Using cellular models to inform diagnoses and predict therapeutic responses of individuals with cystic fibrosis

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Abstract

Cystic fibrosis (CF) continues to be a premier disease model for genetics research by demonstrating many of the successes of molecular genetics research as well as the challenges still facing the genetics research community. Research in CF has elucidated the mechanisms underlying specific DNA variants that cause disease, leading to the development of modulator therapies that are able to very specifically and potently improve the function of specific variant versions of CFTR protein. The eventual goal of providing effective therapy to all individuals with CF will depend on the expansion of the approval of currently available drugs to all individuals who would benefit and the development of novel compounds to treat variants that are unresponsive to current modulators. These two goals will be dependent upon detailed molecular understanding of all variants found on disease associated alleles of CFTR. To this end, we have used cellular models to accurately measure CFTR function *in vitro* and to test the response of missense variants to CFTR small molecule drugs. The current outlook for treating CF is very optimistic as new modulators proceed through clinical trials and extend accessibility of therapies capable of treating the underlying cause of CF to a growing number of individuals.

Preface

This thesis, and the work that it entails, was made possible due to the enormous support and encouragement of many people over more than 10 years. I am thankful for the people and the experiences and this thesis represents the culmination of my professional career thus far, integrating lessons learned from of each of my previous experiences to shape the direction and purpose of my work.

I will be forever thankful for my experiences prior to starting at Hopkins because they prepared me exceptionally well for my time as a graduate student. Drs. Tom and Suzanne Hart at the National Institutes of Health introduced me to the world of patient based research for rare diseases, showing me the clinical potential of genetics research. My time as an analyst working at GeneDx, taught me the monumental importance of accurate genetic testing, further emphasized during my time at Hopkins as Garry Cutting constantly reminds us, “Behind every variant is a patient”. These experiences and the advice from colleagues I have met along the way have shaped my entire career path and I hope will continue to do so, as their advice has been integral to my success.

The IGM provided me with a professional home with continual support and encouragement throughout my 5 years. The faculty who were always willing to talk to me, almost regardless of the topic, and showing that they really knew and cared about the students in the department. Especially Dr. Valle who always made time in his extremely busy schedule to talk to me about science or my future. Sandy who always made sure we were on track for graduation and provided us with everything, especially coffee and candy, to get us through the day. I could not have asked for a better department for graduate school.

The other members of the lab allowed not only enabled my success but made coming to lab an enjoyable experience each and every day. My fellow human genetics students and fantastic lab techs continually provided a light hearted and positive working environment through potlucks, lab outings, and general sense of humor and willingness to put up with my incessant teasing. Melissa, Brianna, Jeenah, and Laura, who welcomed me to the lab in my first year and showed me that the Cutting lab was a place where productive lab work could be balanced with fun. Neeraj Sharma was always able to put a positive spin on any type of data and always willing to help us with whatever we needed. Karen Raraigh was incredibly important to my success by helping me put all of my work in context, and helping me understand what would be most important to the patient. Our many conversations about CF related matters refined my projects, but our many other conversations about genetics and life have helped shape my career overall. This experience blended challenging science with life outside of lab and I could not have asked for a better situation to spend 5 years in.

The person most important for my success in the lab is of course my mentor, Garry Cutting, who gave me the opportunity to pursue projects that aligned with my personal research interests while guiding me to become a well-rounded scientist. He emphasized the need for high quality data, multiple lines of evidence for every conclusion, and for analysis of the big picture goal of every project. Garry also emphasized and respected the need to balance lab life with personal life. This allowed me to have a life outside of lab, where I could get married and move to another city while still having a supportive work environment. Garry allowed me to achieve a work-life

balance that suited me and allowed me to have the professional and personal lives I wanted.

Lastly, I would not have been successful without the support of my family and friends. My parents who have made my education a priority and enabled me to be able to spend 5 years working towards this degree. My friends and fellow students who have always been supportive and helped me enjoy my time in grad school. My wife and best friend Tippa supported me in every way possible throughout this entire time, pushed me to do well in school, but also made me take breaks to eat well and to travel. Tippa shows me every day how to focus on what is important in life and how to live a balanced life and I cannot imagine what it would be like otherwise.

While this thesis represents a significant amount of work done by many individuals during my time as a member of this lab, it was made possible by the interactions and advice of the many people I have been lucky to know.

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Chapter 1. Introduction

1.1 Cystic fibrosis is caused by loss of function of the *CFTR* gene

Cystic fibrosis (CF) is caused by loss of function of the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene and demonstrates autosomal recessive (AR) inheritance. CFTR is a member of the ATP Binding Cassette (ABC) protein family but is unique in that it is the only ABC transporter known to act as an ion channel [1], suggesting that CFTR has a highly specialized role. Moreover, most human cells do not express *CFTR* at detectable levels and recent studies have suggested that only certain subtypes of airway cells express *CFTR* [2]. This specialized role of CFTR is likely the reason that *CFTR* is the only gene that causes CF and that severe mutations in *CFTR* only cause CF; mutations in other genes, such as ENaC and CA12, may result in phenotypes which demonstrate some but not all characteristics with CF. This is in contrast to other Mendelian conditions for which multiple genes within related pathways may result in the same or similar phenotypes or cases when variants in a single gene can cause multiple disorders depending on the nature of the variant.

Loss of CFTR function affects multiple major organ systems by disrupting fluid movement across epithelial tissue layers. This disruption causes chronic dehydration of the surface fluids of secretory epithelia which in turn results in chronic obstruction, infection, inflammation, and ultimate destruction of affected organs [3]. The most severely affected organs with regards to human pathophysiology are the lungs, pancreas, and the gastrointestinal tract; the sweat gland and vas deferens are also affected but have no impact on mortality. The uniform etiology of CF makes it an ideal model for molecular genetics research. Complete interrogation of *CFTR* sequence in individuals with CF should yield pathogenic variants in all individuals with CF and correction of

CFTR function should improve clinical outcomes for all individuals with CF. For these reasons, CF provides an ideal platform for molecular genetics research and many of the lessons learned from studying CF may be applied to many other genetic disorders.

1.2 Many genetic variants have been identified in *CFTR*

More than 2,000 variants have been identified in individuals with CF (www.genet.sickkids.on.ca/cftr/). The most common CF-causing variant is p.Phe508del (F508del) and accounts for approximately 70% of all disease causing alleles in the CF population. Consequently, 49% of individuals with CF have two copies of F508del and 91% of individuals carry at least one copy of F508del. Recommended diagnostic panels by the American College of Medical Genetics (ACMG) included the 23 most common CF causing variants [4]; including F508del, this mutation panel encompasses 87% of CF causing alleles and so is able to identify both pathogenic alleles in approximately 75% of individuals with CF and one pathogenic allele in 98% of individuals [5]. Diagnostic panels designed to interrogate known disease-causing variants have expanded in recent years to include additional variants, but many diagnostic labs perform sequencing of the coding regions of *CFTR*. Sequencing allows for interrogation of every nucleotide position of *CFTR*, and not just the specific variants interrogated by panels, thus the number of interrogated positions has expanded from the common disease-causing variant (F508del) to the entire coding region of *CFTR*, and is likely to expand to include the entire gene in the future. Massively parallel next generation (Next Gen) sequencing has revolutionized genetic testing and molecular diagnostics for all genetic conditions by rapidly decreasing the time and cost of DNA sequencing, allowing for cost efficient sequencing of entire

genes, panels of genes, whole exomes, and whole genomes in many research and clinical settings. This has resulted in the accumulation of *CFTR* sequencing data of individuals with CF and CF related symptoms as well as individuals without CF via whole exome sequencing (WES) or whole genome sequencing (WGS) studies of other unrelated phenotypes. However, interrogation of the entire coding region has resulted in the identification of a significant number of variants of unknown consequence [6].

The CFTR2 project (www.CFTR2.org) was established to collect genotype data from individuals with CF around the world to interpret the clinical implications of this large number of sequence variants. Concurrently, a centralized database was created to improve the annotation of DNA variants with respect to their disease liability [5]. To date the CFTR2 project has assembled data from nearly 90,000 individuals from CF patient registries around the world, has identified 1,641 variants, and has classified 322 variants as CF causing, benign, or of varying clinical consequence. This increase in the number of classified variants over the original 23 ACMG variants has increased the diagnostic yield to 97% of all alleles with a clinical interpretation and 94% of all individuals with CF who have both of their pathogenic alleles identified [www.CFTR2.org; Dec 2017]. Of the 1,641 variants identified, 40% are predicted to result in amino acid substitutions, 36% are predicted to alter RNA processing (including premature termination and splice site variants), 3% are predicted to alter CFTR gene structure (including deletions, duplications, and rearrangements), 1% are predicted to impact promoter activity, 14% appear to be neutral and 6% have unknown effect [3]. Annotation of variants continues to progress with the eventual goal of the annotation of all DNA variants in *CFTR* for their disease liability.

Interpretation of sequence variants that do not cause disease or only cause disease in certain contexts is extremely difficult. The presence of a rare sequence variant in an affected individual does not imply that that variant is pathogenic. Hence, the challenge is in distinguishing pathogenic variants from benign variants. Demonstration of the pathogenicity of a variant, can be achieved by interrogation of the functional consequence *in vitro* or observation that a variant is exclusively found in affected or carrier individuals while simultaneously absent in unaffected individuals. Several databases currently exist for evaluating the natural variation found in the human population; gnomAD is currently the largest of these databases and has collected data from 140,000 WES and WGS samples [7]. These types of databases cataloging *CFTR* variants in the general human population will increase the rate of discovery of rare variants and in some cases aid in the interpretation of those rare variants.

1.3 Disease liability of a *CFTR* variant is determined by its effect on CFTR function

Categorization of sequence variants is not as simple as distribution into pathogenic and non-pathogenic as CF, like many genetic conditions, exist on a phenotypic spectrum of variable expressivity. Variable expressivity has been exceptionally difficult to account for in classification; the clinical spectrum of severe CF, mild CF, and CF related symptoms is an important factor for patients, yet most computational and experimental assays only assess variants in a binary manner as predicted to cause disease or not [8]. The largest category of coding variants, in both affected and unaffected populations, are missense variants. Thus, accurate evaluation of missense variants is one of the most important and one of the most challenging tasks of

molecular diagnostics. This has led to the classification of severe pathogenic variants but the majority of variants that cause mild disease or demonstrate incomplete penetrance are often impossible to classify and are labeled as "Variants of Unknown Significance" (VUS). Rare variants present a significant challenge because many rare variants do not cause disease but exist at population frequencies indistinguishable from pathogenic variants. Interpretation of genetic variants has lagged significantly in comparison to the rate at which variants are discovered [9,10] and repeated sequencing of *CFTR* has identified many genetic variants that have received a VUS classification because they cannot be interpreted based on population frequency alone.

Functional studies are a broad classification of tests that aim to understand the consequences of the genetic variants found in an individual with disease. In biochemical genetics functional testing is primarily performed by measuring the activity of a metabolic enzyme isolated from a patient, thereby directly interrogating the *in vivo* function of the enzyme [11]. For CF, direct *in vivo* functional testing of CFTR activity takes the form of measurements of sweat chloride concentration or nasal potential difference (NPD) [12]. Sweat chloride and NPD have become robust diagnostic tests with the ability to detect CF as well as CF related disorders [13]. While these functional tests will confirm the presence of disease in an individual, they do not identify the genetic variant responsible for the phenotype. Sequencing will identify a portion, dependent on the platform used, of genetic variants carried by an individual and subsequent determination of the pathogenicity of those variants will often require *in vitro* studies.

Analysis of CFTR function *in vitro* relies on the development of relevant model systems. Mouse models have been commonly used in genetics research for decades but

are unfortunately an unsuitable model for studying CF [14]. Other larger mammals, such as ferrets and pigs, better represent the complete CF phenotype but are more difficult and expensive to study and limit the size of possible studies. Cellular models and organoids are significantly cheaper and faster so have the advantages of scalability and reproducibility which are necessary to make detailed assessments of function. However cellular models do not permit study of organismal disease progression or study of tissue specific effects of CFTR deficiency. A perfect model system does not exist for CF, but each of the available systems offers utility for studying specific questions regarding CFTR biology and CF pathogenesis.

Cellular models are especially useful for directly interrogating CFTR function under the assumption that all physiological manifestations of CF are a directly correlated with CFTR function, referred to as the presence of a strong genotype to phenotype correlation. Two cellular models utilized for *in vitro* study of CFTR function are Fischer Rat Thyroid (FRT) and CF Bronchial Epithelial (CFBE) cell lines engineered to express specific alleles of *CFTR* [5,8,15]. These cell lines express *CFTR* in a controlled manner and allow for measurement of CFTR function via chloride conductance by measuring short circuit currents (I_{sc}). The results of these studies have been compared with clinical findings of individuals carrying the studied variants to aid in the interpretation of their disease liability [8]. These studies suggest that CFTR function measured by *in vitro* assays has the ability to predict the disease liability of individual *CFTR* variants in cases when clinical data is lacking, or sample sizes are too small to draw strong conclusions.

1.4 Modulator therapies target specific molecular mechanisms of CFTR dysfunction

Understanding the functional consequences of genetic variants allows for development of therapies designed to specifically correct the molecular defect underlying each genetic variant. As the variants that cause CF have varied molecular effects [28], it follows that there could be a separate class of therapeutic for each molecular mechanism. This paradigm has been successful in recent years with the development of two classes of CFTR modulators that have been approved by the FDA for treatment of individuals with CF with certain genotypes. Potentiators increase CFTR function by increasing the frequency with which CFTR opens [29] while correctors stabilize CFTR folding and biogenesis [30].

Ivacaftor was the first CFTR modulator therapy approved to treat CF and was approved for individuals who carried the p.Gly551Asp (G551D) gating variant. Approval for individuals carrying G551D was based on *in vitro* studies of FRT cells, Human Bronchial Epithelial (HBE) cells, and primary nasal epithelial cells [29] which led to clinical trials demonstrating clinical efficacy [31]. Ivacaftor approval expanded over the following 5 years to include 37 additional variants. The first label expansion was based on the combination of cellular [17] and clinical studies [18] of known gating variants. Further expansion was provided on the basis of cellular or clinical studies [22].

Lumacaftor was the second modulator developed, and improves CFTR function by stabilizing CFTR biogenesis and was designed to target the common F508del variant since it presents a significant folding defect [30]. Unfortunately, lumacaftor alone was not found to be clinically effective for individuals homozygous for F508del [20]. However, when utilized in combination with ivacaftor, homozygous F508del individuals demonstrated clinical improvement [21]. A second corrector compound, tezacaftor, has

recently been shown to effectively treat F508del homozygotes and individuals with residual CFTR function when used in combination with ivacaftor [24,32].

The development and application of potentiators and correctors has revolutionized care for individuals with CF and charged efforts to fully understand the molecular etiology of all *CFTR* variants so that individuals carrying those variants can be prescribed the most effective therapy. However, the approved variants are likely only a small fraction of variants that will respond positively to these modulators. Thus, identifying other responsive variants will provide therapy to additional individuals with CF much more quickly than developing new therapies specific to every remaining variant. Due to the extremely low frequency of most variants, clinical studies are mostly impractical as the few individuals who share a rare variant may be located in disparate parts of the world. However, preliminary data for the response of a rare variant to approved modulators can be collected by *in vitro* assays, including engineered cell lines [17,22,23]. Missense variants have been of particular interest to study *in vitro* to inform therapeutic decisions because *in vitro* assays can study many variants and test multiple potential effects at once.

Many coding variants will result in multiple molecular deficiencies and our ability to predict all of the effects of coding variants remains relatively poor, making it difficult to assign complete functionality to many coding variants. However, CFTR biogenesis is not a fully efficient process, at least to the point that CFTR modulators are able to improve the function of WT CFTR [33,34]. Studies have shown that CFTR modulators have the ability to improve function of coding variants independent of molecular mechanism [22,23], as long as they permit production of some protein which can be

targeted by potentiators and corrector compounds. Thus, it is likely that the vast majority of coding variants that produce CFTR protein can be treated by combination therapy, by increasing the chances of addressing the specific defect conferred by a pathogenic variant as well as improving the efficiency of molecular processes that are not deficient. We have found that the most accurate indicator of protein production in an individual with CF is the presence of moderated symptoms, suggesting that most individuals who have moderate phenotypes should demonstrate positive response to CFTR modulators. This concept has been shown in a clinical trial of individuals carrying 'Residual Function' variants [24] and we have demonstrated this *in vitro* using CFBE and FRT cell lines [23]. For these reasons, we believe combination therapy to be the most efficient route to providing therapy to all individuals with CF.

Chapter 2. Functional Assays Are Essential for Interpretation of Missense Variants Associated with Variable Expressivity

2.1 INTRODUCTION

Determining the phenotypic consequences of DNA variants in genes associated with disease is a major goal for genomic medicine [35]. Variants in the coding region of genes can have a variety of consequences that may affect RNA quantity or processing or may alter the sequence of the encoded protein. Missense changes account for ~38% of variants implicated in single-gene disorders [36] and are particularly challenging to interpret as they can produce a broad array of effects, ranging from loss of protein due to severe instability to no discernible consequence. Accordingly, missense variants can create a spectrum of phenotypic consequences that encompass both variable expressivity and incomplete penetrance of clinical features that constitute Mendelian disorders [37]. Interpreting whether missense variants are responsible for partial expressivity of single gene disorders is a major challenge in the clinical and research setting. Indeed, genetic testing is frequently requested to help diagnose individuals with incomplete features of a Mendelian condition. However, labeling missense changes as variants of unknown significance due to lack of functional information does not resolve diagnostic dilemmas.

Expression of mutants in heterologous cell lines provides a versatile method for assessing the functional effect of a wide range of variants [22,38]. The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) have recognized the importance and utility of heterologous expression systems, and have weighted the results of well-established functional testing heavily within their recommended pipeline for variant classification for all Mendelian diseases [10]. These groups emphasize that studies should be validated, reproducible, and robust, and are most helpful when they are reflective of the biological environment in which a

variant operates. Importantly, the ACMG/AMP guidelines recommend that interpretation of functional testing occur within the context of other available information, thereby avoiding placing too much emphasis on data that, while valuable, may not completely reflect a variant's behavior *in vivo*.

Cystic fibrosis (CF) (MIM: 219700) is caused by variants that lead to reduced function of the CF Transmembrane conductance Regulator (CFTR) (MIM: 602421; NM_000492.3). Full expressivity of CF manifests as dysfunction of epithelial tissues in the lungs, pancreas, and sweat duct [12]. However, not all features are consistently present, thereby giving rise to phenotypes exhibiting partial expressivity that can be difficult to differentiate from other causes of lung and/or pancreatic disease [13]. Missense variants are commonly found in association with partial expressivity of CF, thereby providing an opportunity to evaluate the utility of functional assessment in this situation [39]. Because commonly-performed protein folding studies are poor predictors of overall function [22], we chose to evaluate chloride conductance to determine the functional consequences of 48 *CFTR* missense variants reported in individuals that exhibit full and partial expressivity of CF. We show that functional assessment informs variant annotation when full or partial expressivity is present and that uncertainty of variant effect can be re-interpreted in the context of variable expression of a phenotype. Finally, we illustrate the limitations of algorithms that predict the functional consequences of missense variants.

2.2 RESULTS

Establishing wild-type CFTR standards for mRNA level, protein quantity, and function

To approximate the native context of CFTR in the lungs and to enable repeated measures of the chloride channel function of CFTR, we studied *CFTR* variants that were stably expressed in a well-established human airway cell line (CF Bronchial Epithelia; CFBE41o-) [40]. To determine the degree to which variants altered function relative to wild-type CFTR (WT-CFTR), we independently derived 10 CFBE41o- cell lines expressing a single copy of wild-type *CFTR* cDNA. The level of *CFTR* mRNA expressed in each of the 10 cell lines varied, as previously reported in cell lines with single site integrations [22], as did the level of CFTR protein and CFTR function (**Fig 2.1A-C; Table S3**). Since mRNA expression in each CFBE cell line was stable over time [15], we assessed the degree of correlation between RNA level determined by quantitative reverse transcription PCR (qRT-PCR) and mature (band C) protein level assessed by quantitative Western blotting; the correlation was linear and robust ($R = 0.92$; $p = 1.59 \times 10^{-4}$; **Fig 2.1D, left panel**). Likewise, CFTR protein level and CFTR function determined by short circuit measurement for each of the 10 lines show excellent linear correlation ($R = 0.99$, $p = 1.5 \times 10^{-7}$; **Fig 2.1D, middle panel**). Finally, mRNA levels and CFTR function correlate well ($R = 0.94$; $p = 7.06 \times 10^{-5}$; **Fig 2.1D, right panel**), indicating that *CFTR* mRNA levels could be used to normalize CFTR chloride currents among independent cell lines.

Determining the function of CFTR mutants relative to wild-type

To establish the relationship between mRNA level and function across the full range of WT-CFTR expression, we combined data from 14 additional cell lines with the prior 10 WT-CFTR cell lines (total 24) to derive the slope of the linear correlation between *CFTR* mRNA quantity and CFTR function ($R = 0.84$; $p = 3.37 \times 10^{-7}$; **Fig 2.2A**

and **Table 2.S3**). A minor correction was made so that the slope intersected with the origin, under the assumption that zero mRNA should correspond to zero CFTR chloride current since parental CFBE cells express no detectable *CFTR* mRNA and generate no CFTR chloride current. With the slope set at 100% WT-CFTR, we then calculated slopes representing 25%, 10%, and 1% WT-CFTR function across the range of observed mRNA expression in CFBE cells (**Fig 2.2A**). These functional levels were chosen as they reflect approximate thresholds that transition from fully expressive to partially expressive forms of CF with the 10% to 25% threshold representing the range at which most individuals escape life-limiting lung disease [41–47].

To ensure that expression level of CFTR variants are comparable to native tissues, the mean RNA levels of CFTR relative to the housekeeping gene *HPRT1* were compared using qRT-PCR in the 24 WT-CFTR cell lines or by extracting data from RNA sequencing of a different WT-CFTR and a G551D-CFTR cell line (0.49 vs 0.57; p=0.44; **Fig 2.S1A**). Using the entire set of RNA sequencing data, we determined *CFTR* transcript levels relative to all other RNA transcripts in the CFBE cell lines and compared these levels to *CFTR* mRNA levels in primary tissues affected by CF (study accession numbers in **Table 2.S2**). The mean level of *CFTR* mRNA in the CFBE cell lines is higher than in bronchial and nasal epithelia while lower than its expression in the pancreas (**Fig 2.S1B**). By extrapolation to the qRT-PCR data, we concluded that *CFTR* mRNA levels in the WT-CFTR cell lines are within physiologic ranges of endogenous *CFTR* mRNA levels in airway and pancreatic epithelia.

To better discern *CFTR* variants with low residual function, chloride channel current was plotted on a semi-log chart against *CFTR* mRNA level (**Fig 2.2B**). To

determine the function of CFTR bearing putative CF-causing variants as a fraction of WT, we normalized currents generated by CFTR mutants using the level of mRNA expression in each CFBE cell line. Four examples are presented. Independently-derived cell lines stably expressing F508del (c.1521_1523delCTT; p.Phe508del)-CFTR generated currents ranging from 0.5% to 1.4% of WT-CFTR with a mean of 0.9% WT-CFTR after normalization for mRNA level (**Fig 2.2B**). This estimate of function is consistent with the association of F508del with full expressivity of CF and measurement of F508del-CFTR function in Fischer Rat Thyroid (FRT) cell lines (0.2% WT) and in primary bronchial airway cells (range 0.5% to 3.4% in F508del/F508del individuals) [5,48–51]. CFBE cell lines stably expressing T338I (c.1013C>T; p.Thr338Ile)-CFTR generated currents estimated at 6.4% of WT. The residual function of T338I-CFTR is consistent with full expressivity of CF observed in individuals with this variant, albeit a less severe phenotype than observed with F508del [5,52]. Variant G622D (c.1865G>A; p.Gly622Asp) permitted higher levels of CFTR function at 18.2% of WT-CFTR, consistent with its partial expressivity [53]. Finally, the substitution of cysteine for phenylalanine at codon 508 (F508C [c.1523T>G; p.Phe508Cys]) had no reduction of CFTR function in two CFBE cell lines (mean 114% WT). F508C has been shown to have minimal effect on CFTR folding and function [54], consistent with evidence that F508C does not cause disease when found in healthy CF carriers of F508del [55].

<u>Functional assessment distributes variants according to expressivity and informs assignment of disease liability using expert annotation criteria</u>

Forty-eight missense variants ranging from 0.002% to 0.042% frequency in the CF population were selected from the CFTR2 database (**Table 2.1 and Table 2.S1**). Twenty-nine variants are associated with clinically diagnostic elevations in sweat chloride concentration (≥60 mEq/L) and life-limiting lung disease, consistent with full expressivity of CF. The remaining 19 variants are associated with partial expressivity (elevated but non-diagnostic sweat chloride concentration [31-59 mEq/L] and variably present life-limiting lung disease). Four variants (F508del, G551D, I336K [c.1007T>A; p.Ile336Lys], and T338I) that have been extensively studied in other cell lines and/or primary cells were included to validate our functional assay (total 52 variants). None of the variants are predicted to cause aberrant mRNA splicing using CryptSplice and NNSplice algorithms, each demonstrated to have >80% sensitivity[19,56,57]. CFTR function of the 52 missense variants was determined in CFBE stable cell lines that were normalized for mRNA levels as shown above (**Table 2.1**; data for individual clones are reported in **Table 2.S4)**.

