Expression and Stability of the Ion Channel CFTR in Inflammatory Lung Disease

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Jack Henry Wellmerling

Graduate Program in Biophysics

The Ohio State University

2020

Thesis Committee

Estelle Cormet-Boyaka, Ph. D.

Prosper Boyaka, Ph. D.

Philip Diaz, M.D.

Susheela Tridandapani, Ph. D.

Abstract

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is an ion channel primarily studied in the context of epithelial fluid homeostasis in the lung. CFTR is responsible for maintaining proper ionic composition and pH of the airway surface liquid through its transport of chloride, bicarbonate, and other anions. The airway surface liquid plays an important role in innate immunity, and when its homeostasis is disturbed, the lung becomes highly susceptible to infection. Cystic Fibrosis (CF), the disease after which CFTR is named, is a life-limiting autosomal-recessive disease caused by various loss-of-function mutations in *CFTR*. CF is a multi-organ disorder, but most of its associated morbidity and mortality are caused by recurrent lung infection and inflammation resulting in lung failure. While traditionally thought of as a childhood disease, many CF patients are reaching adulthood thanks to newborn screening and therapeutic advances. However, there remains no cure.

Another airway disease with no cure in which CFTR dysfunction is potentially implicated is Chronic Obstructive Pulmonary Disease (COPD). COPD is primarily caused by tobacco smoking, which has been shown to negatively regulate CFTR protein expression. CFTR expression has also been found to be low in the lungs of severe COPD patients compared to healthy controls. These findings, combined with phenotypic similarities between CF and COPD, have contributed to a growing body of research that

aims to understand COPD as a disease of "acquired" CFTR dysfunction. Traditionally, COPD has been characterized by two primary phenotypes: chronic bronchitis and emphysema, although the disease itself is heterogeneous and there can be considerable overlap. Chronic bronchitis is characterized by airflow restriction in the conducting airways due to excessive mucus production, smooth muscle constriction, and tissue fibrosis. Emphysema encompasses alveolar cell death and tissue destruction, resulting in inefficient gas exchange and gas trapping.

Accordingly, the first chapter of this dissertation will address the question of whether reduced CFTR expression is a potential risk factor of COPD, or merely a consequence. This is important, because approximately 1 in 30 people in the US is an asymptomatic CF carrier. To answer this question, wild-type (WT), *Cftr*$^{+/-}$ (Het), and *Cftr*$^{-/--}$ (KO) mice were subjected to smoking or natural aging, two known COPD risk factors. Lungs were analyzed for histologic signs of COPD, and it was found that in response to smoking, both Het and KO mice displayed an increased mean linear intercept (L_M), indicative of emphysema, compared to WT controls. On the other hand, aging led to increased L_M in KO mice only. Results will be discussed in the context of the biology of emphysema and aging. Following that theme, *in vitro* data identifying a potential calcium-dependent DNA repair deficiency associated with CF will be discussed.

The second chapter will focus on therapeutic strategies to improve CFTR stability in the context of both CF and COPD. Importantly, no current therapeutics target CFTR stability. Literature highlighting the significance of the Epidermal Growth Factor Receptor (EGFR) – Extracellular Regulated Kinase (ERK) axis in both CF and COPD

will be discussed. Data showing that this axis, dependent on the EGFR ligand amphiregulin, negatively regulates CFTR in CF will be presented. Next, it will be shown that ectoine, a natural osmolyte which can target EGFR signaling and benefit COPD patients, can improve CFTR stability and attenuate inflammatory signaling in CF. Another way to stabilize plasma membrane CFTR is by preventing its degradation by interfering with its binding to the CFTR-associated ligand (CAL) protein using PDZ domain peptide inhibitors.

The third chapter will discuss the transcriptome and proteome of CF primary bronchial epithelial cells, and the effects of the clinically approved CFTR corrector VX-661 and ectoine. These data will be used to provide a deeper understanding of CF lung pathology, especially in relation to **chapters 1 and 2**; and to identify potential opportunities for future therapeutic intervention.

Dedication

This dissertation is dedicated to my Parents, Jack R. Wellmerling and Suzana Novak, who have supported me throughout my life.

Acknowledgments

First, I would like to acknowledge my advisor, Dr. Estelle Cormet-Boyaka, whose guidance has been invaluable throughout my graduate career. I would also like to acknowledge my committee members: Drs. Prosper Boyaka, Phil Diaz, and Susheela Tridandapani, who have been incredibly helpful and supportive throughout my education. This work would also not have been possible without our many collaborators, and members of my lab and their labs. I would also like to thank the OSU Proteomics and Genomics shared resources for their help in generation of important data, and Tim Vojt, College of Veterinary Medicine medical illustrator, for help with preparing figures. Finally, I would like to acknowledge the directors and administrators of the Biophysics graduate program, who have been incredibly helpful.

Vita

High School: Dublin Coffman High School (Dublin, OH)……...2009

Undergraduate: Ohio State University (Columbus, OH), B.S. Biomedical Engineering……2013

Graduate: Ohio State University (Columbus, OH), M.S. Biophysics……...2017

Publications

1. Kopp BT, Ortega-García JA, Sadreameli SC, Wellmerling J, Cormet-Boyaka E, Thompson R, McGrath-Morrow S, Groner JA. The Impact of Secondhand Smoke Exposure on Children with Cystic Fibrosis: A Review. *Int J Environ Res Public Health*. 2016 Oct 12;13(10):1003. doi: 10.3390/ijerph13101003. PMID: 27754353; PMCID: PMC5086742.

2. Chang SW, Wellmerling J, Zhang X, Rayner RE, Osman W, Mertz S, Amer AO, Peeples ME, Boyaka PN, Cormet-Boyaka E. The psychoactive substance of cannabis Δ9-tetrahydrocannabinol (THC) negatively regulates CFTR in airway cells. *Biochim Biophys Acta Gen Subj*. 2018 Sep;1862(9):1988-1994. doi: 10.1016/j.bbagen.2018.06.008. Epub 2018 Jun 19. PMID: 29928919.

3. Assani K, Shrestha CL, Rinehardt H, Zhang S, Robledo-Avila F, Wellmerling J, Partida-Sanchez S, Cormet-Boyaka E, Reynolds SD, Schlesinger LS, Kopp BT. AR-13 reduces antibiotic-resistant bacterial burden in cystic fibrosis phagocytes and improves cystic fibrosis transmembrane conductance regulator function. *J Cyst Fibros*. 2019 Sep;18(5):622-629. doi: 10.1016/j.jcf.2018.10.010. Epub 2018 Oct 23. PMID: 30366849.

4. Yeung DA, Shanker N, Sohail A, Weiss BA, Wang C, Wellmerling J, Das S, Ganju RK, Miller JLC, Herr AB, Fridman R, Agarwal G. Clustering, Spatial Distribution, and Phosphorylation of Discoidin Domain Receptors 1 and 2 in Response to Soluble Collagen I. *J Mol Biol*. 2019 Jan 18;431(2):368-390. doi: 10.1016/j.jmb.2018.11.015. Epub 2018 Nov 17. PMID: 30458172; PMCID: PMC6346271.

5. Wellmerling JH, Chang SW, Kim E, Osman WH, Boyaka PN, Borchers MT, Cormet-Boyaka E. Reduced expression of the Ion channel CFTR contributes to airspace enlargement as a consequence of aging and in response to cigarette smoke in mice. *Respir Res*. 2019 Sep 2;20(1):200. doi: 10.1186/s12931-019-1170-3. PMID: 31477092; PMCID: PMC6720379.

6. Rayner RE, Wellmerling J, Osman W, Honesty S, Alfaro M, Peeples ME, Cormet-Boyaka E. In vitro 3D culture lung model from expanded primary cystic fibrosis human airway cells. *J Cyst Fibros*. 2020 Sep;19(5):752-761. doi: 10.1016/j.jcf.2020.05.007. Epub 2020 Jun 18. PMID: 32565193.

7. Daugherty P*, Wellmerling J*, Koley A*, Lukowski J, Hummon A, Cormet-Boyaka E, Pei D. Cyclic peptidyl inhibitors against CAL-CFTR interaction for treatment of cystic fibrosis. *J Med Chem*. (Accepted)

*co-first author

Fields of Study

Major Field: Biophysics

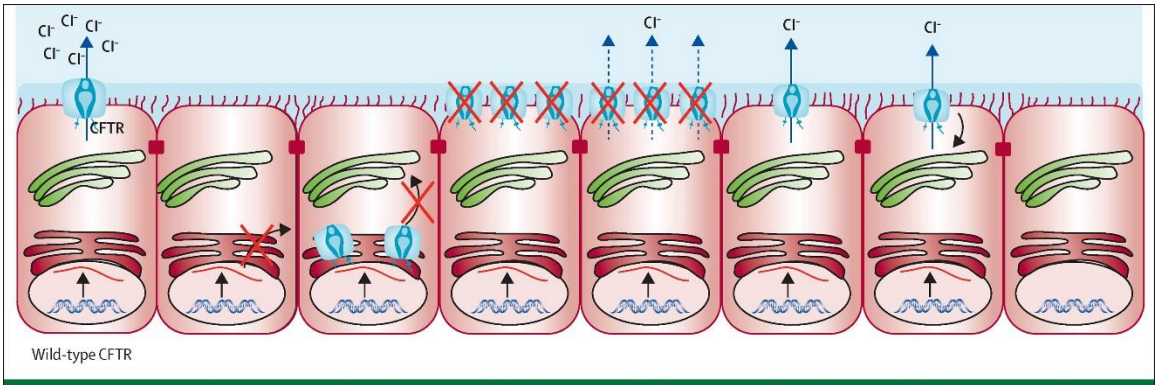
Table of Contents

List of Figures

Introduction

Cystic Fibrosis

Originally termed cystic fibrosis (CF) of the pancreas, CF was first described in 1938 with autopsies identifying pathologies of the lung, pancreas, gastrointestinal tract, and other organs; and a mean survival of approximately 6 months [1]. Life expectancy for people with CF has gradually improved, thanks to pancreatic enzyme supplementation, airway clearance, and antibiotic therapy. Altered ion transport and electrophysiology of CF nasal epithelia was noted in 1983 [2]. In 1989, the CF transmembrane conductance regulator (*CFTR*) gene responsible for CF was discovered [3]. Accordingly, it was shortly thereafter discovered that the gene, *CFTR*, encodes an ion channel responsible for chloride permeation [4]. Over 2000 unique *CFTR* mutations of varying severity have been discovered, with F508del mutation being the most common [5]. Seven different classes of CFTR mutations exist, with F508del belonging to class II mutation, meaning that the ion channel is not trafficked properly [6]. A table detailing the different CFTR mutation classes, adapted from [6] can be found in **Figure 1**.



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	Gly542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A
Corrective therapy	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability	Unrescuable
Drug (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)	Bypass therapies (no)

Figure 1: Diagram detailing different CFTR mutation classes and therapeutic strategies which target them. Adapted from De Boeck and Amaral, 2016. Lancet Respiratory Medicine [6].

While CF is a multi-organ pathology, lung disease represents the major cause of morbidity and mortality, so this dissertation will primarily focus on the role of CFTR in the lung. CFTR is expressed in the bronchial epithelium, which serves as an important physical barrier to respiratory pathogens and pollutants. Here, CFTR transports chloride and bicarbonate ions across the epithelium into the lumen [7]. CFTR-mediated chloride transport is important for maintaining proper ionic composition of the periciliary liquid (PCL), which bathes the epithelial cilia responsible for efficient pathogen clearance. When there is a lack of functional CFTR (as is the case in CF), airway dehydration leads

to impaired mucociliary clearance (MCC) [8]. It has been demonstrated that proper MCC is an important determinant of respiratory health [9], supporting a disease model for CF in which airway surface dehydration impairs host defense. Poor MCC results in chronic lung infections which often result over time in lung failure and is responsible for the majority of deaths in CF [8].

Bicarbonate transport by CFTR is also important for proper mucociliary and antimicrobial function through its action as a biochemical buffer. Acidic pH has been shown to increase the viscosity of the airway surface liquid in CF, likely by strengthening electrostatic interactions between mucin polymers [10]. Indeed, bicarbonate transport by airway epithelia accompanies mucin production to support its release following stimulation by interleukin-4, and it can be seen that this is defective in CF [11]. Acidic pH has also been shown in CF pigs to impair bacterial killing, possibly through an inactivating effect on innate antimicrobial enzymes such as lysozyme and lactoferrin which require specific pH for optimal activity [12].

Mucus trapping and innate immune deficiency lead to persistent infection and inflammation, and indeed, many treatment strategies for CF involve anti-inflammatory and antibiotic drugs [13, 14]. In more recent years however, therapies directly targeting CFTR itself have been developed. The CFTR protein belongs to the ATP binding cassette family, and transports ions at the cost of ATP hydrolysis [15]. CFTR contains two transmembrane domains, two nucleotide binding domains (NBD1 and NBD2), and a regulatory (R) domain; to open the channel, the R domain must be phosphorylated, then, NBD1 and NBD2 must bind ATP [15]. Phenylalanine 508 resides in NBD1, and when

deleted, disrupts an interface between NBD1 and the transmembrane domains, which results in improper folding of the protein [16].

Due to this folding defect, F508del-CFTR is not sufficiently processed in the endoplasmic reticulum (ER), and most of the protein is degraded by the proteasome before it can be trafficked to the plasma membrane [17]. CFTR corrector compounds, such as VX-809 or VX-661 (Vertex Pharmaceuticals) facilitate proper folding and maturation of F508del-CFTR [18, 19]. In addition to its processing defect, F508del-CFTR also displays an insufficient chloride conductance [20]. This has prompted the development of CFTR potentiators such as VX-770 [21]. Current therapies comprise a corrector-potentiator combination to correct and improve F508del-CFTR function. Unfortunately, a third defect exists as a consequence of the F508del mutation, in that the protein's biological half-life when present at the membrane is significantly reduced due to rapid endocytosis and degradation by the lysosomes [22, 23]. To further complicate CFTR pharmacotherapy, potentiators have been demonstrated to counteract the effects of correctors [24]. Nevertheless, VX-661, a CFTR corrector, has shown promising clinical benefit in CF patients with one or two copies of F508del-CFTR, in combination with VX-770 [25, 26]. More recently, a triple combination called "highly effective therapy" comprising two correctors (VX-445 and VX-661) and the potentiator VX-770 has shown impressive clinical results in F508del homozygotes and heterozygotes [27-29]. Still, there remains a need to target CFTR protein stability, and this will be the primary focus of **Chapter 2** of this dissertation.

To target CFTR stability, it is first necessary to understand the biological mechanisms responsible for CFTR instability. As mentioned previously, when F508del-CFTR is able to reach the plasma membrane of airway epithelial cells, it is rapidly endocytosed and degraded by the lysosomes [23]. It has been shown using cigarette smoke extract, that signaling by Extracellular Regulated Kinases 1 and 2 (hereafter referred to as ERK) plays an important role in CFTR degradation [30]. ERK can be activated downstream of many cellular receptors, but one that will receive attention in this dissertation is the Epidermal Growth Factor Receptor (EGFR). EGFR plays an important role in several inflammatory lung diseases, including both CF and COPD [31]. Accordingly, many similarities between CF and COPD have been noted, and in fact, there is substantial evidence that COPD may represent a state of "acquired CFTR dysfunction" [32]. This hypothesis explored in more detail in **Chapter 1** of this dissertation, but first, it is necessary to understand the biology of COPD.

Chronic Obstructive Pulmonary Disease

COPD is the third leading cause of global death [33], caused primarily by tobacco smoking. Historically, COPD has been broken down into two primary phenotypes: chronic bronchitis and emphysema; however, there can be considerable overlap between these two phenotypes, and COPD is often accompanied by several comorbidities [34]. Asthma-COPD overlap [35] and exacerbation-prone COPD [36] have also been suggested to represent distinct phenotypes. Clinically, COPD is diagnosed using spirometry to measure the ratio of forced expiratory volume in 1 second to forced vital

capacity (FEV_1/FVC). An FEV_1/FVC < 70% is required to diagnose COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), with increased airflow limitation and presence of other symptoms indicating increasing stages of severity [37].

Pathological manifestations of chronic bronchitis include increased production of mucus in the airways [38], smooth muscle proliferation [39], and small airway fibrosis [40]; all of which contribute to airflow limitation. Emphysema encompasses alveolar cell death and tissue enlargement [41]. This leads to gas trapping and airflow limitation, and indeed, quantitative computed tomography findings in patients are well-correlated with airflow obstruction and COPD biomarkers [42]. A possible link between CFTR dysfunction and emphysema will be discussed in more detail in **Chapter 1**.

The presentation of COPD can vary considerably between patients, however inflammation and recurrent infections play an integral role. Neutrophils and macrophages are recruited shortly following exposure to noxious gases and particles, and persist throughout the course of COPD, where they release tissue-damaging proteases, in conjunction with various other innate and adaptive immune cells [43]. Different inflammatory profiles have been well-correlated to different COPD phenotypes and severity [44-46], however a full review on inflammation in COPD is beyond the scope of this dissertation. Attempts have been made to define COPD endotypes, in which a molecular mechanism is linked to a disease phenotype, but it has proven difficult to define inflammatory endotypes, mostly due to the complexity and heterogeneity of inflammation in COPD [47]. Two distinct endotypes have been defined however: α1-

antitrypsin deficiency, in which protease-antiprotease imbalance contributes to tissue destruction; and polymorphisms in telomerase reverse transcriptase, in which short telomeres contribute to cellular senescence and inflammation [47].

Noting the causal association between telomere dysfunction and COPD, it is important to mention that COPD has been described by some as a disease of "accelerated aging," wherein many molecular mechanisms associated with the process of aging occur in COPD, including cellular senescence (in which cells stop dividing), oxidative stress, and age-associated inflammation [48, 49]. Cellular senescence can be triggered by telomere shortening, but also by DNA-damaging agents, with double-strand breaks being the most potent inducers of cellular senescence [50]. Interestingly, carbon black, a nanoparticulate found in cigarette smoke, has been shown to damage DNA, which in turn promotes T-helper 17-mediated inflammation and emphysema [51]. DNA damage at telomere-specific sites has also been noted in airway epithelial cells from COPD patients, with telomere damage sites also correlating with markers of senescence and production of interleukin-6 and -8 in cigarette smoke-treated cells [52]. DNA damage accumulation can be a consequence of insufficient DNA repair, and importantly, a cluster of 15 DNA repair genes has been found to be dysregulated in a multi-cohort study of COPD patients, and well-correlated with COPD severity [53].

In a clinical setting, significant efforts are directed toward treating the inflammation associated with COPD, with treatments encompassing drugs that target cellular inflammation such as inhibitors of phosphodiesterase 4 (PDE4) or adenosine receptors, or drugs that target inflammatory mediators, such as various cytokines or

nuclear factor-κB [54]. Bronchodilators, antioxidants, and protease inhibitors are also given to COPD patients [54]. Interestingly one PDE4 inhibitor, roflumilast, has been shown to stimulate CFTR channel activity and improve mucociliary function following its repression by cigarette smoke [55]. CFTR activation by roflumilast has also been suggested to mediate its beneficial effect in chronic bronchitis [56]. Many links between CFTR dysfunction and COPD can be made [32], and these will be elaborated upon in **Chapter 1** of this dissertation, with particular emphasis on the emphysema phenotype.

Overall, this dissertation will first discuss data showing a role for reduced CFTR expression in the development of emphysema. Since DNA damage accumulation is an underlying cause of COPD, evidence suggesting a DNA repair deficiency associated with CFTR dysfunction will then be discussed. From there, the focus will shift to uncovering mechanisms which regulate CFTR stability in the context of CF, and two strategies to target CFTR stability will be presented. Finally, transcriptomic and proteomic data generated from primary CF human airway epithelial cells will be discussed, particularly in the context of chapters 1 and 2.

Chapter 1: Role of CFTR Dysfunction in COPD Susceptibility

Section 1: Reduced CFTR Expression Contributes to Emphysema-like Changes in Response to Aging and Cigarette Smoke

Section 1 of this chapter is modified from the publication "Reduced expression of the Ion channel CFTR contributes to airspace enlargement as a consequence of aging and in response to cigarette smoke in mice" (Wellmerling JH et al, Respir Res. 2019;20(1):200)

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death globally [33]. COPD is primarily caused by tobacco smoking, however other factors such as air pollution, individual genetics, and aging are also thought to play a role. COPD has traditionally been associated with two main phenotypes- chronic bronchitis and emphysema. Chronic bronchitis is accompanied by chronic airway inflammation and encompasses mucus hypersecretion, smooth muscle constriction, and small-airway fibrosis. Emphysema, also associated with inflammation, is characterized by airspace enlargement and tissue destruction in the lung parenchyma which results in alveolar distension and contributes to respiratory decline.

Many mechanistic similarities between aging and the development of COPD have been drawn and reviewed extensively, in which COPD can be considered an "accelerated aging" disorder [49]. While smoking is the primary cause of COPD, the finding that most smokers do not develop the disease has led to considerable interest in genetic factors that may predispose an individual to the effects of tobacco smoke. In accordance with the

accelerated aging hypothesis, mutations in the genes encoding Sirtuin 2 [57] and Telomerase Reverse Transcriptase [58] have been found to be associated with COPD. Another potentially interesting gene in the context of aging and COPD is *CFTR*, encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR).

CFTR is an anion channel involved in airway hydration and mucociliary clearance most commonly studied in the context of Cystic Fibrosis (CF). CF is a life-limiting autosomal-recessive disease in which severely reduced CFTR function increases patients' susceptibility to lung infection and excessive inflammation, resulting in lung damage and ultimately respiratory failure [59]. CF patients also experience several symptoms often associated with aging, including diabetes and bone disease [60]; common comorbidities encountered in COPD [61]. An interesting clinical observation from CF patients undergoing lung transplantation is that they commonly develop emphysema, which becomes more severe with age [62]. Mechanistically, it is not clear whether this is due to the excessive airway inflammation associated with CF, or an alternative phenomenon. Interestingly, several markers of cellular senescence have been found to be increased in CF airways [63]. Additionally, skin fibroblasts from CF patients have been shown to senesce more readily than those from healthy controls [64].

A growing body of research supports the idea that COPD is a disease of acquired CFTR dysfunction, particularly in the context of the chronic bronchitis phenotype [32]. We have previously shown that CFTR expression is reduced in the bronchial epithelium of patients with severe COPD [65], and identified a mechanism by which cigarette smoke exposure leads to CFTR degradation [30]. Considering the similarities between CF and

COPD, we hypothesized that disrupting CFTR expression may promote development of emphysema as a consequence of aging or exposure to tobacco smoke independent of CF airway disease. Mice do not develop the spontaneous infection and inflammation associated with CF airway disease [66, 67], providing a convenient model to address this question. To determine a potential role for CFTR expression levels in developing emphysema, the effects of aging and cigarette smoke exposure on *Cftr*$^{+/+}$ (WT), *Cftr*$^{+/-}$ (Het), and *Cftr*$^{-/-}$ (KO) mice were examined.

Methods

To determine a possible role for CFTR expression in emphysema-like changes, mice lacking CFTR (KO) or heterozygous (Het) for *Cftr*^{tm1Unc} [stop codon in the murine *cftr* gene (S489X)] and homozygous for Tg(FABP-CFTR) [fatty acid-binding protein (FABP)-CFTR]; or wild-type (WT) littermates (*Cftr*^{tm1Unc} FABP-hCFTR-CFTR bitransgenic mice from Jackson Laboratory, Bar Harbor, ME) were aged 14-20 months under standard pathogen-free housing conditions. Mice were considered "Aged" at 14 months, because this age corresponds to the upper limit of a "Middle-aged" mouse (https://www.jax.org/research-and-faculty/research-labs/the-harrison-lab/gerontology/life-span-as-a-biomarker). KO mice express human WT CFTR in the intestine (https://www.jax.org/strain/002364) and thus were able to be fed only standard mouse chow [68]. Seven-nine mice of each genotype were aged. Following euthanasia via CO_2 and cervical dislocation, lungs were inflated with 10% neutral-buffered formalin at a pressure of 20 cm H_2O and fixed for 24 hours, sectioned, and stained with

hematoxylin and eosin for morphometric analysis [69]. Alveolar airspace enlargement was quantified by calculating the mean linear intercept (L_M). The same lungs and lobes were used across all the genotypes. For the aging study, both lungs were analyzed while for the cigarette smoke study, only the right lungs were analyzed. To calculate the L_M, 5 randomly selected images of alveolar tissue per mouse were used. A 10x10 grid was superimposed over each image with ImageJ software (NIH, Bethesda, MD), and the number of alveolar intersections for each line was manually counted. L_M was calculated by dividing the length of lines by the number of intersections and averaged from 5 images for each mouse. Lines intersecting vasculature, bronchioles, or poorly inflated areas of the lung were not used. To determine whether *cftr* genotype contributes to the severity of cigarette smoke-induced emphysema, WT, Het, and KO mice were exposed to smoke from 3R4F research grade cigarettes (University of Kentucky, Lexington, KY) via whole-body exposure as previously described [70]. To model chronic tobacco smoking, the regimen consisted of 4 cigarettes per day, 5 days per week, for 10 months. Control mice of each genotype were exposed to filtered air instead of cigarette smoke. Four-nine mice were used for each group. Following the 10-month exposure, mice were euthanized. To investigate the possibility that CFTR genotype plays a role in the inflammatory response to cigarette smoke, another set of 8 mice from each genotype were subject to the same treatment regimen as above for 4 weeks. Bronchoalveolar lavage (BAL) was conducted by washing lungs twice with 2ml of sterile phosphate-buffered saline (Life Technologies, Grand Island, NY). Cytospin was performed on BAL cells. Following Wright-Giemsa staining (Fischer Scientific, Kalamazoo, MI), numbers of

macrophages/monocytes, neutrophils, and lymphocytes were counted. Studies were approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC, protocol #2015A00000067), in accordance with NIH and OSU IACUC guidelines. Studies conducted at the University of Cincinnati were approved by IACUC protocol 06-04-07-01.

Results

Upon aging, Cftr-knockout mice displayed an increased mean linear intercept (L_M), indicative of alveolar enlargement, compared to Heterozygous or WT mice (**Fig. 2A and 2B**). Among mice aged 14-20 months, there was no correlation between age and L_M ($r^2 = 0.1112$). WT mice exposed to cigarette smoke (CS) for 10 months displayed a L_M that was increased but not significantly different from that of wild-type mice exposed to filtered air (FA). However, airspace enlargement in heterozygous and knockout mice exposed to smoke was significantly greater than their wild-type counterparts.

