Biochemical Interrogation of Rare Cystic Fibrosis Mutations Informs Strategies for Future Therapeutic Intervention

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Biochemistry
University of Toronto

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2016

Abstract
There are over 2000 mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene associated with Cystic Fibrosis (CF) disease, and to date, the two recently FDA-approved drugs Kalydeco™ and Orkambi™ have been effective in rescuing functional expression of the two most common *CFTR* mutations, G551D and ΔF508, respectively, representing approximately 90% of the patient population worldwide. However, potential efficacy of these therapies for the remaining ~2000 mutations is unknown. Thus, biochemical characterization of each rare variant is needed to assess potential clinical benefit. My thesis attempts to understand molecular defects associated with less common *CFTR* mutations, mainly c.3700A>G (causing abberant splicing and subsequent translation of ΔI1234_R1239-CFTR) and c.2052_2053insA (Q685TfsX4-CFTR), with the goal of determining whether current CF therapies (specifically designed for G551D and ΔF508) could be used for these rare variants. Additionally, my work aims to identify unique molecular aberrations for these rare, mutant CFTR proteins in order to facilitate development of mutation-specific therapeutics. Through a collaborative initiative involving clinicians, biochemists and biophysicists, we found that ΔI1234_R1239-CFTR shared certain biochemical attributes with ΔF508-CFTR, however contained intrinsic defects which differed significantly (i.e. poorer interdomain assembly
between the amino- and carboxy-termini, and decreased functional responses to cAMP agonists, CFTR correctors and potentiatators). When CFTR activity was evaluated in nasal epithelial cultures from siblings homozygous for c.3700A>G, we likewise found that ΔI1234_R1239-CFTR responded poorly to small molecules. For Q685TfsX4-CFTR, we found that this variant was prematurely truncated, and therefore did not respond to CFTR-specific therapies. However, a proof-of-concept in vitro strategy to ‘skip’ the exon containing the c.2052_2053insA mutation led to an incomplete, yet partially mature CFTR molecule which exhibited channel activity, albeit this function was unregulated. In parallel to these studies, we generated and functionally characterized several other rare CFTR mutations using novel mutagenesis and high-throughput functional screening methods, allowing for rapid identification and stratification of genotypes that could potentially benefit from current CF therapies. Furthermore, our efforts described here aim to facilitate future mutation-specific CF drug discovery, as well as a ‘personalized medicine’ approach to assist in the development of novel strategies to combat this fatal genetic disease.
Statement of Co-authorship

This thesis is based on research conducted by Steven V. Molinski under the supervision of Dr. Christine E. Bear. All data were generated, analyzed and interpreted by Steven V. Molinski with the assistance of Dr. Christine E. Bear as well as many other colleagues; specific contributions to this thesis by each individual are acknowledged at the beginning of each chapter and in the captions of relevant figures.
Acknowledgements

First and foremost, I would sincerely like to thank my supervisor, Dr. Christine Bear, for providing me with the opportunity to work in her lab, as well as for allowing me to have the intellectual freedom to pursue novel ideas. I would also like to thank Christine for providing the guidance and training to become a well-rounded scientist. Christine has passionately led the pursuit of acquiring therapeutically relevant knowledge to complex, unanswered and clinically pressing questions, and I am grateful to have been a part of her team during this time; I know that our work will one day lead to personalized treatments for individuals suffering from Cystic Fibrosis disease. In addition, I would like to thank the members of my advisory committee, Drs. John Rubinstein and Régis Pomès, for their expert advice and scientific insight during these past six years. Your significant contributions have allowed me to strive for success in attempting to understand the complex protein structure-function relationship of CFTR. Further, I would like to thank all of the collaborators with whom I have worked with, especially Theo Moraes, Tanja Gonska, Kai Du, Wan Ip, Hong Ouyang and Kethika Kulleperuma.

I would also like to thank the place that I called home these past years: the Department of Biochemistry at the University of Toronto, as well as the Department of Molecular Structure and Function at the Hospital for Sick Children. Thank you to Angela Skoutakis for all of her help with administrative work during my studies. I would also like to thank the members of the Bear lab, especially Ling Jun Huan, Drs. Danny Li, Paul Eckford, Mohabir Ramjeesingh, Stan Pasyk, Patrick Kim Chiaw and Leigh Wellhauser for sharing their expertise, and also for their helpful discussions. I would also like to thank Dr. Saumel Ahmadi for his passion and attitude towards science, as well as for his constant assistance and insight into every aspect of my work. He has been a limitless source of ideas, and I really enjoyed working together. I would also like to thank Onofrio Laselva for all of his help with my various projects, as well as for his enthusiasm, ideas and helpful discussions. Also, thank you Michelle Di Paola, Maurita Hung, Stephanie Chin, Randy Kissoon, Ellen Li, Marshall Zhang, Jessica She, Jamie Hu, Wilson Yu and Christina D’Antonio for your helpful insight with research and non-research related subjects alike. It was a pleasure to have worked alongside all of you, thank you for making my experience a
memorable one. Additionally, I would like to say thank you to my family and friends for their support during my time at the University of Toronto. I would especially like to thank my parents, Walter and Luba, as well as my brother, David, and his partner, Diana, for their unconditional love and support. I would also like to thank my ‘new’ family, the Ballas and Skenderis, for their patience, care and support during this stage of my life. Lastly, and most importantly, I would like to express my deepest appreciation and love for my partner in life, Denisa Balla. Your belief in me and my work has provided me with the confidence to strive for success every day. Thank you for being the constant, shining light in my life; I would not be the same man without you by my side. In other words, \textit{ti je dielli në mëngjes që ndriçon tokën time}. Finally, I must say that I have not done it alone. To quote the great Sir Isaac Newton: “If I have seen further, it is by standing on the shoulders of giants.”
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<th>Description</th>
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<tr>
<td>ΔD529_K830</td>
<td>Deletion of residues 529-830</td>
</tr>
<tr>
<td>ΔF508</td>
<td>Deletion of phenylalanine at position 508</td>
</tr>
<tr>
<td>ΔI1234_R1239</td>
<td>Deletion of residues 1234-1239</td>
</tr>
<tr>
<td>2184insA</td>
<td>Insertion of adenine at position 2184 in the mRNA sequence</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCC7</td>
<td>‘C’ subfamily member 7 of the ABC transporter superfamily</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BMD</td>
<td>Becker Muscular Dystrophy</td>
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<tr>
<td>c.2052_2053insA</td>
<td>Insertion of adenine at position 2052 in the c.DNA sequence</td>
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<td>c.3700A&gt;G</td>
<td>Adenine to guanine substitution at position 3700 in the c.DNA sequence</td>
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<tr>
<td>C4</td>
<td>Class 2 CFTR corrector Corr-4a</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CF-1</td>
<td>Cystic Fibrosis patient #1</td>
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<td>CF-2</td>
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<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<td>CFTRinh-172</td>
<td>CFTR-specific inhibitor</td>
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<td>DMD</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERAD</td>
<td>ER-associated degradation</td>
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<td>F508del</td>
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<td>FLIPR</td>
<td>Fluorometric Imaging Plate Reader membrane potential assay</td>
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<td>FSK</td>
<td>Forskolin</td>
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<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>Intracellular loop</td>
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<td>Ivacaftor</td>
<td>Trade name of FDA-approved CFTR potentiator VX-770 (Kalydeco&lt;sup&gt;TM&lt;/sup&gt;)</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
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<td>MSD</td>
<td>Membrane spanning domain</td>
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<td>Nucleotide binding domain</td>
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<td>Brand name of FDA-approved CFTR corrector VX-809 (Lumacaftor) and potentiator VX-770 (Ivacaftor/Kalydeco™) co-therapy</td>
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<td>p.Ile1234Val</td>
<td>Isoleucine to valine substitution at position 1234</td>
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<tr>
<td>p.Phe508del</td>
<td>Deletion of phenylalanine at position 508</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PM</td>
<td>Plasma membrane</td>
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<td>Q685TfsX4</td>
<td>Glutamine to threonine substitution at position 685 with frame-shift and stop codon (X) 4 residues C-terminal</td>
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<td>Regulatory domain</td>
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<td>Transmembrane member 16A (Anoctamin-1, ANO1)</td>
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<td>Wild-type</td>
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Chapter 1

Introduction

1.1 Acknowledgements

Parts of the literature review presented in this chapter have been published:


A letter from the publisher outlining permission to use copyright material is provided in the Appendix, section A1.1.

1.2 ATP-Binding Cassette Transporter Superfamily

ATP-binding cassette (ABC) proteins are polytopic membrane transporters that are found throughout the animal kingdom, requiring ATP to actively and unilaterally translocate structurally diverse endo- and xeno-biotics across the lipid bilayer of cell membranes (Higgins, 1992; Slot *et al.*, 2011). Mammalian ABC transporters are involved in many different physiological processes, including: (1) efflux of signaling molecules, (2) modulating absorption, distribution, and elimination of metabolites, and (3) preventing accumulation, and assisting with the elimination of drugs, toxins and their metabolites (Szakács *et al.*, 2008). Therefore, these proteins are of great physiological, pharmacological, and pathological importance.

The human ABC transporter superfamily is comprised of 49 genes that are distributed among 7 phylogenetic branches designated A through G (Dean and Allikmets, 2001). The human ABC’C’ subfamily consists of 13 members, including the multidrug resistance proteins MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6), MRP7 (ABCC10), MRP8 (ABCC11), MRP9 (ABCC12) and the pseudogene MRP10 (ABCC13), as well as the sulfonylurea receptors SUR1 (ABCC8) and SUR2 (ABCC9), and the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) (*Figure 1.1*) (Dean and Allikmets, 2001). While the nine MRPs are mainly involved in transporting organic anions out of cells (Deeley *et al.*, 2006), and SUR1 and SUR2 are intracellular sensors of ATP and ADP (Bryan *et al.*, 2007),
**Figure 1.1: Phylogenetic tree of the human ABCC transporter subfamily.**

Protein sequence alignments were performed using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The pseudogene ABCC13 has been omitted from this analysis. Modified from Slot *et al.*, 2011.
CFTR is the one exception to the rule among this vast collection of membrane transporters. Rather than being capable of ‘pumping’ various organic substrates across cell membranes, CFTR is instead a bilateral chloride channel, allowing for transmembrane flow of anions down its electrochemical gradient, and thus regulating electrolyte and water homeostasis across epithelial cell membranes within various tissues throughout the body (Riordan et al., 1989; Bear et al., 1992; Li et al., 1993). Further, it has been hypothesized that this structurally similar, yet functionally diverse ABC family member evolved by first starting out as a transporter, and then by acquiring mutations which led to its subsequent ‘broken’ alternating-access translocation mechanism and acquisition of an anion pore (Gadsby et al., 2006). There is some merit to this hypothesis, such that bioinformatics analyses investigating sequence conservation between human ABCC proteins suggests that MRP4 (ABCC4) is a close paralog of CFTR, and further that CFTR acquired mutations in the TMs over an evolutionary timescale to support intraprotein interactions rendering a stable permeation pathway, or open state channel (Jordan et al., 2008). These findings have been supported by significant residue conservation between CFTR orthologs, again suggesting that CFTR functionally diverged very early during evolution to become a unique ABC transporter protein (Jordan et al., 2008). Importantly, mutations in certain human ABC transporters are responsible for a number of genetic diseases (Table 1.1). These include: Harlequin-type ichthyosis (caused by mutations in ABCA12), Dubin-Johnson Syndrome (MRP2/ABCC2), Pseudoxanthoma elasticum (MRP6/ABCC6), and the focus of this doctoral thesis, cystic fibrosis (CFTR/ABCC7) (Akiyama, 2006; Kartenbeck et al., 1996; Ringpfeil et al., 2000; Riordan et al., 1989).

1.3 CFTR and Cystic Fibrosis Disease

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disease in the world, thought to have originated in Europe and subsequently spread to other geographies by human migration patterns as well as heterozygote advantage throughout the past millennia (Figure 1.2) (Gabriel et al., 1994). CF is a multi-system disease primarily affecting epithelial tissues (e.g. lungs, intestine, pancreas), and the main cause of morbidity and mortality is decreased lung function with age (Cutting, 2015). Pathophysiology is caused by mutations in the CFTR/ABCC7 gene (NM_000492.3), and mutations cause a wide array of aberrations, including: CFTR mRNA quantity or quality, CFTR protein biosynthesis, processing and/or channel function.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein (common name)</th>
<th>Genetic Disease</th>
<th>Reference</th>
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<td>Yoshiura et al., 2006</td>
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<td></td>
<td>Sitosterolemia</td>
<td>Rudkowska and Jones, 2008</td>
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Table 1.1: Genetic diseases associated with mutant ABC transporters.
Modified from Slot et al., 2011.
Figure 1.2: Worldwide incidence of Cystic Fibrosis.

(i.e. quantity/quality); ultimately altering CFTR activity in fluid transporting epithelial tissues, such as the airways, gastrointestinal tract and sweat ducts (Figure 1.3) (Cutting, 2015; Sosnay et al., 2013). The clinical phenotype is variable but severely affected individuals typically suffer from airway obstruction with recurrent episodes of inflammation, infection and pancreatic insufficiency (Figure 1.4) (Riordan et al., 1989). Importantly, although significant achievements have been made in CF research since the discovery of the CFTR gene at the Hospital for Sick Children in Toronto in 1989, there are still many unanswered questions, and that is why CF remains fatal (Riordan et al., 1989). However, a major success over the past 50 years in Canada is that the average lifespan of CF patients has increased from 4 to about 50 years (highest in the world)! This is largely attributed to improved clinical care and disease management, although novel CFTR-targeted, drug-based therapies are showing great promise for further enhancement of lifespan and improvement of quality of life in certain patients.

Approximately 2000 different variants in the CFTR gene have been reported in the CF Mutation Database (http://www.genet.sickkids.on.ca/cftr/app), and each mutation is classified based on the predicted nature of the molecular defect; however, validation of predicted biochemical consequences of less than 10% of these variants have been completed (Molinski et al., 2012). Therefore, there is a need to generate these uncharacterized CFTR mutations for further in vitro study, in order to test the response of these mutants to emerging therapeutics, as well as discover novel targeted therapies to enhance the functional expression of rare CF disease-causing mutations. Excitingly, a CFTR-specific small molecule therapy (Ivacaftor, Kalydeco™ or VX-770) has recently been developed by Vertex Pharmaceuticals and is FDA-approved for use in patients bearing the minor CF disease-causing gating mutation, G551D (as well as several other less common CFTR gating mutations: G178R, S549N, S549R, G551S, G970R, G1244E, S1251N, S1255P and G1349D); these mutations in aggregate comprise approximately 5% of the CF patient population (Yu et al., 2012; Okiyoneda et al., 2013). However, although a clinically beneficial compound is now available for CF patients bearing these gating mutations, this CFTR-targeted therapy does not work for the vast majority of individuals with CF, since they bear non-gating mutations.
Figure 1.3: Molecular consequences of mutations in the CFTR gene.

Key steps of CFTR transcription, CFTR translation and forward trafficking through the biosynthetic compartments within a cell are depicted. Events required for the normal life cycle of a CFTR molecule is described on the left. The right side lists events and characteristics which are disrupted via mutations in the CFTR gene; mutations affect quantity (transcription, splicing, folding, stability) and/or quality (activity, conductance) of CFTR mRNA and protein. CFTR protein is shown in the inset (top): MSDs are coloured purple, NBDs are yellow, and the R domain is blue. Modified from Brodlie et al., 2015; Cutting, 2015.
Figure 1.4: Cardinal features of Cystic Fibrosis and Contribution of Genetic Modifiers.
Affected tissues and related consequences are labelled (left and right sides), and the main anatomical site of mortality and morbidity (i.e. lungs) is circled red (center). Modified from Cutting, 2015.
The most common CFTR mutation in Europe and North America, ΔF508, has been studied extensively (Li et al., 1993; Kim Chiaw et al., 2009; Wellhauser et al., 2010; Okiyoneda et al., 2013; Eckford et al., 2014). This mutation causes multiple defects in the CFTR protein, leading to its impaired assembly during synthesis and reduced post-translational stability (Figure 1.5). Recently, it has been argued that a single small molecule compound may be unable to “correct” the defects in conformational maturation, channel activity and folding of the full-length mutant protein within biosynthetic compartments as well as at the cell surface, given the existence of multiple intra- and interdomain defects. Nonetheless, knowledge regarding the molecular defects caused by ΔF508 has driven development of targeted, interventional compounds, such as VX-809 (or Lumacaftor). Although VX-809 has shown promise in partially ameliorating the protein folding defect caused by ΔF508 in vitro, some clinical efficacy (when in combination with G551D-CFTR potentiator VX-770, referred to as Orkambi™ co-therapy) has been validated. However, patient responses are variable; this is partly due to genetic modifiers and environmental factors, as well as the effects of each therapy on mutant CFTR protein stabilities (Hamosh and Cory, 1993; Wright et al., 2011; Sun et al., 2012; Corvol et al., 2015; Viel et al., 2015). Further, development of these drugs highlights the therapeutic relevance of understanding the basic defects caused by each mutation. Unfortunately, a small percentage of the documented CFTR variations have been sufficiently interrogated with respect to their consequences on CFTR protein folding and/or function. Coupled with global excitement about the therapeutic potential of the aforementioned small molecules, VX-809 (Lumacaftor) and VX-770 (Ivacaftor/Kalydeco™; and Orkambi™ co-therapy), there is a growing demand to determine whether patients with rare CFTR variants will be effectively treated with these new drugs.

1.4 Structural Models of CFTR

CFTR is a complex-glycosylated, ATP-dependent and phosphorylation-regulated plasma membrane chloride channel, mediating flux of chloride (and other anions, mainly bicarbonate) across apical membranes of polarized epithelial cells in certain tissues (e.g. lung, gut, pancreas) (Riordan et al., 1989; Bear et al. 1992; Howell et al. 2004; Rowe et al. 2005). The tertiary structure of CFTR is arranged into two membrane-spanning domains (MSDs; six transmembrane helices, TMs, in each MSD), two intracellular nucleotide binding domains
Figure 1.5: Biosynthetic processing of WT-CFTR and ΔF508-CFTR.

Modified from Molinski et al., 2012.
(NBDs) and a regulatory (R) domain (Gadsby et al., 2006). The TMs of MSD1 and MSD2 form the channel pore, while the NBDs form the catalytic heterodimer required for nucleotide-dependent channel gating; the R domain regulates the gating of this ion channel (Figure 1.6) (Hwang et al., 2013). Unfortunately, current methodological limitations make it difficult to crystallize such a large polytopic and post-translationally modified (i.e. glycosylation, phosphorylation) membrane protein as CFTR (which also consists of a disordered Regulatory domain), and as a result, high quality tertiary structural information is lacking; however, some low quality (~30 Å resolution, Rosenberg et al., 2004; ~10 Å resolution, Rosenberg et al., 2011) structures have been determined and have provided insight into domain arrangement, rather than residue side-chain position. Therefore, to provide structures of higher resolution, many homology models based on crystal structure data (3 Å) of the S. aureus ABC transporter Sav1866 were developed (Dawson and Locher, 2006; Dawson and Locher, 2007). Sav1866 is a homodimer, crystallized in the ATP-bound state, and served as a template to build models of the core (MSD1-NBD1 and MSD2-NBD2) of CFTR (Serohijos et al., 2008; Mornon et al., 2009; Dalton et al., 2012; Mornon et al., 2015; Corradi et al., 2015). Importantly, these models have assisted in the elucidation of CFTR structure by validating previous studies, and further, will be an integral tool to guide future biochemical experiments.

Using these and other models of CFTR, several interdomain interfaces have been implicated in maintaining CFTR structure and function. These interfaces include: NBD1:ICL4, NBD1:2, NBD1:2:ICL1:2:4, ICL3:R-domain, and the TMs of MSD1:2. Destabilization of the NBD1:ICL4 interface causes CFTR to misfold, thereby demonstrating the importance of this interdomain interaction; ΔF508 (NBD1) causes this destabilization as previously mentioned (He et al., 2010; Serohijos et al., 2008). Additionally, the NBD1:2 heterodimer interface is required for the enzymatic activity of CFTR (Stratford et al., 2007). The composite NBD1:2:ICL1:2:4 interface is important for coupling the channel pore (MSDs) to the catalytic domains (NBDs) (Pasyk et al., 2009; Kalid et al., 2010). Importantly, the MSD1:2 interface is essential for channel activity, since it comprises the channel pore, and thus mediates chloride flux across biological membranes (Ge et al., 2004; Choi et al., 2005; Fatehi and Linsdell, 2009), while the reported ICL3:R-domain interaction has been shown to regulate activity through an intricate H-bond
**Figure 1.6: Predicted membrane topology and homology models of CFTR.**

A topology model (A) and homology model (Mornon et al., 2009) (B) of CFTR showing the five domains: MSD1, NBD1, MSD2, R domain, and NBD2. MSDs are coloured purple, NBDs are coloured yellow, and the R domain is coloured blue. The ‘Y-shaped’ sticks represent N-glycosylation sites. The plasma membrane is represented as a gray box; intracellular (“in”) and extracellular (“out”) sides are indicated. MSD, membrane spanning domain; TM, transmembrane α-helix; NBD, nucleotide binding domain; R, regulatory domain.
network (Wang et al., 2010). Further identification of interdomain interfaces important for biogenesis and/or functional expression of CFTR is required to further understand this complex ABC protein, and perhaps gain insight into the rational design of therapeutics to repair unique structural defects of rare disease-causing mutant variants.

1.5 Intra-Molecular Defects Conferred by ΔF508 and Considerations for Less Common Disease-Causing Mutations

Several studies have previously shown that the Class 2 mutation ΔF508 (in NBD1) alters the folding and thermostability of NBD1 (when studied in isolation) and disrupts the intramolecular assembly of CFTR, including the structurally relevant interface with ICL4 in MSD2, as well as the catalytic NBD1:2 heterodimer (Figure 1.7) (Serohijos et al., 2010; He et al., 2010; Thibodeau et al., 2010; Mendoza et al., 2012; Rabeh et al., 2012). These conformational defects lead to impaired forward trafficking through the biosynthetic compartments, and retention in the ER (Thibodeau et al., 2010; Molinski et al., 2012; Farinha et al., 2013; Eckford et al., 2014). The limited number of ΔF508-CFTR molecules that reach the cell surface exhibit altered channel activity and reduced cell surface stability at physiological temperature (Serohijos et al., 2010; He et al., 2010; Du et al., 2005; He et al., 2013; Okiyoneda et al., 2013). However, the pharmacological chaperone VX-809 (Lumacaftor) was found to be partially effective in rescuing the functional expression of ΔF508-CFTR at the cell surface in heterologous (e.g. HEK-293 cell overexpression) as well as endogenous (e.g. ex vivo patient epithelial tissues) expression systems (Van Goor et al., 2011; Eckford et al., 2014). Together with VX-770 (Kalydeco™), a drug that enhances CFTR channel activity, VX-809 (Lumacaftor) further enhanced the functional expression of ΔF508-CFTR in pre-clinical studies of primary bronchial cell cultures and rectal biopsy-derived organoids (this combination therapy has been trademarked Orkambi™) (Van Goor et al., 2011; Dekkers et al., 2013; Kopeikin et al., 2014). Finally, in clinical trials, this combination led to significant improvement in lung function (2.6-4% increase), measured as forced expiratory volume (percent predicted) in one second (FEV₁) (Boyle et al., 2014; Wainwright et al., 2015).
Figure 1.7: Structural model of CFTR highlighting the location of F508. (A) Full-length homology model of CFTR (Mornon et al., 2009); MSD1, blue; MSD2, yellow; NBD1, cyan; NBD2, orange; R-domain, green; F508, red. (B) Position of F508 at the ICL4:NBD1 interface. (C) Crystal structure of NBD1 (Lewis et al., 2004); ATP, pink. Republished with permission from Molinski et al., 2012.
Importantly, there are hundreds of other CFTR variants, in addition to ΔF508-CFTR, thought to result from intrinsic defects in folding, assembly and trafficking, although these mutations are relatively rare (Welsh et al., 1993; Sosnay et al., 2013). The prevailing CF drug discovery paradigm suggests that drugs that are effective in targeting ΔF508-CFTR will be effective in rescuing other Class 2 mutations (Awatade et al., 2015; Rapino et al., 2015). It is well known that defective assembly of ΔF508-CFTR is detectable as the marked reduction in the conversion of core-glycosylated ΔF508-CFTR form to the complex-glycosylated form and decreased functional expression on the membrane (Cheng et al., 1990). This hallmark biochemical profile (i.e. reduced complex-glycosylated protein) reports the ER retention of the mutant protein. Lukacs and colleagues were the first to probe the conformational defects of the mutant protein using limited proteolysis (Du et al., 2005; Du and Lukacs, 2009). Protease resistance is known to provide insight into the conformational compactness of proteins folded in cells and the protease resistance of ΔF508-CFTR was shown to be significantly reduced relative to the WT-CFTR protein. Protease digest patterns, analyzed by SDS-PAGE and probed using domain specific antibodies, revealed that the protease resistance of NBD2 was particularly reduced in the context of the full-length ΔF508-CFTR protein, relative to full-length WT-CFTR. These were the first data to reveal the possible consequences of a misfolded ΔF508-NBD1 on assembly with the second half of the CFTR protein during translation.

Misassembly of the full-length ΔF508-CFTR protein as well as other Class 2 CFTR mutants likely occurs at several intramolecular regions (i.e. interdomain interfaces). For ΔF508-CFTR, there are multiples locations at which NBD1 directly interacts with domains in the second half of the full-length protein, including MSD2 (i.e. ICL4) and NBD2. Identification of a pivotal juncture (i.e. NBD1:ICL4) in the ΔF508-CFTR mutant was partly guided by molecular models of the full-length WT-CFTR generated using the crystal structure of the bacterial ABC transporter, Sav1866 as a template. To date, interventions aimed at enhancing this interaction appear to partly, but not completely rescue biosynthesis and processing of ΔF508-CFTR (Thibodeau et al., 2010; Rabeh et al., 2012; Mendoza et al., 2012). Therefore, these findings suggest that perturbation of this interface is not the dominant molecular defect caused by the ΔF508 mutation, since reestablishing this interface did not restore native CFTR folding.
Several other less common CF disease-causing mutations (i.e. L1065P, R1066C and G1069R) have been identified in the coupling helix of ICL4, biochemically interrogated, and found to cause ER retention, further supporting the idea that this region mediates important interactions during biosynthesis (Mendoza et al., 2012). Further, substitution of the arginine at position 1070 with tryptophan (i.e. R1070W) in the context of the WT-CFTR, introduces a bulky group on the face of the coupling helix that interacts with NBD1 and like the substitutions above, this leads to misprocessing. Another piece of evidence for the hypothesis that this helical segment conferred by ICL4, interacts with the NBD1 surface containing F508 in the full-length protein came from chemical cross-linking studies of engineered cysteine pairs. In these studies, deletion of F508 impaired chemical crosslinking of the same cysteine pairs in the full-length protein, supporting the idea that this intramolecular interaction is perturbed in the full-length mutant protein. Lastly, introduction of R1070W (compensating for the loss of the bulky, aromatic side-chain of F508) or V510D (forming an apparent salt bridge with R1070) in the ΔF508-CFTR protein partially corrects folding of the full-length protein, highlighting the idea that even in the absence of F508, assembly of the CFTR can be partially restored through structural changes at key interfaces (Thibodeau et al., 2010; Mendoza et al., 2012).

Clearly, there is still much to learn regarding intra-domain and intra-molecular interactions vital for proper folding, assembly and biosynthesis of CFTR. These challenges could be overcome using biophysical tools directly probing intrinsic properties of folding/unfolding of the full-length WT-CFTR protein, ΔF508-CFTR as well as the remaining ~2000 rare disease-causing mutants; however applicability of these tools may not be feasible for all mutants. To date, the only assay for folding of the full-length protein is assessment of the acquisition of complex-glycosylation, and this biomarker reflects a complex series of events, with a significant number of these processes being mediated by proteins other than CFTR.

### 1.6 Small Molecule Modulators of CFTR and CF Drug Discovery

At least some chemical chaperones, such as sodium butyrate and glycerol, as well as incubation at low temperatures (Denning et al., 1992), can promote increased CFTR trafficking to the cell surface. However, these treatments are highly non-specific for CFTR and otherwise not suitable for use in patients; although surprisingly, recent studies showed that induction of
low internal temperatures \textit{in vivo} partially recapitulated this CFTR-rescuing effect (Zhang \textit{et al.}, 2015). Importantly, at least some first generation CFTR correctors and potentiators interact directly with CFTR to promote its functional rescue (Loo \textit{et al.}, 2009; Wellhauser \textit{et al.}, 2009, Kim Chiaw \textit{et al.}, 2010; Eckford \textit{et al.}, 2012; Eckford \textit{et al.}, 2014) rather than exerting their effect non-specifically by increasing total protein expression and lowering fidelity of ER quality control mechanisms, or by mediated chloride flux via alternative channels. Further, certain corrector compounds may work by modifying aberrant interactions of mutant CFTR with chaperone proteins or degradation pathways (Wang \textit{et al.}, 2006; Younger \textit{et al.}, 2006), while potentiators may work by enhancing intrinsic gating of otherwise gating-incompetent channels.

Further efforts in the identification of correctors and potentiators using high-throughput screening approaches have been very fruitful, and there is much we can learn from each CFTR small molecule. Hundreds of compounds classified as CFTR correctors or potentiators have been identified in literature to date (Pedemonte \textit{et al.}, 2005; Van Goor \textit{et al.}, 2006; Carlile \textit{et al.}, 2007; Kalid \textit{et al.}, 2010, Lin \textit{et al.}, 2010; Van Goor \textit{et al.}, 2009; Van Goor \textit{et al.}, 2011) (Figure 1.8). Most of these molecules are deemed unsuitable for clinical use namely due to low efficacy, cell-type specificity and/or toxicity profiles. However, these compounds provide precedent as a useful scientific tool to probe how an ideal modulator may affect CFTR, and a potential scaffold for future drug designs. An understanding of the mechanism of action and binding site of previous generation CFTR modulators would be an important step towards the rational drug design of best-in-class CFTR correctors and potentiators. To date, no mechanism of action has been entirely resolved for a CFTR-specific modulator, and no clear binding site has been defined. However, through the efforts of many groups over the past decade, we are now aware of some key features of certain compounds.

It seems that corrector binding sites are located at domain-domain interface regions that are critical for intramolecular signal transduction, and these compounds act by promoting native structure and stability at these interfaces in order to allow CFTR protein to escape the quality control machinery of the ER. Evidence to support this comes from recent studies performed by several North American groups, including those by the Riordan, Clarke and Cyr research groups (He \textit{et al.}, 2013; Loo \textit{et al.}, 2013; Ren \textit{et al.}, 2013). Using both \textit{in vitro} and \textit{in silico} techniques,
the Riordan group provided initial evidence to suggest that VX-809 interacted with CFTR at the NBD1:ICL4 interdomain interface; a rationally therapeutic drug-binding site, provided that this interface has been deemed defective in the major mutant, ΔF508-CFTR. However, in the study by Clarke and colleagues, the authors demonstrated that the VX-809 directly interacted with another region of CFTR, MSD1, to enhance its steady-state abundance and further increase its apparent half-life (Loo et al., 2013). Subsequently, Cyr and colleagues validated MSD1 as the VX-809-binding domain, and further identified several key residues in MSD1 which constitute at least part of the small molecule binding site, specifically residues F374 and L375, both of which are proximal to the last TM helix of MSD1 (i.e. TM6; Ren et al., 2013). Taken together, the systematic mapping of these residues to structural models of CFTR suggested that other regions of CFTR likely contribute to a composite binding site, presumably NBD1 and/or MSD2, based on the relative proximity to residues F374/L375 in the tertiary structure.

