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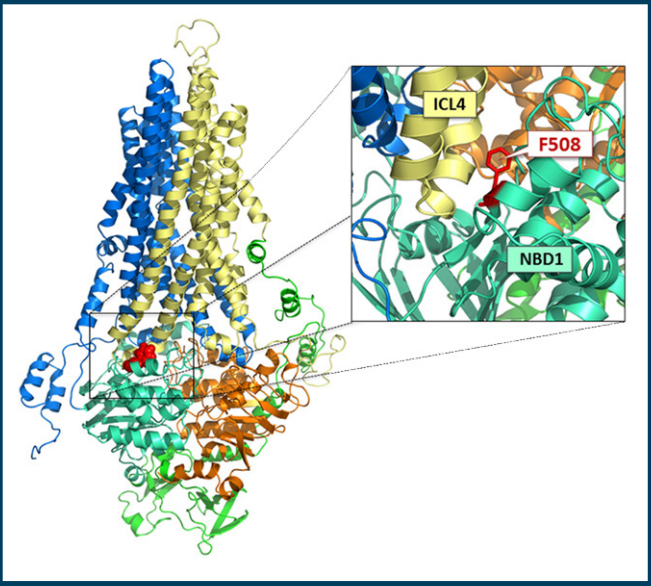

STRATEGIES TO CIRCUMVENT THE CFTR DEFECT IN CYSTIC FIBROSIS

Topic Editors
Frederic Becq and Marc Chanson

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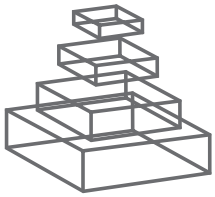

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STRATEGIES TO CIRCUMVENT THE CFTR DEFECT IN CYSTIC FIBROSIS

Topic Editors:
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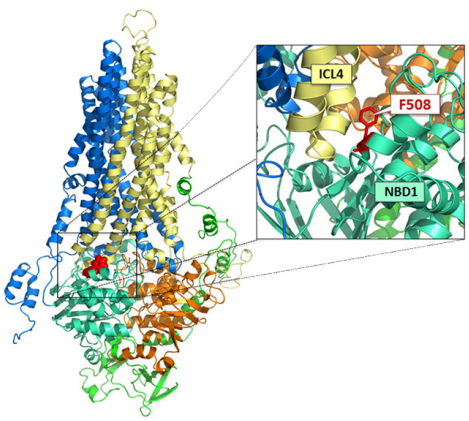

Structural models of CFTR. (A) Full-length homology model of CFTR (Mornon et al., 2009); MSD1, blue; MSD2, yellow; NBD1, cyan; NBD2, orange; R domain, green; F508, red; (B) position of F508 at the ICL4:NBD1 interface.

Mutations within the gene encoding for the chloride ion channel CFTR results in cystic fibrosis, the most common autosomal recessive genetic disease in the Caucasian population. CFTR regulates absorption and secretion mechanisms across intestinal and airway mucosae. Although the intestinal phenotype can be clinically handled, chronic infection and inflammation of the lungs of CF patients remains the principal cause of morbidity and mortality. The aim of this Research Topic is to provide to the readers the most recent information available on "Strategies to circumvent the CFTR defect in cystic fibrosis". The Research Topic is divided in three mains parts: the first part describes the CFTR structure, processing and regulation of the normal and mutant ion channels. Anna Patrick and Philip Thomas (University of Texas Southwestern Medical Center, Dallas, USA) review the molecular interactions leading to the complex folding process of the CFTR protein and how essential steps are disrupted in CFTR mutants. Soo Jung Kim and William Skach (Oregon Health and Science University, Portland, USA) detail the timing and coordination of specific folding steps of the native CFTR in and across the ER membrane. Colleen Weiler and Mitchell Drumm (Case Western Reserve University, Cleveland, USA) broaden our views with genetic studies that have identified variant genes implicated in the clinical manifestations of CF. The second part of the Special topics discusses the molecular targets to rescue CFTR processing. Rebecca Chanoux and Ronald Rubenstein describe the current knowledge of the network of cellular chaperones that facilitate the folding and trafficking of CFTR to

the plasma membrane. Then, a series of papers by the groups of Teresinha Leal (Université Catholique de Louvain, Brussels, Belgium) and John Hanrahan (McGill University, Montreal, Canada) report interesting observations regarding the potential of several inhibitors in correcting the CF phenotype at the level of ion transport and inflammation. Luigi Maiuri and collaborators (European Institute for Research in Cystic Fibrosis, San Raffaele Scientific Institute, Milan, Italy) propose to target the intracellular environment in order to reestablish functional autophagy in CF epithelial cells. The third chapter is dedicated to new molecules that have already been developed or in development and able to rescue CFTR channel function by targeting mutant CFTR at the transcription (Michael Wilschanski, Hadassah Hospitals- Hebrew University, Jerusalem, Israel) and/or at the translation (Nicoletta Pedemonte and Luis Galietta, Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini Genova, Italy) levels. Finally, the Special Topic is concluded by an in-depth review by Christine Bear and collaborators (The Hospital for Sick Children, Toronto, Canada). Importantly, the gaps in our knowledge regarding the mechanism of action of existing correctors, the unmet need to discover compounds which restore proper CFTR structure and function in CF affected tissues and new strategies for therapy development are discussed. We are convinced that we achieved a very interesting Special Topics thanks to the outstanding contributions of all authors. We are especially grateful to the authors for having believed in this project and accepted to share their knowledge. All the manuscripts have been peer-reviewed and we would like to thank the experts for helping us to reach an issue of high standard.

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EDITORIAL
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Strategies to circumvent the CFTR defect in cystic fibrosis

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Mutations within the gene encoding for the chloride ion channel CFTR results in cystic fibrosis, the most common autosomal recessive genetic disease in the Caucasian population. CFTR regulates absorption and secretion mechanisms across intestinal and airway mucosae. Although the intestinal phenotype can be clinically handled, chronic infection and inflammation of the lungs of CF patients remains the principal cause of morbidity and mortality. The aim of this Research Topic is to provide to the readers the most recent information available on "Strategies to circumvent the CFTR defect in cystic fibrosis." The Research Topic is divided in three mains parts: the first part describes the CFTR structure, processing and regulation of the normal and mutant ion channels. Anna Patrick and Philip Thomas (University of Texas Southwestern Medical Center, Dallas, USA) review the molecular interactions leading to the complex folding process of the CFTR protein and how essential steps are disrupted in CFTR mutants. Soo Jung Kim and William Skach (Oregon Health and Science University, Portland, USA) detail the timing and coordination of specific folding steps of the native CFTR in and across the ER membrane. Colleen Weiler and Mitchell Drumm (Case Western Reserve University, Cleveland, USA) broaden our views with genetic studies that have identified variant genes implicated in the clinical manifestations of CF. The second part of the Special topics discusses the molecular targets to rescue CFTR processing. Rebecca Chanoux and Ronald Rubenstein describe the current knowledge of the network of cellular chaperones that facilitate the folding and trafficking of CFTR to the plasma membrane. Then, a series of papers by the groups of Teresinha Leal (Université Catholique de Louvain, Brussels, Belgium) and John Hanrahan (McGill University, Montreal, Canada) report interesting observations regarding the potential of several inhibitors in correcting the CF phenotype at the level of ion transport and inflammation. Luigi Maiuri and collaborators (European Institute for Research in Cystic Fibrosis, San Raffaele Scientific Institute, Milan, Italy) propose to target the intracellular environment in order to reestablish functional autophagy in CF epithelial cells. The third chapter is dedicated to new molecules that have already been developed or in development and able to rescue CFTR channel function by targeting mutant CFTR at the mRNA (Michael Wilschanski, Hadassah Hospitals- Hebrew University, Jerusalem, Israel) and/or at the protein (Nicoletta Pedemonte and Luis Galietta, Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini Genova, Italy) levels. Finally, the Special Topic is concluded by an in-depth review by Christine Bear and collaborators (The Hospital for Sick Children, Toronto, Canada). Importantly, the gaps in our knowledge regarding the mechanism of action of existing correctors, the unmet need to discover compounds which restore proper CFTR structure and function in CF affected tissues and new strategies for therapy development are discussed. We are convinced that we achieved a very interesting Special Topics thanks to the outstanding contributions of all authors. We are especially grateful to the authors for having believed in this project and accepted to share their knowledge. All the manuscripts have been peer-reviewed and we would like to thank the experts for helping us to reach an issue of high standard.

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Development of CFTR structure

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Cystic fibrosis is a lethal genetic disease caused by lack of functional cystic fibrosis transmembrane conductance regulator (CFTR) proteins at the apical surface of secretory epithelia. CFTR is a multidomain protein, containing five domains, and its functional structure is attained in a hierarchical folding process. Most CF-causing mutations in CFTR, including the most common mutation, a deletion of phenylalanine at position 508 (ΔF508), are unable to properly fold into this functional native three dimensional structure. Currently, no high-resolution structural information about full length CFTR exists. However, insight has been gained through examining homologous ABC transporter structures, molecular modeling, and high-resolution structures of individual, isolated CFTR domains. Taken together, these studies indicate that the prevalent ΔF508 mutation disrupts two essential steps during the development of the native structure: folding of the first nucleotide binding domain (NBD1) and its later association with the fourth intracellular loop (ICL4) in the second transmembrane domain (TMD2). Therapeutics to rescue ΔF508 and other mutants in CFTR can be targeted to correct defects that occur during the complex folding process. This article reviews the structural relationships between CFTR and ABC transporters and current knowledge about how CFTR attains its structure–with a focus on how this process is altered by CF-causing mutations in a manner targetable by therapeutics.

Keywords: CFTR, cystic fibrosis, ABC transporter, membrane protein structure, multidomain protein folding

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease affecting more than 70,000 people world-wide. CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). CFTR functions as a regulated chloride channel in the apical membrane of epithelia, where it plays a critical role in maintaining the surface liquid layer. Lack of functional CFTR results in thick secretions that cause gastrointestinal, reproductive, and respiratory system defects. Currently, CF patients most commonly die of respiratory-associated problems.

More than 70% of CF patients have at least one allele with a deletion of phenylalanine at position 508 (ΔF508; Kerem et al., 1989). Further sequencing of CF patient and non-patient CFTR genes has been extensive, and hundreds of mutations have been identified[1]. Many of these mutations have been validated as CF-causing, while others are CF-associated but unstudied. The validated CF-causing mutations are located throughout the CFTR gene, and are inherited in almost all cases (Riordan et al., 1989; Riordan, 2008). ΔF508 (Cheng et al., 1990; Thomas et al., 1992) and many other CF mutations (Gregory et al., 1991) result in mutant CFTR that does not properly fold and is retained in the ER by cell protein quality control. The result is that more than 90% of mutant CFTR alleles produce a misfolded protein that is recognized, mistrafficked, and degraded in the cell.

While we do not have high-resolution three dimensional structural information for full length CFTR, a great deal of correlative information regarding this structure has been obtained *via* homologous structures, domain structures, molecular modeling, and lower resolution techniques.

ABC TRANSPORTERS

Cystic fibrosis transmembrane conductance regulator is a member of the ATP-binding cassette (ABC) transporter superfamily of proteins, which includes membrane spanning proteins that use nucleotide hydrolysis to transport substrates across the membrane bilayer (Holland, 2003). While there is no full length high-resolution structure for CFTR, there are structures for other ABC transporters, providing insight into the structure arrangement and functional mechanisms of CFTR. Most ABC transporters function to move substrates either into the cytoplasm (importers) or out of the cytoplasm (exporters). Exporters are found in both eukaryotes and prokaryotes, while importers have only been found in prokaryotes (Rees et al., 2009). The importance of prokaryotic ABC transporters for cellular functions, such as import of nutrients and export of toxins, is highlighted by their representation as 5% of the *Escherichia coli* genome (Linton and Higgins, 1998). In humans, 48 or 49 distinct ABC transporters have been identified, many of which are implicated in disease (Dean et al., 2001; Gottesman and Ambudkar, 2001; Borst and Elferink, 2002). The core ABC transporter architecture is comprised of two transmembrane spanning domains (TMDs) and two nucleotide binding domains (NBDs). Many transporters also have accessory domains with regulatory functions (Biemans-Oldehinkel et al., 2006). In general, the TMDs are organized as two wings that open and close in response to NBD movements resulting from ATP binding and

[1] www.genet.sickkids.on.ca

hydrolysis (**Figure 1**; Moody et al., 2002; Smith et al., 2002; Locher, 2009; Rees et al., 2009). Additionally, at the external surface, many prokaryotic importers interact with accessory proteins that play a role in substrate transport (Biemans-Oldehinkel et al., 2006). The domains are modular, and are found expressed individually, in combinations, or as a single full length transporter to form the functional protein (Locher, 2009).

ATP-binding cassette transporters have a conserved coupling mechanism, whereby signals from the NBDs are transmitted to the intracellular loops (ICLs) of TMDs to cause substrate transport (Locher, 2009). The conserved NBDs form a sandwich around two ATPs, with each site for ATP binding and hydrolysis requiring both domains (Smith et al., 2002). Two subdomains are present in each NBD. The catalytic subdomain contains the conserved Walker A and B motifs, a Q-loop, and an H-motif, and the alpha-helical subdomain contains the ABC signature motif, LSGGQ (**Figure 1C**; Rees et al., 2009). Each active site is composed of components from the catalytic subunit of one NBD and the alpha-helical components of the other NBD in a head-to-tail arrangement (Smith et al., 2002; Rees et al., 2009). The binding of ATP in these sites drives the association of the NBDs (Moody et al., 2002).

The TMDs are proposed to function in an alternating access model of transport and are the most variable among ABC transporters (Chen et al., 2001; Dawson et al., 2007). ABC transporters can be divided into three classes based on the TMD fold (Locher, 2009). Type I and II ABC importers contain different core transmembrane (TM) span topologies of 10 and 20 TM helices respectively, with the latter tending to facilitate transport of larger substrates (Locher et al., 2002; Hollenstein et al., 2007; Locher, 2009). In both importer types, one TMD interacts with one NBD to form two TMD-NBD units that together form a functional transporter (**Figure 1A**; Locher, 2009). ABC exporters contain a core of 12 TM helices, with each wing of the transporter made of both TMDs, with each TMD interacting with both NBDs in a domain-swapped fashion (**Figure 1B**; Dawson and Locher, 2006; Locher, 2009). In this arrangement, the ICLs extend into the cytoplasm, positioning the NBDs approximately 25 Å from the membrane (**Figure 1B**; Locher, 2009). In exporters, the TMDs and NBDs are expressed as TMD-NBD units, and eukaryotic exporters are most frequently found as full length transporters (Nikles and Tampe, 2007).

CFTR AS AN ABC TRANSPORTER

Cystic fibrosis transmembrane conductance regulator is a member of the ABC C subfamily, and is structurally homologous to the domain-swapped exporters. Structures of homologous ABC exporters such as bacterial Sav1866 (Dawson and Locher, 2006, 2007), bacterial MsbA (Ward et al., 2007), bacterial TM287/288 (Hohl et al., 2012), and mammalian P-glycoprotein (Aller et al., 2009) have been solved. The available structural data in combination with sequence alignments form the basis for homology models of full length CFTR that provide insight into its structure, mechanisms of regulation, and signal transduction (Mendoza and Thomas, 2007; Mornon et al., 2008, 2009; Serohijos et al., 2008). The exporter structures are in both open and closed forms, giving insight into movements within the CFTR protein during a transport cycle (**Figure 2**, open form; Ward et al., 2007; Locher, 2009; Mornon et al., 2009; Rees et al., 2009). The similarity of CFTR movements to other ABC transporters is supported by electron microscopy data in combination with a low resolution crystal structure (Rosenberg et al., 2004, 2011; Zhang et al., 2009,

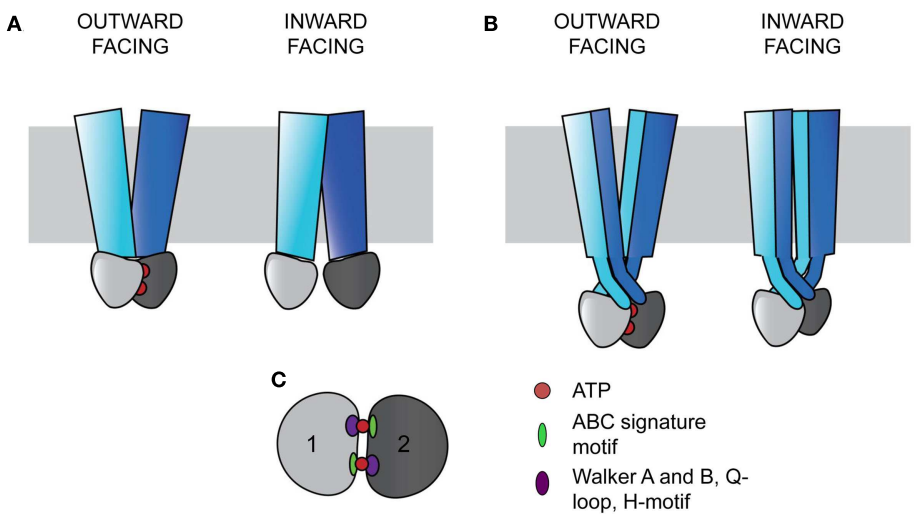

FIGURE 1 | ABC transporters contain a core architecture of two TMDs (blue and cyan) and two NBDs (light and dark gray). Signals from the NBDs relating to ATP binding and hydrolysis result in wing-like TMD movements that transport substrates across the membrane (gray rectangle). In the outward facing configuration, the two NBDs bind ATP (red circles) and are close together, with the TMDs open to the non-cytosolic side. In the inward facing configuration, the NBDs are more distant without ATP bound, and the TMDs are open to the cytosol. **(A)** In ABC importers, each TMD interacts with a single NBD (Examples include BtuCD and MalFGK). **(B)** In ABC exporters, the TMDs wrap around each other in a domain-swapped fashion, with each TMD interacting with both NBDs (Examples include Sav1866 and MsbA). **(C)** The two NBDs sandwich two ATPs in a head-to-tail fashion. Each ATP binding and hydrolysis site is comprised of both NBDs, containing the conserved structural motifs in the catalytic subdomain including the Walker A and B, Q-loop, and H-motif (purple oval), and the ABC signature motif in the alpha-helical subdomain (green oval).

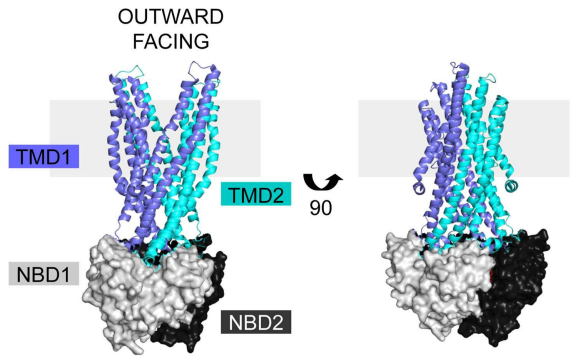

FIGURE 2 | CFTR homology models are based on the ABC exporter structures. The Sav1866 (pdb 2HYD) exporter is shown with domains colored based on representative CFTR domains, with TMD1 (blue), NBD1 (light gray), TMD2 (cyan), and NBD2 (black). There is no homologous structure for the CFTR R domain. The structure is in the outward facing configuration, which for CFTR is the open channel. The view is shown in the plane of the membrane (gray rectangle).

2011). The only high-resolution structures of CFTR domains are of NBD1 (Lewis et al., 2004, 2005, 2010; Thibodeau et al., 2005) and NBD2 (pdb 3GD7). As an ABC transporter, CFTR contains two TMDs, two NBDs, and a unique regulatory R region translated from an mRNA transcript as a single polypeptide chain (Riordan et al., 1989). Sav1866 based CFTR models have extensive interdomain interactions between the TMDs and NBDs, but lack regions without sequence homology, like the R domain (**Figure 2**; Dawson and Locher, 2006; Mendoza and Thomas, 2007).

The NBDs of CFTR, like other ABC transporters (Moody et al., 2002; Smith et al., 2002), interact in a head-to-tail fashion forming two sandwiched ATP binding pockets made of both domains (Vergani et al., 2005; Mense et al., 2006). Each NBD has a catalytic subdomain that contains the Walker A and B motifs and an alpha-helical subdomain that contains the conserved ABC signature motif (Lewis et al., 2004; Thibodeau et al., 2005). However, like several other members of the ABC C subfamily, one ATP binding site is non-hydrolytic (Muallem and Vergani, 2009). In this site, non-conservative mutations, which are located in the NBD1 Walker B and switch motifs and in the NBD2 signature sequence, result in tight binding and inefficient ATP hydrolysis (Aleksandrov et al., 2002; Basso et al., 2003; Gadsby et al., 2006). In general, CFTR ATP driven conformational changes include ATP binding, which results in an NBD dimer that signals the TMDs to open. Then, hydrolysis of one ATP disrupts the NBD interface, the NBDs separate, and the channel closes (Gadsby et al., 2006; Aleksandrov et al., 2007; Muallem and Vergani, 2009). However, the driving forces that control the gating transitions and the signals transmitted by ATP binding and hydrolysis are a matter of debate (Gadsby et al., 2006; Aleksandrov et al., 2007; Muallem and Vergani, 2009). NBD1 also contains two non-conserved regions, a regulatory insert (RI) near the N-terminus and a regulatory extension (RE) near the C-terminus. Of these two regions, studies have focused on the RI. The RI is disordered in the NBD1 crystal structures and plays a role in regulation of CFTR channel gating, but is not required for trafficking in the cell (Lewis et al., 2004; Thibodeau et al., 2005; Aleksandrov et al., 2010). Furthermore, a mechanism wherein RI movements alter ICL1-NBD1 interactions to affect phosphorylation-dependent CFTR gating has been proposed (Kanelis et al., 2010).

Like other domain-swapped exporters, the TMDs form two wings containing TMs from both TMD1 and TMD2, such that the first two TMs and last four TMs of each domain make a wing (**Figure 2**; Dawson and Locher, 2006; Mendoza and Thomas, 2007). Based on the exporter structures, the wings move to open and close the chloride channel for ion transport (Vergani et al., 2005; Mornon et al., 2009). In each TMD, two alpha-helical ICLs extend into the cytoplasm, with each having a distal coupling helix that interacts with the NBDs (Mendoza and Thomas, 2007; Mornon et al., 2008; Serohijos et al., 2008). In combination, the four ICLs form four helix inner and outer bundles that end in the coupling helices (**Figure 2**; Mornon et al., 2008, 2009). Each coupling helix is parallel to the NBD surface, and forms a largely hydrophobic interface (Mendoza and Thomas, 2007). In CFTR, ICL2 interacts with NBD2, ICL4 interacts with NBD1, and ICLs 1 and 3 interact with both NBD1 and NBD2. Importantly, the F508 position in NBD1 is predicted to lie near the interface between NBD1 and ICL4 (Mendoza and Thomas, 2007). Many of the predicted interdomain interactions are also experimentally validated by crosslinking studies (Chen et al., 2004; Mense et al., 2006; He et al., 2008; Loo and Clarke, 2008; Serohijos et al., 2008). Further complexity of the ICL-NBD interactions is generated by phosphorylation-dependent interactions between NBD1 and an ICL1 peptide (Kanelis et al., 2010). Additionally, crosslinks between an ICL and the opposing NBD disrupt channel opening, supporting the essential roles of these components for channel function (He et al., 2008). Models predict specific residues are critical for the interactions between the ICLs and NBDs, including Y275 and W277 which form an interface with NBD2 (He et al., 2008; Mornon et al., 2008); D173, S169, and R170 which are predicted to contact nucleotide and NBD1 (Mornon et al., 2008); and S263 and E267 which stabilize ICL helical bundle structure (Mornon et al., 2009). Notably, the W277 position is equivalent to the R1070 position in ICL4 (Mornon et al., 2008) that when mutated, R1070W, suppresses the ΔF508 mutation (Thibodeau et al., 2010; Mendoza et al., 2012). These positions have not yet been fully tested for their roles in the folding and function of CFTR.

In summary, conformational signals generated in the NBDs in relation to ATP binding and hydrolysis are transmitted by the ICLs in the TMDs, resulting in chloride channel opening and closing (Gadsby et al., 2006; Riordan, 2008). The interactions between CFTR ICLs and NBDs have been validated by crosslinking studies (He et al., 2008; Serohijos et al., 2008) and complementation of a mutant located in an NBD with a mutant in an ICL (Thibodeau et al., 2010). The coupling helices of ABC transporters are architecturally conserved without having a highly conserved sequence (Locher, 2009), making prediction of essential positions and residues difficult without a high-resolution full length CFTR structure.

Cystic fibrosis transmembrane conductance regulator is the only known channel among the ABC transporters. In the alternating access model, ABC transporters are open to one side of the membrane bilayer at a time (Chen et al., 2001; Dawson et al., 2007). In CFTR, channel formation abrogates this model, as one of the gates that would normally block substrate transport must be atrophied or gone to allow chloride flux (Gadsby, 2009). With regard to this, CFTR has been called a broken ABC transporter (Jordan et al., 2008; Muallem and Vergani, 2009). Similar to other chloride channels, CFTR is not very selective among small monovalent anions and has a relatively featureless pore (Gadsby et al., 2006; Gadsby, 2009). Putative residues that make the chloride channel have been identified in TMs and in extracellular loops, with a focus on TM1 and TM6 (Linsdell, 2006). However, it is difficult to validate these residues without better characterizing the TM span positions and TMD structures. Further complicating the TMD structure is a TMD1 N-terminal cytosolic region that regulates CFTR channel activity through interactions with the R domain, neither of which has a homologous structure (Naren et al., 1999; Chappe et al., 2005).

The chloride channel activity of CFTR is regulated by the R domain (Riordan, 2008). The R domain is largely unstructured and has multiple sites that are phosphorylated by PKA, resulting in CFTR channel activation (Gadsby et al., 2006; Baker et al., 2007). Consistent with this, the unphosphorylated R domain has an inhibitory effect on the CFTR channel (Rich et al., 1991; Csanady et al., 2000). The R domain interacts with multiple other regions of CFTR, including NBD1 and the N-terminus of TMD1 (Naren et al., 1999; Baker et al., 2007; Kanelis et al., 2010). This evidence suggests the R domain may act as a signal integrator to regulate channel function via interactions with different regions of CFTR. However, due to its lack of homology and disordered nature, the R domain location within CFTR models remains unclear.

Many different modifications to the CFTR protein that may impact its structure have been identified. For instance, CFTR contains two *N*-linked glycosylation sites, NXS/T (X≠P), within TMD2 that are core glycosylated in the ER lumen. This core glycosylation is then modified in the Golgi to produce complex glycosylated protein (Helenius and Aebi, 2001). The natural sites within CFTR are regularly used to monitor its integration and cellular trafficking by changes in electrophoretic mobility upon core glycosylation, producing Band B at approximately 150 kDa, and complex glycosylation, producing a diffuse Band C above 170 kDa. The natural glycosylation sites are not required for cellular trafficking from the ER and chloride channel function (Howard et al., 1995; Chang et al., 2008; Glozman et al., 2009; Patrick et al., 2011). However, recently, these sites have been found to influence the efficiency of CFTR productive protein folding and early secretory trafficking (Glozman et al., 2009), and cell surface retention and turnover in post-ER cellular compartments (Chang et al., 2008; Glozman et al., 2009). The impact of these and other modifications on the development of CFTR structure is an area of ongoing study.

The combination of experimental and modeling studies provides significant insight into the CFTR structure, which allows formation of models within which mechanochemical mechanisms and the effect of CF-causing folding mutations can be framed. However, since many CF-causing mutations, including ΔF508, result in misfolding of the CFTR protein, the folded full length structure may not adequately describe the relevant defects.

