus far to be pore-lining, including parts of TM helices 2, 7 and 11. In the process of going through the channel to exit the cell, the chloride ion first encounters pore-lining residues on the ICLs (Figure 1.9, black arrows). These residues are predominantly basic (including K190, R248, R303, K370, R1030, K1041, and R1048), and are proposed to play roles in attracting chloride ions into the pore, since charge-eliminating mutations reduce single channel conductance (Aubin and Linsdell, 2006; El Hiani and Linsdell, 2015; Zhou et al., 2008). Some homology models suggest that a subset of these residues may comprise an intracellular “lateral portal” for chloride entry into the pore (Corradi et al., 2015; Mornon et al., 2015).

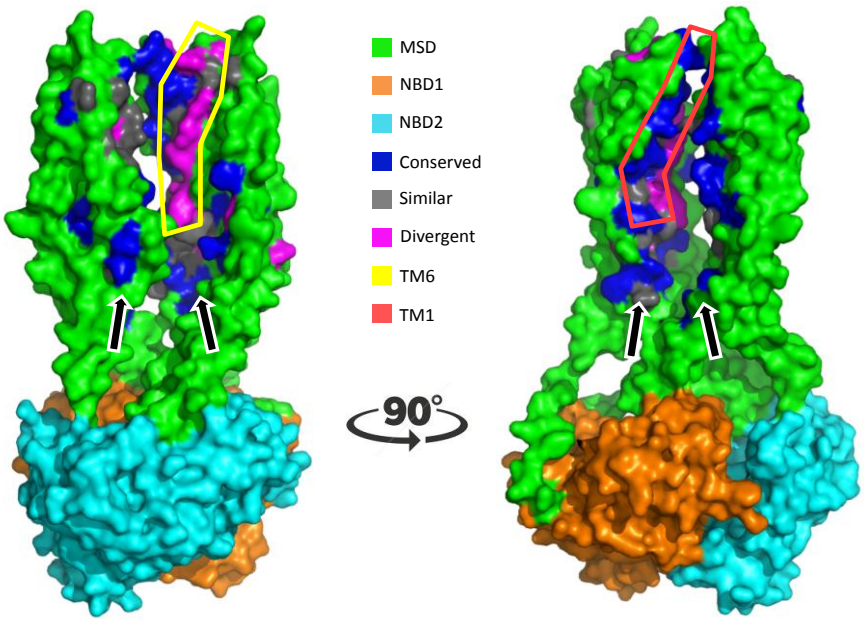


Figure 1.9. Conservation with ABCC4 in residues lining the CFTR channel pore. A) CFTR homology model based on McjD (Corradi et al., 2015), with sections of non-pore lining helices removed in order to visualize the chloride ion permeation pathway. Dark blue residues = identical in consensus mammalian ABCC4, gray residues = biochemically similar, and magenta = biochemically divergent. The highly divergent pore-lining TM6 is circled in yellow, while the highly conserved pore-lining TM1 is circled in red. Black arrows indicate residues highly conserved and proposed to attract chloride ions to the intracellular mouth of the CFTR pore.

Considering that they mediate anion conduction, it is initially surprising that this group of residues is very highly conserved in transporter ABCCs: all 7 residues listed above are basic in ABCC4 and most (5/7) are basic in ABCC5. To our knowledge, the effect of mutations at these positions on the function of ABCC4 or 5 has not been directly tested. However, functional studies of Multidrug Resistance Protein 1 (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), which are transported by the majority of ABCCs, including ABCC4 and 5 (Jansen et al., 2015; Ritter et al., 2005). ABCC1 does not align particularly well to CFTR, ABCC4 or ABCC5. However, at least one region in ICL4 is highly conserved across ABCCs (Figure 1.10). In this alignment, the pore-lining and chloride-attracting K1041 of CFTR (El Hiani and Linsdell, 2015) aligns with K1141 of ABCC1, a residue whose positive charge plays an important role in the binding of organic anionic substrates (Conseil et al., 2006). These data intrigue us because they suggest that one mechanism by which CFTR evolved chloride channel activity was to appropriate residues already functionally important in transport of anionic substrates towards the novel function of conducting anions. In support of this, it has been shown that several substrates of ABCC transporters inhibit CFTR by blocking the pore from the intracellular side (Linsdell and Hanrahan, 1999). Might these residues contribute to a vestigial binding site for these substrates within CFTR?

As the ion travels further up the pore, it encounters pore lining residues contributed by TM helices 1, 5, 6, 9, 11, and 12 (Alexander et al., 2009; Bai et al., 2010;

Bai et al., 2011; Gao et al., 2013; Wang et al., 2014a; Zhang and Hwang, 2015). Strikingly, the pore-lining residues of TM1, 5, 9, 11, and 12 are highly conserved between CFTR and ABCC4; consider in particular the example of TM1 (bounded in red in Figure 1.9), wherein 6 of 7 pore-lining residues in CFTR are identical in the transporter homolog. This is also the case when one considers residues wherein mutation to cysteine strongly affects single channel conductance (Figure 1.11, underlined residues). Though we do not know what role these conserved residues may play in substrate transport, we know that they are compatible with transport activity based on their conservation in ABCC4. In this regard, TM6 is the obvious outlier—it alone accounts for a majority (11/19) of all highly divergent pore-lining residues thus far identified in the TMDs of CFTR (see region bounded in yellow in Figure 1.9). It is unlikely that this perceived divergence is an artifact of poor alignment in this region; recall from above that the two TM6 residues in this region wherein mutations alter energetic signaling in CFTR (F337 and P355) are conserved with other ABCCs. TM6 is also unique in that its substituted cysteine reactivity pattern is anomalous for an alpha helix in a membrane protein; multiple reports agree that the stretch of residues from L331 to V345 is nearly uninterrupted in terms of accessibility of membrane-impermeant reagents (Figure 1.11) (Alexander et al., 2009; Bai et al., 2010). These data are generally not well accounted for in homology models of CFTR based on ABC transporter structures, since these structures are helical in this region (Corradi et al., 2015; Rahman et al., 2013). This is also in contrast to more conserved helices such as TM1 and 11, wherein reactivity

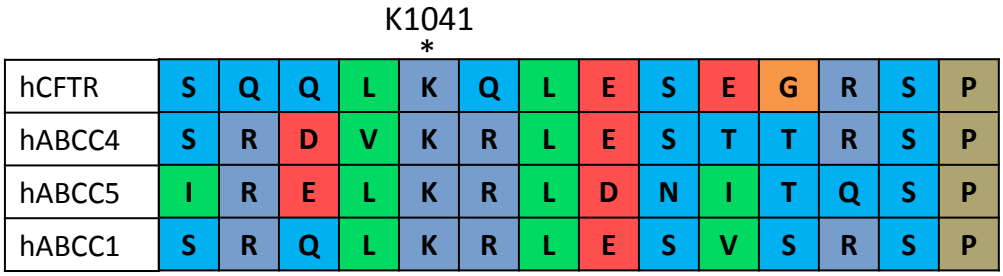


Figure 1.10 Multiple sequence alignment of ICL4, identifying the pore-lining residue K1041, which aligns to a residue responsible for anionic substrate binding in ABCC1 (K1141).

Region	Pore lining residues
TM1	92, 95, 98, 102, 106, 107, 109
ICL1	186, 188, 189, 190
TM3	191, 192, 193, 194, 195, 196, 197, 199, 200, 203, 205, 207, 211, 213, 215
ICL2	241, 243, 244, 248, 252, 299, 303
TM5	306, 307, 310, 311, 326
TM6 + ext	331, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 344, 345, 348, 349, 352, 353, 355, 356, 360, 367, 370
ICL3	986, 988, 989, 990
TM9	993, 1000, 1003, 1008, 1009, 1010
ICL4	1030, 1041, 1048
TM11	1112, 1115, 1118
TM12 + ext	1127, 1129, 1131, 1132, 1134, 1135, 1137, 1138, 1139, 1140, 1141, 1142, 1144, 1145, 1147, 1148, 1150, 1152, 1156

Figure 1.11 Conservation with ABCC4 in residues demonstrated via cysteine substitution to line the CFTR pore. Residues listed have been reported in the literature to line the CFTR pore on the basis of > 20% block of cysteine-substituted mutants by real time application of MTSES or $Au(CN)_2$. Conserved residues = blue, biochemically similar residues = black, and divergent residues = magenta. Residues wherein cysteine substitutions have been reported to alter single channel conductance are underlined.

follows a predictable helical pattern (every three or four residues; Figure 1.11). Divergence in this region of TM6, a highly discriminatory region of the CFTR pore (McCarty and Zhang, 2001), may therefore be responsible for profound differences in the conformation of this region as compared to functionally divergent ABC transporters.

Previously, in an effort to begin to identify important loci of divergence between CFTR and transporters of the ABCC family, we performed Type II divergence analysis between CFTR and ABCC4 sequences among vertebrates and mammals (Jordan et al., 2008). This analysis identifies residues maximally conserved within groups and maximally biochemically divergent between groups. Intuitively, many residues identified as Type II divergent across vertebrate sequences were previously shown to play important channel-specific roles in CFTR, such as selectivity (T338 (Linsdell et al., 1998), R352 (Guinamard and Akabas, 1999)) and electrostatic attraction of anions (R334, K335 (Smith et al., 2001)). We also found that two salt bridges 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 with ABCC4 (R347 in TM6 and D993 in TM9) and one that is Type II divergent (D924 in TM8 and R352 in TM6) (Jordan et al., 2008).

More recently, in our study on the first extracellular loop of CFTR (ECL1), we demonstrated that R117, a relatively common site of mutation in patients with mild CF (Sheppard et al., 1993), may engage in an electrostatic interaction with E1126 in ECL6 that is also critical to maintaining open pore stability and architecture (Cui et al., 2014). R117 was classified as a Type II divergent amino acid with respect to mammalian

sequences of CFTR and ABCC4 (Jordan et al., 2008), while E1126 is biochemically similar among closely related ABCCs (Figure 1.12). Here I note that in all three of these salt bridge interactions, the residue that is biochemically conserved between CFTR and ABCC4 is divergent in ABCC5 (Figure 1.12). Therefore, in each pair, the first residue emerged in a common ancestor of CFTR and ABCC4, and was fixed when the other emerged in CFTR. Notably, the R117-E1126 pair appears not to have been fixed until after the split between jawed and jawless vertebrates, since R117 is conserved within CFTR sequences from jawed vertebrates but absent in that from sea lamprey (Figure 1.12).

1.5.5 CFTR incrementally evolved tight regulation by phosphorylation of its R-domain. Phosphorylation is a form of post-translational modification that is common across cell types and important to a wide range of cellular processes. CFTR is activated by PKA-mediated phosphorylation at consensus sites in the intracellular regulatory "R" domain coded between NBD1 and TMD2 (Hunt et al., 2013). The structural mechanism for phosphorylation-mediated regulation of CFTR is still not completely understood, but evidently involves dynamic, phospho-sensitive interactions between R domain helices and nearby domains of CFTR, including NBD1 and NBD2 (Baker et al., 2007; Bozoky et al., 2013b). Interestingly, although the fully dephosphorylated R domain absolutely precludes channel opening by ATP binding (Rich et al., 1991), biophysical studies strongly suggest that channel activity depends on the degree of PKA-mediated phosphorylation, and that consensus sites play specific roles in "graded" activation of

	R347-D924		R352-D993		R117-E1126	
Charge	+	-	+	-	+	-
Jawed vertebrate CFTR	R	D	R	D	R	E
Sea lamprey CFTR	R	D	R	D	V	E
ABCC4	R	A	L	D	L	D
ABCC5	T	A	V	M	L	P

Figure 1.12: Conservation in residue pairs that maintain CFTR open pore architecture. jvCFTR represents the consensus sequences in CFTR from jawed vertebrates, while slCFTR represents CFTR from sea lamprey.

the channel (Csanady et al., 2005a; Csanady 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, 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, a similar location to the R domain in CFTR (Stolarczyk et al., 2011). However, one should be careful not to analogize these regions too closely to the CFTR R-domain for two reasons. First, there is no clear evidence that mutation or phosphorylation of this region significantly affects the function of transporters, as it profoundly does in CFTR (Stolarczyk et al., 2011). Second, the functionally-relevant PKA consensus sites in the CFTR R-domain are located in an approximately 200 amino acid region that is completely absent in other ABC transporters (including other ABCCs) (Sebastian et al., 2013). This region apparently arose as a result of a lineage-specific intron-to-exon transition in the CFTR gene (Sebastian et al., 2013).

The unique functional phospho-regulation 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 ATPase activity. By contrast, CFTR always

has access to chloride. Since ATP is present in the cell at concentrations well above the EC_{50} for channel opening (Csanady et al., 2000), without some other means of regulation, CFTR would allow uninterrupted chloride flow 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).

The overall sequence of the R domain is poorly conserved across CFTRs, but the PKA consensus sites shown to be functionally relevant in human CFTR are highly conserved across jawed vertebrate orthologs (Sebastian et al., 2013). However, three of nine sites are missing in sea lamprey CFTR, and two more are altered and thus do not match consensus (Figure 1.13). Since several of the sites that are present in sea lamprey CFTR have been shown by themselves to be capable of mediating some degree of phospho-regulation in human CFTR (Hegedus et al., 2009), it is unlikely that sea lamprey CFTR is completely unregulated. Instead, the additional sites may have evolved in jawed vertebrate CFTR as a means of fine tuning the graded PKA-mediated activation inherent to human CFTR.

1.5.6 Conclusions How did CFTR evolve its indispensible channel function? The analyses herein demonstrate that many of the amino acid residues and motifs that bestow on CFTR its overall architecture, inter-subunit energetic signaling, impaired ATPase activity, anion conductance, and phospho-regulation were already present to

PKA Site	S660	S670	S700	S712	S737	S753	S768	S795	S813
Human CFTR	RRNSI	HRFSL	RKNSI	RKFSI	RRLSL	PRISV	RRQSV	RKVSL	RRLSQ
Mouse CFTR	RRSSI	RRFSV	RKNSI	RKISI	KRLSL	PRSNM	RRQSV	RKISL	RRLSQ
Chicken CFTR	RRNSI	RRFSF	RKNSI	RKSLI	RRISL	PRSNM	RRQSV	RKMSV	RRLSR
Xenopus CFTR	RRNSI	RRCSI	RKSSI	RKFSL	RKLSL	PRSNF	RRQSV	RKMSV	RRLSQ
Dogfish CFTR	RRNSI	RRCSV	RKSSL	KKFSL	RHFSL	PRSNI	RRQSV	RKMSM	RRLSE
Lamprey CFTR	RRGSM	RRGST	PQGTQ	QRFSV	RRLSL	PAAGA	RRKSV	HAPHG	PRKSL

Figure 1.13. Lamprey CFTR uniquely lacks three PKA consensus sites that are functionally relevant to the regulation of human CFTR. Amino acids highlighted in red show deviance from the PKA consensus site R(R/K/H)XSΦ, where Φ is a hydrophobic residue.

different degrees in functionally divergent ancestors. Because of this, it is possible to compare the sequence of CFTR to that of increasingly distant homologs, infer what features are common, and, for the first time, describe the molecular evolution of CFTR function in a chronological order (Figure 1.14). From such analysis, I suggest that residues underpinning inter-subunit energetic signaling, partial degeneration of the ATPase activity in ABS1, and intracellular basic residues critical to future CFTR chloride channel activity were present in a common ancestor of the ABCC family (Figure 1.14, point 1). 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 chloride channel pore of CFTR emerged, possibly in use to bind and transport anionic substrates (point 2). Critical channel-specific residues in TM6, pore-stabilizing salt bridges, and some degree of phospho-regulation were present in a common CFTR ancestor (point 3) and fixed in all descendants. Fine tuning of channel regulation and pore architecture continued after the ancient split between jawless and jawed vertebrate CFTR (point 4), but was functionally consolidated before significant additional speciation in jawed vertebrates.

As highlighted in this section, there are many questions that have yet to be answered with respect to the structure-function relationships in CFTR and related true transporters. To the willing and able, many of these questions can be answered through the biochemical and electrophysiological study of revertant mutants between groups. The results of these studies have the potential to shed light on the structures of both channel and non-channel ABC proteins and may also reveal channel-specific features in

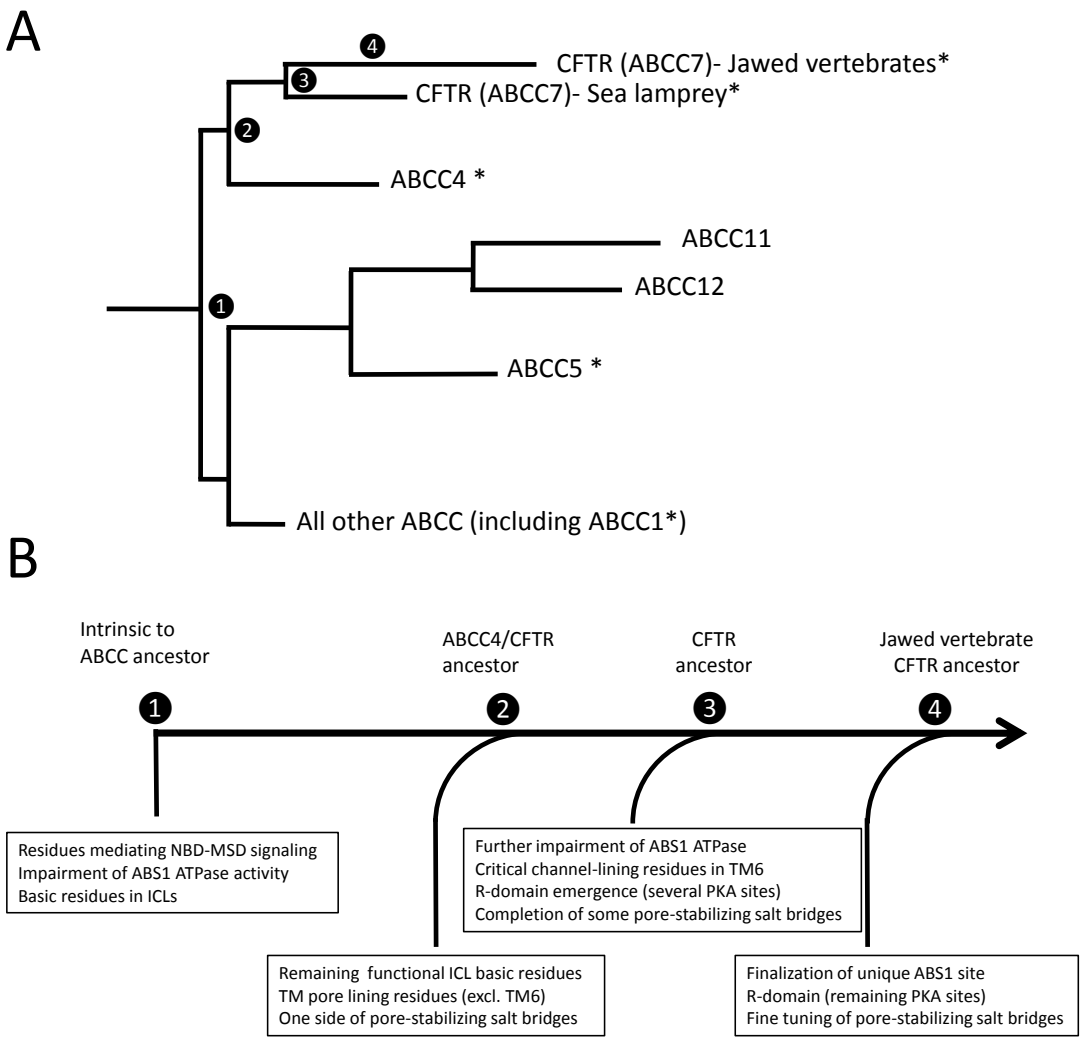


Figure 1.14 Chronology of molecular evolution of CFTR function. A) Dendrogram from Figure 1.6 adapted from two previous studies on CFTR evolution (Jordan et al., 2008; Sebastian et al., 2013). B) Chronology of emergence of functional features of jawed vertebrate CFTR, as supported by the analyses in this review. Ancestors labeled with circled numbers correspond to the dendrogram points in A.

CFTR that may serve as levers for pharmacological repair of mutant channels in CF patients.

1.6 Statement of research questions

In the results chapter, I state the relevant experimental questions pertaining to each experimental section in the context of specific rationale. But since all of the necessary concepts that intersect with these questions have been introduced by this juncture of the Introduction, I believe it helpful to briefly state (as a group) the main questions that are asked via experimentation in this dissertation.

First, as a way to learn more about the structure and evolution of CFTR, I asked if CFTR channel opening entails relative separation between two extracellular loops (ECL1 and ECL4) that are predicted by crystal structures to separate in ABC transport as part of the transition between inward- and outward-facing TMDs. The second question, which follows directly from the results from the first, was to ask whether and how inhibition of CFTR via conformational restriction of ECL1 may affect the modulation of the channel by phosphorylation of the R domain. Finally, the data above as well as findings from contemporary studies of CFTR pharmacology by our group and others, led me to ask whether and how and removal of the regulatory R domain of CFTR may affect the sensitivity of the CFTR to channel to potentiation by the FDA-approved potentiator Ivacaftor.

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CHAPTER 2: METHODS

Ion channel electrophysiology is the central method used by our group. As such, the electrophysiological techniques (and associated equipment) allowing the study of the function of ion channels at various levels of resolution were already in place for me to learn when I joined the lab. However, as is nearly always the case, the application of these techniques to answer the specific questions in this dissertation necessitated some customization. As a result, this research expanded the technical capability of our lab, particularly with respect to the quantitiative measurement of CFTR activity in whole cells during long experiments. This chapter explains the methods used to generate and analyze the data collected in this dissertation. The first section is an account of our efforts to optimize the quality of the *Xenopus laevis* oocytes that we harvest from our colony and use in electrophysiological studies. This includes a case report (submitted last fall to the animal husbandry journal *JAALAS*) detailing our experience in recognizing and eliminating an infection in the colony that was negatively impacting the quality of the oocytes. The second section describes the methodological details pertaining to the electrophysiological experiments conducted as well as how data were analyzed. In the final section, I will briefly discuss the design and construction of an experimental rig that records electrophysiogical and fluorescence data from the same ion channels simultaneously and explain how this rig has been used to generate information on the localization of CFTR in *Xenopus* oocytes.

2.1 Xenopus laevis husbandry and optimization of oocyte quality via eradication of multidrug resistant bacteria

2.1.1 Introduction: The African clawed frog (*Xenopus laevis)* oocyte expression model is used prominently by our group and many others in studies of the structure, regulation, and pharmacology of ion channels and receptors (Dascal, 1987). Many groups have periodically struggled with issues of oocyte quality with respect to their utility in electrophysiological studies. In the case detailed in this report, we addressed an issue of poor oocyte quality first by treating the isolated oocytes with the broad spectrum antibiotic ciprofloxacin (in addition to the standard treatment of penicillin and streptomycin), and subsequently by re-establishing the bacterial biofilter and *Xenopus laevis* colony. Our case serves as a valuable guide toward diagnosis and treatment of bacterial infections for those encountering issues with poor oocyte quality from their *Xenopus* colonies.

2.1.2 Case Report: All work was conducted in accordance with a protocol approved by the institutional animal care and use committee at Emory University. Our colony of between 10 and 15 frogs is housed in a 100 gallon recirculating system incorporating both a bacterial biofilter and particulate filtration (Figure 2.1). We isolate oocytes via partial oophorectomy on one frog per week using aseptic technique. Our water recipe is designed to match the general hardness and alkalinity of South African ponds of which *Xenopus laevis* are endemic, as we, like others, have observed that this recipe correlates with increased heterologous expression of proteins of interest

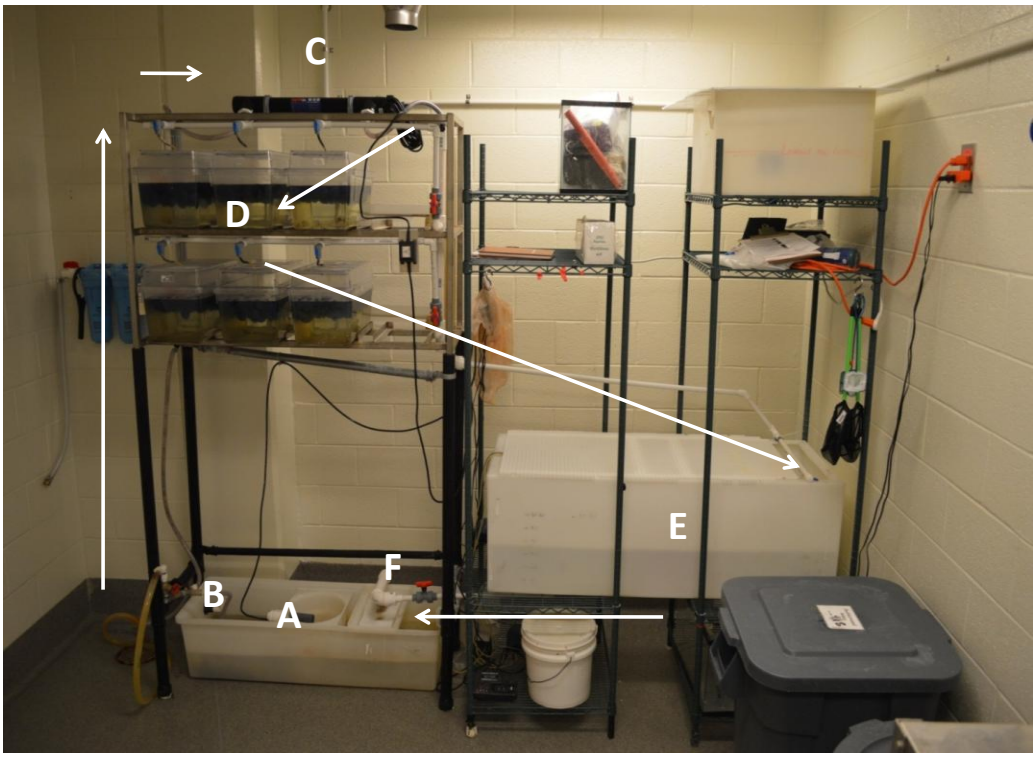


Figure 2.1. Recirculating frog colony housing system in use. Arrows indicate flow of water. A) Water supply tank. B) Carbon filter and pump. C) UV lamp. D) 5 gallon biofilter tanks. E) Frog housing tank. F) Particulate filter.

Table 2.1: *Xenopus laevis* recirculating system pond water recipe

Constituent	**Amount per 10L**
Salts	4.0 g Cichlid lake salts (Seachem) 0.7 g Equilibrium salts (Seachem)
$NaHCO_3$	0.305 g
AmQuel plus (Kordon, Inc.)	5 mL
NovAqua plus (Kordon, Inc.)	5 mL

We monitor the function of the biofilter monthly via testing strips for ammonia, nitrite, and nitrate, and perform three 10% water changes per week to control nitrate levels (Table 2.2). With these maintenance practices in place, we had enjoyed several years of trouble-free oocyte isolation with low frog mortality and high expression of ion channel proteins of interest upon injection of cRNAs encoding those proteins.

However, starting in June of 2014, we noted that several batches of isolated oocytes formed dark spots over their entire surface, and died within 48 hours of isolation when maintained in oocyte storage media containing the antibiotics penicillin and streptomycin (not shown, but indistinguishable in appearance compared to O'Connel *et al*. (O'Connell et al., 2011).) For the next 8 months, we also noted an increase in frog mortality as compared to the same length period in the previous year (5 deaths *vs.* 1 death). Table 2.3 details frog mortality in the period from the point we began to notice the changes in oocyte phenotype, to when we ultimately broke down the system. As shown, primary pathologies observed in the affected frogs were redness on mouth and bloating, both consistent with bacterial infection (Hubbard, 1981). Oocytes with appearance as noted above were useless for electrophysiological recordings; they were characterized by membrane potentials near 0 mV (indicating loss of normal electrochemical gradients) and large leak currents when voltage clamping was attempted.

We noted that during this time, no significant changes were detected in any of the water quality indicators listed previously in Table 2.1 (4 measurements taken between 5/24/14 to 6/24/14). The appearance of the oocytes and timeline of oocyte

Table 2.2: Levels of relevant chemicals in a well controlled recirculating system

Chemical/parameter	**target level**	**readings 5/24/14-6/24/14**
Nitrate	20 ± 20 ppm	30 ±10 ppm
Nitrite	0 ppm	0 ppm
Ammonia	0 ppm	0 ppm
General hardness	300 ± 50 ppm	350 ± 25 ppm
Chlorine	0 ppm	0 ppm
Alkalinity	100	100 ±20
pH	7.5 ± 0.2	7.4 ± 0.1

Table 2.3 Frog mortality during the period of poor oocyte quality

Date Reported	**Issues reported by Veterinarian**	**Result**
6/4/14	No obvious issues	Found dead in tank
7/14/14	Lethargy/bloating	Euthanized
9/3/14	Redness on mouth, secondary bacterial infection	Euthanized
12/24/14	Bloating	Found dead in tank
1/14/15	Bloating	Found dead in tank

symptoms after isolation was remarkably similar to those described in detail by O'Connell and colleagues (O'Connell et al., 2011). In that study, the cause of the oocyte malady was determined to be infection of the frogs by multidrug resistant bacteria. Suspecting our frogs might also be infected, we cultured on antibiotic-free blood agar plates bacteria from the wound of one of the sick frogs before euthanasia (9/4/14). The hemolytic bacteria were subsequently characterized via the Biolog system (Biolog Inc., Hayward, CA), and genetic identities were confirmed via partial 16s ribosomal RNA sequencing by John Varga, PhD (Department of Pediatrics, Emory University). Two bacterial types were identified: *Aeromonas* sp. (most probable identity *Aeromonas allosaccharophilia*) and *Shewanella* sp. (most probable identity *Shewanella xiamenenis*).

The aeromonad bacterium was of particular interest because several species of this genus (particularly *Aeromonas hydrophila*) are known pathogens to freshwater laboratory animals (Hubbard, 1981) and have shown resistance to antimicrobial peptides of *Xenopus laevis* (Schadich and Cole, 2009). In addition, certain strains of *Aeromonas* have been shown to be resistant to penicillin and streptomycin, the antibiotics we had been using in our oocyte storage media (Bondi et al., 2000).

