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Pharmacological rescue of nonsense mutations in Cystic Fibrosis

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ABSTRACT

Cystic fibrosis (CF) is caused by loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel that particularly affects the respiratory system, with bacterial infection and severe inflammation. Patients with the most frequent mutation (F508del) or with many types of missense mutations can be efficiently treated with CFTR modulators, namely correctors and potentiators, i.e. pharmacological agents that improve CFTR protein processing and channel activity. However, such treatments are not appropriate by themselves for people carrying nonsense mutations that lead to CFTR protein truncation. We evaluated the efficacy of combinations of drugs targeting at various levels the effects of nonsense mutations: SMG1i to protect CFTR mRNA from nonsense-mediated decay (NMD); G418 and ELX-02, and the eRF3a degrader CC-90009 for protein read-through (RT); correctors VX-809 and VX-445 to promote protein maturation and function, PTI-428 to enhance CFTR protein synthesis. We found that the extent of rescue and sensitivity to the various agents is largely dependent on the type of mutation, with the W1282X as the most responsive mutation to rescue maneuvers. W1282X-CFTR protein expression and function can be largely rescued by NMD suppression with SMG1i and by protein stabilization with the combination of correctors VX-809/VX-445. In contrast, G542X-CFTR required treatment with RT agents and VX-809 but was insensitive to SMG1i. To identify other types of NMD inhibitors, possibly with better characteristics than SMG1i, we carried out a screening of a chemical library of more than 9000 compounds using a functional assay. We identified three novel kinase inhibitors, CC-115, samotolisib, and elimusertib, that act as potent NMD inhibitors. We also evaluated the efficacy of CC90009 on G542X-CFTR in combination with RT agents (ELX-02) and CFTR correctors (VX-809). Treatment resulted in a significant but relatively modest (three-fold) increase in CFTR function. Importantly, the efficacy of the triple drug combination was largely amplified under inflammatory conditions, achieved by exposing the epithelia to IL-4 or to IL-17A/TNF- α . The large rescue of CFTR function was paralleled by the appearance of full-length CFTR protein and by the increase in CFTR mRNA. The effect of inflammatory stimuli on G542X-

CFTR could be mediated by enhanced translational RT and/or reduced NMD inhibition.

Our results indicate that treatment of CF patients with nonsense mutations requires a precision medicine approach with the design of specific drug combinations for each mutation. Our findings may also lead to the identification of novel targets to correct the effect of nonsense mutations in CF and other genetic diseases. Moreover, and most importantly, our results suggest that pharmacological rescue of CFTR with nonsense mutations could be more effective than expected in vivo due to inflammatory conditions.

1. INTRODUCTION

1.1. The human respiratory system

The respiratory system is a set of different organs and tissues that together contribute to the process of breathing. This complex system is divided into the upper and lower respiratory tract; the first includes the oral and nasal cavities, pharynx and larynx (above the vocal cords). It represents the first point of access of environmental contaminants in the body, thus it has the major function of filter and removal of exogenous noxious agents inhaled with the air. The lower respiratory tract, instead, comprises the conducting airways (trachea, bronchi and bronchioles) and the respiratory zone, with the respiratory bronchioles and the alveoli, that exchange oxygen and carbon dioxide during the pulmonary respiration. (Saint-Criq & Gray, 2017; Bustamante-Marin & Ostrowski, 2017).

The respiratory system is particularly characterized by the presence of specialized epithelial cell populations that line the entire tract from the nasal cavity to the alveoli. The regional differences in cellular composition of each area reflect the specialized functions of each part (BustamanteMarin & Ostrowski, 2017; Bartoszewski et al., 2017).

1.1.1 The human airway epithelium

In the human respiratory tract, the airway epithelium plays a key defensive role against the environmental challenges (including xenobiotics, allergens and pathogens) that arrive by inhalation (Bartoszewski et al., 2017). With the advent of novel, advanced sequencing techniques, the traditional view of the airway epithelium as a uniform barrier between the organism and external environment has been profoundly changed to a more dynamic structure that includes a wide repertoire of highly specialized cells existing in close association with other specialized resident cellular systems, such as the immune and neural cells (Montoro et al., 2018; Plasschaert et al., 2018). Together, this dynamic “community” of cells generate an integrated unit that not only passively filters large particles and react to environmental changes, but also interact with resident microbial populations and cooperate to sense potential dangers

and eliminate them, balancing the risk of damage to the host (Figure 1) (Davis & Wypych, 2021; Hewitt & Lloyd, 2021).

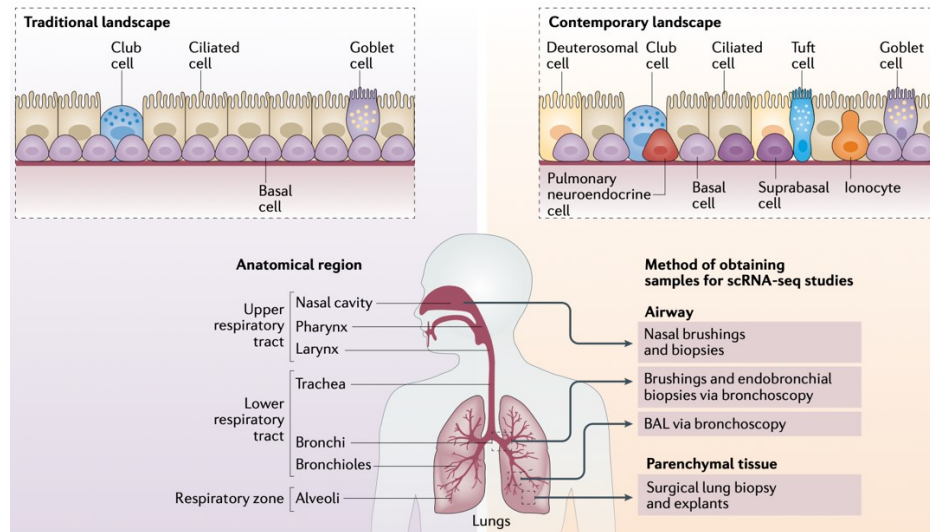


Figure 1. scRNAseq revised the architecture of human airway epithelium. scRNAseq profoundly transformed the view of airways from a uniform barrier to a dynamic and heterogeneous functional unit. The image summarizes the different parts of the respiratory tract and the contemporary landscape of cell types that compose the epithelial airways wall. Image adapted from Hewitt & Lloyd, 2021.

The different epithelial cell types form a continuous layer that lines the entire inner surface of the respiratory tract with the integrity of the layer maintained by tight junctions and other types of intercellular connecting structures. The epithelial layer is sustained by a collagenous lamina propria, a connective tissue containing serous and mucous glands (Bustamante-Marin & Ostrowski, 2017).

The airway epithelium also differs in morphology, between large and small airways. In the large proximal airways, it is pseudostratified, highly ciliated and taller (about 50 μm), with columnar cells (Widdicombe, 2002). Proceeding to the small distal airways, it becomes thinner and cuboidal (Crystal et al., 2008).

The airway epithelium is characterized by two main features essential for transepithelial ion transport and other important processes. First, epithelial cells are polarized, with two distinct apical and basolateral membranes. The apical membrane faces the external environment, and is exposed to air, while the basolateral touches the underlying connective tissue. Apical and basolateral membranes are endowed with a different set of membrane proteins and transporters; such separate localization is essential for the net transport of ions across the two sides of the cells (Saint-Criq & Gray,

2017). Second, ion channels and transporters are not only differently expressed on the two sides of the same cells, but also and most importantly the airway wall presents a specialized pattern of gene expression throughout the respiratory tract, with some cell types expressing particular genes involved in ion transport more than others (Montoro et al., 2018; Plasschaert et al., 2018).

Besides the three main cell types that constitute the airway epithelium, i.e. ciliated cells, goblet cells and basal cells, recent works (Montoro et al., 2018; Plasschaert et al., 2018; Vieira-Braga et al. 2019) identified by scRNAseq novel and rare cell types, such as neuroendocrine cells, tuft cells, and ionocytes, and transitional states between one cellular type and the other (e.g. deuterosomal cells, mucous ciliated cells).

Basal cells. Basal cells are the principal stem cells of the airways, supporting the self-renewal of the epithelium and the ability to generate all other specialized cell types of the epithelium (Rock et al., 2009; Rock & Hogan, 2011). They represent the 30% of the total cells in the upper airways, while their number decreases to around 6% in the distal airways. Basal cells are multipotent stem cells, localized in the deepest part of the epithelial layer in contact with the basal lamina (hence the name “basal”), and characterized by the expression of p63 and KRT5 markers (Ruyssveldt et al., 2021). Recently, also “hillock” cells have been identified as another type of basal progenitor cells, but their function is still unknown (Ruyssveldt et al., 2021).

Club cells. In the small airways the regenerative function of basal cells is probably assumed by club cells (Rock et al., 2009). Secretory/club cells are heterogeneous cells particularly abundant in the small airways and characterized by the presence of secretory granules and by the expression of high levels of SCGB1A1 secretoglobulin. Club cells also produce MUC5B mucin (Zuo et al., 2018).

Ciliated cells. Ciliated cells are the most abundant cell type of the epithelium (around 60%). They are terminally differentiated, and present a characteristic morphology: tall, columnar and obviously with a high number of apical hair-like motile cilia, whose rhythmic beating is crucial for expelling microorganisms and other debris trapped in the mucus, in a process commonly termed mucociliary clearance (MCC). A single ciliated cell can present more than 100 cilia on its apical surface. Each cilium is 6-

7 μm long and has a diameter of 0.1 μm (Bustamante-Marin & Ostrowski, 2017).

Goblet cells. In normal airways, goblet cells account for 20% of all epithelial cells. They are the main mucus producers that, together with ciliated cells, facilitate effective MCC. Goblet cells are highly polarized, with an enlarged apical pole full of mucin granules, that are secreted upon different autocrine and paracrine stimuli, in particular by extracellular ATP (Bustamante-Marin & Ostrowski, 2017), and nucleus and other organelles located towards the basolateral membrane. Mucus contains an assortment of products, namely metabolites, electrolytes, antimicrobial factors, and mucins. Mucins are further divided into membrane-tethered mucins, such as MUC1, MUC4, MUC16 and MUC20, and secreted, gelforming mucins, of which the major examples are MUC5AC and MUC5B. Gel-forming mucins are compacted and stored in intracellular granules, in which they are maintained at a low pH (~ 5.5) and in presence of high concentration of Ca^{2+} ions. Different stimuli can trigger mucus release by exocytosis. Upon release, mucins can largely expand by a process that involves replacement of Ca^{2+} ions by Na^{+} (Ridley & Thornton, 2018). Bicarbonate (HCO_3^{-}) is also considered fundamental, through H^{+} neutralization, to support mucus release and expansion (Garcia et al., 2009; Gustafsson et al., 2012).

Ionocytes and other rare cell types. Besides these major cell types, the detailed single cell transcriptomic analysis recently performed (Montoro et al., 2018; Plasschaert et al., 2018) shed light to rarer cell populations comprising less than 1% of all human airway epithelium. Although their role has not been fully understood yet, these cells have very distinct phenotypes and perform specific tasks in the maintenance of the epithelial landscape (Davis et al., 2021; Hewitt & Lloyd, 2021). The best example is represented by *ionocytes*, termed after their abundant expression of genes involved in ion transport. A specific marker of ionocytes is FOXI1, but they also express high levels of CFTR and vacuolar (V)-ATPase, thus highlighting their importance in airway surface physiology, pH regulation and mucus viscosity. A recent work by Scudieri and colleagues (Scudieri et al., 2020), indicates that ionocytes are more present in the nasal epithelium, suggesting the presence of a proximal-distal gradient through the airways. *Pulmonary Neuroendocrine Cells (PNeC)* and *Tuft cells* are

two other rare cell types of the airways identified by single cell transcriptomics. *PNeCs* are less than 0.5% of cells in the airway epithelium and prevalently present in the proximal tract. They can be best described as innervated resident cells that sense airway environment (Davis et al., 2021; Branchfield, 2016) and secrete neuropeptides to stimulate immune and nervous system responses (Xu et al., 2020). *Tuft* cells are also known as “brush” cells for the presence of brush-like microvilli projecting from their apical surface. Tuft cells have chemosensory, neuronal and immunological functions. They can express a variety of taste receptors and G-protein-coupled receptors and share signaling pathways with taste bud cells (Xu et al., 2020; Lambrecht, 2019). They are also characterized by the expression of several markers, such as POU2F3, TRPM5, and secretion of a specific set of effector molecules, like IL-25, acetylcholine and eicosanoids (Xu et al., 2020; Montoro et al., 2018; Plasschaert et al., 2018).

1.1.2. The Mucociliary Clearance mechanism (MCC)

The first, main feature underlying the protective role of the airway epithelium is the mucociliary clearance (MCC) mechanism. It is the first line of defense of the respiratory system and has the fundamental goal to remove the inhaled harmful particles from the healthy surfaces (Bustamante-Marin & Ostrowski, 2017; Mall, 2008). The MCC consists of three main processes and functional structures, which work in a dynamic and coordinated way:

1. *Ciliated cells that beat rhythmically.* Ciliated cells, as previously introduced, present hair-like cilia that beat in a coordinated metachronal way, with a whip-like movement, all together generating a synchronous wave that moves mucus toward the oro-pharynx (Figure 2). In healthy human airways, the ciliary beat frequency (CBF) is around 10-20 Hz. Different stimuli can modulate CBF, including beta-adrenergic or purinergic stimuli coupled to intracellular cyclic-AMP (cAMP) generation and Ca^{2+} mobilization. Also, physical and chemical stimuli, such as high temperature and intracellular alkaline pH, can increase CBF. Cilia are endowed with chemosensory receptors, that can sense noxious molecules

and respond to them increasing CBF (Bustamante-Marin & Ostrowski, 2017).

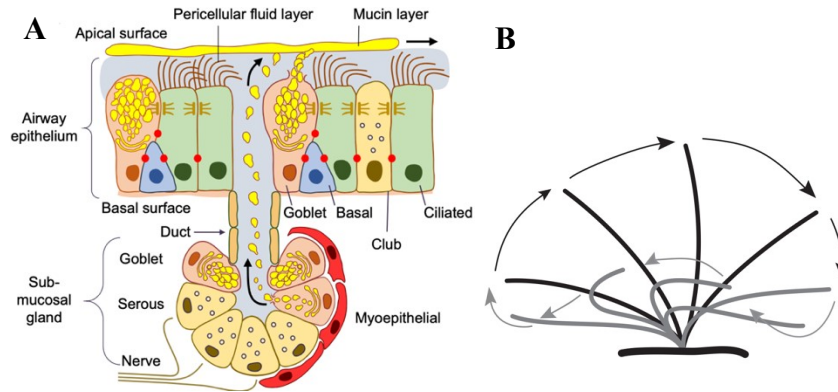


Figure 2. Ciliary beating and mucus secretion as key components of MCC. Panel A shows the main features of the mucociliary clearance mechanism. Adapted from Whitsett, 2018. Panel B illustrates a schematic diagram of a normal ciliary beat cycle. Cilia beat with a strong forward stroke (black) followed by a recovery stroke (grey). Adapted from Raidt et al., 2014.

2. *A thin layer of fluid, namely the airway surface liquid, ASL, that covers and lubricates the airway surfaces.* The ASL can be described through a “gel-on-brush” model (Button et al., 2012), in which beating cilia are immersed in a periciliary liquid (PCL), constituted by membrane-tethered mucins and mucopolysaccharides (Figure 3). This dense brush prevents mucus penetration into the PCL space and causes mucus to form a distinct layer above (mucus layer) (Knowles & Boucher, 2002, Widdicombe et al., 2002; Ridley & Thornton, 2018). The PCL is crucial for an effective cilia beating and as a shield for the underlying epithelium. In fact, PCL possesses a lubricating function that facilitates the mucus movement also during cough (Knowles & Boucher, 2002). To allow the cilia proper beating, the thickness of the ASL must be precisely maintained at a thickness of 5-8 μm through a fine balance between fluid and electrolytes absorption and secretion. Ion transport systems of the cells are the main players of these processes, as will be further explained in the next section (Saint-Criq & Gray, 2017).
3. *A layer of mucus.* Above the PCL there is the mucus layer (ML). It consists in a 2-5 μm thick layer of a tangled net of gel-forming mucins, mainly MUC5B and MUC5AC (Knowles & Boucher, 2002). Differently from the PCL, the ML is discontinuous in human airways, with some tracts in which

it is deeper and some other tracts with mucus-free zones in which PCL is exposed to the air (Knowles & Boucher, 2002).

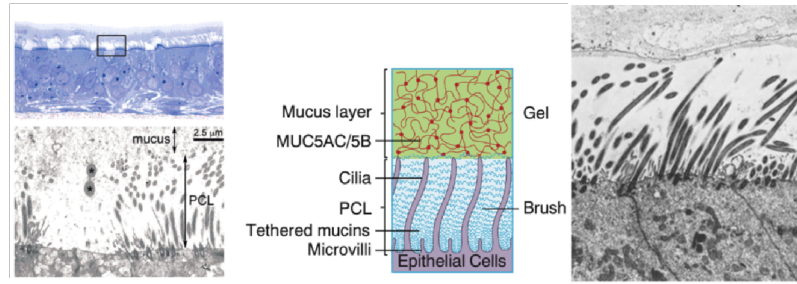


Figure 3. “Gel-on-brush” model describes the ASL composition. Button and colleagues described a model in which tethered macromolecules, such as membranebound mucins and glycoproteins, form a brush-like structure of the PCL that maintains the mucus as a separate layer above. Left and center, adapted from Button et al., 2012. Right image shows TEM image of ciliated cells in the ASL, where a net separation between PCL and ML is clearly visible. Adapted from Widdicombe et al., 2002.

An effective mucociliary clearance mechanism occurs when the motile cilia of the ciliated cells rhythmically beat immersed in the ASL that covers and lubricates the airway surfaces. In this way, the tips of the motile cilia can make contact with the overlying mucus layer that can trap the inanimate particles, bacteria, and other microbes and inhaled pathogens, moving them towards the oropharynx where they can be expectorated or ingested. The average basal rate of mucociliary transport is 5.5 mm/min, but various physiological factors, such as age, sex, posture, sleep, exercise, temperature and humidity conditions, may affect this value. Moreover, the number and length of cilia, the CBF, as well as mucus rheology and hydration, may influence MCC (Bustamante-Marin & Ostrowski, 2017). The rate of MCC seems to be locally regulated by nucleotide release in response to cough-induced shear stress, that act as an autocrine and paracrine stimulus on epithelial ion transport, CBF, and mucus secretion (Button et al., 2013; Knowles & Boucher, 2002).

1.1.3. Ion transport mechanisms in the airways

As mentioned above, transport of ions across the membranes and fluid secretion is indispensable to maintain an efficient mucociliary clearance, since ASL hydration is strictly dependent on salt absorption and secretion. The finely tuned active and passive transport of Na^+ and Cl^- is, in fact, the main driver of water movements across the membrane walls (Saint-Criq

& Gray, 2017; Bartoszewski et al., 2017). Airway epithelial cells are endowed with a complex arsenal of membrane proteins such as ion channels (the epithelial sodium channel ENaC, and the Cl^- channels CFTR, TMEM16A, CLCNKA/B) (Caputo et al., 2008, Elborn, 2016, Moore & Tarran, 2018, Lei et al., 2023), active transporters (e.g. the sodium/potassium-ATPase or the proton/potassium pump ATP12A), and multiple types of ion exchangers and cotransporters (the bumetanidesensitive cotransporter SLC12A2, the sodium/proton exchanger, chloride and bicarbonate transporters of the SLC4 and SLC26 families) (Pedemonte et al., 2007, Caci et al., 2015, Scudieri et al., 2018) that contribute in a coordinated way to maintain the homeostasis of the ASL, regulating its physical (volume, viscosity) and chemical (pH, ion composition) properties (Figure 4).

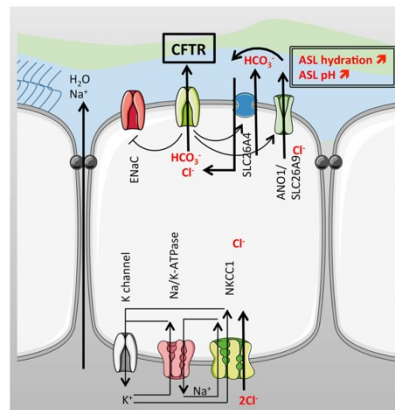


Figure 4. Main ion channels and transporters of the airways. In the airways, salt secretion and absorption are given by the paralleled transport of Na^+ and Cl^- through the membranes. The image sums up the main membrane proteins involved in these mechanisms. For simplicity, the figure only depicts the side of the cell in which proteins are localized, rather than the specific cell type that expresses them. Adapted from SaintCricq & Gray, 2017.

Under normal conditions, the process of salt absorption in the airways is mainly orchestrated by transepithelial absorption of Na^+ . For this purpose, intracellular Na^+ concentration is kept at relatively low levels by the activity of the Na^+/K^+ -ATPase at the basolateral side. This, in turn, favors Na^+ influx from the apical membrane, where Na^+ moves down the electrochemical gradient through the Epithelial Na^+ Channel (ENaC). Passive transport of negatively charged ions (Cl^-) from the apical side to the basolateral side is then allowed by the electrochemical driving force created by Na^+ transport. This movement may occur through the

paracellular pathway, but there are also models postulating a transcellular absorption of anions. The salt concentration gradient across the epithelium generated by NaCl absorption provokes water to be absorbed passively by osmosis. K^+ channels, both at the basolateral and apical sides, are also important since they keep a negative membrane potential suitable for Na^+ influx to occur (Tarran et al., 2001, Knowles & Boucher, 2002). On the contrary, salt secretion depends on active Cl^- transport. This is mediated by the activity of Cl^- transporters on the basolateral membrane that provide the driving force for Cl^- exit at the apical membrane. Basolateral Cl^- transporters particularly include the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1, a.k.a. SLC12A2) that exploits the Na^+ gradient established by the Na^+/K^+ -ATPase to induce accumulation of Cl^- inside the cells (Saint-Criq & Gray, 2017; Bartoszewski et al., 2017). The exit of Cl^- at the apical membrane is mediated by various types of channels that include CFTR, TMEM16A (ANO1), and SLC26A9. A shared feature of CFTR and ANO1 is that, besides Cl^- , they can also conduct bicarbonate (HCO_3^-). In the airways, bicarbonate transport plays a key role in modulating the pH of the secreted fluids (Coakley et al., 2003, Saint-Criq & Gray, 2017). Secretion of anions is paralleled by Na^+ flowing through the paracellular pathway. This leads to water secretion by osmosis.

Absorption and secretion cannot obviously occur at the same time. Absorption and secretion may not occur in the same cell type, too. With the recent unveiling of ionocytes by the works of Montoro et al., 2018 and Plasschaert et al., 2018 how the airways epithelium can switch between absorptive and secretory states became a more puzzling question. A recent study (Lei et al., 2023) proposes a model in which secretion and absorption processes are segregated in different cell types: ionocytes would mediate Cl^- absorption through the coupling of CFTR on the apical membrane with the CLCK/Barttin Cl^- channels on the basolateral membrane (Lei et al., 2023, Romano-Ibarra et al., 2024). Secretory cells, instead, would couple apical CFTR Cl^- exit at the apical membrane with basolateral Cl^- uptake by NKCC1 (Figure 5).

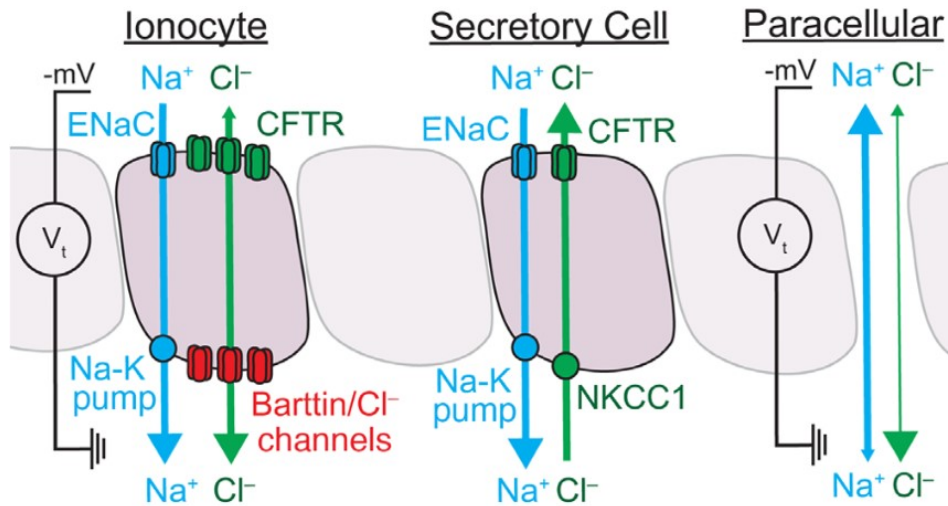


Figure 5. Model for transepithelial ion movement across the airways. Lei and colleagues propose a model in which different cell types are committed to the ion secretion and absorption processes. The peculiar presence of CLCK/Barttin Cl⁻ channels in ionocytes makes them absorptive cell types, since they permit Cl⁻ movements towards the basolateral side; secretory cells couple NKCC1-mediated Cl⁻ import in the cell with CFTR-mediated Cl⁻ secretion. Adapted from Lei et al., 2023.

Other different cell types and stimuli can influence and regulate transepithelial Cl⁻ flow, but they remain less characterized or still unknown (Genovese et al., 2019; Lazarowski & Boucher, 2021).

1.1.4 Impaired MCC is a landmark of airway diseases

As repeatedly underlined, MCC plays a pivotal role in the defense of the respiratory tract. The functional significance of the complex network of airway cells and the MCC mechanism stands out in diseases (Figure 6, panel A). In fact, impairments in the coordinated and dynamic activity of mucus and cilia are common features of several human genetic and nongenetic diseases, including CF (Mall et al., 2024), chronic bronchitis and chronic obstructive pulmonary disease (COPD) (Barnes et al., 2015), and primary ciliary dyskinesia (PCD) (Barbato et al., 2009). Alterations in at least one of the three structural components of the MCC mechanism (ciliary beating, ASL homeostasis and mucus) are the basic defect underlying all these muco-obstructive diseases that lead to lifelong recurrent infections of the entire respiratory tract (Antunes & Cohen, 2007).

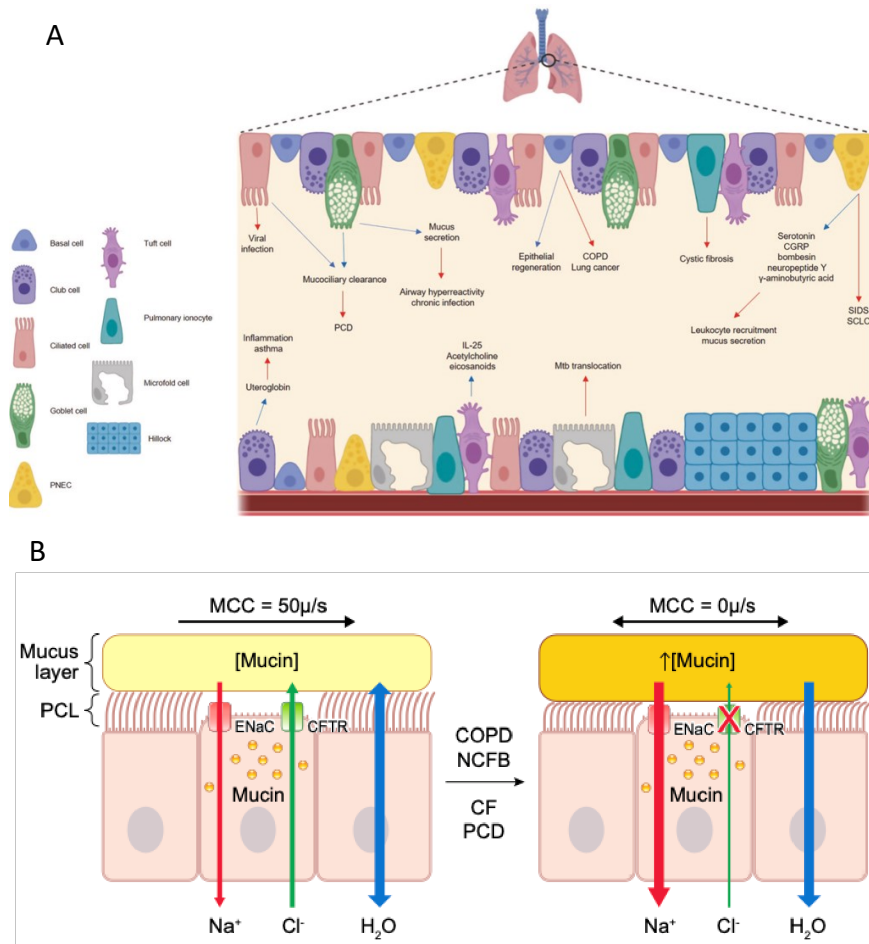


Figure 6. Unbalanced homeostasis of the respiratory epithelium leads to different diseases. As summarized in panel A, in disease state cells contribute differently to the onset of pathologies. As explained throughout the text, the orchestrated activity of goblet and ciliated cells and, ultimately, pulmonary ionocytes, guarantees the correct MCC mechanism. Panel B shows the progression from normal to muco- obstructed airway states. In healthy state (left), effective MCC is allowed by a correct hydration of the ASL thanks to the salt absorption/secretion mechanism. In muco-obstructive lung disease (right), an imbalance of ion/fluid transport together with mucin hypersecretion increases mucin concentrations in the mucus layer, that in turn compresses the PCL impairing ciliary beating. This slows and eventually abolishes MCC. Panel A taken from Davis et al., 2021. Panel B adapted by Hill et al., 2022.

PCD is a severe respiratory disease caused by loss of function mutation in genes responsible for structure and function of motile cilia leading to respiratory, reproductive, and laterality issues such as middle ear infections, chronic airway infection, and male sterility and, in rare cases, hydrocephalus (Kurkowiak et al., 2014; Pereira et al., 2023). More than 40 genes have been associated with the PCD phenotype. Structural abnormalities of the ciliary axoneme are considered as hallmark defects of PCD and leads to impaired cilia motility and CBF (hyperkinetic, stiff or completely immotile cilia). Mutations in genes that account for impaired cilia biogenesis (reduced or absent number of cilia) are also reported (Kurkowiak et al., 2014; Lucas et al., 2020).

An acquired impairment of CBF is also reported in other diseases such as CF, asthma and COPD. A detailed description of the specific mechanisms for each disease is beyond the purposes of this work, but differently from PCD, in other muco-obstructive disease, such as CF, the impaired CBF is secondary to a dehydration of the ASL, with an enhanced mucin content. Impairments in ion transport mechanism of the epithelium, with a defective salt and water secretion or an excessive salt/water absorption determines the collapse of the PCL in which cilia are immersed and beat. In this context, inflammatory conditions induce mucous metaplasia, increasing the number of goblet cells overexpressing and releasing secreted mucins (Lai et al., 2010; Huang et al., 2012; Scudieri et al., 2012). This contributes to expansion of the ML over 10 times in respect to normal conditions. Together, fluid dehydration and ML expansion determines collapse of the PCL, that allows MUC5AC and MUC5B to establish an abnormal contact with the membrane-anchored mucins, such as MUC1 and MUC4, forming a very sticky mucus in which the cilia are trapped and cannot beat. The mucus becomes difficult to eliminate even with cough (Figure 6, panel B), generating static mucus plaques and positive feedback (“vicious”) muco-inflammatory cycles (Knowles & Boucher, 2002; Ridley & Thornton, 2018; Hill et al., 2020). Accumulation and stasis of mucus plaques on the airway surface determines the generation of hypoxic zones, release of soluble pro-inflammatory mediators, and stimulation of additional mucin secretion. Also, resident macrophages of the airway epithelium remain trapped in the thickened mucus, releasing in turn IL-1beta that further accelerates mucin secretion. Other macrophage products (TNF- α , IL-6) and other inflammatory cells also takes part in the process, that worsens with time. Ultimately, severe and persistent mucus plaques become infected by bacterial colonies, which is typical of these diseases. *Pseudomonas aeruginosa*, for example, produces alginate and form biofilms characteristics of chronic infection, a condition that is often observed in CF patients (Worlitzsch et al., 2002; Hill et al., 2020).

1.2 Cystic fibrosis (CF)

CF is one extreme example of how ion transport plays a crucial role in maintaining the proper hydration of the airways and preserves the MCC mechanism. CF is the most common life-threatening autosomal recessively inherited disease that afflicts people of European descent and is estimated to occur in $\sim 1:1,000$ to $\sim 1:30,000$ live births depending on the genetic ancestry of the population (Jia & Taylor-Cousar, 2023). To date, CF patient registries in the United States and Europe include more than 87,000 people who have been diagnosed with CF (ECFS Patient registry 2021, CFF patient registry 2023). Due to autosomal recessive inheritance, a significant part of the world population includes asymptomatic carriers of disease-causing CFTR variants (Martin & Brugel, 2020). CF incidence rates may vary around the world, but a frequency of nearly 1 affected child every 3000 live births rate is mostly associated to Caucasian newborns. The presence of national CF newborn screening programs in countries with robust diagnostic capabilities and patient registries strongly contributed to an earlier identification of the disease among the population. In Europe and the United States about 1000 cases of CF are newly diagnosed every year, with an average age at diagnosis of about 3 months (ECFS Patient registry 2021; CFF Patient registry 2023).

Although CF is recognized as a disease affecting principally Caucasians, the CF population is growing every year. The expanded awareness of the disease in individuals who are Black, Native American, or people of color (BIPOC) allowed the increasing of diagnosis rates also in other countries of non-Caucasian ancestry, particularly in Asia and Africa (Bobbo et al., 2023; CFF Patient registry 2023).

The remarkable progresses that have been achieved during recent years in the pharmacological correction of the basic defect with the use of highly effective modulator therapies (HEMT) is changing the face of CF, from being a disease of children to now being predominantly an adult disorder. Indeed, for the past 5 years, in countries with well-funded health-care systems, there have been more adults than children with CF (Jia & TaylorCousar, 2023; Elborn, 2016). This substantial improvement in life expectancy is also accompanied by a dramatic decrease in the number of

lung transplants reported compared to the numbers prior to the adoption of HEMT (CFF Patient registry 2023). According to the Cystic Fibrosis Foundation 2023 Registry Report, the median predicted survival time of CF patients in the United States is now approximately 61 years, with a decreased mortality rate (from 16,6 to 7 per 1000 people in the last twenty years). The number of adults with CF will continue to increase with almost all deaths occurring in the adult population (Jia & Taylor-Cousar, 2023).

1.2.1 Pathogenic mechanism in CF lung disease

The pivotal role of the CFTR chloride channel in balancing the rates of Na^+ absorption and Cl^- secretion has been explained in the previous sections. However, the precise pathophysiological mechanism by which CFTR dysfunction causes CF lung disease is still debated. According to the *low-volume hypothesis*, the lack of CFTR-dependent Cl^- transport would cause dehydration of airway surface because Na^+ /fluid absorption would prevail due to lack of secretion (Pereira et al., 2023).

In a cascade effect, the dehydration of the PCL would cause the collapse of the ML with entrapment of cilia and impairment of MCC (Tarran et al., 2001; Rowe et al., 2005; Mall & Hartl, 2014) (Figure 7). This would cause a favorable environment for bacteria.

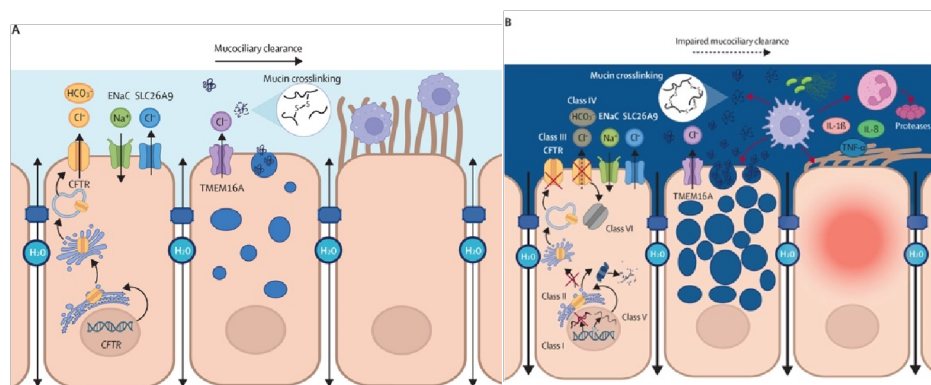


Figure 7. Model for low-volume hypothesis. Panel A: in healthy airways, CFTR channel is essential for a correct hydration of airway surface. Panel B: without functional CFTR, there is an inadequate homeostasis of ion and water transport leading to dehydrated PCL, that causes the collapse of the ML on cilia. Severe mucus plugs formation, resulting from MCC impairment, favors the growth of microbes, with the onset of severe infection and inflammation during time. Adapted from Graeber & Mall, 2023.

Historically, there was also a proposal of a *high-salt hypothesis* in which the absence of a functional CFTR channel imposes an increased ion concentration in the ASL, that in turn inhibits the activity of

broad-spectrum bactericidal peptides normally present in the ASL, fostering colonization of airway mucus by bacteria (Smith et al., 1996; Zabner et al., 1998). However, this hypothesis has been disputed since a high salt concentration in CF airways has not been confirmed.

Another factor contributing to CF lung disease is an altered pH that would impair the bactericidal activity of PCL (McShane et al., 2003) and increase the viscosity of mucus secretion (Garcia et al., 2009; Gustaffson et al., 2012; Hoegger et al., 2014; Shah et al., 2016).

Finally, more than 50% of CFTR transcripts of the entire epithelium have been found concentrated in pulmonary ionocytes (Plasschaert et al., 2018), despite this cellular type comprising less than 1% of the respiratory epithelium (Montoro et al., 2018; Scudieri et al., 2020). It is not yet known if and how this cell type is implicated in the pathogenesis of CF lung disease. However, as previously introduced, these cells rich in membrane ion channels and transporters are being presently described playing an important role in the mechanisms of salt absorption and secretion (Shah et al., 2022; Lei et al., 2023; Romano-Ibarra et al., 2024).

1.2.2. The CFTR chloride channel

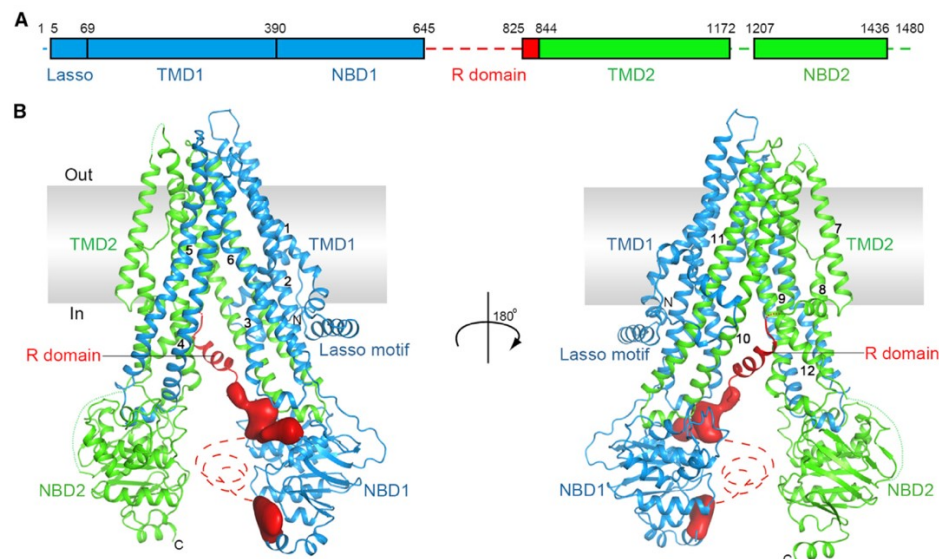


Figure 8. CFTR structure. Panel A: schematic domain structure of CFTR. Panel B: human CFTR in the dephosphorylated, ATP-free conformation described by Liu et al., 2017.

CFTR is a multidomain protein belonging to the ATP-binding cassette (ABC) sub-family C (Moran, 2017). CFTR shares the general protein structure with the other members of the subfamily but, to date, it is the

unique member known to function as an ATP-gated ion channel, thus regulating anion transport, mainly Cl^- but also bicarbonate, across apical membranes of many different epithelia, such as the respiratory tract, digestive and urogenital tracts and lacrimal and sweat glands (Quinton, 2007; Xue et al., 2016; de Souza et al., 2018). Like many other ABC proteins, CFTR is an integral membrane protein, with a characteristic topology in which the intracellular portion accounts for about the 80% of the structure (Moran, 2014; Liu et al., 2017). Two membrane-spanning domains (MSD1 and MSD2) form the ion pore walls. Each MSD is composed of six transmembrane alpha-helices (TM1 to 6 for MSD1 and TM7 to 12 for MSD2) with three short extracellular loops and two long intracellular loops. TM1, 5, 6, 11 and 12 are the segments directly lining the anion pore. MSDs are followed by a nucleotide binding domain (NBD), NBD1 after MSD1 and NBD2 after MSD2, respectively. NBDs are characteristic subunits of all ABC proteins. In CFTR, NBD1 and NBD2 are highly conserved, both at the primary amino acid sequence and 3D structure.

NBDs are subdivided in two subdomains, in which the core subdomain, also known as the “head”, binds ATP, while the alpha-helical subdomain, the “tail”, contains an ABC specific sequence, the “signature sequence”. NBDs dimerize in ABC proteins, in a head-to-tail conformation.

A CFTR characteristic feature, that is different from all other ABC proteins, is the presence of an intrinsically disordered region, the regulatory domain (RD), located between the NBD1 and the MSD2. The RD is about 200 residues long and contains at least 18 putative serines and threonines phosphorylation sites (Figure 8). The gating of the anion pore in CFTR is thus regulated by two processes (Csanády et al., 2019):

- Phosphorylation by protein kinase A (PKA) of the RD is mandatory for activation of the channel. Since activation of the PKA is mediated by the cytosolic messenger cAMP, CFTR is frequently identified as “cAMP-activated channel” (Sheppard & Welsh, 1999; Moran, 2014).
- ATP binds the NBDs of the phosphorylated CFTR and promotes the dimerization of these domains, leading to conformational changes in the transmembrane helices lining that open the channel. Then, the enzymatic activity of the NBD hydrolyses ATP releasing ADP, terminating the cycle. Noteworthy, this cycle is not reversible, and the energy resulting from the hydrolysis is

not used to transport anions across the pore (Sheppard & Welsh, 1999; Moran, 2016).