CFTR FOLDING AS A MULTIDOMAIN PROTEIN

Cystic fibrosis transmembrane conductance regulator, like other ABC transporters, contains extensive interdomain surfaces (Rees et al., 2009) that, in the case of CFTR, likely form during translation (Zhang et al., 1998; Du et al., 2005; Kleizen et al., 2005; Thibodeau et al., 2005). During protein translation, secondary structure can begin to form early, even while the nascent chain is in the tunnel of the ribosome (Kramer et al., 2001; Woolhead et al., 2004). For CFTR, as translation continues each domain folds and can then interact with previously translated domains to form multidomain folding intermediates (**Figure 3**; Lukacs et al., 1994; Du et al., 2005; Kleizen et al., 2005; Thibodeau et al., 2005; Cui et al., 2007; Cheung and Deber, 2008; Du and Lukacs, 2009). The current model of CFTR folding holds that individual domain structures form cotranslationally (Kleizen et al., 2005). Then, intermediate structures form and eventually a TMD1-NBD1-R-TMD2 structure is produced that is required for cellular trafficking (Meacham et al., 1999; Du et al., 2005; Cui et al., 2007; Du and Lukacs, 2009). Finally, NBD2 posttranslationally incorporates into the CFTR structure (**Figure 3**; Du et al., 2005). The addition of NBD2 confers a greater folding efficiency and trafficking from the ER. Thus, although NBD2 is not strictly required for CFTR trafficking (Pollet et al., 2000; Cui et al., 2007; Du and Lukacs, 2009; Thibodeau et al., 2010), its posttranslational association into the CFTR structure (Du et al., 2005) may increase the yield of folded cellular CFTR. Much of this model is based on individual CFTR domains forming protease-resistant structures during translation (Zhang et al., 1998; Kleizen et al., 2005). The order of interdomain interaction formation and whether initial interactions are the same as those in the final CFTR structure is not known.

Cystic fibrosis transmembrane conductance regulator folding occurs during translation as a linear polypeptide (Riordan et al., 1989). However, many ABC transporter domains are expressed separately, and later associate to form a functional transporter (Locher, 2009). To some extent, CFTR retains some ability to fold in this manner. CFTR can be expressed as a split construct, which forms structure that traffics to the cell surface and functions as a chloride channel (Ostedgaard et al., 1997; Chan et al., 2000; Csanady et al., 2000; Du and Lukacs, 2009). Additionally, expression of constructs containing TMD1-NBD1-R or R-TMD2-NBD2 formed chloride channels, likely as multimers (Sheppard et al., 1994; Devidas et al., 1998). Finally, in the cell the minimal construct that traffics from the ER contains TMD1-NBD1-R-TMD2, which forms a chloride channel (Cui et al., 2007). These studies suggest that the domains of CFTR, to a certain extent, can associate posttranslationally to form a functional chloride channel. Yet, CFTR is a linear chain, such that folding requires that each domain attain structure in a more spatially confined manner. The critical role of the primary sequence in the CFTR folding process is highlighted by the multitude of CF-associated folding mutations identified throughout the protein (see text footnote 1).

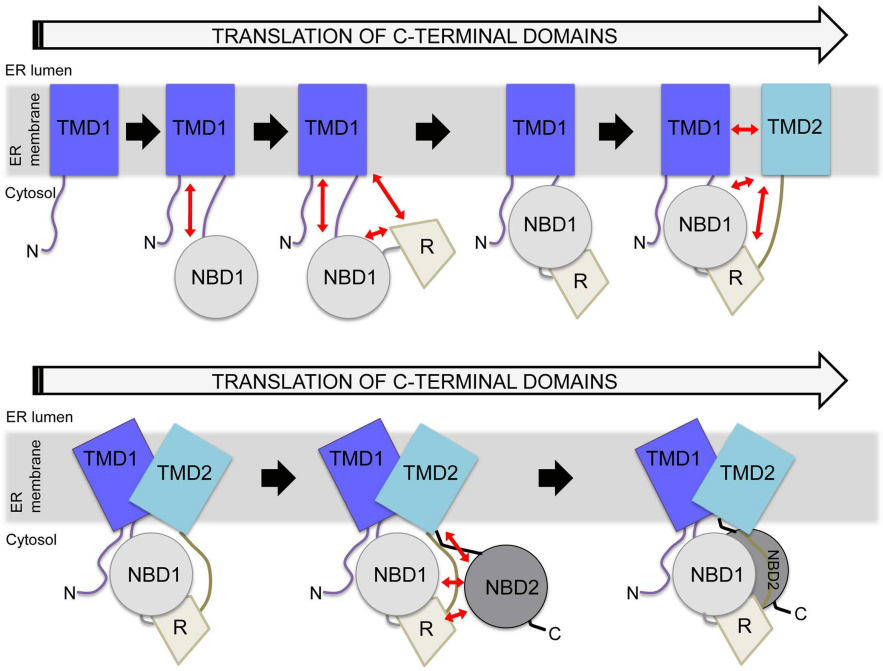

FIGURE 3 | Hierarchical folding model of CFTR. Potential interdomain interactions are indicated by red arrows, with several possible structural units included during the translation process. In the cell, constructs lacking TMD2 do not traffic from the ER, whereas constructs containing TMD2 can traffic from the ER. Reflecting this, a major structural rearrangement is depicted in the presence of TMD2, as shown in the bottom panel. Eventually, NBD2 is incorporated as the final step. There are many points during this process at which the cell may monitor perturbations.

CFTR COTRANSLATIONAL FOLDING INVOLVES INTERACTIONS WITH OTHER PROTEINS

Cystic fibrosis transmembrane conductance regulator folding involves many proteins that act at different stages to aid folding or recognize misfolding. This topic is extensively reviewed elsewhere within this Research Topic. Briefly, the misfolded CFTR is retained in the ER, and eventually degraded (Lukacs et al., 1994) by the proteasome (Jensen et al., 1995; Ward et al., 1995). Many proteins interact with CFTR in the ER lumen, ER membrane, and cytoplasm, suggesting that the domains of CFTR are differentially monitored during the biosynthetic process. Among these identified interacting partners are the cytoplasmic proteins Hsc/p 40, 70, 90, and associated co-chaperones CHIP (Strickland et al., 1997; Meacham et al., 1999, 2001; Younger et al., 2006) and Aha1 (Wang et al., 2006), the ER membrane associated protein RMA1 (Younger et al., 2006; Grove et al., 2011), the ER integral membrane proteins Derlin (Sun et al., 2006; Younger et al., 2006; Wang et al., 2008) and BAP31 (Wang et al., 2008), and the ER luminal-interacting protein calnexin (Pind et al., 1994). After trafficking to the cell surface, CFTR interactions with cytoskeletal proteins are important for its maintenance at this cellular location (Okiyoneda and Lukacs, 2007). Also at the plasma membrane, peripheral protein quality control is involved in the ubiquitination, internalization, and degradation of misfolded CFTR (Okiyoneda et al., 2010). Moreover, a protein interactome for CFTR includes potential interactions far beyond those that have been studied (Wang et al., 2006). However, it is not clear which proteins interact at the earliest stages of folding/maturation and are responsible for initial and irreversible recognition of mutant CFTR. Furthermore, the structural aspects of CFTR during folding that are important for formation of these interactions are unclear. These interactions paint a picture of CFTR biogenesis whereby normal structural formation and interactions are formed with cellular folding and quality control machinery, providing multiple points to monitor CFTR folding.

CF-MUTANTS PERTURB CFTR COTRANSLATIONAL FOLDING

Cystic fibrosis-associated mutations have been found in every domain of CFTR (see text footnote 1). Misfolded CFTR, specifically the ΔF508 mutant protein, is recognized by cellular quality control machinery, accumulates in the ER (Cheng et al., 1990), and is eventually degraded (Lukacs et al., 1994) by the proteasome (Jensen et al., 1995; Ward et al., 1995). Many studies have identified CF-causing mutants that result in accumulation of CFTR in the ER. Mutant effects have been categorized into classes based on the resulting effect on CFTR (Welsh and Smith, 1993; Zielenski and Tsui, 1995). The alterations include lack of protein production (class I), defective protein maturation and early degradation (class II), defective regulation of ATP interactions (class III), reduced chloride transport (class IV), reduced transcripts though splicing or promoter defects (class V), and increased cell surface turnover (class VI; Welsh and Smith, 1993; Zielenski and Tsui, 1995). The ΔF508 mutation accounts for 70% of CF-causing mutant CFTR alleles (Riordan et al., 1989), making class II defects the most common cause of CF.

Mutations within the CFTR protein, including ΔF508, may perturb local protein structure and/or domain structure, or could be surface exposed and perturb interactions with other domains or proteins. For instance, in the NBD-ICL4 interface, mutants in ICL4 including L1065P, R1066C, and A1067T alter trafficking and chloride channel function (Cotten et al., 1996; Seibert et al., 1996). Mutants in different domains alter biogenic intermediates of CFTR, suggesting that misfolding does not require full length CFTR (Du and Lukacs, 2009). Furthermore, in full length CFTR, the proteolytic stability of all domains was reduced for spatially separate mutations, suggesting propagation of one mutant to other domains (Rosser et al., 2008; Du and Lukacs, 2009). The propagation of mutants could occur through a rearrangement step involving multiple domains (Du and Lukacs, 2009), or through coupled folding of the domains. As discussed, various components of cell quality control recognize CFTR as it is created, such that domain and multidomain states are likely differentially monitored (Younger et al., 2006). For each mutation, the effect on individual domain folding and multidomain units plays a fundamental role in determining the mechanisms by which that mutation is recognized and managed within the cell.

An example of mutants similarly located within CFTR with different local mechanisms of misfolding are the G85E and G91R mutations. These mutations are located near or within the TM1 span within TMD1. Both mutations have been demonstrated to disrupt later steps in CFTR folding, including interdomain interactions, which have been proposed to result in mutant recognition by ER quality control machinery (Xiong et al., 1997). Recently, G85E was found to dramatically alter the conformation/integration profile of TM1 (Patrick et al., 2011). Such an alteration would occur at the earliest steps of translation and integration, and could be recognized as a very early misfolding event by ER quality control machinery. The G91R mutant was predicted to have a similar effect on CFTR (Xiong et al., 1997), but this proved not to be true with regards to the TM1 conformation/integration profile (Patrick et al., 2011). Interestingly, the corrector compound four rescues G91R but not G85E-CFTR (Grove et al., 2009), suggesting the differences in the mutant molecular pathologies may be relevant for their ability to benefit from specific treatments to rescue defective CFTR. The detailed mechanistic study of CF-causing mutations provides a better fundamental understanding of membrane protein misfolding and mechanisms for approaching mutant specific therapy for CF patients.

FOLDING OF NBD1 AND ΔF508-NBD1

The best studied disease-causing mutation, ΔF508, alters multiple steps during CFTR folding. Particular focus has been given to folding of NBD1, wherein F508 resides. High-resolution crystal structures of both NBD1 and ΔF508-NBD1 have been solved (Lewis et al., 2004, 2005; Thibodeau et al., 2005; Mendoza et al., 2012). These structures place F508 on the domain surface, and ΔF508 does not cause significant perturbations in the crystal structure (Lewis et al., 2005). However, ΔF508-NBD1 has an increased tendency to aggregate and is destabilized, indicating a disruption during folding that is not represented in these native structures (Qu and Thomas, 1996; Lewis et al., 2005; Thibodeau et al., 2005). Consistent with this, a non-native conformation of NBD1 has been identified that is promoted by ΔF508 and linked to increased aggregation (Hoelen et al., 2010; Richardson, unpublished data). The NBD1 structure is obtained cotranslationally (Kleizen et al., 2005; Hoelen et al., 2010; Khushoo et al., 2011). During translation, a ligand-dependent N-terminal compact structure forms, and upon completion of NBD1 translation another compact structure forms (Khushoo et al., 2011). The compact N-terminal structure is not affected by ΔF508, suggesting that the folding error likely occurs at a later step of NBD1 folding (Khushoo et al., 2011). The ΔF508 misfolding begins in NBD1, making this an attractive target for correcting ΔF508-CFTR. The ΔF508 mutant effects can be partially rescued independently by suppressor mutations within NBD1 (Teem et al., 1993; Qu et al., 1997; DeCarvalho et al., 2002; Hoelen et al., 2010). Importantly, the ΔF508 effects on NBD1 also manifest during translation of the full length CFTR (Kleizen et al., 2005).

In full length CFTR, ΔF508 effects multidomain stability and interdomain interactions. In mammalian cells, the ΔF508-CFTR misfolds, resulting in cellular mistrafficking via its accumulation in the ER (Cheng et al., 1990). As shown by limited proteolysis and pulse chase analysis, the ΔF508 mutation destabilizes NBD1 and multidomain folding intermediates, implying a more global destabilization of the entire ΔF508-CFTR (Zhang et al., 1998; Meacham et al., 1999; Du et al., 2005; Cui et al., 2007; Rosser et al., 2008; Du and Lukacs, 2009). The homology model of CFTR places the F508 position at an interface between NBD1 and ICL4 of TMD2 (Mendoza and Thomas, 2007). Consistent with this, ΔF508 disrupts WT-like crosslinks between ICL4 and NBD1 and within the TMDs (Chen et al., 2004; Serohijos et al., 2008). Additionally, mutations in ICL4 can suppress the effect of ΔF508, further supporting a disruption of this interface (Thibodeau et al., 2010). Recently, the ΔF508-mediated NBD1 misfolding and multidomain assembly were both shown as essential for correction of ΔF508-CFTR (Mendoza et al., 2012; Rabeh et al., 2012). This is consistent with the known ΔF508 effects on NBD1 folding, which is a prerequisite for its interdomain interactions and formation of an NBD1 surface for ICL4 interactions. However, these experiments have not yet been able to identify the timing or mechanism(s) of domain interaction disruption. The point at which ΔF508 effects are detectable and the ability to target multiple steps to rescue the ΔF508 protein emphasizes the multistep misfolding of ΔF508-CFTR. Further details regarding this misfolding are needed to continue to rationally devise new therapeutic interventions.

Other methods to rescue ΔF508-CFTR continue to be explored. For instance, compounds have been identified that rescue ΔF508-CFTR mutation via interactions with the TMDs (Loo et al., 2011). ΔF508 and other mutant CFTRs were also partially rescued by transcomplementation, in which co-expression of parts of CFTR were able to improve trafficking of CF-mutant CFTR from the ER (Cormet-Boyaka et al., 2004; Cebotaru et al., 2008). Insights into the rescue of ΔF508-CFTR also come from the yeast homologous ABC exporter, Yor1p (Pagant et al., 2007, 2008). When a ΔF508 mimic is introduced into Yor1p, consequent mistrafficking and degradation occurs (Pagant et al., 2007). Two Yor1p suppressor mutations in the TM-ICL juncture were found to correct the ΔF508 mimic (Pagant et al., 2010), suggesting that modification of the ICL structures rather than direct stabilization

of the NBD-ICL interface is a potential target for correction of ΔF508-CFTR. Also, a co-expressed Yor1p NBD1 was able to swap into the ΔF508 mimic-Yor1p to replace the defective domain (Louie et al., 2010). Notable differences exist between the Yor1p protein and CFTR; however these findings provide insight into potential mechanisms for ΔF508-CFTR correction that should be investigated directly with CFTR.

RESCUING MUTANT CFTR

It is suggested that only 10–35% of CFTR function is needed to positively impact pulmonary disease (Kerem, 2004), therefore the production and residual activity of mutant CFTR is relevant for clinical outcomes. In CF, there is a focus on rescuing the defective CFTR protein. Ongoing therapeutic developments are aimed at targeting mutations that introduce premature termination codons, decrease chloride channel function, and alter cellular trafficking, which are discussed elsewhere within this Research Topic. For ΔF508 and other missense mutations, two aspects to rescuing mutant CFTR protein are to rescue processing and function, both of which are innately linked to CFTR structure.

Thus far, great success has occurred in rescuing the CF-causing G551D mutant. G551D-CFTR has normal cell surface expression and half-life, but confers a severe defect in channel gating (Welsh and Smith, 1993). The compound VX-770 was initially characterized as a CFTR potentiator in CF airway epithelial cells (Van Goor et al., 2009). This compound has since undergone clinical trials showing efficacy in CF patients (Accurso et al., 2010; Ramsey et al., 2011), has been approved by the FDA for treatment of G551D based CF in patients over 6 years old, and is now marketed as Kalydeco™. These results are promising for CF patients as adults, who already have lung scarring and dysfunction, and for children, who may be able to avoid lung dysfunction with this therapeutic. This success has generated a foundation to guide further progress in CF therapeutic development for other mutants, such as ΔF508.

ΔF508 and other mutants that cause CFTR misfolding, mistrafficking, and disrupted channel function are the largest CF therapeutic target. The ΔF508-CFTR exhibits a temperature sensitive trafficking from the ER, in which it is retained in the ER at 37°C, but partially traffics from the ER at lower temperatures (Denning et al., 1992). This imparts the idea that trafficking correction is feasible for ΔF508 if a chemical compound can mimic the temperature rescue. However, ΔF508-CFTR that is induced to fold/traffic by low temperature or chemical modifier treatments has disrupted chloride channel function (Dalemans et al., 1991) and shorter residence times at the cellular surface (Lukacs et al., 1993), indicating the native structure is not achieved. This makes approaching ΔF508-CFTR a complex problem. Recently, it was found that correction of both the ΔF508-NBD1 defect and the ΔF508-NBD1-ICL4 interaction defect are required to rescue ΔF508, consistent with at least two steps for correction of ΔF508-CFTR (Mendoza et al., 2012; Rabeh et al., 2012). ΔF508 is being targeted pharmacologically by strategies that aim to correct the trafficking defect and potentiate channel function. Currently trials of VX-809 or VX-661, to correct trafficking, and Kalydeco™, to potentiate channel function, are ongoing. However, development of a combination therapy is exponentially more complicated and difficult. Ideally, a single compound to both correct and potentiate mutant CFTR will be identified (Sheppard, 2011). Extensive work has gone into describing ΔF508-CFTR misfolding in order to identify the most pertinent misfolding step(s) for generating the most relevant therapeutic target.

DISCUSSION

Cystic fibrosis transmembrane conductance regulator structural development occurs in a complex manner (**Figure 3**). It requires formation of TMD1, which involves TM span interactions with the translocation machinery in the ER. Then production of two cytosolic domains occurs, first NBD1 and then R. Following this, yet another TMD must be appropriately integrated, with the protein structure completed after the production of cytosolic NBD2. In the final structure, these domains form extensive interdomain interactions, with the later interaction surfaces having no obvious interaction partners prior to formation of the final structure. For instance, during translation, the TM and ICL regions that form later interdomain interactions are present minutes prior to production of their interaction partners. These regions are very hydrophobic and are unlikely to be stable without their partner sequences or other protein interactions. While the ICL helical bundle likely forms only when both TMD1 and TMD2 are present, this has not been tested experimentally. It is not known if ICL structure formation begins in TMD1, or what happens to the coupling helices before both NBDs are present. A requirement of this structure for NBD docking onto the ICLs has not been examined. Knowledge of the timing of this structure formation and its role in TMD-NBD interactions will be required for better understanding development of ABC transporter structure. The interactions required for the formation of native CFTR structure are important for understanding CF-mutant mediated misfolding, which is a therapeutic target for correcting CF-mutant CFTR.

Experimental evidence supports that the first four domains of CFTR undergo a multidomain rearrangement, since a regulated chloride channel that can traffic to the plasma membrane is formed (Cui et al., 2007). The cell is able to monitor and determine whether the TMD2 containing construct should traffic from the ER (Cui et al., 2007; Du and Lukacs, 2009; Thibodeau et al., 2010). This suggests that, upon the translation of TMD2, the protein quality control machinery makes a distinction between folded and unfolded CFTR. A hierarchical folding model also predicts that two and three domain hierarchical interactions also form (**Figure 3**). Though this model is appealing, little evidence exists to support domain associations prior to the translation of TMD2. The most suggestive evidence of interdomain interactions in the first two and three domains of CFTR is the formation of a more stable three domain construct (Meacham et al., 1999; Rosser et al., 2008; Grove et al., 2009). In these studies, the interdomain interactions are implied rather than directly tested. Much of the evidence for formation of multidomain units is forced to rely on the use of modeling and perturbing mutations to detect the structural units. It is clear, however, that the most highly studied mutants, specifically ΔF508, alter domain structure in a manner recognizable by the cell (Du and Lukacs, 2009), convoluting the interpretation of multidomain complexes with domain effects. A continuing

effort to analyze native and mutant CFTR and to develop assays to better study multidomain unit formation are required to continue addressing these specific issues.

It is important to consider that CFTR cotranslational interactions may be directly related to the order of domain translation. If these interactions are required sequentially for structure formation, then a linear peptide should be essential to produce folded CFTR. However, CFTR expressed as two pieces underwent cellular trafficking as monitored by glycosylation (Ostedgaard et al., 1997; Chan et al., 2000; Csanady et al., 2000; Du and Lukacs, 2009), inconsistent with the model. By contrast, the ability of split CFTR to form functional protein is consistent with other ABC transporters within which the modular formation of domain structure indicates that one domain is not required for the formation of other domains (Locher, 2009). Yet, during *in vitro* refolding of the modular ABC transporter, BtuCD, refolding from partially unfolded units resulted in the highest functional measures (Di Bartolo et al., 2011). This suggests that domain interactions during folding may play a role in increasing the production yield of functional protein. For CFTR, these interactions could be potentiated by the linear arrangement and be important for generating enough functional protein to maintain normal physiology. This may play a role in reaching a level of physiologically functional CFTR required to alter the progression of CF therapeutically.

Cystic fibrosis clinically impacts multiple organ systems, such that treatment of the basic defect in CFTR is the best way to address the widespread morbidities. Novel therapeutics show tremendous promise for altering the molecular pathologies of CF, however, implementation of therapeutics designed to correct the most common mutant, ΔF508, is difficult. The ΔF508 molecular pathology is complex and involves multiple levels of misfolding and recognition thereof in the cell. Indeed, ΔF508-CFTR misfolds and is accumulated in the ER (Cheng et al., 1990). Moreover, if the trafficking defect is overcome, cell surface ΔF508-CFTR displays reduced chloride transport (Dalemans et al., 1991) and an accelerated turnover rate (Lukacs et al., 1993). Addressing each effect individually is inadequate, and a successful combination of therapeutics has not yet been identified to effectively rescue the ΔF508 mutation and remains an untraveled therapeutic path. Suppressor mutations of ΔF508 have been identified within NBD1 (Teem et al., 1993) and within ICL4 (Thibodeau et al., 2010), which correct NBD1 folding and/or multidomain folding. But, individually, these suppressors have limited efficacy. It is now established that correction of at least two steps are needed to rescue ΔF508, including NBD1 folding and interdomain interactions (Mendoza et al., 2012; Rabeh et al., 2012). If the effects of the different suppressor mutations for ΔF508 either within NBD1 or distant in the CFTR protein can be mimicked and combined in a small molecule this could prove an effective therapeutic. It is clear from these studies that the identification of disease mechanisms that may be targeted therapeutically requires a global understanding of CFTR structure. Future disease modifying compounds will be more effective if the target is the most relevant biological defect.

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Mechanisms of CFTR folding at the endoplasmic reticulum

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In the past decade much has been learned about how Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) folds and misfolds as the etiologic cause of cystic fibrosis (CF). CFTR folding is complex and hierarchical, takes place in multiple cellular compartments and physical environments, and involves several large networks of folding machineries. Insertion of transmembrane (TM) segments into the endoplasmic reticulum (ER) membrane and tertiary folding of cytosolic domains begin cotranslationally as the nascent polypeptide emerges from the ribosome, whereas posttranslational folding establishes critical domain–domain contacts needed to form a physiologically stable structure. Within the membrane, N- and C-terminal TM helices are sorted into bundles that project from the cytosol to form docking sites for nucleotide binding domains, NBD1 and NBD2, which in turn form a sandwich dimer for ATP binding. While tertiary folding is required for domain assembly, proper domain assembly also reciprocally affects folding of individual domains analogous to a jig-saw puzzle wherein the structure of each interlocking piece influences its neighbors. Superimposed on this process is an elaborate proteostatic network of cellular chaperones and folding machineries that facilitate the timing and coordination of specific folding steps in and across the ER membrane. While the details of this process require further refinement, we finally have a useful framework to understand key folding defect(s) caused by ΔF508 that provides a molecular target(s) for the next generation of CFTR small molecule correctors aimed at the specific defect present in the majority of CF patients.

Keywords: cystic fibrosis, CFTR, membrane protein biogenesis, protein translocation, cotranslational folding, nucleotide binding domain, ABC transporter

INTRODUCTION

Cystic fibrosis (CF) is one of a growing number of human diseases caused by inherited mutations that disrupt protein folding. It is caused by dysfunction of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a cAMP-regulated ion channel that resides in the apical membrane of epithelial cells (Riordan, 2008; Lubamba et al., 2012). CFTR dysfunction can occur by defects in protein synthesis, folding, intracellular trafficking, channel gating, chloride conductance, or plasma membrane stability. In each case, loss of CFTR results in abnormalities of water, chloride, and/or bicarbonate transport that lead to dysfunction of target tissues including: pancreatic insufficiency, increased sweat chloride, intestinal obstruction, and most importantly, chronic pulmonary infection, inflammation, and ultimately death due to respiratory failure (Cohen and Prince, 2012; Ratjen and McColley, 2012). The most prevalent CFTR mutation, Phe508del (ΔF508), is found in ∼90% of CF patients (Riordan et al., 1989) where it impairs CFTR folding, inhibits channel gating, and decreases plasma membrane stability (Lukacs and Verkman, 2012). The mechanisms by which ΔF508 disrupts CFTR folding are beginning to be understood, and small molecule modulators that restore endoplasmic reticulum (ER) trafficking and channel gating hold great promise for new treatments to correct these underlying molecular abnormalities in CF patients.

Cystic fibrosis transmembrane conductance regulator is a 1480 amino acid polytopic glycoprotein in the ABC transporter family (ABCC7) that contains two six-spanning transmembrane (TM) domains (TMD1 and TMD2) that form the channel pore, two cytosolic nucleotide binding domains (NBD1 and NBD2) that drive channel gating, and an intrinsically unstructured regulatory (R) domain that controls channel activity via PKA-mediated phosphorylation (**Figure 1A**). CFTR synthesis has been estimated to take 9–10 min in eukaryotic cells (Ward and Kopito, 1994), suggesting that significant folding occurs cotranslationally. Like most polytopic membrane proteins, CFTR biogenesis occurs at the ER, and requires coordinated folding of individual domains in three distinct cellular compartments: the ER membrane, the ER lumen, and the cytosol. This compartmentalization takes place as the nascent chain emerges from the ribosome. Subsequent assembly of TMDs and NBDs into the final folded structure takes ∼30–120 min and is facilitated by a large cohort of cytosolic and lumenal chaperones including Hsp70, Hsp40, Hsp90, calnexin, and others (Amaral, 2004; Skach, 2006; Wang et al., 2006). If CFTR fails to achieve its native fold, chaperones such as Hsp70 also act to recruit E3 (and/or E4) ubiquitin-ligases that ubiquitinate CFTR and target the mutant protein for degradation by the 26S proteasome. Thus, CFTR folding

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; NBD, nucleotide binding domain; PCC, protein conducting channel; RNC, ribosome nascent chain complex; RTC, ribosome translocon complex; TM, transmembrane segment.

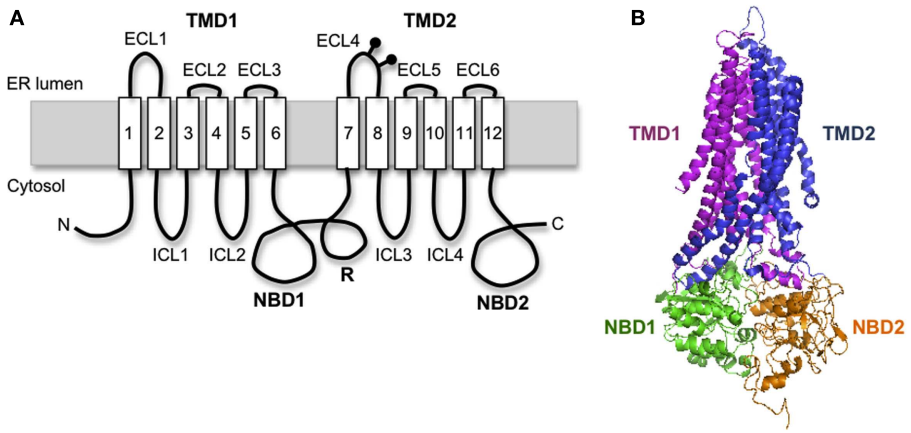

FIGURE 1 | Cystic fibrosis transmembrane conductance regulator structural organization. (A) Schematic diagram of CFTR showing transmembrane topology and domain organization. **(B)** A predicted human CFTR structure based on homology model from Sav1866.

is constantly monitored by cellular quality control machinery throughout its biogenesis.