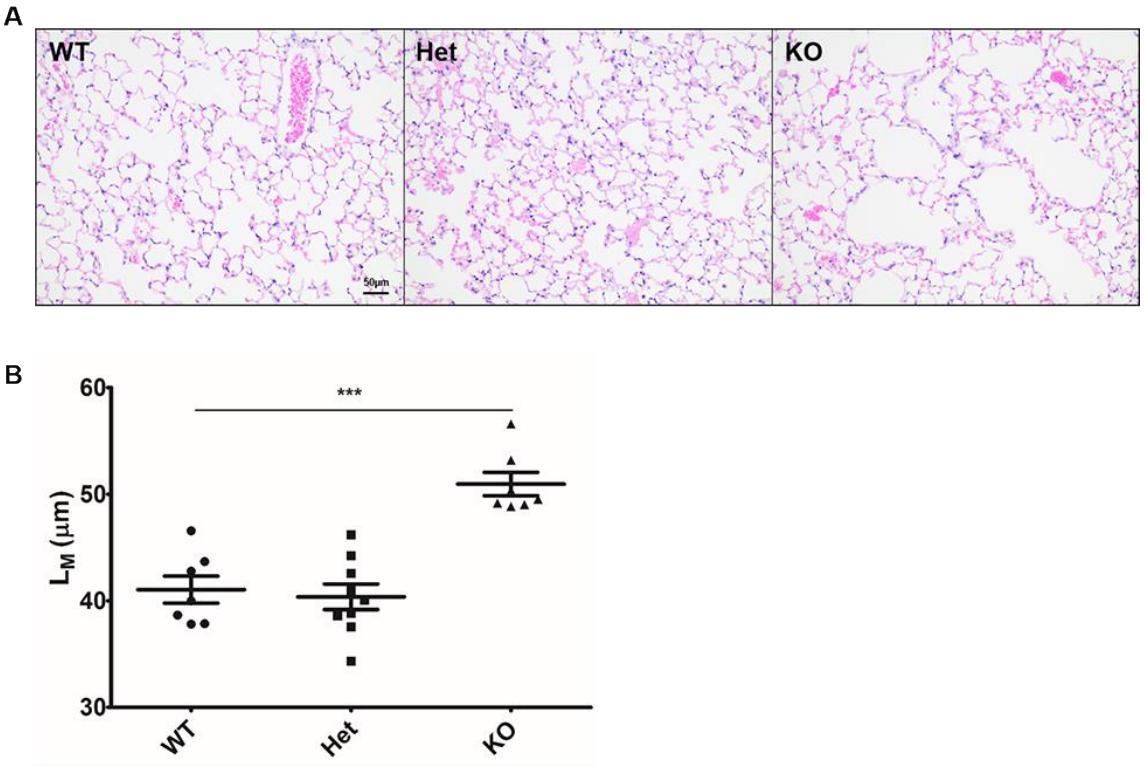


Figure 2: Development of emphysema-like changes in elderly mice. Healthy 14–20 month old wild-type (WT), CFTR-heterozygous (Het), and CFTR-knockout (KO) mice were sacrificed and lungs were fixed and inflated with formalin. (**A**) Representative micrographs of lung parenchyma stained with hematoxylin and eosin; and (**B**) mean linear intercepts (LM) of WT, Het, and KO mice. Magnification: 400X. Scale bar = 50 μm. ***$p < .001$ via one-way ANOVA with Tukey's post-hoc multiple comparison. N = 7–9 mice per group.

Heterozygous and KO mice exposed to CS displayed increased L_M compared to their FA counterparts (**Fig. 3A and 3B**). Periodic Acid-Schiff staining was conducted on lung sections to determine mucus expression and obstruction, however very little mucus was detected in the lungs of all mice. No signs of infection were noted in the lungs of KO mice as evaluated by presence of the key inflammatory mediator IL-1β in BAL, presence of inflammatory cells, altered lung structure, or mouse weight loss. During the smoke

exposure study, one WT mouse exposed to filtered air died from unknown causes (pathology was performed but the cause of death could not be identified). During the aging study, one heterozygous mouse had to be sacrificed due to edema and ocular swelling. In mice exposed to CS for 4 weeks, cytology revealed a modest but statistically significant increase in the percentage of macrophages/monocytes in BAL fluid between WT and both Het and KO mice (Fig. 3C). This increase in the percentage of BAL macrophages/monocytes was accompanied by a decrease in neutrophils (2.95%±1.30 for WT, 1.98%±0.67 for Het, and 1.97%±0.86 for KO mice), without a significant change in the total number of cells ($7.74 \pm 0.43 \times 10^4$ for WT, $7.91 \pm 0.42 \times 10^4$, for Het, and $8.04 \pm 0.42 \times 10^4$ cells/mL for KO mice; p=0.39 via ANOVA).

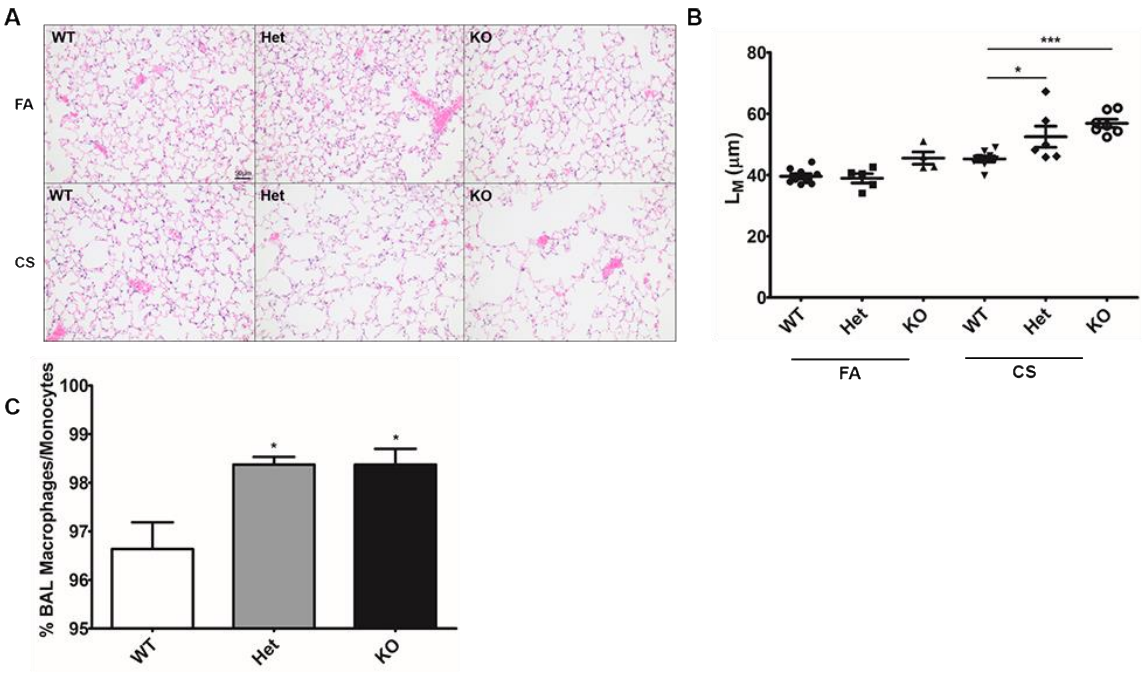


Figure 3: Emphysema-like changes in mice exposed to cigarette smoke. Mice wild-type (WT), CFTR-heterozygous (Het), and CFTR-knockout (KO) were exposed to filtered air (FA) or cigarette smoke (CS) for 10 months. (**A**) Representative micrographs of lung parenchyma stained with hematoxylin and eosin and (**B**) mean linear intercepts (L_M) of wild-type (WT), CFTR-heterozygous

(Het), and CFTR-knockout (KO) C57BL/6 J mice exposed to filtered air (FA) or cigarette smoke (CS) for 10 months. $N = 4$-9 mice per group. (**C**) Percentage of monocytes/macrophages in bronchoalveolar lavage fluid of mice exposed to cigarette smoke for 4 weeks. $N = 8$ from each group. Magnification: 400x. Scale bar = 50 μm. * $p < .05$, *** $p < .001$ between groups indicated by line; ## $p < .01$, ### $p < .001$ compared to FA mouse of same genotype. NS indicates there is no significant difference between FA and CS WT mice. Significance was determined via one-way ANOVA with Tukey's post-hoc multiple comparison

Discussion

In the present study, we show using two different experimental models, aging and cigarette smoking, that lack of CFTR leads to increased airspace enlargement similar to emphysema. The finding of airspace enlargement in aged knockout mice is in accordance with findings in human CF patients [62]. However, we did not detect any mucus over-production or obstruction via periodic acid-Schiff staining. In fact, very little mucus was detected in the lungs of all mice (data not shown). We also did not detect any differences in inflammatory cell counts in the BAL fluid of mice at the time of sacrifice (data not shown), suggesting that complete absence of CFTR promotes emphysema upon aging independent of an external inflammatory or oxidative stimulus. Our finding that only KO aged mice develop emphysema, while both Het and KO mice exposed to cigarette smoke do, may suggest that decreasing CFTR expression sensitizes the lung to the effects of cigarette smoke. This may imply that individuals carrying CFTR mutations might be more prone to developing emphysema.

The mechanisms behind which CFTR dysfunction promotes emphysema are currently unclear. It has been suggested that CFTR dysfunction contributes to emphysema through its regulation of pro-inflammatory ceramide signaling [71]. In

addition, it has been shown that CFTR inhibition increases the permeability of the pulmonary vasculature, and it has been hypothesized that this may lead to increased trafficking of inflammatory cells to the lung [72]. These studies however, were conducted using stressors such as cigarette smoke or lipopolysaccharide. Our novel finding in aged mice may suggest that an alternative, or at least an additional mechanism is playing a role.

The effect of aging on lung architecture has previously been investigated in BALB/c mice [73]. The authors found that while both alveolar surface area and volume increased, L_M did not change after 28 months [73]. L_M represents the alveolar volume to surface area ratio [74]. Thus, our results in WT and Het mice are in agreement with these findings [73]. Interestingly, several mouse models of accelerated aging also display signs of emphysema [75]. Mice homozygous for nonfunctional *klotho* have a maximal lifespan of about 12 weeks [76], and display alveolar epithelial cell apoptosis by 2 weeks of age, and increased L_M by 4 weeks [77]. Klotho is a Fibroblast Growth Factor-23 co-receptor with pleiotropic downstream signaling effects which are potentially related to its "Anti-aging" role, such as suppression of oxidative stress and senescence [78]. Another mouse model of accelerated aging is the Senescence Marker Protein-30 (SMP30) knockout mouse [79]. Compared to WT C57BL/6 controls, SMP30 KO mice displayed increased L_M beginning at 1 month and persisting up to 6 months of age [79]. In another study, SMP30 KO mice displayed increased L_M following 8 weeks of CS exposure, while WT mice did not [80]. Our finding that aged CFTR KO mice display increased L_M are in agreement with those in Klotho- and SMP30-KO mice, as well as several other strains of

Senescence-Accelerated Mouse [75]. However, compared to the accelerated aging mice, CFTR KO mice take considerably longer (14 months) to display increased L_M, and display normal lifespan due to gut correction (expression of human WT-CFTR in the intestinal epithelium under intestinal FABP promoter).

It is worth noting that similarly to the Klotho mouse, the CFTR KO mice display increased L_M later in life that does not further increase with age [77]. This suggests that our findings are not directly caused by a developmental defect associated with knocking out CFTR, which is important to note because it has been suggested that CFTR plays a role in lung development in both mice and humans [81]. It also suggests that our findings represent a discrete occurrence, rather than a continuous process. Thus, all mice in the range of 14-20 months were considered "Aged" for this study. While many signs of cellular senescence have been noted in CF [82], further research will be necessary to determine if this explains our findings in aged CFTR KO mice. The main novelty of our findings in aged mice is that CFTR KOs develop increased L_M, despite only poorly recapitulating the pathophysiology of human CF patients which is characterized by chronic lung infection [66, 67].

Conclusion

To conclude, we report for the first time that mice lacking CFTR develop alveolar remodeling similar to emphysema upon aging, and that genetic reduction of CFTR expression contributes to emphysema-like changes following smoke exposure. Our results in aged mice may have implications for a CF population whose life expectancy is

rapidly increasing [83]. Cystic fibrosis is associated with several age-associated pathologies, including diabetes and bone disease; and now emphysema. This may suggest emphysema as a significant future problem for aging CF patients, as well as foreshadow problems often associated with old age, such as neurological and cardiovascular diseases, which currently receive little basic research attention in the context of CF. Finally, CF carriership is fairly common [84], and our results in heterozygous mice exposed to cigarette smoke suggest that CF carriers may be more susceptible to the effects of cigarette smoke, including secondhand smoke, carrying significant cautionary implications. Our data may also suggest CFTR as an attractive therapeutic target in emphysema, however more research needs to be done.

Section 2: Potential DNA Repair Defect associated with CFTR Dysfunction

Introduction

Taking into consideration the data discussed above, we next asked how, on a mechanistic level, CFTR could contribute to emphysema development. As mentioned previously, DNA damage accumulation is thought to be an important contributing factor to COPD [53], and mechanistic studies have supported a role for DNA damage signaling in its development [51, 52]. Cigarette smoke has long been understood to induce DNA damage, including in human bronchial epithelial cells. [85, 86] Cigarette smoke contains many genotoxic compounds, including those which cause oxidative stress [87]. Reactive oxygen species (ROS) are well-understood to be inducers of various types of DNA damage [88]. This is important, because redox imbalance has been described as playing a potential role in the pathogenesis of CF [89], and in fact, transport of the antioxidant glutathione by CFTR is thought to be important in defense against cigarette smoke [90]. Therefore, we hypothesized that CFTR dysfunction would increase susceptibility to oxidative DNA damage.

Materials and Methods

To address this hypothesis, the CF cell-line CFBE41o- stably overexpressing WT or F508del-CFTR were treated with 50μM H_2O_2 (Sigma-Aldrich, St. Louis, MO) to induce oxidative DNA damage, and phosphorylation of histone 2A fraction X (γH2AX) (Cell Signaling Technology, Danvers, MA) was assessed by western blot and normalized

to the housekeeping protein GAPDH (Santa Cruz Biotechnology, Dallas, TX). Gamma-H2AX is a marker for DNA double-strand breaks [91, 92]. Camptothecin and 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) were purchased from Sigma-Aldrich (St. Louis, MO). Statistical analysis was performed using GrapPad® Prism 5 software. Immunofluorescent detection of γH2AX was performed using the same antibody. Briefly, cells seeded on glass coverslips were permeabilized in methanol at -20°C for 20 minutes, then blocked with 5% bovine serum albumin. Following blocking, the primary solution was incubated at 37°C for 1 hour. After washing, ALEXA-Fluor 488-conjugated rabbit secondary was incubated at 37°C for 45 minutes. After the final wash, coverslips were mounted on glass slides using ProLong™ Gold antifade media containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Waltham, MA) and imaged the next day using an Olympus IX-51 fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Results and Discussion

We first wanted to determine the time required for oxidative stress to induce DNA damage in human bronchial cells. H_2O_2 was added for 2, 8, or 24 hours, and we determined that 2 hours was sufficient to induce H2AX phosphorylation in F508del-CFBE cells, and thus used this time point for experimental replicates (**Fig. 4A**). Following 2 hours of treatment with 50μM H_2O_2, F508del-CFBE cells showed significant increase of H2AX phosphorylation compared to their untreated controls, while

WT-CFBEs did not (**Fig. 4B**). This supports the hypothesis that CFTR dysfunction increases susceptibility to DNA double-strand breaks induced by oxidative stress.

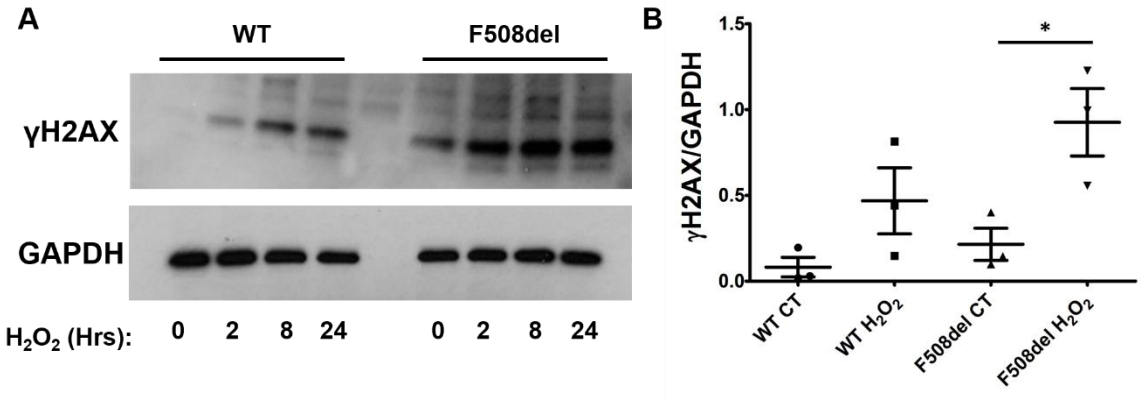


Figure 4: Effect of H_2O_2 on H2AX phosphorylation in CFBE41o- cells stably expressing either WT or F508del-CFTR. WT or CF-CFBE cells were treated with 50μM H2O2 for the indicated time. (**A**) Representative immunoblot and **(B)** quantification from 3 independent experiments at 2hr time-point. *p<.05 by t-test.

However, this finding raises a new question: whether the DNA in F508del-CFBE cells is being damage more easily due to general redox imbalance, or repaired insufficiently. To attempt to reconcile this, we employed the DNA Topoisomerase I inhibitor camptothecin (CPT). Topoisomerase I relaxes DNA supercoils during its replication, and inhibitors such as CPT induce DNA double-strand breaks by inducing replication stress [93].

One μM CPT was added to WT- and F508del-CFBE cells for 2, 6, or 24 hours, and H2AX phosphorylation was measured by western blot as with H_2O_2. Time-course data were fit using a least squares fit to a one-phase association model ($Y = Y_0 +$ (Plateau

– Y_0)*(1 – e^{-kx}), which contains terms for both a plateau, and the rate (k) at which the plateau is reached. Statistical significance was determined using an extra sum-of-squares F test, and a p-value <0.05 was considered to be significant.

It was determined that H2AX phosphorylation in response to CPT in WT- and F508del-CFBE cells was best-fit to curves with different models (**Fig. 5A and 5B**, **Fig. 6**). It can be seen that neither baseline H2AX phosphorylation (the Y_0 term), nor the phosphorylation rate (the K term) differed between WT- and F508del-CFBE cells, however the plateau term varied considerably. For reference, The Y_0 term was 0.15 ± 0.17 for WT and 0.19 ± 0.35 for F508del, k was 0.16 ± 0.10 for WT and 0.14 ± 0.09 for F508del, and the plateau was 1.17 ± 0.22 for WT and 2.60 ± 0.50 for F508del.

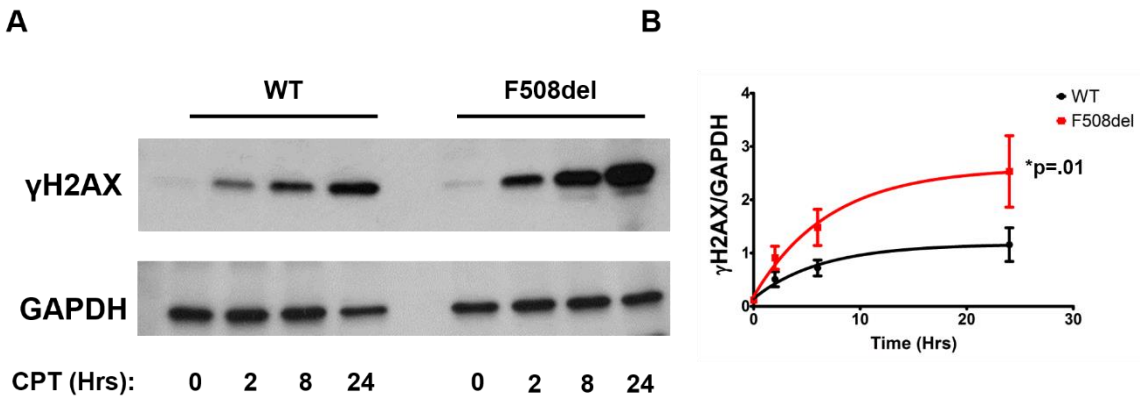


Figure 5: Time course effect of camptothecin (CPT) on H2AX phosphorylation in CFBE41o- cells stably expressing either WT or F508del-CFTR. WT or CF-CFBE cells were treated with 1μM CPT for the indicated time. (**A**) Representative immunoblot and (**B**) Fitting of data to a one phase association kinetic model. Data were generated from three independent experiments. *p<.05.

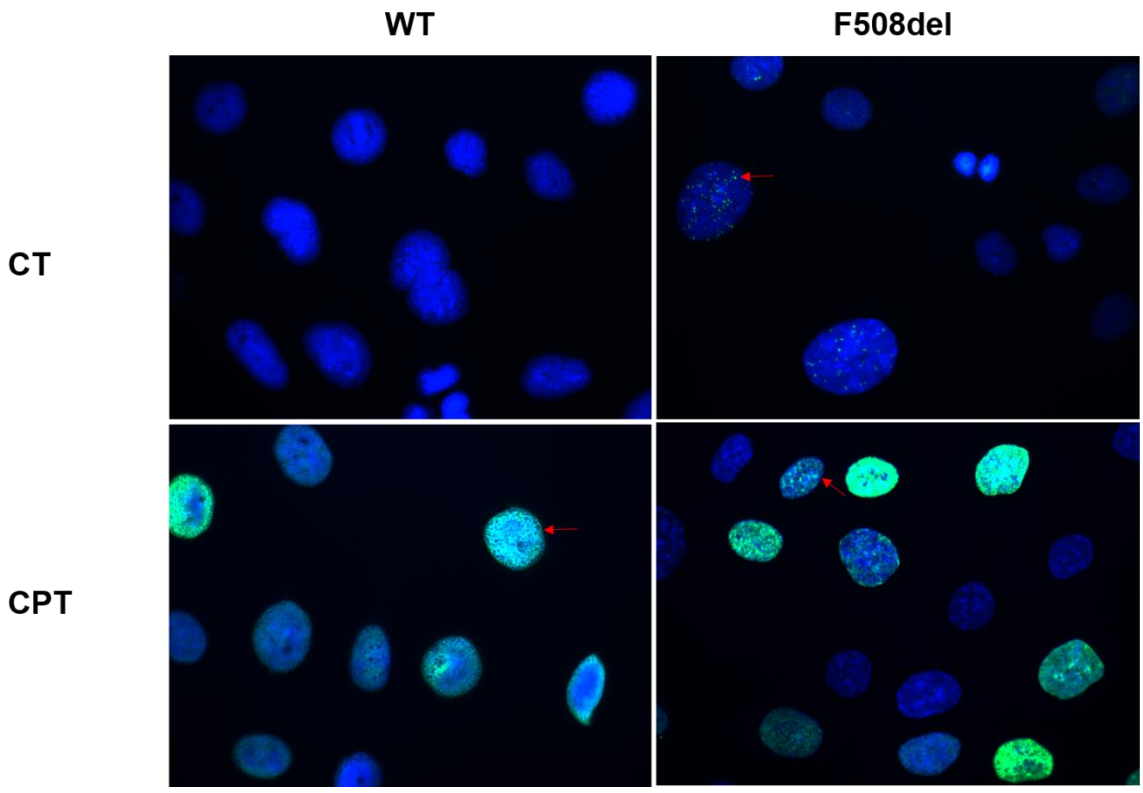


Figure 6: Detection of γH2AX in CFBE41o- cells stably expressing either WT or F508del CFTR in response to camptothecin (CPT) or vehicle. Arrows indicate γH2AX foci.

CPT-induced DNA damage is not unrepairable [93], and in reality, a more comprehensive model would include DNA repair. However, it can be reasoned that after sufficient time, the γH2AX plateau would represent a compromise between the rate at which DNA is damaged and the rate at which it is repaired. Our data fitting does not suggest a difference in the rate at which DNA is damaged by CPT between WT- and F508del-CFBE cells, so this can be taken to infer that DNA is not being repaired as efficiently in cells expressing F508del-CFTR.

We next hypothesized that a DNA repair defect associated with F508del-CFTR may be linked to increased intracellular calcium. Intracellular calcium has been shown to play a role in activation of the enzyme Poly(ADP-ribose) Polymerase (PARP) in response to DNA damage, which depletes the cell of the DNA repair cofactor NAD+, resulting in a greater extent of DNA damage [94]. The F508del mutation has been shown to lead to increased intracellular calcium by at least two separate mechanisms. On one hand, it has been proposed that it maintains some channel activity in the ER where it functions as a counter-ion channel and causes calcium to leak [95]. Increased cellular calcium uptake by the channel Transient Receptor Potential Vanilloid 6 has also been demonstrated in CF [96].

To determine a role for aberrant calcium handling in susceptibility to DNA damage associated with F508del-CFTR, we again treated WT- and F508del-CFBE cells with 1μM CPT for 2 hours, in the presence of either the membrane-permeable calcium chelator BAPTA-AM, the specific CFTR inhibitor CFTR-inh-172, or the appropriate vehicle. CFTR-inh-172 was used to test the hypothesis that CFTR channel activity, specifically in the case of F508del-CFTR is responsible for the increased susceptibility to DNA damage that is seen. Treatment with BAPTA-AM partially reduced the effect of CPT on H2AX phosphorylation in F508del, but not WT-CFBE cells (**Fig. 7**), suggesting that increased intracellular calcium associated with the F508del mutation may contribute in part to DNA repair deficiency. CFTR-inh-172 had no significant effect in either cell lines, suggesting that CFTR channel activity is not influencing DNA damage

susceptibility.

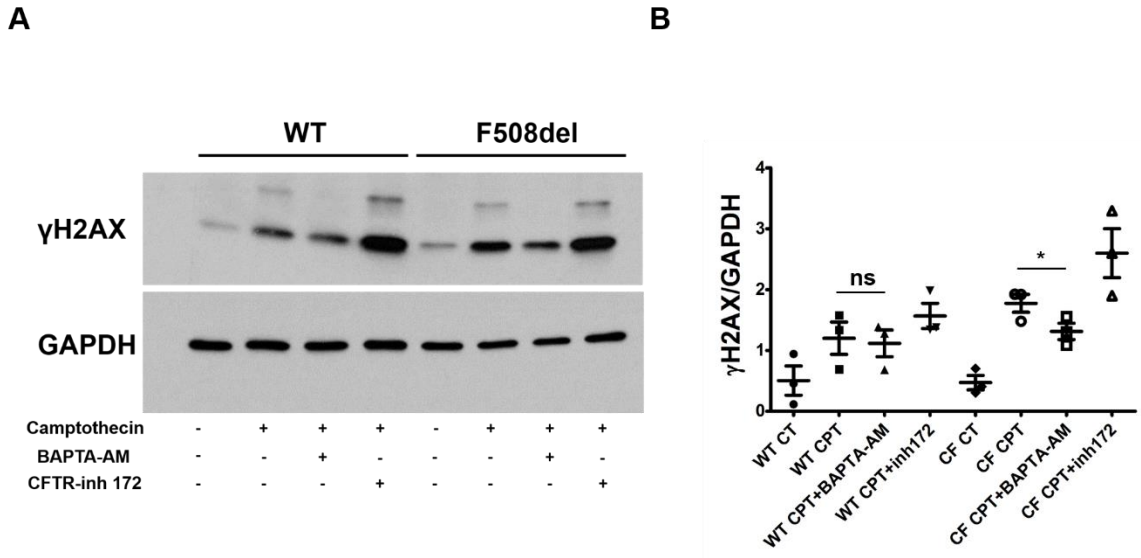


Figure 7: Role of intracellular calcium in CPT-induced DNA damage accumulation. (A) Representative γH2AX immunoblot of CFBE41o- cells stably overexpressing either WT or F508del-CFTR treated with camptothecin (CPT) for 2 hours in the presence of either the membrane permeable calcium chelator BAPTA-AM, or CFTR-inh 172. (B) Quantification of three independent immunoblots as seen in (A).

Conclusion

To conclude, this section of **Chapter 1** aimed to determine if CFTR dysfunction increases susceptibility to DNA damage (specifically double strand breaks). This is because we showed in the first section of this chapter that reduced CFTR expression in mice contributes to emphysema associated with loss of alveolar cells. Because DNA damage and repair deficiency is strongly associated with COPD, we suspected dysfunctional CFTR may contribute to DNA damage accumulation either by increasing susceptibility to oxidative stress or by impairing the cells' ability to repair damaged DNA. We show here that F508del-CFTR expression leads to a greater extent of DNA

double-strand breaks following oxidative stress (H_2O_2), and also present evidence that this may be due in part to decreased repair capacity. Finally, we show that intracellular calcium chelation can partially reduce H2AX phosphorylation in response to CPT in F508del- but not WT-CFBEs, suggesting that the increased intracellular calcium in CF may play a role in this deficiency.