To date, of the 2121 described CFTR genetic variants (CF mutation database, <http://www.genet.sickkids.on.ca/cftr/>), 1085 have been confirmed as CF-causing mutations (cftr2 website, <https://cftr2.org>). CF pathogenic variants have been classically grouped in mutational classes according to the primary defect that they cause (i.e., maturation, expression and function). The first classification was proposed by Welsh and Smith in a land-mark paper (Welsh & Smith, 1993). Currently, at least six major classes are postulated (Zielenski & Tsui, 1995; Rowe et al., 2005).

- Class I includes frameshift, splicing, or nonsense mutations that introduce premature termination codons (PTC), impairing protein synthesis and resulting in severely reduced or absent CFTR expression. Examples are G542X and W1282X.
- Class II mutations lead to misfolding defects, with premature degradation that occurs at the endoplasmic reticulum (ER) level by the ER quality control system, and impaired protein biogenesis, severely reducing the number of CFTR molecules that can correctly traffic to the cell surface. This class includes F508del, that accounts for about 70% of all cases of CF worldwide (Fanen et al., 2014).
- Class III mutations hamper the regulation of the CFTR channel, resulting in abnormal gating characterized by a reduced open probability (G551D).
- Class IV mutations reduce the intrinsic channel conductance, possibly by narrowing the ion pore (R117H).
- Class V includes mutations that downregulate CFTR mRNA expression, by introducing promoter or splicing abnormalities. An example is the 2789+5G->A splicing defect, a residual function mutation (Highsmith et al., 1997).
- Class VI mutations destabilize the conformational stability of the channel and/or generate additional internalization signals post-ER/Golgi processing. This can result in accelerated turnover and reduced expression on the plasma membrane (Haardt et al., 1999; Silvis et al., 2003).

However, this classification remains a simplification, since some mutations can cause pleiotropic molecular defects, irrespectively to the class in which they are included. An extended classification scheme has been proposed (Veit et al., 2016). The expanded classification proposed by Veit and colleagues considers 31 possible classes of mutations, so that the unusually complex, combinatorial molecular phenotypes can be accommodated in 26 new classes resulting from the combination of the six major mechanistic mutational classes, as depicted in the Venn diagram shown in Figure 9.

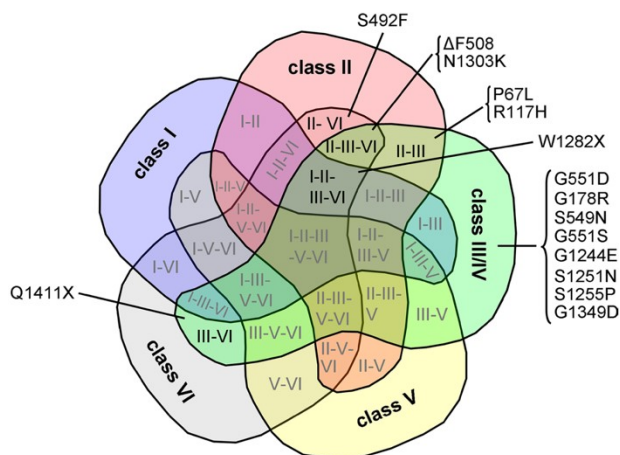


Figure 9. Extended classification of CF mutations. This new refined classification considers complex phenotypes of major CFTR cellular defects. Taken by Veit et al., 2015.

Such classification paved the way for “theratypization” (Cutting, 2015; Clancy, 2018) of complex CFTR mutations, allowing the development of the current modulator therapies based on combination of small molecules, i.e. correctors and potentiators, directly targeting the different underlying CFTR molecular defects (Van Goor, 2009; Van Goor et al., 2011; Farinha et al., 2013; Boyle et al., 2014; Wainwright et al., 2015; Rowe et al., 2017; Keating et al., 2018; Taylor-Cousar et al., 2019).

1.2.3. Pharmacological correction of CFTR basic defect(s)

The classification of CFTR variants has been fundamental to link the phenotypic complexity of the different mutants at the molecular level and their susceptibility to different drugs. Indeed, function of mutant CFTR can be restored in many cases by pharmacological agents (Zegarra-Moran & Galletta, 2017; Veit et al., 2018; Lopes-Pacheco, 2020). For many years, large-scale efforts have been made to identify drugs that can act by directly

binding to and promoting the biogenesis and/or the function of mutated CFTR (Pedemonte, 2005; Van Goor, 2009; Van Goor, 2011; Phuan, 2014; Veit et al., 2018). The description of molecular biology of CFTR mutations allowed the development of small molecule compounds targeting the underlying defect(s) in CF. For example, the VX-770 (a.k.a. Ivacaftor) was the first drug to be approved for treatment of people with CF (pwCF) by the U.S. Food and Drug Administration. It comes after the observation that some CFTR variants belonging to III or IV can be expressed at the apical membranes of the cells at a density similar to that of the wild-type protein, although they are functionally impaired (e.g., G551D) (Veit et al., 2018, Lopes-Pacheco, 2020). Indeed, VX-770 directly targets the gating defect of the class III mutation G551D-CFTR (Van Goor, 2009), so that the approval of this drug was then extended to eight additional class III mutations (Yu et al., 2012; Lopes-Pacheco, 2020). However, as previously introduced, it has been recognized that some mutations have pleiotropic effects on CFTR biology and may impose combinatorial defects. This notion gathered the possibility that the combination of different pharmacotherapies addressing single defects (e.g., transcription, translation, folding, and/or gating) may show improved clinical benefit over available monotherapies with low efficacy (Clancy et al., 2012; Boyle et al., 2014; Lopes-Pacheco, 2020). This is the case of the F508del mutation. The F508del mutation accounts for approximately 70% of CF alleles and is the prototypical class II mutation, since it elicits a complex folding defect that compromises both NBD1 stability and the cooperative domain assembly of the channel (Hoelen et al., 2010; Lukacs & Verkman, 2012). Thus, CFTR conformational maturation is impaired, and the protein is targeted for premature ER-associated degradation (Lukacs et al., 1994; Cyr, 2005; Kim & Skach, 2012). However, a certain amount of F508del-CFTR can escape the ER quality control, either constitutively or following rescue procedures, and accumulates at the plasma membrane where it exhibits a channel-gating defect (a hallmark of class III mutations) as well as accelerated turnover in post ER (a class VI mutation characteristic) (Veit et al., 2018). Thus, the most common mutant has multiple defects that extend beyond the features of a class II mutation, so that pharmacological rescue of the F508del-CFTR must consider both the folding and trafficking defects, and the gating defect (Veit et al., 2018).

From the discovery of the CFTR gene in 1989, research efforts to find pharmacological approaches to correct the basic defects in CFTR function ended up with the successful development of small molecules called CFTR “modulators”, that are essentially divided into potentiators and correctors (Lopes-Pacheco, 2020). Potentiators are compounds that promote the opening of the CFTR channel. They are particularly indicated for the rescue of “gating mutations” (e.g., G551D, G1349D, G178R, class III and IV mutations). The previously mentioned Ivacaftor (VX-770) is the most advanced potentiator. It constitutes the active principle of Kalydeco, the first drug approved for the treatment of pwCF carrying at least one class III or IV mutation.

CFTR correctors, instead, are pharmacological chaperones that target the multiple folding, stability, and trafficking defects caused by F508del and other class II mutations. In the last years, high-throughput screening technologies (HTS) allowed the rapid identification of several small molecules acting as pharmacological chaperones with different mechanisms of action (MOAs), so that a classification of these compounds has been proposed based on their molecular targets in the CFTR structure (Okiyoneda et al., 2013):

- class I correctors stabilize NBD1-TMD1 and/or NBD1TMD2 interfaces;
- class II correctors stabilize NBD2 and its interfaces with other CFTR domains;
- class III correctors directly stabilize NBD1.

The publication of the cryo-EM structures of the human F08del-CFTR together with correctors (Fiedorczuck & Chen, 2022a; Fiedorczuck & Chen, 2022b) updated the precise binding sites of the different compounds on the protein structure and contributed to elucidate the way the copresence of different correctors rectified interdomain assembly during the co-translational folding of the F508del CFTR (Fiedorczuck & Chen, 2022b). Presently, three types of correctors (Lumacaftor, Tezacaftor, Elexacaftor) are included in drugs that have been approved for the treatment of people carrying the F508del mutation (Deeks, 2016; Sala & Jain, 2018; Joshi et al., 2019). Lumacaftor (VX-809; Vertex Pharmaceuticals) is a first-generation corrector that act with a class I corrector mechanism, binding to the TMD1 (Van Goor et al., 2011).

Tezacaftor (VX-661; Vertex Pharmaceuticals), instead, is a second-generation corrector developed upon lumacaftor structure, but with better pharmacokinetic properties and fewer adverse effects. Initial treatments for pwCF carrying the F508del mutation consisted in two combinations of the VX-770 potentiator, with corrector VX-809 named Orkambi (Wainwright et al., 2015) or with corrector VX-661, named Symdeko (Rowe et al., 2017). VX-445, a.k.a. Elexacaftor (E), is a corrector acting with a class III MOA with the attempt to find a compound that could work in synergy with the Tezacaftor/Ivacaftor (TI) combination (Keating et al., 2018). Given the amplitude of the therapeutic response obtained in clinical trials with the triple combination (a.k.a. ETI), in 2019 the Food and Drug Administration approved the triple drug combination of Tezacaftor and Elexacaftor (respectively, class I and III correctors) plus the potentiator Ivacaftor for the treatment of people with one or two F508del alleles (Hoy, 2019) under the name of Trikafta (Kaftrio in Europe) (Figure 10).

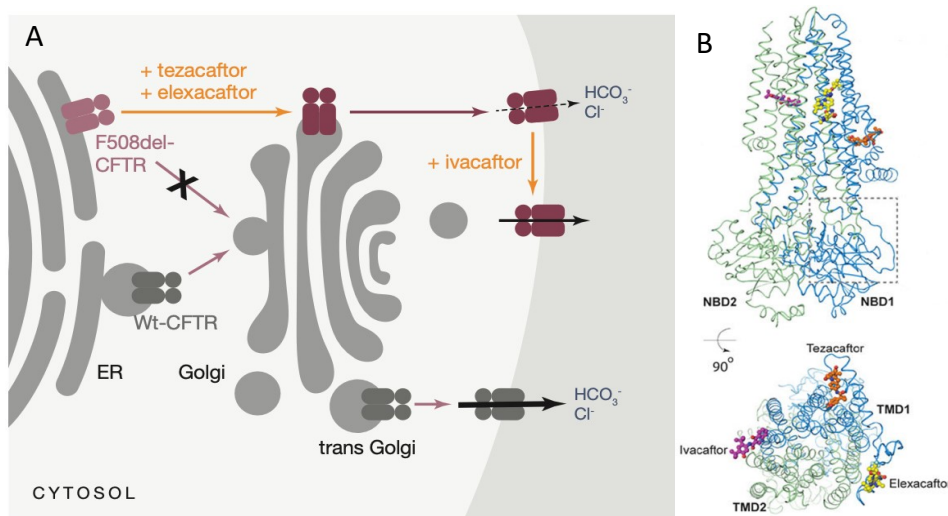


Figure 10. Effects of the ETI on the pleiotropic defects caused by the F508del-CFTR. Panel A. The correctors (ET) act in a complementary manner against misassembly, misprocessing, and mistrafficking of F508del-CFTR. Following proper trafficking to the cell surface, the potentiator, Ivacaftor (I), promotes opening of the CFTR channel. Adapted from Bear, 2020. Panel B. Cryo-EM structure of ETI binding human F508del-CFTR. Adapted from Fiedorczuck & Chen, 2022b.

Thus, highly effective modulator therapy (HEMT) of mutant CFTR is the leading strategy for the treatments of pwCF with at least one copy of F508del-CFTR. To date, approximately 70-90% of pwCF are eligible for an FDA-approved modulator therapy; moreover, there is the possibility of off-label use for mutations other than those for which the drugs were

originally developed (ECFS Patient registry 2021, CFF patient registry 2023).

1.2.4. CFTR class I mutations: nonsense mutations

Despite the undeniable success of CFTR pharmacotherapy, there is a substantial proportion (10-30%, depending on the geographic area) of people who still cannot benefit of these modulator therapies (LopesPacheco, 2020; Mall et al., 2020). Among them, there are people carrying class I mutations, that enclose splice site abnormalities, frameshifts due to insertion or deletions (in/dels), or nonsense mutations, which generate premature termination codons (PTCs), as introduced in the previous sections.

A nonsense mutation is a single point alteration in the DNA sequence that introduces a non-natural stop codon (UAA, UAG, or UGA) in an inappropriate position of the protein-coding region of the corresponding mRNA transcript. Such mutations bring to premature termination of translation leading to abnormally truncated proteins with negligible or totally absent residual function, with consequent disease. Nonsense mutations are responsible for about 10% of CF cases worldwide. However, in specific geographic areas, (e.g. Israel), nonsense mutations are the cause of cystic fibrosis in most patients (Kerem et al., 1997). PTCs can reside throughout the CFTR coding sequence; the G542X and W1282X are the most common nonsense mutations of this class. Both fall in the NBDs of the channel: the G542X hits the NBD1 included in the first half of the protein (exon 11), while the W1282X falls in the exon 20, eliminating part of the NBD2. Similarly to F508del, even though W1282X represents a prototypical class I mutation, recent studies suggest a more complex phenotype (Veit et al., 2016). First, since it deletes part of the NBD2, this defect compromises NBD1-NBD2 dimerization and W1282X-CFTR folding and activity. Further, if the PTCs is corrected either with spontaneous or drug-induced read-through (Spelier et al., 2023), as will be further explained later, the resulting elongated protein would contain aminoacidic substitution that may cause structural defects (as a class II mutation) or gating defects (class III characteristic), as suggested by data from people with missense mutations at residue W1282 (Faucz et al.,

2007; Visca et al., 2008) and by activation of W1282X upon VX-770 stimulation (Xue et al., 2014), respectively.

Another problem caused by W1282X, as well as by other PTCs, is the elimination of the transcript carrying the mutation by the nonsense-mediated RNA decay (NMD) (Hamosh et al., 1992; Linde et al., 2007).

1.3. Nonsense-mediated mRNA decay (NMD)

Nonsense-mediated mRNA decay (NMD) is a physiological translation-coupled mechanism that has the goal to eliminate mRNAs harboring PTCs causing premature abortion of protein translation, producing prematurely truncated proteins with potential harmful effects for the cells due to dominant negative interactions (Brognia & Wen, 2009). Thus, it clearly has a role in mRNA surveillance, since the main targets of NMD are mRNAs that have acquired PTCs because of mutations or errors during transcription or RNA processing (Brognia & Wen, 2009). However, NMD has been also described as an important regulator of the expression of naturally-occurring transcripts (Huang & Wilkinson, 2012). Indeed, the mechanism itself has been pointed out after the observation that cells contain some mRNAs at a concentration unexpectedly low, and they are transcribed from alleles carrying nonsense mutations (Morse & Yanofski, 1969; Maquat, 1995; Moriarty et al., 1998). NMD is an evolutionarily conserved mechanism and all the investigated organisms, from bacteria to mammalian cells, share a plethora of NMD-specific trans-acting factors and protein complexes that are required for an active NMD, together with the translational machinery (Brognia & Wen, 2009). Despite the high level of conservation, NMD can mechanistically differ among species, thus this discussion will be focused on human NMD machinery.

1.3.1. NMD trans-acting proteins

NMD mechanism is mediated by numerous proteins that act in trans on the translating mRNA carrying the PTC. Two are the main protein groups that conduct the NMD machinery: the UPF proteins and the SMG proteins.

UPF proteins. *Up-frameshift* proteins UPF1, 2 and 3 (that is a complex composed by its two paralogues UPF3a and UPF3b), respectively encoded by the *UPF1*, *UPF2* and *UPF3* genes (a.k.a. SMG2, SMG3 and SMG4), represent the core of the NMD pathway. UPF proteins are highly conserved among species: they were first discovered in genetic suppressor screen in yeast (Broгна & Wen, 2009) and their orthologs have been identified in other eukaryotes. Their fundamental role in NMD is underlined by the observation that the deletion or genetic silencing of UPF proteins prevents NMD in humans and all the studied organisms (Broгна & Wen, 2009). Studies of UPF proteins interactions in humans are derived from yeast. In yeast, UPF proteins are known to form a complex in which UPF2 serves as a bridge between UPF1 and UPF3 to trigger NMD (Yi et al., 2021). However, there are different branches leading to NMD activation, and degradation of some mammalian targets can proceed independently of UPF2 and/or UPF3 (Yi et al., 2021). A similar behaviour exists between human UPF proteins. In general, UPF1 is considered the master regulator factor of the NMD mechanism. As in other species, in humans it links translation termination to mRNA degradation, because it takes part to most, if not all, steps of the process, from the recognition of the target mRNAs to their enzymatic digestion. UPF1 indeed has an ATPase and ATP-dependent RNA helicase activity, by which its ability to selectively target mRNAs containing PTCs depends. Additionally, the C-terminus of hUPF1 protein contains several serine-rich repeats in which multiple serine residues (S1073, S1077, S1096, S1116) are targets of phosphorylation by hSMG1, a phosphatidylinositol 3-kinase (PI3K) related protein kinase, as will be further explained. Phosphorylation of UPF1 is essential for NMD to occur. Triggering of NMD, indeed, directly relies on UPF1 phosphorylation, since starting from there, translation initiation will be inhibited, and subsequent NMD-specific mRNA degradation machinery components will be recruited. Dephosphorylation of UPF1 is critical: failure to recruit phosphatase PP2A has been shown to lead to an inhibition of NMD. The N-terminus of hUPF1, instead, is rich in negatively charged residues and, in humans, has been shown to undergo dephosphorylating cycles required for UPF1 dissociation from mRNA and the recycling of NMD machinery (Yi et al., 2021) (Figure 11).

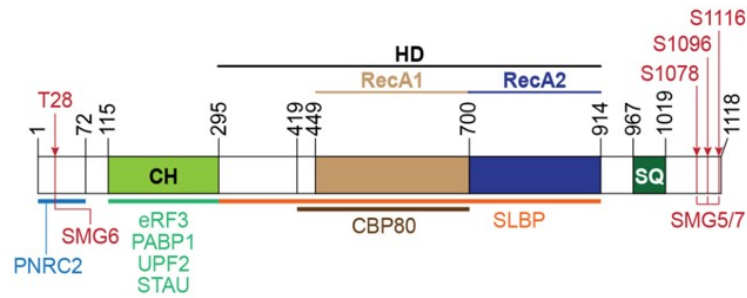


Figure 11. Structure of hUpf1 protein. The figure illustrates the functional domains of human Upf1 protein and binding regions with principal interactors. Black numbers indicate amino acid positions, while red number and arrows indicate phospho-serine residues. CH, cysteine- and histidine-rich domain; HD, helicase domain; SQ, serine- and glutamine-rich domain. Taken from Yoon & Maquat, 2019.

Given its protein structure and the variety of interactions that UPF1 can take with different proteins, UPF core is involved in different cellular processes, making NMD at the crossroad of many cellular pathways (Lejeune, 2017). Besides NMD, in fact, UPF1 has a role in different kind of RNA decay mechanisms, such as replication-dependent histone mRNA decay (HMD) or structure- mediated mRNA decay (SMD); it can also take part in degradation of viral RNAs, for example Rous sarcoma virus (RSV), flaviviruses Hepatitis C virus (HCV) and Zika virus (ZIKV) (Lejeune, 2017). Moreover, in the formation of the NMD-initiating machinery it interacts with proteins of the translation termination complex, such as eRF3 proteins, modulating at different levels the natural occurring mechanism of translation. The central role of the UPF core is underlined by the fact that UPF proteins deletions affect viability in mammals. For example, in mice, Upf1 is essential for embryonic development, while Upf2 depletion in hematopoietic cells causes the loss of all hematopoietic stem and progenitor populations (Weischenfeldt et al., 2008). Also, mutations that lead to aberrant forms of the UPF core have been implicated in numerous types of cancer (Staszewski et al., 2023), as well as neurodevelopmental and degenerative disorders (Staszewski et al., 2023).

SMG proteins. *Suppressor of morphogenesis in genitalia* (SMG) proteins were first identified as allele-specific suppressors of mutations in various *C. elegans* genes (Brognia & Wen, 2009). There are seven SMG proteins that play important roles in the triggering, regulation and working out of the NMD process. SMG1 is a 400 kDa kinase protein that belongs to the phosphatidylinositol-3-kinase related kinase (PIKK) family, that also includes the ATM, ATR, DNA-PKc, and mTOR kinases. They share a

common architecture, in which the C-terminal region is the catalytic module and comprises the so-called FAT, kinase, and FATC domains, and the N-terminal moiety works as a protein-protein interacting module (Yamashita et al., 2005) (Figure 12).

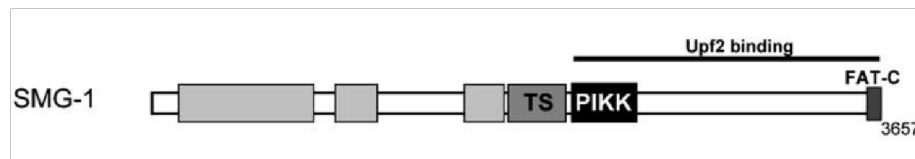


Figure 12. Structure of SMG1 protein. Schematic structure of the SMG1 protein domains. The black boxes indicate the PIKK and FAT-C domains. SMG1-specific sequences are depicted in light gray. Adapted from Yamashita et al., 2005.

As a typical PIKK, indeed, SMG binds to and is regulated by interacting proteins. SMG1 works in association with two subunits, SMG8 and SMG9, that physically associate with its N-terminus forming the SMG1 complex (SMG1c) and keep it inactive in an auto-phosphorylated manner. SMG5-SMG6-SMG7, instead, form protein complexes recruited after UPF1 phosphorylation that can induce RNA decay with up to four different mechanisms. SMG5 and SMG7 regulate UPF1 dephosphorylation cycles, that are thought to be important for proceeding of the NMD towards RNA degradation and recycling of NMD machinery components. SMG6 initiates mRNA degradation by endonucleolytic cleavage; the SMG5-SMG7 dimer, instead, is involved in mRNA degradation after deadenylation and decapping by the carbon catabolite repressor protein 4 (CCR4)–NOT deadenylase and by the decapping complexes, respectively (Yamashita et al., 2005).

1.3.2. Mechanism of NMD

Nonsense mediated mRNA decay is one of the best characterized posttranscriptional quality control mechanisms that cells have evolved to ensure transcriptome fidelity (Popp & Maquat, 2013). As other polymer quality control pathways, such as protein quality control, it generally consists of three main steps: substrate recognition, triggering of the mechanism, and substrate degradation (Popp & Maquat, 2013). It is intimately connected with translation termination process, with whom it also shares numerous components of the protein machinery.

Substrate recognition. To ensure that a NMD event occurs, the translation termination must be recognised as an abnormal or “aberrant” event. mRNAs that can be targeted to NMD arise in a variety of way (Yi et al., 2021). Since NMD is a translational surveillance mechanism, the most obvious targets are mRNAs that contains NMD-inducing PTCs generated via nonsense or frameshift mutations, or random transcriptional errors (Yi et al., 2021). A nonsense mutation is a point mutation responsible of a single amino acidic change that converts a codon specifying for amino acid to a translational termination codon that will arise prematurely in the protein coding sequence (PTC). Frameshift mutations, instead, could be caused by in/dels events that alter the normal open reading frame (ORF) of a transcribed gene, with the downstream creation of a PTC as well. NMD substrates can be also generated by naturally occurring physiological events, such as the somatic recombination at the basis of the VDJ recombination to produce the highly diverse repertoire of antibodies/immunoglobulins and T cell receptors (Lambert et al., 2019). There are other cases in which termination at normal stop codons can be sensed as “abnormal” owing to the presence of naturally occurring features, such as longer 3' untranslated regions (3'UTRs) than average and upstream open reading frames (uORFs) resulting from alternative splicing events, reinforcing the important role of NMD in regulating also endogenous transcripts (Yi et al., 2021).

NMD activation. The precise sequence of the events that trigger the activation of the NMD pathway after substrate recognition are still poorly understood and constantly debated, despite the extensive studies and description of the molecular machineries involved (Kim & Maquat, 2019; Carrard & Lejeune, 2023). In general, newly synthesized pre-mRNAs undergo splicing events that remove introns from the protein coding sequence and connect exons to produce mature, intron-less mRNAs. Therefore, multimeric complexes, namely the exon-junction complexes (EJCs), are deposited onto mature mRNAs each ~20–24 nt upstream of the resulting exon–exon junctions (Kim & Maquat, 2019). Most of the exon-exon junction receives an EJC, and their composition is complex and heterogeneous. EJC core components include the RNA helicase eIF4A3, that functions as an anchor for the EJC and the Y14–MAGOH heterodimer, that regulates eIF4a3 inhibiting its ATPase activity. eIF4a3

activity is also regulated by Barentsz (BTZ a.k.a. MLN51 or CASC3), which enhances its helicase activity. These core components are helped by additional factors including RNA-binding proteins, the DEAD-box protein UAP56, mRNA export factors, and, importantly, UPF2, which is bound to the EJC via UPF3. Other recently identified *trans*-effectors, such as the DEAH- box protein 34 (Dhx34) have been shown to function in human NMD, but their precise molecular roles remain to be determined (Popp & Maquat, 2013).

The resulting mature mRNAs are then exported from the nucleus to cytoplasm carrying the nuclear cap-binding complex (CBC), the EJCs, and other RNA binding proteins with protective and structural functions. Once in the cytoplasm, the mRNAs undergo protein translation with dramatic remodeling of the associated protein complexes. They also contribute to recruit ribosomes to support the pioneer round of mRNA translation (Kim & Maquat, 2019). In a translation event, when the ribosome reaches a termination codon that does not trigger NMD, translation termination is relatively efficient. A complex consisting of two eukaryotic release factors (eRF1 and eRF3) is recruited to the ribosomal A site. eRF3 induces a conformational change in eRF1 and activates hydrolysis of the polypeptide from the poly-peptidyl-tRNA in the ribosomal P site. These processes are promoted by cytoplasmic poly(A)binding protein (PABPC) binding to the amino-terminal region of eRF3 (Carrard & Lejeune, 2023). The PABPC and the 5' cap-bound eukaryotic translation initiation factor 4G (eIF4G), that is associated with the CBC or forms part of the cap-binding translation initiation complex eIF4F, interact to circularize the mRNA, as shown in Figure 13.

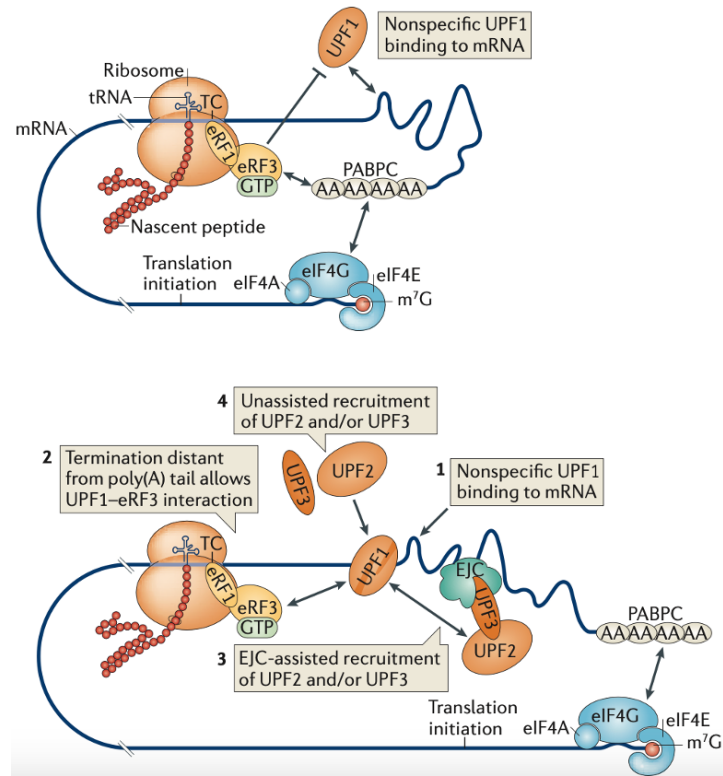


Figure 13. Normal translation of termination (top) vs NMD activation (bottom). On a normal termination of translation event, NMD is prevented by the interactions between the PABPC protein and the eRF3 termination factor that penalize a tight binding of the UPF1 helicase on the targeted mRNA. Thus, an efficient translation termination can occur. In the combined model for NMD activation, when a ribosome encounters a PTC different branches lead to the activation of UPF1 that accumulates on the 3'-UTR, starting NMD and subsequent degradation of the targeted mRNA. At least two important mechanisms are involved in UPF1 recruitment: the presence of an EJC 20-24 nt upstream of the PTC, and a high distance between the PTC and the PABPC. Adapted from LykkeAndersen & Jensen, 2015.

This interaction prevents ATP-dependent RNA helicase activity of UPF1, which only binds transiently and nonspecifically to the mRNA, from interacting with eRF3 when the termination codon is proximal to the 3' of the mRNA (Lykke-Andersen & Jensen, 2015). Indeed, possibly every termination event during the pioneer round of translation has the potential to involve UPF1. UPF1 has been shown to bind transiently to RNAs, with no particular sequence specificity and with no apparent discrimination between PTC-containing mRNAs and other RNAs (Kurosaki et al., 2013, Lykke-Andersen & Jensen, 2015). As introduced before, the UPF1 helicase domain has RNA binding, ATPase, and RNA helicase activities. Thanks to its intra-molecular interactions and protein-protein interactions with its regulator complexes, UPF1 can undergo cycles of ATP binding and hydrolysis that determine conformational changes that make it switching from a 'loosely' RNA-bound state to more tightly RNA-bound form. (Yi et al., 2021). Even if UPF1 binds indiscriminately various kinds

of RNA, UPF1 interactions are primarily detected in the 3' UTR of actively translated mRNAs, where it binds the eRF1-eRF3 complex at a termination codon (Kurosaki et al., 2013; Lykke-Andersen & Jensen, 2015). Target discrimination may occur thanks to the ATPase activity that promotes UPF1 dissociation from non-NMD target RNAs (Kurosaki et al., 2013). Without the tight bind of UPF1 to the eRF3 complex, the elongating ribosome can easily displace the EJC that remain after RNA splicing. In this way, efficient translation can occur. At least two models of NMD activation have been described: the EJC-dependent model and the faux 3'UTR model (Lejeune, 2017; Carrard et al., 2023). In the EJCdependent model, mammalian NMD is proposed to occur during the early rounds of translation when transcripts remain associated with the nuclear CBC; the faux 3'-UTR model, instead, will occur whenever the CBC has been replaced by the cytoplasmic cap-binding protein EIF4E (Amrani, 2001; Ishigaki et al., 2001; Rufener & Mühlemann, 2013). These two nonmutually exclusive NMD-promoting features may function by increasing the probability that ribosome pauses at PTCs significantly longer than it does at normal termination codons, thus increasing the likelihood that a termination event is recognized as aberrant during each round of translation (Yi et al., 2021). The current version of the 'EJC-dependent model' relies on the fact that if the PTC is located more than 50-55 nt upstream of the last exon-exon junction, it will enhance NMD (Brognia & Wen, 2009, Lykke-Andersen & Jensen, 2015; Lejeune 2017; Yi et al., 2021). The EJC placed 20-24 nt upstream of the exon-exon junction after the splicing event will be in close contact with the stalled ribosome on the PTC. Through UPF3 and UPF2 factors, EJC promotes recruitment and activation of UPF1 on the ribosome, initiating the formation of the so-called SURF complex, composed by SMG1c (SMG1 plus its regulatory subunits SMG8 and SMG9), UPF1 and eRF3, the NMD-initiating complex. This model of NMD is consistent with the proposal that in mammalian cells the mRNA is susceptible to NMD only during the pioneer round of translation, since it has been shown that UPF1 is recruited by the EJC either directly as part of the SURF complex or interacting with the cap binding proteins of the CBC (CBP20/80) (Lejeune, 2017, Yi et al., 2021). Although very descriptive, this model does not fit all cases: for example, EJCs cannot be present upstream of all

exon-exon junctions; moreover, some PTCs located less than 50 nt upstream trigger NMD, while PTCs located more than 50 nt do not. So, a second model has been proposed, based on the distance between the PTC and the PABPC1 (Figure 13, panel B) (Broga & Wen, 2009; Lejeune, 2017). The presence of a PTC on a mRNA sequence determines a 3'-UTR artificially longer than the normal, so that the abnormal distance between the stalled ribosome with the eRF complex and the PABPC1 protein constitute a signal for the degradation of the targeted mRNA. In this model UPF1 directly binds to mRNA concentrating on the 3'-UTR, thus competing with the PABPC1 protein for the binding to eRF3. This will determine the switching from a normal translation event to NMD. In this case, NMD can occur during all cycles of translation, independently from the presence of an EJC (Lejeune, 2017). However, this model suffers of some experimental paradoxes: for example, such distance-dependence would suggest a gradient of NMD efficiency, that would decrease with the increasing in proximity of the PTC with the physiological termination codon (and thus with the PABPC). However, such gradient has never been observed. So, a combinatory model has been proposed, in which NMD is triggered by many branches that all converge to recruitment and activation of UPF1 (Lejeune, 2017; Yi et al., 2021). Thus, both the persistence of EJCs and the distance between the PTC and the PABPC protein are two successive steps that initiates NMD. EJCs will recruit UPF1 at an earlier stage during the first round of translation, while later the accumulated UPF1 at the 3'-UTR will further enhance NMD displacing translating ribosomes from the eRF3 factor (Lykke-Andersen & Jensen, 2015).

Substrate degradation. As summarized in Figure 14, regardless the way NMD is triggered, the essential feature of the mechanism is the activation of UPF1 through phosphorylation at its several serine/threonine/glutamine motifs (S/T)Q, which is carried out by the SMG1 kinase. UPF1, together with eRF1 and eRF3, interacts with SMG1c to form the SURF complex, that hence is composed by UPF1, SMG1 together with its SMG8-SMG9 regulatory heterodimer and the scaffold protein DHX34. This latter protein facilitates interactions between the SURF complex and UPF2-UPF3 complexes, leading to formation of the decay-inducing (DECID) complex and further UPF1 phosphorylation.

Phosphorylated UPF1 binds to EIF4E and thus inhibits new cap-dependent translation while functioning as a binding platform for SMG5/SMG7 and SMG6 proteins, the effectors that initiate mRNA decay and mediate UPF1 dephosphorylation via PP2A phosphatase (Lykke-Andersen & Jensen, 2015, Yi et al., 2021). mRNA degradation can occur by endonucleolytic cleavage (by SMG6), deadenylation (by the CCR4–NOT deadenylase complex via the SMG5–SMG7 dimer) and/or decapping by the decapping complex (DCPC). Either endonucleolytic cleavage, decapping and/or deadenylation are followed by complete mRNA degradation by general cellular 5'–3' and 3'–5' exonucleolytic activities (Lykke-Andersen & Jensen, 2015).

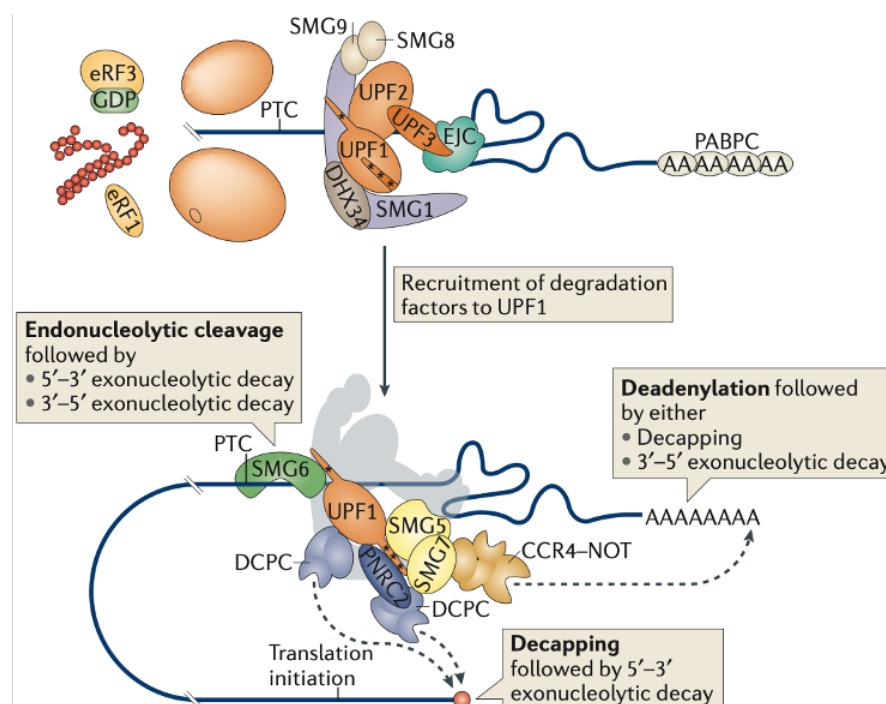


Figure 14. UPF1 hyperphosphorylation leads to mRNA degradation. The ultimate task of the SURF and DECID complex together is the activation of Upf1 helicase activity by phosphorylation on multiple serine/threonine residues (indicated with asterisks in the figure). Among the numerous functions of phosphorylated UPF1 there is the recruitment of degradation factors that will lead to mRNA degradation by multiple routes, as depicted in the figure. Adapted from Lykke-Andersen & Jensen, 2015.

1.4. Pharmacological strategies to treat nonsense mutations in cystic fibrosis

Nonsense mutations are often associated with the most severe form of CF due to the complete abrogation of CFTR protein. Current HEMT relies on the possibility to combine different CFTR modulators (i.e. correctors and potentiator) that require at least partial CFTR expression (Sharma et al., 2020). Thus, while current HEMT represent a feasible therapeutic option for many CF patients, no pharmacological treatments are available for individuals who carry nonsense mutations. Targeting PTCs still represents an unmet clinical need in CF. However, the therapeutic interest in rescuing of PTCs goes beyond CF, since other severe genetic diseases interested by nonsense mutations (long-QT syndrome, Duchenne muscular dystrophy, Usher syndrome, and Miyoshi myopathies among others) are currently orphans of interventional strategies.

1.4.1. Translational read-through

A possible strategy to overcome PTCs is the pharmacological stimulation of translational read-through (RT) (Figure 15).

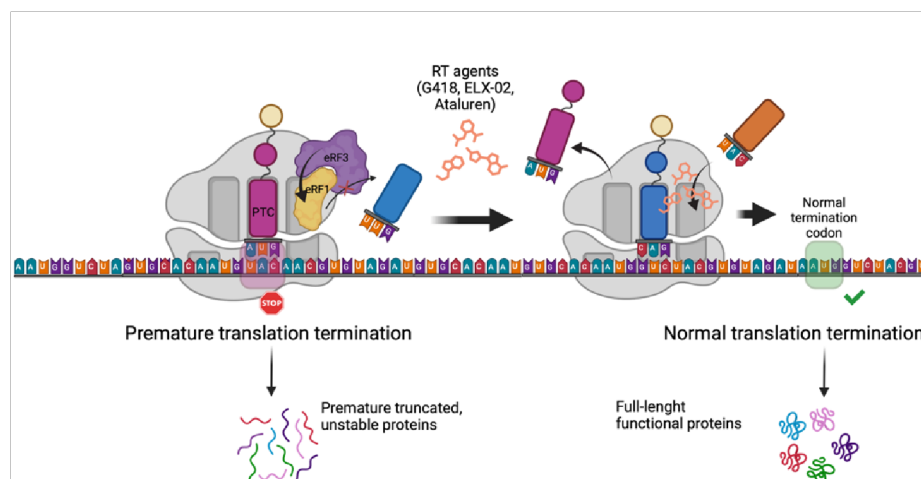


Figure 15. Pharmacological RT agents over-read PTCs. Read-through of PTCs can be pharmacologically stimulated by compounds that act on the stalled ribosome, reducing its proofreading ability and increasing the probability it can insert in the elongating polypeptide a near-cognate aminoacyl-tRNA in the place of the stop codon, allowing the continuation of the protein synthesis. Image created with Biorender.com.