Since the effects of infection in the study by O'Connell and colleagues were ameliorated to their satisfaction via addition of broad-spectrum antibiotics to the oocyte storage media, we similarly treated the isolated oocytes with the same dose (100 mg/L) of ciprofloxacin. Oocytes maintained in the presence of this antibiotic were absent of dark spots, and typically survived ≥ 6 days in storage medium. Oocyte quality was increased to the point where some experiments could be performed, although

greater than 40% of oocytes tested still failed to withstand voltage clamping to V_m = -60 mV within the first five minutes of experimentation during this period (Figure 2.2). Additionally, there remained histological indications that the treated oocytes were suboptimal. In the majority of oocyte batches, we observed that a high fraction of isolated oocytes had follicular tissue attached after collagenase digestion. The micrograph in Figure 2.3 shows a batch of oocytes with follicular tissue attached, indicated by constrictions of the oocyte by the edge of the follicular membrane (white arrows). This follicular tissue persisted even after we increased both the concentration and duration of collagenase exposure by 30%, relative to our normal protocol. These oocytes could not be used for electrophysiological recordings, because attached follicular tissue has been shown to confound results of pharmacological studies in oocytes (Madeja et al., 1997).

After an extended period of ordering oocytes from an outside company (Ecocyte Inc., Austin, TX), we decided to rebuild our recirculating system and re-establish our biofilter and *Xenopus* colony. We replaced all of the plastic and PVC parts (sourced via Grainger Industrial Supply Inc., Chicago, IL) with the exception of the 5-gallon biofilter tanks, the pump and large water tanks (see Figure 2.1). The reused parts were washed twice in a sterilizing high temperature cage washing machine and then wiped down with ethanol. We filled the system with water adulterated according to our *Xenopus* recipe (detailed in Table 2.1), and left it to cycle for 7 days with the UV sterilization lamp on, while at this point the biofilter tanks were disconnected from the system.

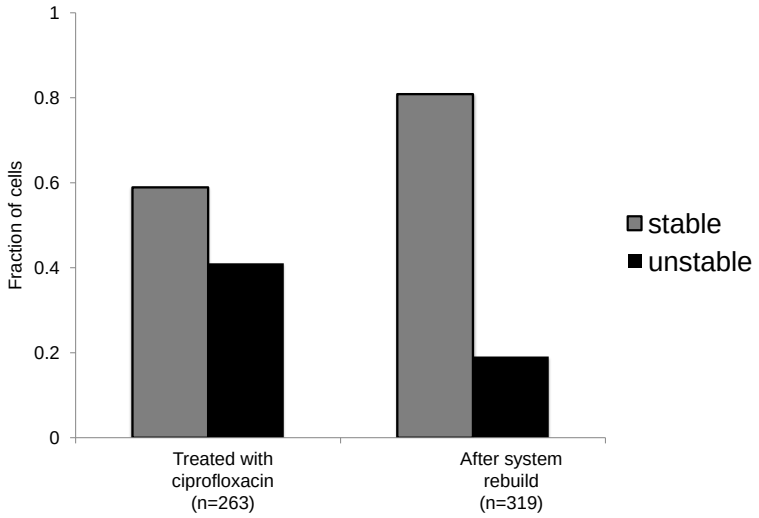


Figure 2.2. Effect of intervention on usefulness of *Xenopus* oocytes in electrophysiological experiments. Oocyte was considered "stable" if still alive after 5 minutes of voltage clamping to V_m = -60 mV.

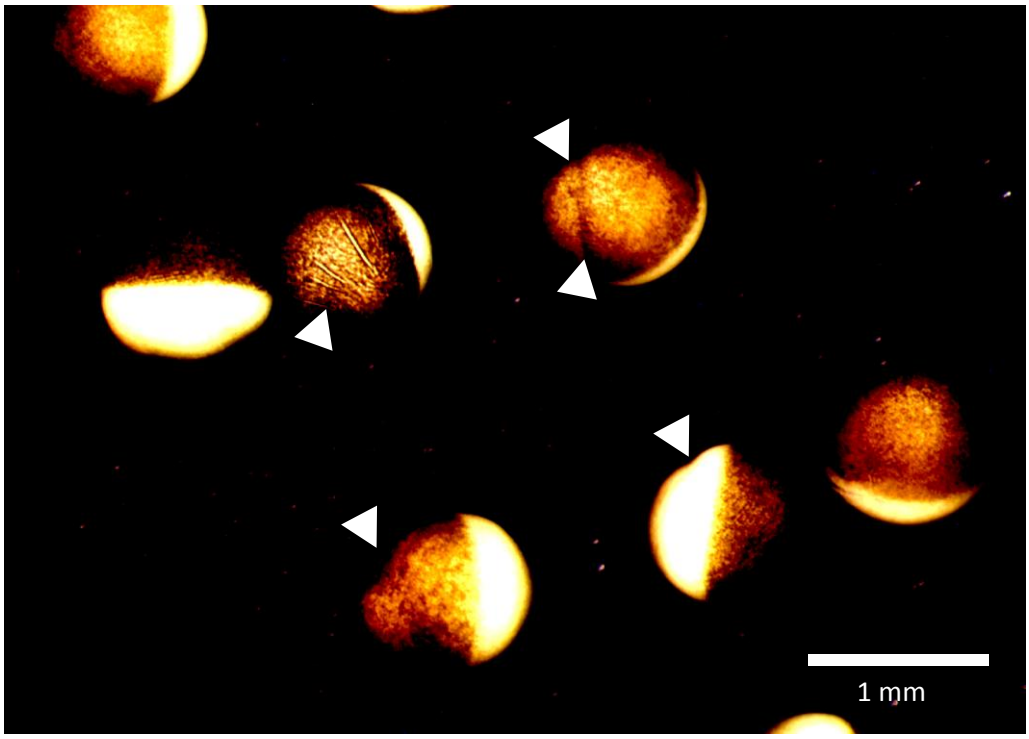


Figure 2.3. Micrograph of oocytes with persistent follicular tissue after collagenase treatment. White arrowheads show constrictions in oocyte resulting from follicular tissue.

In parallel, we re-established the biofilter. Specifically, we filled each of the 5 gallon biofilter tanks with clean bioballs (Amiracle Plastics Inc., Englewood, CO) and water adulterated according to our *Xenopus* recipe detailed in Table 2.1. We then added ammonia to reach initial levels of 3 ppm as indicated via test strips. After two days of measuring ammonia levels to confirm stability, we introduced the bacterial formulation Nutrafin Cycle (Hagen Inc., Mansfield, MA) according to the manufacturer's instructions. As this bacterial mixture has been formulated to accomplish both conversion of ammonia to nitrite and of nitrite to nitrate, we followed the levels of these chemicals over time using test strips. The ammonia-fixing bacteria were established within one week of introduction, after which they rapidly neutralized a second dose of ammonia. Within 3 weeks, a steady increase in nitrate confirmed that the bacterial population was effectively converting ammonia to the nitrate end product.

At this point, we connected the biofilter to the rest of the recirculating system and let water circulate for one week, with the UV sterilization lamp off. We then turned the UV lamp on and stocked the tank with 5 new frogs (Xenopus One Inc., Ann Arbor, MI).

We tested the quality of the oocytes from these frogs in two ways. Twelve weeks after the frogs were stocked, we checked to see if the oocytes would remain healthy in our standard penicillin and streptomycin antibiotic cocktail or if dark spots would form in the absence of ciprofloxacin as they had before re-establishment. Oocytes from all batches of the new frog colony were free of dark spots in the absence of ciprofloxacin (Figure 2.4), suggesting that the infection resident in the initial frog

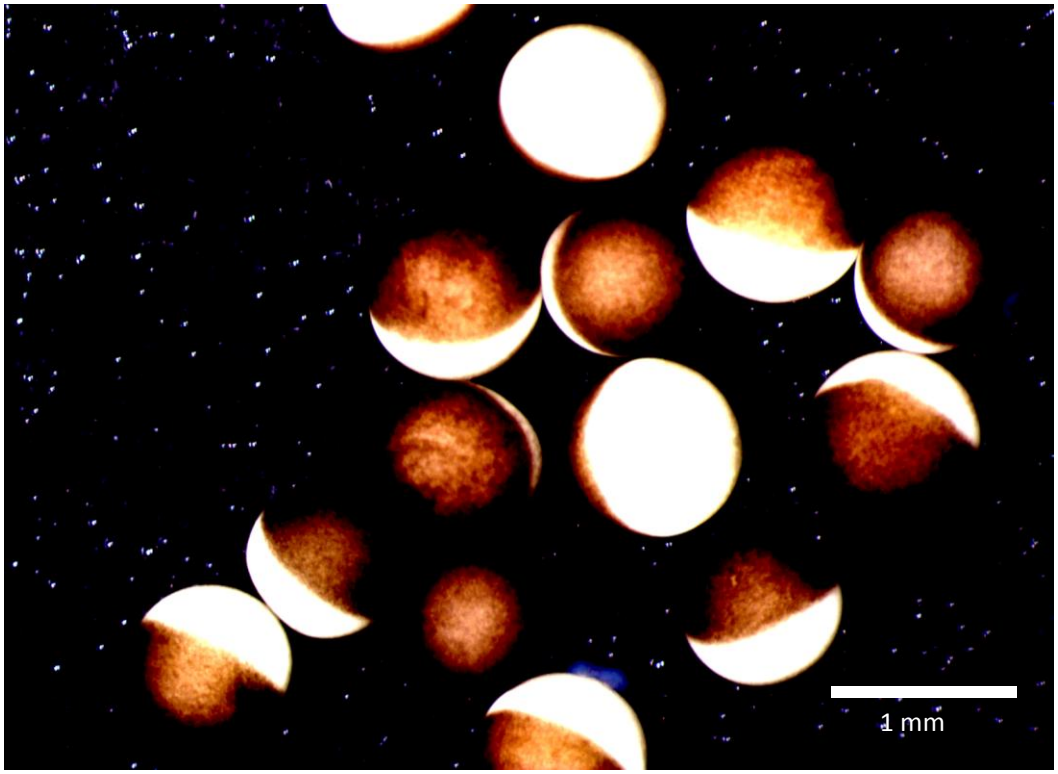


Figure 2.4. Micrograph of oocytes from *Xenopus* following rebuild. Oocytes lack any signs of infection in standard penicillin/streptomycin antibiotic, absent of ciprofloxacin.

colony (and therefore the oocytes) was no longer present. Second, we tested the oocytes functionally. In terms of utility for our electrophysiological experiments, these oocytes have been exceptional in the surgeries performed over 14 weeks since re-establishing the frog colony. As shown in Figure 2.2, fewer than 20% of the oocytes were unstable during voltage-clamp experimentation without having been maintained with ciprofloxacin in the storage media, whereas before rebuild, greater than 40% were unstable with the aid of ciprofloxacin and none were useable without it.

2.1.3 Discussion: In this report, we have detailed our experience combating a pen/strep-resistant bacterial infection in our *Xenopus laevis* colony that caused increased mortality in the colony and adverse effects on the oocytes isolated from the frogs, in terms of their usefulness in electrophysiological assays. The infection of *Xenopus laevis* by bacteria such as *Aeromonas hydrophila* and other pathogens has been previously described (Hill et al., 2010; Hubbard, 1981) but in our case the disease course was somewhat different. While frog mortality was overall increased as compared to analogous previous periods, mortality was not acutely catastrophic or widespread in the tank at any one time. In our case, the chronic condition most strikingly manifested itself symptomatically in the poor quality of oocytes isolated from the frogs. Furthermore, the species of bacteria we isolated from our infected frog (*Aeromonas allosaccharophilia* and *Shewanella xiamenenis*) have not, to our knowledge, been previously isolated from infected *Xenopus laevis.* In fact, the literature on these bacteria is limited: *Aeromonas allosaccharophilia* has been islolated from diseased European eels

(Martinez-Murcia et al., 1992), while *Shewanella xiamenenis* has been isolated from an infected human patient (Antonelli et al., 2015).

Our experience is similar to that noted in the previously mentioned study by O'connell and colleagues, particularly in the appearance of dark spots that appeared several days after isolation (see (O'Connell et al., 2011)). However, unique characteristics distinguish our study, in addition to the different bacterial species (*Pseudomonas fluorescens*) isolated in that study. These differences pertain primarily to the condition of our oocytes in the presence of ciprofloxacin. Whereas in their study, addition of broad-spectrum antibiotics to treat the isolated oocytes completely rescued oocyte quality, we found that while treating the oocytes with the same antibiotic dose (ciprofloxacin 100 μg/L) improved the oocytes, it did not completely ameliorate the quality issues (Figure 2.2-2.3). The partial improvement may be explained by reports that ciprofloxacin functions either as bactericidal or bacteriostatic depending on dose and bacterial target (Silva et al., 2011).

We noted an apparent effect of frog health condition on follicular tissue tightness and adherence following collagenase treatment (Figure 2.4). The effect of infection and/or immune status on follicular tissue physiology of *Xenopus laevis* is poorly understood; however, it has been reported that follicular tissue serves an innate immunological role in mammals (Herath et al., 2007). Given that the phenotype of tightly adhering follicular tissue was readily observed in oocytes isolated during the time we believe the frogs were contending with elevated levels of pathogenic bacteria in the

tanks (as opposed to after the system was rebuilt), we speculate that this undesirable phenotype of the oocytes may be attributable to a protective reproductive mechanism.

The most valuable lessons from our experience most directly relate to the health of our *Xenopus* colony and its impact on the quality of isolated oocytes. First, we learned that if observed and reviewed critically (for even subtle changes), data on oocyte quality and overall frog mortality/morbidity have the potential to inform on each other. As causes of *Xenopus* oocyte quality issues are notoriously mysterious (at least in the ion channel field), this principle is of high potential value to investigators. Second, when confronted with aquaculture issues, one needs to consider not only the function of the biofilter, which in this case was unaffected (Table 2.2), but also the potential for changing bacterial composition in the frog housing system. This is particularly important since potentially pathogenic bacteria such as the aeromonads are ubiquitous at low levels in the environment (Hanninen et al., 1997). Finally, although treating isolated oocytes from frogs with confirmed or suspected health issues with antibiotics may suffice for certain applications and types of experiments (as achieved in (O'Connell et al., 2011) and (Elsner et al., 2000)), in our experience, the most prudent (although most laborious and time-consuming) course of action has been the elimination of the root cause of frog morbidity via re-establishment of the colony and biofilter.

2.2 Electrophysiological methods used to study the structure and pharmacology of CFTR

2.2.1 Use of cysteine trapping to study protein structure and conformational change.

Cysteine trapping has been successfully used in numerous studies of the structure and function of membrane proteins such as CFTR (El Hiani and Linsdell, 2014; Linsdell, 2015; Wang and Linsdell, 2012). The technique takes advantage of both the relative rarity and physiological reactivity of cysteine residues in proteins to allow the elucidation of both structural proximity and conformational dynamics between different regions of proteins. In general, the demonstration that two cysteine residues interact is indicative of their close proximity in a given state of a protein; when engaged in a disulfide bond, the sulfhydryl groups of cysteine residues approach within 2.2 Å of each other (Bosnjak et al., 2014), while linkage via a soft metal such as cadmium suggests proximity within 5 Å (Rulisek and Vondrasek, 1998). In this dissertation, we couple chemical modulation of engineered cysteine residues with electrophysiology, observing the functional effects in real time of DTT (to reduce disulfide bonds) or cadmium (to trap cysteine residues in metal coordination complexes).

2.2.2 Methodological details:

Oocyte preparation and molecular biology: Human wild type (WT) CFTR (V470M/V1470M variant) subcloned into the pGEM-HE oocyte expression vector was previously provided by Dr. David Gadsby (The Rockefeller University, New York, NY). Human WT beta-2 adrenergic receptor (β2AR) subcloned into the pSP65 vector was previously provided by Dr. Brian Kobilka (Stanford University, Stanford, CA). Mutations

were made using the Quikchange XL mutagenesis kit (Stratagene) according to the manufacturer's instructions and sequences were verified through the open reading frame. Complementary RNA was transcribed using the Ambion *in vitro* RNA transcription kit according to the manufacturer's instructions. *Xenopus laevis* oocytes were injected with 1-10 ng of CFTR cRNA. For some two-electrode voltage clamp (TEVC) experiments, cRNA encoding β2AR was premixed with CFTR cRNA at a mass-to-mass ratio of 1:20. Oocytes were incubated in modified Leibovitz's L-15 media plus HEPES pH 7.5, penicillin, streptomycin, and (during some periods) ciprofloxacin. The oocyte isolation procedure was in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Emory University. Recordings were typically made 48-96 hours after cRNA injection.

Electrophysiology:

Two electrode voltage clamp recording. All recordings were made at room temperature in Ringer's solution (ND96) consisting of (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES, pH 7.5. For cadmium dose response experiments, ND96 was made up using new plastic vessels to minimize the effect of contaminating trace metals leaching from glassware. Recordings were made using a Geneclamp 500 amplifier (Molecular Devices). Intracellular electrode resistances averaged 0.5 MΩ when filled with 3 M KCl solution. Different voltage protocols were utilized depending on application. For recordings measuring conductance, the membrane voltage was held at V_m = -20 mV (approximating the resting membrane potential for an expressing cell) and either pulsed to -80 mV, -60 mV, -40 mV, -20 mV, and 0 mV for 200 ms every 2 seconds or ramped

from -60 mV to 0 mV over the course of 500 ms. In all cases, cell conductance was calculated as the slope between -40 mV and 0 mV. For each CFTR variant studied, the data generated an I/V slope that was linear from -60 to 0 mV. The design of these protocols allowed even highly expressing oocytes to be recorded at 10 second time resolution while limiting rundown from intracellular chloride depletion. In experiments with higher desired resolution, the membrane potential was held at -20 mV and pulsed to -60 mV for 200 ms every 2 seconds, and current at -60 mV was reported, or cells were held at V_m = -40 mV and gap-free recordings were digitized at 500 Hz.

In most experiments, CFTR was activated through the beta-2 adrenergic receptor (β2AR) with isoproterenol to elicit robust Gαs signaling and increase intracellular cAMP. Physiological activation of CFTR via β2AR has been demonstrated in lung (Bossard et al., 2011), sweat glands (Quinton et al., 2012), and nasal epithelia (Rowe et al., 2011). This activation mode has been used successfully by our group and others in several studies to probe CFTR structure in oocytes via cysteine accessibility and modification, including those using DTT as a reducing agent (Alexander et al., 2009; Cui et al., 2014; McCarty et al., 1993; Norimatsu et al., 2012). In experiments wherein precisely graded activation was desired, CFTR was activated by 10 μM of the adenylyl kinase activator forskolin and various concentrations of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) to reach previously described quasi-plateaus (Drumm et al., 1991; Smit et al., 1993; Wilkinson et al., 1996).

Single channel recording. Excised, inside-out patches containing several CFTR channels were recorded at room temperature as previously described (Fuller et al.,

2007). Manual removal of the oocyte vitelline membrane was performed after shrinking in hypertonic solution containing (in mM): 200 monopotassium aspartate, 20 KCl, 1 $MgCl_2$, 10 EGTA, and 10 HEPES, pH 7.2 adjusted with KOH. Pipettes were pulled from borosilicate glass using a Sutter laser puller (model P-2000). Pipette resistances averaged 10 MΩ when filled with pipette solution containing (in mM): 150 NMDG-Cl, 5 $MgCl_2$, 10 TES, pH 7.5. Channels were activated and recorded in intracellular solution containing (in mM): 150 NMDG-Cl, 1.1 $MgCl_2$, 2 Tris-EGTA, 10 TES, 1 MgATP, and 127 U/ml PKA, pH 7.5. CFTR currents were measured using an Axon Instruments 200B patch-clamp amplifier (Molecular Devices) holding in all cases at V_m = -100 mV, low pass Bessel filtered at 10 kHz, and recorded to DAT tape. Traces were played back to pClamp 10.2 software, filtered at 100 Hz, digitized at 500 Hz, and baseline was manually adjusted. To quantify open channel burst durations, at least 100 openings pooled from at least 4 independent patches per condition were manually analyzed. Open bursts were separated by closures of at least 10 ms. All points amplitude histograms corresponding to each individual displayed record were fit to Gaussian distributions and provided next to the associated traces.

Generation of CFTR model images: Coordinates for amino acids were based on the 2.5 nanosecond snapshot of a recently published molecular dynamics simulation of CFTR gating (Rahman et al., 2013). Images were generated using Pymol 0.99 software.

Statistics and analyses: Groups of data were compared via Student's t-test. P-values are indicated in the figure legends. Error bars indicate standard deviation in all

cases except for single channel mean burst duration, where error bars indicate standard error of the mean. Percent inhibition and fold increase were calculated as:

$$\text{Inh \%} = (A_{INITIAL} - A_{FINAL})/(A_{INITIAL})$$

$$\text{Fold increase} = A_{FINAL} / A_{INITIAL}$$

where A_{FINAL} is the activity level of the channel population (current or conductance) at the end of treatment and $A_{INITIAL}$ is the activity level directly before treatment.

Source of chemicals and reagents: Purified PKA catalytic subunit was purchased from Promega, and L-15 Media was purchased from Gibco. The CFTR inhibitor Inh_{172} and the potentiator VX-770 (Ivacaftor) were purchased from Cayman Chemicals and diluted to working concentrations from frozen 20 mM stocks in DMSO. All other chemicals were purchased from Sigma Aldrich. DTT stocks (1 M) in deionized ddH_2O were made fresh at the beginning of each experiment day and kept on ice. IBMX stocks (1 M) in DMSO were made fresh at the beginning of each experiment day and kept at room temperature. Cadmium solutions were made from serial dilutions of a 1 M $CdCl_2$ stock solution in deionized ddH_2O.

Finally, I note that it was discovered in pilot experiments using Ivacaftor that the drug was particularly prone to “carry-over” effects. This is to say, after using the drug on one cell, its effect would be blunted when applied to subsequent cells, despite thorough rinsing of the recording chamber between experiments. This phenomenon likely arises from the high lipophilicity and potency of the drug (Hadida et al., 2014). To circumvent this, it was necessary to disassemble the recording chamber and perfusion

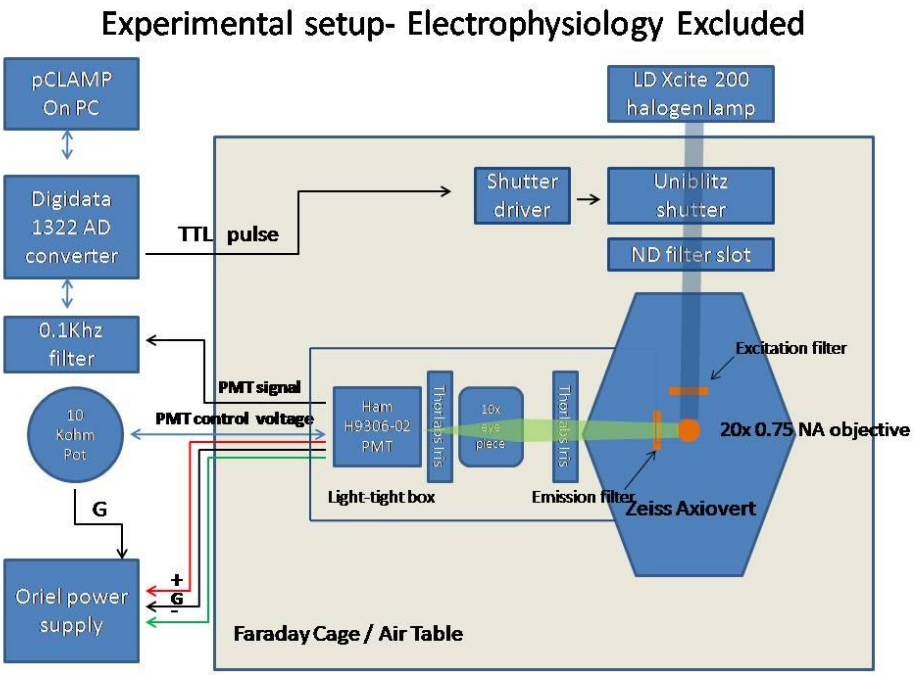
manifold, wash all parts thoroughly (removing all vacuum grease), and allow parts to soak for ≥ 2 hours in detergent. To save time, multiple modules consisting of clean perfusion manifolds and recording chambers were assembled at the beginning of each experimental day and replaced as needed.

2.3 Development of an assay to report on CFTR localization in Xenopus oocytes

An early iteration of my dissertation project aimed to study the structure and function of CFTR using quantitative fluorescence techniques. At that time, I built an experimental rig that allowed the simultaneous and sensitive recording of fluorescence and electrophysiological data, by combining a conventional TEVC system to the optical system schematized in Figure 2.5A, with details on relevant parts in Table 2.4. This rig utilizes a conventional metal halide fluorescence light source which is reduced in intensity via neutral density filters and shuttered via TTL pulses from pCLAMP to minimize photo-bleaching of the sample. The oocyte is mounted with its animal pole facing downwards in a custom designed recording chamber that suspends the oocyte above the unidirectional flow of solution (Figure 2.5B). The fluorescence signal is amplified, recorded as a voltage via a photomultiplier tube (PMT), and digitized to pCLAMP 10.2 software.

The experimental rig was not ultimately successfully used in studies of the conformational dynamics of CFTR; however, we have employed it towards a different purpose that takes advantage of the intrinsic pH sensitivity of the fluorescence of green

A.



B.

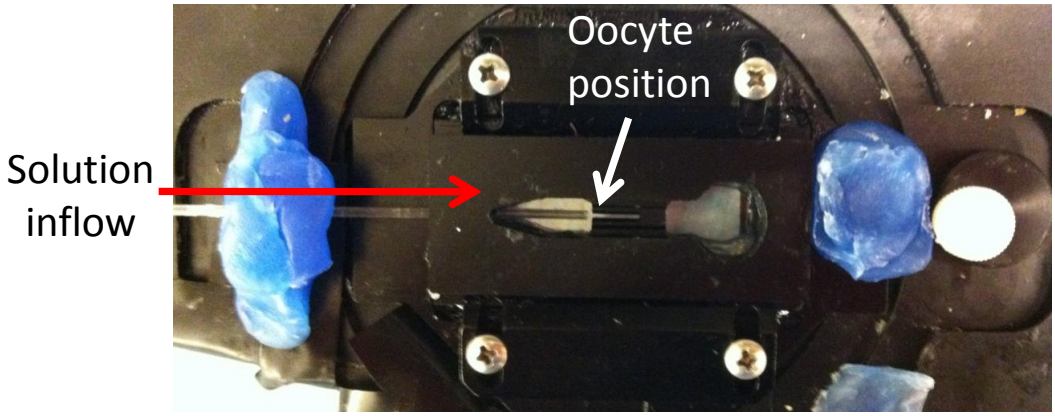


Figure 2.5. Design of an experimental rig that measures fluorescence from *Xenopus* oocytes in real time. A) Schematic diagram showing signal inputs and outputs. B) Top-view photograph of the custom designed recording chamber used in this rig. The chamber utilizes a glass bottom not visible in this photograph.

Table 2.4 Identity of non-standard parts in the oocyte optical rig in Figure 2.5

Label in Figure 2.5	**Manufacturer**	**Part number**
LD Xcite 200	Lumen Dynamics	Xcite 120Q
Uniblitz shutter	Vincent Associates	9003-0212
ND filter	Thorlabs	NE03B
Thorlabs Iris	Thorlabs	8203206060
Ham PMT	Hamamatsu	H9306-02
Shutter Driver	Vincent Associates	VCM-D1
0.1 kHz filter	Warner Instruments	LPF-100B
Oriel Power supply	Newport Instruments	70703
10 kOhm pot	PEC	KU2531S28
Zeiss Axiovert	Zeiss	1021858676

fluorescent protein (GFP) (Ashby et al., 2004; Kneen et al., 1998). Specifically, I generated a version of CFTR wherein GFP was subcloned into the fourth extracellular loop of CFTR (exGFP-CFTR, Figure 2.6A), and found that the fluorescence measured from oocytes expressing this CFTR variant was rapidly and reversibly responsive to changes in the pH of the Ringer's solution (Figure 2.6B,D). The fluorescence of oocytes expressing a CFTR variant tagged with intracellular GFP (inGFP-CFTR, Figure 2.6C) was essentially insensitive to changes in extracellular pH (Figure 2.6D), suggesting that the previously observed reduction of macroscopic fluorescence from exGFP-CFTR is due to the quenching of extracellular GFP from plasma membrane-localized CFTR channels. This assay is currently being used by another member of our group (B. Stauffer) to ask whether silencing of CFTR channels by bacterial sphingomyelinase (Ramu et al., 2007; Ramu et al., 2014) arises from internalization of CFTR from the plasma membranes of *Xenopus* oocytes. In addition, in the future, this system could be used to test the expression and plasma membrane localization of CFTR mutants that do not generate currents in electrophysiological assays.

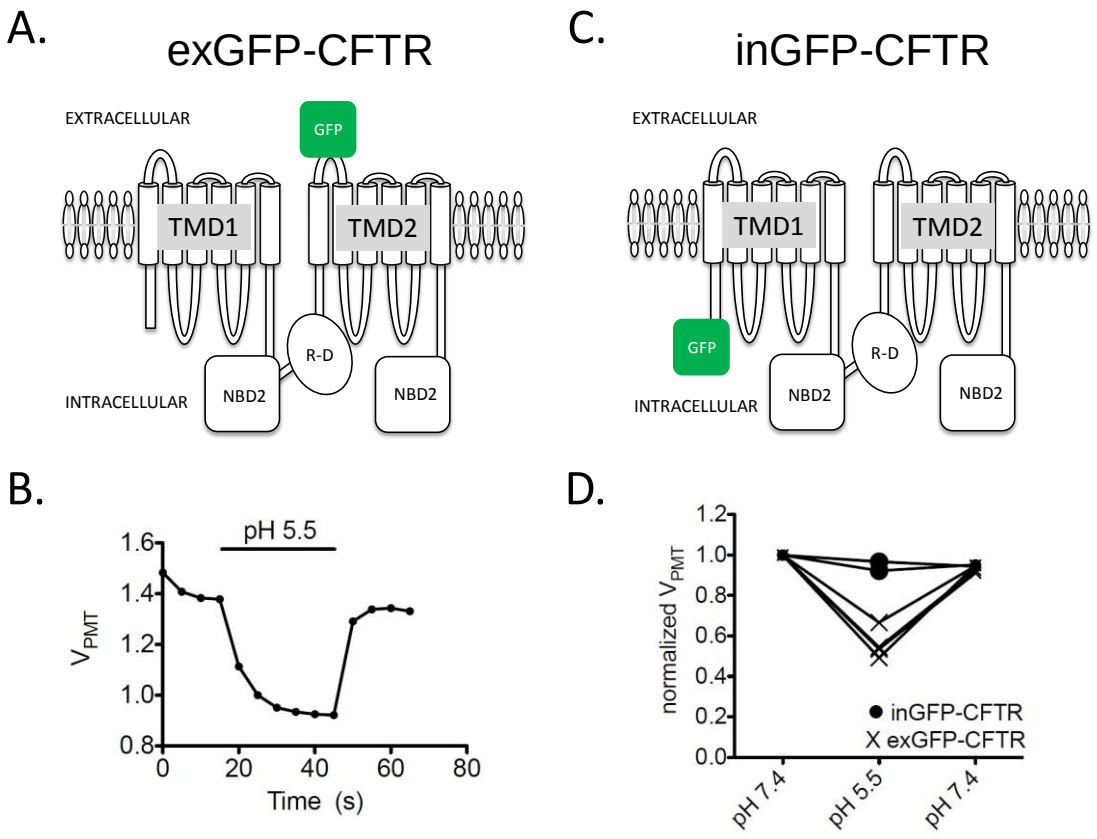


Figure 2.6. Fluorescence quenching-based assay of CFTR localization in *Xenopus* oocytes. A) Cartoon layout of a version of CFTR featuring GFP subcloned into its fourth extracellular loop. B) Example trace showing rapid quenching and de-quenching of fluorescence from exGFP-CFTR upon changes from pH 7.4 to pH 5.5. C) Cartoon layout of a version of CFTR featuring GFP subcloned at the intracellular N-terminus. D) Summary data demonstrating the apparent insensitivity of inGFP-CFTR relative to exGFP-CFTR in experiments wherein oocytes injected with either variant are exposed to Ringer's solution with neutral, acidic, and neutral pH in sequence. V_{PMT} = the voltage recorded by the photomultiplier tube.