Chapter 2: Targeting CFTR Stability to Improve CFTR Function

Introduction

In this chapter, we will primarily discuss factors which regulate CFTR stability, and means to target them. This chapter will be broken up into two sections. The first will discuss regulation of CFTR stability by AREG-EGFR-ERK signaling, and how this axis can be targeted with the natural osmolyte ectoine. The second section will discuss a cyclic cell-permeable peptide that can improve CFTR stability through inhibiting CAL-CFTR protein interactions. Mutant CFTR folding defects can be improved through pharmacological correctors, and function can be improved through potentiator drugs, as seen in **Figure 8**. Importantly, no therapeutic compounds exist which target stability. **Figure 8** shows a diagram of the two signaling axes that will be the focuses of this chapter.

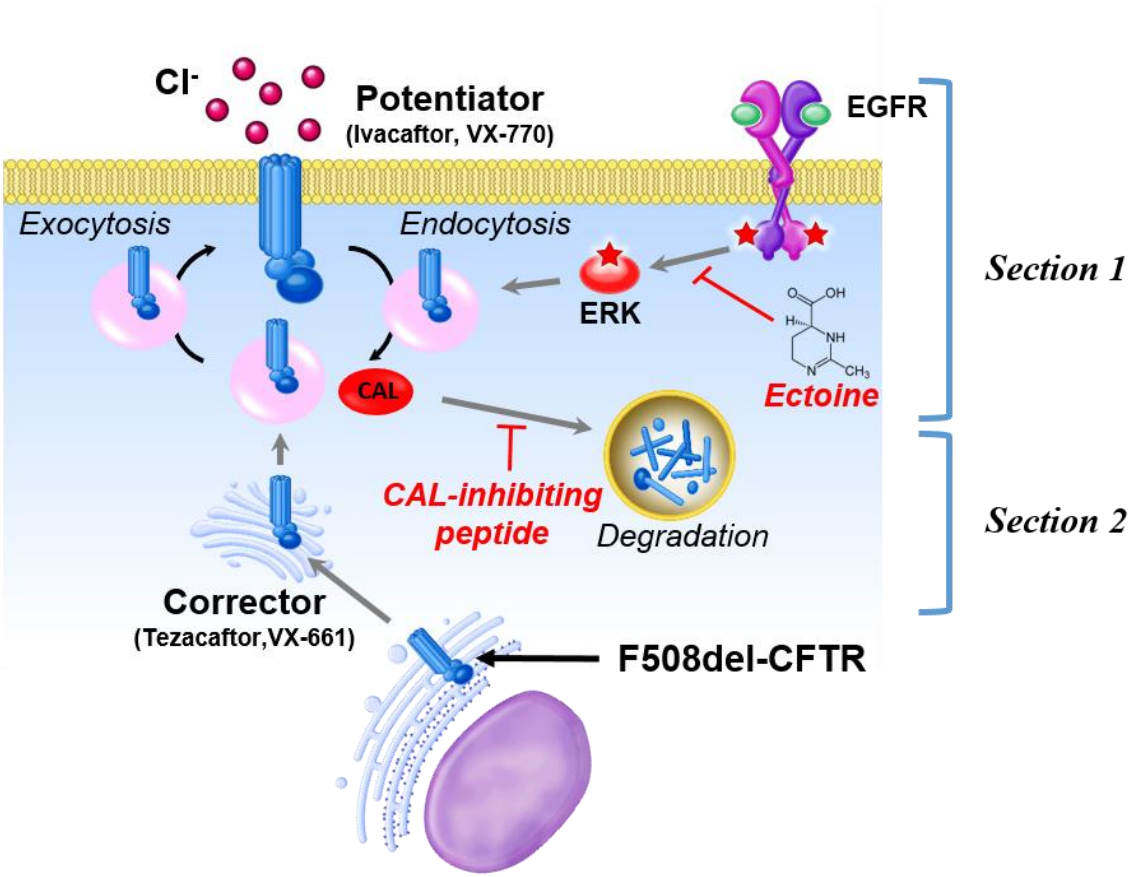


Figure 8: Diagram showing the two therapeutic avenues that will be explored in **Chapter 2**. F508del-CFTR folding can be improved by corrector drugs such as VX-661, which allows it to be trafficked to the plasma membrane. Here, its channel activity can be improved by potentiator drugs such as VX-770. However, F508del-CFTR still displays a short plasma membrane half-life due to its instability, and no drugs currently target this. Section 1 will focus on targeting CFTR regulation by ERK with ectoine. Section 2 will focus on inhibiting CAL-CFTR interactions through cyclic cell-permeable peptide drugs.

Section 1: Targeting the EGFR-ERK axis to stabilize plasma membrane CFTR

This section is modified from a publication which is currently in preparation: "Targeting the EGFR-ERK axis using the compatible solute ectoine to stabilize F508del CFTR"

Introduction

Cystic Fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CFTR is a chloride channel that plays a critical role in the lung by regulating airway fluid homeostasis allowing cilia to beat and clear pathogens [97]. Absence of functional CFTR is associated with chronic infection and inflammation [98] and results in lung failure. The most common CF-causing mutation is deletion of phenylalanine at position 508 (F508del) and is present in about 70% of CF patients [99]. CFTR correctors and potentiators are currently being developed to correct the trafficking and functional defects of CFTR mutants, respectively. The potentiator VX-770 (Ivacaftor: trade name Kalydeco) has been approved by the FDA for CF patients carrying the G551D mutation. This latter mutation results in CFTR protein that can traffic properly to the plasma membrane of airway cells but exhibits reduced activity. On the other hand, the F508del-CFTR mutant protein is trapped in the ER and then targeted to degradation via the ubiquitin-proteasome pathway [100]. VX-661 (Tezacaftor) is a recently FDA-approved CFTR corrector that facilitates folding and membrane trafficking of F508del-CFTR [101]. In addition to its trafficking defect, F508del-CFTR, if corrected, exhibits reduced chloride conductance [20]. A combination therapy of VX-661 and VX-

770 (trade name: Symdeko) has been developed. A clinical trial of Symdeko in F508del-homozygous patients showed a modest improvement in lung function and a considerable decrease in the rate of pulmonary exacerbations [26]. The new highly effective triple combination therapy Trikafta (VX-445 plus VX-661 plus VX-770) has been recently approved by the FDA. While there is significant improvement in lung function for many CF patients, this increase remains very modest (<5% of FEV_1) for about 30% of CF patients on the trial [28]. Another defect with F508del-CFTR is that even when corrected, it displays greatly reduced plasma membrane stability [23]. It is therefore envisioned that a triple-combination therapy targeting protein trafficking, ion channel conductance, and membrane stability will prove beneficial for patients with the F508del mutation.

Using cigarette smoke, we previously identified a role for the Extracellular Signal-Regulated (ERK) Mitogen-Activated Protein Kinase (MAPK) pathway in degradation of CFTR, and determined that this was due to oxidative stress [102]. The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor tyrosine kinase that plays a critical role in the activation of MAPK signaling in response to agents of oxidative stress in the lung epithelium, such as cigarette smoke and bacterial infection [103]. EGFR can be activated by several different ligands, including EGF, Transforming Growth Factor–Alpha (TGF-α), heparin-binding EGF, amphiregulin, epiregulin, betacellulin, and epigen [104]. Oxidative stress has also been implicated in the pathogenesis of CF lung disease [89, 105]. Accordingly, a recent report has identified increased activation of EGFR by amphiregulin in CF airway epithelial cells as a consequence of reduced transport of the antioxidant glutathione by CFTR [106]. We therefore hypothesized that targeting EGFR-ERK

signaling may offer a way to prevent degradation of corrected CFTR and therefore further improve its function.

In the lung, EGFR is important for mucus production and inflammation, but also epithelial repair [31, 107]. EGF was initially identified as a hormone involved in development and organ growth [108, 109], with receptors present in many cell types and tissues [110]. In the lung specifically, EGFR was identified to induce expression of the mucin MUC5AC by its ligands EGF and TGF-α [111]. Upon activation by one of its ligands, EGFR undergoes homo- or heterodimerization and signals to its downstream targets [112]. EGFR activation results in initiation of the Ras-Raf-MEK-ERK signaling cascade [113]. Upon stimulation by 12-myristate 13-acetate (PMA), EGFR has been shown to promote production of the neutrophil chemoattractant interleukin-8 (IL-8) by airway epithelial cells through signaling to ERK [114]. EGFR ligands require shedding by metalloproteinases such as A disintegrin and metalloproteinase 17 (ADAM17), and interestingly, ADAM17 inhibition reduced IL-8 production by these cells, likely due to decreased TGF-α cleavage [114]. On the other hand, EGFR has been found to be important for epithelial wound repair [107] and in fact EGFR inhibition has been shown to worsen acute lung injury in a naphthalene-induced mouse model [115], and also enhanced inflammation and apoptosis tumor necrosis factor-α (TNF-α) overexpressing mice, which model emphysema and pulmonary fibrosis [116]. Infection and inflammation cause significant damage to the airways in CF, which is not repaired efficiently [117], and CFTR itself has been shown to play a role in airway repair [118]. Therefore, targeting EGFR directly may be counterproductive.

EGFR can also undergo ligand-independent activation, and this is often an inhibitor resistance mechanism that develops in various cancers as a result of EGFR mutation [119] or overexpression of the wild-type form [120]. Additionally, EGFR can undergo ligand-independent activation following high oxidative stress, which has been shown to happen following cigarette smoke exposure [121]. In this case, EGFR does not form dimers, and remains active upon internalization [121]. An important step in ligand-independent EGFR signaling is its translocation from caveolin-rich lipid domains ("rafts"), and interestingly, the compatible solute ectoine has been shown to attenuate EGFR translocation from rafts in response to carbon nanoparticles, which negatively impacts ERK signaling without directly inhibiting EGFR [122]. In nature, compatible solutes such as ectoine exert a protective effect via increasing the native conformational stability of macromolecules [123]. Ectoine has been shown to restore neutrophil apoptosis in human primary cells and rats exposed to carbon nanoparticles; likely due in part to a reduction of ERK signaling [124]. Ectoine is well-tolerated in human subjects, and has afforded antioxidant or anti-inflammatory benefit in several respiratory diseases [125-127].

We thus hypothesized that EGFR-ERK signaling is an important regulator of CFTR stability in CF, and that ectoine could offer a viable therapeutic option to target stability, in addition to potentially conferring an anti-inflammatory effect that may be beneficial to CF patients.

Materials and methods

Chemical Reagents

The CFTR inhibitor Inh-172 was purchased from Sigma-Aldrich (St. Louis, MO). UO126, was from Calbiochem (La Jolla, CA). Epidermal growth factor receptor (EGFR) inhibitors, AZD9291 and erlotinib, TAPI-1, and CFTR corrector VX-661 were purchased from Selleckchem (Houston, TX). Forskolin was purchased from Abcam (Cambridge, MA). Recombinant human TGF-α was purchased from R&D Systems (Minneapolis, MN). Ectoine was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

16HBE14o- and CFBE41o- are immortalized human bronchial epithelial cell lines from normal (non-CF) and CF patients (homozygous for F508del mutation), respectively. CFBE41o- expressing either wild-type-CFTR (WT-CFBE) or F508del (F508del-CFBE) were purchased from EMD Millipore (Burlington, MA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 µg/ml), and hygromycin B (300 µg/ml). The tissue culture plates were coated using human fibronectin (1 mg/ml), collagen I bovine (3 mg/ml), and bovine serum albumin (1 mg/ml).

Primary human bronchial epithelial cells (HBE) were isolated either from lungs rejected for lung transplant for non-CF (NHBE), or from CF patients receiving lung transplant (CF-HBE) following a protocol approved by Nationwide Children's Hospital (Columbus, Ohio). Primary NHBE and CF-HBE were isolated as previously described by our group using a modified protocol from Dr. Randell [128-130]. Cells were seeded onto collagen-

coated, semipermeable membranes (0.33 cm^2; Transwell, Corning) and grown at an air-liquid interface in Pneumacult-ALI medium (StemCell Technologies, Tukwila, WA). All the cells were grown and maintained at 37 °C in a 5% CO_2 humidified incubator. One CF donor was F508del/1078delT and all others were F508del/F508del.

Immunoblotting

Cells were lysed in TN1 lysis buffer [50mM Tris pH 8.0, 10mM EDTA, 10mM $Na_4P_2O_7$, 10mM NaF, 1% Triton-X 100, 125mM NaCl, 3mM Na_3VO_4, and a cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN)]. Western blotting was performed as previously described [131]. In brief, 20-40 μg of total proteins were separated with SDS-PAGE in 4-15% polyacrylamide gel and then transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk or BSA in PBS-Tween 20 and immunoblotted with primary antibodies against CFTR (24-1, R&D Systems, Minneapolis, MN; or 769, from the UNC distribution program), phospho-Erk1/2, Erk1/2, phospho-EGFR and EGFR (Cell Signaling, Danvers, MA), β-actin or GAPDH (Santa Cruz Biotechnology, Dallas, TX) followed by treatment with appropriate HRP-conjugated secondary antibody (Pierce, Rockford, IL). The signals were detected with enhanced chemiluminescence (Super Signal West Pico; Thermo Scientific, Waltham MA). The protein bands were scanned and band density was measured using ImageJ software (NIH). For the cycloheximide chase assay, cells were incubated with 100μg/ml cycloheximide for intervals up to two hours prior to lysis and process for western blotting. The fraction of remaining CFTR was calculated by normalizing the ratio of CFTR to GAPDH at a specific time point to the ratio calculated in

cells which were not treated with cycloheximide. Data were fit to a one-phase exponential decay using GraphPad Prism software.

Short-circuit current measurements using Ussing chambers

Ussing chambers were performed as previously described [129, 131, 132]. Briefly, the inserts were mounted in Ussing chambers in Ringer's buffer (115 mM NaCl, 25 mM NaHCO3, 2.4 KH2PO4, 1.24 K2HPO4, 1.2 CaCl2, 1.2 mM MgCl2, and 10 mM D-glucose; pH 7.4). Ringer's buffer was replaced by low chloride (1.2 mM NaCl and 115 mM Na gluconate replacing 115 mM NaCl) on the apical chamber as previously described [129, 132]. The epithelial sodium channel ENaC was inhibited by addition of the ENaC inhibitor amiloride (100μM). CFTR channels were activated using forskolin (to increase intracellular cAMP, since CFTR is a cAMP-activated chloride channel). At the end of the experiment CFTR currents were inhibited using 10μM specific CFTR inhibitor Inh-172.

Quantitative real time PCR (qRT-PCR)

Quantitative RT-PCR was employed to measure the transcript levels of the EGFR ligands AREG, EREG, EGF, HB-EGF, and TGF-α mRNA and was performed as previously described [133]. The mRNA levels were normalized to the expression of the housekeeping gene (CAP-1) and expressed as relative copy number (RCN). $RCN=2^{\Delta Ct}$ X 100 where ΔCt = Cycle threshold (Ct) of gene−Ct of the housekeeping gene (CAP-1).

Enzyme-linked immunosorbent assay (ELISA)

AREG, TGF-α, and IL-8 present in supernatants were measured using ELISA kits (R&D systems, Minneapolis, MN) and experiments were performed according to the manufacturer's instructions.

Lentiviral vector, transduction, and gene editing of primary CF-HBE

Primary airway cells from CF patients were infected with lentiviral particles containing single-guide RNA (sgRNA) targeting AREG or control sgRNA at a multiplicity of infection of 5 (5 particles per cell). Cells containing the lentivirus were selected using puromycin. When 75% confluent cells were seeded onto inserts switched to Pneumacult-ALI cells were allowed to differentiate as described above.

Transcriptomics and Pathway analysis

RNA was extracted from well-differentiated CF-HBE cells using RNeasy Micro Kit (Qaiagen, Germantown, MD). Input RNA quality and quantity were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit Fluorometer, respectively. Samples with RNA integrity numbers greater than 7, and RNA concentration greater than 100ng/μL were sequenced utilizing the OSU Genomics Shared Resource. Messenger RNA (mRNA) sequence libraries were generated with NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NEB #E7760L) and NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) with an input amount of 200ng total RNA per sample. Libraries were pooled and sequenced on an Illumina NovaSeq SP flowcell in paired-end 150bp format (Illumina, San Diego CA) to read yield between 35 – 40 million reads. Differentially Expressed Genes were identified using the DESeq2 algorithm [134]. Genes with an absolute $\log_2$ fold-change greater than 1, and a Benjamini-Hochberg adjusted p-value less than .05 were considered to be differentially expressed. Pathway enrichment analysis was performed using the software Reactome® [135].

Statistical Analysis

Data are expressed as mean ± SD of at least three independent experiments. Statistical analysis was performed using GraphPad Prism. The results of the experiments were analyzed by unpaired *t* tests or one-way ANOVA with appropriate *post hoc* comparison. In all cases, a p value of ≤ 0.05 was considered statistically significant.

Results

The ERK MAPK pathway is active in human CF bronchial epithelial cells due to EGFR signaling

We previously reported that Erk1/2 (referred as ERK) activation induces lysosomal degradation of CFTR in airway epithelial cells upon cigarette smoke exposure [102]. We first wanted to determine if basal ERK activation was increased in CF human bronchial epithelial cells when compared to non-CF cells. To address this, we cultured primary HBE cells from CF (CF-HBE) and non-CF (NHBE) donors to differentiation, and assessed ERK phosphorylation via western blot. ERK phosphorylation was significantly increased in CF-HBE compared to NHBE cells (**Fig. 9A**). Upon phosphorylation, ERK initiates expression of MAP kinase phosphatase-3 (MKP-3) at the gene level, which in turn dephosphorylates ERK in a negative feedback control mechanism [136]. Conversely, strong ERK activation also stimulates degradation of MKP-3 protein [136]. We found significantly decreased expression of MKP-3 in primary CF-HBE cells compared to NHBE cells (**Fig. 9B**), further supporting the case for constitutive ERK activation in CF airway cells.

Since ERK activation can occur downstream of EGFR, and EGFR signaling has previously been reported to be enhanced in CF [106], we aimed to determine the contribution of EGFR to the ERK activation observed above in human CF bronchial epithelial cells. First, we determined via western blot that EGFR phosphorylation was increased in F508del-CFBE cells compared to 16HBE14o- cells (**Fig. 9C**). Next, we showed that two different specific EGFR inhibitors, AZD9291 and Erlotinib, offer near-complete inhibition of ERK phosphorylation in F508del-CFBE cells (**Fig. 9D**). EGFR is activated by the binding of its specific ligands, which require cleavage by metalloproteinases such as ADAM17. Addition of TNF-α protease inhibitor-1 (TAPI-1), an ADAM17 inhibitor, also attenuated ERK phosphorylation (**Fig. 9D**).

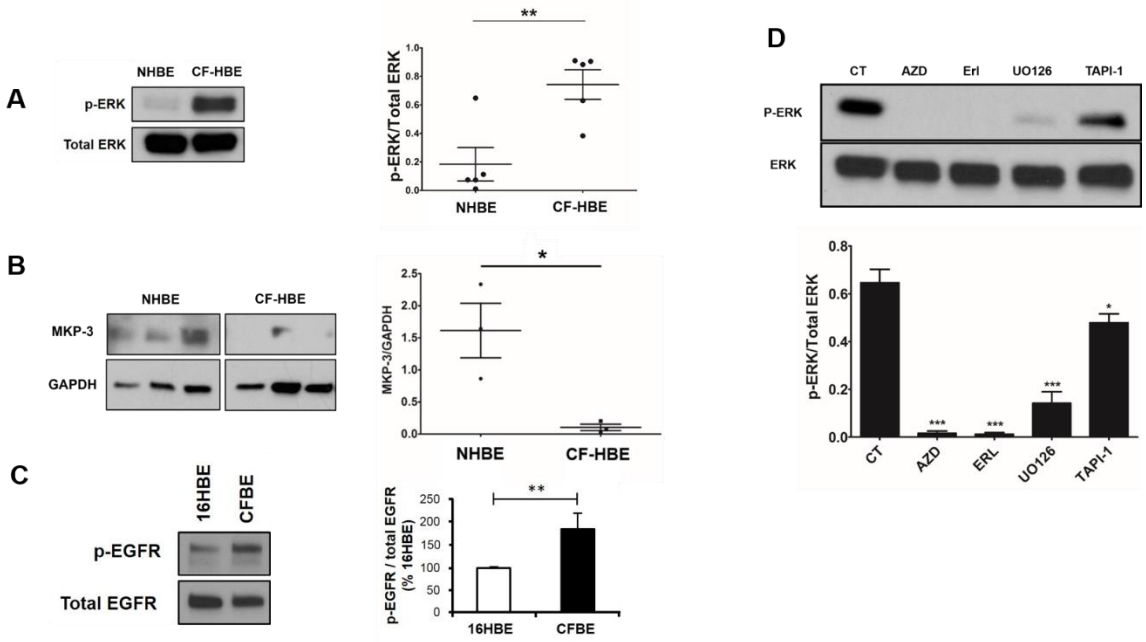


Figure 9: EGFR-ERK signaling is increased in CF bronchial epithelial cells. (**A**) Representative immunoblot and quantification of p-ERK in primary CF-HBE (CF) and NHBE (non-CF) cells. Data were generated from 4 F508del and 4 non-CF donors. (**B**) Representative immunoblot and quantification of the ERK phosphatase MKP-3 in CF-HBE and NHBE cells. Data were generated from 3

F508del and 3 non-CF donors. (**C**) Representative immunoblot and quantification of p-EGFR (Y1173) in 16HBE14o- and CFBE41o- cells. Data represent three independent experiments. (**D**) Representative immunoblot and quantification of three independent experiments showing the effects of EGFR inhbitors AZD9291 (AZD; 5μM) and Erlotinib (Erl; 5μM), the MEK inhibitor UO126 (5μM), and the ADAM17 inhibitor TAPI-1 (5μM) on ERK phosphorylation in CFBE41o- cells. All inhibitors were added for 24 hours. *p<0.05; **p<0.01.

EGFR signaling negatively regulates CFTR

After determining that EGFR plays an important role in sustaining ERK activation in CF, we hypothesized that activation of EGFR in CF cells would lead to CFTR degradation. To test this, we added recombinant human TGF-α (one of the main EGFR ligands) to 16HBE14o- cells and measured CFTR protein expression by western blotting. Treatment with TGF-α caused a dose-dependent decrease in CFTR protein expression (**Fig. 10**).

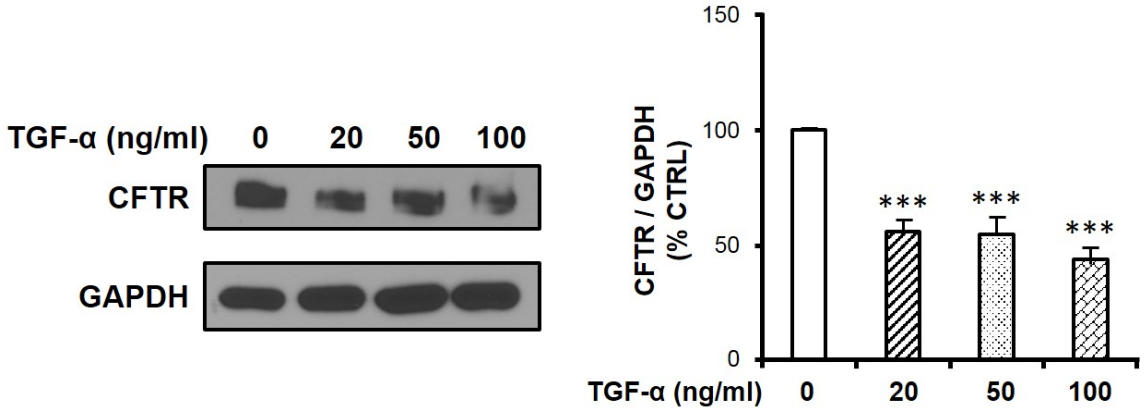


Figure 10: The EGFR ligand TGF-α negatively regulates CFTR. 16HBE cells were treated with the indicated concentrations of TGF-α. Representative immunoblot (left panel) and quantification (right panel) of CFTR expression. Data are representative of three independent experiments.

This latter result strongly suggests that ERK activation observed in CF airway cells interferes with rescue of F508del-CFTR. CFTR correctors such as VX-661 have been recently approved by the FDA to rescue the most-common CF mutant F508del but the improvement in FEV_1 remains modest. Therefore, we next aimed to answer a more translationally relevant question: whether EGFR blockade could enhance rescue of F508del CFTR by correctors. In CFBE cells stably overexpressing F508del CFTR, addition of an EGFR inhibitor increased expression of mature ("Band C") CFTR **Fig. 11A**); as well as CFTR-mediated chloride transport as measured by Ussing chambers (**Fig. 11B**). EGFR inhibition on its own caused an increase in both expression and function, which worked additively with the CFTR corrector VX-661.

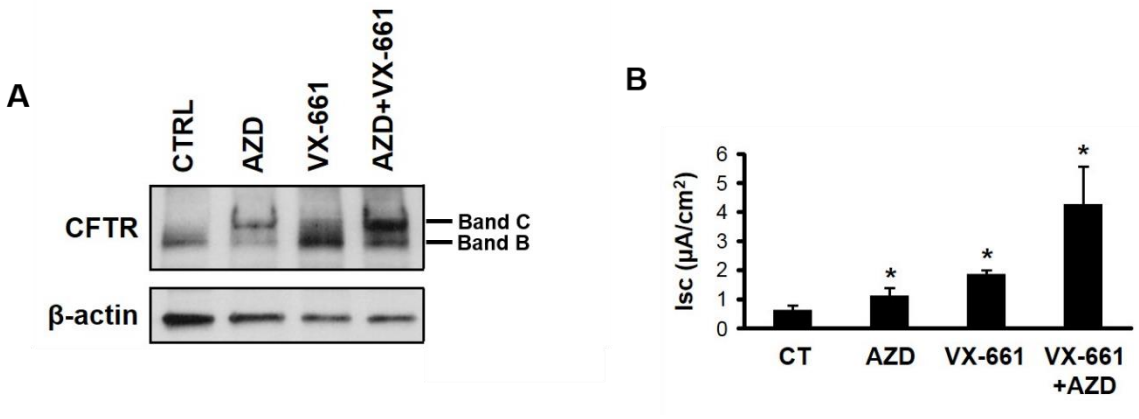


Figure 11: Targeting EGFR facilitates F508del CFTR rescue. (**A**) F508del-CFBE cells were treated with the EGFR inhibitor AZD9291 (AZD), the CFTR corrector VX-661 or a combination of the two. Representative immunoblot demonstrating the additive effects of the EGFR inhibitor AZD9291 (AZD; 5μM) and the CFTR corrector VX-661 (5μM) on expression of mature (Band C) CFTR in CFBE41o- cells. (**B**) Forskolin-activated change in short-circuit current (I_{SC}) measured with Ussing chambers in CFBE41o- cells treated with VX-661 (5μM), AZD (5μM), or a combination of the two. VX-661 was added for 48 hours, and AZD was added for 24 hours. *p<.05.