In order to elucidate which regions and residues of CFTR comprise the composite VX-809 binding site (as well as sites for other correctors), one experimental approach worth pursuing involves photo-labeling (i.e. ultraviolet cross-linking) full-length CFTR with a ‘tagged’ (e.g. radiolabelled) analogue of VX-809, followed by trypsin digestion and autoradiography to identify interacting peptides and thus participating CFTR residues. This approach would facilitate binding site identification in theory, yet the precision would be dependent of the relative position of arginine and lysine amino acids, as these constitute enzymatic cleavage sites for trypsin, and therefore would provide positional information of higher accuracy if cleavage sites were closely flanking photo-labelled amino acids. This strategy has exhibited some success (i.e. few, but likely not all residues could be mapped) in identifying small molecule binding sites in other, related ABC proteins, namely MRP1 (ABCC1) and P-gp (ABCB1) (Yoshimura et al., 1989; Daoud et al., 2000; 2001). This knowledge could allow for medicinal chemists to design ‘better’, CFTR-specific correctors, having enhanced binding affinities, as well as improved pharmacodynamic and/or pharmacokinetic properties to list a few. Similarly, potentiator compounds seem to stabilize the open state of the channel, allowing for the MSD1:MSD2-based pore to function properly. It is interesting to note that previous corrector and potentiator screens have used chemical libraries of 2,000 - 200,000 compounds, and typically the hit rate is approximately 0.01-0.03% (Lin et al., 2010). This low yield suggests
Figure 1.8: Small molecule modulators of CFTR.

CFTR-specific correctors, potentiators and inhibitors are labelled blue, red and green, respectively.
that larger libraries would be more successful. Furthermore, successful compounds found from HTS must be drug-like, and be able to have therapeutic properties once administered to patients. Thus, any compounds which do not abide by Lipinski’s Rule of Five need to be discarded or optimized at the outset of a screen (Lipinski et al., 2001). Molecules which could become a drug or pro-drug, are retained and tested for corrector activity. Hits from such high-throughput screens must then be validated using more rigorous assays of biological activity, usually involving purified CFTR protein (Eckford et al., 2014). Such leads are then derivatized, optimized, and subjected to further validation.

Further, Lin and colleagues used a library containing >3,000 FDA-approved drugs to search for small molecule correctors and potentiators in cell-based assays, and approximately 40 chemicals with ΔF508-CFTR corrector activity were identified (Lin et al., 2010). Their choice to screen previously approved drugs is advantageous, since it would streamline application from bench to bedside, saving many years it would normally take to become approved for human indications. Additionally, since CF is a disease in which few therapeutic interventions exist, the Orphan Drug Act allows the approval process to be facilitated, reaching market much sooner than other drugs at the same stage of development (Thorat et al., 2012).

Another approach which is less resource intensive than in vitro and in vivo studies, yet has had successful applications in identifying bioactive small molecules is that of in silico drug discovery (Varady et al., 2003; Klebe et al., 2004; Evers et al., 2005). In silico compound libraries can include naturally occurring molecules from flora and fauna, chemicals from de novo synthesis, those which do not physically exist but have been computationally designed, and more importantly small molecules which have been rationally designed from protein structures. Since compound structures can be easily modified in silico, this approach can be a powerful tool for finding novel therapeutics which satisfies allostERIC and electrostatic requirements of the receptor (i.e. ΔF508-CFTR and other rare CFTR mutants) binding site(s).

Based on results reported throughout the literature this past decade, it is apparent that a CFTR corrector would require the ability to repair at least three major conformational defects in order to be maximally effective in treating CF disease clinically. These include: (1) efficient
rescue of trafficking to the cell surface, ideally to at least 50% of WT-CFTR levels, as heterozygous individuals do not have CF disease; (2) increases in channel activity to levels near WT-CFTR, while retaining ATP- and phospho-regulation of this activity; (3) increases in stability and residency on the cell surface to near WT-CFTR levels (Figure 1.9). Further, correctors should be highly specific for CFTR to avoid off-target effects, and work via direct binding to the protein to restore proper folding and assembly. These small molecules must repair both inter- and intra-domain folding defects in order to permit proper biosynthetic processing, including typical post-translational modification to that observed in WT-CFTR, which should result in normal trafficking to the cell surface and subsequent channel activity. However, if a small molecule is not CFTR-specific, not only would it have potentially toxic effects on other proteins that could render them unsuitable for sustained patient use, they could be rapidly removed from the cell via activity of intracellular enzymes or efflux pumps (Loo et al., 2012).

Understanding drug responses for each CFTR mutation will help elucidate mechanism of action, and furthermore assist in the design of mutation-specific therapies (i.e. personalized CF medicine). Ivacaftor and Lumacaftor taught us that repurposing compounds is not straightforward, that one size does not fit all. It is clear that there are future challenges for CF drug discovery, however, there is hope for repurposing certain CF drugs towards mutations within the same dysfunctional class; for example, Ivacaftor is being approved to treat 9 additional CFTR gating mutations. Therefore, by building on this innovation via future clinical testing, it may still be possible to further expand the number of mutations (population size) in which Ivacaftor and Lumacaftor have therapeutic benefits, or in other words, enhance the size in which these drugs fit.

1.7 Strategies for Personalized CF Medicine

As previously described, several structural and functional aberrations require correction in order to restore activity of ΔF508-CFTR, and this is likely the case for other less common mutations which have not been fully studied. These structural defects involve intra- and interdomain interfaces, and subsequently constitute multiple therapeutic targets. Repair of these interfaces can potentially be achieved using pharmacological chaperones (i.e. small
Figure 1.9: Molecular defects requiring repair for functional rescue of misfolded CFTR mutants. Republished with permission from Molinski et al., 2012.
molecules) which repair each aberrant site. However, a more desirable therapy would be to identify a single small molecule that intrinsically repairs multiple defects. This would avoid any adverse drug interactions a regimen of multiple therapeutic drugs could incur. For example, it is not enough to repair NBD1 of ΔF508-CFTR since it has been shown that improved stability of this domain is not sufficient to produce a globally stable protein (Rabeh et al., 2010). Therefore, targeting multiple interfacial defects in the full-length protein is necessary to restore the biosynthesis, stability and activity back to wild-type levels.

Although targeting mutant CFTR proteins directly is desirable, therapeutics which promote trafficking, repress degradation, and increase synthesis/stability via modulation of chaperones could also be useful. However, this approach would likely have non-specific and toxic effects, since these chaperones are necessary for proper folding of many other proteins. Additionally, it has been shown that even after low temperature rescue, the peripheral protein quality control machinery removes structurally compromised CFTR variants (e.g. ΔF508-CFTR) from the plasma membrane, and thus could be another target for therapeutics (Lukacs et al., 1993; Heda et al., 2001). Likewise, removal of mutant CFTR from the cell surface is ubiquination-dependent and involves several E3 ubiquitin ligases (e.g. CHIP, gp78) which could also be targeted for therapeutic repair (Meacham et al., 2001; Morito et al., 2008).

Interestingly, many solubilizing mutations which enhance the biosynthesis of ΔF508-CFTR mainly, have been identified, and could provide insight into functional interfaces which need to be repaired for restoration of functional activity in this and other rare CFTR mutations. Drugs that mimic the structural consequences of these stabilizing mutations could be of therapeutic use. For example, a peptide containing a diarginine (RXR)-based ER retention motif was found to compete with the aberrantly exposed R553AR555 within NBD1 of ΔF508-CFTR, subsequently preventing its ER retention and promoting anterograde trafficking to the cell surface (Kim Chiaw et al., 2009). By targeting intracellular pathways which compete with mutant CFTR biosynthesis, enhanced protein expression could be functionally rescued to the cell surface.

Another potential route of functional rescue could involve targeting CFTR mRNA. RNA as a drug target has been shown to improve the outcome of type 1 muscular dystrophy in vitro (Parkesh
et al., 2012; Childs-Disney et al., 2012). Likewise, Bartoszewski and colleagues showed that the trinucleotide deletion causing ΔF508, which is found in the majority of patients with CF (i.e. the out-of-frame CTT deletion between amino acids I507 and F508) and rendering a synonymous single nucleotide polymorphism at I507, caused instability of ΔF508-CFTR mRNA due to the enhanced size of hairpin loops relative to wild-type CFTR mRNA (Bartoszewski et al., 2010). These larger hairpins increased the rate of degradation, and resulted in less mRNA being retained in the cell for translation. In this same study, the authors generated ΔF508 by deleting the trinucleotide corresponding to amino acid F508 directly (i.e. TTTdel) and showed that although this same deletion causes ΔF508, the RNA primary sequence differed from (CTTdel)-ΔF508-CFTR and was sufficient to retain wild-type mRNA loop secondary structure. An abundance of (TTTdel)-ΔF508-CFTR was present at physiological temperature relative to (CTTdel)-ΔF508-CFTR, and allowed for enhanced low temperature rescue at the protein level. This demonstrates the fragility of the naturally occurring (CTTdel)-ΔF508-CFTR mRNA, in addition to the well documented instability at the protein level. Thus, if the loop structure of the naturally occurring ΔF508-CFTR mRNA could be induced to mimic that of wild-type CFTR (or even TTTdel) with therapeutics, sufficient transcript would be available for translation, even though the underlying mutation remains. This approach could enhance the half-life of the misfolded mRNA, increase the synthesis of nascent ΔF508-CFTR, and establish a novel pool of therapeutic targets which could then be corrected with small molecule protein correctors. Although this approach could improve downstream protein synthesis of ΔF508-CFTR, it would not directly address the underlying protein folding defects which cause disease.

Importantly, although not a small molecule modulator, CFTR gene therapy, in which the wild-type CFTR gene is introduced into the target tissues (e.g. lung, gut), could be another potential approach to treating all CFTR mutations, especially those in which the biochemical consequences have not yet been interrogated. This delivery method has been under investigation as a CF therapy for over 20 years, and although it may seem straightforward in principle, gene transfer into the lungs has proven to be a problematic endeavor (Griesenbach and Alton, 2012). Gene therapy involves the introduction of foreign DNA using liposomal or viral vectors, and as a result, most approaches have had modest to poor clinical outcomes, having issues with low transfer efficiency and immunoreactivity, respectively (Cao et al., 2011;
Alton et al., 2015). Therefore, a current approach involves pluripotent stem cell therapy using human amniotic mesenchymal stem cells which are reprogrammed into the required cell type (e.g. bronchial epithelial cells) and which contain wild-type CFTR (Paracchini et al., 2012). This method could allow for functional tissue regeneration by means of topical and systemic administration of stem cells, with the goal of replacing dysfunctional tissues containing ΔF508-CFTR or other CFTR mutants. However, this approach is still in the investigational stage, and additional favorable pre-clinical results are needed to allow further pursuit at the clinical level (Wong et al., 2012; Firth et al., 2015).

1.8 Rationale, Hypotheses, and Objectives

As described above, of the 2000 CF disease-causing mutations that have been identified to date, very few have been biochemically interrogated. This is partly due to the fact that most of these are extremely rare, affecting few individuals, and therefore the cost-benefit ratio may not favour the pharmaceutical company or research organization studying this disease. However, it is these rare, previously uncharacterized mutations which would arguably add most value towards fully understanding the molecular nature of CFTR genetics and biochemistry, as well as CF pathophysiology as a whole. Therefore, this substantial subset of CFTR variants could provide insight into the structure-function relationship of WT-CFTR, and more importantly the major disease-causing mutation: ΔF508. Further, our current understanding of the molecular consequences of rare CF disease-causing mutations is mainly based on low resolution molecular models as well as bioinformatics predictions, and therefore may be inaccurate. By biochemically characterizing each and every CF disease-causing mutation in systematic manner, predicted molecular consequences would be clarified, and importantly, therapeutic approaches to repair structural and/or functional defects could be developed.

In this thesis, the primary aim was to understand the molecular consequences of two rare, previously uncharacterized CF disease-causing mutations: c.3700A>G (ΔI1234_R1239-CFTR) and c.2052_2053insA (Q685TfsX4-CFTR), as well as develop novel methods for rapid functional screening of previously uncharacterized, rare CF mutations to determine effectiveness of current CF drugs. Accordingly, it was first hypothesized that ΔI1234_R1239-CFTR and
Q685TsX4-CFTR would be misprocessed and truncated, respectively, and therefore prohibit functional CFTR channels on the apical membrane of epithelial cells. Secondly, it was also hypothesized that current CF therapies (i.e. KalydecoTM and Orkambi™), rationally designed for G551D and ΔF508, would not be efficacious for these two less common CFTR variants. Thirdly, it was hypothesized that some, but not all rare mutations would respond to these small molecules, and therefore a ‘personalized medicine’ approach would be needed to facilitate future mutation-specific CF drug discovery.

Thus, to test these hypotheses the following objectives were pursued:

1. c.3700A>G (ΔI1234_R1239-CFTR) and c.2052_2053insA (Q685TsX4-CFTR) were generated in CFTR cDNA, overexpressed in HEK-293 cells, and relative levels of mutant CFTR biosynthesis, stability and function were biochemically determined.

2. For each CFTR variant, molecular mechanisms of dysfunction were characterized, and responses to current CFTR modulators (e.g. VX-770 and VX-809) were determined.

3. In the case that current small molecules did not efficiently repair conformational defects, other avenues of potential therapeutic repair which could address these unique aberrations were evaluated.

4. Development of novel methods to generate and functionally screen rare disease-causing mutations, to facilitate identification and stratification of potential responders to current CF therapies.

In this thesis, the materials and methods used for all of the studies listed in the objectives above are described in Chapter 2. The results obtained from the identification and molecular characterization of the rare disease-causing mutation c.3700A>G (ΔI1234_R1239-CFTR) are presented and discussed in Chapters 3 and 4, while novel mutagenesis and functional screening approaches developed to further assess efficacy of current CF small molecules (e.g. response to Kalydeco™, Ivacaftor or VX-770) for this and other previously uncharacterized and thus rare CF disease-causing mutations are outlined in Chapter 5. Additionally, results obtained from the characterization of c.2052_2053insA (Q685TsX4-CFTR) are presented and discussed in Chapter 6. Finally, Chapter 7 includes an overall discussion and conclusion from results described in Chapters 3 to 6, as well as proposes future directions of research.
Chapter 2

Materials and Methods

2.1 Generation of mutant CFTR constructs

p.Ile1234_Arg1239del-CFTR, p.Gln685ThrfsX4-CFTR, p.Asp529_Lys830del-CFTR, p.Gln2_Trp846del-CFTR, p.Glu1172-3Gly-6His*-CFTR and p.Glu1172*-CFTR cDNA were generated using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Woburn, MA) according to the manufacturer’s Standard PCR Protocol with high quality (>300 ng/µL, 260/280 nm ratio of 1.8) plasmid DNA containing WT-CFTR cDNA (in pcDNA3.1) as the template. For p.Ile1234_Arg1239del-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used: 5'- CAT ATT AGA GAA CAT TTC CTT CTC A (del) GT GGG CCT CTT GGG AAG AAC TGG ATC -3' (sense); 5'- GAT CCA GTT CTT CCC AAG AGG CCC AC (del) T GAG AAG GAA ATG TTC TCT AAT ATG -3' (antisense); 18 nucleotides deleted from the template sequence are highlighted as "del" in bold italics. For p.Gln685ThrfsX4-CFTR the following PCR primers were used: 5'- GGA CAG AAA CAA AAA AAA CAA TCT TTT AAA CA G AC-3' (sense); 5'- GTG TGT TTA AAA GAT TGT TTT TTT TGT TTC TGT CC-3' (antisense). For p.Asp529_Lys830del-CFTR the following primers were used: 5'- GTC ATC AAA GCC TG C CAA CTA GAA GAG TGC TTT TTT GAT GAT ATG GAG AGC-3' (sense); 5'- GCT CTC CAT ATC ATC AAA AAA GCA CTC CTC TTC TAG TTG GCA TGC TTT GAT GAC-3' (antisense). For p.Glu1172-3Gly-6His*-CFTR the following PCR primers were used: 5'- GAC ATG CCA ACA GAA GGT GGT GGT CAT CAT CAT CAT CAT TAG AAA CCT ACC AAG TCA ACC -3' (sense); 5'- GGT TGA CCT TGT AAG TTT CTA ATG ATG ATG ATG ATG ACC ACC ACC TTC TGT TGG CAT GTC -3' (antisense); nucleotides in bold represent the insertion which codes for 2 Gly residues, 6 His residues and one stop codon (i.e. 2Gly-6His*); the third Gly residue in the final construct was from the native amino acid at position 1173. For p.Glu1172*-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used: 5'- GAC
ATG CCA ACA TAA GGT AAA CCT ACC -3' (sense); 5'- GGT AGG TTT ACC TTA TGT TGG CAT GTC -3' (antisense); the nucleotide in bold represents the codon change from GAA (Glu) to TAA (stop, * or X). For PCR amplification, 1 or 10 ng of template plasmid and 0.3 µM (final concentration) of each sense and antisense primers were added to 2X KAPA HiFi HotStart ReadyMix (containing KAPA HiFi DNA polymerase, deoxynucleotide triphosphates, magnesium chloride and a proprietary buffer; 25 µL final volume). The reaction mixture was incubated in a TP遥控ermocycler (Biometra, Goettingen, Germany) as per the KAPA HiFi HotStart Standard PCR Protocol. Briefly, PCR amplifications (taking approximately 3 hrs) for each mutation were as follows: (1) initial denaturation for 3.5 min at 95 °C; (2) further denaturation for 20 s at 98 °C; (3) primer annealing for 15 s at actual Tm-5 or Tm-10 °C; (4) DNA polymerase extension for 5 min (30 s/kb) at 72 °C; (5) cycle back to step 2, 25 times; (6) final round of DNA polymerase extension for 3 min at 72 °C, followed by incubation at 4 °C to stop the reaction. Following PCR, 1 µL FastDigest DpnI (10U; Thermo) was added to each tube for 60 min at 37 °C to remove methylated (and hemi-methylated) parental DNA; 10 µL of the PCR product post DpnI-digestion was run on 0.8% agarose gels (cast with GelRed; Biotium) to determine the size(s) of the PCR product(s). Next, 50 µL of DH5α competent cells (Life Technologies) were transformed with 10 µL of the PCR product (without heat inactivation following DpnI digestion) according to the manufacturer’s protocol, and subsequently grown on LB-Ampicillin (100 µg/mL) selective agar plates at 37 °C overnight. Colonies were picked, and after 17 h of growth in liquid culture (supplemented with 100 µg/mL Ampicillin) at 37 °C with shaking (250 rpm), plasmid DNA was prepared using the GenElute™ Plasmid Miniprep Kit (Sigma). The presence of site-directed mutations, as well as the integrity of full-length CFTR cDNA (nucleotides 1-4443 with stop codon), was confirmed by DNA sequencing (TCAG Inc., Toronto, ON).

2.2 Studies of CFTR protein processing

Human embryonic kidney (HEK) cells (GripTite™, kindly provided as a gift from Dr. Daniela Rotin, Hospital for Sick Children, Toronto, ON) were grown at 37 °C in 24-well (clear, flat bottom; Sarstedt) plates to 50% confluence and transiently transfected with p.Ile1234_Arg1239del-CFTR, p.Ser700_Asp835del-CFTR, p.Gln2_Trp846del-CFTR, p.Glu1172-3Gly-6His*-CFTR, p.Glu1172*-CFTR or WT-CFTR (positive control) cDNA constructs (pcDNA3.1)
using PolyFect Transfection Reagent (Qiagen), according to the manufacturer’s protocol. HEK cells transiently expressing CFTR proteins were maintained in DMEM (Wisent) supplemented with non-essential amino acids (Life Technologies) and 10% FBS (Wisent) at 37 °C with 5% CO2 (HEPA incubator, Thermo Electron Corporation) and processed as previously described (Molinski et al., 2015). Briefly, following incubation at 37 °C for 24 h, HEK cells transiently expressing CFTR proteins were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% Tris-Glycine gels (Life Technologies). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated in 5% (w/v) milk, and CFTR bands were detected with human CFTR-NBD1-specific (amino acids 484-589) murine mAb 660 (1:10000, University of North Carolina Chapel Hill, NC; Cui et al., 2007) for NBD1-containing constructs (i.e. p.Ile1234_Arg1239del-CFTR, p.Glu1172-3Gly-6His*-CFTR, p.Glu1172*-CFTR or WT-CFTR) or human CFTR-NBD2-specific (amino acids 1204-1211) murine mAb 596 (1:10000, University of North Carolina Chapel Hill, NC; Cui et al., 2007) for NBD2-containing constructs (i.e. p.Ser700_Asp835del-CFTR, p.Gln2_Trp846del-CFTR or WT-CFTR) at 4 °C overnight, horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10000, Pierce) at room temperature for 1 hr, Amersham ECL (GE Healthcare), and exposure to film (Denville Scientific) for 0.5 to 5 min as required. Importantly, when blots containing p.Glu1172-3Gly-6His*-CFTR or p.Glu1172*-CFTR were probed with the CFTR-NBD2-specific (amino acids 1204-1211) murine mAb 596 (Cui et al., 2007) (1:10000, University of North Carolina Chapel Hill, NC), or p.Gln2_Trp846del-CFTR probed with the CFTR-N-terminus-specific (amino acids 25-36) murine mAb MM13-4 (1:1000, EMD Millipore; Cui et al., 2007) no signals were detected (the respective mAb epitopes are not expressed in these deletion mutants). Calnexin was used as a protein loading control, and detected with a Calnexin-specific rabbit pAb (1:10000, Sigma-Aldrich) at 4 °C overnight, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1: 10000, Pierce) at room temperature for 1 hr, Amersham ECL, and exposure to film for 0.5 to 5 min as required. Relative expression levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ software version 1.46 (National Institutes of Health).
2.3 Studies of CFTR protein degradation
HEK cells were transiently transfected with ΔI1234_R1239-CFTR at 37 °C, as described previously (Molinski et al., 2014). Following transfection, HEK cells overexpressing ΔI1234_R1239-CFTR were treated with 2 µM of the proteasomal inhibitor epoxomicin (Santa Cruz), 100 nM of the lysosomal inhibitor Bafilomycin A1 (Santa Cruz), or both, for 7 hours at 37°C. Cells treated with either proteasomal, lysosomal, or both inhibitors were lysed following the respective treatments and soluble proteins were analyzed by SDS-PAGE and immunoblotting as described previously (Molinski et al., 2014; Molinski et al., 2015). The accumulation of ΔI1234_R1239-CFTR was compared in the presence of the treatments relative to vehicle (0.1% DMSO).

2.4 Studies of CFTR protein glycosylation
To evaluate protein glycosylation status, HEK or BHK cells expressing WT-CFTR, p.Phe508del-CFTR or p.Ile1234_Arg1239del-CFTR were grown at 37 °C for 24 h, and subsequently lysed in modified radioimmunoprecipitation assay buffer as described above. Lysates were treated with either Endoglycosidase H or Peptide-N-Glycosidase F (both from New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol. Immunoblots were performed using the human CFTR-NBD2-specific murine mAb 596 as described above.

2.5 Preparation of crude membranes, and limited proteolysis of CFTR
Crude membranes were prepared from HEK cells transiently expressing WT-CFTR, ΔF508-CFTR, as well as ΔI1234_R1239-CFTR treated with either VX-809 (3 µM), Corr-4a (10 µM), VX-809 and Corr-4a (3 µM and 10 µM, respectively), or vehicle (0.1% DMSO) for 24 h at 37 °C as previously described (Eckford et al., 2014). Briefly, cell pellets were resuspended in cell lysis buffer (10 mM HEPES, 1 mM EDTA, pH 7.2), and cells were lysed using a cell disruptor (10,000 psi, 4 °C, 5 min). The cell suspension was centrifuged at 800 x g for 10 min at 4 °C to pellet unbroken cells, and crude membranes were isolated from the resulting supernatant after centrifugation at 100,000 x g for 60 min at 4 °C. The crude membrane pellet was resuspended in buffer (40 mM Tris-HCl, 5 mM MgCl₂, 0.1 mM EGTA, pH 7.4) by passage through a 1 mL syringe 20 times with a 27-gauge needle.
For limited proteolysis studies, 20 µg crude membranes were resuspended in buffer (40 mM Tris-HCl, 5 mM MgCl₂, 0.1 mM EGTA, pH 7.4) and sonicated. Samples were kept on ice, and trypsin (Promega) was added at the following concentrations: 0, 1.6, 3.1, 6.3, 12.5, 25, 50 µg/mL. Samples were again sonicated and incubated at 4°C for 15 min. Proteolysis was terminated by addition of 0.5 mg/mL trypsin soybean inhibitor (Sigma). Membranes were solubilized in modified RIPA for 15 min, and the soluble fraction was analyzed by SDS-PAGE on a 4-12% gradient gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE), and protein bands were detected using the human CFTR-NBD1-specific murine mAb L12B4 (1:1000, EMD Millipore) or the human CFTR-NBD2-specific murine mAb M3A7 (1:1000, EMD Millipore). Fluorescence was detected using the secondary antibody IRDye-800 (goat anti-mouse IgG: 1:15,000, Rockland Immunochemicals, Gilbertsville, PA). Blots were imaged, and band intensities were detected using the Odyssey infrared imaging system (LI-COR Biosciences). Relative levels of full-length ΔI1234_R1239-CFTR resulting from trypsin digestion were measured using ImageJ 1.42 Q software (National Institutes of Health).

2.6 Studies of CFTR protein expression in primary nasal epithelial cells

Nasal cultures were grown at 37°C for 48 h in the absence or presence of small molecules (3 µM VX-809 or 1 µM VX-661) as required. Cells were then lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and CFTR bands were detected using the human CFTR-MSD1-specific (amino acids 25-36) murine mAb MM13-4 (1:200, Abcam), horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:2500) and exposure to film for 0.5 to 30 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were
quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

2.7 Studies of TMEM16A protein expression in primary nasal epithelial cells

Nasal epithelial cells from confluent transwells (6.5 mm diameter, 0.4 µm pore size) were each lysed in 150 µL modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions (20 µL) were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and TMEM16A bands were detected using the human anti-TMEM16A rabbit mAb SP31 (1:100, Abcam), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2500) and exposure to film for 0.5 to 5 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

2.8 Statistical analysis

All data are represented as mean ± S.E. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Non-paired Student's t tests, one-way analysis of variance (ANOVA), and two-way ANOVA were conducted as appropriate, and P values less than 0.05 were considered significant. Each experiment was replicated at least three times.
Chapter 3

Identification of the rare CFTR mutation c.3700A>G
(p.Ile1234_Arg1239del)

3.1 Acknowledgements

The results presented in this chapter have been published:


A letter from the publisher outlining permission to use copyright material is provided in the Appendix, section A1.1.

3.2 Abstract

Purpose: To determine the molecular consequences of the variant: c.3700 A>G in the CFTR gene, a variant which has been predicted to cause a missense mutation in CFTR protein (p.Ile1234Val). Methods: Clinical assays of CFTR function were performed and genomic DNA from patients homozygous for c.3700 A>G and family members was sequenced. Total RNA was extracted from epithelial cells of the patients, transcribed into cDNA and sequenced. CFTR cDNA clones were constructed containing the missense mutation p.Ile1234Val or a truncated exon 19 (p.Ile1234_Arg1239del) and heterologously expressed to test CFTR protein synthesis and processing. Results: In-vivo functional measurements revealed that the individuals homozygous for the variant c.3700 A>G exhibited defective CFTR function. We show that this mutation in exon 19 activates a cryptic donor splice-site 18bp upstream of the original donor splice-site, resulting in deletion of 6 amino acids (r.3700_3717del; p.Ile1234_Arg1239del). This deletion, like p.Phe508del caused a primary defect in folding and processing. Importantly, Lumacaftor (VX-809), currently in clinical trial for CF patients with the major CF causing mutation, p.Phe508del partially ameliorated the processing defect exhibited by
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p.Ile1234_Arg1239del. **Conclusion:** These studies highlight the need to verify molecular and clinical consequences of CFTR variants in order to define possible therapeutic strategies.

### 3.3 Introduction

A recent, large scale study showed that individuals in North America and in Europe bearing the rare variant: c.3700 A>G, predicted to cause the missense mutation p.Ile1234Val or alternative splicing, exhibited variable CF disease severity (Sosnay *et al.*, 2013). This variant, while rare in North America (present in only fifteen patients, http://cftr2.org), is relatively common in the Middle East. Importantly, statistics from the World Health Organization (www.who.int/genomics/publications) report p.Ile1234Val as the second most common CF-causing mutation in the Middle East (12.3% occurrence in patients from Jordan, Kuwait, Lebanon, Oman, Qatar, Saudi Arabia, United Arab Emirates) with the exception of two countries, Bahrain and Israel, in which the occurrence is less than 3.8% and 0.06%, respectively. The mutation 1548delG is most common in these seven countries (17.2%), while 2043delG is most common in Bahrain (30.8%), and W1282X is most common in Israel (36.1%). Further, the c.3700 A>G (p.Ile1234Val) mutation is specific to Middle Eastern individuals originating from Bedouin tribes, and although diagnostic tests are sensitive enough to identify this mutation at an early age, currently there is no effective treatment for patients with this CF-causing genotype (Blackman *et al.*, 2013).

Interestingly, Sosnay and colleagues recently showed that there were no functional consequences of introducing the missense mutation (p.Ile1234Val) in CFTR cDNA with respect to CFTR protein synthesis, processing and/or function (Sosnay *et al.*, 2013). As these cell biological findings are discordant with the clinical phenotype and defective CFTR function in *in vivo* measurements, we were prompted to test the effect of this variant on splicing.

In this study, we showed that the c.3700 A>G variant caused aberrant splicing leading to the in-frame deletion of six amino acids (p.Ile1234_Arg1239del) in a conserved region of the CFTR protein, and that this deletion leads to its misfolding during synthesis as in the case of p.Phe508del. These insights led us to test the efficacy of an investigational compound in trials for p.Phe508del and found that, in cell culture, VX-809 partially ameliorated the folding defect
of p.Ile1234_Arg1239del-CFTR. Hence, these studies support the rationale for defining the molecular consequences of rare CFTR mutations in guiding decisions regarding future therapeutic interventions.

3.4 Materials and Methods

3.4.1 Clinical Studies

The pancreas status was determined by measuring fecal elastase concentration in stool. The sweat chloride test was performed using standardized protocol (Wescor®). Nasal potential difference (NPD) measurements allowed assessment of CFTR function as change (Δ) in potential difference following chloride-free and isoproterenol perfusion (ΔCl-free+Iso) in the presence of amiloride. Sweat gland potential difference was measured between a topical placed electrode and subcutaneous inserted needle following sweat stimulation by pilocarpine iontophoresis. Lastly, the β-adrenergic sweat secretion rate was assessed using an evaporimeter (CyberDerm RG-1; Dasylab, Broomall, PA) and following a sweat stimulation sequence of 1) cholinergic secretion with carbachol (0.01 mg) (Alcon, Mississauga, Ontario, Canada); (2) inhibition of cholinergic secretion with atropine (8.8 mg) (Sandoz, Boucherville, Quebec, Canada); and (3) pure β-adrenergic secretion with a cocktail containing 8.8 mg atropine, 4.4 mg isoproterenol hydrochloride (Sandoz), and 0.93 mg aminophylline (Hospira, Saint-Laurent, Quebec, Canada), as described previously (Quinton et al., 2012).

3.4.2 Genomic Mutation Analysis

Genomic DNA from peripheral leukocytes from the patient was analyzed by direct sequence analysis. All exons and flanking intron sequences of CFTR (NM_000492.3) were sequenced both in forward and reverse directions. Sanger sequencing was performed according to standard protocols using BigDye terminator v1.1 (Life Technologies, Carlsbad, CA) and sequencing products were separated on an ABI model 3730 Capillary Sequencer (Life Technologies, Carlsbad, CA) and analyzed using SeqPilot software (JSI Medical systems, Germany). Exons are numbered based on traditional numbering. Sequence nomenclature is based on HGVS.
3.4.3 RT-PCR Analysis

Total RNA was extracted from nasal epithelial cells of the patient homozygous for c.3700 A>G using PerfectPure RNA cultured cell kit (5Prime GmbH, Hamburg, Germany) according to manufacturer’s protocol. RNA was then transcribed into cDNA using SuperScript first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) using manufacturer’s protocol. Primers amplifying the entire CFTR transcript as a series of overlapping fragments were designed. Standard PCR conditions were used. Primer sequences are available upon request.