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CHAPTER 3: RESULTS

3.1 The position of ECL1 significantly affects the pore gating of CFTR

NOTE: the data in this section are published in (Infield et al., 2015).

As discussed in Section 1.5, several recent reports have provided evidence that the conformational changes intrinsic to CFTR channel gating mirror those of the transport cycle of evolutionarily-related ABC transporters (Bai et al., 2011; Gadsby et al., 2006; Mense et al., 2006; Wang et al., 2014). Particularly, ATP-mediated CFTR channel opening is proposed to entail an inward- to outward-facing transition of TMDs that is driven by ATP-mediated dimerization of the NBDs (Figure 1.5). In crystal structures of both homodimeric and monomeric ABC exporters, a structural hallmark of this transition is the separation of two apposing extracellular loops: extracellular loop 1 and extracellular loop 4 (ECL1 and ECL4) (Dawson and Locher, 2007; Li et al., 2014; Ward et al., 2007). We and others had previously studied several disease-relevant residue positions on ECL1 and found that mutation of several of these residues caused profound effects on the gating of the channel pore (Cui et al., 2014; Hammerle et al., 2001), highlighting a vital functional role of this region of the channel. In parallel, molecular dynamics simulations of ABC-transporter based homology models of CFTR suggested that *if* CFTR gating "looks like" ABC transport, the N-terminal end of ECL1 of CFTR may be closely positioned to ECL4 in a closed state (Rahman et al., 2013).

3.1.1 DTT reduces a putative disulfide bond between D110C and K892C, highly potentiating channel activity. I initially asked whether linking a substituted cysteine

residue in ECL1 to one in ECL4 (expected to lie across the channel pore) would stabilize the channel's closed state in whole oocytes, using a protocol designed to minimize chloride depletion-mediated rundown during long experiments. When co-expressed in *Xenopus laevis* oocytes with the beta-2 adrenergic receptor (β2AR), D110C/K892C-CFTR was activated with a saturating concentration of isoproterenol (10 μM ISO) to reach a conductance plateau, typically within 5-10 minutes. Addition of 1 mM of the reducing agent DTT after maximal activation by ISO elicited a large increase in conductance that failed to reach plateau even after 5 minutes of continued perfusion (13.0 +/- 3.2 fold increase, n=10; Figure 3.1A). Wild type CFTR channels activated in the same way were not apparently potentiated by DTT (n=6; Figure 3.1B), in agreement with reports from our lab and others (Beck et al., 2008; Cui et al., 2014; Liu et al., 2006), nor were K892C-CFTR channels potentiated by DTT (n=4; Figure 3.1C). There was no apparent effect of DTT or ISO on the conductance of uninjected oocytes (n=4; not shown). Interestingly, D110C-CFTR single mutant channels were stably potentiated 2.87 +/- 1.05 fold by DTT (n=18; Figure 3.1D), typically reaching plateau within 5 minutes. D110A-CFTR channels were unaffected by DTT, directly implicating the reactive cysteine substitution in the potentiation of D110C-CFTR by DTT (n=4; Figure 3.1E). Lack of significant potentiation of D110C/K892C-CFTR by 500 μM of the potent metal chelator EGTA in pilot experiments indicated that the effect of DTT was due to cysteine reduction rather than metal chelation (n=2; Figure 3.1F). These experiments, summarized in Figure 3.1G, provide evidence that D110C and K892C form a spontaneous disulfide in CFTR, and the

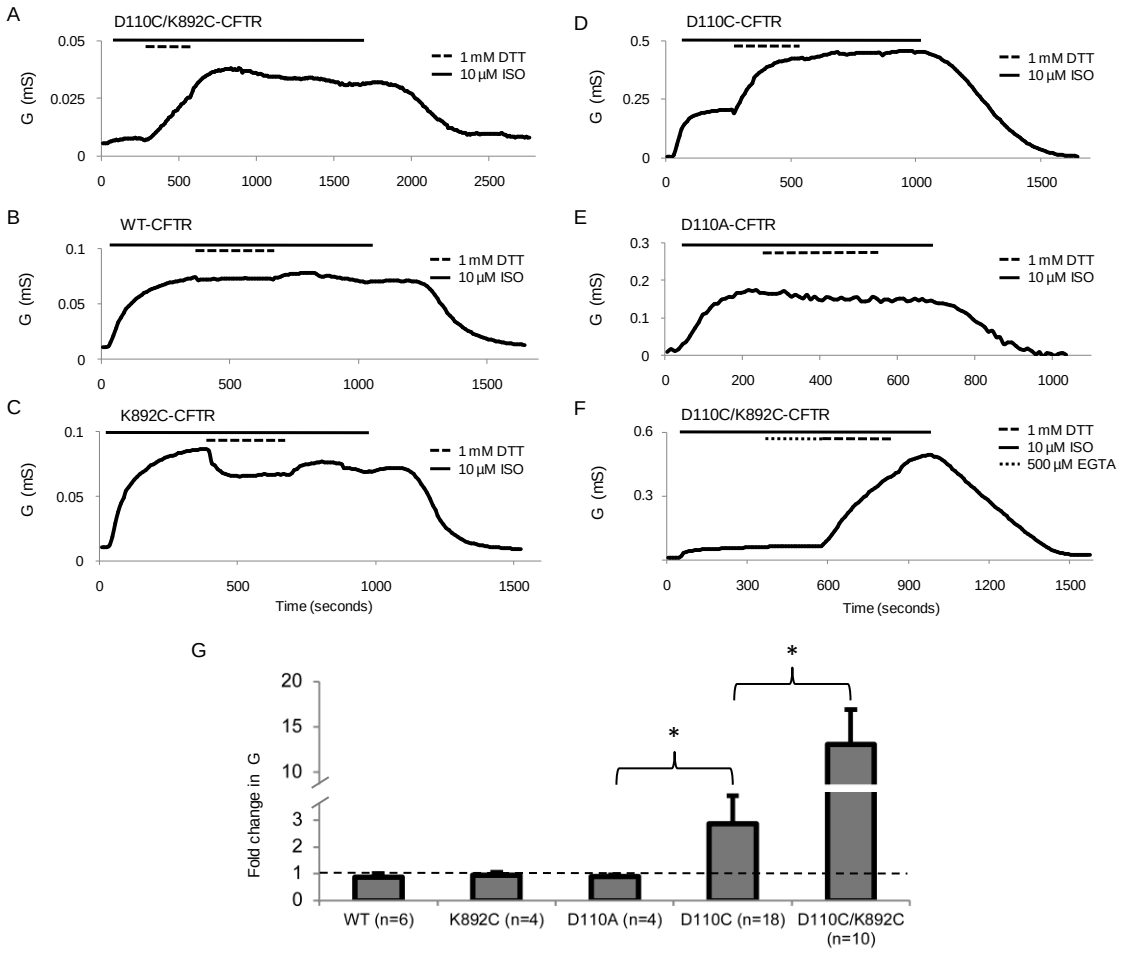


Figure 3.1. D110C/K892C-CFTR macroscopic currents were highly potentiated by DTT. Traces show change in whole-cell conductance (G) over time, during activation of CFTR by stimulation of coexpressed β2AR using isoproterenol (ISO), and subsequent exposure to DTT in the continuing presence of ISO. A) D110C/K892C-CFTR, B) WT-CFTR, C) K892C-CFTR, D) D110C-CFTR, E) D110A-CFTR, all activated by 10 µM ISO and then exposed to 1 mM DTT for 5 minutes. F) Treatment with the metal chelator EGTA neither potentiated D110C/K892C-CFTR nor affected its subsequent potentiation by DTT. Quantification of fold increase of conductance (G; slope between V_m = -40 mV and 0 mV) after 5 minutes of DTT application for all variants tested, with N-values for each variant as indicated. The dashed line indicates level of no change (i.e., "fold change of 1"). An asterisk (*) indicates $p < 0.05$ between groups.

presence of this close linkage between ECL1 and ECL4 markedly perturbs channel activity.

3.1.3 D110C/K892C-CFTR channels rapidly coordinate cadmium, which inhibits channel macroscopic conductance. Since cysteine residues are engineered via coding into the protein of interest, it is possible that a disulfide bond between them could irreversibly form at any time between protein expression/folding and performing the experiment—a time course of multiple days. For this reason, it has been suggested that engineered disulfide bonds can "trap" residues into rare and otherwise unnatural conformations (El Hiani and Linsdell, 2014). To better understand the kinetics of the C110-C892 linkage, I performed metal bridging experiments using acute applications of the soft metal cadmium, as successfully used to probe cysteine proximity in previous studies of CFTR (El Hiani and Linsdell, 2014; Linsdell, 2015; Wang and Linsdell, 2012) and many other types of ion channels. Note that, consistent with the idea that the vast majority of D110C/K892C-CFTR channels form a disulfide bond between C110 and C892 in cells, this mutant was negligibly inhibited in the presence of a high concentration (2 mM) of cadmium, until after incubation with 1 mM DTT liberated the cysteine residues (Figure 3.2). Therefore, in all subsequent experiments using cadmium, all variants were first treated with DTT to ensure that engineered cysteine residues of these channels were free to coordinate metals.

Cells were clamped to V_m = -40 mV, exposed to ISO, and at current plateau, 20 µM cadmium was applied for 30 seconds, followed by 30 seconds of cadmium washout in the continuing presence of ISO. D110C/K892C-CFTR channels were rapidly inhibited

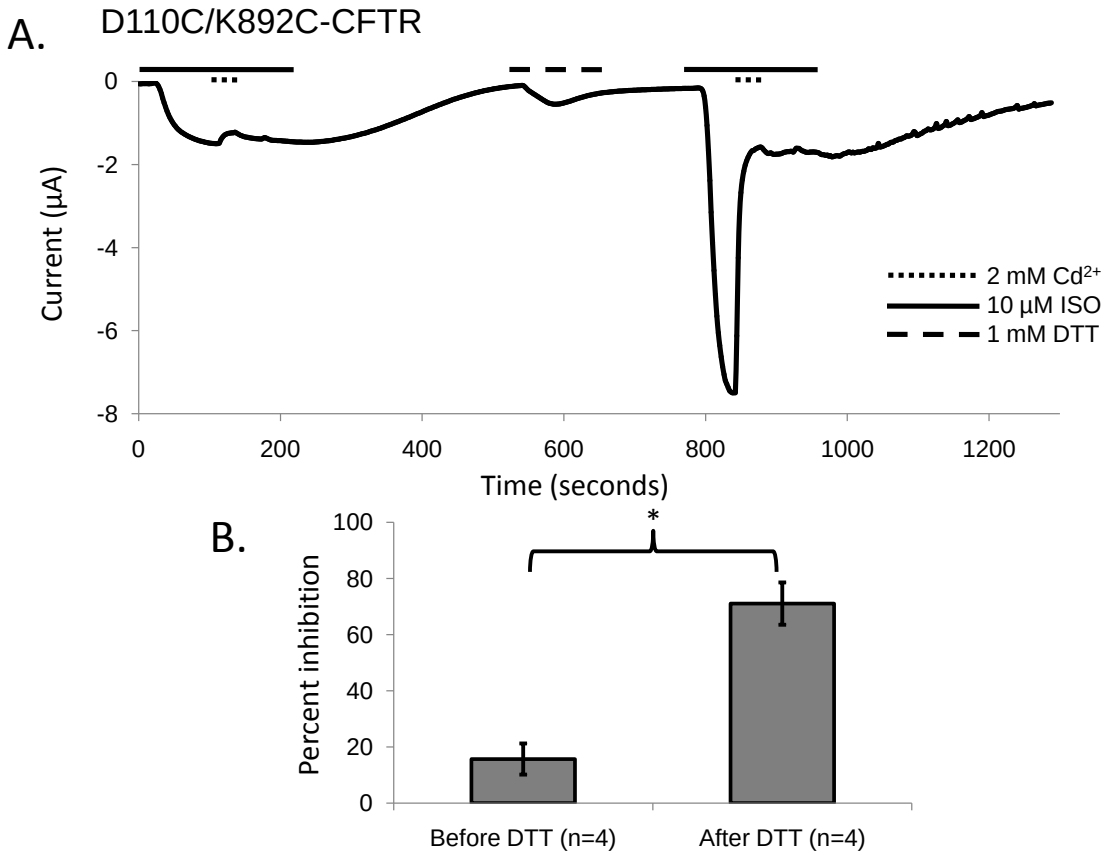


Figure 3.2 D110C/K892C-CFTR is inhibited by cadmium after reduction of cysteine residues with DTT. Traces show change in macroscopic current (μA) over time, during activation by stimulation of coexpressed β2AR using isoproterenol (ISO). A) Example trace showing minimal inhibition by 2 mM cadmium before DTT and high inhibition after DTT. B) Quantification of inhibition in both conditions.

by cadmium, with negligible reversibility in the subsequent 30 seconds of ISO alone (Figure 3.3A). By contrast, D110C-CFTR channels were reversibly inhibited by 20 μM cadmium (Figure 3.3B). There were no apparent significant effects of cadmium on WT-CFTR (Figure 3.3C), in agreement with previous studies using millimolar (Beck et al., 2008) or micromolar (Wang and Linsdell, 2012) extracellular cadmium; nor was there an apparent effect on K892C-CFTR channels (Figure 3.3D). The difference in extent of recovery after removal of cadmium (quantified in Figure 3.3E) between D110C-CFTR and D110C/K892C-CFTR suggested a large apparent difference in the affinity for cadmium in these mutants.

Consistent with these results, dose response experiments carried out over a seven $\log_{10}$ range of cadmium concentrations revealed that the affinity of D110C/K892C-CFTR for cadmium was approximately 1000-fold higher than that of D110C-CFTR (Figure 3.4 A,B). A saturating cadmium concentration (20 μM) reversed a large fraction of the increase elicited in D110C/K892C-CFTR upon exposure to DTT (Figure 3.4C), consistent with the idea that linkage of C110 and C892 via cadmium may inhibit channel activity in a similar way to linkage via a disulfide. The data in Figure 3.4 also indicate that the previously observed slow recovery of D110C/K892C-CFTR from cadmium inhibition upon washout (Figure 3.3A) is due to high affinity coordination of the metal in the double mutant, relative to the single mutant. Therefore, the washout data in Figure 3.3 demonstrate that within the time scale of a 30-second application of 20 μM cadmium to D110C/K892C-CFTR, essentially all of the cadmium that bound to the variant bound to a high affinity site contributed to by both C110 and C892, as opposed to the low affinity

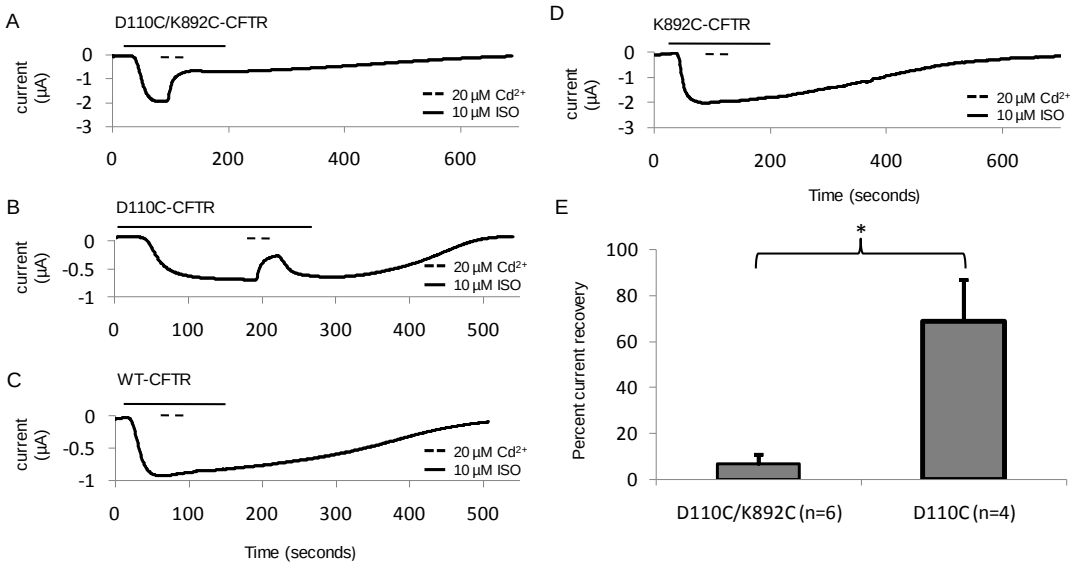


Figure 3.3. Inhibition of D110C-CFTR and D110C/K892C-CFTR by cadmium demonstrated differences in reversibility. Traces show change in macroscopic current (μA) over time, during activation by stimulation of coexpressed β2AR using isoproterenol (ISO). All cells were preincubated with 1 mM DTT for five minutes. A) D110C/K892C-CFTR, B) D110C-CFTR, C) WT- CFTR, D) K892C-CFTR, all with 30-second exposures to 20 μM cadmium followed by 30 seconds of cadmium washout in the presence of ISO. Current reported at V_m = -40 mV; increased activation results in downward deflection. N = 4 to 7 per variant. E) Comparison of D110C-CFTR and D110C/K892C-CFTR percentage current recovery after 30 seconds of washout of cadmium in the continuing presence of ISO. An asterisk (*) indicates $p < 0.05$ between groups.

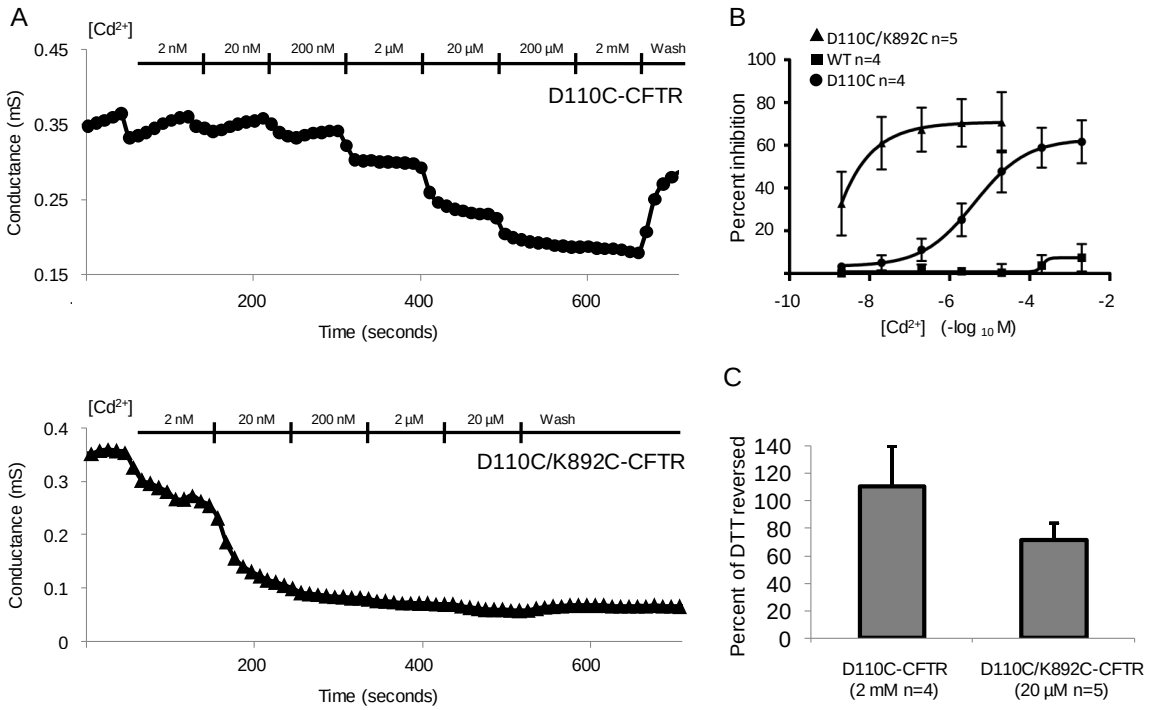
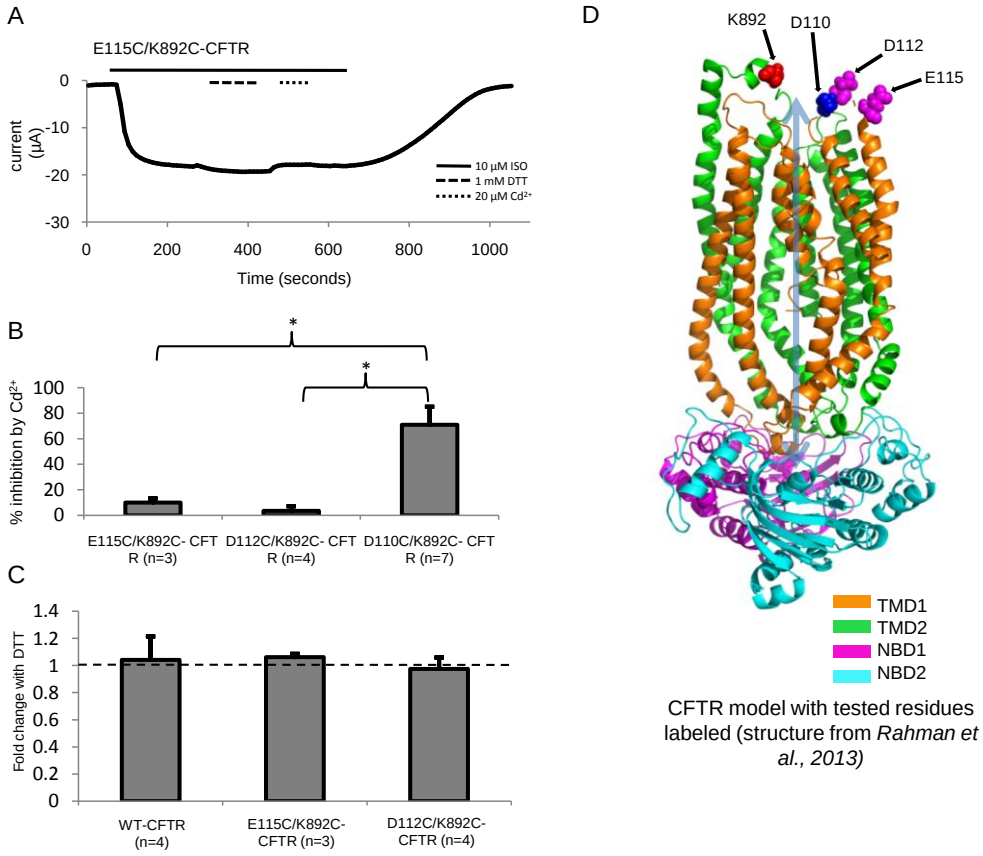


Figure 3.4. Dose response experiments for D110C-CFTR and D110C/K892C-CFTR after DTT and in the constant presence of 10 µM isoproterenol demonstrate that D110C-K892C-CFTR was ~ 1000-fold more sensitive to cadmium. A) Representative records for D110C-CFTR (top) and D110C/K892C-CFTR (bottom) wherein cadmium was applied in the constant presence of 10 µM ISO after 4 minutes of 1 mM DTT. The electrophysiological protocol was identical to that used in Figure 3.1. B) Plot of cadmium dose response; K_d for cadmium in the single mutant D110C-CFTR = 4.49 +/- 1.65 µM; K_d in the double mutant was ≈ 2 nM. C) Quantification of percent of DTT-induced increase that was reversed by maximal cadmium concentration for both D110C-CFTR (2 mM cadmium) and D110C/K892C-CFTR (20 µM cadmium). N-values for each variant as indicated.

(reversible) site contributed only by C110 whose reversibility is observed in the D110C-CFTR traces (Figure 3.3B). These data therefore suggest that, at least in this mutant, ECL1 and ECL4 frequently come within close enough proximity in actively gating channels to enable the coordination of cadmium.

3.1.4 K892C in ECL4 fails to form spontaneous disulfide bonds or coordinate cadmium with two substituted cysteine residues near D110. I next asked whether cysteine linkages could be formed between K892C and other ECL1 cysteine substitutions via either disulfide formation or cadmium coordination. In Figure 3.5A, E115C/K892C-CFTR is shown first exposed to 1 mM DTT, and next to 20 μM cadmium. There was no significant effect of either chemical on macroscopic currents (Figure 3.5B). There also was no significant effect of either DTT or cadmium on D112C/K892C-CFTR (Figure 3.5B,C). Since D110C/K892C-CFTR was sensitive to DTT and cadmium but D112C/K892C- and E115C/K892C-CFTR were sensitive to neither chemical, these results suggest that ECL1 of CFTR is oriented such that N-terminal end positioned closer to ECL4 than the C-terminal end (Figure 3.5D).

3.1.5 A loss-of-charge mutation at D110 affects CFTR open pore stability, but not single channel amplitude. To better understand the mechanisms of potentiation and inhibition of the DTT and cadmium-sensitive CFTR variants, I aimed to record the behavior of single channels in the excised inside-out patch configuration. In order to characterize the effect of loss of the native aspartic acid at D110 on single channel behavior, I first recorded the cystic fibrosis-related mutant, D110H-CFTR. Channels in excised patches were directly activated by bath application of 1 mM ATP and 127 U/ml



CFTR model with tested residues labeled (structure from *Rahman et al., 2013)*

Figure 3.5. Neither 1 mM DTT nor 20 μM cadmium significantly affected the function of other ECL1/ECL4 cysteine double mutants. A) Example trace showing that neither 1 mM DTT nor 20 μM cadmium significantly affected E115C/K892C-CFTR. B) Quantification of inhibition of current by cadmium at V_m = -60 mV after 60 seconds of treatment; an asterisk (*) indicates $p < 0.05$. C) Quantification of potentiation of current at V_m = -60 mV by 1 mM DTT after 3 min of treatment. N-values for each variant as indicated. D) Snapshot at 2.5 ns of a molecular dynamics simulation from our recent study (Rahman et al., 2013) with D110, D112, E115, and K892 highlighted, demonstrates that the orientation of ECL1 is such that D112 and E115 are further from K892 than is D110.

PKA (without co-expression of β2AR). D110H-CFTR opened to a full conductance similar to that of WT-CFTR (Figure 3.6A), a result consistent with that found by another group recording this mutant in lipid bilayers (Hammerle et al., 2001). D110H-CFTR displayed a significantly shorter mean burst duration relative to WT-CFTR; interestingly, the mean burst duration of this variant was very similar to what we recently reported for D110E-CFTR (Figure 3.6B) (Cui et al., 2014). These data, combined with our finding that D110C-CFTR macroscopic currents are unaffected by the positively charged Cys-reactive MTS reagent MTSET (Cui et al., 2014), strongly suggest that D110 does not itself play a direct role in permeation through the CFTR channel. Since mutations at D110 significantly shortened the open burst duration, the residue is clearly involved in stabilizing the open CFTR pore; however, since both a charge-sparing (D110E) and charge neutralizing (D110H) mutation appear to affect open burst stability similarly, there appears to be a specific role for the geometry of the native aspartic acid that is not merely related to charge. Consistent with this idea, Van Goor and colleagues recently demonstrated that D110H-CFTR and D110E-CFTR are modulated by the FDA-approved CFTR potentiator Ivacaftor with nearly identical potency and efficacy (Van Goor et al., 2014) .