Twenty-one of the 29 variants associated with full expressivity generated less than 10% WT-CFTR function, a conservative threshold for the development of life limiting lung disease, and were assigned as CF-causing using expert annotation criteria as previously described [5]. Another 4 variants (P5L [c.14C>T; p.Pro5Leu], D110E [c.330C>A; p.Asp110Glu], F1099L [c.3297C>A; p.Phe1099Leu], and T1246I [c.3737C>T; p.Thr1246Ile]) reduced function to 10-25% of WT-CFTR, a range consistent with their less severe phenotype. Since expert annotation uses a highly conservative 10% threshold to define CF-causing, these 4 variants were assigned as varying clinical consequences (VCC). Of the 4 remaining variants observed in

individuals with full expressivity of CF, one (E588V [c.1763A>T; p.Glu588Val]) had 27.5 ± 6% WT-CFTR function, which overlaps with the 10-25% WT-CFTR functional range described above and consistent with a less severe phenotype. However, the remaining three (V562I [c.1684G>A; p.Val562Ile], D836Y [c.2506G>T; p.Asp836Tyr], and S912L [c.2735C>T; p.Ser912Leu]) allowed 71.2-121.1% WT-CFTR function (**Table 2.1**). Even though these variants were presumed disease-causing and were reported as such by clinicians, functional evidence indicates that they are not deleterious. Notably, all three variants have been reported to occur as part of complex alleles (**Table 2.S5**) involving other known or likely deleterious variants *in cis* [26,58–60], hence potentially explaining their presence in individuals with CF.

None of the 19 variants associated with partial expressivity of CF had less than 10% WT-CFTR function, as expected (**Fig 2.3)**. Five variants generated 10-25% WT-CFTR function, consistent with variable disease presentation, and were assigned as VCC. However, 10 variants generated between 25 and 75% WT-CFTR function, which should be sufficient to escape life-limiting lung disease. Six of these 10 variants were reported in individuals with clinical features consistent with a diagnosis of CF and were characterized as VCC. The remaining 4 in this group were reported in individuals who did *not* have clinical features consistent with a diagnosis of CF; however, because they reduce CFTR function by more than 25%, their role in the development of CF disease processes is unclear and their disease liability is unable to be determined. A further 4 variants associated with partial expressivity of CF had minimal to no effect on CFTR function (75-100% WT-CFTR) and were assigned as non CF-causing, including one variant (F508C) with previously-published evidence of lack of CF phenotype when

present *in trans* with F508del [55,61]. Of the 14 variants associated with >25% WT-CFTR function, 8 are associated with previously-reported complex alleles (**Table 2.S5**), which complicates our ability to directly correlate variant function with phenotype. We suspect that the identification of these 'indeterminate' or 'non CF-causing' missense variants in individuals with CF may have led to the erroneous assumption that they were deleterious (http://www.genet.sickkids.on.ca). Thus, functional testing was informative for assessment of variants associated with full expressivity and essential for interpretation of variants found in individuals with partial expressivity of CF.

Functional data informs assignment of disease liability using ACMG/AMP criteria

Use of ACMG/AMP criteria for disease assignment, which incorporates evidence from a variety of sources, demonstrated good correlation with expert annotation (**Table 2.2;** individual variant annotations in **Table 2.S6**). Inclusion of functional data in the ACMG/AMP algorithm enabled assignment of 4 VUS as likely pathogenic and 17 likely pathogenic variants could be assigned as pathogenic (**Table 2.2, upper panel**). At the other end of the spectrum, 2 of the 6 variants assigned as non CF-causing by CFTR2 criteria could be moved from VUS to likely benign or from likely benign to benign. The ACMG/AMP criteria also distributed the 16 variants of varying clinical consequence more precisely when applying a 25% threshold for defining a deleterious variant. Thus, functional data were particularly useful for verifying and excluding pathogenicity, thereby improving the assignment of variants as fully expressive or benign.

To confirm that ACMG/AMP classifications maintained good correlation with expert annotation for more common variants, we also applied the guidelines to the 74

missense variants previously characterized by the CFTR2 team, many of which occur more frequently than those reported in this manuscript and some of which have been widely studied by other groups. ACMG/AMP annotations were reviewed by a CAP-accredited and CLIA-certified clinical laboratory director with multiple years of experience in variant classification (M.B.S). Excellent correlation between ACMG/AMP and CFTR2 annotation was again observed, with all 50 CF-causing variants being assigned as pathogenic or likely pathogenic and 10 of 11 non CF-causing variants being assigned as benign or likely benign using ACMG/AMP guidelines when functional data was considered (**Table 2.2, lower panel;** individual variant annotations in **Table 2.S6**). Inclusion of functional data again enabled distribution of the variants annotated as causing varying clinical consequences and confirms its utility in determining pathogenicity.

Algorithms fail to distinguish variants associated with variable expressivity

To assess the ability of algorithms to predict the effect of the missense variants upon protein function, we evaluated four methods (CADD, REVEL, SIFT, and PolyPhen-2) commonly used in diagnostic and research settings. To ensure that variant scores for CADD and REVEL could reliably predict well-studied, relatively common, fully penetrant missense variants as deleterious, we first tested 6 *CFTR* missense variants that are included in the ACMG-recommended panel of CF-causing variants (A455E [c.1364C>A; p.Ala455Glu], G551D, G85E [c.254G>A; p.Gly85Glu], N1303K [c.3909C>G; p.Asn1303Lys], R334W [c.1000C>T; p.Arg334Trp], and R347P [c.1040G>C; p.Arg347Pro]). All six variants exceeded the recommended threshold score

for assignment as deleterious by CADD (PHRED score of 15) and scored highly (mean score of 0.88 out of 1) using REVEL, which does not have a specific recommended cutoff for deleteriousness. Next, an additional 30 variants previously classified as CF-causing [5] were scored as deleterious using CADD, confirming its ability to correctly predict CF-causing variants even at lower frequencies. This group of 30 and the previously-described 6 ACMG variants were used to determine the appropriate REVEL cutoff for deleterious, which was set at 0.659 (two standard deviations below the mean score of all 36 variants) and which corresponds to 62% specificity and 95% sensitivity [62] (**Table 2.S7**).

We next scored the 48 missense variants functionally studied here using REVEL and CADD and used the thresholds described to assign pathogenicity. Two other commonly used methods that provide categorical assignments (SIFT and PolyPhen-2) were also employed (**Table 2.3**). Each method classified the majority of variants as deleterious (**Table 2.4**). Consequently, accuracy was quite high when calling variants that reduced function below 10% of WT, the level at which full expressivity is present. Likewise, accuracy remained high if 25% of WT-CFTR function was used, although there is less evidence that reduction in function to between 10% and 25% is fully expressive for CF (see above). However, all four methods over-called variants as deleterious that were shown to have greater than 25% WT-CFTR function, leading to high numbers of false positives and low specificity, which ranged from 28% (REVEL) to 6% (CADD). Finally, all four methods were inaccurate in predicting the consequences of the 6 variants that allowed CFTR to function in the normal range (>75% WT-CFTR) (**Table 2.4**). Two programs (CADD and SIFT) predicted all 6 variants to be deleterious

while REVEL and PolyPhen-2 predicted 5 of the 6 to be deleterious. Conversely, while false negative rates were low, SIFT was a notable exception as the algorithm called 6 of 30 variants tolerated, even though functional testing found that 4 of the 6 had less than 10% and the remaining 2 had less than 25% WT-CFTR function.

To further assess the reliability of the predictive algorithms with variants known to be benign for CF, we also evaluated five relatively frequent CFTR variants which have minimal effect on function and multiple lines of evidence of non-penetrance. All four tools predicted at least 2 variants to be deleterious, meaning that they over-called 40% (SIFT and Poly-Phen2) or 60% (CADD and REVEL) of variants as deleterious though they are accepted as benign (**Table 2.S8**). Moreover, three of the five variants (G576A [c.1727G>C; p.Gly576Ala], R668C [c.2002C>T; p.Arg668Cys], and S1235R [c.3705T>G; p.Ser1235Arg]) were inconsistently predicted by the four tools; only one variant (V470M [c.1408G>A; p.Val470Met]) was consistently and correctly deemed non-deleterious and another (R75Q [c.224G>A; p.Arg75Gln]) was consistently but incorrectly deemed deleterious by all four tools.

2.3 DISCUSSION

As the number of identified variants continues to increase, interpretation of missense variants and their contribution to expressivity of Mendelian diseases presents a growing challenge to both researchers and clinicians. To inform this process, broader use of laboratory-based functional assays has been advocated [10,63]. Wide application of functional tests could increase the accuracy of variant interpretation in genes with both known and unknown association with disease, generate information and reagents

necessary for testing therapeutic agents, and inform the development of analytic tools for predicting variant effect [35]. Each of the aforementioned benefits was evident from our analysis of 48 *CFTR* missense variants associated with variable expressivity of CF.

Assaying effects of missense variants upon protein function is a well-established approach to interpreting disease liability [64,65]. A desirable situation is to test mutant function in primary cells obtained from subjects, but this is challenging because: 1) samples are not easily collected from difficult-to-access tissues; 2) extremely rare variants may exist only in a few individuals worldwide; 3) primary cells undergo few cell divisions, limiting culturing and expansion; and 4) individual factors (e.g. background variation in the cellular genome) may confound the interpretation of variant effect. Together, these factors limit the number of variants that can be reliably measured in primary cells. High-throughput assays that utilize uniform measures of function, such as assessments of enzyme activity or protein binding, are useful for testing many variants but may not provide detailed functional information for non-soluble proteins or those with complex functions [66,67]. Most non-secreted proteins can be analyzed in cell-based systems that discern severity of defect by comparing the mutant function to wild-type [68]. Thus, heterologous cell-based systems provide a viable option for variant interpretation in the research and potentially the clinical laboratory [6,26]. Such systems are not without challenges; transient expression of mutants may have variability in transfection rates, cell growth, and other factors that lead to significant differences in expression level and complicate assessment of mutant function. As shown here, many of these problems can be addressed in systems that enable stable expression of a mutant.

Numerous *CFTR* mutants have been functionally assessed by stable expression in Fischer Rat Thyroid (FRT) cells; this system has garnered FDA approval for drug label expansion to new variants. While FRT cells have provided a useful platform, they are from a different species and tissue type than human airway epithelia, in which *CFTR* defects cause life-shortening lung disease. For this reason, we elected to use a CF airway epithelial cell line shown to be a viable substitute for primary airway cells [15,69,70]. By using isogenic cell lines stably expressing CFTR, we were able to normalize for the level of expression among cell lines. This step was critical to interpreting partially functional variants expressed at different levels among individual clones. Without this normalization, minimally functional variants expressed at high levels and high-functioning variants expressed at low levels could appear to have the same effect on function. Our approach to functional studies can be applied to almost any protein of interest since many different cell lines with targetable integration sites are available from academic and commercial sources.

Functional characterization revealed expected and unexpected results. Thirty variants associated with full or partial expressivity of the CF phenotype generated 25% or less WT-CFTR function. This result is consistent with our current understanding of the level of CFTR function associated with a CF phenotype [44–47]. Notably, the 21 variants with less than 10% WT-CFTR function were associated with full expressivity of CF. Conversely, 6 of 48 variants did not affect the assayed function of CFTR, despite being reported as putative disease-causing variants in individuals with CF. With no meaningful reduction in function, these variants are likely *in cis* with other deleterious variants that may be unidentified by the genotyping methodology used. Indeed, 5 of the 6 variants

with function >75% WT-CFTR exist *in cis* with known or likely deleterious *CFTR* variants [26,59,60,71]. This observation emphasizes that individuals with an unambiguous clinical diagnosis of a recessive loss-of-function disorder who carry high-function variants should have sequencing and deletion/duplication analysis to search for deleterious *in cis* variants. Evaluation of a variant found to exist as part of a complex allele may warrant further functional studies to accurately assess overall allele contribution to disease.

In the absence of other deleterious *in cis* changes, alternative mechanisms of action not detectable by our methodology should be considered for missense variants with minimal reduction in chloride conductance. It is possible that these variants affect an untested function of CFTR such as bicarbonate transport, as seen in individuals presenting with pancreatitis [72]. However, the clinical features of some individuals bearing these variants are consistent with CF and all are followed by a CF specialty care center. We also believe that all variants should demonstrate the same relationship between mRNA level and CFTR function as WT-CFTR (as suggested by **Fig 2.2**), and that none of the missense variants tested had a significant impact on mRNA stability, though this mechanism has been suggested by others studying a synonymous change at codon 507 [73]. Another possibility is an effect on translation speed by use of rare tRNAs, as shown with the synonymous change T854T (c.2562T>G; p.Thr854T) [74], or up- or down-regulation of other genes resulting from changes within CFTR [75]; however, these effects are rarely reported to result from amino acid substitutions. Given the relative frequency of *in cis* changes within *CFTR*, complex alleles remain the most

likely explanation for the observation of variants with high function in individuals with CF [26,59,60,71].

Twelve variants caused a modest decrease in CFTR function (25% to 75% of WT-CFTR), a level generally viewed as not causative for CF, but which may be influenced by other factors and result in partially or fully expressive CF in some individuals. Emerging data, such as the presence of additional *in cis* variants reported in the literature for 5 of these 12 variants, or that genetic and/or environmental modifiers conspire to the development of a range of CF symptoms when CFTR function is modestly compromised [76], may aid in further refinement of disease liability in the future. These findings illustrate that functional studies can disclose unexpected discrepancies that require further investigation to explain mechanism of disease and prevent mis-assignment of disease liability, even in a well-studied disorder such as CF. Functional analysis is likely to be of equal or potentially greater utility for more recently discovered disease-associated genes[64,65].

The ACMG/AMP guidelines for the interpretation of sequence variants generally had good correlation with expert annotation, particularly for variants with <25% WT-CFTR function. However, one must carefully define the phenotype of interest so that the terms ‘pathogenic’ and ‘benign’ are used appropriately. For example, a variant associated with a CFTR-related disorder such as isolated male infertility could be deemed pathogenic for that phenotype, but may be benign in the context of fully expressive CF [77]. Clinical laboratories are often not provided detailed phenotype information, making it challenging to decide in what disease context to evaluate a variant [6]. Indeed, an investigation into annotation discrepancies identified functional data and population data

as frequent contributors to differing interpretations [78]. Our study also revealed that correlation between ACMG/AMP and CFTR2 annotation is incomplete since there is no defined category for variants causing partial expressivity using ACMG/AMP guidelines. CFTR2 uses the term 'varying clinical consequence' for variants that may cause CF in some people but not others, but which typically have enough of a functional deficit that they cannot be considered non CF-causing. As shown here, these variants have reduced CFTR function and are associated with CFTR-related symptoms that do not meet the diagnostic criteria for CF in all individuals bearing them. If CF is the defined phenotype for which ACMG/AMP guidelines are being applied, it may be difficult to assign these variants as pathogenic or likely pathogenic (without clear evidence of CF in some people), but the term VUS is not appropriate because, in many cases, clinical significance is *not* uncertain. A similar dilemma is faced in annotation of *BRCA2* variants in which a probability threshold and annotation for deleterious variants associated with only moderate risk for cancer have yet to be determined [79]. The ongoing challenge of annotation of variants with moderate functional effects lends itself well to the incorporation of quantitative functional results and their associated endophenotypes (continuous-valued quantitative traits) [80] into a variant classification scheme.

Considerable effort has been devoted to the development of computational tools that predict variant effect. Most tools consider evolutionary conservation, properties of the native and variant amino acids and their possible impact on structure and stability, or both [81]. Machine learning using a subset of annotated variants can incorporate more and varied types of information, but is limited by data quality, quantity, and relevance of the training set, often leading to low sensitivity, low specificity, and incongruent variant

calls [79,82–84]. The *in silico* tools evaluated here, selected because they are either commonly used individual predictors (SIFT and PolyPhen-2) [85] or are more recently-developed ensemble predictors (CADD and REVEL), had good performance for functionally deleterious variants that lead to full expressivity of CF. However, they could not discern functional levels that distinguish full from partial expressivity of CF. The main issue lies within the binary output of most models, which predict *whether or not* a variant has an effect but not its *magnitude* [86]. This likely leads to the over-calling of benign variants as deleterious, some of which may minimally affect protein function but at a level that does not impact phenotype. When we compared functional results and disease liability assignments to the variant effect predictions of four *in silico* tools, we found that the tools overestimate deleteriousness, a trend also seen in other genes with well-annotated or functionally-assessed variants [79,87,88]. In the current exome and genome sequencing era, clinicians and researchers using these *in silico* tools to assess the pathogenicity of missense variants discovered in putative disease-causing genes in the absence of other supporting data should be cautious[6,89].

In summary, investigation into the effects of 48 missense variants in *CFTR* has filled a gap in knowledge regarding the function associated with partial expressivity of a recessive Mendelian phenotype. This work will also inform the testing of newly-developed therapeutics on rare *CFTR* variants that are not well-suited for clinical trials.

2.4 Materials and Methods

Selection of CFTR variants

CFTR variants for study were selected from de-identified demographic and clinical data collected by the CFTR2 project from 88,664 individuals currently or previously alive and followed by a CF specialty center in 41 countries. Data were provided by national CF patient registries and by major clinical centers in countries with no registry (**Table 2.S1** [contributors of variants reported in this manuscript] and **Acknowledgments** [all CFTR2 contributors]). *CFTR* genotype and sweat chloride concentration were obtained from the individual's clinical record as previously reported [5]. Missense variants of unknown effect were selected for study if they were reported in at least three individuals in the CFTR2 database and if the average sweat chloride concentration of individuals bearing the variant of interest in *trans* with a severe CF-causing variant fell within a range of minimal to moderate elevation of 31 to 102 mEq/L.

RNA sequencing analysis

RNA sequencing raw reads of CF bronchial epithelial (CFBE) cells expressing wild-type CFTR (WT-CFTR) and G551D (c.1652G>A; p.Gly551Asp)-CFTR [15] (n=6), pancreas (n=3), lung tissues (n=3), nasal epithelia (n=3), and ALI bronchial epithelia (n=3) were obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (study accession numbers in **Table 2.S2**). Transcripts were assembled and abundances were estimated with the Tuxedo software suite. Raw reads were mapped to the reference genome (hg19) with TopHat (v2.0.13), using Bowtie2 (version 2.1.0.0). Mapped sequences were assembled with Cufflinks (v2.2.1). CuffQuant was used to estimate the relative abundances of gene transcripts among samples and CuffDiff was then used to determine differential expression values

among samples. For comparison with RT-PCR fold-change values, *CFTR* expression was measured as a fold-change relative to *HPRT1* (*CFTR* FPKM / *HPRT1* FPKM).

Derivation and analysis of CFBE cell lines expressing CFTR variants

Site-directed mutagenesis

Site-directed mutagenesis of pEF5/FRT plasmid, utilizing human eF1α promoter coupled to WT-*CFTR* cDNA sequence, was performed using mutagenic primers designed using the Agilent QuikChange primer design website. PCR products were digested with Dpn1 to remove parental plasmid and then ethanol precipitated, rehydrated, and used to transform XL10-Gold Ultracompetent cells (Agilent). DNA minipreps were prepared (Denville Spinsmart Plasmid Miniprep DNA Purification Kit) and Sanger sequencing confirmed the presence of the variant of interest. Sequence-confirmed miniprep plasmid was used to transform DH5α competent cells (Invitrogen), and DNA maxipreps were prepared (Qiagen Plasmid Plus Maxi Kit). Sanger sequencing of the entire *CFTR* cDNA confirmed the presence of the variant of interest and the absence of secondary changes.

Generation of human airway epithelial cell lines with integrated CFTR variants

CF bronchial epithelial (CFBE41o-) cells containing a Flp Recombinase Target (FRT) integration site [15] were grown in complete media supplemented with 100µg/ml Zeocin (Gibco or ThermoFisher). Prior to transfection, cells were seeded in collagen-coated 6-well plates and grown to >70% confluency. Collagen coating was achieved by applying a mixture of 5ml of 0.1% bovine serum albumin (MilliporeSigma), 500µl rat tail Collagen I (Life Technologies – 3mg/ml), and 500µl of human fibronectin (Sigma-Adrich – 1mg/mL) diluted in 44 ml MEM to each well, before aspirating the mixture and allowing the plates to dry for at least 1 hour. 0.5µg of *CFTR* plasmid combined with

4.5µg of pOG44 Flp-recombinase plasmid was transfected using Lipofectamine LTX (Life Technologies). Cells were incubated for 48 hours and then split 1:4 into collagen coated 6-well plates. Media was changed after 48 hours to include 50µg/ml Hygromycin B for 24-48 hours then changed again with media containing 100µg/ml Hygromycin B. Cells remained under Hygromycin selection until distinct clones were observed in transfection wells and all cells in mock-transfected wells had died. Individual clones were isolated using 8x8mm sterile cloning cylinders (Millipore) and grown in collagen-coated 24-well plates until confluency, when they were expanded to uncoated vessels for characterization.

Verification of CFTR cDNA integration by PCR

Genomic DNA was extracted from Hygromycin-resistant cells using Qiagen DNeasy Blood & Tissue Kit and full length *CFTR* cDNA was PCR amplified to confirm plasmid integration. PCR of the Flp-In site was also performed to confirm disruption of the target FRT site. PCR fragments containing the sequence variant of each cell line were sequenced to confirm the presence of the variant in the genomic DNA of the cell line.

Quantification of CFTR RNA by real-time PCR

Total RNA was isolated from cultured cells on the same day short-circuit current (I_{sc}) measurements were taken (see below). Cells were washed with PBS made with DEPC-treated H_2O and lysed by addition of 250µl of TRIzol reagent (Life Technologies) followed by centrifugation through a shredder column (Denville). One hundred µl chloroform was added to cell lysates and were centrifuged at 12,000xg for 5 minutes at 4C. Clear supernatant was kept for RNA purification using the Total RNA Mini Purification Kit (Denville). 500ng of total RNA was used to generate first strand cDNA

libraries using the iScript™ cDNA Synthesis Kit (Bio-Rad). RNA and cDNA preparations were made each day that short circuit current measurements were taken. cDNA samples were amplified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on the CFX Connect Real-Time System (Bio-Rad). Two sets of PCR primers were designed to generate short fragments (121bp and 102bp) of *CFTR* cDNA which spanned exon-exon junctions. PCR primers for housekeeping genes were purchased from BioRad. Reactions were performed in triplicate for 30 cycles and ΔCt was calculated for each sample.

Quantification of CFTR protein by Western blot

Whole cell lysate was purified from WT-CFTR CFBE stable cell lines using 250µl of RIPA protein lysis buffer (MilliporeSigma) supplemented with 1% protease inhibitor cocktail (MilliporeSigma) and 0.1% serine protease inhibitor, PMSF (MilliporeSigma). Cells were further lysed by vortexing and lysates were collected after centrifugation. 100µg of protein lysate was diluted to 21µl in PBS and incubated with 7µl dye solution containing a 1:5 dilution of DTT to 4X Laemmli Sample Buffer (BioRad) at 37C for 15 minutes. Samples were run in a 7.5% Tris-HCl, 1.0 mm BIO-RAD Criterion Precast Gel with running buffer composed of 25mM Tris, 250mM electrophoresis grade glycine (pH 8.3), 0.1% SDS in dH_2O. Protein was transferred to a PVDF membrane by electrophoresis using the Trans-Blot Turbo Transfer System (BioRad) at 2.5A, 25V for 10 minutes. Membranes were cut above the 100kDa band, reserving the upper half for visualization of CFTR protein and the lower half for visualization of Na^+/K^+-ATPase. Membranes were blocked for one hour in 5% non-fat dry milk reconstituted in PBS containing 0.1% tween-20 (PBST). Membranes were washed in PBST and then incubated

for 1 hour at room temperature with primary antibody: anti-CFTR m596 antibody (UNC) diluted 1:1000 or anti-Na^+/K^+ antibody (abcam – ab76020) diluted to 1:100,000. Primary antibody was removed by washing with PBST for 30 minutes. Membranes were incubated for 1 hour at room temperature with secondary antibody: anti-mouse (CFTR) (GE Healthcare) diluted 1:150,000 or anti-rabbit (Na^+/K^+) (GE Healthcare) diluted 1:150,000. Secondary antibody was removed by washing with PBST for 45 minutes. Membranes were imaged on high performance chemiluminescence film (GE Healthcare) using ECL Prime Detection Reagent (GE Healthcare) and the Kodak X-Omat 2000A Processor.

CFTR protein was quantified using the ImageJ software by dividing the intensity of CFTR C-band by the intensity of the Na^+/K^+-ATPase band for exposures that had not reached saturation. CFTR protein quantity represents an average of 3 of Western blots, with 5-21 exposures measured for each cell line.

Assessment of CFTR function by short-circuit current measurement

$1x10^5$ cells were plated onto Snapwell filters (12 mm filter diameter with 0.4 μm pore diameter; Corning Costar #3407) for 6 days with daily feeding, resulting in a transepithelial resistance of at least $200\Omega \cdot cm^2$, although the specific resistance achieved was variable between cell lines. Filters were mounted into Ussing chambers and short circuit currents (I_{sc}) were measured with a VCC MC6 or VCC MC8 multichannel voltage-current clamp amplifier (Physiologic Instruments). Asymmetric apical and basolateral buffers were used to create a chloride gradient, with the apical buffer composed of 145mM NaGluconate, 1.2mM $MgCl_2$, 1.2mM $CaCl_2$, 10mM dextrose, and 10mM HEPES, and the basolateral buffer composed of 145mM NaCl, 1.2 $MgCl_2$mM,

1.2 $CaCl_2$mM, 10mM dextrose, and 10mM HEPES. Buffers were maintained at 37°C and air was bubbled in to introduce circulation. After stabilization of transepithelial current, 10μM forskolin (Selleckchem) was added to the basolateral chamber to stimulate generation of cAMP and activation of CFTR, followed by administration of 10μM CFTR inhibitor-172 (Selleckchem) in the apical chamber to block CFTR-mediated currents. Data were acquired with the software Acquire and Analyze (Physiologic Instruments). I_{sc} changes (ΔI_{sc}) were calculated by taking the difference in the I_{sc} recorded after adding Inh-172.

In silico *prediction models and ACMG/AMP classification criteria*

Four *in silico* prediction models were applied to determine the predicted effect of missense variants on protein function: CADD [90], REVEL [62], SIFT [91], and PolyPhen-2 (HumVar model) [92]. These programs were accessed via their respective online tools. CADD and REVEL had specific thresholds for deleteriousness applied (CADD PHRED score of 15, as recommended by the program developers, and REVEL score of 0.659, as determined by a subset of previously-defined CF-causing variants). SIFT and PolyPhen-2 distinguish between damaging and tolerated and benign, possibly damaging, and probably damaging, respectively. All missense variants (total 122), including those reported in this manuscript and those previously-published on the CFTR2 website, were also characterized according to the ACMG/AMP variant classification guidelines as pathogenic, likely pathogenic, uncertain significance (VUS), likely benign, or benign [10]. Interpretations were assigned with and without the inclusion of functional testing data and were reviewed by the director of a CAP-accredited and CLIA-certified clinical laboratory.