3.4.4 Generation of Mutant CFTR Constructs

p.Ile1234Val-CFTR was generated in human wild-type (WT)-CFTR cDNA (pcDNA3.1) by Norclone Biotech Laboratories (London, ON, Canada). p.Ile1234_Arg1239del-CFTR was generated in human WT-CFTR cDNA (pcDNA3.1), using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Woburn, MA). The deletion was generated using the following primers:

5’-CAT ATT AGA GAA CAT TTC CTT CCT GCT GGG CCT CTT GGG AAG AAC TGG ATC-3’ (sense); 5’-GAT CCA GTT CTT CCC AAG AGG CCC ACT GAG AAG GAA ATG TTC TCT AAT ATG-3’ (antisense).

Plasmid DNA was prepared using the GenElute™ Plasmid Miniprep Kit (Sigma), and the presence of the deletion, as well as the integrity of CFTR cDNA, was confirmed by DNA sequencing (TCAG Inc., Toronto, ON).

3.4.5 Studies of CFTR Protein Processing and Function

Human embryonic kidney (HEK) cells were transiently transfected with WT-CFTR or p.Ile1234Val-CFTR using PolyFect Transfection Reagent, according to the manufacturer’s protocol (Qiagen). Baby hamster kidney (BHK) cells were transiently transfected with WT-CFTR or p.Ile1234_Arg1239del-CFTR using GenJet™, according to the manufacturer’s protocol (SignaGen Laboratories, Rockville, MD). HEK cells transiently expressing CFTR proteins were maintained in DMEM (Wisent) supplemented with non-essential amino acids (Life Technologies) and 10% FBS (Wisent) at 37 °C with 5% CO₂ (HEPA incubator, Thermo Electron Corporation), and BHK cells transiently expressing CFTR proteins were maintained in DMEM/F12 (Wisent) supplemented with 10% FBS at 37 °C with 5% CO₂. p.Phe508del-CFTR was stably expressed in BHK cells (obtained from Dr. Lukacs at McGill University, Montreal, QC) and
maintained DMEM/F12 supplemented with 10% FBS and 200 µg/mL methotrexate (Sigma) at 37 °C with 5% CO₂.

HEK cells expressing WT-CFTR or p.Ile1234Val-CFTR, and BHK cells expressing WT-CFTR, p.Phe508del-CFTR or p.Ile1234_Arg1239del-CFTR were grown at 37 °C for 24 h, and subsequently lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and CFTR bands were detected using the human CFTR-NBD2-specific murine mAb 596 (1:30000, University of North Carolina Chapel Hill, NC), horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

To evaluate protein glycosylation status, BHK cells expressing WT-CFTR, p.Phe508del-CFTR or p.Ile1234_Arg1239del-CFTR were grown at 37 °C for 24 h, and subsequently lysed in modified radioimmunoprecipitation assay buffer as described above. Lysates were treated with either Endoglycosidase H or Peptide-N-Glycosidase F (both from New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol. Immunoblots were performed using the human CFTR-NBD2-specific murine mAb 596 as described above.

To test function, HEK cells over-expressing WT-CFTR or p.Ile1234Val-CFTR were grown in 12 well plates, and upon formation of a mono-layer, the cells were incubated overnight with 10 mM of the halide sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) (Invitrogen Molecular Probes Inc.), at 37 °C and 5% CO₂. Next day, the cells were washed 3 times with phosphate buffered saline to make the extra-cellular fluid free of SPQ. Cells were
then kept in chloride containing physiological solution, Hank’s buffered saline solution. Fluorescence measurements were made using Gemini EM Fluorescence microplate reader (Molecular devices). Intracellular SPQ was excited at 350 nm wavelength and the emission was measured at 450 nm, reporting chloride concentration. After reading baseline fluorescence in presence of physiological solution, a chloride gradient was established via exchange of the extracellular solution with a chloride free, nitrate containing buffer (NaNO₃ 136 mM, KNO₃ 3 mM, Ca(NO₃)₂ 2 mM, Glucose 11 mM, HEPES 20 mM, pH 7.2 and osmolarity 300 mOsm). CFTR was stimulated using cAMP agonist forskolin (10 μM). After reaching equilibrium for chloride flux, the extra-cellular solution was replaced with physiological solution containing chloride, allowing SPQ to quench. The fluorescence measurements were expressed as the change in fluorescence relative to the fluorescence measurement just before CFTR stimulation. Data is summarized as initial rate of change in relative fluorescence units in the first 4 minutes of stimulation. Each condition was repeated with at least 4 technical replicates on the same plate, and a total of at least 3 biological replicates were per condition.

3.4.6 Data Analysis

All data are represented as mean ± S.E. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Non-paired Student’s t tests, one-way analysis of variance (ANOVA), and two-way ANOVA were conducted as appropriate, and P values less than 0.05 were considered significant. Each experiment was replicated at least three times.

3.5 Results

3.5.1 Siblings in a Qatari Family Homozygous for the CFTR Variant c.3700 A>G Exhibit Clinical Features of CF and lack of CFTR Function in in-vivo Measurements

A 27 year old man and his 15 year old sister, both from Qatar and homozygous for the mutation c.3700 A>G (p.Ile1234Val), presented for further CF diagnostic evaluation. The man, patient #1, was diagnosed at 4 months of age and had experienced recurrent lung infection over the years. He was hospitalized twice at the age of 13 years and 15 years for acute pulmonary exacerbation. In addition, he was recently admitted at the age of 27 years for mild
hemoptysis. Sputum cultures grew *Staphylococcus aureus* and *Haemophilus influenzae* repeatedly. More recently his sputum grew *Mycobacterium abscessus*. His lung function has always been within normal ranges. The pancreas function has been progressively worsening as indicated by decreased fecal elastase levels (122 μg EL/g stool). His sweat chloride concentration was 90 mM (CF diagnostic cut-off >60 mM; Farrell et al., 2008).

The woman, patient #2, was diagnosed at 2 month of age due to family history, persistent cough and failure to thrive. She shows a similar disease course and remained pancreatic sufficient. She had experienced multiple chest infections and has been hospitalized several times for more severe pulmonary exacerbations. Her sputum cultures grew *Staphylococcus aureus, Haemophilus influenzae* and *Streptococcus pneumoniae* repeatedly. Her lung function has been maintained in the normal range as well. Her sweat chloride concentration was 74 mM, above the CF diagnostic cut-off (Farrell et al., 2008).

Due to the discrepant findings between clinical presentation and the biological evaluation of the CFTR mutant c.3700 A>G, we decided to perform additional functional tests. NPD measurements demonstrated a ΔCl-free+Iso result of 1.8 mV in patient #1 and of -7.4 in patient #2 compatible with ΔCl-free+Iso seen in CF (Figure 3.1a). However, the ΔCl-free+Iso result of patient #2 was close to our institutional borderline cut-off of -7.5 mV suggesting presence of some residual CFTR function. The potential difference measured in the sweat glands was hyperpolarized as demonstrated for patients with CF. However, it was more negative in patient #2 (-47.2 mV) compared patient #1 (-35.8 mV) where it was in a more borderline range. The β-adrenergic sweat secretory test demonstrated complete absence of β-adrenergic sweat secretion for patient #1 and patient #2 (Figure 3.1b).

### 3.5.2 Determining the Consequences of p.Ile1234Val on CFTR Folding, Processing and Function

The CFTR variant c.3700 A>G has been predicted to create a missense mutation (p.Ile1234Val). We expressed a missense mutant construct in mammalian cells and found that CFTR protein processing was similar to WT-CFTR in that the protein was expressed as both the Golgi-
Figure 3.1  Qatari siblings with *CFTR* variant c.3700 A>G (predicted to cause the CFTR missense mutation: p.Ile1234Val) exhibit loss of CFTR function in NPD and sweat secretion assays.

(a) The original NPD tracing demonstrates absence of any CFTR-mediated response in patient #1 (ΔCl-free+Iso = 1.8 mV) and a somewhat borderline CFTR-mediated response (ΔCl-free+Iso = -7.4 mV) in patient #2. Amiloride (Amil.) inhibited the epithelial sodium channel (ENaC), thereby limiting sodium reabsorption and allowing for measurement of the CFTR-mediated chloride response. (b) Evaporimeter tracings from healthy controls (β-adrenergic (β) peak = cholinergic (Chol.) peak), obligate heterozygote (β peak = ½ Chol. peak) and CF (no β peak) are presented to illustrate range of responses with this assay. Tracings from patients #1 and #2 do not show any β-adrenergic response. Short-lived responses following injection of β-adrenergic drugs represent experimental artifact caused by necessary movement of the evaporimeter. These studies were conducted by Tanja Gonska.
modified, complex glycosylated, band C (migrating as broad band of 170 kDa) as well as endoplasmic reticulum-modified, core glycosylated, band B (migrating as a sharper band of 150 kDa) (Figure 3.2a). Function of the missense mutation as a cyclic AMP activated chloride channel was compared to that of the WT-CFTR using a fluorescence based assay. The fluorescence intensity of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) is sensitive to halide concentrations and can be loaded into cells to monitor transmembrane chloride ion fluxes in response to agonists of cyclic AMP, namely forskolin and the phosphodiesterase inhibitor: 3-Isobutyl-1-methylxanthine (IBMX). In the presence of an outwardly-directed chloride gradient, the addition of the above agonists caused an increase in SPQ fluorescence in cells expressing WT-CFTR (Figure 3.2b). Therefore, this mutation caused no change in the CFTR protein folding, processing or function, arguing that it does not account for the disease phenotype.

Routine diagnostic testing by direct sequence analysis of genomic DNA from peripheral leukocytes revealed a homozygous sequence alteration (c.3700 A>G) in the patients with no additional known disease causing mutations. While sequence change was predicted to cause an isoleucine to valine substitution at position 1234, in silico splicing analysis suggested the mutation might affect splicing of exon 19 to exon 20, with deletion of 18 bp (5’-ATA AGT CCT GGC CAG AGG-3’), coding for residues 1234-1239. To evaluate if this sequence variant had a functional impact on splicing, we performed mRNA analysis from nasal epithelial cells from the patients.

3.5.3 Identification of Alternative Splicing Leading to the Deletion of six Amino Acids (p.Ile1234_Arg1239del) in the Second Nucleotide Binding Domain (NBD2) of CFTR

Reverse transcriptase PCR was performed using primers that amplified all exons in overlapping fragments of the CFTR transcript. One of the fragments containing exons 18–21 showed a slightly smaller size compared to that of a normal control visualized on an agarose gel (Figures 3.3a & 3.3b). Direct sequence analysis of the smaller size fragment generated from the patient revealed a deletion of the last 18 bp of exon 19, r.3700_3717del with little or no wild-type transcript (Figure 3.3c). This indicates that the c.3700 A>G substitution identified in the
Figure 3.2 *In vitro* characterization of p.Ile1234Val missense mutation introduced into CFTR cDNA and expressed in heterologous expression system.

(a) Immunoblots show the processing of WT-CFTR and p.Ile1234Val-CFTR in HEK cells after 24 h at 37 °C. WT-CFTR and p.Ile1234Val-CFTR were expressed as both the Golgi-modified, complex glycosylated (mature) band C form (broad 170 kDa band) as well as the endoplasmic reticulum-modified, core glycosylated (immature), band B form (sharp 150 kDa band). Maturation (expressed as percent band C/(band B + band C)) of WT-CFTR and p.Ile1234Val-CFTR was quantified for three independent trials, and there is no significant difference between the two (P > 0.05). (b) Fluorescence based anion flux assay in HEK cells show WT-CFTR and p.Ile1234Val-CFTR function after stimulation using a cAMP agonist (grey bar, forskolin, 10 μM) or vehicle (DMSO) alone (empty bar). Flux responses in the first 4 min after cAMP stimulation (reported as an initial rate of change in relative fluorescence units) for WT-CFTR and p.Ile1234Val-CFTR were quantified for three independent trials (four technical replicates each trial), and there is no significant difference between the two (P > 0.05). These studies were conducted by Ling Jun Huan.
a

18  19  20  21

499 bp

b

Marker  Control  Patient H1

499 bp  ≈480 bp

600 bp
500 bp
400 bp
300 bp
200 bp
100 bp

c

Exon 19  Exon 20

del 18bp

d

c.3700A>G

Ex 19  TTCTCAGTAAGTCTGGCCAGAGG  GT  //  AG  ex 20  gDNA

Ex 19  Exon 20

r.3700_3717del
(p.Ile1234_Arg1239del)

mRNA
Figure 3.3 Aberrant splicing due to c.3700 A>G mutation in CFTR predicts a deletion of six amino acids (p.Ile1234_Arg1239del).

(a) Strategy for RT-PCR amplification of a region containing exon 19. (b) The amplified product visualized on an agarose gel. The expected size of 499 bp is seen in an unaffected control, whereas a slightly smaller band was seen in the patient. (c) Direct sequence analysis of the fragment in the patient revealed a deletion of the last 18 bp of exon 19. (d) A schematic overview of the gDNA with activation of a cryptic donor splice-site in exon 19. The c.3700 A>G mutation in exon 19 activates a cryptic donor splice-site 18bp upstream of the original donor splice-site. This results in a truncated exon 19 with a predicted deletion of 6 amino acids (r.3700_3717del; p.Ile1234_Arg1239del) in CFTR. These studies were conducted by Berivan Baskin and Peter Ray.
genomic DNA results in aberrant splicing of the CFTR transcript, by activating a cryptic donor splice-site 18 bp upstream of the original donor splice-site (Figure 3.3d). The 18 bp deletion removes six amino acids (p.Ile1234_Arg1239del) in the second nucleotide binding domain (NBD2) of CFTR protein. To determine the structural and functional consequences of this deletion, it was introduced in human wild-type CFTR cDNA and expressed in mammalian cells. We found that like p.Phe508del, p.Ile1234_Arg1239del-CFTR is misprocessed and likely retained in the endoplasmic reticulum as it fails to express complex glycosylation (Figure 3.4a). However, the band C/(band B + band C) ratio, an indicator of CFTR processing, suggested that p.Ile1234_Arg1239del-CFTR is less severe than p.Phe508del, since it had a significantly ($P < 0.01$) greater ratio (Figure 3.4a). Like p.Phe508del, the major form of p.Ile1234_Arg1239del-CFTR was found to be sensitive to Endoglycosidase H, indicative of core-glycosylation and retention in the endoplasmic reticulum (Figure 3.4b). However, like WT-CFTR, the heavier form of p.Ile1234_Arg1239del-CFTR was insensitive to Endoglycosidase H yet sensitive to Peptide-N-Glycosidase F, indicative of complex glycosylation (Figure 3.4b). Further, we tested the effect of a therapeutic small molecule currently in clinical trials for p.Phe508del patients (a corrector compound, VX-809 or Lumacaftor) and found that it partially rescued the p.Ile1234_Arg1239del processing defect to a level comparable to that of p.Phe508del rescued by Lumacaftor ($P < 0.01$) (Figure 3.4c).

3.6 Discussion
This study highlights the importance of interrogating the consequences of CF causing mutations using multiple research tools in order to develop rational therapeutic strategies. Adjunctive functional in-vivo tests, i.e. nasal potential difference measurements and the novel β-adrenergic sweat secretion assay (Quinton et al., 2012), confirmed that the variant c.3700 A>G led to reduced CFTR channel function on the apical membrane of these CF affected tissues. This clinical phenotype is incompatible with the prediction that this variant caused the missense mutation, p.Ile1234Val. Cell-based studies of the predicted missense mutation introduced into CFTR cDNA showed that the predicted missense mutation, p.Ile1234Val, caused no apparent defects in protein folding, processing or function. These findings prompted a detailed analysis of the entire CFTR gene and CFTR mRNA obtained from the nasal epithelium of a homozygous patient with the detection of aberrant splicing. We found that this variant
Figure 3.4  p.Ile1234_Arg1239del-CFTR is misprocessed, retained in the ER, and partially rescued by Lumacaftor (currently in clinical trials for p.Phe508del patients).

(a) Immunoblots show steady-state levels of WT-CFTR, p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR in BHK cells after 24 h at 37 °C. WT-CFTR was expressed as both the golgi-modified, complex glycosylated (mature) band C form (broad 170 kDa band) as well as the endoplasmic reticulum-modified, core glycosylated (immature), band B form (sharp 150 kDa band). p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR were expressed primarily as the immature, band B form. Maturation (expressed as percent band C/(band B + band C)) of WT-CFTR, p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR was quantified for three independent trials, and a significant difference was found between the mutant proteins ($P < 0.01$). (b) To evaluate glycosylation status, immunoblots show the sensitivity of WT-CFTR, p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR to Endoglycosidase H and Peptide-N-Glycosidase F. White arrowhead, complex-glycosylated; black arrowhead, core-glycosylated; grey arrowhead, deglycosylated. (c) Immunoblots show the processing of WT-CFTR, p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR in BHK cells in the absence (DMSO) and presence of 3 µM Lumacaftor (or VX-809) after 24 h at 37 °C. Equivalent sample loading was confirmed by immunoblots of calnexin protein expression. Maturation (expressed as percent band C/(band B + band C)) of p.Phe508del-CFTR and p.Ile1234_Arg1239del-CFTR was quantified for three independent trials, and there is a significant difference between untreated (DMSO) or Lumacaftor (VX-809) treated cultures as indicated with the asterisks (**, $P < 0.01$).
caused aberrant splicing with the deletion of six residues (p.Ile1234_Arg1239del) in a conserved region of CFTR. Interestingly, deletion of this region resulted in defective folding and processing, a protein processing defect similar to that exhibited by p.Phe508del. This comparison motivated studies of Lumacaftor, a compound currently in clinical trial for patients with the p.Phe508del mutation and shown in cell culture to partially rescue the processing defect of p.Phe508del-CFTR. Importantly, a partial rescue was also induced by Lumacaftor of the p.Ile1234_Arg1239del-CFTR mutant in a heterologous expression system and this finding motivates our future work to determine the extent of functional rescue that can be mediated by this compound in epithelial tissues derived from patients with this mutation.

3.6.1 The Effect of p.Ile1234_Arg1239del on Protein Folding and Processing is Consistent with Bioinformatics Predictions

Examination of homology models and bioinformatics analyses predict that p.Ile1234_Arg1239del will cause significant conformational defects in the full length CFTR protein. As shown in Figure 3.5, residues 1234-1239 are modeled to constitute a β-turn between anti-parallel β-strands (β2 and β3) in NBD2. Residues p.Ile1235_Gln1238 are integral to the putative β-turn structural motif as predicted by Chou and Fasman (based on 408 β-turns in 29 proteins; Chou and Fasman, 1979). p.Ile1234_Arg1239 is N-terminal of NBD2 functional motifs (Walker A, residues 1244-1251; Q-loop, 1291-1297; signature C motif, 1346-1350; Walker B, 1366-1370; D-loop, 1374-1377; H-loop, 1401-1403), and therefore deletion of this sequence likely results in an altered and non-native assembly of CFTR.

Interestingly, the CF Mutation Database (genet.sickkids.on.ca/cftr) contains several disease-associated mutations within the p.Ile1234_Arg1239 sequence, and includes: c.3705T>G (p.Ser1235Arg), c.3709G>A (p.Gly1237Ser), c.3713A>G (p.Gln1238Arg), c.3712C>T (p.Gln1238X), and c.3717G>C (p.Arg1239Ser). These mutations were each found in one or two individuals of European descent (i.e. Belgian, Spanish, French and English), and the clinical presentation varies from a mild phenotype where the mutation was detected at 40 years of age (p.Gly1237Ser, with pancreatic sufficiency and FEV1>70%) to a more severe CF phenotype (p.Gln1238X, in trans with p.Phe508del, diagnosed at birth and with pancreatic insufficiency).
Figure 3.5  Inspection of a CFTR homology model predicts secondary structure of p.Ile1234_Arg1239.

(a) Since NBD2 of CFTR has yet to be crystallized, a recent CFTR homology model (Dalton et al., 2012) was inspected to evaluate the structural contribution of p.Ile1234_Arg1239 to CFTR assembly. In concordance with bioinformatics predictions, p.Ile1234_Arg1239 (red) putatively forms a β-turn between β strands (β2 and β3) in NBD2 (orange), which is N-terminal to several functional motifs, including Walker A (residues 1244-1251, black), Q-loop (1291-1297, magenta), signature C motif (1346-1350, blue), Walker B (1366-1370, yellow), D-loop (1374-1377, pink) and H-loop (1401-1403, gray). p.Phe508 (pale red), located in NBD1 (cyan), is also highlighted. Membrane Spanning Domains 1 and 2 (light blue and pale yellow, respectively) and the Regulatory domain (green) are also shown. This figure was prepared using PyMOL software (Schrödinger, Portland, OR).
These findings suggest that this region of NBD2 is mutation-sensitive. Importantly, as the current studies show that the variant c.3700 A>G caused CF disease because it led to aberrant splicing and not the missense mutation predicted, we suggest that the molecular consequences for all of these variants should be interrogated in detail.

### 3.6.2 Future Studies are Required to Understand the Genotype-Phenotype Relationship

Both individuals exhibited pancreatic sufficiency at the time of the study, consistent with a relatively mild form of CF and possibly modest residual CFTR function. The capacity for patients bearing the mutation, c.3700 A>G to exhibit residual CFTR function has yet to be rigorously tested. We tested the propensity for normal RNA splicing in one of the patients as shown in Figure 3.3. Only the aberrantly spliced CFTR mRNA was detected, hence, the mild pancreatic disease exhibited by the homozygous patients described in this study is unlikely to be conferred by modest expression of the normal transcript. This does not rule out the possibility that the propensity for normal RNA splicing may be different in pancreatic tissue, or that other patients with this mutation may exhibit some disposition for normal RNA processing. In addition, modifier genes have been shown to be important contributors to lung and pancreatic disease (Blackman et al., 2012; Sun et al., 2012). Polymorphisms in SLC26A9 have been shown to be associated with CF-related diabetes and meconium ileus (Blackman et al., 2012; Sun et al., 2012). In our future work, it will also be important to determine the SLC26A9 genotype in a larger number of patients with the c.3700 A>G mutation in CFTR in order to examine the contribution of this modifier gene to exocrine pancreas function.

Currently, we are developing the methods necessary to measure the residual function in epithelial cultures from patients with this rare mutation. Whereas Ussing chamber studies of residual function conferred by p.Phe508del (or e.g. 3849+10kbC>T) in respiratory epithelia are possible using primary cultures derived from transplanted lungs, this is not possible for studies of the c.3700 A>G mutation as lung transplantation is not performed for CF patients in Qatar (Chiba-Falek et al., 1999). There is optimism that recent advances in stem cell biology will enable the generation of epithelial tissues from patients with rare CFTR mutations and that
such cultures will provide insight into consequences of rare CFTR mutation in epithelial transport function.

In summary, these findings highlight the importance of experimentally verifying the clinical phenotype and the functional consequences of CF-causing variations as such studies will ultimately inform effective medical intervention. The discovery of aberrant splicing leading to deletion of six residues, and the encouraging findings in cell culture studies that Lumacaftor (VX-809) partially rescues defective protein folding, may have a significant impact on medical treatment of CF in the Middle East where this mutation affects most patients. Furthermore, we predict that the anticipated consequences of other CF-causing mutations which have not yet been fully characterized in vitro may require investigation into the status of alternative splicing, in order to more effectively treat CF disease in these patients.
Chapter 4

Biochemical interrogation of ΔI1234_R1239-CFTR predicts modest response to ΔF508-CFTR modulators

4.1 Acknowledgements

We thank the family of study participants for their contribution to this work. We also thank Dr. Felix Ratjen (Head, Division of Respiratory Medicine at the Hospital for Sick Children, Toronto, Ontario) for helpful discussions throughout the study, Dr. Peter N. Ray (Head, Molecular Genetics at the Hospital for Sick Children, Toronto, Ontario) for contributions in interrogating the CFTR sequence of affected family members, and Dr. Luis Galietta (U.O.C. Genetica Medica, Istituto Giannina Gaslini, Genova, Italy) for helpful comments regarding the TMEM16A studies.

The results presented in this chapter have been submitted for publication:


4.2 Abstract

Orkambi™ combination therapy (Lumacaftor plus Kalydeco™) was approved for treatment of patients bearing the prevalent Cystic Fibrosis (CF) mutation: c.1521_1523delCTT (or ΔF508-CFTR). This approval ignited hope that the use of this therapy could be expanded to treat patients bearing other CFTR mutations causing similar protein defects. However, a strategy for estimating patient-specific response magnitude prior to clinical testing does not exist. Here we introduce such a strategy, using a specific CF-causing mutation (c.3700 A>G), as an example. This mutation causes aberrant splicing and deletion of six residues in nucleotide binding domain 2 (NBD2; i.e. ΔI1234_R1239-CFTR). We found that in silico methods predicted the deleterious effect of this mutation on the stable assembly of the full length protein and its channel function. As expected based on the putative mechanism of action, the Orkambi™ drug combination was less effective in improving the functional expression of ΔI1234_R1239-CFTR
than ΔF508-CFTR in a heterologous expression system. Similarly, the Orkambi™ combination failed to significantly enhance the functional expression of ΔI1234_R1239-CFTR in primary cultures derived from affected siblings to the level achieved in preclinical trials for ΔF508-CFTR. Interestingly, relatively low residual CFTR function as measured in primary cultures from sibling CF-2 could be improved to the superior levels measured in the other sibling (CF-1) using a triple combination of compounds. Here, we’ve described an in vitro strategy for estimating drug response in individual patients and future clinical studies are necessary to test its potential to inform precision medicine for patients with rare CF-causing mutations.

4.3 Introduction

Several studies have previously shown that ΔF508 (in NBD1) alters the folding and thermostability of NBD1 (when studied in isolation) and disrupts the intramolecular assembly of CFTR, including the structurally relevant interface with the fourth intracellular loop (ICL4; linking transmembrane helix 10, i.e. TM10, to TM11) within MSD2, as well as the catalytic NBD1:NBD2 heterodimer (Serohijos et al., 2008; He et al., 2010; Thibodeau et al., 2010; Mendoza et al., 2012). These conformational defects lead to impaired forward trafficking through the biosynthetic compartments, and retention in the endoplasmic reticulum (previously defined as a Class 2 mutation) (Molinski et al., 2012; Farinha et al., 2013; Eckford et al., 2014). The limited number of ΔF508-CFTR molecules that reach the cell surface exhibit altered channel activity and reduced cell surface stability at physiological temperature (Du et al., 2005; Serohijos et al., 2008; He et al., 2010; He et al., 2013; Okiyoneda et al., 2013). Recently, a pharmacological chaperone (VX-809 or Lumacaftor) was found to be partially effective in rescuing the functional expression of ΔF508-CFTR at the cell surface in heterologous expression systems (Van Goor et al., 2011; Eckford et al., 2014). Together with Kalydeco™ (VX-770 or Ivacaftor), a drug that enhances channel activity, Lumacaftor significantly enhanced the functional expression of ΔF508-CFTR in pre-clinical studies of primary bronchial cell cultures and rectal biopsy-derived organoids (this combination therapy has recently been trademarked Orkambi™) (Van Goor et al., 2011; Dekkers et al., 2013; Kopeikin et al., 2014). Finally, in clinical trials, this combination led to significant improvement in lung function (2.6-4% increase), measured as forced expiratory volume (percent predicted) in one second (FEV1) (Boyle et al., 2014; Wainwright et al., 2015).
There are multiple CF-causing Class 2 mutations in addition to \( \Delta F508 \)-CFTR thought to result from intrinsic defects in folding, assembly and trafficking, although these mutations are relatively rare (Welsh and Smith, 1993; Sosnay et al., 2013). The prevailing CF drug discovery paradigm suggests that drugs that are effective in targeting \( \Delta F508 \)-CFTR will be effective in rescuing other Class 2 mutations (Awatade et al., 2015; Rapino et al., 2015). In the current study, we tested this paradigm and present a strategy to predict the efficacy of the co-therapy (Orkambi\textsuperscript{TM}) in treating patients possessing other Class 2 mutations using c.3700 A>G (p.Ile1234_Arg1239del-CFTR or \( \Delta I1234_R1239 \)-CFTR) as an example (Molinski et al., 2014).

4.4 Materials and Methods

4.4.1 Study Design

The goal of this study was to define the conformational defect(s) conferred by a rare CF-causing mutation \( \Delta I1234_R1239 \)-CFTR, which like \( \Delta F508 \)-CFTR leads to misprocessing with loss of functional expression. Towards this goal, we employed molecular dynamics simulations and biochemical (protease susceptibility) studies of the mutant protein after heterologous expression in HEK-293 cells. Biochemical (protease susceptibility) and functional, fluorescence-based studies of halide flux studies were conducted to determine if VX-809 or C4, correctors initially identified to target the defects inherent in \( \Delta F508 \)-CFTR were also partially effective in repairing the defects of the \( \Delta I1234_R1239 \)-CFTR mutant. Primary nasal epithelial cultures were studied from two siblings, homozygous for the \( \Delta I1234_R1239 \) and five non-CF family members in order to determine if the minor rescue effect of chronic treatment with the correctors (VX-809 or VX-661) and acute treatment with the potentiator (Kalydeco\textsuperscript{TM}) in the heterologous system translates to significant rescue of CFTR channel function in primary tissues. Finally, we present a novel approach wherein drug responses in tissues in affected individuals were benchmarked relative to the family-specific range for normal CFTR expression as defined using electrophysiological and immunolabeling studies.

4.4.2 Molecular dynamics system preparation and simulation conditions

The WT-CFTR homology model published in Dalton et al., 2012 was embedded in a membrane-mimetic \( n \)-octane slab with an approximate thickness of 3.5 nm. The system was 14.5 x 12.4 x
17 nm$^3$ in size with 2511 octane molecules and was hydrated by 68915 water molecules. A salt concentration of approximately 75 mM was achieved by adding 277 Cl$^-$ and 258 Na$^+$ ions to the aqueous solution. All calculations were performed using GROMACS version 4.5.5 (Hess et al., 2008) with the TIP3P water model (Jorgensen et al., 1983) and the OPLS-AA force field (Jorgensen et al., 1996) for protein and octane. The integration time step was 2 fs. A twin-range cutoff of 10 Å for van der Waals interactions and for direct electrostatic interactions calculated by particle-mesh Ewald (Essmann et al., 1995) was used, updating the neighbor list every 10 steps. Constant NPT conditions were applied using Parrinello-Rahman (Parrinello et al., 1980) semi-isotropic pressure coupling in the plane of the membrane, with a constant pressure of one bar applied via a coupling constant of $\tau_p = 2.0$ and zero compressibility in the direction of the membrane normal. The aqueous solution, octane, and the protein were coupled separately to a temperature bath at 300 K with a coupling constant of $\tau_T = 0.1$ ps using the Nosé-Hoover algorithm (Nose et al., 1984). The LINCS algorithm was used to constrain bond lengths (Hess et al., 1997). The molecular graphics in Figures 1 and S1 were produced using Visual Molecular Dynamics (VMD) (Humphrey et al., 1996).