3.1.6 Potentiation of D110C-CFTR and D110C/K892C-CFTR by DTT and inhibition by cadmium involve modulation of both the mean burst duration and unitary conductance of the channel. To lay further groundwork for the investigation of the dramatic effects of DTT and cadmium on D110C/K892C-CFTR, we next investigated the mechanism underlying the effects of these chemicals on the single mutant D110C-CFTR via excised patch recordings with single channel resolution. Once again, channels in

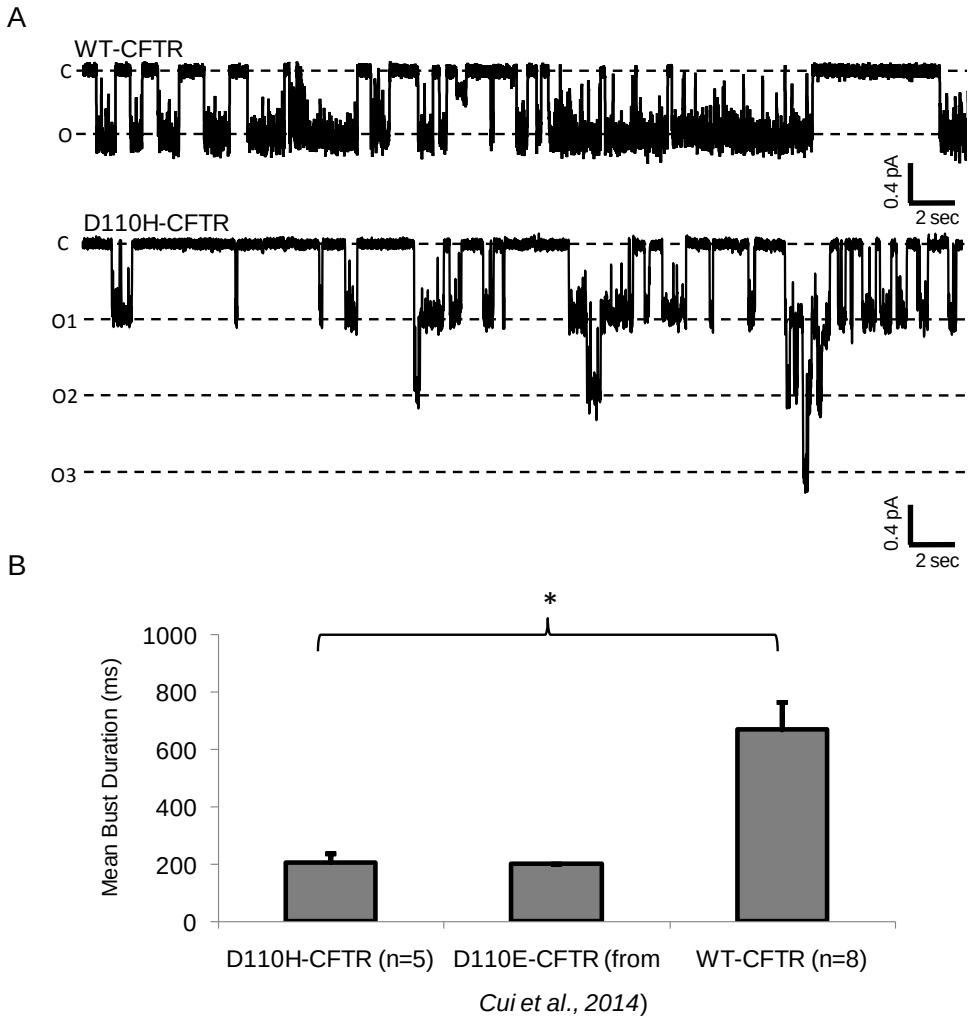


Figure 3.6. D110H-CFTR channels exhibited reduced mean burst durations but conductance levels similar to WT-CFTR. A) Example traces for both WT-CFTR and D110H-CFTR activated by 1 mM ATP and 127 U/ml PKA. Recordings were made at V_m = -100 mV. B) Comparison of mean burst duration among D110 variants; an asterisk (*) indicates $p < 0.05$. Charge-neutralized D110H-CFTR had a similar mean burst duration to charge-sparing D110E-CFTR (reported in (Cui et al., 2014)). N-values for each variant as indicated. c: closed current level; o_1 –o_3 open current levels for one to three active channels in the patch.

excised patches were directly activated by bath application of 1 mM ATP and 127 U/ml PKA (without co-expression of β2AR). Naïve D110C-CFTR channels demonstrated a reduced mean burst duration (154.2 ± 6.1 ms; n=5 patches; Figure 3.7 A,B) compared to WT-CFTR ($p < 0.05$), and a prevalent subconductance of approximately ≈ 80% of full conductance alongside full conductance openings in the same patches (Fig. 3.7A, black *v*s. gray arrowheads, respectively, in upper trace). When D110C-CFTR channels were recorded in the presence of 1 mM DTT in the pipette, I observed an increase in overall mean open burst duration (369.5 ± 16.8 ms; n=4 patches; Figure 3.7B). In addition, the prevalence of the full conductance openings was increased by DTT, as indicated by a comparison of the all-points amplitude histograms. Since long, full conductance openings are favored in the presence of DTT, we suggest that this single channel behavior is indicative of D110C-CFTR channels in the reduced chemical state.

I also investigated the mechanism underlying the reversible cadmium-mediated inhibition of D110C-CFTR via multichannel patch recording. Oocytes were first incubated in 1 mM DTT during shrinking in hypertonic stripping solution with periodic agitation (>10 minutes), then briefly washed in intracellular solution and recorded in the presence of 20 μM cadmium in the patch pipette. As evidenced in the channel records (Figure 3.7A, bottom two traces) and amplitude histogram, cadmium apparently induced a range of subconductance openings in D110C-CFTR channels. Additionally, in the presence of cadmium, D110C-CFTR channels displayed a reduced mean burst duration compared to that in the presence of DTT (134.5 ± 5.8 ms; n = 5 patches; Figure

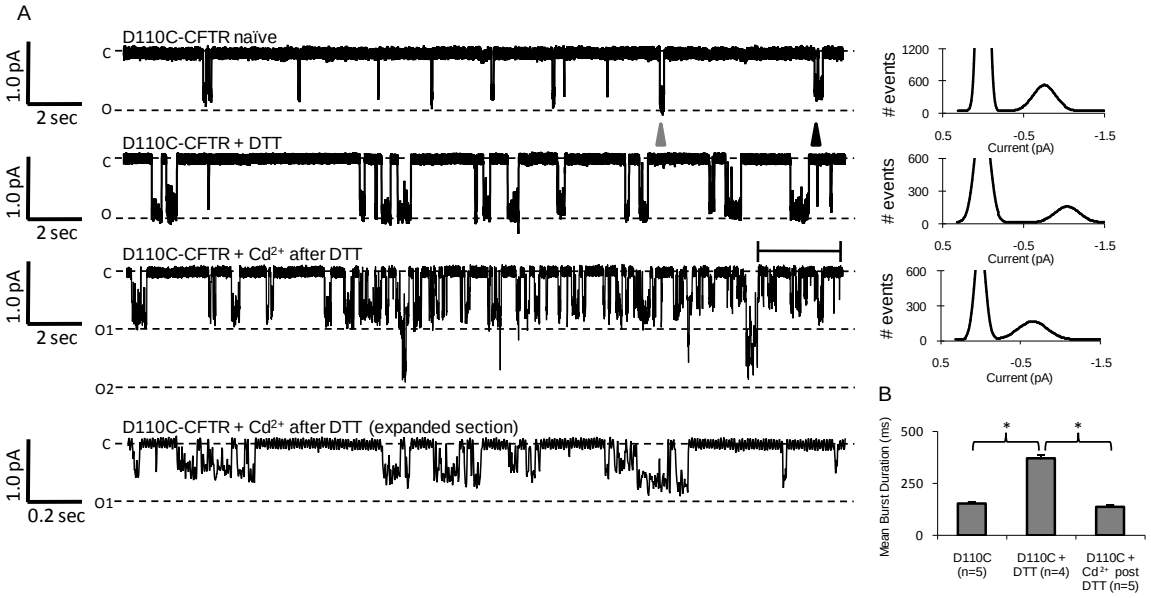


Figure 3.7. D110C-CFTR gating was modulated by DTT and cadmium in a manner consistent with the macroscopic results. CFTR variants were activated by 1 mM ATP and 127 U/ml PKA, and recordings were made at V_m = -100 mV. A) Single channel traces of D110C-CFTR activated by intracellular ATP and PKA and either standard pipette solution (top trace), pipette solution containing 1 mM DTT (middle trace), or pipette solution containing 20 µM cadmium after pre-incubation of the oocyte with 1 mM DTT, all with corresponding amplitude histograms fit to Gaussian distributions. The bottom trace is an expanded representation of the area in the above trace indicated by a bracket. In the naïve channels, subconductance (black arrow) and full conductance (gray arrow) openings were observed in the same patches. B) Quantification of mean open burst duration of D110C-CFTR with each pipette solution condition with N-values for each condition as indicated; an asterisk (*) indicates $p < 0.05$.

3.7B). In general terms, the effects of the inhibitory cadmium on naïve D110C-CFTR channels appear to be opposite of the effects of DTT, which lengthened the burst duration and increased the prevalence of full conductance openings.

In whole oocytes, DTT treatment of D110C/K892C-CFTR channels resulted in a large potentiation in macroscopic conductance (Figure 3.1). When we recorded single D110C/K892C-CFTR channels directly activated by bath application of 1 mM ATP and 127 U/ml PKA (again without co-expression of β2AR) in the absence of DTT, the mean burst duration of untreated channels (155 ± 7.6 ms, n=4 patches; Figure 3.8A,B) was significantly less than that of WT-CFTR ($p < 0.05$). The conductance of the naïve channel varied significantly; there existed full conductance openings, openings of the size of the dominant subconductance from D110C-CFTR channels, and subconductances of lower amplitudes (Figure 3.8A, top trace). D110C/K892C-CFTR channels were also recorded with solution wherein 1 mM DTT was present throughout the pipette. These channels displayed an increased mean burst duration (440 ± 44.1 ms; n=4 patches) compared to naïve channels, and typically opened to a full conductance, as seen in the representative record (Figure 3.8A, middle trace) and corresponding amplitude histogram. Unsurprisingly, these data show that the DTT-treated D110C/K892C-CFTR channels behaved very similarly to DTT-treated D110C-CFTR channels; this is to say, both D110C/K892C- and D110C-CFTR channels opened to a reasonably stable, full conductance state in the presence of DTT, when their cysteine residues were likely reduced. When, after 1 mM DTT exposure, D110C/K892C-CFTR channels were recorded with a concentration of cadmium in the pipette (20 nM) that selectively

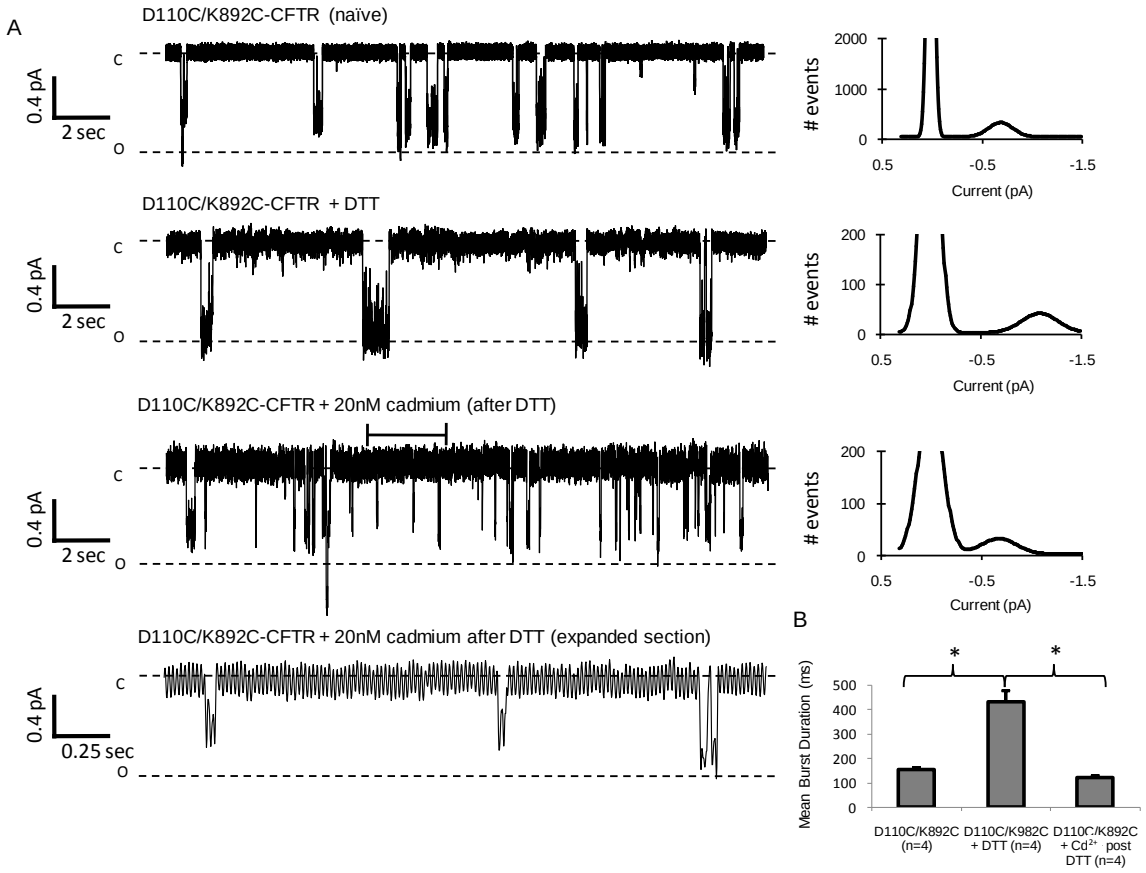


Figure 3.8. DTT and cadmium modulated the single channel behavior of D110C/K892C-CFTR channels. CFTR variants were activated by 1 mM ATP and 127 U/ml PKA, and recordings were made at V_m = -100 mV. A) D110C/K892C-CFTR channels recorded with standard pipette solution opened briefly and to a variable conductance level (top trace). D110C/K892C-CFTR channels in the presence of 1 mM DTT opened stably to a full conductance (middle trace). D110C/K892C-CFTR channels in the presence of 20 nM cadmium opened briefly to a range of subconductances (bottom trace with expanded presentation of bracketed section below). A corresponding amplitude histogram fit to a Gaussian distribution accompanies each trace. B) Quantification of mean open burst duration for each condition with N-values for each condition as indicated; an asterisk (*) indicates $p < 0.05$.

inhibits this double mutant (Figure 3.4), the channels demonstrated a decreased burst duration (122.2 ± 7.0 ms, n = 4 patches) when compared to DTT-treated channels, and exhibited frequent subconductance openings of variable levels (Fig. 3.8A, bottom two traces). The burst duration results strongly suggest that both DTT and cadmium modulate the pore gating kinetics of D110C/K892C-CFTR.

3.2 Conformational restriction between ECL1 and ECL4 alters the sensitivity of CFTR to activation by phosphorylation

Note: The data in this section are published in (Infield et al., 2015).

3.2.1 Naïve D110C/K892C-CFTR channels display a reduced sensitivity to IBMX stimulation that is countered by breakage of the disulfide bond between C110 and C892. As described in the Methods, in all of the whole cell experiments mentioned above, CFTR was activated via robust Gαs signaling through the co-expressed beta 2 adrenergic receptor via isoproterenol. This mode of PKA signaling is highly physiological with respect to CFTR (Bossard et al., 2011; Gonska et al., 2009; Mutlu and Factor, 2008; Quinton et al., 2012; Rowe et al., 2014), but it does require the introduction of another protein and therefore of another variable into the experimental design. One of the reviewers of my manuscript requested that we provide some data confirming that the large macroscopic potentiation of D110C/K892C-CFTR by DTT is independent of β2AR. To address this, we aimed to perform TEVC experiments wherein we activated channels with the phosphodiesterase inhibitor IBMX in the continuing presence of 10 μM of the adenylyl cyclase activator forskolin, as done in several previous studies to elicit

activation of CFTR channels expressed in oocytes (Drumm et al., 1991; Smit et al., 1993; Wilkinson et al., 1996; Wilkinson et al., 1997). In this regard, one advantage of the *Xenopus* oocyte system is that it allows "graded" activation of WT-CFTR in response to increasing concentrations of IBMX in the presence of forskolin (Drumm et al., 1991; Smit et al., 1993), likely due to the higher phosphatase activity in *Xenopus* oocytes relative to mammalian cells ((Weinreich et al., 1997) *vs.* (Al-Nakkash and Hwang, 1999)). I had also recently become aware of data suggesting that mutations disrupting ABC-transporter-conserved tetrahelix bundle formation in CFTR altered the sensitivity of CFTR to PKA (Wang et al., 2014). These data led me to ask whether, in whole oocytes, a conformational restriction such as that mediated by the engineered disulfide bond between ECL1 and ECL4 might affect the sensitivity of CFTR to phosphorylation stimuli.

Interestingly, I found that whereas WT-CFTR was nearly maximally activated by 10 µM forskolin and 0.5 mM IBMX (comparable to previous studies; see (Drumm et al., 1991)), naïve D110C/K892C-CFTR channels were potentiated linearly as [IBMX] was increased up to the highest concentration tested (2 mM; Figure 3.9 A,B). This lack of saturation suggested a lower overall sensitivity of this channel population to the activation stimulus, as has been previously observed in some CF-related CFTR mutants (Drumm et al., 1991). This reduced sensitivity was mirrored by a much faster deactivation rate upon washout of stimuli in naïve D110C/K892C-CFTR channels as opposed to WT-CFTR channels (Figure 3.10).

Importantly, D110C/K892C-CFTR channels activated by 10 µM forskolin and 2 mM IBMX were still highly potentiated by 1 mM DTT without reaching plateau after five

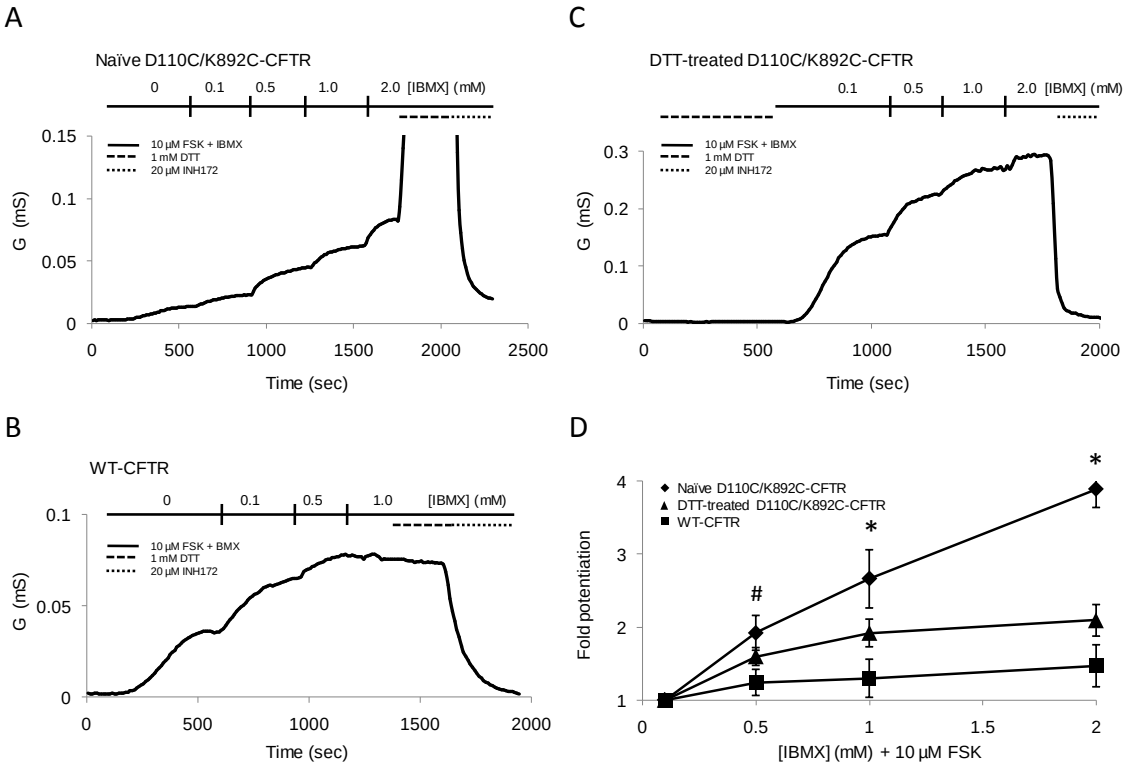


Figure 3.9. An intact inhibitory disulfide bond between ECL1 and ECL4 reduces the sensitivity of D110C/K892C-CFTR to phosphorylation stimulus by IBMX. CFTR currents in whole oocytes were activated by 10 µM forskolin (FSK) and increasing concentrations of IBMX, and 1 mM DTT and 20 µM of the CFTR-specific inhibitor Inh_{172} were added at the indicated times. Conductance is calculated as in Figures 3.1 and 3.4. A) Conductance from naïve D110C/K892C-CFTR channels potentiated by increasing concentrations of IBMX and further potentiated by subsequent application of 1 mM DTT. B) Conductance from WT-CFTR channels showing saturation above 0.5 mM IBMX and lack of potentiation by 1 mM DTT. C) Conductance from DTT-treated D110C/K892C-CFTR channels with increasing concentrations of IBMX. D) Quantification of potentiation of each variant normalized to 0.1 mM IBMX + 10 µM forskolin. N ≥ 3 for each variant. An asterisk (*) indicates $p < 0.05$ between naïve D110C/K892C-CFTR and both DTT-treated D110C/K892C-CFTR and WT CFTR; a pound sign (#) indicates $p < 0.05$ between naïve D110C/K892C-CFTR and WT-CFTR and $p = 0.087$ between naïve D110C/K892C-CFTR and DTT- treated D110C/K892-CFTR.

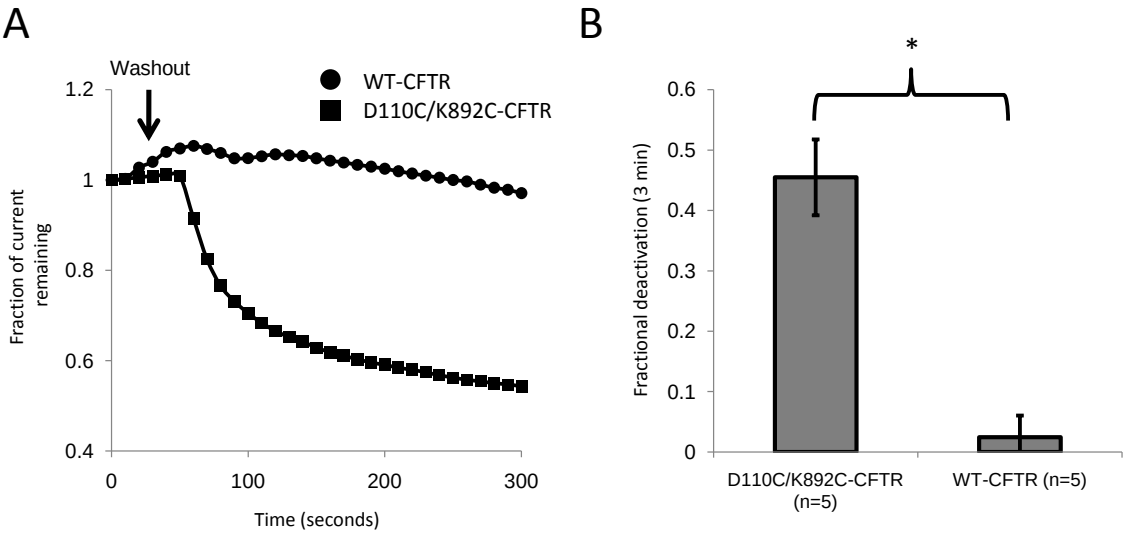


Figure 3.10. Naïve (disulfided) D110C/K892C-CFTR channels deactivated faster than WT-CFTR channels upon washout of activating stimulus. A) At current plateau (arrow), 2 mM IBMX + 10 µM Forskolin was washed out in the presence of ND96 Ringer's solution. B) Quantification of deactivation rates as the fraction of current deactivated after 180 seconds of agonist washout. An asterisk indicates $p < 0.05$

minutes of exposure; there was no significant difference in the degree of potentiation compared to when channels were activated via the β2AR (10.3 +/- 2.5 fold *vs.* 13.0 +/- 3.2 fold, respectively). This suggested that at least the majority of the channel population remained in the disulfided form prior to DTT; *ie.* that high phosphorylation stimulus was not able to break the disulfide bond.

The above results led me to ask whether the sensitivity of this mutant to IBMX would be modulated by DTT. To this end, I treated D110C/K892C-CFTR cells with 1 mM DTT for 10 minutes and then immediately activated with 10 μM forskolin and IBMX, starting with 0.1 mM IBMX to minimize the amount of time between DTT reduction of the disulfide bond and channel activation. DTT-treated channels showed an increased IBMX sensitivity relative to naïve channels, as evidenced by the lower degree of potentiation of macroscopic conductance with increasing concentrations of IBMX (Figure 3.9 C,D). A straightforward interpretation of this normalized IBMX sensitivity relative to WT-CFTR is that the disulfided channels were somehow rendered less sensitive to a given level of phosphorylation stimulus, and that breakage of the disulfide partially restored sensitivity. Additionally, the apparent change in the IBMX sensitivity of D110C/K892C-CFTR upon DTT treatment is further evidence (along with changes in cadmium sensitivity (Figure 3.2) and single channel behavior (Figure 3.8) of this mutant) that disulfided D110C/K892C-CFTR channels *are able to open* (however infrequently and briefly), because if all of the current we measured in naïve cells across the IBMX concentrations arose from a fraction of non-disulfided channels in the population, we would not expect their sensitivity to have been altered by DTT. Altogether, these

results demonstrate that the conformational restriction between ECL1 and ECL4 not only affects the frequency and stability of CFTR opening, but also the requirement of phosphorylation stimulus required for opening. The implications of these data to a model of CFTR regulation are discussed in Section 4.3.

3.3 CFTR lacking the Regulatory R-domain is highly potentiated by the FDA-approved CFTR potentiator Ivacaftor.

Note: the data in this section are not yet published.

Our group has recently become interested in investigating the mechanism of action of Ivacaftor, which is currently the only functional modulator of CFTR demonstrated to show efficacy in CF-patients (Accurso et al., 2010). As discussed in detail in Section 1.4, this drug was discovered through high throughput screening, and as a result very little is known about where the drug binds on CFTR or how it works.

It has been demonstrated that Ivacaftor does not raise cAMP levels in cells (Pyle et al., 2011). Of course, this was already strongly implied by electrophysiological experiments establishing that the drug potentiates CFTR, rather than activating it (Van Goor et al., 2009). However, the dependence of Ivacaftor on the degree of CFTR phosphorylation is not known. In the near future, our group intends to perform IBMX dose-response experiments (similar to the ones I performed in collecting the data in Section 3.2), wherein we will test the effect of Ivacaftor and other drugs on WT and mutant CFTR under different levels of stimulation. These experiments will be coupled to direct biochemical measurements of the phosphorylation status of specific PKA sites

under these different levels of stimulation, yielding powerful data to elucidate this aspect of the mechanism of CFTR modulators.

As a first step to inform these future studies, we reasoned it would be highly informative to test, in our system, the effect of Ivacaftor on a version of CFTR wherein the Regulatory R domain has been removed (Csanady et al., 2000). This so-called "ΔR-CFTR" is still dependent on ATP for gating, but it is not absolutely dependent on PKA-mediated phosphorylation (Chan et al., 2000). Therefore, by using ΔR-CFTR, we can discern what effects the drug may have on the channel that are unrelated to its regulation by the R domain.

To do these experiments, I expressed ΔR-CFTR in *Xenopus* oocytes over a course of three weeks, to mitigate concerns over oocyte batch-specific effects. Consistent with its PKA-independent activity, cells expressing ΔR-CFTR displayed a stable and constitutive chloride conductance (Figure 3.11). Ivacaftor, applied at a concentration of 1 μM, elicited a rapid increase in current that reached plateau in approximately 3 minutes on average (Figure 3.11). The kinetics of potentiation were consistent with those elicited by Ivacaftor on WT-CFTR in *Xenopus* oocytes (Cui and McCarty, 2015). Currents were potentiated 6.35 ± 2.39- fold on average (n=5), and potentiated current was confirmed to be carried by CFTR on the basis of nearly complete inhibition by the CFTR-specific inhibitor Inh_{172} (Figure 3.11). These data demonstrate that the effect of Ivacaftor on WT-CFTR is dependent on neither the existence nor phosphorylation of the R domain.

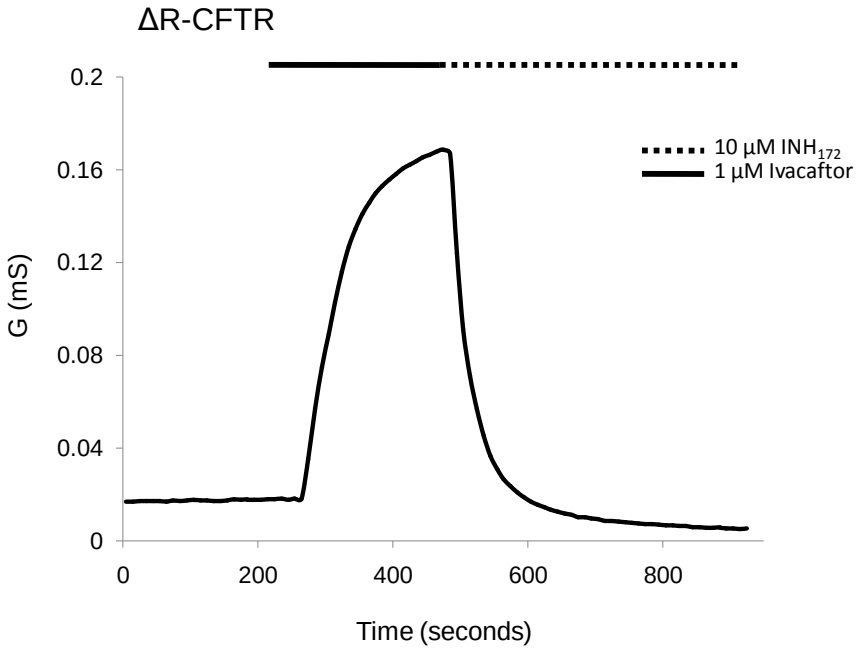


Figure 3.11 Effect of Ivacaftor on an R domain-less version of WT-CFTR. Currents were potentiated 6.35 ± 2.39 fold (n=5), and potentiated current was inhibited by the specific CFTR inhibitor CFTR INH_{172}.

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CHAPTER 4: DISCUSSION

Except for some investigation into the mechanisms of glucose regulation in the airway (Appendix A), my dissertation research has concerned the structure, function, and pharmacology of CFTR. In the following Discussion, I will contextualize these results towards four main conceptual areas. In the first section, I interpret the data concerning the inhibition of CFTR activity through conformational restriction between ECL1 and ECL4 both in terms of what we know about this aspect of the conformational change in ABC transporters, and in terms of a structural model of the CFTR pore. Secondly, I interpret the modulation of single channel behavior of the ECL1 pore mutants in context of an energetic model of pore gating. Third, I discuss the consequence of conformational restriction between ECL1 and 4 with respect to the allosteric modulation of CFTR gating by phosphorylation of its R domain. Fourth, I discuss the relevant mechanistic implications of the observation that the FDA-approved drug Ivacaftor potentiates a version of CFTR lacking its lineage-specific regulatory R domain. In conclusion, I take a step back and consider the possibility, supported by results herein and across the literature, that advances in the understanding of the structural, evolutionary, and regulatory relationships between CFTR and related ABC transporters may unlock keys to the pharmacology of these highly medically relevant proteins.

4.1 A structural model of CFTR pore gating that relates it to the transport cycle of ABC exporters

4.1.1 Both CFTR channel opening and ABC transporter function require relative separation between two extracellular loops. To understand how the conformational changes intrinsic to CFTR gating may relate to ABC transporter function, I conducted cysteine-trapping experiments between regions found to be in close proximity in multiple ATP-free "inward-facing" crystal structures of ABC transporters (ECL1 and 4) (Li et al., 2014; Ward et al., 2007). I found that when D110 in ECL1 and K892 in ECL4 are mutated to cysteine residues, the resulting CFTR variant was highly (>13-fold) potentiated by the reducing agent DTT (Figure 3.1), presumably through breakage of a spontaneous disulfide bond that strongly stabilized a closed state of CFTR. After liberation of these cysteine residues via DTT, D110C/K892C-CFTR rapidly coordinated low concentrations of the soft metal cadmium (Figure 3.4), suggesting that, at least in this variant, C110 and C892 frequently come in reasonably close proximity in actively gating channels. At the single channel level, it was shown that inhibition of D110C/K892C-CFTR via disulfide or cadmium entailed modulation of the gating kinetics and subconductance behavior of the channel (Figure 3.8). A straightforward interpretation of this data is that normal channel opening in CFTR entails some degree of separation between these loops. If so, this constitutes a fifth major area of conformational change known to be shared by CFTR gating and ABC transporter function, added to the four others discussed in Section 1.5 (Figure 4.1).