Amphiregulin production is upregulated in CF

After determining that EGFR-ERK signaling is upregulated in CF and negatively regulates CFTR, we aimed to determine the basis of its activation. The main ligands leading to activation of EGFR are EGF, Transforming Growth Factor–Alpha (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (AREG), and epiregulin (EREG) [104]. We therefore asked whether there were differences in expression of EGFR ligands or the enzymes which cleave them associated with CF. Upon comparing gene expression of EGFR ligands and ADAMs in CFBE cells expressing WT or F508del CFTR, we noted increased expression of the EGFR ligands AREG and TGF-α (**Fig. 12A**), and the metalloproteinase ADAM10 (**Fig. 12B**). On the other hand, EGF and EREG were below detection levels. In primary HBE cells, mRNA expression of both AREG and TGF-α were higher in cells cultured from CF than from non-CF donors (**Fig. 12C**). Using an ELISA approach, we detected increased AREG secretion in apical washes of CF-HBE compared to NHBE cells (**Fig. 12D**).

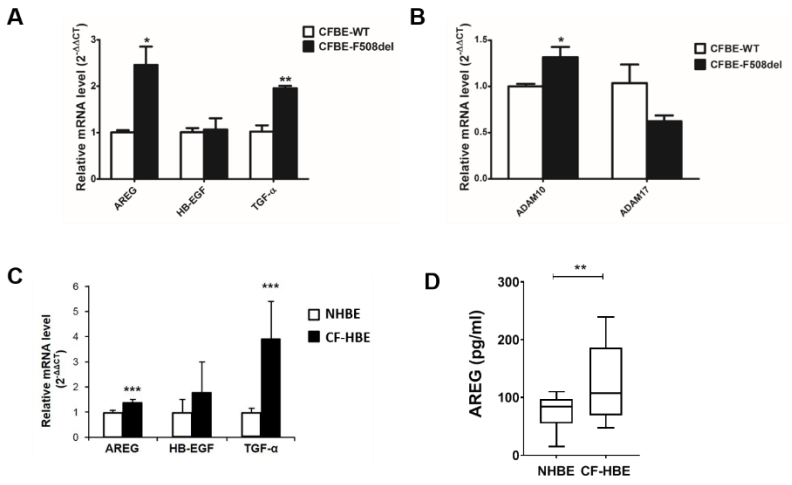


Figure 12: AREG production is up-regulated in CF. Gene expression of the main EGFR ligands (**A**) and the enzymes which cleave them, ADAM10 and ADAM17 (**B**) were detected by qRT-PCR in CFBE41o- cells stably expressing either WT or F508del CFTR. (**C**) Gene expression of the main EGFR ligands were detected by qRT-PCR in primary HBE cells from three separate F508del/F508del donors or non-CF controls. (**D**) AREG secretion into apical media by NHBE and F508del CF-HBE cells (3 donors each) measured by ELISA. AREG: Amphiregulin, HB-EGF: Heparin-binding epidermal growth factor, TGF-α: Transforming growth factor alpha. *p<.05, **p<.01, ***p<.001.

Amphiregulin knockout facilitates CFTR rescue

Our results above show that the EGFR ligand AREG is increased in primary CF-HBE cells when compared to NHBE cells (see **Fig. 10**). To further demonstrate the importance of EGFR ligands in regulating CFTR in primary airway cells, we tested whether down-regulation of AREG would improve CFTR rescue and function with the corrector VX-661. AREG was knocked out in primary CF-HBE cells (F508del/F508del) using a lentiviral vector containing sgRNA targeting AREG and a CRISPR-Cas9 knockout system. **Figure 13A** shows that AREG expression was decreased with the tested sgRNA.

Primary CF-HBE cells were incubated with the corrector VX-661 resulting in increased mature F508del-CFTR (Band C). Rescue of F508del-CFTR was further increased in cells with reduced AREG (**Fig. 13A**). CFTR function was then measured in the presence or absence of VX-661 using Ussing chambers. Compared to control sgRNA, AREG-knock-down cultures demonstrated a significantly greater forskolin-mediated change in short-circuit current after treatment with VX-661, suggesting that targeting AREG improves CFTR rescue **Fig. 13B**).

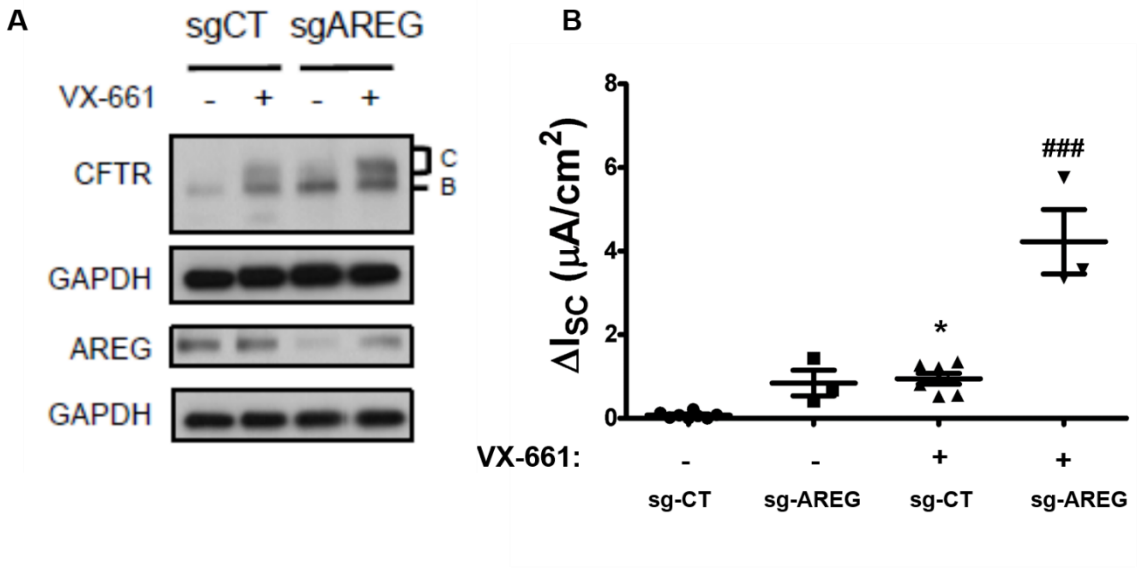


Figure 13: AREG knockout facilitates CFTR rescue. Primary CF-HBE cells were transduced with a lentiviral vector encoding Cas9 and a single guide (sg) RNA targeting AREG (sgAREG), or a control sgRNA (sgCT). Cells were treated with VX-661 or vehicle. (**A**) Expression of CFTR and AREG in primary F508del/F508del CF-HBE transduced with sgCT or sgAREG. GAPDH shows equal protein loading. (**B**) Forskolin-activated short-circuit current (I_{SC}) measured by Ussing chambers. ***p<0.001 compared to untreated sgCT , #p<0.05 compared to sgCT treated with VX-661. N=3-6 filters per condition.

Ectoine decreases ERK signaling in CF, and improves CFTR stability and function

To target EGFR-ERK signaling in CF, we also employed the biologically inert osmolyte ectoine. Ectoine has been shown to prevent EGFR phosphorylation and ERK activation in response to carbon nanoparticles [122, 124]. First, we wanted to evaluate the effect of ectoine on ERK activation in CF-HBE cells. We found that ectoine decreases ERK phosphorylation in primary CF-HBE cells from F508del/F508del donors (**Fig. 14A**). Since we previously reported that cigarette smoke-induced ERK activation leads to degradation of plasma membrane CFTR [102], and we know that F508del-CFTR has reduced plasma membrane stability [100], we wanted to test whether ectoine could stabilize rescued F508del-CFTR. Next, we measured the biological half-life of F508del-CFTR rescued with VX-661 in CFBE cells. Ectoine significantly increased the half-life of CFTR from about 42 to 111 minutes (**Fig. 14B**). To determine if this increase in stability corresponds to increased chloride transport, primary CF-HBE cells were treated with ectoine, VX-661, a combination of the two, or vehicle (control). As expected, VX-661 significantly increased chloride transport. Ectoine in combination with VX-661 further increased chloride transport, while ectoine alone had no effect (**Fig. 14C**).

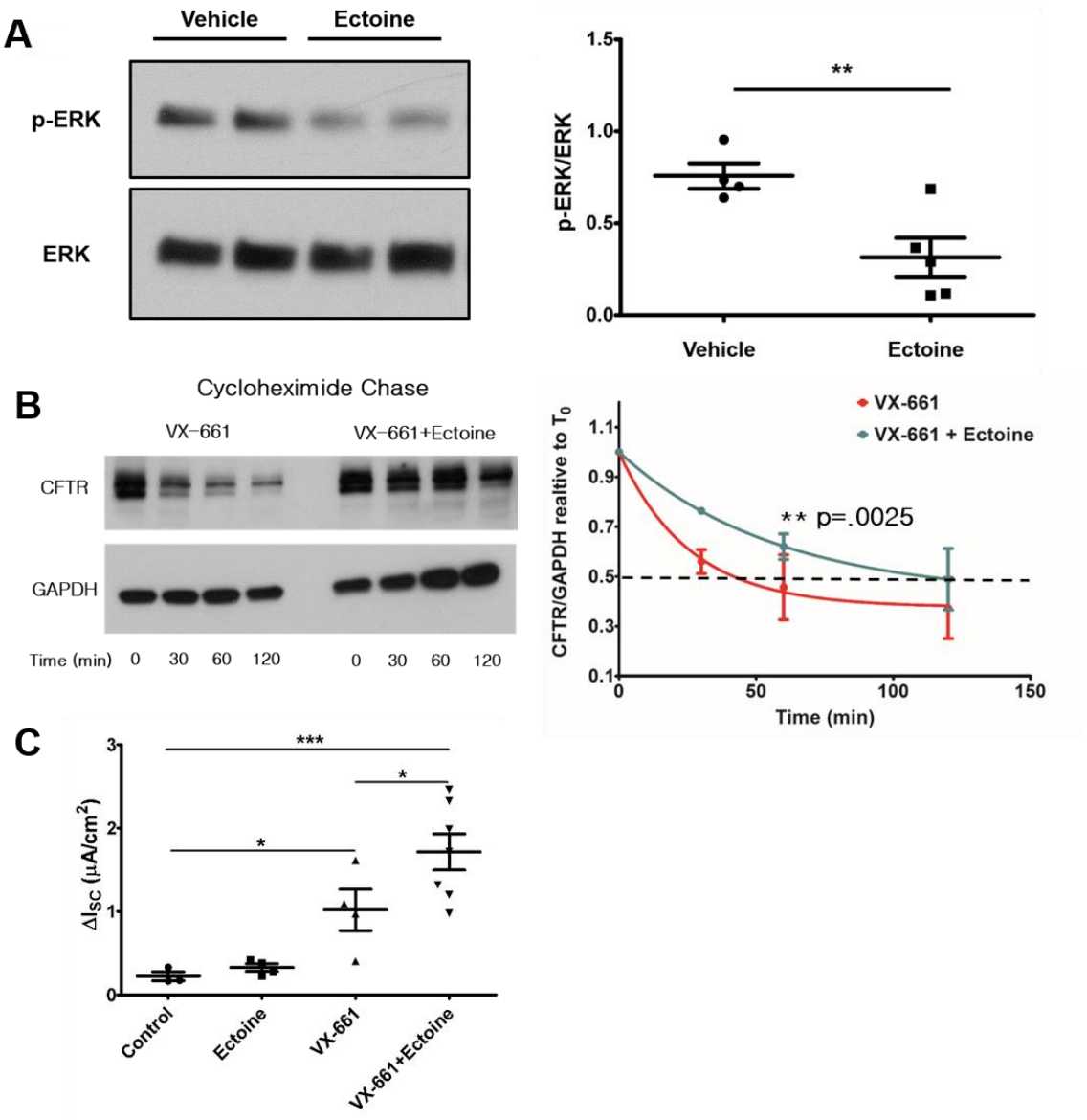


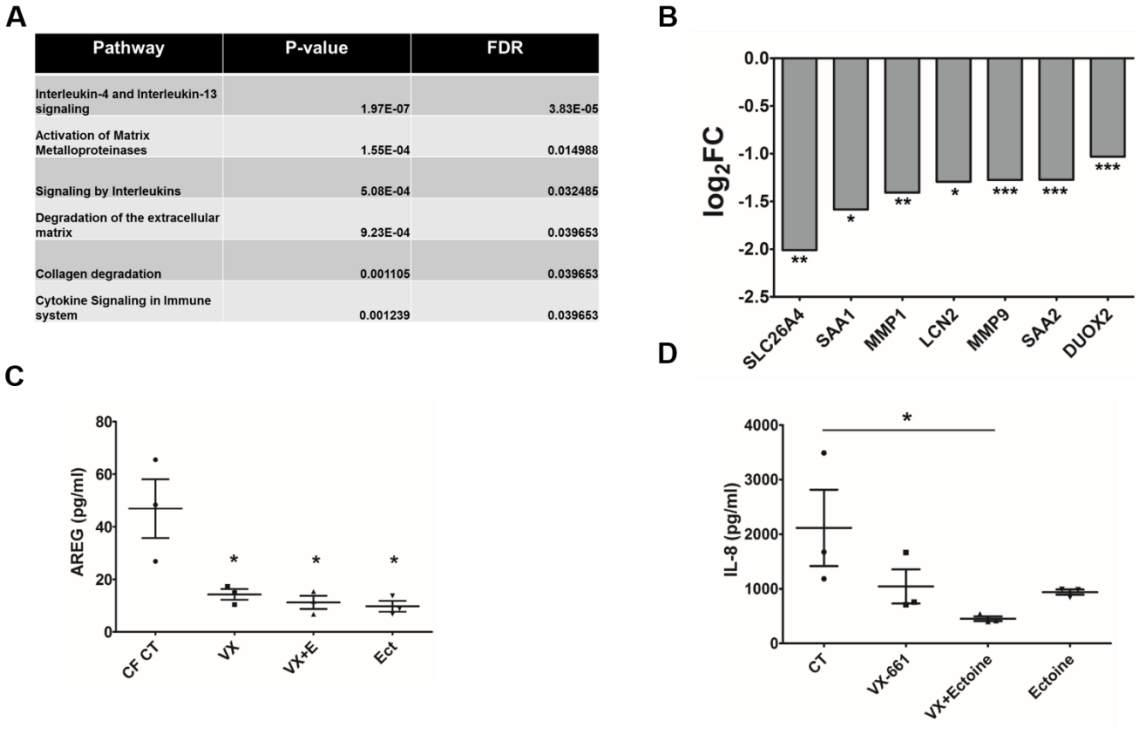
Figure 14: Ectoine decreases ERK phosphorylation and increases CFTR stability. (**A**) Representative immunoblot and quantification for p-ERK in primary F508del/F508del CF-HBE cells treated with ectoine or vehicle.
(**B**) Representative immunoblot and measurement of CFTR biological half-life via cycloheximide chase in CFBE41o- cells overexpressing F508del CFTR. Data were generated from three independent experiments. Cells were treated with VX-661 for 48 hours and ectoine for 24 hours. (**C**) Forskolin-mediated short-circuit current (I_{SC}) indicative of CFTR function in primary F508del/F508del CF-HBE cells treated with ectoine, VX-661, a combination of the two, or vehicle (control). N=3-7 from 3 independent donors. *p<0.05, ***p<0.001.

Ectoine dampens inflammatory signaling in CF-HBEs

Due to ectoine's negative effect on ERK signaling, we next investigated a possible effect on inflammatory signaling. To better understand the potential effects of ectoine in CF, we analyzed how the bulk transcriptome of CF-HBE cells changes when treated with VX-661 and ectoine. We performed pathway enrichment analysis on genes up- or down-regulated by combination treatment. Pathways enriched among genes down-regulated by treatment were mostly related to extracellular matrix turnover and interleukin signaling (**Fig. 15A**). Genes related to interleukin signaling were down-regulated by ectoine, including various cytokines and metalloproteinases. Interestingly, combination treatment also downregulated the NADPH oxidase DUOX2, and the electroneutral chloride/bicarbonate transporter Pendrin (SLC26A4) (**Fig. 15B**). Pendrin and DUOX2 are important for hypothiocyanite production, which can in turn stimulate the pro-inflammatory transcription factor NF-κB [137]. Surprisingly, no genes were determined to be differentially regulated by VX-661 on its own. There is evidence that AREG can promote expression of matrix metalloproteinase-9[138], which is down-regulated by treatment. Therefore, we hypothesized that ectoine itself may be reducing AREG production. Indeed, ectoine significantly decreased shedding of the EGFR ligand AREG by primary CF-HBE cells (**Fig. 15C**). VX-661 alone also decreased release of AREG. However, combination of ectoine and VX-661 did not further decrease release of AREG (**Fig. 15C**).

CF patients also have excessive neutrophil infiltration in their lungs due to secretion of the potent chemoattractant IL-8 by airway cells [139]. Since the ERK pathway

contributes to IL-8 secretion by airway epithelial cells [140] we wanted to assess the effect of ectoine on IL-8 secretion. Ectoine in combination with VX-661 significantly decreased secretion of IL-8, whereas each compound on its own decreased IL-8 but the effect was not statistically significant (**Fig. 15D**).



Pathway	P-value	FDR
Interleukin-4 and Interleukin-13 signaling	1.97E-07	3.83E-05
Activation of Matrix Metalloproteinases	1.55E-04	0.014988
Signaling by Interleukins	5.08E-04	0.032485
Degradation of the extracellular matrix	9.23E-04	0.039653
Collagen degradation	0.001105	0.039653
Cytokine Signaling in Immune system	0.001239	0.039653

Figure 15: Anti-inflammatory effects of ectoine in primary CF-HBE cells. (**A**) Reactome® pathways significantly enriched (FDR<.05) among the fraction of genes downregulated by VX-661 and ectoine together in CF-HBE cells compared to vehicle-treated CF-HBE controls. (**B**) Log fold-change plot of genes of interest differentially regulated by VX-661 and ectoine related to inflammatory signaling. Data were generated from four separate F508del/F508del donors. (**C**) The EGFR ligand AREG and the neutrophil chemoattractant interleukin-8 (IL-8) (**D**) were detected by ELISA in apical washes from primary F508del/F508del CF–HBE cells treated with ectoine, VX-661, a combination, or vehicle (control). N=3 from 3 independent donors. *p<0.05.

Discussion

Improving rescue of CFTR mutants remains challenging. This is particularly true for F508del-CFTR due to its various effects altering CFTR protein trafficking, ion channel function, and stability at the plasma membrane. Drugs currently available for F508del target the trafficking defect and channel function, but not protein instability. Here we show that the EGFR/ERK axis is up-regulated in CF airway cells at least partially due to increased release of the EGFR ligand AREG. We also show that targeting this axis using specific inhibitors or by modulating AREG expression increased rescue and function of CFTR in human CF (F508del/F508del) bronchial epithelial cells. Finally, we found that the osmoprotectant ectoine could reduce ERK activation resulting in increased half-life of F508del-CFTR, improved rescue and enhanced chloride transport. Interestingly, ectoine could also reduce secretion of the neutrophil chemoattractant IL-8 by primary CF-HBE cells.

An important finding of our study is that ERK signaling is constitutively active in CF airway cells. We have previously shown that ERK activation by cigarette smoke induces lysosomal degradation of CFTR [102], so ERK's constitutive activity in CF airway epithelial cells makes it an attractive therapeutic target. The molecular mechanisms responsible for ERK-mediated CFTR degradation are currently unclear. An RNA interference screen identified many kinases that can activate ERK to suppress CFTR rescue, and the authors proposed that ERK's inhibition of several chaperone proteins was important for this [141]. The ring finger and FYVE-like domain containing E3 ubiquitin protein ligase (RFFL) has also been shown to mediate lysosomal degradation of F508del-

CFTR independent of chaperones, and interestingly, RFFL is transcribed following ERK activation [143].

As noted by others, many kinases, several of which involve ERK, negatively regulate CFTR; including EGFR, fibroblast growth factor receptors, and TGF-β receptors [141, 144]. Our data using inhibitors of EGFR and ADAMs suggest an important role for this axis in regulating CFTR, but we cannot rule out the importance of other signaling mechanisms. The difficulty in determining the contribution of various receptors further supports the use of ectoine to target ERK in CF, since ectoine's stabilizing effect is not specific to EGFR. In fact, using single-molecule force spectroscopy, it has been shown that ectoine and other compatible solutes increase the force necessary to pull bacteriorhodopsin out of the cell membrane [145]. Taking this into consideration, it is also possible that the effect we report of ectoine on CFTR stability could be due to a direct stabilizing effect on CFTR. If this were the case, then it would also be conceivable that ectoine's inhibitory effect on ERK could be mediated by CFTR, since EGFR-ADAM17 signaling in CF is likely a direct consequence of decreased glutathione transport by CFTR [106]. Our data showing that VX-661 decreases AREG secretion could support this, since ERK has been shown to be involved in ADAM17 activation [146]. However, the effect of VX-661 alone on ERK phosphorylation was inconsistent (not shown).

Slight discrepancies exist between our findings and those of others, which could likely be explained by differences in the experimental systems employed. Stolarczyk *et al*. showed increased AREG production, but not gene expression in CFBE cells expressing F508del compared to WT CFTR, but only after culturing the cells at air-liquid interface. It

can be seen that switching the culture system to air-liquid causes a change in AREG mRNA expression [106]. In our experiments, CFBE cells were cultured under liquid-liquid conditions, so this could explain these differences. Our failure to detect differences in protein expression of TGF-α or AREG between CFBE cells expressing WT- or F508del-CFTR could also be a consequence of culturing the cells at liquid-liquid interface. In primary HBE cells, we detected differences at the gene level in both AREG and TGF-α. However, we only detected differences in AREG secretion between cells from CF and non-CF donors. We did not detect TGF-α via ELISA in apical washings of these cells.

The importance of the ligand AREG is further demonstrated by our finding that reducing AREG expression using a CRISPR-Cas9 system can improve CFTR rescue and function in primary CF-HBE cells treated with VX-661. While both AREG and TGF-α were found to be up-regulated at the mRNA level in CF airway cells, we decided to focus on AREG because TGF-α protein was not detected in apical washes. CFTR knockdown with shRNA has also been reported to increase the expression of the EGFR ligand EREG at the gene and protein level in the intestinal epithelial cell line Caco-2 [147], however we did not detect any differences in this EGFR ligand in either of our experimental systems. Others have reported that AREG is markedly increased by WT-CFTR at the transcription level via the long non-coding RNA SUMF1-2 [148], which is contradictory to our findings. Future studies will be necessary to understand this difference, but it is likely due to differences in the experimental systems used.

Regardless, our results have identified AREG-EGFR-ERK signaling as an intrinsic regulator of CFTR in CF, and shown that the natural osmolyte ectoine can target this

pathway; resulting in increased CFTR stability in addition to its anti-inflammatory effects (Summarized in **Fig. 16**). We suspect that ectoine, in combination with CFTR modulators, would benefit CF patients. Our results showed a synergistic effect between ectoine and VX-661, however future studies will be necessary to determine whether ectoine complements the effects of other CFTR modulators. The CFTR potentiator VX-770 has been reported to counteract the beneficial effects of the CFTR modulators VX-809 and VX-661 by decreasing bulk CFTR stability [24, 149]. Evidence exists that at the single-channel level, a small population of CFTR molecules are in fact stabilized, possibly offering an explanation for the clinical benefits observed with combination therapy [150]. It was speculated by the authors that this stabilized CFTR population corresponds to CFTR localized to cholesterol-rich lipid domains (rafts) [150]. If this were the case, it is then conceivable that ectoine could counteract the destabilizing effects of VX-770 by enriching membrane- or raft-fraction CFTR, in a similar fashion to its effect on EGFR membrane and raft localization [122], and further improve CFTR rescue. Finally, the newly approved CFTR modulator VX-445 has been shown to robustly increase CFTR function in triple combination with VX-661 and VX-770, as well as drastically improve clinical outcomes [27, 28]. However, this increase remains very modest (<5% of FEV1) for about 30% of CF patients on the trial. Whether ectoine, or other drugs targeting EGFR-ERK will afford any additional benefits in combination with VX-445 will need to be investigated in the future.

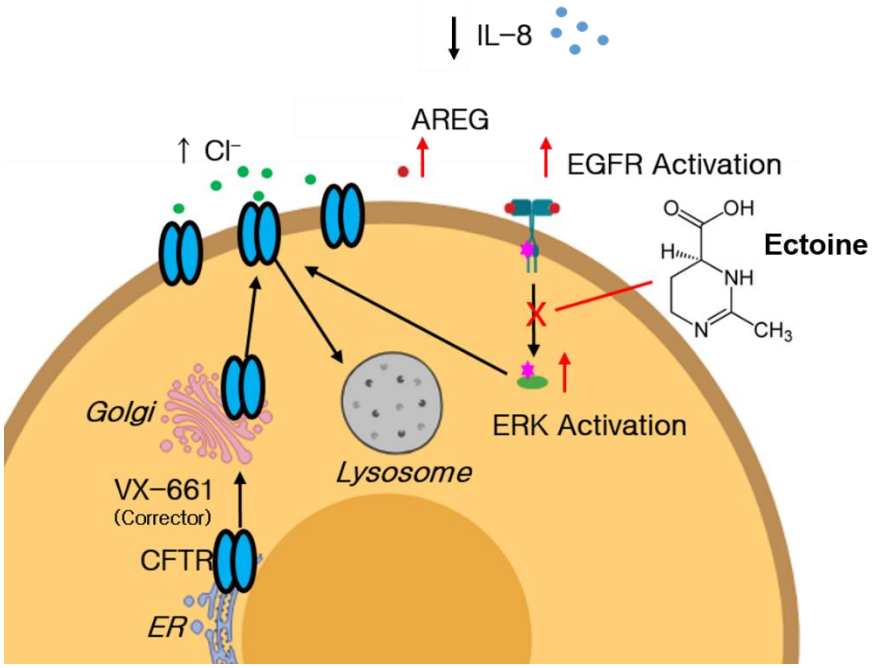


Figure 16: Graphical abstract showing the role for AREG-EGFR-ERK signaling in regulation of CFTR. AREG production is up-regulated in CF and activates EGFR, signals downstream to ERK. ERK signaling causes lysosomal CFTR degradation. Ectoine can attenuate ERK signaling, likely through interfering with EGFR translocation. This has a preventative effect on degradation of CFTR that has been trafficked through the membrane through correction with VX-661. Increased plasma membrane CFTR expression results in increased chloride transport. Ectoine also reduced production of IL-8, and likely other inflammatory cytokines.

Section 2: Stabilizing plasma membrane CFTR by targeting the CFTR-associated ligand (CAL) protein using PDZ domain peptide inhibitors.

This section is modified from a publication that is currently in press: "Cyclic Peptidyl Inhibitors against CAL-CFTR Interaction for Treatment of Cystic Fibrosis"(Dougherty, Wellmerling* et al, Journal of Medicinal Chemistry)*

**Co-first author*

Introduction

The severity and life altering nature of CF has made the search for therapeutic interventions of the utmost importance. Symptomatic treatment options, such as antibiotics and inhaled corticosteroids, are helpful but do not address the underlying pathophysiology [151]. Direct modulation of aberrant CFTR biochemical function has been challenging because CFTR mutants are nontraditional drug targets and highly heterogeneous; each therapeutic is likely to be only effective against a specific class of CFTR mutations. To date, three different classes of modalities have been developed to rescue the function of CFTR mutants, namely potentiators, correctors, and stabilizers (Figure 1a). Potentiators, as exemplified by the small molecule Ivacaftor (VX-770) [21], act by increasing the probability for the ion channel to stay in the open state and are most effective against Class III mutations (e.g., G551D). Correctors such as Tezacaftor (VX-661) [152] and the newly FDA approved Elexacaftor (VX-445) [27] function to promote proper folding and stabilize Class II CFTR mutants (e.g., F508del). They generally lack single-agent efficacy and must be used in combination with potentiators. The third class,

stabilizers, function to increase the membrane expression of CFTR by preventing it from lysosomal trafficking and degradation [153]. Stabilizers represent a very attractive modality for the treatment of CF as they should be effective across the entire CFTR mutational landscape and synergize with correctors and/or potentiators. Unfortunately, such a compound is not currently available to CF patients.