This review will focus on the current state of knowledge as to how CFTR domains fold, how they interact, how mutations alter this process, and how misfolded conformations are distinguished from native structure by cellular chaperone machinery.

MULTISPANNING MEMBRANE PROTEIN BIOGENESIS AT THE ER

To understand specialized aspects of CFTR biogenesis, it is helpful to first consider general mechanisms. In eukaryotic cells, membrane proteins are targeted to the ER during synthesis by the cytosolic signal recognition particle (SRP; Walter and Blobel, 1981), which brings the ribosome nascent chain complex (RNC) to the Sec61 translocon (**Figure 2A**). As the RNC docks onto the translocon, the insertion of the signal sequence into Sec61α opens the protein conducting channel (PCC) and establishes a continuous aqueous pathway from the ribosome exit tunnel into the ER lumen (**Figure 2**; Crowley et al., 1993, 1994). Extracellular peptide loops generally pass through the PCC cotranslationally until synthesis of a hydrophobic TM segment (i.e., stop transfer sequence) terminates nascent chain translocation (Haigh and Johnson, 2002; Woolhead et al., 2004; Alder et al., 2005) and relaxes the ribosome translocon junction to allow the downstream peptide region access to the cytosol (Liao et al., 1997). TM segments also move laterally out of the translocon as they integrate into the lipid bilayer. In some cases, integration occurs via a passive thermodynamic partitioning (Martoglio et al., 1995; Heinrich et al., 2000), whereas in others, it appears to be mechanistically controlled by the ribosome translocon complex (RTC; Do et al., 1996; Pitonzo et al., 2009). Indeed, TMs may be released from the translocon individually, in pairs, or even groups depending on specific properties and folding requirements of the substrate (Meacock et al., 2002; McCormick et al., 2003; Sadlish et al., 2005). Crystal structures of the Sec61αβγ homolog from *M. jannaschii* (SecYEβ) have suggested that TMs exit the translocon via a lateral cleft between Sec61α TMs2-3 and TMs7-8 along one side of the PCC (Van den Berg et al., 2004). Functional mammalian translocons also contain additional translocon-associated proteins including the translocation-associated membrane protein (TRAM), translocon-associated membrane protein (TRAP) complex, signal peptidase complex, oligosaccharyltransferase (OST), and others that modulate translocation, integration, and early processing events (Schröder et al., 1999; Wang and Dobberstein, 1999; Shibatani et al., 2005). Thus, the Sec61αβγ PCC functions as part of a large integrated molecular machine.

In the simplest model, polytopic protein topology could be established by alternating TMs (encoding signal or stop transfer activity) that sequentially open the translocon pore into the ER lumen to initiate translocation and close the pore to terminate translocation and direct peptide segments into the cytosol. Such a mechanism would maintain ER integrity while essentially stitching TM segments into the bilayer via coordinated structural changes at the lumenal and the cytosolic faces of the RTC (Johnson, 2003; Sadlish and Skach, 2004; Pitonzo and Skach, 2006; Skach, 2009).

CFTR FOLDING

CFTR TM INSERTION AND TMD FORMATION

Homology models predict that CFTR exhibits a complex domain swap structure in which two six-spanning helical bundles containing TMs1-2, 9-12 and TMs7-8, 3-6 are twisted around a central ion-conducting pore (Locher et al., 2002; Dawson and Locher, 2006; Aller et al., 2009). Helical extensions of the TMs form intracellular loops (ICL1-4) that project nearly 40 Å into the cytosol and form docking sites for NBD1 and NBD2 (**Figure 1B**). It is currently believed that ATP binding and hydrolysis at the interface between the two NBDs transmits an allosteric conformational change along the ICLs to the TMDs that controls channel gating. This elegant structure immediately raises several important questions when considered from a biosynthetic viewpoint. First, how do CFTR TMs acquire their proper topology as they are oriented and integrated into the ER membrane? Second, how do TMs interact during TMD assembly? Third, where do domain swapping and assembly occur in relation to the translocon, i.e., where do TMs1-2

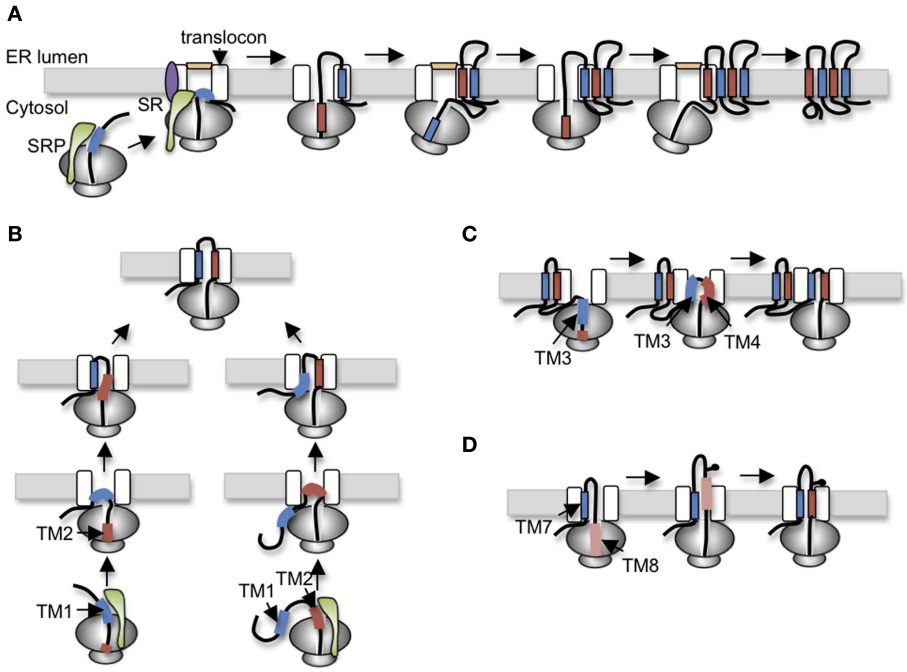

FIGURE 2 | Variations of polytopic protein topogenesis. (A) Simplest cotranslational topogenesis model in which ER targeting begins as signal recognition particle (SRP) recognizes an emerging signal sequence (TM), binds its receptor (SR) at the ER membrane, and transfers the RNC to the Sec61 translocon. TM topology is achieved through alternating signal and stop transfer activities that sequentially open and close the translocon pore. Careful orchestration of ribosome translocon junction ensures delivery of soluble domains into either cytosol or ER lumen and integration of TMs into the bilayer. **(B)** For CFTR, topology of TM1 and TM2 is established by two alternate pathways in which translocation is initiated by either TM1 (left) or TM2 (right). Most CFTR nascent chains utilized a posttranslational mechanism in which TM2 insertion drags TM1 into the translocon. **(C)** The short loop between TM3 and TM4 (five residues) suggests that TM3 and TM4 simultaneously insert into the translocon as a helical hairpin. A similar mechanism is also proposed for TM5-6, TM9-10, and TM11-12. **(D)** Stop transfer activity of TM8 is weakened by Asp924 which results in transient exposure of TM8 in the ER lumen before acquiring its final membrane spanning topology.

and TMs3-6 transiently reside for the several minutes that it takes to synthesize TMs7-8 and TMs9-12? An important consideration is that CFTR TMs contain an unusually large number of potentially ionizable residues (4 Arg, 2 Lys, 3 Glu, 1 Asp, and 1 His), which likely establish a network of polar interactions within the membrane. However, such residues would be predicted to delay or destabilize integration of individual TMs in the bilayer (Hessa et al., 2005). In addition, mutagenesis studies have revealed that TMD assembly influences folding of cytosolic NBDs and visa versa (Chen et al., 2004; Loo et al., 2008), such that domain folding and domain–domain assembly exhibit a high degree of cooperativity.

One of the first identifiable features of CFTR folding involves the orientation and integration of TMs into the ER membrane. Early work from our group established that ER targeting occurs as TM1 and TM2 emerge from the ribosome, bind SRP (Carlson et al., 2005), and engage the Sec61 translocon via a novel mechanism that involves two alternative folding pathways (Lu et al., 1998). Notably, TM1 lacks efficient signal anchor activity due to the presence of two ionizable residues, Glu92 and Lys95, within its membrane spanning region. As a result, TM1 initiates translocation for only ∼25% of nascent CFTR polypeptides. For the remaining 75% of chains, topology of the TM1-2 loop is established by type I signal anchor activity of TM2. In this case, the energy of TM2 insertion into the translocon, essentially "drags" the first extracellular loop (ECL1) into the ER lumen, thereby establishing the type II topology of TM1 (**Figure 2B**). While the final outcome of the two pathways is identical, the latter differs from the simple cotranslational model because TM1 acquires its topology after TM2. Such a mechanism suggests that both TMs are accommodated simultaneously either within or closely adjacent to the translocon channel.

An important implication of this topogenesis mechanism is highlighted by two CF-causing mutations, G85E and G91R, each of which introduces an additional ionizable residue into TM1. Both mutants completely block TM1 signal anchor activity but do not affect TMD1 topology because TM1 can still be inserted into the membrane by TM2 (Xiong et al., 1997). Despite achieving correct topology, however, G85E and G91R still disrupt CFTR folding and trafficking (Xiong et al., 1997; Patrick et al., 2011). Analysis of TM1-2 topogenesis gave rise to the early prediction that disease related mutations in different regions of CFTR might disrupt folding via a common mechanism, namely by preventing higher order tertiary domain–domain interactions (Xiong et al., 1997; Skach, 2000). In the case of TM1, we proposed that insertion of an additional polar residue disrupted the arrangement of helical bundles and subsequent interactions between helical extensions

and cytosolic NBDs (Xiong et al., 1997; Skach, 2000). This finding led to the early proposal that normal CFTR folding requires precise formation of domain–domain contacts, similar to a molecular jig-saw puzzle, which has recently been shown by several groups to be a major defect in the ΔF508 mutation as well (Serohijos et al., 2008; Mendoza et al., 2012; Rabeh et al., 2012).

TM3 also encodes an inefficient signal sequence that cooperates with TM4 to translocate the intervening extracellular loop, ECL2. Because ECL2 contains only five residues it is likely that TM3 and TM4 insert simultaneously into the translocon pore as a helical hairpin (**Figure 2C**). Similarly, TM5 and TM6, which function as signal anchor and stop transfer sequences, are separated by only a single charged lysine residue, indicating that their topology is also established together as ECL3 is translocated into the ER lumen. This feature of coincident translocation by TM helical hairpins is a common feature of native polytopic proteins (Sadlish et al., 2005) and suggests that two closely spaced TMs could be considered as a single functional topogenic determinant. However, few studies have investigated the mechanism by which such determinants interact with the translocon to establish topology.

From these results, we propose a general, although admittedly incomplete model in which TM1-2 topology is acquired through the combined actions of weak type II SA (TM1) and strong type I SA (TM2) activities. Subsequently, TM3-4 and TM5-6 insert into Sec61 as helical hairpins to translocate short ECL2 and ECL3 loops. An interesting and currently unresolved question is whether TMs or TM pairs partition freely into the bilayer from the small Sec61αβγ pore, if they remain associated with Sec61 and/or other translocon proteins during subsequent helical packing. This question is particularly relevant in light of the mature domain swap structure where TM1-2 ultimately bundles with TM9-12 and TM3-6 with TM7-8. Given the prevalence of ionizable residues in TMs1, 2, 3, and 6, final assembly likely requires precise alignment of TMs prior to complete integration into the lipid bilayer.

When TM6 terminates translocation NBD1 cotranslationally passes beneath the base of the ribosome into the cytosol. Several features of CFTR suggest that during this process, the ribosome transiently disengages from Sec61 to allow folding of the cytosolic domains. CFTR TMD1 helices are predicted to extend ∼40–50 Å from the membrane, and these extensions appear to provide a preliminary docking site for NBD1 prior to synthesis of TMD2 (Xiong et al., 1997; Kleizen et al., 2005; Du and Lukacs, 2009). The TMD extension plus bound NBD1 would therefore extend nearly 80 Å from the membrane surface, requiring that this region must move away from the ribosome to avoid a major steric clash. It is unknown whether a new translocon is recruited for TMD2 topogenesis, or whether preliminary assembly of the N-terminal half of CFTR occurs within or adjacent to the translocon. Precisely where these early folding events might take place and the factors involved remain important as yet unanswered questions.

NOVEL MECHANISMS OF CFTR TMD2 TOPOGENESIS AND FOLDING

After completion of R-domain synthesis, TMD2 topology is established in a cotranslational manner by alternating signal (TM7, 9, and 11) and stop transfer (TM8, 10, and 12) sequences. As TM7 emerges from the ribosome, it efficiently directs membrane targeting and ECL4 translocation. TM8, which is separated from TM7 by ∼31 residues, terminates translocation, and redirects ICL3 in the cytosol as expected. Interestingly, TM8 functions as an efficient stop transfer only when it is normally paired with TM7, but not in a heterologous context (Carveth et al., 2002; Enquist et al., 2009). This suggests that TM7 either influences TM8 stop transfer activity inside the translocon or alternatively, that TM7 affects recognition of TM8 within the ribosome exit tunnel. To date, this type of cooperativity appears unique to CFTR, although few proteins have been studied at this level of detail. The remaining TM pairs, TM9-10 and TM11-12, each encode signal anchor and stop transfer sequences with short extracellular loops, and it is likely that they insert into the translocon as helical hairpins much like TM3-4 and TM5-6 (Carveth et al., 2002).

TMD2 exhibits several additional unusual folding behaviors. It is well known that N-linked glycosylation sites must be at least 12–14 residues from the lipid bilayer to be accessible to OST (Popov et al., 1997; Nilsson and von Heijne, 2000). The CF mutation T908N, however, creates a glycosylation site that is recognized by OST even though it is only four-residues from the predicted N-terminus of TM8. Given that the precise boundaries of CFTR TMs are not yet known, one possible explanation for these findings is that residues within TM8 that actually span the membrane bilayer may differ from current predictions. Alternatively, if TM8 membrane boundaries are accurately predicted by homology models, then this finding suggests that TM8 transiently extends into the ER lumen during CFTR synthesis and is then repositioned within the membrane during subsequent folding and helical packing (Hammerle et al., 2000; Carveth et al., 2002; **Figure 2D**). Such behavior could be due to either altered interactions with translocon components that fail to recognize TM8 during synthesis, altered timing of TM8 helix formation, or both. Interestingly, removal of an aspartate residue from TM8 (D924V) prevents transient lumenal exposure and at the same time confers independent stop transfer activity. Although the original observation that TM8 might transiently sample the lumenal environment was unexpected, there is growing appreciation that other weakly hydrophobic TMs in polytopic proteins do indeed undergo repositioning within the membrane, either through interactions with neighboring TMs during tertiary folding, or due to differences in membrane thickness and/or composition that occur at various locations along the secretory pathway (Meindl-Beinker et al., 2006; Hessa et al., 2007; Skach, 2009; Nörholm et al., 2011).

Cystic fibrosis transmembrane conductance regulator also exhibits a distinct mechanism of membrane integration. The first clue came from the observation that after synthesis is completed, CFTR remains transiently bound to a large protein complex with properties similar to the RTC (Oberdorf et al., 2005). Release from this complex into the bilayer requires both cytosol and energy. *In vitro* photocrosslinking experiments further demonstrated that TM8 can maintain stable interactions with Sec61α after cleavage of peptidyl tRNA bond, and that release from the translocon also requires ATP (Pitonzo et al., 2009). Surprisingly, Asp924, which influences TM8 stop transfer activity, is also responsible for retaining TM8 within Sec61, suggesting that polar interactions can rigidly hold a TM within the translocon structure (Pitonzo et al., 2009). These results demonstrate that the translocon has the capacity to regulate the timing of TM integration via specific

protein–protein interactions and thereby potentially facilitate early steps of TMD assembly (Do et al., 1996; Liao et al., 1997; Skach, 2009).

In summary, CFTR TMD biogenesis utilizes multiple mechanisms that deviate from a cotranslational topogenesis model including: alternate co- and post-translational translocation pathways (TM1-2), coincident insertion of helical hairpins (TM3-4, TM5-6, TM9-10, and TM11-12), cooperativity for topogenic determinant function (TM7-8), and regulated integration into the ER membrane. Reasons underlying these distinct translocation mechanisms are only beginning to be understood, but evidence suggests that different folding pathways have functional implications. For example, replacement of ionizable residues in TM1 (E92A and K95A) converts TM1 to a strong signal anchor sequence, thus favoring cotranslational topogenesis, but disrupts CFTR function (Lu et al., 1998; Patrick et al., 2011). Similarly, the D924V mutation converts TM8 to a strong strop transfer sequence and facilitates cotranslational membrane integration, but decreases CFTR chloride conductance (our observations). These results are mirrored in the mammalian aquaporin family and suggest that by facilitating different topogenesis mechanisms, eukaryotic translocon machinery has allowed TM segments to accommodate key functional residues that would otherwise disrupt cotranslational membrane insertion (Skach, 2009). An obvious but profound implication is that folding and function are closely intertwined such that structural elements needed for higher order folding ultimately dictate which topogenesis mechanisms prevail.

CFTR CYTOPLASMIC DOMAIN FOLDING AND THE DEFECT OF ΔF508

It is now evident that correct folding of individual CFTR domains is required for proper domain assembly, and that proper domain assembly reciprocally influences domain folding (Qu and Thomas, 1996; Younger et al., 2006; Loo et al., 2008; Du and Lukacs, 2009; Thibodeau et al., 2010). Among these processes, NBD1 folding and mechanism(s) by which folding is disrupted by ΔF508 have received intense attention. NBD1 is composed of three subdomains: an N-terminal subdomain that contains the ATP binding site (Khushoo et al., 2011), an α-helical subdomain containing Phe508, and a central α/β core analogous to the F1-type ATPase containing a six-stranded, largely parallel β-sheet (**Figure 3**). NBD1 also contains the canonical LSGGQ signature motif (residue 548–552), a unique unstructured regulatory insertion (residues 404–436), a structurally diverse region (residues 526–547), and a C-terminal regulatory extension (RE; **Figure 3A**). Given its profound effect on CFTR folding, it was initially surprising that the ΔF508 mutation has little effect on NBD1 crystal structure (Lewis et al., 2004, 2005). However, recent work has revealed that ΔF508 significantly disrupts both kinetic and thermodynamic stability of NBD1 as well as increasing local backbone dynamics at residues 507–511 (Hoelen et al., 2010; Lewis et al., 2010; Wang et al., 2010; Rabeh et al., 2012). Moreover, the specific folding defect induced by ΔF508 appears to reside at least in part within the α-helical subdomain (Hoelen et al., 2010; Wang et al., 2010) as well as C-terminal β-strands, S9 and S10 (Hudson et al., 2012). ΔF508 also eliminates a hydrophobic contact between NBD1 and TMD2 that is required for trafficking and channel gating (Serohijos et al., 2008).

Mutations that increase NBD1 solubility and/or thermodynamic stability (I539T, G550E, R553Q, and others; Teem et al., 1993; DeCarvalho et al., 2002; Roxo-Rosa et al., 2006; Pissarra et al., 2008; Hoelen et al., 2010) and/or decrease backbone flexibility (Aleksandrov et al., 2012) can enhance both NBD1 folding yield in cells and trafficking efficiency of full length WT as well as ΔF508 CFTR (**Figure 3B**). Thus NBD1 folding *per se*, is a liming step in both WT and ΔF508 CFTR biogenesis. Mutations within ICL4 or NBD1 that restore NBD1–TMD2 interaction also improve ER export and chloride channel function (Serohijos et al., 2008; He et al., 2010; Loo et al., 2010; Thibodeau et al., 2010; Aleksandrov et al., 2012). However, correction of both the NBD1–TMD2 interface and NBD1 thermodynamic stability are required to restore ΔF508 processing to near wild-type levels (Mendoza et al., 2012; Rabeh et al., 2012). A major goal in CF, therefore, is to identify small molecules that act at both of these folding steps and thereby increase channel function in CF patients.

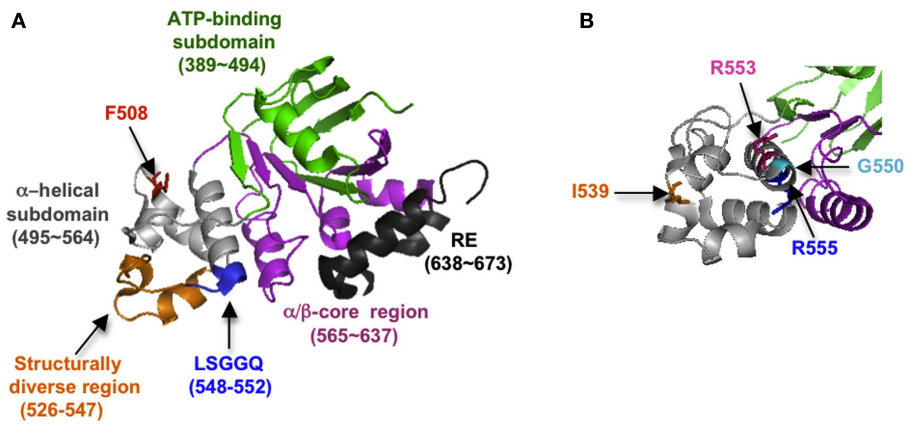

FIGURE 3 | (A) Crystal structure of NBD1 showing subdomain organization and location of key structural elements (2BBO). **(B)** Slightly different view of **(A)**, showing location of suppressor mutations (1XMI).

How then does NBD1 fold in cells, and which limiting steps might provide a target for small molecule correction? NBD1 folding begins cotranslationally after TM6 terminates translocation, and the elongating nascent chain moves into the cytosol through the relaxed ribosome translocon junction (Carveth et al., 2002). It is estimated to take roughly 1 min to synthesize NBD1 in eukaryotic cells, and significant folding (as well as mis-folding of ΔF508) occurs during this time (Kleizen et al., 2005; Hoelen et al., 2010). However, understanding NBD1 cotranslational folding has been technically challenging because of the complex biological folding environment. For example, cotranslational folding is influenced by the rate and vectorial nature of translation (Fedorov and Baldwin, 1997; Siller et al., 2010), the ribosome, and geometry of the ribosome exit tunnel (Woolhead et al., 2004; Lu and Deutsch, 2005; Ziv et al., 2005; Kaiser et al., 2011), molecular crowding (Ellis, 2001), and interaction with several cellular chaperone networks (Frydman, 2001; Ellis, 2007; Hartl and Hayer-Hartl, 2009).

One promising method to define folding transitions as the nascent chain emerges from the ribosome is to measure fluorescence energy transfer (FRET) between Donor and Acceptor probes that are cotranslationally incorporated at distant sites in primary sequence but which become proximal to one another as the protein folds. Because FRET efficiency is highly sensitive to changes in distance on a scale of ∼10–80 Å, changes in FRET that occur at increasing chain lengths provide a sensitive readout for nascent chain compaction and folding. Using this approach, Khushoo et al. (2011) showed that NBD1 folding begins cotranslationally and proceeds via discrete steps as individual subdomains emerge from the ribosome. The first step involves abrupt compaction of the N-terminal ATP binding subdomain (residues 389–500), which occurs on a time scale similar to or exceeding the predicted rate of translation. Because NBD1 has a very high contact order characterized by a large number of long-distance intrachain interactions, it is likely that the N-terminal subdomain provides a template or scaffold upon which the α-helical subdomain and α/β-core assemble. Finally, ΔF508 does not measurably influence N-terminal subdomain folding, indicating that the ΔF508 defect occurs during later folding of α-helical and/or α/β-core subdomains.

CFTR DOMAIN–DOMAIN ASSEMBLY

Based on the time required for CFTR to exit the ER, CFTR domain assembly takes ∼30–120 min. This suggests a hierarchical process in which domain folding begins cotranslationally and is followed by posttranslational formation of domain–domain contacts (Ostedgaard et al., 1997; Du et al., 2005; Cui et al., 2007; **Figure 4**). During this time CFTR undergoes at least two distinct folding events that require ATP. The first involves release of full length CFTR from a large biosynthetic complex that likely includes the RTC and cellular chaperones, and appears to coincide with CFTR integration (i.e., release) into the bilayer of the ER membrane

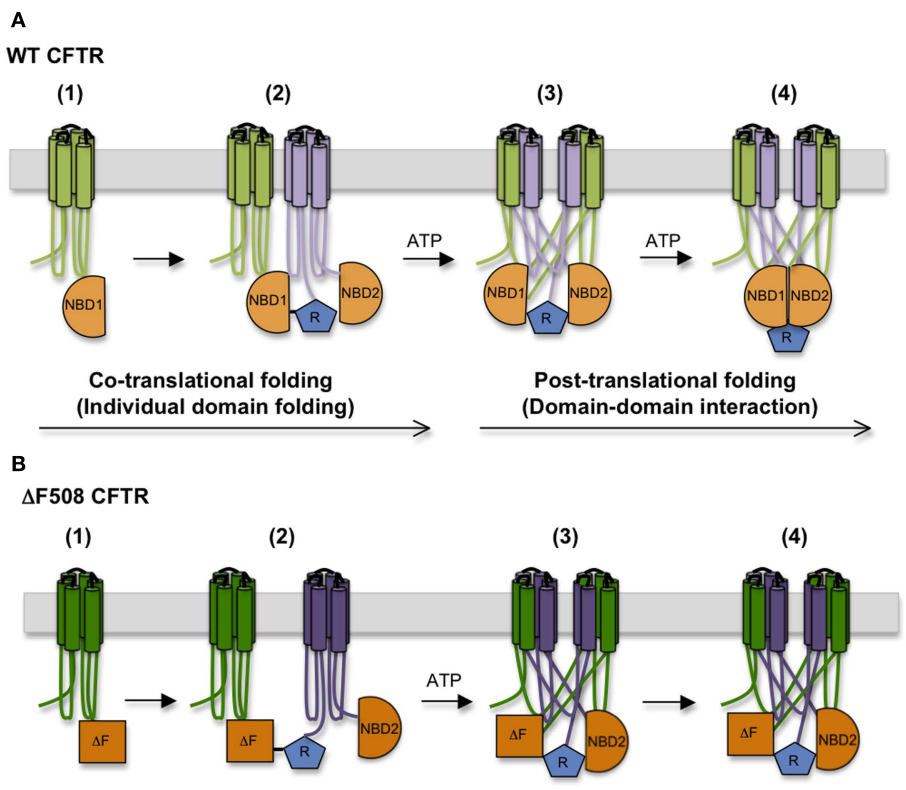

FIGURE 4 | Step-wise CFTR folding pathway. (A) CFTR folding begins cotranslationally as individual domains are synthesized, and proceeds as domains assemble into a mature tertiary structure. **(B)** The ΔF508 mutation destabilizes NBD1 structure, interferes with the TMD1, TMD2, and NBD2 folding, and disturbs interactions between NBD1 and ICL4, compromising domain–domain assembly.

(Meacham et al., 1999; Oberdorf et al., 2005). Both WT and ΔF508 CFTR undergo this step with equal efficiency (Oberdorf et al., 2005). As discussed above, delayed integration of TMDs may reflect the time required to establish the complex contacts within the domain swap structure. It is not known how ATP hydrolysis facilitates membrane integration, however, as no known translocon components hydrolyze ATP. The second maturation step involves conversion of CFTR from an immature, incompletely folded, ER-associated conformation (typically designated as Band B) to a properly folded, mature conformation that is competent to exit the ER and undergo Golgi processing into the Band C form (Lukacs et al., 1994). Interestingly, trapping CFTR in the ER with Brefeldin A results in accumulation of a stable, "mature" Band B form that is able to exit the ER upon Brefeldin A washout, indicating that the key folding step is distinct from Golgi processing. This folding transition also involves reorganization and/or release of cytosolic chaperones (Yang et al., 1993; Meacham et al., 1999) and results in a substantial change in CFTR structure as demonstrated by limited proteolysis (Zhang et al., 1998). Importantly, the ΔF508 mutation prevents this latter step.