The system was first energy minimized using steepest descent followed by an initial pre-equilibration phase of 5 ns with position restraints on protein backbone and water oxygen atoms with a force constant of 1,000 kJ mol$^{-1}$nm$^{-2}$. This procedure allowed octane molecules to relax around the protein and at the water-octane interface until the octane density was stabilized. The second equilibration phase included position restraints on protein backbone atoms C, C$\alpha$ and N for another 20 ns while relaxing the octane and water molecules around the protein. After pre-equilibration, 18 independent replicas for WT were set up by randomizing the starting velocities to initiate production runs each of which consisted of an unrestrained simulation of 40 ns long. Following a procedure detailed in (Kulleperuma et al., 2013) to perform cluster analysis, three representative structures that correspond to times frames at 14 ns (2 structures) and 10 ns (1 structure), and that also belong to the most populated cluster and obtained after at 14 ns (2 structures) and 10 ns (1 structure) of simulation were chosen to generate the ΔI1234_R1239 mutation.
The six-residue (I1234_R1239) long loop was manually deleted followed by an energy minimization to close the gap between residue numbers 1233 and 1240. The above-mentioned pre-equilibration phases were performed on the ΔI1234_R1239 mutant system. After pre-equilibration, 18 independent replicas (6 for each of the three starting conformations) of the ΔI1234_R1239 mutant were set up by randomizing the starting velocities to initiate production runs each of which consisted of a 30-ns unrestrained simulation.

All simulations were generated using GROMACS version 4.5.5 (Hess et al., 2008) with the OPLS-AA force field (Jorgensen et al., 1996) for protein and octane, and the TIP3P model for water (Jorgensen et al., 1983). The integration time step was 2 fs. A twin-range cutoff of 10 Å for the van der Waals interactions and 10 Å for direct electrostatic interactions calculated by particle-mesh Ewald (Essmann et al., 1995) was used, updating the neighbor list every 10 steps. Constant NPT conditions were applied using Parrinello-Rahman (Parrinello et al., 1980) semi-isotropic pressure coupling in XY directions, with a constant pressure of one bar applied via a coupling constant of $\tau_p = 2.0$ and zero compressibility in the z direction. The aqueous solution, octane, and the protein were coupled separately to a temperature bath at 300 K with a coupling constant of $\tau_T = 0.1$ ps using the Nosé-Hoover algorithm (Nose et al., 1984). The LINCS algorithm was used to constrain bond lengths (Hess et al., 1997). Graphics related MD simulations are generated using VMD (Humphrey et al., 1996).

### 4.4.3 Generation of ΔNBD2 mutant CFTR construct

E1172X-CFTR (ΔNBD2) was generated in human WT-CFTR cDNA (pcDNA3.1) using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Woburn, MA). The NBD2 deletion construct was generated by introducing a stop codon at position E1172 using the following primers:

- 5'- GAC ATG CCA ACA TAA GGT AAA CCT ACC -3' (sense);
- 5'- GGT AGG TTT ACC TTA TGT TGG CAT GTC -3' (antisense).  

Plasmid DNA was prepared using the GenElute™ Plasmid Miniprep Kit (Sigma), and the presence of the stop codon, as well as the integrity of CFTR cDNA, was confirmed by DNA sequencing (TCAG Inc., Toronto, ON).
4.4.4 Studies of CFTR protein processing

ΔI1234_R1239-CFTR was generated in human WT-CFTR cDNA (pcDNA3.1) as previously described (Molinski et al., 2014). Human embryonic kidney (HEK) cells were transiently transfected with WT-CFTR or mutant CFTR constructs using PolyFect Transfection Reagent, according to the manufacturer’s protocol (Qiagen). HEK cells transiently expressing CFTR proteins were maintained in DMEM (Wisent) supplemented with non-essential amino acids (Life Technologies) and 10% FBS (Wisent) at 37 °C with 5% CO₂ (HEPA incubator, Thermo Electron Corporation).

HEK cells expressing WT-CFTR or mutant CFTR constructs were grown at 37 °C for 24 h in the absence or presence of small molecules (3 µM VX-809 or 1 µM VX-661, Selleck Chemicals; 10 µM C4, 6 µM C18, 10 µM VRT-325 or 10 µM VRT-532; Cystic Fibrosis Foundation Therapeutics) as required. Cells were subsequently lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and CFTR bands were detected using the human CFTR-NBD2-specific (Cui et al., 2007) (amino acids 1204-1211) murine mAb 596 (1:20000, University of North Carolina Chapel Hill, NC), horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

4.4.5 Studies of ΔI1234_R1239-CFTR protein degradation

HEK cells were transiently transfected with ΔI1234_R1239-CFTR at 37 °C, as described previously (Molinski et al., 2014). Following transfection, HEK cells overexpressing ΔI1234_R1239-CFTR were treated with 2 µM of the proteasomal inhibitor epoximicin (Santa
Cruz), 100 nM of the lysosomal inhibitor Bafilomycin A1 (Santa Cruz), or both, for 7 hours at 37°C. Cells treated with either proteasomal, lysosomal, or both inhibitors were lysed following the respective treatments and soluble proteins were analyzed by SDS-PAGE and immunoblotting as described previously (Molinski et al., 2014). The accumulation of ΔI1234_R1239-CFTR was compared in the presence of the treatments relative to vehicle (0.1% DMSO).

4.4.6 Preparation of crude membranes, and limited proteolysis of ΔI1234_R1239-CFTR

Crude membranes were prepared from HEK cells transiently expressing WT-CFTR, ΔF508-CFTR, as well as ΔI1234_R1239-CFTR treated with either VX-809 (3 µM), Corr-4a (10 µM), VX-809 and Corr-4a (3 µM and 10 µM, respectively), or vehicle (0.1% DMSO) for 24 h at 37°C as previously described (Yu et al., 2011; Eckford et al., 2014). Briefly, cell pellets were resuspended in cell lysis buffer (10 mM HEPES, 1 mM EDTA, pH 7.2), and cells were lysed using a cell disruptor (10,000 psi, 4°C, 5 min). The cell suspension was centrifuged at 800 x g for 10 min at 4°C to pellet unbroken cells, and crude membranes were isolated from the resulting supernatant after centrifugation at 100,000 x g for 60 min at 4°C. The crude membrane pellet was resuspended in buffer (40 mM Tris-HCl, 5 mM MgCl₂, 0.1 mM EGTA, pH 7.4) by passage through a 1 mL syringe 20 times with a 27-gauge needle.

For limited proteolysis studies, 20 µg crude membranes were resuspended in buffer (40 mM Tris-HCl, 5 mM MgCl₂, 0.1 mM EGTA, pH 7.4) and sonicated. Samples were kept on ice, and trypsin (Promega) was added at the following concentrations: 0, 1.6, 3.1, 6.3, 12.5, 25, 50 µg/mL. Samples were again sonicated and incubated at 4°C for 15 min. Proteolysis was terminated by addition of 0.5 mg/mL trypsin soybean inhibitor (Sigma). Membranes were solubilized in modified RIPA for 15 min, and the soluble fraction was analyzed by SDS-PAGE on a 4-12% gradient gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, NE), and protein bands were detected using the human CFTR-NBD1-specific murine mAb L12B4 (1:1000, EMD Millipore) or the human CFTR-NBD2-specific murine mAb M3A7 (1:1000, EMD Millipore).
Fluorescence was detected using the secondary antibody IRDye-800 (goat anti-mouse IgG: 1:15,000, Rockland Immunochemicals, Gilbertsville, PA). Blots were imaged, and band intensities were detected using the Odyssey infrared imaging system (LI-COR Biosciences). Relative levels of full-length ΔI1234_R1239-CFTR resulting from trypsin digestion were measured using ImageJ 1.42 Q software (National Institutes of Health).

4.4.7 Studies of CFTR-mediated fluorescence dequenching

To test function, HEK cells over-expressing CFTR constructs were grown in 12 well plates, and upon formation of a mono-layer, the cells were incubated overnight with 10 mM of the halide sensitive fluorophore 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) (Invitrogen Molecular Probes Inc.), at 37 °C and 5% CO₂. Next day, the cells were washed 3 times with phosphate buffered saline to make the extra-cellular fluid free of SPQ. Cells were then kept in chloride containing physiological solution, Hank’s buffered saline solution. Fluorescence measurements were made using Gemini EM Fluorescence microplate reader (Molecular devices). Intracellular SPQ was excited at 350 nm wavelength and the emission was measured at 450 nm, reporting chloride concentration. After reading baseline fluorescence in presence of physiological solution, a chloride gradient was established via exchange of the extracellular solution with a chloride free, nitrate containing buffer (NaNO₃ 136 mM, KNO₃ 3 mM, Ca(NO₃)₂ 2 mM, Glucose 11 mM, HEPES 20 mM, pH 7.2 and osmolarity 300 mOsm). CFTR was stimulated using cAMP agonist forskolin (10 μM). After reaching equilibrium for chloride flux, the extra-cellular solution was replaced with physiological solution containing chloride, allowing SPQ to quench. The fluorescence measurements were expressed as the change in fluorescence relative to the fluorescence measurement just before CFTR stimulation (Eckford et al., 2014). Data is summarized as initial rate of change in relative fluorescence units in the first 5 minutes of stimulation. Each condition was repeated with at least 2 technical replicates on the same plate, and a total of at least 3 biological replicates per condition.

4.4.8 Studies of CFTR-mediated membrane depolarization

CFTR-mediated membrane depolarization was measured as previously described (Maitra et al., 2013; Molinski et al., 2015). Briefly, HEK cells over-expressing CFTR constructs were grown to
100% confluence in 24-well plates (Costar), washed with PBS, and the blue membrane potential dye (dissolved in chloride free buffer containing 136 mM sodium gluconate, 3 mM potassium gluconate, 10 mM glucose, 20 mM HEPES, pH 7.35, 300 mOsm, at a concentration of 0.5 mg/ml; Molecular devices), which can detect changes in transmembrane potential, was added to the cells for 1 hr at 27 °C or 37 °C as required. The plate was then read in a fluorescence plate reader (Molecular devices – Gemini EM) at the required temperature, and after reading the baseline fluorescence, CFTR was stimulated using the cAMP agonist forskolin (10 μM; Sigma) or forskolin with VX-770 (1 μM; Selleck Chemicals). CFTR-mediated depolarization of the membrane was detected as an increase in fluorescence, and repolarization or hyperpolarization as a decrease (Maitra et al., 2013). To terminate the assay, CFTR specific inhibitor Inh-172 (CFTRinh-172, 10 μM; Cystic Fibrosis Foundation Therapeutics) was added. Changes in membrane potential were normalized to the addition of agonists.

4.4.9 Generation and characterization of primary nasal epithelial cells from a family with c.3700 A>G (ΔI1234_R1239-CFTR)

Nasal brushing was performed on family members (after obtaining informed consent) by a research nurse with procedural experience. A 3-mm diameter sterile cytology brush (MP Corporation, Camarillo, CA) was used. The inferior turbinate was visualized and the brush inserted into the nares and rotated to brush the turbinate. The brush was then placed in warm basal epithelial growth media (BEGM, Lonza, Walkersville, MD). Cells were dissociated with gentle agitation and seeded on a collagen coated flask (P0 or passage number = 0). Cultures were maintained at 37 °C in BEGM with antibiotics and an atmosphere of 5% CO2 in air. Cells were subsequently expanded into a larger flask (defined as P1) and passaged once 70% to 80% confluent. To generate air-liquid interface cultures, P2 cells were seeded on collagen coated Transwell inserts (6.5 mm diameter, 0.4 µm pore size) at a density of $10^5$ cells per insert. Unused cells were frozen for later use. Cells were maintained in BEGM but once confluent, the media was changed to air liquid interface (ALI) with basal differentiation media (PneumaCult, StemCell Tech., Vancouver, Canada). Basal media was changed every day for the first week and then every 2 days subsequent to that. The apical surface was washed weekly with PBS. By 3 weeks, cells had a ciliated phenotype.
4.4.10 Studies of CFTR protein expression in primary nasal epithelial cells

Nasal cultures were grown at 37 °C for 48 h in the absence or presence of small molecules (3 µM VX-809 or 1 µM VX-661) as required. Cells were then lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and CFTR bands were detected using the human CFTR-MSD1-specific (amino acids 25-36) murine mAb MM13-4 (1:200, Abcam), horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:2500) and exposure to film for 0.5 to 30 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

4.4.11 Ussing chamber studies of primary nasal epithelial cells

Nasal cell transwells were mounted in a circulating Ussing chamber (Physiological instruments Inc. San Diego, CA), continuously perfused with buffer (126 mM NaCl, 24 mM NaHCO3, 2.13 mM K2HPO4, 0.38 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 10 mM glucose) with symmetrical chloride concentrations, and gassed with 5%CO2 and 95%O2 to maintain at pH 7.4. Transepithelial voltage (Vte) and resistance (Rte), following brief 1 µA current pulses every 30 seconds, were recorded in open–circuit mode. Results are presented as calculated equivalent short-circuit (Ieq). Following inhibition of epithelial Na+ channel channel with Amiloride, CFTR function was assessed as Forskolin-activated current (Ieq-Fsk) and as CFTRinh-172–sensitive current (Ieq-CFTRinh-172). To test efficacy of corrector compounds, nasal cells were treated for 48 hrs with VX-809 (3 µM) or VX-661 (3 µM) prior to experiments, VX-770 as potentiator compound was applied acutely during the experiments.
4.4.12 Immunofluorescence detection of CFTR
Air-liquid interface (ALI) cultured nasal epithelial cells on filter membranes were cut into 1-2 mm pieces and embedded in OCT compound (Sakura Finetek). Cryosections were cut at 6-8 micron. They were then fixed in cold 90% methanol/10% PBS solution at -20 °C for 5 minutes. For en face imaging, epithelial cultures were fixed in a mixture of 2% paraformaldehyde, 0.01% glutaraldehyde, in 0.1M phosphate buffer, pH7.2 for 1 hour. The cell layer was then scraped from the filter membrane using a razor blade. The released cell patches were neutralized in 0.15M glycine, 80 mM NH₄Cl, 0.1M phosphate buffer, pH7.2 for 10 minutes, washed 3 times with 0.15M glycine in phosphate buffer, pH7.2 for 5 minutes, and permeabilized in 0.2% Triton X-100, in 0.15M glycine, 0.5% BSA, 0.1M phosphate buffer, pH7.2 for 20 minutes, with solution changing every 4 minutes. To block nonspecific staining, the samples were incubated in 4% BSA in PBS for 30 minutes. Incubation with primary and secondary antibodies was done in the same blocking solution. DAPI was used as a nuclear stain. Images were captured with the Nikon ECLIPSE Ti inverted microscope and NIS-Elements 3.10 software for fluorescence imaging.

4.4.13 Studies of TMEM16A protein expression in primary nasal epithelial cells
Nasal epithelial cells from confluent transwells (6.5 mm diameter, 0.4 µm pore size) were each lysed in 150 µL modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions (20 µL) were analyzed by SDS-PAGE on 6% gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and TMEM16A bands were detected using the human anti-TMEM16A rabbit mAb SP31 (1:100, Abcam), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2500) and exposure to film for 0.5 to 5 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1: 5000) and exposure to film for 0.5 to 5 min as required. Relative levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.
4.4.14 Statistical analysis

All data are represented as mean ± S.E. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Non-paired Student's t tests, one-way analysis of variance (ANOVA), and two-way ANOVA were conducted as appropriate, and P values less than 0.05 were considered significant. Each experiment was replicated at least three times.

4.5 Results

As previously shown, when heterologously expressed in HEK-293 cells, the major form of the ΔI1234_R1239-CFTR mutant is retained in the endoplasmic reticulum (ER) (immature form, called Band B) and lacks the complex glycosylation conferred in the Golgi (i.e. mature, Band C form) (Figure 4.S1) (Molinski et al., 2014). Similar to ΔF508-CFTR, the ER-retained ΔI1234_R1239-CFTR mutant protein undergoes proteosomal degradation (Figure 4.S2). Interestingly, in contrast to the ΔF508-CFTR protein (Denning et al., 1992), low temperature (27°C) incubation and chemical chaperones such as sodium butyrate, while effective in enhancing the total abundance of the mutant protein, are not effective in enhancing the maturation of ΔI1234_R1239-CFTR, suggesting that there may be distinct defects in biosynthesis inherent in this mutant (Figure 4.S2) (Cheng et al., 1995; Moyer et al., 1999).

4.5.1 ΔI1234_R1239-CFTR exhibits conformational defects that are only partially rescued by small molecule modulators of ΔF508-CFTR

Compounds are described as correctors for ΔF508-CFTR if they are effective in promoting native folding and assembly with appearance of Band C, the mature, complex glycosylated form of the mutant protein (Okiyoneda et al., 2013). Lukacs and colleagues categorized ΔF508-CFTR corrector compounds based on their putative mechanism of action with Class 1 compounds acting to repair aberrant intramolecular interactions between ICL4 and NBD1 (as well as stabilize MSD1 (Ren et al., 2013; Loo et al., 2013), Class 2 compounds enhancing stability of NBD2, and Class 3 compounds promoting stability of NBD1 (He et al., 2013; Okiyoneda et al., 2013). For ΔF508-CFTR, the combination of correctors from each class led to full correction of its protein processing defect and the appearance of wild-type levels of
Figure 4.51. Location of I1234_R1239 in the WT-CFTR homology model and relative protein expression levels between clinically relevant CFTR variants.

(A) WT-CFTR homology model (Dalton et al., 2012) showing the predicted secondary structure of I1234_R1239. MSD1, blue; NBD1, cyan; R-domain, omitted from model; MSD2, yellow; NBD2, orange; I1234_R1239 sequence, red. (B) Immunoblots and (C) quantitative analysis showing steady state expression levels of ΔI1234_R1239-CFTR compared with other mutant CFTR proteins (i.e. ΔF508-CFTR, G551D-CFTR, ΔNBD2-CFTR). ***, p<0.001; n≥3 biological replicates.
Figure 4.S2. ΔI1234_R1239-CFTR is degraded by the proteasome, and abundance, but not maturation of ΔI1234_R1239-CFTR is enhanced by a transcriptional modulator and low temperature incubation.

(A) Immunoblots and (B) quantitation of ΔI1234_R1239-CFTR following treatments with epoxomicin (proteasome inhibitor) or Bafilomycin A1 (lysosomal degradation inhibitor). (C) Immunoblots of ΔI1234_R1239-CFTR following treatments with sodium butyrate (NaBu, transcriptional modulator) or low temperature incubation (27 °C); (D) Quantitation of maturation and (E) relative abundance of ΔI1234_R1239-CFTR. *, p<0.05; **, p<0.01; n≥3 biological replicates.
mature protein (Okiyoneda et al., 2013). In the case of ΔI1234_R1239-CFTR, Class 1 corrector compounds (VX-661, C18, VRT-325 and VX-809) and the Class 2 corrector: Corr-4a (C4) were effective in enhancing the maturation of ΔI1234_R1239-CFTR (Figures 4.1A and 4.1B). Interestingly, co-treatment with Class 1 and Class 2 correctors (i.e. VX-809 and C4) did not lead to an additive or synergistic response in processing of the ΔI1234_R1239-CFTR mutant as reported for ΔF508-CFTR (Okiyoneda et al., 2013). We could not assess the effect of the Class 3 chemical chaperone: glycerol, as it exerted a deleterious effect on total protein expression in the current studies. The lack of synergy between corrector classes suggests that the intrinsic defects in protein folding and assembly caused by ΔI1234_R1239 are not identical to those caused by ΔF508 in CFTR.

Since the deleted region: I1234_R1239, is proximal to functional motifs located in NBD2 (i.e. Walkers A and B; signature C; D-, H- and Q-loops) we hypothesized that deletion of this sequence likely disrupts the native, channel-competent conformation of CFTR protein (Gadsby et al., 2006; Deeley et al., 2006). To test this idea, we studied the channel activity of ΔI1234_R1239-CFTR in HEK-293 cells using a halide flux assay (Mansoura et al., 1999). First, we were prompted to evaluate the function of ΔI1234_R1239-CFTR in the absence of pharmacological intervention. As expected, at physiological temperature (37°C) the low percentage of mature, plasma membrane-localized ΔI1234_R1239-CFTR protein was insufficient to mediate significant chloride flux after activation by CFTR agonists, forskolin and isobutylmethyloxanthine (IBMX); findings in agreement with the recent work by Ramalho and colleagues (Figure 4.1C) (Ramalho et al., 2015). Long-term (24 hour) treatment with the Class 1 corrector VX-809 or the Class 2 corrector C4 (to a lesser extent) conferred the appearance of cAMP-activated chloride flux (Figure 4.1C). Interestingly, the magnitude of the VX-809-rescued channel function for ΔI1234_R1239-CFTR was approximately half of that observed for VX-809-rescued ΔF508-CFTR despite similar rescue of the processing defect (Figures 4.1B and 4.1C). The relatively severe channel function defect for ΔI1234_R1239-CFTR may relate to aberrant ATP dependent gating, predicted based on the proximity of the deletion to the functionally important Walker A motif of NBD2. We then assessed the response of ΔI1234_R1239-CFTR channels having undergone correction using VX-809 for their response to the channel
Figure 4.1. Maturation and conformational stability of ΔI1234_R1239-CFTR is enhanced by several ΔF508-CFTR correctors in HEK-293 cells.

(A) Immunoblots of steady-state expression of ΔI1234_R1239-CFTR following treatments with ΔF508-CFTR modulators. Class 1 and Class 2 correctors are labeled in red and blue, respectively. ΔF508-CFTR and WT-CFTR are included as controls. (B) Quantitation of maturation by most efficacious modulators; VX-809 was used as a representative Class 1 corrector. Maturation was benchmarked to ΔF508-CFTR and WT-CFTR. (C) Quantitation of rate of activation (chloride efflux assay) of ΔI1234_R1239-CFTR following chronic treatment with ΔF508-CFTR correctors and acute activation (forskolin/IBMX). Activation rate was benchmarked to ΔF508-CFTR and WT-CFTR. (D) Representative traces (membrane depolarization assay) of ΔI1234_R1239-CFTR and ΔF508-CFTR function following chronic treatment with VX-809 and acute activation (forskolin/VX-770). (E) Quantitation of relative activated responses of ΔI1234_R1239-CFTR and ΔF508-CFTR, benchmarked to WT-CFTR without VX-809 or VX-770. (F) Immunoblots of proteolytic digestion of full-length WT-CFTR, ΔF508-CFTR and ΔI1234_R1239-CFTR. (G) Quantitation of proteolytic digestion of NBD1 and NBD2 fragments of ΔI1234_R1239-CFTR in the presence of small molecule correctors (VX-809, C4). *, p<0.05; **, p<0.01; ***, p<0.001; n≥3 biological replicates. Functional studies were conducted in collaboration with Saumel Ahmadi.
potentiator VX-770 (Ivacaftor or Kalydeco™) as this potentiator can act independently of the Walker A motif in NBD2 (Yeh et al., 2015). As in the case of ΔF508-CFTR (ΔF), VX-809-corrected ΔI1234_R1239-CFTR (ΔIR) channels were potentiated by acute treatment with VX-770 (1 μM) after pre-treatment with forskolin (Figure 4.1D). However, paired experiments showed that this potentiation was significantly less (approximately 50%) than that observed in cells expressing ΔF508-CFTR (Figure 4.1D and 4.1E). These data support the hypothesis that distinct conformational defects persist in VX-809-rescued ΔI1234_R1239-CFTR protein leading to impaired channel potentiation.

### 4.5.2 ΔI1234_R1239-CFTR exhibits conformational defects in NBD1 and NBD2

In order to better define the conformational defects induced by ΔI1234_R1239-CFTR, we conducted limited proteolysis studies of the mutant CFTR expressed in HEK-293 cells. First we validated our methods by confirming previous studies showing that the full-length, as well as NBD2 (the 36 kDa fragment monitored using the NBD2 specific antibody: M3A7) of ΔF508-CFTR protein exhibited enhanced protease (trypsin) susceptibility relative to WT-CFTR (Figures 4.1F and 4.3A) (Du et al., 2005; Cui et al., 2007; Yu et al., 2011; Eckford et al., 2014). Applying these methods to the full-length ΔI1234_R1239-CFTR protein, we found that this mutant protein also exhibited enhanced protease sensitivity supporting the claim that its conformation is altered (Figures 4.1F and 4.3A). Interestingly, in the context of the full length protein, the domain in which the mutation is located, ΔI1234_R1239 in NBD2 is stable and trypsin resistant even at high trypsin concentrations (Figures 4.1F and 4.1G). On the other hand, the fragmentation pattern for the amino-terminal half of the ΔI1234_R1239-CFTR mutant is strikingly different than that detected for WT-CFTR and ΔF508-CFTR (Figures 4.1F and 4.3A). For example, a dominant 36 kDa fragment corresponding to NBD1 that is trypsin-resistant in WT-CFTR or ΔF508-CFTR is not detectable for ΔI1234_R1239-CFTR. Although this approach does not provide molecular detail, these results suggest that in the context of the full-length CFTR protein, the ΔI1234_R1239 mutation does not significantly affect the overall stability of NBD2 where the mutation is located, rather it confers an allosteric, de-stabilizing effect on the contralateral half of the CFTR protein containing NBD1.
Figure 4.S3. Quantitative analysis of limited proteolysis studies of Δl1234_R1239-CFTR.

(A) Quantitation of proteolytic susceptibility of full-length WT-CFTR, ΔF508-CFTR and Δl1234_R1239-CFTR via relative protein abundances (mature + immature forms) at increasing trypsin concentrations. (B) Quantitation of proteolytic susceptibility (via relative protein abundances of mature + immature forms) of full-length Δl1234_R1239-CFTR in the absence (DMSO) or presence of small molecule correctors of ΔF508-CFTR (i.e. VX-809, C4, or VX-809+C4). *, p<0.05; n≥3 biological replicates.
Figure 4.S4. Proteolytic digestion and probing of amino-terminal fragments of ΔI1234_R1239-CFTR in HEK cells using mAb 660 in the absence (DMSO) or presence of VX-809.

NBD1-specific mAb 660 did not detect the dominant 36 kDa NBD1 fragment of ΔI1234_R1239-CFTR, which is observed in ΔF508-CFTR and WT-CFTR (15, 44); the proteolytic digestion profile of ΔI1234_R1239-CFTR using mAb 660 is comparable to the profile using L12B4.
As in the processing studies in Figure 4.1A, treatment with VX-809 plus C4 was maximally effective in increasing the stability of ΔI1234_R1239 (evident as trypsin resistance) (Figures 4.1F, 4.1G and 4.53B). This effect was mediated by directly or indirectly stabilizing the amino terminal half of the mutant protein. However, as previously shown, the combination treatment with C4 and VX-809 was less effective in promoting functional expression than the single treatment: VX-809, suggesting that C4 induced rigidity in this mutant protein which was inhibitory to its proper channel activity. Hence, VX-809 was added as a single treatment in future correction studies on validation studies using patient-specific tissues because it is permissive to the enhanced processing and channel function.

4.5.3 Molecular dynamic simulations were effective in predicting consequences of the mutation: ΔI1234_R1239, on full length protein structure and function

We developed a molecular model of the full-length ΔI1234_R1239-CFTR mutant protein to gain greater insight into the structural and functional defects we observed in the above biochemical studies. We conducted all-atom molecular dynamics (MD) simulations, adopting a ground-up approach, using homology models of the full-length WT-CFTR protein and generated ΔI1234_R1239-CFTR from the WT-CFTR template based on the Sav1866 crystal structure (Figure 4.S1A) (Dawson and Locher, 2006; Dawson and Locher, 2007). Multiple simulations with multiple repeats were performed both on the WT-CFTR and on ΔI1234_R1239-CFTR, starting from the equilibrated WT-CFTR system. The total simulation time for each system was over 0.5 μs-long simulation time for each system. The residues deleted in the mutant are predicted to constitute a loop connecting two anti-parallel β-strands (β2 and β3) in NBD2, which form part of an anti-parallel three-stranded β-sheet and a parallel four-stranded β-sheet, respectively (Figure 4.2A). After 30 ns of unrestrained simulation, the loss of secondary structure in β-strands β2 and β3 with varying intensities was evident in multiple simulations of ΔI1234_R1239-CFTR. While both of these strands remained intact in our simulations of WT-CFTR, one or both of the two β strands (β2 and β3) unraveled in 2/3 of the simulations of ΔI1234_R1239-CFTR (Figure 4.2A). We then analyzed how these local perturbations may have affected the overall conformation of NBD2. To this end, the root mean square deviation (RMSD) of the backbone of each NBD2 residue from the starting conformation of NBD2 as a
Figure 4.2. Change in secondary structure and structural deviation in NBD2 of ΔI1234_R1239-CFTR.

(A) Representative snapshots of NBD2 in WT-CFTR (left panel) and ΔI1234_R1239-CFTR (right panel) systems following 30 ns of simulation. Each secondary structure element of the labelled β-strands is represented by a unique color as follows; isolated-bridge: brown, coil: grey, β-strand: yellow, 3-10 helix: dark blue, α-helix: cyan, turn: green; Walker A is in magenta, while the rest of NBD2 is in transparent representation. The amide N and carbonyl C atoms of residues 1233 and 1240 respectively are shown as red spheres and the backbone atoms between the same residue pair is shown in red cartoon representation. (B) Replica-averaged root mean square deviation (RMSD) of backbone atoms of NBD2 from the starting conformation of WT-CFTR and ΔI1234_R1239-CFTR as a function of the residue number is represented in red and blue thick lines, respectively. The six-residue deletion (ΔI1234_R1239) is labelled “del”. The thick black and magenta lines denote β-strands and the Walker A motif, respectively. Studies were conducted by Kethika Kulleperuma and Régis Pomès.
function of its residue number was compared between WT-CFTR and ΔI1234_R1239-CFTR systems (Figures 4.2B). The deviation of all regions in ΔI1234_R1239-CFTR, fall within the error of the WT-CFTR system. These findings suggest that in this model of the full-length protein, the effect of ΔI1234_R1239 was localized to the immediate vicinity of the deletion and did not modify the global fold of the whole NBD2 during the relatively short simulation time of 30 ns.

To examine the effect of ΔI1234_R1239 on the downstream, canonical ATP binding site of NBD2 (which contains the Walker A lysine, K1250), we compared the conformational ensemble of the replicas of the WT-CFTR and mutant protein; the ATP-analog-bound crystal structure of Sav1866 is used as the reference for comparison (Figure 4.55) (Dawson and Locher, 2006; Dawson and Locher, 2007; Dalton et al., 2012). The superimposition of backbone atoms of Walker A in WT-CFTR and ΔI1234_R1239-CFTR on the crystal structure revealed somewhat more structural heterogeneity in the mutant protein than in WT-CFTR. Moreover, the spatial distribution of the ammonium N atom of K1250 of the Walker A motif in NBD2 which is critical for ATP binding, shows a wider dispersion within the mutant ensemble compared to WT-CFTR, for which more replicas are comparable to the reference. These findings suggest that the canonical ATP binding site of NBD2 that is engaged in ATP-dependent hetero-dimerization with NBD1 and ATP-dependent channel gating is structurally perturbed in ΔI1234_R1239-CFTR.

Together, the MD simulation studies were predictive of the protein defects seen in ΔI1234_R1239-CFTR by biochemical and functional assays. Specifically, the MD studies predicted that the structural consequences of this mutation would be localized and have minimal consequences on the overall stability of NBD2 in the context of the full-length protein. Similarly, the MD studies predicted that the canonical ATP binding site in NBD2 would exhibit an altered orientation thereby perturbing the ATP-dependent heterodimerization of NBD2 with NBD1. Such a perturbation of the NBD1:NBD2 interface would account for the deleterious effect of the deletion of I1234_R1239 in NBD2 on the stability of the contralateral half of the intact protein, containing NBD1 as observed in our biochemical, limited proteolysis studies. Further, the predicted destabilization of this interface would account for the aberrant
Figure 4.S5. Structural heterogeneity of Walker A in WT-NBD2 and ΔI1234_R1239-NBD2.

Distribution of pairwise RMSDs for final states of the backbone atoms of the Walker A motif (residues 1244-1251) in WT-NBD2 (red) and ΔI1234_R1239-NBD2 (blue). Studies were conducted by Kethika Kulleperuma and Régis Pomès.
channel function exhibited by ΔI1234_R1239-CFTR, as channel opening is associated with NBD heterodimerization (Csanady et al., 2010).