The most common mutation, corresponding to a deletion of phenylalanine 508 (F508del), is present in an estimated 90% of CF patients.16 As a Class II mutant, F508del-CFTR is conformationally unstable and upon rescue subject to rapid lysosomal degradation. While combination therapies of correctors and potentiators have substantially improved lung function of CF patients with one or two F508del alleles, they cannot address the underlying issue of rapid membrane turnover conferred by the F508del mutation. The membrane expression of CFTR is controlled through interactions between its C-terminus and two different classes of PDZ domain-containing proteins [154, 155]. While binding to PDZ domain proteins Na+/H+-exchanger regulatory factor isoform-1 and 2 (NHERF-1 and 2) stabilizes CFTR on the plasma membrane [156, 157], interaction with CFTR-associated ligand (CAL) targets CFTR for lysosomal degradation (Figure 1a) [158, 159]. These observations suggest that selective inhibitors against the CAL PDZ-CFTR interaction should provide a novel class of CFTR stabilizers. This approach was initially validated by the discovery of a peptidyl inhibitor of the CAL PDZ domain, iCAL36, which enhanced CFTR membrane half-life in cultured airway epithelial cells [160]. Since then, a number of other peptidyl inhibitors have been reported, with CAL binding affinities typically in the low μM to high nM range [161]. There have also

been efforts in developing covalent small-molecule inhibitors of this interaction [162]. Peptides are well suited to target the CAL-CFTR interaction because of their potential for high affinity and specificity, but they generally lack cell-permeability and metabolic stability. To overcome these limitations, we previously designed a cell-permeable, reversibly cyclized peptidyl inhibitor, by fusing a cell-penetrating peptide (CPP) with a CAL PDZ domain-binding sequence [163]. When outside the cell, peptide 1 exists as a disulfide-cyclized macrocycle, which has improved proteolytic stability and cell-permeability. Upon entering the cell, peptide 1 is converted into the linear, active form by intracellular thiols [e.g., glutathione (GSH)]. Peptide 1 increased the ion channel activity of F508del-CFTR in CF bronchial epithelial (CFBE) cells by 77%, but required high concentrations (50 μM) owning to its modest binding affinity for the CAL PDZ domain (KD = 0.5 μM), limited cell-permeability, as well as proteolytic stability (serum $t_{1/2}$ ~5 h). In this work, we carried out a modeling-guided medicinal chemistry campaign and greatly improved the potency, specificity, cell-permeability, and metabolic stability of the parent peptide. The optimized inhibitor greatly improved the ion channel activity of F508del-CFTR in CFBE cell line and CF patient-derived primary lung epithelial cells at low nanomolar concentrations.

Results and Discussion

Selective Binding to CAL Is Required for Improving the Function of Rescued F508del-CFTR.

We evaluated peptides 19c and 22c for their efficacy in improving the chloride ion channel activity of primary CF human bronchial epithelial (CF-HBE) cells from a patient homozygous for the F508del mutation. In this assay, CFTR function is assessed by measuring the short-circuit current (I_{SC}) flowing across differentiated primary CF-HBE cultures grown at air/liquid interface using Ussing chambers [129, 164]. CF-HBE cells were incubated with 5 μM VX-809, a small-molecule corrector [18], and 100 nM peptide before measuring CFTR function. Peptide 19c increased the I_{SC} value by ~50% relative to VX-809 alone, suggesting effective rescue of F508del-CFTR on the apical membrane (**Fig. 17**). Much to our surprise, peptide 22c had no significant effect on I_{SC}, despite its similar CAL binding affinity and 18-fold higher cellular entry efficiency. We reasoned that the lack of efficacy by peptide 22c might be caused by off-target effects. Specifically, the C-terminus of CFTR interacts with the PDZ domain(s) of several different proteins, including CAL, NHERF1, and NHERF2. [22] While binding to CAL targets CFTR for lysosomal degradation, interaction with NHERF1/2 facilitates CFTR trafficking to the plasma membrane and stabilizes CFTR on the membrane. Thus, simultaneous inhibition of NHERF1/2 PDZ domains would offset the beneficial effects of inhibiting the CAL-CFTR interaction. To test this hypothesis, we determined the binding affinities of peptides 19 and 22 to CAL PDZ, NHERF1 PDZ domain 1, and NHERF2 PDZ domains 1 and 2 by fluorescence polarization. Our attempt to produce

soluble NHERF1 PDZ domain 2 was unsuccessful. Peptide 19 showed modest selectivity for CAL-PDZ (14- to 36-fold) over NHERF1/2 PDZ domains. In contrast, peptide 22 exhibited essentially no selectivity for CAL vs NHERF1/2 domains (2- to 12-fold). These results suggest that selective inhibition of CAL PDZ domain is essential for increasing F508del-CFTR function.

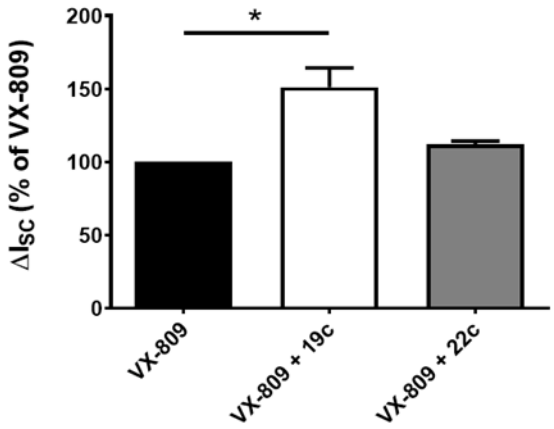


Figure 17: Comparison of peptides 19c and 22c for their effi-cacy in increasing CFTR-mediated short-circuit currents in CF-HBE cells. Cells were treated with 5 μM VX-809 for 48 h, followed by 100 nM peptide for 2 h before measuring short-circuit current (Isc) in an Ussing chamber. Values are scaled relative to treatment with VX-809 alone (100%) and represent the mean ± SD (2-3 replicates/condition), one donor. *, $p < 0.05$.

PGD97 Increases Surface Expression of Rescued F508del-CFTR.

Previous studies have shown that knock-down of CAL expression by siRNA increases the plasma membrane population of F508del CFTR by 4.4-fold.37 Inhibition of the CAL-CFTR interaction by PGD97 is therefore expected to reduce lysosomal degradation of plasma membrane CFTR, thereby increasing the overall cellular level and cell surface expression of CFTR [160, 163]. To test this hypothesis, we treated CFBE41o- cells,38 which stably express F508del-CFTR, with vehicle (DMSO), 5 μM

VX-661 (a corrector), or 5 µM VX-661 in combination with 100 nM PGD97 and immunostained the cells with an antibody against CFTR. In vehicle treated cells, the F508del-CFTR fluorescence was weak and largely perinuclear (**Fig. 18A**). Treatment with VX-661 alone increased the overall fluorescence intensity, although the fluorescence remained mostly perinuclear with some staining extending to the surface of the cells (**Fig. 18A and 18B**). In contrast, cells treated with both VX-661 and PGD97 showed much brighter CFTR fluorescence, which was also more diffused throughout the cytoplasmic region. These observations indicate that PGD97 and VX-661 act synergistically to increase the surface expression of F508del-CFTR.

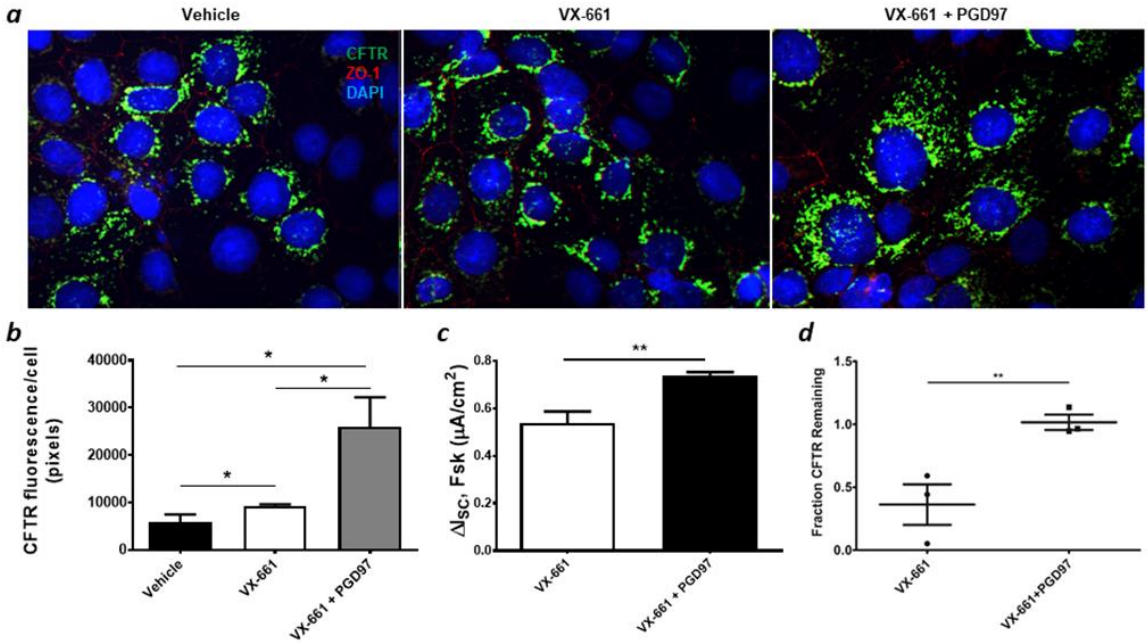


Figure 18: PGD97 improves CFTR function in CFBE41o- cells stably overexpressing F508del-CFTR. (**A**) Immunofluorescence staining of CFBE41o- cells after treatment with vehicle (DMSO), 5 µM VX-661, or 5 µM VX-661 + 100 nM PGD97. CFTR is stained green, the tight junction marker Zona Occludins-1 (ZO-1) is stained red, and nuclei are stained blue. (**B**) Quantification of CFTR staining in (a) of 3 independent experiments. *p<0.05; ***p<0.001. (**C**) Change in short-circuit currents (Isc) in CFBE41o- cells after treatment with VX-661 (5 µM) or VX-661 (5 µM) + PGD97 (100 nM). (**D**) Fraction of CFTR

remaining in CFBE41o- cells pretreated with VX-661 (5 μM) or VX-661 (5 μM) + PGD97 (100 nM) after 2 h of cycloheximide treatment as assessed by western blot quantification. Data shown are relative to that of cells which were not treated with cycloheximide. *, $p<0.05$; **, $p<0.01$ from n = 3 independent experiments.

PGD97 Improves Function of Rescued F508del-CFTR.

We next evaluated the capacity for PGD97 to restore F508del-CFTR function in CFBE41o- cells. Co-treatment with corrector VX-661 (5 μM) and 100 nM PGD97 resulted in ~50% increases in ISC over VX-661 as measured both with CFTR activation and inhibition (**Fig. 18C** and data not shown). As membrane localized F508del-CFTR is still only marginally functional and the membrane current may not accurately reflect the efficacy in inhibiting the CAL-CFTR interaction, we evaluated if our compound was effective for preventing CAL-mediated F508del-CFTR degradation. Use of a cycloheximide chase to arrest de novo protein synthesis and probe the fraction of extant CFTR remaining following treatment with 100 nM PGD97 indicated a ~60% improvement in CFTR over VX-661 alone (**Fig. 18D**). Taken together, these results show that PGD97 increases the stability of the rescued CFTR population resulting in increased channel function.

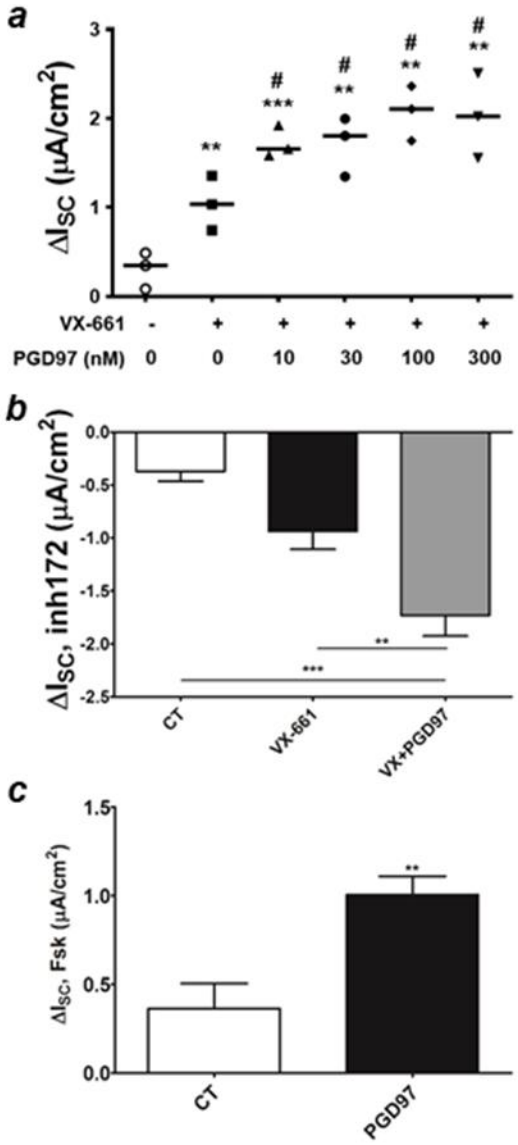


Figure 19: Ex vivo efficacy of PGD97 in CF patient-derived primary HBE cells (CF-HBE). (**A**) Dose response of PGD97 in increasing the short-circuit currents (Isc) in the presence of 0 or 5 μM VX-661. (**B**) Comparison of CFTR-mediated Isc using the specific CFTR inhibitor Inh-172 (100 μM) in CF-HBE cells treated with vehicle (CT), VX-661 alone (5 μM), or VX-661 (5 μM) + PGD97 (100 nM). (**C**) Change in Isc after treatment with 100 nM PGD97 alone. Primary CF-HBE cells from 3 CF donors were used in the experiments. Data shown were from five independent experiments (n = 5 filters). *, $p<0.05$, **, $p<0.01$, and ***, $p<0.001$ via ANOVA with Tukey's posthoc multiple comparison.

Encouraged by the results obtained with the immortal-ized CFBE41o- cells, we next tested PGD97 in patient-derived primary cells from multiple CF donors homozygous for F508del (CF-HBE). In the presence of a constant con-centration of VX-661 (5 μM), PGD97 dose-dependently in-creased the ion channel activity (ISC), with an estimated EC50 value of ~10 nM and reaching the maximal activity at ~100 nM concentration (**Fig. 19A**). Importantly, at the saturating concentration (100 nM), PGD97 increased the ion channel activity by ~2-fold, relative to the VX-661 only control as observed using a CFTR specific inhibitor (**Fig. 19B**). A greater negative change in current upon addition of Inh-172 signifies more chloride transport by CFTR. Interestingly, PGD97 also demonstrated efficacy as a single agent (in absence of a corrector); treatment of primary CF-HBE cells with 100 nM PGD97 alone increased the ion current by ~3-fold, relative to the control (no treatment) (**Fig. 19C**). The magnitude of improvement by 100 nM PGD97 was comparable to that achieved by 5 μM VX-661.

Conclusion

To conclude this section of **Chapter 2**, we developed, through close collaboration with the Pei lab (OSU), a cyclic cell-permeable peptide drug which targets the PDZ-binding domain of CAL. Inhibiting the CAL-CFTR interaction through targeting this domain, we show that stability of F508del CFTR can be improved significantly in a CF bronchial cell line and in donor-derived primary bronchial epithelial cells. This stabilizing effect has a positive effect on CFTR-mediated chloride transport, which is

synergistic with the corrector VX-661. In the future, we aim to test this type of peptide drug in combination with the potentiator VX-770, and the next-generation corrector VX-445. Ideally, this sort of peptide-based strategy design will represent a mutation-agnostic therapeutic that will benefit the vast majority of CF patients.

Chapter 3: Transcriptomic Analysis of Primary CF Human Bronchial Epithelial Cells and the Effects of VX-661 and Ectoine

Introduction

Extending upon the data presented in **Chapter 2**, we aimed to gain a deeper understanding of the basic biology of CF and the effects of pharmacological CFTR correction, in addition to the novel therapeutic compound ectoine. To do this, we employed both transcriptomic and proteomic approaches using donor-derived HBE cells as described in **Chapter 2**. Previous efforts have been undertaken to characterize the CF transcriptome utilizing different cell types, including epithelial and immune tissues; with many studies identifying an enrichment of genes related to innate immunity and inflammation [165]. Proteomic approaches have also been employed to characterize CF, including exhaled exosomes [166], brocnhoalveolar lavage fluid [167], and primary bronchial epithelial cells [168]; with many dysregulated proteins in these studies being related to innate immunity, extracellular matrix remodeling, and mucus homeostasis.

Several studies have employed a transcriptomics approach to better understand the effects of CFTR modulators. The effects of the potentiator VX-770, which increases CFTR function, on the transcriptome of monocytes from patients with one copy of R117H-CFTR (a gating mutant) have been investigated. These results show that VX-770 promotes an increase in innate immune and inflammatory genes (which can be understood as a reversal of the immune defect in CF), cell cycle control genes, and oxidative phosphorylation [169]. The whole-blood transcriptome of patients receiving a

combination of the corrector VX-809 and the potentiator VX-770 has also been assessed [170]. In this study, it was found that compared to non-CF controls, the transcriptome of CF blood cells reveals an increase in inflammatory and apoptotic genes, with a deficiency in genes related to T-cells and natural killer cells. Combination therapy was found to promote normalization of cell death genes, and responder status was associated with changes in genes related to protein synthesis, oxidative phosphorylation, and IL-17 signaling [170]. To our knowledge, no studies have investigated the transcriptomic or proteomic effects of CFTR modulators in primary HBE cells. Additionally, since ectoine exerts its biological effects through indirect biophysical mechanisms and could potentially influence multiple cellular process, these approaches are useful in gaining a deeper understanding of its effects.

Methods

Cell culture and transcriptomic analysis were performed as described in Chapter 2

Proteomic analysis

Well differentiated primary CF- and non-CF human bronchial epithelial cells (CF-HBEs and NHBEs) were treated with the corrector VX-661 (Selleckchem, Houston, TX), Ectoine (Sigma-Aldrich, St. Loius, MO), or vehicle. VX-661 was added for 48 hours, and ectoine was added for 24 hours. Following treatment, cells were frozen at -80°C and sent for lysis and liquid chromatography with tandem mass spectrometry (LC-MS/MS) that was performed utilizing The Ohio State University Proteomics Shared Resource. Spectral data were normalized using Scaffold®

(http://www.proteomesoftware.com/products/scaffold/). Differential protein expression was determined using the webtool ProtExA® to perform linear models for mixed microarray (LIMMA) analysis [171], and pathway enrichment analysis was performed using Reactome® [135]. Three independent F508del-homozygous donors were used for each CF condition, and 3 donors homozygous for non-mutant CFTR were used as controls.

Results and discussion

When comparing the transcriptomes of primary CF-HBE and NHBE (non-CF) cells, we identified 29 genes to be significantly up-regulated in CF, while 39 were down-regulated (**Fig. 20**). Among the up-regulated gene fraction, several genes closely linked to inflammation were noted. These include *ANO1*, *CCL20*, *CXCL6*, and *NFAT5* (**Fig. 21A**)

	CF	VX-661	VX-661 + Ectoine
Genes	29 Up, 39 Down	No change	21 Up, 32 Down
Proteins	20 Up, 51 Down	133 Up, 22 Down	124 Up, 32 Down

Figure 20: Table showing the number of genes and proteins that are differentially expressed in CF-HBE cells compared to NHBE cells (first column); and the number of genes and proteins that change when treated with either VX-661 or VX-661 in combination with ectoine compared to vehicle-treated CF-HBE controls (right two columns).

The *ANO1* gene encodes the calcium-activated chloride channel Transmembrane Protein 16A (TMEM16A). TMEM16A expression is robustly induced following stimulation by the type II cytokines IL-4 and IL-13, where it is thought to play an

important role in mucin expression and release [11, 172]. TMEM16A has received considerable attention in CF. On one hand, small molecule activators of TMEM16A have been shown to attenuated production of the inflammatory cytokines IL-6, IL-8, and CXCL1/2 [173]. Activation of TMEM16A has also been extensively explored as a means of restoring chloride transport in CF [174]. However, there is debate regarding whether TMEM16A activation or inhibition would be beneficial in CF [175].

TMEM16A inhibition has been shown to attenuate mucus secretion in an ovalbumin-induced guinea pig asthma model [176], and a prodrug of niflumic acid (which inhibits TMEM16A) promoted increased survival of CF mice [177]. TMEM16A can also promote activation of EGFR and ERK, likely through its effect on intracellular calcium signaling [178, 179], which we describe in **Chapter 2** to be important regulators of CFTR that can also promote pro-inflammatory signaling. TMEM16A plasma membrane expression and function were found to be reduced in CF donor-derived airway cells and mouse lungs [180], and CFTR and TMEM16A currents have been suggested to depend on each other [181, 182]. Regardless, in our study, *ANO1* was found to be one of the most up-regulated genes in CF, suggesting a native type II inflammatory state in CF airway epithelial cells.

C-C Motif Chemokine Ligand 20 (CCL20) was also found to be highly up-regulated in primary CF-HBEs. This chemokine has been shown to be important in the recruitment of T_H17 cells in several different disorders through its binding to chemokine receptor 6 [183-185]. T_H17 cells produce IL-17 cytokines, which can stimulate epithelial cells to produce IL-8, ultimately resulting in neutrophil recruitment [186]. T_H17 cells

have been shown to be increased in the lungs of newly diagnosed CF patients compared to non-CF controls [187]. Blood T_H17 cell percentage has also been found to be negatively associated with lung function in CF, highlighting their importance [188]. Interestingly, naïve T-cells from CF patients have shown an increased tendency to differentiate toward the T_H17 phenotype upon stimulation [189]. High intrinsic CCL20 production by epithelial cells could conceivably contribute to inflammation in CF in concert with CF T-cells' intrinsic T_H17 tendency, and would be an interesting avenue to explore in the future. Further supporting the intrinsic pro-inflammatory role of CF airway epithelial cells, we detected increased gene expression of Chemokine (C-X-C motif) Ligand 6 (CCL6) in CF. The protein-product of this gene, also referred to as granulocyte chemotactic protein 2, is a neutrophil recruiter that has previously been found in the airways of CF patients [190], further supporting the role of CF airway epithelial cells in promoting inflammation.

Finally, we detected increased transcription of the gene encoding Nuclear Factor of Activated T-Cells 5 (*NFAT5*) in CF. Often discussed in the context of renal biology, NFAT5 is a transcription factor that is induced by hypertonicity, and interestingly, can promote T_H17 inflammation [191]. The expression of *NFAT5* has also been demonstrated in airway epithelial cells [192]. Interestingly, in corneal epithelial cells, the CFTR activator genistein has been shown to attenuate induction of NFAT5 under hyperosmotic conditions; and CFTR inhibition led to increased NFAT5 expression under normal conditions [193]. It is conceivable that increased fluid absorption by CF-HBE cells would lead to a hyperosmotic airway surface liquid (ASL), however, conflicting results exist

regarding the ionic composition of the ASL, and discrepancies likely arise due to differences in experimental models [194]. Nevertheless, it is intriguing to consider the possibility that a hyperosmotic ASL may cause CF epithelial cells to become pro-inflammatory through inducing NFAT5.

Using Reactome® to perform pathway enrichment analysis among up-regulated genes in CF, we noted only five pathways that were significantly enriched (FDR<0.05). Four out of these five pathways were related to mitochondrial respiratory electron transport, and one was related to cholesterol uptake (**Fig. 21B**). Mitochondrial dysfunction has been reported in CF airway epithelial cells [195], and evidence also exists suggesting CF mitochondrial dysfunction is responsible for increased susceptibility to colonization of lungs by *Pseudomonas aeruginosa* [196]. Taken in the context of our data, it is conceivable that some genes related to respiratory electron transport may be up-regulated in response to mitochondrial defects.

Among genes down-regulated in CF, we did not detect any statistically significant pathways (FDR<.05) that were overrepresented, even though more genes were down-regulated in CF than up-regulated. Still, the top enriched pathways among down-regulated genes were pathways linked to mucin synthesis (*MUC5AC* and *B3GNT6*), as well as pathways linked to the genes *KRT16, KRT13,* and *TUBA1C* (**Fig. 21C**).

KRT13 and *KRT16* both encode type I cytokeratins, and were mapped to the pathway "formation of the cornified envelope." The cornified envelope is the outermost layer of epithelial tissues, and forms as a result of programmed cell death in terminally-differentiated epithelial cells [197]. Mostly studied in the context of skin biology, the

cornified envelope forms a protective physical barrier, which also contains proteins with antibacterial and antioxidant functions [198, 199]. Bacterial infection in CF is well-described to be an important contributing factor to morbidity and mortality. Oxidative stress is also thought to contribute to CF airway pathology [200, 201]. Whether the cornified envelope plays a similarly protective role in the lung remains to be determined, however it is plausible that a defect in this barrier contributes significantly to the pathogenesis of CF.

Mucins are another core component of the lung's physical barrier against infection, with MUC5AC and MUC5B representing the primary airway mucins [202]. Mucus obstruction is a core aspect of CF lung pathology, and evidence suggests that mucus accumulation (MUC5AC and MUC5B) precedes infection in CF children [203]. It may therefore seem counterintuitive that *MUC5AC* transcription would be decreased in CF airway cells, however it has been shown before that *MUC5AC* is decreased in nasal epithelial cells from CF donors compared to non-CF individuals [204]. We speculate that *MUC5AC* may be decreased to an extent in response to its accumulation. *B3GNT6* encodes the O-glycan synthesis enzyme Core 3 Synthase, and as such is important for mucin biosynthesis [205]. The finding that *B3GNT6* is decreased at the gene level in CF-HBE cells supports the hypothesis that there is a tendency to reduce mucus production in response to its accumulation.

TUBA1C encodes an alpha tubulin chain. Tubulins form microtubules, and acetylation of alpha tubulin is specific to ciliary structures [206]. While decreased *TUBA1C* does not necessarily imply a reduced number of ciliated cells, it may potentially

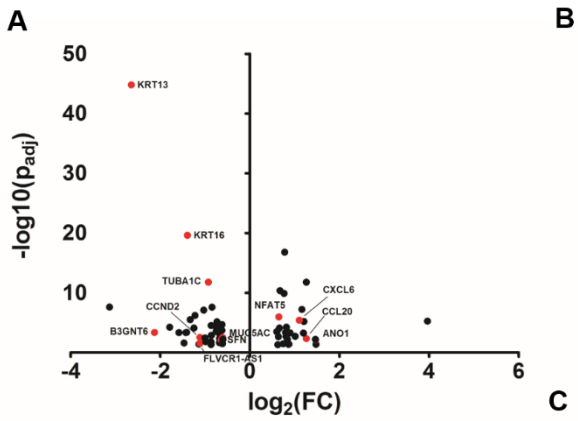
suggest it. Acetylation of alpha tubulin has been shown to regulate ciliary beating [207], and interestingly, we have shown previously that ciliary beat frequency is reduced in CF-HBE cells compared to NHBE (non-CF) cells [164]. The pathogenesis of primary ciliary dyskinesia, in which airway cilia do not beat properly, is surprisingly similar to CF [208].