Given the redundancy of the genetic code, it can happen that a termination event can be suppressed at a stop codon site by the misleading

incorporation of aminoacyl-tRNAs into the ribosomal A-site. At a low frequency, indeed, near-cognate tRNAs similar to stop codons can outcompete the translation termination factor, eRF1 for binding to A-site bound termination codon. This brings a random amino acid to be incorporated into the polypeptide chain in the place of the stop codon, allowing the translation elongation to continue in the correct reading frame until the next in-frame stop codon (Sharma et al., 2020). The frequency of RT at a PTC site is approximately 10-fold greater (0.01–1%) than the readthrough rate at NTCs (0.001–0.1%) (Sharma et al., 2020). Such a natural “over-reading” of termination codons (Lopes-Pacheco, 2020) can be artificially enhanced for therapeutic purposes to correct PTCs by drugs and compounds that act on the stalled ribosome at the PTC site. Several small molecules have been identified acting as pharmacological RT agents through various MoA (Spelier et al., 2023). Aminoglycosides (AAGs) antibiotics, such as gentamycin and geneticin (G418), represent the first proof-of-principle pharmacological RT agents (Burke & Mogg, 1985; Bedwell et al., 1997). CF is the first genetic disease model in which this approach has been tested (Sharma et al., 2020). Gentamicin has been and is, indeed, commonly used to eradicate *P. aeruginosa* infection in CF patients. In cell lines and transgenic mouse models, AAGs promoted the expression of full-length CFTR and partially restore chloride function (Howard et al., 1996; Du et al., 2002; Lopes-Pacheco 2020). In people carrying PTCs in the CFTR chloride channel gentamicin improved nasal potential difference after administration in the nasal mucosa or intravenously (Lopes-Pacheco, 2020). AAGs demonstrated PTC suppression and increased protein expression also in people with Duchenne muscular dystrophy (DMD) carrying nonsense mutations in the dystrophin gene (Malik et al., 2010). However, AAGs treatment showed no long-term clinical benefit in pilot clinical trials in CF (Sharma et al., 2020). Moreover, pharmacological activity of AAGs also involve off-target proteins, such as mitochondrial ribosomes and lysosomal membranes, leading to renal and ototoxicity effects that are not compatible with prolonged administration for treating of life-long genetic diseases (Sharma et al., 2020). Ataluren (a.k.a. PTC124 or Translarna) is a small molecule identified in HTS (Welch et al., 2007) in an attempt to find more potent and safer RT agents to treat CF and other genetic

diseases. Ataluren showed good safety profile and absence of antibacterial activity; it demonstrated to restore dystrophin expression in human primary muscular cells and mouse models of DMD (Mercuri et al., 2023) but failed to restore enough CFTR function to improve lung function in pwCF in different phase III studies (Lopes-Pacheco, 2020; Spelier et al., 2023). Although their precise MoA is still not clear, RT agents possibly act reducing the proof-reading ability of the ribosome and increasing the time it spends at PTC site and insert a near cognate tRNA in the place of the stop codon, allowing continuation of the protein synthesis. Ng et al. proposed different MoAs for both Ataluren and G418: Ataluren is proposed to inhibit the activity of eRFs, while G418 promotes the interaction between the PTC mRNA and the near-cognate tRNA (Ng et al., 2021; Morais et al., 2024). More recently, a medicinal chemistry approach has been used to synthesize aminoglycoside derivatives with less adverse effects. Eloxx Pharmaceuticals promoted the clinical development of ELX-02, formerly identified as NB124 (Xue et al., 2014). This molecule show less toxicity than conventional aminoglycosides; it also rescued around 7% of the CFTR-WT function in human bronchial epithelial cells (Lopes-Pacheco, 2020, Sharma et al., 2020). From 2019, phase II clinical trials are in progress to evaluate the effects of multiple doses of ELX-02 in CF patients carrying the G542X mutation in at least one allele (NCT04126473, NCT04135495).

Pharmacological RT is further challenged by multiple factors that influence the efficacy of most drugs irrespectively of their MoA. The main determinant is the NMD process that degrades aberrant mRNAs containing PTCs, reducing the pool of PTC-containing transcripts subjected to RT correction (Sharma et al., 2020). PTC identity, location, and sequence context, together with the characteristics of the affected protein also influences RT efficacy (Spelier et al., 2023, Morais et al., 2024). For example, the pattern of amino acid incorporation at a PTC site is influenced by the local mRNA surrounding sequence. Furthermore, the random aminoacidic insertion can affect the protein stability, folding and/or function of the RT product (Sharma et al., 2020). CFTR is a seminal example. Indeed, different aminoacidic incorporations take place during G418-mediated RT of the CFTR protein, with cysteine, tryptophane and arginine as the preferentially inserted at the G542X position, while

leucine, cysteine or tryptophane at the W1282X, both sharing TGA as stop-codon (Roy et al., 2016, Xue et al., 2017). Thus, beside the intrinsic efficacy of the distinct drugs acting as RT compound, pharmacological interventions must consider these limitations to RT activity. For this reason, combinations of different approaches are being tested to overcome these challenges.

1.4.2. Nonsense mediated mRNA decay inhibitors

Ideally, inhibition of NMD could potentially enhance the availability of mRNAs with PTCs substrate of RT action. NMD inhibition could hence have an additive effect when combined with RT agents, leading to an increased production of full-length proteins (Morais et al., 2024). Currently, strategies inhibiting NMD encloses small molecules used as pharmacological inhibitors, like amlexanox (Gonzalez-Hilarion et al., 2012), NMDI-1 (Durand et al., 2007), NMDI-14 (Martin et al., 2014) SMG1i (Gopalsamy et al., 2012), and nucleic acid-based approaches, such as small interfering RNAs (siRNA), tRNA suppressors and antisense oligonucleotides (ASOs) that specifically target NMD factors (Sharma et al., 2020). Multiple evidence of synergy between NMD inhibitors and RT agents have been shown for mouse models of mucopolysaccharidosis IHurler (MPS I-H) and hemophilia, and in immortalized human bronchial cell line genetically modified to express the CFTR- W1282X mutation (16HBEge cell line). However, as previously explained, caution must be taken in considering strategies that suppress NMD, since this mechanism also regulates the levels of many physiological transcript, taking part to different cellular pathways as well as embryonic development (Sharma et al., 2020).

1.4.3. Small molecules with novel mechanisms

Novel investigational drugs that target protein truncation caused by PTC are continuously being identified. A promising example is represented by the so-called molecular glues (Tan et al., 2007). This term generally refers to a class of chemical compounds that can bind and stabilize proteinprotein interactions in biological systems just acting as "glue", by enhancing the

affinity between proteins and ultimately influencing various cellular processes. Molecular glues have specific binding sites for their target proteins and can encourage their association to proteins with which they do not usually interact. Molecular glue can also enhance the stability of protein complexes, making them more resistant to dissociation (Sasso et al., 2023). In the last years such compounds have gained increasing attention, since they have the potential for valuable therapeutic options regarding disease-causing conditions previously thought to be undruggable. In particular, they have become a widely utilized approach for targeted protein degradation (Lu et al., 2024). The recognition of substrates by E3 ubiquitin ligases, governed by protein-protein interactions, plays a critical role in cellular function. In 2013, the mechanism of thalidomide analogs as molecular glue degraders was revealed. Indeed, thalidomide and its derivative lenalidomide have been discovered as a cereblon (CRBN)-E3 ubiquitin ligase modulator. Since then, many different chemical derivatives of such immunomodulatory (IMiDs) drugs have been proposed as molecular glues acting on CRBN ligase (Nishiguchi et al., 2021; Hansen et al., 2021; Coelho et al., 2024). Two of them have been showed having PTCs RT activity. BaradaranHeravi and his group identified two molecular glue compounds, CC-885 and CC-90009, acting as CRBN modulators capable of promoting proteasomal degradation of eRF3 factors participating both to termination of translation and NMD process (SURF complex), as summarized in Figure 16 (Baradaran-Heravi et al., 2021). CC-90009 was recently found to rescue PTC-CFTR, particularly in combination with RT agents, thus suggesting a possible action as an NMD inhibitor (NMDi) (Lee et al., 2022).

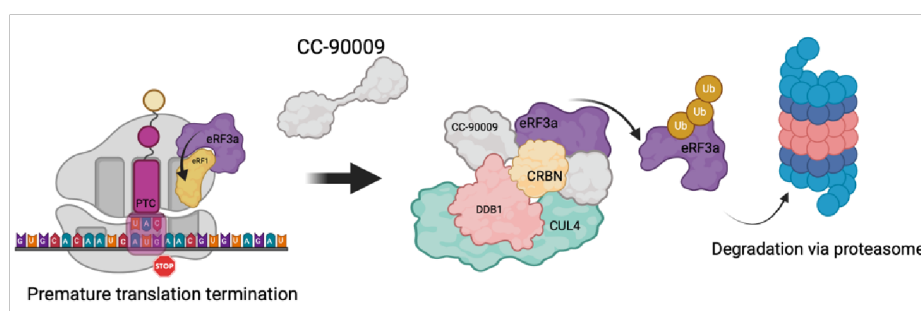


Figure 16. CC-90009 is a novel identified CRBN modulator with PTC suppression activity. Compounds that act as molecular glues have been showed having RT activities. CC-90009 is a CRBN modulator that favors the binding of eRF3a factor of the translation termination machinery to CRBN E3 ligase. This leads to eRF3a ubiquitination and subsequent degradation, with suppression of PTCs. Image created with Biorender.com.

In another study, Coelho and colleagues recently proposed that the small molecule SRI-41315 molecule identified as an eRF1 degrader can promote translational RT of PTCs (Coelho et al., 2024). Sharma et al. showed that SRI-41315 depletes eRF1 in immortalized and primary human bronchial cell lines, thus demonstrating to rescue PTC-CFTR function (Sharma et al., 2021).

1.5. Inflammation in CF

Although the pathophysiology of the CFTR loss of function is well established, it is still not clear if the functional abnormalities of the airways of pwCF are a direct consequence of the primary defect or are more effects of subsequent infections and inflammation. CF airways are characterized by persistent inflammation sustained by the release of proinflammatory mediators. Recently, CF has been defined as a Th17-related disease, since IL-17A has been found elevated in the sputum and in the lung of pwCF and correlated with bacterial infection, neutrophilic infiltration, lung damage and lung function decline (Decraene et al., 2010; Hagner et al., 2021). Recently, several studies started to evaluate the remodeling of epithelial properties by Th17 inflammatory cytokines, particularly the combination of IL-17A with TNF- α (Rehman et al., 2021; Guidone et al., 2022). It is well known, indeed, that the airway epithelium can reshape its physiological properties in response to inflammatory stimuli mediated by cytokines and chemokines to accomplish its defense functions. Studies on Th2 cytokines showed that IL-4 and IL-13, that are involved in allergic pulmonary inflammation and airway hyperreactivity in asthma, share the same IL-4R α receptor, that leads to the same signaling cascade and biological activities (Galietta et al., 2002). These include change in the overall architecture of the epithelium, with a major hypersecretory phenotype, characterized by strongly reduced ciliation and goblet cell metaplasia with mucus hypersecretion (Galietta et al., 2002; Danahay et al., 2002; Lachowicz-Scroggins et al., 2010; Scudieri et al., 2012). Moreover, these cytokines promote the activity of Cl⁻ and HCO₃⁻ transporters. IL-4 upregulates NKCC1 in the basolateral membrane, coupled with upregulation of SLC26A4, CFTR and

TMEM16A in the apical membrane (Galiotta et al., 2002; Pedemonte et al., 2007; Gorrieri et al., 2016) and downregulation of the γ and β ENaC subunits, thus decreasing Na^+ absorption. These changes in the channel composition of the epithelium lead to a general ASL hydration and net HCO_3^- secretion, that may favor mucus expansion and release. Overall, MCC and airway clearance is improved together with alkalinization of ASL pH, in order to remove pathogens in the secreted mucus supported by antimicrobial activity (Gustaffson et al., 2012; Hoegger et al., 2014; Gorrieri et al., 2016; Scudieri et al., 2018). Acidification of ASL in CF lungs is still an open question. Enhanced ATP12A H^+/K^+ pump expression has been showed in vitro after treatment with IL-4/IL-13 (Gorrieri et al., 2016; Scudieri et al., 2018; Lennox et al., 2018). The resulting H^+ secretion mediates ASL acidification, that seems in contrast with the high HCO_3^- secretion rate in IL-4 treated epithelia (Gorrieri et al., 2016).

However, it has been reported that ASL is acidic in CF neonates (Abou Alaiwa et al., 2014) and newborn CF pigs compared to non-CF, but it normalizes after few months (Schultz et al., 2017). Rehman and colleagues hypothesized that Th17 inflammation increases ASL pH over time, enhancing HCO_3^- secretion (Rehman et al., 2021). The same group demonstrated that IL-17 plus TNF- α combination alkalinizes ASL pH by increasing HCO_3^- secretion (Rehman et al., 2020; Rehman et al., 2021). Importantly, upon stimulation with cytokines, alkalinization is driven also by enhanced CFTR activity (Rehman et al., 2020) and an increase in pH is also achieved in F508del-CFTR bronchial cells treated with the triple modulator combination (Rehman et al., 2021). Similar results were shown by a recent work by our group, where in vitro treatment of human bronchial epithelial cells with IL-17A plus TNF- α combination resulted in hyper viscosity of the airway surface. Importantly, stimulation with a β adrenergic agonist switched airway surface to a low-viscosity state in non-

CF but not in CF epithelia (Guidone et al., 2022). These findings intriguingly suggest that, despite excessive inflammation may lead to lung damage (Higgins et al., 2015), suppressing inflammation may impact on modulators efficacy. Accordingly, several studies are now starting to evaluate modulators efficacy upon inflammatory stimuli on different CFTR mutations. A recent work by Gentzsch and colleagues further

confirm the enhancing effect of inflammatory environment on the pharmacological correction by ETI on N1303K-CFTR (Gentzsch et al., 2024). In the conclusions, the authors point out the necessity to consider the impact of inflammation on the efficacy of CFTR correction by modulators and intriguingly speculate that this could potentially increase the number of mutations that qualify for HEMT (Gentzsch et al., 2024). Regarding nonsense mutations, there are no indications of whether inflammation could influence pharmacological correction of PTCs-CFTR. To knowledge, only two recent studies report direct involvement of inflammatory cytokines (IL-1 β , INF γ and TNF- α) in the regulation of the NMD mechanism in INS1(832/13), EndoC-bH3 pancreatic cell lines or dispersed human islet cells (Ghiasi et al., 2018, Ghiasi et al., 2024).

1.6. Aim of the thesis

The complexity and the partial knowledge of the RT mechanism and the NMD machinery makes it very difficult to identify the most adequate pharmacological strategy to rescue PTCs in CF. The overall aim of my thesis was the study of pharmacological small molecules to identify novel treatments to restore CFTR defects due to nonsense mutations. Initially, we tested several combinations of compounds on a panel of CF-causing PTCs localized at different positions of the *CFTR* coding sequence. Our goal was to identify the minimal combination of drugs producing the most effective rescue of CFTR function for each mutation. As result of our work, we published a paper in which we conclude that PTC rescue requires a mutation-specific approach (Venturini et al., 2021). Indeed, we showed that PTCs arising at different positions of the CFTR coding sequence responded differently to the same pharmacological treatments. Results were particularly interesting for the G542X-CFTR and W1282XCFTR mutations. For the W1282X-CFTR we obtained the maximal rescue combining correctors with NMD inhibitors (NMDi) in absence of RT agents. This means that NMDi alone can rescue W1282X-CFTR mRNA level at an extent such that, even if mutated, it could still synthesize a functional channel protein if adequately supported by correctors and potentiators. This notion led us to envisage the possibility to set up a screening of large chemical libraries to find novel small molecules acting

as NMDi. As introduced before, a total blockade of the NMD machinery is not compatible with life. The first specific aim of my thesis is then to find novel drugs that achieve a partial modulation of this machinery that may contribute to PTC rescue avoiding the undesired effects associated with total knockdown of the process. Identification of novel NMDi acting through alternative mechanisms could be also more compatible with RT agents achieving synergistic effect. On the other side, combination of RT agents and NMD inhibitor with CFTR modulators resulted in a significant but relatively modest increase in G542X-CFTR function, where unexpectedly the NMD inhibitor did not contribute to improving CFTR activity when added to the combination, despite a large effect on mRNA levels was detected by real time RT-PCR. Given the recent works on the effects of inflammatory stimuli on CFTR function, as a second aim of the thesis, we asked if and how inflammation could influence also pharmacological correction of PTCs in CFTR.

2. MATERIALS AND METHODS

Part of the following materials and methods are included in Venturini et al., 2021.

2.1. Compounds and chemicals

SMG1 inhibitor (SMG1i) was provided by the Cystic Fibrosis Foundation (CFF) and dissolved in dimethyl sulfoxide (DMSO). ELX-02 disulfate (HY-114231B) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). For each experiment, the precise quantity of ELX-02 powder was weighed and dissolved directly in the culture medium to reach a final concentration of 0.5 mg/ml (720 μ M) or 0.138 mg/ml (200 μ M) where indicated. G418 sulphate powder (Euroclone, Pero, Italy) was dissolved in Dulbecco's phosphate-buffered saline (Euroclone) at a concentration of 50 mg/ml, filtered and stored at +4 °C. For cell treatment, G418 stock was diluted in the medium to a final concentration of 0.5 mg/ml. VX-809 (Lumacaftor), VX-445 (Elexacaftor), and PTI-428 were purchased from MedChemExpress. VX-770 (Ivacaftor) and CFTR_{inh}-172

were purchased from Selleckchem (Houston, TX, USA). CC-90009 (CAY-33692-5) was purchased from Cayman Chemicals (Vinci- Biochem). All these compounds were dissolved in DMSO at a concentration of 10 mM. The Bioactive Molecule Library (MCE HY-LD000002384) was purchased by MedChemExpress. Compounds were dissolved in DMSO as detailed described in section 2.5. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), including IL-17A (used at a final concentration of 20 ng/ml, SRP3080, Sigma-Aldrich), TNF- α (10 ng/ml, T6674, Sigma-Aldrich) and IL-4 (10 ng/ml, 14269, Sigma-Aldrich).

2.2. Cell Culture

16HBE14o⁻ immortalized bronchial epithelial cells gene-edited to express different PTCs (i.e.: Y122X, G542X, R553X, R1162X, and W1282X) were obtained from the Cystic Fibrosis Foundation (CFF). The introduction of different nonsense mutations by gene editing directly at the CFTR locus makes them an ideal model, since the normal gene expression regulation and the exon-intron structure are preserved, and these features are considered necessary to achieve reliable PTC correction (Valley et al. 2019; Lejeune, 2019). Also, these cells can form electrically tight epithelia suitable for short-circuit current (I_{sc}) recording studies to investigate CFTR function (Gianotti et al., 2018). Cells were cultured at 37 °C in 5% CO₂ in minimal essential medium (MEM, Euroclone) supplemented with 10% fetal bovine serum (Euroclone), 2 mM Lglutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. All flasks and plastic supports were coated with a solution consisting of LHC-8 basal medium, 1.34 µl/ml of bovine serum albumin (7.5%), and 10 µl/ml bovine collagen solution Type I (Advanced BioMatrix, Carlsbad, CA, USA). The coating solution was left for 2–3 h at 37 °C and then removed. Coated flasks and all the plastic supports were stored at 4 °C until use. For 16HBE14o⁻ freezing and sub-culturing, we followed the instructions provided by CFF.

HEK-293 cells were cultured in a mixture of Dulbecco's Modified Eagle Medium (DMEM, high glucose version) and Ham's F12 (1:1), supplemented with 10% fetal bovine serum (Euroclone), 2 mM Lglutamine, 100 U/ml penicillin, 100 µg/mL streptomycin.

Generation and culture of CFBE41o⁻ bronchial epithelial cells expressing

F508del- CFTR and the halide-sensitive yellow fluorescent protein (HSYFP) was previously described (Sondo et al., 2011).

Primary human bronchial epithelial cells (HBECs) are considered an excellent system model to study many aspects of airway epithelium function and diseases (Fulcher et al., 2005). When cultured on porous filters and allowed to differentiate under air liquid interface (ALI) condition (i.e. no culture medium on the apical side), they can form a pseudostratified epithelium that maintains most of the morphological and functional characteristics of the airway epithelium *in vivo*, thus serving as a more physiological tissue model for *in vitro* studies (Gianotti et al. 2018). Previous studies (Scudieri et al. 2012; Gianotti et al. 2018; Scudieri et al., 2020) optimized protocols describing the complete procedure and key steps for isolation and expansion of basal airway stem cells (p63+/KRT5+) with a serum-free medium. HBECs from non-CF individuals and G542X/1717-1G >A and G542X/G542X patients were isolated in our laboratory. HBECs from a W1282X/W1282X patient were kindly provided by the Cystic Fibrosis Foundation Laboratory. Bronchial cells were cultured in a serum-free medium (LHC basal medium/RPMI 1640, Thermo Fisher Scientific, Waltham, MA, USA) with the addition of a cocktail of various hormones and supplements (a detailed preparation of this medium is described in Scudieri et al., 2012). To further promote proliferation of basal stem cells, the medium was supplemented with a BMP antagonist (DMH-1; Tocris, Bristol, UK), a TGF- β antagonist (A 83-01; Tocris), and a ROCK1 inhibitor (Y-27632; Tocris). After four to five passages, cells were seeded at high density (5×10^5 cells/cm²) onto Snapwell or Transwell porous inserts (cc3801 and cc3470, respectively, Corning Costar). After 24 h from seeding, the proliferative medium was switched to differentiation medium (PneumaCult ALI, Stemcell Technologies) to induce mucociliary differentiation and the medium on the apical side was removed to obtain the ALI condition. The cells were directly kept ALI for 2–3 weeks. When needed, cells were treated basolaterally with drug combinations and for 72 h with IL-17A (20 ng/ml) plus/minus TNF- α (10 ng/ml) and IL-4 (10 ng/ml). Nasal epithelial cells were collected by nasal brushing at the Bambino Gesù Pediatric hospital (Rome) and shipped to our laboratory. Nasal cells were expanded and used

for the generation of differentiated epithelia similarly to bronchial epithelial cells.

2.3. Short-Circuit Current (I_{sc}) Recordings

16HBE14o⁻ cells were seeded at high density ($5 \times 10^5/\text{cm}^2$) onto Snapwell permeable supports, with 12 mm diameter, 0.4 μm pore size (3801, Corning Costar). Submerged cultures of cells were kept for a week by changing the medium daily on both sides of the Snapwell supports. After 6 days, transepithelial resistance (R_t) was measured to assess formation of electrically tight epithelia. Epithelia with high R_t , comprised between 400 and 1000 Ω/cm^2 were used for experiments. Twenty-four hours before the experiments, cells were treated with compounds or vehicle added in the culture medium on both sides (apical and basolateral) of epithelia. After treatment (day 7 after plating), the Snapwell supports carrying 16HBE14o⁻ epithelia were inserted in P2300 vertical chambers and mounted in an 8Channel EasyMount Ussing Chamber System (Physiologic Instruments, San Diego, CA, USA) as depicted in Figure 17. The apical and basolateral hemi-chambers were filled with solutions of different composition to create a Cl^- gradient. The apical chamber was filled with a 5 ml Ringer solution containing (in mM): 63 NaCl, 63 sodium gluconate, 0.38 KH_2PO_4 , 2.13 K_2HPO_4 , 2 CaCl_2 , 1 MgSO_4 , 20 Na-HEPES, 10 glucose and phenol red. The basolateral chamber was instead filled with 5 ml of a solution containing (in mM): 126 NaCl, 0.38 KH_2PO_4 , 2.13 K_2HPO_4 , 1 CaCl_2 , 1 MgSO_4 , 20 Na-HEPES, 10 glucose and phenol red. Both solutions were adjusted to a pH of 7.4. The apical and the basolateral solutions were continuously bubbled with air, and the temperature of the solution was kept at 37 °C.

For HBECs, symmetrical bicarbonate-buffered solution was used. The composition of this solution was (in mM): 126 NaCl, 0.38 KH_2PO_4 , 2.13 K_2HPO_4 , 1 MgSO_4 , 1 CaCl_2 , 24 NaHCO_3 , 10 glucose and phenol red. Both sides were continuously bubbled with a gas mixture containing 5% CO_2 /95% air, and the temperature of the solution was kept at 37 °C.

Transepithelial voltage was short-circuited with a voltage clamp (VCC/MC8, Physiologic Instruments, San Diego, CA, USA) connected to the apical and basolateral chambers via Ag/AgCl electrodes and agar

bridges (1 M KCl in 2% agar). The offset between voltage electrodes and the fluid resistance was cancelled before experiments. The short-circuit current was recorded with an Acquire & Analyze U100 (Physiologic Instruments, San Diego, CA, USA) analog-to-digital converter connected to a computer and visualized by the Acquire & Analyze 2.3 software.

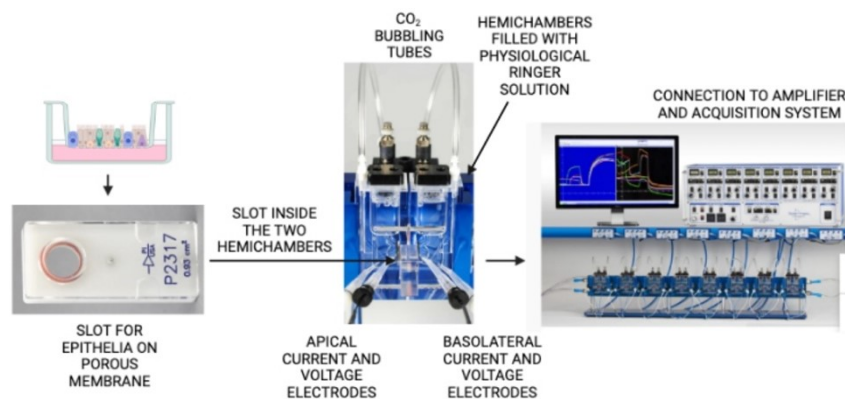


Figure 17. Instruments for short-circuit current recordings. The porous supports carrying the differentiated airway epithelia were mounted on a specific slot for Transwell or Snapwell and inserted in the vertical Ussing-like chamber, between the apical and basolateral hemi-chambers, both filled with HEPES- or bicarbonate-buffered Ringer solutions and connected to voltage-sensing and current-passing electrodes. Two connecting tubes allowed 5% CO₂/95% air bubbling, and the chambers were heated to 37 °C. Every chamber was connected to a pre-amplifier and to the acquisition system. Created with Biorender.com

2.4. Halide-sensitive YFP (HS-YFP) assay

We generated a fluorescent version of the 16HBE14o⁻ carrying W1282XCFTR by stably expressing the HS-YFP through lentiviral infection. Either the 16HBE14o⁻ cells with the W1282X-CFTR or CFBE41o⁻ with the F508del-CFTR, both also expressing HS-YFP, were plated at high density on bottom clear, black wall 96-well microplates (3603, Corning Costar).

After 24 h, cells were treated with the indicated compounds or vehicle. After a further 24 h, CFTR activity was determined with the HS-YFP assay as previously described (Sondo et al., 2011, Pedemonte et al., 2020). The experimental scheme is summarized in Figure 18. In brief, culture medium was removed, and cells were washed three times with complete PBS. Then, to maximally stimulate CFTR activity, 60 µl of PBS solution supplemented with forskolin (FSK) (20 µM) or CPT-cAMP (100 µM) plus VX-770 (1 µM) were added cells in each well and the plate was incubated for 20–25 min at 37 °C. The microplates were then transferred to a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg,

Germany) for CFTR activity study. The plate reader was equipped with high-quality excitation and emission filters for YFP (ET500/20x and ET535/30m, respectively) (Chroma Technology, Rockingham, VT, USA). HS-YFP fluorescence was recorded, with 0.2 s sampling time, for 2 s before and 12 s after injection of 165 μ l of an iodide-containing solution (PBS with Cl^- replaced by I^- ; final I^- concentration: 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine the fluorescence quenching rate (QR), caused by iodide influx through CFTR, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope (dF/dt).

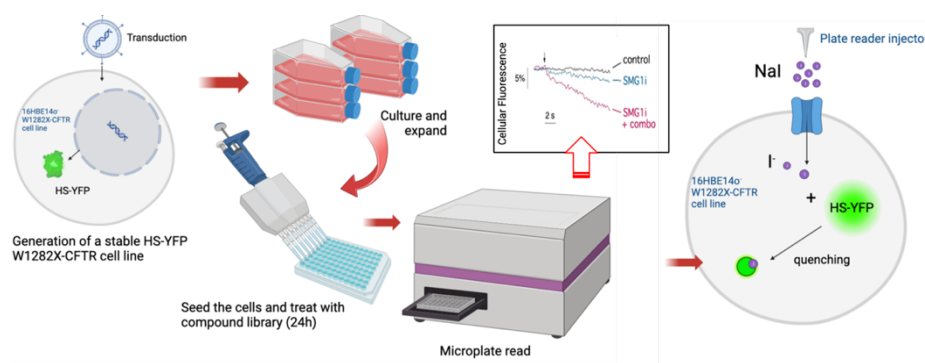


Figure 18. Scheme of HS-YFP assay for 16HBE14o⁻ cells with W1282X-CFTR. A fluorescent version of the 16HBE14o⁻ cells with W1282X-CFTR was created by stably expressing the HS-YFP protein through lentiviral infection. Cells were expanded and seeded at high density onto 96-well microplates. After 24 h treatment with desired compound combinations, CFTR activity was determined with a microplate reader. Briefly, CFTR function is estimated as the quenching rate of HS-YFP resulting from iodide uptake. Image created with Biorender.com

2.5. Screening of the MedChem-Express Library

We screened the MedChemExpress Bioactive Compounds Library (9,351 small molecules) already available in our laboratory. The library is arranged as 80 compounds per plate in 96-well microplates with a concentration of 10 mM in DMSO. The library encloses approved drugs, investigational drugs evaluated in clinical trials, natural compounds, and many pharmacological modulators with known biological targets (i.e., mechanistic probes). In detail, to screen the Bioactive Compounds Library, 16HBE14o⁻ with W1282X-CFTR stably expressing the HS-YFP protein were seeded at high density in 96-well microplates. After 24 h, cells received test compounds (5 μ M final concentration; 80 compounds/plate), vehicle (DMSO 0,28% as negative control; 8 wells), or 1 μ M SMG1i (as

positive control; 8 wells). The 24 h treatment also included the combination of VX-445 (2.5 μM) and VX-809 (1 μM) to support mutant CFTR folding/stability and trafficking to the plasma membrane. To dilute all required compounds at the desired final concentrations, we prepared two v-bottom shaped 96 micro-well plates containing all the drugs diluted in MEM medium at a concentration four times higher than the final one. The first plate, named “4X-combo plate”, contained in each well 150 μl MEM medium with VX-809 (4 μM) and VX-445 (10 μM) correctors. The second plate, the “4X-compounds plate”, contained 80 compounds of the library (20 μM in 150 μl MEM medium) distributed in the ten central columns of the microplate (Figure 19). The “4X-compounds plate” also contained the positive control (SMG1i 4 μM in MEM) and the negative control (DMSO 1.12% in MEM) in the two side columns of the microplate as shown in Figure 19. Then, the content of the two plates was mixed together with a 96-channel liquid handling system to generate the “2X-mix plate”. From each “2X-mix plate”, a volume of 100 μl was transferred to the 96-well microplate (clear bottom, black wall) containing the cells (in 100 μl of MEM) in order to achieve the desired final concentration of all compounds. Each set of compounds from the library was tested in duplicate.

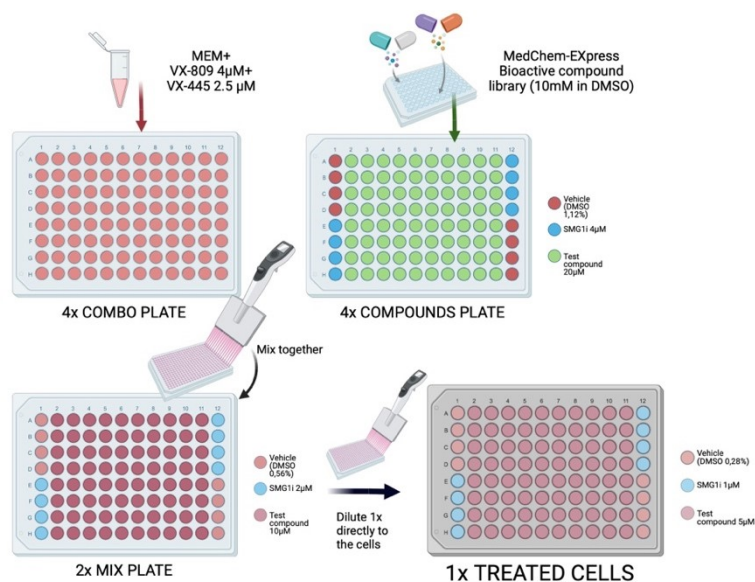


Figure 19. Library screening protocol. Schematic representation of microplate preparation for the screening showing the compound dilution procedure. Image created with Biorender.com.

After 24 h treatment, the HS-YFP assay was carried on a plate reader with the usual conditions (14 s reading per well; 0.2 s sampling time; injection

of iodide-rich solution at 2 s). The fluorescence quenching rate of SMG1i plus VX-809/VX-445-treated cells is relatively small and slow. In this respect, it should be considered that this cell line has an endogenous expression of CFTR, and the 16HBE14o⁻ cells are not hyper-expressing cells. Accordingly, we determined the HS-YFP quenching rate calculating the fluorescence difference between 3.2 s (after injection of iodide solution) and 13.8 s (endpoint of the lecture) and not fitting the decay with an exponential function, as usually done (Pedemonte et al. 2005; Sondo et al., 2011; Pedemonte et al., 2020). The use of this simple parameter is more precise and allow us to reliably detect the relatively small activity of the positive control. We calculated the Z' factor for this assay. The Z' factor is a measurement of the “size effect” that defines the quality of the assay; a Z'-factor between 0.5 and 1.0 is indicative of an excellent assay (Zhang et al., 1999). The Z' factor calculated for this test was 0.5. We considered as hits all the compounds whose activity exceeded a threshold corresponding to 50% of the difference between positive and negative controls.

2.6. Western Blot

16HBE14o⁻ cells carrying the different CFTR mutations were seeded at a concentration of 400,000 cells per well on coated 6-well culture plates (Corning Costar) two days before the experiment and then treated with vehicle or indicated compounds the day after.

HEK293T cells were also grown on 6-well culture plates until 60% of confluence and then transfected the day prior the experiment with 2 µg total plasmidic DNA encoding one of the three different aminoacidic substitutions resulting from the RT event caused by G418 on G542XCFTR. After 6 h from transfection, medium was changed, and cells were treated with vehicle or the indicated treatment combinations. Cells were then lysed in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor Cocktail (Roche) and supplemented with 100 µM PMS, 1:100 Merck Phosphatase inhibitor cocktail III (P0044) and II (P5726) 1:100, NaF 1 mM, 1 mM Na-Orthovanadate to better allow detection of phosphorylated

proteins. Samples were then centrifuged at $14,500 \times g$ at 4°C for 15 min. Total protein concentration of the centrifuged supernatant was calculated using the BCA assay (Euroclone), following the manufacturer's instructions. Equal amounts of protein (20 μg to detect CFTR and GAPDH for HEK293T transfected cells and 40 μg to detect CFTR and GAPDH in 16HBE14o⁻ cells) were then separated onto gradient (4-15%) Criterion TGX Precast gels (Bio-Rad, Hercules, CA, USA), transferred to nitrocellulose membrane with Trans-Blot Turbo system (Bio-Rad) and analyzed by Western blotting. CFTR protein was detected with mouse monoclonal anti-CFTR antibody (596, kindly provided by Cystic Fibrosis Foundation and University of North Carolina, Chapel Hill) diluted 1:2500 or rabbit IgG anti-CFTR antibody (D6W6L, Cell Signaling Technology) diluted 1:1000; human SMG1/RENT1 was detected with polyclonal rabbit anti-SMG1/RENT1 ab30916 (Abcam) diluted 1:1000; a phosphorylated form of UPF1 at Ser1107 was detected using polyclonal rabbit antiphospho-UPF1(Ser1107) BK84283S (Cell Signaling Technology) diluted 1:1000; phosphorylated- UPF1 at Ser1127 was revealed with polyclonal rabbit anti-phospho-UPF1 (Ser1127) 7-1016 (Merck), diluted 1:1000; unphosphorylated UPF1 was detected with rabbit polyclonal anti-UPF1 ab86057 (Abcam) diluted 1:1000, GAPDH was instead immunodetected with anti-GAPDH antibody MAB374 (Merck-Millipore) diluted 1:5000. Anti-CFTR 596 and anti-GAPDH were followed by polyclonal antimouse HR-conjugated secondary antibody diluted 1:10000 (P0447, Dako, Agilent); anti-CFTR D6W6L, anti-phospho UPF1 (Ser1107 and Ser1127), anti-UPF1 and anti-SMG1i were followed by polyclonal antirabbit HR-conjugated secondary antibody diluted 1:10000 (P0448, Dako,) and subsequently revealed by chemiluminescence using the SuperSignal West Femto Substrate (Thermo Fisher Scientific). Direct imaging of the chemiluminescence was obtained using the Molecular Imager UVITEC Cambridge System. Images were analyzed with Fiji software (National Institutes of Health, Bethesda, MD, USA). Total band C was analyzed as a region of interest normalized against the GAPDH control.

2.7. Immunofluorescence

The 16HBE14o⁻ cells expressing G542X- or W1282X-CFTR were seeded on previously coated μ -Slide 12-well removable chamber supports (Ibidi) in a total volume of 200 μ l of complete supplemented MEM medium (20,000 cells/well). Immunostaining was performed as previously described (Capurro et al., 2021). The day before the experiment, cells were treated with vehicle and the indicated treatment combinations. After 24 h, cells were fixed with 10% neutral buffered formalin (0501005Q, BioOptica, Milan, Italy) for 5 min at room temperature. After three washings in PBS, cells were permeabilized and blocked by adding a blocking buffer (BB) containing saponin as a permeabilizing agent and BSA (1X PBS, 0.5% BSA, 50 mM NH₄Cl, 0.02% NaNH₃, 0.05% saponin) for 30 min at room temperature. After blocking, cells were incubated overnight at 4 °C with primary antibody diluted in the BB. Rabbit IgG anti-CFTR (D6W6L, Cell Signaling Technology) at 1:400 was used as the primary antibody.

Following incubation with primary antibody, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) diluted 1:200 in BB for 1 h in the dark. After 3 washes in PBS, the silicone chamber was removed, and cells were mounted with Fluoroshield with DAPI (Sigma-Aldrich) to stain cell nuclei. Image acquisition was performed using a laser scanning confocal microscope (Zeiss LSM 700). Image analysis was performed using ImageJ (NIH) software.

2.8. RNA extraction and real time Reverse Transcription PCR (RTqPCR)

The 16HBE14o⁻ cells expressing the various mutations were seeded in coated 6-well plates at a density of 400,000 cells/well. Twenty-four hours after seeding, cells were treated with vehicle or the desired compounds. After 24 h of treatment, cells were lysed in Trizol (Thermo Fisher Scientific), and total RNA was extracted using the ReliaPrep RNA cell Miniprep System (Z6011, Promega). cDNAs were synthesized using LunaScript® RT SuperMix kit (E3010L, New England BioLabs). Realtime PCR was carried out using TaqMan™ Gene Expression Assays (Thermo Fischer Scientific) for CFTR (Assay ID: Hs00357011_m1) and

for B2M (Assay ID: Hs00187842_m1). The real-time PCR reaction was run in a Light Cycler 96 (Roche). We performed a relative quantitation of CFTR transcript in cells treated with G418 and/or SMG1i compared with control. CFTR expression levels were calculated by subtracting reference gene (B2M) cycle threshold (Ct) values from target gene (CFTR) Ct values to normalize for total input, resulting in Δ Ct levels. Relative CFTR transcript abundance was computed as $2^{-\Delta\text{Ct}}$.

Primary human bronchial or nasal epithelial cells were seeded onto porous support (Snapwell, Corning 3801) at a density of 500,000 cells/well. After two weeks at air-liquid interface configuration, to allow the formation of a fully differentiated epithelia, cells were treated with pro-inflammatory cytokines for 72 h: IL-4 (10 ng/ml) or IL-17A (20 ng/ml) plus TNF- α (10 ng/ml). The last 24 h, cells were also treated with vehicle (DMSO) or with the combination of ELX-02 (200 μ M), VX-809 (1 μ M) and CC-90009 (0.1 μ M). Then, cells were lysed in 200 μ l of RLT Buffer, and total RNA was extracted using the RNeasy Mini Kit (74106, QIAGEN). One μ g of RNA for each sample was retrotranscribed to cDNAs using LunaScript® RT SuperMix kit (E3010L, New England BioLabs). Real-time PCR and analysis of results was carried out as described for 16HBE14o- cells.

For statistical analysis, we evaluated the CFTR transcript fold-change using one-sample t-test and considering the CFTR expression value in DMSO condition as 1.

2.9. RNAscope

To specifically detect CFTR mRNA we customized RNAscope™ protocols (Advanced Cell Diagnostic) according to the manufacturer's protocol (Wang et al., 2012). RNA in situ hybridization (ISH) was performed using the the RNAscope Multiplex Fluorescent v2 reagent kit (ACD, no. 323285). Pre-fixed cells well were treated with RNAscope Protease III (ACD, no. 322380). CFTR probe (Hs-CFTR 603291) was applied on sections; then the amplification cascade was performed as stated in the manufacturer's protocol and detected by using TSA Vivid Fluorophor 520 (ACD.323271, for RNAscope). After RNAscope protocols, sections were counterstained with DAPI and mounted with

GEL/MOUNT (Biomeda). Image acquisition was performed with Nikon Eclipse T2 Confocal Microscope (Nikon Instruments inc).

2.10. Bulk-RNA sequencing

Transcriptome analysis of 16HBE14o⁻ cells expressing the WT-CFTR and W1282X-CFTR in presence of compound hits from the screening was performed on four different biological replicates. Both cell lines were equally treated with vehicle (DMSO as negative control), 1 μ M SMG1i as positive control, and two selected compound hits from the screening, 2 μ M CC-115 and 3 μ M samotolisib. Total RNA was extracted using the RNeasy Mini Kit (74106, QIAGEN) according to the manufacturer's instructions. RNAseq was performed at NEGEDIA using proprietary protocols. The results were analyzed by the Bioinformatics Facility at TIGEM.