4.5.4 Chronic treatment with VX-770 (Ivacaftor, Kalydeco™) exerts deleterious effect on modest correction of ΔI1234_R1239-CFTR by VX-809

In light of recent reports demonstrating that long term (24-48 hours) treatment with VX-770 in vitro diminishes the pharmacological correction of ΔF508-CFTR, we were prompted to investigate the consequences of long term VX-770 treatment on the functional correction of ΔI1234_R1239-CFTR mediated by VX-809 (Cholon et al., 2014; Veit et al., 2014). Similar to the findings with ΔF508-CFTR, we found that a 24 hr treatment with VX-770 (10 µM) significantly reduced correction of the processing defect in ΔI1234_R1239-CFTR by VX-809 (Figures 4.3A and 4.3B), and exerted a significant deleterious effect on its functional expression in this over-expression system (Figures 4.3C and 4.3D). Hence, ΔI1234_R1239-CFTR protein (and possibly other Class 2 mutants) may be similar to ΔF508-CFTR with respect to the detrimental effect of chronic in vitro dosing of high concentrations of VX-770 (10 µM) on its stability.

4.5.5 ΔF508-CFTR correctors induce modest rescue in primary nasal epithelial cells from individuals homozygous for c.3700 A>G (ΔI1234_R1239) relative to non-CF family members

Our next goal was to determine if the modest functional rescue observed in our heterologous expression system translated to a detectable response in patient tissue. Therefore, we generated differentiated nasal epithelial cultures from two CF individuals (siblings homozygous for ΔI1234_R1239-CFTR) as well as cultures from four other non-CF family members (homozygous or heterozygous for WT-CFTR), excluding two individuals who are heavy smokers as cigarette smoke is known to be deleterious to CFTR expression (Figure 4.4A) (Cantin et al., 2006). Immunofluorescence studies of cultures generated from family members show well-polarized epithelium with apically localized WT-CFTR for cultures derived from a non-CF individual (Figure 4.5C).
Figure 4.3. Chronic VX-770 reduces functional expression of pharmacologically repaired ΔI1234_R1239-CFTR.

(A) Immunoblot and (B) quantitation of ΔI1234_R1239-CFTR expression following pharmacological correction (VX-809) in the absence (DMSO) and presence of chronic (24 h) VX-770 treatment (0.1, 1 and 10 µM). (C) Representative traces (membrane depolarization assay) and (D) quantitation of ΔI1234_R1239-CFTR function following pharmacological correction (VX-809) in the absence (DMSO) and presence of chronic (24 h) VX-770 treatment (10 µM). *, p<0.05; **, p<0.01; ***, p<0.001; n≥3 biological replicates. Functional studies were conducted in collaboration with Saumel Ahmadi.
Figure 4.4. Electrophysiological studies of differentiated primary nasal epithelial cultures reveal defective function of endogenous ΔI1234_R1239-CFTR.

(A) Pedigree of the relationship of family members toward the index patients and their genotype status. Relative generation levels are marked as I and II; the proband is marked with an arrow; heterozygote carriers are represented by a half-filled square (father), circle (mother) or diamond (three siblings), while homozygous WT (two siblings) or c.3700 A>G individuals (CF-1 and CF-2) are unfilled or filled, respectively. (B) A representative tracing shows Ussing chamber measurements of CFTR function in nasal cell cultures from the non-CF father. (C) Bar graph showing mean leq for nasal cultures derived from all family members depicted in panel A except for two brothers who are heavy smokers. CFTR function presented as leq-CFTRinh-172 inhibition after forskolin activation. Each bar represents measurement from a single family member except for the male heterozygotes (two individuals showing the mean, and standard deviation as an error bar). (D) Representative tracings show Ussing chamber measurements of CFTR function in nasal cell cultures from affected patients (CF-1 and CF-2) in the absence and presence of the small molecule corrector VX-809. (E) Bar graph showing mean (+SD) CFTRinh-172 sensitive leq for nasal cultures from CF-1 and CF-2 after pre-treatment with VX-809 (48 hours, 3 μM), activation with forskolin and acute potentiation with VX-770 (1 μM); replicate measurements of n=4 (CF-1) and n=3 (CF-2). After treatment, the in vitro responses of the CF subjects failed to reach the lower range (25th quartile) for in vitro CFTRinh-172 sensitive leq exhibited by the family members. Studies were conducted by Wan Ip and Tanja Gonska.
Figure 4.5. Immunoblotting and immunofluorescence of differentiated primary nasal epithelial cultures reveal defective processing of endogenous ΔI1234_R1239-CFTR.

(A) Immunoblot of steady-state expression of WT-CFTR (non-CF) and ΔI1234_R1239-CFTR (CF-1 and CF-2) following treatment with VX-809 or VX-661. (B) Quantitation of total CFTR (Band C and Band B forms in non-CF and CF individuals, respectively) expressed in the absence of small molecules (*, p<0.05; n≥3). (C) Top panel: immunofluorescence showing expression and localization of WT-CFTR (green) on primary nasal tissue obtained from a non-CF family member. Tight junction protein ZO-1 (red) and nuclei (DAPI) are also labeled; scale bar represents 10 µm. Bottom panel: expression and localization of ΔI1234_R1239-CFTR (CF-1, green). (D) Immunoblot of steady-state expression of ΔI1234_R1239-CFTR (from CF-2) following treatment with VX-809, transcriptional modulator (HDAC inhibitor) 4-phenylbutyrate (4-PBA), or 4-PBA + VX-809, compared to a non-CF family member. (E) Quantitation of total CFTR expression (top) and Band C expression (bottom) for each treatment relative to a non-CF family member (n=2). Immunofluorescence studies were performed by Kai Du.
As expected, forskolin treatment led to a robust change in $I_{eq}$ in monolayers of nasal cells from a non-CF individual which was sensitive to inhibition by CFTRinh-172, confirming that it is mediated by the CFTR channel (Figure 4.4B). Interestingly, one non-CF family member failed to exhibit a forskolin response but did exhibit a robust CFTRinh-172 response (22.45 µA/cm$^2$), arguing that CFTR had been basally active in this nasal epithelial culture. This observation prompted us to consider $I_{eq}$-CFTRinh-172 sensitive currents (after forskolin stimulation) as indicative of the family-specific range of maximal CFTR function in epithelial cultures (Figure 4.4C).

Next, we determined that both CF affected individuals: CF-1 and CF-2, exhibited abrogated CFTR channel function relative to those mediated by non-CF family members (Figures 4.4D, 4.56 and 4.57). Interestingly, there was a significant difference ($p=0.002$) in the residual CFTR function measured as $I_{eq}$-CFTRinh-172 between the two siblings, with the female (CF-2) showing less residual function compared to the male (Figures 4.4C and 4.4D).

In contrast to our observation for a nasal culture from a patient homozygous for the F508del mutation (Figure 4.57), a 48 hour treatment with Lumacaftor (VX-809), induced only a minor increase in VX-770 potentiated CFTR activity in the nasal cultures of CF-2 ($p=0.02$, n=3) and no significant rescue in the nasal cultures of CF-1 ($p=0.05$, n=4), even (Figures 4.4D). We compared the magnitude of the functional rescue in cultures from affected individuals to those responses in unaffected family members in order to benchmark the efficacy of pharmacological response. Unfortunately, the maximum achievable CFTR function after treatment with VX-809 and VX-770 (i.e. Orkambi$^\text{TM}$ co-therapy) for subjects CF-1 and CF-2, corresponded to only 10% and 3% of the median CFTR function measured in the unaffected family members, respectively (Figure 4.4E). This rescue effect is small relative to that observed in preclinical studies of the Orkambi$^\text{TM}$ treatment effect for $\Delta F508$-CFTR of 30-32% the median CFTR function in non-CF primary cultures (Van Goor et al., 2011).
Figure 4.S6. Forskolin and CFTRinh-172 responses in nasal epithelial cells expressing ΔI1234_R1239-CFTR from patients CF-1 and CF-2 in the absence of ΔF508-CFTR modulators, and CF-2 in the presence of 4-PBA, VX-809 and VX-770.

Representative tracings show Ussing chamber measurements of CFTR function in nasal cultures from affected patients CF-1 (left panel) and CF-2 (middle panel) in the absence of ΔF508-CFTR modulators, and CF-2 in the presence of small molecules (corrector VX-809, transcriptional modulator 4-PBA and potentiator VX-770; right panel). Studies performed by Wan Ip and Tanja Gonska.
Figure 4.57. VX-809 correction and VX-770 potentiation response in nasal epithelial cells from a patient homozygous for ΔF508-CFTR.

Representative tracings show Ussing chamber measurements of CFTR function (Fsk-stimulated and CFTRinh-172-inhibitable activity) in nasal cell cultures from a patient homozygous for ΔF508-CFTR in the absence of ΔF508-CFTR modulators (left panel) or presence of the small molecule corrector VX-809 and potentiator VX-770 (right panel). Studies performed by Wan Ip and Tanja Gonska.
In order to understand why the residual CFTR activity is low in the CF individuals with this mutation, and particularly low in CF-2, we analyzed CFTR protein expression by immunoblotting of primary nasal epithelial cultures. Immunoblots showed that in native tissues, mutant CFTR protein was misprocessed as expected (predominantly immature, Band B) and its total abundance was drastically reduced relative to the total abundance (Bands B and C) detected in cultures from a non-CF family member (heterozygous for the mutation) (Figure 4.5A). Similarly, in paired immunofluorescence studies of primary nasal cultures, we observed that ΔI1234_R1239-CFTR protein was absent from the apical membrane (Figure 4.5C). Interestingly, the total abundance of mutant CFTR protein detected in cultures from CF-2 by immunoblotting was significantly less than that detected for CF-1 (Figures 4.5A and 4.5B).

Hence, low mutant protein abundance partially accounts for the low level of residual mutant CFTR function detected for these CF patients (Figure 4.5b). On the other hand, abundance of an alternative, apical chloride channel (TMEM16A) is similar for all cultures, arguing that the previously declared differences in CFTR expression amongst nasal cultures are dictated by CFTR genotype rather than difference in culture quality (Figure 4.6A).

As detected by immunoblots, VX-809 or VX-661 increased the abundance of the immature (Band B) form of ΔI1234_R1239-CFTR in nasal cultures from CF-1 by approximately 2-fold, however rescue of the mature form (Band C) was marginal (Figure 4.5A). The cultures from CF-2 showed little total CFTR protein even after treatment with VX-809 or VX-661 (Figure 4.5A). This prompted us to test the idea that cultures from CF-2 exhibited differential transcriptional regulation and/or biosynthetic chaperoning relative to CF-1, and we therefore assessed CFTR protein abundance after treatment with the histone deacetylase (HDAC) inhibitor and heat shock protein (Hsp70) inducer: 4-phenylbutyrate (4-PBA) (Rubenstein et al., 1997; Choo-Kang et al., 2001). Interestingly, 4-PBA treatment of CF-2 cultures increased total immature, Band B protein to the level detected in CF-1 cultures (Figures 4.5A and 4.5B). The combination of 4-PBA and VX-809 increased total protein abundance to the level detected in the non-CF cultures, with the percentage of the mature (Band C) protein reaching approximately 15-20% that of the non-CF cultures (Figures 4.5A and 4.5B). Pretreatment with the combination of 4-PBA together with VX-809 also enabled the potentiated channel function.
Figure 4.6. Alternative chloride channels as potential therapeutic targets in epithelial cells. 

(A) Immunoblots detect the presence of an alternative chloride channel TMEM16A (ANO1) in CF-1, CF-2 and a non-CF family member in the absence (DMSO) and presence of small molecule correctors (VX-809 or VX-661). (B) Representative tracing shows Ussing chamber measurements of CaCC activity in nasal cell cultures from CF-2 in response to the P2Y2 receptor agonist ATP. Functional studies were performed by Wan Ip and Tanja Gonska.
Figure 4.S8. Quantitation of changes in CFTR abundance in nasal epithelial cells from a non-CF family member and CF-1 following treatment with VX-809 or VX-661.

(A) Quantitation of the increase in band C (mature form) of WT-CFTR by VX-809 or VX-661 in cultures from the non-CF individual. (B) Quantitation of the increase in band B (immature form) of ΔI1234_R1239-CFTR by VX-809 or VX-661 in cultures from CF-1. (C) Immunoblot (long exposure) of steady-state expression of mature and immature forms (Bands C and B, respectively) of ΔI1234_R1239-CFTR (CF-1) following treatment with VX-809 or VX-661. (D) Quantitation of maturation (Band C/B+C) by VX-809 or VX-661. *, p<0.05; ***, p<0.001; n≥3 biological replicates.
mediated by CF-2 cultures to approach the residual function exhibited by CF-1 (Figure 4.5.6). These studies suggest that there is differential transcriptional and/or translational regulation of Δl1234_R1239-CFTR in CF-2 relative to CF-1.

4.5.6 An alternative chloride channel is functionally expressed in nasal cultures generated from the affected family members

As previously mentioned, the protein thought to mediate calcium-activated chloride currents across the apical membrane of respiratory epithelial tissues (Anoctamin-1, ANO1; also called transmembrane protein 16A, TMEM16A) is expressed in the primary nasal cultures as determined by immunoblotting (Figure 4.6A) (Caputo et al., 2008; Schroeder et al., 2008; Huang et al., 2009). Given the poor response of ΔF508-CFTR modulators on Δl1234_R1239-CFTR, we were prompted to determine if a putative, alternative therapeutic target, i.e. the Ca\(^{2+}\)-activated chloride channel (CaCC), was functionally expressed in nasal cultures derived from the two affected individuals (Caputo et al., 2008; Kunzelmann et al., 2012). We found that nasal cell cultures from CF-2 exhibited robust responses to ATP, an agonist of the P2Y2 receptor (Figure 4.6B) (Rajagopal et al., 2011). These studies suggest that the nasal cultures generated from both CF patients were capable of exhibiting epithelial chloride secretion, providing rationale for developing activator compounds for TMEM16A as a potential therapy for these affected CF patients (Namkung et al., 2011).

4.6 Discussion

The results of this study suggest that the corrector and potentiator combination (VX-809 and VX-770, Orkambi™) developed to be a therapy targeting patients homozygous for the ΔF508 mutation, will not have universal efficacy for the entire Class 2 of CF-causing mutations (Boyle et al., 2014; Wainwright et al., 2015). Our in silico and in vitro studies of a rare Class 2 mutation (c.3700 A>G, Δl1234_R1239) revealed that it manifests distinct conformational defects relative to ΔF508-CFTR and these are relatively refractory to Orkambi™. Further, in primary nasal epithelial cultures, we found that the steady-state levels of Δl1234_R1239-CFTR protein were variable between affected individuals and very low relative to levels in non-CF family members.
Modulation with Orkambi™ was ineffective in enhancing ΔI1234_R1239-CFTR activity in primary tissues to the minimum functional level measured for healthy family members. Our studies suggest that this failure reflects both the inadequate repair of intrinsic defects in the mutant protein by Orkambi™ as well the deficiency in protein substrate for modulation by Lumacaftor and Kalydeco™. These findings should encourage caution in translating small molecule modulators of ΔF508-CFTR to clinical use in patients bearing other CFTR ‘misfolding’ mutations.

Our in vitro studies support a new concept wherein NBD2 has an important role for the biosynthetic assembly of CFTR. Whereas deletion of the entire NBD2 does not negatively impact CFTR folding and processing, our in silico and biochemical studies show that the perturbation of NBD2 structure caused by mutation can cause a deleterious effect (Cui et al., 2007). The ΔI1234_R1239 mutation in NBD2, caused misassembly of the full-length protein, possibly by distorting the NBD1:NBD2 interface and allosterically, reducing the conformational stability of the amino-terminal half of CFTR. In our future work, we will test the role for NBD2 in post-translational assembly of the full-length protein in studies of other Class 2 mutations in NBD2. We will also test the capacity of the in silico methods described in this study, effective in predicting the consequences of the ΔI1234_R1239 mutation on NBD2 structure in the context of the full-length CFTR protein, to predict the molecular consequences of other disease-causing mutations.

The corrector compounds, VX-809 and C4, were both partially effective in promoting the appearance of the mature, properly processed form of ΔI1234_R1239-CFTR in HEK-293 cells. However, as revealed by limited proteolysis studies of corrector-treated ΔI1234_R1239-CFTR protein, neither compound was fully effective in rescuing the conformational defect in the amino-terminal half. Specifically, while the steady-state abundance of fragments containing NBD1 increased relative to the full-length protein, their fragmentation pattern did not recapitulate that observed for WT-CFTR (Eckford et al., 2014; Du et al., 2005; Cui et al., 2007; Yu et al., 2011). Further, channel activity assays show that VX-809-corrected ΔI1234_R1239-CFTR exhibited modest activation by forskolin and VX-770, approximately half the response
observed for VX-809-corrected ΔF508-CFTR. These findings are consistent with our interpretation of the proteolysis studies and suggest that the conformational defects in ΔI1234_R1239-CFTR are relatively resistant to repair using these small molecules.

To date, it is unclear what level of *in vitro* CFTR channel activity correlates with, or predicts a meaningful clinical response (Cutting, 2015). In this study, we employed a novel approach to benchmark responses to compounds in patient-derived nasal epithelial cultures. Specifically, we compared the CFTR channel activity in nasal epithelial cultures from CF family members to CFTR channel activity in nasal cultures derived from non-CF family members. Although, it has been widely reported that there is considerable variability in *in vitro* CFTR-mediated responses amongst epithelial cultures generated from CF patients, even those with the same mutations (Van Goor *et al.*, 2011; Awatade *et al.*, 2015; Dekkers *et al.*, 2015), we were surprised to observe the extent of this variability amongst the four non-CF family members. This variability was observed despite selecting cultures with uniformly high, transepithelial resistances of 1000-1500 ohms/cm$^2$. Hence, we are currently investigating the hypothesis that this variation amongst non-CF nasal cultures is due in part to differential CFTR gene expression. Finally, we propose that the lowest CFTR-mediated currents measured in nasal cultures from unaffected family members correlates with the lowest CFTR expression required for respiratory health within that family. Unfortunately, the *in vitro* CFTR responses in tissues from both CF individuals remained much lower than that observed for non-CF cultures from family members (≈10%). We anticipate that this study will stimulate similar intra-family comparisons and encourage discussion about meaningful and predictive *in vitro* metrics of pharmacological rescue in respiratory epithelial tissues.

From *in vitro* studies of primary nasal cultures, one of the two subjects (CF-2) exhibited a lower level of total CFTR protein and residual CFTR channel function than the other (CF-1). Interestingly, the differential *in vitro* responses of these two subjects mirrored their relative respiratory health (Molinski *et al.*, 2014). We confirmed that the poorer residual function exhibited in the cultures generated from patient CF-2 did not result from compound CFTR gene mutations. Both individuals (CF-1 and CF-2) were tested by multiplex-PCR analysis for 39
recurrent CFTR mutations, by gene dosage using MLPA and by direct sequence analysis of the coding and flanking region of the CFTR gene [NCBI ref. NC_000007.13; NM_000492.3]. These tests confirmed that both individuals have identical CFTR mutation and polymorphism status (Table 4.1). On the other hand, expression of non-CFTR genes, including modifier genes (Sun et al., 2012; Li et al., 2014) may account for the differential functional expression of Δl1234_R1239-CFTR in the two individuals and this will be tested in our future work.

The lack of robust responses by tissues derived from both affected individuals to the Vertex combination: Orkambi™, highlights the need for alternatives to protein repair modulators; these may involve genetic editing strategies and/or pre-mRNA splicing modulation. Future studies aim to repair the aberrant splice site introduced by c.3700 A>G. Hypothetically, this could be achieved by steric blockade of aberrant splicing via masking of the c.3700 A>G mutation in the pre-mRNA transcript (e.g. using antisense oligonucleotides) in order to promote normal splicing at the original donor splice site. The feasibility of this approach as a therapeutic intervention is unclear at present but may become viable as delivery systems for genetic material evolve.

This in-depth study of a relatively rare Class 2, CF-causing mutation highlights several important issues important for therapy development. First, we present a possible in vitro model with which to predict efficacy of emerging therapeutic compounds on patient-specific tissues. We suggest that electrophysiological studies of nasal epithelial cultures from non-CF family members, showing a broad range of CFTR function, may provide a meaningful benchmark for functional rescue of CF affected family members. As more clinical data is obtained from treated subjects who have undergone nasal brushing, this prediction will no doubt be refined. Second, not all mutations classified as Class 2, like ΔF508-CFTR, will exhibit similar conformational defects and hence, will not be effectively rescued using compounds developed to target ΔF508-CFTR. Third, subjects (in this case two siblings) with the same CFTR mutation may have different residual CFTR function and responses to small molecules when tested in vitro. These last two points implicitly suggest that as we attempt to restore functional CFTR to all those afflicted with CF, a personalized approach to both drug discovery
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<th>CF-1</th>
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<td>Genetic Analysis</td>
<td>Genetic Variant</td>
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<tr>
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<td>Genomic DNA analysis of poly-T-tract</td>
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**Table 4.1. Identical CFTR mutations and polymorphisms detected in both CF Subjects.**

Both CF affected individuals tested by multiple-PCR analysis for 39 recurrent CFTR mutations, by gene dosage using MLPA and by direct sequence analysis of the coding and flanking region of the CFTR gene [NCBI reference sequence NC_000007.13; NM_000492.3].

Recurrent mutations tested: G542X; G85E; R334W; Y122X; R560T; 3905insT; 3876delA; 2789+5G>A; G551D; R117H; A455E; S549R; R347H; 1078delT; 1717-1G>A; 3120+1G>A; S1255X; A559T; Y1092X; M1101K; 2307insA; 2183AA>G; 1898+5G>T. These data were generated by Peter Ray.
and testing will be needed for CFTR modulators. Unfortunately, a ‘one size fits all’ approach does not apply to CFTR modulators and we will test the power of patient-specific \textit{in vitro} assays to predict patient-specific clinical outcomes. We are encouraged to suggest on the basis of this small cohort, that residual CFTR function observed \textit{in vitro} does correlate with relative lung health, and further that respiratory function for patient CF-2 may improve with a therapeutic cocktail including Orkambi\textsuperscript{TM} together with a compound that acts like 4-PBA to enhance total CFTR expression.
Chapter 5

Functional Screening of drug responses in rare CFTR mutations informs strategies for personalized Cystic Fibrosis medicine

5.1 Acknowledgements

The results presented in this chapter have been published:


A letter from the publisher outlining permission to use copyright material is provided in the Appendix, section A1.1.

5.2 Abstract

There are nearly 2000 mutations in the CFTR gene associated with Cystic Fibrosis disease and to date, the one approved drug Kalydeo™ has been effective in rescuing the functional expression of a small subset of these mutant proteins with defects in channel activation. However, there is currently an urgent need to assess other mutations for possible “rescue” by Kalydeo™, and further, definition of the binding site of such modulators on CFTR would enhance our understanding of the mechanism of action of such therapeutics. Here, we describe a simple and rapid one-step PCR-based site-directed mutagenesis method to generate mutations in the CFTR gene. This method was used to generate CFTR mutants bearing deletions (p.Gln2_Trp846del, p.Ser700_As p835del, p.Ile1234_Arg1239del) and truncation with polyhistidine-tag insertion (p.Glu1172-3Gly-6-His*) which either recapitulate a disease phenotype or render tools for modulator binding site identification, with subsequent evaluation of drug responses using a high-throughput (384-well) membrane potential-sensitive fluorescence assay of CFTR channel activity within a one week time frame. This proof-of-concept study shows that these methods enable rapid and quantitative comparison of
multiple CFTR mutants to emerging drugs, facilitating future large-scale efforts to stratify mutants according to their “theratope” or most promising targeted therapy.

5.3 Introduction

Although approximately 2000 CFTR mutations have been identified to date (CFTR Mutation Database: www.genet.sickkids.on.ca), the molecular characterization of only a few (<10%) have been investigated (Sosnay et al., 2013). Therefore, there is a need to generate these uncharacterized CFTR mutations for further in vitro study, in order to test the response of these mutants to emerging therapeutics, as well as discover targeted therapeutics to enhance the functional expression of CF disease-causing mutations. This could subsequently lead to the development of therapies earmarked for each genotype, rather than a one-size-fits-all approach which is the current paradigm in the CF field (e.g. using pharmacological potentiators to treat multiple mutations affecting channel gating or corrector compounds to target all mutations leading to CFTR misprocessing).

Site-directed mutagenesis has been a staple of molecular biology and genetic research, allowing for the generation of nucleotide substitutions to recapitulate disease-causing mutations in an artificial expression system to further understand the molecular consequences leading to pathophysiology (De Fusco et al., 2003; D’Antonio et al., 2013). Polymerase chain reaction (PCR)-based site-directed mutagenesis has shed light on countless genetic diseases, by allowing for the qualitative and quantitative analysis of the structure-function relationship of a vast number of disease-causing mutant proteins (Kato et al., 2003). Most often, this is done in double stranded plasmid DNA which includes the complementary DNA (cDNA) of the exonic open reading frame sequence of the gene of interest. Subsequently, there are several approaches to obtain site-directed cDNA mutations using PCR, and traditional methods include overlap extension PCR, inverted PCR, megaprimer PCR and recombination PCR. However, one of the first commercial mutagenesis kits (QuikChange™ by Stratagene) has become the method of choice over the past two decades to generate a countless number of engineered mutations, for further characterization using the tools of choice within any given laboratory. Other mutagenesis kits are commercially available, but most are variations of QuikChange™ (Liu et al., 2008). Although QuikChange™ may be the most common site-directed mutagenesis
strategy, it is not capable of performing deletion and insertion mutagenesis because it is designed and optimized for single or multiple site (primer-based) modifications (Liu et al., 2008). Additionally, insertion/deletion mutagenesis is incompatible with the one-step QuickChange™ approach as well as other one-step PCR-based site-directed mutagenesis kits, due to the relatively high error rate of their DNA polymerases (usually Taq DNA polymerase) preventing high fidelity replication of the template; DNA mismatch repair proteins are not included in PCR kits since they cannot survive thermocycling (Tindall et al., 1988). Therefore, to reduce second-site errors, molecular biologists typically perform site-directed mutagenesis in smaller constructs containing a fragment of their gene (cDNA) of interest, with the aim of subcloning the mutated fragment into an unadulterated full-length template plasmid.

Using site-directed mutagenesis methods we have generated over 100 CFTR missense mutant constructs to date, several of which have been recently reported (Molinski et al., 2014; Pasyk et al., 2015), while the vast majority of these are critical tools within many ongoing projects. However, there are a large number of CF-associated mutations predicted to cause deletions, insertions or truncations (i.e. stop mutations) and generation of these mutations is relatively time-consuming (Table 5.1) (Sosnay et al., 2013; also refer to the CFTR Mutation Database; Clinical and Functional Translation of CFTR, CFTR2: www.cftr2.org; Universal Mutation Database – CFTR, UMD-CFTR: www.umdb.org/CFTR). In recent studies, we described a CF-associated mutation common in the Middle East (c.3700 A>G) that leads to deletion of six amino acids, p.Ile1234_Arg1239del-CFTR, through an alternative splicing mechanism (Molinski et al., 2014). In addition, there is a large subset of at least forty disease-causing mutations which fully or partially truncate NBD2 (residues 1173-1480); including: p.Met1157_Arg1239del, p.Lys1177*, p.Tyr1182*, p.Gln1186*, p.Ser1196*, p.Val1198*, p.Trp1204*, p.Ser1206*, p.Gln1238*, p.Leu1254*, p.Ser1255*, p.Trp1274*, p.Ile1277*, p.Gln1281*, p.Trp1282*, p.Gln1291*, p.Gly1298*, p.Tyr1307*, p.Glu1308*, p.Trp1310*, p.Gln1313*, p.Trp1316*, p.Val1322_Leu1414del, p.Val1322_Leu1480del, p.Leu1353*, p.Lys1363*, p.Glu1371*, p.Tyr1381*, p.Gln1382*, p.Ile1384*, p.Gln1390*, p.Cys1400*, p.Glu1401*, p.Gln1411*, p.Gln1412*, p.Glu1418*, p.Tyr1424*, p.Ser1455*, p.Glu1473* and p.Gln1476* (Cui et al., 2007; CFTR Mutation Database; CFTR2; UMD-CFTR). Future studies of the molecular defects caused by these mutations and assessment of their response to emerging therapies required the
<table>
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<tr>
<th>CFTR Mutation Database</th>
<th># of Alleles</th>
<th>Allele Frequency (%)</th>
<th># of Mutations</th>
<th>Mutation Frequency (%)</th>
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<tr>
<td><strong>UMD-CFTR</strong> <em>(European)</em></td>
<td>Total counts</td>
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<td>100</td>
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<td>Small insertions (&lt;1 exon)</td>
<td>20</td>
<td>1.5</td>
<td>15</td>
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</tbody>
</table>

Table 5.1: Frequency of CFTR mutations predicted to cause truncations (stop mutations), deletions or insertions.

Complete counts and frequencies from the CFTR Mutation Database (http://www.genet.sickkids.on.ca; comprising a worldwide dataset) are not available. However, from the 2001 mutations described in this database, 167 (8.4%) are truncations (stop mutations), 52 (2.6%) are large deletions or insertions, and 39 (2.0%) are in-frame deletions or insertions. CFTR2: www.cftr2.org; UMD-CFTR: www.umd.be/CFTR. The remainder of mutation frequencies (approximately 50%) not reported in this table represents missense mutations.
development of rapid and efficient methods for mutagenesis and functional assessment – ideally using a multi-well high-throughput format.

To assess functional responses of CFTR variants in a high-throughput fashion, the recently described rapid and simple mix-and-read Fluorometric Imaging Plate Reader (FLIPR) membrane potential assay will be advantageous (Maitra et al., 2013). This method allows for kinetic resolution comparable to electrophysiological measurements, allowing for detection of rapid changes in membrane potential, however, FLIPR is a faster, less labour intensive and higher throughput approach (Bridal et al., 2010; Ettorre et al., 2014). Additionally, the FLIPR system eliminates wash steps, which translates to healthier (and a higher number of) cells due to less manipulation, as well as shorter read times due to the simple mix-and-read protocol. Several other membrane potential-sensitive dyes exist (e.g. DiBAC), but they have slower response times (10X slower for DiBAC) and can be sensitive to temperature variations; therefore FLIPR is more robust, allowing for high quality screening results that yield high signal-to-noise ratios (Wolff et al., 2003). Importantly, this method has several advantages over traditional assays for CFTR function, such that FLIPR can measure both activation and inhibition, whereas CFTR assays using fluorescence-based halide dequenching (i.e. SPQ or MQAE dyes, halide-sensitive YFP) and iodide-selective probes are unidirectional and can only detect anion channel activation (Trzcinska-Daneluti et al., 2009; Eckford et al., 2014). A potential disadvantage of this method is that non-CFTR ion channels may also affect changes in membrane potential. However, specificity of the FLIPR response for CFTR can be assessed by confirming several signature features of the CFTR channel, including: activation by agonists of cyclic AMP (cAMP), sensitivity to electrochemical anion gradients and inhibition by CFTRinh-172.

In the present study, we developed an improved PCR-based site-directed insertion and deletion mutagenesis method to generate disease-causing mutations in CFTR cDNA. This technique is rapid, inexpensive and simple. Importantly, it can be used for replicating large vectors (>10 kb) without the need for subcloning (a time-consuming yet previously necessary approach). Here, we incorporated our desired insertion into the primers (or omitted it for the deletion construct) and employed the KAPA HiFi HotStart PCR Kit, containing a high fidelity (engineered B-family, proofreading) DNA polymerase, to generate four relatively complex CFTR
mutants in a one-step PCR process. The deletion constructs removed 18 nucleotides (in-frame deletion of 6 amino acids in NBD2) rendering p.Ile1234_Arg1239del-CFTR, 405 nucleotides (in-frame deletion of residues 700-835 comprising the R domain) rendering p.Ser700_As835del-CFTR, and 2532 nucleotides (in-frame deletion of residues 2-846 comprising the MSD1-NBD1-R domain sequence) rendering p.Gln2_Trp846del-CFTR (i.e. MSD2-NBD2 construct). The insertion construct added 27 nucleotides (2 Gly, 6 His residues and one stop codon), yielding p.Glu1172-3Gly-6His*-CFTR, a deletion mutant lacking 308 C-terminal residues (amino acids 1173-1480) and containing an engineered C-terminal poly-His-tag. When transiently expressed in human embryonic kidney (HEK)-293 GripTite™ cells (abbreviated to HEK in this study) and paired with a rapid, multi-well (384-well) plate reader assay of channel function (FLIPR, a membrane potential-sensitive dye-based system), these methods permitted comparison of the functional consequences of emerging CFTR therapies on these rare deletion mutants of CFTR.