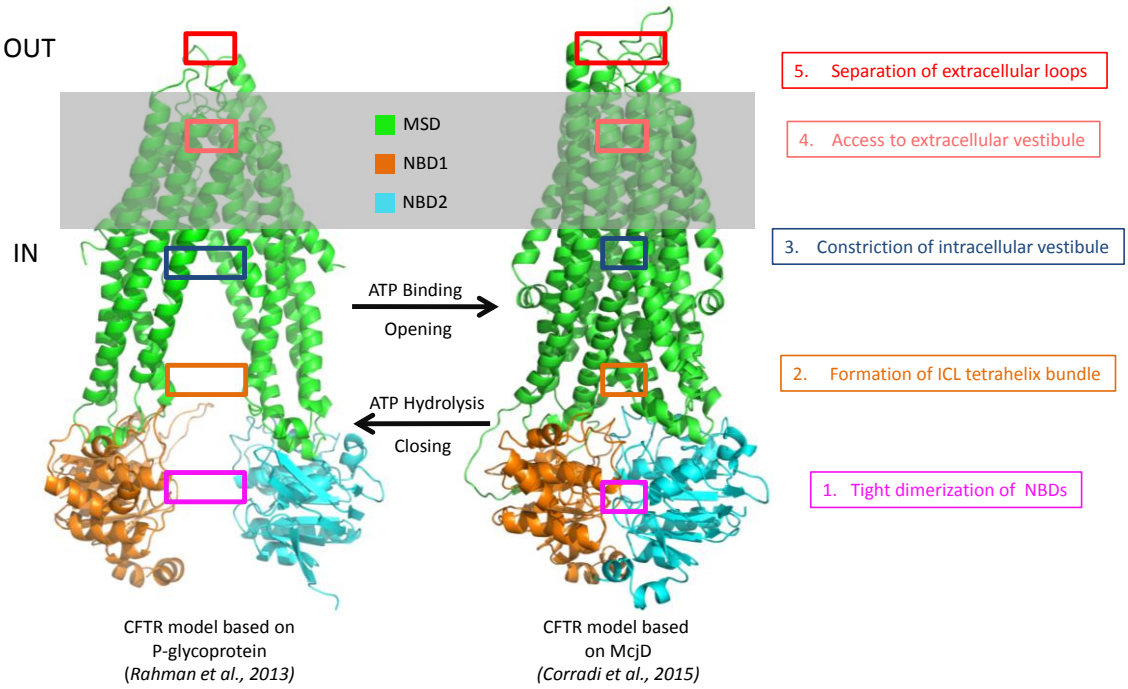


Figure 4.1 Conformational changes shared between gating in CFTR and transport in ABC Transporters. The inward-facing (closed) CFTR model is based on the ATP-free mouse P-glycoprotein structure (Li et al., 2014; Rahman et al., 2013), and the outward-facing (open) CFTR model is based on the substrate-occluded structure of McjD (Choudhury et al., 2014; Corradi et al., 2015). Five loci of conformational change are boxed, adding in the separation of extracellular loops, as established by the work herein. Images generated with PyMol 0.99.

However, with this interpretation, it is important to also consider the limitations of cysteine trapping to study structural proximity. First, since the technique requires cysteine residues to be engineered into the protein sequence, there exists the possibility that these mutations themselves will affect channel function in a way that colors the interpretation of the observed effects of chemical treatments. This is particularly relevant in this study, since D110 is a site of CF mutations D110H and D110E (Van Goor et al., 2014). However, in the presence of reducing agents, the single channel behavior of both cysteine mutants for which effects of chemicals were investigated (D110C-CFTR and D110C/K892C-CFTR) was similar to WT-CFTR, with the exception of a decrease in open burst duration less severe than the CF mutations (*see* Section 3.1). In addition, positive results from this method only directly associate the close proximity of two residues with a particular state; they cannot report the degree or nature of conformational change that occurs between states. Therefore, the data in this dissertation do not tell us the distance of separation of ECL1 and ECL4 that entails channel opening; only that channel opening is not compatible with the close proximity of ECL1 and ECL4 characteristic of the closed state, and thus that some degree of relative movement must occur between them to allow channel opening.

By nature, our data support the functional relevance of close proximity between ECL1 and ECL4 in CFTR. However, I must note that despite having been observed in the crystal structures of P-glycoprotein and MsbA, the physiological relevance of the proximity of these ECLs (and indeed, of the inward-facing ATP-free conformation of ABC exporters as a whole) has been questioned, primarily on the basis that ATP is ever-

present in the cell (George and Jones, 2012). However, recent functional studies have demonstrated that, similarly to CFTR, spontaneous disulfide bonds between ECL1 and ECL4 inhibit the function of both of the above ABC transporters (Doshi et al., 2013; Loo and Clarke, 2014). These functional studies provide further evidence that these ECLs do come into close proximity in cells and that their relative separation is required for substrate transport.

4.1.2 The orientation of ECL1 in CFTR may match that of ABC exporters. In several crystal structures of ABC exporters with 12 transmembrane helices, the helixes are arranged such that the N-terminal end of the ECL1 loop is facing the substrate pathway (the protein interior), while the C-terminal end is distal to the substrate pathway. This orientation of ECL1 is observed in both inward- and outward-facing crystal structures of these proteins (Hohl et al., 2012; Hohl et al., 2014; Li et al., 2014; Ward et al., 2007). In the experiments done herein, neither potentiation by DTT nor inhibition by cadmium was observed in double cysteine mutants wherein the ECL1 cysteine residue was engineered in a position C-terminal to D110 (Figure 3.5). Assuming that these negative results are due to lack of proximity of these pairs and not to the less likely alternative that their proximity is without functional effect, the data suggest that only the N-terminal (pore-facing) end of ECL1 of CFTR ever comes close enough to ECL4 to be linked (Figure 4.2). This orientation may also explain why, in addition to its possible engagement in a pore-stabilizing electrostatic interaction with E1126 (Section 1.2.4), R117 does not act to electrostatically attract chloride into the pore; it is likely positioned too far from the permeation pathway to elicit a direct effect on permeation (Figure 4.2).

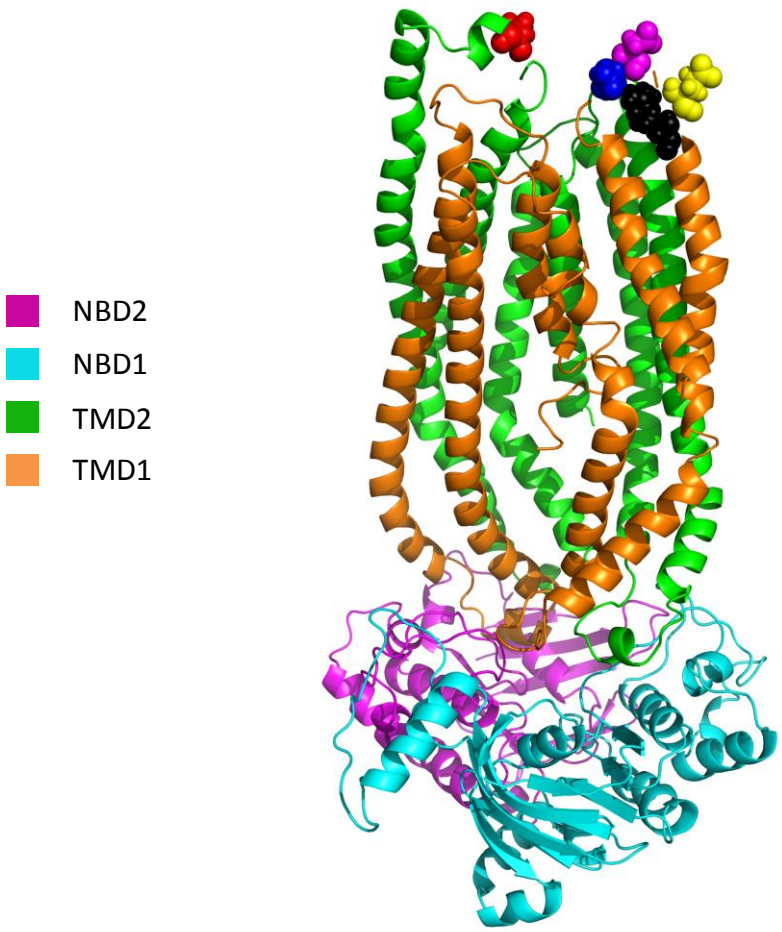
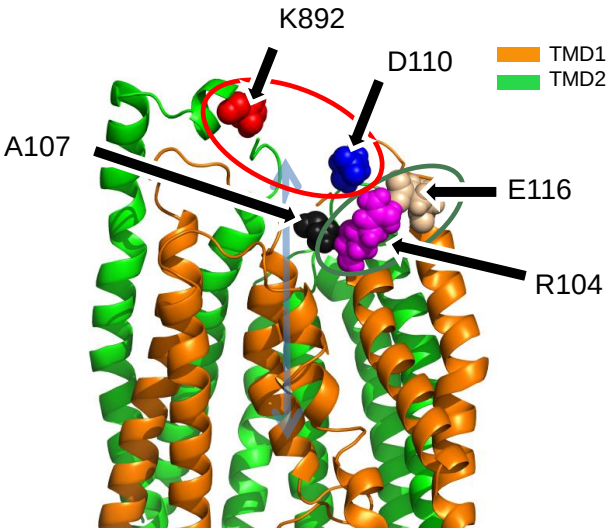


Figure 4.2. Relative position of ECL residues in a homology model of CFTR. Snapshot at 2.5 ns of a molecular dynamics simulation of CFTR (Rahman et al., 2013) with D110 (dark blue), D112 (magenta), E115 (yellow), R117 (black) and K892 (red) highlighted, suggests that the orientation of ECL1 is such that D112 and E115 are further from K892 than is D110.

4.1.3 A potential structural mechanism for the impact of ECL1 positioning on pore gating. Inhibition of CFTR by constriction of ECL1 and ECL4 allows us to draw analogies between the conformational dynamics of CFTR and ABC exporters. But from a structural standpoint, but it is not immediately obvious how modulating the position of an extracellular loop, from a residue position (110) neither intrinsically important for permeation nor appreciably affected by single residue modification (Cui et al., 2014; Gao et al., 2013), so profoundly affects pore gating. It is possible, but hard to envision, that one linkage formed between apposing extracellular loops could alone preclude the gross transition from inward- to outward-facing TMDs proposed to occur as a result of ATP binding and NBD dimerization (Figure 4.1).

A clue into the specific role of ECL1 may be found in the recent characterization of transmembrane helix 1 (TM1) residues positioned immediately cytoplasmic to D110. Specifically, Gao and coworkers (Gao et al., 2013) found that the conductance of A107C-CFTR was sensitive to pH, a trait normally characteristic of cysteine substitutions of residues intimately involved in chloride permeation through CFTR, such as T338C (Liu et al., 2004). If the pH sensitivity of A107C-CFTR is similarly indicative of an important role of A107 in the permeation pathway through CFTR, then a perturbation of the position of this part of TM1, perhaps as modulated by the position of the adjacent ECL1, may be expected to significantly affect the state of the pore. Therefore, it is certainly reasonable that introducing a strong interaction between nearby C110 and another amino acid, particularly C892 across the pore, would perturb the pore in such a way as to disrupt chloride flux (Figure 4.3).



CFTR pore model with discussed residues labeled (structure from *Rahman et al., 2013)*

Figure 4.3. Structural interpretation of cross-linking results in model of pore gating. The structural model is built from the coordinates of a 2.5 ns snapshot of a molecular dynamics simulation of CFTR previously published by our group (Rahman et al., 2013). This study suggests that close proximity between cysteine substituted residues at positions 892 and 110 (red circle) stabilizes a closed state of the pore, while we previously demonstrated that close proximity between cysteine residues at positions 104 and 116 (green circle) stabilizes an open state of the pore. Both linkages modify pore gating behavior via impacting the position of the extracellular end of the first transmembrane helix of CFTR, possibly by altering the position of A107 (black), an amino acid predicted to lie in the permeation pathway for chloride ions.

Further, in our 2014 study, we found that a spontaneous disulfide bond between R104C and E116C resulted in R104C/E116C-CFTR channels being "locked open" unless the disulfide bond was broken with DTT (Cui et al., 2014); these residues are located in TM1 and ECL1, respectively. Considering that these residues likely participate in an electrostatic interaction in the WT-channel (as suggested by charge-swapping experiments (Cui et al., 2014)), one structural consequence of the substitution of the interaction between the large endogenous side chains with a cysteine disulfide would be to bring the associated regions of the protein closer together, with the functional effect of "pinning the pore open." Overall, the antagonistic effects of a disulfide or cadmium linkage between C110 and C892 (Figure 4.3; red circle) stabilizing a *closed* state of CFTR, versus a disulfide between C104 and C116 (Figure 4.3; green circle) stabilizing an *open* state of CFTR, support a pore gating model wherein the position of the ECLs and the extracellular end of TM1 serves as a critical determinant of permeation through the CFTR pore.

Finally, note also that in general, the results for the single mutant D110C-CFTR (mild potentiation by DTT and reversible inhibition by cadmium) also independently support the importance of the position of this region of ECL1 on CFTR pore gating and architecture, since both treatments affected the mean open burst duration and incidence of subconductances in this variant (Figure 3.7). The potentiation of D110C-CFTR by DTT is not likely due to an interaction between C110 and an endogenous cysteine residue, as experimentally supported alignments fail to locate any endogenous cysteine residues near the extracellular side of the CFTR channel (Alexander et al., 2009;

Rahman et al., 2013). It is important to note that the effects of these chemicals on D110C-CFTR were quantitatively different to that on D110C/K892C-CFTR (*see* Figure 3.1 and 3.4). As such, they do not confound the interpretation of the double mutant data discussed above. But since we were unable to conclusively determine (among many chemical possibilities (Eiamphungporn et al., 2009; Lee et al., 2007; Liu et al., 2006; Zhou et al., 2009)) the nature of the modulation of D110C-CFTR by DTT, nor in exploratory experiments to identify any endogenous amino acids participating in these effects, we limit the structural interpretation of the effects of DTT and cadmium on the single mutant to the conservative suggestion that in the absence of the K892C mutation, an interaction between C110 and an as yet unidentified part of the protein destabilizes the channel open state and distorts the pore.

4.2. Integrating the present studies into an energetic model of CFTR pore gating.

4.2.1 The behavior of CFTR ECL1 mutants is characterized by equilibrium gating of an unstable pore. The gating behavior of ligand-gated ion channels has been modeled in terms of reversible transitions between open and closed conformations at given free energy levels (G°) (Grosman et al., 2000). Figure 4.4 shows a plot of an idealized energy landscape for a ligand-gated channel in the presence of agonist and the presence, or absence, of a structural perturbation (such as that introduced by a positive allosteric modulator) that stabilizes the open state relative to the closed state of the channel. Although electrophysiology cannot generate information as to the absolute energy levels of different channel states, several observable parameters of single

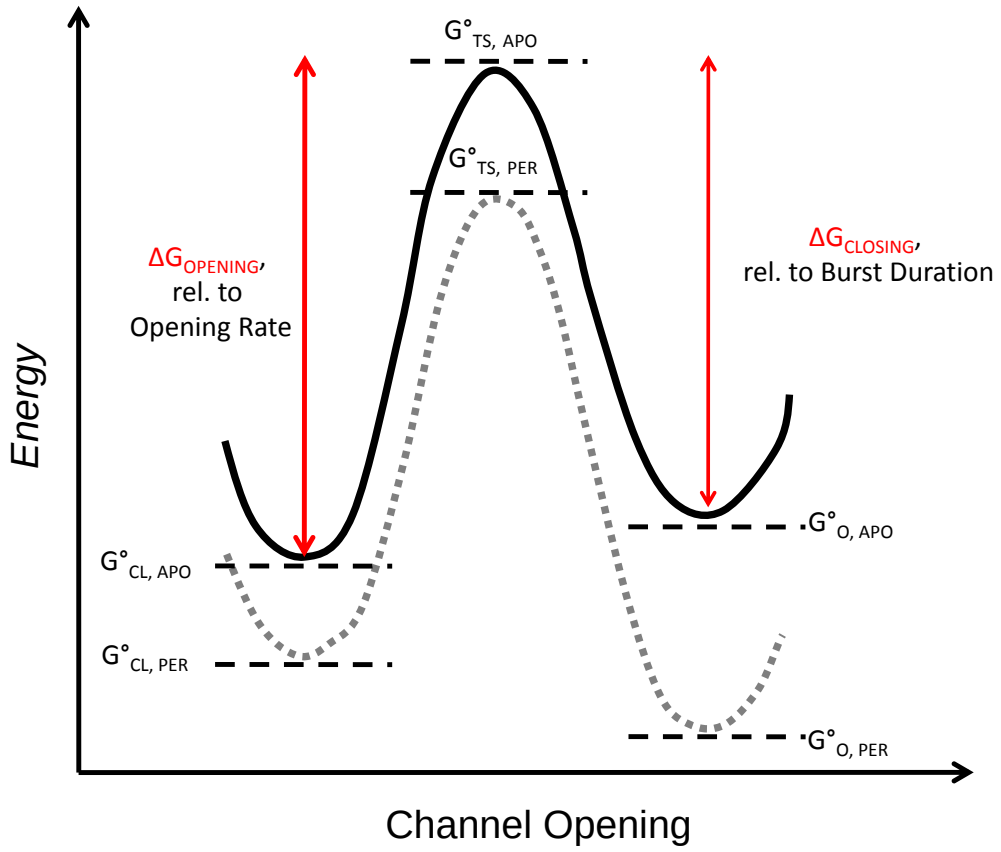


Figure 4.4. Energy landscape of equilibrium ion channel gating in the presence and absence of structural perturbation. Adapted from (Grosman et al., 2000). $G°_{CL}$ = free energy of closed state, $G°_{O}$ = free energy of open state, $G°_{TS}$ = free energy of transition state. APO = absence of perturbation, PER = presence of perturbation. $\Delta G_{CLOSING}$= free energy change for channel closure; proportional to closing rate, which, in turn, is proportional to the observed open burst duration of the channel. $\Delta G_{OPENING}$ = free energy change for channel opening; proportional to the observed opening rate of the channel.

channel behavior relate proportionally to ΔG° values that characterize the energy required for the channel to transition between states (Figure 4.4). Studies utilizing these models have provided insights into the conformational dynamics intrinsic to ion channel gating (Grosman et al., 2000; Mitra et al., 2005a; Mitra et al., 2005b), which may be altered by mutations and drugs.

As mentioned in the introduction, one of the ways that the gating of CFTR is unique is that, at least in the WT channel, closure is highly dependent on the hydrolysis of bound ATP at the competent ABS2 site (Section 1.2.4, Section 1.5.2). It has been emphasized throughout this dissertation that the dependence of CFTR NBD de-dimerization and channel closure on ATP hydrolysis has provided a useful basis for relating the structures and conformational changes between CFTR and ABC Transporters. However, the enzymatic closure CFTR has also complicated analyses of the energetics of CFTR function. Since CFTR channel closing is dependent on the enzymatic process of ATP hydrolysis, channel gating cannot be thought of as a truly equilibrium process compatible with free energy modeling.

Recently, Sorum and Csanady overcame this hurdle to studying the energetics intrinsic to CFTR gating by recording from CFTR channels wherein ATP hydrolysis was abolished via a mutation in the NBDs (Sorum et al., 2015). In order to be able to observe channel closure in the absence of competent hydrolysis, they made a mutation (D1370N) that both abolished ATP hydrolysis and destabilized the NBD dimer relative to WT-CFTR (Sorum et al., 2015). Despite this caveat, Sorum and Csanady's study provided very interesting evidence of a longitudinal conformational wave intrinsic to

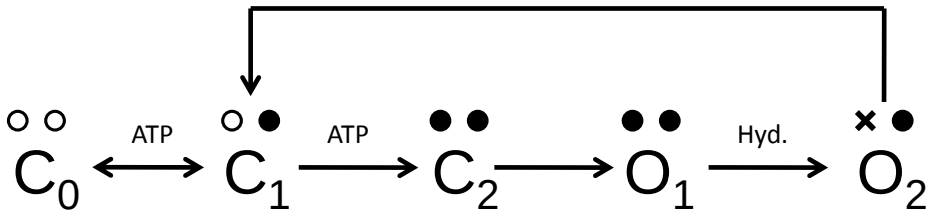
CFTR opening that begins in the intracellular NBDs and is transmitted to the TMDs (Sorum et al., 2015), first suggested by computational work in the McCarty lab (Rahman et al., 2013). However, it also highlighted the utility of a complementary approach wherein CFTR gating may be converted to equilibrium in the context of normal NBD function.

Many mutants of CFTR display very brief open burst durations as compared to WT-CFTR. We previously found that application of the poorly-hydrolyzable ATP analog AMP-PNP increases the overall open probability of ECL1 mutants D110R-, E116R, and R117A-CFTR, without lengthening their brief burst durations (Cui et al., 2014). These data suggest that, in these mutants, the NBDs dimerize as normal, but that most observed closures are not dependent on (or due to) ATP hydrolysis. Therefore, in channel variants wherein open burst durations are brief compared to WT-CFTR (due to defects in pore stability), it seems reasonable to argue that the gating observed is in fact *at equilibrium* (Figure 4.5B) between an unstable open pore and a relatively more stable closed pore. It is therefore possible to model the dominant reversible gating behavior of these mutants in terms of an energy landscape.

4.2.2 Modeling the changes in the energetics of pore gating induced by conformational restriction at ECL1/ECL4. Figure 4.6 shows proposed energy landscapes of D110C/K892C-CFTR in both its cysteine-reduced state and disulfided state. Since I observed changes in the gating kinetics of this variant in the presence and absence of DTT, we know that in both conformations, it is possible for this mutant to open; *i.e.,* to

A

WT-CFTR (non-equillibrium gating dominant)



B

Pore gating mutants of CFTR (equillibrium gating dominant)

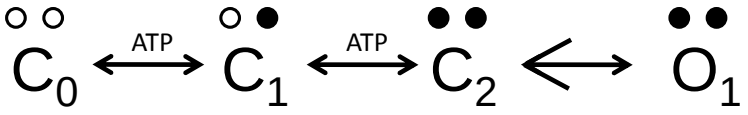


Figure 4.5 Schemes of hydrolytic *vs.* non-hydrolytic pore gating in CFTR. A) Non-equillibrium, hydrolytic gating of WT-CFTR, as supported by the studies discussed in Section 1.2.3. B) In mutants with unstable pores, nucleotides bind the NBDs of the channel, which elicits dimerization and pore opening, but these openings are unstable (even in the presence of non-hydrolyzable analogs), so the majority of closures are independent of hydrolysis.

inhabit free energy minima corresponding to both the open and closed states (Figure 4.6, $G°_{CL}$ and $G°_{O}$). An intact disulfide between C110 and C892 renders the open pore more unstable relative to the reduced channel, as evidenced by the reduced open burst durations in single channel recordings of DTT-naïve channels (Figure 3.8). I can model this behavior in terms of the energy barrier that must be overcome for pore closure; this change is smaller in disulfided versus reduced channels (Figure 4.6, $\Delta G_{CLOSING,\ S=S}$ *vs.* $\Delta G_{CLOSING,\ RED}$).

In addition, consider that the observed >13-fold increase of macroscopic D110C/K892C-CFTR conductance in the presence of DTT cannot be accounted for solely by changes observed in the open channel behavior between naïve and DTT-treated channels (an approximately 3-fold increase in open burst duration and 25% increase in single channel conductance), especially when one considers that the effects of DTT upon macroscopic currents reported here were limited to that elicited by 5 minutes of exposure (Figures 3.1 and 3.9). In a previous report, we found that when D110C/K892C-CFTR channels were exposed to DTT backfilled in the pipette of a multichannel patch, the number of channel openings observed in the presence of ATP and PKA was significantly increased late in the recording (upon DTT presumably reaching the channels) (Cui et al., 2014). In combination, these results strongly suggest that in addition to destabilizing the open state of D110C/K892C-CFTR, a disulfide between C110 and C892 significantly lowers the opening rate of the variant relative to that observed after the disulfide is broken with DTT. This is expressed in the model by a larger

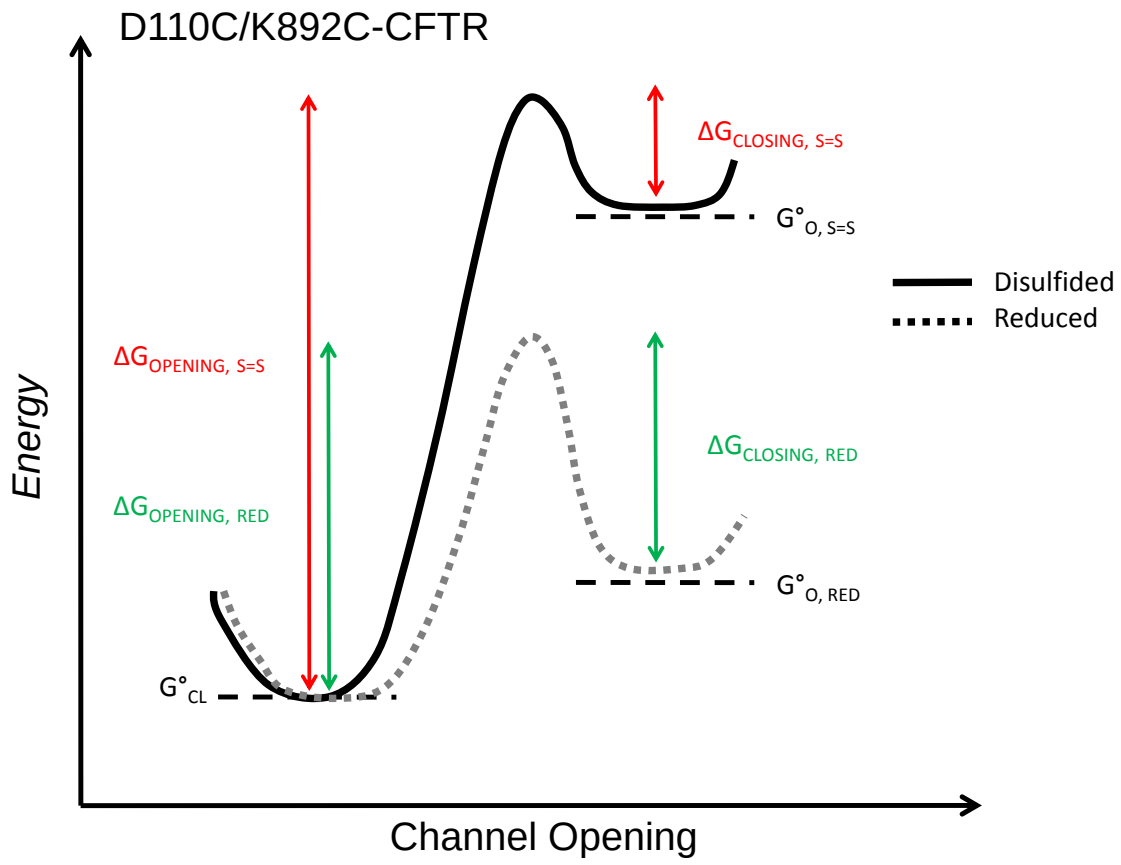


Figure 4.6. Energy landscape of D110C/K892C-CFTR in the presence and absence of conformational restriction between ECL1 and ECL4. G°_{CL} = free energy of closed state, G°_{O} = free energy of open state. S=S = disulfided, RED = reduced. $\Delta G_{CLOSING}$= free energy change for channel closure. $\Delta G_{OPENING}$ = free energy change for channel opening.

for disulfided channels than for reduced channels (Figure 4.6, $\Delta G_{OPENING,\ S=S}$ *vs.* $\Delta G_{OPENING,\ RED}$). It is more difficult and less important to determine where the two curves are positioned relative to each other overall on the Y-axis, because we do not know what effect the disulfide bond has in stabilizing the overall conformation of the protein. One might argue that the extra disulfide bond might increase the overall stability of the protein, bringing the entire curve down somewhat (similar to that of the allosteric modulator in the model in Figure 4.4). However, this is an engineered rather than endogenous disulfide bond, and it is possible that its introduction destabilizes another area of the protein, which would raise G° overall. Therefore, to express both curves on the same axes, I am assuming a negligible difference in the free energy of the closed states of the disulfided and reduced channels (Figure 4.6, $G°_{CL,\ S=S}$ *vs.* $G°_{CL,\ RED}$). Putting all of this together allows us to express the effect of conformational restriction at ECL1 and ECL4 in terms of the energetics of the pore gating of CFTR.

A major consideration to studying CFTR gating via methods that may require making additional mutations into a background of a channel with an unstable pore is that the information gained may be specific to the (defective) pore gating of the background and may not be easily translatable to the wildtype channel; but considering the abundance of class IV mutants known to cause disease that display defective pore gating (Table 1.1), this is not a crippling limitation. Although the model above relates to a cysteine cross-linking experiment, there is no reason why a similar approach could not be used to investigate the energetic signaling inherent to the pore gating of CF-related CFTR mutants such as D110H-CFTR (Figure 3.6) or R117H-CFTR, as well as to investigate

the modulation of these mutants as elicited by drugs such as Ivacaftor (Van Goor et al., 2014).

4.3 Evidence for allosteric modulation of CFTR activity by R domain phosphorylation.

As compared to our understanding of how CFTR is gated by ATP, the mechanism of regulation of CFTR by the R domain remains poorly understood, despite its vital physiological role. In experiments presented in Section 3.2 of the results, I observed an apparent reduction in the sensitivity of naïve D110C/K892C-CFTR channels to IBMX relative to WT-CFTR. Interestingly, after treatment with DTT, the channels were rendered apparently more sensitive to stimulation by IBMX (Figure 3.9). IBMX is thought to elicit CFTR current by inhibiting phosphodiesterases and therefore raising levels of cAMP in cells, leading to stimulation of endogenous PKA (Drumm et al., 1991), although it also may inhibit the phosphatases that dephosphorylate CFTR (Becq et al., 1993). The net effect of both of these functions of IBMX is to increase the phosphorylation of the channel. Of note, IBMX has been demonstrated to block and possibly directly potentiate CFTR, although neither effect is significant at IBMX concentrations wherein differences between variants were observed in the present study (Schultz et al., 1999; Wilkinson et al., 1996) (Figure 3.9).