While the precise details of CFTR maturation remain a mystery, WT and ΔF508 conformations differ in several important aspects. First, the complement of bound chaperones changes significantly; Hsp/c70 is released from WT CFTR prior to ER export, but remains bound to ΔF508 CFTR and may stimulate degradation (Yang et al., 1993; Matsumura et al., 2011). Second, the biological stability (as measured by half-life) of ΔF508 CFTR is more temperature sensitive than fully folded WT CFTR both in the ER and at the plasma membrane (Zhang et al., 1998). Structural differences between WT and mutant proteins can therefore be readily distinguished both by ER and peripheral quality control machinery (Okiyoneda et al., 2010). Third, channel activity of ΔF508 CFTR is more thermolabile than WT and rapidly declines at physiological temperatures (37˚C; Aleksandrov et al., 2010; Wang et al., 2011; Liu et al., 2012). Fourth, ΔF508 CFTR following maximal stimulation by PKA is less biologically stable than quiescent channels (Liu et al., 2012), indicating that features of the ΔF508 defect are mechanistically linked to conformational changes that take place during the gating cycle. Finally, different mechanisms of ΔF508 correction (e.g., low-temperature rescue, suppressor mutations, or small molecules) can be accomplished by a variety of structural changes that give rise to channels with different physical properties.

In addition to directly destabilizing NBD1 and weakening the interface between NBD1 and TMD2, limited proteolysis and cysteine crosslinking studies indicate that ΔF508 also causes conformational abnormalities in TMD1, TMD2, and NBD2 and misassembly of TMD1/TMD2 and NBD1/NBD2 interfaces (Du et al., 2005; Cui et al., 2007; Loo et al., 2008; Rosser et al., 2008; Du and Lukacs, 2009; He et al., 2010; Thibodeau et al., 2010). This high degree of cooperativity in CFTR domain folding is further supported by CF-related mutations in TMD1 and TMD2 that also reciprocally affect the conformation of other domains (Du and Lukacs, 2009).

THE ROLE OF CHAPERONES IN CFTR FOLDING

Because CFTR folding takes place in three different compartments, the ER lumen, the ER membrane, and the cytosol, CFTR interacts with several large cellular chaperone and co-chaperone networks (at least 31 components) at various stages of folding (Skach, 2006; Wang et al., 2006). Major chaperone families include cytosolic Hsp70, Hsp90, and their co-chaperones (Yang et al., 1993; Loo et al., 1998; Meacham et al., 1999; Younger et al., 2004; Grove et al., 2011), as well as ER lumenal lectins calnexin and possibly calreticulin (Pind et al., 1994; Harada et al., 2006).

Cytosolic chaperone interactions begin cotranslationally during synthesis as Hsp/c70 binds and presumably shields extended hydrophobic regions of the nascent chain to prevent aggregation (Yang et al., 1993; Meacham et al., 1999; Oberdorf et al., 2005; Kampinga and Craig, 2010). Hsp/c70 binds substrate in the ATP-bound state, and binding is stabilized by ATP hydrolysis, which is stimulated by DnaJ (Hsp40) cofactors. Substrate is released upon nucleotide exchange, which can be either spontaneous, or stimulated by nucleotide exchange factors (NEFs) such as Bag-1 and HspBP1. While details of Hsp/c70-CFTR interactions are far from complete, peptide binding studies have identified potential binding sites in NBD1, and have shown that Hsc70 decreases ΔF508 NBD1 aggregation *in vitro* possibly by reducing off-pathway folding events (Strickland et al., 1997; **Figure 5**). In cells, both Hsc70 and Hdj-2 interact with CFTR after the NBD1 synthesis but are released in the presence of the R-domain (Meacham et al., 1999). In addition, Hsp70 and Hdj-1 coexpression stabilizes WT CFTR *in vivo* (Farinha et al., 2002), pointing out the critical role of Hsp70 in CFTR NBD1 folding in the cytosol. Later stages of TMD2 folding and TMD1 and TMD2 assembly appear to require calnexin (Rosser et al., 2008) which likely binds TMD2 via N-linked glycans attached to ECL4. This interaction may stabilize TMD2 and/or assist in orienting TMs during domain swapping. Taken together, these findings suggest that CFTR utilizes a carefully orchestrated complement of chaperones at numerous sequential and interdependent folding steps (**Figure 5**).

Paradoxically, chaperones that facilitate CFTR folding also play a direct role in degradation. The best understood example is Hsp70, which resides squarely at the intersection of folding and quality control. Pro-folding activities of Hsp/c70 are mediated through its N-terminal ATPase domain, which controls affinity of the central peptide binding cleft. The C-terminus of Hsc70, however, contains a tetratricopeptide binding motif that interacts with at least one E3 ubiquitin ligase, CHIP, that functions in concert with the E2 ubiquitin conjugating enzyme, UbcH5 (Meacham et al., 2001; Younger et al., 2004). While other E3 ligases are also implicated in CFTR ubiquitination (e.g., Nedd4-2, RMA1, and gp78; Younger et al., 2006; Morito et al., 2008; Caohuy et al., 2009; Grove et al., 2011), Hsc70-CHIP seems to play a major role in recognizing cytosolic structural perturbations caused by ΔF508 (Meacham et al., 2001; Younger et al., 2004).

A non-trivial question therefore is how Hsp/c70 carries out two diametrically opposed actions, on the one hand protecting proteins from aggregation and facilitating folding, while on the other identifying terminally misfolded proteins and targeting them for degradation. An important clue was recently provided by Matsumura et al. (2011) who used a C-terminal fragment of Bag-1 to stimulate Hsc70 nucleotide exchange (Höhfeld and Jentsch, 1997; Takayama et al., 1997). Addition of cBag during CFTR translation slightly increased degradation, consistent with predictions that

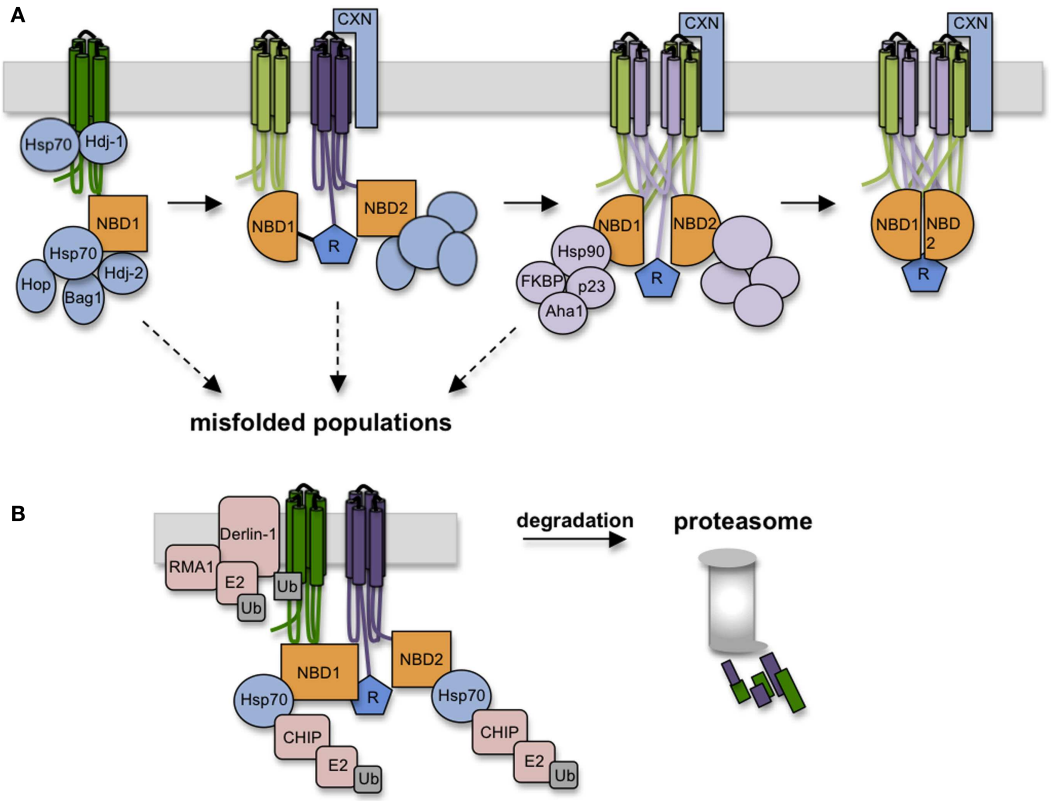

FIGURE 5 | Chaperones assist CFTR folding and target misfolded CFTR for degradation. (A) As CFTR is synthesized, numerous chaperones and co-chaperones (some depicted here) decorate the nascent polypeptide on both lumenal and cytosolic sides of the ER membrane. Hsp70 and co-chaperones interact with NBD1, followed by calnexin association with TMD2. Hsp70-Hop interactions recruits Hsp90 complexes which likely aid domain assembly in conjunction with calnexin. **(B)** Failure to achieve productive folding at any step in the folding pathway is detected by persistent binding of Hsp70, which serves to recruit E3 ligases (i.e., RMA1 and CHIP) that ubiquitinate CFTR and target it to the 26S proteasome.

Hsp70-client interactions stimulate *de novo* folding (Meacham et al., 1999; Younger et al., 2004; Grove et al., 2011), whereas similar levels of cBag completely blocked CFTR degradation, consistent with studies in yeast (Zhang et al., 2001) and mammalian cells (Farinha et al., 2002). Kinetic analysis revealed that shortening the time required for CFTR-Hsc70 dissociation from roughly 3 min to less than 1 min resulted in a marked decrease in CFTR ubiquitination and degradation. Thus, the timing of the Hsp70 binding cycle, rather than binding *per se*, appears to be a critical decision point in the degradation process.

Hsp/c70 also recruits Hsp90 complexes through the intermediate linker protein p60 (Hop; Frydman and Höhfeld, 1997). In contrast to Hsp/c70, Hsp90 appears to primarily enhance CFTR folding (Loo et al., 1998). During its binding cycle, conformational shifts in Hsp90's client binding interface likely induce structural changes in substrate that mediate conversion from immature to mature conformations. Hsp90-client binding is also regulated by a variety of co-chaperones that include p23, cyclophilins (i.e., FKBPs), and Aha1, each of which associates with CFTR in cells (Wang et al., 2006; Hutt et al., 2012). While it is not yet known precisely how Hsp90 affects CFTR folding, overexpression of the co-chaperone Aha1, which stimulates Hsp90 ATPase activity and client release, decreases ΔF508 CFTR stability, and Aha1 knockdown enhances ΔF508 processing. Thus, stabilization of CFTR Hsp90 binding increases the dwell time of CFTR in the Hsp90 complex, which may overcome a kinetic bock in CFTR folding (Qu et al., 1997; Skach, 2006; Koulov et al., 2010). A recent study has also shown that an additional Hsp90 co-chaperone, a peptidylprolyl isomerase, FKBP8, interacts with and stabilizes both WT and ΔF508 CFTR in the ER via a mechanism that requires prolyl-isomerase activity (Hutt et al., 2012). Thus the Hsp90 axis is a potentially attractive target for CFTR correction.

SUMMARY

In summary, research in the past decade has revealed much about how CFTR folds and misfolds in cells. Membrane insertion and tertiary folding of cytosolic domains begin cotranslationally during CFTR synthesis, whereas posttranslational folding involves assembly of TM helical bundles that provide critical domain–domain contacts needed to form a physiologically stable structure. While the details of this process require further refinement at the molecular level, the model that emerges from these studies provides a useful framework to understand the key folding defect(s) caused by ΔF508 in the majority of CF patients. Within NBD1 itself, removal of Phe508 decreases

folding efficiency and renders the domain susceptible to unfolding, denaturation, and aggregation at physiologic temperatures, possibly as a direct result of destabilizing the α-helical subdomain. Absence of Phe508 also disrupts the interaction between NBD1 and ICL4 (within TMD2), which distorts TMD structure and interferes with channel gating. Defects in NBD1 and the NBD1–ICL4 interface are both recognized by quality control machinery, and correction of both is necessary and sufficient to restore trafficking and function to near WT levels. Importantly, partial correction of ΔF508 CFTR folding can be achieved by a variety of means: cis-acting suppressor mutations, manipulation of the proteostatic network, or small molecule correctors. Moreover, combinations of these maneuvers are now able to achieve near WT levels of surface expression and function. Thus, it is increasingly attractive to target the next generation of CFTR small molecule correctors to specific defects that will optimize synergy in correction mechanisms. While the most precise targets reside within the CFTR molecule itself, i.e., NBD1 and the NBD1–TMD2 interface, it is also possible that other clinically beneficial targets will be developed in the years to come, which will undoubtedly be driven by increasing resolution of the folding problem.

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Genetic influences on cystic fibrosis lung disease severity

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Understanding the causes of variation in clinical manifestations of disease should allow for design of new or improved therapeutic strategies to treat the disease. If variation is caused by genetic differences between individuals, identifying the genes involved should present therapeutic targets, either in the proteins encoded by those genes or the pathways in which they function. The technology to identify and genotype the millions of variants present in the human genome has evolved rapidly over the past two decades. Originally only a small number of polymorphisms in a small number of subjects could be studied realistically, but speed and scope have increased nearly as dramatically as cost has decreased, making it feasible to determine genotypes of hundreds of thousands of polymorphisms in thousands of subjects. The use of such genetic technology has been applied to cystic fibrosis (CF) to identify genetic variation that alters the outcome of this single gene disorder. Candidate gene strategies to identify these variants, referred to as "modifier genes," has yielded several genes that act in pathways known to be important in CF and for these the clinical implications are relatively clear. More recently, whole-genome surveys that probe hundreds of thousands of variants have been carried out and have identified genes and chromosomal regions for which a role in CF is not at all clear. Identification of these genes is exciting, as it provides the possibility for new areas of therapeutic development.

Keywords: polymorphism, genotype, phenotype

CYSTIC FIBROSIS BACKGROUND

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in Caucasians, affecting an estimated 1 in 3,300 live-born infants (Davis et al., 1996). Affected individuals have variants in both copies of the 230-kb CF transmembrane conductance regulator gene (CFTR), that result in significant reduction or absence of CFTR function. The *CFTR* gene is located on the long arm of chromosome 7 at position 7q31and encodes a 1,480 amino acid protein (Riordan et al., 1989; Rommens et al., 1989) with cAMP-dependent anion channel activity (Bear et al., 1992) found in the apical membranes of epithelial cells in the lungs, olfactory sinuses, pancreas, intestines, vas deferens, and sweat ducts, as well as non-epithelial cells such as immune cells (myeloid and lymphocytes) and various muscle cell types (Yoshimura et al., 1991; Krauss et al., 1992; McDonald et al., 1992; Dong et al., 1995; Moss et al., 2000; Robert et al., 2005; Di et al., 2006; Vandebrouck et al., 2006; Divangahi et al., 2009; Lamhonwah et al., 2010). Low or absent CFTR function in the airway epithelium not only results in decreased chloride permeability, but also in increased sodium absorption across the epithelium, impairing hydration of the airway mucosal surface and resulting in thick, sticky mucus and an environment for bacteria to thrive. Thus, typical clinical features of CF include chronic infection and inflammation of the airways. Accordingly, a hallmark characteristic of the CF airways is progressive bronchiectasis; this destruction and dilation of the airways is the primary cause of morbidity and mortality of CF patients. In addition to the airway manifestations, most CF patients will experience exocrine pancreatic insufficiency, males are most often sterile, and other co-morbidities such as liver disease and diabetes are common as well. Previously considered almost exclusively a pediatric disease, CF babies now have a predicted median survival of nearly 40 years (Cystic Fibrosis Foundation Patient Registry, 2009).

HETEROGENEITY OF *CFTR*

To date, over 1,800 CF-associated mutations have been described[1] and the effects of these mutations have been grouped into six general classes based on the consequence to CFTR message and/or protein (Zielenski, 2000). These range from complete absence of full-length, functional CFTR protein (class I), proteins that do not traffic to the membrane well due to misfolding (class II), proteins that reach the membrane but do not respond to activation stimuli such as phosphorylation (class III), proteins that reach the membrane and activate, but do not conduct anions sufficiently to prevent disease (class IV), mutations that reduce the amount of functional CFTR, such as by gene expression regulation or protein trafficking (class V), and proteins that are unstable and experience increased turnover in the plasma membrane (class VI). It should be noted that these classes are not mutually exclusive, as a single change may have multiple effects on the protein.

Given the diversity of mutations, it is perhaps not surprising that there is a wide range of phenotypic variability in CF simply due to variation in *CFTR*. Many reports of correlations between *CFTR* genotype and clinical phenotype exist (Kerem et al., 1990a; Stuhrmann et al., 1991; The Cystic Fibrosis Genotype-Phenotype Consortium, 1993; Tsui and Durie, 1997; Zielenski,

[1] http://www.genet.sickkids.on.ca

2000), with the most extensive catalog to date carried out as an international effort[2] and currently includes data on over 35,000 patients. Because most CF mutations are rare, surveying such a large number of individuals makes it possible to most reliably assess the phenotypic effects associated with a genotype, rather than extrapolate from individual cases.

In addition to *CFTR* genotype, there is evidence that gender contributes to phenotypic variability (Davis, 1999). Females are reported to have a reduced median survival age (by approximately 3 years), an earlier average age of *Pseudomonas aeruginosa* infection in the lungs, greater rates of pulmonary decline, and elevated resting energy expenditure when compared to males (Demko et al., 1995; Corey et al., 1997; Allen et al., 2003). Although some current studies replicate these findings (Barr et al., 2011; Reid et al., 2011), others show no evidence of a gender gap and propose that phenotypic variability could be attributed to non-uniformity of care or the need to account for other factors such as body habitus, presence of diabetes, or the finding that females are more likely to be diagnosed later in life than males (Widerman et al., 2000; Milla et al., 2005; Rodman et al., 2005; Verma et al., 2005; Stern et al., 2008; Fogarty et al., 2012).

GENOMIC HETEROGENEITY AND CLINICAL VARIATION

Even among patients with the same *CFTR* genotype, there is a wide range of phenotypic variability (Kerem et al., 1990a; Tsui and Durie, 1997). Perhaps most notably, there is remarkable variation of pulmonary phenotype, with some patients maintaining normal lung function well into adolescence and adulthood while others do quite poorly even at a very young age (Kerem et al., 1990a). Understanding the causes of this variation is important, as it provides insight into developing new therapies, or improving existing ones.

Clearly environmental factors contribute to clinical variation; exposure to tobacco smoke, bacterial infections, and socioeconomic status have all been implicated as having detrimental effects on pulmonary phenotype of CF patients (Kerem et al., 1990b; Rubin, 1990; Corey and Farewell, 1996; Schechter et al., 2001; O'Connor et al., 2003) while improvement of nutritional status, through aggressive treatment, has been associated with improvements in pulmonary phenotype (Steinkamp and von der Hardt, 1994). Each of the environmental sources of clinical variation provide potential intervention points, but it is also clear that there are heritable sources (Mekus et al., 2000; Vanscoy et al., 2007) of variation as well and that may provide insight into even more therapeutic targets.

EVIDENCE OF GENETIC MODIFIERS OF DISEASE

Human twin and sibling studies have been useful in verifying the role of modifier genes, and quantifying their contribution to phenotypic variation. Mekus et al. (2000) found in a survey of 277 sibling pairs, with 29 monozygous and 12 dizygous pairs, that a combined index of lung function and body mass was more concordant among monozygous twins (sharing 100% of genetic material) than dizygous twins or other sibling pairs (sharing 50% of genetic material), pointing to a genetic etiology of variation. Similarly, Vanscoy et al. (2007) examined the pulmonary phenotype of 57 twin pairs and 231 sibling pairs with CF. Lung function measurements were significantly more concordant between monozygous twins than dizygous twins, also indicating the presence of genetic modifiers. The similarity in lung function between sibling pairs was compared to the similarity in lung function in unrelated patients, and again was found to be more similar. Heritability estimates were calculated from these data, and it was determined that non-*CFTR* genetic variation could account for approximately 50–80% of the pulmonary phenotypic variability in CF patients with the same *CFTR* genotype (homozygous F508del) (Vanscoy et al., 2007).

GENETIC APPROACHES

With a genetic component established, the next task at hand was to identify the genes responsible. There are two fundamental strategies by which to accomplish this. One requires family information and is often referred to as linkage analysis. Through this approach, one determines whether a polymorphism's genotype is concordant in siblings with similar clinical profiles, discordant when clinical features are discordant or show no pattern. The other approach is association, determining if particular alleles of a polymorphism are distributed randomly among patients or have skewed distributions that track with clinical characteristics. These two approaches are outlined in **Figure 1** and the findings that these strategies have produced are listed in **Table 1** with several examples described in more detail below.

The vast majority of studies have been of the association design, predominantly due to the small number of families with multiple, affected children. These studies have evolved over time; cost and time restricted most early studies to screen for potential disease-modifying genes by candidate gene approaches with later studies utilizing array-based methods and soon whole-genome sequencing will be the state of the art. These three approaches are compared in **Figure 2**.

PHENOTYPIC CONSIDERATIONS

As lung disease is the major source of CF-related mortality, most studies have focused on some measure of lung function as a phenotype to examine for association. As most CF care centers carry out standard pulmonary function tests, spirometry has most commonly been used. Other tests may, in fact, be more specific for particular modifying functions, such as lung clearance index, but these are not as widely used and thus less practical for multi-center studies.

CANDIDATE GENES

Candidate genes are those suspected to have a role in some aspect of CF pathophysiology and variants in those genes are then tested for association with disease manifestations. Those traits may be represented by a continuum of values (lung disease severity, for example) or discrete traits, such as the occurrence of intestinal obstruction. Candidate gene selections for study involved many areas because of the complex pathophysiology of CF, including bacterial infections, inflammation, and lung remodeling/deterioration. This approach yielded multiple reports of putative modifiers of the CF pulmonary phenotype. For example, mannose-binding lectin (*MBL*), a gene involved in innate

[2]http://www.cftr2.org

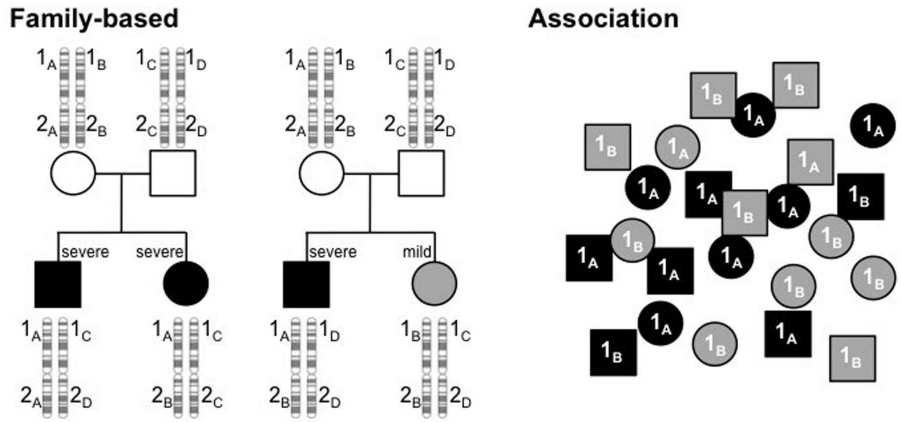

FIGURE 1 | Linkage analysis tracks alleles of polymorphisms through families to determine if an allele is linked to a phenotype. In this example, alleles of gene 1, 1_A, 1_B, 1_C, and 1_D, track with severity (black, severe; gray, mild), showing concordant genotypes between siblings with similar phenotypes (left pedigree) and discordant genotypes when phenotypes are dissimilar (right pedigree). In contrast, genotype and phenotype show no relationship at polymorphism 2. Association studies examine a population of unrelated individuals to determine if particular alleles of a polymorphism are found in different proportions, depending on the disease profile. In the example here, alleles 1_A and 1_B have equal frequencies in the population, but 1_A is much higher in the severely affected subjects (black) and 1_B higher in the mildly affected subset (gray).

immunity, was one of the first potential modifier genes described. Low-expressing *MBL* alleles were found to associate with a more severe pulmonary disease course than those with higher expression (Garred et al., 1999). *HLA* haplotypes were also investigated as modifiers due to the role of the genes in this complex in innate defense and inflammation. Carriers of the *HLA* II DR7 haplotype were found to have a higher incidence of *P. aeruginosa* colonization (Aron et al., 1999).

Polymorphisms within cytokines and other inflammatory mediators were investigated as potential modifiers of CF pulmonary disease due to their role in immune response as well. Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that is stimulated by NF-κB as a first line of defense against infection. The minor allele of a *TNF*α promoter polymorphism associated with worse pulmonary function in a small set of CF patients (Hull and Thomson, 1998). Interestingly, the *TNF*α minor allele that associated with a worse CF prognosis was also associated with an increase in mRNA expression level when measured using a reporter construct (Wilson et al., 1992). Interleukin-10 (IL-10), an anti-inflammatory cytokine was also investigated. Like *TNF*α, an *IL-10* promoter polymorphism was also associated with differences in IL-10 expression (Turner et al., 1997). In this case, the lower expressing *IL-10* allele was associated with worse CF disease. These studies supported a model in which higher levels of the pro-inflammatory cytokine TNFα, and lower levels of the anti-inflammatory cytokine IL-10 contribute to more severe CF lung disease.

CHALLENGES OF EARLY CANDIDATE GENE MODIFIER STUDIES

Early studies that attempted to identify potential modifiers were challenged by small numbers of study subjects. Typically, pulmonary function data using standard spirometry are not available on children younger than age 6, and multiple measures over time are needed to assess a subject's trajectory, as an indicator of current and future disease severity. Nonetheless, numerous studies compared pulmonary function of subjects over a range of ages, statistically adjusting for age. Younger patients were included in order to maximize participation, but epidemiologic studies indicated that much of the pulmonary phenotypic variability was not present until after puberty (Zemel et al., 2000).

An additional constraint is that not all mutations in *CFTR* have the same consequences on protein function and thus it is likely to confound interpretation if *CFTR* genotype is not accounted for. Consequently, after limiting to patients with sufficient lung function measurements and comparable *CFTR* genotypes, the number of available subjects is low, making it unfeasible for any single center to carry out an association study that would have the statistical power to detect anything but a very major effect of a modifier gene.

CONSORTIUM APPROACHES

The ability to effectively carry out genetic studies is limited by numbers of subjects. As a means to increase numbers, the European CF Twin and Sibling Study mentioned earlier was conceived and compared morphometric and pulmonary function indices of sib pairs. Using lung function measurements from patients in North America and Europe, this study was the first to compare lung function using a CF population for reference (Mekus et al., 2000).

Subsequently, the CF Gene Modifier Study (GMS) was conceived in 1999 to carry out a genetic study on a large group of patients for which longitudinal lung function data were available and genotype was restricted. In its inception, the study design was to use a candidate gene approach to search for potential genetic modifiers of CF pulmonary disease. The unique study design reduced genetic heterogeneity by using only patients who were homozygous for F508del (commonly referred to as ΔF508), and maximized the number of patients available by including patients from CF centers nationwide, comparing the most mild and most

Table 1 | Summary of published cystic fibrosis pulmonary modifiers.