A common theme among the above-mentioned down-regulated genes is that they are all markers of well-differentiated epithelial cells. *KRT13* and *KRT16* mark terminally-differentiated epithelia, *MUC5AC* marks mucus-producing goblet cells, and *TUBA1C*, if acetylated, marks ciliated cells. In agreement with these findings, there is a considerable body of evidence suggesting that CFTR plays a role in development and differentiation [209], and other genes we detect as differentially expressed in CF may support this role.

An interesting gene in this context is *CCND2*, encoding the cell cycle control protein Cyclin D2. Cyclin D2 promotes progression from the G_1 to the S phase of the cell cycle [210]. We also detect decreased expression of the long non-coding RNA (lncRNA) *FLVCR1-AS1* (Feline Leukemia Virus Subgroup C Cellular Receptor 1 – Antisense 1). This is interesting, because it has been shown that silencing of *FLVCR1-AS1* results in decreased *CCND2* mRNA [211], however the exact mechanism is not known. Antisense oligonucleotides can "sponge" microRNAs, thereby regulating their target genes [212], so it is possible that this is a natural regulatory mechanism that is aberrant in CF. Cyclin D2 repression has been shown to promote a switch from proliferation to differentiation [213]. There is also evidence that "basal-like" airway cells rapidly proliferate in CF airways [214]. These proliferating cells also highly express EGFR [214], in agreement

with the constitutive ERK activation in CF we discuss in **Chapter 2** (ERK is activated by EGFR and promotes proliferation).

Taken together with our finding that several markers of differentiated cell types are decreased in CF, this may seem contradictory to what would be expected to occur as a consequence of decreased *FLVCR1-AS1* and *CCND2*. However, this could be taken to infer that cell cycle arrest is attempted, but not successful. Cell cycle arrest often occurs in response to unrepaired DNA, and can occur at checkpoints between the G_1 and S, or G_2 and M phases. The *SFN* gene, encoding the protein 14-3-3σ, prevents G_2/M progression in response to DNA damage [215], and we detect decreased transcription of this gene in CF. In **Chapter 1**, we provide evidence that there is a DNA repair defect associated with the F508del mutation. Under normal conditions, it would be expected that *SFN* would be induced in response to DNA damage and promote cell cycle arrest. To attempt to reconcile this, we will examine the proteome of CF-HBE cells in comparison to NHBE cells.



B

Pathway Name	P-value	FDR
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.	1.76E-05	0.004487
Respiratory electron transport	1.12E-04	0.01191
The citric acid (TCA) cycle and respiratory electron transport	1.40E-04	0.01191
NR1H2 & NR1H3 regulate gene expression to limit cholesterol uptake	2.26E-04	0.013505
Complex I biogenesis	2.65E-04	0.013505
SCF-beta-TrCP mediated degradation of Emi1	0.006428	0.165529
Chemokine receptors bind chemokines	0.006886	0.165529
APC/C:Cdc20 mediated degradation of Securin	0.009656	0.165529
Cdc20:Phospho-APC/C mediated degradation of Cyclin A	0.011053	0.165529
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfation of the cell cycle checkpoint	0.011343	0.165529

C

Pathway Name	P-value	FDR
O-linked glycosylation	7.19E-04	0.065776
Formation of the cornified envelope	8.47E-04	0.065776
O-linked glycosylation of mucins	0.001481	0.065776
Translocation of SLC2A4 (GLUT4) to the plasma membrane	0.001854	0.065776
TP53 Regulates Transcription of Genes Involved in G2 Cell Cycle Arrest	0.001935	0.065776
Keratinization	0.005016	0.140459
Essential pentosuria	0.012156	0.255282
Defective LARGE causes MDDGA6 and MDDGB6	0.012156	0.255282
TP53 Regulates Transcription of Cell Cycle Genes	0.017025	0.266442
Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	0.021146	0.266442

Figure 21: Differentially expressed genes and overrepresented pathways in CF-HBE cells. (**A**) Volcano plot showing genes that were differentially expressed in CF-HBE cells compared to NHBE (non-CF) cells. Genes discussed in the text are highlighted in red. Tables show the top 10 enirched Reactome® pathways among the up-regulated (**B**) and down-regulated (**C**) gene fractions. FDR: False Discovery Rate

In CF, we detected a total of 71 differentially expressed proteins. Twenty proteins were up-regulated, while 51 were down-regulated **Fig. 22A**). Interestingly, many of the top pathways among the up-regulated protein fraction were linked to the proteasome subunits PSMB6 and PSMF1 and histone proteins, including G2/M checkpoints and ubiquitination processes (**Fig. 22B**). This is important to consider, because the *SFN* gene is induced by the tumor suppressor p53 [216]. P53 signals to promote DNA repair, cell cycle arrest, and apoptosis in response to various stress events, and is regulated by

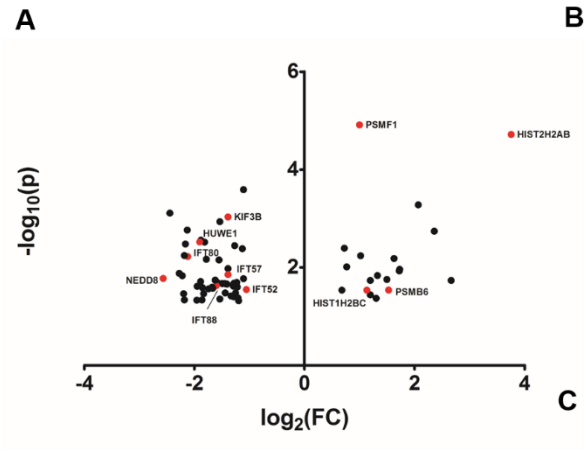
ubiquitination and proteasomal degradation [217]. Misfolded CFTR is poly-ubiquitinated and degraded by the proteasome [218], so it is not surprising that pathways related to these proteins are up-regulated in CF-HBE cells. In this case, it would be expected that p53-mediated signaling is defective in CF due to its degradation by the proteasome. Taken together, and in the context of data discussed in **Chapter 1** (DNA damage) and **Chapter 2** (ERK), it is likely that cell cycle arrest at the G_1/S and G_2/M checkpoints is attempted in CF, but unsuccessful due to degradation of p53. Unfortunately, our LC-MS/MS approach did not detect p53 in either NHBE cells or CF-HBE cells, possibly due to its localization in the nucleus, so future experimental verification will be necessary.

P53 deficiency in the airway leads to an abundance of rapidly proliferating basal-like cells with decreased ciliation [219], not unlike what is seen in CF [214]. Finally, it is worth mentioning that a p53 deficiency would be well in-line with the increased risk of several types of cancer seen in CF patients [220], however more research needs to be done in this regard.

Among down-regulated proteins in CF, only two pathways were significantly enriched (FDR<.05), despite more proteins being down-regulated than up-regulated (Fig. 15C). One of these pathways was “cilium assembly,” which being down-regulated, is in agreement with what is seen at the mRNA level (**Fig. 21A**). Proteins associated with this pathway were intraflagellar transport (IFT) proteins and the microtubule motor Kinesis Family Member 3B (KIF3B) (**Fig. 22A**).

An interesting protein in the down-regulated fraction that should be mentioned is the HECT, UBA, and WWE domain containing E3 ubiquitin protein ligase 1 (Huwe1)

(**Fig. 22A**). In **Chapter 1**, we showed that the DNA double-strand break marker γH2AX was increased in F508del-CFTR CFBE cells compared to WT-CFTR CFBE cells in response to either H_2O_2 or camptothecin. Huwe1 has been shown to mono-ubiquitinate H2AX, which is important to promote its downstream DNA repair signaling [221]. It would be expected that low Huwe1 expression results in γH2AX accumulation in response to DNA damage, which is in agreement with data presented in **Chapter 1**. Interestingly, Huwe1 also neddylates DNA-dependent protein kinase catalytic subunit (DNA-PKcs) with the NEDD8 ubiquitin-like modifier, which promotes efficient non-homologous end-joining, a type of DNA double strand break repair [222]. Indeed, our proteomics data also show decreased expression of NEDD8 in CF (**Fig. 22A**), possibly offering an explanation for a DNA repair deficiency in CF. Finally, Huwe1 also ubiquitinates EGFR to promote its degradation [223], so low Huwe1 expression could potentially be in agreement with data discussed in **Chapter 2**, where we identified increased EGFR activation in CF airway epithelial cells.



Pathway Name	P-value	FDR
Ub-specific processing proteases	4.03E-05	0.008105
RUNX1 regulates transcription of genes involved in differentiation of HSCs	4.80E-05	0.008105
Deubiquitination	1.94E-04	0.016859
G2/M Checkpoints	2.01E-04	0.016859
HDACs deacetylate histones	2.84E-04	0.01901
TCF dependent signaling in response to WNT	7.16E-04	0.021809
UCH proteinases	8.54E-04	0.021809
HATs acetylate histones	0.001189	0.021809
TP53 regulates transcription of several additional cell death genes whose specific roles in p53-dependent apoptosis remain uncertain	0.001324	0.021809
Signaling by NOTCH	0.00138	0.021809

Pathway Name	P-value	FDR
Intraflagellar transport	1.27E-07	2.37E-05
Cilium Assembly	2.07E-04	0.019291
Apoptotic cleavage of cell adhesion proteins	9.60E-04	0.059547
Organelle biogenesis and maintenance	0.002432	0.111871
Beta-oxidation of pristanoyl-CoA	0.00724	0.267872
Apoptotic cleavage of cellular proteins	0.010682	0.299314
Hedgehog 'off' state	0.014319	0.299314
Apoptotic execution phase	0.020695	0.299314
Signaling by Hedgehog	0.031428	0.299314
Kinesins	0.031652	0.299314

Figure 22: Differentially expressed proteins and enriched pathways in CF-HBE cells. (**A**) Volcano plot showing proteins that were differentially expressed in CF-HBEs compared to NHBE (non-CF) cells. Proteins discussed in the text are highlighted in red. Tables show Reactome® pathways enriched among the up-regulated (**B**) and down-regulated (**C**) protein fractions. FDR: False Discovery Rate.

Effects of the CFTR corrector VX-661

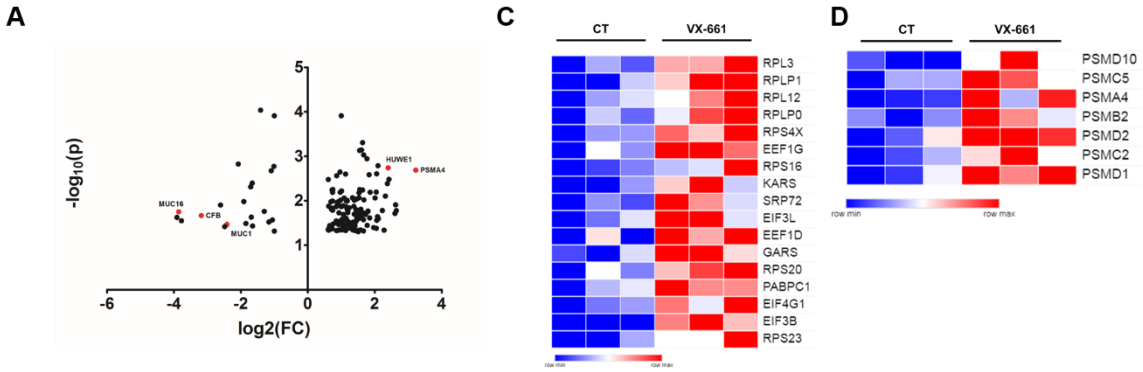
So far, the data have characterized CF-HBE cells as pro-inflammatory, proliferative, and deficient in differentiation capacity. Next, it is necessary to understand the effects of CFTR correction with the modulator VX-661.

Surprisingly, no genes were differentially regulated by VX-661. However, at the protein level, we found that VX-661 up-regulated 133 proteins and down-regulated 22 (**Fig. 21A and 23A**). This likely means that after 48h of treatment with VX-661, gene expression sufficiently returns to its pre-treatment state.

Pathway enrichment analysis of proteins up-regulated by VX-661 identified many pathways related to ribosomal subunits, eukaryotic initiation factors, and eukaryotic elongation factors; suggesting an increase in protein synthesis (**Fig. 23B and 23C**). This finding is in agreement with a transcriptomic study of CF leukocytes, which showed increased protein synthesis pathways in patients who responded to combination therapy [170]. This is also in agreement with the finding that overall protein expression is increased by VX-661. Synthesis of proteins and organelles such as ribosomes occurs during the G_1 and G_2 phases of the cell cycle, so this could indicate that the theorized attempts to halt the cell cycle at these stages mentioned above are successful upon correction of CFTR folding, however significant experimental verification would need to be done to confirm this. It is worth mentioning that a transcriptomics study of monocytes from CF patients with two copies of *CFTR* encoding gating mutants showed an increase in cell cycle control genes following administration of the CFTR potentiator VX-770 [169].

Many subunits of the proteasome were also found to be up-regulated by VX-661 (**Fig. 23D**). At first, this may seem counterintuitive since some proteasomal subunits were found to be increased in CF already. This could reflect a restoration of proteostasis upon correction of CFTR folding, since misfolded CFTR has been found to accumulate in aggresomes [224]. Increased protein degradation would also likely be an expected consequence of increased general protein synthesis. Interestingly, Huwe1, mentioned above, was increased by treatment with VX-661 (**Fig. 23A**).

Top-enriched pathways among proteins down-regulated by VX-661 were related to surfactant metabolism and innate immune signaling, including the complement system (**Fig. 23E**). Proteins mapped to these pathways included Complement Factor B (CFB), as well as the mucins Muc1 and Muc16 (**Fig. 23A**). The complement system is part of the innate immune system, and is responsible for opsonization of foreign particles and pathogens [225]. Interestingly, another proteomics study showed complement protein C3 to be one of the most abundant proteins in CF-HBE cells [168]. The complement protein C3a has been shown to positively correlate with FEV_1 in CF, while C5a was negatively correlated with FEV_1 [226]. We suspect that normalization of complement signaling would be a consequence of CFTR correction, and likely beneficial to CF patients, however this would have to be verified experimentally.



B

Pathway Name	P-value	FDR
Regulation of expression of SLITs and ROBOs	1.91E-13	1.12E-10
Signaling by ROBO receptors	1.55E-12	4.53E-10
L13a-mediated translational silencing of Ceruloplasmin expression	7.33E-11	1.43E-08
Cap-dependent Translation Initiation	2.05E-10	2.40E-08
Eukaryotic Translation Initiation	2.05E-10	2.40E-08
GTP hydrolysis and joining of the 60S ribosomal subunit	8.67E-10	8.41E-08
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	1.42E-09	1.16E-07
Eukaryotic Translation Elongation	1.59E-09	1.16E-07
Formation of a pool of free 40S subunits	2.43E-09	1.58E-07
Translation	5.68E-09	3.29E-07

E

Pathway Name	P-value	FDR
Surfactant metabolism	1.91E-13	1.12E-10
Defective CSF2RA causes SMDP4	1.55E-12	4.53E-10
Defective CSF2RB causes SMDP5	7.33E-11	1.43E-08
Diseases associated with surfactant metabolism	2.05E-10	2.40E-08
Diseases of metabolism	2.05E-10	2.40E-08
Activation of C3 and C5	8.67E-10	8.41E-08
Innate Immune System	1.42E-09	1.16E-07
Signal regulatory protein family interactions	1.59E-09	1.16E-07
Defective GALNT12 causes colorectal cancer 1 (CRCS1)	2.43E-09	1.58E-07
Defective GALNT3 causes familial hyperphosphatemic tumoral calcinosis (HFTC)	5.68E-09	3.29E-07

Figure 23: Proteins differentially regulated by VX-661. (**A**) Volcano plot showing proteins that were differentially regulated by VX-661 in CF-HBE cells

compared to vehicle-treated controls. (**B**) Table showing the top 10 Reactome® pathways enriched among the up-regulated protein fraction. (**C**) Heatmap indicating relative expression of proteins linked to the Reactome® pathway "Translation." (**D**) Heatmap showing relative expression of proteasome subunits. Each column in the heatmap corresponds to a separate F508del-homozygous donor. (**E**) Table showing the top 10 Reactome® pathways enriched among the down-regulated protein fraction.

Effects of VX-661 and Ectoine in Combination

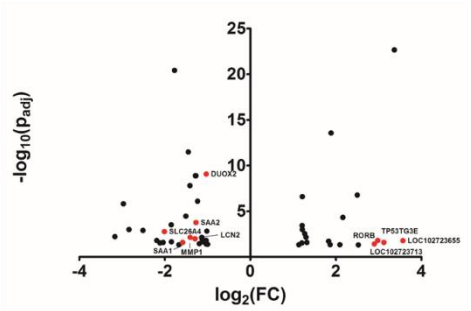
Ectoine is a natural osmolyte that we have shown in **Chapter 2** to improve CFTR stability and function in combination with the corrector VX-661. We also showed that ectoine in combination with VX-661 led to reduced expression of several genes and pathways related to inflammation from this dataset (**Fig. 24A and Chapter 2**). In addition to the 32 genes down-regulated by VX-661 and ectoine which have been described in **Chapter 2**, combination treatment led to up-regulation of 21 genes (**Fig. 21A and 24A**). Pathway enrichment analysis did not reveal any pathways that were significantly enriched among this gene fraction (not shown), however, several of these up-regulated genes are still worth mentioning. Three of the top five most up-regulated genes were target genes of p53, with as of yet undescribed functions (*LOC102723655*, *TP53TG3E*, and *LOC102723713*) (**Fig. 24A**). This would likely suggest increased activity of p53. *RORB*, encoding the Retinoic Acid-Related Orphan Receptor Beta, was also among the top five up-regulated genes (**Fig. 24A**). *RORB* is mostly expressed in nervous tissue, where it plays a role in promoting differentiation, among other functions [227]. This could potentially indicate an increase in the number of neuroendocrine cells (by due to promoting cellular differentiation) following treatment, although this would

require verification. Interestingly, ectoine on its own has been shown to have a pro-differentiation effect on periodontal ligament mesenchymal stem cells [228].

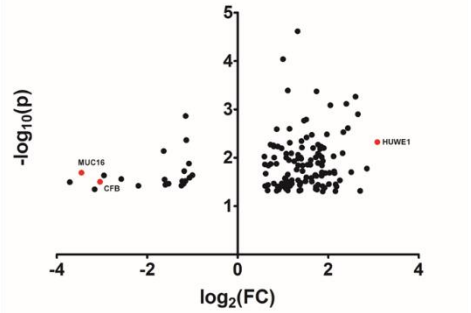
At the protein level, the effects of VX-661 and ectoine in combination were similar to those of VX-661 on its own. We detected 156 differentially expressed proteins following treatment with VX-661 and ectoine in combination. 124 proteins were up-regulated, while 32 were down-regulated (**Fig. 21A and 24B**). Pathway enrichment analysis showed results that were highly similar to what was seen with VX-661 on its own, with top enriched pathways among both protein fractions being nearly identical to what was seen with VX-661 on its own (**Fig. 24C and 24D**). Interestingly, Huwe1 was the most up-regulated protein by treatment with VX-661 and ectoine, although this was not significantly different from the extent to which it was up-regulated by VX-661 on its own (**Fig. 24B**).

It is not entirely clear why differences exist at the gene level when comparing the effects of the VX-661 – ectoine combination, yet its effects at the protein level are almost identical to those of VX-661 on its own. We can speculate that changes on gene expression induced by VX-661 probably normalize by 48 hours, preceding changes at the protein level which are still detected. Based on data presented in **Chapter 2**, ectoine likely enhances the effects of VX-661, so it is sensible that changes in the transcriptome are still seen, while effects on the proteome are similar.

A



B



C

Pathway Name	P-value	FDR
Regulation of expression of SLITs and ROBOs	1.11E-16	3.72E-14
Signaling by ROBO receptors	1.11E-16	3.72E-14
GTP hydrolysis and joining of the 60S ribosomal subunit	1.11E-15	1.79E-13
L13a-mediated translational silencing of Ceruloplasmin expression	1.11E-15	1.79E-13
Eukaryotic Translation Elongation	1.33E-15	1.79E-13
Translation	3.00E-15	3.33E-13
Cap-dependent Translation Initiation	4.55E-15	3.78E-13
Eukaryotic Translation Initiation	4.55E-15	3.78E-13
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	2.11E-14	1.56E-12
Nonsense-Mediated Decay (NMD)	3.15E-14	1.92E-12

D

Pathway Name	P-value	FDR
Surfactant metabolism	1.89E-07	3.03E-05
Defective CSF2RA causes SMDP4	1.13E-06	5.99E-05
Defective CSF2RB causes SMDP5	1.13E-06	5.99E-05
Diseases associated with surfactant metabolism	8.93E-06	3.57E-04
Diseases of metabolism	5.17E-05	0.001655
Activation of C3 and C5	1.41E-04	0.003665
Innate Immune System	2.33E-04	0.005118
Signal regulatory protein family interactions	9.16E-04	0.017383
Defective GALNT12 causes colorectal cancer 1 (CRCS1)	0.001128	0.017383
Defective GALNT3 causes familial hyperphosphatemic tumoral calcinosis (HFTC)	0.001128	0.017383

Figure 24: Effects of VX-661 and ectoine in combination. (**A**) Volcano plot showing genes that were differentially regulated following treatment of F508del-homozygous CF-HBE cells with VX-661 and ectoine together, compared to vehicle-treated controls. (**B**) Volcano plot showing proteins that were differentially regulated following treatment of F508del-homozygous CF-HBE cells with VX-661 and ectoine together. Genes and proteins discussed in the text are highlighted in red. Tables show the top 10 enriched Reactome® pathways among the up-regulated (**C**) and down-regulated (**D**) protein fractions.

Conclusion

To conclude, we present transcriptomic and proteomic analysis of primary CF-HBE cells that support literature reports of the CF respiratory epithelium as being pro-inflammatory, hyper-proliferative, and altered differentiation. Differentially expressed genes and proteins in CF point to a deficiency in p53 signaling due to disturbed proteostasis, that likely results in an inability to exit or arrest the cell cycle to promote differentiation or DNA repair, respectively. However, we must emphasize that this will

require further experimental verification. Correction of CFTR with VX-661 seems to promote proteostasis, which likely has several downstream benefits. Ectoine, when added with VX-661, likely enhances its effects, and also promotes differentiation and dampens inflammation, as discussed in **Chapter 2**.

Conclusions and Future Directions

In this dissertation, we discussed CFTR expression and stability in the context of inflammatory lung diseases, namely, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). In **Chapter 1**, we hypothesized that reduced CFTR expression would increase the susceptibility to emphysema. This is due to the significant phenotypic overlap between the diseases CF and COPD, as well as findings that cigarette smoke negatively regulates CFTR, as discussed earlier. We first showed that mice lacking CFTR develop emphysema in response to natural aging. We also showed that in response to cigarette smoke, CFTR-haploinsufficient mice develop emphysema, as do mice knocked out for CFTR. Taking our findings into consideration in the context of a large body of evidence in the literature which suggests DNA damage may be a driving factor we next hypothesized that CFTR deficiency may lead to DNA damage accumulation.

In **Section 2 of chapter 1**, we addressed this hypothesis using a model human CF bronchial epithelial cell line which overexpresses either wild-type or F508del-CFTR. Data presented in this section suggest that the F508del mutation leads to a DNA repair deficiency, however this will need to be further investigated in more detail in the future. Such a deficiency would however, have important consequences. First, it could potentially explain the data presented in **Section 1 of Chapter 1**. From a translational standpoint, this could potentially implicate therapies which involve restoring CFTR function as viable options to reduce the burden of emphysema. Additionally, many other diseases are associated with DNA damage. Cancer is an example that quickly comes to

mind. On one hand, CF patients are at a notably increased risk of cancer compared to the general population [220]. Less described is the disease burden associated with CF carriership. We showed in **Chapter 1** that CFTR-haploinsufficient mice were more susceptible to emphysema following exposure to cigarette smoke. In the human population recent studies have shown that CF carriers are in fact at an increased risk of many diseases, including several which affect the lung, as well as cancers of the gastro-intestinal tract [229]. Of course, it is unlikely that all of these could simply be explained by a DNA repair deficiency. However, we believe uncovering more mechanistically, a role for CFTR dysfunction in DNA repair deficiency would be highly worthwhile. On the other hand, it also must be considered that our findings may implicate CFTR inhibition as a strategy to sensitize cancer cells to therapeutics that work by damaging DNA, such as chemotherapeutics (for example, camptothecin) or radiation-based approaches.

In **Chapter 2**, attention was shifted to restoring CFTR stability in the context of CF. We identified a novel signaling axis that regulates CFTR stability (AREG-EGFR-ERK), and developed the natural osmolyte ectoine as a potential therapeutic to target this signaling axis. Indeed, we demonstrated that ectoine could improve CFTR stability and function, and also had anti-inflammatory effects that we suspect would be beneficial for CF patients. In the future, it will be necessary to determine how ectoine, or other therapeutic strategies that target the AREG-EGFR-ERK, axis will interact with other CFTR therapeutics. Namely, the CFTR potentiator VX-770, and the next generation corrector VX-445. Another future avenue to investigate in the regard would be the regulation of less common CFTR mutations. While potentiator and corrector drug

combinations are highly promising for a majority of CF patients (since F508del is the most common mutation), factors which regulate other CFTR mutants are less explored. Stability is known to be impaired in several different CFTR mutations (as shown in Fig. 1), and understanding factors which regulate stability of these mutants would be highly desirable. The anti-inflammatory effects of ectoine we see are likely not specific to F508del CFTR, or even CF for that matter. Ectoine could potentially represent a mutation-agnostic anti-inflammatory strategy for CF, and this should be investigated in the future. We also showed that a cell-permeable peptide that targets CAL-CFTR interaction can improve CFTR stability, and therefore function.

In **Chapter 3**, we aimed to better understand the basic biology of CF, as well as the effects of VX-661 and ectoine. To do this, we conducted both transcriptomic and proteomic studies of primary human bronchial epithelial cells. Much of these data are supportive of findings in **Chapter 1** and **Chapter 2**. Broadly, these data support CF bronchial epithelial cells as being pro-inflammatory and deficient in their ability to differentiate. Mechanistically, these data also point to an inability of CF cells to exit the cell cycle and stop proliferating, which is in agreement with a differentiation deficiency. This is also in agreement with findings in **Chapter 2** that show increased ERK activation in CF, since ERK is well-described to promote proliferation. More speculatively, some of the data generated suggest that p53 deficiency may be playing a role, however we must stress that this would require experimental validation, and would be an interesting future direction for CF research. This would also be supportive of data presented in both sections of **Chapter 1**, since p53 can signal to promote DNA repair, and p53 knockout

has also been shown to exacerbate elastase-induced emphysema in mice [230]. This could be an interesting avenue to explore in the future, as p53 in CF is largely unexplored.

These data also showed that CFTR correction had a mostly-normalizing effect on proteostasis, as evidenced by increased expression of many proteins involved in translations, as well as many subunits of the proteasome. Several anti-inflammatory pathways were shown to be down-regulated with CFTR correction by VX-661. With ectoine, several inflammatory genes were down-regulated, as shown in **Chapter 2**. This is also in agreement with ELISA data in that chapter. Ectoine, in combination with VX-661, also increased transcription of several gene targets of p53 which as of yet, do not have described functions. This is somewhat supportive of aberrant p53 activity in CF that can be rescued by correcting CFTR, however much more experimental validation is required to make such a claim. In addition, these genes may potentially play important roles in the biology of CF, and we believe characterizing their function in the future may be beneficial.