The difference in IBMX sensitivity could be due to a difference in propensity of the WT-, disulfided D110C/K892C-, and DTT-treated D110C/K892C-CFTR conformations to be phosphorylated. This possibility can only be truly tested via direct biochemical measurement of the extent of R domain phosphorylation elicited in each variant at

different concentrations of compounds such as IBMX. But lacking evidence of any kind that mutations in the pore of CFTR affect the propensity of the channel to be phosphorylated, we interpret the data in light of CFTR's "graded" activation by phosphorylation (Section 1.2.2) in that disulfide-mediated restriction of ECL1 and ECL4 alters the degree of phospohorylation (*i.e.,* fraction of sites phosphorylated) that is necessary to reach a given level of activity. A concordant interpretation was advanced in a recent study by the Kirk group, wherein they observed that molecular disruption of an important electrostatic interaction in another area of CFTR (the intracellular loops) rendered the channel far less sensitive to PKA than WT-CFTR (Wang et al., 2014).

4.4 Insights into the mechanism of action of Ivacaftor enabled by WT-ΔR-CFTR

As discussed in Section 3.3, the data I collected implicating an effect of conformational restriction between ECLs on sensitivity to PKA-mediated activation led me to investigate the literature regarding allosteric models of CFTR regulation by phosphorylation of its R domain. Several contemporary reports had suggested that the efficacy of several related drugs was observed to be greater in weakly-phosphorylated as opposed to strongly-phosphorylated channels (Wang et al., 2005; Wang et al., 2014). Moreover, a member of our group had recently found that this also appeared to be characteristic of the mechanism of action of two investigational CFTR potentiators *(Cui et al., 2016 submitted)*. I became interested in characterizing the phosphorylation dependence of the action of the FDA-approved CFTR potentiator Ivacaftor, and as a first step I decided to test the effect of this drug on a version of CFTR lacking the R-domain.

4.4.1 Mechanistic implications of the potentiation of ΔR-CFTR by Ivacaftor. I found that ΔR-CFTR was indeed potentiated by Ivacaftor (Figure 3.11). This observation has two straightforward implications. First, the R domain is likely not required to mediate binding between Ivacaftor and CFTR and therefore likely not a significant contributor to the binding site of the drug in the channel. Secondly, wherever Ivacaftor does bind (on the rest of the protein), it likely does not function to alter (block or facilitate) the previously described phospho-sensitive interactions between the R domain and the rest of the protein proposed to be involved in channel regulation (Baker et al., 2007; Bozoky et al., 2013). This is an important point, because it suggests that the proposed allosteric effects of Ivacaftor and of high phosphorylation of the R-domain (Wang et al., 2014; Wang et al., 2010) are likely complementary, rather than redundant.

4.2.3 Degree of potentiation of ΔR-CFTR by Ivacaftor suggests that the efficacy of the drug may be dependent on the initial activity of the CFTR variant.

Two related factors led me to initially hypothesize that ΔR-CFTR would be, at best, very weakly potentiated by Ivacaftor. First, both our group and others had previously reported that highly phosphorylated human WT-CFTR channels were only weakly potentiated by Ivacaftor (1.1 to 1.45-fold), either when recorded from cells (Cui and McCarty, 2015; Van Goor et al., 2014) or excised patches (Cui and McCarty, 2015). Second, it had been previously shown that ΔR-CFTR channels mimic phosphorylated WT-CFTR channels in that they are essentially constitutively active and their activity is only very weakly stimulated by PKA (Bompadre et al., 2005; Csanady et al., 2000). Contrary to my hypothesis, I observed that ΔR-CFTR channels were highly (as much as 7-fold)

potentiated by Ivacaftor (Figure 3.11); this was much higher than we or others had previously observed for WT-CFTR. What accounts for this difference? At least in oocytes, although ΔR-CFTR activity is essentially independent of PKA, its maximum open probability is still significantly lower than that of WT-CFTR (Csanady et al., 2000). The reduction in open probability appears to be the result of defective pore stability in ΔR-CFTR relative to WT-CFTR, because while the maximal opening rate of the variants is equivalent, ΔR-CFTR displays a reduced mean burst duration and ability to be locked open by AMP-PNP (Csanady et al., 2000). Therefore, whereas ΔR-CFTR reasonably mimics PKA phosphorylated WT-CFTR, its gating is still impaired.

The data demonstrating that the gating-impaired ΔR-CFTR is more highly potentiated than WT-CFTR by Ivacaftor suggests on its surface that the efficacy of Ivacaftor may be inversely related the open probability of a CFTR variant in the absence of the drug. This idea is supported by data from two recent studies of the effect of Ivacaftor on macroscopic currents of 44 relatively normally-processed CF-related missense mutants of CFTR expressed in Fisher Rat Thyroid (FRT) cells (Van Goor et al., 2014; Yu et al., 2012). In Figure 4.7, I have plotted the data collected in these studies with the degree of potentiation by Ivacaftor as a function of the initial activity level of a given mutant. A pattern is seen to emerge wherein mutants with lower initial activity were, in general, more highly potentiated than those whose initial activity was observed to be closer to WT-CFTR. In fact, if one considers as a group the all the mutants for a given initial activity level wherein Ivacaftor is reportedly most efficacious (Figure 4.7,

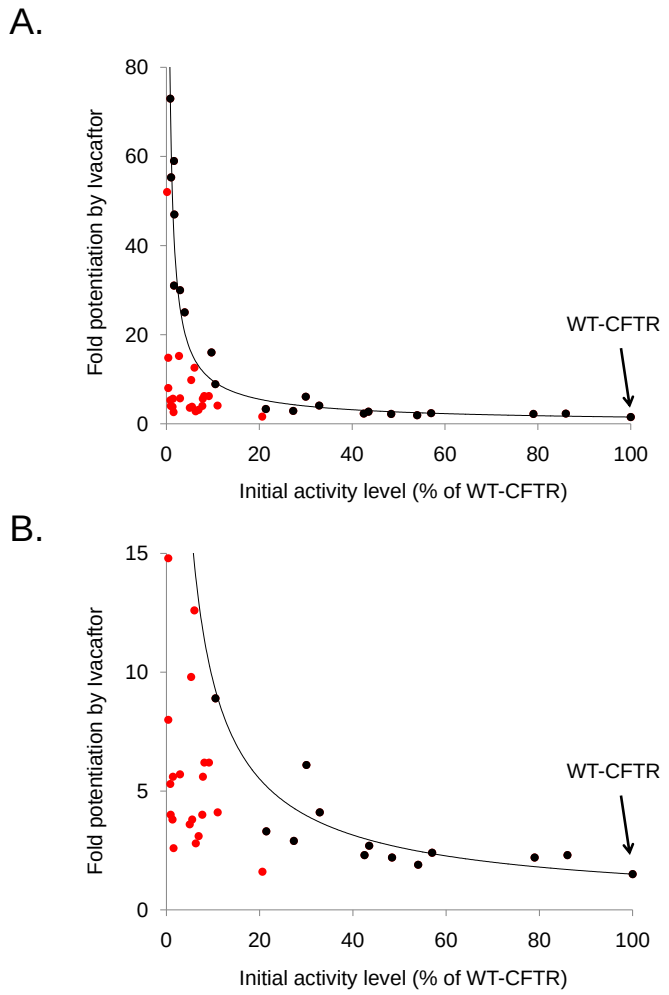


Figure 4.7. Maximal efficacy of Ivacaftor on a panel of missense mutants with activity below WT-CFTR in the absence of the drug. The data used in this graph were culled from two studies of Ivacaftor wherein multiple missense mutants of CFTR were expressed in FRT cells and recorded in Ussing chambers (Van Goor et al., 2014; Yu et al., 2012). CFTR was activated by 10 µM forskolin in all cases (Van Goor et al., 2014; Yu et al., 2012). A) Entire view of range of potentiation elicited by various mutants, with data points corresponding to most highly potentiated mutants at a given initial activity level (black) fit to a rational function. B) Same data as in A, but the ordinate is truncated to better visualize weakly potentiated variants. Variants whose potentiation by Ivacaftor is suboptimal relative to other variants with similar initial activity level are colored as red points and are listed in Table 4.1.

black data points), these data fit very well to a simple rational function that would be expected to characterize an inverse relationship between drug efficacy and intrinsic activity (Figure 4.7, black line, $R^2 = 0.965$).

This observation has two possible implications. First, Ivacaftor may bind to a (more or less) common site in all mutants and promote the adoption or stabilization of a similar open state conformation in those mutants. If so, efficacy may be theoretically dependent on the dynamic range established by the severity of the gating defect in each mutant. Second, some of the mutants that display efficacy lower than predicted by their initial activity level (Figure 4.7, red data points) may feature structural defects that preclude them from being "fully repaired" by the drug. These mutants still apparently bind Ivacaftor (Table 4.1), and thus may inform future studies of drug binding and modulation of the single channel behavior of CFTR. In this regard, most informative would be variants like D110H-CFTR, wherein mutation affects neither single channel conductance (Figure 3.6) nor protein processing (Hammerle et al., 2001).

4.5 Conclusions:

The experimental studies in this dissertation began with a question pertaining to the structural relationship between CFTR and related ABC transporters (Section 3.1). The results of the experiments testing that question ultimately led to other insights pertaining to the regulation (Section 3.2) and pharmacology (Section 3.3) of CFTR. In part motivated by my own experience, I would like to conclude with a "big picture"

Table 4.1 Binding affinities of suboptimally potentiated CFTR mutants for Ivacaftor

Variant	**EC_{50} for Ivacaftor (nM)** [a]
R1070Q	162 +/- 12
D110E	164 +/- 20
D110H	249 +/- 59
R1070W	158 +/- 48
P67L	195 +/- 40
E56K	123 +/- 33
F1074L	141 +/- 19
A455E	170 +/- 44
S945L	181 +/- 36
S977F	283 +/- 36
R347H	280 +/- 35
L206W	101 +/- 13
R117C	380 +/- 136
R352Q	287 +/- 75
R1066H	390 +/- 179
T338I	334 +/- 38
R334W	259 +/- 103
I336K	735 +/- 204
H1054D	187 +/- 20
F508del	**129 +/- 38**[b]
E92K	198 +/- 46
L927P	313 +/- 66

a. All data values culled from (Van Goor et al., 2014).
b. F508del-CFTR was the variant of CFTR that Ivacaftor was screened and optimized against. This affinity may therefore be thought of as the “ideal” for the drug.

discussion of why approaching the study of proteins such as CFTR from the perspective of their evolution is more than a purely academic exercise and is useful in a biomedical context.

Although the research in this dissertation focuses on only one member of the ABC transporter family, CFTR (ABCC7), many others have been implicated in disease, including close relatives, such as P-glycoprotein (ABCB1), and multidrug resistance proteins 1, 4, and 5 (ABCC1, 4, 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 ported to another is an important consideration in both the discovery and mechanistic understanding of therapeutics directed against these proteins.

As no high resolution structures of any ABCC protein currently exist, one obvious application of this knowledge is in the construction and refinement of ABC Transporter-based homology models that will be used as templates for *in silico* drug screening. Indeed, by screening against the critical NBD1-ICL4 interaction thought to be disrupted by deletion of F508 in CFTR, a homology model based on Sav1866 has been used to identify chemicals that correct the aberrant trafficking of F508del-CFTR *in vitro* (Kalid et al., 2010). Of course, the high conservation across ABC proteins in the NBD-ICL interactions mediating protein folding and energetic signaling is fortuitous for modeling and screening related to these regions. By contrast, screening for functional modulators, such as potentiators of CFTR or inhibitors of ABCCs, may be more

dependent on refinement of these models, as aided by subfamily or protein-specific functional data such as that discussed in Section 1.5.

However, the utility of improved understanding of the structural relationships between these groups is not limited to computational approaches, but may also be relevant to the investigation of the mechanisms of action of drugs discovered through high throughput screening. In fact, related non-channel transporters may potentially help define the mechanisms and binding sites of both of the FDA-approved CFTR-directed therapeutic compounds, Lumacaftor (VX809) and Ivacaftor (VX770). Despite data suggesting that many pharmacological agents correct the folding of trafficking mutants of both CFTR (ABCC7) and P-glycoprotein (ABCB1) (Loo et al., 2012), Lumacaftor (which appears to bind MSD1 of CFTR (Loo et al., 2013)) is unable to correct P-glycoprotein (Loo et al., 2012). However, the drug is able to correct trafficking mutants of ABCA4 associated with macular degeneration (Sabirzhanova et al., 2015). Comparative pharmacology approaches may therefore reveal details on the structural determinants of the binding and action of these “corrector” drugs.

The mechanism of the clinically efficacious CFTR gating potentiator Ivacaftor is poorly understood. It has been shown to potentiate (and therefore likely directly bind) CFTR from multiple species, including human, murine (Cui and McCarty, 2015; Van Goor et al., 2009) and *Xenopus* (*Cui et al., submitted 2016)* orthologs. In studies reported herein, I have now also shown that Ivacaftor potentiates a version of CFTR lacking its lineage-specific R-domain (Section 3.3). Interestingly, it also has been reported that Ivacaftor may inhibit P-glycoprotein (ABCB1) with high affinity *in vitro* (Robertson et al.,

2015), although the molecular mechanism of inhibition has not been elucidated. All of these data, combined with findings that VX770 increases the opening rate of CFTR especially strongly (≈ 50 fold) in mutants wherein the transporter-conserved energetic signaling network (Section 1.5.3) is selectively disrupted (Wang et al., 2014; Wei et al., 2015), lead to an intriguing and motivating possibility. Potentiators of CFTR, which, like Ivacaftor, stabilize the open state of the channel (Van Goor et al., 2009), may also bind *bona fide* ABC transporters and inhibit them by preventing the conformational transitions necessary to the process of alternating access. Conversely, inhibitors of ABC transporters that stabilize one particular conformation of their TMDs may function as modulators, even potentiators, upon binding CFTR. 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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Appendix A: Localization of Insulin receptor in primary airway cells

NOTE: the data in Appendix A.1.3 are included in a manuscript due to be submitted to the journal *Procedures of the National Academy of Sciences* in March of 2016 (I will be third author).

A.1.1 Brief Introduction:

As described in Section 1.3, CF-related diabetes (CFRD) is a common co-morbidity of CF that is correlated with accelerated lung function decline [1]. There is some evidence that CFRD is correlated with increased glucose levels in the airway lumen [2-4]. This glucose may serve as a nutrient source for pathogenic bacteria [5]. In collaboration with the Koval lab at the Emory University Department of Cell Biology, we have investigated the mechanisms of glucose regulation in the airway. We have found evidence that cultured primary airway cells exhibit insulin-dependent glucose uptake and that this is blunted in cells from CF patients. Interestingly, confocal microscopy of immuno-stained primary airway epithelial cells performed by myself and others has localized the insulin-dependent glucose transporter (GLUT4) to the *apical* side of the airway, suggesting that insulin-dependent glucose uptake may be a mechanism for removal of glucose from the airway lumen, which the correlating functional glucose uptake data suggest is defective in CF.

To determine where the insulin receptor (IR) is localized, I optimized and performed immunofluorescence experiments for this protein on polarized primary airway cells cultured on Transwell filters. In addition to the IR, the cells are concurrently

stained for nuclei (DAPI) and for a junctional marker that allows the visualization of cell borders (β-catenin).

A1.2 Materials (make fresh for each experimental day):

1. DPBS: Sterile DPBS + Ca^{2+} and Mg^{2+}
2. TX-100: 0.5 % Triton X-100 in DPBS
3. 4% PFA: 4% paraformaldehyde diluted from fresh ampule of 20% PFA
4. DPBS + GS: 2% Goat serum in DPBS
5. TX-100 + GS: 2% Goat serum + 0.5 % Triton X-100 in DPBS
6. AB solutions: 2% Goat serum in DPBS + prescribed antibody
    a. Primary Insulin receptor AB mix: (rIR) (Abcam cat # ab5500) 1:250 in AB solution
    b. Primary β-catenin AB mix: (mBCAT) (BD Biosciences cat # BD610153) 1:200 in AB solution
    c. Secondary AB mix: rCy2 1:1000 + rCy3 1:1000 in AB solution.
7. DAPI solution: dilute Hoescht 33258 1:1000 in DPBS

A1.2 Protocol:

1. Receive Transwells containing polarized airway epithelial cells maintained at air-liquid interface ≥ 3 weeks from the Cell Cultures Core of the Emory + Children's Center for CF and Airways Disease Research (contact mhkoval@emory.edu ; s.a.molina@emory.edu).
2. Rinse each filter 4 X with DPBS
3. Visualize filters by light microscope; verify that all mucous (dark gray mass) has been washed off of the cells.
4. Wash each filter 3 X for 5 minutes with DPBS at room temperature
5. Fix each filter with 4% PFA 10 min at room temperature
6. Wash each filter with 3 X for 5 minutes in DPBS at room temperature
7. Permeabilize each filter for 5 minutes in TX-100 at room temperature
8. Block/permeabilize by washing each filter 2 X for 5 minutes with TX-100 + GS at room temperature
9. Make up primary insulin receptor AB (0.75 ml of each per filter)
10. Add primary insulin receptor AB (0.25 mL above filter, 0.5 mL below filter), incubate O/N at room temperature
11. Wash 3 X for 5 minutes in DPBS + GS at room temperature
12. Make up primary β-catenin AB (0.75 ml of each per filter)

13. Add primary β-catenin AB (0.25 ml above filter, 0.5 mL below filter), incubate 2 hours at room temperature
14. Wash 3 X for 5 minutes with DPBS + GS at room temperature
15. Make secondary AB mixture (0.75 ml of each per filter)
16. Add secondary AB mixture, incubate 1 hour at room temperature
17. Wash 3 X for 5 minutes with DPBS + GS at room temperature
18. Incubate in DAPI solution for 10 minutes
19. Wash 3 X for 5 minutes with DPBS + GS
20. Wash 3 X for 5 minutes with DPBS.
21. Carefully cut the filters from Transwells using a sterile razor blade
22. Mount filters cell side up on labeled microscope slides with Slow-fade mounting media.
23. Image through the Z-stack of the filter via confocal microscopy utilizing 100x oil immersion objective and 0.25 μM slices. For presentation, the Z-stack can be reconstituted as a 3-D pavement view of the epithelium, as shown in Figure A1.1

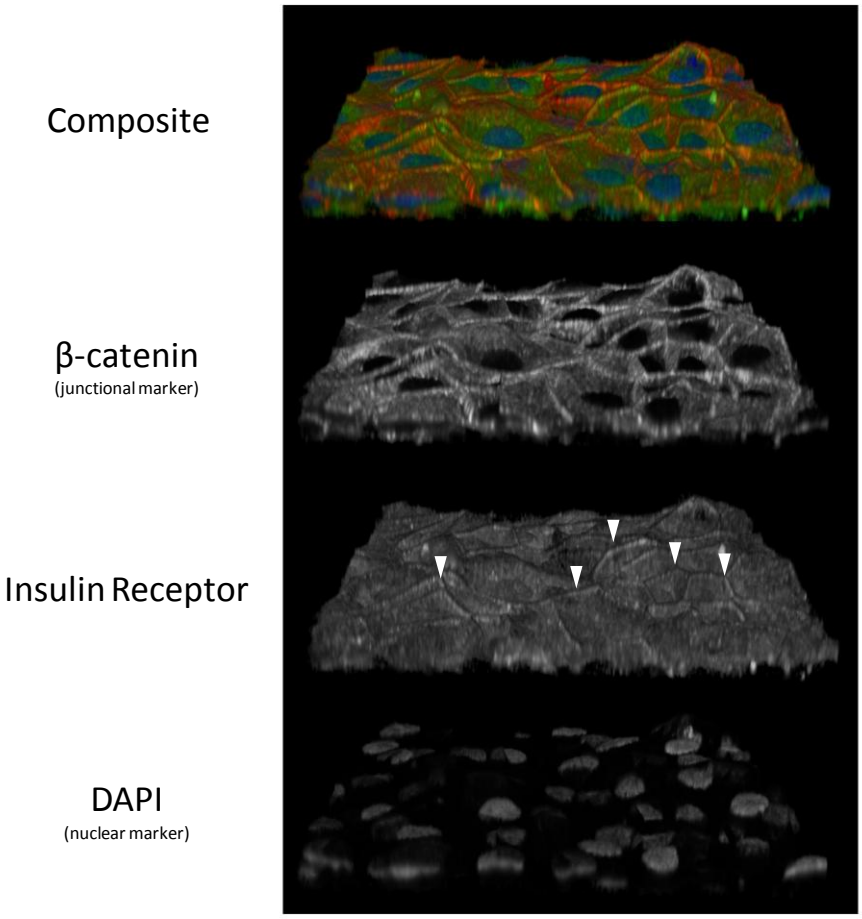

Figure A1.1. The above is a 3D projection reconstituted from a z-stack of primary human bronchial epithelial cells from a CF patient. The insulin receptor (green in top panel, isolated in the second from bottom panel) shows strong signal at cell borders, including the apical junctional complex (white arrowheads). Beta catenin protein (red in top panel, isolated in second from top panel) serves as a cell-cell junctional marker. A special thanks to Darryl Hanover from the Santangelo lab (Georgia Institute of Technology) for assistance in imaging the airway epithelium filters.

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Appendix B: Multiple Sequence Alignments used for analysis in Section 1.5 (Accession numbers follow)