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
8.1AH	*LTA* *TNF* *HSP70-2* *RAGE*	+252 A > G −308 G > A 1267 A > G −429 T > C	909253 1800629 106158 1800625	FEV_1 % pred Chronic *P. aeruginosa* colonization	<0.04 0.99	404 (Corvol et al., 2012)		
8.1MHC	*AGER* *HSP70-2* *TNFA*	−429 T > C 1267 A > G G-308A	106158	Age at onset of colonization Frequency of colonization	0.036 0.012	72 (Laki et al., 2006)		
11p13	*APIP* *EHF*		12793173	FEV_1 % pred (adjusted)	3.34×10^{-8}	1,978 (Wright et al., 2011)	557 (Wright et al., 2011)	
19q13	*hCFM1*	APOC2, D19S219, D19S112 haplotype		FEV_1 % pred	0.779	197 sib pairs (Zielenski et al., 1999)		
A1AT	*SERPINA1*	1237 G > A	11568814	FEV_1 % pred CXR score Age at onset of *P. aeruginosa*	0.368 0.813 0.146	157 (Mahadeva et al., 1998b)	716 (Frangolias et al., 2003)	124 (Henry et al., 2001) 320(Courtney et al., 2006) 808 (Drumm et al., 2005)
		S allele Z allele	17580 28929474	FEV_1 % pred CXR score Age at onset of *P. aeruginosa*	0.043 0.127 0.899	157 (Mahadeva et al., 1998b)	215 (Doring et al., 1994) 79 (Mahadeva et al., 1998a)	124 (Henry et al., 2001) 269 (Meyer et al., 2002) 808 (Drumm et al., 2005)
ABCC1	*MRP-1*	4741 C > G	504348	Age at onset of *P. aeruginosa* Age at which $FEV_1 < 60\%$ FEV_1 % pred	0.0644 <0.05 0.52	203 (Mafficini et al., 2011)		
ABO			8176719	Pulmonary disease severity	No association	778 (Taylor-Cousar et al., 2009)		
		T99T	8176720	Age at onset of *P. aeruginosa*	No association			
		21404 C > A	1053878					
		R176G	7853989					
		21583 T > A	8176740					
		H219H	8176741					
		P227P	8176742					
			816750					
		66119 G > A	8176472					

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
ACE		Insertion or deletion		Age of first *P. aeruginosa* infection	0.9	261 (Arkwright et al., 2003)		808 (Drumm et al., 2005)
				Age at which $FEV_1 < 50\%$	0.03 (0.04)[§]			
				Age of death	No association			
ADRB2		Arg16Gly	1042713	FEV_1 % pred	<0.05	126 (Buscher et al., 2002)		808 (Drumm et al., 2005)
				FVC	<0.05			
				Flows at lower lung volumes	<0.01			
				5 year decline in pulmonary function	<0.01			
		Gln27Glu	1042714	Bronchodilator responses to albuterol	NS			
		Thr164Ile	1800888					
				Pulmonary function	Reduced			
AGER		−429T > C	1800625	FEV1	0.02	967 (Beucher et al., 2012)		
				Kulich CF-specific percentile z-score	0.03			
				KNoRMA	0.03			
AGTR2			1403543	FEV_1 % pred (adjusted)	1.61×10^{-5}	1,978 (Wright et al., 2011)		557 (Wright et al., 2011)
AHRR			12188164	FEV_1 % pred (adjusted)	5.92×10^{-4}	1,978 (Wright et al., 2011)		557 (Wright et al., 2011)
C3		31778 G > A	393770	FEV_1 % pred	0.75 (0.05)[⌘]	755 (Park et al., 2011)		
		4023 T > G	11569393		0.66 (0.03)[⌘]			
		39718 G > A	7257062		0.78 (0.52)[⌘]			
CD14		−159 C > T		Pulmonary disease severity	No association	105 (Faria et al., 2009)		
CDH8			11645366	FEV_1 % pred (adjusted)	1.23×10^{-5}	1,978 (Wright et al., 2011)		557 (Wright et al., 2011)
CEACAM3	*19q13*		6508999–10414823	Disease severity	0.0469	37 nuclear families (Stanke et al., 2010)		
CEACAM6	*19q13*		1549960–11548735	Disease severity	0.0106	37 nuclear families (Stanke et al., 2010)		

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
CFB		7680 A > G 10858 A > G	537160 2072633	FEV_1 % pred	0.50 (0.83)[⌘] 0.68 (0.74)[⌘]	755 (Park et al., 2011)		
CLCN2	*CLC-2*	−693 A > G 358 G > C 427 A > G 1089 T > C 1909 G > C		FEV_1 % pred	0.72 0.32 0.32 0.21 0.22	74 (Blaisdell et al., 2004)		
DCTN4		Any missense variant	11954652	Age at onset of chronic *P. aeruginosa* infection	0.05 0.002	91 (Emond et al., 2012)		
			35772018	Age of first *P. aeruginosa* infection	0.01		645 (Emond et al., 2012)[⊙]	
				Age at onset of chronic *P. aeruginosa* infection	0.004		530°	
				Age at onset of mucoid *P. aeruginosa* infection	0.03			
				Time from first detection of *P. aeruginosa* infection to mucoid *P. aeruginosa*	0.01			
DEFB1		Frequent polymorphisms		Age of first *P. aeruginosa* infection	No association	210 (Vankeerberghen et al., 2005)	62 (Segat et al., 2010)	224 (Tesse et al., 2008) 92 (Crovella et al., 2011)[+]
				FEV_1 %	No association			
DEFB4		Genomic copy number (2–12) of repeat unit		Pulmonary disease (mean and current FEV_1, mean and current FVC)	No association	355 (Hollox et al., 2005)		
EDNRA		6672 G > C	5335	Pulmonary function (FEV_1)	0.002	1,577 (Darrah et al., 2010)		
EEA1			4760506	FEV_1 % pred (adjusted)	6.77×10^{-6}	1,978 (Wright et al., 2011)		557 (Wright et al., 2011)
FCGR2	*FcγRII*	R131H		Chronic *P. aeruginosa* colonization	0.042	167 (De Rose et al., 2005)		
FUT2		G428A	601338	Impairment of lung function (FEV_1)	0.569	806 (Taylor-Cousar et al., 2009)		

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
FUT3		T59G T202C C314T T1067A	28362459 812936 778986 3894326	Impairment of lung function (FEV_1)	0.544 0.491 0.615 0.792	707 (Taylor-Cousar et al., 2009)		
GCLC		$(GAG)_n$		FEV_1 % pred	0.097 0.001 (mild) 0.533 (severe)	440 (McKone et al., 2006)		
GSTM1		GSTM1*0/ GSTM1*0		FEV_1 % pred Chrispin–Norman score Shwachman score Positive for *P. aeruginosa* No. of ΔF508 homozygotes	0.16 0.02 0.04 0.12 0.43	53 (Hull and Thomson, 1998)	194 (Baranov et al., 1996) 60 (Korytina et al., 2004)	146 (Flamant et al., 2004) 808 (Drumm et al., 2005)
GSTM3		GSTM3*A GSTM3*B	1799735	FEV_1 FVC	0.01 0.002	146 (Flamant et al., 2004)		
GSTP1		1375 A > G I105V	947894	Spirometry	NS	146 (Flamant et al., 2004)	808 (Drumm et al., 2005)	60 (Korytina et al., 2004)
GSTT1		GSTT1*0/ GSTT1*0		Spirometry	NS	146 (Flamant et al., 2004)		
HFE		C282Y and/or H63D	1800562 and/or 1799945	Positive for *P. aeruginosa* FEV_1 % pred FVC% pred Annual change in FEV_1 % pred Annual change in FVC% pred	0.81 0.03 0.02 0.003 0.001	82 (Pratap et al., 2010)		
HLA		DRA	9268905	FEV_1 % pred (adjusted)	1.42×10^{-5}	1,978 (Wright et al., 2011)	557 (Wright et al., 2011)	
		DR4		Chronic *P. aeruginosa* colonization	≤0.03	98 (Aron et al., 1999)		72 (Laki et al., 2006)
		DR7/DQA*0201		Chronic *P. aeruginosa* colonization	<0.03			
HMOX1		11354 A > G 4613 A > T	2071749 2071746	FEV_1 % pred	0.01 (0.29)[⌘] 0.40 (0.03)[⌘]	755 (Park et al., 2011)		
IFNG	*IFNγ*	+874 A > T		Age of first *P. aeruginosa* infection Age at which $FEV_1 < 50\%$ Age of death	No association 0.09 No association	261 (Arkwright et al., 2003)		

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
IFRD1		57460 C > T	7817	Cross-sectional measures of lung function	0.004 (0.0168)[£]	320 (Gu et al., 2009)		
				Longitudinal measures of lung function	0.016 (0.0187)[£]			
				FEV_1 % pred (adjusted)	No association	1,978 (Wright et al., 2011)		
		47556 G > T	3807213	Longitudinal measures of lung function	0.080			
		38923 C > T	6968084	Cross-sectional measures of lung function	0.082			
IL8			2227306	Pulmonary disease severity	0.19	737	385 (Hillian et al., 2008)	
			2227307		0.04	727		
			2227543		0.06	732	329 (Corvol et al., 2008)[♦]	
		−251 A > T	4073		0.07	733 (Hillian et al., 2008)		
IL-10		−592 CC/-	1800872	Pulmonary function decline	No association	261 (Arkwright et al., 2003)		
		−592 CC/TA		Age of first *P. aeruginosa* or *B. cepacia* infection	No association			
				Age of death	No association			
		−1082 G > A	1800896	Colonization with *A. fumigatus*	0.06 (0.03)[§]	378 (Brouard et al., 2005)		808 (Drumm et al., 2005)
					0.02 (0.01)[§]			
				Development of ABPA	No association			
				Colonization with *P. aeruginosa*				
KRT8/ KRT18			1907671	Disease severity	Associates	49 (24 sib pairs) (Stanke et al., 2011)		
			4300473		Associates			
			8608		Associates			
		7952 T > C	2035875		0.00131			
			1907671-4300473-2035878-2035875 haplotype	Disease severity	0.0051			
			2638526	Disease severity	NS			
			2070876		NS			
KRT19		c.90T > C	11550883	Effective specific airway resistance	0.0093	95 (Gisler et al., 2012)		
		c.179G > C	4602		0.0052			
			11550883-4602 haplotype		0.0097			

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
MASP-2		Exon 3 A > G, D120G	72550870	Pulmonary function Need for transplantation Colonization with *P. aeruginosa* Lung function in patients colonized with *S. aureus*	No association No association 0.04 0.04	112 (Carlsson et al., 2005)	109 (Olesen et al., 2006)	
MBL2		X1 – B (A > G) X1 – C (A > G) X1 – D (C > T) (*A/A, A/O, O/O*) −221 G > C (*X/Y*)	1800450 1800451 5030737 7096206	FEV_1 % FVC% Age of onset of *P. aeruginosa*	0.003 0.03 0.07	149 (Garred et al., 1999)	164 (Gabolde et al., 1999) 179 (Yarden et al., 2004) 298 (Davies et al., 2004)[Θ] 47 (Trevisiol et al., 2005)[❖] 135 (Choi et al., 2006) 254 (Buranawuti et al., 2007)	112 (Carlsson et al., 2005) 260 (Davies et al., 2004)[Θ] 47 (Trevisiol et al., 2005)[❖] 808 (Drumm et al., 2005) 105 (Faria et al., 2009) 788 (McDougal et al., 2010) 123 (Olesen et al., 2006)
		−550 G > C (*H/L*)		Lung function Colonization	No association No association	112 (Carlsson et al., 2005)	105 (Faria et al., 2009)	
MIF		−794 presence of absence of 5-CATT		Colonization with *P. aeruginosa* Colonization with *S. aureus* Colonization with *Candida* $FEV_1 \geq 80\%$	0.004 0.50 0.36 0.14	167 (Plant et al., 2005)		
NOS1		$(AAT)_{9–15}$ $(GT)_{18–36}$		Colonization with *P. aeruginosa* Mean FE_{NO} Colonization with *A. fumigatus* 5 year decline of pulmonary function	0.0358 0.027 0.8505 0.025	75 (Grasemann et al., 2000) 59 (Texereau et al., 2004)	40 (Grasemann et al., 2002)	

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
NOS3		894 G >T		FE_{NO}	0.07 (0.02 in females)	70 (Grasemann et al., 2003)		
				FEV_1	0.08 (in females)			
				Colonization with *P. aeruginosa*	<0.05			
		T5220G	1799983	Impairment of lung function (FEV_1)	0.54	808 (Drumm et al., 2005)		
PPP2R1A		c.*465T > A	2162779	Functional residual capacity	0.0033	95 (Gisler et al., 2012)		
PPP2R4		c.−185A > C	3118625	FEV1	0.0048	95 (Gisler et al., 2012)		
				Lung clearance index	0.0059			
				Effective specific airway resistance	0.0064			
SCNN1B	*ENaC*β	T313M		Disease severity		56 (Viel et al., 2008)		
		938 C >T						
		G589S						
		1765 G > A						
SCNN1G	*ENaC*γ	L481G	5735–5723 haplotype	Disease severity		56 (Viel et al., 2008)		
		1442 T > A						
		V546I						
		1636 G > A						
SERPINA3	*ACT, A1ACT*	T-15A	4934	FEV_1 % pred	0.04	157 (Mahadeva et al., 2001)		
				Radiography score	0.03			
SFTPA1		$6A^3$ (and $6A^3/1A^1$ haplotype)		FEV_1 % pred	0.01	135 (Choi et al., 2006)		
				DLCO	0.10			
				ATS score	0.006			
				AMA score	0.02			
				Dyspnea score	0.20			
				Physical score	0.002			
				Severity score	0.005			

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
SFTPA2		$1A^1$ (and $6A^3/1A^1$ haplotype)		FEV_1 % pred	0.009	135 (Choi et al., 2006)		
				DLCO	0.13			
				ATS score	0.007			
				AMA score	0.06			
				Dyspnea score	0.07			
				Physical score	0.12			
				Severity score	0.10			
SLC8A3			12883884	FEV_1 % pred (adjusted)	1.20×10^{-6}	1,978 (Wright et al., 2011)		557 (Wright et al., 2011)
SLC9A3		521096 C > T	4957061	Age of first *P. aeruginosa* infection	0.02	1,004		
				Decline of lung function (FEV_1)	0.05	752 (Dorfman et al., 2011)		
SNAP23		c.267-9T > C	9302112	FEF_{50}	0.0088	95 (Gisler et al., 2012)		
				Functional residual capacity	0.011			
				Volume of trapped gas	0.0043			
TGFB1		codon 10 C29T	1800470	Age at which $FEV_1 < 50\%$	<0.02	171 (Arkwright et al., 2000)*	261 (Arkwright et al., 2003)	118 (Brazova et al., 2006)
				Age at which FVC < 70%	<0.005			1,978 (Wright et al., 2011)
		codon 25 G74C	1800471	Age at which $FEV_1 < 50\%$	NS		808 (Drumm et al., 2005)*	
				Age at which FVC < 70%	NS			
		C-509T	1800469	Impairment of lung function (FEV_1)	0.006	808 (Drumm et al., 2005)	498 (Drumm et al., 2005) 329 (Corvol et al., 2008) 105 (Faria et al., 2009) 472 (Bremer et al., 2008)	254 (Buranawuti et al., 2007)
TLR4		D299G	4986790	Mean FEV_1 % pred	0.55	100 (Urquhart et al., 2006)		
				Mean FVC% pred	0.52			
				Age of first *P. aeruginosa* infection	0.78			
				Chrispin–Norman X-ray score	0.16			
		2688 G > A	10759931	Rate of change of FEV_1 % pred per year	0.12			
				FEV_1 % pred	0.84 (0.55)[⌘]	755 (Park et al., 2011)		

(Continued)

Table 1 | Continued

Gene/ locus	Genes involved	Variant aliases	Variant position (rs no.)	Phenotypes tested	Association *p*-value	Source *n* (reference)	Replication *n* (reference)	Tested, not replicated *n* (reference)
TLR5		R392X	5744168	Mean FEV_1 % pred	0.77	2219 (Blohmke et al., 2010)		
TNFA	*TNFa*	G-308A (TNF2)	1800629	Mean FEV_1% pred	0.02	53 (Hull and Thomson, 1998)		261 (Arkwright et al., 2003)
				Mean Chrispin–Norman X-ray score	0.17	180 (Yarden et al., 2005)		180 (Yarden et al., 2005)
				Mean Shwachman score	0.17			53 (Schmitt-Grohe et al., 2006)
				No. positive for *P. aeruginosa*	0.72			808 (Drumm et al., 2005)
		C-851T		Mean FEV_1% pred	0.25			
				Age of first *P. aeruginosa* infection	0.60			
		G-238A		Mean FEV_1% pred	0.8			
				Age of first *P. aeruginosa* infection	0.64			
		+691g ins/del		Mean FEV_1% pred	0.008			
				Age of first *P. aeruginosa* infection	0.018			
TNFR1	*TNFRSF1A*	intron 1 haplotype		Disease severity	Associates	37 sib pairs (Stanke et al., 2006)		

[§]*The number in parenthesis indicates the p-value for the association found in F508del homozygotes.*

[⌘]*Only multivariate p-values are reported. The number outside the parenthesis is the p-value for pediatrics and the number in parenthesis is the p-value for adults.*

[⊙]*The association of missense variants with age at first P. aeruginosa-positive culture and age at onset of chronic P. aeruginosa was replicated in a population of only European American patients.*

[◦]*The association of missense variants with age at first P. aeruginosa-positive culture and age at onset of chronic P. aeruginosa was replicated in a population excluding patients with non-European ancestry.*

[+]*Found that only the c.−20G > A SNP associated with disease severity.*

[£]*The number in parenthesis indicates the p-value after a Bonferroni correction.*

[♦]*Found that −251 TT, +396TT, and +781CC may be associated with an earlier occurrence of chronic P. aeruginosa colonization, which is an indicator of disease severity, but this was not examined in the study by Hillian et al. (2008).*

[⊖]*The association of MBL2 deficiency alleles with indicators of pulmonary disease severity was replicated in a population of 298 adults, but refuted in a population of 260 children.*

[❖]*The Trevisiol et al. (2005) study replicated an association of MBL2 deficiency alleles with pulmonary function, but not with PA colonization.*

**The study by Arkwright et al. (2000) found the severe variant at codon 10 to be T/T, but the study by Drumm et al. (2005) found the severe variant to be C/C at codon 10. A more detailed discussion of the TGB1 association with CF can be found in the text.*

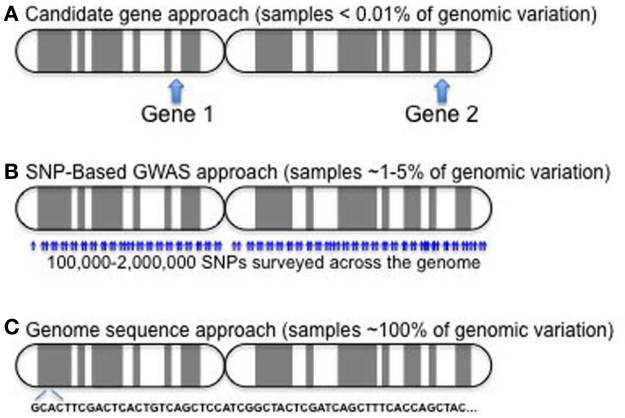

FIGURE 2 | Candidate gene approaches (A) have only involved a few variants in one to several dozen genes. Given a genome of roughly 25,000 genes, this represents a very small sampling (∼0.01% or less). GWAS **(B)** samples a much larger component of the genome, probing more than 90% of the genes, but it still only examines less than 5% of the over 50 million reference SNPs (http://www.ncbi.nlm.nih.gov/mailman/pipermail/dbsnp-announce/2012q2/000123.html) curated as of June, 2012. As costs come down, exome (not shown) and whole-genome sequencing **(C)** provide the potential to capture all variation in study subjects.

severe patients for differences in allele or genotype frequencies of single nucleotide polymorphisms (SNPs) or other gene-associated variants as markers of potential modifier genes.

Phenotypic categories of disease severity were defined using a patient's forced expiratory volume in 1 s (FEV_1), a pulmonary function index based on age, sex, and height, and used clinically to monitor CF disease progression and therapeutic efficacy. Subjects with FEV_1 values in the upper quintile were classified as "mild" and those in the lower quintile as "severe." Those subjects surviving beyond the age of 34 were classified as mild regardless of pulmonary function, as they represented the upper quintile of their birth cohort (Schluchter, 1992; Schluchter et al., 2002). DNA was obtained from these individuals and genotyped for a variety of variants in or near genes that were considered candidate modifiers.

In the initial candidate gene approach, 1,064 SNPs were tested in over 300 genes/gene regions that were chosen in the following ways: (1) they were SNPs that had previously been reported in the literature as associating with CF phenotype, (2) they were SNPs that were reportedly associated with similar pulmonary disease phenotypes, (3) they were genes that were known to play a key role in CF pathophysiology (Drumm et al., 2005).

Experience using this approach has shed light on the challenges involved in conducting modifier studies. Early studies struggled to achieve statistical power due to small sample sizes. Long and Langley (1999) calculated that the sample size must include at least 500 individuals in order to detect a causative polymorphism and for its association to be replicable. To accommodate the ability to replicate and maximize power, the GMS expanded to a North American Consortium that included a family-based genetic study at the Johns Hopkins University and a population-based study of Canadian CF patients being led by investigators at the University of Toronto and the Hospital for Sick Kids (Taylor et al., 2006). This consortium grew from the need to increase sample size and carry out replication studies and demonstrated its utility in a report that showed variants in the *TGFB1* gene associate with pulmonary disease (Drumm et al., 2005) (discussed in more detail below).

The union of the three large studies provided a cohort of unprecedented size for studying modifiers of a single gene disorder, but also presented logistical issues due to the nature of the designs as each group had developed their own methods for assessing pulmonary phenotypes. Kulich et al. (2005) generated CF-specific reference equations for FEV_1 that compare a CF subject's lung function to CF subjects of the same age, sex, and height, as a more appropriate reference than the non-CF population and those values, adjusted for survival, were used to develop a phenotypic index that all three designs could incorporate.

The candidate gene approach showed the effectiveness of genetic studies, but a limitation is that it does not identify genetic locations other than those suspected to influence disease. That is, it will not detect modifying genes or pathways beyond those involved in our limited understanding of the disease. Understanding the functional effects of a modifier and its protein product fuel future studies to provide mechanistic insight of disease pathophysiology and how it might be dealt with (Cutting, 2010).

ASSOCIATING GENES AND INSIGHT INTO THEIR MODIFYING MECHANISMS

One of the powerful attributes of genetics is that it allows one to identify clinically relevant genes, proteins, or pathways by virtue of the effect that variation in the gene produces on a clinical trait. However, the mechanisms by which genetic variation acts on the phenotype is not necessarily obvious. Thus, for any associating gene an obligatory step is to carry out functional studies to understand how it imparts its effect on disease presentation or outcome. Some examples are given below.

ASSOCIATING GENES: MBL

Mannose-binding lectin is a serum protein involved in innate immunity. MBL enhances phagocytosis of infectious organisms, especially during infancy, when adaptive immune response is immature (Eisen and Minchinton, 2003). Variant alleles that decrease MBL serum levels increase risk for many different infections (Garred et al., 1995, 1997; Summerfield et al., 1995, 1997) and have been shown to play a role in autoimmune diseases (Davies et al., 1995; Graudal et al., 1998). MBL has been suggested to regulate inflammatory responses, perhaps by delaying one of the first steps in inflammation or by reducing the levels of inflammatory cytokines (Jack et al., 2001). *MBL* is an attractive CF modifier candidate because it protects against infection and has some role in modulating inflammation.

Three amino acid substitutions in exon 1 (alleles B, C, and D) each contribute to decreased MBL plasma concentrations and are collectively referred to as 0, or null, alleles with the functional allele, containing none of the above variants, designated *A*. There are also variants with quantitative effects on mRNA expression, termed *X*, that also result in low MBL serum levels. Genotypes resulting in low MBL levels are designated low-producing or deficient alleles, but there are also genotype combinations associated with high and intermediate serum levels of MBL as well. Using the rationale that

MBL protects against bacterial infection or somehow suppresses inflammation, then *MBL* deficiency alleles would be predicted to associate with a more severe CF lung disease.

In support of such a model, Garred et al. (1999) found that patients with higher expression *MBL* genotypes had a higher FEV_1 and forced vital capacity (FVC). In other words, there was an additive effect of poor pulmonary function in the presence of an *X* allele. After further analysis, the cumulative adverse effects of low expression alleles were restricted to patients with chronic *P. aeruginosa* and were more pronounced in adults. MBL deficiency did not significantly associate with chronic colonization of *P. aeruginosa*. A study by Gabolde et al. found that cirrhosis of the liver was more common in CF patients carrying deficiency alleles, but other sources are conflicting about the association with CF liver disease (Gabolde et al., 2001; Bartlett et al., 2009; Tomaiuolo et al., 2009).

Several studies agree that *MBL* low expression alleles associate with lung function (Gabolde et al., 1999; Davies et al., 2004; Yarden et al., 2004; Trevisiol et al., 2005; Choi et al., 2006; Buranawuti et al., 2007; Dorfman et al., 2008), but there is no consensus as to whether this effect is only seen in patients colonized with *P. aeruginosa*, and whether a heterozygous genotype is sufficient to cause such impairment. Two studies found an association with chronic *P. aeruginosa* colonization (Trevisiol et al., 2005; McDougal et al., 2010), whereas others failed to detect an association between *MBL* alleles and colonization of any kind. Buranawuti et al. (2007) found that *MBL* high expression alleles predicted survival; the null genotype was underrepresented in adult populations and over represented in patients who died late in adolescence. This is consistent with multiple observations that the adverse effect of deficiency alleles is more pronounced in adults (Garred et al., 1999; Yarden et al., 2004; Buranawuti et al., 2007). In fact, a study by Davies et al. (2004) found no association between pulmonary function and *MBL* genotype in children. Despite replications, not all studies have detected associations between *MBL* alleles and lung disease severity (Carlsson et al., 2005; Drumm et al., 2005; Faria et al., 2009; McDougal et al., 2010).

ASSOCIATING GENES: *TGFB1*

As alluded to above, the first significant association identified by the consortium approach demonstrated that severity of pulmonary disease tracked with variants in the *TGFB1* gene (Drumm et al., 2005). *TGFB1* encodes transforming growth factor beta-1 (TGFβ1), a protein with complex function, involved in several cellular processes from differentiation and proliferation to innate immunity, and has been studied in relation to many disorders including Alzheimer's disease, cancer, Marfan disease, and heart disease (Waltenberger et al., 1993; Yamamoto et al., 1993; Dickson et al., 2005; Brooke et al., 2008). Interest in investigating *TGFβ1* as a potential modifier of CF pulmonary disease stemmed from both its biologic plausibility, and its identification as a modifier of asthma and chronic obstructive pulmonary disease (COPD) (Pulleyn et al., 2001; Celedon et al., 2004; Silverman et al., 2004; Wu et al., 2004).

TGFβ1 is biologically relevant to CF for several reasons. Leukocytes secrete TGFβ1 in response to infectious agents. TGFβ1 participates in the immune process by regulating the production of cytokines, and is generally thought to be pro-inflammatory in nature (Omer et al., 2003). TGFβ1 also increases the formation of extracellular tissue during injury repair by increasing production of connective tissue by altered gene regulation (Bartram and Speer, 2004). Post-injury repair in the lung is a delicate balance; inadequate remodeling leads to poor wound healing, whereas excessive remodeling leads to pathogenic fibrosis and scarring. There is strong evidence to suggest that the difference between these outcomes is at least in part related to *TGFβ1* expression levels (Bartram and Speer, 2004).

Variation in *TGFβ1* has been shown to modify asthma and COPD. A variant in the promoter region (C-509T), thought to be associated with increased *TGFβ1* expression, was studied as a potential contributor to asthma disease severity. In two separate studies homozygosity for the T allele (associated with increased TGFβ1 production) was found to be more common among severe asthmatics when compared to mild asthmatics or healthy controls (Pulleyn et al., 2001; Silverman et al., 2004). Variation in codon 10 was studied in patients with COPD. In this case, the allele associated with increased TGFβ1 production was found more commonly in control patients, suggesting a protective role for TGFβ1 in COPD (Wu et al., 2004). Contrasting with associations found in asthma patients, the T allele of -509 was more prevalent in those with mild COPD (Celedon et al., 2004).

The *TGFβ1* variants that have been implicated in other airway diseases have become a source of interest in CF as well. A study by Arkwright et al. (2000) found that the T allele (high producer genotype) in codon 10 associated with more rapid deterioration in lung function, while the genotype at codon 25 did not correlate with survival or lung function. Another study confirmed the codon 10 association found by Arkwright but interestingly, it was the C allele (low producer genotype) that prevailed in severe patients (Drumm et al., 2005). This finding, replicated in a second population of 498 patients, is counterintuitive given the protective role of TGFβ1 in COPD. The same study, by Drumm et al. found that the -509 T allele also associated with a severe pulmonary phenotype, which is the same adverse effect seen in asthma populations. There have been several attempts to resolve these conflicting data (Arkwright et al., 2000, 2003; Drumm et al., 2005; Brazova et al., 2006; Buranawuti et al., 2007; Bremer et al., 2008; Corvol et al., 2008; Faria et al., 2009), but only one study has used a relatively large cohort to accommodate the statistical power needed. It found that a haplotype of a 3′ C allele (rs8179181), -509 C, and codon 10 T associated with improved lung function to a greater degree than any SNP alone (Bremer et al., 2008). It would appear from these studies that CF more closely mimics the type of disease seen in asthma and that the same polymorphisms may be protective or adverse, depending on the genetic and environmental context.