While the three chapters presented in this dissertation may have different focuses, they can be tied together, and suggest CFTR as an important regulator of lung homeostasis through regulating many core biological processes.

Bibliography

1. Andersen D: **Cystic fibrosis of the pancreas and its relation to celiac disease: a clinical and pathological study.** *Am J Dis Child* 1938, **56:**344-399.
2. Knowles MR, Stutts MJ, Spock A, Fischer N, Gatzy JT, Boucher RC: **Abnormal ion permeation through cystic fibrosis respiratory epithelium.** *Science* 1983, **221:**1067-1070.
3. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: **Identification of the cystic fibrosis gene: genetic analysis.** *Science* 1989, **245:**1073-1080.
4. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al.: **Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.** *Science* 1989, **245:**1066-1073.
5. Sosnay PR, Siklosi KR, Van Goor F, Kaniecki K, Yu H, Sharma N, Ramalho AS, Amaral MD, Dorfman R, Zielenski J, et al: **Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene.** *Nat Genet* 2013, **45:**1160-1167.
6. De Boeck K, Amaral MD: **Progress in therapies for cystic fibrosis.** *Lancet Respir Med* 2016, **4:**662-674.
7. Webster MJ, Tarran R: **Slippery When Wet: Airway Surface Liquid Homeostasis and Mucus Hydration.** *Curr Top Membr* 2018, **81:**293-335.
8. Boucher RC: **Airway surface dehydration in cystic fibrosis: pathogenesis and therapy.** *Annu Rev Med* 2007, **58:**157-170.
9. Randell SH, Boucher RC, Grp UNCVL: **Effective mucus clearance is essential for respiratory health.** *American Journal of Respiratory Cell and Molecular Biology* 2006, **35:**20-28.
10. Tang XX, Ostedgaard LS, Hoegger MJ, Moninger TO, Karp PH, McMenimen JD, Choudhury B, Varki A, Stoltz DA, Welsh MJ: **Acidic pH increases airway surface liquid viscosity in cystic fibrosis.** *J Clin Invest* 2016, **126:**879-891.
11. Gorrieri G, Scudieri P, Caci E, Schiavon M, Tomati V, Sirci F, Napolitano F, Carrella D, Gianotti A, Musante I, et al: **Goblet Cell Hyperplasia Requires High Bicarbonate Transport To Support Mucin Release.** *Sci Rep* 2016, **6:**36016.
12. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M, et al:

Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 2012, **487:**109-113.
13. Cantin AM, Hartl D, Konstan MW, Chmiel JF: **Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy.** *J Cyst Fibros* 2015, **14:**419-430.
14. Bhatt JM: **Treatment of pulmonary exacerbations in cystic fibrosis.** *Eur Respir Rev* 2013, **22:**205-216.
15. Csanady L, Vergani P, Gadsby DC: **Structure, Gating, and Regulation of the Cftr Anion Channel.** *Physiol Rev* 2019, **99:**707-738.
16. Zhang Z, Chen J: **Atomic Structure of the Cystic Fibrosis Transmembrane Conductance Regulator.** *Cell* 2016, **167:**1586-1597 e1589.
17. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: **Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis.** *Cell* 1990, **63:**827-834.
18. Van Goor F, Hadida S, Grootenhuis PD, Burton B, Stack JH, Straley KS, Decker CJ, Miller M, McCartney J, Olson ER, et al: **Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809.** *Proc Natl Acad Sci U S A* 2011, **108:**18843-18848.
19. Ren HY, Grove DE, De La Rosa O, Houck SA, Sopha P, Van Goor F, Hoffman BJ, Cyr DM: **VX-809 corrects folding defects in cystic fibrosis transmembrane conductance regulator protein through action on membrane-spanning domain 1.** *Mol Biol Cell* 2013, **24:**3016-3024.
20. Dalemans W, Barbry P, Champigny G, Jallat S, Dott K, Dreyer D, Crystal RG, Pavirani A, Lecocq JP, Lazdunski M: **Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation.** *Nature* 1991, **354:**526-528.
21. Van Goor F, Hadida S, Grootenhuis PD, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubran J, Hazlewood A, et al: **Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770.** *Proc Natl Acad Sci U S A* 2009, **106:**18825-18830.
22. Heda GD, Tanwani M, Marino CR: **The Delta F508 mutation shortens the biochemical half-life of plasma membrane CFTR in polarized epithelial cells.** *Am J Physiol Cell Physiol* 2001, **280:**C166-174.
23. Swiatecka-Urban A, Brown A, Moreau-Marquis S, Renuka J, Coutermarsh B, Barnaby R, Karlson KH, Flotte TR, Fukuda M, Langford GM, Stanton BA: **The short apical membrane half-life of rescued {Delta}F508-cystic fibrosis transmembrane conductance regulator (CFTR) results from accelerated endocytosis of {Delta}F508-CFTR in polarized human airway epithelial cells.** *J Biol Chem* 2005, **280:**36762-36772.
24. Veit G, Avramescu RG, Perdomo D, Phuan PW, Bagdany M, Apaja PM, Borot F, Szollosi D, Wu YS, Finkbeiner WE, et al: **Some gating potentiators, including VX-770, diminish DeltaF508-CFTR functional expression.** *Sci Transl Med* 2014, **6:**246ra297.

25. Rowe SM, Daines C, Ringshausen FC, Kerem E, Wilson J, Tullis E, Nair N, Simard C, Han L, Ingenito EP, et al: **Tezacaftor-Ivacaftor in Residual-Function Heterozygotes with Cystic Fibrosis.** *N Engl J Med* 2017, **377:**2024-2035.
26. Taylor-Cousar JL, Munck A, McKone EF, van der Ent CK, Moeller A, Simard C, Wang LT, Ingenito EP, McKee C, Lu Y, et al: **Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del.** *N Engl J Med* 2017, **377:**2013-2023.
27. Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, Ramsey BW, Rowe SM, Sass LA, Tullis E, et al: **VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles.** *N Engl J Med* 2018, **379:**1612-1620.
28. Heijerman HGM, McKone EF, Downey DG, Van Braeckel E, Rowe SM, Tullis E, Mall MA, Welter JJ, Ramsey BW, McKee CM, et al: **Efficacy and safety of the elexacaftor plus tezacaftor plus ivacaftor combination regimen in people with cystic fibrosis homozygous for the F508del mutation: a double-blind, randomised, phase 3 trial.** *Lancet* 2019, **394:**1940-1948.
29. Middleton PG, Mall MA, Drevinek P, Lands LC, McKone EF, Polineni D, Ramsey BW, Taylor-Cousar JL, Tullis E, Vermeulen F, et al: **Elexacaftor-Tezacaftor-Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele.** *N Engl J Med* 2019, **381:**1809-1819.
30. Xu X, Balsiger R, Tyrrell J, Boyaka PN, Tarran R, Cormet-Boyaka E: **Cigarette smoke exposure reveals a novel role for the MEK/ERK1/2 MAPK pathway in regulation of CFTR.** *Biochim Biophys Acta* 2015, **1850:**1224-1232.
31. Stolarczyk M, Scholte BJ: **The EGFR-ADAM17 Axis in Chronic Obstructive Pulmonary Disease and Cystic Fibrosis Lung Pathology.** *Mediators Inflamm* 2018, **2018:**1067134.
32. Rab A, Rowe SM, Raju SV, Bebok Z, Matalon S, Collawn JF: **Cigarette smoke and CFTR: implications in the pathogenesis of COPD.** *Am J Physiol Lung Cell Mol Physiol* 2013, **305:**L530-541.
33. Burney PG, Patel J, Newson R, Minelli C, Naghavi M: **Global and regional trends in COPD mortality, 1990-2010.** *Eur Respir J* 2015, **45:**1239-1247.
34. Vestbo J: **COPD: definition and phenotypes.** *Clin Chest Med* 2014, **35:**1-6.
35. Soler-Cataluna JJ, Cosio B, Izquierdo JL, Lopez-Campos JL, Marin JM, Aguero R, Baloira A, Carrizo S, Esteban C, Galdiz JB, et al: **Consensus document on the overlap phenotype COPD-asthma in COPD.** *Arch Bronconeumol* 2012, **48:**331-337.
36. Hurst JR, Vestbo J, Anzueto A, Locantore N, Mullerova H, Tal-Singer R, Miller B, Lomas DA, Agusti A, Macnee W, et al: **Susceptibility to exacerbation in chronic obstructive pulmonary disease.** *N Engl J Med* 2010, **363:**1128-1138.
37. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS, Committee GS: **Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary.** *Am J Respir Crit Care Med* 2001, **163:**1256-1276.

38. Kesimer M, Ford AA, Ceppe A, Radicioni G, Cao R, Davis CW, Doerschuk CM, Alexis NE, Anderson WH, Henderson AG, et al: **Airway Mucin Concentration as a Marker of Chronic Bronchitis.** *New England Journal of Medicine* 2017, **377:**911-922.
39. Chung KF: **The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease.** *Proc Am Thorac Soc* 2005, **2:**347-354; discussion 371-342.
40. Barnes PJ: **Small airway fibrosis in COPD.** *Int J Biochem Cell Biol* 2019, **116:**105598.
41. Sharafkhaneh A, Hanania NA, Kim V: **Pathogenesis of emphysema: from the bench to the bedside.** *Proc Am Thorac Soc* 2008, **5:**475-477.
42. Ostridge K, Wilkinson TM: **Present and future utility of computed tomography scanning in the assessment and management of COPD.** *Eur Respir J* 2016, **48:**216-228.
43. Brightling C, Greening N: **Airway inflammation in COPD: progress to precision medicine.** *Eur Respir J* 2019, **54**.
44. Bafadhel M, McKenna S, Terry S, Mistry V, Reid C, Haldar P, McCormick M, Haldar K, Kebadze T, Duvoix A, et al: **Acute exacerbations of chronic obstructive pulmonary disease: identification of biologic clusters and their biomarkers.** *Am J Respir Crit Care Med* 2011, **184:**662-671.
45. Hastie AT, Martinez FJ, Curtis JL, Doerschuk CM, Hansel NN, Christenson S, Putcha N, Ortega VE, Li X, Barr RG, et al: **Association of sputum and blood eosinophil concentrations with clinical measures of COPD severity: an analysis of the SPIROMICS cohort.** *Lancet Respir Med* 2017, **5:**956-967.
46. Suzuki M, Sze MA, Campbell JD, Brothers JF, 2nd, Lenburg ME, McDonough JE, Elliott WM, Cooper JD, Spira A, Hogg JC: **The cellular and molecular determinants of emphysematous destruction in COPD.** *Sci Rep* 2017, **7:**9562.
47. Barnes PJ: **Inflammatory endotypes in COPD.** *Allergy* 2019, **74:**1249-1256.
48. Kumar M, Seeger W, Voswinckel R: **Senescence-associated secretory phenotype and its possible role in chronic obstructive pulmonary disease.** *Am J Respir Cell Mol Biol* 2014, **51:**323-333.
49. Mercado N, Ito K, Barnes PJ: **Accelerated ageing of the lung in COPD: new concepts.** *Thorax* 2015, **70:**482-489.
50. Campisi J, d'Adda di Fagagna F: **Cellular senescence: when bad things happen to good cells.** *Nat Rev Mol Cell Biol* 2007, **8:**729-740.
51. You R, Lu W, Shan M, Berlin JM, Samuel EL, Marcano DC, Sun Z, Sikkema WK, Yuan X, Song L, et al: **Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema.** *Elife* 2015, **4:**e09623.
52. Birch J, Anderson RK, Correia-Melo C, Jurk D, Hewitt G, Marques FM, Green NJ, Moisey E, Birrell MA, Belvisi MG, et al: **DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease.** *Am J Physiol Lung Cell Mol Physiol* 2015, **309:**L1124-1137.

53. Sauler M, Lamontagne M, Finnemore E, Herazo-Maya JD, Tedrow J, Zhang X, Morneau JE, Sciurba F, Timens W, Pare PD, et al: **The DNA repair transcriptome in severe COPD.** *Eur Respir J* 2018, **52**.
54. Cazzola M, Page CP, Calzetta L, Matera MG: **Emerging anti-inflammatory strategies for COPD.** *Eur Respir J* 2012, **40:**724-741.
55. Schmid A, Baumlin N, Ivonnet P, Dennis JS, Campos M, Krick S, Salathe M: **Roflumilast partially reverses smoke-induced mucociliary dysfunction.** *Respir Res* 2015, **16:**135.
56. Lambert JA, Raju SV, Tang LP, McNicholas CM, Li Y, Courville CA, Farris RF, Coricor GE, Smoot LH, Mazur MM, et al: **Cystic fibrosis transmembrane conductance regulator activation by roflumilast contributes to therapeutic benefit in chronic bronchitis.** *Am J Respir Cell Mol Biol* 2014, **50:**549-558.
57. Bakke PS, Zhu G, Gulsvik A, Kong X, Agusti AG, Calverley PM, Donner CF, Levy RD, Make BJ, Pare PD, et al: **Candidate genes for COPD in two large data sets.** *Eur Respir J* 2011, **37:**255-263.
58. Stanley SE, Chen JJ, Podlevsky JD, Alder JK, Hansel NN, Mathias RA, Qi X, Rafaels NM, Wise RA, Silverman EK, et al: **Telomerase mutations in smokers with severe emphysema.** *J Clin Invest* 2015, **125:**563-570.
59. Stoltz DA, Meyerholz DK, Welsh MJ: **Origins of cystic fibrosis lung disease.** *N Engl J Med* 2015, **372:**1574-1575.
60. Plant BJ, Goss CH, Plant WD, Bell SC: **Management of comorbidities in older patients with cystic fibrosis.** *Lancet Respir Med* 2013, **1:**164-174.
61. Cavailles A, Brinchault-Rabin G, Dixmier A, Goupil F, Gut-Gobert C, Marchand-Adam S, Meurice JC, Morel H, Person-Tacnet C, Leroyer C, Diot P: **Comorbidities of COPD.** *Eur Respir Rev* 2013, **22:**454-475.
62. Mets OM, Roothaan SM, Bronsveld I, Luijk B, van de Graaf EA, Vink A, de Jong PA: **Emphysema Is Common in Lungs of Cystic Fibrosis Lung Transplantation Patients: A Histopathological and Computed Tomography Study.** *PLoS One* 2015, **10:**e0128062.
63. Fischer BM, Wong JK, Degan S, Kummarapurugu AB, Zheng S, Haridass P, Voynow JA: **Increased expression of senescence markers in cystic fibrosis airways.** *Am J Physiol Lung Cell Mol Physiol* 2013, **304:**L394-400.
64. Shapiro BL, Lam LF, Fast LH: **Premature senescence in cultured skin fibroblasts from subjects with cystic fibrosis.** *Science* 1979, **203:**1251-1253.
65. Hassan F, Xu X, Nuovo G, Killilea DW, Tyrrell J, Da Tan C, Tarran R, Diaz P, Jee J, Knoell D, et al: **Accumulation of metals in GOLD4 COPD lungs is associated with decreased CFTR levels.** *Respir Res* 2014, **15:**69.
66. Guilbault C, Saeed Z, Downey GP, Radzioch D: **Cystic fibrosis mouse models.** *Am J Respir Cell Mol Biol* 2007, **36:**1-7.
67. Grubb BR, Boucher RC: **Pathophysiology of gene-targeted mouse models for cystic fibrosis.** *Physiol Rev* 1999, **79:**S193-214.
68. Zhou L, Dey CR, Wert SE, DuVall MD, Frizzell RA, Whitsett JA: **Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human CFTR.** *Science* 1994, **266:**1705-1708.

69. Motz GT, Eppert BL, Sun G, Wesselkamper SC, Linke MJ, Deka R, Borchers MT: **Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema.** *J Immunol* 2008, **181:**8036-8043.
70. Motz GT, Eppert BL, Wesselkamper SC, Flury JL, Borchers MT: **Chronic cigarette smoke exposure generates pathogenic T cells capable of driving COPD-like disease in Rag2-/- mice.** *Am J Respir Crit Care Med* 2010, **181:**1223-1233.
71. Bodas M, Min T, Vij N: **Critical role of CFTR-dependent lipid rafts in cigarette smoke-induced lung epithelial injury.** *Am J Physiol Lung Cell Mol Physiol* 2011, **300:**L811-820.
72. Brown MB, Hunt WR, Noe JE, Rush NI, Schweitzer KS, Leece TC, Moldobaeva A, Wagner EM, Dudek SM, Poirier C, et al: **Loss of cystic fibrosis transmembrane conductance regulator impairs lung endothelial cell barrier function and increases susceptibility to microvascular damage from cigarette smoke.** *Pulm Circ* 2014, **4:**260-268.
73. Kawakami M, Paul JL, Thurlbeck WM: **The effect of age on lung structure in male BALB/cNNia inbred mice.** *Am J Anat* 1984, **170:**1-21.
74. Jacob RE, Carson JP, Gideon KM, Amidan BG, Smith CL, Lee KM: **Comparison of two quantitative methods of discerning airspace enlargement in smoke-exposed mice.** *PLoS One* 2009, **4:**e6670.
75. Fukuchi Y: **The aging lung and chronic obstructive pulmonary disease: similarity and difference.** *Proc Am Thorac Soc* 2009, **6:**570-572.
76. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, et al: **Mutation of the mouse klotho gene leads to a syndrome resembling ageing.** *Nature* 1997, **390:**45-51.
77. Ishii M, Yamaguchi Y, Yamamoto H, Hanaoka Y, Ouchi Y: **Airspace enlargement with airway cell apoptosis in klotho mice: a model of aging lung.** *J Gerontol A Biol Sci Med Sci* 2008, **63:**1289-1298.
78. Kuro-o M: **Klotho as a regulator of oxidative stress and senescence.** *Biol Chem* 2008, **389:**233-241.
79. Mori T, Ishigami A, Seyama K, Onai R, Kubo S, Shimizu K, Maruyama N, Fukuchi Y: **Senescence marker protein-30 knockout mouse as a novel murine model of senile lung.** *Pathol Int* 2004, **54:**167-173.
80. Sato T, Seyama K, Sato Y, Mori H, Souma S, Akiyoshi T, Kodama Y, Mori T, Goto S, Takahashi K, et al: **Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking.** *Am J Respir Crit Care Med* 2006, **174:**530-537.
81. Brennan SC, Wilkinson WJ, Tseng HE, Finney B, Monk B, Dibble H, Quilliam S, Warburton D, Galietta LJ, Kemp PJ, Riccardi D: **The extracellular calcium-sensing receptor regulates human fetal lung development via CFTR.** *Sci Rep* 2016, **6:**21975.
82. Bezzerri V, Piacenza F, Caporelli N, Malavolta M, Provinciali M, Cipolli M: **Is cellular senescence involved in cystic fibrosis?** *Respir Res* 2019, **20:**32.

83. Cohen-Cymberknoh M, Shoseyov D, Kerem E: **Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life.** *Am J Respir Crit Care Med* 2011, **183:**1463-1471.
84. Strom CM, Crossley B, Buller-Buerkle A, Jarvis M, Quan F, Peng M, Muralidharan K, Pratt V, Redman JB, Sun W: **Cystic fibrosis testing 8 years on: lessons learned from carrier screening and sequencing analysis.** *Genet Med* 2011, **13:**166-172.
85. Nakayama T, Kaneko M, Kodama M, Nagata C: **Cigarette smoke induces DNA single-strand breaks in human cells.** *Nature* 1985, **314:**462-464.
86. Liu X, Conner H, Kobayashi T, Kim H, Wen F, Abe S, Fang Q, Wang X, Hashimoto M, Bitterman P, Rennard SI: **Cigarette smoke extract induces DNA damage but not apoptosis in human bronchial epithelial cells.** *Am J Respir Cell Mol Biol* 2005, **33:**121-129.
87. DeMarini DM: **Genotoxicity of tobacco smoke and tobacco smoke condensate: a review.** *Mutat Res* 2004, **567:**447-474.
88. Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD: **ROS and the DNA damage response in cancer.** *Redox Biol* 2019, **25:**101084.
89. Ziady AG, Hansen J: **Redox balance in cystic fibrosis.** *Int J Biochem Cell Biol* 2014, **52:**113-123.
90. Gould NS, Min E, Martin RJ, Day BJ: **CFTR is the primary known apical glutathione transporter involved in cigarette smoke-induced adaptive responses in the lung.** *Free Radic Biol Med* 2012, **52:**1201-1206.
91. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ: **ATM phosphorylates histone H2AX in response to DNA double-strand breaks.** *J Biol Chem* 2001, **276:**42462-42467.
92. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM: **DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139.** *J Biol Chem* 1998, **273:**5858-5868.
93. Pommier Y: **Topoisomerase I inhibitors: camptothecins and beyond.** *Nat Rev Cancer* 2006, **6:**789-802.
94. Bentle MS, Reinicke KE, Bey EA, Spitz DR, Boothman DA: **Calcium-dependent modulation of poly(ADP-ribose) polymerase-1 alters cellular metabolism and DNA repair.** *J Biol Chem* 2006, **281:**33684-33696.
95. Martins JR, Kongsuphol P, Sammels E, Dahimene S, Aldehni F, Clarke LA, Schreiber R, de Smedt H, Amaral MD, Kunzelmann K: **F508del-CFTR increases intracellular Ca(2+) signaling that causes enhanced calcium-dependent Cl(-) conductance in cystic fibrosis.** *Biochim Biophys Acta* 2011, **1812:**1385-1392.
96. Vachel L, Norez C, Jayle C, Becq F, Vandebrouck C: **The low PLC-delta1 expression in cystic fibrosis bronchial epithelial cells induces upregulation of TRPV6 channel activity.** *Cell Calcium* 2015, **57:**38-48.
97. Boucher RC: **Cystic fibrosis: a disease of vulnerability to airway surface dehydration.** *Trends Mol Med* 2007, **13:**231-240.

98. Boucher R: **New concepts of the pathogenesis of cystic fibrosis lung disease.** *European Respiratory Journal* 2004, **23:**146-158.
99. Zielenski J: **Genotype and phenotype in cystic fibrosis.** *Respiration* 2000, **67:**117-133.
100. Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H, Lukacs GL: **Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes.** *J Cell Biol* 2004, **164:**923-933.
101. Amin R, Ratjen F: **Emerging drugs for cystic fibrosis.** *Expert Opin Emerg Drugs* 2014, **19:**143-155.
102. Xu X, Balsiger R, Tyrrell J, Boyaka PN, Tarran R, Cormet-Boyaka E: **Cigarette smoke exposure reveals a novel role for the MEK/ERK1/2 MAPK pathway in regulation of CFTR.** *Biochimica et Biophysica Acta (BBA)-General Subjects* 2015, **1850:**1224-1232.
103. Mossman BT, Lounsbury KM, Reddy SP: **Oxidants and signaling by mitogen-activated protein kinases in lung epithelium.** *Am J Respir Cell Mol Biol* 2006, **34:**666-669.
104. Singh B, Carpenter G, Coffey RJ: **EGF receptor ligands: recent advances.** *F1000Res* 2016, **5**.
105. Hull J, Vervaart P, Grimwood K, Phelan P: **Pulmonary oxidative stress response in young children with cystic fibrosis.** *Thorax* 1997, **52:**557-560.
106. Stolarczyk M, Veit G, Schnur A, Veltman M, Lukacs GL, Scholte BJ: **Extracellular oxidation in cystic fibrosis airway epithelium causes enhanced EGFR/ADAM17 activity.** *Am J Physiol Lung Cell Mol Physiol* 2018, **314:**L555-L568.
107. Burgel PR, Nadel JA: **Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium.** *Thorax* 2004, **59:**992-996.
108. Cohen S: **Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal.** *J Biol Chem* 1962, **237:**1555-1562.
109. Cohen S: **The stimulation of epidermal proliferation by a specific protein (EGF).** *Dev Biol* 1965, **12:**394-407.
110. Carpenter G, Cohen S: **Epidermal growth factor.** *Annu Rev Biochem* 1979, **48:**193-216.
111. Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA: **Epidermal growth factor system regulates mucin production in airways.** *Proc Natl Acad Sci U S A* 1999, **96:**3081-3086.
112. Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TP, Leahy DJ, Lemmon MA, Sliwkowski MX, Ward CW, Yokoyama S: **An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors.** *Mol Cell* 2003, **12:**541-552.
113. Lemmon MA, Schlessinger J: **Cell signaling by receptor tyrosine kinases.** *Cell* 2010, **141:**1117-1134.

114. Nakanaga T, Nadel JA, Ueki IF, Koff JL, Shao MX: **Regulation of interleukin-8 via an airway epithelial signaling cascade.** *Am J Physiol Lung Cell Mol Physiol* 2007, **292:**L1289-1296.
115. Harada C, Kawaguchi T, Ogata-Suetsugu S, Yamada M, Hamada N, Maeyama T, Souzaki R, Tajiri T, Taguchi T, Kuwano K, Nakanishi Y: **EGFR tyrosine kinase inhibition worsens acute lung injury in mice with repairing airway epithelium.** *Am J Respir Crit Care Med* 2011, **183:**743-751.
116. Yamaoka T, Arata S, Homma M, Homma T, Kusumoto S, Ando K, Manabe R, Kishino Y, Ohba M, Tsurutani J, et al: **Blockade of EGFR Activation Promotes TNF-Induced Lung Epithelial Cell Apoptosis and Pulmonary Injury.** *Int J Mol Sci* 2019, **20**.
117. Hajj R, Lesimple P, Nawrocki-Raby B, Birembaut P, Puchelle E, Coraux C: **Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis.** *J Pathol* 2007, **211:**340-350.
118. Schiller KR, Maniak PJ, O'Grady SM: **Cystic fibrosis transmembrane conductance regulator is involved in airway epithelial wound repair.** *Am J Physiol Cell Physiol* 2010, **299:**C912-921.
119. Pines G, Kostler WJ, Yarden Y: **Oncogenic mutant forms of EGFR: lessons in signal transduction and targets for cancer therapy.** *FEBS Lett* 2010, **584:**2699-2706.
120. Endres NF, Das R, Smith AW, Arkhipov A, Kovacs E, Huang Y, Pelton JG, Shan Y, Shaw DE, Wemmer DE, et al: **Conformational coupling across the plasma membrane in activation of the EGF receptor.** *Cell* 2013, **152:**543-556.
121. Filosto S, Becker CR, Goldkorn T: **Cigarette smoke induces aberrant EGF receptor activation that mediates lung cancer development and resistance to tyrosine kinase inhibitors.** *Mol Cancer Ther* 2012, **11:**795-804.
122. Peuschel H, Sydlik U, Grether-Beck S, Felsner I, Stockmann D, Jakob S, Kroker M, Haendeler J, Gotic M, Bieschke C, et al: **Carbon nanoparticles induce ceramide- and lipid raft-dependent signalling in lung epithelial cells: a target for a preventive strategy against environmentally-induced lung inflammation.** *Part Fibre Toxicol* 2012, **9:**48.
123. Arakawa T, Timasheff SN: **The stabilization of proteins by osmolytes.** *Biophys J* 1985, **47:**411-414.
124. Sydlik U, Peuschel H, Paunel-Gorgulu A, Keymel S, Kramer U, Weissenberg A, Kroker M, Seghrouchni S, Heiss C, Windolf J, et al: **Recovery of neutrophil apoptosis by ectoine: a new strategy against lung inflammation.** *Eur Respir J* 2013, **41:**433-442.
125. Unfried K, Kramer U, Sydlik U, Autengruber A, Bilstein A, Stolz S, Marini A, Schikowski T, Keymel S, Krutmann J: **Reduction of neutrophilic lung inflammation by inhalation of the compatible solute ectoine: a randomized trial with elderly individuals.** *Int J Chron Obstruct Pulmon Dis* 2016, **11:**2573-2583.