1. CFTR orthologs

```
humanCFTR       -----------------------mqrsplekasvvsklffswtrpilrkgyrqrlelsdi        37
MouseCFTR       -----------------------mqksplekasfisklffswstailrkgyrqhlelsdi        37
ChickenCFTR     -----------------------mqrsplekanifsklffrwtkpilkkgyrqrlelsdi        37
XenopusCFTR     -----------------------mqktplekasifsqiffswtkpilwkgyrqrlelsdi        37
DogfishCFTR     -----------------------mqrspiekanafsklffrwprpilkkgyrqklelsdi        37
LampreyCFTR     mseappstagnrltapmsghnlkmqaspfekagifsriffrwprhvlrkgykhriersdi        60
                                      ** :*:***  .*::** *   :* ***::::* ***

humanCFTR       yqipsvdsadnlseklerewdrelas-kknpklinalrrcffwrfmfygiflylgevtka        96
MouseCFTR       yqapsadsadhlseklerewdreqas-kknpqlihalrrcffwrflfygillylgevtka        96
ChickenCFTR     yqipsadsadnlseklerewdrelatskkkpklinalrrcffwkfmfygillylgevtks        97
XenopusCFTR     yqihpgdsadnlserlerewdrevatskknpklinalkrcffwkflfygillylgevtka        97
DogfishCFTR     yqipssdsadelsemlerewdrelatskknpklvnalrrcffwrflfygillyfveftka        97
LampreyCFTR     hrtpqqdqadtlserlerewdreisqsenrphlltairrcflwrfvglgvimllaeftkv        120
                ::    *.** *** ******** :  ::.*:*: *::***:*:*:  *::: : *.**

humanCFTR       vqplllgriiasydpdnkeersiaiylgiglcllfivrtlllhpaifglhhigmqmriam        156
MouseCFTR       vqpvllgriiasydpenkversiaiylgiglcllfivrtlllhpaifglhrigmqmrtam        156
ChickenCFTR     vqplllgriiasydpdnssersiayylgiglcllflvrtllihpsifglhhigmqirial        157
XenopusCFTR     vqplllgriiasydrdnehersiayylaiglcllfvvrmlllhpaifglhhigmqmriam        157
DogfishCFTR     vqplclgriiasynaknтyereiayylalglcllfvvrtlflhpavfglqhlgmqmrial        157
LampreyCFTR     vqpfflggiiasfnpstvdv-qepllalglsacfvvrtlllhpavfglqhlgmdvriat        179
                ***. **:****:: ..    .    *.:**.  *:** *::**::***:::**::* *

humanCFTR       fsliykktlklssrvldkisigqlvsllsnnlnkfdeglalahfvwiaplqvallmgliw        216
MouseCFTR       fsliykktlklssrvldkisigqlvsllsnnlnkfdeglalahfiwiaplqvtllmgllw        216
ChickenCFTR     fsliykktlklsskvldkistgqlvsllsnnlnkfdeglalahfvwiaplqvallmgllw        217
XenopusCFTR     fsliykktlklsskvldkistgqlvsllsnnlnkfdeglalahfvwiaplqvlllmgllw        217
DogfishCFTR     fsliykkilkmssrvldkidtgqlvsllsnnlnkfdegvavahfvwiapvqvvllmgliw        217
LampreyCFTR     fcliykkimklsssvldkitvgqlvsllsnnlnkfdesltqahfiwiapiqcvgllaflw        239
                *.***** :*:** *****  ****************.:: ***:****:*   *:.::*

humanCFTR       ellqasafcglgflivlalfqaglgrmmmkyrdqragkiserlvitsemieniqsvkayc        276
MouseCFTR       dllqfsafcglglliilvifqailgkmmvkyrdqraakinerlvitseiidniysvkayc        276
ChickenCFTR     dmlqasafaglaflivmaffqawlgqmmmkyrdkragkinerlvitseiieniqsvkayc        277
XenopusCFTR     dllqasafcglgfliilslfqarlgrmmmkykdkragkinerlvitsqiieniqsvkayc        277
DogfishCFTR     neltefvfcglgflimlalfqawlgkkmmqyrdkragkinerlaitseiidniqsvkvyc        277
LampreyCFTR     relgpfclvgfaillivtllqawlghkinsyrdkrggkinerialtsevlenieaikvhg        299
                  *    : *:.:*::: ::** **: : .*:*:*:..**.**:.:**:::** ::*.:

humanCFTR       weeamekmienlrqtelkltrkaayvryfnssafffsgffvvflsvlpyalikgiilrki        336
MouseCFTR       wesamekmienlrevelkmtrkaaymrfftssafffsgffvvflsvlpytvingivlrki        336
ChickenCFTR     wedamekmieslretelkltrkaayvryfnssafffsgffvvflavvpyavtkgiilrki        337
XenopusCFTR     wenamekiietiretelkltrkaayvryfnssafffsgffvvflsivphllldgislrki        337
DogfishCFTR     wedamekiiddirqvelkltrkvaycryfsssafffsgffvvflsvvpyafihtiklrri        337
LampreyCFTR     weeamglidelrkqelkltsrasysryfnsaafffsgvfvvfasilpyavyneltlrrv        359
                **.** *:*: :*: ***:* :.:* *:*.*:******.**** :::*: . . : **::

humanCFTR       fttisfcivlrmavtrqfpwavqtwydslgainkiqdflqkqeyktleynltttevvmen        396
MouseCFTR       fttisfcivlrmsvtrqfptavqiwydsfgmirkiqdflqkqeykvleynlmttgiimen        396
ChickenCFTR     fttisfcivlrmtvtrqfpgsvqtwydsigainkiqdfllkeeykaleynltttgvevdk        397
XenopusCFTR     fttisfsivlrmavtrqfpwavqtwydslgvinkiqeflqkeeyksleynltttevamen        397
DogfishCFTR     fttisynivlrmtvtrqfpsaiqtwydslgairkiqdflhkdehktveynlttkevemvn        397
LampreyCFTR     ltsvsffnvlrmaitrslpwslqmwvdatasirkiqdflllkeyrpldynlstteveivs        419
                :*::*:  ****::**.:* ::* * *: . *.***:**  .*:: ::*** *. : : .

humanCFTR       vtafweegfgelfekakqnnnnrktsngddslffsnfsllgtpvlkdinfkiergqllav        456
MouseCFTR       vtafweegfgellekvqqsngdrkhssdennvsfshlclvgnpvlkninlniekgemlai        456
ChickenCFTR     vtafwdegigelfvkakqennnskapsndnnlffsnfplhaspvlqdinfkiekgellav        457
XenopusCFTR     vsaswdegigeffekaklevnggnisnedpsaffsnfslhvapvlrninfkiekgqllai        457
DogfishCFTR     vtaswdegigelfekvkqndserkmangddglffsnfslhvtpvlknisfklekgellai        457
LampreyCFTR     itaswdqidgivpeqg------skvkngiinvaatdlpfyksyvlsdinfklnkgemliv        473
                ::* *::  * .  :        :  .       :.: :    ** :*.::::::*::* :

humanCFTR       agstgagktsllmvimgelepsegkikhsgrisfcsqfswimpgtikeniifgvsydeyr        516
MouseCFTR       tgstgsgktsllmlilgeleasegiikhsgrvsfcsqfswimpgtikeniifgvsydeyr        516
ChickenCFTR     sgstgsgktsllmlimgelepsegkikhsgrisfspqvswimpgtikeniifgvsydeyr        517
XenopusCFTR     agstgagktsllmmimgelepsagkikhsgrisfspqvswimpgtikenivfgvsydqyr        517
DogfishCFTR     agstgsgkssllmmimgelepsdgkikhsgrisyspqvpwimpgtikdniifglsydeyr        517
LampreyCFTR     mgsfgsgkssllllmlgellpwdgrvrhsgrlsfssqqpwiinasvqenitlglhldkal        533
                 ** *:**:***::::***    * ::****:*:. *  **: .::::** :*:  *:

humanCFTR       yrsvikacqleediskfaekdnivlgeggitlsggqrarislaravykdadlylldspfg        576
MouseCFTR       yksvvkacqlqqditkfaeqdntvlgeggvtlsggqrarislaravykdadlylldspfg        576
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```
ChickenCFTR      yksviqacqleedilkfpdkdytvlgeggiilsgggqrarislaravykdadlylmdspfg        577
XenopusCFTR      ylsvikacqleediskfpekdntvlgeggitlsgggqrarislaravykdadlylldspfs        577
DogfishCFTR      ytsvvnacqleeditvfpnkdktvlgdggitlsgggqrarislaralykdadlylldspfs        577
LampreyCFTR      lwqvlrscglqeeimnlpqkektligesgfnlsgggqrarislaravyreadlylldspfs        593
                   .*:.:* *:::*  : :::  ::*:.*. **************:*::*****:****.

humanCFTR        yldvltekeifescvcklmanktrilvtskmehlkkadkililhegssyfygtfselqnl        636
MouseCFTR        yldvfteeqvfescvcklmanktrilvtskmehlrkadkililhqgssyfygtfselqsl        636
ChickenCFTR      yldiftekeifescvcklmanktrilvtsklehlkiadkililhegscyfygtfselqgq        637
XenopusCFTR      yldlftekeifescvcklmanktrilvtskveqlkkadkvlilhegscyfygtfseledq        637
DogfishCFTR      hldvttekdifesclcklmvnktrilvtsklehlkkadkilllheghcyfygtfselqge        637
LampreyCFTR      yldvstekqvfescidgflakktrilvtskvehlqradkvlilndgvvyfygtfpelqks        653
                 :**: **:::****:  ::.:*********:*:*: ***:*:*.:*  ****** **:

humanCFTR        qpdfssklmgcdsfdqfsaerrnsiltetlhrfsleg---dapvswtetkkqsfkqt-ge        692
MouseCFTR        rpdfssklmgydtfdqfteerrssiltetlrrfsvdd---ss-apws-kpkqsfrqt-ge        690
ChickenCFTR      rpdfsselmgfdsfdqfsaerrnsiitetlrrfsfeg---esmgsrnemkkqsfkqt-sd        693
XenopusCFTR      rpefsshligfd---hfnaerrnsiitetlrrcsids---dpsavrnevknksfkqv-ad        690
DogfishCFTR      kpdfssqllgsvhfdsfsaerrnsiltetfrrcsvssgdgaglgsysetrkasfkqpppe        697
LampreyCFTR      kleilslilqaerfdscssdrrgsmitgvlrrgsthsgdvatppntasv-----aeppqg        708
                 : :: * ::        . :** *::* .::* *                      :

humanCFTR        fgekrknsi-lnpinsirkfsivqktpl--qmngieeds--deplerrlslvpdseqgea        747
MouseCFTR        vgekrknsi-lnsfssvrkisivqktpl--cidges-----ddlqekrlslvpdseqgea        742
ChickenCFTR      fndkrknsiiinplnagrklsimqkngt--qvngledgh--idsperrislvpdleqgdv        749
XenopusCFTR      ftekrkssi-inprkssrkfslmqksqp--qmsgieeedmpaeqgerklslvpeseqgea        747
DogfishCFTR      fnekrksslivnpitsnkkfslvqtamsypqtngme--datsepgerhfslipenelgep        755
LampreyCFTR      t---------qfpfq-aqrfsvvsagas---vvvaiptagphepedrrlslipdtedgkq        755
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MouseCFTR        alprsnmiatgptfpgrrrqsvldlmtftpn-sgssnlqrt-------rtsirkislvpq        794
ChickenCFTR      glprsnmlnsdhmlqsrrrqsvlslmtgtsvnqgphvskkg-------stsfrkmsvvpq        802
XenopusCFTR      slprsnflntgptfqgrrrqsvlnlmtrtsisqgsnafatr-------nasvrkmsvnsy        800
DogfishCFTR      tkprsnifkselpfqahrrqsvlalmthssts--pnkihar-------rsavrkmsmlsq        806
LampreyCFTR      llpaa---gagplhaskrrksvlhlmlgvsagyentrrmariqqhaphgdpfllpsvvpq        812
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humanCFTR        anl--teldiysrrlsqetgleise-eineedlkecffddmesipavttwntylryitvh        856
MouseCFTR        isl--nevdvysrrlsqdstlnite-eineedlkecflddvikippvttwntylryftlh        851
ChickenCFTR      tnl-sseidiytrrlsrdsilditd-eineedlkecftddaesmgtvttwntyfryitih        860
XenopusCFTR      sns-sfdldiynrrlsqdsilevse-eineedlkecflddtdsqspttwntylrfltah        858
DogfishCFTR      tnfasseidiysrrlsedgsfeise-eineedlkecfadeeeiqnvtttwstylryvttn        865
LampreyCFTR      r----------pprksltsvaeesddvpeddnikdcfvdvdgeegelaswttyrryfgss        862
                              * *     : ::   :::::*:** *        ::*.** *:.

humanCFTR        kslifvliwclviflaevaaslvvlwllgntplq-dkg--nsth--srnnsyaviitsts        911
MouseCFTR        kglllvliwcvlvflvevaaslfvlwllknnpvn-sgn--ngtk--isnssyvviitsts        906
ChickenCFTR      kslifvlilcvtifllevaaslvlllflqkaaqi-nat--qpen--atsdnppviitdts        915
XenopusCFTR      knfifilvfclviffvevaassawlwiikrnapainmtsnenvs--evsdtlsvivthts        916
DogfishCFTR      rnlvfvlilclviflaevaaslaglwiisglaintgsqtndtstdlshlsvfskfitngs        925
LampreyCFTR      tlfgivlclnlvlfaiqvmvygvglwnlrsqedrvnttrpen--------gtggvhsftd        914
                   : ::*   : :*  :* .    *  :            :             . :  .

humanCFTR        syyvfyiyvgvadtllamgffrglplvhtlitvskilhhkmlhsvlqapmstlntlkagg        971
MouseCFTR        fyyifyiyvgvadtllalslfrglplvhtlitaskilhrkmlhsilhapmstisklkagg        966
ChickenCFTR      syymiyiyvgiadtllamgifrglplvhtlitvsktlhqkmvhavlyapmstfnslkagg        975
XenopusCFTR      fyyvfyiyvgvadslllalgifrglplvhslisvskvlhkkmlhailhapmstfntmragr        976
DogfishCFTR      hyyifyiyvgladsflalgvirglplvhtlvtvskdlhkqmlhsvlqgpmtafnkmkagr        985
LampreyCFTR      nyyyfyiyvgladsffvldpirglllihssirvsdtlsrgmlrailhapasfflekqpgy        974
                  ** :*****:**:::.:  :*** *:*: : .*. * : *::::* .* : :   : *

humanCFTR        ilnrfskdiailddllpltifdfiqlllivigaiavvavlqpyifvatvpvivafimlra        1031
MouseCFTR        ilnrfskdiailddflpltifdfiqlvfivigaiivvsalqpyiflatvpglvvfillra        1026
ChickenCFTR      ilnrfskdtailddllpltvfdliqlililvigaitvvsilqpyiflasvpviaafivlra        1035
XenopusCFTR      ilnrfskdtailddilplsifdltqlvlivigaitvvsllepyiflatvpvivafillrs        1036
DogfishCFTR      ilnrfikdtaiiddmlpltvfdfvqlilivvgaicvvsvlqpytllaaipvavifimlra        1045
LampreyCFTR      iinrfskdvaitddqlplaifdyfqlflivlgavtvvsamipwtmlvtlplgiscmvlrh        1034
                 *:*** ** ** ** ***::**  **.:**:**: **: : *: ::.::*     ::**

humanCFTR        yflqtsqqlkqlesegrspifthlvtslkglwtlrafgrqpyfetlfhkalnlhtanwfl        1091
MouseCFTR        yflhtaqqlkqlesegrspifthlvtslkglwtlrafrrqtyfetlfhkalnlhtanwfm        1086
ChickenCFTR      yflhtsqqlkqlesearspifthlvtslkglwtlrafgrqpyfetlfhkalnlhtanwfl        1095
XenopusCFTR      yflhtsqqlkqleskarspifahlitslkglwtlrafgrqpyfetlfhkalnlhtanwfl        1096
DogfishCFTR      yflrtsqqlkqlesearspifshlitslrglwtvrafgrqsyfetlfhkalnlhtanwfl        1105
LampreyCFTR      yflrtfrqlkqmeseaknpifahivatlkglwtirafsrddyfenifhqaldihtatwfl        1094
                 ***:* :****:**:.:.***:*::::*:****:*** *: ***.:**:**::***.**:

humanCFTR        ylstlrwfqmriemifviffiavtfisilttgegegrvgiiltlamnimstlqwavnssi        1151
MouseCFTR        ylatlrwfqmridmifvlffivvtfisilttgegegtagiiltlamnimstlqwavnssi        1146
ChickenCFTR      ylstlrwfqmriemifvvffsavafisiittgdgpgrvgiiltlamnimgtlqwavnssi        1155
```

```
XenopusCFTR      ylstlrwfqmtiemifviffiavsfisiatsgageekvgivltlamnimntlqwavnasi        1156
DogfishCFTR      ylstlrwfqmridivfvlffiavtfiaiathdvgegqvgiiltlamnitstlqwavnssi        1165
LampreyCFTR      ylsalrwfqmridiiftlfitavtfisvgvkgysegsiaialtlamnimstfqwaintsi        1154
                 **::****** *:::*.:*: .*:**:: .   .    .* *******  *:***:*:**

humanCFTR        dvdslmrsvsrvfkfidmptegkp-tkstkpykngqlskvmiienshvkkddiwpsggqm        1210
MouseCFTR        dtdslmrsvsrvfkfidiqteesmytqiikelpregssdvlviknehvkksdiwpsggem        1206
ChickenCFTR      dvdslmrsvsrifkfidmpteem---ktikpqknnqfsdaliienrhvkdeknwpsggqm        1212
XenopusCFTR      dvdslmrsvsrifridlpveeline---nknkeeqlsevliyendyvkktqvwpsggqm        1213
DogfishCFTR      dvdglmrsvsrvfkyidippegsetk---nrhnannpsdvlvienkhlt--kewpsggqm        1220
LampreyCFTR      evegmmrsterilrfmdipeessgfvvt----------------------ppppdwpsaghl        1194
                 :.:.:***..*:::::*:  *                                ***.*.:

humanCFTR        tvkdltakyteggnailenisfsispgqrvgllgrtgsgkstllsaflrllntegeiqid        1270
MouseCFTR        vvkdltvkymddgnavlenisfsispgqrvgllgrtgsgkstllsaflrmlnikgdieid        1266
ChickenCFTR      tvtdltaryteggtavlenisfsissgqtvgllgrtgsgkstllfaflrllntegdiqid        1272
XenopusCFTR      tvknlsanyidggntvlenisfslspgqrvgllgrtgsgkstllsaflrllstqgdiqid        1273
DogfishCFTR      mvnnltakytsdgravlqdlsfsvnaggqrvgllgrtgagkstllsallrllstegeiqid        1280
LampreyCFTR      evvnlsmrhsptapyvlqnisfnvqqggkmailgrtgagkstllaclllqrdvegevrid        1254
                  * :*: .:   .  :*:::**.:. ** :.:*****:****** .:*   . :*::.**

humanCFTR        gvswdsitlqqwrkafgvipqkvfifsgtfrknldpyeqwsdqeiwkvadevglrsvieq        1330
MouseCFTR        gvswnsvtlqewrkafgvitqkvfifsgtfrqnldpngkwkdeeiwkvadevglksvieq        1326
ChickenCFTR      gvswntvslqqwrkafgvipqkvfifsgtfrknldpygqwndeeiwkvaeevglksvieq        1332
XenopusCFTR      gvswqtiplqkwrkafgvipqkvfifsgsirknldpygkwsdeellkvteevglkliidq        1333
DogfishCFTR      giswnsvslqkwrkafgvipqkvfvfsgtfrknldpyeqwsdeeiwkvteevglksmieq        1340
LampreyCFTR      glsarnvapytwrsafqvitqkififtgtlrknldpygrcndeeiwkaidlaglrdvvea        1314
                 *:*  .:    **.** ** **:*:*:*::*:****  : .*:*: *. : .**: :::

humanCFTR        fpgkldfvlvdggcvlshghkqlmclarsvlskakillldepsahldpvtyqiirrtlkq        1390
MouseCFTR        fpgqlnftlvdggyvlshghkqlmclarsvlskakiilldepsahldpityqvirrvlkq        1386
ChickenCFTR      fpgqldfvlvdggcvlshghkqlmclarsvlskakillldepsahldpitsqvirktlkh        1392
XenopusCFTR      fpgqldfvlldggcvlshghkqlvclarsvlskakillldepsahldpitfqiirktlkh        1393
DogfishCFTR      fpdklnfvlvdggyilsnghkqlmclarsilskakillldeptahldpvtfqiirktlkh        1400
LampreyCFTR      lpggldvmlveggwvlseggkqllslvrcllykarillldeptanldqitaqtmmriirk        1374
                 :*  *:. *::** :**.*:***:.*.*.:* **:*:*****:*.** :* * : : :::

humanCFTR        afadctvilcehrieamlecqqflvieenkvrqydsiqkllnerslfrqaispsdrvklf        1450
MouseCFTR        afagctvilcehrieamldcqrflvieesnvwqydslqallseksifqqaisssekmrff        1446
ChickenCFTR      afadctvvlsehrleailecqrflviednkmrqyesiqkllseksslrqaishadrlkll        1452
XenopusCFTR      afadctvilsehrleamlecqrflviedntvrqydsiqklvneksffkqaishsdrlklf        1453
DogfishCFTR      tfsnctvilsehrveallecqqflviegcsvkqfdalqkllteaslfkqvfghldraklf        1460
LampreyCFTR      efkdctilvsehriraiydcnqilvledgkvreygtaqvvfkksalfrefvpdtgsfrgh        1434
                  *  **:::.***:.*: :*:::**:*  .: :: : * :..: : ::: .      :

humanCFTR        ph--rnsskckskpqiaalkeet--eeevqdtrl  1480
MouseCFTR        qg--rhsskhkprtqitalkeet--eeevqetrl  1476
ChickenCFTR      pahrrnsskrkprpqicalqeet--eeevqetrl  1484
XenopusCFTR      plhrrnsskrksrpqisalqeet--eeevqdtrl  1485
DogfishCFTR      tahrrnsskrktrpkisalqeea--eedlqetrl  1492
LampreyCFTR      pvr--ssmtlrhsikhislqeelaeddiiqetrl  1466
                       * . :   :  :*:**   :: :*:***
```

2. <u>CFTR and ABCC4 homologs</u>

```
HumanCFTR        -------mqrsplekasvvsklffswtrpilrkgyrqrlelsdiyqipsvdsadnlsekl        53
MouseCFTR        -------mqksplekasfisklffswstailrkgyrqhlelsdiyqapsadsadhlsekl        53
ChickenCFTR      -------mqrsplekanifsklffrwtkpilkkgyrqrlelsdiyqipsadsadnlsekl        53
HumanABCC4       mlpvyqevkpnplqdanlcsrvffwwlnplfkighkrrleeddmysvlpedrsqhlgeel        60
MouseABCC4       mlpvhtevkpnplqdanlcsrvffwwlnplfktghkrrleeddmfsvlpedrskhlgeel        60
RatABCC4         mlpvhtevkpnplqdanlcsrlffwwlnplfkaghkrrleeddmfsvlpedrskhlgeel        60
                        :: .**:.*.. *::** *   ::: *::::** .*::.    * :..*.*:*

HumanCFTR        erewdrelas-k---knpklinalrrcffwrfmfygiflylgevtkavqplllgriia--        107
MouseCFTR        erewdreqas-k---knpqlihalrrcffwrflfygillylgevtkavqpvllgriia--        107
ChickenCFTR      erewdrelatsk---kkpklinalrrcffwkfmfygillylgevtksvqplllgriia--        108
HumanABCC4       qgfwdkevlraendaqkpsltraiikcywksylvlgiftlieesakviqpiflgkiinyf        120
MouseABCC4       qrywdkellrakkdsrkpsltkaiikcywksylilgiftlieegtrvvqpflgkiieyf        120
RatABCC4         qgywdkevlrakkdarkpsltkaivkcywksylilgiftlieettrvvqpiflgkiidyf        120
                 :  **:*    :   ::*.* .*: :*::  ::. **:  : * :: :**::**:**

HumanCFTR        -sydpdnkeersiaiylgiglcllfivrtlllhpaifglhhigmqmriamfsliykktlk        166
MouseCFTR        -sydpenkversiaiylgiglcllfivrtlllhpaifglhrigmqmrtamfsliykktlk        166
ChickenCFTR      -sydpdnssersiayylgiglcllflvrtllihpsifglhhigmqirialfsliykktlk        167
HumanABCC4       enydpmdsvalntayayatvltfctlilailhhlyfyhvqcagmrlrvamchmiyrkalr        180
MouseABCC4       ekydpddsvalhtaygyaavlsmctlilailhhlyfyhvqcagmrlrvamchmiyrkalr        180
RatABCC4         ekydsddsaalhtaygyaavlslctlilailhhlyfyhvqcagmrirvamchmiyrkalr        180
                  .**  :.     *   .  * :  :: ::* *  :: ::  **::* *:  :**:*:*:

HumanCFTR        lssrvldkisigqlvsllsnnlnkfdeglalahfvwiaplqvallmgliwellqasafcg        226
MouseCFTR        lssrvldkisigqlvsllsnnlnkfdeglalahfiwiaplqvtllmgllwdllqfsafcg        226
```

```
ChickenCFTR     lsskvldkistgqlvsllsnnlnkfdeglalahfvwiaplqvallmgllwdmlqasafag        227
HumanABCC4      lsnmamgktttgqivnllsndvnkfdqvtvflhflwagplqaiavtallwmeigisclag        240
MouseABCC4      lsnsamgktttgqivnllsndvnkfdqvtiflhflwagplqaiavtvllwveigisclag        240
RatABCC4        lsnsamgktttgqivnllsndvnkfdqvtiflhflwagplqaigvtillwveigisclag        240
                **. .: * : **:*.****::****:   : **:* .***.  :  *:*  :  *.:.*

HumanCFTR       lgflivlalfqaglgrmmmkyrdqragkiserlvitsemieniqsvkaycweeamekmie        286
MouseCFTR       lglliilvifqailgkmmvkyrdqraakinerlvitseiidniysvkaycwesamekmie        286
ChickenCFTR     laflivmaffqawlgqmmmkyrdkragkinerlvitseiieniqsvkaycwedamekmie        287
HumanABCC4      mavliillplqscfgklfsslrsktatftdarirtmnevitgiriikmyaweksfsnlit        300
MouseABCC4      lavlvillplqscigklfsslrsktaaftdarirtmnevitgmriikmyaweksfadlia        300
RatABCC4        lailvillplqscigklfsslrsktaaftdarfrtmnevitgmriikmyaweksfadlit        300
                :..*:::  :*: :*::: . *.: *   . *:   .*:*  :  :* *.**.:: .:*

HumanCFTR       nlrqtelkltrkaayvryfnssafffsgffvvflsvlpyalikgi-ilrkifttisfciv        345
MouseCFTR       nlrevelkmtrkaaymrfftssafffsgffvvflsvlpytvingi-vlrkifttisfciv        345
ChickenCFTR     slretelkltrkaayvryfnssafffsgffvvflavvpyavtkgi-ilrkifttisfciv        346
HumanABCC4      nlrkkeiskilrssclrgmnlasffsaskiivfvtfttyvllgsvitasrvfvavtlyga        360
MouseABCC4      nlrkkeiskilgssylrgmnmasffiankvilfvtftsyvllgneitashvfvamtlyga        360
RatABCC4        nlrkkeiskilgssylrgmnmasffiankvilfvtfttyvllgnkitsshvfvamtlyga        360
                .**: *:.    :: :* :. ::** :  .::*::.  *.:        ::*.::::  .

HumanCFTR       lrmavtrqfpwavqtwydslgainkiqdflqkqeyktleynltttev---vmenvtafwe        402
MouseCFTR       lrmsvtrqfptavqiwydsfgmirkiqdflqkqeykvleynlmttgi---imenvtafwe        402
ChickenCFTR     lrmtvtrqfpgsvqtwydsigainkiqdfllkeeykaleynltttgv---evdkvtafwd        403
HumanABCC4      vrltvtlffpsaiervseaivsirriqtfllldeisqrnrqlpsdgkkmvhvqdftafwd        420
MouseABCC4      vrltvtlffpsaiergseaivsirriknfllldelpqrkahvpsdgkaivhvqdftafwd        420
RatABCC4        vrltvtlffpsaiervseavvsvrriknfllldelperkaqepsdgkaivhvqdftafwd        420
                :*::**  ** :::   ::.  :.:*: **  :*    : .  :       ::..****:

HumanCFTR       egfgelfekakqnnnnrktsngddslffsnfsllgtpvlkdinfkiergqllavagstga        462
MouseCFTR       egfgellekvqqsngdrkhssdennvsfshlclvgnpvlkninlniekgemlaitgstgs        462
ChickenCFTR     egigelfvkakqennnskapsndnnlffsnfplhaspvlqdinfkiekgellavsgstgs        463
HumanABCC4      ka------------------------------setptlqglsftvrpgellavvgpvga        449
MouseABCC4      ka------------------------------ldsptlqglsfiarpgellavvgpvga        449
RatABCC4        ka------------------------------ldtptlqglsftarpgellavvgpvga        449
                :.                              .*.*: :.:  . *::**: * .*:

HumanCFTR       gktsllmvimgelepsegkikhsgrisfcsqfswimpgtikeniifgvsydeyryrsvik        522
MouseCFTR       gktsllmlilgeleasegiikhsgrvsfcsqfswimpgtikeniifgvsydeyryksvvk        522
ChickenCFTR     gktsllmlimgelepsegkikhsgrisfspqvswimpgtikeniifgvsydeyryksviq        523
HumanABCC4      gkssllsavlgelapshglvsvhgriayvsqqpwvfsgtlrsnilfgkkyekeryekvik        509
MouseABCC4      gkssllsavlgelppasglvsvhgriayvsqqpwvfsgtvrsnilfgkkyekeryekvik        509
RatABCC4        gkssllsavlgelpptsglvsvhgriayvsqqpwvfsgtvrsnilfgrkyekeryekvik        509
                **:***  ::***  : * :.  **:::  *  *:: **::.**:** .*:: **..*::

HumanCFTR       acqleediskfaekdnivlgeggitlsgggrarislaravykdadlylldspfgyldvlt        582
MouseCFTR       acqlqqditkfaeqdntvlgeggvtlsgggrarislaravykdadlylldspfgyldvft        582
ChickenCFTR     acqleedilkfpdkdytvlgeggiilsgggrarislaravykdadlylmdspfgyldift        583
HumanABCC4      acalkkdlqlledgdltvigdrgttlsgggkarvnlaravyqdadiyllddplsavdaev        569
MouseABCC4      acalkkdlqlledgdltvigdrgatlsgggkarvnlaravyqdadiylldplsavdaev        569
RatABCC4        acalkkdlqlledgdltvigdrgatlsgggkarvnlaravyqdadiylldplsavdaev        569
                ** *::*:  : : *  *:*: *  *****:**:.******:***:**:*.*:. :*  .

HumanCFTR       ekeifescvcklmanktrilvtskmehlkkadkililhegssyfygtfselqnlqpdfss        642
MouseCFTR       eeqvfescvcklmanktrilvtskmehlrkadkililhqgssyfygtfselqslrpdfss        642
ChickenCFTR     ekeifescvcklmanktrilvtsklehlkiadkililhegscyfygtfselqgqrpdfss        643
HumanABCC4      srhlfelcicqilhekitilvthqlqylkaasqililkdgkmvqkgtyteflksgidfgs        629
MouseABCC4      gkhlfqlcicqalhekitilvthqlqylkaashililkdgemvqkgtyteflksgvdfgs        629
RatABCC4        gkhlfqlcicqtlhekitilvthqlqylkaashililkdgemvqkgtyteflksgvdfgs        629
                 ..:*: *:*: : :*  **** ::::*: *.:****::*.    **::*:     **.*

HumanCFTR       klmgcdsfdqfsaerrnsiltetlhrfslegdapvswtetkkqsfkqtgefgekrknsi-        701
MouseCFTR       klmgydtfdqfteerrssiltetlrrfsvddss-apws-kpkqsfrqtgevgekrknsi-        699
ChickenCFTR     elmgfdsfdqfsaerrnsiitetlrrfsfegesmgsrnemkkqsfkqtsdfndkrknsii        703
HumanABCC4      llkkdnees----eqppvpgtptlrn----------------rtfsessvwsq-------        662
MouseABCC4      llkkeneea----epstapgtptlrk----------------rtfseasiwsq-------        662
RatABCC4        llkkeneea----epspvpgtptlrn----------------rtfseasiwsq-------        662
                 *   :       *      * **:.               ::* ::.   :

HumanCFTR       lnpinsirkfsivqktplqmngieedsdeplerrlslvpdseqgeailprisvistgptl        761
MouseCFTR       lnsfssvrkisivqktplcidges---ddlqekrlslvpdseqgeaalprsnmiatgptf        756
ChickenCFTR     inplnagrklsimqkngtqvngledghidsperrislvpdleqgdvglprsnmlnsdhml        763
HumanABCC4      ------------qssrpslkdgalesqd-------------------------------        678
MouseABCC4      ------------qssrpslkdgapegqdae-----------------------------        680
RatABCC4        ------------qssrpslkdgvpdaqdae-----------------------------        680
                            ..     :*

HumanCFTR       qarrrqsvlnlmth-svnqgqnihrkttastrkvslapqanl-teldiysrrlsqetgle        819
MouseCFTR       pgrrrqsvldlmtftpn-sgssnlqrtrtsirkislvpqisl-nevdvysrrlsqdstln        814
ChickenCFTR     qsrrrqsvlslmtgtsvnqgphvskkgstsfrkmsvvpqtnlsseidiytrrlsrdsild        823
```

```
HumanABCC4      ------------------------------------------------------------        678
MouseABCC4      ----------------------nt---------qavqpee--------------------        689
RatABCC4        ----------------------nt---------qaaqpee--------------------        689


HumanCFTR       iseeineedlkecffddmesipavttwntylryitvhkslifvliwclviflaevaaslv        879
MouseCFTR       iteeineedlkecfldd vikippvttwntylryftlhkglllvliwcvlvflvevaaslf        874
ChickenCFTR     itdeineedlkecftddaesmgtvttwntyfryitihkslifvlilcvtifllevaaslv        883
HumanABCC4      ------------------------------------------------------vayvlq        684
MouseABCC4      ------------------srsegrigfkayknyfsagaswffiiflvllnmvgqvfyvlq        731
RatABCC4        ------------------srsegrigfkayknyfsagaswffiiflvllnlmgqvfyvlq        731
                                                                     *   *

HumanCFTR       vlwllgntplqdkgnsthsrnnsyaviitstssyyvfyiyvgvadtllamgffrglplvh        939
MouseCFTR       vlwllknnpvnsgnngtkisnssyvviitstsfyyifyiyvgvadtllalslfrglplvh        934
ChickenCFTR     lllflqkaaqinatqpenatsdnppviitdtssyymiyiyvgiadtllamgifrglplvh        943
HumanABCC4      dwwlsywankqsmlnvtvngg---gnvtekldlnwylgiysgltvatvlfgiarsllvfy        741
MouseABCC4      dwwlshwankqgalnntrnan---gnitetldlswylgiyagltavtvlfgiarsllvfy        788
RatABCC4        dwwlshwanrqgalndtknan---gnvtgtldlswylgiytgltavtvlfgiarsllvfy        788
                   :          :           :    .  : : ** *:: . : :.: *.* :.:

HumanCFTR       tlitvskilhhkmlhsvlqapmstlntlkaggilnrfskdiailddllpltifdfiqlll        999
MouseCFTR       tlitaskilhrkmlhsilhapmstisklkaggilnrfskdiailddflpltifdfiqlvf        994
ChickenCFTR     tlitvsktlhqkmvhavlyapmstfnslkaggilnrfskdtailddllpltvfdliqlil        1003
HumanABCC4      vlvnssqtlhnkmfesilkapvlffdrnpigrilnrfskdighlddllpltfldfiqtll        801
MouseABCC4      ilvnasqtlhnrmfesilkapvlffdrnpigrilnrfskdighmddllpltfldfiqtll        848
RatABCC4        vlvnasqtlhnrmfesilkapvlffdrnpigrilnrfskdighmddllpltfldfiqtll        848
                 *:. *: **.:*..::* **:  :.    * ******** . :**:****.:*:** ::

HumanCFTR       ivigaiavvavlqpyifvatvpvivafimlrayflqtsqqlkqlesegrspifthlvtsl        1059
MouseCFTR       ivigaiivvsalqpyiflatvpglvvfillrayflhtaqqlkqlesegrspifthlvtsl        1054
ChickenCFTR     ivigaitvvsilqpyiflasvpviaafivlrayflhtsqqlkqlesearspifthlvtsl        1063
HumanABCC4      qvvgvvsvavavipwiaiplvplgiififlrryfletsrdvkrlesttrspvfshlsssl        861
MouseABCC4      lvvsviavaaavipwiliplvplsvvflvlrryfletsrdvkrlesttrspvfshlsssl        908
RatABCC4        lvvsviavaaavipwiliplvplsiifvvlrryfletsrdvkrlesttrspvfshlsssl        908
                 *:..: *.  : *:* :  **    *:.** ***.*::::*:***  ***:*:** :**

HumanCFTR       kglwtlrafgrqpyfetlfhkalnlhtanwflylstlrwfqmriemifviffiavtfisi        1119
MouseCFTR       kglwtlrafrrqtyfetlfhkalnlhtanwfmylatlrwfqmridmifvlffivvtfisi        1114
ChickenCFTR     kglwtlrafgrqpyfetlfhkalnlhtanwflylstlrwfqmriemifvvffsavafisi        1123
HumanABCC4      qglwtiraykaeercqelfdahqdlhseawflflttsrwfavrldaicamfviivafgsl        921
MouseABCC4      qglwtiraykaeercqelfdahqdlhseawflflttsrwfavrldaicaifvivvafgsl        968
RatABCC4        qglwtiraykaeercqelfdahqdlhseawflflttsrwfavrldaicavfvivvafgsl        968
                :****:**:  :   : **.   :**:  **::*:* *** :*:: * .:*.  *:* *:

HumanCFTR       lttgege-grvgiiltlamnimstlqwavnssidvdslmrsvsrvfkfidmptegkp-tk        1177
MouseCFTR       lttgege-gtagiiltlamnimstlqwavnssidtdslmrsvsrvfkfidiqteesmytq        1173
ChickenCFTR     ittgdgp-grvgiiltlamnimgtlqwavnssidvdslmrsvsrifkfidmpteem---k        1179
HumanABCC4      ilaktldagqvglalsyaltlmgmfqwcvrqsaevenmmisvervieytdlekeapwey-        980
MouseABCC4      vlaktlnagqvglalsyaltlmgmfqwsvrqsaevenmmisvervieytdlekeapwec-        1027
RatABCC4        vlaktldagqvglalsysltlmgmfqwsvrqsaevenmmisvervieytdlekeapwec-        1027
                :  .    * .*: *: ::.:*. :**.*..* :.:.:* **.*:::: *: .*

HumanCFTR       stkpykngqlskvmiienshvkkddiwpsggqmtvkdltakyteggnailenisfsispg        1237
MouseCFTR       iikelpregssdvlviknehvkksdiwpsggemvvkdltvkymddgnavlenisfsispg        1233
ChickenCFTR     tikpqknnqfsdaliienrhvkdeknwpsggqmtvtdltaryteggtavlenisfsissg        1239
HumanABCC4      -------------------qkrpppawphegviifdnvnfmyspggplvlkhltaliksq        1021
MouseABCC4      -------------------kkrpppgwphegvivfdnvnftysldgplvlkhltaliksr        1068
RatABCC4        -------------------rkrpppgwphegvivfdnvnftysldgplvlkhltaliksr        1068
                                  : :    **  * : . ::.  *   *  :*:.::  *.

HumanCFTR       qrvgllgrtgsgkstllsaflrllntegeiqidgvswdsitlqqwrkafgvipqkvfifs        1297
MouseCFTR       qrvgllgrtgsgkstllsaflrmlnikgdieidgvswnsvtlqewrkafgvitqkvfifs        1293
ChickenCFTR     qtvgllgrtgsgkstllfaflrllntegdiqidgvswntvslqqwrkafgvipqkvfifs        1299
HumanABCC4      ekvgivgrtgagksslisalfrlsepegkiwidkiltteiglhdlrkkmsiipqepvlft        1081
MouseABCC4      ekvgivgrtgagksslisalfrlsepegkiwidkiltteiglhdlrkkmsiipqepvlft        1128
RatABCC4        ekvgivgrtgagksslisalfrlsepegkiwidkiltteiglhdlrkkmsiipqepvlft        1128
                : **::****:***:*: *::*: : :*.* ** :    : *:: ** :.:* *: .:*:

HumanCFTR       gtfrknldpyeqwsdqeiwkvadevglrsvieqfpgkldfvlvdggcvlshghkqlmcla        1357
MouseCFTR       gtfrqnldpngkwkdeeiwkvadevglksvieqfpgqlnftlvdggyvlshghkqlmcla        1353
ChickenCFTR     gtfrknldpygqwndeeiwkvaeevglksvieqfpgqldfvlvdggcvlshghkqlmcla        1359
HumanABCC4      gtmrknldpfnehtdeelwnalqevqlketiedlpgkmdtelaesgsnfsvgqrqlvcla        1141
MouseABCC4      gtmrknldpfnehtdeelwraleevqlkeaiedlpgkmdtelaesgsnfsvgqrqlvcla        1188
RatABCC4        gtmrknldpfnehsdeelwkaleevqlkeaiedlpgkmdtelaesgsnfsvgqrqlvcla        1188
                **:*:****  : .*:*:*.. :** *:..**::**:::  *.:.*  :* *::**:***

HumanCFTR       rsvlskakillldepsahldpvtyqiirrtlkqafadctvilcehrieamlecqqflvie        1417
MouseCFTR       rsvlskakilldepsahldpityqvirrvlkqafagctvilcehrieamldcqrflvie        1413
ChickenCFTR     rsvlskakillldepsahldpitsqvirktlkhafadctvvlsehrleailecqrflvie        1419
HumanABCC4      railrknqiliideatanvdprtdeliqkkirekfahctvltiahrlntiidsdkimvld        1201
```

```
MouseABCC4       railknnriliideatanvdprtdeliqqkirekfaqctvltiahrlntiidsdkimvld        1248
RatABCC4         railkknriliideatanvdprtdeliqqkirekfaqctvltiahrlntiidsdkimvld        1248
                 *::* : :*:::** :*.:** * ::*:: ::. ** ***:   **:::::::::*::

HumanCFTR        enkvrqydsiqkllnerslf-rqaisp---sdrvklfph--rnsskckskpqiaalke--        1469
MouseCFTR        esnvwqydslqallseksif-qqaiss---sekmrffqg--rhsskhkprtqitalke--        1465
ChickenCFTR      dnkmrqyesiqkllsekssl-rqaish---adrlkllpahrrnsskrkprpqicalqe--        1473
HumanABCC4       sgrlkeydepyvllqnkeslfykmvqqlgkaeaaalteta-kqvyfkrnyphightdhmv        1260
MouseABCC4       sgrlkeydepyvllqnpeslfykmvqqlgkgeaaalteta-kqvyfrrnypditftspav        1307
RatABCC4         sgrlreydepyvllqnpeslfykmvqqlgkgeaaalteta-kqvyfrrnypdiafsspav        1307
                 . .: :*:.   **.: . :  : :.    .:   :     :.    :   .*   .

HumanCFTR        -------eteeevqdtrl      1480
MouseCFTR        -------eteeevqetrl      1476
ChickenCFTR      -------eteeevqetrl      1484
HumanABCC4       tntsngqpstltifetal      1278
MouseABCC4       mntsngqpsaltifetal      1325
RatABCC4         mstsngqpsaltifetal      1325
                        :   : :* *
```