ASSOCIATING GENES: *IFRD1*

Gu et al. (2009) applied a novel strategy by pooling equal amounts of DNA from similarly affected subjects into "mild" and "severe" pools and examined 320 patients in the GMS population (160 with severe lung disease, 160 with mild lung disease) with much lower cost and time than the other efforts. By quantifying the signal for each allele (rather than a yes/no output) the genotyping arrays were used to estimate allele frequencies in the pools. Discordant allele frequencies were identified between the pools using this

strategy (Gu et al., 2009) and indicated that alleles of *IFRD1* may contribute to pulmonary disease severity. In a subsequent study, however, *IFRD1* variants did not significantly associate with lung disease (Wright et al., 2011).

The IFRD1 protein acts in a histone deacetylase (HDAC)-dependent manner to regulate gene expression (Vietor et al., 2002) and the *IFRD1* gene is up-regulated during cell differentiation and regeneration in response to stress (Vietor and Huber, 2007). Previous studies found high expression in human blood cells (SymAtlas, 2008) and Gu et al. found highest expression in neutrophils, where up-regulation occurs during the final differentiation steps (Ehrnhoefer, 2009; Gu et al., 2009). The authors suggested that IFRD1 modulates CF lung disease through the regulation of neutrophil effector function, but that other explanations, involving different cell types, should not be ignored.

GENOME-WIDE ASSOCIATION STUDIES

Although the cost of large-scale genotyping had fallen more than a 1000-fold since these studies were initiated, genome sequencing was still well out of range by price and feasibility. Thus, it became feasible to think about whole genome, or genome-wide association studies (GWAS). A GWAS would rapidly interrogate hundreds of thousands of SNPs for association in large populations (Manolio, 2010) without bias imposed by pre-existing models and provide the opportunity to identify novel genes, regulatory loci, and pathways not previously considered. The disadvantage to testing so many variants is that there are statistical penalties that increase as the number of comparisons rises, and thus power is a major limitation (Cutting, 2010). This is less of a concern if the effect of a locus is large, but as common population variants are being examined in these studies, it is likely that the effects of any one locus are not large, perhaps with each accounting for only a few percent of the variation, for example (Long and Langley, 1999). It is an important concept to understand that these studies are conceptually analogous to those designed to find disease-causing genes, which would have major effects if they do, in fact, cause disease.

GWAS-IDENTIFIED ASSOCIATIONS

In a combined GWAS and family-based (linkage) study, 3,467 CF patients were tested for associations between lung disease severity and more than half a million SNPs (Wright et al., 2011). To accommodate the various study designs and data acquisition protocols, yet another method to examine pulmonary function, with age-specific CF percentile values of FEV_1 (Kulich et al., 2005; Taylor et al., 2011), was developed and which accounted for mortality and longitudinal changes. With this phenotype and over 500,000 common genetic variants to assess for association, two new loci, one on chromosome 11p13 and one on chromosome 20q13 were identified as having variants that associate with lung function in CF.

The region on chromosome 11p13 of most significant association lies between two annotated genes, *APIP* and *EHF*. *APIP* encodes Apaf-1-interacting protein and EHF is a member of the epithelial-specific Ets transcription factors, both of which provide interesting candidates as disease modifiers, but through very different models, all of which must yet be worked out. It is important to understand that despite the power of genetics to identify such disease-relevant locations in the genome, it does not provide information regarding mechanisms and these must be examined empirically. APIP, for example, has been shown to suppress apoptosis in the presence of hypoxia (Cho et al., 2007), a context experienced by CF tissues. At this point, it is not clear if the adverse allele provides less or greater activity than the protective allele, but one could construct models either way. For example, one hypothesis is that excessive anti-apoptotic activity, resulting from increased APIP, could prolong neutrophilic inflammation and therefore lead to more severe lung disease (Wright et al., 2011). Similarly, EHF is reported to serve as a regulator of epithelial cell differentiation under conditions of stress and inflammation (Tugores et al., 2001; Wright et al., 2011) and thus could be modeled to have very important effects during airway development or remodeling from disease-related damage. Finally, it must be considered that the modifying locus could be working at a distance, involving a regulatory site such as a transcriptional enhancer or non-coding RNA.

The other associating region on chromosome 20 was detected by linkage analysis and then refined by association. The linkage signal includes several genes including *MC3R*, encoding the melanocortin-3 receptor, *CBLN4* encoding cerebellin-like 4, *CASS4*, encoding Crk-associated substrate scaffolding (CASS) 4, and *AURKA*, encoding Aurora kinase A (Wright et al., 2011). With the exception of MC3R, which is a receptor involved in metabolic control, models to explain the other candidates are not presently clear.

Certainly functional studies will help sort out which genes in these associating intervals are responsible for their modifying effects, but these findings illustrate both the power and some of the challenges of genetic studies. On one hand, the unbiased approach provides the opportunity to identify novel disease modulators, but on the other hand identifying the source of the modifying effect and the mechanisms through with it acts are challenging tasks.

THE IMPACT OF DISEASE-MODIFYING GENES

The implications of disease-modifying genes are multiple. First, understanding the genetic contribution to phenotypic variation has the potential to provide insight into prognosis. Second, understanding the mechanisms by which these genes and their alleles are exerting their effects will likely suggest new therapeutic approaches or ways to optimize existing ones. Third, it opens the door to personalized medicine, as a given patient's treatment regimen could conceivably be developed around a genetic profile. Using inflammation as an example, one could imagine a patient whose modifier panel predicts a lessened inflammatory response, and another patient whose modifier panel predicts a heightened inflammatory response. Inflammation is part of the immune response that is necessary to fight infection, however its prolonged state in CF patients can cause lung damage. The patient with the heightened response may benefit from anti-inflammatory drugs earlier, and the patient with the reduced inflammatory response may benefit from increased antibiotic usage. Both are common treatments for CF, but they may be used more beneficially with the help of modifier identification and mechanistic understanding.

SUMMARY

Cystic fibrosis is a simple, Mendelian disorder with complex clinical manifestations that are consequences of *CFTR* genotype, environmental factors (Boyle, 2007), and heterogeneity throughout the entire genome. The discovery of genetic modifiers may help account for the broad spectrum of disease severity observed in patients, especially those with the same *CFTR* genotype. Modifying loci identified thus far each appear to contribute only a small percentage to overall disease profile and thus it is likely the combination of these variants in different permutations shape an individual's outcome, an outcome that is also significantly influenced by non-genetic factors, as well as the interaction of genetic and non-genetic factors. There are few genes whose modifying effects withstand the test of replication and further studies must elucidate the role of each one in CF. Additional research about gene-environment interactions and gene–gene interactions will certainly demonstrate how complex these genetic effects are. With the careful use of candidate gene approaches and now, genome-wide scans (and soon whole-genome sequencing), it is realistic to believe that modifiers of CF disease will be identified and from which interventions tailored around an individual's genetic profile will be developed. This fine-tuning of therapeutic strategies could contribute to better quality of life and ultimately, improved survival in CF.

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Molecular chaperones as targets to circumvent the CFTR defect in cystic fibrosis

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Cystic Fibrosis (CF) is the most common autosomal recessive lethal disorder among Caucasian populations. CF results from mutations and resulting dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). CFTR is a cyclic AMP-dependent chloride channel that is localized to the apical membrane in epithelial cells where it plays a key role in salt and water homeostasis. An intricate network of molecular chaperone proteins regulates CFTR's proper maturation and trafficking to the apical membrane. Understanding and manipulation of this network may lead to therapeutics for CF in cases where mutant CFTR has aberrant trafficking.

Keywords: CFTR, chaperone, endoplasmic reticulum, ERAD, heat shock protein, phenylbutyrate

INTRODUCTION

The most common disease-causing mutation in cystic fibrosis transmembrane conductance regulator (CFTR) is the deletion of a single phenylalanine at position 508, ΔF508-CFTR. This mutation is present in one or both alleles of ∼90% of people with CF (Riordan, 2008), making it an attractive target for therapeutics. In contrast to wild type CFTR, which reaches the apical cell surface after its N-linked oligosaccharides are modified in the Golgi to an endoglycosidase H digestion-resistant form, ΔF508-CFTR does not acquire endoglycosidase H resistance (Cheng et al., 1990). These data suggested that ΔF508-CFTR is retained in the endoplasmic reticulum (ER; Kerem et al., 1989; Collins, 1992; Riordan, 1999; Bobadilla et al., 2002). Interestingly, ΔF508-CFTR appears to retain some ability to transport chloride when in the ER (Pasyk and Foskett, 1995), suggesting that the deletion of phenylalanine interferes with proper biogenesis and promotes degradation of the mutant protein (Ward and Kopito, 1994; Ward et al., 1995; Okiyoneda et al., 2010).

Because ΔF508-CFTR retains the ability to transport chloride, it is widely hypothesized that correction of the mutant protein's trafficking would lead to functional CFTR at the apical cell surface (Denning et al., 1992b; Li et al., 1993; Pasyk and Foskett, 1995). This premise was supported by early data from Drumm et al. (1991), indicating that ΔF508-CFTR was functional in *Xenopus* oocytes, which are typically incubated at room temperature. Studying mammalian cells, Denning et al. (1992a) found that decreasing the cell incubation temperature led to an increase in both expression and function of ΔF508-CFTR at the cell surface. Overcoming this kinetic trafficking defect of ΔF508-CFTR would be an important step in developing therapeutics for people with CF.

CFTR BIOGENESIS

Proper biogenesis of the CFTR protein is not a trivial task. CFTR is synthesized as a ∼140 kDa protein (comprising 1480 amino acids) and requires a number of processing steps to progress to a mature, ∼180 kDa form. The protein contains two nucleotide binding domains (NBD1 and NBD2), two membrane-spanning domains (MSD1 and MSD2), and an intervening regulatory domain (R; Riordan et al., 1989). During translation, MSD1 is synthesized first, followed by NBD1, R, MSD2, and finally NBD2; folding of the nascent peptide appears to occurs both co-translationally and post-translationally (Du et al., 2005; Kleizen et al., 2005).

F508 is located in NBD1, and while the crystal structures of wild type and ΔF508 NBD1 are quite similar, deletion of F508 appears to cause NBD1 to have a more unfolded solution conformation, as assessed by proton-deuterium exchange (Lewis et al., 2005, 2010). Furthermore, deletion of F508 appears to destabilize a critical interaction of NBD1/MSD2 interaction (Thibodeau et al., 2005; Serohijos et al., 2008). Du et al. (2005) also suggested that phenylalanine 508 provides an important interaction with NBD2 that assists in proper post-translational folding of this domain. Together, these data suggest that newly synthesized ΔF508-CFTR is less appropriately folded, and therefore more readily recognized by ER quality control mechanisms and targeted for degradation.

Interestingly, Cui et al. (2007) found that a wild type CFTR construct lacking the NBD2 domain escaped degradation and trafficked to the cell membrane where it had similar stability to full-length CFTR, but had a very low open probability. These data suggest that, though important for CFTR activity, NBD2 is not essential for CFTR biogenesis and exit from the ER. Consistent with this notion, when this group introduced the ΔF508 mutation into their NBD2-deficient construct, the resulting protein did

not reach the plasma membrane, supporting the earlier hypothesis that ΔF508 impacts aspects of CFTR folding and biogenesis other than the NBD1/NBD2 interaction.

MOLECULAR CHAPERONES

To better understand the difficulties of ΔF508-CFTR biogenesis, it is important to examine the cellular context in which CFTR biogenesis occurs. The folding and trafficking environment, referred to by Wang et al. (2006) as the "CFTR interactome," contains over 200 proteins that co-immunoprecipitate with either wild type or ΔF508-CFTR in model systems. These co-precipitating proteins, a number of which are implicated in proper folding, trafficking, and function of CFTR, include a number of molecular chaperone proteins. Molecular chaperones are proteins that aid in the folding of other proteins, but do not become part of the final product (Ellis, 1987). Instead, they promote self-assembly of their client proteins and prevent non-productive folding. Historically, the functions of many molecular chaperones are defined by their ability to assist in the refolding of denatured proteins, such as luciferase, *in vitro* (Schroder et al., 1993; Barral et al., 2004).

Molecular chaperones appear to interact with CFTR during many stages of biogenesis. Nascent peptides of membrane proteins, such as CFTR, are synthesized at the ER, where co-translational folding occurs (Hartl, 1996). Because CFTR is inserted into the ER membrane, its folding is monitored by chaperone proteins within both the ER and cytoplasm. If CFTR folding is delayed or prolonged, interaction with molecular chaperones (Loo et al., 1998; Meacham et al., 1999) can cause improperly folded proteins to be transported back to the cytoplasm, where they are targeted for degradation by the proteasome (reviewed in Rivett, 1993). This process, known as ER-associated degradation (ERAD), also involves a number of molecular chaperones. These interactions and processes are discussed in detail below.

Appropriately folded CFTR exits the ER and is transported to the Golgi where its N-linked glycosyl modification is further processed into the mature form before trafficking to the apical cell surface. The ΔF508-CFTR mutant is unable to reach the Golgi, though it is able to transport chloride in reconstituted systems (Li et al., 1993; Lukacs et al., 1993). A number of data suggest differing and not mutually exclusive mechanisms by which ΔF508-CFTR is retained in the ER. One proposed mechanism suggests that recognition of an ER exit sequence within NBD1 of the CFTR protein by Coat Complex II (COP II) ER → Golgi transport machinery is impaired in the ΔF508 protein (Chang et al., 1999; Wang et al., 2004). Other works cite improper and/or more robust chaperone binding as the mechanism by which ΔF508-CFTR is retained in the ER (Pind et al., 1994; Wang et al., 2006). Hypothetically, excessive chaperone binding could inhibit COP II's access to the ER exit motif within NBD1. To address this question, Wendeler et al. (2007) affixed a strong ER exit signal to the wild type CFTR protein. This signal did not disrupt protein localization or expression, but did enhance wild type CFTR maturation by two-fold. In contrast, this ER exit signal did not enhance the maturation of the ΔF508 protein, thereby contradicting the hypothesis that a primary defect in the ER exit sequence is responsible for failure in ΔF508-CFTR trafficking. Instead, these data support the hypothesis that molecular chaperone proteins may play a key role in the quality control of wild type CFTR.

CFTR AND ERAD

Accumulated non-functional membrane or ER luminal proteins can aggregate and interfere with the production or function of other newly synthesized proteins, as well as cause an ER and/or cellular stress response. To prevent this, aberrant proteins are recognized, shuttled out of the ER, and targeted for degradation by ERAD.

Ciechanover and colleagues demonstrated that Hsc70, the constitutively expressed 70 kDa heat shock protein, is required for the ubiquitin-directed proteasome-mediated degradation of a number of cellular proteins (Bercovich et al., 1997); this ubiquitin-proteasome pathway is also operative in ERAD. Hsc70 has a variety of roles in the cell, including uncoating clathrin-coated pits and promoting protein ubiquitination and both proteasomal and lysosomal degradation (Chiang et al., 1989; DeLuca-Flaherty et al., 1990; Bercovich et al., 1997; Morgan et al., 2001). Because improperly folded CFTR undergoes ubiquitination-mediated degradation (Jensen et al., 1995; Ward et al., 1995), it was hypothesized that Hsc70 promotes ERAD of ΔF508-CFTR. In fact, ΔF508-CFTR associates more robustly with Hsc70 than wild type CFTR (Strickland et al., 1997; Meacham et al., 1999; Rubenstein and Zeitlin, 2000). Furthermore, pharmacologic disruption of Hsc70 binding to either wild type or ΔF508-CFTR decreases CFTR ubiquitination (Fuller and Cuthbert, 2000), stabilizes the ER (immature band B) form of CFTR (Fuller and Cuthbert, 2000), and can promote CFTR maturation (Jiang et al., 1998).

Investigations in our group have focused on the mechanism by which 4-phenylbutyrate (4PBA) enhances ΔF508-CFTR trafficking (Rubenstein et al., 1997). We found that 4PBA decreased Hsc70 mRNA and protein expression in CF epithelial cells, as well as decreased recovery of ΔF508-CFTR when Hsc70 was immunoprecipitated (Rubenstein and Zeitlin, 2000; Rubenstein and Lyons, 2001). These data support the hypothesis that Hsc70 inhibits ΔF508-CFTR maturation, likely by promoting its ERAD (see **Figure 1**).

Hsc70's promotion of ERAD involves a co-chaperone known as CHIP (C-terminus of Hsc70-interacting protein), an E3 ubiquitin ligase (Wiederkehr et al., 2002; Murata et al., 2003). Meacham et al. (2001) demonstrated that CHIP and Hsc70 cooperate to target the immature (band B) form for ubiquitination and degradation; overexpression of CHIP decreased whole cell and surface expression of CFTR. Simplistically, association of Hsc70 with a client (like CFTR) would bring CHIP into proximity where it could catalyze ubiquitination of the client. A more robust association of Hsc70 with client, as was demonstrated by our group for ΔF508 vs. wild type CFTR (Rubenstein and Zeitlin, 2000), would portend greater ubiquitination and likelihood for ERAD.

Additional co-chaperone proteins interact with the Hsc70/CHIP complex to modulate their client interaction. HspBP1 binds Hsc70 and this binding decreases the ubiquitin ligase activity of CHIP (Alberti et al., 2004). This, in turn, decreases the ubiquitin-mediated degradation of CFTR and increased the steady-state expression of either wild type or ΔF508-CFTR in an *in vitro* assay. Similarly, Bag-2 interacts with CHIP and inhibits its ubiquitin

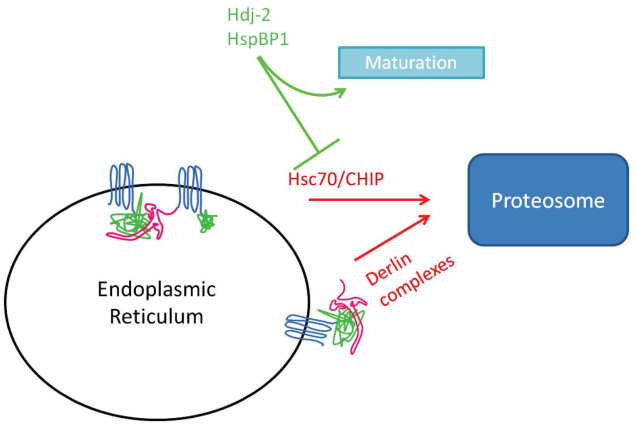

FIGURE 1 | Complexes implicated in ERAD.

ligase activity (Arndt et al., 2005). With regards to CFTR, increased Bag-2 expression increases steady-state expression of both immature and mature CFTR in heterologous cells (Arndt et al., 2005). Bag-2 appears to stabilize the NBD1 domain of CFTR and prevent its aggregation while unfolded. Matsumura et al. (2011) performed experiments in a cell-free system to discern the role of Hsc70 in promoting biogenesis from its role in promoting ubiquitination. Using a fragment of the Bag-1 protein to destabilize the interaction between Hsc70 and CFTR led to a decrease in CFTR ubiquitination, but no effect on protein biogenesis (Matsumura et al., 2011). Similarly, Meacham et al. (1999) found that the interaction between Hsc70 and Hdj-2 promotes stabilization of a folding-competent CFTR intermediate and prevents aggregation of NBD1, while Zhang et al. (2006) also found that Hdj-2/Hsc70 promoted stabilization of mature CFTR and prevented aggregation. Together, these data suggest that Hsc70 and CHIP primarily cooperate to promote ERAD of clients, and that this interaction can be modified by co-chaperones. In the case of ΔF508-CFTR, a more robust association with Hsc70/CHIP portents increased ERAD.

In addition to Hsc70, degradation of newly synthesized ΔF508-CFTR is also controlled by Derlin, an ER membrane-associated complex comprised of RMA1 (an E3 ubiquitin ligase), Ubc6e (an E2 ubiquitin-conjugating enzyme), and Derlin-1 (Younger et al., 2006). Derlin-1 appears to retain ΔF508-CFTR at the ER membrane and allow its recognition by Ubc6e and RMA1. Derlin-1 can interact with p97, the ATPase that extracts proteins from the ER during ERAD, within a separate complex that also targets CFTR for degradation (Sun et al., 2006). Derlin-1 overexpression leads to decreased wild type and ΔF508-CFTR expression, while RNAi-mediated depletion of Derlin-1 had the opposite effect. Interestingly, the Derlin complex can ubiquitinate proteins co-translationally (Younger et al., 2006), which is known to occur for CFTR (Sato et al., 1998) while CHIP/Hsc70 primarily recognizes misfolded proteins post-translationally (Younger et al., 2006). Derlin-1 degrades the CFTR fragment containing only MSD1, but not longer forms of the protein, possibly because partial CFTR folding prevents binding of Derlin-1 (Sun et al., 2006). Together, these data suggest that Derlin and CHIP/Hsc70 have complementary roles in surveillance of newly synthesized proteins to prevent accumulation of misfolded proteins.

CFTR AND CHAPERONES IN THE CYTOPLASM

Folding of the cytosolic domains of CFTR requires coordinated effort of heat shock proteins (Hsps), a large family of functionally related chaperones that promote folding and prevent aggregation of new proteins. ΔF508-CFTR demonstrates prolonged interaction with cytosolic Hsps (Yang et al., 1993; Loo et al., 1998; Rubenstein and Zeitlin, 2000; Choo-Kang and Zeitlin, 2001), indicating that these chaperones also represent potential therapeutic targets in improving ΔF508-CFTR trafficking.

Hsp70, the stress induced 70 kDa heat shock protein, and the aforementioned Hsc70, are two extensively studied members of this family. They are more than 85% identical on an amino acid level, which has led many to hypothesize that Hsp70 and Hsc70 have similar functions. Interestingly, however, Hsp70 function does not always overlap with Hsc70's, and the two often have opposite cellular effects (Gething and Sambrook, 1992; Goldfarb et al., 2006). Experimentally, Hsc70 inhibition has been shown to lead to an increase in Hsp70 expression (Aquino et al., 1996); this may represent cellular stress, as Hsp70 expression is induced by such stress (reviewed in Mayer and Bukau, 2005).

The exact role of Hsp70 in CFTR function and expression remains controversial. Choo-Kang and Zeitlin examined the effect of increased Hsp70 expression on CFTR in CF epithelial cells. In contrast to previous data (Rubenstein and Zeitlin, 2000), their data suggested that 4PBA increased Hsp70 expression and increased Hsp70/CFTR interaction (Choo-Kang and Zeitlin, 2001). They also found that overexpression of Hsp70 enhanced the interaction between Hsp70 and ΔF508-CFTR, which promoted ΔF508-CFTR maturation (see **Figure 2**). Suaud et al. (2011b) recently reconciled these data and demonstrated that 4PBA causes a transient increase in Hsp70 expression by a mechanism that involves the STAT-3 transcription factor and its interacting protein, Elongator Protein 2 (Elp2). This transient increase in Hsp70 expression with 4PBA is consistent with that suggested by gene expression profiling experiments (Wright et al., 2004). Taken together, these data support a model in which Hsp70 promotes proper trafficking of ΔF508-CFTR; this contrasts the role of its homolog, Hsc70, which, as discussed above, appears to promote ΔF508-CFTR degradation by ERAD.

In contrast, Farinha et al. (2002) found no increase in either wild type or ΔF508-CFTR maturation when both CFTR and Hsp70 were overexpressed in Chinese Hamster Ovary (CHO) cells. Instead, they saw increased wild type CFTR maturation only when Hsp70's co-chaperone, Hdj-1, was also overexpressed, but did not see a similar increase in maturation of ΔF508-CFTR. They found that Hsp70/Hdj-1 could slow the degradation rate of wild type CFTR, but not the mutant protein, possibly because of the folded state of ΔF508-CFTR. Farinha et al. also examined 4PBA treatment of cells to determine if the effect was similar to the results of their transient Hsp70/Hdj-1 overexpression. They observed a more rapid degradation of ΔF508-CFTR with 4PBA treatment, but no effect on wild type CFTR. This is contradictory to what was seen in previous reports, which suggest 4PBA promotes ΔF508-CFTR trafficking (Rubenstein et al., 1997; Choo-Kang and Zeitlin,

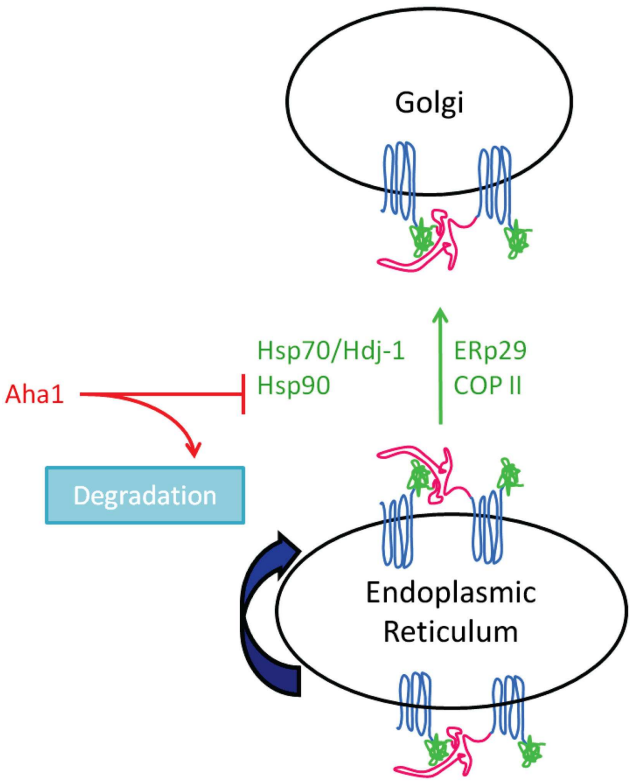

FIGURE 2 | Chaperone proteins implicated in ΔF50S-CFTR biogenesis and maturation.

2001; Suaud et al., 2011b). This apparent disparity may result from the model systems under study. Farinha et al. used heterologous CHO cells where CFTR (wild type or ΔF508) was overexpressed, while others (Rubenstein et al., 1997; Choo-Kang and Zeitlin, 2001; Suaud et al., 2011b) used IB3-1 CF bronchiolar epithelial cells where ΔF508-CFTR is endogenously expressed.

Another heat shock protein, Hsp90, also plays a key role in protein homeostasis and folding of a variety of proteins in a number of organisms (reviewed in Balch et al., 2008; Hutt et al., 2009; Powers et al., 2009). CFTR folding intermediates are stabilized by binding to Hsp90, which prolongs their half-life and aids in their trafficking and maturation (Loo et al., 1998; Fuller and Cuthbert, 2000; Wang et al., 2006). Hsp90 binding to client depends on its ATPase activity, and both client binding and Hsp90 ATPase activity are enhanced by the presence of co-chaperones, such as Aha1 (Pearl and Prodromou, 2006). Recently, Aha1 was suggested to regulate CFTR interaction with Hsp90, leading to increased interest in this co-chaperone (Wang et al., 2006). Sun et al. (2008) examined chaperone binding of wild type and ΔF508-CFTR and found that both proteins interacted similarly with Hsp90. Interestingly, they found that Aha1 interacted with ΔF508-CFTR at almost twice the affinity of wild type CFTR (Sun et al., 2008). They also expressed CFTR fragments in an attempt to rescue ΔF508-CFTR trafficking, as was reported in previous studies (Owsianik et al., 2003; Clarke et al., 2004; Cormet-Boyaka et al., 2004). With one such fragment of CFTR, they saw the predicted increase in ΔF508-CFTR maturation and a corresponding decrease in Aha1 binding to ΔF508-CFTR. These data suggest that Aha1 plays an important role in the Hsp90-mediated stabilization of CFTR. Koulov et al. (2010) recently extended these findings by demonstrating that mutations introduced in both the N- and C-terminal structures of Aha1 decreased binding of Aha1 to Hsp90, which in turn decreased the ATPase activity of Hsp90 and its ability to bind client proteins. Taken together, these data suggest that Aha1 promotes the binding of Hsp90 to client proteins by increasing the Hsp90's ATPase activity.