Figure 2.1 *CFTR* mRNA, protein level, and function are variable but correlated. **(A)** Mean and standard deviations of *CFTR* mRNA transcript quantity relative to *HPRT1* (n =3 for each cell line) for 10 independent cell lines expressing WT-CFTR. (**B**) Western blot detecting varying quantities of mature (Band C) CFTR protein from whole cell lysates of 10 cell lines expressing WT-CFTR. Controls include cell lines expressing the CF-causing variants F508del that causes a folding defect (Band B only) and G551D (Band C), non-transfected CFBE cells (no signal), and WT-CFTR transiently expressed in HEK293 cells (Band C). Loading controls for protein quantity (Na+/K+ ATPase) are shown below. Plot on right shows mean and standard deviations of CFTR protein quantities for each cell line relative to the WT-CFTR 5 cell line assessed from at least 3 Western blots. (**C**) Representative recordings of CFTR function measured by I_{sc} for 10 WT-CFTR cell lines. Forskolin (10 μM) activates CFTR chloride current and the amount of current inhibited by the CFTR-specific inhibitor inh-172 (10 μM) determines the level of CFTR function. Plot on right shows mean and standard deviations for I_{sc} derived from at least 3 measurements for the 10 WT-CFTR cell lines. (**D**) Correlations of the quantity of *CFTR* mRNA with quantity of mature CFTR protein (left panel); quantity of mature CFTR protein with CFTR function (center panel); and quantity of *CFTR* mRNA with CFTR function (right panel) for 10 independent cell lines expressing WT-CFTR.

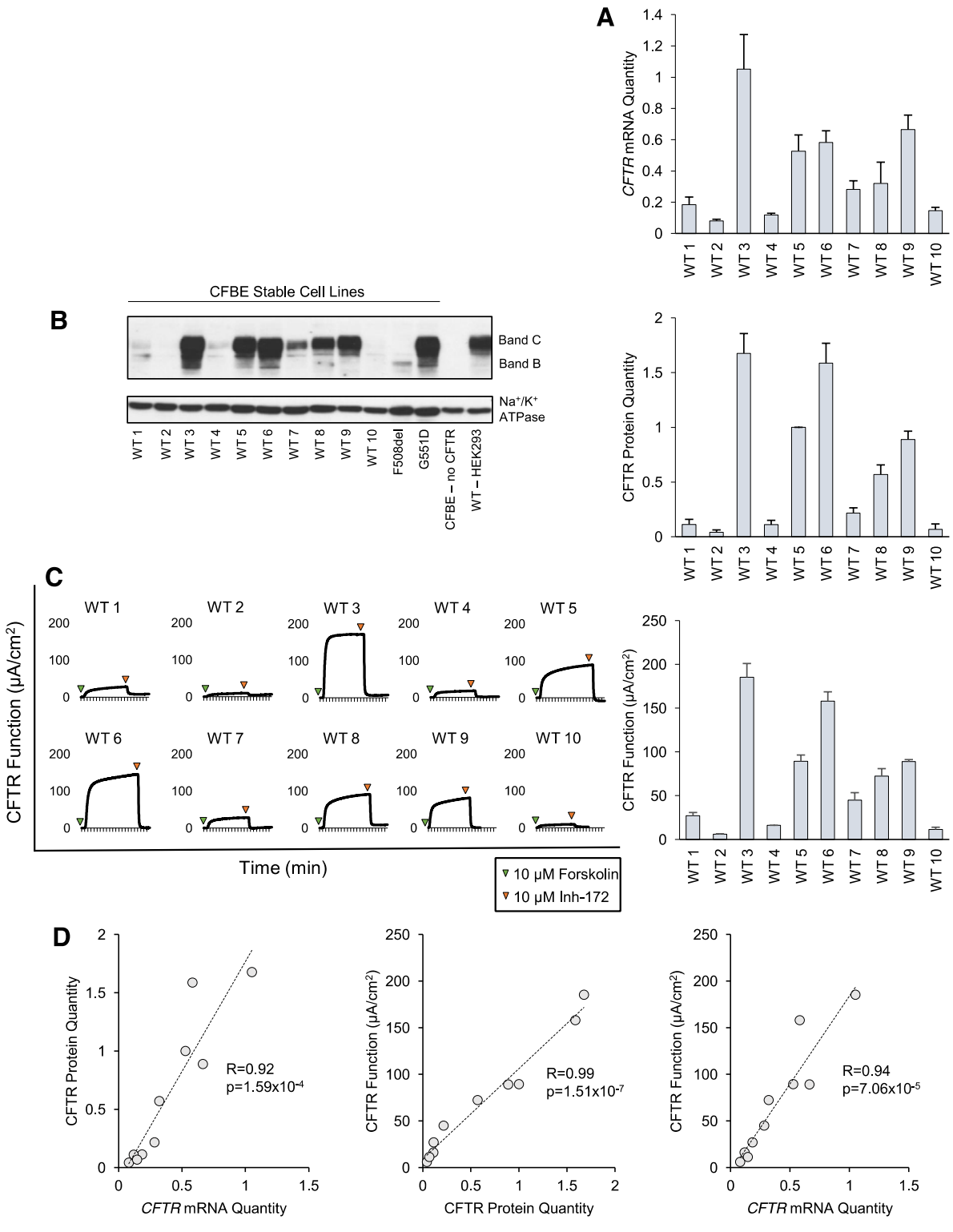
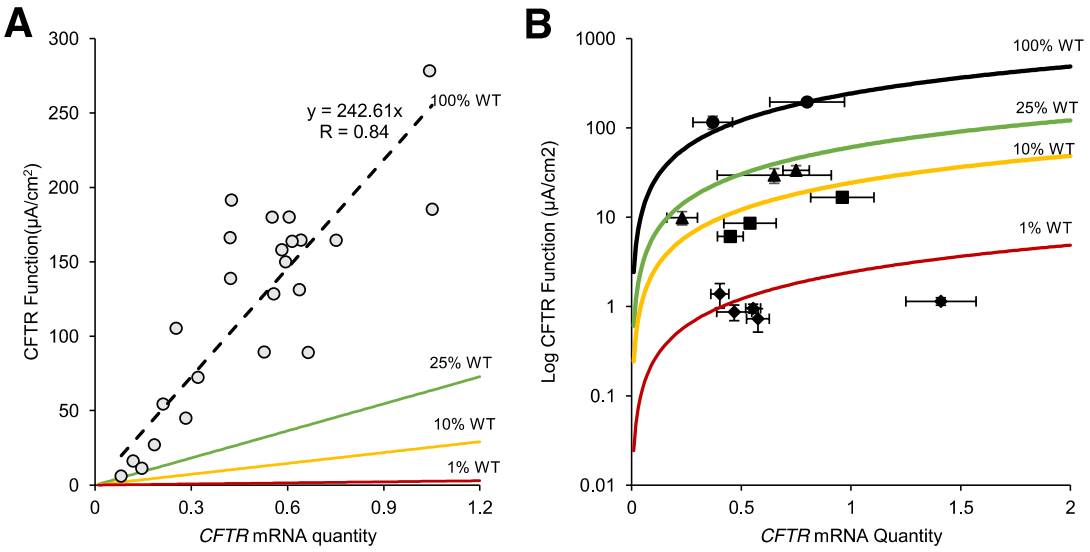

A
CFTR mRNA Quantity
WT 1
WT 2
WT 3
WT 4
WT 5
WT 6
WT 7
WT 8
WT 9
WT 10
B
CFBE Stable Cell Lines
Band C
Band B
Na+/K+ ATPase
F508del
G551D
CFBE – no CFTR
WT – HEK293
CFTR Protein Quantity
C
CFTR Function (µA/cm2)
Time (min)
10 µM Forskolin
10 µM Inh-172
D
R=0.92
p=1.59x10-4
R=0.99
p=1.51x10-7
R=0.94
p=7.06x10-5

Figure 2.2 Independently derived cell lines of *CFTR* missense variants yield consistent interpretation.

(**A**) Standard curve (dashed line) for 100% WT-CFTR function derived using *CFTR* mRNA and CFTR function from 24 independent cell lines expressing WT-CFTR. Predicted CFTR function corresponding to 25% (green), 10% (gold), and 1% (red) of WT-CFTR function across the range of mRNA expression observed in WT-CFTR cell lines. (**B**) Plot of log CFTR function against *CFTR* mRNA quantity derived from correlation shown in (**A**). After normalization for mRNA levels, CFTR variants expressed in multiple independent cell lines show consistent levels of residual CFTR function. Four variants illustrate the range of CFTR function observed.



Variant	Cell line 1	Cell line 2	Cell line 3	Cell line 4	Cell line 5	Average %WT ± SD
◆ F508del	1.4	0.8	0.7	0.5	0.3	0.7% ± 0.4
■ T338I	7.2	5.6	6.5			6.4% ± 0.8
▲ G622D	17.7	18.6	18.3			18.2% ± 0.4
● F508C	99.9	128.2				114.0% ± 20.1

Figure 2.3 Distinct distributions of the residual CFTR function of variants associated with full or partial expressivity of CF.

The majority of variants associated with full expressivity of CF allow less than 10% WT-CFTR function while the remainder distribute across three higher ranges of function. None of the variants associated with partial expressivity of CF have less than 10% CFTR function.

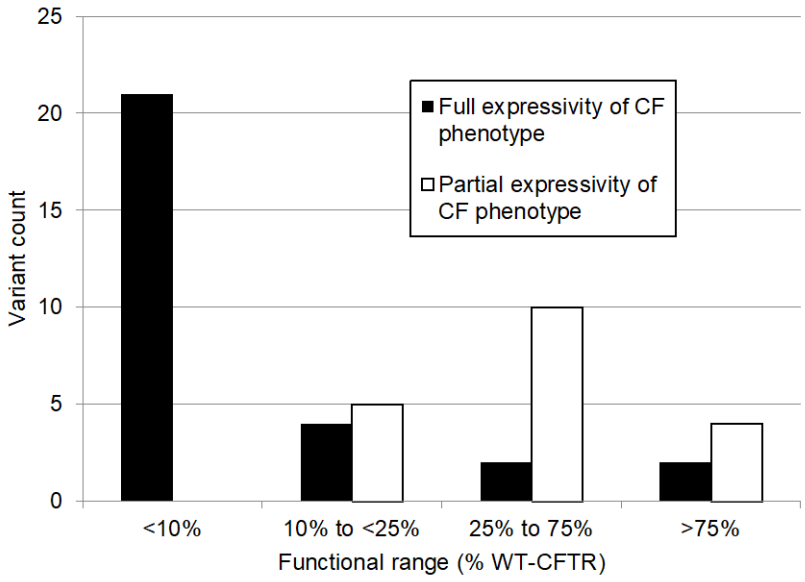

25
20
15
10
5
0
Variant count
Full expressivity of CF phenotype
Partial expressivity of CF phenotype
<10%
10% to <25%
25% to 75%
>75%
Functional range (% WT-CFTR)

Table 2.1 Functional results and disease liability determination for missense variants

Variant (legacy)	Variant (cDNA)	Variant (protein)	n alleles in CFTR2	Allele freq. in CFTR2	Mean sweat [Cl-] in CFTR2	%WT function, +/-SD	CFTR2 final determination
Variants with function <10% WT-CFTR							
F508del	c.1521_1523delCTT	p.Phe508del	98735	69.856%	≥60	0.7 ± 0.4	CF[b]
W57G	c.169T>G	p.Trp57Gly	10	0.007%	≥60	1 ± 0.2	CF
L558S	c.1673T>C	p.Leu558Ser	34	0.024%	≥60	1.2 ± 1.6	CF
Y563D	c.1687T>G	p.Tyr563Asp	7	0.005%	≥60	1.3 ± 0.7	CF
Y563N	c.1687T>A	p.Tyr563Asn	33	0.023%	≥60	1.9 ± 1	CF
H609R	c.1826A>G	p.His609Arg	10	0.007%	≥60	2.2 ± 0.4	CF
A613T	c.1837G>A	p.Ala613Thr	6	0.0042%	≥60	2.3 ± 0.9	CF
L1335P	c.4004T>C	p.Leu1335Pro	19	0.013%	≥60	2.4 ± 1.2	CF
I336K	c.1007T>A	p.Ile336Lys	55	0.0389%	≥60	2.4 ± 0.7	CF[b]
L165S	c.494T>C	p.Leu165Ser	21	0.015%	≥60	2.7 ± 1.3	CF
G551D	c.1652G>A	p.Gly551Asp	2984	2.111%	≥60	2.9 ± 1.6	CF[b]
P574H	c.1721C>A	p.Pro574His	25	0.018%	≥60	3 ± 0.5	CF
A1006E	c.3017C>A	p.Ala1006Glu	8	0.0057%	≥60	3.4 ± 1.5	CF
R334L	c.1001G>T	p.Arg334Leu	15	0.011%	≥60	3.6 ± 0.9	CF
P99L	c.296C>T	p.Pro99Leu	7	0.005%	≥60	3.6 ± 0.8	CF
V456A	c.1367T>C	p.Val456Ala	27	0.019%	≥60	4.1 ± 1.4	CF
S1159F	c.3476C>T	p.Ser1159Phe	13	0.009%	≥60	4.7 ± 0.5	CF
D513G	c.1538A>G	p.Asp513Gly	7	0.005%	≥60	4.8 ± 1.7	CF
Q98R	c.293A>G	p.Gln98Arg	16	0.011%	≥60	5.4 ± 0.7	CF
S1118F	c.3353C>T	p.Ser1118Phe	7	0.005%	≥60	5.8 ± 3.1	CF
T338I	c.1013C>T	p.Thr338Ile	52	0.0368%	≥60	6.4 ± 0.8	CF[b]
R1283M	c.3848G>T	p.Arg1283Met	7	0.005%	≥60	6.7 ± 3.9	CF
E116K	c.346G>A	p.Glu116Lys	8	0.006%	≥60[a]	6.7 ± 2	CF
D979V	c.2936A>T	p.Asp979Val	3	0.002%	≥60	7 ± 3.7	CF
F311L	c.933C>G	p.Phe311Leu	9	0.006%	≥60	7.6 ± 3.3	CF
Variants with function 10 to <25% WT-CFTR							
T1246I	c.3737C>T	p.Thr1246Ile	23	0.016%	≥60	12.9 ± 4.1	VCC
F1099L	c.3297C>A	p.Phe1099Leu	7	0.005%	≥60	15.1 ± 6.4	VCC
F575Y	c.1724T>A	p.Phe575Tyr	7	0.005%	<60	17.1 ± 3.1	VCC
G622D	c.1865G>A	p.Gly622Asp	8	0.006%	<60	18.2 ± 0.4	VCC
V1153E	c.3458T>A	p.Val1153Glu	6	0.004%	<60	18.7 ± 5.6	VCC
M265R	c.794T>G	p.Met265Arg	7	0.005%	<60	19.1 ± 1.9	VCC
D110E	c.330C>A	p.Asp110Glu	14	0.010%	≥60	19.9 ± 4.6	VCC
Y1032C	c.3095A>G	p.Tyr1032Cys	16	0.011%	<60	20.6 ± 4.7	VCC
P5L	c.14C>T	p.Pro5Leu	60	0.042%	≥60	22.4 ± 3.1	VCC
Variants with function 25 to 75% WT-CFTR							
R334Q	c.1001G>A	p.Arg334Gln	8	0.006%	<60	26.6 ± 4.2	VCC
E588V	c.1763A>T	p.Glu588Val	6	0.004%	≥60	27.5 ± 6	VCC
R117G	c.349C>G	p.Arg117Gly	8	0.006%	<60	34.7 ± 4.7	VCC
A349V	c.1046C>T	p.Ala349Val	11	0.008%	<60	44.9 ± 10.3	IND
V201M	c.601G>A	p.Val201Met	11	0.008%	<60	47.5 ± 15.1	IND
P750L	c.2249C>T	p.Pro750Leu	13	0.009%	<60	48.6 ± 6.6	VCC
D443Y	c.1327G>T	p.Asp443Tyr	8	0.006%	<60	53.2 ± 10.8	VCC
R31L	c.92G>T	p.Arg31Leu	7	0.005%	<60	56.3 ± 16	IND
Q1291R	c.3872A>G	p.Gln1291Arg	9	0.006%	<60	62.3 ± 43.3	VCC
S912L	c.2735C>T	p.Ser912Leu	6	0.004%	≥60	71.2 ± 18.2	IND
Y1014C	c.3041A>G	p.Tyr1014Cys	6	0.004%	<60	73.8 ± 27.7	IND
L967S	c.2900T>C	p.Leu967Ser	20	0.014%	<60	74.4 ± 12.2	VCC
Variants with function >75% WT-CFTR							
T1053I	c.3158C>T	p.Thr1053Ile	9	0.006%	<60	78.8 ± 27.2	Non CF
F508C	c.1523T>G	p.Phe508Cys	8	0.006%	<60	114 ± 20.1	Non CF
I807M	c.2421A>G	p.Ile807Met	9	0.006%	<60	115.2 ± 27	Non CF
V562I	c.1684G>A	p.Val562Ile	20	0.014%	≥60	116.4 ± 30.5	Non CF
D836Y	c.2506G>T	p.Asp836Tyr	9	0.006%	≥60	122.1 ± 36.4	Non CF
R170H	c.509G>A	p.Arg170His	11	0.008%	<60	150.4 ± 97.1	Non CF

[a]Sweat chloride value is based on n=2, which is below CFTR2 standards (minimum requirement of sweat chloride n = 3) to use as clinical evidence for disease. Consequently, this variant will not be published on the CFTR2 website.

[b]Variant disease liability previously determined and published in Sosnay et al. [5]

CF = CF-causing; VCC = Varying clinical consequences; Non CF = Non CF-causing; IND = Indeterminate

Table 2.2 Comparison of variant annotation using ACMG/AMP criteria (with and without inclusion of functional data) and expert annotation with functional data (CFTR2)

	CFTR2 interpretation	Inclusion of functional data in classification[a]	ACMG/AMP classification				
			Pathogenic	Likely Pathogenic	Variant of Uncertain Significance	Likely Benign	Benign
Variants reported in this manuscript	CF-causing n=21	-	-	17	4	-	-
		+, 10%	17	4	-	-	-
		+, 25%	17	4	-	-	-
	VCC n=16	-	-	7	9	-	-
		+, 10%	-	7	9	-	-
		+, 25%	5	6	5	-	
	Non CF-causing n=6	-	-	-	5	1	-
		+, 10%	-	-	4	1	1
		+, 25%	-	-	4	1	1
	IND n=5	-	-	-	5	-	-
		+, 10%	-	-	5	-	-
		+, 25%	-	-	5	-	-
Variants previously reported on cftr2.org	CF-causing n=50	-	1	47	2	-	-
		+, 10%	46	4	-	-	-
		+, 25%	46	4	-	-	-
	VCC n=12	-	-	5	7	-	-
		+, 10%	2	3	6	1	-
		+, 25%	4	1	6	1	-
	Non CF-causing n=11	-	-	-	3	7	1
		+, 10%	-	-	1	2	8
		+, 25%	-	-	1	2	8
	IND n=1	-	-	-	1	-	-
		+, 10%	-	-	-	1	-
		+, 25%	-	-	-	1	-

[a]Function was incorporated using different thresholds for pathogenicity; 10% (more conservative, considered the threshold for life-limiting lung disease) and 25% (less conservative, may be the threshold for CFTR-related symptoms). VCC = Varying Clinical Consequence; IND = Indeterminate

Table 2.3 Predicted effects of 48 missense variants using four algorithms

Variant (legacy)	Variant (cDNA)	Variant (protein)	CFTR2 determination	% WT function	CADD PHRED	REVEL score	SIFT	PolyPhen-2
Variants with function <10% WT-CFTR								
W57G	c.169T>G	p.Trp57Gly	CF	1.0	29.9	0.834	Damaging	Benign
L558S	c.1673T>C	p.Leu558Ser	CF	1.2	30	0.98	Damaging	Probably damaging
Y563D	c.1687T>G	p.Tyr563Asp	CF	1.3	31	0.975	Damaging	Probably damaging
Y563N	c.1687T>A	p.Tyr563Asn	CF	1.9	31	0.969	Damaging	Probably damaging
H609R	c.1826A>G	p.His609Arg	CF	2.2	18.85	0.809	Tolerated	Probably damaging
A613T	c.1837G>A	p.Ala613Thr	CF	2.3	24.9	0.83	Tolerated	Probably damaging
L1335P	c.4004T>C	p.Leu1335Pro	CF	2.4	29	0.948	Damaging	Probably damaging
L165S	c.494T>C	p.Leu165Ser	CF	2.7	27.4	0.929	Damaging	Probably damaging
P574H	c.1721C>A	p.Pro574His	CF	3.0	32	0.98	Damaging	Probably damaging
A1006E	c.3017C>A	p.Ala1006Glu	CF	3.4	23.2	0.58	Damaging	Benign
R334L	c.1001G>T	p.Arg334Leu	CF	3.6	23.2	0.726	Damaging	Probably damaging
P99L	c.296C>T	p.Pro99Leu	CF	3.6	34	0.945	Damaging	Probably damaging
V456A	c.1367T>C	p.Val456Ala	CF	4.1	25.4	0.916	Damaging	Possibly damaging
S1159F	c.3476C>T	p.Ser1159Phe	CF	4.7	33	0.919	Damaging	Probably damaging
D513G	c.1538A>G	p.Asp513Gly	CF	4.8	24.2	0.963	Damaging	Probably damaging
Q98R	c.293A>G	p.Gln98Arg	CF	5.4	27.1	0.946	Damaging	Probably damaging
S1118F	c.3353C>T	p.Ser1118Phe	CF	5.8	25	0.085	Damaging	Probably damaging
R1283M	c.3848G>T	p.Arg1283Met	CF	6.7	33	0.962	Damaging	Probably damaging
E116K	c.346G>A	p.Glu116Lys	CF	6.7	26.5	0.782	Tolerated	Probably damaging
D979V	c.2936A>T	p.Asp979Val	CF	7.0	28.7	0.985	Damaging	Probably damaging
F311L	c.933C>G	p.Phe311Leu	CF	7.6	22.8	0.712	Tolerated	Possibly damaging
Variants with function 10% to <25% WT-CFTR								
T1246I	c.3737C>T	p.Thr1246Ile	VCC	12.9	32	0.925	Damaging	Probably damaging
F1099L	c.3297C>A	p.Phe1099Leu	VCC	15.1	23.3	0.619	Tolerated	Probably damaging
F575Y	c.1724T>A	p.Phe575Tyr	VCC	17.1	29.6	0.868	Damaging	Probably damaging
G622D	c.1865G>A	p.Gly622Asp	VCC	18.2	29.7	0.964	Damaging	Probably damaging
V1153E	c.3458T>A	p.Val1153Glu	VCC	18.7	33	0.939	Damaging	Possibly damaging
M265R	c.794T>G	p.Met265Arg	VCC	19.1	24.3	0.659	Damaging	Benign
D110E	c.330C>A	p.Asp110Glu	VCC	19.9	16.39	0.733	Tolerated	Possibly damaging
Y1032C	c.3095A>G	p.Tyr1032Cys	VCC	20.6	25.1	0.887	Damaging	Probably damaging
P5L	c.14C>T	p.Pro5Leu	VCC	22.4	33	0.887	Damaging	Probably damaging
Variants with function 25% to 75% WT-CFTR								
R334Q	c.1001G>A	p.Arg334Gln	VCC	26.6	19.78	0.692	Tolerated	Probably damaging
E588V	c.1763A>T	p.Glu588Val	VCC	27.5	29.3	0.97	Damaging	Possibly damaging
R117G	c.349C>G	p.Arg117Gly	VCC	34.7	26.4	0.773	Damaging	Probably damaging
A349V	c.1046C>T	p.Ala349Val	IND	44.9	27.5	0.632	Tolerated	Possibly damaging
V201M	c.601G>A	p.Val201Met	IND	47.5	27.1	0.676	Damaging	Possibly damaging
P750L	c.2249C>T	p.Pro750Leu	VCC	48.6	22.9	0.655	Damaging	Benign
D443Y	c.1327G>T	p.Asp443Tyr	VCC	53.2	26	0.83	Damaging	Possibly damaging
R31L	c.92G>T	p.Arg31Leu	IND	56.3	22.9	0.546	Tolerated	Benign
Q1291R	c.3872A>G	p.Gln1291Arg	VCC	62.3	23.2	0.871	Damaging	Benign
S912L	c.2735C>T	p.Ser912Leu	IND	71.2	9.977	0.543	Tolerated	Benign
Y1014C	c.3041A>G	p.Tyr1014Cys	IND	73.8	28.5	0.889	Damaging	Probably damaging
L967S	c.2900T>C	p.Leu967Ser	VCC	74.4	24.7	0.659	Damaging	Possibly damaging
Variants with function >75% WT-CFTR								
T1053I	c.3158C>T	p.Thr1053Ile	Non CF	78.8	26	0.899	Damaging	Possibly damaging
F508C	c.1523T>G	p.Phe508Cys	Non CF	114.0	29.4	0.865	Damaging	Probably damaging
I807M	c.2421A>G	p.Ile807Met	Non CF	115.2	21.9	0.738	Damaging	Probably damaging
V562I	c.1684G>A	p.Val562Ile	Non CF	116.4	23.8	0.637	Damaging	Benign
D836Y	c.2506G>T	p.Asp836Tyr	Non CF	122.1	32	0.924	Damaging	Probably damaging
R170H	c.509G>A	p.Arg170His	Non CF	150.4	34	0.829	Damaging	Probably damaging

CF = CF-causing; VCC = Varying Clinical Consequence; Non CF = Non CF-causing; IND = Indeterminate

Table 2.4 Predicted functional consequences of missense variants

		CADD		REVEL		SIFT		PolyPhen-2	
Functional grouping	n	Del	Not Del	Del	Not Del	Dam	Tol	Pr/Po Dam	Ben
<10%	21	21	-	19	2	17	4	19	2
10% to <25%	9	9	-	8	1	7	2	8	1
25% to 75%	12	11	1	8	4	8	4	8	4
>75%	6	6	-	5	1	6	-	5	1

Del = Deleterious; Not Del = Not deleterious; Dam = Damaging; Tol = Tolerated; Pr/Po Dam = Probably damaging or possibly damaging; Ben = Benign

Chapter 3. Residual Function of Cystic Fibrosis Mutants Predicts Response to Small Molecule CFTR Modulators

3.1 Introduction

Treatment of individuals with cystic fibrosis (CF) has been transformed by modulator therapies that target select molecular mechanisms affecting function of the CF transmembrane conductance regulator (CFTR). However, allelic heterogeneity and low frequency of alleles present significant challenges to therapeutic classification of pathogenic variants. Approximately 1,650 variants have been identified in individuals with CF; most variants have been observed in fewer than 10 individuals worldwide and only 39 genotypes are FDA approved for CFTR modulator therapy. To generate data useful for expansion of treatment eligibility, we stably expressed 43 *CFTR* variants from a single integration site in the genome of CF Bronchial Epithelial (CFBE) cells and evaluated CFTR function by measuring transepithelial ion transport. We found that most variants demonstrated a modest elevation of CFTR function in response to all treatments tested. Importantly, we found that the magnitude of therapeutic response was highly correlated with residual CFTR function for the potentiator ivacaftor ($r = 0.96$), the corrector lumacaftor ($r = 0.83$), and the ivacaftor-lumacaftor combination ($r = 0.93$). Response of a partially overlapping set of 16 variants studied in a similar manner using Fisher Rat Thyroid (FRT) cells, a robust system for studying CFTR biology, showed nearly identical correlations. As expected, only a small subset of variants demonstrated statistically significantly greater response to specific treatments, presumably due to direct interaction between modulator action and molecular mechanism of disease. Furthermore, 40/43 variants studied in CFBE cells and 13/16 variants studied in FRT cells demonstrated highest response to combination therapy. The variants studied here represent 87% of individuals in the CFTR2 database who carry at least one missense

variant, indicating that most individuals with CF caused by missense variants are likely to demonstrate modest response to currently available modulator therapy while a small fraction will show significantly elevated responses. Additionally, our results indicate that most individuals with missense variants should derive greatest benefit from combination therapy and we found no variant for which monotherapy was more effective than combination therapy. These findings provide a path to expanding modulator therapy to a significant number of individuals with CF who have rare genotypes that cannot be studied by clinical trials.