3. <u>CFTR, P-glycoportein (ABCB1) and ABCC4</u>

```
HumanCFTR        -------mqrsplekasvvsklffswtrpilrkgyrqrlelsdiyqipsvdsadnlsekl         53
HumanP-gp        ---------------------------------------------mdlegdrnggakk         13
HumanABCC4       mlpvyqevkpnplqdanlcsrvffwwlnplfkighkrrleeddmysvlpedrsqhlgeel         60
                                                                :   . . :

HumanCFTR        erewdrelas-k----knpkl--inalrrcffw--rfmfygiflylge-vtkavqplllg         103
HumanP-gp        knffklnnksekdkkekkptvsvfsmfrysnwldklymvvgtlaaiihgaglplmmlvfg         73
HumanABCC4       qgfwdkevlr-aendaqkpsl--traiikcywk--sylvlgiftliee-sakviqpiflg         114
                 :  :. :         ::*.:     :  . :    ::. * :  : .     :  :.:*

HumanCFTR        riiasydpdnk---e-----------------------ersiaiylgiglcllfivrtll         137
HumanP-gp        emtdifanagnledlmsnitnrsdindtgffmnleedmtryayyysgigagvlv--aay-         130
HumanABCC4       kiinyfenydpmdsv----------------------alntayayatvltfctlilail         151
                 .:   :                                        .    .     :

HumanCFTR        lhpaifglh--higmqmriamfsliykktlklssrvldkisigqlvsllsnnlnkfdegl         195
HumanP-gp        iqvsfwclaagrqihkirkqffhaimrqei----gwfdvhdvgelntrltddvskinevi         186
HumanABCC4       hhlyfyhvq--cagmrlrvamchmiyrkalrlsnmamgktttgqivnllsndvnkfdqvt         209
                  :  :: :       ::*  :   * :: :      :     *:: . *::::.*:::

HumanCFTR        alahfvwiapl---qvallmg--liwe----llqasafcglgflivlalfqaglgrmmmk         246
HumanP-gp        gdkigmffqsmatfftgfivgftrgwkltlvilaispvlg--------lsaavwakilss         238
HumanABCC4       vflhflwagpl---qaiavta--llwm----eigisclagmavliillplqscfgklfss         260
                      ::   :    .  : .    *      :  * . *           :  .::: .

HumanCFTR        yrdqragkiserlvitsemieniqsvkaycwee-amekmienlrq---telkltrkaayv         302
HumanP-gp        ftdkellayakagavaeevlaairtviafggqkkelerynknleeakrigikkaitanis         298
HumanABCC4       lrsktatftdarirtmnevitgiriikmyawek-sfsnlitnlrk---keiskilrsscl         316
                   .:           .*::  *: :  :  ::  :..   **.:     :.    :

HumanCFTR        ryfnssafffsgffvvflsvlpyalikgiil-rkifttisfcivlrmavtrqfpwavqtw         361
HumanP-gp        igaa-flliyasyalafwygttlvlsgeysi-gqvlt-vffsvligafsvgqaspsieaf         355
HumanABCC4       rgmnlasffsaskiivfvtfttyvllgsvitasrvfvavtlygavrltvtlffpsaierv         376
                        :: :.  :.*      .*        :::. : :   :    .     :::

HumanCFTR        ydslgainkiqdflqkqeyktleynlttte---vvmenvtafweegfgelfekakqnnnn         418
HumanP-gp        anargaayeifkii--d---------------------------nkpsidsysksghkpdn         387
HumanABCC4       seaivsirriqtflldeisqrnrqlpsdgkkmvhvqdftafwdk---------------         421
                  ::  :  .*  ::  :                          ::

HumanCFTR        rktsngddslffsnfsllgtpvlkdinfkiergqllavagstgagktsllmvimgeleps         478
HumanP-gp        ikgnlefrnvhfsypsrkevkilkglnlkvqsgqtvalvgnsgcgksttvqlmqrlydpt         447
HumanABCC4       ----------------asetptlqglsftvrpgellavvgpvgagkssllsavlgelaps         465
                                .  *: :.:.:. *: :*:.*  *.**:: :  :     *:

HumanCFTR        egkikhsgr-------------isfcsqfswimpgtikeniifgvsyde-yryrsvikac         524
HumanP-gp        egmvsvdgqdirtinvrflreiigvvsqepvlfattiaenirygrenvtmdeiekavkea         507
HumanABCC4       hglvsvhgr-------------iayvsqqpwvfsgtlrsnilfgkkyek-eryekvikac         511
                 .* :.  *:             *.  **   ::  *: .** :* .     . ...:* .

HumanCFTR        qleediskfaekdnivlgeggitlsggqrarislaravykdadlylldspfgyldvltek         584
HumanP-gp        naydfimklphkfdtlvgergaqlsggqkqriaiaralvrnpkillldeatsaldtesea         567
HumanABCC4       alkkdlqlledgdltvigdrgttlsggqkarvnlaravyqdadiylldplsavdaevsr         571
                    . :  : .    ::*: *  *****: *: :***: :: .: ***.  . :*.  .
```

```
HumanCFTR       eifescvcklmanktrilvtskmehlkkadkililhegssyfygtfselqnlqpdfsskl         644
HumanP-gp       -vvqvaldkarkgrttiviahrlstvrnadviagfddgvivekgnhdelmkekgiyfklv         626
HumanABCC4      hlfelcicqilhekitilvthqlqylkaasqililkdgkmvqkgtyteflksgidfgsll         631
                 :.: .: :    :  *::: ::. :: *. *  :.:*     *.. *: :    : . :

HumanCFTR       mgcdsfdqfsaerrnsiltetlhrfslegdapvswtetkkqsfkqtgefgekrknsilnp         704
HumanP-gp       tmqtagneve----------------lenaa-----------------------------         641
HumanABCC4      kkdnee-------------------------------------------------------         637


HumanCFTR       insirkfsivqktplqmngieedsdeplerrlslvpdseqgeailprisvistgptlqar         764
HumanP-gp       ---------------------deskseid-alemssnds-------------rsslirkr         666
HumanABCC4      ----------------------seqppv-------p----------------gtptlrnr         652
                                    ...  :                          :: *

HumanCFTR       rrqsvlnlmthsvnqgqnihrkttastrkvslapqanlteldiysrrlsqetgleiseei         824
HumanP-gp       str-------rsv-------------------------rgsqaqdrklst----------         684
HumanABCC4      tf-----------------------------------sessvwsqqssr----------         666
                                                  .  .:: *

HumanCFTR       needlkecffddmesipavttwntylryitvhkslifvliwclviflaevaa-slvvlwl         883
HumanP-gp       -----ke---aldesippvsfwrimklnlte-wpyfvvgvfcaiingglqpafaiifski         735
HumanABCC4      --pslkdgalesqdvayvlqdw--wlsywankqsml------------------------         698
                     *:      :    :  *       :     :

HumanCFTR       lgntplqdkgnsthsrnnsyaviitstssyyvfyiyvgvadtllamgffrglplvhtlit         943
HumanP-gp       igvftriddpetkrqnsnlfsll----------flalgiis--fitfflqgftfgkagei         783
HumanABCC4      --nvtvngggnvtek-----------ldlnwylgiysgltvatvlfgiarsllvfyvlvn         745
                          : ...                 :  *:    .   : :.: .  .

HumanCFTR       vskilhhkmlhsvlqapmstlntl--kaggilnrfskdiailddllpltifdfiqlliv         1001
HumanP-gp       ltkrlrymvfrsmlrqdvswfddpknttgalttrlandaaqvkgaigsrlavitqn-ian         842
HumanABCC4      ssqtlhnkmfesilkapvlffdrn--pigrilnrfskdighlddllpltfldfiqtllqv         803
                 :: *:  ::.*:*:  :  ::      * : .*:::* . :.  :   :  : *  :

HumanCFTR       igaiavvavlqpy----ifvatvpvivafimlrayf--lqtsqqlkqlesegrspifthl         1055
HumanP-gp       lgtgiiisfiygwqltllllaivpiiaiagvvemkmlsgqalkdkkelegagk--iatea         900
HumanABCC4      vgvvsvavavipw----iaiplvplgiififlrryf--letsrdvkrlesttrspvfshl         857
                :*.  :   :  :    : :  **:     .:.  :   :: :: *.**.  :  : :.

HumanCFTR       vtslkglwtlrafgrqpyfetlfhkalnlhtanwflylstlrwfqmri----emifviff         1111
HumanP-gp       ---ienfrtvvsltqeqkfehmyaqslqvpyrns---lrkahifgitfsftqammyfsya         954
HumanABCC4      ssslqglwtiraykaeercqelfdahqdlhseawflflttsrwfavrl----daicamfv         913
                   :: : *: :   :   : ::    ::        * . : * : :      :   :

HumanCFTR       iavtfisil-ttgegegrvgiiltlamnimstlqwavnssidvdslmrsvsrvfkfidmp         1170
HumanP-gp       gcfrfgaylvahklms-fedvllvfsavvfgama---------------vgqvssf----         994
HumanABCC4      iivafgslilaktldagqvglalsyaltlmgmfqwcvrqsaevenmmisservieytdle         973
                  . * : : :         : *  :  ::. :                * :* .:

HumanCFTR       tegkptkstkpykngqlskvmiiensh------vkkddiwpsggqmtvkdltakyteg-g         1223
HumanP-gp       ---apdyakaki--saahiimiiektplidsysteglmpntlegnvtfgevvfnyptrpd         1049
HumanABCC4      keapwey-------------------q------krpppawphegviifdnvnfmyspg-g         1007
                                           .       * : . ::   *

HumanCFTR       nailenisfsispgqrvgllgrtgsgkstllsaflrllnt-egeiqidgvswdsitlqqw         1282
HumanP-gp       ipvlqglslevkkgqtlalvgssgcgkstvvqllerfydplagkvlldgkeikrlnvqwl         1109
HumanABCC4      plvlkhltaliksqekvgivgrtgagksslisalfrlsep-egkiwidkiltteiglhdl         1066
                  :*: ::  :.  : :.::* :*.***:::. : *: :   *:: :*      : ::

HumanCFTR       rkafgvipqkvfifsgtfrknldp---yeqwsdqeiwkvadevglrsvieqfpgkldfvl         1339
HumanP-gp       rahlgivsqepilfdcsiaeniaygdnsrvvsqeeivraakeanihafieslpnkystkv         1169
HumanABCC4      rkkmsiipqepvlftgtmrknldp---fnehtdeelwnalqevqlketiedlpgkmdtel         1123
                *  :.:: *: .:*  :: :*:      .  :::*: .. .*. ::  **.:* * .  :

HumanCFTR       vdggcvlshghkqlmclarsvlskakillldepsahldpvtyqiirrtlkqafadctvil         1399
HumanP-gp       gdkgtqlsggqkqriaiaralvrqphilldeatsaldtesekvvqealdkaregrtciv         1229
HumanABCC4      aesgsnfsvgqrqlvclarailrknqiliideatanvdprtdeliqkkirekfahctvlt         1183
                 : *  :* *::* :.:**::: : :**::** :: :*  : ::::. : :     * :

HumanCFTR       cehrieamlecqqflvieenkvrqydsiqkllner-slfrqaispsdrvklfphrnssk-         1457
HumanP-gp       iahrlstiqnadlivvfqngrvkehgthqqllaqkgiyfsmvsvqagtkrq---------         1280
HumanABCC4      iahrlntiidsdkimvldsgrlkeydepyvllqnkeslfykmvqqlgkaeaaaltetakq         1243
                  **:.:: :.: ::*::. :::::     ** ::   *          .

HumanCFTR       ---ckskpqiaalkeetee--evq-------dtrl  1480
HumanP-gp       ----------------------------------- 1280
HumanABCC4      vyfkrnyphightdhmvtntsngqpstltifetal  1278
```

4. ABCC4 aligned to ABCC5

```
HumanABCC4      ------------------------------------------------------------  0
MouseABCC4      ------------------------------------------------------------  0
humanABCC5      mkdidigkeyiipspgyrsvrertstsgthrdredskfrrtrplecqdaletaaraegls  60
MouseABCC5      mkdidmgkeyiipspgyrsdrdrsavpgqhrdpeeprfrrtrslecqdaletaarvegls  60


HumanABCC4      ---------------------------mlpvy-qevkpnplqdanlcsrvffwwlnplfk  32
MouseABCC4      ---------------------------mlpvh-tevkpnplqdanlcsrvffwwlnplfk  32
humanABCC5      ldasmhsqlrildeehpkgkyhhglsalkpirttskhqhpvdnaglfscmtfswlsslar  120
MouseABCC5      ldisvhshlqildeehskgkyhhglsvlkpfrtttkhqhpvdnaglfsymtfswlsplar  120
                                  : *.     : .*:::* * * : * **. * :

HumanABCC4      ighkr-rleeddmysvlpedrsqhlgeelqgfwdkevlraendaqkpsltraiikcywks  91
MouseABCC4      tghkr-rleeddmfsvlpedrskhlgeelqrywdkellrakkdsrkpsltkaiikcywks  91
humanABCC5      vahkkgelsmedvwslskhessdvncrrlerlwqeelnevgpdaas--lr-rvvwifcrt  177
MouseABCC5      vvhkkgellmedvwplskyessdvnsrrlerlwqeelnevgpdaas--lr-rvvwifcrt  177
                  **: .*  :*:: :   : *.   ..*:  *::*: ..  *: .  *   ::  : ::

HumanABCC4      ylvlgiftli-eesakviqpiflgk-iinyfenydpmdsvalntayayatvltfctlila  149
MouseABCC4      ylilgiftli-eegtrvvqplflgk-iieyfekydpddsvalhtaygyaavlsmctlila  149
humanABCC5      rlilsivclmitqlagfsgpafmvkhlleytqate--------snlqysllvlglllte  229
MouseABCC5      rlilsivclmitqlagfsgpafvvkhlleytqate--------snlqysllvlglllte  229
                 *:*.*. *:  : : .  * *: * :::* :  :        :   *: :* :  *:

HumanABCC4      ilh----hlyfyhvqcagmrlrvamchmiyrkalrlsnmamgktttgqivnllsndvnkf  205
MouseABCC4      ilh----hlyfyhvqcagmrlrvamchmiyrkalrlsnsamgktttgqivnllsndvnkf  205
humanABCC5      ivrswslaltwalnyrtgvrlrgailtmafkkilklkni--kekslgelinicsndgqrm  287
MouseABCC5      vvrswslaltwalnyrtgvrlrgailtmafkkilklkni--kekslgelinicsndgqrm  287
                :::     * :     :*:*** *:  * ::* *:*.*    :.: *:::*: *** :::

HumanABCC4      dqvtvflhflwagplqaiavtallwmeigisclagmavliillplqscfgklfsslrskt  265
MouseABCC4      dqvtiflhflwagplqaiavtvllwveigisclaglavlvillplqscigklfsslrskt  265
humanABCC5      feaaavgsllaggpvvailgmiynviilgptgflgsavfilfypammfasrltayfrrkc  347
MouseABCC5      feaaavgsllaggpvvailgmiynviilgptgflgsavfilfypammfvsrltayfrrkc  347
                 :.: .  :* .**: **       : :* : : * **::: *     .:* : :* *

HumanABCC4      atftdarirtmnevitgiriikmyaweksfsnlitnlrkkeiskilrssclrgmnlasff  325
MouseABCC4      aaftdarirtmnevitgmriikmyaweksfadlianlrkkeiskilgssylrgmnmasff  325
humanABCC5      vaatdervqkmnevltyikfikmyawvkafsqsvqkireeerrilekagyfqsitvgvap  407
MouseABCC5      vaatddrvqkmnevltyikfikmyawvkafsqcvqkireeerrilekagyfqsitvgvap  407
                .: ** *::.****:* :::******* *:*:: : ::*::*   :  :. ::.:.:.

HumanABCC4      saskiivfvtfttyvllgsvitasrvfvavtlygavrltvtlffpsaiervseaivsirr  385
MouseABCC4      iankvilfvtftsyvllgneitashvfvamtlygavrltvtlffpsaiergseaivsirr  385
humanABCC5      ivvviasvvtfsvhmtlgfdltaaqaftvvtvfnsmtfalkv-tpfsvkslseasvavdr  466
MouseABCC5      ivvviasvvtfsvhmtlgfhltaaqaftvvtvfnsmtfalkv-tpfsvkslseasvavdr  466
                 .  :  .***: :: **  :**::.*..:*:: :: :::.:  * :::  *** *:: *

HumanABCC4      iqtfllldeisqrnrqlpsdgkkmvhvqdftafwdk------------------------  421
MouseABCC4      iknfllldelpqrkahvpsdgkaivhvqdftafwdk------------------------  421
humanABCC5      fkslflmeevhmiknkpa-sphikiemknatlawdsshssiqnspkltpkmkkdkrasrg  525
MouseABCC5      fkslflmeevhmiknkpa-sphikiemknatlawdsshssiqnspkltpkmkkdkratrg  525
                ::.::*::*:   : :   . :  :.::: *  **.

HumanABCC4      ------------------------------------------------asetptlqglsftv  435
MouseABCC4      ------------------------------------------------aldsptlqglsfia  435
humanABCC5      kkekvrqlqrtehqavlaeqkghllldsderpspeeeegkhihlghlrlqrtlhsidlei  585
MouseABCC5      kkeksrqlqhtehqavlaeqkghllldsderpspeeeegkqihtgslrlqrtlynidlei  585
                                                              **  :.:

HumanABCC4      rpgellavvgpvgagkssllsavlgelapshglvsvhgriayvsqqpwvfsgtlrsnilf  495
MouseABCC4      rpgellavvgpvgagkssllsavlgelppasglvsvhgriayvsqqpwvfsgtvrsnilf  495
humanABCC5      qegklvgicgsvgsgktslisailgqmtllegsiaisgtfayvaqqawilnatlrdnilf  645
MouseABCC5      eegklvgicgsvgsgktslvsailgqmtllegsiavsgtfayvaqqawilnatlrdnilf  645
                . *:*:.: * **:**:**:**:**::    * ::: * :***:** *::..*:*.****

HumanABCC4      gkkyekeryekvikacalkkdlqlledgdltvigdrgttlsgggkarvnlaravyqdadi  555
MouseABCC4      gkkyekeryekvikacalkkdlqlledgdltvigdrgatlsgggkarvnlaravyqdadi  555
humanABCC5      gkeydeerynsvlnscclrpdlailpssdlteigerganlsgggrqrislaralysdrsi  705
MouseABCC5      gkefdeerynsvlnscclrpdlailpnsdlteigerganlsgggrqrislaralysdrsi  705
                **:::***:.*:::*.*: ** :* ..*** **:**:.*****: *:.****:*.* .*

HumanABCC4      yllddplsavdaevsrhlfelcicqilhekitilvthqlqylkaasqililkdgkmvqkg  615
MouseABCC4      yllddplsavdaevgkhlfqlcicqalhekitilvthqlqylkaashililkdgemvqkg  615
humanABCC5      yilddplsaldahvgnhifnsairkhlksktvlfvthqlqylvdcdevifmkegciterg  765
MouseABCC5      yilddplsaldahvgnhifnsairkrlksktvlfvthqlqylvdcdevifmkegciterg  765
```

```
                *:*******:**.*..*:*: .* : *:.* .::********  ...::::*:* :.::*

HumanABCC4      tyteflksgidfgsllkkdneeseqppvpgtptlrnrtfsessvwsqqssrpslkdgale         675
MouseABCC4      tyteflksgvdfgsllkkeneeaepstapgtptlrkrtfseasiwsqqssrpslkdgape         675
humanABCC5      theelmnlngdyatifnnlllgetppvein-------skketsg-----sqkksqdkgpk         813
MouseABCC5      theelmnlngdyatifnnlllgetppvein-------skkeatg-----sq-ksqdkgpk         812
                *: *:::   *:.::::::                  : .*::      *: . :* . :

HumanABCC4      sqd-------------------------------------------------------vay         681
MouseABCC4      gqdaentqavqpe-------esrsegrigfkayknyfsagaswffiiflvllnmvgqvfy         728
humanABCC5      tgsvkkekavkpeegqlvqleekgqgsvpwsvygvyiqaaggplaflvimalfmlnvgst         873
MouseABCC5      pgsvkkekavkseegqlvqveekgqgsvpwsvywvyiqaaggplaflvimvlfmlnvgst         872
                  .

HumanABCC4      vlqdwwlsywankqsmlnvtvngg-----gnvtekldlnwylgiysgltvatvlfgiars         736
MouseABCC4      vlqdwwlshwankqgalnntrnan-----gnitetldlswylgiyagltavtvlfgiars         783
humanABCC5      afstwwlsywikqgsgnttvtrgnetsvsdsmkdnphmqyyasiyalsmavmlilkairg         933
MouseABCC5      afstwwlsywikqgsgnstvyqgnrsfvsdsmkdnpfmqyyasiyalsmavmlilkairg         932
                .:. ****:* :: .  . . ..       .:.:.  :.:* .**:   .. :::   *.

HumanABCC4      llvfyvlvnssqtlhnkmfesilkapvlffdrnpigrilnrfskdighlddllpltfldf         796
MouseABCC4      llvfyilvnasqtlhnrmfesilkapvlffdrnpigrilnrfskdighmddllpltfldf         843
humanABCC5      vvfvkgtlrassrlhdelfrrilrspmkffdttptgrilnrfskdmdevdvrlpfqaemf         993
MouseABCC5      vvfvkgtlrassrlhdelfrrilrspmkffdttptgrilnrfskdmdevdvrlpfqaemf         992
                ::..   :.:*. **:.:*. **::*: *** .* **********: .:*  **:    *

HumanABCC4      iqtllqvvgvvsvavavipwiaiplvplgiififlrryfletsrdvkrlesttrspvfsh         856
MouseABCC4      iqtlllvvsviavaaavipwiliplvplsvvflvlrryfletsrdvkrlesttrspvfsh         903
humanABCC5      iqnvilvffcvgmiagvfpwflvavgplvilfsvlhivsrvlirelkrldnitqspflsh         1053
MouseABCC5      iqnvilvffcvgmiagvfpwflvavgpllilfsllhivsrvlirelkrldnitqspflsh         1052
                **.:: *.  :.: ..*:**: : : ** ::* .*:       *::***:. *:**.:**

HumanABCC4      lssslqglwtiraykaeercqelfdahqdlhseawflflttsrwfavrldaicamfviiv         916
MouseABCC4      lssslqglwtiraykaeercqelfdahqdlhseawflflttsrwfavrldaicaifvivv         963
humanABCC5      itssiqglatihaynkgqeflhryqellddnqapfflftcamrwlavrldlisialittt         1113
MouseABCC5      itssiqglatihaynkrqeflhryqellddnqapfflftcamrwlavrldlisialittt         1112
                ::**:*** **:**:  :.  . ::   * ..  :***  : **:***** *.  ::  .

HumanABCC4      afgslilaktldagqvglalsyaltlmgmfqwcvrqsaevenmmisvervieytdl-eke         975
MouseABCC4      afgslvlaktlnagqvglalsyaltlmgmfqwsvrqsaevenmmisvervieytdl-eke         1022
humanABCC5      glmivlmhgqippayaglaisyavqltglfqftvrlasetearftsverinhyiktlsle         1173
MouseABCC5      glmivlmhgqipsayaglaisyavqltglfqftvrlasetearftsverinhyiktlsle         1172
                .:  :::   :  . .***:***: * *:**: ** ::*.*  : ****: .* .  . *

HumanABCC4      apwe-yqkrpppawphegviifdnvnfmyspggplvlkhltaliksqekvgivgrtgagk         1034
MouseABCC4      apwe-ckkrpppgwphegvivfdnvnftysldgplvlkhltaliksrekvgivgrtgagk         1081
humanABCC5      apariknkapspdwpqegevtfenaemryrenlplvlkkvsftikpkekigivgrtgsgk         1233
MouseABCC5      apariknkapphdwpqegevtfenaemryrenlplvlkkvsftikpkekigivgrtgsgk         1232
                ** .  :* *   **:** : *:*.:: *    *****:::  ** :**:*******:**

HumanABCC4      sslisalfrlsepe-gkiwidkiltteiglhdlrkkmsiipqepvlftgtmrknldpfne         1093
MouseABCC4      sslisalfrlsepe-gkiwidkiltteiglhdlrkkmsiipqepvlftgtmrknldpfne         1140
humanABCC5      sslgmalfrlvelsggcikidgvrisdigladlrsklsiipqepvlfsgtvrsnldpfnq         1293
MouseABCC5      sslgmalfrlvelsggcikidgirisdigladlrsklaiipqepvlfsgtvrsnldpfnq         1292
                ***  ***** * . * * ** :  ::*** ***.*::*********:**:*.******:

HumanABCC4      htdeelwnalqevqlketiedlpgkmdtelaesgsnfsvgqrqlvclarailrknqilii         1153
MouseABCC4      htdeelwraleevqlkeaiedlpgkmdtelaesgsnfsvgqrqlvclarailknnrilii         1200
humanABCC5      ytedqiwdalerthmkeciaqlplklesevmengdnfsvgerqllciarallrhckilil         1353
MouseABCC5      ytedqiwdalerthmkeciaqlplklesevmengdnfsvgerqllciarallrhckilil         1352
                :*::::* **:..::** * :** *:::*: *.*.*****:***:*:***:*:. :***:

HumanABCC4      deatanvdprtdeliqkkirekfahctvltiahrlntiidsdkimvldsgrlkeydepyv         1213
MouseABCC4      deatanvdprtdeliqqkirekfaqctvltiahrlntiidsdkimvldsgrlkeydepyv         1260
humanABCC5      deataamdtetdlliqetireafadctmltiahrlhtvlgsdrimvlaqgqvvefdtpsv         1413
MouseABCC5      deataamdtetdlliqetireafadctmltiahrlhtvlgsdrimvlaqgqvvefdtpsv         1412
                ***** :* .** ***:.*** **.**:*******.*:: **:**** .*:: *:* * *

HumanABCC4      llqnkeslfykmvqqlgkaeaaaltetakqvyfkrnyphightdhmvtntsngqpstlti         1273
MouseABCC4      llqnpeslfykmvqqlgkgeaaaltetakqvyfrrnypditftspavmntsngqpsalti         1320
humanABCC5      llsndssrfyamfaaaenkvavkg-------------------------------------         1437
MouseABCC5      llsndssrfyamfaaaenkvavkg-------------------------------------         1436
                **.* .* ** *.    :  *.

HumanABCC4      fetal         1278
MouseABCC4      fetal         1325
humanABCC5      -----         1437
MouseABCC5      -----         1436
```

Human CFTR NP_000483.3
Mouse CFTR AAR16297.1
Chicken CFTR ABK34432.1
Xenopus CFTR CAA46348.1
Dogfish CFTR AAA49616.1
Sea lamprey CFTR AKC42149.1
Human ABCC4 NP_001288758.1
Mouse ABCC4 NP_001157148.1
Rat ABCC4 NP_596902.1
Human ABCC5 NP_005679.2
Mouse ABCC5 NP_038818.2
Human P-glycoprotein AAA59575.1