While initial studies using Hsp90 inhibitors, such as geldanamycin, suggested that Hsp90 promotes ΔF508-CFTR maturation and trafficking (Loo et al., 1998; Wegele et al., 2004), studies focused on Hsp90 and Aha1 suggest an alternate mechanism (Wang et al., 2006; Koulov et al., 2010). It is likely that, similar to Hsc70, the Hsp90/CFTR interaction is complex. Perhaps initial binding between Hsp90 and CFTR lead to productive biogenesis. However, if the interaction is prolonged by CFTR's inability to fold, CFTR is targeted for degradation instead.

While many studies focus on correcting the trafficking of ΔF508-CFTR to the apical cell surface, there is evidence that regulation of this mutant's endocytic trafficking is also abnormal. In fact, wild type CFTR is efficiently recycled back to the apical cell membrane after endocytosis. In contrast, ΔF508-CFTR that is delivered to the membrane using low temperature is removed from the surface more rapidly and is recycled less efficiently than the wild type CFTR (Cholon et al., 2009). These data suggest that increasing the fraction of ΔF508-CFTR that arrives at the apical cell surface, while important, may not be sufficient to increase the functional expression of this mutant protein. Interestingly, because Hsc70 is involved in endocytosis and the uncoating of clathrin-coated vesicles (DeLuca-Flaherty et al., 1990; Morgan et al., 2001), and for targeting proteins for degradation by the lysosomes (Gething and Sambrook, 1992), it seems likely that Hsc70 may also influence the stability of the wild type and mutant CFTR proteins that are expressed on the apical cell surface. These data also suggest that therapeutics which modulate the effect of Hsc70 on clathrin-mediated endocytosis may lead to increased apical membrane stability of ΔF508-CFTR.

CFTR AND CHAPERONES IN THE ENDOPLASMIC RETICULUM

The role of ER luminal chaperones in CFTR biogenesis is less well delineated. CFTR biogenesis appears influenced by additional molecular chaperone proteins in the ER, including calreticulin and calnexin. These proteins recognize terminal oligosaccharides on proteins modified with high mannose N-linked glycosylation and promote ER retention of "folding intermediates" until they either fold properly or undergo ERAD. As such, Harada et al. (2006, 2007) found that CFTR expression and function were enhanced by RNAi-mediated depletion of calreticulin in both cultured cells and mouse models, suggesting that calreticulin negatively regulates CFTR. Because previous reports indicated that curcumin, a SERCA pump inhibitor, corrected ΔF508-CFTR trafficking to the apical plasma membrane (Egan et al., 2004), Harada et al. (2007) examined the mechanism by which this occurs. They found that curcumin downregulates calreticulin expression, leading to enhanced CFTR expression. Though curcumin alone could not

activate ΔF508-CFTR in their experiments, in combination with calreticulin knockdown they showed enhanced activity of mutant CFTR, again consistent with calreticulin negatively regulating CFTR.

Calnexin's role in regulating CFTR biogenesis is less clear. Initial reports suggest that calnexin binds to immature CFTR, and the interaction with ΔF508-CFTR is prolonged, compared to wild type CFTR (Pind et al., 1994). Based on these data, it is reasonable to hypothesize that calnexin is responsible for ER retention of ΔF508-CFTR, and may therefore represent a viable target for therapeutics to rescue ΔF508-CFTR. However, recent studies suggest a more complex picture of CFTR regulation by calnexin. One study suggested that calnexin actually decreased ERAD of ΔF508-CFTR (Okiyoneda et al., 2004), and depletion of calnexin using RNAi did not improve trafficking of newly synthesized ΔF508-CFTR (Farinha and Amaral, 2005). While calnexin might not influence CFTR trafficking as predicted, this study may have been limited by incomplete calnexin depletion. To address this possibility, a follow-up study examined CFTR trafficking in calnexin-deficient cells, or cells containing calnexin mutant proteins (Okiyoneda et al., 2008). One calnexin mutant, a truncated form that is exported from the ER, was able to bind to ΔF508-CFTR with similar affinity to wild type. However, this mutant failed to increase the amount of ΔF508-CFTR in the Golgi, suggesting that calnexin may not be responsible for ER retention of ΔF508-CFTR. In complimentary experiments, the group also employed wild type and calnexin knockout murine embryonic fibroblasts (MEFs) to address caveats of earlier RNAi experiments. They found that wild type CFTR protein was decreased in calnexin knockout MEFs, compared to MEFs containing wild type calnexin. Consistent with the RNAi experiments, they found that neither ΔF508-CFTR trafficking nor chloride transport was affected by calnexin knockout. These data suggest that calnexin is not sufficient for ER retention and degradation of the ΔF508-CFTR protein. Instead, other ER chaperone proteins may represent a stronger therapeutic target for CF patients.

Endoplasmic reticulum luminal chaperones involved in the unfolded protein response (UPR) work closely with the ERAD system. When protein folding in the ER is delayed, the UPR is activated to reestablish homeostasis within the ER by increasing the protein folding capacity of the cell and/or decreasing biosynthesis (reviewed in Schroder and Kaufman, 2005). The UPR is comprised of the regulator protein Grp78/BiP and a number of signal transducers, including ATF6 and PERK (Bertolotti et al., 2000; Lee, 2005). Under non-stress conditions, Grp78/BiP binds ATF6 and maintains it in an inactive state. Under ER stress, such as an excess of unfolded protein, Grp78/BiP preferentially binds to the luminal unfolded protein, which releases and allows activation of ATF6 and PERK, leading to initiation of the UPR.

Because ΔF508-CFTR is a misfolded protein, Kerbiriou et al. hypothesized that ΔF508-CFTR-expressing cells would activate the UPR. Using ATF6 and Grp78/BiP as markers of the UPR, they found that protein levels of both Grp78/BiP and activated ATF6 were increased in ΔF508-CFTR-containing cells (Kerbiriou et al., 2007). Interestingly, RNAi-mediated depletion of ATF6, but not Grp78/BiP, corrected ΔF508-CFTR trafficking, as evidenced by increased ΔF508-CFTR-mediated chloride transport and surface expression. These data suggest that the UPR pathway is involved in the retention of ΔF508-CFTR in the ER, but that Grp78/BiP is not involved directly in CFTR biogenesis. This is also consistent with earlier data from Yang et al. (1993) and Pind et al. (1994), which found no interaction between CFTR and Grp78/BiP, and no effect of Grp78/BiP on the trafficking of ΔF508-CFTR. In contrast to Kerbiriou et al. others have not found increased Grp78/BiP expression in cells expressing ΔF508-CFTR (Nanua et al., 2006). These seemingly contradictory findings may indicate a potentially transient interaction between unfolded proteins and Grp78/BiP. In addition, ERAD may be the predominant mechanism by which the cell responds to unfolded CFTR, meaning that Grp78/BiP's role in the response to ΔF508-CFTR is small, leading to a small or negligible activation of the UPR. Based on these data, it remains unclear what role the UPR plays in trafficking or internal retention of ΔF508-CFTR.

Our group has recently focused on another ER chaperone and its potential role in regulating CFTR trafficking. ERp29 (ER luminal protein of 29 kDa) is ubiquitously expressed, but is especially prominent in brain and lung (Demmer et al., 1997). Its function is not entirely clear, but is suggested to promote thyroglobulin secretion and regulate assembly of connexin hemichannels (Sargsyan et al., 2002; Hubbard et al., 2004; Baryshev et al., 2006; Das et al., 2009), and it also seems to play a role in CFTR trafficking. Our group recently demonstrated that 4PBA increased ERp29 mRNA and protein expression (Suaud et al., 2011a). We also demonstrated that overexpression of ERp29 in *Xenopus* oocytes and mammalian cells increased the functional and surface expression of wild type and ΔF508-CFTR, while RNAi-mediated depletion of ERp29 decreased wild type CFTR in bronchial epithelial cells (Suaud et al., 2011a). These data suggested that ERp29 protein acts to promote biogenesis of both ΔF508 and wild type CFTR, and is the first ER luminal protein described to have this role. While additional studies are necessary, these data suggest an additional mechanism by which 4PBA may correct ΔF508-CFTR biogenesis and trafficking.

MOLECULAR CHAPERONES AS PHARMACOLOGIC TARGETS

To improve the function of ΔF508-CFTR, it is important to consider the many molecular chaperones in the CFTR "interactome" as potential therapeutic targets. Though 4PBA is a prototype ΔF508-CFTR corrector, its effects are only partial. While most reports suggest that 4PBA promotes ΔF508-CFTR trafficking by decreasing Hsc70 and increasing Hsp70 (Rubenstein et al., 1997; Rubenstein and Zeitlin, 1998, 2000; Choo-Kang and Zeitlin, 2001; Rubenstein and Lyons, 2001; Suaud et al., 2011b), another found no 4PBA effect on these chaperones or on ΔF508-CFTR (Farinha et al., 2002). Early phase clinical trials showed a partial improvement in CFTR-mediated chloride transport in ΔF508-CFTR homozygous subjects with CF (Rubenstein and Zeitlin, 1998; Zeitlin et al., 2002), but the amount of improvement suggested that more efficacious correctors would be necessary to achieve meaningful clinical improvements.

In addition to 4PBA, a variety of Hsc70 inhibitors are being examined as potential correctors of ΔF508-CFTR trafficking and may also represent therapeutic targets for treatment of CF (see **Figure 3**). Apoptazole is one such drug that interferes with Hsc70.

Cho et al. (2011) found that apoptazole has the potential to promote ΔF508-CFTR trafficking and activity. Apoptazole appears to disrupt the ATPase activity of Hsc70 and decreases the ubiquitination of ΔF508-CFTR by blocking the interaction between Hsc70 and CHIP.

Matrine, a quinolizidine alkaloid, also downregulates Hsc70 expression, leading to an increase in ΔF508-CFTR protein levels (Basile et al., 2012). It also allows ΔF508-CFTR to exit the ER and localize to the plasma membrane, as evidenced by an increase in interaction between ΔF508-CFTR and BAG3, a co-chaperone located at the apical cell surface.

Deoxyspergualin is a drug that targets both Hsc70 and Hsp90 (Nadler et al., 1992; Nadeau et al., 1994), but has no apparent effect on Hsp70. Jiang et al. (1998) found that deoxyspergualin treatment increased CFTR activity in ΔF508-CFTR-expressing cells, suggesting this drug may provide an alternate mechanism by which to affect Hsc70 and indirectly increase ΔF508-CFTR trafficking. Clinically, there are many potential problems with deoxyspergualin treatment, however, likely because Hsc70 and Hsp90 are ubiquitously expressed proteins with many functions. Recently, Norez et al. explored a potential solution to this problem by constructing a form of the molecule with an adjuvant. When they generated a human serum albumin/deoxyspergualin construct, they were able to deliver the drug at lower doses, with lower toxicity, and achieve even better correction of ΔF508-CFTR trafficking than they saw with deoxyspergualin alone (Norez et al., 2008). This is a promising method by which drugs could be delivered to patients with lower toxicity.

Pharmacologic agents that specifically target Hsp90 are also being studied to understand their effects on ΔF508-CFTR. Early studies showed that geldanamycin, as well as other members of the ansamycin family, target Hsp90, and disrupt binding to CFTR (Loo et al., 1998). However, geldanamycin increased turnover of CFTR by increasing CFTR's susceptibility to ERAD. Based on these data, it seems that geldanamycin would be detrimental, rather than helpful, in CF patients. However, more recent data provided a completely different picture. Using an *in vitro* system, Fuller and Cuthbert (2000) found that geldanamycin interferes with degradation of ΔF508-CFTR by disrupting ubiquitination. The caveat of this study is that it was conducted using rabbit reticulocyte lysates, rather than cell or animal models. Further investigation into geldanamycin or other Hsp90 inhibitors is needed and would provide a more complete picture of the role that these agents play in maturation of the mutant CFTR protein.

The identification of ER luminal chaperones, such as ERp29, that modulate CFTR and ΔF508-CFTR biogenesis is an exciting new development. These chaperones may be useful targets for development of novel ΔF508-CFTR corrector strategies.

CONCLUSION

Patients currently receive therapeutics primarily aimed at treating symptoms of Cystic Fibrosis (CF; Ashlock and Olson, 2011; Cuthbert, 2011), although the first mechanism-based therapy for CF patients harboring a CFTR gating mutation like G551D was recently approved. For most people with CF this is not a permanent solution, thus new therapies that can target the underlying pathology of the defect are needed. This is a difficult task, as ΔF508-CFTR correctors tested thus far have had only limited efficacy (Rubenstein and Zeitlin, 1998), likely due to the complexities of CFTR folding and trafficking. Targeting chaperone proteins that influence CFTR, rather than CFTR itself holds promise for success. Because of their ubiquitous expression and interactions with so many cellular proteins, small changes in chaperone level or function may have dramatic effects on client proteins, such as CFTR.

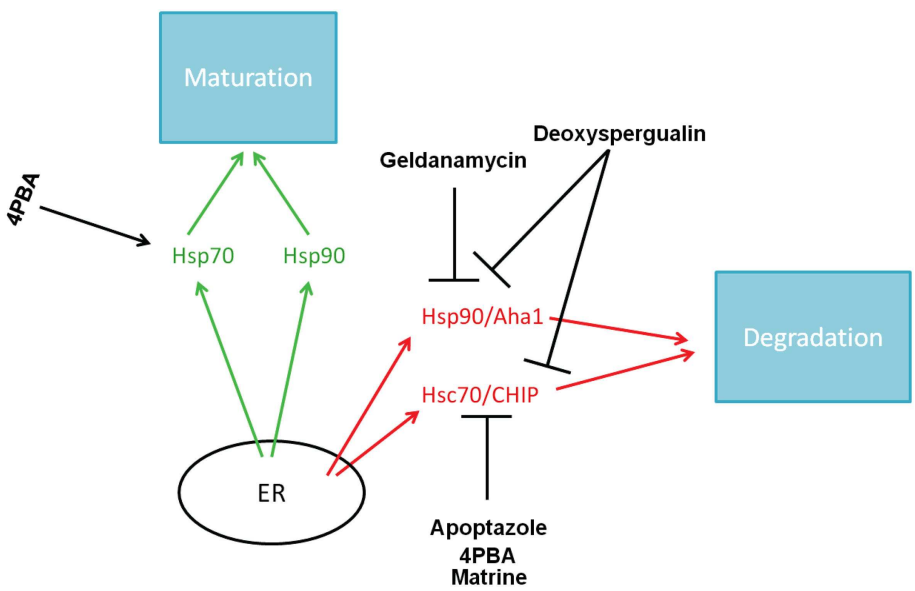

FIGURE 3 | AF508-CFTR correctors and their molecular targets.

It is important to keep in mind that the molecular chaperone functions described here (ERAD, UPR, folding, etc.) are tightly regulated and highly evolved to prevent the prolonged existence of unfolded or improperly folded proteins. In order to overcome the ΔF508-CFTR trafficking defect, it is necessary to find ways to bypass and/or change the set point of these quality control mechanisms. The system redundancy, highlighted by chaperone proteins with similar or overlapping roles (e.g., Hsc70/CHIP and Derlin), adds a level of security which is essential to the cell, but difficult to overcome, from a scientific perspective. A very delicate balance must be struck if a highly efficient therapeutic agent is to be found. The compound must prolong the lifetime of the misfolded ΔF508-CFTR protein, in order to allow proper folding. However, increased half-life might also lead to increased chaperone binding, which, as in the case of Hsp90, can counterproductively force the cell to degrade misfolded proteins (Koulov et al., 2010).

Because a large fraction of newly synthesized ΔF508-CFTR is degraded by the ubiquitin-proteasome pathway, inhibition of the proteasome inhibitors might seem like an attractive therapeutic strategy. However, inhibiting proteasomal degradation does not increase the functional ΔF508-CFTR at the apical cell surface (Ward and Kopito, 1994; Ward et al., 1995). Instead, inhibiting the proteasome led to intracellular accumulation of ubiquitinated immature ΔF508-CFTR without increasing surface expression and function. In addition, proteasomal inhibition leads to increased cellular stress due to accumulation of misfolded proteins, which in turn induces expression of heat shock proteins, such as Hsp70, Hsc70, and Hsp90 (Liao et al., 2006), and may lead to cell apoptosis/death (Fribley et al., 2004; Park et al., 2011). These data suggest that inhibition of the proteasome is not a viable therapeutic option for correcting ΔF508-CFTR trafficking.

Unfortunately, there are a number of difficulties that scientists face in designing therapeutics to correct ΔF508-CFTR. Many of the studies on CFTR and chaperones have been conducted using overexpression systems. This, of course, is necessary for detection of the extremely low-level expression of ΔF508-CFTR in cells where the protein is not overexpressed. However, this overexpression makes interpretation of the results somewhat more difficult. In addition, while often used non-epithelial cell models facilitate the overexpression of wild type and ΔF508-CFTR, non-epithelial cells do not endogenously express CFTR, so their responses to overexpression my not be physiologically relevant (as discussed above, Farinha et al., 2002). Studies performed in these models must be validated using epithelial cells.

CFTR expression varies between epithelial tissue types. Kalin et al. examined samples from CF patients as well as healthy human samples using immunohistochemistry. They found that the wild type CFTR protein could be detected in sweat glands, lung epithelia, and villi and goblet cells in the intestine (Kalin et al., 1999). In contrast, ΔF508-CFTR could not be detected in sweat glands, but expression in the lung and intestine were very similar to wild type CFTR. While this study did not address the functional activity of ΔF508-CFTR in these tissues, these data suggest that CFTR processing defects may be tissue type-specific and that ΔF508-CFTR processing may affect some tissues more than others. Further study of chaperone function in a range of epithelial tissues is required to fully understand their role in CFTR trafficking and activity.

Recent generation of novel animal models of CF, such as the ferret and pig, and their disease pathology is of great benefit to the advancement of this field as a whole (reviewed in Fisher et al., 2011) and (Keiser and Engelhardt, 2011). While the role of chaperones in CFTR trafficking have yet to be investigated in these models, future interrogations of epithelial cells from these models will undoubtedly yield a great deal of insights into both underlying physiology and therapeutic approaches.

Many chaperone proteins are upregulated in response to cellular stress, which may result from overexpression of exogenous proteins or increased abundance of misfolded proteins in the ER. Overexpressing ΔF508-CFTR may lead to a specific activation of proteins needed to fold the mutant, or instead cause a global upregulation of chaperone proteins involved in ERAD or the UPR, simply by increasing cellular stress. Studies examining overexpression of both wild type and ΔF508-CFTR lend credence to the hypothesis that the response is specific to the mutant protein, but this is still a concern that needs to be addressed when designing therapeutics.

Many pharmacologic agents that correct ΔF508-CFTR trafficking do so by an as yet unknown mechanism. Though many chaperones have been extensively studied, there are still aspects of our understanding that are lacking. This is evidenced by studies with seemingly contradictory data, discussed above. As an additional caveat, chaperone proteins have many targets and interact with an abundance of proteins in response to cellular stress. While changes in chaperone expression may positively influence ΔF508-CFTR expression, the effects on other important protein pathways could have unforeseen negative consequences. The use of these pharmacologic agents must be understood in the context of these other roles for chaperones within the cell. Building an even greater knowledge base of molecular chaperones and ΔF508-CFTR, in the context of the CFTR "interactome," will help to fill in the gaps and lead to a better understanding of the pharmacologic agents, as well as the proteins that they target.

Finally, ΔF508-CFTR interacts with many other proteins during its lifetime, and it may not be possible to design a single molecule to correct all its potentially problematic interactions. Instead, a combination of therapeutics may be more appropriate and effective. Targeting multiple chaperones may allow therapies to avoid the trap of decreasing a single molecular chaperone protein too much. Small changes in multiple chaperones may provide the balance needed to prolong the life of ΔF508-CFTR enough to allow proper folding, but not so much that it is recognized by ERAD or the UPR. These sorts of small changes to multiple chaperones may also help create therapies with less toxic side effects.

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PDE5 inhibitors as potential tools in the treatment of cystic fibrosis

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Despite great advances in the understanding of the genetics and pathophysiology of cystic fibrosis (CF), there is still no cure for the disease. Using phosphodiesterase type 5 (PDE5) inhibitors, we and others have provided evidence of rescued F508del-CFTR trafficking and corrected deficient chloride transport activity. Studies using PDE5 inhibitors in mice homozygous for the clinically relevant F508del mutation have been conducted with the aim of restoring F508del-CFTR protein function. We demonstrated, by measuring transepithelial nasal potential difference in F508del mice following intraperitoneal injection of sildenafil, vardenafil, or taladafil at clinical doses are able to restore the decreased CFTR-dependent chloride transport across the nasal mucosa. Moreover, vardenafil, but not sildenafil, stimulates chloride transport through the normal CFTR protein. We developed a specific nebulizer setup for mice, with which we demonstrated, through a single inhalation of PDE5 inhibitors, local activation of CFTR protein in CF. Significant potential advantages of inhalation drug therapy over oral or intravenous routes include rapid onset of pharmacological action, reduced systemic secondary effects, and reduced effective drug doses compared to the drug delivered orally; this underlines the relevance and impact of our work for translational science. More recently, we analyzed the bronchoalveolar lavage of CF and wild-type mice for cell infiltrates and expression of pro-inflammatory cytokines and chemokines; we found that the CFTR activating effect of vardenafil, selected as a representative long-lasting PDE5 inhibitor, breaks the vicious circle of lung inflammation which plays a major role in morbi-mortality in CF. Our data highlight the potential use of PDE5 inhibitors in CF. Therapeutic approaches using clinically approved PDE5 inhibitors to address F508del-CFTR defects could speed up the development of new therapies for CF.

Keywords: CFTR, cystic fibrosis, PDE5 inhibitors, sildenafil, vardenafil, taladafil

INTRODUCTION

Approximately 80,000 people in the world are diagnosed with Cystic Fibrosis (CF), the most common, life-threatening, recessively inherited disease in Caucasian populations. Affecting about one newborn in every 2,500 live births, CF is due to mutations in the *CF transmembrane conductance regulator* (*CFTR*) gene (Kerem et al., 1989; Riordan et al., 1989) which encodes the main chloride channel expressed in epithelia. CF disease causes abnormal mucociliary clearance mainly in the lungs, leading to a vicious cycle of obstruction/infection/inflammation that progressively and irreversibly damages lung tissue and architecture. Many organs are affected in CF but pulmonary disease is the major cause of morbidity and mortality (Rowe et al., 2005; Davis, 2006). Although life expectancy and quality of life have progressively improved over time, there is still no cure for CF.

The most common disease allele, F508del, corresponding to a deletion of a single phenylalanine residue at position 508 of a single polypeptide chain of 1480 amino acids, prevents the efficient folding of the CFTR protein. The F508del-CFTR protein is correctly translated but it is retained in the endoplasmic reticulum and directed toward proteosomal degradation (Lukacs et al., 1994). As a consequence, expression of the misfolded, immature, partly glycosylated F508del-CFTR protein at apical membranes is reduced, leading to a loss-of-function of transepithelial chloride transport.

Recent research in CF basic science has focused on the discovery of pharmacological therapies directed to treat mutation-specific changes (for review, Lubamba et al., 2012a). In the case of the F508del-CFTR mutation, efforts have been made to correct localization of the mutant protein by favoring its expression at the apical membrane of cells. However, it has been recognized that rescuing F508del-CFTR to the plasma membrane does not completely correct chloride transport abnormalities as it also displays reduced channel activity (Amaral, 2004). Therefore, finding a compound that also promotes CFTR channel activity would be of a great benefit. Searching for such compounds, we and others have demonstrated the potential of inhibitors of phosphodiesterase type 5 (PDE5), such as sildenafil, vardenafil, and taladafil, for the treatment of CF. Indeed, recent findings have evidenced that the drugs, already in clinical use for the treatment of erectile dysfunction and of pulmonary arterial hypertension, are able to rescue F508del-CFTR trafficking (Dormer et al., 2005; Robert et al., 2008) and to improve its channel activity (Lubamba et al., 2008, 2011).

CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

PDE activity is found in all cells, but with a distinct cellular and subcellular distribution of the 11 mammalian isoforms (Beavo et al., 1970). By catalyzing the hydrolysis of 3′ cyclic phosphate bonds of adenosine and/or guanosine 3′5′ cyclic monophosphate (cAMP and/or cGMP) the enzyme regulates the intracellular levels of the second messengers. The multiple isoforms of PDEs and their 50 or so subtypes, displaying different kinetics and regulatory properties (Cheung, 1970; Conti, 2000; Soderling and Beavo, 2000; Francis et al., 2001; Mehats et al., 2002), are characterized by their specificity and sensitivity to calcium-calmodulin and their affinity for cAMP or cGMP (**Figure 1**).

Eleven families of PDE have been identified in mammalian tissues (Cheung, 1970; Conti, 2000; Soderling and Beavo, 2000; Francis et al., 2001; Mehats et al., 2002) and are classified on the basis of their amino acid sequences, substrate specificities, pharmacological properties, and tissue distributions (**Table 1**).

PDE INHIBITORS: MAIN CHARACTERISTICS AND CLINICAL APPLICATIONS

Inhibition of PDEs leads to increasing intracellular concentrations of endogenous cAMP/cGMP (Bender and Beavo, 2006). Therefore, inhibition of PDE can mediate a variety of physiological mechanisms at different cell and organ levels. Strategies directed to promote inhibition of PDE activity have been applied as therapeutic tools in a variety of lung and inflammatory disorders, such as asthma and chronic obstructive pulmonary disease (COPD) but also in neuronal, cardiovascular, and other conditions (**Table 1**).

Many selective and non-selective PDE inhibitors have been explored as therapeutic agents. PDE1s are calcium- and calmodulin-dependent activators or regulators (Ahn et al., 1991; Yan et al., 1995; Loughney et al., 1996; Yu et al., 1997). Several isoforms have been recognized exhibiting different affinities for cAMP and cGMP. PDE1 inhibition has been investigated in treating neuronal plasticity (Medina et al., 2006; Menniti et al., 2006), detrusor instablities and urgency incontinence (Truss et al., 2001), memory loss (Zhang et al., 2004), reversal of the effects of early alcohol exposure in learning performance in the water maze (Jeon et al., 2010) and Parkinson and Alzheimer diseases (Reed et al., 2002). It was recently demonstrated that vinpocetine has a strong anti-inflammatory effect (Filgueiras et al., 2010; Medina, 2010).

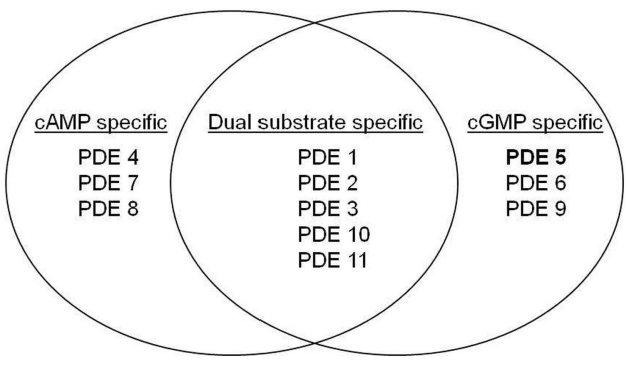

FIGURE 1 | Substrate specificity of the different families of PDE. Although a high degree of homology has been observed within the catalytic domain of PDEs, slight structural differences in these domains determine the specificity of substrate of PDEs (Xu et al., 2004).

PDE2, which metabolizes both cGMP and cAMP (Rosman et al., 1997), is highly expressed in heart (Rivet-Bastide et al., 1997) and brain but lower expression levels are found in a variety of organs (Sadhu et al., 1999). PDE2 inhibitors identified so far lack therapeutic actions (Repaske et al., 1992; Podzuweit et al., 1995; Suvarna and O'Donnell, 2002; Boess et al., 2004; Rutten et al., 2009).

It is well known that methylxanthines, non-selective PDEs found in tea, coffee, and cocoa, stimulate the central nervous system, relax the bronchial smooth muscle, and stimulate cardiac muscle. Methylxanthines have long been used as therapeutic agents in respiratory diseases (Sullivan et al., 1994; Barnes, 2003a,b,c; Bhatt-Mehta and Schumacher, 2003; Barnes and Stockley, 2005; Muller and Jacobson, 2011). Indeed theophylline (1,3-dimethylxanthine) and other methylxanthines have been used in medical practice long before they were identified as PDE inhibitors. Caffeine has long been used as a bronchodilating agent. It has been perceived that theophylline has additional anti-inflammatory properties for use in asthma or COPD, diseases characterized by inflammatory and immune responses. Paraxanthine (1,7-dimethylxanthine), the primary metabolite of caffeine (1,3,7-trimethylxanthine), acts through the ryanodine receptor to elevate intracellular calcium concentration and increases viability of neuronal cells in culture (Guerreiro et al., 2008). The synthesized 3-isobutyl-1-methylxanthine (IBMX) has a much higher affinity for PDEs and, at low concentrations, it preferentially inhibits cGMP-dependent over cAMP-dependent PDEs (Wells et al., 1975). Moreover, methyxanthines are potent antagonists of adenosine receptors (Muller and Jacobson, 2011).