2.11. Automated capillary protein separation and immunodetection

Differentiated epithelia on Snapwell inserts treated with vehicle or desired compounds were lysed in RAS buffer (10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.5% Np40, 0.5% Na deoxycholate, 0.1% SDS), containing Complete Protease Inhibitor Cocktail (Roche), 100 μ M PMSF, Merck Phosphatase inhibitor cocktails III (P0044) and II (P5726) diluted 1:100, 1 mM NaF, and 1 mM Na-orthovanadate. Cells were kept on ice for 30 min and then syringed with an insulin syringe. Then, lysates were centrifuged at 4 °C for 15 min at 14500 x g. Protein suspensions were collected in clean tubes on ice and stored at -80 °C or directly used. Supernatant protein concentration was calculated using the Bio-Rad DC Protein Assay as usually done in our lab. After quantification, lysates were briefly vortexed and spun at 4 °C and then mixtures of cell lysates and the fluorescent master mix were prepared according to EZ kit manufacturer's instructions. To allow detection of CFTR protein, samples were heated at 37 °C for 15 to 20 minutes. After heat treatment, samples were again briefly vortexed and spun. The samples, blocking and chemiluminescent reagents, primary and secondary antibodies, and wash buffer were loaded onto the microplates and capillary electrophoresis western analysis was

performed using the ProteinSimple JESS instrument according to manufacturer's instructions (Biotechne, Minneapolis, USA). The mastermix EZ kit, the blocking solution, the wash buffer, the 12-230 kDa matrix microplates and the capillary cartridges were purchased all included in the same ProteinSimple Separation Module (SMW-004, Biotechne,

Minneapolis, USA). The blocking solution, the milk-free antibody diluent solution and the chemiluminescent reagents were purchased together with the ready-to-use HRP-conjugated secondary antibody (ProteinSimple Anti-rabbit Detection Module, DM-002, Biotechne). The following primary antibodies were all diluted in the milk-free antibody diluent and used at the indicated concentrations to detect their respective protein target: rabbit anti-CFTR (D6W6L, CellSignaling Technology) 1:10; rabbit anti- β actin (A2066, Merck) 1:250; rabbit anti-GPST1 (eRF3a) (PA5-62621, ThermoFisher) 1:20. Data acquisition and analysis were carried out using the inbuilt Compass software (ProteinSimple) with the high dynamic range detection profile, which collects multiple exposure times. The software allows to convert the electropherograms of the detected protein targets as virtual western-blot images, that we presented in figures for ease of visualization.

2.12. Fluorescence recovery after photobleaching (FRAP) assay

This method was set up by Daniela Guidone in our lab and described in detail elsewhere (Guidone et al., 2022). Briefly, HBECs were seeded in an inverted configuration on the bottom part of “flipped” Snapwell inserts. Cells were let adhere for at least 3 h, then Snapwell inserts were returned to their normal position and fresh LHC proliferative medium was added to the top and bottom sides of the Snapwell. After 24 h, PneumaCult ALI differentiating medium was added to the top side of the Snapwell (basolateral with respect to cells) and medium was removed from the bottom side. Epithelia were then allowed to differentiate (2–3 weeks) and treated with vehicle or the indicated conditions. The day of the experiment, the apical epithelial surface was stained with 5 μ l PBS containing FITC-Dextran (70 kDa, 2 μ g/ml, Thermo Fisher Scientific). After 3 h, Snapwells with epithelia were mounted on the stage of a Nikon Eclipse Ti-E Spinning Disk inverted microscope within a chamber that allowed control of

temperature (37 °C) and atmosphere (humidified 5% CO₂/95% air or pure air depending on the presence of bicarbonate in the basolateral medium). Images of the stained epithelial surface were sequentially acquired for 5 s before and 60 s after photobleaching of a preselected circular ROI (50 µm diameter). After normalization for the initial value, the recovery of fluorescence at 30 s from bleach pulse was measured (Figure 20).

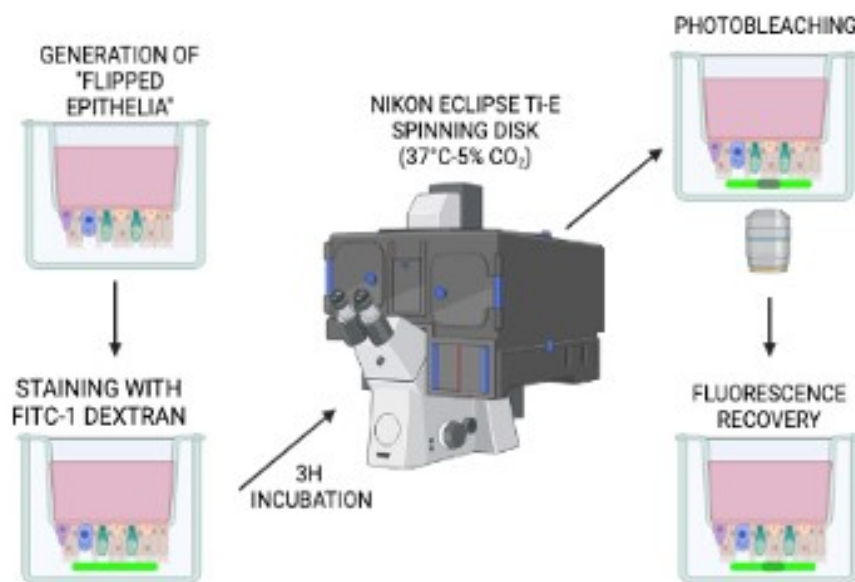


Figure 20. Experimental workflow of a FRAP experiment. “Flipped” human bronchial epithelia were generated by seeding the cells on porous supports in upside-down configuration. The apical surface was stained with FITC-dextran and, after incubation, they were mounted on a spinning disk microscope in controlled atmosphere conditions. The photobleaching of a circular ROI was applied and the fluorescence recovery was recorded. Created with Biorender.com.

2.13. Statistics and data visualization

Venn diagrams were generated using custom annotation scripts. All graphs and figures were prepared with Igor Pro (WaveMetrics). Quantitative data are shown as scatter dot plots with mean $\pm$ SD. Each symbol in the plots represents the result of an independent experiment. To assess significant differences between normally distributed data, we used one-way ANOVA for more than two groups. ANOVA was followed by Dunnett’s or Tukey’s post hoc tests, as appropriate. Statistical analysis was performed with PRISM software (GraphPad).

2.14. Study approval

The collection of nasal epithelial cells by brushing and the collection of human bronchi from explanted lungs to generate cultures of epithelial cells were approved by the relevant Ethical Committee with the registration numbers CER 28/2020 and ANTECER 042-09/07/2018.

3. RESULTS

Part of the following results were published in Venturini et al., 2021. All other results are being included in two manuscripts under preparation.

3.1. Pharmacological rescue of the G542X-CFTR mutant

We started analyzing the rescue effect of different compounds on PTCCFTR on 16HBE14o⁻ cells with G542X-CFTR, which is the most frequent

PTC in CF. We chose this cell line, provided by the Cystic Fibrosis Foundation, since it has endogenous expression of CFTR protein. To obtain this mutant, the CFTR gene locus has been edited to introduce the nonsense mutation in the G542 residue by Crispr-Cas9 technology (Valley et al., 2019). Moreover, these cells form polarized and electrically tight epithelia when seeded at high density on porous supports. For these reasons they are a reliable model to study CFTR-dependent Cl⁻ transport by short I_{sc} experiments. We generated G542X-CFTR epithelia and treated them for 24 h with selected compounds and then mounted in Ussing chambers for transepithelial I_{sc} recordings. During recordings, we maximally stimulated CFTR channel activity with the membranepermeable cAMP analog CPT-cAMP. Then, CFTR activity was further potentiated with VX-770. We finally blocked CFTR-dependent Cl⁻ transport with the specific CFTR inhibitor-172 (inh-172) (Caci et al., 2008). We determined the extent of CFTR activity by measuring the amplitude of the current drop caused by the inhibitor.

As shown in Figure 21, G542X cells showed negligible CFTR activity under control conditions. Treatment with the RT agents significantly enhanced function. We chose G418 or ELX-02 as RT agents at an optimal concentration of 0.5 mg/ml (i.e. 722 and 736 μ M, respectively), based on

preliminary experiments. The effect of ELX-02 was further enhanced by the combination of VX-809 and VX-445 correctors, while the G418 effect was enhanced in presence of the VX-809, but not with VX-445. Unexpectedly, the inclusion of the well-known NMD inhibitor SMG1i to the triple combinations G418/VX-809/VX-445 or ELX-02/VX-809/VX445 did not further increase G542X-CFTR function. The combination of RT agents and SMG1i was also tested in the absence of CFTR correctors but, again, we did not observe any additivity.

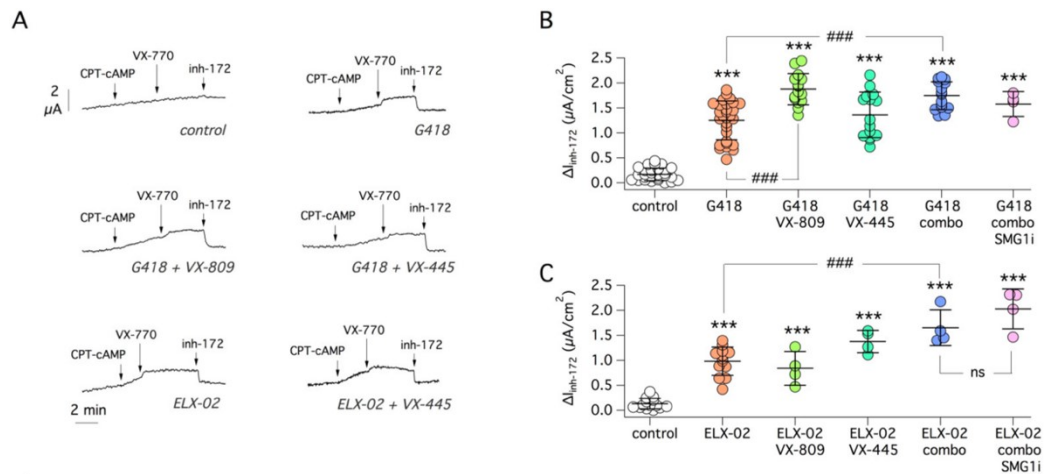


Figure 21. I_{sc} recordings of G542X-CFTR activity upon stimulation of RT agents and SMG1i. Representative traces (Panel A) and summary of data (Panel B and C) from I_{sc} experiments done on the 16HBE14o- bronchial epithelial cell line expressing G542XCFTR. Before recordings, cells were treated for 24 h with 0.5 mg/ml of G418 or ELX-02 alone or in combination with VX-809 (1 μM), VX-445 (5 μM), the two correctors together (combo), with/without SMG1i (1 μM). During recordings, cells were sequentially stimulated with the cAMP analog (CPT-cAMP, 100 μM) and with VX-770 (1 μM). CFTR activity was then inhibited with inh-172 (10 μM). Pharmacological rescue results in a higher CFTR activity as indicated by the larger amplitude of inh-172 effect. The scatter dot plots (panels B and C) report the amplitude of the current drop elicited by inh-172 for the indicated treatments. ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. G418/ELX-02. Adapted from Venturini et al., 2021.

To investigate the stabilizing effects of CFTR correctors on the G542X mutant, we investigated the rescue at the protein level. We tested both compounds, VX-809 and VX-445, on the expression of the G542C-CFTR variant, that is considered the most favored outcome after a RT event on the G542X-CFTR (Xue et al., 2017). We transfected HEK293 cells with the G542C-CFTR expression vector, kindly provided by David M. Bedwell (University of Alabama at Birmingham), treated them with or without correctors, and lysed. Then, CFTR protein was analyzed by immunoblot. Treatment with VX-809, but not with VX-445, significantly enhanced the expression of G542C-CFTR, as summarized in Figure 22. For comparison, we carried out similar experiments with wild-type CFTR,

where we observed that treatment with correctors did not significantly change CFTR expression.

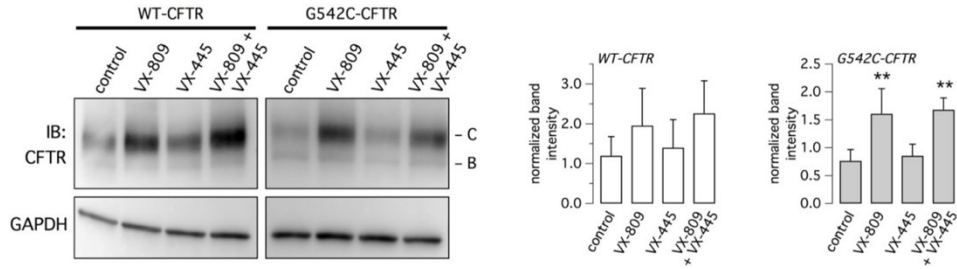


Figure 22. CFTR correctors stabilize the G542C-CFTR variant. Immunoblot analysis of CFTR protein processing in HEK293 cells transiently transfected with wildtype or G542C-CFTR plasmids. After transfection, cells were treated for 24 h with VX809 (1 μ M) and/or VX-445 (5 μ M). Left: representative images showing CFTR and GAPDH detection. VX-809, but not VX-445, increases the expression of the C band, corresponding to fully glycosylated form of CFTR protein, in G542C-CFTR expressing cells. Right: summary of results reporting C band intensity (normalized for GAPDH expression) from multiple immunoblot experiments. **, $p < 0.01$ vs. control ($n = 4$). Adapted from Venturini et al., 2021.

We also tested the effects of VX-661, a second-generation class I corrector that is present in the triple drug combination Trikafta. We tested VX-661 in combination with G418 by I_{sc} . In contrast to VX-809, VX-661 did not significantly improve the rescue by G418. This result can be explained with a reduced corrector efficacy of VX-661 compared with VX-809 (Capurro et al., 2021) (data not shown).

Amplifiers are small molecules described as general-purpose modulators, since they promote enhanced CFTR protein expression by stabilizing CFTR transcript and for this reason, they are similarly effective on wildtype and mutant CFTR (Dukovski et al., 2020). Thus, we explored the possibility of improving rescue of G542X-CFTR using PTI-428, that has been described as a CFTR amplifier (Giuliano et al., 2018; Dukovski et al., 2020). Surprisingly, in our I_{sc} experiments PTI-428 did not increase CFTR function above the level achieved with G418 alone (Figure 23).

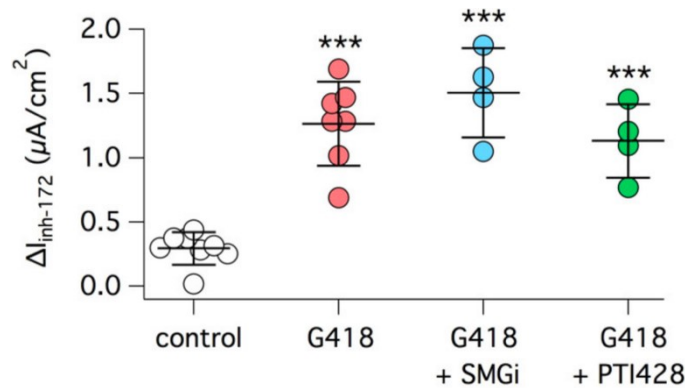


Figure 23. Functional analysis of PTI-428 and SMG1i. The scatter dot plot summarizes CFTR function (i.e., current sensitive to inh-172) in 16HBE14o⁻ cells expressing G542X-CFTR treated for 24 h with G418 (0.5 mg/mL) alone or in combination with SMG1i (1 μ M) or PTI-428 (30 μ M). SMG1i and PTI-428 did not further increase G418- mediated CFTR rescue. ***, $p < 0.001$ vs. control. Adapted from Venturini et al., 2021.

Accordingly, we evaluated the effect of PTI-428 by real-time RT-PCR. PTI-428 did not increase CFTR mRNA (Figure 24), with a modest increase observed only when combined with G418.

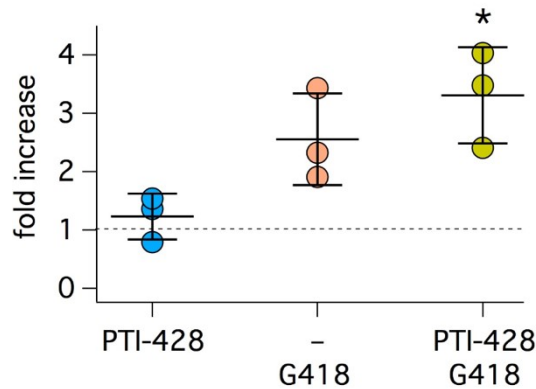


Figure 24. Effect of PTI-428 on CFTR mRNA. 16HBE14o⁻ cells expressing G542XCFTR were treated for 24 hours with PTI-428 (30 μ M), G418 (0.5 mg/ml) or both compounds. After treatment, RNA was extracted, converted to cDNA, and CFTR transcript quantified by real time RT-PCR. Results in each sample were normalized to B2M expression. The scatter dot-plot shows CFTR transcripts fold-change relative to control condition. *, $p < 0.05$ vs. vehicle treated cells (dashed line). Adapted from Venturini et al., 2021.

We then tested the efficacy of PTI-428 on F508del-CFTR and WT-CFTR. CFTR correction levels in CFBE41o⁻ cells expressing F508del-CFTR were evaluated with the HS-YFP assay. PTI-428 was tested at different concentrations, alone or in combination with VX-809. Treatment with PTI-428 for 24 h increased F508del-CFTR function in a dose-dependent manner. In combination with VX-809, PTI-428 activity was nearly 3 times larger than when tested alone, suggesting a synergistic effect in the presence of the CFTR corrector, as shown in Figure 25.

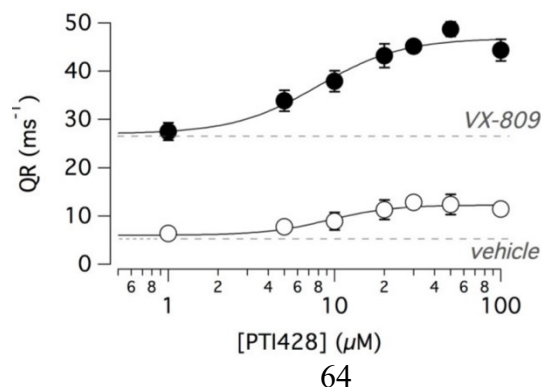


Figure 25. Rescue of F508del-CFTR function by PTI-428. PTI-428 was tested for 24 h at increasing concentrations on CFBE41o⁻ cells expressing F508del-CFTR, in the presence or absence of VX-809 (1 μ M). The graph reports the quenching rate (QR) deriving from the HS-YFP functional assay. Data were fitted with a Hill function. Adapted from Venturini et al., 2021.

To test the activity of PTI-428 on WT-CFTR, we studied non-CF primary bronchial epithelia with I_{sc} recordings. As expected from the postulated activity of PTI-428 as a CFTR amplifier, a 24 h treatment (30 μ M PTI428) increased CFTR function, as shown by the larger amplitude of the block caused by inh-172 in Figure 26. Intriguingly, we also observed an “amplifier” effect on other electrogenic processes. Indeed, the activity of the epithelial Na^+ channel ENaC was augmented by the treatment with PTI-428, as indicated by the increased inhibitory effect of amiloride. The amplifier also markedly increased the response to UTP, a purinergic agonist that activates the Ca^{2+} -dependent TMEM16A (ANO1) Cl^- channel (Figure 26).

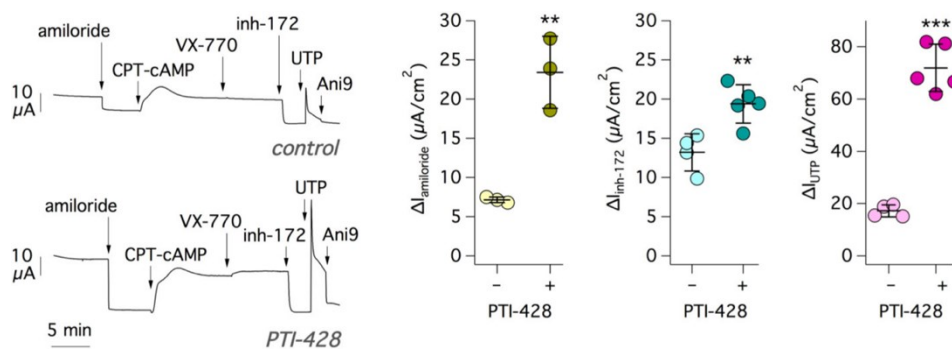


Figure 26. Effect of PTI-428 on electrogenic transport mechanisms of bronchial epithelia. Representative traces (left) and scatter dot plots (right) showing data Ussing chamber experiments (I_{sc} recordings) done on non-CF primary bronchial epithelial cells. Cells were treated for 24 h with the CFTR amplifier PTI-428 (30 μ M) or vehicle (DMSO) alone. During recordings, epithelia were sequentially exposed to: amiloride (10 μ M) to inhibit ENaC channel, CPT- cAMP (100 μ M) and VX-770 (1 μ M) to stimulate CFTR channel, CFTR_{inh-172} (inh-172, 20 μ M) to inhibit CFTR activity, UTP (100 μ M), and Ani9 (5 μ M) respectively to activate and then inhibit TMEM16A channel. Scatter dot plots show the amplitude of the amiloride effect (left graph), the amplitude of the inh172 block (middle graph), the amplitude of the current activated by UTP (right graph). **, p < 0.01; ***, p < 0.001 vs. control. Adapted from Venturini et al., 2021.

To further investigate the feasibility of G542X-CFTR rescue with combinations of small molecules, we carried out experiments on native bronchial epithelial cells from pwCF carrying the G542X mutation. We took advantage from cells heterozygous for the G542X-CFTR and 17171G > A mutations. The second mutation, which targets a canonical splicing site leading to full disruption of CF protein synthesis, can be

considered un-rescuable (Guillermi et al., 1990; Marson et al., 2016). In agreement with data obtained in 16HBE14o⁻ cells, I_{sc} recordings on bronchial epithelia revealed a significant rescue of G542X-CFTR following treatment with RT agents and the rescue was further enhanced by VX-809 (Figure 27).

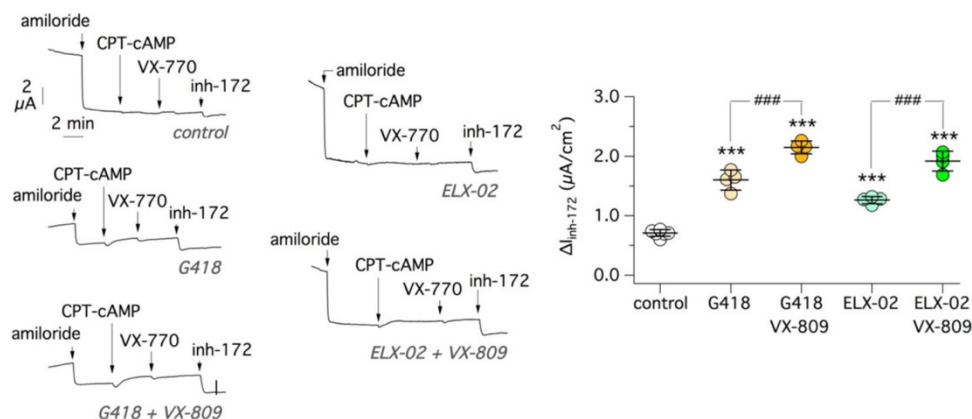


Figure 27. Rescue of G542X-CFTR in primary bronchial epithelia. Representative short-circuit current traces (left) and summary of data (right) obtained from bronchial epithelia generated from cells of a G542X/1717-1G > A patient. Epithelia were treated with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μ M VX-809). ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. readthrough agent alone. Adapted from Venturini et al., 2021.

3.2. Pharmacological rescue of the Y122X, R553X and R1162X-CFTR mutants

By I_{sc} experiments, we evaluated the rescue maneuvers tested on G542X on PTCs localized in other regions of the CFTR coding sequence. Such PTCs included: i) Y122X, that resides in the N-terminal portion of the protein, precisely in the TMD1; ii) R553X that, similarly to G542X and F508del, is localized in the NBD1 domain; iii) R1162X, localized in the linker region between the second transmembrane domain (TMD2) and the second nucleotide binding domain (NBD2).

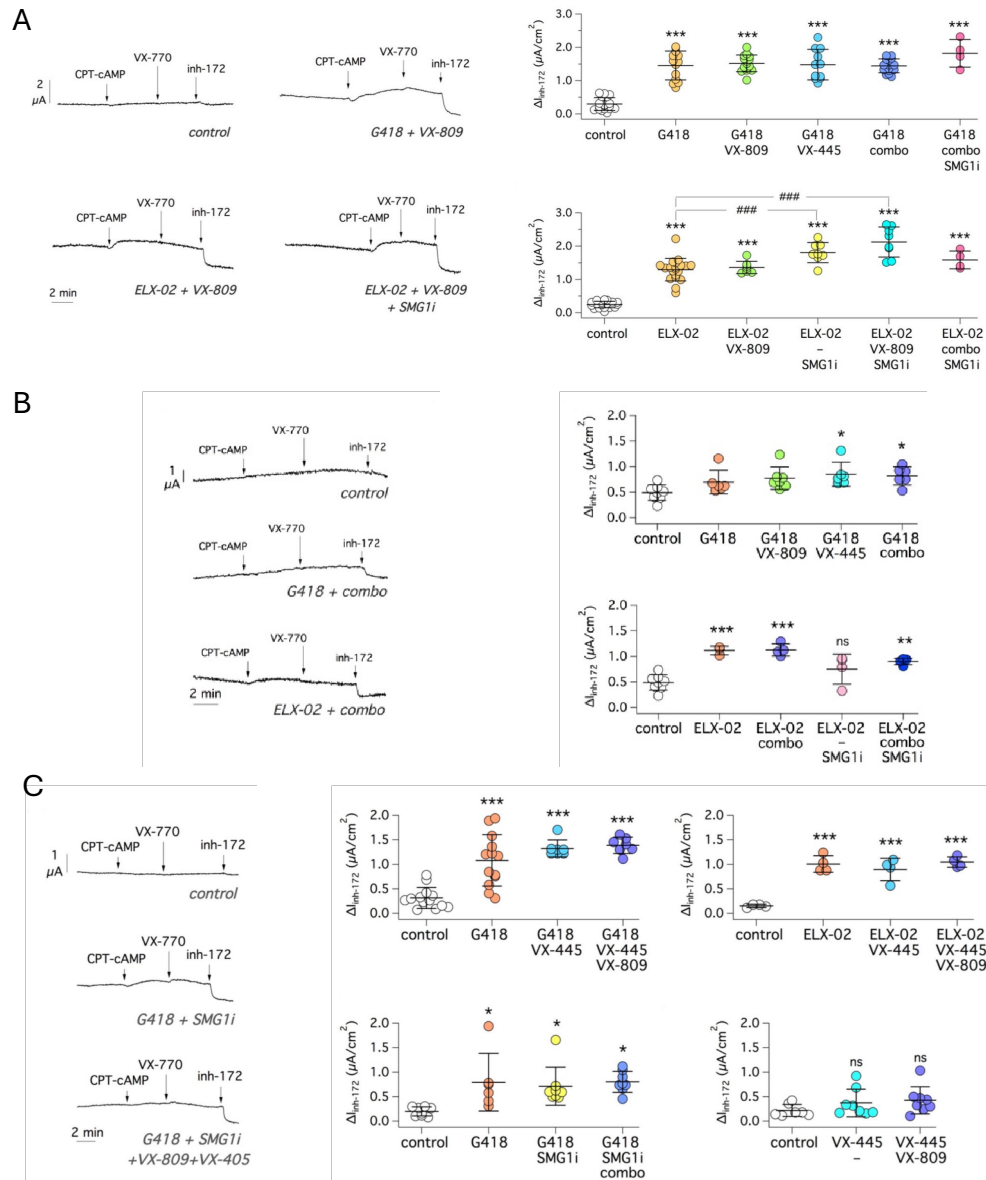


Figure 28. Pharmacological rescue of Y122X, R553X, and R1162X. Each panel shows representative I_{sc} traces (left) and summary graphs (right) obtained from 16HBE14o⁻ cells expressing Y122X-CFTR (A), R553X-CFTR (B) and R1162X-CFTR (C). Epithelia were treated with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μ M VX-809, 5 μ M VX-445, 1 μ M SMG1i). *, $p < 0.05$ vs. control, ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. ELX-02 alone. Adapted from Venturini et al., 2021.

As summarized in Figure 28, under control conditions CFTR-dependent Cl^- current was nearly negligible for all the CFTR mutants. We found that the Y122X-CFTR mutant is sensitive to RT agents, since both G418 and ELX-02 induced a significant increase in CFTR currents, as indicated by the appearance of a current blocked by the CFTR inhibitor (Figure 28A). Similarly to G542X-CFTR, inclusion of SMG1i did not improve the effect of G418, while a modest but significant effect was observed by testing SMG1i together with ELX-02 alone or in combination with VX-809. It is interesting to note that with ELX-02, CFTR appeared to be active under

basal conditions, before stimulation with the cAMP agonist. This is a behavior similar to that of wild-type CFTR.

Regarding R553X-CFTR, the effect of G418 alone was negligible but a very modest rescue was obtained when including CFTR correctors (Figure 28B). In contrast, ELX-02 alone elicited a small but significant rescue, but no further rescue was detected by including correctors or SMG1i.

Finally, for R1162X, we found rescue by both G418 and ELX-02 but correctors did not elicit a further increase in CFTR function (Figure 28C). Similarly to the other mutants, inclusion of SMG1i did not enhance the efficacy of the RT agent. We also tested correctors by themselves assuming that a CFTR protein truncated at position 1162 has the ability to show some function. However, treatment with correctors alone was ineffective (Figure 28C).

3.3. Pharmacological correction of W1282X-CFTR

In general, W1282X appeared to be more sensitive to rescue maneuvers compared to the other PTCs tested. W1282X, the second most frequent nonsense mutation in CF, is close to the carboxy terminus, residing precisely in the NBD2 domain of the CFTR protein. The activity of W1282X-CFTR was not increased by RT agents alone but required the inclusion of the VX-809/VX-445 correctors (Figure 29A). When taken singularly, VX-445 appeared to contribute more to the rescue with respect to VX-809.

Similarly to R1162X-CFTR, we also tested correctors in the absence of RT agents. As for R1162X, the rationale was that truncation of the protein at a terminal position could allow some residual function, potentially improved by correctors/potentiators. We found that W1282X-CFTR can be significantly rescued with correctors, particularly by VX-445 (Figure 29B), differently to what happens for the R1162X mutant. Noteworthy, comparing the results obtained by RT agents, correctors, and combination of both, it appears that RT is essentially ineffective on W1282X. Like G542X, we found no additional rescue by including PTI-428.

The most interesting results were obtained with SMG1i. Surprisingly, functional rescue of W1282X-CFTR appeared markedly sensitive to this compound, a finding that is in contrast with all other PTCs. The strong

effect of SMG1i was observed only when combined with CFTR correctors (Figure 29B). A response to VX-770 potentiator was also clearly visible. The total rescue effect was equal to a 7-fold increase relative to vehicle-treated cells. Importantly, we found no improvement when G418 as RT agent was included in SMG1i-based treatments (Figure 29B).

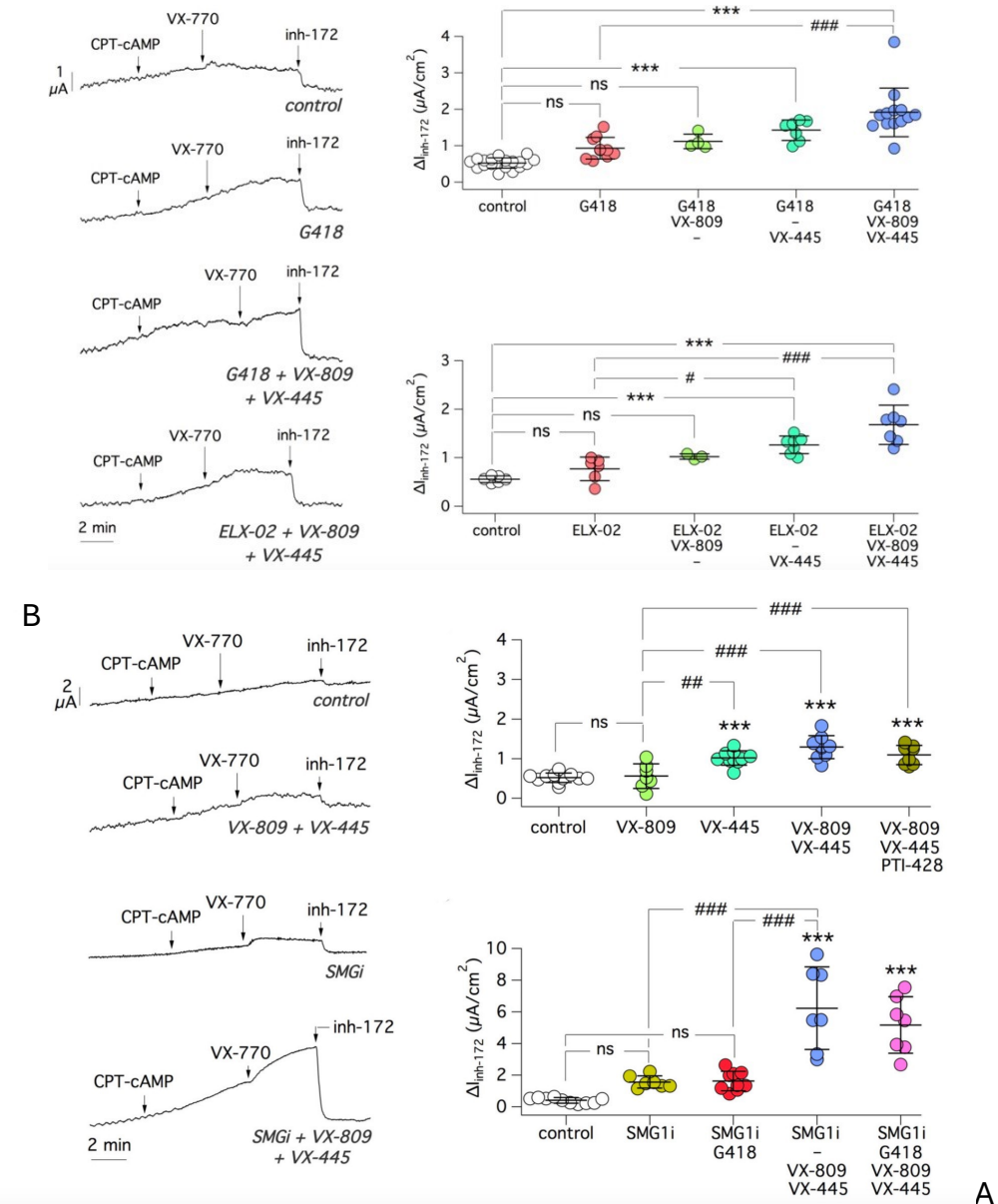


Figure 29. Sensitivity of the W1282X-CFTR mutant to RT agents and SMG1i. Each panel shows representative I_{sc} traces (left) and summary graphs (right) obtained from experiments on 16HBE14o⁻ cells expressing W1282X-CFTR. Panel A collects data obtained with RT agents, panel B includes traces and currents obtained with SMG1i-based treatments. Epithelia were treated for 24 h with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μM VX-809, 5 μM VX-445, 1 μM SMG1i, 30 μM PTI428). Panel A: ***, p < 0.001 vs. control. #, p < 0.05 and ###, p < 0.001 vs. G418/ELX02, ns not significant. Panel B: ***, p < 0.001 vs. control. ##, p < 0.01 and ###, p < 0.001 vs. VX-809 (up); p < 0.001 vs. control. ###, p < 0.01 and ###, p < 0.001 vs. SMG1i with or without G418 (down); ns not significant. Adapted from Venturini et al., 2021.

As done for the G542X-CFTR, we confirmed our results in primary bronchial epithelia. In particular, we used bronchial epithelial cells from a patient with the W1282X/W1282X genotype. In agreement with data obtained in 16HBE14o- cells, a significant recovery of CFTR function was detected by treatment with SMG1i plus VX-809/VX-445 correctors as shown in Figure 30. Of notice, the mean value of CFTR current recorded in bronchial epithelia with G542X and W1282X mutations after most effective treatments was 1.9 (as showed in Figure 27) and 2.5 $\mu\text{A}/\text{cm}^2$, respectively. Taking in consideration data relative to WT-CFTR currents recorded in non-CF epithelia ($\sim 13 \mu\text{A}/\text{cm}^2$, see Figure 26, trace on the bottom left), such values correspond to $\sim 15\%$ and 19% of normal CFTR function.

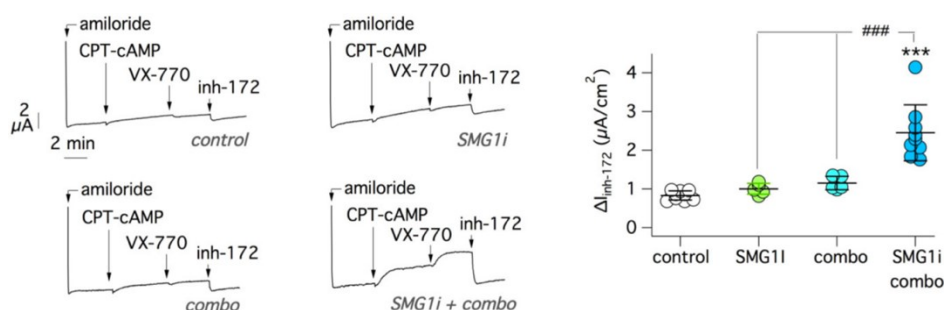


Figure 30. Effect of SMG1i on primary bronchial epithelia expressing W1282XCFTR. Short-circuit current traces (left) and summary of data (right) obtained from epithelia generated from cells of a W1282X/W1282X patient. Epithelia were treated with the indicated compounds (1 μM SMG1i; combo: 1 μM VX-809 plus 5 μM VX-445). ***, $p < 0.001$ vs. control. ####, $p < 0.001$ vs. indicated treatments. Adapted from Venturini et al., 2021.

We investigated the molecular basis of the large functional rescue of W1282X-CFTR obtained with SMG1i. First, we evaluated the effect of SMG1i on W1282X-CFTR protein processing by immunoblot (Figure 31). Upon treatment with SMG1i, we observed the appearance of the two characteristic bands that correspond to the different glycosylation states of the CFTR protein (Figure 30). Importantly, we found no evidence of a shift to a higher molecular weight when G418 was included with SMG1i, thus confirming the lack of readthrough for this mutation.

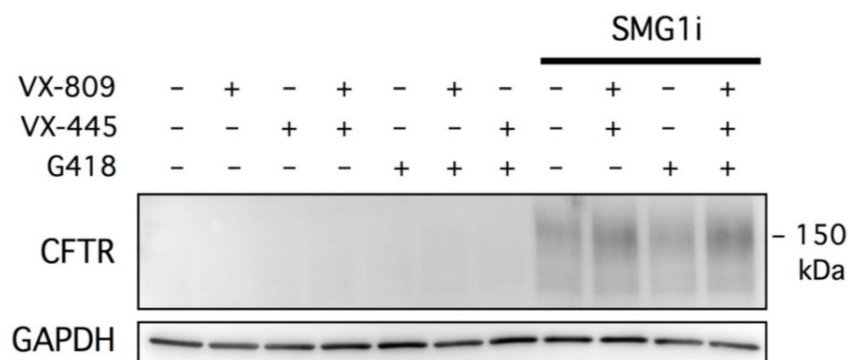


Figure 31. Molecular analysis of SMG1i effects on W1282X-CFTR protein processing. Representative Western blot experiment performed on 16HBE14o⁻ cells expressing W1282X-CFTR. Treatment with SMG1i (1 μ M) was required to evoke the appearance of CFTR protein. The molecular size of the rescued protein (150 kDa) is smaller than that of wild-type CFTR (180 kDa), in agreement with the effect of the premature truncation and the lack of readthrough when G418 is included. Adapted from Venturini et al., 2021.

We also investigated W1282X-CFTR protein expression/trafficking by immunofluorescence. Treatment of W1282X-CFTR cells with SMG1i plus correctors caused the appearance of a signal corresponding to CFTR partially visible at the cell periphery, consistent with plasma membrane localization (Figure 32). Importantly, the localization at the cell periphery was also visible with SMG1i alone, without correctors. In contrast, no signal was found for G542X-CFTR cells treated with G418 plus correctors, which indicates that the rescue of this mutant was below the level of detection with this technique.

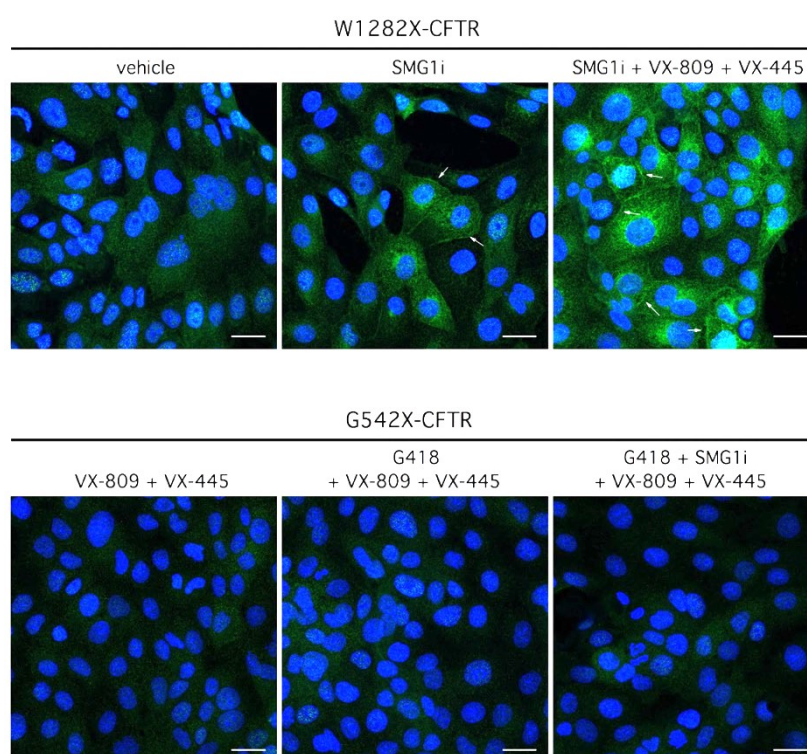


Figure 32. Analysis of W1282X- and G542X-CFTR by immunofluorescence. Representative images reporting immunodetection of CFTR protein in 16HBE14o- cells expressing W1282X- and G542X-CFTR. Cells were treated with SMG1i (1 μ M) and G418 (0.5 mg/ml) alone or in combination with VX- 809 (1 μ M) plus VX-445 (5 μ M). Cells expressing the W1282X mutant show appearance of immunoreactive CFTR (green) following pharmacological treatment. Arrows: appearance of CFTR signal at cell borders. Scale bar: 20 μ m. Adapted from Venturini et al., 2021.

3.4. Evaluation of SMG1i activity on CFTR mRNA

By real time RT-PCR, we investigated the effect of SMG1i on the levels of CFTR mRNAs carrying the various PTCs. Interestingly, SMG1i significantly increased CFTR mRNA levels in all 16HBE14o- cells expressing PTCs. For Y122X cells, the increase was approximately 5-fold whereas for all other mutations the increase was larger than 10-fold. We also tested the effect of the G418 alone and plus SMG1i. The readthrough agent did not enhance mRNA levels by itself, nor did it appreciably alter the effect of SMG1i (Figure 33).