Targeting specific mutant forms of defective CFTR with orally bioavailable modulators has revolutionized treatment of cystic fibrosis (CF) [3,93–95]. Ivacaftor, the first drug to achieve FDA approval, potentiates function by altering chloride channel gating and increasing the open probability (P_o) of CFTR[29,96,97]. Ivacaftor was originally approved for individuals with CF who carried at least one copy of the p.Gly551Asp (G551D) variant. Subsequent clinical trials demonstrated benefit for individuals with CF who carried at least one copy of any one of eight variants that affected the gating of CFTR [18,98]. The second drug, lumacaftor, was developed to correct protein folding defects caused by the most common CF causing variant, p.Phe508del (F508del) [30,99]. While lumacaftor alone was not clinically effective for individuals homozygous for F508del [20] in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation}, co-administration of lumacaftor and ivacaftor improved clinical outcome measures for individuals homozygous for F508del [21]. Recently, tezacaftor, a second corrector compound demonstrated clinical efficacy in F508del homozygotes when combined with ivacaftor [32]. The same combination of CFTR-

targeted drugs also proved efficacious in a clinical trial of individuals with CF who carried F508del *in trans* with a select set of 'residual' function variants [24].

Ivacaftor alone or in combination with either lumacaftor or tezacaftor has demonstrated efficacy for 39 of the ~1640 *CFTR* variants associated with CF (http://www.CFTR2.org). This leaves thousands of individuals with CF who carry variants that have not been approved or in many cases even experimentally tested for response to these three drugs. Review of the untested variants indicates that approximately 50% are predicted to generate CFTR protein, and therefore could potentially be targeted with the currently available drugs [3]. Unfortunately, clinical trials of uncommon variants are difficult to conduct due to the wide geographic dispersion of the small number of individuals carrying these variants. Moreover, the high cost of CFTR modulators has made off-label prescription problematic. Even if an individual with a rare variant responds well in the clinic, insurers may not support the cost of treatment unless the modulator is FDA-approved for that particular genotype. Thus, alternative approaches are needed to assess the response to CFTR modulators for rare variants. Cell-based functional assays represent an avenue for evaluating rare variants in cases where clinical studies or assessment of primary tissues are impractical, provided these systems are well vetted and generate reproducible results. Fischer Rat Thyroid (FRT) cells have been extensively used as a model cell line for studying the role of CFTR in epithelial ion transport [100,101] and FRT cell lines expressing *CFTR* cDNA have been used in a number of studies to generate response data that have provided preliminary evidence to proceed to clinical trials [17,22,29,30], and more recently, to facilitate drug label expansion [102]. CF Bronchial Epithelial CFBE41o$^-$ (CFBE) cells provide an opportunity

to test the effects of *CFTR* variants in a human cell line from a relevant tissue type with a transcriptome that is very similar to that of primary airway epithelial cells [15]. These two cell lines offer complementary platforms to evaluate the functional consequences and responses to modulators of CFTR missense variants in a standardized and reproducible manner.

In this study, we utilized CFBE cells stably expressing *CFTR* missense variants to extend our understanding of drug responses to *CFTR* bearing rare (minor allele frequency (MAF) <1% in the CF population) missense variants. Our initial goal was to identify variants with either positive or less favorable responses to ivacaftor, lumacaftor, or ivacaftor-lumacaftor combination treatment to inform clinical applications. However, we discovered that response to the modulators was closely correlated with residual function of the mutant forms of CFTR for most variants expressed in CFBE cells. This observation was replicated with a different set of missense variants expressed stably in FRT cells and was also apparent upon retrospective analysis of previously published ivacaftor studies using another independent set of FRT cells [22]. Using these results, we devised a statistically valid approach to identify robust responders to ivacaftor and lumacaftor based on the fold change in CFTR function. Furthermore, we showed that the combination of the two modulators produces a greater response for most missense variants, including high response variants, than either drug alone. These observations, in concert with the recent demonstration that combinatorial treatment was efficacious for residual function variants [24], suggest that CFTR-targeted treatment may be appropriate for most individuals with CF carrying residual function missense variants.

3.2 Results

To assess the response of CFTR variants associated with a wide range of CF phenotypes to FDA approved CFTR modulators, we studied 57 missense variants reported in individuals with CF using two cellular expression systems. Forty-three rare missense variants that were associated with a range of phenotypes measured by modest increases in sweat chloride concentration (40-90 mM) and/or pancreatic exocrine sufficiency (PS) prevalence greater than 50%, were selected from the CFTR2 database (**Table 3.1**) and reported previously [8]. Due to the moderate pancreatic disease, each variant was expected to allow residual CFTR function [103,104] and was associated with less severe disease. To approximate the native environment of CFTR in the lung, the rare variants were studied in CF bronchial epithelial (CFBE41o$^-$) cells that are devoid of endogenous *CFTR* expression [15,105]. These CFBE cells have been used to generate cell lines expressing *CFTR* missense variants at a level comparable to primary human tissues in order to measure their effect on CFTR function and aid in interpretation of disease liability [8]. A second set of 16 variants were expressed in FRT cells (**Table 3.2**), a cell type that has been employed to evaluate the response of CFTR to FDA approved drugs [102], although the studies presented here were performed on cell lines selected within a more narrow range of mRNA expression. These variants are generally more frequent in individuals with CF and are associated with more severe disease (60-106 mM and 2-80% PS). Two variants, p.Arg334Trp (R334W) and p.Thr338Ile (T338I), were common to both groups yielding a total study set of 57 unique variants. Variants F508del and G551D that have been extensively studied previously were also analyzed in both cell lines [3], bringing the total number of cell lines studied here to 63.

In each cell system, *CFTR* bearing each of the missense variants was stably expressed from a single site of integration, as previously described [8,15,22,106]. Residual CFTR function of each missense variant in CFBE cells was measured as the magnitude of forskolin-mediated (10 μM) cAMP-stimulated current inhibited by $CFTR_{inh}$-172 (inh-172; 10 μM), a widely used potent inhibitor of CFTR [107], normalized to wild type (WT) function based on mRNA expression level as previously described [8] to calculate a precise %WT function. CFTR function in FRT cells was measured as the magnitude of forskolin-mediated (5 μM) cAMP-stimulated current (with specificity confirmed by inh-172 (10 μM)) compared to WT. All FRT cell lines selected for study expressed CFTR mRNA within a narrow range (0.5 – 1.5 fold) of CFTR mRNA compared to a WT-expressing line generated by the same method [106]. The four variants studied in both CFBE and FRT cells, (R334W, T338I, F508del, and G551D) produced comparable levels of CFTR function: F508del (CFBE, 0.5% WT; FRT, 1.6% WT); G551D (CFBE, 3.2% WT; FRT 4.0% WT); R334W (CFBE, 2.0% WT; FRT 3.9% WT); T338I (CFBE, 6.4% WT; FRT, 7.6% WT) (**Table 3.1 and Table 3.2**).

CFTR missense variants expressed in CFBE cells were first tested for their response to 10 μM ivacaftor, a compound that is efficacious for variants that affect channel gating [29]. G551D-CFTR in CFBE cells demonstrated a dose dependent response to ivacaftor from 0.1 μM to 100 μM, similar to previously reported findings in FRT cells [29] (data not shown). Ivacaftor enhanced the forskolin (10 μM) -stimulated function of many of the variants tested in CFBE cells regardless of whether or not they were known to impair CFTR channel gating. Furthermore, the magnitude of ivacaftor enhanced CFTR activity correlated with the magnitude of forskolin activated CFTR

activity (r = 0.96) (**Figure 3.1A; Table 3.1; Supplemental Table 3.1**). Data were plotted on logarithmic scale to visualize the entire functional range of variants studied. To determine whether the observed correlation between ivacaftor response and residual function was cell-line independent, we analyzed the ivacaftor response of 16 *CFTR* variants and the G551D and F508del variants stably expressed in FRT cells. The response to ivacaftor also correlated with forskolin activated CFTR function for all 18 variants expressed in FRT cells (r = 0.93) (**Figure 3.1B; Table 3.2**).

When data from CFBE and FRT studies were combined and evaluated as fold change over baseline function, four cell lines (G551D expressed in both CFBE and FRT cells, p.Ser1159Phe (S1159F) and p.Ser1159Pro (S1159P) in CFBE cells) were found to be outliers (defined here as greater than 2SD above the mean of all variants tested) and labeled as 'high response'. G551D is the prototype for highly-responsive gating variants [29,108] while S1159F and S1159P are two previously uncharacterized variants. When these outliers were removed, mean and SD were recalculated for all remaining data and four outliers from this analysis (p.Phe311Leu (F311L), p.Met348Val (M348V), p.Thr1246Ile (T1246I), and p.Asn1303Lys (N1303K)) were separated and labeled as 'intermediate response'; all other variants were labeled as 'modest response' variants, which accounted for the vast majority of variants (**Figure 3.1C**). F311L, M348V, and T1246I are three previously untested variants while N1303K is a variant of particular interest. Single-channel studies revealed that once delivered to the plasma membrane, CFTR bearing N1303K exhibited a severe gating defect characterized by infrequent brief channel openings reminiscent of the G551D variant (**Supplemental Figure 3.1A**) [109,110]. Ivacaftor (1 μM) modestly enhanced open probability (P_o) of the N1303K

variant by increasing the frequency and duration of channel openings (**Supplemental Figure 3.1B-E**). Taken together, the I_{sc} and single-channel studies indicate that ivacaftor can elicit an increase in N1303K function although the extremely low residual function prevents a large absolute change CFTR function after ivacaftor treatment.

We next evaluated previously published ivacaftor response data generated by 54 *CFTR* variants stably expressed in a separate FRT cell line established by Vertex Pharmaceuticals (noted here as FRT*) that overlapped with the 16 variants expressed in FRT cells in this study [22]. Experimental conditions for measuring CFTR function were comparable to those used for the CFBE [15] and FRT [22] stable cell lines studied here. Correlation was evident for the variants studied in the FRT* cell lines (**Figure 3.1D**). Although drug doses differed between data sets, the CFBE and FRT data from this study were normalized to cells expressing WT-CFTR treated with the same drug doses, allowing for comparison across cell types. When high and intermediate response variants were removed from analysis, the regression functions for all 3 independent studies had very similar slopes, intercepts, and high correlation (**Figure 3.1E**). These results indicate that ivacaftor increases CFTR function for most variants regardless of the specific molecular defect as the variants studied confer a range of defects in CFTR function.

Overall, the variants shared between studies yielded the same quality of ivacaftor response across systems, i.e. high response variants in one system were high response variants in other systems. The fold increase in current following ivacaftor treatment was similar in both cell lines for F508del (1.76 in CFBE and 1.31 in FRT), G551D (6.81 fold in CFBE and 6.02 fold in FRT), R334W (1.03 fold in CFBE and 1.24 fold in FRT), and T338I (2.21 fold in CFBE and 1.58 fold in FRT).

Identification of variants that allow CFTR to fold to a mature form but cause it to be poorly responsive to ivacaftor could be informative for future drug design and optimization. To screen for potential poor responders, we plotted the published folding and chloride channel function effects of 54 *CFTR* variants [17,22] (**Figure 3.2A**). We noted that a group of variants p.Ile336Lys (I336K), T338I, p.Ser341Pro (S341P), R334W, and p.Arg347Pro (R347P) located in transmembrane segment 6 (TM6) in membrane-spanning domain 1 (MSD1) of CFTR permitted partial folding, but severely disrupted channel function. TM6 is notable for its role in ion conductance [111] as it is predicted to line the channel pore by homology models and cryo-electron microscopy structures[112–117], measurements of conductance properties [118–120], and evaluation of solvent accessibility [121–124]. Based on these data, we hypothesized that variants which alter residues lining the channel pore might be resistant to CFTR potentiators, such as ivacaftor [17,22]. The nine naturally occurring TM6 variants generated detectable mature (band C) CFTR which suggested that each variant may allow at least partial CFTR maturation and residual CFTR function (**Figure 3.2B**). Furthermore, the ivacaftor response of TM6 variants increased as residual function increased from <1% WT to 78% WT, indicating that the two measurements are correlated (**Figure 3.2C**). The responses of the TM6 variants to ivacaftor were modest and not different from the total set of modest response variants (**Figure 3.2D**). Furthermore, 2 TM6 variants, p.Arg334Gln (R334Q) and T338I, responded very similarly to a separate collection of 10 CFTR potentiator compounds obtained from CF Foundation Therapeutics (CFFT) CFTR Chemical Compound Program (**Supplemental Figure 3.2**). Although TM6 variants had minimal residual CFTR function despite some of them having efficient protein folding, a

characteristic shared with 'gating' variants, they are distinct in that generated a modest response to potentiators which was correlated with residual function similar to non-gating missense variants.

Our next step was to test the response of the missense variants to the CFTR corrector lumacaftor. Lumacaftor improves the biogenesis of CFTR protein and has been primarily targeted to the common misfolding variant F508del [30]. Importantly, lumacaftor has been reported to increase the function of WT CFTR and a few missense variants [33,34] and protein folding of several other variants [125,126]. Accordingly, we tested the effect of lumacaftor on the variants studied here. As with ivacaftor, the magnitude of forskolin stimulated CFTR function following incubation with 6 μM lumacaftor correlated with the forskolin stimulated function of each variant when incubated with an equal volume of DMSO for the 45 variants studied in CFBE cells (**Figure 3.3A, Table 3.1**). Correlation between residual function and lumacaftor response was also observed in 18 variants expressed in FRT cell lines (**Figure 3.3B, Table 3.2**). Like the ivacaftor response, data from CFBE and FRT cells were combined and evaluated as fold response. Outliers for the lumacaftor response were determined by measuring 2SD above the mean; p.Gly91Arg (G91R), p.Glu92Lys (E92K), p.Leu138insLeu (L138ins), p.Leu145His (L145H), and p.Leu206Trp (L206W) were designated as high response variants, while p.Gly27Arg (G27R), p.Pro67Leu (P67L), p.Ile340Asn (I340N), and p.Tyr1032Cys (Y1032C) were designated as intermediate response variants (**Figure 3.3C**). When high and intermediate response variants were removed from the analysis, the regression functions for CFBE and FRT modest response variants demonstrated robust correlation (CFBE r = 0.95, FRT r = 0.98) with similar slopes (**Figure 3.3D**).

These results indicate that lumacaftor increases CFTR function for most variants regardless of the specific molecular defect as the 59 variants studied confer a range of folding defects in CFTR from mild to severe.

Lumacaftor has been shown to be effective at improving the protein processing of variants located in MSD1 [125] and the intracellular loops [126]. We were intrigued that two variants (I336K and I340N) located in TM6 within MSD1 demonstrated elevated responses to lumacaftor, which prompted further studies to verify and explain the responses of these two TM6 variants. Lumacaftor increased the total amount of mature CFTR protein expressed in stable CFBE cell lines for all variants (**Figure 3.4A**), even though many of them are naturally fully folded and processed (note absence of immature band B protein for most variants). We confirmed that this increase in protein quantity was not due to an increase in *CFTR* mRNA (**Supplemental Figure 3.3**). Given the variability in the fold response to lumacaftor (see **Figure 3.3A, 3.3B**), we tested up to 4 independently derived CFBE cell lines stably expressing each of the nine TM6 variants (**Figure 3.4B**). Multiple measurements of the different clones of TM6 variants demonstrated that the response to lumacaftor becomes more pronounced at higher levels of residual function (**Figure 3.4C**). Notably, two TM6 variants (I336K and I340N) had significantly higher fold responses than the remaining 7 TM6 variants (**Figure 3.4D**). To explore whether the less responsive TM6 variants respond to other CFTR correctors, we evaluated R334Q and T338I alongside the highly responsive variant I340N using a series of 18 compounds obtained from the CFFT CFTR Chemical Compound Program. Only two compounds generated a greater response compared to the vehicle control (DMSO) for all 4 variants tested (C17 and C18), but neither approached the efficacy of lumacaftor

(**Supplemental Figure 3.4**). Since the TM6 variants are located on an alpha helix that spans the cell membrane, we speculated that the robust corrector activity might be related to the orientation of the side chains of the mutated residues relative to the proposed ion pore. Using the predicted alpha helical structure of TM6 when CFTR is in its open conformation, the side chains of residues R334, T338, S341, and M348 are predicted to reside in the channel pore, while I336, I340, and A349 are oriented such that they are embedded in either the protein or the lipid bilayer (**Figure 3.4E**). The results of arginine scanning mutagenesis are consistent with this orientation for these residues [127] as are the cryo-EM structures of CFTR in the ATP-bound state [117]. Grouping of the responses of TM6 variants by predicted orientation revealed a significant correlation with lumacaftor response (**Figure 3.4F**). These results suggest that amino acid substitutions at embedded residues in TM6 are particularly responsive to lumacaftor.

The combination therapy of ivacaftor and lumacaftor (iva/lum) and ivacaftor paired with a different corrector, tezacaftor, has demonstrated clinical benefit in individuals homozygous for F508del [21,32], and the latter combination is effective for individuals carrying one copy of F508del and a variant that allows partial CFTR function [24]. Based on these observations and the responses we observed with potentiator and corrector alone, we sought to determine the effects of acute administration of ivacaftor after incubation (24 h in CFBE cells or 48 h in FRT cells) with lumacaftor (iva/lum) on the 59 variants tested here. The response of 45 variants stably expressed in CFBE cells to iva/lum correlated with CFTR function without modulators (**Figure 3.5A**). Results obtained from 18 variants expressed in FRT cells demonstrated a similar relationship (**Figure 3.5B**). High response and intermediate response variants were separated from

modest response variants (**Figure 3.5C**); G91R, E92K, L138ins, L145H, and G551D (CFBE) were designated high response variants, while L206W, I336K, G551D, and N1303K were designated as intermediate response variants (note absolute magnitude of N1303K activity is quite low, although the relative modulator enhancement is substantial). Trend lines of modest response variants were again found to be very similar between CFBE and FRT cell lines (**Figure 3.5D**). Plotting of all variants studied revealed robust correlation between forskolin stimulated CFTR function and iva/lum enhanced CFTR function illustrating that this relationship is independent of cell type and pathologic mechanism for each variant (**Figure 3.5D**).

To formally test whether the combination of ivacaftor and lumacaftor produces higher responses than either compound alone, we performed detailed analysis of the 45 variants expressed in CFBE cells. Iva/lum combination treatment generated an equal or better response than either ivacaftor or lumacaftor for 30 out of 33 variants that demonstrated modest responses to both ivacaftor and lumacaftor (**Figure 3.6A**). The variants which did not demonstrate significantly better function with the iva/lum combination were p.His139Arg (H139R), R334W, and p.Asp513Gly (D513G) and these variants also did not demonstrate a significant response to either ivacaftor or lumacaftor. Five rare variants were found to have an intermediate or high response to ivacaftor; for these five variants iva/lum combination treatment was greater or equal to either ivacaftor or lumacaftor treatment (**Figure 3.6B**). Six variants were intermediate or high response variants to lumacaftor treatment; for these six variants iva/lum combination treatment was greater or equal to either ivacaftor or lumacaftor treatment (**Figure 3.6C**). Notably, the iva/lum combination was never worse than ivacaftor or lumacaftor alone. Out of the

45 rare variants evaluated, the response was not statistically significantly higher than forskolin stimulated function only for H139R, R334W, and D513G.

Although the trends for modest response variants are the results that apply to the majority of variants and individuals with CF, the responses of the intermediate and high response variants also warrant evaluation. We compared the regression lines for modest, intermediate, and high response variants for ivacaftor, lumacaftor, and iva/lum combination (**Figure 3.7A**). This analysis demonstrated that each response group yielded a steeper slope of the regression for all three treatments. These steeper slopes suggest that higher response variants were distinguished by more effective modulator responses and were not the simple top end of a normal distribution; we believe that the regressions would be parallel but elevated if the latter were the case. When we compared across each tier of response, modest, intermediate, and high, we found that the iva/lum combination generated the steepest regression for all three response levels (**Figure 3.7B**). Taken together, these results suggest that high response variants are mechanistically distinct from modest response variants and that iva/lum combination therapy is the best option for all variants regardless of whether they are a modest or high response variant.

3.3 Discussion

The CFTR modulators ivacaftor and lumacaftor have been approved for treatment of individuals with CF who carry a select set of variants based on the specific mechanistic defects of those variants. However, clinical studies of individuals with variants for which aberrant channel gating or folding is not the sole molecular defect, such as p.Pro67Leu (P67L) [128], p.Arg117His (R117H) [98], and other residual function variants [24,129], have all shown clinical benefit from ivacaftor in a mechanism

independent manner. Using a wide range of variants expressed in two cell types, we show that residual CFTR function and drug response are highly correlated. Furthermore, we demonstrate that almost all tested variants generate higher currents in response to ivacaftor combined with lumacaftor than when treated with either compound alone. Following the common CF-causing variant F508del [130], missense variants represent the largest category of remaining variants associated with CF [3]. Although the number of missense variants studied here encompass a small fraction of all missense variants reported in CFTR (57 out of >800), they represent a significant portion of CF causing alleles in the CF population. In total, the missense variants evaluated by this study here represent 10% of all alleles reported to CFTR2 and 13,062 out of 15,082 individuals (87%) with at least one missense variant. Of added significance to the CF community moving forward, our results predict that greater clinical benefit would likely be derived from combinatorial therapy for most individuals with CF who carry missense variants and are given modulator compounds. The utility of this approach was recently demonstrated by a clinical trial employing modulator combinations (ivacaftor and tezacaftor) for individuals with residual CFTR function [24].

Heterologous expression of CFTR mutants in FRT cells has been used extensively to test modulator response, particularly to ivacaftor [17,22,29,30,125]. Based on the clinical efficacy and safety of ivacaftor, the FDA elected to accept results of CFTR testing in FRT cells as evidence to allow expansion of ivacaftor use without requiring clinical trials [102]. This decision was reached based on the strong correlation between *in vitro* studies using FRT cells and clinical trials. Importantly, the results of those studies agreed despite using a non-human cell line expressing a single allele of CFTR. This

forwarding thinking approach provides a path to treat every individual with CF who carries *CFTR* variants that are sufficiently responsive to modulators and provides a mechanism to address barriers of formal clinical testing for CF individuals with ultra-rare or even private mutations. However, the possibility remains that certain variants or certain modulators will require CFTR to function in its native human airway cellular context. To this end, we tested variants in CFBE cells and compared our results to the FRT model that has been well established as a system for studying CFTR modulators. The CFBE and FRT cell lines used here expressed *CFTR* in a controlled manner by stable expression from a single locus which guarded against artefactual interpretation of variants due to overexpression of the allele. For example, the finding of N1303K response to ivacaftor, if taken on its own, could be suggestive of clinical benefit, but the function in the expression controlled FRT cell line demonstrates that the response is quite low and less likely to reach levels sufficient for clinical benefit. mRNA levels of *CFTR* expressed in CFBE cells were also determined to be within normal physiological limits of native human tissues by RNAseq studies. Despite their differences, we believe that our efforts to maintain controlled expression allowed CFBE and FRT cells to generate highly consistent results across a wide range of variants. The slopes and intercepts of correlation between residual function and modulator responses were remarkably similar, suggesting that the observed relationships were highly likely to be due to CFTR rather than cell-specific factors.