5.4 Materials and Methods

5.4.1 Generation of Mutant CFTR Constructs

p.Ile1234_Arg1239del-CFTR (site-directed deletion mutagenesis), p.Ser700_As835del-CFTR (deletion), p.Gln2_Trp846del-CFTR (deletion), p.Glu1172-3Gly-6His*-CFTR (insertion) and p.Glu1172*-CFTR (point mutation) cDNA were generated using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Woburn, MA) according to the manufacturer’s Standard PCR Protocol with high quality (>300 ng/µL, 260/280 nm ratio of 1.8) plasmid DNA containing WT-CFTR cDNA (in pcDNA3.1) as the template. For p.Ile1234_Arg1239del-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used: 5'- CAT ATT AGA GAA CAT TTC CTT CTC A (del) GT GGG CCT CTT GGG AAG AAC TGG ATC -3' (sense); 5'- GAT CCA GTT CTT CCC AAG AGG CCC AC (del) T GAG AAG GAA ATG TTC TCT AAT ATG -3' (antisense); 18 nucleotides deleted from the template sequence are highlighted as "del" in bold italics. For p.Ser700_As835del-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used: 5'- CAT ATT AGA GAA CAT TTC CTT CTC A (del) GT GGG CCT CTT GGG AAG AAC TGG ATC -3' (sense); 5'- GAT CCA GTT CTT CCC AAG AGG CCC AC (del) T GAG AAG GAA ATG TTC TCT AAT ATG -3' (antisense); 18 nucleotides deleted from the template sequence are highlighted as "del" in bold italics. For p.Gln2_Trp846del-CFTR the following PCR primers (synthesized by ACGT Corp.,
Toronto, ON) were used: 5’- CTA GCG GAT CGG AGC TCG ATC GAG ATG (del) AAC ACA TAC CTT CGA TAT ATT ACT GTC -3’ (sense); 5’- GAC AGT AAT ATA TCG AAG GTA TGT GTT (del) CAT CTC GAT CGA GCT ATC CGC TAG -3’ (antisense); 2532 nucleotides deleted from the template sequence are highlighted as "del" in bold italics. For p.Glu1172-3Gly-6His*-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used:

5’- GAC ATG CCA ACA GAA GGT GGT GGT CAT CAT CAT CAT CAT CAT CAT TAG AAA CCT ACC AAG TCA ACC -3’ (sense); 5’- GGT TGA CTT GGT AGG TTT CTA ATG ATG ATG ATG ATG ATG ACC ACC ACC TTC TGT TGG CAT GTC -3’ (antisense); nucleotides in bold represent the insertion which codes for 2 Gly residues, 6 His residues and one stop codon (i.e. 2Gly-6His*); the third Gly residue in the final construct was from the native amino acid at position 1173. For p.Glu1172*-CFTR the following PCR primers (synthesized by ACGT Corp., Toronto, ON) were used: 5’- GAC ATG CCA ACA TAA GGT AAA CCT ACC -3’ (sense); 5’- GGT AGG TTT ACC TT A TGT TGG CAT GTC -3’ (antisense); the nucleotide in bold represents the codon change from GAA (Glu) to TAA (stop, * or X). For PCR amplification, 1 or 10 ng of template plasmid and 0.3 µM (final concentration) of each sense and antisense primers were added to 2X KAPA HiFi HotStart ReadyMix (containing KAPA HiFi DNA polymerase, deoxynucleotide triphosphates, magnesium chloride and a proprietary buffer; 25 µL final volume). The reaction mixture was incubated in a TPersonal Thermocycler (Biometra, Goettingen, Germany) as per the KAPA HiFi HotStart Standard PCR Protocol. Briefly, PCR amplifications (taking approximately 3 hrs) for each mutation were as follows: (1) initial denaturation for 3.5 min at 95 °C; (2) further denaturation for 20 s at 98 °C; (3) primer annealing for 15 s at actual T<sub>m</sub>-5 or T<sub>m</sub>-10 °C; (4) DNA polymerase extension for 5 min (30 s/kb) at 72 °C; (5) cycle back to step 2, 25 times; (6) final round of DNA polymerase extension for 3 min at 72 °C, followed by incubation at 4 °C to stop the reaction. Following PCR, 1 µL FastDigest DpnI (10U; Thermo) was added to each tube for 60 min at 37 °C to remove methylated (and hemi-methylated) parental DNA; 10 µL of the PCR product post DpnI-digestion was run on 0.8% agarose gels (cast with GelRed; Biotium) to determine the size(s) of the PCR product(s). Next, 50 µL of DH5α competent cells (Life Technologies) were transformed with 10 µL of the PCR product (without heat inactivation following DpnI digestion) according to the manufacturer’s protocol, and subsequently grown on LB-Ampicillin (100 µg/mL) selective agar plates at 37 °C overnight. Colonies were picked, and after 17 h of growth in liquid culture (supplemented with 100 µg/mL Ampicillin) at 37 °C with shaking (250 rpm),
plasmid DNA was prepared using the GenElute™ Plasmid Miniprep Kit (Sigma). The presence of site-directed mutations, as well as the integrity of full-length CFTR cDNA (nucleotides 1-4443 with stop codon), was confirmed by DNA sequencing (TCAG Inc., Toronto, ON).

5.4.2 Studies of CFTR Protein Expression

Human embryonic kidney (HEK)-293 GripTite™ cells (abbreviated to HEK in this study, and kindly provided as a gift from Dr. Daniela Rotin, Hospital for Sick Children, Toronto, ON) were grown at 37 °C in 24-well (clear, flat bottom; Sarstedt) plates to 50% confluence and transiently transfected with p.Ile1234_Arg1239del-CFTR, p.Ser700_As835del-CFTR, p.Gln2_Trp846del-CFTR, p.Glu1172-3Gly-6His*-CFTR, p.Glu1172*-CFTR or WT-CFTR (positive control) cDNA constructs (pcDNA3.1) using PolyFect Transfection Reagent (Qiagen), according to the manufacturer’s protocol. HEK cells transiently expressing CFTR proteins were maintained in DMEM (Wisent) supplemented with non-essential amino acids (Life Technologies) and 10% FBS (Wisent) at 37 °C with 5% CO₂ (HEPA incubator, Thermo Electron Corporation) and processed as previously described. Briefly, following incubation at 37 °C for 24 h, HEK cells transiently expressing CFTR proteins were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% or 4-12% Tris-Glycine gels (Life Technologies) as appropriate. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated in 5% (w/v) milk, and CFTR bands were detected with human CFTR-NBD1-specific (amino acids 484-589) murine mAb 660 (1:10000, University of North Carolina Chapel Hill, NC; Cui et al., 2007) for NBD1-containing constructs (i.e. p.Ile1234_Arg1239del-CFTR, p.Glu1172-3Gly-6His*-CFTR, p.Glu1172*-CFTR or WT-CFTR) or human CFTR-NBD2-specific (amino acids 1204-1211) murine mAb 596 (1:10000, University of North Carolina Chapel Hill, NC; Cui et al., 2007) for NBD2-containing constructs (i.e. p.Ser700_As835del-CFTR, p.Gln2_Trp846del-CFTR or WT-CFTR) at 4 °C overnight, horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10000, Pierce) at room temperature for 1 hr, Amersham ECL (GE Healthcare), and exposure to film (Denville Scientific) for 0.5 to 5 min as required. Importantly, when blots containing p.Glu1172-3Gly-6His*-CFTR or p.Glu1172*-CFTR were probed with the CFTR-NBD2-specific (amino acids 1204-1211) murine mAb 596 (1:10000, University of North
Carolina Chapel Hill, NC; Cui et al., 2007), or p.Gln2_Trp846del-CFTR probed with the CFTR-N-terminus-specific (amino acids 25-36) murine mAb MM13-4 (1:1000, EMD Millipore; Cui et al., 2007) no signals were detected (the respective mAb epitopes are not expressed in these deletion mutants; data not shown). Calnexin was used as a protein loading control, and detected with a Calnexin-specific rabbit pAb (1:10000, Sigma-Aldrich) at 4 °C overnight, horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1: 10000, Pierce) at room temperature for 1 hr, Amersham ECL, and exposure to film for 0.5 to 5 min as required. Relative expression levels of CFTR proteins were quantitated by densitometry of immunoblots using ImageJ software version 1.46 (National Institutes of Health).

5.4.3 Studies of p.Gln2_Trp846del-CFTR Glycosylation Status
To evaluate protein glycosylation, HEK cells expressing p.Gln2_Trp846del-CFTR were grown at 37 °C for 24 h, and then lysed in modified radioimmunoprecipitation assay buffer as described above and previously (Molinski et al., 2014). Lysates were then treated with either Endoglycosidase H (EndoH) or Peptide-N-Glycosidase F (PNGaseF) (NEB, Ipswich, MA) endoglycosidases according to the manufacturer’s protocol. Samples were analyzed by SDS-PAGE using 4-12% Tris-glycine gradient gels (Life Technologies), and immunoblots were performed using the human CFTR-NBD2-specific murine mAb 596 as described above.

5.4.4 Studies of CFTR Function using a Membrane Potential Assay
HEK cells transiently overexpressing p.Ile1234_Arg1239del-CFTR were grown at 37 °C to 90-100% confluence in 384-well (black, flat bottom; Greiner) plates. Following 24 h incubation in the presence of the pharmacological corrector VX-809 (3 µM; Selleck Chemicals, Houston, TX), the cells were washed with PBS, and blue membrane potential dye (dissolved in chloride free buffer containing 136 mM sodium gluconate, 3 mM potassium gluconate, 10 mM glucose, 20 mM HEPES, pH 7.35, 300 mOsm, supplemented with 3 µM VX-809, at a concentration of 0.5 mg/ml; Molecular devices), which can detect changes in transmembrane potential, was added to the cells for 1 hr at 37 °C. HEK cells transiently overexpressing p.Glu1172-3Gly-6His*-CFTR, p.Ser700_Asp835del-CFTR or WT-CFTR were grown and prepared in the same manner, but did not require pharmacological correction using VX-809. The plate was then read in a
fluorescence plate reader (Molecular devices – SpectraMax i3) at 37 °C, and after reading the baseline fluorescence (excitation: 530 nm, emission: 560 nm) for 10 min, CFTR was stimulated using the cAMP agonist forskolin (10 μM; Sigma) and the small molecule potentiator VX-770 (1 μM; Selleck Chemicals); dimethylsulfoxide (DMSO) vehicle was used as a negative control. CFTR-mediated depolarization of the plasma membrane was detected as an increase in fluorescence, and hyperpolarization (or repolarization) as a decrease. To terminate the functional assay, the CFTR inhibitor CFTRinh-172 (10 μM; Cystic Fibrosis Foundation Therapeutics) was added to each well of the 384-well plate. Changes in transmembrane potential were normalized to the measurement taken at the time of agonist (i.e. DMSO, or forskolin and VX-770) addition and prior to activation.

5.4.5 Data Analysis

All data are represented as mean ± S.E.M. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis (non-paired Student's t tests), and P values less than 0.05 were considered significant. Each experiment was repeated at least three times.

5.5 Results and Discussion

The major advantage of mutagenesis methods described here is that the KAPA HiFi DNA polymerase (engineered B-family, proofreading) has a >100X lower second-site error rate compared with conventional DNA polymerases (e.g. Taq), allowing for direct PCR on the destination vector, rather than PCR with subcloning from an intermediate vector (Quail et al., 2012). Additionally, this approach does not require complex primer design (a complementary pair is used) or phosphorylation of primers/oligos (typical for mutagenic primers required for traditional primer-‘tail’-based insertion/deletion), nor does it require subcloning or enzymatic ligation steps. Furthermore, this PCR-based mutagenesis method is minimalist on many levels, from its reaction volume (25 μL) and required DNA template (1 ng, up to 10 ng), to the time required for PCR cycling (approximately 3 hrs for a 10 kb vector), which can lead to positive clones identified (from primer design to plasmid sequencing) in as little as 4 to 5 days. Subsequently, these newly generated rare CFTR mutants can be functionally expressed and characterized in a high-throughput fashion within an additionally 2 to 3 days, allowing for
potential identification of disease-causing genotypes which could benefit from current CFTR-specific small molecule therapies (i.e. VX-809 and VX-770) within approximately a single week.

5.5.1 Generation of Site-directed Insertion and Deletion Variants of CFTR

The strategies for site-directed insertion and deletion mutagenesis are illustrated in Figure 5.1, and the characteristics of mutagenesis primers are noted in Figure 5.2; primers for p.Glu1172* (point mutation) are also included. For insertions (27 base pairs in this study, i.e. p.Glu1172-3Gly-6His*), the insert segment is included in the primer pair and directly incorporated into the template plasmid DNA by means of PCR cycling, while for deletions (18, 405 and 2532 base pairs in this study, i.e. p.Ile1234_Arg1239del, p.Ser700_Asp835del-CFTR and p.Gln2_Trp846del-CFTR, respectively) the segment to be removed is excluded from the primer pair. This one-step site-directed insertion and deletion mutagenesis approach takes advantage of the secondary structures of the primers and template for insertion and deletions, respectively, such that a ‘loop’ or ‘hairpin’ structure of the non-complementary sequence forms during PCR to generate the desired change (Figure 5.1). Following PCR cycling, the insert becomes integrated or the sequence to be deleted is removed. Although each amplified mutant construct becomes the major fraction present, the remaining starting template (i.e. methylated parental template DNA) is enough to contaminate downstream steps, and therefore must be removed in order to enrich the synthesized mutant construct. To do this, PCR products are incubated with DpnI endonuclease; the mutant plasmid is then used to transform competent E. coli cells, which allows for amplification and selection of positive clones. Furthermore, it must be noted that for the deletion mutagenesis, the actual T_m of the full-length primer can be used to determine the annealing temperature during PCR amplification; however the annealing temperature for insertion mutagenesis must not take into account the sequence to be added (otherwise the apparent annealing T_m will be too high). In this case, only the actual (or calculated) T_m of the complementary sequence of the primer pair should be used to determine annealing temperature.

Following PCR and DpnI digestion, 10 µL of each reaction was run on 0.8% agarose gels, and PCR products from site-directed deletion (p.Ile1234_Arg1239del) and insertion (p.Glu1172-3Gly-6His*) mutagenesis are shown in Figure 5.3 and Figure 5.4, respectively. In Figure 5.3,
A  **Insertion**  

- Primer
- PCR
- Minor fraction (<0.01%)
- Major fraction (>99.9%)

B  **Deletion**  

- Primer
- PCR
- Minor fraction (<0.01%)
- Major fraction (>99.9%)
Figure 5.1. Schematic illustration of the PCR-based method for site-directed insertion and deletion mutagenesis.

**A** Overview of insertion mutagenesis strategy. The segment to be inserted (red) is included in the sense and antisense (not shown) primer pair, and upon annealing of the complementary segments of the primer (black) to the template plasmid (black circle, labelled ‘T’), the non-complementary insert nucleotides form a hairpin or ‘loop’ structure. Following subsequent (25) PCR cycles, the insert becomes integrated and amplified, and forms the major fraction following PCR. To remove the non-mutant template plasmid DNA, *DpnI* restriction enzyme is added, leaving the mutant plasmid (black circle, labelled ‘M’) as the only remaining form.

**B** Overview of deletion mutagenesis strategy. The segment to be deleted (red) from the template plasmid (black circle, labelled ‘T’) is incubated with a sense and antisense (not shown) primer pair which omits the sequence to be deleted, and instead bridges the nucleotides immediately preceding and following the template segment to be deleted. Upon primer annealing, the segment to be deleted forms a hairpin or ‘loop’ structure. Following subsequent (25) PCR cycles, the deletion segment becomes omitted and the mutant construct amplified, forming the major fraction following PCR. To remove the non-mutant template plasmid DNA containing the deletion segment, *DpnI* restriction enzyme is added, leaving the deletion mutant plasmid (black circle, labelled ‘M’) as the only remaining form.
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<tr>
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<th>Strategy Illustration</th>
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<th>Complementary Sequence</th>
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<td></td>
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<td></td>
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<tr>
<td>p.Glu1172-3Gly-6His* (insertion)</td>
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<tr>
<td></td>
<td>Primer +27 18→18</td>
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<td>p.Ser700_As835del (R domain deletion)</td>
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<td>Template 26→26</td>
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</table>
Figure 5.2. Characteristics of primers used in this study for PCR-based site-directed insertion and deletion mutagenesis.

Sense and antisense primer pairs for p.Ile1234_Arg1239del (deletion), p.Glu1172-3Gly-6His* (insertion), p.Ser700_As p835del (deletion) and p.Gln2_Trp846del (deletion) are described in the Materials and Methods. Primer and template structures illustrating mutagenesis strategies are also shown (the number of complementary, deleted [-] and inserted [+] nucleotides are noted). For the full-length and complementary sequences of each primer pair, the number of base pairs (bp), percent guanine-cytosine content (GC), number of calculated primer hairpins (hp; calculated using OligoAnalyzer, Integrated DNA Technologies, Inc., Coralville, IA), and the calculated and actual melting temperatures (T_m in °C; ACGT Corp., Toronto, ON) are tabulated.
Figure 5.3. PCR amplification and analysis of deletion mutagenesis generating p.Ile1234_Arg1239del-CFTR cDNA.

A) Agarose gel electrophoresis of PCR products following amplification of plasmid DNA using deletion mutagenesis primers. The actual Tₘ (71.4 °C) of the sense/antisense primer pair was used to calculate annealing temperatures (i.e. Tₘ-5 °C and Tₘ-10 °C). Template (wild-type CFTR cDNA) plasmid DNA (500 ng) was included on the same gel as a positive control, and appears as both open circular (white arrowhead) and supercoiled (black arrowhead) forms. PCR products are observed (open circular form) only in the Tₘ-10 °C conditions. A summary of total colonies, those picked, plasmid DNA isolated, and constructs where the deletion was found is provided underneath the gel. 

B) Electrophoretogram of the cDNA sequence in the region of the deletion (image generated using FinchTV, Geospiza). The Black arrowhead and dotted vertical line denote the precise location of the 18 nucleotide deletion.

C) Sequence alignment (NCBI Nucleotide BLAST) of the template (wild-type CFTR cDNA) and the mutant plasmid in the region of the deletion. Nucleotides c.3700_3717 are absent from the mutated plasmid, which translates to deletion of six amino acids in the protein sequence: p.Ile1234_Arg1239 (shown in red).

D) Homology model of wild-type CFTR protein highlighting the location of the six amino acids (p.Ile1234_Arg1239) deleted in this mutant construct (image generated using PyMOL, Schrödinger; Mornon et al., 2009). MSD1, MSD2, NBD1, NBD2 and the R domain are shown in blue, yellow, cyan, orange and magenta, respectively. The approximate location of CFTR relative to the phospholipid bilayer is also shown.
Figure 5.4. PCR amplification and analysis of insertion mutagenesis generating p.Glu1172-3Gly-6His*-CFTR cDNA.

A) Agarose gel electrophoresis of PCR products following amplification of plasmid DNA using insertion mutagenesis primers. The actual \( T_m \) (68.4 °C) of the complementary sequence of sense/antisense primer pair was used to calculate annealing temperatures (i.e. \( T_m-5 \) °C and \( T_m-10 \) °C). PCR products are observed (open circular form) only in the \( T_m-5 \) °C (complementary sequence) conditions. A summary of total colonies, those picked, plasmid DNA isolated, and constructs where the deletion was found is provided underneath the gel.  

B) Electrophoretogram of the cDNA sequence in the region of the insertion (image generated using FinchTV, Geospiza). The Black arrowheads and dotted vertical lines denote the precise location of the 27 nucleotide insertion (also on a blue background).  

C) Sequence alignment (NCBI Nucleotide BLAST) of the template (wild-type CFTR cDNA) and the mutant plasmid in the region of the insertion. Nucleotides c.3520_3546 are inserted into the plasmid DNA (coding for 2 Gly and 6 His residues, as well as a stop codon (TAG; denoted by X or *), yielding a mutant protein: p.Glu1172-3Gly-6His*-CFTR (shown in red), which lacks the second nucleotide binding domain (NBD2, residues 1173-1480) and contains a 3Gly-6His-tag at the C-terminus (i.e. non-native residues 1173-1181).  

D) Homology model of wild-type CFTR protein highlighting the location of the 3Gly-6His-tag (red spheres) as well as deletion of NBD2 (omitted domain; orange in Figure 5.3D) in this mutant construct (image generated using PyMOL, Schrödinger; Mornon et al., 2009). MSD1, MSD2, NBD1 and the R domain are shown in blue, yellow, cyan and magenta, respectively. The approximate location of CFTR relative to the phospholipid bilayer is also shown.
PCR for the deletion mutant was performed using 1 and 10 ng of template DNA at two annealing temperatures (T<sub>m</sub>-10 °C and T<sub>m</sub>-5 °C) (**Figure 5.3A**). T<sub>m</sub>-5 °C reactions did not yield a PCR product likely because the temperature (66.4 °C) was too close to the extension temperature of the DNA polymerase, and therefore successful primer annealing could not occur. However, the PCR products from the T<sub>m</sub>-10 °C (i.e. 61.4 °C) reactions yielded a major band at ~10 kb (the expected size of the full-length vector containing CFTR cDNA; 500 ng template DNA was included as a control), which was the desired product. This PCR product was then transformed into competent cells, and the tabulation underneath the agarose gel shows that although many colonies grew on antibiotic selective agar plates, from the colonies that were picked (10 and 15 from PCR reactions starting with 1 ng and 10 ng template DNA, respectively) only a few produced high quality DNA from minipreps. From the six successful DNA preps, only two had the desired mutation (33.3% efficiency in terms of the presence of the mutation in isolated DNA, while 8% efficiency in terms of colonies picked). Although these efficiency rates are low, it must be noted that one successful mutant clone is sufficient for all downstream structure-function applications; therefore, in order to isolate one successful deletion clone at this efficiency, we suggest that at least 12 colonies are grown from each site-directed deletion mutagenesis attempt. Furthermore, it must be noted that these relatively low efficiencies are at least partially caused by the direct transformation of PCR reaction mixtures, rather than transformation of gel purified PCR products; this would likely increase the efficiencies several-fold (as has been shown for other CFTR mutant constructs by our lab group, data not shown).

To further characterize the nucleotide sequence of the site-directed deletion mutant, we sequenced the entire cDNA of CFTR (nucleotides 1-4443, with stop codon), and show a short segment of the electrophoretogram containing the desired mutation (**Figure 5.3B**). Here, the sequence omits the wild-type nucleotides, denoted by a black arrowhead and a vertical dashed line, suggesting that the deletion is present; this is more apparent when aligned with the cDNA sequence of wild-type CFTR (**Figure 5.3C**). Further, we identify the location of the consequence of this 18 base pair deletion (i.e. p.Ile1234_Arg1239del) and highlight these six amino acids (within NBD2) in the tertiary structure of a protein homology model of wild-type CFTR, which is
based on the structure of a related bacterial ABC transporter protein, Sav1866 (Figure 5.3D; Mornon et al., 2009).

Similarly, PCR for the insertion construct was performed using 1 and 10 ng of template DNA at two annealing temperatures ($T_m$-10 °C and $T_m$-5 °C in terms of the full-length primers; $T_m$-5 °C and $T_m$-0 °C in terms of the complementary primer sequences) (Figure 5.4A). $T_m$-0 °C (complementary sequence of primers) reactions did not yield a PCR product because the temperature used (68.4 °C) was not below the $T_m$, and therefore could not anneal to the template; this reaction acted as a control. However, the PCR products from the $T_m$-5 °C (complementary sequence of primers, i.e. 62.1 °C) reactions yielded a major band at ~10 kb (larger yield with 10 ng template). PCR products were then transformed, and the tabulation underneath the DNA gel shows that few colonies (4) grew on antibiotic selective agar plates. When these colonies were picked and grown in liquid culture supplemented with antibiotics, only one grew, leading to the isolation of just one DNA miniprep clone. Fortunately, this clone was sequenced and found to contain the desired insertion mutation (25% efficiency in terms of colonies picked). Although this efficiency rate is low, again it must be emphasized that one mutant clone is sufficient for further biochemical analyses. Therefore, we suggest that in order to isolate one successful insertion clone at this rate of efficiency, screening of at least 4 colonies is necessary.

To further characterize the nucleotide sequence of the site-directed insertion construct, we sequenced the entire cDNA of CFTR (nucleotides 1-4443, with stop codon), and show a short segment of the electrophoretogram containing the desired mutation (Figure 5.4B). Here, the sequence contains additional nucleotides which are not present in wild-type, denoted by black arrowheads and vertical dashed lines (on a blue background), suggesting that the insertion is present; this is more apparent when aligned with the cDNA sequence of wild-type CFTR (Figure 5.4C). Further, we highlight the location of the consequence of this 27 base pair insertion (i.e. p.Glu1172-3Gly-6His*) by colouring the poly-His tag in red (immediately following MSD2), as well as omitting NBD2 (since this mutation causes deletion of this domain; for reference, NBD2 is shown in orange in Figure 5.3D) in the CFTR protein structural model (Figure 5.4D; Mornon et al., 2009).
Finally, although not extensively reported in this work, p.Glu1172*-CFTR (point mutation) was also generated in this study (Figure 5.2). Using an annealing temperature of $T_m - 5 \, ^\circ C$ (55.0 $^\circ C$), 1 ng and 10 ng of template DNA yielded 2 and 5 colonies after transformation, respectively, which led to the successful growth of 2 and 3 antibiotic-selective liquid cultures, respectively, and sufficient miniprep DNA for sequencing (2 from each). DNA sequencing determined that all four clones were positive for the desired point (stop) mutation, yielding a truncated CFTR variant lacking NBD2 and without a poly-His tag (57% efficiency in terms of colonies picked, 100% efficiency in terms of mutation within isolated DNA preps). These higher efficiencies are likely correlated to yield, and probably due to the relatively simpler mutagenesis strategy involving a single nucleotide change (i.e. GAA to TAA) for this mutation, rather than a larger insertion or deletion of 27 or 18 nucleotides, respectively.

5.5.2 Biochemical and Functional Analysis of Mutant CFTR Constructs

Following generation of the deletion (p.Ile1234_Arg1239del-CFTR) and insertion (p.Glu1172-3Gly-6His*-CFTR) constructs, we transiently transfected HEK cells and used immunoblots to evaluate steady-state levels of CFTR protein following incubation at physiological temperature ($37 \, ^\circ C$) for 24 hours (Figure 5.5A). Compared to wild-type CFTR, we found that p.Ile1234_Arg1239del-CFTR is misprocessed as previously reported$^{13}$, yielding immature, core-glycosylated protein as the major form of this CFTR variant, while p.Glu1172-3Gly-6His*-CFTR appears to have similar levels of mature and immature protein as that of wild-type CFTR, which is also comparable to previous reports (Cui et al., 2007); however, p.Glu1172-3Gly-6His*-CFTR lacks NBD2 (approximately $\sim 30 \, kDa$) and therefore appears to migrate faster on SDS-PAGE, having a lower apparent molecular weight than wild-type CFTR (Figures 5.5A and 5.5B). When compared with the NBD2 deletion mutant lacking the poly-His tag (i.e. p.Glu1172*-CFTR), p.Glu1172-3Gly-6His*-CFTR appears to migrate slightly slower on SDS-PAGE (Figure 5.5C, denoted with asterisks). Although the tagged variant contains 9 additional amino acids (apparent $\sim 1kDa$ increase in molecular weight), we speculate that this alone does not account for the difference in migration; instead, the nature of the amino acids (i.e. 6 His residues which are positively charged during SDS-PAGE), together with the additional number of residues, collectively contributes to this difference.
Figure 5.5. Relative expression levels of CFTR mutant constructs: p.Ile1234_Arg1239del-CFTR, p.Glu1172-3Gly-6His*-CFTR, p.Ser700_Asp835del-CFTR and p.Gln2_Trp846del-CFTR.

A) Representative immunoblot (using anti-CFTR-NBD1 antibody: mAb 660) compares the processing of p.Ile1234_Arg1239del-CFTR and p.Glu1172-3Gly-6His*-CFTR with WT-CFTR following incubation at 37 °C for 24 hours. Mature (complex glycosylated) and immature (core-glycosylated) forms are indicated as white and black triangles, respectively. Calnexin expression was probed to confirm equal protein loading.

B) Bar graphs show mean (± SEM) densitometry measurements for at least three independent experiments, reported as the calculated ratio of mature/(mature + immature) CFTR protein.

C) Representative immunoblot (anti-CFTR antibody: mAb 660) comparing the processing of p.Glu1172-3Gly-6His*-CFTR and p.Glu1172*-CFTR (WT-CFTR as a control) following incubation at 37 °C for 24 hours. Mature (complex glycosylated) and immature (core-glycosylated) forms are indicated as white and black triangles, respectively. Calnexin expression was probed to confirm equal protein loading. Asterisks denotes the slower migrating p.Glu1172-3Gly-6His*-CFTR bands, when compared to p.Glu1172*-CFTR bands.

D,E) Homology models of wild-type CFTR protein highlighting the location of the R domain (p.Ser700_Asp835, red) deleted in the p.Ser700_Asp835del-CFTR mutant construct, as well as the domains remaining (MSD2-NBD2, residues 847-1480) in the
MSD1-NBD1-R domain deletion (p.Gln2_Trp846del-CFTR) mutant construct (images generated using PyMOL, Schrödinger; Mornon et al., 2009). MSD1, MSD2, NBD1 and NBD2 are shown in blue, yellow, cyan and orange, respectively. The approximate location of CFTR relative to the phospholipid bilayer is also shown. F) Representative immunoblot (using anti-CFTR-NBD2 antibody: mAb 596) compares the processing of p.Ser700_As835del-CFTR and p.Gln2_Trp846del-CFTR with WT-CFTR following incubation at 37 °C for 24 hours. Mature (complex glycosylated) and immature (core-glycosylated) forms are indicated as white and black triangles, respectively, while CFTR forms of unknown glycosylation are indicated as a gray triangle. Calnexin expression was probed to confirm equal protein loading, and asterisks denote non-specific bands. G) Representative immunoblot (using anti-CFTR-NBD2 antibody: mAb 596) compares glycosidase sensitivity of p.Gln2_Trp846del-CFTR to EndoH (recognizes core-glycosylation) and PNGase F (recognizes complex-glycosylation) enzymes. Mature (i.e. complex-glycosylated) and immature (core-glycosylated) CFTR bands could not be precisely discerned and are indicated by a gray triangle, while deglycosylated forms are indicated by a red triangle. Calnexin expression was probed to confirm equal protein loading, and asterisks denote non-specific bands. H) Bar graphs show mean (± SEM) densitometry measurements for at least three independent experiments, reported as the calculated ratio of mature/(mature + immature) CFTR protein. Data in panel C was generated by Maurita Hung.
Additionally, to assess whether our deletion mutagenesis strategy was robust, we generated p.Ser700_Asp835del-CFTR and p.Gln2_Trp846del-CFTR mutant constructs, which lack the R domain or MSD1-NBD1-R domain, respectively; these deletions are highlighted in the CFTR protein structural model (Figures 5.5D and 5.5E; Mornon et al., 2009). We then transiently transfected HEK cells and used immunoblots to evaluate steady-state levels of CFTR protein following incubation at physiological temperature (37 °C) for 24 hours (Figures 5.5F and 5.5H). Compared to wild-type CFTR, we found that p.Ser700_Asp835del-CFTR migrates slightly faster (due to the loss of 135 residues) and is misprocessed, yielding immature, core-glycosylated protein as the major form of this CFTR variant as previously described.24 We also found that p.Gln2_Trp846del-CFTR appeared to have one major band that migrated substantially faster on SDS-PAGE (due to the loss of 844 residues), which we determined to contain both core- and complex-glycosylated forms due to relative endoglycosidase sensitivity to either EndoH (recognizes immature, core-glycosylation) or PNGase F (mature, complex-glycosylation) glycosidases (Figures 5.5F and 5.5G). However, we were not able to precisely discern and thus quantify the relative abundance of each form, but speculate that the core-glycosylated form is more abundant based on these studies.