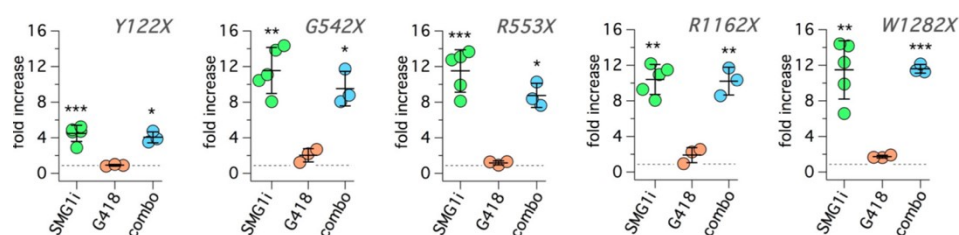


Figure 33. Analysis of CFTR mRNA by real-time RT-PCR. 16HBE14o- cells expressing the indicated PTCs were treated for 24 h with G418 (0.5 mg/ml), SMG1i (1 μ M), or with both compounds together (combo), then RNA was extracted. The scatter dot plots summarize CFTR transcript fold-change relative to control condition for every mutation. All values were normalized to B2M. The p value was determined using onesample t-test. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. control (dashed line). Adapted from Venturini et al., 2021.

3.5. Generation of 16HBE14o- W1282X-CFTR cells stably expressing HS-YFP

Compared to the other CFTR mutant, W1282X-CFTR showed a very large response to rescue maneuvers. We asked whether it was possible to detect the rescue also with the HS-YFP assay, which allows a highthroughput determination of CFTR activity. For this purpose, 16HBE14o- cells expressing W1282X-CFTR cells were transduced through lentiviral infection with the HS-YFP coding sequence to obtain its stable expression.

After 24 h treatment with different compound combinations, HS-YFP assay was performed and CFTR function was determined by measuring the HS-YFP quenching rate (Sondo et al., 2011; Pedemonte et al., 2020). In agreement with data obtained with I_{sc} , we found a significant rescue of the CFTR function in cells treated with SMG1i plus correctors (Figure 34, top). We assessed the specific contribution of each CFTR corrector to the functional rescue: VX-445, and not VX-809, was essentially responsible for the rescue of W1282X-CFTR in the presence of SMG1i. Again, we observed that inclusion of G418 as a RT agent did not further improve the extent of CFTR rescue obtained by SMG1i plus correctors. We also tested SMG1i at different concentrations, with and without correctors. An SMG1i concentration as low as 0.25 μ M was still effective in the presence of correctors (Figure 34, bottom).

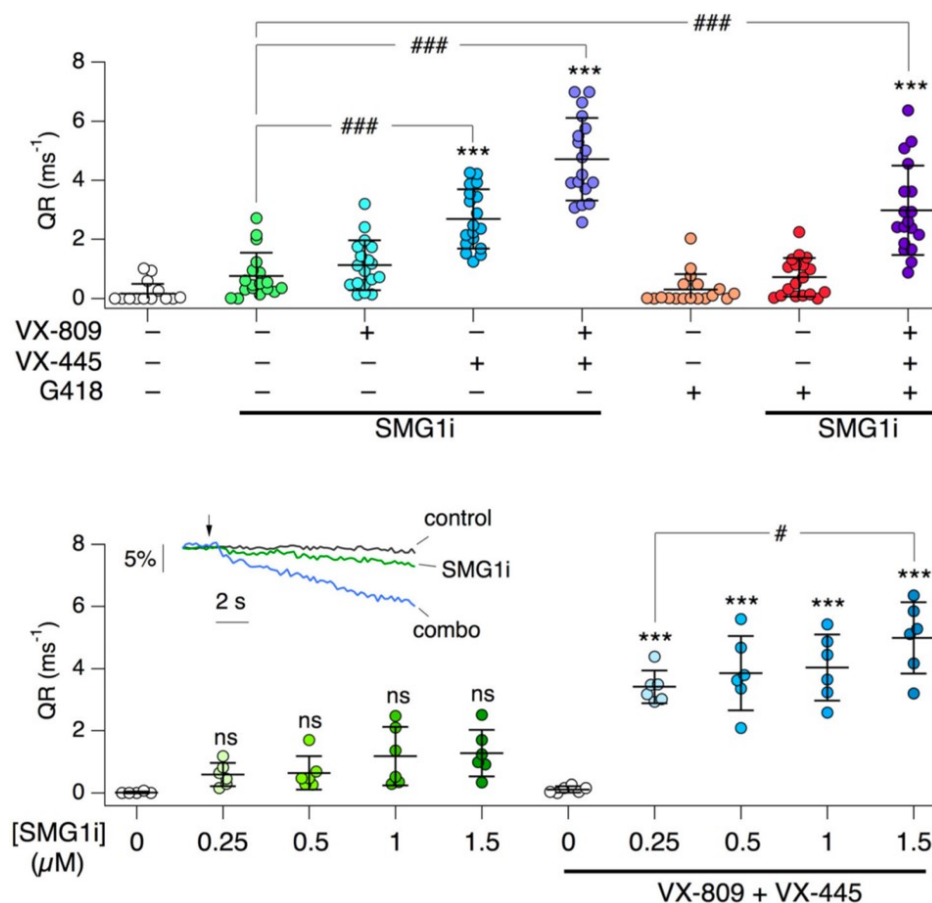


Figure 34. Functional analysis of W1282X-CFTR rescue with the HS-YFP assay. In the upper panel, the scatter dot plot reports W1282X-CFTR activity (expressed as quenching rate, QR) in cells treated with the indicated combinations of compounds: SMG1i (1 μ M), VX-809 (1 μ M), VX-445 (5 μ M), G418 (0.5 mg/ml). ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. SMG1i alone. In the bottom panel, a dose-response relationship for SMG1i in the absence and in the presence of CFTR correctors is shown. ***, $p < 0.001$ vs. no SMG1i. #, $p < 0.05$ between indicated conditions. The inset reports representative traces from the HS-YFP assay. Adapted from Venturini et al., 2021.

3.6. Functional screening of the MedChem-Express Bioactive Compounds Library

Results obtained with the W1282X-CFTR cells were interesting. As shown so far, our findings indicate that block of NMD by SMG1i, which is an inhibitor of UPF1 phosphorylation by SMG1, is not compatible with RT agents, suggesting that SMG1 activity could be necessary for both NMD and RT mechanisms (Lejeune, 2017; Jia et al., 2017). Accordingly, SMG1 inhibition should be avoided in combinatorial strategies including RT agents. We postulated that high-throughput screening of a large chemical library could lead to NMD inhibitors with a MoA other than that of SMG1i, possibly more compatible with RT agents.

We took advantage of the 16HBE14o- cell line co-expressing W1282XCFTR and the HS-YFP (Figure 34) to screen the MedChemExpress Bioactive Compounds Library (9,351 small molecules), that includes approved drugs, investigational drugs and compounds with known biological activity. As explained in detail in the Materials and Methods section, cells were seeded in 96-well plates and treated for 24 h with test compounds (5 μ M), DMSO (negative control), or SMG1i 1 μ M (positive control) plus the combination of correctors VX-809 (1 μ M) and VX-445 (2.5 μ M). After treatment and before the HS-YFP assay, cells were stimulated for 20 min with CPT-cAMP (100 μ M) plus the CFTR potentiator VX-770 (1 μ M) to fully activate CFTR channel. Then, CFTR activity was evaluated in a fluorescence microplate reader (Figure 35A). We considered as positive hits those compounds whose activity exceeded a threshold corresponding to 50% of the difference between positive and negative controls. With this primary functional assay, we identified several compounds able to increase iodide uptake, potentially reflecting the rescue of W1282X-CFTR. Active compounds belonged to different main groups: proteasome and kinase inhibitors, cyclin-dependent kinases (CDKs) inhibitors, eukaryotic initiation factors (eIFs), and topoisomerases inhibitors among others. After the primary screening, we performed a re-test of all hits to confirm activity. Compounds passing this step were then tested at multiple concentrations in the range 0.1 – 20 μ M to generate dose-response relationships and calculate EC50 and maximal efficacy (Figure 35B). Finally, all compounds were tested at a single

concentration giving maximal effect in the presence and absence of CFTR inh-172 (Figure 35C). This important experiment allowed to distinguish compounds rescuing W1282X-CFTR from those increasing iodide uptake with other mechanisms. We considered for further analysis those compounds whose effect was significantly reduced by CFTR_{inh}-172 (Figure 35C).

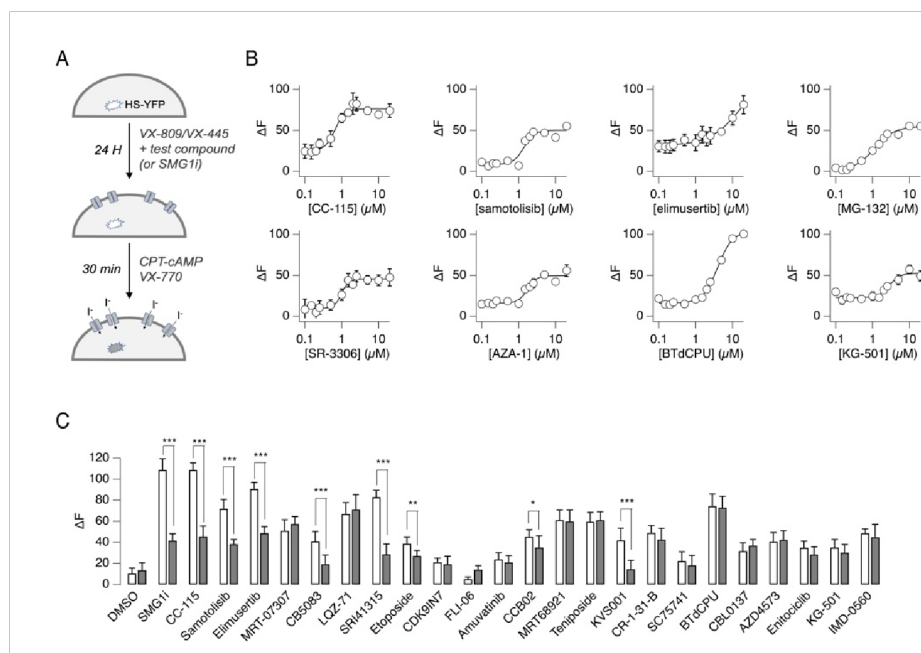


Figure 35. Functional screening of the MedChemExpress Bioactive Compounds library. (A) Scheme of the screening based on the HS-YFP assay. 16HBE14o⁻ cells coexpressing W1282X-CFTR and HS-YFP were treated with test compounds or SMG1i plus VX-809/VX-445 correctors. After treatment, iodide uptake was measured with HS-YFP. (B) Representative dose-response curves for compounds with different biological activities. Compounds were all tested at concentrations in the 0.1 – 20 μM range, in the presence of VX-809 (1 μM) plus VX-445 (5 μM). (C) HS-YFP data obtained in 16HBE14o⁻ cells treated with indicated compounds in presence (filled bars) or absence (empty bars) of the CFTR_{inh}-172 (10 μM). *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. no CFTR_{inh}-172 (Student's t test).

Three compounds, CC-115, samotolisib, and elimusertib, known as kinase inhibitors, emerged for their greater effect of CFTR activity. CC-115, a DNA-PK and mTOR kinase inhibitor (Tsuji et al., 2017), increased CFTR activity with an EC₅₀ = 0.65 μM. Samotolisib inhibits class I PI3K isoforms, DNA-PK, and mTORC1/2 at low nanomolar concentrations (Smith et al., 2016). Instead, elimusertib is a potent, orally active and selective ATR inhibitor with anti-tumor activity showing an IC₅₀ of 7 nM (Lücking et al., 2020). We tested these three compounds in other assays. To confirm the functional rescue of W1282X-CFTR, we evaluated the efficacy of CC-115, samotolisib and elimusertib in I_{sc} recordings on 16HBE14o⁻ cells (Figure 36). All three compounds largely increased

CFTR function in combination with correctors. Importantly, elimusertib induced a significant effect also in the absence of correctors.

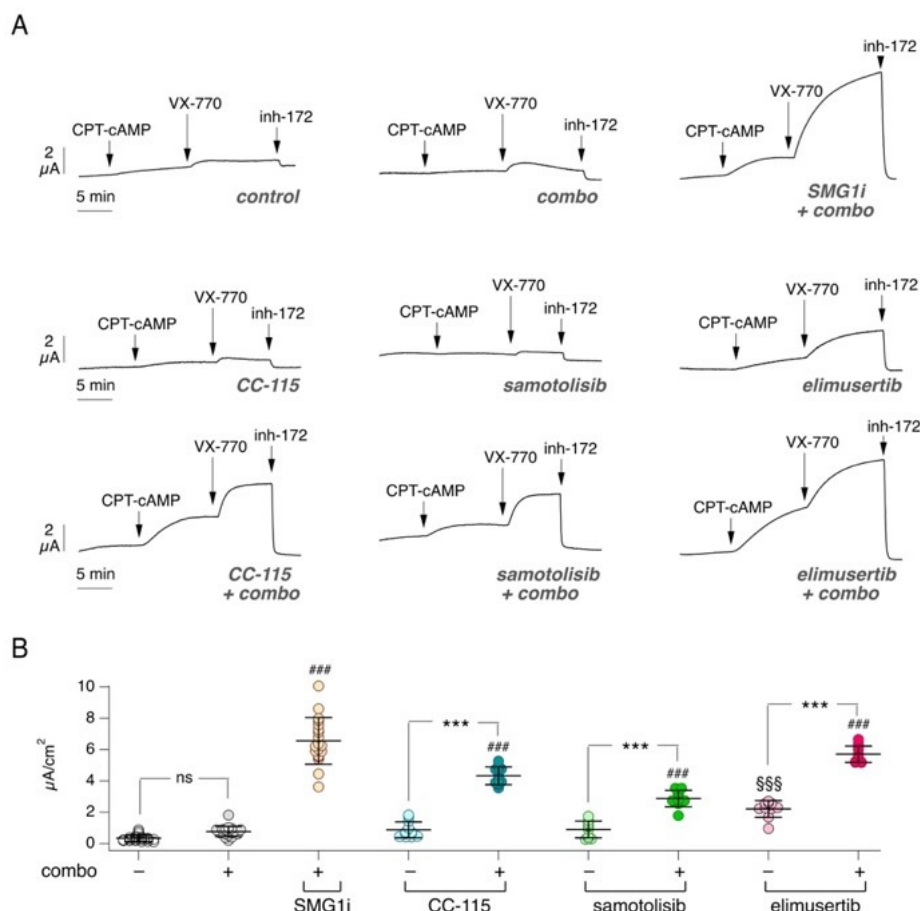


Figure 36. Evaluation of CC-115, samotolisib and elimusertib in I_{sc} recordings. The figure shows representative I_{sc} recordings (top) and summary of data (bottom) obtained from experiments on 16HBE14o⁻ cells expressing W1282X-CFTR. Cells were treated for 24 h with CC-115 (2 μM), samotolisib (3 μM) or elimusertib (20 μM) with/without CFTR correctors (combo: 1 μM VX-809 plus 5 μM VX-445) or vehicle alone (control). During recordings, cells were sequentially exposed to: CPT-cAMP (100 μM), VX-770 (1 μM), CFTR_{inh}-172 (10 μM). The scatter dot plot reports the amplitude of the current drop caused by CFTR_{inh}-172. ***, p < 0.001 vs. vehicle; ####, p < 0.001 vs CC-115, samotolisib or elimusertib alone. (ANOVA with Tukey's post hoc test).

3.7. Characterization of confirmed hits from screening

We evaluated the effect of CC-115, samotolisib, and elimusertib at the transcriptional and protein expression levels in 16HBE14o⁻ cells. By real time RT-PCR, we found that CFTR mRNA was increased nearly 15-fold by CC-115 and elimusertib and nearly 7-fold by samotolisib. The inclusion in the treatments of G418 did not enhance their effect (Figure 37).

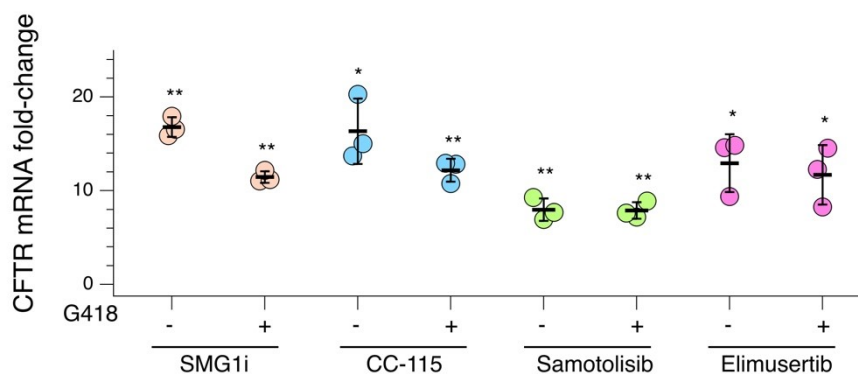


Figure 37. Evaluation of confirmed hits from the screening on W1282X-CFTR transcript levels. The graph represents the fold-change in CFTR mRNA compared to control condition (DMSO). 16HBE14o- cells expressing W1282X-CFTR were treated for 24 h with CC-115 (2 μ M), samotolisib (3 μ M), or elimusertib (20 μ M), with/without G418 (0.5 mg/ml). ***, $p < 0.001$ vs control condition.

We also took advantage of RNAscope technology to visualize the effect of compounds on CFTR transcripts. CC-115 and samotolisib, as well as SMG1i, induced a strong increase of bright dots corresponding to single CFTR mRNA molecules. These dots appeared in the perinuclear region of cells (Figure 38). Elimusertib effect is currently under investigation.

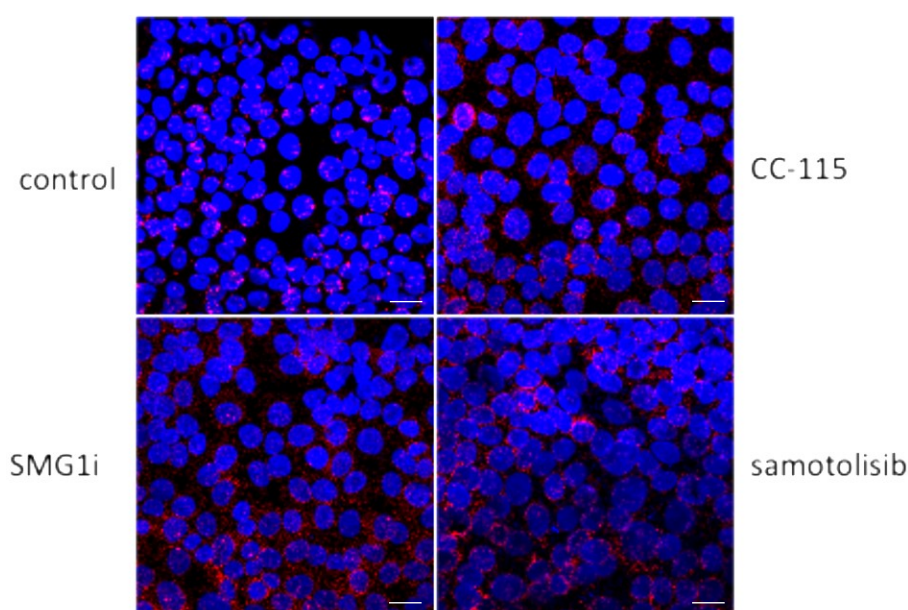


Figure 38. Detection of CFTR mRNA by RNAscope. The figure shows representative images of 16HBE14o- cells expressing W1282X-CFTR cells after 24 h treatment with DMSO (as negative control), SMG1i (1 μ M, as positive control), CC-115 (2 μ M), and samotolisib (3 μ M). After fixation and permeabilization, cells were exposed to specific probes that hybridize with endogenous CFTR mRNA. Images were acquired with a Nikon T2-Eclipse confocal microscope. The increase in single CFTR RNA molecules can be clearly detected as the appearance of red spots. Scale bar: 20 μ m.

To check if increased CFTR mRNA levels correspond to an increased amount of synthesized CFTR protein, we carried out immunoblot experiments on the same cell line treated in presence of the selected hits, together with vehicle (DMSO) and SMG1i as negative and positive control conditions, respectively. With the use of a highly sensitive antiCFTR antibody, we clearly detected the appearance of a signal for CFTR protein with the two characteristic bands (band B, corresponding to the core-glycosylated CFTR and band C, corresponding to the fully glycosylated form) for all the compounds tested at selected concentrations (Figure 39).

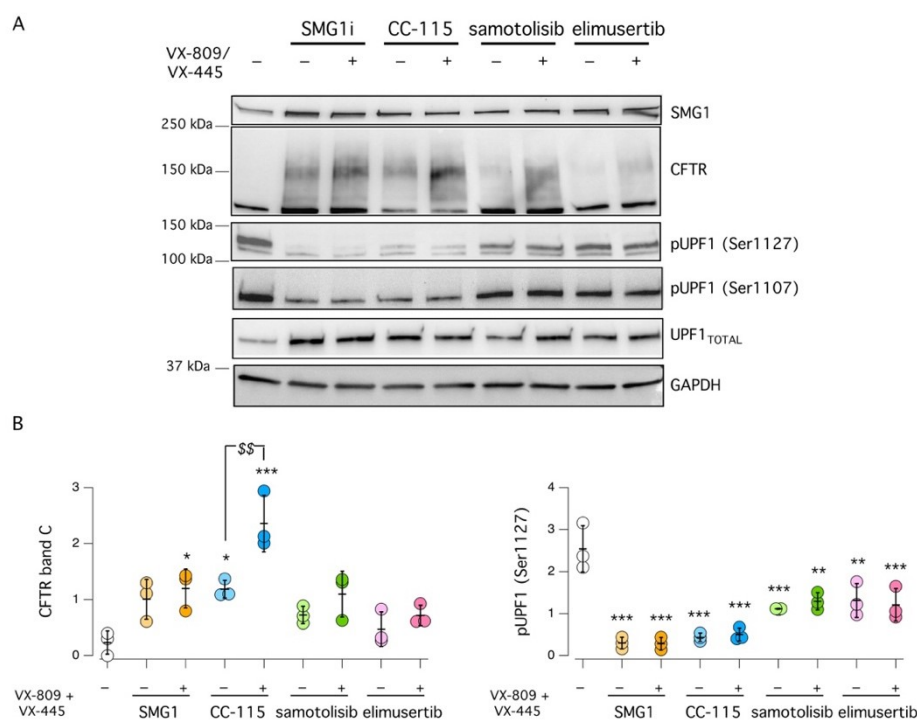


Figure 39. Effects of CC-115, samotolisib, and elimusertib on protein processing. Panel A: representative western blot image of 16HBE14o- treated with the indicated conditions. Protein targets are indicated on the right. Panel B: the scatter dot-plots summarize densitometry of the bands corresponding to the protein target under indicated treatments. CC-115 (2 μ M), samotolisib (3 μ M) and elimusertib (20 μ M) were analysed with or without VX-809 (1 μ M) plus VX-445 (5 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. totally untreated (no compounds) cells. §, $p < 0.05$; §§ $p < 0.01$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

To better understand the impact on the NMD process, we also checked by immunoblot the expression levels of key-proteins of this mechanism. As stated previously, SMG1i is a specific inhibitor of the SMG1i kinase that phosphorylates UPF1. Therefore, we investigated the expression of the phosphorylated and total form of the UPF1 protein. We expect that SMG1 inhibitors will result as compounds that decrease the phosphorylation of UPF1. We used two different antibodies targeting two different

phosphorylated serine residues, S1107 and S1127, of the human UPF1 protein. All confirmed hits from the screening, namely CC-115, samotolisib, and elimusertib, behaved similarly to SMG1i, decreasing the levels of the phosphorylated UPF1. It has been observed that silencing of key components of the NMD mechanism has an impact on feedback mechanisms that controls the physiological levels of such proteins (Nguyen et al., 2014, Huth et al., 2022). We observed a parallel increase in total UPF1 protein compared to non-treated cells, suggesting that compounds are affecting its expression. We also observed an increase in SMG levels with treatment with SMG1i, that is coherently suggestive of an increased production of its target protein as a control feedback mechanism. Compounds resulting from the screening also modestly increased SMG1 protein.

The observed effects are compatible with strong NMD inhibition. We came back to the HS-YFP assay to test if there is additivity/synergy with SMG1i. A lack of additivity would suggest that compounds are likely acting with the same MoA of SMG1i, while additivity would be informative that SMG1i and hit compounds have different MoAs, potentiating each other's effects. In Figure 40, data from the functional HS-YFP assay show no increment of CFTR function when kinase inhibitors found in the screening are combined with SMG1i, compared to SMG1i treatment alone.

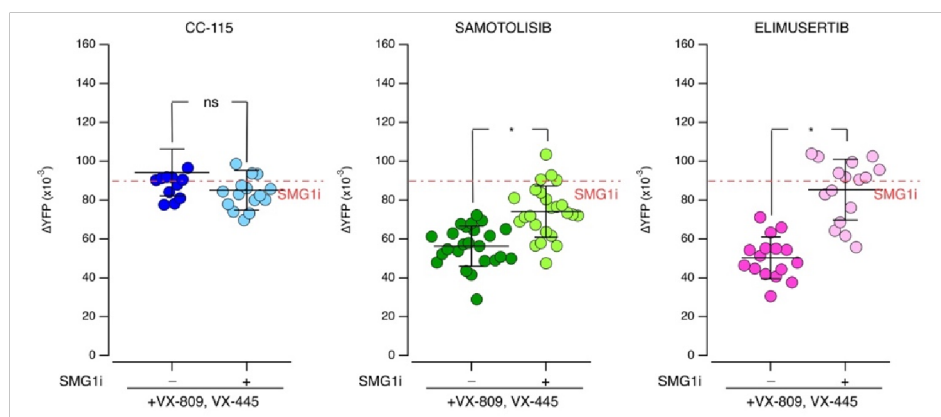


Figure 40. Effect of CC-115, samotolisib, and elimusertib in combination with SMG1i. The scatter dot-plots summarize HS-YFP assay data obtained with the treatment of CC-115 (2 μ M), samotolisib (3 μ M) and elimusertib (20 μ M), with or without SMG1i, in presence of VX-809 (1 μ M) plus VX-445 (5 μ M). The activity with SMG1i plus CFTR correctors is indicated in the graphs as a dashed red line. ***, $p < 0.001$ vs compounds plus VX-809/VX-445.

To obtain further information about the mechanism of action of active compounds, we performed analysis of their effect on cell transcriptome.

We treated 16HBE14o⁻ cells, both the WT- and W1282X-CFTR versions, for 24 h with SMG1i, CC-115, or samotolisib. After treatment, RNA was extracted, and transcripts were analyzed by bulk RNAseq. The results show that, as expected for NMD inhibitors, a large number of transcripts were similarly upregulated by the treatment in both wild type and W1282X cells. The exception was CFTR transcript, which was upregulated in W1282X cells, but not in WT-CFTR cells. This result is in agreement with NMD specifically affecting the CFTR mRNA with the PTC (Figure 41). A deep bioinformatic analysis is being carried out to analyze the data obtained by RNA seq. The goal is to identify common and compound-specific signatures elicited by treatments.

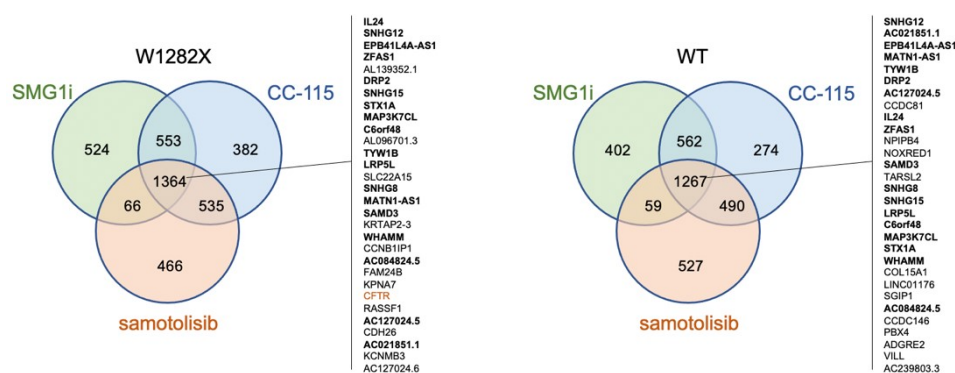


Figure 41. Effect of compounds on cell transcriptome. Venn diagrams reporting data from bulk RNAseq of 16HBE14o⁻ cells expressing W1282X- (left) or wild type CFTR (right), treated for 24 h with SMG1i, CC-115, or samotolisib. The list of the top 30 genes commonly upregulated by the three treatments is reported on the side of each Venn diagram. In bold are the genes that are upregulated in both wild type and W1282X cells. In red is CFTR.

3.8. Pharmacological correction of PTC-CFTR is enhanced under inflammatory stimuli

The eRF3a degrader CC-90009 has been recently proposed to be effective on G542X-CFTR in human bronchial epithelial cells (Lee et al., 2022). We decided to test CC-90009 in combination with ELX-02 and VX-809. For these experiments, we used epithelia generated from cells of a G542X/G542X homozygous patient. In contrast to previous results from our lab, the treatment with ELX-02 plus VX-809 did not induce a significant CFTR rescue. Instead, a significant 3-fold increase of the current sensitive to CFTR_{inh}-172 was observed upon inclusion of CC90009 (Figure 42 A, B).

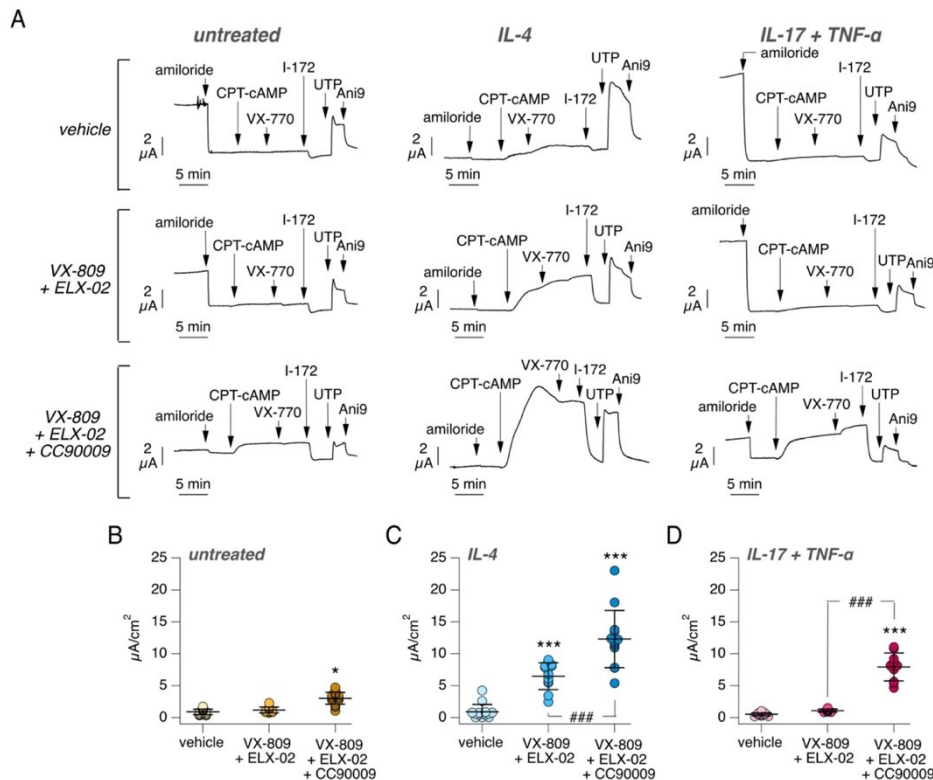


Figure 42. Effect of CFTR rescue manoeuvres and cytokines on G542X/G542X bronchial epithelia. (A) Representative I_{sc} traces and data (B-D) reporting results obtained from epithelia treated for 24 h with vehicle alone, VX-809 (1 μM) and ELX-02 (200 μM), or the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μM). Where indicated, the epithelia were previously treated for 72 h with IL-4 (10 ng/ml) (C) or IL-17A (20 ng/ml) plus TNF- α (10 ng/ml) (D). *, $p < 0.05$; ***, $p < 0.001$ vs. vehicle. ###, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

Recently, it has been reported that treatment of bronchial epithelia in vitro with IL-17A and TNF- α , cytokines that are involved in the inflammation of CF airways, enhances the efficacy of CFTR modulators in rescuing F508del-CFTR (Rehman et al., 2021). We asked if inflammatory

mediators also have effects on the pharmacological correction of the CFTR PTCs. Thus, we treated the epithelia for 72 h in presence of two different pro-inflammatory stimuli, namely IL-4 or IL-17A plus TNF- α . IL-4 is a Th2 cytokine that induces goblet cell hyperplasia with abundant MUC5AC production, upregulation of CFTR- and TMEM16A-dependent chloride secretion and downregulation of ENaC function (Gorrieri et al., 2016). IL-17A is a Th17 cytokine, involved in the immune response against extracellular bacteria and with an emerging role in CF lung disease (Hagner et al., 2021). The combination of IL-17A with TNF- α is considered to be responsible for promoting neutrophil infiltration and to induce an inflammatory response of the airway epithelium mimicking the situation in vivo. Surprisingly, we found that the effect of agents used to rescue G542X-CFTR was greatly amplified in presence of IL-4 treatment, as indicated by the large response to the activating stimulus, CPT-cAMP, and the subsequent inhibition by CFTR_{inh}-172 (Figure 42A). Indeed, under this condition we obtained a nearly 8-fold increase in CFTR function with the combination of ELX-02 with VX-809 compared to untreated cells (Figure 42C). Inclusion of CC-90009 further improved CFTR rescue with a nearly 15-fold increase (Figure 42C). Similarly, the treatment with IL-17A plus TNF- α was also effective, but surprisingly it required the presence of CC-90009 together with ELX-02 and VX-809. Under this condition, there was a 9-fold increase compared to the cells without cytokine treatment (Figure 42A and D). We confirmed these data on epithelia from a second patient in which the G542X mutation is in heterozygous condition with the 1717-1G>A mutation that causes total loss of CFTR protein and is insensitive to pharmacological treatments (Guillermi et al., 1990). In I_{sc} experiments, these cells, similarly to those with the G542X/G542X genotype, showed a strong increase in CFTR function by combining the triple drug treatment (ELX-02, CC-90009, VX809) with IL-4 or IL-17A/TNF- α (Figure 43).

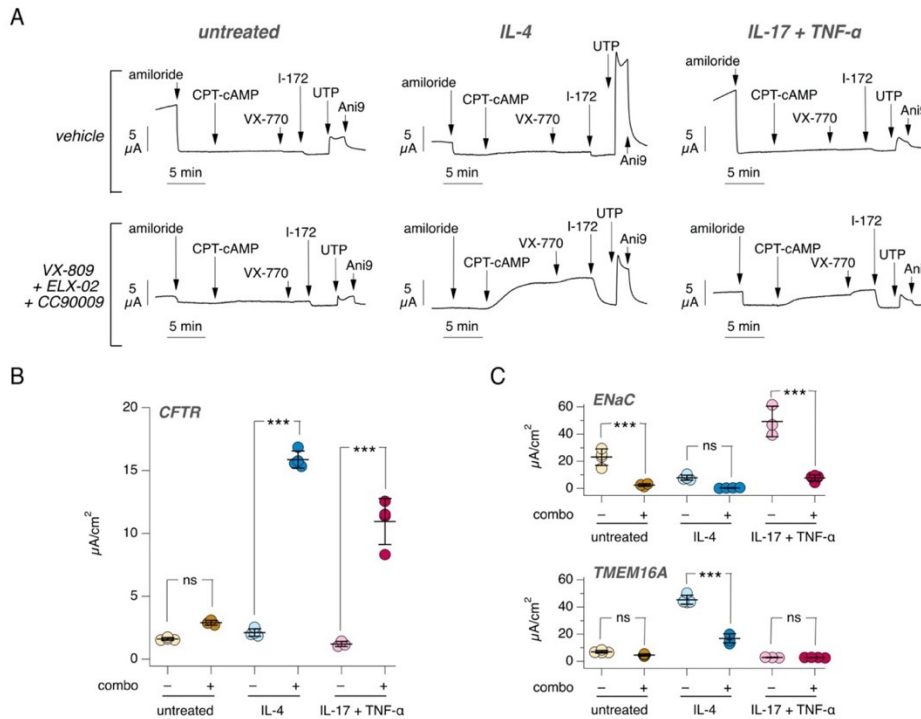


Figure 43. Effect of CFTR rescue manoeuvres and cytokines on G542X/1717-1G>A bronchial epithelia. (A-B) The figure shows the same set of experiments of the previous figure carried on a patient with a different genotype. (C) The scatter dot plots report the effect of treatments on the function of ENaC (top) and TMEM16A function (bottom) as indicated by the response to amiloride and UTP, respectively. ***, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test). "Combo" indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μM).

As previously described, pro-inflammatory cytokines have broad transcriptional effects on various channel expressed by the airway epithelium. ENaC activity, for example, is decreased by IL-4 and enhanced by IL-17A/TNF- α (Gorrieri et al., 2016; Guidone et al., 2022). IL-4 also markedly upregulates TMEM16A channel function (Gorrieri et al., 2016). We found that treatment of epithelia with the triple drug combination markedly reduced ENaC function in epithelia kept under control conditions or treated with IL-17A/TNF- α , as indicated by the current deflection elicited by the amiloride (Figure 42A and 43A). According to a previous study, this decrease in ENaC function may be attributed to CC-90009 (Lee et al., 2022). The triple drug combination containing the CC-90009 also significantly downregulated TMEM16A function (response to UTP) in epithelia treated by IL-4 (Figure 43C). As a further confirmatory test, we investigated nasal epithelial cells. We collected by brushing nasal epithelial cells of a patient with the G542X/R1162X genotype. Cells were expanded and used to generate differentiated epithelia. Epithelia were then treated with the same experimental conditions used on bronchial epithelia. The triple drug

combination (ELX-02, CC-90009, VX-809) was ineffective by itself, but resulted in a high CFTR activity (indicated by the large response to cAMP stimulus and block by CFTR_{inh}-172) when epithelia were also treated with IL-4 or IL-17A/TNF- α (Figure 44).

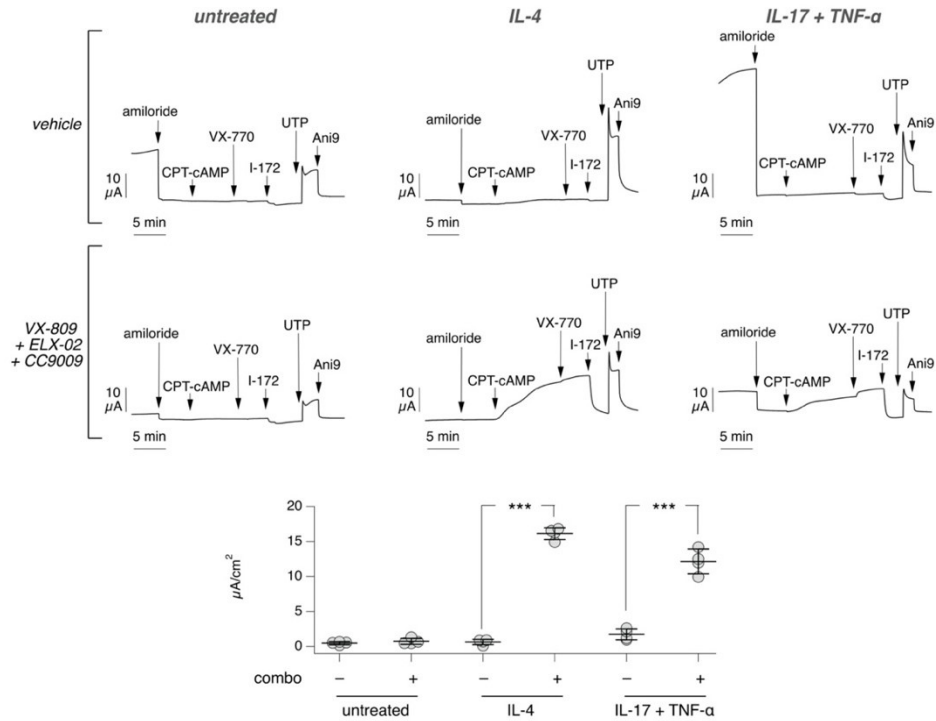


Figure 44. Effect of CFTR rescue maneuvers and cytokines on G542X/R1162X nasal epithelia. Top: representative traces from I_{sc} experiments. Bottom: scatter dot plots reporting the effect of treatments on the function of CFTR as indicated by the response to CFTR_{inh}-172. ***, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test). "Combo" indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M).

3.9. Molecular analysis of pro-inflammatory cytokine effects

The large effect on CFTR function when the triple drug combination was combined with inflammatory stimuli was surprising. We investigated the consequences at the protein level. We took advantage of capillary-based protein separation and immunodetection, that provides a good sensitivity. In this system, immunoreactivity of the target proteins results in the recording of an intensity profile of the chemiluminescent signal. Such intensity profile can be recapitulated as a virtual western blot image for ease of visualization (Figure 45A, B).