Ivacaftor increased chloride currents of G551D-CFTR by almost 7-fold in both cell lines, which is significantly less than was observed in previous studies in FRT cells [17,22], but more consistent with studies performed in primary cells [29] and other

cellular contexts [96,97]. The rare variants S1159F and S1159P, located prior to the second nucleotide-binding domain (NBD2) [131], also exhibited a high response to ivacaftor. Even after removing high response variants from the analysis, a second set of variants demonstrated a response to ivacaftor that was significantly elevated compared to the remaining variants. The high degree of correlation between residual function and modulator response for modest response variants ($r = 0.94$) suggests that baseline function might predict ivacaftor response for all variants and not just the ones tested here. Although absolute response to modulators may not be sufficient for very low function variants to demonstrate significant clinical benefit, i.e. crossing a 10% WT function disease threshold, evaluation by fold change could inform mechanism of action. Such mechanisms may be distinct from potentiator compounds that promote NBD dimerization [132], and guide rationale design of more effective potentiators. In addition, differentiating variants that have exceptional responses will be important to establish accurate expectations for clinical outcomes.

The effect of lumacaftor on *CFTR* bearing missense variants has been less extensively studied than for ivacaftor, and the present study reports the largest collection of variants tested for their response to lumacaftor. Lumacaftor was designed to target the common F508del variant, and we observe increased CFTR function in cell lines expressing F508del when treated with this compound. Although we were unable to fully recapitulate the magnitude of effect previously published [30], we believe our findings of a 4 fold increase in function from 0.5% to 2% WT function are concordant with results from clinical studies demonstrating the ability of lumacaftor to reduce sweat chloride in a dose dependent manner up to a reduction of 6.6 mmol/l while being unable to

significantly improve lung function [20] as well as improving of biomarkers of CFTR function [133]. Correlation between residual CFTR function and lumacaftor response had previously been observed in studies of primary nasal epithelial cells bearing a small series of missense variants [51]. Furthermore, *in vitro* and *ex vivo* studies of missense variants demonstrate increased CFTR function following treatment with lumacaftor [33,134]. Thus, it is reasonable to expect that the observations in the CFBE and FRT cell lines will be relevant *in vivo*. We also identified missense variants which respond significantly better to lumacaftor than F508del (i.e. greater than 2SD above the mean). These results suggest that individuals carrying certain variants might respond better to lumacaftor than those who carry F508del. Although intermediate and high lumacaftor responses applied only to 9 variants, lumacaftor increased the CFTR function of most variants tested, regardless of the mechanism underlying their CFTR dysfunction. Thus, many individuals with CF who carry variants that allow protein production may also benefit from lumacaftor treatment. Given that tezacaftor is functionally similar to lumacaftor [135], we predict that the same benefit would be obtained when using this CFTR corrector, similar to what has been observed in studies of primary cells [51].

The location of the residues that confer high lumacaftor responses could inform the future development of correctors. Of the six variants that demonstrated robust responses to lumacaftor, four are located in the TMDs (E92K, L206W, I336K, and I340N), while the two variants with the highest responses are located in the intracellular loops (ICLs): L138ins and L145H near the first intracellular loop (ICL1) and Y1032C near the interface of TM10 and ICL4. Recent evidence indicates that lumacaftor promotes interactions between ICL1 and NBD1 [136] as well as between ICL4 and

NBD1 [126]. Thus, the significant increases in current above baseline exhibited by CFTR bearing L138ins and L145H upon treatment with lumacaftor might be a consequence of their locations. As lumacaftor is thought to rescue CFTR function by improving protein folding and hence, structure, we mapped the location of all TM6 variants studied here onto models [112] and structures [116] of the CFTR protein. I336K and I340N are found in TM6 at residues where cryo-EM structures [116,117], homology modeling [112], and experimental evidence predicts that they are embedded [122,123,127], whereas all other variants, except for p.Ala349Val (A349V), are predicted to be oriented into the open channel pore. Although the lumacaftor response of A349V was not found to be significantly different from all other missense variants, it was significantly higher than other TM6 variants ($p<0.05$). These results indicate that missense variants that alter ion conductance by disruption of the structure of the pore are mechanistically distinct from those which more directly impede ion flow. Amelioration of local folding defects imposed on the channel pore by variants such as I336K and I340N might underlie the prominent response of these variants to lumacaftor.

Our extensive evaluation of responses of variants expressed in CFBE cells confirmed that the majority of variants (42/45) demonstrated increased CFTR function when treated with the combination of ivacaftor and lumacaftor. Moreover, 26 of 45 variants demonstrated greater responses to the combination than to ivacaftor or lumacaftor alone, while 16 others showed an equal response to ivacaftor or lumacaftor that was higher than baseline function, and the combination was not found to be less effective than either individual modulator for any variant tested. Although some studies have observed antagonism between ivacaftor and lumacaftor for some genotypes

[134,137], we saw no evidence of antagonistic effects between these compounds in either CFBE or FRT systems. The regression functions suggest that a minimum (~2% WT) amount of residual function is required for ivacaftor action, likely due to the requirement of well folded and processed protein localized to the cell surface, while lumacaftor has no such requirement. The regression function for iva/lum combination treatment removes the minimum function requirement for response, suggesting that lumacaftor action results in ivacaftor response. It is known that F508del results in multiple molecular defects that require correction [138,139] and so combination therapy should therefore be expected to be the most effective treatment for F508del [140] and other variants that have folding and activity defects. Combination therapy was also effective for variants with high responses to ivacaftor or lumacaftor, including G551D, indicating that individuals bearing these variants could additionally benefit from combinatorial therapy.

Of significant note, the modest response variants treated with the iva/lum combination crossed 10% WT function for variants with 3% baseline function. The threshold of 3% residual WT function as a differentiator of predicted alleviation of lung disease for combinatorial modulator therapy is important as this level of CFTR activity also coincides with an approximate threshold for exocrine pancreatic sufficiency [141]. Pancreatic sufficiency of an individual with CF can be determined independent of genetic testing, thus potentially removing hurdles to interpretation of genetic variants including incomplete genotyping, variants of uncertain significance, errors in genetic test reports, and extreme rarity of missense variants, among other considerations. Given the high correlation between baseline function and modulator response for modest response variants ($r = 0.94$), residual function of an individual may be a reasonable predictor of

drug response, which has been postulated previously based on studies of primary cells of a limited number of genotypes [51].

The exceptions to the modest responses to ivacaftor and lumacaftor are significant for individuals carrying those variants as well as for informing mechanism and design of future CFTR modulators. Individuals who carry these highly responsive variants may be able to achieve significant levels of CFTR function and improved clinical outcomes even if their CFTR function without treatment is poor. Secondly, understanding the mechanism underlying the strong response may inform development of more efficient versions of these modulators which can then confer larger responses to individuals carrying modest response variants as well. While these high response variants might lead to significant improvements in clinical outcome, it is important to note different tiers of response when discussing therapeutic outcomes with patients. The specific level of function required to escape lung disease will vary to some degree among individuals due to modifier genes, environmental factors, and/or stochastic factors unique to each individual, as noted in studies of twins and siblings [142]. The level of CFTR function required to demonstrate clinical benefit which may in some cases be as low as 5%, as implied by RNA studies [44] or possibly as high as 25%, which was necessary to restore full ciliary function [47]. Of note, the conservative threshold of 10% WT function is higher than what was achieved for combinatorial treatment of F508del in both FRT and CFBE systems. This is important, since the combination of ivacaftor and lumacaftor has achieved clinical benefit and FDA approval for individuals who carry two copies of F508del [21]. If the response of mutant CFTR to newer derivatives and novel classes of

modulators continues to correlate with residual function, it is possible that therapeutic response might be achieved in individuals with very minimal residual function.

3.4 Materials and Methods

Study design

Rare missense variants from the CFTR2 database which had moderate sweat chloride (40-90 mM) were selected for study in CFBE cells. The set of moderate sweat variants were ranked in priority based on frequency within the CF population. Variants which had <10% WT function were selected to evaluate drug responses. Additionally, several of the first cell lines made were also tested for drug response even if their residual function was greater than 10% WT (D979V, Y1032C, G622D, P5L). Missense variants from TM6 were based on previous studies [22,111] (R334W, I336K, T338I, and S341P) or naturally occurring variants at positions of interest (R334L, R334Q, I340N, M348V, A349V). All TM6 variants were tested for drug response regardless of residual function. In total, variants were selected across a range of clinical and molecular phenotypes to identify trends which might be useful for predicting the drug response of novel missense variants identified in individuals without the need for *in vitro* testing. Functional testing was measured with a minimum of n = 3 for each cell line tested for each drug condition as well as a minimum of n = 3 for measurement of CFTR expression by qRT-PCR. Drug doses were assigned based on previous studies [15,17,22,29,30] and compared to equivalent treatment with the drug vehicle (DMSO in all cases).

Generation of variant plasmids

A pEF/FRT expression plasmid (Life Technologies) containing WT-CFTR cDNA sequence was used as a template for generation of variant alleles of *CFTR* by site directed

mutagenesis. Mutagenesis was confirmed by sequencing of the complete *CFTR* cDNA sequence to guard against secondary mutations. Plasmids were confirmed to be competent for transfection by transient transfection of HEK293 cells and subsequent Western blotting to evaluate production of protein as well as the effect of each variant on CFTR processing.

Generation of stable cell lines

A CFBE derived cell line containing an FLP-recombinase recognition target (*FRT)* integration site [15] were transfected with *CFTR* variant plasmids in the presence of the *pOG44* recombinase plasmid. Cells were grown under hygromycin selection and individual clones were picked using cloning cylinders and grown/expanded separately. Isogenic Fisher Rat thyroid (FRT) cells (a generous gift from Dr. M. Welsh, University of Iowa) encoding CFTR variants were generated using the Flp-In™ system (Thermo) following previously published methods [22,106]. CFTR cDNAs encoding variants were cloned between NotI and XhoI restriction sites of the pcDNA5/FRT expression vector. All variants encoded the common polymorphism methionine at position 470 except three (G85E, R347P, and M1101K), which were instead generated with valine at this position to reflect haplotypes commonly associated with these complex alleles. Multiple clones of FRT cells expressing each variant were isolated and CFTR mRNA levels compared to an established wild-type CFTR line obtained by the same protocol. Only cells with mRNA levels comparable to wild-type (within 0.5-1.5 fold) as determined by quantitative RT-PCR were selected for further study.

Confirmation of CFTR expression

Integration of CFTR variant plasmids was evaluated by extraction of genomic DNA from hygromycin resistant cell lines and PCR amplification of the entire *CFTR* cDNA as well as of an empty FRT integration site. Cell lines that contained the entire *CFTR* cDNA and had an interrupted FRT site were expanded for further testing, all other cell lines were discarded. To confirm expression from the integrated plasmid, cells were plated to confluency and grown for 6 days. Total RNA was extracted and 500 ng was used for generation of a first strand cDNA library. qRT-PCR was performed using 2 primer sets within the *CFTR* cDNA sequence as well as internal primers for the housekeeping gene *HPRT1*; *B2M*, *GUSB*, *GAPDH*, and *TBP* were also evaluated as housekeeping genes, but *HPRT1* was determined to have the most stable expression under our test conditions. Cells were discarded if they failed to demonstrate measurable CFTR expression, lower limit of detection was approximately 7 cycles higher than *HPRT1*.

Measurement of CFTR function in epithelia

CFBE analysis

CFTR-mediated transepithelial Cl^- currents were recorded using a large Cl^- concentration gradient to magnify current size without permeabilizing the basolateral membrane as previously described [15,33]. To test the function of CFBE cell lines, two aliquots of cells were plated to confluency, one was used to determine function by short circuit current (I_{sc}) measurements and the other was used to measure mRNA at the time of I_{sc} measurements. CFBE cells ($1x10^5$) were plated on Snapwell filters (Corning) and cultured while submerged to a minimum transepithelial resistance (R_t) of 200 $\Omega \bullet cm^2$

while cells for RNA extraction were plated in flat bottom 6 well plates. Cells were grown in DMEM with 10% FBS without hygromycin and media was changed daily for 6 days. I_{sc} measurements were performed with EasyMount Ussing chambers (Physiologic Instruments, CA) using a chloride concentration gradient with high chloride buffer in the basolateral chamber and low chloride buffer in the apical chamber. The apical buffer contained 145 mM NaGluconate, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM dextrose, 10 mM HEPES, and the basolateral buffer was composed of 145 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM dextrose, 10 mM HEPES. Buffers were pH adjusted to 7.3 using NaOH and warmed to 37 °C prior to use. Air was gently bubbled into the buffers to promote circulation. After cancelling voltage offsets, transepithelial voltage was clamped (referenced to the basolateral solution) at 0 mV and I_{sc} recorded continuously. Once currents had stabilized (10-30 min after mounting filters in Ussing chambers), forskolin (Selleckchem) was administered to the basolateral chamber at a final concentration of 10 μM. Once stabilized at the maximal forskolin-stimulated current level, the inhibitor $CFTR_{inh}$-172 (Inh-172, Selleckchem) was administered to the apical chamber at a final concentration of 10 μM. The contribution of CFTR function to overall current was measured as the decline in current following administration of Inh-172 (ΔI_{sc}). Assessment of the functional consequence of CFTR missense variants was performed by normalizing the ΔI_{sc} based on mRNA quantity to generate a % of WT function value as described previously. Briefly, the slope derived from the correlation between mRNA level (x) and short circuit current (I_{sc}) in $\mu A/cm^2$ (y) for 24 cell lines expressing WT-CFTR is expressed as y=242.61(x) [8]. This is the slope for 100% function. The CFTR mRNA level of each variant ($mRNA_{var}$) is determined by normalizing against the mRNA

level of the housekeeping gene *HPRT1* which are each expressed at similar levels in all cell lines. Using the I_{sc} generated by each variant ($I_{sc\text{-}var}$), the % CFTR function of a variant relative to WT-CFTR (F_{var}) is calculated as follows:

$$F_{var}=100\% \text{ X } (I_{sc\text{-}var}/242.61\ (mRNA_{var})) \qquad \text{Eq. 1}$$

FRT analysis

FRT cells ($1.5x10^5$) were seeded and cultured on Transwell permeable supports (Corning) for 5 days to form well polarized monolayers with a minimum transepithelial resistance (R_t) of 400 Ω•cm^2. I_{sc} was evaluated in the presence of basolateral to apical chloride concentration gradient with an EasyMount Ussing Chamber System (Physiologic Instruments, CA) and Acquire and Analyze software. Briefly, transwell inserts were mounted into chambers bathed in low chloride Ringer`s solution (1.2 mM NaCl, 140 mM Na-gluconate, 25 mM $NaHCO_3$, 3.33 mM KH_2PO_4, 0.83 mM K_2HPO_4, 1.2mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose) bathing the apical surface and physiological Ringer's solution (120 mM NaCl, 25 mM $NaHCO_3$, 3.33 mM KH_2PO_4, 0.83 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose) bathing the basolateral surface. Once current had stabilized, 100 μM amiloride was applied to both the apical and basolateral sides of FRT epithelia to inhibit ENaC (epithelial sodium channel). To activate CFTR specific chloride current, 5 μM forskolin (Sigma) was added to both sides, followed by 5 μM ivacaftor (Selleckchem), to the apical side only. Finally, 10 μM $CFTR_{inh}$-172 (Sigma) was applied to the apical side to inhibit CFTR-mediated current. The process for determining %WT function in FRT cells was similar to that previously reported [5] but a much narrower range of mRNA expression was used. Briefly, cell lines were selected that had a very similar level of mRNA expression as cells

lines expressing WT-CFTR (0.5 – 1.5 fold) so that %WT function of the variant was derived after dividing the current generated in the cell line expressing the variant by the current generated by the cell line expressing WT CFTR.

Western blotting

Biochemical effects of small molecules on the expression of CFTR protein were evaluated by Western blot analysis [15,33]. For Western blots of transiently transfected CFTR expression plasmids, we used 40 μg of total lysate collected from HEK293 cells 48 hours post transfection. CFTR was detected using the mouse anti-CFTR monoclonal antibody (596), which recognizes NBD2 of human CFTR [143]. Blots were probed with 596 at a concentration of 1:5,000 and secondary anti-mouse antibody at a concentration of 1:100,000.

For Western blots of stably transfected CFTR expression plasmids, we used 100 μg of total lysate collected from CFBE cells. Blots were probed with 596 at a concentration of 1:1,000 and secondary anti-mouse antibody at a concentration of 1:100,000. The sodium potassium (Na^+/K^+) ATPase (Abcam) was used as a loading control for all blots; primary antibody concentration was 1:50,000 and secondary antibody concentration was 1:200,000. All blots were imaged using ECL reagent (GE) and x-ray film.

Potentiator testing

CFBE cells were prepared for I_{sc} measurements as described above (*Measurement of CFTR function in epithelia*). Following stabilization of the forskolin-stimulated current, ivacaftor (Selleckchem) was administered to the apical chamber at a final concentration of 10 μM. Potentiators from the CFFT CFTR Chemical Compound

Program were also administered into the apical chambers at concentrations corresponding to their published EC_{50} values [144–148]. Inh-172 was applied to measure the CFTR portion of the current. The effects of potentiators were measured as the Inh-172 inhibited portion (ΔI_{sc}) of the current when stimulated by forskolin and then enhanced by the potentiator compared to the ΔI_{sc} when stimulated by forskolin only.

FRT cells were prepared for I_{sc} measurements as described above, 5 μM forskolin (Sigma) was added to both sides, followed by 5 μM ivacaftor (Selleckchem), to the apical side only. Finally, 10 μM $CFTR_{inh}$-172 (Sigma) was applied to the apical side to inhibit CFTR-mediated current. Ivacaftor enhanced function was measured as the ΔI_{sc} of ivacaftor enhanced forskolin activated current and compared to ΔI_{sc} when stimulated by forskolin only.

Corrector testing

CFBE cells were grown in the presence of corrector compound or equivalent volume of DMSO for 24 h prior to measurements. Lumacaftor (Selleckchem) was administered at a final concentration of 6 μM while corrector compounds obtained from the CFFT CFTR Chemical Compound Program were tested at concentrations corresponding to their EC_{50} values [149–154]. The effects of correctors on CFTR function were evaluated by I_{sc} comparison of forskolin-stimulated CFTR currents of cells treated with each compound compared to the DMSO treated negative control. To evaluate the effect of lumacaftor on protein processing, total protein lysates were collected from stable cells after 24 h incubation with 3 μM lumacaftor or an equivalent volume of DMSO and Western blots were performed as described above (*Western blotting*). FRT cells were prepared for I_{sc} measurements as described above. Forty-eight

hours prior to assay, cells were treated with 3 μM lumacaftor (Selleckchem) or vehicle (DMSO) from both apical and basolateral surfaces of the epithelia. The effects of correctors on CFTR function were evaluated by I_{sc} comparison of forskolin-stimulated CFTR currents of cells treated with each compound compared to the DMSO treated negative control.

CFTR single-channel studies

CFTR Cl^- channels were recorded in excised inside-out membrane patches from CHO cells co-expressing CFTR and GFP voltage-clamped at –50 mV as described previously [155]. Prior to experiments, the plasma membrane expression of N1303K-CFTR was rescued by incubating transfected CHO cells at 27 °C for 3 – 7 days. The pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine (NMDG), 140 aspartic acid, 5 $CaCl_2$, 2 $MgSO_4$ and 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) adjusted to pH 7.3 with Tris ([Cl^-], 10 mM). The bath (intracellular) solution contained (mM): 140 NMDG, 3 $MgCl_2$, 1 CsEGTA and 10 TES, adjusted to pH 7.3 with HCl ([Cl^-], 147 mM; free [Ca^{2+}], < 10^{-8} M) and was maintained at 37 °C.

CFTR Cl^- channels were activated promptly following membrane patch excision using the catalytic subunit of protein kinase A (PKA [purified from bovine heart] 75 nM; Calbiochem) and ATP (1 mM; Sigma-Aldrich). To minimize channel rundown, PKA and ATP were added to all intracellular solutions. Because of the difficulty removing ivacaftor from the recording chamber [156] specific interventions with the drug were compared with pre-intervention controls. In this study, membrane patches contained ≤ 5 active channels determined using the maximum number of simultaneous channel

openings observing the precautions described previously to minimize errors counting channels.

After recording, filtering and digitizing data, single-channel current amplitude (i), P_o, mean burst duration (MBD) and interburst interval (IBI) were determined as described previously [109,155]. For wild-type CFTR, we used only membrane patches with a single active CFTR Cl^- channel for burst analyses, whereas for N1303K-CFTR, we used only bursts of single-channel openings with no superimposed openings from membrane patches with ≤ 4 active channels [157].

Structural analysis

Cryo-EM structures and homology models of the open conformation of CFTR [112,116,117] were used to map the position and orientation of the residues studied here using Pymol software and publicly available PDB files (5W81,5UAK, 2ONJ, 2HYD).

Statistics

Designation of modulator response outliers was performed by designating those responses with fold changes greater than 2SD above the mean as “high response”. High response variants were removed from the data set and outliers from the remaining dataset (greater than 2SD above the mean) were designated as “intermediate response” variants; all other responses were designated as “modest response”. Results are presented as mean values ± SEM for bar plots. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values. Correlations were calculated by Pearson linear correlations. Statistical significance was calculated by Student’s unpaired two tailed t-tests. Differences were

considered statistically significant when $p < 0.05$. Complete individual data sets for each cell line are available in Supplemental Table 3.1.

Figure 3.1. **Ivacaftor response correlates with residual function.**

(**A**) Ivacaftor (10 μM) enhanced CFTR function compared to residual forskolin (10 μM) stimulated CFTR function for 45 variants expressed in CF Bronchial Epithelial (CFBE) cells. Each variant measured $n \geq 3$ and plotted as mean ± SEM. (**B**) Ivacaftor (5 μM) enhanced CFTR function compared to residual forskolin (5 μM) stimulated CFTR function for 18 variants expressed in Fisher Rat Thyroid (FRT) cells. Each variant measured $n \geq 3$ and plotted as mean ± SEM. (**C**) Separation of variants based on their fold response to ivacaftor. Response of cell lines expressing G551D (CFBE), G551D (FRT), S1159F, and S1159P were designated as outliers by demonstrating fold response greater than 2 SD beyond the mean fold response of all variants studied in CFBE and FRT cells, and labeled as high response variants. Intermediate response variants were those that remained outliers when high response variants were removed from the comparison. All remaining variants were classified as modest response. Lines through data points represent the mean value ± 1 SD of each group. (**D**) Previously published data collected from FRT* cells (*18, 19*) of non-gating variants plotted as in A and B. (**E**) Comparison of trend lines of modest response variants identified in CFBE and FRT studies compared to non-gating variants identified in FRT* cells.

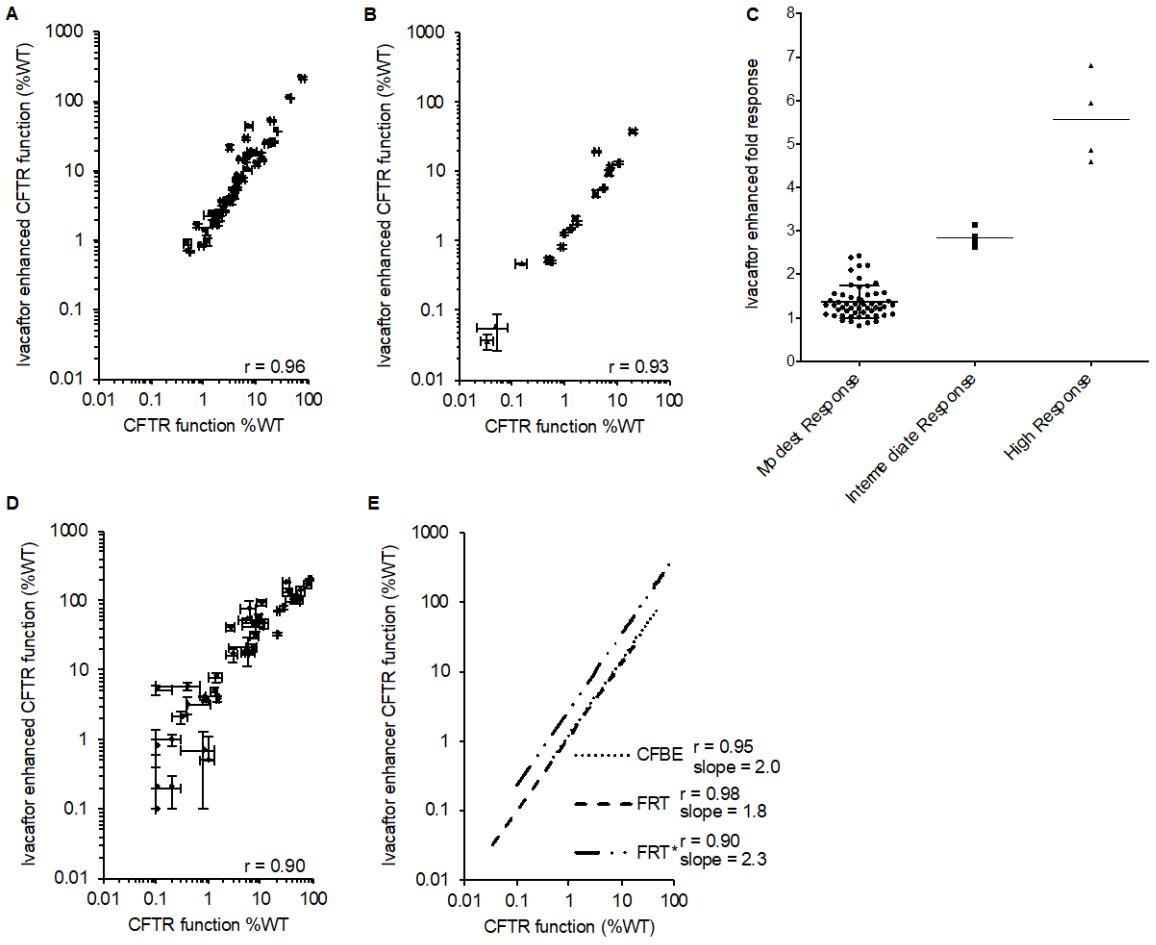

A
Ivacaftor enhanced CFTR function (%WT)
CFTR function %WT
r = 0.96
B
Ivacaftor enhanced CFTR function (%WT)
CFTR function %WT
r = 0.93
C
Ivacaftor enhanced fold response
Moderate Response
Intermediate Response
High Response
D
Ivacaftor enhanced CFTR function (%WT)
CFTR function %WT
r = 0.90
E
Ivacaftor enhancer CFTR function (%WT)
CFBE r = 0.95 slope = 2.0
FRT r = 0.98 slope = 1.8
FRT* r = 0.90 slope = 2.3
CFTR function (%WT)

Figure 3.2 Variants located in the 6th transmembrane domain (TM6) show modest response to ivacaftor.