Using the FLIPR system, agonist-mediated (e.g. forskolin) CFTR activation is detected as membrane depolarization using a plate reader, while CFTR inhibition (mediated by CFTRInh-172) is detected as membrane repolarization (or hyperpolarization), both of which are probed using the same fluorescent membrane potential sensor dye within the same experiment. In Figure 5.6A, we evaluated the channel activity of p.Ile1234_Arg1239del-CFTR (pharmacologically rescued with the p.Phe508del-CFTR corrector VX-809), p.Glu1172-3Gly-6His*-CFTR and p.Ser700_Asp835del, with HEK cells (without transfection of CFTR, as a negative control) and WT-CFTR (positive control) together on 384-well plates (Eckford et al., 2014). We tested function of p.Gln2_Trp846del-CFTR but it did not support chloride channel activity, presumably because this mutant protein lacks a substantial portion of CFTR which is required for function (data not shown); these findings were similar to HEK cells not expressing CFTR (Figure 5.6B) and therefore significantly different than responses of WT-CFTR (Figure 5.6C). Since p.Ile1234_Arg1239del-CFTR is misprocessed, like the major CF disease-causing mutation p.Phe508del, we pharmacologically (partially) repaired the trafficking defect as
Figure 5.6. High throughput functional analysis of CFTR mutants using the FLIPR membrane depolarization assay.

A) Functional analysis of p.Ile1234_Arg1239del-CFTR (VX-809 corrected, 24 hours at 37 °C) p.Glu1172-3Gly-6His*-CFTR and p.Ser700_As835del-CFTR, with HEK cells (without CFTR expression, as a negative control) and WT-CFTR (positive control) together on a multi-well (384-well) plate format. Following a 10 min baseline measurement, vehicle (DMSO) or a CFTR activation cocktail (forskolin and VX-770) was added (black arrow). After 10 min incubation, a CFTR inhibitor (CFTRinh-172) was added to deactivate CFTR (gray dashed arrow). B-F) Bar graphs show mean (± SEM) activation rates of DMSO, or forskolin and VX-770 treatments from three independent experiments (n≥8 for each) with HEK cells not expressing CFTR (B), WT-CFTR (C), p.Ile1234_Arg1239del-CFTR (D), p.Glu1172-3Gly-6His*-CFTR (E) and p.Ser700_As835del-CFTR (F). These studies were conducted in collaboration with Saumel Ahmadi.
previously reported (Molinski et al., 2014), in order to assess functional activity of mature, plasma membrane-associated p.Ile1234_Arg1239del-CFTR. Subsequently, we found that the adenylate cyclase activator forskolin, together with the CFTR-specific potentiator VX-770, activated both p.Ile1234_Arg1239del-CFTR (Figure 5.6D) and p.Glu1172-3Gly-6His*-CFTR (Figure 5.6E) to comparable levels; VX-770-dependent activation of the CFTR mutant lacking NBD2 is in agreement with recent reports (Yeh et al., 2015). Additionally, we found that the CFTR inhibitor CFTRinh-172 inhibited both mutant proteins, and reduced functional activation of both back to near baseline levels. Taken together, these findings suggest that each CFTR variant can be activated and inhibited with CFTR-specific modulators, and that repurposing modulators of p.Phe508del-CFTR (i.e. VX-809) or p.Gly551Asp-CFTR and other CFTR gating mutations (VX-770) could be useful for other CFTR mutations. Importantly, for p.Ser700_Asp835del-CFTR we found that the basal (forskolin- and VX-770-independent) channel activity was higher than that of wild-type CFTR (data not shown), which is in agreement with previous reports (Rich et al., 1991). Interestingly, this mutant protein could be stimulated with forskolin and VX-770, suggesting that the R domain does not comprise the VX-770 binding site (Figure 5.6F). These data suggest that p.Ser700_Asp835del-CFTR forms a partially unregulated chloride channel which is sensitive to CFTR-specific modulators, and furthermore that this mutant protein may be a useful tool for identification of binding sites for certain small molecules modulators. Furthermore, we found that the kinetics of inhibition by CFTRinh-172 was variable between CFTR mutants, and the mechanism of these responses is currently being investigated.

Using this high-throughput approach, future screening efforts may identify novel, potent, efficacious and mutation-specific compounds for these and other rare and previously uncharacterized CFTR variants. Additionally, this method has the potential to be sufficiently different yet equally advantageous when compared with the current ‘blind’ drug screening paradigm employed by academics and pharmaceutical companies (previously reviewed by our group in Molinski et al., 2012), such that instead of screening large libraries of compounds on a single CFTR mutant, we could now screen a cohort of rare CF disease-causing mutations against the most efficacious and CFTR-specific small molecules (i.e. VX-809 and VX-770) to identify genotypes which could potentially benefit from the current therapeutic regimen.
5.5.3 Application towards Drug Discovery in rare Cystic Fibrosis Disease-causing Mutations

One major advantage of this work is the ability to rapidly screen multiple rare CFTR variants for compounds that functionally rescue mature CFTR protein in a high-throughput assay. Here, we validated our site-directed insertion/deletion mutagenesis and functional assay using four CFTR variants, by employing previously identified modulators of CFTR activity (i.e. those for p.Phe508del-CFTR, as well as p.Gly551Asp and other CFTR gating mutations, VX-809 and VX-770, respectively). It will be possible in the future to translate this strategy to evaluate the most efficacious CFTR-specific small molecules (i.e. VX-770 and VX-809) against all of the rare CF disease-causing insertion, deletion and truncation mutations simultaneously (i.e. on 384-well plates); although it may soon be necessary to increase the throughput of our mutagenesis strategy, using tools which have been recently described (Bill et al., 2014). This novel approach would allow for rapid determination of potential mutation-specific responders to the current therapeutic regimen for individuals with CF. Likewise, it may also be feasible to compare responses of the most common 100-200 missense and deletion mutations (e.g. those described in CFTR2: www.cftr2.org) to existing and/or emerging corrector and potentiator compounds. For example, by simultaneously evaluating novel translational ‘read-through’ agents for CF disease-causing mutations conferred by premature termination codons (e.g. p.Gly542*, p.Arg553*, p.Arg1162* and p.Trp1282*); these CFTR mutants can be readily generated and functionally characterized for responders within a short time frame (Xue et al., 2014). Further, the necessary next steps would include functional analysis of primary tissues from patients with these rare CF disease-causing mutations, pending compound validation in our high-throughput overexpression system. However, studies involving a cohort of patient mutations in our HEK system will facilitate stratification into groups which may have potential therapeutic response, thereby justifying the acquisition of biopsies for in vitro analysis. Finally, this integrated mutagenesis method with functional screening strategy may also hold potential application towards other protein folding diseases as well, by screening for novel therapeutic modulators and personalized medicines for patients with rare and uncharacterized mutations in various channelopathies (including those of K+, Na+, Ca2+ and other Cl- channels), which cause a wide variety of neurological, cardiac, skeletal muscle, renal, and endocrine diseases.
Chapter 6

Functional repair of the CFTR nonsense mutation c.2052_2053insA (2184insA, Q685TfsX4) in vitro using a multi-exon skipping strategy

6.1 Acknowledgements

The results presented in this chapter are in preparation for publication:


6.2 Abstract

Purpose: To evaluate molecular consequences of the Cystic Fibrosis mutation c.2052_2053insA (2184insA) in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, a variant predicted to cause a frame-shift and nonsense mutation in CFTR protein (Q685TfsX4).

Methods: CFTR c.DNA clones were generated containing the frame-shift mutant Q685TfsX4 or a multi-exon (exons 12-14) ‘skipped’ variant which lacked part of Nucleotide Binding Domain 1, most of the Regulatory Domain and importantly the c.2052_2053insA mutation (i.e. ΔD529_K830), and then heterologously expressed to test CFTR protein biosynthesis and channel function using immunoblots and a membrane potential-sensitive fluorescence assay of CFTR activity, respectively. Results: When expressed in HEK-293 cells, we found that the truncated variant (Q685TfsX4-CFTR) could not be repaired using small molecule modulators of CFTR. However, we found that by skipping exon 14 in our c.DNA construct which contains the c.2052_2053insA mutation (in addition to skipping of exons 12 and 13 to maintain transcript frame) a larger, incomplete yet partially mature form of CFTR (i.e. ΔD529_K830-CFTR) yielded an unregulated (ATP- and phosphorylation-independent) but inhibitor-sensitive (CFTRinh-172) and therefore functional chloride channel. Conclusion: These in vitro findings suggest that exon skipping may be a therapeutic strategy for individuals with this CFTR mutation. Further, this strategy may provide proof-of-concept for use in other rare CFTR mutations which respond poorly to current Cystic Fibrosis therapies.
6.3 Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) is the lone channel protein among the 49 members of the human ATP-Binding Cassette (ABC) transporter superfamily (Riordan et al., 1989; Gadsby et al., 2006; Slot et al., 2011). Mutations in the CFTR gene cause the fatal, autosomal recessive genetic disease Cystic Fibrosis (CF) which affects over 80,000 people worldwide; the major disease-causing mutation, ΔF508, accounts for approximately 70% of all cases (Mickle & Cutting, 2000; Sosnay et al., 2013). CFTR is an ATP- and PKA-dependent chloride channel, regulating chloride ion flux across apical membranes of polarized epithelial and endothelial cells within the so-called ‘hollow organs’ of the body (e.g. lungs, intestines, bile ducts), and mutations, including ΔF508, cause structural and/or functional perturbations with subsequent loss of fluid homeostasis across these membranes (Howell et al., 2004). Importantly, although most research efforts aim to understand CF disease in patients bearing the dominant disease-causing mutation (i.e. ΔF508), there is a significant unmet need to evaluate the approximately 2000 additional CFTR mutations that have been detected throughout the past decades via genetic testing, since these are of much lower frequency and therefore remain biochemically uncharacterized (Molinski et al., 2012; Pasyk et al., 2012; Molinski et al., 2015).

One such mutation, originally reported as 2184insA (legacy nomenclature, referring to adenine insertion at position 2184 in the mRNA sequence, within a polyA-tract) but is now more precisely referred to as c.2052_2053insA (i.e. c.DNA insertion) or Q685TfsX4 at the protein level (i.e. missense of glutamine ‘Q’ to threonine ‘T’ with a stop-codon ‘X’ inducing frame-shift 4 residues downstream or 3’). This mutation has been previously reported in patients of Slavic ancestry (Petrova et al., 1997; Witt et al., 1999; Aznarez et al., 2000; Bobadilla et al., 2002; Minarowska et al., 2007), but is likely of Galician origin and second most common after ΔF508 in Western Ukraine (Makukh et al., 2010). As reported by Makukh and colleagues, this mutation is more often found in trans with ΔF508, and the severity of symptoms (i.e. lung, liver and pancreatic disease) is highly variable, although within CF diagnostic cutoffs (Makukh et al., 2010). Interestingly, several disease-causing mutations appear within this polyA-tract, including 2183AA>G (2183delAA>G), 2184delA and 2185insC, and therefore this region seems to be a local ‘hot-spot’ (Bobadilla et al., 2002; Ferec et al., 2006; CFTR2.org). Furthermore, as
reported in the CFTR2 Mutation Database (http://www.cftr2.org/), c.2052_2053insA (2184insA) has been identified in 319 patients, representing approximately 0.36% of the CF population in this database (88,664 individuals) and supporting the classification as being rare (Sosnay et al., 2013).

In this work, we generated and biochemically interrogated the protein product of the rare CFTR mutation c.2052_2053insA (2184insA). When expressed in HEK-293 cells, we found that this mutation caused a frame-shift (with Q685T and S686I missense mutations) and inclusion of a newly formed in-frame stop codon at position 688 (i.e. K688X), yielding Q685TfsX4-CFTR. This truncated CFTR protein was of approximately 60 kDa, and lacked most of the R-domain and two C-terminal domains, MSD2 and NBD2. We found that restoration of biosynthetic processing or, more importantly, function of this overexpressed, truncated CFTR variant could not be repaired using the transcriptional modulator sodium butyrate, low temperature incubation, CFTR corrector Lumacaftor (VX-809), read-through agent Ataluren (PTC-124) or potentiator Kalydeco (Ivacaftor, VX-770). However, we found that by ‘skipping’ exon 14 in our c.DNA construct which contains the c.2052_2053insA mutation (in addition to skipping of exons 12 and 13 to maintain transcript frame) using site-directed deletion mutagenesis, a larger molecular weight CFTR form (~120 kDa) was translated, yielding an incomplete CFTR molecule lacking part of NBD1 and most of the R-domain (i.e. ΔD529_K830-CFTR). When immunoblots were exposed for longer periods of time, a low abundance of a higher molecular weight (>120 kDa) form of this variant could be detected. We then determined that this heavier form was EndoH-insensitive and therefore represented the mature, complex-glycosylated form. Surprisingly, we found that this multi-exon skipped mutant CFTR protein yielded an unregulated (ATP- and phospho-independent) chloride channel in the plasma membrane, since basal channel activity was sensitive to the CFTR inhibitor: CFTRinh172.

6.4 Materials and Methods

6.4.1 Small Molecule Modulators
Lumacaftor (VX-809; 3 µM), Kalydeco (VX-770; 1 µM) and Ataluren (PTC-124; 12 µM) were from Selleck Chemicals, sodium butyrate (5 mM) and forskolin (10 µM) were from Sigma, and CFTRinh-172 (10 µM) was from Cystic Fibrosis Foundation Therapeutics.

6.4.2 Generation of Mutant CFTR Constructs
Q685TfsX4-CFTR (c.2052_2053insA, 2184insA) and ΔD52 9_K830-CFTR were generated in human WT-CFTR c.DNA (pcDNA3.1), using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Woburn, MA) as previously described (Molinski et al., 2015). Q685TfsX4-CFTR was generated using the following primers: 5'-GGA CAG AAA CAA AAA AAA CAA TCT TTT AAA CAG AC-3' (sense); 5'- GTC TGT TTA AAA GAT TGT TTT TTT TGT TTC TGT CC-3' (antisense). ΔD529_K830-CFTR was generated using the following primers: 5'-GTC ATC AAA GCA TGC CAA CTA GAA GAG GAG TGC TTT TTT GAT GAC-3' (sense); 5'-GCT CTC CAT ATC ATC AAA AAA GCA CTC CTC TTC TTG GCA TGC TTT GAT GAC-3' (antisense). Plasmid DNA was prepared for each using the GenElute™ Plasmid Miniprep Kit (Sigma), and the presence of the designed mutations, as well as the integrity of CFTR c.DNA, was confirmed by DNA sequencing (TCAG Inc., Toronto, ON). WT-CFTR, ΔF508-CFTR and ΔR-CFTR were generated as previously described (Molinski et al., 2015).

6.4.3 Studies of CFTR Protein Processing
Human embryonic kidney (HEK-293) cells were transiently transfected with Q685TfsX4-CFTR or ΔD529_K830-CFTR, as well as WT-CFTR, ΔF508-CFTR and ΔR-CFTR constructs as controls, using PolyFect Transfection Reagent, according to the manufacturer’s protocol (Qiagen) as previously described (Molinski et al., 2015). HEK-293 cells transiently expressing CFTR proteins were maintained in DMEM (Wisent) supplemented with non-essential amino acids (Life Technologies) and 10% FBS (Wisent) at 37 °C with 5% CO₂ (HEPA incubator, Thermo Electron Corporation). HEK-293 cells expressing WT-CFTR or mutant CFTR constructs were grown at 37 °C for 24 h in the absence or presence of small molecules as required. Cells were subsequently lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail (Roche) for 10 min, and the soluble fractions were analyzed by SDS-PAGE on 6% or 4-
12% Tris-glycine gels (Life Technologies) as appropriate and as previously described (Eckford et al., 2014). After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% (w/v) milk, and CFTR bands were detected using either the human CFTR-MSD1-specific (Cui et al., 2007) (amino acids 25-36) murine mAb MM13-4 (1:1000, EMD Millipore) or the human CFTR-NBD2-specific (Cui et al., 2007) (amino acids 1204-1211) murine mAb 596 (1:10000, University of North Carolina Chapel Hill), horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Calnexin was used as a loading control, and detected using a Calnexin-specific rabbit Ab (1:5000, Sigma-Aldrich), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000) and exposure to film for 0.5 to 5 min as required. Relative CFTR levels were quantitated by densitometry of immunoblots using ImageJ 1.42 Q software (National Institutes of Health) and reported values are normalized to Calnexin expression levels.

6.4.4 Studies of ΔD529_K830-CFTR Glycosylation Status

To evaluate protein glycosylation, HEK-293 cells expressing ΔD529_K830-CFTR were grown at 37 °C for 24 h, and then lysed in modified radioimmunoprecipitation assay buffer as described above and previously (Molinski et al., 2014). Lysates were then treated with either Endoglycosidase H (EndoH) or Peptide-N-Glycosidase F (PNGaseF) (NEB, Ipswich, MA) endoglycosidases according to the manufacturer’s protocol. Samples were analyzed by SDS-PAGE using 6% or 4-12% Tris-glycine gels (Life Technologies) as appropriate, and immunoblots were performed using the human CFTR-NBD2-specific murine mAb 596 as described above.

6.4.5 Studies of CFTR-mediated Membrane Depolarization

CFTR-mediated membrane depolarization was measured as previously described (Maitra et al., 2013; Molinski et al., 2015). Briefly, HEK-293 cells over-expressing CFTR constructs were grown to 100% confluence in 96-well or 384-well plates (black, flat bottom; Greiner), washed with PBS, and the blue membrane potential dye (dissolved in chloride free buffer containing 136 mM sodium gluconate, 3 mM potassium gluconate, 10 mM glucose, 20 mM HEPES, pH 7.35, 300 mOsm, at a concentration of 0.5 mg/ml; Molecular devices), which can detect changes in transmembrane potential, was added to the cells for 1 hr at 27 °C or 37 °C as required. The
plate was then read in a fluorescence plate reader (Molecular devices – Gemini EM) at the required temperature, and after reading the baseline fluorescence, CFTR was stimulated using the cAMP agonist forskolin (10 μM; Sigma) or forskolin with VX-770 (1 μM; Selleck Chemicals). CFTR-mediated depolarization of the membrane was detected as an increase in fluorescence, and repolarization or hyperpolarization as a decrease (Maitra et al., 2013; Molinski et al., 2015). To terminate the assay, CFTR specific inhibitor Inh-172 (CFTRinh-172, 10 μM; Cystic Fibrosis Foundation Therapeutics) was added to all the wells. Changes in membrane potential were normalized to the addition of agonists.

6.4.6 Statistical Analysis

All data are represented as mean ± S.D. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Non-paired Student’s t tests, one-way analysis of variance (ANOVA), and two-way ANOVA were conducted as appropriate, and P values less than 0.05 were considered significant. Each experiment was repeated at least three times.

6.5 Results and Discussion

6.5.1 Biochemical and Functional Analysis of Q685TfsX4-CFTR

Following generation of c.2052_2053insA in CFTR c.DNA, we expressed the mutant protein in HEK-293 cells and as predicted, the protein product was a truncated version of CFTR: Q685TfsX4-CFTR. This mutation caused a frame-shift (with Q685T and S686I missense mutations) and inclusion of a newly formed in-frame stop codon at position 688 (i.e. K688X; Figures 6.1a, 6.1b and 6.2a-d). This truncated variant was approximately 60 kDa and lacked most of the R-domain and two C-terminal domains, MSD2 and NBD2 (Figure 6.2a-d). Next, we tested known modulators of ΔF508-CFTR: the chemical chaperone sodium butyrate (Moyer et al., 1999), low temperature incubation (Denning et al., 1992), CFTR corrector Lumacaftor (VX-809; Van Goor et al., 2011), read-through agent Ataluren (PTC-124; Kerem et al., 2014) or potentiator Kalydeco (VX-770; Van Goor et al., 2009) for pontential repair of this CFTR mutant. We found that although sodium butyrate and lumacaftor increased the abundance of this approximate “half-channel” (i.e. Q685TfsX4-CFTR), none of these interventions could restore processing towards the full-length WT-CFTR form (i.e. 130 kDa without glycosylation) or exhibit
a

WT-CFTR

Amino Acids

N – 681 682 683 684 685 686 687 688 – C
Glu Thr Lys Lys Gln Ser Phe Lys

c.DNA

5’ – GAA ACA AAA AAA CAA TCT TTT AAA – 3’

b

Q685TfsX4-CFTR

Amino Acids

N – 681 682 683 684 685 686 687 688 – C
Glu Thr Lys Lys **Thr Ile Phe** *

c.DNA

5’ – GAA ACA AAA AAA ACA ATC TTT TAA – 3’

↑

c.2052_2053insA
(2184insA)
Figure 6.1  c.2052_2053insA (2184insA) renders a frame-shift with two missense mutations and a stop codon. (a) WT and (b) c.2052_2053insA c.DNA and protein sequences in the region of the mutation. PolyA-tract is underlined; * denotes a stop codon.
Figure 6.2  Gene product of c.2052_2053insA (2184insA) is a truncated CFTR protein: Q685TfsX4-CFTR.

Predicted translation of full-length WT-CFTR (a) and Q685TfsX4-CFTR (b) proteins. Amino acids on a pink background denote an in-frame polypeptide; red asterisks denote stop codons. (c) CFTR homology model (Mornon et al., 2009) showing the truncated variant Q685TfsX4-CFTR in gray, and the untranslated portion of CFTR (present in WT) is depicted in transparent surface representation (orange). Red spheres, Q685T; magenta spheres, S686I. (d) Immunoblots of short and long exposures comparing WT-CFTR, ΔF508-CFTR and Q685TfsX4-CFTR. Mature, Band C forms of CFTR are indicated with a white triangle, while immature, Band B forms are indicated with a black triangle; Q685TfsX4-CFTR, which lacks glycosylation, is indicated with a gray triangle; asterisks denote non-specific bands; Calnexin was used as a loading control.
CFTR-dependent activity (Figures 6.3a-f). Importantly, for Ataluren, it was hypothesized that read-through of K688X would promote inclusion of more C-terminal amino acids until the next in-frame stop codon (shown in Figure 6.2b) and therefore be detected on an immunoblot; however, these residues are not derived from the CFTR sequence and thus are inconsequential. Further, for the other modulators, including Lumacaftor, restoration of a mature CFTR form was not expected, but rather enhanced abundance of the “half-channel” (i.e. Q685TfsX4-CFTR) form which may lead to a partially functional CFTR molecule; achieved by dimerization, as has been reported for other ABC proteins, including Sav1866 (Dawson and Locher, 2006).

6.5.2 Biochemical and Functional Analysis of Multi-Exon Skipped Variant ΔD529_K830-CFTR

An alternative strategy to partially ‘repair’ the CFTR gene could involve ‘skipping’ of the exon containing the mutation (Forrest et al., 2010; Veltrop and Aartsma-Rus, 2014). This strategy has been validated in several disease models both in vitro and in vivo, as well as shown recent clinical efficacy mainly for the recessive, X-linked disorder Duchenne Muscular Dystrophy (DMD) (Wang et al., 2010; Yang et al., 2013; Cao et al., 2014; Kole and Krieg, 2015). In DMD there are many disease-causing mutations which either create a frame-shift or direct missense to a stop codon, leading to a truncated dystrophin protein product and clinical manifestation of DMD (Ray et al., 1985; Worton, 1992; Biggar et al., 2002). However, by skipping the exon containing the mutation using antisense oligonucleotide technology, an incomplete, but larger molecular weight form of dystrophin is produced which subsequently causes a less severe DMD phenotype, referred to as Becker Muscular Dystrophy (BMD) (Worton, 1992; Chakkalakal et al., 2005). Here, we tested whether skipping of exon 14 in our c.DNA construct, containing the c.2052_2053insA mutation (in addition to skipping of exons 12 and 13 to maintain transcript frame), would yield a larger molecular weight form of CFTR that could be functional.

Following site-directed deletion mutagenesis to remove exons 12-14 in our CFTR c.DNA construct (Figure 6.4a), we found that when expressed in HEK-293 cells the protein product was approximately 120 kDa (2-fold larger than the original mutation), yielding an incomplete CFTR molecule lacking part of NBD1 and most of the R-domain (i.e. ΔD529_K830-CFTR;
Figure 6.3  Sodium butyrate, low temperature incubation, Lumacaftor (VX-809) and Ataluren (PTC-124) cannot enhance functional maturation of Q685TfsX4-CFTR.

Immunoblots (a) and analysis (b) of Q685TfsX4-CFTR abundance following sodium butyrate and low temperature incubation. CFTR forms without glycosylation are indicated with a gray triangle; Calnexin was used as a loading control. Immunoblots (c) and analysis (d) of Q685TfsX4-CFTR abundance following lumacaftor (VX-809) or Ataluren (PTC-124) treatments. CFTR forms without glycosylation are indicated with a gray triangle; Calnexin was used as a loading control. Representative traces (e) and analysis (f) of functional activation of Q685TfsX4-CFTR following small molecule treatments compared to controls (WT-CFTR, positive; HEK-293 cells, negative) in a 384-well plate format. The colours of traces in (e) correspond to the colour of bars in (f). *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; n.s., not significant. Functional studies performed in collaboration with Michelle Di Paola and Saumel Ahmadi.
Figure 6.4 Multi-exon skipping strategy yields a larger, incomplete but partially mature form of CFTR: ΔD529_K830-CFTR.

(a) CFTR exons, amino acid sequence, and location of c.2052_2053insA (2184insA). The dotted red box and scissors indicate amino acids (residues 529-830, highlighted as a blue bar, corresponding to exons 12-14) deleted from the c.DNA construct. The gray bar corresponds to residues 1-528, present in the original truncated mutant CFTR variant, while the orange bar corresponds to residues 831-1480, gained from excision of the frame-shift mutation. (b) CFTR homology model (Mornon et al., 2009) highlighting amino acids deleted (i.e. D529_K830) using this strategy. Colours correspond to those in panel (a). (c) Immunoblot comparing relative molecular weights of Q685TfsX4-CFTR and ΔD529_K830-CFTR. CFTR bands of unknown glycosylation are indicated with a red triangle; CFTR bands without glycosylation are indicated with a gray triangle; Calnexin was used as a loading control. (d) Deglycosylation of ΔD529_K830-CFTR using endoglycosidases Endo H (recognizes immature, core-glycosylated form) and PNGase F (recognizes mature, complex-glycosylated form). Mature, Band C forms of CFTR are indicated with a white triangle, while immature, Band B forms are indicated with a black triangle; CFTR forms without glycosylation are indicated with a gray triangle. Immunoblots (e) and analysis (f) of ΔD529_K830-CFTR processing (mature/total CFTR forms) relative to controls (WT-CFTR and ΔR-CFTR) following chronic incubation with Lumacaftor (VX-809). Mature, Band C forms of CFTR are indicated with a white triangle, while immature, Band B forms are indicated with a black triangle. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; n.s., not significant. Studies performed by Onofrio Laselva.
Figures 6.4b and 6.4c). Importantly, when immunoblots were exposed for longer periods of time, a higher molecular weight (>120 kDa) form of this variant was detected, although the abundance was significantly less than WT-CFTR (Figures 6.4d and 6.4e). Using deglycosylating enzymes, we determined that this higher molecular weight form was Endoglycosidase H-insensitive, suggesting that it was the mature, complex-glycosylated form of ΔD529_K830-CFTR; however, this band was the minor fraction, comprising <10% of the total (mature and immature) form of this mutant (Figures 6.4d and 6.4e). Interestingly, chronic treatment with the ΔF508-CFTR corrector Lumacaftor (VX-809) increased the abundance of the mature form by approximately 3-fold, while the ratio of mature/total CFTR increased from <10% to ~20% (Figures 6.4e and 6.4f).

By inspection of CFTR homology models, ΔD529_K830-CFTR was predicted to lack both ATP- and phosphorylation-dependent channel activity since NBD1 and the R-domain, respectively, are substantially truncated in this deletion mutant, and therefore may subsequently cause unregulated CFTR function (Mornon et al., 2009; Pasyk et al., 2015). Accordingly, when CFTR channel activity was assessed, it appeared that the relatively low abundance of the mature form of ΔD529_K830-CFTR (i.e. ~10% mature/total CFTR as described above) exhibited basal activity since the CFTR inhibitor (CFTRinh-172) affected changes in membrane-potential; such changes were not observed in HEK-293 cells lacking CFTR expression (Figures 6.5a-d). Interestingly, comparable to the phosphorylation-independent CFTR mutant: ΔR-CFTR, ΔD529_K830-CFTR was only partially stimulated (less than WT-CFTR) using CFTR-specific agonists (i.e. forskolin and VX-770), likewise suggesting that these variants possess basal channel activity; again, such changes were not observed in HEK-293 cells lacking CFTR expression (Figures 6.5a and 6.5b; Figure 5.6F). Taken together, these data suggest that the multi-exon skipped variant ΔD529_K830-CFTR, lacking part of NBD1 and most of the R-domain, yields an unregulated (ATP- and phosphorylation-independent) chloride channel or ‘pore’ in the plasma membrane, comprised of the remaining domains of CFTR: mainly MSD1 and MSD2, as well as the N-terminal part of NBD1 and the entirety of NBD2.
Figure 6.5 Functional analysis of ΔD529_K830-CFTR determines unregulated (ATP- and phosphorylation-independent) but CFTR inhibitor-sensitive chloride channel activity.

Representative traces (a) and analysis (b) of functional activation (forskolin and VX-770) of ΔD529_K830-CFTR compared to controls (WT-CFTR, positive; HEK-293 cells and ΔR-CFTR, negative) in a 96-well plate format. Representative traces (c) and analysis (d) of functional inhibition (CFTRinh-172) of ΔD529_K830-CFTR compared to controls (WT-CFTR and ΔR-CFTR, positive; HEK-293 cells, negative) in a 96-well plate format. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; n.s., not significant. Studies performed by Onofrio Laselva.
6.5.3 Exon Skipping as a Potential Future Therapeutic Strategy for Less Common CFTR mutations

These *in vitro* findings using CFTR c.DNA constructs in an overexpression system provide further rationale to investigate the feasibility of this strategy in *ex vivo* epithelial tissue from patients bearing the c.2052_2053insA CFTR mutation. Future studies aim to investigate the efficacy of antisense oligonucleotides designed to specifically block splicing and inclusion of exons 12-14 in the mRNA transcript, in order to prevent incorporation and thus translation of this CF disease-causing mutation. Although the protein product would not be of full-length, we show that it may be advantageous to express ΔD529_K830-CFTR *in vivo* rather than a truncated CFTR variant (i.e. Q685TfsX4-CFTR) which presumably undergoes nonsense medicated decay (mRNA) and proteasomal degradation (protein).