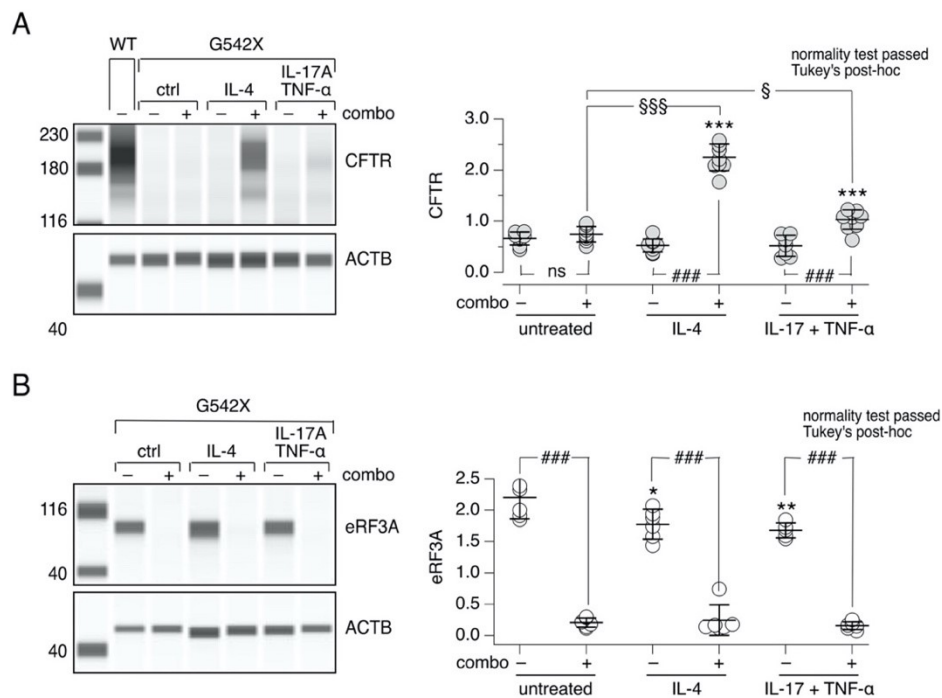


Figure 45. Analysis of CFTR and eRF3A protein levels in presence of triple drug combination and cytokine treatment. Results from capillary-based protein separation and immunodetection experiment are presented as western blot images (left) and densitometry analysis (right). Panel A shows detection of CFTR protein from lysates of bronchial epithelia (G542X/G542X genotype) treated with/without cytokines (IL-4, IL17A/TNF- α) and with/without the triple combination VX-809, ELX-02, CC90009 (combo). Panel B summarizes the detection of eRF3A protein. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. totally untreated (no compounds, no cytokines) epithelia. §, $p < 0.05$; §§§ and ###, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

A strong signal corresponding to the full-length mature form of CFTR protein appeared when bronchial epithelia (G542X/G542X genotype) were treated with IL-4 plus the drug combination (Figure 45A). A rescue of CFTR protein, although less marked, was also observed in epithelia treated with IL-17A/TNF- α plus drugs. We then checked for the expression of eRF3A protein, that is the target of CC-90009. As expected,

eRF3A protein nearly disappeared upon treatment with the drug combination that includes CC-90009 (Figure 45B). Interestingly, we also found a modest but significant decrease in eRF3A signal by treating epithelia with IL-4 or IL-17A/TNF- α in the absence of drugs.

Since PTCs also affects mRNA stability through the NMD process, we interrogated CFTR mRNA levels in presence of cytokines together with the triple drug combination by real time RT-PCR. We found that the amount of CFTR transcript is not altered by the triple drug combination by itself. Treatment of epithelia with IL-4 alone induced an increase of nearly 1.7-fold. Unexpectedly, treatment with IL-17A/TNF- α decreased CFTR mRNA by nearly half. Importantly, a substantial increase in CFTR mRNA levels was reached when epithelia were treated with cytokines together with the triple drug combination, in particular $\sim$ 4-fold for IL-4 and $\sim$ 6-fold for IL-17A/TNF- α (Figure 46).

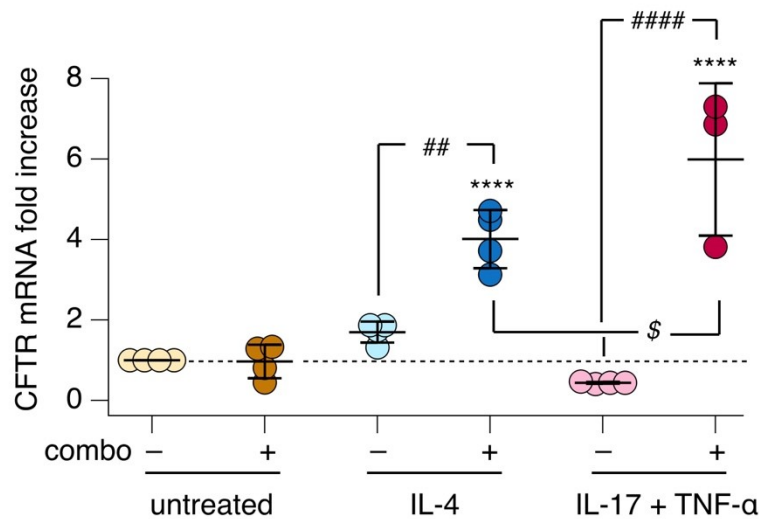


Figure 46. Effect of treatments on CFTR mRNA levels. The graph reports the results of CFTR mRNA quantification by real time RT-PCR (fold-increase with respect to untreated epithelia). RNA was extracted from four separate preparations of G542X/G542X bronchial epithelia. *, $p < 0.05$; **, $p < 0.01$ vs. untreated epithelia (one sample t and Wilcoxon test). “Combo” indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M).

3.10. Pharmacological rescue of PTC-CFTR can restore apical surface fluidity after treatment with IL-17A plus TNF- α

Recently, our group reported that in vitro treatment of bronchial epithelial cells by combination of IL-17A plus TNF- α induces a hyper-viscous state. Changes in fluidity of the ASL can be assessed with the Fluorescence Recovery After Photobleaching (FRAP) technique (Guidone et al., 2022). We found that non-CF epithelia expressing wild type CFTR can be switched to a fluid state after beta-adrenergic activation of CFTR with basolateral application of isoproterenol. A significant recovery from the viscous state was also observed in epithelia expressing F508del-CFTR following treatment with correctors (Guidone et al., 2022). We asked whether rescue of the G542X-CFTR by the triple compound combination ELX-02/CC-90009/VX-809 is enough to achieve a similar restoration of the ASL fluidity. The surface of epithelia was treated as usual for 72 h with IL-17A/TNF- α and, coherently, displayed the high viscosity previously reported, as indicated by the very slow recovery of the photobleached area (Figure 47, top, panel on the right). When the triple compound combination was included, we observed a significantly accelerated rate of fluorescence recovery that is suggestive of a reduced viscosity. We also studied the viscosity of epithelia treated with IL-4 with or without the triple drug combination. Despite the abundant mucus production previously described (Scudieri et al., 2012; Gorrieri et al., 2016), we did not observe any significant change in viscosity in epithelia treated with IL-4 with respect to untreated epithelia (Figure 47, top, middle panel).

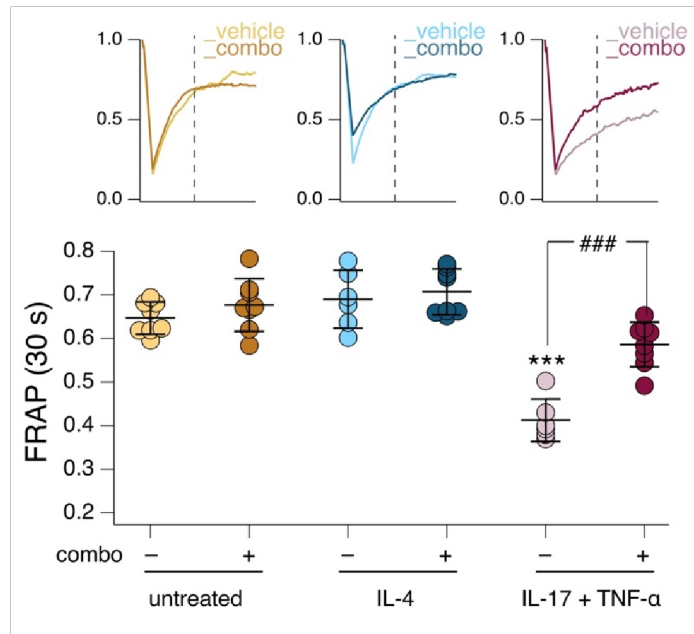


Figure 47. Epithelial surface viscosity measured with FRAP technique. Top: representative traces showing fluorescence recovery after photobleaching of a selected area in epithelia in control conditions (untreated) or treated with IL-4 or IL-17A/TNF- α with/without VX-809/ELX-02/CC90009 (combo). Bottom: scatter dot plots reporting the extent of fluorescence recovery at 30 s after photobleaching. ***, $p < 0.001$ vs. totally untreated epithelia (no compounds, no cytokines). ###, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

4. DISCUSSION

The present thesis work is focused on the comprehensive analysis of pharmacological approaches for the correction of nonsense mutations impairing the function of the CFTR channel. This is an important topic since most CF patients carrying F508del or a large number of missense mutations can presently benefit from CFTR correctors and potentiators. In contrast, patients with nonsense mutations are orphan of treatments targeting the basic defect.

4.1. Comprehensive analysis of PTC sensitivity to pharmacological treatments

The strong point of the study is the systematic analysis of multiple drugs and the evaluation of several PTC mutants of the CFTR channel within a common framework of functional assays and cell models. The availability of a bronchial cell line (16HBE14o-) in which the endogenous *CFTR* locus has been edited to introduce a selected panel of nonsense mutations was crucial for the study, since the use of isogenic cell lines minimizes confounding factors due to different genetic background. Further, and most importantly, since NMD machinery exploits some protein components that remains on the mRNA after the splicing event, study of the PTC rescue in the context of the NMD requires the presence of the endogenous gene, rather than an over-expressed cDNA deprived of its introns and exon-exon junctions. Moreover, the availability of the same cell line encoding PTCs localized in different domains of the CFTR protein gave us insights on the positional effects of the PTCs. In the first part of the study, we studied five different versions of the 16HBE14o- cell line modified with CRISPR/Cas9 technology to express five different PTCs: the Y122X in TMD1, the G542X and R553X in NBD1, the R1162X in the TMD2-NBD2 linker region, and the W1282X in NBD2. In our study, we evaluated some compounds that are already used for the treatment of pwCF (such as CFTR correctors VX-809, VX-445 and VX770) or that are being tested in clinical trials (ELX-02). We also included G418 as another type of RT agent.

Essentially, we found a significant effect of the RT agents, G418 and ELX-02, with various degrees of efficacy, on all the examined PTCs, with the

striking exception of the W1282X-CFTR, that in our experimental conditions resulted totally insensitive to RT-mediated correction. Another important finding of our work is that, for mutants responding to rescue maneuvers, the addition of clinically approved CFTR modulators to the treatment appeared to be fundamental to achieve a proper functional rescue of the CFTR channel. This is quite represented by G542X-CFTR, in which the class I corrector VX-809 improved the rescue obtained with G418 and ELX-02, probably because it contributes to enhance the stability of the full-length CFTR protein that could contain a random amino acidic substitution resulting from the RT event. In this respect, we tested CFTR correctors on G542C-CFTR, which is the most probable outcome of G542X RT (Xue et al., 2017). In agreement with previous results (Xue et al., 2017), we found that VX-809 significantly enhances G542C-CFTR protein expression. This could probably explain the efficacy of the combination of the CFTR corrector with the RT agent on G542X. We also tried the combination of RT agents with VX-445, a class III corrector very active on other CFTR variants, particularly the F508del. Intriguingly, we found that VX-445 was ineffective on G542X-CFTR rescued by RT agents and on G542C-CFTR. Another important, and unexpected feature of G542X was the insensitivity to NMD suppression. Despite causing a large increase in CFTR mRNA levels, SMG1i did not significantly increase the function of G542X-CFTR when combined with ELX02/G418 and VX-809. A similar lack of efficacy of NMD inhibition was found for other two mutations, namely R553X and R1162X. In theory, we would expect a cooperative activity between RT agents and NMD inhibitors, since inhibition of the NMD process prevents mRNA degradation, thus providing more CFTR mRNA susceptible to RT agent action. However, we never observed additivity between SMG1i and G418 or ELX-02. Further studies are needed to understand why there is a lack of synergy/additivity between RT agents and the NMD inhibitor. A possible explanation is that NMD and RT processes shares key protein, so that suppression of NMD could also affect RT, and vice versa. Previous studies have found that NMD suppression has a negative impact on RT, since UPF1 and other related proteins are required for both processes (Jia et al., 2017). Moreover, a proposed model for NMD activation involves the exon-exon junction complexes (EJC) that cannot be displaced and remain

downstream of a ribosome stalled at a PTC site. Continuation of the protein synthesis by RT should theoretically result in EJC displacement and thus prevention of the NMD. However, we did not observe this effect in our experiment, since G418 treatment did not result in increased mRNA levels (Figure 33). It is tempting to speculate that NMD suppression impedes mRNA degradation, but the resulting mRNA is under a condition or localization in the cell that is not suitable for RT action.

Compared to G542X, R553X, and R1162X, Y122X was the only mutation that displayed some sensitivity to the combination of a RT agent and SMG1i. Indeed, a very modest but significant increase in Y122X-CFTR activity was obtained when SMG1i was combined with ELX-02. Interestingly, Y122X cells were the least responsive to SMG1i on CFTR mRNA levels (4-fold increase compared to 10-fold for the other mutants). It has been reported that Y122X is less severely affected by NMD than the other nonsense mutations (Sharma et al., 2018), a characteristic that could explain the lower efficacy of the NMD inhibitor. Moreover, the protein truncating effect of the Y122X mutation can also be circumvented by alternative translation start sites occurring downstream the mutation (Sharma et al., 2018). Therefore, despite its localization at the very beginning of the CFTR coding sequence, various factors can contribute to explain the lesser severity of this mutation.

In contrast to the other mutations, W1282X appeared very sensitive to NMD suppression and not to RT agents. A large rescue of W1282X-CFTR function could be achieved with SMG1i plus correctors. This functional rescue was in agreement with appearance of a substantial amount of CFTR protein in immunoblot and immunofluorescence experiments. In particular, immunofluorescence analysis showed a significant localization of the protein at the plasma membrane with SMG1i alone, in the absence of correctors, which is suggestive that W1282X-CFTR, once adequately synthesized in larger amounts by overcoming NMD, can traffic to the cell surface.

In our search for the best combination of drugs to rescue PTCs, we also included PTI-428, described as a CFTR amplifier that is potentially able to enhance the synthesis of CFTR protein irrespective of the presence and type of mutations (Giuliano et al., 2018, Dukovski et al., 2020). However, we found no effect of PTI-428 on G542X-CFTR or W1282X-CFTR

rescue. PTI-428 activity was instead effective on the F508del mutation. The amplifier was effective by itself and further improved the rescue by VX-809 in a synergistic way. The efficacy of PTI-428 on CFTR function was also confirmed in primary HBECs. It is known that CFTR amplifiers act through PCBP1, a protein that interacts with CFTR mRNA (Dukovski et al., 2020). It is possible that, unlike other CF mutations, PTCs interfere with the function of PCBP1. Our experiments in primary cells also revealed that PTI-428 significantly enhances the ion currents mediated by ENaC and TMEM16A channels. Such results suggest that CFTR amplifiers may alter the expression/function of other proteins involved in transepithelial ion transport with possible undesired effects.

4.2. Identification of other NMD inhibitors by high-throughput screening

Given the large efficacy of NMD inhibition on W1282X-CFTR rescue, we decided to look for other small molecules with better characteristic of SMG1i. This decision was also based on the satisfactory results obtained by generating a stable cell line co-expressing W1282X-CFTR and HSYFP. Initial results demonstrated that this cell line was suitable for highthroughput screening of small molecule libraries. In particular, data obtained with the HS-YFP assay were perfectly in agreement with transepithelial current recordings. The assay confirmed the sensitivity of this mutant to VX-445 and the lack of synergy between SMG1i and RT agents.

There is a rationale for the search of novel inhibitors of NMD, possibly acting with a MoA different from SMG1i. Indeed, SMG1i action may generate undesired side effects in vivo (Keenan et al., 2019). In this respect, a complete shutdown of NMD factors, in particular SMG1, is not compatible with life, since they play an important general role in gene expression regulation (Han et al., 2018). We speculated that milder modulators of NMD process, still effective in rescuing CFTR mRNA to a sufficient level, could be better tolerated by the organism.

The primary screening identified a large number of hits, all belonging to different families of drugs, such as proteasome and kinase inhibitors, cyclin-dependent kinases (CDKs) inhibitors, eukaryotic initiation factors (eIFs) and topoisomerases inhibitors. We carried out a series of secondary assays

to investigate and prioritize hits. Importantly, we used an assay based on the CFTR pharmacological inhibitor CFTR_{inh}-172 (Caci et al., 2008) to exclude those compounds inducing enhanced iodide uptake in the HS-YFP uptake but irrespective of mutant CFTR rescue. Indeed, we found that many screening hits had to be excluded because their effect was insensitive to CFTR_{inh}-172. At the end of the secondary analysis, three compounds emerged as the best outcome of the screening, namely CC115, samotolisib and elimusertib. All of them greatly enhanced W1282XCFTR-mediated Cl⁻ current in I_{sc} recordings at micromolar concentrations when combined to CFTR correctors. Interestingly, elimusertib per se elicited a significant increase in CFTR current without the use of correctors. Activity of kinase inhibitors is generally promiscuous. Since the same compounds have effects on a broad panel of PI3K-related kinases (i.e. mTOR for CC-115 and samotolisib and ATM/ATR for elimusertib) we also tested other known inhibitors of the same kinases such as Torin for mTOR, Akti172 for Akt and Nu774 for ATM/ATR to exclude their involvement. None of these other agents fully reproduced the effects of active compounds identified in the screening. In particular, they did not increase CFTR activity when combined with CFTR correctors (data not shown), suggesting absence of protein rescue. These results are suggestive that the effect on mutant CFTR rescue of the kinase inhibitors resulting from our screening is not dependent on their nominal target.

To investigate the MoA of CC-115, samotolisib, and elimusertib, we analysed their effect on mRNA stability and protein processing. The three compounds increased CFTR mRNA expression in 16HBE14o⁻ cells, as demonstrated by RNAscope and real time RT-PCR, in a manner comparable to SMG1i action. Importantly, addition of G418 did not further contribute to stabilize mRNA, as previously shown for SMG1i. The increased mRNA content was paralleled by an increased expression of a protein signal corresponding to a fully glycosylated form of CFTR (C band), but with reduced molecular weight, in agreement with increased protein expression but in the absence of RT overcoming W1282X. Of note, addition of VX-809/VX-445 combination further increased the glycosylated form of CFTR. Summarizing, the results obtained with CC115, samotolisib, and elimusertib are highly suggestive of NMD inhibition. We tested the compounds in the presence of SMG1. None of

the three compounds showed synergy/additivity with SMG1i, indicating that they are possibly acting on the same molecular target or, at least, modulating the same pathway. In this respect, while carrying our primary screening, a paper appeared describing CC-115 having effects similar to SMG1i (Leeksa et al., 2022).

We are currently performing further analysis to clearly define the MoA of our identified compounds. Indeed, the efficacy of elimusertib when tested alone without correctors would be suggesting of a MoA that could slightly differs from the others. In this respect, similarly to SMG1i and CC-115, also samotolisib and elimusertib determined a reduction in the phosphorylated form of the UPF1 protein, the phospho-target of the SMG1 kinase, but with a milder effect. It is tempting to speculate that such compounds could have pleiotropic effects on the NMD pathway, modulating other components of the SURF complex necessary to UPF1 phosphorylation and activation (Chamieh et al., 2008, Langer et al., 2024). Another possibility is that such compounds somehow affect UPF1 phosphorylation at different serine residues (Yamashita, 2013, Isken et al., 2008). Indeed, it has been demonstrated in vitro that the Upf1 protein can be differentially phosphorylated in several Ser/Thr phospho-sites accordingly to the task it has to perform (Okada-Katsuhata et al., 2011, Durand et al., 2016, Langer et al., 2024). We are also currently studying the MoA and effects of other compounds belonging to different pharmacological families to also identify novel biological pathways linked to NMD and/or, in general, to PTC rescue. Finally, we performed bulk mRNA sequencing of 16HBE14o⁻ cells. By now, we found that, as expected for NMD inhibitors, many transcripts were similarly upregulated by the treatment in both wild type and W1282X cells. The exception was CFTR transcript, which was specifically upregulated in W1282X cells. This result is in agreement with NMD specifically affecting the CFTR mRNA when carrying a PTC. Our goal is to identify common and compound-specific signatures elicited by treatments, with the ambitious aim to define the impact of NMD inhibition at the transcriptomic level. This information could be useful to outline a transcriptional “fingerprinting” of how NMD inhibitors behave in cells. The results reported in this chapter are included in a manuscript under preparation

(Borrelli et al., “Identification of novel NMD inhibitors by highthroughput screening”).

4.3. Effect of inflammatory conditions of G542X-CFTR rescue

ELX-02, formerly known as NB-124, is a proof of principle RT agent, able to induce the continuation of protein synthesis bypassing the PTC (Xue et al., 2014; Crawford et al., 2020; Kerem, 2020). As discussed above, ELX-02 caused modest effects on a variety of CF-causing PTCs. We asked whether the activity of ELX-02 could be improved by CC90009. Indeed, CC-90009, a “molecular glue” that works as an eRF3a degrader, has been demonstrated to correct G542X-CFTR function in HBECs (Baradaran-Heravi et al., 2021; Lee et al., 2022). In our experiments, we found that combination of ELX-02 with CC-90009, plus VX-809, improves the rescue of G542X-CFTR. Surprisingly, we found that the effect of the triple drug combination is largely amplified under pro-inflammatory conditions, i.e. by treating the epithelia with IL-4 or with the combination IL-17A/TNF- α . These two types of stimuli have been shown to remarkably modify airway epithelia at the molecular and functional levels (Gorrieri et al., 2016; Guidone et al., 2022), with broad changes in gene expression. The marked functional rescue was also paralleled by the appearance of full-length CFTR protein, particularly with IL-4.

There are various mechanisms, possibly acting in a cooperative way, that could explain the effects of cytokines on G542X-CFTR rescue. A first intriguing possibility is that cytokine treatment somehow suppresses the NMD process. This would contribute to increase CFTR mRNA thus providing more substrate for RT and synthesis of full-length CFTR protein. It should be noticed that IL-4 and IL-17A/TNF- α are already known to modestly increase CFTR mRNA in non-CF epithelia, probably at the level of transcription (Gorrieri et al., 2016; Guidone et al., 2022). Interestingly, in our experiment both IL-4 and IL-17A/TNF- α did not upregulate CFTR transcript levels per se, but they stabilize G542X-CFTR mRNA particularly when combined with ELX-02/CC-90009/VX-809. Therefore, an inhibition of NMD can be claimed. In this respect, a recent work by Ghiasi and colleagues, has proposed that proinflammatory cytokines that are implicated in pancreatic β cell failure in type 1 and type

2 diabetes attenuate NMD activity through activation of ER stress, UPR response and downregulation of the UPF3B factor (Ghiasi et al., 2024). By investigation of our previous published transcriptomic dataset in non-CF bronchial epithelial cells, we found that treatment with IL-4 and IL17A/TNF- α induced downregulation of factors involved in this biological process (e.g. PABPC1, PPA1A2, RUVBL proteins). However, further studies are needed to elucidate the molecular basis of this effect. A second possibility, that does not exclude NMD suppression, is the generation by cytokines of an environment that is more favorable for RT. Finally, we must consider that cAMP-activated chloride secretion does not only depend on CFTR but also relies on the proteins that generate the driving force for Cl⁻ exit at the apical membrane. In this respect, we showed that IL-4 upregulates expression of many ion channels and transporters such as SCL12A2, the Na⁺/K⁺/Cl⁻ cotransporter, that is responsible for Cl⁻ uptake at the basolateral membrane (Gorrieri et al., 2016). Upregulation of SLC12A2, at least in IL-4 treated epithelia, could contribute to enhanced chloride secretion in G542X epithelia treated for CFTR rescue.

In this respect, we wondered whether the extent of CFTR rescue achieved in our experiments could also influence ASL properties. We previously reported that treatment of epithelia with the IL-17A/TNF- α combination induces a strong increase in viscosity as revealed by FRAP experiments (Guidone et al., 2022). This condition can be reverted by CFTR activation in non-CF epithelia. In the present study, we found that the expected hyper viscosity induced by the IL-17A/TNF- α treatment can be actually decreased in the presence of the triple drug combination (VX-809/ELX02/CC90009). This result suggests that the amount of CFTR function restored by the drugs is enough to improve airway surface properties. However, we cannot exclude that part of the effect on viscosity is due to a decrease in ENaC activity since Na⁺ absorption is another process controlling epithelial surface hydration. The results in this chapter are included in a second paper under preparation (Borrelli et al., “Rescue of CFTR chloride channel with nonsense mutations is markedly improved under inflammatory conditions”).

5. CONCLUSIONS

Summarizing, with our study we obtained several important indications that may guide a precision medicine approach for treating patients with PTCs (Figure 48).

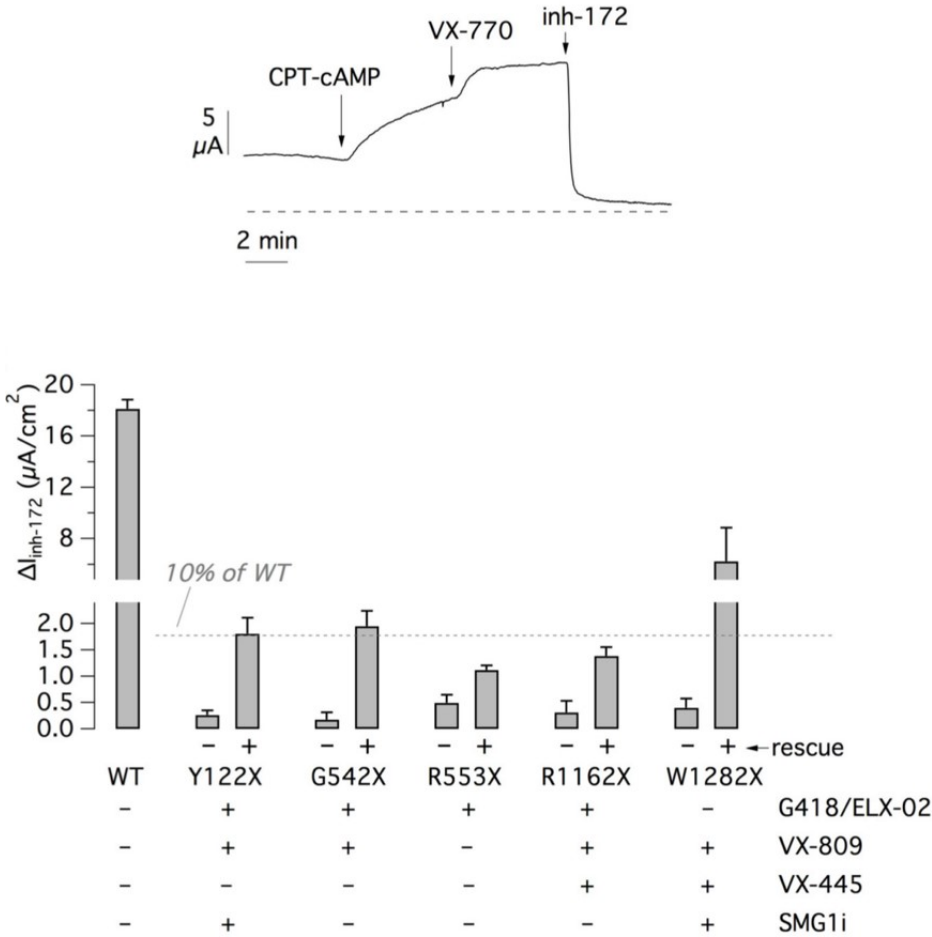


Figure 48. Rescue of different CFTR mutants requires different pharmacological treatments. Upper: representative short-circuit current recording from 16HBE14o- cells expressing wild-type CFTR. Lower: bar graph showing the comparison of CFTR function in 16HBE14o- cells expressing wild-type or mutant CFTR. For cells carrying mutant CFTR, the bars report CFTR function (mean plus SD) under control conditions and after rescue with the most effective treatment for each mutation as indicated.

W1282X appears as the most promising mutation to treat, given the large extent of rescue obtained at the functional level, as also indicated by previous studies (Keenan et al., 2019, Laselva et al., 2020). Rescue of this mutant only requires the combination of NMD suppression and CFTR correction, without inclusion of a RT that could even exert detrimental effects (Laselva et al., 2020). We also observed in 16HBE14o- cells a small but significant response to correctors in the absence of NMD inhibition,

thus suggesting that a certain level of mRNA could be insensitive to NMD, possibly escaping the mechanism. Our study indicates that the presence of CFTR corrector is required to achieve appreciable rescue of CFTR function. Therefore, from a translational point of view, treatments including these agents, as the triple drug Trikafta, are expected to be beneficial for patients carrying nonsense mutations, also taking into consideration the potentiation effect achieved with VX-770. However, treatment approach for nonsense mutations must consider the important general role of NMD in physiological gene expression regulation (Lejeune, 2017, Han et al., 2018). In this respect, NMD factors cannot be completely shut down since the total inhibition of the NMD process generate undesired side effects in vivo. This is the case of the SMG1, that is essential for mouse embryonic development (Medghalchi et al., 2001, Hwang & Maquat, 2011). A possible strategy is to modulate regulatory steps of NMD that, instead of totally blocking this pathway, induce a partial inhibition that is tolerated by the organism but is still effective in rescuing CFTR mRNA from degradation. In this respect, we performed a cell-based high throughput functional screening on 16HBE14o⁻ cells expressing W1282X-CFTR to find novel NMD inhibitors acting with a MoA more acceptable for PTC rescue and in vivo use. Future analysis will indicate if CC-115, samotolisib, or elimusertib have the appropriate characteristics.

Finally, our results with inflammatory stimuli show that the effect of compounds targeting PTCs can be largely increased under inflammatory conditions. The results point out to changes in gene expression that lead to an intracellular environment that is more permissive for PTC correction. Analysis of these events can identify novel therapeutic targets whose pharmacological modulation allows an improved rescue of mutant CFTR as well of other proteins affected by PTCs.

6. BIBLIOGRAPHY

<https://www.ecfs.eu/ecfspr>

CF patient registry annual data report 2021

<http://www.genet.sickkids.on.ca/cftr/>

CF mutation database

<https://cftr2.org>

<https://www.cff.org/medical-professionals/patient-registry>

CFF patient registry annual data report 2023

Abou Alaiwa, M. H., Beer, A. M., Pezzulo, A. A., Launsbach, J. L., Horan, R. A., Stoltz, D. A., Starner, T. D., Welsh, M. J., & Zabner, J. (2014). Neonates with cystic fibrosis have a reduced nasal liquid pH; a small pilot study. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 13(4), 373–377. <https://doi.org/10.1016/j.jcf.2013.12.006>

Amrani, N., Ganesan, R., Kervestin, S., Mangus, D. A., Ghosh, S., & Jacobson, A. (2004). A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. *Nature*, 432(7013), 112–118. <https://doi.org/10.1038/nature03060>

Antunes, M. B., & Cohen, N. A. (2007). Mucociliary clearance--a critical upper airway host defense mechanism and methods of assessment. *Current opinion in allergy and clinical immunology*, 7(1), 5–10. <https://doi.org/10.1097/ACI.0b013e3280114eef>

Baradaran-Heravi, A., Balgi, A. D., Hosseini-Farahabadi, S., Choi, K., Has, C., & Roberge, M. (2021). Effect of small molecule eRF3 degraders on premature termination codon readthrough. *Nucleic acids research*, 49(7), 3692–3708. <https://doi.org/10.1093/nar/gkab194>

Barbato, A., Frischer, T., Kuehni, C.E., Snijders, D., Azevedo, I., Baktai, G., Bartoloni, L., Eber, E., Escribano, A., Haarman, E., Hesselmar, B., Hogg, C., Jorissen, M., Lucas, J., Nielsen, K. G., O'Callaghan, C., Omran, H., Pohunek, P., Strippoli, M-P. F., Bush, A. (2009) Primary ciliary dyskinesia: a consensus statement on diagnostic and treatment approaches in children. *European Respiratory Journal*. 34(6): 1264-1276; DOI: <https://doi.org/10.1183/09031936.00176608>

Barnes, P., Burney, P., Silverman, E. et al. (2015) Chronic obstructive pulmonary disease. *Nat Rev Dis Primers* 1, 15076. <https://doi.org/10.1038/nrdp.2015.76>

Bartoszewski, R., Matalon, S., & Collawn, J. F. (2017). Ion channels of the lung and their role in disease pathogenesis. *American journal of physiology. Lung cellular and molecular physiology*, 313(5), L859–L872.

<https://doi.org/10.1152/ajplung.00285.2017>

Bear C. E. (2020). A Therapy for Most with Cystic Fibrosis. *Cell*, 180(2), 211. <https://doi.org/10.1016/j.cell.2019.12.032>

Bedwell, D. M., Kaenjak, A., Benos, D. J., Bebok, Z., Bubien, J. K., Hong, J., Tousson, A., Clancy, J. P., & Sorscher, E. J. (1997). Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nature medicine*, 3(11), 1280–1284. <https://doi.org/10.1038/nm1197-1280>

Bobbo, A.K., Ahmad U., Chau, D.-M., Nordin, N., Abdullah, S. (2023). A comprehensive review of cystic fibrosis in Africa and Asia, Saudi Journal of Biological Sciences, Volume 30, Issue 7, 103685, ISSN 1319562X, <https://doi.org/10.1016/j.sjbs.2023.103685>.

Boyle, M. P., Bell, S. C., Konstan, M. W., McColley, S. A., Rowe, S. M., Rietschel, E., Huang, X., Waltz, D., Patel, N. R., Rodman, D., & VX09809-102 study group (2014). A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial. *The Lancet. Respiratory medicine*, 2(7), 527–538. [https://doi.org/10.1016/S2213-2600\(14\)70132-8](https://doi.org/10.1016/S2213-2600(14)70132-8)

Branchfield, K., Nantie, L., Verheyden, J. M., Sui, P., Wienhold, M. D., & Sun, X. (2016). Pulmonary neuroendocrine cells function as airway sensors to control lung immune response. *Science (New York, N.Y.)*, 351(6274), 707–710. <https://doi.org/10.1126/science.aad7969>

Brogna, S., & Wen, J. (2009). Nonsense-mediated mRNA decay (NMD) mechanisms. *Nature structural & molecular biology*, 16(2), 107–113. <https://doi.org/10.1038/nsmb.1550>

Burke, J. F., & Mogg, A. E. (1985). Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic acids research*, 13(17), 6265–6272. <https://doi.org/10.1093/nar/13.17.6265>

Button, B., Cai, L. H., Ehre, C., Kesimer, M., Hill, D. B., Sheehan, J. K., Boucher, R. C., & Rubinstein, M. (2012). A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. *Science (New York, N.Y.)*, 337(6097), 937–941. <https://doi.org/10.1126/science.1223012>

Button, B., Okada, S. F., Frederick, C. B., Thelin, W. R., & Boucher, R. C. (2013). Mechanosensitive ATP release maintains proper mucus hydration of airways. *Science signaling*, 6(279), ra46. <https://doi.org/10.1126/scisignal.2003755>

Bustamante-Marin, X. M., & Ostrowski, L. E. (2017). Cilia and Mucociliary Clearance. *Cold Spring Harbor perspectives in biology*, 9(4), a028241. <https://doi.org/10.1101/cshperspect.a028241>

Caci, E., Caputo, A., Hinzpeter, A., Arous, N., Fanen, P., Sonawane, N., Verkman, A. S., Ravazzolo, R., Zegarra-Moran, O., & Galiotta, L. J. (2008). Evidence for direct CFTR inhibition by CFTR(inh)-172 based on Arg347 mutagenesis. *The Biochemical journal*, 413(1), 135–142. <https://doi.org/10.1042/BJ20080029>

Caci, E., Scudieri, P., Di Carlo, E., Morelli, P., Bruno, S., De Fino, I., Bragonzi, A., Gianotti, A., Sondo, E., Ferrera, L., Palleschi, A., Santambrogio, L., Ravazzolo, R., & Galiotta, L. J. (2015). Upregulation of TMEM16A Protein in Bronchial Epithelial Cells by Bacterial Pyocyanin. *PloS one*, 10(6), e0131775. <https://doi.org/10.1371/journal.pone.0131775>

Capurro, V., Tomati, V., Sondo, E., Renda, M., Borrelli, A., Pastorino, C., Guidone, D., Venturini, A., Giraudo, A., Mandrup Bertozzi, S., Musante, I., Bertozzi, F., Bandiera, T., Zara, F., Galiotta, L. J. V., & Pedemonte, N. (2021). Partial Rescue of F508del-CFTR Stability and Trafficking Defects by Double Corrector Treatment. *International journal of molecular sciences*, 22(10), 5262. <https://doi.org/10.3390/ijms22105262>

Caputo, A., Caci, E., Ferrera, L., Pedemonte, N., Barsanti, C., Sondo, E., Pfeffer, U., Ravazzolo, R., Zegarra-Moran, O., & Galiotta, L. J. (2008). TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. *Science (New York, N.Y.)*, 322(5901), 590–594. <https://doi.org/10.1126/science.1163518>

Carrard, J., & Lejeune, F. (2023). Nonsense-mediated mRNA decay, a simplified view of a complex mechanism. *BMB reports*, 56(12), 625–632. <https://doi.org/10.5483/BMBRep.2023-0190>

Chamieh, H., Ballut, L., Bonneau, F., & Le Hir, H. (2008). NMD factors UPF2 and UPF3 bridge UPF1 to the exon junction complex and stimulate its RNA helicase activity. *Nature structural & molecular biology*, 15(1), 85–93. <https://doi.org/10.1038/nsmb1330>

Clancy, J. P., Rowe, S. M., Accurso, F. J., Aitken, M. L., Amin, R. S., Ashlock, M. A., Ballmann, M., Boyle, M. P., Bronsveld, I., Campbell, P. W., De Boeck, K., Donaldson, S. H., Dorkin, H. L., Dunitz, J. M., Durie, P. R., Jain, M., Leonard, A., McCoy, K. S., Moss, R. B., Pilewski, J. M., ... Konstan, M. W. (2012). Results of a phase IIa study of VX-809, an investigational CFTR corrector compound, in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation. *Thorax*, 67(1), 12–18. <https://doi.org/10.1136/thoraxjnl-2011-200393>

Clancy J. P. (2018). Rapid therapeutic advances in CFTR modulator science. *Pediatric pulmonology*, 53(S3), S4–S11. <https://doi.org/10.1002/ppul.24157>

Coakley, R. D., Grubb, B. R., Paradiso, A. M., Gatzky, J. T., Johnson, L. G., Kreda, S. M., O'Neal, W. K., & Boucher, R. C. (2003). Abnormal surface

liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, 100(26), 16083–16088. <https://doi.org/10.1073/pnas.2634339100>

Coelho, J. P. L., Yip, M. C. J., Olton, K., Taunton, J., & Shao, S. (2024). The eRF1 degrader SRI-41315 acts as a molecular glue at the ribosomal decoding center. *Nature chemical biology*, 20(7), 877–884. <https://doi.org/10.1038/s41589-023-01521-0>

Csanády, L., Vergani, P., & Gadsby, D. C. (2019). STRUCTURE, GATING, AND REGULATION OF THE CFTR ANION CHANNEL. *Physiological reviews*, 99(1), 707–738. <https://doi.org/10.1152/physrev.00007.2018>

Crawford, D. K., Alroy, I., Sharpe, N., Goddeeris, M. M., & Williams, G. (2020). ELX-02 Generates Protein via Premature Stop Codon ReadThrough without Inducing Native Stop Codon Read-Through Proteins. *The Journal of pharmacology and experimental therapeutics*, 374(2), 264–272. <https://doi.org/10.1124/jpet.120.265595>

Crystal, R. G., Randell, S. H., Engelhardt, J. F., Voynow, J., & Sunday, M. E. (2008). Airway epithelial cells: current concepts and challenges. *Proceedings of the American Thoracic Society*, 5(7), 772–777. <https://doi.org/10.1513/pats.200805-041HR>

Cutting G. R. (2015). Cystic fibrosis genetics: from molecular understanding to clinical application. *Nature reviews. Genetics*, 16(1), 45–56. <https://doi.org/10.1038/nrg3849>

Cyr D. M. (2005). Arrest of CFTRDeltaF508 folding. *Nature structural & molecular biology*, 12(1), 2–3. <https://doi.org/10.1038/nsmb0105-2>

Danahay, H., Atherton, H., Jones, G., Bridges, R. J., & Poll, C. T. (2002). Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *American journal of physiology. Lung cellular and molecular physiology*, 282(2), L226–L236. <https://doi.org/10.1152/ajplung.00311.2001>

Davis, J.D., Wypych, T. P. (2021). Cellular and functional heterogeneity of the airway epithelium. *Mucosal Immunology*. 14:978–990; <https://doi.org/10.1038/s41385-020-00370-7>

Decraene, A., Willems-Widyastuti, A., Kasran, A., De Boeck, K., Bullens, D. M., & Dupont, L. J. (2010). Elevated expression of both mRNA and protein levels of IL-17A in sputum of stable Cystic Fibrosis patients. *Respiratory research*, 11(1), 177. <https://doi.org/10.1186/1465-9921-11177>

de Souza, D. A. S., Faucz, F. R., Pereira-Ferrari, L., Sotomaior, V. S., & Raskin, S. (2018). Congenital bilateral absence of the vas deferens as an

atypical form of cystic fibrosis: reproductive implications and genetic counseling. *Andrology*, 6(1), 127–135. <https://doi.org/10.1111/andr.12450>

Deeks E. D. (2016). Lumacaftor/Ivacaftor: A Review in Cystic Fibrosis. *Drugs*, 76(12), 1191–1201. <https://doi.org/10.1007/s40265-016-0611-2>

Du, M., Jones, J. R., Lanier, J., Keeling, K. M., Lindsey, J. R., Tousson, A., Bebök, Z., Whitsett, J. A., Dey, C. R., Colledge, W. H., Evans, M. J., Sorscher, E. J., & Bedwell, D. M. (2002). Aminoglycoside suppression of a premature stop mutation in a *Cftr*^{-/-} mouse carrying a human CFTRG542X transgene. *Journal of molecular medicine (Berlin, Germany)*, 80(9), 595–604. <https://doi.org/10.1007/s00109-002-0363-1>

Durand, S., Cougot, N., Mahuteau-Betzer, F., Nguyen, C. H., Grierson, D. S., Bertrand, E., Tazi, J., & Lejeune, F. (2007). Inhibition of nonsensemediated mRNA decay (NMD) by a new chemical molecule reveals the dynamic of NMD factors in P-bodies. *The Journal of cell biology*, 178(7), 1145–1160. <https://doi.org/10.1083/jcb.200611086>

Durand, S., Franks, T. M., & Lykke-Andersen, J. (2016). Hyperphosphorylation amplifies UPF1 activity to resolve stalls in nonsense-mediated mRNA decay. *Nature communications*, 7, 12434. <https://doi.org/10.1038/ncomms12434>

Dukovski, D., Villella, A., Bastos, C., King, R., Finley, D., Kelly, J. W., Morimoto, R. I., Hartl, F. U., Munoz, B., Lee, P. S., Zecevic, M., & Miller, J. P. (2020). Amplifiers co-translationally enhance CFTR biosynthesis via PCBP1-mediated regulation of CFTR mRNA. *Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society*, 19(5), 733–741. <https://doi.org/10.1016/j.jcf.2020.02.006>

Elborn J. S. (2016). Cystic fibrosis. *Lancet (London, England)*, 388(10059), 2519–2531. [https://doi.org/10.1016/S0140-6736\(16\)005766](https://doi.org/10.1016/S0140-6736(16)005766)

Fanen, P., Wohlhuter-Haddad, A., & Hinzpeter, A. (2014). Genetics of cystic fibrosis: CFTR mutation classifications toward genotype-based CF therapies. *The international journal of biochemistry & cell biology*, 52, 94–102. <https://doi.org/10.1016/j.biocel.2014.02.023>

Farinha, C. M., King-Underwood, J., Sousa, M., Correia, A. R., Henriques, B. J., Roxo-Rosa, M., Da Paula, A. C., Williams, J., Hirst, S., Gomes, C. M., & Amaral, M. D. (2013). Revertants, low temperature, and correctors reveal the mechanism of F508del-CFTR rescue by VX-809 and suggest multiple agents for full correction. *Chemistry & biology*, 20(7), 943–955. <https://doi.org/10.1016/j.chembiol.2013.06.004>

Faucz, F. R., Gimenez, J., Ramos, M. D., Pereira-Ferrari, L., Estivill, X., Raskin, S., Casals, T., & Culpi, L. (2007). Cystic fibrosis in a southern Brazilian population: characteristics of 90% of the alleles. *Clinical*

- genetics, 72(3), 218–223.
<https://doi.org/10.1111/j.13990004.2007.00854.x>
- Fiedorczuk, K., & Chen, J. (2022a). Mechanism of CFTR correction by type I folding correctors. *Cell*, 185(1), 158–168.e11.
<https://doi.org/10.1016/j.cell.2021.12.009>
- Fiedorczuk, K., & Chen, J. (2022b). Molecular structures reveal synergistic rescue of $\Delta 508$ CFTR by Trikafta modulators. *Science (New York, N.Y.)*, 378(6617), 284–290.
<https://doi.org/10.1126/science.ade2216>
- Fulcher, M. L., Gabriel, S., Burns, K. A., Yankaskas, J. R., & Randell, S. H. (2005). Well-differentiated human airway epithelial cell cultures. *Methods in molecular medicine*, 107, 183–206.
<https://doi.org/10.1385/159259-861-7:183>
- Galletta, L. J., Pagesy, P., Folli, C., Caci, E., Romio, L., Costes, B., Nicolis, E., Cabrini, G., Goossens, M., Ravazzolo, R., & Zegarra-Moran, O. (2002). IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *Journal of immunology (Baltimore, Md.: 1950)*, 168(2), 839–845. <https://doi.org/10.4049/jimmunol.168.2.839>
- Garcia, M. A., Yang, N., & Quinton, P. M. (2009). Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *The Journal of clinical investigation*, 119(9), 2613–2622. <https://doi.org/10.1172/JCI38662>
- Gentzsch, M., Baker, B., Cholon, D. M., Kam, C. W., McKinzie, C. J., Despotes, K. A., Boyles, S. E., Quinney, N. L., Esther, C. R., Jr, & Ribeiro, C. M. P. (2024). Cystic fibrosis airway inflammation enables elxacaftor/tezacaftor/ivacaftor-mediated rescue of N1303K CFTR mutation. *ERJ open research*, 10(1), 00746-2023.
<https://doi.org/10.1183/23120541.00746-2023>
- Ghiasi, S. M., Krogh, N., Tyrberg, B., & Mandrup-Poulsen, T. (2018). The No-Go and Nonsense-Mediated RNA Decay Pathways Are Regulated by Inflammatory Cytokines in Insulin-Producing Cells and Human Islets and Determine β -Cell Insulin Biosynthesis and Survival. *Diabetes*, 67(10), 2019–2037. <https://doi.org/10.2337/db18-0073>
- Ghiasi, S. M., Marchetti, P., Piemonti, L., Nielsen, J. H., Porse, B. T., Mandrup-Poulsen, T., & Rutter, G. A. (2024). Proinflammatory cytokines suppress nonsense-mediated RNA decay to impair regulated transcript isoform processing in pancreatic β cells. *Frontiers in endocrinology*, 15, 1359147. <https://doi.org/10.3389/fendo.2024.1359147>
- Gianotti, A., Delpiano, L., & Caci, E. (2018). *In vitro* Methods for the Development and Analysis of Human Primary Airway Epithelia. *Frontiers in pharmacology*, 9, 1176.
<https://doi.org/10.3389/fphar.2018.01176>

Giuliano, K. A., Wachi, S., Drew, L., Dukovski, D., Green, O., Bastos, C., Cullen, M. D., Hauck, S., Tait, B. D., Munoz, B., Lee, P. S., & Miller, J. P. (2018). Use of a High-Throughput Phenotypic Screening Strategy to Identify Amplifiers, a Novel Pharmacological Class of Small Molecules That Exhibit Functional Synergy with Potentiators and Correctors. *SLAS discovery : advancing life sciences R & D*, 23(2), 111–121.