(**A**) Plot of CFTR processing versus residual function for 54 variants expressed in FRT* cells (*18*) reveals heterogeneous response to 10 μM ivacaftor of partially or well processed low residual function missense variants (labeled). Filled green circles represent variants approved by FDA for ivacaftor treatment, G551D labeled in bold. (**B**) Western blot demonstrating that all TM6 variants produce mature C band CFTR protein when transiently expressed in HEK293 cells and representative I_{sc} tracings of all TM6 variants stably expressed in CFBE cells demonstrating response to acute treatment with 10 μM ivacaftor, recorded as area corrected current ($\mu A/cm^2$), over time, measured in minutes represented by tick marks in 1 min intervals. Data are representative of $n \geq 3$ for each variant. (**C**) Summary data for response of TM6 variants to acute treatment with 10 μM ivacaftor expressed as %WT function. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values. (**D**) Fold response for acute treatment with 10 μM ivacaftor calculated over residual function (10 μM forskolin) of TM6 variants compared to modest responsive variants identified in this study. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values.

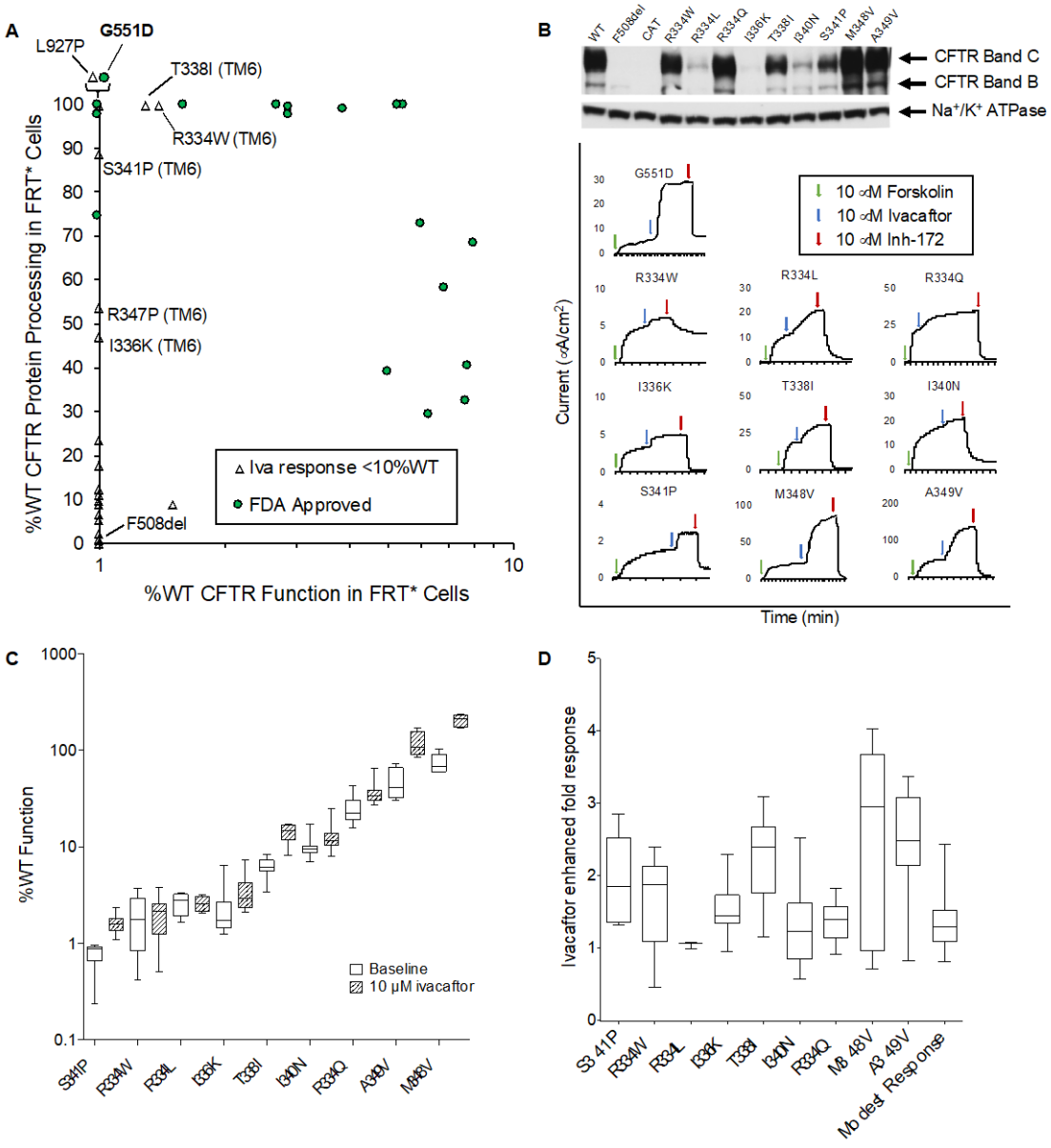

A
G551D
L927P
T338I (TM6)
R334W (TM6)
S341P (TM6)
R347P (TM6)
I336K (TM6)
F508del
Iva response <10%WT
FDA Approved
%WT CFTR Protein Processing in FRT* Cells
%WT CFTR Function in FRT* Cells
B
WT
F508del
CAT
R334W
R334L
R334Q
I336K
T338I
I340N
S341P
M348V
A349V
CFTR Band C
CFTR Band B
Na+/K+ ATPase
G551D
10 μM Forskolin
10 μM Ivacaftor
10 μM Inh-172
R334W
R334L
R334Q
I336K
T338I
I340N
S341P
M348V
A349V
Current (μA/cm2)
Time (min)
C
%WT Function
Baseline
10 μM ivacaftor
S341P
R334W
R334L
I336K
T338I
I340N
R334Q
A349V
M348V
D
Ivacaftor enhanced fold response
S341P
R334W
R334L
I336K
T338I
I340N
R334Q
M348V
A349V
Modest Response

Figure 3.3 Lumacaftor response correlates with residual function.

(**A**) Forskolin (10 μM) stimulated CFTR function of 45 missense variants expressed in CF Broncial Epithelial (CFBE) cells treated for 24 h with 6 μM lumacaftor compared to residual forskolin (10 μM) stimulated CFTR function when incubated for 24 h with an equal volume DMSO. Each variant was measured $n \geq 3$ and plotted as mean ± SEM. (**B**) Forskolin (5 μM) stimulated CFTR function of 18 missense variants expressed in Fisher Rat Thyroid (FRT) cells treated for 48 h with 3 μM lumacaftor compared to residual forskolin (5 μM) stimulated CFTR function when incubated for 48 h with an equal volume DMSO. Each variant was measured $n \geq 3$ and plotted as mean ± SEM. (**C**) Separation of variants based on their fold response to lumacaftor. Response of cell lines expressing G91R, E92K, L138ins, L145H, and L206W were designated as outliers by demonstrating fold response greater than 2SD beyond the mean fold response of all variants studied in CFBE and FRT cells, and are labeled as high response variants. Intermediate response variants were those that remained outliers when high response variants were removed from the comparison. All remaining variants were classified as modest response. Lines through data points represent the mean value ±1SD of each group. (**D**) Comparison of best fit functions for variants expressed in CFBE and FRT cells which demonstrated modest response to lumacaftor.

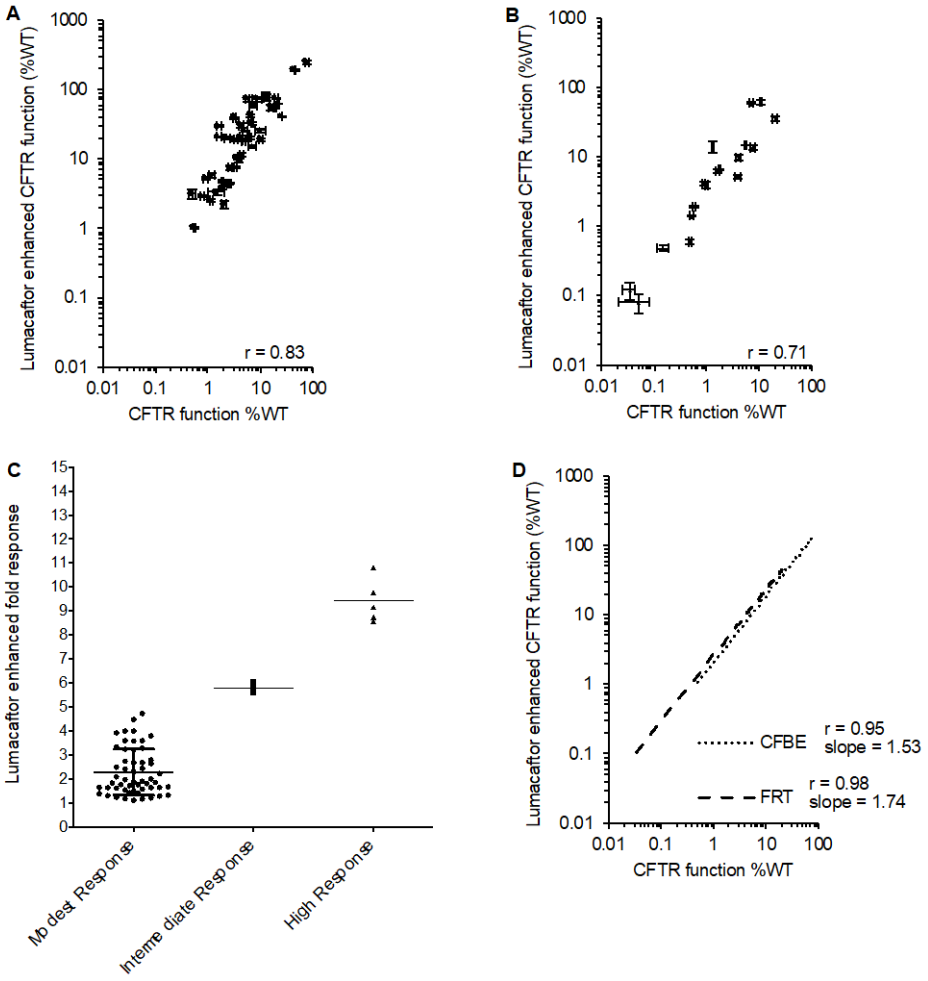

A
Lumacaftor enhanced CFTR function (%WT)
1000
100
10
1
0.1
0.01
r = 0.83
0.01 0.1 1 10 100
CFTR function %WT
B
Lumacaftor enhanced CFTR function (%WT)
r = 0.71
CFTR function %WT
C
Lumacaftor enhanced fold response
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Modest Response
Intermediate Response
High Response
D
Lumacaftor enhanced CFTR function (%WT)
CFBE
r = 0.95
slope = 1.53
FRT
r = 0.98
slope = 1.74
CFTR function %WT

Figure 3.4 TM6 variants with exceptional response to lumacaftor corresponds to embedded side chain orientation within channel pore.

(**A**) Western blots of whole cell lysates from CF Bronchial Epithelial (CFBE) cell lines stably expressing TM6 variants show that lumacaftor increases the quantity of mature CFTR protein for all TM6 variants. (**B**) I_{sc} tracings of TM6 variants reveal that a subset of TM6 variants have an increased response to lumacaftor (lum) recorded as area corrected current ($\mu A/cm^2$), over time, measured in minutes represented by tick marks in 1 min intervals. (**C**) Lumacaftor response of TM6 variants following 24 h treatment with 6 μM lumacaftor or an equal volume of DMSO. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values. (**D**) Lumacaftor response for each TM6 variant calculated as fold response over residual function compared to all modest response variants identified in CFBE cells. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values. (**E**) Predicted orientation of TM6 residues within the CFTR conductance pore when in the open conformation (32). (**F**) Lumacaftor response relative to residual function for TM6 variants based on predicted orientation within the pore. Box plots divide the data by quartile with the median value indicated by a horizontal line within the box and whiskers extend to minimum and maximum values.

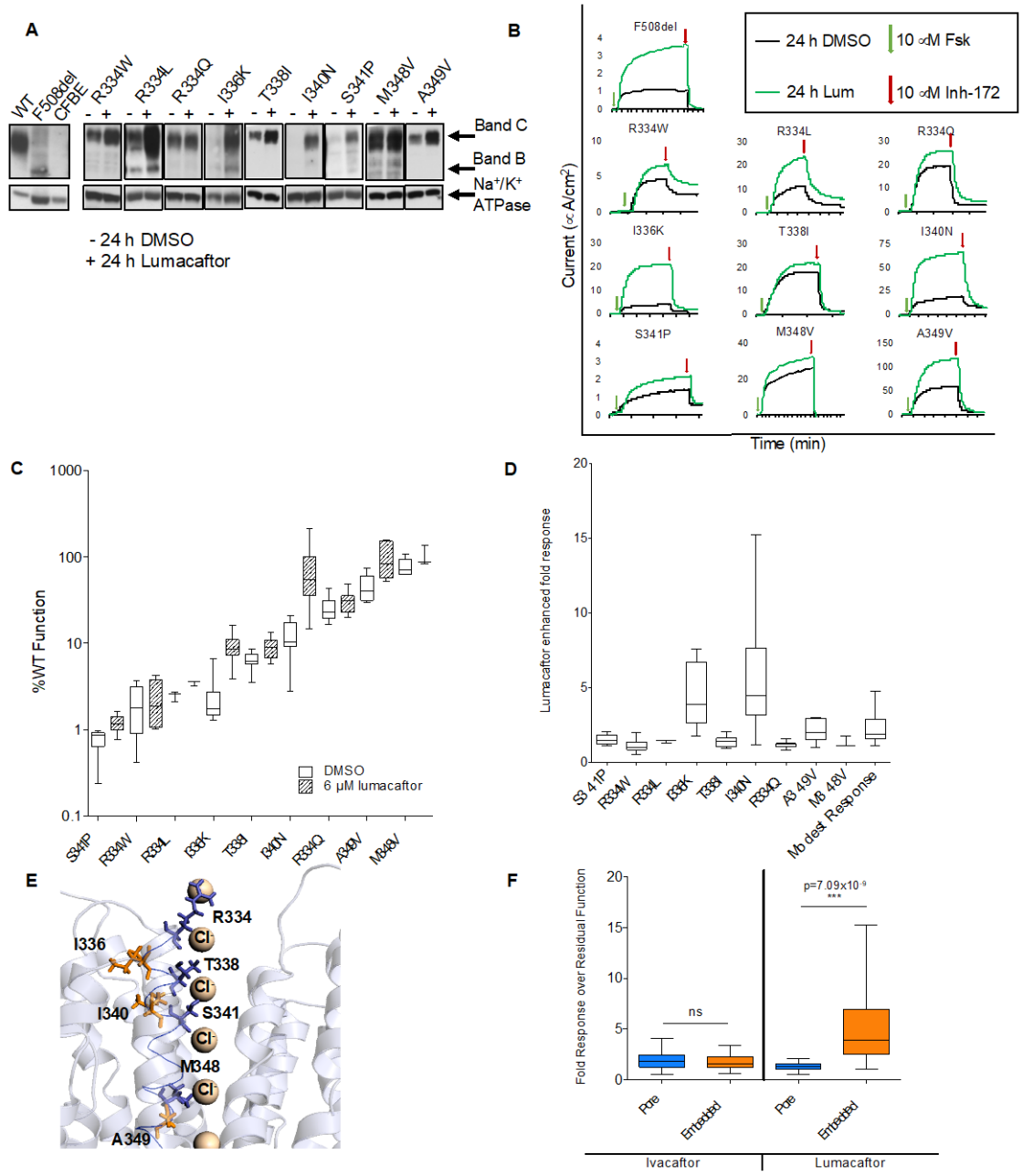

A
WT
F508del
CFBE
R334W
R334L
R334Q
I336K
T338I
I340N
S341P
M348V
A349V
Band C
Band B
Na+/K+ ATPase
- 24 h DMSO
+ 24 h Lumacaftor
B
F508del
24 h DMSO
10 μM Fsk
24 h Lum
10 μM Inh-172
R334W
R334L
R334Q
I336K
T338I
I340N
S341P
M348V
A349V
Current (μA/cm²)
Time (min)
C
%WT Function
DMSO
6 μM lumacaftor
D
Lumacaftor enhanced fold response
Modest Response
E
R334
I336
T338
I340
S341
M348
A349
F
Fold Response over Residual Function
p=7.09x10⁻⁹
***
ns
Pre
Enhanced
Ivacaftor
Lumacaftor

Figure 3.5 Ivacaftor/lumacaftor (iva/lum) response correlates with residual function. (**A**) Ivacaftor (10 μM) enhanced CFTR function of 45 missense variants expressed in CFBE cells following for 24 h incubation with 6 μM lumacaftor compared to residual forskolin (10 μM) stimulated CFTR function when incubated for 24 h with DMSO. Each variant measured n ≥ 3 and plotted as mean ± SEM. (**B**) Ivacaftor (5 μM) enhanced CFTR function of 18 missense variants expressed in FRT cells following 24 h incubation with 3 μM lumacaftor compared to residual forskolin (5 μM) stimulated CFTR function when incubated for 24 h with DMSO. Each variant measured n ≥ 3 and plotted as mean ± SEM. (**C**) Separation of variants based on their fold response to lumacaftor. Response of cell lines expressing G91R, E92K, L138ins, L145H, and G551D (CFBE) were designated as outliers by demonstrating a fold response greater than 2 SD beyond the mean fold response of all variants studied in CFBE and FRT cells, and are labeled as high response variants. Intermediate response variants were those that remained outliers when high response variants were removed from the comparison. All remaining variants were classified as modest response. Lines through the data points represent the mean value ± 1 SD of each group. (**D**) Comparison of best fit functions for variants expressed in CFBE and FRT cells which demonstrated modest response to ivacaftor/lumacaftor combination treatment.

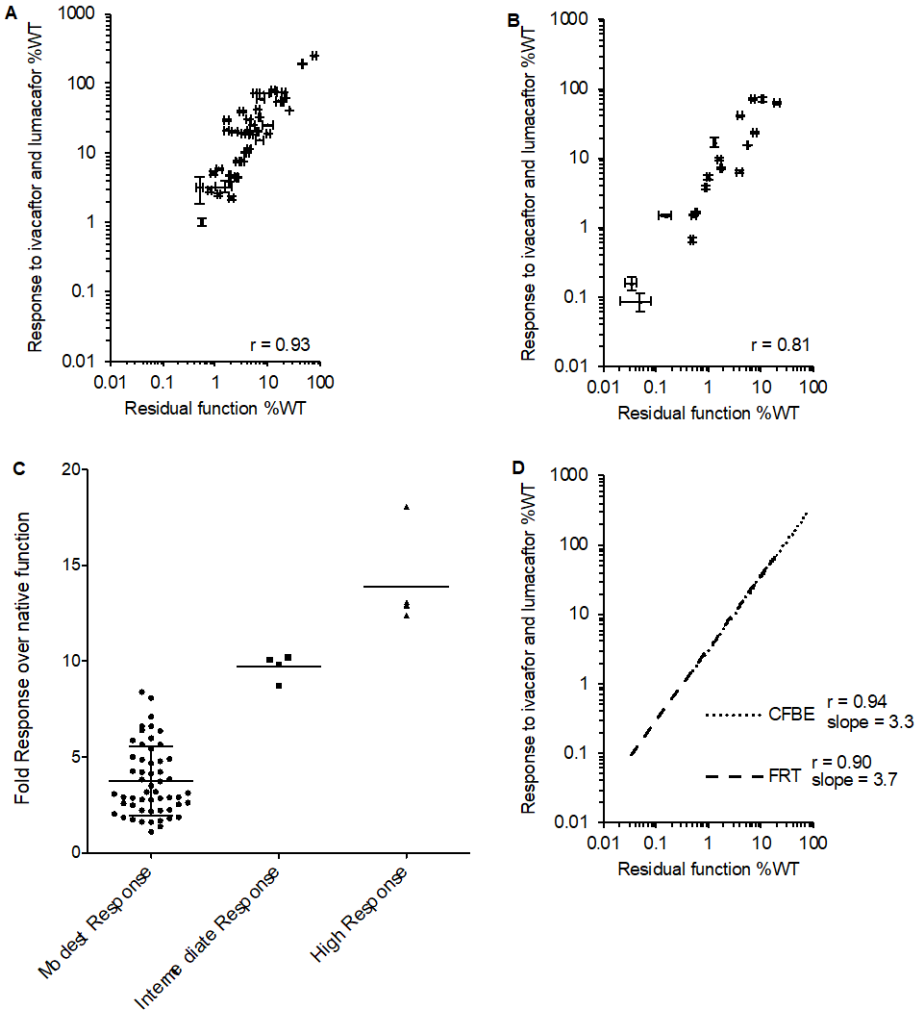

A
Response to ivacaftor and lumacaftor %WT
Residual function %WT
r = 0.93
B
Response to ivacaftor and lumacaftor %WT
Residual function %WT
r = 0.81
C
Fold Response over native function
Mo dest Response
Interme diate Response
High Response
D
Response to ivacaftor and lumacaftor %WT
Residual function %WT
CFBE
r = 0.94
slope = 3.3
FRT
r = 0.90
slope = 3.7

Figure 3.6 Summary of response of missense variants to CFTR modulators.

(**A**) CFTR function for variants that were modest response variants for ivacaftor and lumacaftor when treated with forskolin (baseline), ivacaftor, lumacaftor, or iva/lum combination. Error bars represent ± SEM. (**B**) Response to ivacaftor, lumacaftor, or ivacaftor/lumacaftor combination for variants designated as intermediate or high response to ivacaftor. (**C**) Response to ivacaftor, lumacaftor or iva/lum combination for variants designated as intermediate or high response to lumacaftor.

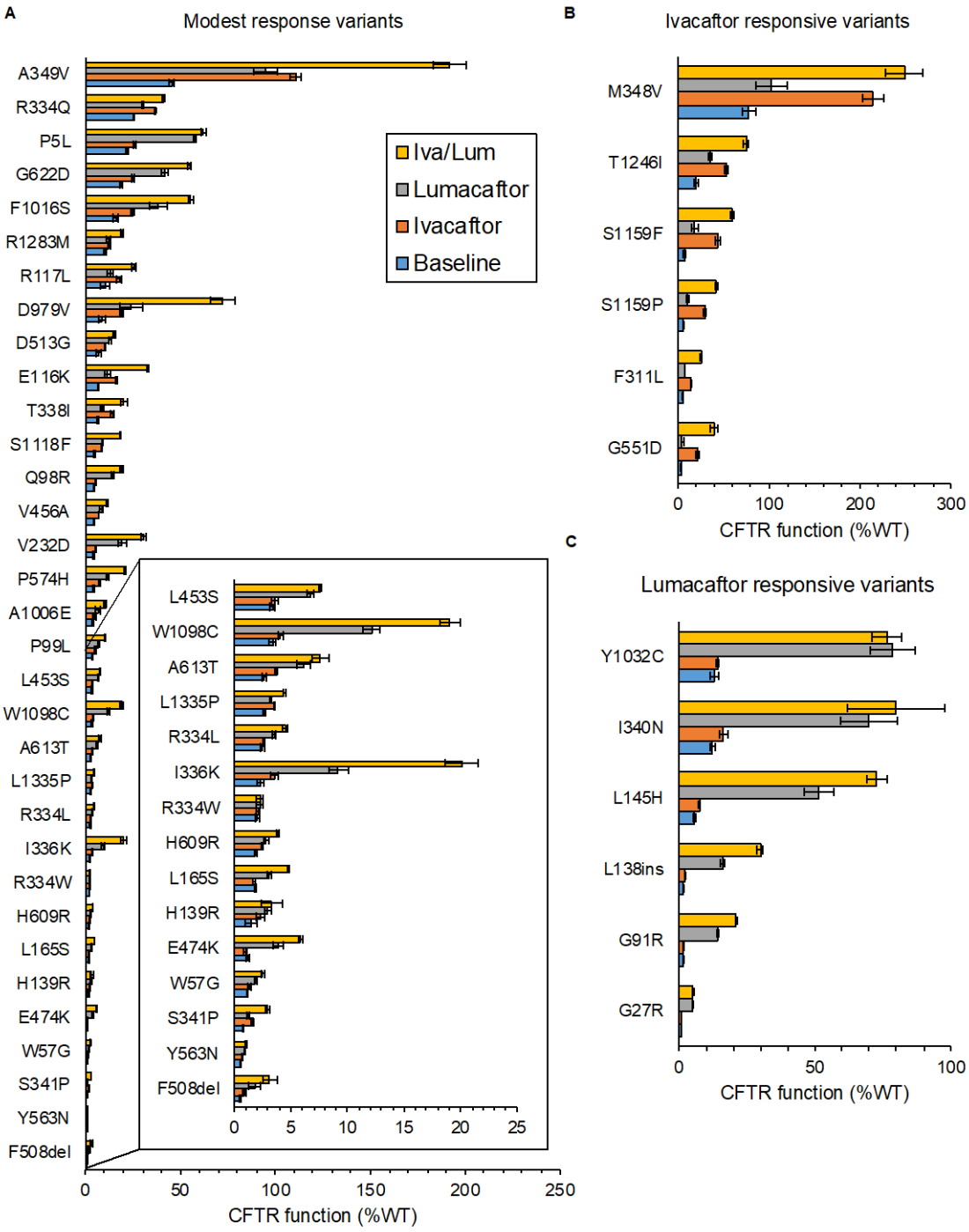

A
Modest response variants
A349V
R334Q
P5L
G622D
F1016S
R1283M
R117L
D979V
D513G
E116K
T338I
S1118F
Q98R
V456A
V232D
P574H
A1006E
P99L
L453S
W1098C
A613T
L1335P
R334L
I336K
R334W
H609R
L165S
H139R
E474K
W57G
S341P
Y563N
F508del
Iva/Lum
Lumacaftor
Ivacaftor
Baseline
0
50
100
150
200
250
CFTR function (%WT)
0
5
10
15
20
25
B
Ivacaftor responsive variants
M348V
T1246I
S1159F
S1159P
F311L
G551D
0
100
200
300
CFTR function (%WT)
C
Lumacaftor responsive variants
Y1032C
I340N
L145H
L138ins
G91R
G27R
0
50
100
CFTR function (%WT)

Figure 3.7 Combination therapy yields larger response than monotherapy across all response tiers.