Based on our results, we suggest that ΔD529_K830-CFTR allows for enhanced chloride flux, albeit this mutant CFTR channel activity would be unregulated (independent of ATP and phosphorylation); importantly, this activity appears similar to ΔR-CFTR in these studies, as both ΔD529_K830-CFTR and ΔR-CFTR lack the R-domain. Further, having an unregulated CFTR variant expressed at the apical membrane of epithelial cells lining disease-affected tissues (i.e. lungs, pancreas, intestines) may be of clinical benefit for individuals homozygous for c.2052_2053insA, a mutation in which the recently FDA-approved therapies Kalydeco™ (Ivacaftor) and Orkambi™ (Lumacaftor and Ivacaftor co-therapy) likely do not enhance chloride flux. Additionally, since several other CF disease-causing mutations are found within exons 12-14 (e.g. the second and seventh most common CFTR mutations, G542X and R553X, respectively), this multi-exon skipping strategy could have broader application for a greater subpopulation of CF patients as well. Finally, this *in vitro* strategy provides proof-of-concept and rationale for investigating translational efficacy in *ex vivo* tissues for other rare CFTR mutations, nonsense mutations in particular, in other exons which respond poorly to current CF therapeutics.
Chapter 7

Conclusions and Future Directions

Although ΔF508 is the most common CF disease-causing mutation, it is clear that, in aggregate, the ~2000 other CFTR mutations comprise a significant portion of the CF patient population. However, current advances in CF research and development mostly aim to understand and subsequently repair molecular defects associated with ΔF508. This perspective does not directly address the therapeutic needs of the remaining individuals with rare mutations, although much is being done to ‘reposition’ small molecules designed to repair ΔF508 for non-ΔF508 variants. Importantly, this strategy has had limited success for several other CFTR mutations; most notably the rare disease-causing mutation described in Chapters 3 and 4 of this thesis (i.e. c.3700A>G, ΔI1234_R1239). Therefore, through the results shown here, as well as those described by other laboratories and pharmaceutical companies worldwide, it has become clear that mutation- and even patient-specific therapies or personalized CF medicines need to be rationally designed on a mutation-by-mutation, as well as a patient-by-patient basis (taking into account non-CFTR modifier genes) in order to have the best chance at a positive therapeutic outcome (Figure 7.1). In this regard, direct implications of reported discoveries made within this thesis, as well as conclusions and future directions for pre-clinical studies of patients bearing these mutations are discussed in this chapter; putative directions for the CF field as a whole is also considered.

7.1 Towards Personalized Medicine for Rare Cystic Fibrosis Mutations

7.1.1 Strategies to Repair c.3700A>G (ΔI1234_R1239-CFTR)

It may have been anticipated that compounds effective in functionally rescuing ΔF508-CFTR (i.e. Lumacaftor and Kalydeco™, or Orkambi™ co-therapy), will also be effective in rescuing rarer mutations that exhibit similar defects. However, we determined that another CF-causing mutation (c.3700 A>G, ΔI1234_R1239), which also exhibited misprocessing and loss of channel function, could not be efficiently repaired by ΔF508 small molecules. Structural defects between these two genotypes substantially diverged once investigations into the molecular mechanism of disease were performed. For example, protease susceptibility studies of
Figure 7.1. Personalized CF medicine must target affected tissues (e.g. lung epithelia) at the mutation- and patient-specific level.

**Bottom:** CF population with various disease-causing mutations, having diverse set(s) of modifier genes and genetic backgrounds, as well as variable clinical presentations. **Right inset:** Immunofluorescence cross-sectional image of lung epithelial from a patient with a rare CFTR mutation. CFTR (misfolded, ER-retained) is labelled green; nuclei are blue; plasma membrane is stained red. These data were provided by Kai Du (SickKids). **Left inset:** CFTR homology model depicting the location of a rare disease-causing mutation (magenta). The unique molecular defects caused by this mutation, as well as the context of the whole individual must be addressed in order to effectively treat their disease.
ΔI1234_R1239-CFTR showed that this mutation caused local structural defects in NBD2 and allosterically, altered stability of the first half of the molecule; whereas ΔF508 did not alter stability of the first half of CFTR. These structural differences likely contribute to the reported differences in sensitivity to modulators.

In order to repair intramolecular defects caused by the ΔI1234_R1239 mutation, insight into unique structural aberrations will play a large role. For ΔI1234_R1239-CFTR, proteolytic digestion studies suggested that altering the stability of NBD2 (site of the mutation) and/or increasing the stability of the C-terminal half of CFTR toward a more wild-type-like phenotype may provide superior repair compared to Class I modulators designed for ΔF508-CFTR; Class I modulators essentially repair the interfaces perturbed by the ΔF508 mutation, i.e. ICL1:NBD1:ICL4. Further, in Chapter 4 of this thesis it was also determined that Class 2 modulators, which repair interfaces involving NBD2, provided superior rescue of ΔI1234_R1239-CFTR trafficking through the biosynthetic compartments, although did not allow for competent channel gating; this phenotype was intriguing, and became the source of much contemplation.

The working hypothesis is that the specific Class 2 modulator tested (i.e. Corr-4a, C4) could directly repair the local structural defects of ΔI1234_R1239-CFTR (i.e. the NBD2-binding compound could rescue the NBD2 misfolding mutation) and improve protein conformation and thus maturation. However, the nature of the chemical structure of this small molecule (specifically, the high degree of freedom and thus rotamers) may not allow for channel gating perhaps since this compound may form an elongated, flexible rotamer and ‘wrap’ around the NBD(s) to prevent catalytic activity. Whereas VX-809, a small molecule which does not bind directly to NBD2, could partially enhance conformational maturation (although to a lesser degree than C4), yet did not prevent channel gating; for this reason VX-809 was deemed superior to C4. Taken together, it may be beneficial to have a Class 2 corrector, able to repair the primary defect caused by ΔI1234_R1239, but which allows for enhanced channel activity. To do this, perhaps a C4-like small molecule with less degrees of freedom (fewer potential rotatmers) would bind without assuming a putative elongated and thus CFTR-inhibitory conformation.
From the literature, Verkman and colleagues have identified and further rationally synthesized derivatives of C4 which have less degrees of freedom, being structurally constrained and therefore less flexible (deemed a constrained bizthiazole). Their studies suggested that the constrained variant was the likely ‘binding mode’ assumed by the parent compound, C4, when found in the ΔF508-CFTR binding pocket. For ΔI1234_R1239-CFTR, we were interested in determining whether constrained C4 derivatives (e.g. core-corr-II, compound 10b) could rescue both processing and gating. Preliminary studies in our lab may support this idea, where functional assessment of ΔI1234_R1239-CFTR chronically incubated with compound 10b exhibited greater CFTR activation than when incubated with C4 in paired experiments (Figure 7.2). Interestingly, when compared to functional rescue by VX-809 in paired experiments, compound 10b allowed for greater CFTR activity (which was further enhanced with combined with VX-809), suggesting that the rotameric conformation of certain small molecules is indeed important for rescue of specific conformational defects, i.e. either processing or gating, and further that these parameters must be independently considered when designing CFTR mutation-specific therapies.

From the studies presented in Chapter 4, it is clear that neither Kalydeco nor the Orkambi co-therapy can sufficiently repair ΔI1234_R1239 in vitro to provide rationale for future clinical trials; importantly, these therapies work best for the mutations they were designed for, G551D and ΔF508, respectively (Figure 7.3). Therefore, alternate and defect-specific strategies are needed to address the underlying causes of CF disease in individuals expressing ΔI1234_R1239-CFTR. Perhaps constrained Class 2 modulators could be derivatized to be more druggable as well as more CFTR-specific, since there is some in vitro evidence suggesting off-target effects (i.e. G-protein-coupled receptor modulation). Additionally, high-throughput screening of existing small molecule libraries may yield hits which repair ΔI1234_R1239-CFTR processing and channel activity. Accordingly, this work is currently being pursued in our laboratory, initially by screening the ~1300 FDA-approved compounds in the Prestwick library using our HEK overexpression system, with the end goal of identifying protein modulators (correctors, potentiators or stabilizers) which can then be validated using primary, patient-derived tissues.
Figure 7.2. Functional analysis of ΔI1234_R1239-CFTR in the presence of C4-like compound “10b” using the FLIPR membrane depolarization assay.

(A) Corrector-4a (C4) and its derivative core-corr-II or 10b. Site of ‘locking’ via carbon-carbon bonds is shown in red. (B) Functional analysis in the presence or absence of these correctors. Studies were conducted in collaboration with Onofrio Laselva (SickKids).
Figure 7.3. Mutation- and tissue-specific efficacy of current CF therapies.

Differences between tissues (within the same mutation) may yield a predictive trendline to guide future pre-clinical trials and further assess potential clinical benefit. Ovals represent ranges of responses within each tissue and functional assay. Purple ovals and trendline suggest that responses greater than those measured for G551D and ΔF508 are required for a novel drug in a rare CFTR mutation to have a high probability of future clinical efficacy (i.e. near levels of an unaffected heterozygote, or CF carrier).
Another strategy which is concurrently being pursued by our group, uses antisense oligonucleotides (e.g. Morpholinos) to target and repair the underlying defect causing disease by c.3700A>G: aberrant splicing. The rationale of using antisense technology is that it can sterically block the splicing machinery (i.e. ASF/SF2 protein) from recognizing the premature splice site caused by the c.3700A>G mutation; the aberrant site is masked, and proper translation (without exclusion of residues I1234_R1239) can occur. Here, the oligonucleotide is synthesized having a phosphorodiamidate backbone, which significantly enhances the half-life and delivery of this molecule; essentially, this chemistry cannot be degraded by intracellular proteins, and therefore could become a long-lasting therapeutic molecule if proof-of-concept studies are validated. Importantly, this custom designed molecule would be highly specific for the aberrant splice site, without any off-target effects. If successful, premature splicing will be blocked, and the wild-type transcript will be translated, allowing for functional chloride channel activity proportional to the extent of splicing machinery blockade. This effort aims to rescue the issues of CFTR quantity and quality caused by c.3700A>G, by re-established proper translation. Currently, delivery studies are being performed on nasal epithelial cells from non-CF individuals in order to assess feasibility of delivery on precious patient-derived tissues. Furthermore, readouts in our assays will include: delivery of fluorescently-tagged oligos (immunofluorescence), increase in mature CFTR protein (immunoblots) and increase in CFTR channel activity (membrane depolarization).

Lastly, long-term strategies to efficiently repair this genetic defect could involve regenerative medicine (i.e. stem cell therapy) as well as gene replacement/repair therapies. These approaches would use each patient’s own cells to allow for genetic ‘correction’ of the mutation back to the wild-type nucleotide, either in vivo or ex vivo with transplantation. This strategy aims to modify the CFTR gene alone, without modulating any other intracellular components. If successful, this could then provide individuals with their own CFTR-corrected tissues as a replacement for their diseased tissue. Importantly however, this approach is still many years away from showing clinical efficacy in unhealthy individuals, and furthermore, the regulatory and ethical barriers would need to be considered for regenerative medicine and gene replacements tools to become an essential component of the clinician’s toolkit.
7.1.2 Strategies to Repair c.2052_2053insA (Q685TfsX4-CFTR)

From our studies in HEK cells, the c.2052_2053insA (2184insA) mutation led to a truncated CFTR protein (Q685TfsX4-CFTR) which, as expected, was not amenable to current FDA-approved small molecule regimens (i.e. Kalydeco™ or Orkambi™) or other modulators of ΔF508-CFTR. However, we found that by ‘skipping’ the exon which contained the c.2052_2053insA mutation (as well as two additional exons to maintain transcript frame) in our c.DNA construct, a larger but incomplete CFTR protein (i.e. ΔD529_K830-CFTR) was translated. Importantly, although the activity of this protein was unregulated, it was shown to be CFTR inhibitor-sensitive and therefore constituted a functional chloride channel. These findings are relevant since they propose a new therapeutic strategy in which to repair this genetic defect.

As mentioned in Chapter 6, this strategy has been demonstrated in vitro, and further shown to be clinically relevant in Duchenne Muscular Dystrophy (DMD). Using antisense oligonucleotide technology to skip aberrant exons, containing frame-shifting and thus disease-causing mutations, an incomplete but larger molecular weight form of dystrophin is produced which subsequently causes a less severe clinical manifestation, i.e. Becker Muscular Dystrophy (BMD). Preliminary results have demonstrated efficacy of these splicing modulators in patients, and currently, the medical community is eagerly awaiting the final results of recent clinical trials by Sarepta Therapeutics and Prosensa Therapeutics to determine whether a novel, beneficial therapy will come to market in the near future. Furthermore, these Phase III results may provide proof-of-concept and rationale for related studies in other genetic diseases including Cystic Fibrosis.

Importantly, if our findings from heterologous overexpression studies are validated in ex vivo patient tissue using antisense oligonucleotides (i.e. three Morpholinos targeting the intron-exon boundaries of exons 12, 13 and 14), this strategy could have broader implications for treating those with rare CFTR mutations. Since the repair strategy for the c.2052_2053insA mutation involved skipping three exons, it is hypothesized that this approach may be useful for all CFTR mutations contained within this stretch. For example, the second, seventh and thirteenth most commons CFTR mutations, c.1624G>T (G542X), c.1657C>T (R553X) and c.2051_2052delAAinsG (K684SfsX38), respectively, are found within these exons, and thus this
strategy could have clinical significance for the thousands of individuals suffering from this fatal genetic disease and bearing these three mutations (www.CFTR2.org). However, there is also an abundance of less common CFTR mutations within these exons, and therefore this approach could be amenable to treating a greater subpopulation of CF patients as well.

The studies performed in Chapter 6 may provide proof-of-concept for generation of other cDNA deletion constructs to evaluate efficacy of exon skipping for other CFTR mutations which reside in the other 24 exons and respond poorly to current CF therapeutics. Accordingly, CFTR cDNA constructs lacking either exon 22 (residues M1157_R1239) or exon 23 (residues V1240_Q1291) have been generated in order to assess potential exon skipping benefit for the fifth and twelfth most common CFTR mutations: c.3846G>A (W1282X) and c.3484C>T (R1162X), respectively; preliminary results from these studies are provided in Figure 7.4. If successful, patients bearing these as well as any other CFTR mutation within exons 22 or 23 could have a potential novel strategy to treat their disease. Furthermore, as alluded to in this Chapter, four of the top twelve CF disease-causing mutations involve generation of an in-frame stop codon. Therefore, if found to be successful *in vitro* and *ex vivo*, exon skipping using antisense oligonucleotides could become a standard of care for mutations that cannot be repaired by pharmacological means, since these defective transcripts and proteins undergo irrepairable nonsense mediated decay and misfolding, respectively.

### 7.1.3 Strategies to Repair Uncharacterized, Rare CFTR Mutations

In order to apply what has been learned to treat those with previously uncharacterized and thus rare CF disease-causing mutations, understanding the molecular defects of each and every mutation will be critical. However, although current therapies and approaches may not be amenable for all other mutations, it is at least worth evaluating the potential for CFTR modulation. With this in mind, it would be advantageous to generate each and every disease-causing mutation in a heterologous overexpression system, and further test the efficacy of current therapies (mainly Kalydeco™ and Orkambi™) towards a functional CFTR response; this could be done in a high-throughput manner as discussed in Chapter 5. Here, each and every CFTR variant could be represented within a single well of a 384-well plate, and using multiple 384-well plates the functional response to CFTR correctors and potentiators could be assessed.
Figure 7.4 Exon skipping strategy to repair c.3846 G>A (W1282X) yields a larger, incomplete but partially mature form of CFTR: ΔV1240_Q1291-CFTR.

(a) CFTR exons, amino acid sequence, and location of c.3846 G>A (W1282X). The dotted red box and scissors indicate amino acids (residues 1240-1291, highlighted as a blue bar, corresponding to exon 23) deleted from the c.DNA construct. The gray bar corresponds to residues 1-1239, present in the original truncated mutant CFTR variant, while the orange bar corresponds to residues 1292-1480, gained from excision of the nonsense mutation. (b) CFTR homology model (Mornon et al., 2009) highlighting amino acids deleted (i.e. V1240_Q1291) using this strategy. Colours correspond to those in panel (a). (c) Immunoblot of ΔV1240_Q1291-CFTR in the presence or absence of Lumacaftor (VX-809). CFTR bands of unknown glycosylation are indicated with a red triangle; CFTR bands of complex glycosylation are indicated with a white triangle; Calnexin was used as a loading control. (d) Deglycosylation of ΔV1240_Q1291-CFTR using endoglycosidases Endo H (recognizes immature, core-glycosylated form) and PNGase F (recognizes mature, complex-glycosylated form). Mature, Band C forms of CFTR are indicated with a white triangle, while immature, Band B forms are indicated with a black triangle; CFTR forms without glycosylation are indicated with a gray triangle. (e) Analysis of ΔV1240_Q1291-CFTR processing (mature/total CFTR forms) relative to WT-CFTR following chronic incubation with Lumacaftor (VX-809). Studies performed by Onofrio Laselva (SickKids).
by current functional tools developed by our lab (i.e. FLIPR membrane depolarization assay). This undertaking would be a large effort, but it is feasible. The number of uncharacterized mutations (~1800-1900, since approximately 100-200 disease-causing mutations have been reported in the literature) is somewhat manageable, and methods have been optimized for rapid generation and functional evaluation of CFTR c.DNA constructs transiently expressed in an HEK cell system. However, a potential concern could be the logistics of such a large-scale approach, and therefore a collaborative team would be necessary for this effort. Additionally, a prioritization scheme would be needed to facilitate stratification of mutations (e.g. by grouping CFTR variants bearing molecular defects contained to a certain region of the protein) and further streamline the process of drug discovery.

If current CF therapies do not enhance the functional response of previously uncharacterized CFTR mutants, each rare variation must then be assessed one-by-one, or at least stratified based on known parameters: i.e. mutation consequence (e.g. stop codon), domain-boundaries (e.g. within MSD1, MSD2, R domain, NBD1 or NBD2) or perhaps even clinical phenotype (e.g. mild vs. severe disease within an affected tissue). Stratification could establish a ‘theratype’ or targeted therapy for a group of \textit{CFTR} mutations. Accordingly, if attempts to categorize mutations into novel classes, or theratypes, based on their response to small molecule therapies are successful, early stratification would also be advantageous for future clinical trials in patients bearing such mutations. By increasing the CF population in which novel therapies could be tested, greater significance and thereby clinical indication could be achieved.

As previously mentioned throughout this thesis, by understanding how rare mutations alter the conformation, stability and function of CFTR, insight into potential mechanisms of repair of the major mutation may become clearer. In other words, by elucidating consequences of each molecular defect of each rare mutant CFTR protein one at a time, the CF field will inch closer to fully understanding how ΔF508 can be efficiently repaired by small molecules or other therapies as well. Also, by understanding how each rare mutation causes CF disease, predictive power could be demonstrated by making correlations to other similarly defective CFTR variants,
and furthermore, rationally designed structure-based CFTR therapeutics could become a reality.

In addition to the aforementioned mechanism-based repair of CFTR protein quantity (i.e. steady-state abundance, conformational maturation and plasma membrane localization) and quality (i.e. gating competent), aberrant homeostasis of certain pathways (e.g. proteostasis) within each rare CF genotype could also be discovered, and thus may suggest requirement for modulation with potential translational application as a repair strategy for ΔF508. For example, other (non-CFTR) differences in the disease-state could be identified, including: altered gene expression/regulation, epigenetics, mRNA stability, translation rate, intercompartmental trafficking competency as well as residency time within each compartment, and finally, maintainance of native intermolecular interactions with other intracellular proteins which are required for regulation and activity of CFTR. All of these potential defects could become additional targets for small molecules modulators to rescue and repair dysfunctional, rare CFTR variants. Alternatively, if CFTR-specific therapies for rare variants cannot be rapidly developed, targeted modulation of other apical membrane proteins (e.g. the calcium-activated chloride channel ANO-1 or TMEM16A) may compensate for the loss of CFTR activity. A major advantage of this alternative approach is that it involves modulation of a natively expressed and relatively abundant cell surface target, and is therefore less difficult to target using small molecules compared with the requirement of repair strategies for defective biosynthesis, trafficking and/or function of certain disease-causing CFTR mutants.

Lastly, an alternative strategy to evaluating one or two small molecule modulators on each and every CFTR mutant is the approach that is commonly pursued by academia and industry alike: evaluating many compounds on one mutation. This has been the norm for many years in the CF field, as well as across most fields within the realm of life science research, and is one of the main reasons why there are currently FDA-approved therapies for ΔF508 and G551D. By focusing on drug discovery one mutation at a time, greater, more diverse sets of chemical library could be screened for specific functional repair properties. However, these efforts would require vast and dedicated resources, and would likely only identify small molecules which uniquely repair the screened mutation, but which do not have broader application
towards other variants; although this was the case for ΔF508, importantly, small molecules (mainly Kalydeco™) discovered from screening efforts on G551D-CFTR have demonstrated efficacy on other CFTR mutants which have similar gating defects. These findings suggest that the classical approach of screening all known chemical entities on a single mutant protein may in fact yield a novel therapy for the screened protein, as well as provide hope for subsequent translation towards other similarly defective variants.

7.2 Concluding Remarks

In summary, the data presented in this thesis suggest the importance of understanding the molecular defects associated with each CF disease-causing mutation on a case-by-case basis. Unfortunately, it appears that small molecule therapies discovered and designed for the major disease-causing mutation (ΔF508) cannot be readily used to repair less common CFTR mutations; in other words, one size does not fit all. Therefore, this work establishes that there is a real, unmet therapeutic need for many individuals suffering from rare CF disease-causing mutations, and further that only biochemical interrogation of each CFTR variant will provide insight into the required mechanism of repair.

The efforts described in this work aim to facilitate future mutation-specific CF drug discovery, as well as a ‘personalized medicine’ approach to develop novel strategies to combat this fatal genetic disease for each individual sufferer. Importantly, understanding the molecular defects caused by rare mutations will also provide insight into CFTR structure-function as a whole, and possibly assist in enhancing pharmacological repair of the major mutation: ΔF508, as well as provide predictive power for repair strategies of previously uncharacterized CFTR variants. Future discoveries may aid in the rational, structure-based design of therapeutics which repair the primary aberrations causing misfolding or dysfunction for all CF disease-causing mutations. Likewise, by employing the integrated mutagenesis method with functional screening strategy towards other protein folding diseases as well, novel therapeutic modulators and personalized medicines for patients with rare and uncharacterized mutations in various channelopathies (including those of K⁺, Na⁺, Ca²⁺ and other Cl⁻ channels), which cause a wide variety of neurological, cardiac, skeletal muscle, renal and endocrine diseases may be identified.
In conclusion, understanding drug responses for each CFTR mutation will help elucidate mechanism of action, and assist in the design of mutation-specific therapies (i.e. personalized CF medicine). By also screening less common and previously uncharacterized mutant CFTR proteins to current therapies, identification of genotypes that could potentially benefit from these compounds may be identified. It is clear that there are future challenges for CF drug discovery, however, there is some evidence and thus hope for ‘repurposing’ certain small molecule CFTR modulators towards mutations within the same dysfunctional class; for example, Kalydeco™ has been recently approved to treat 9 additional CFTR gating mutations. Therefore, by building on this innovation via future clinical testing, it may still be possible to further expand the number of mutations (population size) in which Kalydeco™, Lumacaftor and Orkambi™ have therapeutic benefits, or in other words, enhance the size in which these drugs fit. However, in order to facilitate future treatment of all individuals with CF disease, especially those with the rare, uncharacterized, or even yet to be discovered disease-causing mutations, personalization and thus a case-by-case strategy will provide relevant insight into efficacious repair of the unique molecular defects associated with each rare CFTR mutation.
References


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Appendix

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Title: Genetic, cell biological, and clinical interrogation of the CFTR mutation c.3700 A>G (p.Ile1234Val) informs strategies for future medical intervention

Author: Steven V. Molinski, Tanja Gonska, Ling Jun Huan, Berivan Baskin, Ibrahim A. Janahe et al.

Publication: Genetics in Medicine
Publisher: Nature Publishing Group
Date: Feb 20, 2014

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### List of CFTR Mutant Constructs Generated (last updated: October 2014)

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**All mutants generated by S. Moulinski**

**ALL CONSTRUCTS ARE IN pDNA3.1(+) WITH 10X-HIS AT C-TERMINUS UNLESS OTHERWISE NOTED**

#### MUTATIONS IN WT-CFTR

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<td>381</td>
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<td>Lys</td>
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<tr>
<td>401</td>
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<td>Ser, Trp</td>
<td>Pro, Gly</td>
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<td>Phe</td>
<td>del</td>
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<tr>
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<td>Cys</td>
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<td>R</td>
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<td>Ser</td>
<td>Asp</td>
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<tr>
<td>660, 508</td>
<td>S660D/F508del-easy</td>
<td>Ser</td>
<td>Asp</td>
<td>R</td>
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<tr>
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<td>Ser, Ser</td>
<td>Asp, Ala</td>
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MUTATIONS IN CYS-LESS-CFTR (pIRES with N-term HA-tag)

<table>
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<tr>
<th>Position</th>
<th>Mutation</th>
<th>Native Residue</th>
<th>New Substitution</th>
<th>Location</th>
<th>Expression level: G/C/B/C (%)</th>
<th>Activity</th>
<th>Disease Severity</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>WT-Cys-less</td>
<td>18 Cys</td>
<td>16 Ser, 2 Leu (NBD1)</td>
<td>Throughout</td>
<td>n/a</td>
<td>18 Cys mutated, no V510A (from Gascoy lab)</td>
<td>WT-Cys-less with suppressor mutation, increases expression</td>
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<tr>
<td>VS10A</td>
<td>Val</td>
<td>Ala</td>
<td>NBD1</td>
<td>n/a</td>
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<tr>
<td>170</td>
<td>R170C</td>
<td>Arg</td>
<td>Cys</td>
<td>IC1</td>
<td></td>
<td></td>
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<td>(from Clancy lab; no His tag)</td>
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R-domain construct
C-term GFP tag
<table>
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<tr>
<th>170/401</th>
<th>R170C/W401C</th>
<th>Arg, Thr</th>
<th>Cys, Cys</th>
<th>ICL1, IBD1</th>
<th>R170G/C/H exist, R170G is severe; W401X is severe</th>
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</thead>
<tbody>
<tr>
<td>177</td>
<td>F508del/V510A/?</td>
<td>Ile</td>
<td>Cys</td>
<td>ICL1</td>
<td>Major mutation</td>
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<tr>
<td>177</td>
<td>F508del/V510A</td>
<td>Ile</td>
<td>Cys</td>
<td>ICL1</td>
<td>I177T is severe (MII and MII);</td>
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<td>177, 973</td>
<td>I177C/V1393C/V510A</td>
<td>Ile, Leu</td>
<td>Cys, Cys</td>
<td>ICL1, ICL3</td>
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<td>Leu</td>
<td>Cys</td>
<td>ICL2</td>
<td>Not disease-causing</td>
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<td>Leu, Val</td>
<td>Cys, Cys</td>
<td>ICL2, ICL4</td>
<td>Not disease-causing</td>
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<tr>
<td>401</td>
<td>W401C</td>
<td>Thr</td>
<td>Cys</td>
<td>NHERS</td>
<td>W401X is severe</td>
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N-term HA tag removed: n/a

**NON-CFTR CONSTRUCTS**

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<tr>
<td>vGFP</td>
<td>Enhanced GFP (F64L); from Salk lab</td>
</tr>
<tr>
<td>SLC6A14 C-term FLAG tag</td>
<td>Modifier gene; from Origitne</td>
</tr>
<tr>
<td>SLC6A14 N-term mCherry tag</td>
<td>Modifier gene; from SIDNET</td>
</tr>
<tr>
<td>SLC26A4 C-term FLAG tag</td>
<td>Modifier gene; from Romero lab</td>
</tr>
</tbody>
</table>
A1.3 List of Publications and Patent Co-authored During Doctoral Studies


A1.4 List of Awards Received During Doctoral Studies

- Trainee Travel Award ($500), Cystic Fibrosis Foundation, 2015
- PhD Lyons Poster Award, Dept. Biochemistry Research Conference, University of Toronto, 2015
- RTC Trainee Travel Award ($1000), Research Training Centre, Hospital for Sick Children, 2015
- Ontario Graduate Scholarship ($15000/year), University of Toronto & Province of Ontario, 2013-2015
- Dr. Joe A. Connolly Memorial Award ($8400/year), Faculty of Medicine, University of Toronto, 2014-2015
- Hilda and William Courtney Clayton Paediatric Research Award ($4100/year), Faculty of Medicine, University of Toronto, 2014-2015
- Peterborough K.M. Hunter Graduate Studentship ($15000/year), Faculty of Medicine, University of Toronto, 2013-2014
- Dr. Goran Enhorning Award in Pulmonary Research ($1600/year), Faculty of Medicine, University of Toronto, 2012-2014
- Conference Grant ($1210), School of Graduate Studies, University of Toronto, 2012
- Travel Award ($1000), European Cystic Fibrosis Society, 2012
- ICS Travel Award ($1225), Institute of Nutrition, Metabolism and Diabetes, Canadian Institutes of Health Research, 2011
- Conference Grant ($500), School of Graduate Studies, University of Toronto, 2011

A1.5 List of Invited Talks During Doctoral Studies

The Somerset Lecture Series, Hospital for Sick Children
Toronto, ON, September 10, 2015

Cystic Fibrosis Research Rounds, Hospital for Sick Children
Toronto, ON, October 20, 2014

37th European Cystic Fibrosis Society Conference
Gothenburg, Sweden, June 11-14, 2014

10th Annual European Cystic Fibrosis Basic Science Conference
Málaga, Spain, March 20-23, 2013

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S. Molinski and C.E. Bear. ICL1 Facilitates Conformational Maturation of CFTR Through an Interdomain Interface with NBD1.

9th Annual European Cystic Fibrosis Basic Science Conference
Sainte-Maxime, France, March 28 - April 1, 2012
S. Molinski and C.E. Bear. Structure-Function Analysis of Interhelical Interfaces Comprised of the Intracellular Loops of CFTR.

A1.6 List of Remaining Hypotheses

• Core-corr-II and related constrained bithiazoles, but not unconstrained derivatives (i.e. Corr-4a), repair gating defects in ΔI1234_R1239-CFTR by allowing for competent gating due to less degrees of freedom within the chemical structure of the small molecule (i.e. Core-corr-II does not ‘wrap’ around NBD2 to prevent channel gating)
• Specifically designed antisense oligos (morpholinos) will block abberant splicing caused by c.3700A>G and restore WT protein and thus CFTR function
• R1066C can be repaired using ΔF508 modulators (i.e. Orkambi)
• c.2052_2053insA (2184insA) will be functionally repaired by CFTR exon skipping using three antisense oligonucleotides targeted against the intron-exon boundary of exons 12, 13 and 14 (CFTRinh-172 co-therapy may be required to regulate channel activity)
• Skipping of exon 22, containing the CFTR nonsense mutation c.3472C>T (R1158X), using antisense oligonucleotides (at either the 5’ acceptor site, 3’ donor site, or a combination of both) will yield an incomplete, but partially mature CFTR variant: ΔM1157_R1239-CFTR, which will be misfolded but amenable to functional repair by Orkambi
• Skipping of exon 23, containing the CFTR nonsense mutation c.3846G>A (W1282X), using antisense oligonucleotides (at either the 5’ acceptor site, 3’ donor site, or a combination of both) will yield an incomplete, but partially mature CFTR variant: ΔV1240_Q1291-CFTR, which will be amenable to functional repair by Orkambi
• VX-770 binds to MSD2 of CFTR (hypothesized via chronic treatment studies using CFTR toxicity as a biomarker of binding, where only CFTR truncation constructs containing MSD2 were sensitive to VX-770 toxicity) – K892, R899 and R1128 are ‘best’ amino acid candidates as VX-770-interacting residues, based on sequence alignments with mouse CFTR (mouse CFTR is differently/hyper sensitive to VX-770)
• Lys mutation of N1303K creates a di-lysine retention motif in NBD2 which is recognized by COPI and partially retained in the ER (VX-809 and other correctors cannot overcome this retention defect); overexpression of 14-3-3 or fusicoccin treatment could overcome this effect; further, VX-770 does not potentiate this mutant protein because the binding site in MSD2 is altered via interdomain interaction with mutated NBD2