<https://doi.org/10.1177/2472555217729790>

Gonzalez-Hilarion, S., Beghyn, T., Jia, J., Debreuck, N., Berte, G., Mamchaoui, K., Mouly, V., Gruenert, D. C., Déprez, B., & Lejeune, F. (2012). Rescue of nonsense mutations by amlexanox in human cells.

Orphanet journal of rare diseases, 7, 58. <https://doi.org/10.1186/17501172-7-58>

Gopalsamy, A., Bennett, E. M., Shi, M., Zhang, W. G., Bard, J., & Yu, K. (2012). Identification of pyrimidine derivatives as hSMG-1 inhibitors. *Bioorganic & medicinal chemistry letters*, 22(21), 6636–6641.

<https://doi.org/10.1016/j.bmcl.2012.08.107>

Gorrieri, G., Scudieri, P., Caci, E., Schiavon, M., Tomati, V., Sirci, F., Napolitano, F., Carrella, D., Gianotti, A., Musante, I., Favia, M., Casavola, V., Guerra, L., Rea, F., Ravazzolo, R., Di Bernardo, D., & Galletta, L. J. (2016). Goblet Cell Hyperplasia Requires High Bicarbonate Transport To Support Mucin Release. *Scientific reports*, 6, 36016.

<https://doi.org/10.1038/srep36016>

Graeber, S. Y., & Mall, M. A. (2023). The future of cystic fibrosis treatment: from disease mechanisms to novel therapeutic approaches.

Lancet (London, England), 402(10408), 1185–1198.

[https://doi.org/10.1016/S0140-6736\(23\)01608-2](https://doi.org/10.1016/S0140-6736(23)01608-2)

Guidone, D., Buccirosi, M., Scudieri, P., Genovese, M., Sarnataro, S., De Cegli, R., Cresta, F., Terlizzi, V., Planelles, G., Crambert, G., Sermet, I., & Galletta, L. J. (2022). Airway surface hyperviscosity and defective mucociliary transport by IL-17/TNF- α are corrected by β -adrenergic stimulus. *JCI insight*, 7(22), e164944.

<https://doi.org/10.1172/jci.insight.164944>

Guillermi, H., Fanen, P., & Ferec, C. (1990). A 3' splice site consensus sequence mutation in the cystic fibrosis gene. *Human genetics*, 85(4), 450–453.

<https://doi.org/10.1007/BF02428306>

Gustafsson, J. K., Ermund, A., Ambort, D., Johansson, M. E., Nilsson, H. E., Thorell, K., Hebert, H., Sjövall, H., & Hansson, G. C. (2012). Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *The Journal of experimental medicine*, 209(7), 1263–1272.

<https://doi.org/10.1084/jem.20120562>

Haardt, M., Benharouga, M., Lechardeur, D., Kartner, N., & Lukacs, G. L. (1999). C-terminal truncations destabilize the cystic fibrosis

transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. *The Journal of biological chemistry*, 274(31), 21873–21877. <https://doi.org/10.1074/jbc.274.31.21873>

Hagner, M., Albrecht, M., Guerra, M., Braubach, P., Halle, O., ZhouSuckow, Z., Butz, S., Jonigk, D., Hansen, G., Schultz, C., Dittrich, A. M., & Mall, M. A. (2021). IL-17A from innate and adaptive lymphocytes contributes to inflammation and damage in cystic fibrosis lung disease. *The European respiratory journal*, 57(6), 1900716. <https://doi.org/10.1183/13993003.00716-2019>

Hamosh, A., Rosenstein, B. J., & Cutting, G. R. (1992). CFTR nonsense mutations G542X and W1282X associated with severe reduction of CFTR mRNA in nasal epithelial cells. *Human molecular genetics*, 1(7), 542–544. <https://doi.org/10.1093/hmg/1.7.542>

Han, X., Wei, Y., Wang, H., Wang, F., Ju, Z., & Li, T. (2018). Nonsensemediated mRNA decay: a 'nonsense' pathway makes sense in stem cell biology. *Nucleic acids research*, 46(3), 1038–1051. <https://doi.org/10.1093/nar/gkx1272>

Hansen, J. D., Correa, M., Alexander, M., Nagy, M., Huang, D., Sapienza, J., Lu, G., LeBrun, L. A., Cathers, B. E., Zhang, W., Tang, Y., Ammirante, M., Narla, R. K., Piccotti, J. R., Pourdehnad, M., & Lopez-Girona, A. (2021). CC-90009: A Cereblon E3 Ligase Modulating Drug That Promotes Selective Degradation of GSPT1 for the Treatment of Acute Myeloid Leukemia. *Journal of medicinal chemistry*, 64(4), 1835–1843. <https://doi.org/10.1021/acs.jmedchem.0c01489>

Hewitt, R. J., & Lloyd, C. M. (2021). Regulation of immune responses by the airway epithelial cell landscape. *Nature reviews. Immunology*, 21(6), 347–362. <https://doi.org/10.1038/s41577-020-00477-9>

Higgins, G., Ringholz, F., Buchanan, P., McNally, P., & Urbach, V. (2015). Physiological impact of abnormal lipoxin A4 production on cystic fibrosis airway epithelium and therapeutic potential. *BioMed research international*, 2015, 781087. <https://doi.org/10.1155/2015/781087>

Highsmith, W. E., Jr, Burch, L. H., Zhou, Z., Olsen, J. C., Strong, T. V., Smith, T., Friedman, K. J., Silverman, L. M., Boucher, R. C., Collins, F. S., & Knowles, M. R. (1997). Identification of a splice site mutation (2789 +5 G > A) associated with small amounts of normal CFTR mRNA and mild cystic fibrosis. *Human mutation*, 9(4), 332–338. [https://doi.org/10.1002/\(SICI\)1098-1004\(1997\)9:4<332::AIDHUMU5>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1098-1004(1997)9:4<332::AIDHUMU5>3.0.CO;2-7)

Hill, D. B., Button, B., Rubinstein, M., & Boucher, R. C. (2022). Physiology and pathophysiology of human airway mucus. *Physiological reviews*, 102(4), 1757–1836. <https://doi.org/10.1152/physrev.00004.2021>

Hoegger, M. J., Fischer, A. J., McMenimen, J. D., Ostedgaard, L. S., Tucker, A. J., Awadalla, M. A., Moninger, T. O., Michalski, A. S., Hoffman, E. A., Zabner, J., Stoltz, D. A., & Welsh, M. J. (2014). Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. *Science (New York, N.Y.)*, 345(6198), 818–822.
<https://doi.org/10.1126/science.1255825>

Hoelen, H., Kleizen, B., Schmidt, A., Richardson, J., Charitou, P., Thomas, P. J., & Braakman, I. (2010). The primary folding defect and rescue of $\Delta F508$ CFTR emerge during translation of the mutant domain. *PloS one*, 5(11), e15458. <https://doi.org/10.1371/journal.pone.0015458>

Howard, M., Frizzell, R. A., & Bedwell, D. M. (1996). Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nature medicine*, 2(4), 467–469.
<https://doi.org/10.1038/nm0496-467>

Hoy S. M. (2019). Elexacaftor/Ivacaftor/Tezacaftor: First Approval. *Drugs*, 79(18), 2001–2007. <https://doi.org/10.1007/s40265-019-01233-7>
Huang, F., Zhang, H., Wu, M., Yang, H., Kudo, M., Peters, C. J., Woodruff, P. G., Solberg, O. D., Donne, M. L., Huang, X., Sheppard, D., Fahy, J. V., Wolters, P. J., Hogan, B. L., Finkbeiner, W. E., Li, M., Jan, Y. N., Jan, L. Y., & Rock, J. R. (2012). Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction. *Proceedings of the National Academy of Sciences of the United States of America*, 109(40), 16354–16359.
<https://doi.org/10.1073/pnas.1214596109>

Huang, L., & Wilkinson, M. F. (2012). Regulation of nonsense-mediated mRNA decay. *Wiley interdisciplinary reviews. RNA*, 3(6), 807–828.
<https://doi.org/10.1002/wrna.1137>

Huth, M., Santini, L., Galimberti, E., Ramesmayer, J., Titz-Teixeira, F., Sehlke, R., Oberhuemer, M., Stummer, S., Herzog, V., Garmhausen, M., Romeike, M., Chugunova, A., Leesch, F., Holcik, L., Weipoltshammer, K., Lackner, A., Schoefer, C., von Haeseler, A., Buecker, C., Pauli, A., ... Leeb, M. (2022). NMD is required for timely cell fate transitions by finetuning gene expression and regulating translation. *Genes & development*, 36(5-6), 348–367. <https://doi.org/10.1101/gad.347690.120>

Hwang, J., & Maquat, L. E. (2011). Nonsense-mediated mRNA decay (NMD) in animal embryogenesis: to die or not to die, that is the question. *Current opinion in genetics & development*, 21(4), 422–430.
<https://doi.org/10.1016/j.gde.2011.03.008>

Ishigaki, Y., Li, X., Serin, G., & Maquat, L. E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsensemediated decay in mammalian cells are bound by CBP80 and CBP20. *Cell*, 106(5), 607–617. [https://doi.org/10.1016/s0092-8674\(01\)00475-5](https://doi.org/10.1016/s0092-8674(01)00475-5)

Isken, O., Kim, Y. K., Hosoda, N., Mayeur, G. L., Hershey, J. W., & Maquat, L. E. (2008). Upf1 phosphorylation triggers translational repression during nonsense-mediated mRNA decay. *Cell*, 133(2), 314–327. <https://doi.org/10.1016/j.cell.2008.02.030>

Jia, S., & Taylor-Cousar, J. L. (2023). Cystic Fibrosis Modulator Therapies. *Annual review of medicine*, 74, 413–426. <https://doi.org/10.1146/annurev-med-042921-021447>

Joshi, D., Ehrhardt, A., Hong, J. S., & Sorscher, E. J. (2019). Cystic fibrosis precision therapeutics: Emerging considerations. *Pediatric pulmonology*, 54 Suppl 3(Suppl 3), S13–S17. <https://doi.org/10.1002/ppul.24547>

Keating, D., Marigowda, G., Burr, L., Daines, C., Mall, M. A., McKone, E. F., Ramsey, B. W., Rowe, S. M., Sass, L. A., Tullis, E., McKee, C. M., Moskowitz, S. M., Robertson, S., Savage, J., Simard, C., Van Goor, F., Waltz, D., Xuan, F., Young, T., Taylor-Cousar, J. L., ... VX16-445-001 Study Group (2018). VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *The New England journal of medicine*, 379(17), 1612–1620. <https://doi.org/10.1056/NEJMoA1807120>

Keenan, M. M., Huang, L., Jordan, N. J., Wong, E., Cheng, Y., Valley, H. C., Mahiou, J., Liang, F., Bihler, H., Mense, M., Guo, S., & Monia, B. P. (2019). Nonsense-mediated RNA Decay Pathway Inhibition Restores Expression and Function of W1282X CFTR. *American journal of respiratory cell and molecular biology*, 61(3), 290–300. <https://doi.org/10.1165/rcmb.2018->

Kerem, B., Chiba-Falek, O., & Kerem, E. (1997). Cystic fibrosis in Jews: frequency and mutation distribution. *Genetic testing*, 1(1), 35–39. <https://doi.org/10.1089/gte.1997.1.35>

Kerem E. (2020). ELX-02: an investigational read-through agent for the treatment of nonsense mutation-related genetic disease. *Expert opinion on investigational drugs*, 29(12), 1347–1354. <https://doi.org/10.1080/13543784.2020.1828862>

Kim, S. J., & Skach, W. R. (2012). Mechanisms of CFTR Folding at the Endoplasmic Reticulum. *Frontiers in pharmacology*, 3, 201. <https://doi.org/10.3389/fphar.2012.00201>

Kim, Y. K., & Maquat, L. E. (2019). UPFRONT and center in RNA decay: UPF1 in nonsense-mediated mRNA decay and beyond. *RNA (New York, N.Y.)*, 25(4), 407–422. <https://doi.org/10.1261/rna.070136.118>

Knowles, M. R., & Boucher, R. C. (2002). Mucus clearance as a primary innate defense mechanism for mammalian airways. *The Journal of clinical investigation*, 109(5), 571–577. <https://doi.org/10.1172/JCI15217>

- Kurosaki, T., Popp, M. W., & Maquat, L. E. (2019). Quality and quantity control of gene expression by nonsense-mediated mRNA decay. *Nature reviews. Molecular cell biology*, 20(7), 406–420. <https://doi.org/10.1038/s41580-019-0126-2>
- Kurkowiak, M., Ziętkiewicz, E., & Witt, M. (2015). Recent advances in primary ciliary dyskinesia genetics. *Journal of medical genetics*, 52(1), 1–9. <https://doi.org/10.1136/jmedgenet-2014-102755>
- Lachowicz-Scroggins, M. E., Boushey, H. A., Finkbeiner, W. E., & Widdicombe, J. H. (2010). Interleukin-13-induced mucous metaplasia increases susceptibility of human airway epithelium to rhinovirus infection. *American journal of respiratory cell and molecular biology*, 43(6), 652–661. <https://doi.org/10.1165/rcmb.2009-0244OC>
- Lai, H. Y., & Rogers, D. F. (2010). Mucus hypersecretion in asthma: intracellular signalling pathways as targets for pharmacotherapy. *Current opinion in allergy and clinical immunology*, 10(1), 67–76. <https://doi.org/10.1097/ACI.0b013e328334643a>
- Lambert, J. M., Srour, N., & Delpy, L. (2019). The Yin and Yang of RNA surveillance in B lymphocytes and antibody-secreting plasma cells. *BMB reports*, 52(12), 671–678. <https://doi.org/10.5483/BMBRep.2019.52.12.232>
- Lambrecht, B. N., Hammad, H., & Fahy, J. V. (2019). The Cytokines of Asthma. *Immunity*, 50(4), 975–991. <https://doi.org/10.1016/j.immuni.2019.03.018>
- Langer, L. M., Kurscheidt, K., Basquin, J., Bonneau, F., Iermak, I., Basquin, C., & Conti, E. (2024). UPF1 helicase orchestrates mutually exclusive interactions with the SMG6 endonuclease and UPF2. *Nucleic acids research*, 52(10), 6036–6048. <https://doi.org/10.1093/nar/gkae323>
- Laselva, O., Eckford, P. D., Bartlett, C., Ouyang, H., Gunawardena, T. N., Gonska, T., Moraes, T. J., & Bear, C. E. (2020). Functional rescue of c.3846G>A (W1282X) in patient-derived nasal cultures achieved by inhibition of nonsense mediated decay and protein modulators with complementary mechanisms of action. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 19(5), 717–727. <https://doi.org/10.1016/j.jcf.2019.12.001>
- Lazarowski, E. R., & Boucher, R. C. (2021). Purinergic receptors in airway hydration. *Biochemical pharmacology*, 187, 114387. <https://doi.org/10.1016/j.bcp.2020.114387>
- Lee, R. E., Lewis, C. A., He, L., Bulik-Sullivan, E. C., Gallant, S. C., Mascenik, T. M., Dang, H., Cholon, D. M., Gentzsch, M., Morton, L. C., Minges, J. T., Theile, J. W., Castle, N. A., Knowles, M. R., Kimple, A. J., & Randell, S. H. (2022). Small molecule eRF3a degraders rescue CFTR nonsense mutations by promoting premature termination codon readthrough. *The Journal of clinical investigation*, 132(18), e154571.

Leeksa, A. C., Derks, I. A. M., Garrick, B., Jongejan, A., Colombo, M., Bloedjes, T., Trowe, T., Leisten, J. C., Howarth, M., Malek, M., Mortensen, D. S., Blease, K., Groza, M. C., Narla, R. K., Loos, R., Kersten, M. J., Moerland, P. D., Guikema, J. E. J., Kater, A. P., Eldering, E., ... Filvaroff, E. H. (2023). SMG1, a nonsense-mediated mRNA decay (NMD) regulator, as a candidate therapeutic target in multiple myeloma. *Molecular oncology*, 17(2), 284–297. <https://doi.org/10.1002/1878-0261.13343>

Lennox, A. T., Coburn, S. L., Leech, J. A., Heidrich, E. M., Kleyman, T. R., Wenzel, S. E., Pilewski, J. M., Corcoran, T. E., & Myerburg, M. M. (2018). ATP12A promotes mucus dysfunction during Type 2 airway inflammation. *Scientific reports*, 8(1), 2109. <https://doi.org/10.1038/s41598-018-20444-8>

Lei, L., Traore, S., Romano Ibarra, G. S., Karp, P. H., Rehman, T., Meyerholz, D. K., Zabner, J., Stoltz, D. A., Sinn, P. L., Welsh, M. J., McCray, P. B., Jr, & Thornell, I. M. (2023). CFTR-rich ionocytes mediate chloride absorption across airway epithelia. *The Journal of clinical investigation*, 133(20), e171268. <https://doi.org/10.1172/JCI171268>.

Lejeune F. (2017). Nonsense-mediated mRNA decay at the crossroads of many cellular pathways. *BMB reports*, 50(4), 175–185. <https://doi.org/10.5483/bmbrep.2017.50.4.015>

Liu, F., Zhang, Z., Csanády, L., Gadsby, D. C., & Chen, J. (2017). Molecular Structure of the Human CFTR Ion Channel. *Cell*, 169(1), 85–95.e8. <https://doi.org/10.1016/j.cell.2017.02.024>

Linde, L., Boelz, S., Neu-Yilik, G., Kulozik, A. E., & Kerem, B. (2007). The efficiency of nonsense-mediated mRNA decay is an inherent character and varies among different cells. *European journal of human genetics: EJHG*, 15(11), 1156–1162. <https://doi.org/10.1038/sj.ejhg.5201889>

Lu, P., Cheng, Y., Xue, L., Ren, X., Xu, X., Chen, C., Cao, L., Li, J., Wu, Q., Sun, S., Hou, J., Jia, W., Wang, W., Ma, Y., Jiang, Z., Li, C., Qi, X., Huang, N., & Han, T. (2024). Selective degradation of multimeric proteins by TRIM21-based molecular glue and PROTAC degraders. *Cell*, S00928674(24)01197-8. Advance online publication. <https://doi.org/10.1016/j.cell.2024.10.015>

Lucas, J. S., Davis, S. D., Omran, H., & Shoemark, A. (2020). Primary ciliary dyskinesia in the genomics age. *The Lancet. Respiratory medicine*, 8(2), 202–216. [https://doi.org/10.1016/S2213-2600\(19\)30374-1](https://doi.org/10.1016/S2213-2600(19)30374-1)

Lücking, U., Wortmann, L., Wengner, A. M., Lefranc, J., Lienau, P., Briem, H., Siemeister, G., Bömer, U., Denner, K., Schäfer, M., Koppitz, M., Eis, K., Bartels, F., Bader, B., Bone, W., Moosmayer, D., Holton, S.

J., Eberspächer, U., Grudzinska-Goebel, J., Schatz, C., ... von Nussbaum, F. (2020). Damage Incorporated: Discovery of the Potent, Highly Selective, Orally Available ATR Inhibitor BAY 1895344 with Favorable Pharmacokinetic Properties and Promising Efficacy in Monotherapy and in Combination Treatments in Preclinical Tumor Models. *Journal of medicinal chemistry*, 63(13), 7293–7325.

<https://doi.org/10.1021/acs.jmedchem.0c00369>

Lukacs, G. L., Mohamed, A., Kartner, N., Chang, X. B., Riordan, J. R., & Grinstein, S. (1994). Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and requires ATP. *The EMBO journal*, 13(24), 6076–6086.

<https://doi.org/10.1002/j.1460-2075.1994.tb06954.x>

Lukacs, G. L., & Verkman, A. S. (2012). CFTR: folding, misfolding and correcting the $\Delta F508$ conformational defect. *Trends in molecular medicine*, 18(2), 81–91. <https://doi.org/10.1016/j.molmed.2011.10.003>

Lykke-Andersen, S., & Jensen, T. H. (2015). Nonsense-mediated mRNA decay: an intricate machinery that shapes transcriptomes. *Nature reviews. Molecular cell biology*, 16(11), 665–677. <https://doi.org/10.1038/nrm4063>

McShane, D., Davies, J. C., Davies, M. G., Bush, A., Geddes, D. M., & Alton, E. W. (2003). Airway surface pH in subjects with cystic fibrosis. *The European respiratory journal*, 21(1), 37–42. <https://doi.org/10.1183/09031936.03.00027603>

Malik, V., Rodino-Klapac, L. R., Viollet, L., & Mendell, J. R. (2010). Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy. *Therapeutic advances in neurological disorders*, 3(6), 379–389.

<https://doi.org/10.1177/1756285610388693>

Mall M. A. (2008). Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *Journal of aerosol medicine and pulmonary drug delivery*, 21(1), 13–24.

<https://doi.org/10.1089/jamp.2007.0659>

Mall, M. A., & Hartl, D. (2014). CFTR: cystic fibrosis and beyond. *The European respiratory journal*, 44(4), 1042–1054.

<https://doi.org/10.1183/09031936.00228013>

Mall, M. A., Mayer-Hamblett, N., & Rowe, S. M. (2020). Cystic Fibrosis: Emergence of Highly Effective Targeted Therapeutics and Potential Clinical Implications. *American journal of respiratory and critical care medicine*, 201(10), 1193–1208.

<https://doi.org/10.1164/rccm.2019101943SO>

Mall, M.A., Burgel, P.R., Castellani, C. et al. Cystic fibrosis. (2024) *Nature Reviews Disease Primers* 10, 53.

<https://doi.org/10.1038/s41572024-00538-6>

Marson, F. A. L., Bertuzzo, C. S., & Ribeiro, J. D. (2016). Classification of CFTR mutation classes. *The Lancet. Respiratory medicine*, 4(8), e37–e38. [https://doi.org/10.1016/S2213-2600\(16\)30188-6](https://doi.org/10.1016/S2213-2600(16)30188-6)

Martin, L., Grigoryan, A., Wang, D., Wang, J., Breda, L., Rivella, S., Cardozo, T., & Gardner, L. B. (2014). Identification and characterization of small molecules that inhibit nonsense-mediated RNA decay and suppress nonsense p53 mutations. *Cancer research*, 74(11), 3104–3113. <https://doi.org/10.1158/0008-5472.CAN-13-2235>

Martin, C. Burgel, P.R., (2020). Carriers of a single CFTR mutation are asymptomatic: an evolving dogma? *European Respiratory Journal*; 56(3): 2002645; DOI: <https://doi.org/10.1183/13993003.02645-2020>

Maquat L. E. (1995). When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA (New York, N.Y.)*, 1(5), 453–465.

Medghalchi, S. M., Frischmeyer, P. A., Mendell, J. T., Kelly, A. G., Lawler, A. M., & Dietz, H. C. (2001). Rent1, a trans-effector of nonsense-mediated mRNA decay, is essential for mammalian embryonic viability. *Human molecular genetics*, 10(2), 99–105. <https://doi.org/10.1093/hmg/10.2.99>

Mercuri, E., Osorio, A. N., Muntoni, F., Buccella, F., Desguerre, I., Kirschner, J., Tulinius, M., de Resende, M. B. D., Morgenroth, L. P., Gordish-Dressman, H., Johnson, S., Kristensen, A., Werner, C., Trifillis, P., Henricson, E. K., McDonald, C. M., & STRIDE and CINRG DNHS investigators (2023). Safety and effectiveness of ataluren in patients with nonsense mutation DMD in the STRIDE Registry compared with the CINRG Duchenne Natural History Study (2015-2022): 2022 interim analysis. *Journal of neurology*, 270(8), 3896–3913. <https://doi.org/10.1007/s00415-023-11687-1>

Moore, P. J., & Tarran, R. (2018). The epithelial sodium channel (ENaC) as a therapeutic target for cystic fibrosis lung disease. *Expert Opinion on Therapeutic Targets*, 22(8), 687–701. <https://doi.org/10.1080/14728222.2018.1501361>

Montoro, D. T., Haber, A. L., Biton, M., Vinarsky, V., Lin, B., Birket, S. E., Yuan, F., Chen, S., Leung, H. M., Villoria, J., Rogel, N., Burgin, G., Tsankov, A. M., Waghray, A., Slyper, M., Waldman, J., Nguyen, L., Dionne, D., Rozenblatt-Rosen, O., Tata, P. R., ... Rajagopal, J. (2018). A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature*, 560(7718), 319–324. <https://doi.org/10.1038/s41586018-0393-7>

Morais, P., Zhang, R., & Yu, Y. T. (2024). Therapeutic Nonsense Suppression Modalities: From Small Molecules to Nucleic Acid-Based Approaches. *Biomedicines*, 12(6), 1284. <https://doi.org/10.3390/biomedicines12061284>

- Moran O. (2014). On the structural organization of the intracellular domains of CFTR. *The international journal of biochemistry & cell biology*, 52, 7–14. <https://doi.org/10.1016/j.biocel.2014.01.024>
- Moriarty, P. M., Reddy, C. C., & Maquat, L. E. (1998). Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. *Molecular and cellular biology*, 18(5), 2932–2939. <https://doi.org/10.1128/MCB.18.5.2932>
- Morse, D. E., & Yanofsky, C. (1969). Polarity and the degradation of mRNA. *Nature*, 224(5217), 329–331. <https://doi.org/10.1038/224329a0>
- Nishiguchi, G., Keramatnia, F., Min, J., Chang, Y., Jonchere, B., Das, S., Actis, M., Price, J., Chepyala, D., Young, B., McGowan, K., Slavish, P. J., Mayasundari, A., Jarusiewicz, J. A., Yang, L., Li, Y., Fu, X., Garrett, S. H., Papizan, J. B., Kodali, K., ... Rankovic, Z. (2021). Identification of Potent, Selective, and Orally Bioavailable Small-Molecule GSPT1/2 Degradors from a Focused Library of Cereblon Modulators. *Journal of medicinal chemistry*, 64(11), 7296–7311. <https://doi.org/10.1021/acs.jmedchem.0c01313>
- Ng, M. Y., Li, H., Ghelfi, M. D., Goldman, Y. E., & Cooperman, B. S. (2021). Ataluren and aminoglycosides stimulate read-through of nonsense codons by orthogonal mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, 118(2), e2020599118. <https://doi.org/10.1073/pnas.2020599118>
- Nguyen, L. S., Wilkinson, M. F., & Gecz, J. (2014). Nonsense-mediated mRNA decay: inter-individual variability and human disease. *Neuroscience and biobehavioral reviews*, 46 Pt 2, 175–186. <https://doi.org/10.1016/j.neubiorev.2013.10.016>
- Okada-Katsuhata, Y., Yamashita, A., Kutsuzawa, K., Izumi, N., Hirahara, F., & Ohno, S. (2012). N- and C-terminal Upf1 phosphorylations create binding platforms for SMG-6 and SMG-5: SMG-7 during NMD. *Nucleic acids research*, 40(3), 1251–1266. <https://doi.org/10.1093/nar/gkr791>
- Okiyoneda, T., Veit, G., Dekkers, J. F., Bagdany, M., Soya, N., Xu, H., Roldan, A., Verkman, A. S., Kurth, M., Simon, A., Hegedus, T., Beekman, J. M., & Lukacs, G. L. (2013). Mechanism-based corrector combination restores $\Delta F508$ -CFTR folding and function. *Nature chemical biology*, 9(7), 444–454. <https://doi.org/10.1038/nchembio.1253>
- Pedemonte, N., Sonawane, N. D., Taddei, A., Hu, J., Zegarra-Moran, O., Suen, Y. F., Robins, L. I., Dicus, C. W., Willenbring, D., Nantz, M. H., Kurth, M. J., Galiotta, L. J., & Verkman, A. S. (2005). Phenylglycine and sulfonamide correctors of defective delta F508 and G551D cystic fibrosis transmembrane conductance regulator chloride-channel gating. *Molecular pharmacology*, 67(5), 1797–1807.

<https://doi.org/10.1124/mol.105.010959>

Pedemonte, N., Caci, E., Sondo, E., Caputo, A., Rhoden, K., Pfeffer, U., Di Candia, M., Bandettini, R., Ravazzolo, R., Zegarra-Moran, O., & Galiotta, L. J. (2007). Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: role of pendrin and anion channels. *Journal of immunology* (Baltimore, Md.: 1950), 178(8), 5144–5153.

<https://doi.org/10.4049/jimmunol.178.8.5144>

Pedemonte, N., Bertozzi, F., Caci, E., Sorana, F., Di Fruscia, P., Tomati, V., Ferrera, L., Rodríguez-Gimeno, A., Berti, F., Pesce, E., Sondo, E., Gianotti, A., Scudieri, P., Bandiera, T., & Galiotta, L. J. V. (2020). Discovery of a picomolar potency pharmacological corrector of the mutant CFTR chloride channel. *Science advances*, 6(8), eaay9669.

<https://doi.org/10.1126/sciadv.aay9669>

Pereira, P., Barbosa, T., Cardoso, A.L., Sá, R., Sousa, M. (2023) Cystic fibrosis and primary ciliary dyskinesia: Similarities and differences, *Respiratory Medicine*, Volume 209, 107169, ISSN 0954-6111, <https://doi.org/10.1016/j.rmed.2023.107169>.

Plasschaert, L. W., Žilionis, R., Choo-Wing, R., Savova, V., Knehr, J., Roma, G., Klein, A. M., & Jaffe, A. B. (2018). A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature*, 560(7718), 377–381. <https://doi.org/10.1038/s41586-018-0394-6>

Popp, M. W., & Maquat, L. E. (2013). Organizing principles of mammalian nonsense-mediated mRNA decay. *Annual review of genetics*, 47, 139–165. <https://doi.org/10.1146/annurev-genet-111212-133424>

Quinton P. M. (2007). Cystic fibrosis: lessons from the sweat gland. *Physiology (Bethesda, Md.)*, 22, 212–225. <https://doi.org/10.1152/physiol.00041.2006>

Raidt, J., Wallmeier, J., Hjej, R., Onnebrink, J. G., Pennekamp, P., Loges, N. T., Olbrich, H., Häffner, K., G. W., Omran, H., & Werner, C. (2014). Ciliary beat pattern and frequency in genetic variants of primary ciliary dyskinesia. *The European respiratory journal*, 44(6), 1579–1588.

<https://doi.org/10.1183/09031936.00052014>

Rehman, T., Thornell, I. M., Pezzulo, A. A., Thurman, A. L., Romano Ibarra, G. S., Karp, P. H., Tan, P., Duffey, M. E., & Welsh, M. J. (2020). TNF α and IL-17 alkalinize airway surface liquid through CFTR and pendrin. *American journal of physiology. Cell physiology*, 319(2), C331–C344. <https://doi.org/10.1152/ajpcell.00112.2020>

Rehman, T., Karp, P. H., Tan, P., Goodell, B. J., Pezzulo, A. A., Thurman, A. L., Thornell, I. M., Duffey, S. L., Duffey, M. E., Stoltz, D. A., McKone, E. F., Singh, P. K., & Welsh, M. J. (2021). Inflammatory cytokines TNF α and IL-17 enhance the efficacy of cystic fibrosis transmembrane conductance regulator modulators. *The Journal of clinical investigation*, 131(16), e150398. <https://doi.org/10.1172/JCI150398>

Ridley, C., & Thornton, D. J. (2018). Mucins: the frontline defence of the lung. *Biochemical Society transactions*, 46(5), 1099–1106.
<https://doi.org/10.1042/BST20170402>

Rock, J. R., & Hogan, B. L. (2011). Epithelial progenitor cells in lung development, maintenance, repair, and disease. *Annual review of cell and developmental biology*, 27, 493–512.
<https://doi.org/10.1146/annurevcellbio-100109-104040>

Rock, J. R., Onaitis, M. W., Rawlins, E. L., Lu, Y., Clark, C. P., Xue, Y., Randell, S. H., & Hogan, B. L. (2009). Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, 106(31), 12771–12775. <https://doi.org/10.1073/pnas.0906850106>

Romano Ibarra, G.S., Lei, L., Yu, W., Thurman, A.L., Gansemer, N.D., Meyerholz, D.K., Pezzulo, A. A., McCray, P.B., Thornell, I.M., Stoltz, D.A. (2024) IL-13 induces loss of CFTR in ionocytes and reduces airway epithelial fluid absorption. *Journal of clinical investigation*. 134(21):e181995. <https://doi.org/10.1172/JCI181995>.

Roy, B., Friesen, W. J., Tomizawa, Y., Leszyk, J. D., Zhuo, J., Johnson, B., Dakka, J., Trotta, C. R., Xue, X., Mutyam, V., Keeling, K. M., Mobley, J. A., Rowe, S. M., Bedwell, D. M., Welch, E. M., & Jacobson, A. (2016). Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression. *Proceedings of the National Academy of Sciences of the United States of America*, 113(44), 12508–12513.
<https://doi.org/10.1073/pnas.1605336113>

Rowe, S. M., Miller, S., & Sorscher, E. J. (2005). Cystic fibrosis. *The New England journal of medicine*, 352(19), 1992–2001.
<https://doi.org/10.1056/NEJMra043184>

Rowe, S. M., Daines, C., Ringshausen, F. C., Kerem, E., Wilson, J., Tullis, E., Nair, N., Simard, C., Han, L., Ingenito, E. P., McKee, C., Lekstrom-Himes, J., & Davies, J. C. (2017). Tezacaftor-Ivacaftor in ResidualFunction Heterozygotes with Cystic Fibrosis. *The New England journal of medicine*, 377(21), 2024–2035.
<https://doi.org/10.1056/NEJMoal709847>

Rufener, S. C., & Mühlemann, O. (2013). eIF4E-bound mRNPs are substrates for nonsense-mediated mRNA decay in mammalian cells. *Nature structural & molecular biology*, 20(6), 710–717.
<https://doi.org/10.1038/nsmb.2576>

Ruyssseveldt, E., Martens, K., & Steelant, B. (2021). Airway Basal Cells, Protectors of Epithelial Walls in Health and Respiratory Diseases. *Frontiers in allergy*, 2, 787128. <https://doi.org/10.3389/falgy.2021.787128>

Saint-Criq, V., & Gray, M. A. (2017). Role of CFTR in epithelial physiology. *Cellular and molecular life sciences: CMLS*, 74(1), 93–115.
<https://doi.org/10.1007/s00018-016-2391-y>

Sala, M. A., & Jain, M. (2018). Tezacaftor for the treatment of cystic fibrosis. *Expert review of respiratory medicine*, 12(9), 725–732.
<https://doi.org/10.1080/17476348.2018.1507741>

Sasso, J. M., Tenchov, R., Wang, D., Johnson, L. S., Wang, X., & Zhou, Q. A. (2023). Molecular Glues: The Adhesive Connecting Targeted Protein Degradation to the Clinic. *Biochemistry*, 62(3), 601–623.
<https://doi.org/10.1021/acs.biochem.2c00245>

Schultz, A., Puvvadi, R., Borisov, S. M., Shaw, N. C., Klimant, I., Berry, L. J., Montgomery, S. T., Nguyen, T., Kreda, S. M., Kicic, A., Noble, P. B., Button, B., & Stick, S. M. (2017). Airway surface liquid pH is not acidic in children with cystic fibrosis. *Nature communications*, 8(1), 1409.
<https://doi.org/10.1038/s41467-017-00532-5>

Scudieri, P., Caci, E., Bruno, S., Ferrera, L., Schiavon, M., Sondo, E., Tomati, V., Gianotti, A., Zegarra- Moran, O., Pedemonte, N., Rea, F., Ravazzolo, R., & Galletta, L. J. (2012). Association of TMEM16A chloride channel overexpression with airway goblet cell metaplasia. *The Journal of physiology*, 590(23), 6141–6155.
<https://doi.org/10.1113/jphysiol.2012.240838>

Scudieri, P., Musante, I., Caci, E., Venturini, A., Morelli, P., Walter, C., Tosi, D., Palleschi, A., Martin-Vasallo, P., Sermet-Gaudelus, I., Planelles, G., Crambert, G., & Galletta, L. J. (2018). Increased expression of ATP12A proton pump in cystic fibrosis airways. *JCI insight*, 3(20), e123616. <https://doi.org/10.1172/jci.insight.123616>

Scudieri, P., Musante, I., Venturini, A., Guidone, D., Genovese, M., Cresta, F., Caci, E., Palleschi, A., Poeta, M., Santamaria, F., Ciciriello, F., Lucidi, V., & Galletta, L. J. V. (2020). Ionocytes and CFTR Chloride Channel Expression in Normal and Cystic Fibrosis Nasal and Bronchial Epithelial Cells. *Cells*, 9(9), 2090. <https://doi.org/10.3390/cells9092090>
Shah, V. S., Meyerholz, D. K., Tang, X. X., Reznikov, L., Abou Alaiwa, M., Ernst, S. E., Karp, P. H., Wohlford-Lenane, C. L., Heilmann, K. P., Leidinger, M. R., Allen, P. D., Zabner, J., McCray, P. B., Jr, Ostedgaard, L. S., Stoltz, D. A., Randak, C. O., & Welsh, M. J. (2016). Airway acidification initiates host defense abnormalities in cystic fibrosis mice. *Science (New York, N.Y.)*, 351(6272), 503–507.
<https://doi.org/10.1126/science.aad5589>

Shah, V. S., Chivukula, R. R., Lin, B., Waghray, A., & Rajagopal, J. (2022). Cystic Fibrosis and the Cells of the Airway Epithelium: What Are Ionocytes and What Do They Do?. *Annual review of pathology*, 17, 23–46. <https://doi.org/10.1146/annurev-pathol-042420-094031>

Sharma, N., Evans, T. A., Pellicore, M. J., Davis, E., Aksit, M. A., McCague, A. F., Joynt, A. T., Lu, Z., Han, S. T., Anzmann, A. F., Lam, A.