(**A**) Comparison of response trends for ivacaftor (left), lumacaftor (middle), and ivacaftor/lumacaftor combination (right) of all variants studied in CF Bronchial Epithelial (CFBE) and Fisher Rat Thyroid (FRT) cell lines. (**B**) Comparison of response trends for all 3 treatment strategies for modest response variants (left), intermediate response variants (middle), and high response variants (right).

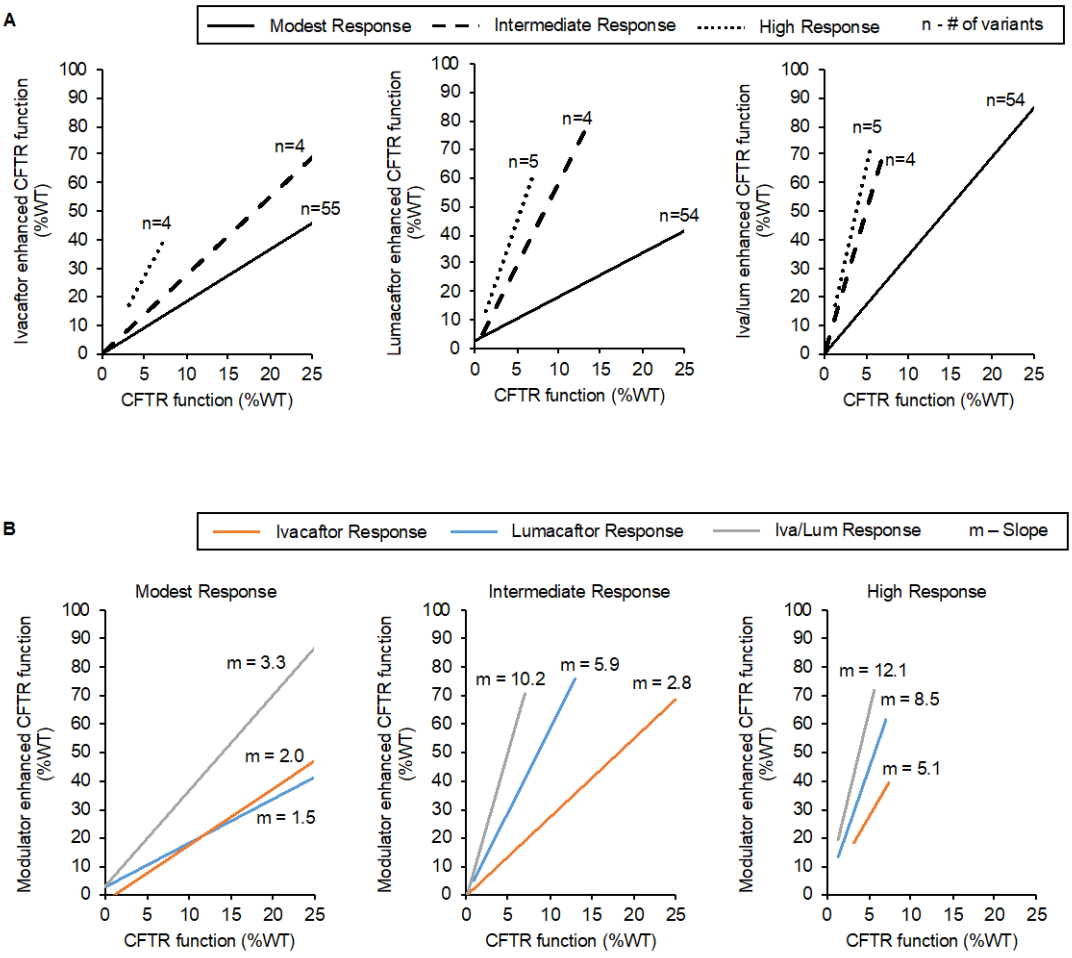

A
Modest Response
Intermediate Response
High Response
n - # of variants
Ivacaftor enhanced CFTR function (%WT)
Lumacaftor enhanced CFTR function (%WT)
Iva/lum enhanced CFTR function (%WT)
CFTR function (%WT)
n=4
n=55
n=5
n=54
B
Ivacaftor Response
Lumacaftor Response
Iva/Lum Response
m – Slope
Modest Response
Intermediate Response
High Response
Modulator enhanced CFTR function (%WT)
m = 3.3
m = 2.0
m = 1.5
m = 10.2
m = 5.9
m = 2.8
m = 12.1
m = 8.5
m = 5.1

Table 3.1 Residual function and drug response for CF Bronchial Epithelial (CFBE) cell lines

Variant	HGVS	cDNA	Residual function (n)	10 μM Ivacaftor (n)	6 μM Lumacaftor (n)	10 μM ivacaftor 6 μM lumacaftor (n)
P5L	p.Pro5Leu	c.14C>T	21.9 ± 2 (3)	25.9 ± 0.6 (3)	57.72 ± 0.6 (3)	62.2 ± 1.4 (3)
G27R	p.Gly27Arg	c.79G>A	0.9 ± 0.1 (3)	0.8 ± 0 (3)	5.14 ± 0.07 (3)	5.2 ± 0.3 (3)
W57G	p.Trp57Gly	c.169T>G	1.1 ± 0.1 (3)	1.3 ± 0.2 (3)	1.89 ± 0.11 (3)	2.5 ± 0.1 (3)
G91R	p.Gly91Arg	c.271G>A	1.6 ± 0.2 (3)	1.7 ± 0.1 (3)	14.29 ± 0.31 (3)	21.1 ± 0.2 (3)
Q98R	p.Gln98Arg	c.293A>G	4.5 ± 0.1 (3)	5.5 ± 0.2 (3)	14.32 ± 0.51 (3)	18.8 ± 0.9 (3)
P99L	p.Pro99Leu	c.296C>T	3.7 ± 0.1 (3)	5.1 ± 0.6 (3)	7 ± 0.47 (3)	10.2 ± 0.3 (3)
E116K	p.Glu116Lys	c.346G>A	6.9 ± 0.3 (3)	16.4 ± 0.5 (3)	11.59 ± 1.61 (3)	32.9 ± 0.5 (3)
R117L	p.Arg117Leu	c.350G>T	10.2 ± 2.5 (3)	17.8 ± 1.3 (3)	13.16 ± 1.53 (3)	25.4 ± 1.3 (3)
L138ins	p.Leu138insLeu	c.413_415dupTAC	1.6 ± 0.2 (3)	2.2 ± 0.2 (3)	16.1 ± 0.86 (3)	29.8 ± 1.1 (3)
H139R	p.His139Arg	c.416A>G	1.5 ± 0.5 (3)	2.3 ± 0.4 (3)	2.96 ± 0.29 (3)	3.3 ± 0.9 (3)
L145H	p.Leu145His	c.434T>A	5.6 ± 0.4 (3)	7.5 ± 0.4 (3)	51.48 ± 5.36 (3)	72.8 ± 3.6 (3)
L165S	p.Leu165Ser	c.494T>C	1.9 ± 0.1 (3)	1.7 ± 0.1 (3)	3.1 ± 0.19 (3)	4.8 ± 0.1 (3)
V232D	p.Val232Asp	c.695T>A	4.3 ± 0.4 (3)	5.5 ± 0.2 (3)	19.26 ± 2.25 (3)	30.5 ± 1.5 (3)
F311L	p.Phe311Leu	c.933C>G	5.1 ± 0.6 (3)	14.7 ± 0.4 (3)	7.31 ± 0.27 (0)	25.1 ± 0.7 (3)
R334W	p.Arg334Trp	c.1000C>T	2 ± 0.3 (13)	2.1 ± 0.2 (21)	2.2 ± 0.5 (8)	2.2 ± 0.3 (10)
R334L	p.Arg334Leu	c.1001G>T	2.5 ± 0.2 (3)	2.6 ± 0.1 (3)	3.5 ± 0.13 (0)	4.5 ± 0.3 (3)
R334Q	p.Arg334Gln	c.1001G>A	25.5 ± 1.4 (29)	34.2 ± 2.3 (30)	30.2 ± 2.1 (15)	40.9 ± 2.3 (15)
I336K	p.Ile336Lys	c.1007T>A	2.3 ± 0.3 (20)	3.2 ± 0.4 (24)	9.2 ± 0.9 (13)	20.1 ± 1.5 (26)
T338I	p.Thr338Ile	c.1013C>T	6.4 ± 0.3 (18)	14.2 ± 0.8 (18)	8.9 ± 0.8 (9)	20.3 ± 1.8 (9)
I340N	p.Ile340Asn	c.1019T>A	12.5 ± 0.9 (33)	16.5 ± 1.6 (21)	70 ± 10.4 (20)	79.8 ± 17.7 (14)
S341P	p.Ser341Pro	c.1021T>C	0.8 ± 0.1 (11)	1.5 ± 0.1 (13)	1.2 ± 0.1 (11)	2.9 ± 0.2 (13)
M348V	p.Met348Val	c.1042A>G	77.8 ± 7.4 (6)	138.8 ± 7.3 (6)	103 ± 17.3 (3)	124.2 ± 56.3 (6)
A349V	p.Ala349Val	c.1046C>T	45.5 ± 4.7 (13)	110.7 ± 11.2 (14)	94.9 ± 16.3 (7)	139.5 ± 32.3 (11)
L453S	p.Leu453Ser	c.1358T>C	3.4 ± 0.2 (3)	3.6 ± 0.3 (3)	6.7 ± 0.3 (3)	7.6 ± 0.1 (3)
V456A	p.Val456Ala	c.1367T>C	4.4 ± 0.4 (3)	6.9 ± 0 (3)	8.2 ± 0.7 (3)	11.5 ± 0.4 (3)
E474K	p.Glu474Lys	c.1420G>A	1.2 ± 0.1 (3)	1 ± 0.1 (3)	3.9 ± 0.5 (3)	5.9 ± 0.2 (3)
F508del	p.Phe508del	c.1521_1523del	0.5 ± 0.1 (15)	0.9 ± 0.2 (11)	1.8 ± 0.5 (10)	3.2 ± 0.7 (10)
D513G	p.Asp513Gly	c.1538A>G	7 ± 1.3 (3)	10.4 ± 0.3 (3)	12.9 ± 0.6 (3)	15.2 ± 0.9 (3)
G551D	p.Gly551Asp	c.1652G>A	3.2 ± 0.3 (38)	21.6 ± 1.8 (30)	4.9 ± 1.3 (14)	39.4 ± 4.2 (13)
Y563N	p.Tyr563Asn	c.1687T>A	0.6 ± 0 (3)	0.7 ± 0 (3)	0.9 ± 0 (3)	1 ± 0.1 (3)
P574H	p.Pro574His	c.1721C>A	4.3 ± 0.3 (3)	7.3 ± 0.3 (3)	11.5 ± 0.7 (3)	20.8 ± 0.5 (3)
H609R	p.His609Arg	c.1826A>G	1.9 ± 0.1 (3)	2.5 ± 0.1 (3)	2.8 ± 0.2 (3)	3.9 ± 0.1 (3)
A613T	p.Ala613Thr	c.1837G>A	2.7 ± 0.2 (3)	3.7 ± 0.1 (3)	6.1 ± 0.6 (3)	7.6 ± 0.7 (3)
G622D	p.Gly622Asp	c.1865G>A	18.8 ± 0.8 (3)	24.9 ± 0.8 (3)	41.7 ± 1.7 (3)	54.7 ± 0.8 (3)
D979V	p.Asp979Val	c.2936A>T	8.6 ± 1.8 (3)	19 ± 0.9 (3)	24.1 ± 5.9 (3)	72.4 ± 6.4 (3)
A1006E	p.Ala1006Glu	c.3017C>A	3.9 ± 0.4 (3)	4.8 ± 0.9 (3)	6.4 ± 1.4 (3)	10.2 ± 0.5 (3)
F1016S	p.Phe1016Ser	c.3047T>C	15.8 ± 1.5 (3)	24.7 ± 0.7 (3)	38.2 ± 4.7 (3)	55.4 ± 1.3 (3)
Y1032C	p.Tyr1032Cys	c.3095A>G	13 ± 1.6 (3)	14.2 ± 0.4 (3)	78.5 ± 8.3 (3)	76.4 ± 5.5 (3)
W1098C	p.Trp1098Cys	c.3294G>C	3.4 ± 0.3 (6)	4.1 ± 0.3 (6)	12.1 ± 0.7 (6)	19.1 ± 0.9 (6)
S1118F	p.Ser1118Phe	c.3353C>T	4.8 ± 0.5 (3)	8.6 ± 0.3 (3)	8.9 ± 0.2 (3)	18.3 ± 0.4 (3)
S1159F	p.Ser1159Phe	c.3476C>T	7.4 ± 1.3 (3)	43.8 ± 2.4 (3)	18.4 ± 3.8 (3)	59.5 ± 2 (3)
S1159P	p.Ser1159Pro	c.3475T>C	6.4 ± 0.5 (4)	29.3 ± 1.3 (4)	10.9 ± 1.6 (4)	42.2 ± 1.4 (4)
T1246I	p.Thr1246Ile	c.3737C>T	20 ± 2 (3)	52.9 ± 1.8 (3)	35.2 ± 1.8 (3)	74.7 ± 2.7 (3)
R1283M	p.Arg1283Met	c.3848G>T	10.3 ± 0.9 (3)	12.5 ± 0.7 (3)	12.1 ± 1 (3)	19.1 ± 0.7 (3)
L1335P	p.Leu1335Pro	c.4004T>C	2.6 ± 0.1 (3)	3.5 ± 0.1 (3)	3.2 ± 0.1 (3)	4.4 ± 0.1 (3)

Table 3.2 Residual function and drug response for Fisher Rat Thyroid (FRT) cell lines

Variant	HGVS	cDNA	Residual function (n)	5 μM Ivacaftor (n)	3 μM Lumacaftor (n)	5 μM ivacaftor 3 μM lumacaftor (n)
P67L	p.Pro67Leu	c.200C>T	10.7 ± 0.5 (3)	13.5 ± 0.7 (3)	62.1 ± 3.1 (3)	70.8 ± 4.8 (3)
G85E	p.Gly85Glu	c.245G>A	0.5 ± 0 (11)	0.5 ± 0 (11)	0.6 ± 0 (11)	0.7 ± 0.1 (11)
E92K	p.Glu92Lys	c.274G>A	1.3 ± 0.1 (6)	1.5 ± 0.1 (6)	14.2 ± 2.2 (6)	17.2 ± 2.6 (6)
R117H	p.Arg117His	c.350G>A	20.1 ± 2.4 (6)	38.3 ± 1.9 (6)	35.6 ± 2.6 (6)	62.9 ± 2.9 (6)
L206W	p.Leu206Trp	c.617T>G	7 ± 0.6 (6)	9.8 ± 0.9 (6)	60.1 ± 2.2 (6)	70.7 ± 2.3 (6)
R334W	p.Arg334Trp	c.1000C>T	3.9 ± 0.5 (5)	4.9 ± 0.5 (5)	5.1 ± 0.3 (5)	6.4 ± 0.3 (5)
T338I	p.Thr338Ile	c.1013C>T	7.6 ± 0.7 (6)	12 ± 0.5 (6)	13.7 ± 0.7 (6)	23.4 ± 1.1 (6)
R347P	p.Arg347Pro	c.1040G>C	1 ± 0.1 (9)	1.3 ± 0.1 (9)	4 ± 0.4 (9)	5.4 ± 0.4 (9)
A455E	p.Ala455Glu	c.1364C>A	5.6 ± 0.2 (3)	5.8 ± 0.2 (3)	14.8 ± 0.5 (3)	15.4 ± 0.2 (3)
S492F	p.Ser492Phe	c.1475C>T	1.7 ± 0.1 (3)	1.8 ± 0.1 (3)	6.6 ± 0.1 (3)	7.2 ± 0.2 (3)
F508del	p.Phe508del	c.1521_1523del	1.6 ± 0.1 (5)	2.1 ± 0.2 (5)	6.3 ± 0.4 (5)	9.6 ± 0.4 (5)
V520F	p.Val520Phe	c.1558G>T	0.5 ± 0 (5)	0.5 ± 0.1 (5)	1.4 ± 0 (6)	1.5 ± 0 (6)
G551D	p.Gly551Asp	c.1652G>A	4 ± 0.4 (5)	19.7 ± 0.7 (5)	9.9 ± 2 (6)	41.1 ± 0.8 (6)
R560T	p.Arg560Thr	c.1679G>C	0.1 ± 0 (7)	0.1 ± 0 (7)	0.1 ± 0 (9)	0.1 ± 0 (9)
A561E	p.Ala561Glu	c.1682C>A	0 ± 0 (6)	0 ± 0 (6)	0.1 ± 0 (6)	0.2 ± 0 (6)
L1077P	p.Leu1077Pro	c.3230T>C	0.9 ± 0 (6)	0.8 ± 0.1 (6)	4.2 ± 0.3 (6)	3.8 ± 0.3 (6)
M1101K	p.Met1101Lys	c.3302T>A	0.6 ± 0 (6)	0.5 ± 0 (6)	1.9 ± 0.1 (6)	1.7 ± 0 (6)
N1303K	p.Asn1303Lys	c.3909C>G	0.2 ± 0 (5)	0.5 ± 0 (5)	0.5 ± 0.1 (4)	1.5 ± 0 (4)

Chapter 4. Transformative Therapies for Rare CFTR Missense Alleles

4.1 Introduction

Cystic Fibrosis (CF) is an autosomal recessive disorder with nearly 90,000 reported cases worldwide, and highest incidence occurs among white individuals of Northern European descent. The disease results from loss-of-function of the CF transmembrane conductance regulator (CFTR), a chloride and bicarbonate channel expressed at the apical surface of exocrine secretory epithelia. In the absence of functional CFTR, hyperviscous luminal secretions accumulate within respiratory, pancreatic, gastrointestinal and reproductive systems, ultimately leading to chronic inflammation, severe tissue damage, and multi-organ destruction.

The *CFTR* gene was mapped in 1989, allowing extensive study and characterization of the biochemistry and functional role of CFTR protein. More than 1,900 variants within *CFTR* have been observed to date, many of which have been shown to elicit one or more defects of steps comprising biogenesis, ion transport, or plasma membrane (PM) turnover [158–160]. *CFTR* variants have traditionally been grouped into six classes based on features associated with molecular pathogenesis: class I – defective protein synthesis (e.g. premature termination codons); class II – aberrant protein maturation and premature degradation (e.g. 'processing' defects); class III – abnormal channel regulation (e.g. 'gating' defects); class IV – improper formation of the channel pore (e.g. 'conductance' defects); class V – decreased levels of protein synthesis (e.g. splicing defects); and class VI – accelerated internalization or faulty recycling from the PM (e.g. 'turnover' defects) [www.cftr2.org] (**Fig. 1**).

Although these subcategories provide a valuable means of profiling the panoply of CFTR abnormalities, molecular complexity of individual *CFTR* variants has become

increasingly evident. *CFTR* defects exist across a spectrum of severity at the molecular level, which correlate with a range of clinical presentations. For example, mild variants result in male infertility, deleterious variants result in chronic lung infection with mucus obstruction and progressive deterioration, whereas the most severe variants often lead to pancreatic and/or hepatic insufficiency. The challenge, therefore, is to understand mechanisms underlying various *CFTR* variants and develop precision medicine approaches tailored to all forms of the disease. This review will focus on missense variants (i.e. amino acid substitutions), which represent the largest category of *CFTR* defects (~40%) [3]. We will discuss strategies that have proven successful for rescuing CFTR function, methods for predicting therapeutic responsiveness, challenges faced with the current design of clinical trials, and cutting-edge tools utilized to develop more efficient interventions.

4.2 Success story for p.G551D

The first CFTR-targeted compound resulted from a revolutionary partnership between the U.S. CF Foundation and the pharmaceutical industry. Using high-throughput, cell-based fluorescence membrane potential assays in recombinant Fischer rat thyroid (FRT) cells, the small molecule VX-770 (Ivacaftor, Kalydeco^{TM}) was discovered as a robust potentiator of gating activity in the *CFTR* class III variant, p.G551D. Ivacaftor was shown to improve multiple measures of clinical utility in patients carrying at least one G551D-CFTR allele, including: (1) enhanced forced expiratory volume in one second (FEV_1), (2) increased body mass index (BMI), (3) fewer respiratory infections, (4) decreased hospitalizations, and (5) reduced sweat chloride

levels [31,161].

The Food and Drug Administration (FDA) approved Ivacaftor in 2012 as a pharmacologic treatment for CF patients 12 years and older who carry at least one copy of the p.G551D variant. In the years following, trials conducted in younger patients with the p.G551D variant (i.e. 6-12 years old), suggested that starting Ivacaftor at an earlier age may slow or even prevent lung disease progression [162]. Today, children two years and older carrying certain CFTR gating variants are approved to receive the drug, and longevity studies among all ages indicate clinical benefits can be maintained for years [161,163,164].

4.3 Ivacaftor spectrum of activity

Based on compelling response of G551D-CFTR achieved with Ivacaftor, additional studies were undertaken to determine whether this compound could rescue other CFTR molecular phenotypes similar to p.G551D (i.e. class III or IV defects, including p.R117H, p.G178R, p.S549N, p.S549R, p.G551S, p.G1244E, p.G1349D, p.S1251N, and p.S1255P). *In vitro* analysis conducted on this cohort of gating/conductance variants revealed the compound greatly augmented resident channel activity [17], and subsequent clinical trial results showed significant improvement in FEV_1, BMI, and sweat chloride levels [18,98]. As a consequence, Ivacaftor was FDA-approved in 2014 for individuals two years and older carrying R117H-CFTR, and the following year, gained approval for the eight other variants listed above.

While this strategy has proven highly successful for a number of *CFTR* variants, similar approaches may not be sufficient to identify therapeutic interventions for all

missense mutations. Many CF variants exist at extremely low frequencies in the patient population, presenting a significant challenge to clinical evaluation. Informative human trials may require large numbers of subjects and controls, and are impractical for rare variants occurring in only 2 or 3 individuals worldwide. In general, ultra-orphan diseases such as CF may require alternative strategies for establishing clinical efficacy [165].

4.4 Improved understanding of *CFTR* variant complexity

To bring molecular-based therapy and precision medicine to CF patients of all genotypes, two significant hurdles must be overcome: (1) the large number of variants known to exist in *CFTR*, and (2) the low frequency at which many of these occur. Of the 796 missense variants reported in the CF mutation database [www.genet.sickkids.on.ca/app], 81 have been functionally described, and 57 of those have been classified as disease-causing [www.cftr2.org]. The primary goal of the CFTR2 project is to characterize and determine disease liability for all mutant *CFTR* alleles [5].

Distinguishing CF disease phenotypes through use of traditional categories has proven beneficial for studying individual *CFTR* variants (**Fig. 1**). However, variants that affect several processes require a more complex classification protocol involving multiple categories, each of which might require a separate class of compound [28]. Missense variants frequently fall into the aforementioned group. *In silico* tools such as molecular modeling and dynamics simulations [114] can help predict functional consequences of missense variants, but are far from comprehensive.

Sequencing of the entire *CFTR* coding region has become nearly commonplace due to substantial reduction in cost [10]. The increasing number of individuals sequenced

by whole-exome, whole-genome, and carrier screening, has dramatically enhanced the volume of sequence data derived from CF patients, asymptomatic carriers, and the general population. As with other genetic diseases [166], this has led to significant increases in the number of *CFTR* variants identified, necessitating more sophisticated methods for variant interpretation. Sequence analysis of all *CFTR* exons has augmented discovery of complex alleles, which contain multiple variants *in cis*. Complex alleles may behave differently than those with single variants in terms of function and/or drug response. This can lead to incorrect labeling of a variant as disease-causing until segregation analysis and/or functional studies exclude deleterious effects, as occurred with p.I148T [167]. Consequently, it may become imperative to identify all variants within *CFTR* when considering appropriate therapy for a particular CF patient. The establishment of large general databanks for genetic variants (ClinVar, ExAC, etc.), as well as expertly curated databases (e.g. CFTR2), provides important tools for differentiating detrimental versus benign variants based on population frequency [168].

4.5 Challenges evaluating therapeutic responsiveness

As the number of reported rare *CFTR* variants increases, model systems will become significantly more valuable for experimental evaluation of underlying molecular defects. The ability of cell-based platforms to generate robust, reliable data in an efficient manner will determine the rate at which new therapies can be delivered to patients. Investigations using an immortalized cell line (FRT) contributed to the FDA-approval process for Ivacaftor [17,29] and Orkambi™ (combination of Ivacaftor with the corrector

Lumacaftor) [30][22], and have been useful for interpreting variant phenotypes studied thus far [22][23].

In vitro systems can be utilized to identify additional variants that respond well to Ivacaftor alone or with Lumicaftor. For example, p.P67L is a rare variant for which clinical trials are less likely to occur, since there are ~240 patients worldwide [www.cftr2.org] who carry this defect. Notably, studies conducted in FRT cells have shown p.P67L responds robustly to both compounds, reaching nearly wild-type levels of CFTR activity [33], suggesting that patients would benefit from these drugs. Conversely, the p.N1303K variant is 8 times more common (reported in over 2,000 individuals), but it is unresponsive to VX-770 or VX-809 in FRT cells [22] and primary airway epithelia [49]. Although sufficient numbers of patients may be available for a robust clinical study, preclinical evidence strongly suggests against clinical improvement in this setting.

Of the missense variants reported to CFTR2 still requiring clinical classification, more than 500 have been noted in 10 individuals or fewer worldwide (personal communication, Karen S. Raraigh, Johns Hopkins). The rarity of these patients precludes routine collection of primary cells for functional and drug studies. Thus, *in vitro* studies are expected to be primary generators of preliminary data for classification and analysis of the variants. Development of cell-based systems that more closely approximate primary human airway cells provides an opportunity to study rare CFTR defects and their response to FDA-approved compounds in a near-native context [15]. Novel strategies must also be employed to perform clinical efficacy trials in individuals with ultra-rare variants, and “N-of-1” or “N-of-a-few” are among methods currently under consideration [169].