126. Dao VA, Overhagen S, Bilstein A, Kolot C, Sonnemann U, Mosges R: **Ectoine lozenges in the treatment of acute viral pharyngitis: a prospective, active-controlled clinical study.** *Eur Arch Otorhinolaryngol* 2019, **276:**775-783.
127. Tran BH, Dao VA, Bilstein A, Unfried K, Shah-Hosseini K, Mosges R: **Ectoine-Containing Inhalation Solution versus Saline Inhalation Solution in the Treatment of Acute Bronchitis and Acute Respiratory Infections: A Prospective, Controlled, Observational Study.** *Biomed Res Int* 2019, **2019:**7945091.
128. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, Boucher RC: **Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease.** *Cell* 1998, **95:**1005-1015.
129. Rayner RE, Makena P, Prasad GL, Cormet-Boyaka E: **Optimization of Normal Human Bronchial Epithelial (NHBE) Cell 3D Cultures for in vitro Lung Model Studies.** *Sci Rep* 2019, **9:**500.
130. Rayner RE, Wellmerling J, Osman W, Honesty S, Alfaro M, Peeples ME, Cormet-Boyaka E: **In vitro 3D culture lung model from expanded primary cystic fibrosis human airway cells.** *J Cyst Fibros* 2020.
131. Rennolds J, Butler S, Maloney K, Boyaka PN, Davis IC, Knoell DL, Parinandi NL, Cormet-Boyaka E: **Cadmium regulates the expression of the CFTR chloride channel in human airway epithelial cells.** *Toxicological sciences* 2010, **116:**349-358.
132. Rayner RE, Makena P, Prasad GL, Cormet-Boyaka E: **Cigarette and ENDS preparations differentially regulate ion channels and mucociliary clearance in primary normal human bronchial 3D cultures.** *Am J Physiol Lung Cell Mol Physiol* 2019, **317:**L295-L302.
133. Hassan F, Xu X, Nuovo G, Killilea DW, Tyrrell J, Da Tan C, Tarran R, Diaz P, Jee J, Knoell D: **Accumulation of metals in GOLD4 COPD lungs is associated with decreased CFTR levels.** *Respiratory research* 2014, **15:**69.
134. Love MI, Huber W, Anders S: **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.** *Genome Biol* 2014, **15:**550.
135. Fabregat A, Sidiropoulos K, Viteri G, Forner O, Marin-Garcia P, Arnau V, D'Eustachio P, Stein L, Hermjakob H: **Reactome pathway analysis: a high-performance in-memory approach.** *BMC Bioinformatics* 2017, **18:**142.
136. Zeliadt NA, Mauro LJ, Wattenberg EV: **Reciprocal regulation of extracellular signal regulated kinase 1/2 and mitogen activated protein kinase phosphatase-3.** *Toxicol Appl Pharmacol* 2008, **232:**408-417.
137. Izuhara K, Suzuki S, Ogawa M, Nunomura S, Nanri Y, Mitamura Y, Yoshihara T: **The Significance of Hypothiocyanite Production via the Pendrin/DUOX/Peroxidase Pathway in the Pathogenesis of Asthma.** *Oxid Med Cell Longev* 2017, **2017:**1054801.
138. Kondapaka SB, Fridman R, Reddy KB: **Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells.** *Int J Cancer* 1997, **70:**722-726.

139. Chmiel JF, Berger M, Konstan MW: **The role of inflammation in the pathophysiology of CF lung disease.** *Clin Rev Allergy Immunol* 2002, **23:**5-27.
140. Cormet-Boyaka E, Jolivette K, Bonnegarde-Bernard A, Rennolds J, Hassan F, Mehta P, Tridandapani S, Webster-Marketon J, Boyaka PN: **An NF-kappaB-independent and Erk1/2-dependent mechanism controls CXCL8/IL-8 responses of airway epithelial cells to cadmium.** *Toxicol Sci* 2012, **125:**418-429.
141. Trzcinska-Daneluti AM, Chen A, Nguyen L, Murchie R, Jiang C, Moffat J, Pelletier L, Rotin D: **RNA Interference Screen to Identify Kinases That Suppress Rescue of DeltaF508-CFTR.** *Mol Cell Proteomics* 2015, **14:**1569-1583.
142. Okiyoneda T, Veit G, Sakai R, Aki M, Fujihara T, Higashi M, Susuki-Miyata S, Miyata M, Fukuda N, Yoshida A, et al: **Chaperone-Independent Peripheral Quality Control of CFTR by RFFL E3 Ligase.** *Dev Cell* 2018, **44:**694-708 e697.
143. Gan X, Wang C, Patel M, Kreutz B, Zhou M, Kozasa T, Wu D: **Different Raf protein kinases mediate different signaling pathways to stimulate E3 ligase RFFL gene expression in cell migration regulation.** *J Biol Chem* 2013, **288:**33978-33984.
144. Trzcinska-Daneluti AM, Nguyen L, Jiang C, Fladd C, Uehling D, Prakesch M, Al-awar R, Rotin D: **Use of kinase inhibitors to correct DeltaF508-CFTR function.** *Mol Cell Proteomics* 2012, **11:**745-757.
145. Roychoudhury A, Bieker A, Haussinger D, Oesterhelt F: **Membrane protein stability depends on the concentration of compatible solutes--a single molecule force spectroscopic study.** *Biol Chem* 2013, **394:**1465-1474.
146. Bell HL, Gooz M: **ADAM-17 is activated by the mitogenic protein kinase ERK in a model of kidney fibrosis.** *Am J Med Sci* 2010, **339:**105-107.
147. Massip-Copiz M, Clauzure M, Valdivieso AG, Santa-Coloma TA: **Epiregulin (EREG) is upregulated through an IL-1beta autocrine loop in Caco-2 epithelial cells with reduced CFTR function.** *J Cell Biochem* 2018, **119:**2911-2922.
148. Kamei S, Maruta K, Fujikawa H, Nohara H, Ueno-Shuto K, Tasaki Y, Nakashima R, Kawakami T, Eto Y, Suico MA, et al: **Integrative expression analysis identifies a novel interplay between CFTR and linc-SUMF1-2 that involves CF-associated gene dysregulation.** *Biochem Biophys Res Commun* 2019, **509:**521-528.
149. Cholon DM, Quinney NL, Fulcher ML, Esther CR, Jr., Das J, Dokholyan NV, Randell SH, Boucher RC, Gentzsch M: **Potentiator ivacaftor abrogates pharmacological correction of DeltaF508 CFTR in cystic fibrosis.** *Sci Transl Med* 2014, **6:**246ra296.
150. Meng X, Wang Y, Wang X, Wrennall JA, Rimington TL, Li H, Cai Z, Ford RC, Sheppard DN: **Two Small Molecules Restore Stability to a Subpopulation of the Cystic Fibrosis Transmembrane Conductance Regulator with the Predominant Disease-causing Mutation.** *J Biol Chem* 2017, **292:**3706-3719.

151. Edmondson C, Davies JC: **Current and future treatment options for cystic fibrosis lung disease: latest evidence and clinical implications.** *Ther Adv Chronic Dis* 2016, **7:**170-183.
152. Bagdany M, Veit G, Fukuda R, Avramescu RG, Okiyoneda T, Baaklini I, Singh J, Sovak G, Xu H, Apaja PM, et al: **Chaperones rescue the energetic landscape of mutant CFTR at single molecule and in cell.** *Nat Commun* 2017, **8:**398.
153. Fukuda R, Okiyoneda T: **Peripheral Protein Quality Control as a Novel Drug Target for CFTR Stabilizer.** *Front Pharmacol* 2018, **9:**1100.
154. Farinha CM, Canato S: **From the endoplasmic reticulum to the plasma membrane: mechanisms of CFTR folding and trafficking.** *Cell Mol Life Sci* 2017, **74:**39-55.
155. Harris BZ, Lim WA: **Mechanism and role of PDZ domains in signaling complex assembly.** *J Cell Sci* 2001, **114:**3219-3231.
156. Wang S, Raab RW, Schatz PJ, Guggino WB, Li M: **Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR).** *FEBS Lett* 1998, **427:**103-108.
157. Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL: **An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton.** *J Biol Chem* 1998, **273:**19797-19801.
158. Cushing PR, Fellows A, Villone D, Boisguerin P, Madden DR: **The relative binding affinities of PDZ partners for CFTR: a biochemical basis for efficient endocytic recycling.** *Biochemistry* 2008, **47:**10084-10098.
159. Cheng J, Wang H, Guggino WB: **Modulation of mature cystic fibrosis transmembrane regulator protein by the PDZ domain protein CAL.** *J Biol Chem* 2004, **279:**1892-1898.
160. Cushing PR, Vouilleme L, Pellegrini M, Boisguerin P, Madden DR: **A stabilizing influence: CAL PDZ inhibition extends the half-life of DeltaF508-CFTR.** *Angew Chem Int Ed Engl* 2010, **49:**9907-9911.
161. Vouilleme L, Cushing PR, Volkmer R, Madden DR, Boisguerin P: **Engineering peptide inhibitors to overcome PDZ binding promiscuity.** *Angew Chem Int Ed Engl* 2010, **49:**9912-9916.
162. Zhao Y, Cushing PR, Smithson DC, Pellegrini M, Pletnev AA, Al-Ayyoubi S, Grassetti AV, Gerber SA, Guy RK, Madden DR: **Cysteine modifiers suggest an allosteric inhibitory site on the CAL PDZ domain.** *Biosci Rep* 2018, **38**.
163. Qian Z, Xu X, Amacher JF, Madden DR, Cormet-Boyaka E, Pei D: **Intracellular Delivery of Peptidyl Ligands by Reversible Cyclization: Discovery of a PDZ Domain Inhibitor that Rescues CFTR Activity.** *Angew Chem Int Ed Engl* 2015, **54:**5874-5878.
164. Rayner RE, Wellmerling J, Osman W, Honesty S, Alfaro M, Peeples ME, Cormet-Boyaka E: **In vitro 3D culture lung model from expanded primary cystic fibrosis human airway cells.** *J Cyst Fibros* 2020, **19:**752-761.

165. Ideozu JE, Zhang X, McColley S, Levy H: **Transcriptome Profiling and Molecular Therapeutic Advances in Cystic Fibrosis: Recent Insights.** *Genes (Basel)* 2019, **10**.
166. Rollet-Cohen V, Bourderioux M, Lipecka J, Chhuon C, Jung VA, Mesbahi M, Nguyen-Khoa T, Guérin-Pfyffer S, Schmitt A, Edelman A, et al: **Comparative proteomics of respiratory exosomes in cystic fibrosis, primary ciliary dyskinesia and asthma.** *J Proteomics* 2018, **185:**1-7.
167. Gharib SA, Vaisar T, Aitken ML, Park DR, Heinecke JW, Fu X: **Mapping the lung proteome in cystic fibrosis.** *J Proteome Res* 2009, **8:**3020-3028.
168. Peters-Hall JR, Brown KJ, Pillai DK, Tomney A, Garvin LM, Wu X, Rose MC: **Quantitative proteomics reveals an altered cystic fibrosis in vitro bronchial epithelial secretome.** *Am J Respir Cell Mol Biol* 2015, **53:**22-32.
169. Hisert KB, Birkland TP, Schoenfelt KQ, Long ME, Grogan B, Carter S, Liles WC, McKone EF, Becker L, Manicone AM, Gharib SA: **CFTR Modulator Therapy Enhances Peripheral Blood Monocyte Contributions to Immune Responses in People With Cystic Fibrosis.** *Front Pharmacol* 2020, **11:**1219.
170. Kopp BT, Fitch J, Jaramillo L, Shrestha CL, Robledo-Avila F, Zhang S, Palacios S, Woodley F, Hayes D, Jr., Partida-Sanchez S, et al: **Whole-blood transcriptomic responses to lumacaftor/ivacaftor therapy in cystic fibrosis.** *J Cyst Fibros* 2020, **19:**245-254.
171. Minadakis G, Sokratous K, Spyrou GM: **ProtExA: A tool for post-processing proteomics data providing differential expression metrics, co-expression networks and functional analytics.** *Comput Struct Biotechnol J* 2020, **18:**1695-1703.
172. Qin Y, Jiang Y, Sheikh AS, Shen S, Liu J, Jiang D: **Interleukin-13 stimulates MUC5AC expression via a STAT6-TMEM16A-ERK1/2 pathway in human airway epithelial cells.** *Int Immunopharmacol* 2016, **40:**106-114.
173. Veit G, Bossard F, Goepp J, Verkman AS, Galietta LJ, Hanrahan JW, Lukacs GL: **Proinflammatory cytokine secretion is suppressed by TMEM16A or CFTR channel activity in human cystic fibrosis bronchial epithelia.** *Mol Biol Cell* 2012, **23:**4188-4202.
174. Danahay HL, Lilley S, Fox R, Charlton H, Sabater J, Button B, McCarthy C, Collingwood SP, Gosling M: **TMEM16A Potentiation: A Novel Therapeutic Approach for the Treatment of Cystic Fibrosis.** *Am J Respir Crit Care Med* 2020, **201:**946-954.
175. Kunzelmann K, Ousingsawat J, Cabrita I, Dousova T, Bahr A, Janda M, Schreiber R, Benedetto R: **TMEM16A in Cystic Fibrosis: Activating or Inhibiting?** *Front Pharmacol* 2019, **10:**3.
176. Kondo M, Tsuji M, Hara K, Arimura K, Yagi O, Tagaya E, Takeyama K, Tamaoki J: **Chloride ion transport and overexpression of TMEM16A in a guinea-pig asthma model.** *Clin Exp Allergy* 2017, **47:**795-804.
177. Walker NM, Simpson JE, Levitt RC, Boyle KT, Clarke LL: **Talniflumate increases survival in a cystic fibrosis mouse model of distal intestinal obstructive syndrome.** *J Pharmacol Exp Ther* 2006, **317:**275-283.

178. Britschgi A, Bill A, Brinkhaus H, Rothwell C, Clay I, Duss S, Rebhan M, Raman P, Guy CT, Wetzel K, et al: **Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling.** *Proc Natl Acad Sci U S A* 2013, **110:**E1026-1034.
179. Duvvuri U, Shiwarski DJ, Xiao D, Bertrand C, Huang X, Edinger RS, Rock JR, Harfe BD, Henson BJ, Kunzelmann K, et al: **TMEM16A induces MAPK and contributes directly to tumorigenesis and cancer progression.** *Cancer Res* 2012, **72:**3270-3281.
180. Ruffin M, Voland M, Marie S, Bonora M, Blanchard E, Blouquit-Laye S, Naline E, Puyo P, Le Rouzic P, Guillot L, et al: **Anoctamin 1 dysregulation alters bronchial epithelial repair in cystic fibrosis.** *Biochim Biophys Acta* 2013, **1832:**2340-2351.
181. Lerias J, Pinto M, Benedetto R, Schreiber R, Amaral M, Aureli M, Kunzelmann K: **Compartmentalized crosstalk of CFTR and TMEM16A (ANO1) through EPAC1 and ADCY1.** *Cell Signal* 2018, **44:**10-19.
182. Benedetto R, Ousingsawat J, Wanitchakool P, Zhang Y, Holtzman MJ, Amaral M, Rock JR, Schreiber R, Kunzelmann K: **Epithelial Chloride Transport by CFTR Requires TMEM16A.** *Sci Rep* 2017, **7:**12397.
183. Yu M, Hu J, Zhu MX, Zhao T, Liang W, Wen S, Li HH, Long Q, Wang M, Guo HP, et al: **Cardiac fibroblasts recruit Th17 cells infiltration into myocardium by secreting CCL20 in CVB3-induced acute viral myocarditis.** *Cell Physiol Biochem* 2013, **32:**1437-1450.
184. Koga T, Otomo K, Mizui M, Yoshida N, Umeda M, Ichinose K, Kawakami A, Tsokos GC: **Calcium/Calmodulin-Dependent Kinase IV Facilitates the Recruitment of Interleukin-17-Producing Cells to Target Organs Through the CCR6/CCL20 Axis in Th17 Cell-Driven Inflammatory Diseases.** *Arthritis Rheumatol* 2016, **68:**1981-1988.
185. Kaneko S, Kondo Y, Yokosawa M, Furuyama K, Segawa S, Tsuboi H, Kanamori A, Matsumoto I, Yamazaki M, Sumida T: **The RORgammat-CCR6-CCL20 axis augments Th17 cells invasion into the synovia of rheumatoid arthritis patients.** *Mod Rheumatol* 2018, **28:**814-825.
186. Weaver CT, Elson CO, Fouser LA, Kolls JK: **The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin.** *Annu Rev Pathol* 2013, **8:**477-512.
187. Tan HL, Regamey N, Brown S, Bush A, Lloyd CM, Davies JC: **The Th17 pathway in cystic fibrosis lung disease.** *Am J Respir Crit Care Med* 2011, **184:**252-258.
188. Mulcahy EM, Hudson JB, Beggs SA, Reid DW, Roddam LF, Cooley MA: **High peripheral blood th17 percent associated with poor lung function in cystic fibrosis.** *PLoS One* 2015, **10:**e0120912.
189. Kushwah R, Gagnon S, Sweezey NB: **Intrinsic predisposition of naive cystic fibrosis T cells to differentiate towards a Th17 phenotype.** *Respir Res* 2013, **14:**138.

190. Jovic S, Linge HM, Shikhagaie MM, Olin AI, Lannefors L, Erjefalt JS, Morgelin M, Egesten A: **The neutrophil-recruiting chemokine GCP-2/CXCL6 is expressed in cystic fibrosis airways and retains its functional properties after binding to extracellular DNA.** *Mucosal Immunol* 2016, **9:**112-123.
191. Lee N, Kim D, Kim WU: **Role of NFAT5 in the Immune System and Pathogenesis of Autoimmune Diseases.** *Front Immunol* 2019, **10:**270.
192. Neuringer IP, Sloan J, Budd S, Chalermskulrat W, Park RC, Stonebraker JR, O'Neal WK, Aris RM, Randell SH: **Calcineurin inhibitor effects on growth and phenotype of human airway epithelial cells in vitro.** *Am J Transplant* 2005, **5:**2660-2670.
193. Panigrahi T, D'Souza S, Shetty R, Padmanabhan Nair A, Ghosh A, Jacob Remington Nelson E, Ghosh A, Sethu S: **Genistein-Calcitriol Mitigates Hyperosmotic Stress-Induced TonEBP, CFTR Dysfunction, VDR Degradation and Inflammation in Dry Eye Disease.** *Clin Transl Sci* 2020.
194. Zabner J, Seiler MP, Launspach JL, Karp PH, Kearney WR, Look DC, Smith JJ, Welsh MJ: **The osmolyte xylitol reduces the salt concentration of airway surface liquid and may enhance bacterial killing.** *Proc Natl Acad Sci U S A* 2000, **97:**11614-11619.
195. Antigny F, Girardin N, Raveau D, Frieden M, Becq F, Vandebrouck C: **Dysfunction of mitochondria Ca2+ uptake in cystic fibrosis airway epithelial cells.** *Mitochondrion* 2009, **9:**232-241.
196. Riquelme SA, Lozano C, Moustafa AM, Liimatta K, Tomlinson KL, Britto C, Khanal S, Gill SK, Narechania A, Azcona-Gutierrez JM, et al: **CFTR-PTEN-dependent mitochondrial metabolic dysfunction promotes Pseudomonas aeruginosa airway infection.** *Sci Transl Med* 2019, **11**.
197. Candi E, Schmidt R, Melino G: **The cornified envelope: a model of cell death in the skin.** *Nat Rev Mol Cell Biol* 2005, **6:**328-340.
198. Vermeij WP, Alia A, Backendorf C: **ROS quenching potential of the epidermal cornified cell envelope.** *J Invest Dermatol* 2011, **131:**1435-1441.
199. Niehues H, Tsoi LC, van der Krieken DA, Jansen PAM, Oortveld MAW, Rodijk-Olthuis D, van Vlijmen I, Hendriks W, Helder RW, Bouwstra JA, et al: **Psoriasis-Associated Late Cornified Envelope (LCE) Proteins Have Antibacterial Activity.** *J Invest Dermatol* 2017, **137:**2380-2388.
200. Scholte BJ, Horati H, Veltman M, Vreeken RJ, Garratt LW, Tiddens H, Janssens HM, Stick SM, Australian Respiratory Early Surveillance Team for Cystic F: **Oxidative stress and abnormal bioactive lipids in early cystic fibrosis lung disease.** *J Cyst Fibros* 2019, **18:**781-789.
201. Dickerhof N, Pearson JF, Hoskin TS, Berry LJ, Turner R, Sly PD, Kettle AJ, Arest CF: **Oxidative stress in early cystic fibrosis lung disease is exacerbated by airway glutathione deficiency.** *Free Radic Biol Med* 2017, **113:**236-243.
202. Ridley C, Thornton DJ: **Mucins: the frontline defence of the lung.** *Biochem Soc Trans* 2018, **46:**1099-1106.
203. Esther CR, Jr., Muhlebach MS, Ehre C, Hill DB, Wolfgang MC, Kesimer M, Ramsey KA, Markovetz MR, Garbarine IC, Forest MG, et al: **Mucus**

accumulation in the lungs precedes structural changes and infection in children with cystic fibrosis. *Sci Transl Med* 2019, **11**.
204. Voynow JA, Selby DM, Rose MC: **Mucin gene expression (MUC1, MUC2, and MUC5/5AC) in nasal epithelial cells of cystic fibrosis, allergic rhinitis, and normal individuals.** *Lung* 1998, **176:**345-354.
205. Iwai T, Inaba N, Naundorf A, Zhang Y, Gotoh M, Iwasaki H, Kudo T, Togayachi A, Ishizuka Y, Nakanishi H, Narimatsu H: **Molecular cloning and characterization of a novel UDP-GlcNAc:GalNAc-peptide beta1,3-N-acetylglucosaminyltransferase (beta 3Gn-T6), an enzyme synthesizing the core 3 structure of O-glycans.** *J Biol Chem* 2002, **277:**12802-12809.
206. Wloga D, Joachimiak E, Louka P, Gaertig J: **Posttranslational Modifications of Tubulin and Cilia.** *Cold Spring Harb Perspect Biol* 2017, **9**.
207. Alper JD, Decker F, Agana B, Howard J: **The motility of axonemal dynein is regulated by the tubulin code.** *Biophys J* 2014, **107:**2872-2880.
208. Cockx M, Gouwy M, Van Damme J, Struyf S: **Chemoattractants and cytokines in primary ciliary dyskinesia and cystic fibrosis: key players in chronic respiratory diseases.** *Cell Mol Immunol* 2018, **15:**312-323.
209. Amaral MD, Quaresma MC, Pankonien I: **What Role Does CFTR Play in Development, Differentiation, Regeneration and Cancer?** *Int J Mol Sci* 2020, **21**.
210. Lukas J, Bartkova J, Welcker M, Petersen OW, Peters G, Strauss M, Bartek J: **Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types.** *Oncogene* 1995, **10:**2125-2134.
211. Lin H, Shangguan Z, Zhu M, Bao L, Zhang Q, Pan S: **lncRNA FLVCR1-AS1 silencing inhibits lung cancer cell proliferation, migration, and invasion by inhibiting the activity of the Wnt/beta-catenin signaling pathway.** *J Cell Biochem* 2019, **120:**10625-10632.
212. Quemener AM, Bachelot L, Forestier A, Donnou-Fournet E, Gilot D, Galibert MD: **The powerful world of antisense oligonucleotides: From bench to bedside.** *Wiley Interdiscip Rev RNA* 2020, **11:**e1594.
213. Gay MS, Dasgupta C, Li Y, Kanna A, Zhang L: **Dexamethasone Induces Cardiomyocyte Terminal Differentiation via Epigenetic Repression of Cyclin D2 Gene.** *J Pharmacol Exp Ther* 2016, **358:**190-198.
214. Voynow JA, Fischer BM, Roberts BC, Proia AD: **Basal-like cells constitute the proliferating cell population in cystic fibrosis airways.** *Am J Respir Crit Care Med* 2005, **172:**1013-1018.
215. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B: **14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage.** *Nature* 1999, **401:**616-620.
216. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B: **14-3-3sigma is a p53-regulated inhibitor of G2/M progression.** *Mol Cell* 1997, **1:**3-11.

217. Kwon SK, Saindane M, Baek KH: **p53 stability is regulated by diverse deubiquitinating enzymes.** *Biochim Biophys Acta Rev Cancer* 2017, **1868:**404-411.
218. Turnbull EL, Rosser MF, Cyr DM: **The role of the UPS in cystic fibrosis.** *BMC Biochem* 2007, **8 Suppl 1:**S11.
219. McConnell AM, Yao C, Yeckes AR, Wang Y, Selvaggio AS, Tang J, Kirsch DG, Stripp BR: **p53 Regulates Progenitor Cell Quiescence and Differentiation in the Airway.** *Cell Rep* 2016, **17:**2173-2182.
220. Zhang J, Wang Y, Jiang X, Chan HC: **Cystic fibrosis transmembrane conductance regulator-emerging regulator of cancer.** *Cell Mol Life Sci* 2018, **75:**1737-1756.
221. Choe KN, Nicolae CM, Constantin D, Imamura Kawasawa Y, Delgado-Diaz MR, De S, Freire R, Smits VA, Moldovan GL: **HUWE1 interacts with PCNA to alleviate replication stress.** *EMBO Rep* 2016, **17:**874-886.
222. Guo Z, Wang S, Xie Y, Han Y, Hu S, Guan H, Xie D, Bai C, Liu X, Gu Y, et al: **HUWE1-dependent DNA-PKcs neddylation modulates its autophosphorylation in DNA damage response.** *Cell Death Dis* 2020, **11:**400.
223. Zhu Q, Dong H, Bukhari AA, Zhao A, Li M, Sun Y, Zhang X, Cao C, Su D, Liang X: **HUWE1 promotes EGFR ubiquitination and degradation to protect against renal tubulointerstitial fibrosis.** *FASEB J* 2020, **34:**4591-4601.
224. Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M, et al: **Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition.** *Nat Cell Biol* 2010, **12:**863-875.
225. Sarma JV, Ward PA: **The complement system.** *Cell Tissue Res* 2011, **343:**227-235.
226. Hair PS, Sass LA, Vazifedan T, Shah TA, Krishna NK, Cunnion KM: **Complement effectors, C5a and C3a, in cystic fibrosis lung fluid correlate with disease severity.** *PLoS One* 2017, **12:**e0173257.
227. Jetten AM, Joo JH: **Retinoid-related Orphan Receptors (RORs): Roles in Cellular Differentiation and Development.** *Adv Dev Biol* 2006, **16:**313-355.
228. Tuncer Budanur D, Zibandeh N, Genc D, Gokalp M, Kasali K, Akkoc T, Sepet E: **Effect of cDMEM media containing Ectoine on human periodontal ligament mesenchymal stem cell survival and differentiation.** *Dent Traumatol* 2018, **34:**188-200.
229. Miller AC, Comellas AP, Hornick DB, Stoltz DA, Cavanaugh JE, Gerke AK, Welsh MJ, Zabner J, Polgreen PM: **Cystic fibrosis carriers are at increased risk for a wide range of cystic fibrosis-related conditions.** *Proc Natl Acad Sci U S A* 2020, **117:**1621-1627.
230. Chrusciel S, Zysman M, Caramelle P, Tiendrebeogo A, Baskara I, Le Gouvello S, Chabot F, Giraudier S, Boczkowski J, Boyer L: **Lack of Transcription Factor p53 Exacerbates Elastase-Induced Emphysema in Mice.** *Am J Respir Cell Mol Biol* 2016, **54:**188-199.