N., Thaxton, A., West, N., Merlo, C., Gottschalk, L. B., Raraigh, K. S., Sosnay, P. R., Cotton, C. U., & Cutting, G. R. (2018). Capitalizing on the heterogeneous effects of CFTR nonsense and frameshift variants to inform therapeutic strategy for cystic fibrosis. *PLoS genetics*, 14(11), e1007723. <https://doi.org/10.1371/journal.pgen.1007723>

Sharma, J., Keeling, K. M., & Rowe, S. M. (2020). Pharmacological approaches for targeting cystic fibrosis nonsense mutations. *European journal of medicinal chemistry*, 200, 112436. <https://doi.org/10.1016/j.ejmech.2020.112436>

Sharma, J., Du, M., Wong, E., Mutyam, V., Li, Y., Chen, J., Wangen, J., Thrasher, K., Fu, L., Peng, N., Tang, L., Liu, K., Mathew, B., Bostwick, R. J., Augelli-Szafran, C. E., Bihler, H., Liang, F., Mahiou, J., Saltz, J., Rab, A., ... Bedwell, D. M. (2021). A small molecule that induces translational readthrough of CFTR nonsense mutations by eRF1 depletion. *Nature communications*, 12(1), 4358. <https://doi.org/10.1038/s41467-021-24575-x>

Sheppard, D. N., & Welsh, M. J. (1999). Structure and function of the CFTR chloride channel. *Physiological reviews*, 79(1 Suppl), S23–S45. <https://doi.org/10.1152/physrev.1999.79.1.S23>

Silvis, M. R., Picciano, J. A., Bertrand, C., Weixel, K., Bridges, R. J., & Bradbury, N. A. (2003). A mutation in the cystic fibrosis transmembrane conductance regulator generates a novel internalization sequence and enhances endocytic rates. *The Journal of biological chemistry*, 278(13), 11554–11560. <https://doi.org/10.1074/jbc.M212843200>

Smith, J. J., Travis, S. M., Greenberg, E. P., & Welsh, M. J. (1996). Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell*, 85(2), 229–236. [https://doi.org/10.1016/s00928674\(00\)81099-5](https://doi.org/10.1016/s00928674(00)81099-5)

Smith, M. C., Mader, M. M., Cook, J. A., Iversen, P., Ajamie, R., Perkins, E., Bloem, L., Yip, Y. Y., Barda, D. A., Waid, P. P., Zeckner, D. J., Young, D. A., Sanchez-Felix, M., Donoho, G. P., & Wacheck, V. (2016). Characterization of LY3023414, a Novel PI3K/mTOR Dual Inhibitor Eliciting Transient Target Modulation to Impede Tumor Growth. *Molecular cancer therapeutics*, 15(10), 2344–2356. <https://doi.org/10.1158/1535-7163.MCT-15-0996>

Sondo, E., Tomati, V., Caci, E., Esposito, A. I., Pfeffer, U., Pedemonte, N., & Galletta, L. J. (2011). Rescue of the mutant CFTR chloride channel by pharmacological correctors and low temperature analyzed by gene expression profiling. *American journal of physiology. Cell physiology*, 301(4), C872–C885. <https://doi.org/10.1152/ajpcell.00507.2010>

Spelier, S., van Doorn, E. P. M., van der Ent, C. K., Beekman, J. M., & Koppens, M. A. J. (2023). Readthrough compounds for nonsense mutations: bridging the translational gap. *Trends in molecular medicine*, 29(4), 297–314. <https://doi.org/10.1016/j.molmed.2023.01.004>

Staszewski, J., Lazarewicz, N., Konczak, J., Migdal, I., & MaciaszczykDziubinska, E. (2023). UPF1-From mRNA Degradation to Human Disorders. *Cells*, 12(3), 419. <https://doi.org/10.3390/cells12030419>

Taylor-Cousar, J. L., Mall, M. A., Ramsey, B. W., McKone, E. F., Tullis, E., Marigowda, G., McKee, C. M., Waltz, D., Moskowitz, S. M., Savage, J., Xuan, F., & Rowe, S. M. (2019). Clinical development of triplecombination CFTR modulators for cystic fibrosis patients with one or two *F508del* alleles. *ERJ open research*, 5(2), 00082-2019. <https://doi.org/10.1183/23120541.00082-2019>

Tan, X., Calderon-Villalobos, L. I., Sharon, M., Zheng, C., Robinson, C. V., Estelle, M., & Zheng, N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature*, 446(7136), 640–645. <https://doi.org/10.1038/nature05731>

Tarran, R., Grubb, B. R., Gatzky, J. T., Davis, C. W., & Boucher, R. C. (2001). The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. *The Journal of general physiology*, 118(2), 223–236. <https://doi.org/10.1085/jgp.118.2.223>

Tsuji, T., Sapinoso, L. M., Tran, T., Gaffney, B., Wong, L., Sankar, S., Raymon, H. K., Mortensen, D. S., & Xu, S. (2017). CC-115, a dual inhibitor of mTOR kinase and DNA-PK, blocks DNA damage repair pathways and selectively inhibits ATM-deficient cell growth in vitro. *Oncotarget*, 8(43), 74688–74702. <https://doi.org/10.18632/oncotarget.20342>

Van Goor, F., Hadida, S., Grootenhuis, P. D., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M., ... Negulescu, P. (2009). Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proceedings of the National Academy of Sciences of the United States of America*, 106(44), 18825–18830. <https://doi.org/10.1073/pnas.0904709106>

Van Goor, F., Hadida, S., Grootenhuis, P. D., Burton, B., Stack, J. H., Straley, K. S., Decker, C. J., Miller, M., McCartney, J., Olson, E. R., Wine, J. J., Frizzell, R. A., Ashlock, M., & Negulescu, P. A. (2011). Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proceedings of the National Academy of Sciences of the United States of America*, 108(46), 18843–18848. <https://doi.org/10.1073/pnas.1105787108>

Veit, G., Avramescu, R. G., Chiang, A. N., Houck, S. A., Cai, Z., Peters, K. W., Hong, J. S., Pollard, H. B., Guggino, W. B., Balch, W. E., Skach, W. R., Cutting, G. R., Frizzell, R. A., Sheppard, D. N., Cyr, D. M., Sorscher, E. J., Brodsky, J. L., & Lukacs, G. L. (2016). From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Molecular biology of the cell*, 27(3), 424–433. <https://doi.org/10.1091/mbc.E14-04-0935>

Veit, G., Xu, H., Dreano, E., Avramescu, R. G., Bagdany, M., Beitel, L. K., Roldan, A., Hancock, M. A., Lay, C., Li, W., Morin, K., Gao, S., Mak, P. A., Ainscow, E., Orth, A. P., McNamara, P., Edelman, A., Frenkiel, S., Matouk, E., Sermet-Gaudelus, I., ... Lukacs, G. L. (2018). Structure-guided combination therapy to potentially improve the function of mutant CFTRs. *Nature medicine*, 24(11), 1732–1742. <https://doi.org/10.1038/s41591-018-0200-x>

Vieira Braga, F. A., Kar, G., Berg, M., Carpaij, O. A., Polanski, K., Simon, L. M., Brouwer, S., Gomes, T., Hesse, L., Jiang, J., Fasouli, E. S., Efremova, M., Vento-Tormo, R., Talavera-López, C., Jonker, M. R., Affleck, K., Palit, S., Strzelecka, P. M., Firth, H. V., Mahbubani, K. T., ... Teichmann, S. A. (2019). A cellular census of human lungs identifies novel cell states in health and in asthma. *Nature medicine*, 25(7), 1153–1163. <https://doi.org/10.1038/s41591-019-0468-5>

Visca, A., Bishop, C. T., Hilton, S. C., & Hudson, V. M. (2008). Improvement in clinical markers in CF patients using a reduced glutathione regimen: an uncontrolled, observational study. *Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society*, 7(5), 433–436. <https://doi.org/10.1016/j.jcf.2008.03.006>

Wainwright, C. E., Elborn, J. S., Ramsey, B. W., Marigowda, G., Huang, X., Cipolli, M., Colombo, C., Davies, J. C., De Boeck, K., Flume, P. A., Konstan, M. W., McColley, S. A., McCoy, K., McKone, E. F., Munck, A., Ratjen, F., Rowe, S. M., Waltz, D., Boyle, M. P., TRAFFIC Study Group, ... TRANSPORT Study Group (2015). Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *The New England journal of medicine*, 373(3), 220–231. <https://doi.org/10.1056/NEJMoa1409547>

Weischenfeldt, J., Damgaard, I., Bryder, D., Theilgaard-Mönch, K., Thoren, L. A., Nielsen, F. C., Jacobsen, S. E., Nerlov, C., & Porse, B. T. (2008). NMD is essential for hematopoietic stem and progenitor cells and for eliminating by-products of programmed DNA rearrangements. *Genes & development*, 22(10), 1381–1396. <https://doi.org/10.1101/gad.468808>
Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C. R., Hwang, S., Wilde, R. G., Karp, G., Takasugi, J., Chen, G., Jones, S., Ren, H., Moon, Y. C., Corson, D., Turpoff, A. A., Campbell, J. A., ... Sweeney, H. L. (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature*, 447(7140), 87–91. <https://doi.org/10.1038/nature05756>

Welsh, M. J., & Smith, A. E. (1993). Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*, 73(7), 1251–1254.
[https://doi.org/10.1016/0092-8674\(93\)90353-r](https://doi.org/10.1016/0092-8674(93)90353-r)

Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo, H. T., Ma, X. J., & Luo, Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of molecular diagnostics : JMD*, 14(1), 22–29.
<https://doi.org/10.1016/j.jmoldx.2011.08.002>

Widdicombe, J.H. (2002). Regulation of the depth and composition of airway surface liquid. *Journal of Anatomy* (2002) 201, pp313–318.
<https://doi.org/10.1046/j.1469-7580.2002.00098.x>

Worlitzsch, D., Tarran, R., Ulrich, M., Schwab, U., Cekici, A., Meyer, K. C., Birrer, P., Bellon, G., Berger, J., Weiss, T., Botzenhart, K., Yankaskas, J. R., Randell, S., Boucher, R. C., & Döring, G. (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *The Journal of clinical investigation*, 109(3), 317–325.
<https://doi.org/10.1172/JCI13870>

Xu, J., Yu, H., & Sun, X. (2020). Less Is More: Rare Pulmonary Neuroendocrine Cells Function as Critical Sensors in Lung. *Developmental cell*, 55(2), 123–132.
<https://doi.org/10.1016/j.devcel.2020.09.024>

Xue, X., Mutyam, V., Tang, L., Biswas, S., Du, M., Jackson, L. A., Dai, Y., Belakhov, V., Shalev, M., Chen, F., Schacht, J., J Bridges, R., Baasov, T., Hong, J., Bedwell, D. M., & Rowe, S. M. (2014). Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor. *American journal of respiratory cell and molecular biology*, 50(4), 805–816. <https://doi.org/10.1165/rcmb.2013-0282OC>

Xue, R., Gu, H., Qiu, Y., Guo, Y., Korteweg, C., Huang, J., & Gu, J. (2016). Expression of Cystic Fibrosis Transmembrane Conductance Regulator in Ganglia of Human Gastrointestinal Tract. *Scientific reports*, 6, 30926.
<https://doi.org/10.1038/srep30926>

Yamashita, A., Kashima, I., & Ohno, S. (2005). The role of SMG-1 in nonsense-mediated mRNA decay. *Biochimica et biophysica acta*, 1754(12), 305–315. <https://doi.org/10.1016/j.bbapap.2005.10.002>

Yamashita A. (2013). Role of SMG-1-mediated Upf1 phosphorylation in mammalian nonsense-mediated mRNA decay. *Genes to cells : devoted to molecular & cellular mechanisms*, 18(3), 161–175.
<https://doi.org/10.1111/gtc.12033>

Yi, Z., Sanjeev, M., & Singh, G. (2021). The Branched Nature of the Nonsense-Mediated mRNA Decay Pathway. *Trends in genetics: TIG*,

Yu, H., Burton, B., Huang, C. J., Worley, J., Cao, D., Johnson, J. P., Jr, Urrutia, A., Joubran, J., Seepersaud, S., Sussky, K., Hoffman, B. J., & Van Goor, F. (2012). Ivacaftor potentiation of multiple CFTR channels with gating mutations. *Journal of cystic fibrosis: official journal of the European Cystic Fibrosis Society*, 11(3), 237–245. <https://doi.org/10.1016/j.jcf.2011.12.005>

Zabner, J., Smith, J. J., Karp, P. H., Widdicombe, J. H., & Welsh, M. J. (1998). Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. *Molecular cell*, 2(3), 397–403. [https://doi.org/10.1016/s1097-2765\(00\)80284-1](https://doi.org/10.1016/s1097-2765(00)80284-1)

Zegarra-Moran, O., & Galletta, L. J. (2017). CFTR pharmacology. *Cellular and molecular life sciences : CMLS*, 74(1), 117–128. <https://doi.org/10.1007/s00018-016-2392-x>

Zielenski, J., & Tsui, L. C. (1995). Cystic fibrosis: genotypic and phenotypic variations. *Annual review of genetics*, 29, 777–807. <https://doi.org/10.1146/annurev.ge.29.120195.004021>

Zhang, J. H., Chung, T. D., & Oldenburg, K. R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of biomolecular screening*, 4(2), 67–73. <https://doi.org/10.1177/108705719900400206>

Zuo, W. L., Shenoy, S. A., Li, S., O'Beirne, S. L., Strulovici-Barel, Y., Leopold, P. L., Wang, G., Staudt, M. R., Walters, M. S., Mason, C., Kaner, R. J., Mezey, J. G., & Crystal, R. G. (2018). Ontogeny and Biology of Human Small Airway Epithelial Club Cells. *American journal of respiratory and critical care medicine*, 198(11), 1375–1388. <https://doi.org/10.1164/rccm.201710-2107OC>

7. FIGURE LEGENDS

Figure 1. scRNAseq revised the architecture of human airway epithelium. scRNAseq profoundly transformed the view of airways from a uniform barrier to a dynamic and heterogeneous functional unit. The image summarizes the different parts of the respiratory tract and the contemporary landscape of cell types that compose the epithelial airways wall. Image adapted from Hewitt & Lloyd, 2021.

Figure 2. Ciliary beating and mucus secretion as key components of MCC. Panel A shows the main features of the mucociliary clearance mechanism. Adapted from Whitsett, 2018. Panel B illustrates a schematic diagram of a normal ciliary beat cycle. Cilia beat with a strong forward stroke (black) followed by a recovery stroke (grey). Adapted from Raidt et al., 2014.

Figure 3. “Gel-on-brush” model describes the ASL composition. Button and colleagues described a model in which tethered macromolecules, such as membranebound mucins and glycoproteins, form a brush-like structure of the PCL that maintains the mucus as a separate layer above. Left and center, adapted from Button et al., 2012. Right image shows TEM image of ciliated cells in the ASL, where a net separation between PCL and ML is clearly visible. Adapted from Widdicombe et al., 2002.

Figure 4. Main ion channels and transporters of the airways. In the airways, salt secretion and absorption are given by the paralleled transport of Na^+ and Cl^- through the membranes. The image sums up the main membrane proteins involved in these mechanisms. For simplicity, the figure only depicts the side of the cell in which proteins are localized, rather than the specific cell type that expresses them. Adapted from SaintCricq & Gray, 2017.

Figure 5. Model for transepithelial ion movement across the airways. Lei and colleagues propose a model in which different cell types are committed to the ion secretion and absorption processes. The peculiar presence of CLCK/Barttin Cl^- channels in ionocytes makes them absorptive cell types, since they permit Cl^- movements towards the basolateral side; secretory cells couple NKCC1-mediated Cl^- import in the cell with CFTR-mediated Cl^- secretion. Adapted from Lei et al., 2023.

Figure 6. Unbalanced homeostasis of the respiratory epithelium leads to different diseases. As summarized in panel A, in disease state cells contribute differently to the onset of pathologies. As explained throughout the text, the orchestrated activity of goblet and ciliated cells and, ultimately, pulmonary ionocytes, guarantees the correct MCC mechanism. Panel B shows the progression from normal to muco- obstructed airway states. In healthy state (left), effective MCC is allowed by a correct hydration of the ASL thanks to the salt absorption/secretion mechanism. In muco-obstructive lung disease (right), an imbalance of ion/fluid transport together with mucin hypersecretion increases mucin concentrations in the mucus layer, that in turn compresses the PCL impairing ciliary beating. This slows and eventually abolishes MCC. Panel A taken from Davis et al., 2021. Panel B adapted by Hill et al., 2022.

Figure 7. Model for low-volume hypothesis. Panel A: in healthy airways, CFTR channel is essential for a correct hydration of airway surface. Panel B: without functional CFTR, there is an inadequate homeostasis of ion and water transport leading to dehydrated PCL, that causes the collapse of the ML on cilia. Severe mucus plugs formation, resulting from MCC impairment, favors the growth of microbes, with the onset of severe infection and inflammation during time. Adapted from Graeber & Mall, 2023.

Figure 8. CFTR structure. Panel A: schematic domain structure of CFTR. Panel B: human CFTR in the dephosphorylated, ATP-free conformation described by Liu et al., 2017.

Figure 9. Extended classification of CF mutations. This new refined classification considers complex phenotypes of major CFTR cellular defects. Taken by Veit et al., 2015.

Figure 10. Effects of the ETI on the pleiotropic defects caused by the F508del-CFTR. Panel A. The correctors (ET) act in a complementary manner against misassembly, misprocessing, and mistrafficking of F508del-CFTR. Following proper trafficking to the cell surface, the potentiator, Ivacaftor (I), promotes opening of the CFTR channel. Adapted from Bear, 2020. Panel B. Cryo-EM structure of ETI binding human F508del-CFTR. Adapted from Fiedorczuck & Chen, 2022b.

Figure 11. Structure of hUpf1 protein. The figure illustrates the functional domains of human Upf1 protein and binding regions with principal interactors. Black numbers indicate amino acidic positions, while red number and arrows indicate phospho-serine residues. CH, cysteine- and histidine-rich domain; HD, helicase domain; SQ, serine- and glutamine-rich domain. Taken from Yoon & Maquat, 2019.

Figure 12. Structure of SMG1 protein. Schematic structure of the SMG1 protein domains. The black boxes indicate the PIKK and FAT-C domains. SMG1-specific sequences are depicted in light gray. Adapted from Yamashita et al., 2005.

Figure 13. Normal translation of termination (top) vs NMD activation (bottom). On a normal termination of translation event, NMD is prevented by the interactions between the PABPC protein and the eRF3 termination factor that penalize a tight binding of the UPF1 helicase on the targeted mRNA. Thus, an efficient translation termination can occur. In the combined model for NMD activation, when a ribosome encounters a PTC different branches lead to the activation of UPF1 that accumulates on the 3'-UTR, starting NMD and subsequent degradation of the targeted mRNA. At least two important mechanisms are involved in UPF1 recruitment: the presence of an EJC 20-24 nt upstream of the PTC, and a high distance between the PTC and the PABPC. Adapted from LykkeAndersen & Jensen, 2015.

Figure 14. UPF1 hyperphosphorylation leads to mRNA degradation. The ultimate task of the SURF and DECID complex together is the activation of Upf1 helicase activity by phosphorylation on multiple serine/threonine residues (indicated with asterisks in the figure). Among the numerous functions of phosphorylated UPF1 there is the recruitment of degradation factors that will lead to mRNA degradation by multiple routes, as depicted in the figure. Adapted from Lykke-Andersen & Jensen, 2015.

Figure 15. Pharmacological RT agents over-read PTCs. Read-through of PTCs can be pharmacologically stimulated by compounds that act on the stalled ribosome, reducing its proofreading ability and increasing the probability it can insert in the elongating polypeptide a near-cognate aminoacyl-tRNA in the place of the stop codon, allowing the continuation of the protein synthesis. Image created with Biorender.com.

Figure 16. CC-90009 is a novel identified CRBN modulator with PTC suppression activity. Compounds that act as molecular glues have been showed having RT activities. CC-90009 is a CRBN modulator that favors the binding of eRF3a factor of the translation termination machinery to CRBN E3 ligase. This leads to eRF3a ubiquitination and subsequent degradation, with suppression of PTCs. Image created with Biorender.com.

Figure 17. Instruments for short-circuit current recordings. The porous supports carrying the differentiated airway epithelia were mounted on a specific slot for Transwell or Snapwell and inserted in the vertical Ussing-like chamber, between the apical and basolateral hemi-chambers, both filled with HEPES- or bicarbonate-buffered Ringer solutions and connected to voltage-sensing and current-passing electrodes. Two connecting tubes allowed 5% CO₂/95% air bubbling, and the chambers were heated to 37 °C. Every chamber was connected to a pre-amplifier and to the acquisition system. Created with Biorender.com

Figure 18. Scheme of HS-YFP assay for 16HBE14o⁻ cells with W1282X-CFTR. A fluorescent version of the 16HBE14o⁻ cells with W1282X-CFTR was created by stably expressing the HS-YFP protein through lentiviral infection. Cells were expanded and seeded at high density onto 96-well microplates. After 24 h treatment with desired compound combinations, CFTR activity was determined with a microplate reader. Briefly, CFTR function is estimated as the quenching rate of HS-YFP resulting from iodide uptake. Image created with Biorender.com

Figure 19. Library screening protocol. Schematic representation of microplate preparation for the screening showing the compound dilution procedure. Image created with Biorender.com

Figure 20. Experimental workflow of a FRAP experiment. “Flipped” human bronchial epithelia were generated by seeding the cells on porous supports in upsidedown configuration. The apical surface was stained with FITC-dextran and, after incubation, they were mounted on a spinning disk microscope in controlled atmosphere conditions. The photobleaching of a circular ROI was applied and the fluorescence recovery was recorded. Created with Biorender.com.

Figure 21. I_{sc} recordings of G542X-CFTR activity upon stimulation of RT agents and SMGi. Representative traces (Panel A) and summary of data (Panel B and C) from I_{sc} experiments done on the 16HBE14o⁻ bronchial epithelial cell line expressing G542XCFTR. Before recordings, cells were treated for 24 h with 0.5 mg/ml of G418 or ELX-02 alone or in combination with VX-809 (1 μM), VX-445 (5 μM), the two correctors together (combo), with/without SMG1i (1 μM). During recordings, cells were sequentially stimulated with the cAMP analog (CPT-cAMP, 100 μM) and with VX-770 (1 μM). CFTR activity was then inhibited with inh-172 (10 μM). Pharmacological rescue results in a higher CFTR activity as indicated by the larger amplitude of inh-172 effect. The scatter dot plots (panels B and C) report the amplitude of the current drop elicited by inh-172 for the indicated treatments. ***, p < 0.001 vs. control. ###, p < 0.001 vs. G418/ELX-02. Adapted from Venturini et al., 2021.

Figure 22. CFTR correctors stabilize the G542C-CFTR variant. Immunoblot analysis of CFTR protein processing in HEK293 cells transiently transfected with wildtype or G542C-CFTR plasmids. After transfection, cells were treated for 24 h with VX809 (1 μM) and/or VX-445 (5 μM). Left: representative images showing CFTR and GAPDH detection. VX-809, but not VX-445, increases the expression of the C band, corresponding to fully glycosylated form of CFTR protein, in G542C-CFTR expressing cells. Right: summary of results reporting C band intensity (normalized for GAPDH expression) from multiple immunoblot experiments. **, p < 0.01 vs. control (n = 4). Adapted from Venturini et al., 2021.

Figure 23. Functional analysis of PTI-428 and SMG1i. The scatter dot plot summarizes CFTR function (i.e., current sensitive to inh-172) in 16HBE14o⁻ cells expressing G542X-CFTR treated for 24 h with G418 (0.5 mg/mL) alone or in combination with SMG1i (1 μM) or PTI-428 (30 μM). SMG1i and PTI-428 did not further increase G418- mediated CFTR rescue. ***, p < 0.001 vs. control. Adapted from Venturini et al., 2021.

Figure 24. Effect of PTI-428 on CFTR mRNA. 16HBE14o⁻ cells expressing G542XCFTR were treated for 24 hours with PTI-428 (30 μM), G418 (0.5 mg/ml) or both compounds. After treatment, RNA was extracted, converted to cDNA, and CFTR transcript quantified by real time RT-PCR. Results in each sample were normalized to B2M expression. The scatter dot-plot shows CFTR transcripts fold-change relative to control condition. *, p < 0.05 vs. vehicle treated cells (dashed line). Adapted from Venturini et al., 2021

Figure 25. Rescue of F508del-CFTR function by PTI-428. PTI-428 was tested for 24 h at increasing concentrations on CFBE41o⁻ cells expressing F508del-CFTR, in the presence or absence of VX-809 (1 μM). The graph reports the quenching rate (QR) deriving from the HS-YFP functional assay. Data were fitted with a Hill function. Adapted from Venturini et al., 2021.

Figure 26. Effect of PTI-428 on electrogenic transport mechanisms of bronchial epithelia. Representative traces (left) and scatter dot plots (right) showing data Ussing chamber experiments (I_{sc} recordings) done on non-CF primary bronchial epithelial cells. Cells were treated for 24 h with the CFTR amplifier PTI-428 (30 μ M) or vehicle (DMSO) alone. During recordings, epithelia were sequentially exposed to: amiloride (10 μ M) to inhibit ENaC channel, CPT- cAMP (100 μ M) and VX-770 (1 μ M) to stimulate CFTR channel, CFTR_{inh}-172 (inh-172, 20 μ M) to inhibit CFTR activity, UTP (100 μ M), and Ani9 (5 μ M) respectively to activate and then inhibit TMEM16A channel. Scatter dot plots show the amplitude of the amiloride effect (left graph), the amplitude of the inh172 block (middle graph), the amplitude of the current activated by UTP (right graph). **, $p < 0.01$; ***, $p < 0.001$ vs. control. Adapted from Venturini et al., 2021.

Figure 27. Rescue of G542X-CFTR in primary bronchial epithelia. Representative short-circuit current traces (left) and summary of data (right) obtained from bronchial epithelia generated from cells of a G542X/1717-1G > A patient. Epithelia were treated with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μ M VX-809). ***, $p < 0.001$ vs. control. ####, $p < 0.001$ vs. readthrough agent alone. Adapted from Venturini et al., 2021.

Figure 28. Pharmacological rescue of Y122X, R553X, and R1162X. Each panel shows representative I_{sc} traces (left) and summary graphs (right) obtained from 16HBE14o⁻ cells expressing Y122X-CFTR (A), R553X-CFTR (B) and R1162X-CFTR (C). Epithelia were treated with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μ M VX-809, 5 μ M VX-445, 1 μ M SMG1i). *, $p < 0.05$ vs. control, ***, $p < 0.001$ vs. control. ####, $p < 0.001$ vs. ELX-02 alone. Adapted from Venturini et al., 2021

Figure 29. Sensitivity of the W1282X-CFTR mutant to RT agents and SMG1i. Each panel shows representative I_{sc} traces (left) and summary graphs (right) obtained from experiments on 16HBE14o⁻ cells expressing W1282X-CFTR. Panel A collects data obtained with RT agents, panel B includes traces and currents obtained with SMG1ibased treatments. Epithelia were treated. for 24 h with the indicated compounds (0.5 mg/ml G418 and ELX-02, 1 μ M VX-809, 5 μ M VX-445, 1 μ M SMG1i, 30 μ M PTI428). Panel A: ***, $p < 0.001$ vs. control. #, $p < 0.05$ and ####, $p < 0.001$ vs. G418/ELX02, ns not significant. Panel B: ***, $p < 0.001$ vs. control. ##, $p < 0.01$ and ####, $p < 0.001$ vs. VX-809 (up); $p < 0.001$ vs. control. ##, $p < 0.01$ and ####, $p < 0.001$ vs. SMG1i with or without G418 (down); ns not significant. Adapted from Venturini et al., 2021.

Figure 30. Effect of SMG1i on primary bronchial epithelia expressing W1282XCFTR. Short-circuit current traces (left) and summary of data (right) obtained from epithelia generated from cells of a W1282X/W1282X patient. Epithelia were treated with the indicated compounds (1 μ M SMG1i; combo: 1 μ M VX-809 plus 5 μ M VX-445). ***, $p < 0.001$ vs. control. ####, $p < 0.001$ vs. indicated treatments. Adapted from Venturini et al., 2021

Figure 31. Molecular analysis of SMG1i effects on W1282X-CFTR protein processing. Representative Western blot experiment performed on 16HBE14o⁻ cells expressing W1282X-CFTR. Treatment with SMG1i (1 μ M) was required to evoke the appearance of CFTR protein. The molecular size of the rescued protein (150 kDa) is smaller than that of wild-type CFTR (180 kDa), in agreement with the effect of the premature truncation and the lack of readthrough when G418 is included. Adapted from Venturini et al., 2021.

Figure 32. Analysis of W1282X- and G542X-CFTR by immunofluorescence. Representative images reporting immunodetection of CFTR protein in 16HBE14o⁻ cells expressing W1282X- and G542X-CFTR. Cells were treated with SMG1i (1 μ M) and G418 (0.5 mg/ml) alone or in combination with VX- 809 (1 μ M) plus VX-445 (5 μ M). Cells expressing the W1282X mutant show appearance of immunoreactive CFTR (green) following pharmacological treatment. Arrows: appearance of CFTR signal at cell borders. Scale bar: 20 μ m. Adapted from Venturini et al., 2021.

Figure 33. Analysis of CFTR mRNA by real-time RT-PCR. 16HBE14o- cells expressing the indicated PTCs were treated for 24 h with G418 (0.5 mg/ml), SMG1i (1 μ M), or with both compounds together (combo), then RNA was extracted. The scatter dot plots summarize CFTR transcript fold-change relative to control condition for every mutation. All values were normalized to B2M. The p value was determined using onesample t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control (dashed line). Adapted from Venturini et al., 2021.

Figure 34. Functional analysis of W1282X-CFTR rescue with the HS-YFP assay. In the upper panel, the scatter dot plot reports W1282X-CFTR activity (expressed as quenching rate, QR) in cells treated with the indicated combinations of compounds: SMG1i (1 μ M), VX-809 (1 μ M), VX-445 (5 μ M), G418 (0.5 mg/ml). ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. SMG1i alone. In the bottom panel, a dose-response relationship for SMG1i in the absence and in the presence of CFTR correctors is shown. ***, $p < 0.001$ vs. no SMG1i. #, $p < 0.05$ between indicated conditions. The inset reports representative traces from the HS-YFP assay. Adapted from Venturini et al., 2021.

Figure 35. Functional screening of the MedChemExpress Bioactive Compounds library. (A) Scheme of the screening based on the HS-YFP assay. 16HBE14o- cells coexpressing W1282X-CFTR and HS-YFP were treated with test compounds or SMG1i plus VX-809/VX-445 correctors. After treatment, iodide uptake was measured with HSYFP. (B) Representative dose-response curves for compounds with different biological activities. Compounds were all tested at concentrations in the 0.1 – 20 μ M range, in the presence of VX-809 (1 μ M) plus VX-445 (5 μ M). (C) HS-YFP data obtained in 16HBE14o- cells treated with indicated compounds in presence (filled bars) or absence (empty bars) of the CFTR_{inh}-172 (10 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. no CFTR_{inh}-172 (Student's t test).

Figure 36. Evaluation of CC-115, samotolisib and elimusertib in I_{sc} recordings. The figure shows representative I_{sc} recordings (top) and summary of data (bottom) obtained from experiments on 16HBE14o- cells expressing W1282X-CFTR. Cells were treated for 24 h with CC-115 (2 μ M), samotolisib (3 μ M) or elimusertib (20 μ M) with/without CFTR correctors (combo: 1 μ M VX-809 plus 5 μ M VX-445) or vehicle alone (control). During recordings, cells were sequentially exposed to: CPT-cAMP (100 μ M), VX-770 (1 μ M), CFTR_{inh}-172 (10 μ M). The scatter dot plot reports the amplitude of the current drop caused by CFTR_{inh}-172. ***, $p < 0.001$ vs. vehicle; #####, $p < 0.001$ vs CC-115, samotolisib or elimusertib alone. (ANOVA with Tukey's post hoc test).

Figure 37. Evaluation of confirmed hits from the screening on W1282X-CFTR transcript levels. The graph represents the fold-change in CFTR mRNA compared to control condition (DMSO). 16HBE14o- cells expressing W1282X-CFTR were treated for 24 h with CC-115 (2 μ M), samotolisib (3 μ M), or elimusertib (20 μ M), with/without G418 (0.5 mg/ml). ***, $p < 0.001$ vs control condition.

Figure 38. Detection of CFTR mRNA by RNAscope. The figure shows representative images of 16HBE14o- cells expressing W1282X-CFTR cells after 24 h treatment with DMSO (as negative control), SMG1i (1 μ M, as positive control), CC-115 (2 μ M), and samotolisib (3 μ M). After fixation and permeabilization, cells were exposed to specific probes that hybridize with endogenous CFTR mRNA. Images were acquired with a Nikon T2-Eclipse confocal microscope. The increase in single CFTR RNA molecules can be clearly detected as the appearance of red spots. Scale bar: 20 μ m.

Figure 39. Effects of CC-115, samotolisib, and elimusertib on protein processing. Panel A: representative western blot image of 16HBE14o- treated with the indicated conditions. Protein targets are indicated on the right. Panel B: the scatter dot-plots summarize densitometry of the bands corresponding to the protein target under indicated treatments. CC-115 (2 μ M), samotolisib (3 μ M) and elimusertib (20 μ M) were analysed with or without VX-809 (1 μ M) plus VX-445 (5 μ M). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. totally untreated (no compounds) cells. §, $p < 0.05$; §§ $p < 0.01$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

Figure 40. Effect of CC-115, samotolisib, and elimusertib in combination with SMG1i. The scatter dot-plots summarize HS-YFP assay data obtained with the treatment of CC-115 (2 μ M), samotolisib (3 μ M) and elimusertib (20 μ M), with or without SMG1i, in presence of VX-809 (1 μ M) plus VX-445 (5 μ M). The activity with SMG1i plus CFTR correctors is indicated in the graphs as a dashed red line. ***, $p < 0.001$ vs compounds plus VX-809/VX-445.

Figure 41. Effect of compounds on cell transcriptome. Venn diagrams reporting data from bulk RNAseq of 16HBE14o- cells expressing W1282X- (left) or wild type CFTR (right), treated for 24 h with SMG1i, CC-115, or samotolisib. The list of the top 30 genes commonly upregulated by the three treatments is reported on the side of each Venn diagram. In bold are the genes that are upregulated in both wild type and W1282X cells. In red is CFTR

Figure 42. Effect of CFTR rescue manoeuvres and cytokines on G542X/G542X bronchial epithelia. (A) Representative I_{sc} traces and data (B-D) reporting results obtained from epithelia treated for 24 h with vehicle alone, VX-809 (1 μ M) and ELX-02 (200 μ M), or the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M). Where indicated, the epithelia were previously treated for 72 h with IL-4 (10 ng/ml) (C) or IL-17A (20 ng/ml) plus TNF- α (10 ng/ml) (D). *, $p < 0.05$; ***, $p < 0.001$ vs. vehicle. ###, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

Figure 43. Effect of CFTR rescue manoeuvres and cytokines on G542X/1717-1G>A bronchial epithelia. (A-B) The figure shows the same set of experiments of the previous figure carried on a patient with a different genotype. (C) The scatter dot plots report the effect of treatments on the function of ENaC (top) and TMEM16A function (bottom) as indicated by the response to amiloride and UTP, respectively. ***, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test). "Combo" indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M).

Figure 44. Effect of CFTR rescue manoeuvres and cytokines on G542X/R1162X nasal epithelia. Top: representative traces from I_{sc} experiments. Bottom: scatter dot plots reporting the effect of treatments on the function of CFTR as indicated by the response to CFTR_{inh}-172. ***, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test). "Combo" indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M).

Figure 45. Analysis of CFTR and eRF3a protein levels in presence of triple drug combination and cytokine treatment. Results from capillary-based protein separation and immunodetection experiment are presented as western blot images (left) and densitometry analysis (right). Panel A shows detection of CFTR protein from lysates of bronchial epithelia (G542X/G542X genotype) treated with/without cytokines (IL-4, IL17A/TNF- α) and with/without the triple combination VX-809, ELX-02, CC90009 (combo). Panel B summarizes the detection of eRF3A protein. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. totally untreated (no compounds, no cytokines) epithelia. §, $p < 0.05$; §§§ and ####, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

Figure 46. Effect of treatments on CFTR mRNA levels. The graph reports the results of CFTR mRNA quantification by real time RT-PCR (fold-increase with respect to untreated epithelia). RNA was extracted from four separate preparations of G542X/G542X bronchial epithelia. *, $p < 0.05$; **, $p < 0.01$ vs. untreated epithelia (one sample t and Wilcoxon test). "Combo" indicates the triple combination with VX-809, ELX-02, and CC-90009 (0.1 μ M).

Figure 47. Epithelial surface viscosity measured with FRAP technique. Top: representative traces showing fluorescence recovery after photobleaching of a selected area in epithelia in control conditions (untreated) or treated with IL-4 or IL-17A/TNF- α with/without VX-809/ELX-02/CC90009 (combo). Bottom: scatter dot plots reporting the

extent of fluorescence recovery at 30 s after photobleaching. ***, $p < 0.001$ vs. totally untreated epithelia (no compounds, no cytokines). ###, $p < 0.001$ for the indicated groups of data (ANOVA with Tukey's post-hoc test).

Figure 48. Rescue of different CFTR mutants requires different pharmacological treatments. Upper: representative short-circuit current recording from 16HBE14o- cells expressing wild-type CFTR. Lower: bar graph showing the comparison of CFTR function in 16HBE14o- cells expressing wild-type or mutant CFTR. For cells carrying mutant CFTR, the bars report CFTR function (mean plus SD) under control conditions and after rescue with the most effective treatment for each mutation as indicated.

8. LIST OF ABBREVIATIONS

air liquid interface (ALI) airway surface liquid
(ASL) aminoglycosides (AAGs) apical
membrane (AM) ATP-Binding Cassette (ABC)
bicarbonate (HCO_3^-) blocking buffer (BB)
calcium (Ca^{2+}) cap-binding complex (CBC)
cereblon (CRBN) CFTR inhibitor 172 (inh-
172) chloride (Cl^-) chronic obstructive
pulmonary disease (COPD) ciliary beat
frequency (CBF) cyclic-AMP (cAMP) cyclin-
dependent kinases (CDKs) cystic fibrosis (CF)
Cystic Fibrosis Foundation (CFF)
Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)
deletion of phenylalanine 508 (F508del) dimethyl-sulfoxide
(DMSO) Duchenne muscular dystrophy (DMD)
Elexacaftor/Tezacaftor/Ivacaftor (ETI) endoplasmic reticulum
(ER) eukaryotic initiation factors (eIFs) eukaryotic translation
initiation factor 4G (eIF4G), European Cystic Fibrosis Society
(ECFS) exon-junction complexes (EJCs) flaviviruses Hepatitis
C virus (HCV) fluorescence recovery after photobleaching
(FRAP) forskolin (FSK) halide-sensitive YFP (HS-YFP) high-
throughput screening (HTS) highly effective modulator
therapies (HEMT)
histone mRNA decay (HMD) human
bronchial epithelial cells (HBECs)
insertion/deletions (in/dels) interferon
(IFN) interleukin (IL) iodide (I^-)
mechanisms of action (MOA) membrane-spanning
domain (MSD) mucociliary clearance (MCC)
mucopolysaccharidosis I-Hurler (MPS I-H) mucus
layer (ML) NMD inhibitor (NMDi) nonsense
mediated mRNA decay (NMD) nucleotide binding
domain (NBD) open reading frame (ORF) people
with CF (pwCF) periciliary liquid (PCL)
phosphatidylinositol 3-kinase (PI3K)

phosphatidylinositol-3-kinase related kinase (PIKK)
 poly(A)-binding protein (PABPC) potassium (K^+)
 premature termination codon (PTC) primary ciliary
 dyskinesia (PCD) protons (H^+)
 Pulmonary Neuroendocrine Cells (PNeC)
 quenching rate (QR) radioimmune precipitation
 assay (RIPA) read through (RT) real time
 Reverse Transcription PCR (RT-qPCR) region of
 interest (ROI) regulatory domain (RD) rho
 kinase (ROCK) RNA-sequencing (RNAseq)
 Rous sarcoma virus (RSV) short circuit current
 (Isc)
 SMG1 complex (SMG1c)
 smg1 inhibitor (SMG1i) sodium (Na^+)
 structure- mediated mRNA decay (SMD)
 suppressor of morphogenesis in genitalia (SMG)
 T helper type-1 (Th1)
 T helper type-17 (Th17) T helper type-
 2 (Th2) thiocyanate (SCN-)
 transepithelial resistance (Rt)
 transmembrane (TM) tumor necrosis
 factor- α (TNF- α) up-frameshift proteins
 (UPF) upstream open reading frames
 (uORFs) vacuolar-ATPase (V-ATPase)
 Zika virus (ZIKV)