In vitro experiments, *in silico* predictions, and pre-clinical testing are distinct branches of research employed for characterizing individual variants and predicting response to pharmacological agents. As *CFTR* missense variants under study are increasingly rare and functionally complex, strengthening the quality and quantity of evidence generated by these strategies will be paramount.

4.6 Recent advances in model systems

Characterization of disease-associated *CFTR* variants, including assessment of therapeutic responsiveness, has been performed in cell-based models for decades. Many immortalized mammalian cell lines have proven essential for distinguishing specific features of CFTR biogenesis, as well as mechanisms invoked by investigational compounds [17,105]. Recently, primary human nasal or bronchial epithelia [49,170] and induced pluripotent stem cells [171–173] have emerged as strong predictive tools. Organoids generated from patients represent another topical area of progress with the potential to predict individual response to a therapeutic strategy (i.e. precision medicine), but the validity of this postulate remains to be determined [174,175]. Additionally, six animal models expressing a variety of *CFTR* variants are now available (e.g. zebrafish, mouse, rat, rabbit, ferret, pig), although each has limitations regarding ease of use or degree to which human disease is recapitulated [14,176]. Finally, yeast phenomic screening has emerged as a means for discovery of gene-gene interaction networks and other features of *CFTR* class II and III variants [177–179], including identification and targeting of novel CFTR modulators in patient-derived epithelia [180].

4.7 Future directions relevant to CF therapeutics

Based on the success of Ivacaftor, pharmaceutical companies have begun developing other small molecules that partially restore CFTR function, the most advanced of which are second generation correctors that improve intracellular processing and cell surface activity of class II variants (P Grootenhuis et al, abstract 188, 30th North American Cystic Fibrosis Conference, Orlanda FL, October 2016), i.e. to levels above those achieved by the combination of Ivacaftor and Lumicaftor. There are at least 20 clinical trials underway that utilize such pharmacological interventions, with examples of phase II and III studies currently under enrollment shown in **Fig. 2** [www.clinicaltrials.gov].

The overarching goal of translational CF science is to develop therapeutic strategies that will benefit all individuals with CF, irrespective of genotype. Basic and clinical investigations are in progress to explore feasibility of innovative genetic and genomic medicine technologies, including transfer of nucleic acids by airway stem/progenitor cells [181,182], zinc finger nuclease- or CRISPR/Cas9-edited human pluripotent stem cells [171–173], and nanoparticles [183–185], as well as protein replacement via mRNA transfer [186]. Recently, enhanced adenoviral and lentiviral vectors were used to show functional *CFTR* gene delivery to airways of the CF porcine model [187,188], and the first lentivirus-based clinical trial is scheduled for 2017 [189].

4.8 Conclusions

Therapeutic benefit for individuals with CF harboring missense variants can be achieved by improving function of existing, partially-processed CFTR protein. As such,

missense alleles represent “low hanging fruit” for small molecule intervention. Variants that result in complete loss of CFTR protein share a potential therapeutic mechanism, in that they require insertion and expression of an entirely new or repaired *CFTR* allele. This is no small task, as gene transfer therapy has been in development for CF and other monogenic diseases since the 1990s. While the medical genetics community awaits technological progress to allow for sufficient *CFTR* gene delivery, effective therapy for most individuals with CF carrying missense variants is expected much sooner.

Figure 4.1 Classification scheme and cellular localization of *CFTR* variants.

Class I and V defects result in diminished protein production, whereas class II and VI yield reduced stability of CFTR. In addition, class III and IV variants inhibit channel function or activity of cell surface associated CFTR. Molecular-based therapeutic strategies target each of these categories and include the following: (1) 'synthesizers', which rescue CFTR protein production (e.g. suppression of premature truncation codons, or PTCs), (2) 'correctors', which augment maturation and decelerate turnover of CFTR (e.g. VX-809, VX-661, VX-152, VX-440, CTP-656, Miglustat, Riociguat), and (3) 'potentiators', which increase open channel probability and/or gating potential of apically localized CFTR (e.g. VX-770).

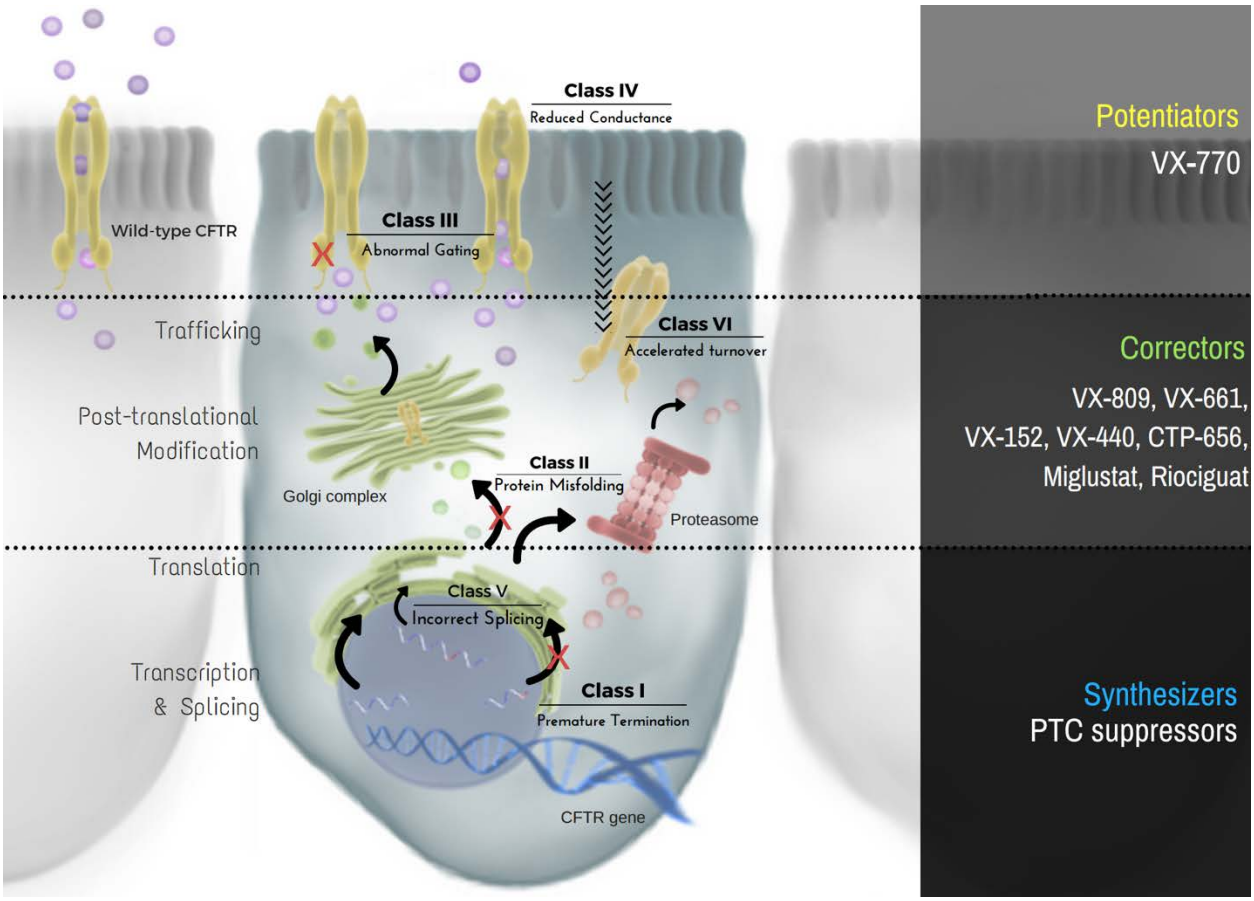
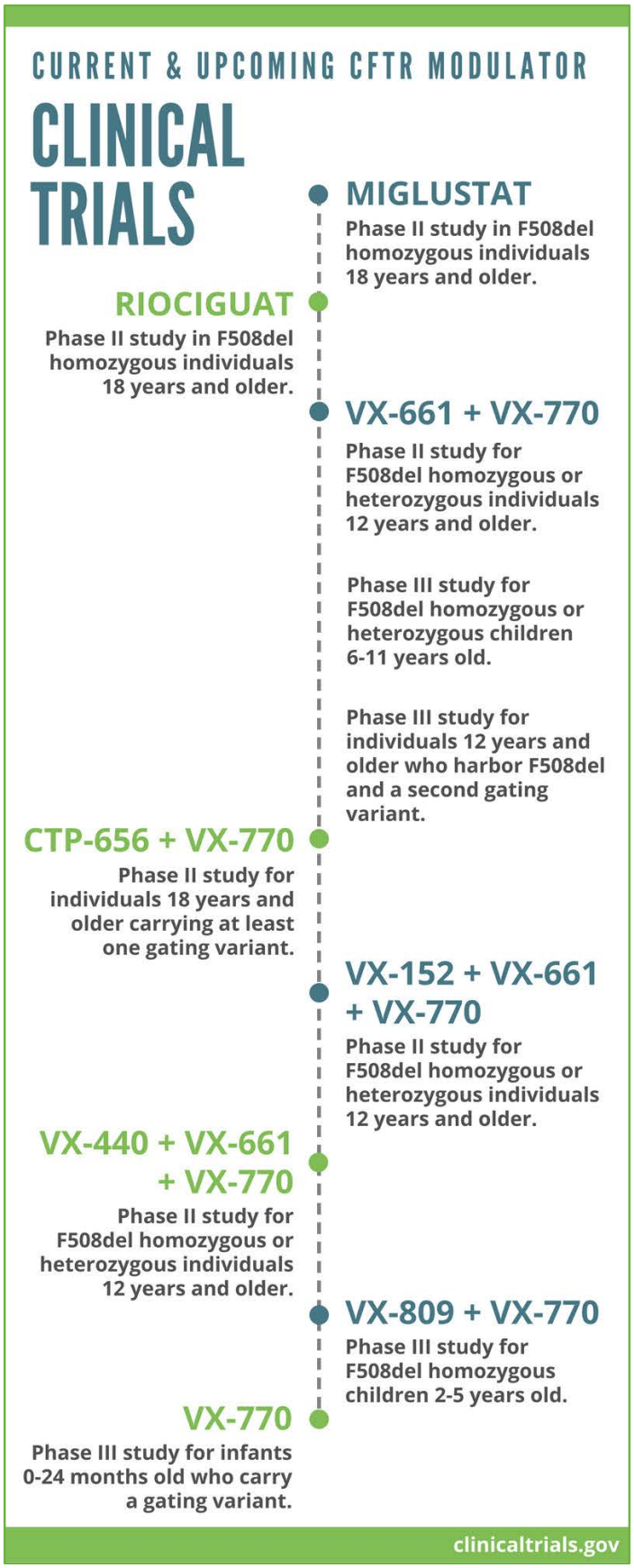

Class IV
Reduced Conductance
Potentiators
VX-770
Wild-type CFTR
Class III
Abnormal Gating
Trafficking
Class VI
Accelerated turnover
Correctors
VX-809, VX-661,
VX-152, VX-440, CTP-656,
Miglustat, Riociguat
Post-translational
Modification
Golgi complex
Class II
Protein Misfolding
Proteasome
Translation
Class V
Incorrect Splicing
Transcription
& Splicing
Class I
Premature Termination
Synthesizers
PTC suppressors
CFTR gene

Figure 4.2 Examples of current phase II or III clinical trials under enrollment in the United States and Europe (see also www.clinicaltrials.gov).

Various strategies outlined above intend to test safety, tolerability, and efficacy of CFTR modulators administered as single agents or combinatorial treatments. In the majority of cases, eligible patients must be homozygous or heterozygous for the most prevalent *CFTR* variant, F508del, or carry a gating or partial function defect.


CURRENT & UPCOMING CFTR MODULATOR
CLINICAL TRIALS
MIGLUSTAT
Phase II study in F508del homozygous individuals 18 years and older.
RIOCIGUAT
Phase II study in F508del homozygous individuals 18 years and older.
VX-661 + VX-770
Phase II study for F508del homozygous or heterozygous individuals 12 years and older.
Phase III study for F508del homozygous or heterozygous children 6-11 years old.
Phase III study for individuals 12 years and older who harbor F508del and a second gating variant.
CTP-656 + VX-770
Phase II study for individuals 18 years and older carrying at least one gating variant.
VX-152 + VX-661 + VX-770
Phase II study for F508del homozygous or heterozygous individuals 12 years and older.
VX-440 + VX-661 + VX-770
Phase II study for F508del homozygous or heterozygous individuals 12 years and older.
VX-809 + VX-770
Phase III study for F508del homozygous children 2-5 years old.
VX-770
Phase III study for infants 0-24 months old who carry a gating variant.
clinicaltrials.gov

Chapter 5 Conclusions

The studies detailed here demonstrate the utility of cellular models to generate data that can be used to determine the functional consequence of *CFTR* missense variants, which in turn can be used to inform diagnosis of individuals carrying those variants. Secondly, these cell lines were used to test the response of missense variants to small molecule drugs to generate preliminary data which is especially useful for individuals carrying variants that are too rare to be tested in traditional clinical settings. Lastly, these studies uncovered that currently available CFTR modulators can improve CFTR function for the majority of missense variants, potentially allowing for modulator therapy to be expanded to many more individuals with CF and aiding in the development of future CFTR therapies.

In vitro expression and testing of CFTR missense variants using CFBE cells has yielded results that are well correlated with clinical presentation of individuals carrying those variants. These results can be used to inform diagnosis of individuals with CF, as they aid in the classification of a variant as disease causing or not. This is especially important for rare variants as their associated clinical information is often relatively shallow. The functional data collected here also has the accuracy to resolve the variable expressivity of the CF phenotype as conferred by level of residual function. Functional evaluation of genetic variants by *in vitro* testing is wholly dependent on the strength of correlation with phenotype, and our CFBE system provides robust correlation with phenotype based on *in vivo* sweat chloride measurements and clinical presentation.

We also demonstrated that these CFBE cell lines can be used to test the response of missense variants to the CFTR modulators ivacaftor and lumacaftor. This was first demonstrated by showing that the effects of those modulators on the G551D and F508del

variants were similar to previously published results. Subsequent testing of other missense variants demonstrated that ivacaftor and lumacaftor were able to increase CFTR activity for most missense variants. While ivacaftor and lumacaftor were developed against specific genetic variants, the mechanisms they improve are intrinsic to CFTR function itself, and thus have the potential to improve the activity of many other variants of CFTR protein. Variants that are specifically defective in the mechanism targeted by a modulator appear as high response variants demonstrating that modulators with high response variants are capable of targeting a specific molecular process. Developing compounds to target additional molecular mechanisms will require studying variants specifically deficient in targeted mechanisms, analogous to the development of potentiators against the gating variant G551D and development of correctors against the folding variants F508del.

While searching for high response variants will yield novel modulators, treatment of individuals with CF will not be limited to those who carry variants with modulators developed specifically for them. Biological systems often have functional buffers such that function of a process or pathway can be upregulated or downregulated within certain limits in response to physiological needs in order to maintain functional homeostasis. Thus, in the same way that the function of WT CFTR protein can be increased by potentiators and correctors, variants that yield CFTR protein that are not deficient in channel gating or protein folding can also benefit from these modulators. This suggests that treatment of individuals with these modulators may not be limited to those carrying variants specifically targeted by those modulators. However, it is important to recognize the difference in magnitude of response when considering therapy because while CFTR

function may be improved at the molecular level, individuals who have very low residual CFTR activity may not recover enough function to demonstrate clinical utility.

We found that combination therapy was more effective than treatment with ivacaftor or lumacaftor alone for the majority of variants and was never worse than monotherapy. Given these findings paired with the mechanistic understanding that CFTR modulators act within homeostatic bounds of normal function, combination therapy is likely the best path to pursue for future treatment of individuals with CF, so long as they have variants that allow for production of CFTR protein.

The findings from these studies have resolved one of the key challenges to delivering therapy to all individuals with CF: the large number of missense variants found in *CFTR*. These findings demonstrate that all missense variants that have no defect in RNA processing can be targeted by small molecule therapies. Moreover, it appears that the response to CFTR modulators is highly predictable due to the strong correlations that exist between residual function and modulator enhanced function. These correlations suggest that it may be possible to predict clinical response based on severity of clinical presentation. The *in vitro* findings presented here predict that all variants with greater than 3% WT function will achieve the 10% theoretical threshold for clinical benefit, which correlates with variants associated with a clinical presentation of exocrine pancreatic sufficiency. If true, this means that pancreatic status could be used as a biomarker for expected therapeutic response to the ivacaftor-lumacaftor combination therapy.

Despite these advances, two major goals still exist for developing therapy for individuals with CF. First is developing therapy for individuals who make no CFTR

protein, either due to large deletions of *CFTR* sequence or DNA sequence variants which introduce a PTC. Second is developing more complete combination therapies targeting additional molecular mechanisms to treat those individuals who are able to make CFTR protein so that even those who have very low residual function can benefit from combination therapy. Addressing those individuals who do not generate any CFTR protein from either of their *CFTR* alleles present the most significant challenge, but one that is not unique to CF. These types of therapies will necessitate either correction of genomic DNA *in vivo* via gene editing or some form of gene replacement therapy, both therapeutic avenues are under investigation for several genetic diseases. Developing more complete combination therapies for individuals who make CFTR protein is a process specific to CF. This will require a strong understanding of all of the molecular mechanisms involved in CFTR biogenesis, possibly by identification of archetypical variants for each mechanism as G551D is for channel gating. Furthermore, developing compounds to target additional mechanisms may be a more effective route to delivering therapy to more individuals with CF than developing better versions of currently available compounds. If these two goals can be achieved, then robust therapeutic intervention should be possible for all individuals with CF.

These CFTR modulators have already had a significant impact on the CF community and individuals with CF are able to live longer healthier lives. The major limitation of these modulators appears to be their limited ability to recover lung function as there is likely some portion of irreversible damage done by chronic infection and inflammation of diseased tissues. However, beginning treatment at a younger age will prevent significant disease progression and further increasing quality of life and lifespan

for individuals born with CF today. Molecular genetics research in CF has shown the promise and utility of understanding the consequences of DNA sequence variants for developing therapy targeted not only to the defective gene but to the specific molecular defect conferred by specific variants.

We hope that the findings presented here aid in delivering molecular diagnoses and effective therapy to many more individuals with CF than are currently available.

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Higgins, T. Hironaka, L. Hyndman, G. McLachlan, M. Inoue, S.C. Hyde, J.A. Innes, T.M. Maher, C. Moran, C. Meng, M.C. Paul-Smith, I.A. Pringle, K.M. Pytel, A. Rodriguez-Martinez, A.C. Schmidt, B.J. Stevenson, S.G. Sumner-Jones, R. Toshner, S. Tsugumine, M.W. Wasowicz, J. Zhu, Preparation for a first-in-man lentivirus trial in patients with cystic fibrosis, Thorax. 72 (2017) 137–147. doi:10.1136/thoraxjnl-2016-208406.

Curriculum Vitae

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Education

2003-2007 B.S. Chemistry, Emory University, Atlanta GA

2009-2010 M.S. Molecular Biology and Biochemistry, Georgetown University, Washington DC

2013-2018 PhD Human Genetics, Johns Hopkins University School of Medicine, Baltimore MD

Professional Experience

2013-present Human Genetics graduate student, Johns Hopkins University School of Medicine, Baltimore MD
Development of a cellular model to measure CFTR function to measure the effects of rare missense variants on protein function and response to small molecule drugs. These studies allowed for accurate assessment of disease liability and generation of primary molecular data for response to small molecule drugs for variants that are too rare to be included in clinical trials.

2010-2013 Senior Analyst, NextGen Sequencing Department, GeneDx, Gaithersburg MD
DNA sequence analyst and test manager overseeing the operation of multiple clinical sequencing panels in a CLIA/CAP certified DNA diagnostic lab.

2010 Intern, National Human Genome Research Institute, National Institutes of Health, Bethesda MD
Confirmation and fine mapping of a double recombination event detected by SNP array in a region that would be the smallest observed double recombination event.

2007-2009 Post-Baccalaureate Intramural Research Training Fellow, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda MD
Elucidating the genetics underlying gingival hyperplasia and amelogenesis imperfecta.

Professional Society Memberships

2008 to Present	American Society of Human Genetics
2015 to Present	American College of Medical Genetics
2017 to Present	American Society of Human Genetics Training and Development Committee

Peer Reviewed Publications:

1. Hart PS, Becerik S, Cogulu D, Emingil G, Ozdemir-Ozenen D, **Han ST**, Sulima P, Firatli E, Hart TC. Novel FAM83H mutations in Turkish families with autosomal dominant hypocalcified amelogenesis imperfecta. Clin Genet. 2009 Apr;75(4):401-4
2. Wright JT, Frazier-Bowers S, Simmons D, Alexander K, Crawford P, **Han ST**, Hart PS, Hart TC. Phenotypic variation in FAM83H-associated amelogenesis imperfecta. J Dent Res. 2009 Apr;88(4):356-60.
3. Becerik S, Cogulu D, Emingil G, **Han T**, Hart PS, Hart TC. Exclusion of candidate genes in seven Turkish families with autosomal recessive amelogenesis imperfecta Am J Med Genet A. 2009 Jul;149A(7):1392-8.
4. Markello TC, **Han T**, Carlson-Donohoe H, Ahaghotu C, Harper U, Jones M, Chandrasekharappa S, Anikster Y, Adams DR; NISC Comparative Sequencing Program, Gahl WA, Boerkoel CF. Recombination mapping using Boolean logic and high-density SNP genotyping for exome sequence filtering. Mol Genet Metab. 2012 Mar;105(3):382-9.
5. Denadai R, Raposo-Amaral CE, Bertola D, Kim C, Alonso N, Hart T, **Han S**, Stelini RF, Buzzo CL, Raposo-Amaral CA, Hart PS. Identification of 2 novel ANTXR2 mutations in patients with hyaline fibromatosis syndrome and proposal of a modified grading system. Am J Med Genet A. 2012 Apr;158A(4):732-42
6. Lee M, Vecchio-Pagán B, Sharma N, Waheed A, Li X, Raraigh KS, Robbins S, **Han ST**, Franca AL, Pellicore MJ, Evans TA, Arcara KM, Nguyen H, Luan S, Belchis D, Hertecant J, Zabner J, Sly WS, Cutting GR. Loss of carbonic anhydrase XII function in individuals with elevated sweat chloride concentration and pulmonary airway disease. Hum Mol Genet. 2016 May 15;25(10):1923-1933

7. Gottschalk LB, Vecchio-Pagan B, Sharma N, **Han ST**, Franca A, Wohler ES, Batista DA, Goff LA, Cutting GR. Creation and characterization of an airway epithelial cell line for stable expression of CFTR variants. J Cyst Fibros. 2016 May; 15(3):285-94
8. Oliver KE*, **Han ST***, Sorscher EJ, Cutting GR, Transformative therapies for rare CFTR missense alleles. Current Opinion in Pharmacology 2017 Jun;34:76–82
9. Raraigh KS*, **Han ST***, Davis E, Evans T, Pellicore M, McCague A, Joynt A, Zhongzhou L, Atalar M, Sharma N, Sheridan M, Sosnay P, Cutting GR. Functional assays are essential for interpretation of missense variants associated with variable expressivity. Am J Hum Genet 2018 Jun 7,102(6):1062-1077
10. **Han ST***, Rab A*, Pellicore MJ, Davis EF, McCague AF, Evans TA, Joynt AT, Lu Z, Cai Z, Raraigh KS, Hong JS, Sheppard DN, Sorscher EJ, Cutting GR. Residual Function of Cystic Fibrosis Mutants Predicts Response to Small Molecule CFTR Modulators. JCI Insight. 2018 Jul 25;3(14)

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Funding Awards

2016-2018 Title: Analysis of complex alleles in CFTR
Identification number: CUTTIN16I0
Sponsor: The Cystic Fibrosis Foundation
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Awards/Honors

2016 ASHG/Charles J. Epstein Trainee Award for Excellence in Human Genetics Research – Semifinalist.

Meeting Platform Presentations

2016 **Han ST,** Characterization of a new class of disease-causing variants unresponsive to current CFTR targeted therapies; American Society of Human Genetics Annual Meeting, Vancouver, Oct 17-21, 2017

Meeting Poster Presentations

2008 **Han ST**, Hart PS, Sulima P, Turner C, Jang SI, Hart TC. Genetic Characterization of Zimmerman Laband syndrome; American Society of Human Genetics Annual Meeting, Philadelphia, Nov 11-15

2015 **Han ST**, Bjornsson HT, Hamosh A, Valle D; An Ornithine Transcarbamylase Promoter Variant and Surrounding Haplotype Associated with Late Onset Hyperammonemia; American College of Medical Genetics Annual Meeting, Salt Lake City, Mar 24-28

2016 **Han ST**, Pellicore MJ, Evans TA, Davis EF, Raraigh KS, Cutting GR. Amino acid substitutions of pore lining residues may comprise a distinct theratype of CF causing CFTR variants; North American Cystic Fibrosis Conference, Orlando, Oct 27-29

2017 **Han ST**, McCague, AF, Atalar M, Pellicore MJ, Evans TA, Davis EF, Sharma N, Raraigh KS, Sosnay PR, Cutting GR. Functional assessment of rare CFTR missense variants in human airway cells informs disease liability and drug therapy, North American Cystic Fibrosis Conference, Indianapolis, Nov 2-4

2018 **Han ST**, Raraigh KS, Pellicore MJ, Davis EF, McCague AF, Evans TA, Cutting GR. Residual Function Predicts Response to CFTR Modulators, American College of Medical Genetics Annual Meeting, Charlotte, North Carolina, Apr 10-14 Reviewers' Choice Selection

2018 **Han ST**, Rab A, Pellicore MJ, Davis EF, McCague AF, Evans TA, Joynt A, Lu Z, Cai Z, Raraigh KS, Hong JS, Sheppard DN, Sorscher EJ, Cutting GR Residual Function of Cystic Fibrosis Mutants Predicts Therapeutic Response, American Society of Human Genetics Annual Meeting, San Diego, California, Oct 15-20 Reviewers' Choice Selection