PDE3 are non-selective enzymes with high affinity for both cAMP and cGMP (Palmer and Maurice, 2000). A large number of selective PDE3 inhibitors including milrinone, cilostamide, and cilostazol have been identified as potential therapeutic tools for cardiovascular diseases and asthma (Vandecasteele et al., 2001; Nohria et al., 2003; Shin et al., 2007; Carev et al., 2010).

PDE4s have high affinity for cAMP, they are expressed in inflammatory cells such as T and B lymphocytes, eosinophils, neutrophils, airway epithelial cells and endothelial cells (Tenor et al., 1995a,b,c), cardiovascular tissues, and smooth muscles. PDE4 inhibitors have been developed for the treatment of asthma and COPD (Essayan, 2001). Rolipram, a highly selective first generation PDE4 inhibitor, has been used for many years as a research tool to investigate the role of PDE4. Rolipram inhibits neutrophilic and eosinophilic inflammation; it proved to be an effective antidepressant, but side effects such as nausea and gastro-intestinal disturbance terminated its clinical development (Scott et al., 1991). Roflumilast was beneficial, as assessed by improvement in lung function, even when added to a long acting β_2 agonist or a long acting inhaled antimuscarinic (O'Byrne and Gauvreau, 2009).

PDE5 has a higher affinity for cGMP and was identified in rat platelets (Hamet and Coquil, 1978; Coquil et al., 1980) and rat lung (Francis et al., 1980; Francis and Corbin, 1988). It is known to be abundant in smooth muscle cells (Moncada and Martin, 1993) and high expression levels have been found in pulmonary vascular

Table 1 | Main characteristics of phosphodiesterase families, corresponding substrates and specific inhibitors, and their clinical applications.

PDE	Main substrate	Km (μM) cAMP	Km (μM) GMP	Tissue expression	Specific inhibitors	Reference
1	Ca2+/ calmodulin-stimulated cAMP ≤ cGMP	70–120	0.6–6.0	Heart, brain, lung, smooth muscle, T lymphocytes, sperm	KS505a, bepril, Vinpocetine, Flunarizine, Amiodarone[a,b]	Bender and Beavo (2006), Yan et al. (1995), Loughney et al. (1996), Yu et al. (1997), Ahn et al. (1991), Medina et al. (2006), Menniti et al. (2006), Truss et al. (2001), Zhang et al. (2004), Jeon et al. (2010), Reed et al. (2002), Filgueiras et al. (2010), Medina (2010)
2	cAMP = cGMP	30	10–24	Adrenal gland, heart, lung, liver, platelets	EHNA, BAY 60–7550, Oxindole, PDP[c]	Rosman et al. (1997), Rivet-Bastide et al. (1997), Sadhu et al. (1999), Suvarna and O'Donnell (2002), Podzuweit et al. (1995), Repaske et al. (1992), Boess et al. (2004), Rutten et al. (2009)
3	cAMP > cGMP	0.2–0.4	0.02–0.2	Heart, lung, liver, kidney, oocytes, adipocytes, T lymphocytes, platelets, inflammatory cells	Cilostamide, Cilostazol, Enoxamone, Milrinone, Siguazodan[b,d]	Palmer and Maurice (2000), Vandecasteele et al. (2001), Shin et al. (2007), Nohria et al. (2003), Carev et al. (2010)
4	cAMP	1.5–10	–	Kidney, brain, liver, lung, smooth muscle, cardiovascular tissues, Sertoli cells inflammatory cells	Rolipram, Roflumilast, Cilomilast, Drotaverine, ibudilast[b,d,e]	Tenor et al. (1995a,b,c), Essayan (2001), Scott et al. (1991), O'Byrne and Gauvreau (2009)
5	cGMP	290	2.9–6.2	Lung, platelets, vascular, smooth muscle	Sildenafil, Vardenafil, Tadalafil, Zaprinast[b,d,e]	Hamet and Coquil (1978), Coquil et al. (1980), Francis et al. (1980), Francis and Corbin (1988), Moncada and Martin (1993), Sebkhi et al. (2003), Ghofrani et al. (2006), Milligan et al. (2002), Nichols et al. (2002), Muirhead et al. (2002), Burgess et al. (2008), Klotz et al. (2001), Gresser and Gleiter (2002), Stark et al. (2001), Ormrod et al. (2002), Eardley and Cartledge (2002), Bella and Brock (2003), Staab et al. (2004), Brock (2003), Porst et al. (2003), Curran and Keating (2003), Corbin et al. (2005), Wharton et al. (2005), Prickaerts et al. (2002), Baratti and Boccia (1999)
6	cGMP	610–700	15–17	Photoreceptor	Dipyridamole	Zhang et al. (2005), Estrade et al. (1998)
7	cAMP	0.03–0.2	–	Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes, eosinophils, neutrophils	BRL-50481, BC30[b]	Gardner et al. (2000), Sasaki et al. (2000), Hetman et al. (2000a), Smith et al. (2003), Pitts et al. (2004), Vergne et al. (2004), Zhang et al. (2008)
8	cAMP	0.06	–	Testis, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes	PF-04957325[f]	Perez-Torres et al. (2003), Wang et al. (2001), Hayashi et al. (2007), Kobayashi et al. (2003), Glavas et al. (2001), Dong et al. (2006), Vasta et al. (2006), Vang et al. (2010), Tsai et al. (2011), Dov et al. (2008)
9	cGMP	230	0.2–0.7	Kidney, liver, lung, brain, spleen, small intestine	BAY 73-6691	Soderling et al. (1998a,b), van der Staay et al. (2008)
10	cAMP < cGMP	0.2–1.0	13–14	Testis, brain	pyrazoloquinoline analogs	Soderling et al. (1999), Fujishige et al. (1999), Loughney et al. (1999), Hebb et al. (2004), Yang et al. (2012)
11	cAMP = cGMP	2.0–3.2	0.95–2.1	Skeletal muscle, prostate, kidney, liver, pituitary, testis, salivary glands	BC 11-38	Fawcett et al. (2000), Hetman et al. (2000b), Weeks et al. (2007), Ceyhan et al. (2012)

[a]Therapeutic action with neuronal effects (neural plasticity, memory loss, detrusor instabilities, and urgency incontinence,...); [b]Therapeutic action with anti-inflammatory effects; [c]Without therapeutic action; [d]therapeutic action for lung diseases (asthma, COPD); [e]Therapeutic action with cardiovascular effects (inotopic, vasodilator,...); [f]Therapeutic action for adrenal insufficiency.

smooth muscle, bronchial blood vessels, and airway smooth muscle (Francis et al., 1980; Francis and Corbin, 1988). Recent data have shown that PDE5 may modulate pressure-induced cardiac hypertrophy and fibrosis (Sebkhi et al., 2003). Several compounds that potently inhibit PDE5 have been synthesized recently, and three of these are currently in clinical use for male erectile dysfunction (**Figure 2**). Sildenafil (Viagra; Pfizer Inc., USA), the first compound of this class to be marketed, provides well-tolerated pharmacotherapy for erectile dysfunction (Milligan et al., 2002; Muirhead et al., 2002; Nichols et al., 2002; Ghofrani et al., 2006; Burgess et al., 2008). Two newer selective PDE5 inhibitors, vardenafil (Levitra; GlaxoSmithKline, UK; Klotz et al., 2001; Stark et al., 2001; Gresser and Gleiter, 2002; Ormrod et al., 2002), and taladafil (Cialis; Eli Lilly, US; Eardley and Cartledge, 2002; Bella and Brock, 2003; Brock, 2003; Curran and Keating, 2003; Porst et al., 2003; Staab et al., 2004) have the same mechanism of action, as they specifically bind to the catalytic site of the enzyme catalyzing the breakdown to 5′-GMP of cGMP, the second messenger of the nitric oxide (NO) pathway in vascular smooth muscle cells (Moncada and Martin, 1993). Sildenafil (under the tradename Revatio) and taladafil (under the tradename Adcirca) have also been approved for the treatment of ailments related to smooth muscle tissues, such as pulmonary arterial hypertension (Sebkhi et al., 2003; Corbin et al., 2005; Wharton et al., 2005). It has been reported that sildenafil and vardenafil raise hippocampal cGMP levels and improve memory in aged rats (Prickaerts et al., 2002) and mice (Baratti and Boccia, 1999).

PDE6s display high affinity for cGMP and are expressed in the photoreceptor outer segments of the mammalian retina, in which they mediate transduction of the light signal into an electrical signal (Zhang et al., 2005). Dipyridamole has been described to be a very potent cGMP-specific PDE inhibitor of visual transduction by cGMP accumulation (Estrade et al., 1998).

PDE7s are characterized by their high affinity and selectivity for cAMP as a substrate (Gardner et al., 2000; Hetman et al., 2000a; Sasaki et al., 2000; Smith et al., 2003; Pitts et al., 2004; Vergne et al., 2004; Zhang et al., 2008). Expression is abundant in T cells, eosinophils and neutrophils, epithelial cells, vascular smooth muscle cells, and lung fibroblasts (Smith et al., 2003). Several distinct PDE7 inhibitors have been reported (Pitts et al., 2004; Vergne et al., 2004). As PDE7 is simultaneously expressed in inflammatory cells and in the brain highlights the potential role of PDE7 as drug target for neuroinflammation. It has been shown that selective PDE7 inhibition or dual PDE4/7 inhibition may provide a novel therapeutic approach for the treatment of chronic lymphocytic leukemia (CLL) by enhancing killing and increasing specificity for CLL cells (Zhang et al., 2008).

PDE8s are cAMP specific, widely distributed in various tissues (Glavas et al., 2001; Wang et al., 2001; Kobayashi et al., 2003; Perez-Torres et al., 2003; Dong et al., 2006; Hayashi et al., 2007) and abundant in testis (Vasta et al., 2006). The company Pfizer reported on a small molecule called PF-04957325 that selectively inhibits PDE8 at very low doses (Vang et al., 2010). PDE8-selective inhibitors might be used to correct adrenal insufficiency, and a PDE8 activator might be used to treat Cushing's syndrome (Tsai et al., 2011). It has also been shown that inhibiting PDE8 potentiates the biphasic insulin response to glucose (Dov et al., 2008).

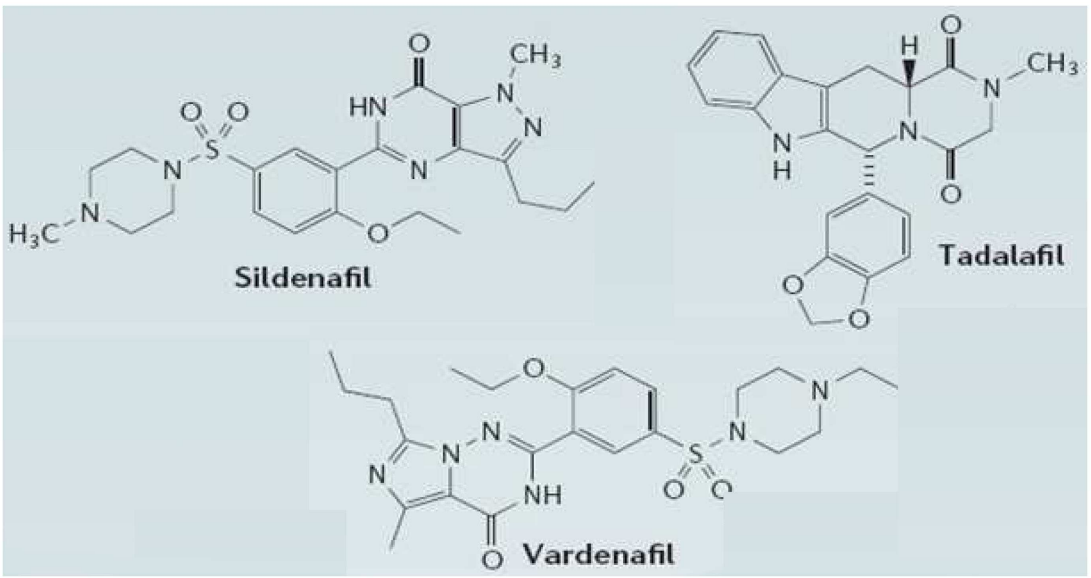

FIGURE 2 | Structures of the three clinically approved phosphodiesterase type 5 inhibitors. Sildenafil, vardenafil, and tadalafil have been approved for treatment of erectile dysfunction. Sildenafil and taladafil have also been approved as a treatment for pulmonary arterial hypertension. Sildenafil citrate is designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate. Vardenafil HCl is designated chemically as piperazine, 1-[[3-(1,4-dihydro-5-methyl-4-oxo-7-propylimidazo[5,1-*f*][1,2,4]triazin-2-yl)-4-ethoxyphenyl]sulfonyl]-4-ethyl-, monohydrochloride. Taladafil is designated chemically as pyrazino[1′,2′:1,6]pyrido[3,4-b]indole-1,4-dione,6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-, (6R,12aR)-.

PDE9 is one of the most recently discovered PDE families. It has a very high affinity for cGMP and it is expressed in a variety of tissues (Soderling et al., 1998a,b). Compared to the other cGMP-specific PDEs, PDE9 apparently lacks the non-catalytic cGMP-binding domain present in the cGMP-specific PDE5 and PDE6 and also in the dually specific PDE2. BAY 73-6691, acting as a PDE inhibitor selective for the PDE9A subtype, is a drug developed by Bayer for the treatment of Alzheimer's disease (van der Staay et al., 2008).

PDE10 was isolated and characterized as a dual-substrate gene family distributed in fetal lungs and brain (Fujishige et al., 1999; Loughney et al., 1999; Soderling et al., 1999). The finding that striatal PDE10 mRNA and protein levels have been found to be reduced in Huntington's disease (Hebb et al., 2004) would impact on the development of PDE10 agonists. Based on their high expression levels in the brain, PDE10s have become a target for central nervous system research, especially concerning cognitive deficits related to schizophrenia and psychotic statuses. A series of pyrazoloquinoline analogs have been synthesized and shown to bind with high affinity to PDE10 (Yang et al., 2012).

PDE11 are characterized by their high affinity for both cAMP and cGMP, although kinetic characteristics for the variants are different (Fawcett et al., 2000; Hetman et al., 2000b; Weeks et al., 2007). BC 11–38 is a recently identified potent and selective PDE11 inhibitor ($IC_{50} = 0.28\,\mu M$) with potential application for adrenal insufficiency (Ceyhan et al., 2012).

PDE INHIBITORS AS POTENTIAL TOOLS IN THE TREATMENT OF CYSTIC FIBROSIS

As an important second messenger signaling molecule, cAMP controls a wide variety of eukaryotic and prokaryotic responses to extracellular cues (Antoni, 2000). As CF is characterized by a defective cAMP-dependent chloride conductance in epithelial cells, it could be expected that modulating intracellular levels of the second messenger would bring beneficial therapeutic effects for patients with CF.

NON-SELECTIVE PDE INHIBITORS

Non-specific PDE inhibitors such as IBMX, theophylline, and DPMX (7-methyl-1,3-dipropylxanthine) have been shown to activate normal and mutated CFTR chloride channels in epithelia (Chappe et al., 1998). Due to impact on the cAMP pathway and activity at low concentrations, studies have looked at the effect of methylxanthines on the cAMP activated CFTR channel. It has been reported that IBMX increases CFTR chloride current in *Xenopus* oocytes expressing F508del-CFTR (Drumm et al., 1991). In nasal bronchial epithelial tissues expressing the mutant F508del-CFTR, treatment with IBMX associated with a potent adenylate cyclase agonist, forskolin was unable to stimulate chloride efflux (Grubb et al., 1993). However, stably transfected F508del-CFTR cells (Haws et al., 1996) showed a sevenfold increase in cAMP levels following IBMX treatment but not after cyclopentyl-1,3-dipropylxanthine (CPX), another non-specific PDE inhibitor. Interestingly both IBMX and CPX potentiated the effect of forskolin on CFTR-mediated efflux of ^{125}I by 2.5-fold (Haws et al., 1996). A potentiation by IBMX of prostaglandin E (PGE)-induced bicarbonate secretion has been reported in the rat duodenum *in vivo* (Takeuchi et al., 1997; Aoi et al., 2004).

SELECTIVE PDE INHIBITORS

PDE inhibitors increase cAMP by inhibiting one or more enzymes involved in cAMP degradation. Cyclic AMP-activated PKA mediates phosphorylation of CFTR and increases the open probability of the CFTR channel. PDE3 inhibitors, amrinone, and milrinone, also cause vasodilation, which may be beneficial for CF airways. Drumm et al. showed that inhibiting PDE had a larger effect on CFTR activation than have adenylate cyclase stimulants (Kelley et al., 1995). Using airway epithelial cell lines expressing wild-type CFTR, Calu-3, and 16HBE cells, it has been found that, at 100 μM concentrations, milrinone, or amrinone applied in the absence of adenylate cyclase activators, stimulate chloride efflux by 13.7-fold (Kelley et al., 1995). No effect on chloride efflux was found under stimulation with IBMX, rolipram, or dipyridamole. The increase of channel efflux by PDE3 inhibitor, amrinone, or milrinone, was not associated with a significant rise in cAMP concentrations but it was inhibited by protein kinase A inhibitors (H-8 and Rp-cAMPS), suggesting that it might work through a more distal signal. Kelley et al. (1996) also looked at endogenous CFTR in transformed nasal polyp tissue of patients homozygous for F508del (CF-T43). They found that, when administered in the presence of a β-agonist (isoproterenol) and protein kinase A activator, milrinone, and amrinone, at 100 μM concentrations, increased chloride efflux by 19–61% from baseline. Mice homozygous for F508del-CFTR were administered with a combination of milrinone (100 μM) and forskolin (10 μM; Kelley et al., 1997). This combination of drugs resulted in an increased magnitude of the nasal potential difference. The implications of this study are exciting; but the effect was confirmed in mice but not in humans (Smith et al., 1999).

It has been shown that CFTR has a major role in the regulation of duodenal bicarbonate secretion (Hogan et al., 1997). Furthermore, O'Grady et al. (2002) showed that both PDE1 and PDE3 are involved in the activation of CFTR in T84 cells and human colonic epithelial cells. Hayashi et al. (2007) suggested that PDE1 and PDE3 are involved in the regulation of duodenal bicarbonate secretion and that the response to PGE2 is associated with both PDE1 and PDE3, while the response to NO is mainly modulated by PDE1 (Hayashi et al., 2007). McPherson et al. (1999) showed that a selective cyclic nucleotide PDE5 inhibitor partially corrected defective L-adrenergic stimulation of mucin secretion in CFTR antibody-inhibited submandibular cells. The PDE5 inhibitor did not increase cAMP levels, nor did it potentiate isoproterenol-induced cAMP rise (McPherson et al., 1999). Of note, Dormer et al. (2005) demonstrated that the PDE5 inhibitor sildenafil also acts as a pharmacological chaperone. Because sildenafil is approved for clinical use, they speculated that their data might speed up the development of new therapies for CF (Dormer et al., 2005).

COMPARISON OF THE PDE5 INHIBITORS

There are distinct differences between the three clinically approved PDE5 inhibitors, sildenafil, vardenafil, and tadalafil, regarding their selectivity and specificity for PDE inhibition, with consequences on safety profile but also on biopharmaceutic and

pharmacokinetic disparities that largely affect efficacy of the compounds (Klotz et al., 2001; Gresser and Gleiter, 2002; Milligan et al., 2002; Muirhead et al., 2002; Nichols et al., 2002; Burgess et al., 2008). Sildenafil and vardenafil are very similar in terms of chemical structure, whereas tadalafil, with a methyldione structure, differs markedly (**Figure 2**). These chemical properties are also reflected in similarities and dissimilarities of their clinical pharmacokinetics.

PDE5 inhibitors are rapidly absorbed after oral administration, with peak concentrations reached slightly earlier for vardenafil compared to sildenafil and tadalafil (Klotz et al., 2001; Gresser and Gleiter, 2002; Milligan et al., 2002; Muirhead et al., 2002; Nichols et al., 2002; Burgess et al., 2008). Although no clear concentration-effect relationships have been established for any of the three PDE5 inhibitors, rapid absorption is considered essential for a rapid onset of efficacy. Administration of a high-fat meal had no significant effect on the rate and extent of absorption of tadalafil but decreased the rate of absorption for sildenafil and vardenafil. All three drugs are lipophilic and have a volume of distribution larger than the volume of total body water, indicating tissue uptake and binding. Furthermore, the three compounds are highly protein bound, with free plasma concentration fractions of only 4–6%.

The major route of elimination for all PDE5 inhibitors is hepatic metabolism, with renal excretion of unchanged drug accounting for 1% or less of the elimination pathways. Based on their relatively high systemic clearance after intravenous administration, sildenafil, and vardenafil can be classified as non-restrictively cleared drugs with intermediate to high hepatic extraction ratio. The relatively comparable distribution volumes together with the substantial differences in systemic clearance among the PDE5 inhibitors result in distinct differences of the elimination half-life, 3–5 h for sildenafil and vardenafil compared to 17.5 h for tadalafil. Tadalafil, however, has been detected in plasma even 5 days after oral administration, in line with its long half-life. This suggests the possibility of accumulation if taken regularly and in short intervals, which may result in an increased risk of side effects with excessive use.

PDE5 INHIBITORS FOR THE TREATMENT OF CYSTIC FIBROSIS

So far, many efforts have been focused on CFTR pharmacotherapy to target the abnormal protein pharmacologically by various approaches such as direct correction of stop codon mutations, CFTR channel activation, or trafficking defects. High-throughput screening has been used to identify molecules that increase F508del-CFTR activity (Pedemonte et al., 2005; Van Goor et al., 2006; Carlile et al., 2007). Such molecules have been categorized according to whether they improve the folding/cellular processing defect (correctors) or increase the responsiveness of F508del-CFTR channels already present in the membrane to cAMP activation (potentiators). Sildenafil has been initially shown to correct F508del-CFTR processing when used at supratherapeutic doses (Dormer et al., 2005).

PDE5 INIBITORS CORRECT TRANSEPITHELIAL CHLORIDE TRANSPORT IN CYSTIC FIBROSIS: PARENTERAL ADMINISTRATION

To test the hypothesis that PDE5 inhibitors sildenafil, vardenafil, and taladafil, when applied at therapeutic doses, are able to restore transepithelial ion transport abnormalities of the F508del-CFTR protein, we have conducted experimental studies (Lubamba et al., 2008, 2011) in CF mice homozygous for the F508del mutation (van Doorninck et al., 1995) and in their corresponding wild-type homozygous normal mice. The F508del-CFTR mouse model has been chosen because F508del is the most common and one of the most severe CF mutations and because the mouse model recapitulates, although with different degrees of severity in the different systems, the human disease phenotype. Epithelia of the F508del-CF mouse model are characterized by defective electrolyte transport, and *Pseudomonas aeruginosa* lipopolysaccharide (LPS) exposure mimics several aspects of CF airway epithelial inflammation such as increased pro-inflammatory cytokines, most notably interleukin (IL)-8, IL-6, and Tumor Necrosis Factor (TNF)-α, and the predominant neutrophil infiltration.

In our protocols, CFTR function has been assessed *in vivo* by measuring the transepithelial nasal potential difference, a diagnostic technique that has been more recently used as an index of therapeutic efficacy in novel fundamental therapies, either in animal models (Lubamba et al., 2008, 2009, 2011) or in CF patients (Sermet-Gaudelus et al., 2010; Leonard et al., 2012a,b). Our results provide clear evidence that intraperitoneal injection of PDE5 inhibitors (**Figure 3**), at therapeutic doses, to F508del-CF mice interact with CFTR, propping open the mutant protein to allow a normal flow of chloride ions across the epithelium of nasal mucosa, thereby restoring the decreased or even abolished CFTR-dependent chloride transport (Lubamba et al., 2008). In F508del mice, but not in *cftr* knockout mice, the chloride conductance, evaluated by perfusing the nasal mucosa with a chloride-free solution in the presence of amiloride (to inhibit sodium entry through the epithelial sodium channel ENaC) and with forskolin, is corrected 1 h after a single sildenafil administration (**Figure 4A**). A more prolonged effect, persisting for at least 24 h, is observed with vardenafil (**Figure 4B**). Moreover, vardenafil, but not sildenafil, is able to stimulate chloride transport associated with normal wild-type CFTR protein (**Figure 4B**). The forskolin response is increased after treatment with sildenafil or vardenafil in wild-type and in F508del mutant animals. In F508del mice, the chloride conductance in the presence of 200 μM DIDS (4-4′-diisothiocyanostilbene-2,2′-disulphonic acid), an inhibitor of alternative chloride channels, was much higher after sildenafil injection than following placebo treatment. This observation, in addition to the finding that no activating effect of chloride transport can be observed after treatment with PDE5 inhibitors in animals knockout for the CFTR protein, indicates that the action of PDE5 inhibitors on chloride transport across the respiratory epithelium involves F508del-CFTR and not a CFTR bypass channel. No effect on the sodium conductance was detected in any group of animals.

PDE5 INHIBITORS CORRECT TRANSEPITHELIAL CHLORIDE TRANSPORT IN CYSTIC FIBROSIS: INHALATIONAL ADMINISTRATION

More recently, animal studies have shown that nebulizing F508del-CF mice with any of the PDE5 inhibitors sildenafil, vardenafil, or taladafil led to correction of the nasal chloride transport (Lubamba et al., 2011). Correction is largest with taladafil and smallest, but still highly significant, with sildenafil. The effect of vardenafil, but

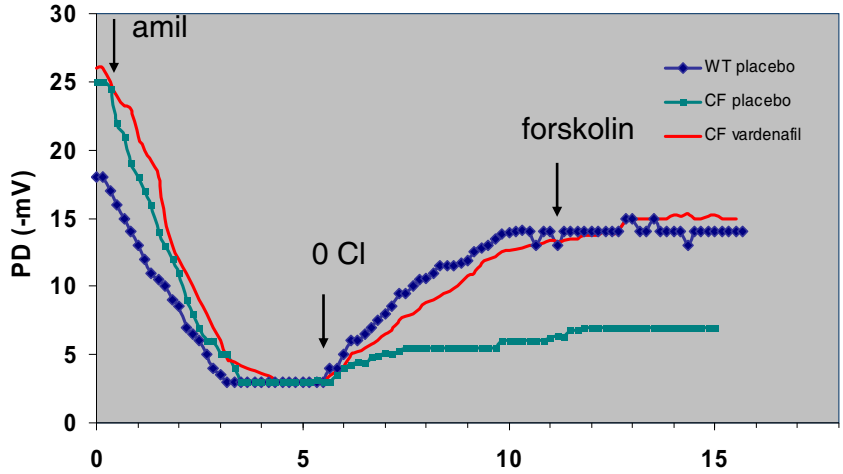

FIGURE 3 | Representative tracings of nasal potential difference (PD) measurements in wild-type (WT) and F508del-CF (CF) mice 24 h after placebo (saline) or vardenafil (single i.p. dose of 0.14 mg/kg body weight). Tracings show sequential response of the nasal surface to perfusion successively with basal solution, basal solution with 10^{-4} M amiloride (amil), chloride-free solution plus amiloride (0 Cl), and chloride-free solution with amiloride plus 10–5 M forskolin (forskolin). Arrows indicate change of solutions. As illustrated, basal values and amiloride response are not influenced by vardenafil treatment. However, chloride secretion (difference between values obtained at the end of the test and the end of the amiloride phase) is restored in CF animals and the effect lasts at least 24 h after vardenafil treatment (Lubamba et al., 2008).

not sildenafil, lasts at least 8 h after a single inhaled therapeutic dose. These findings clearly identify the inhalational route as a potential therapy for PDE5 inhibitors in CF which is clinically relevant taking into account the cost of systemic side effects of the drugs (Dalby and Suman, 2003).

Consistent with our results, it has recently been demonstrated that the inhalation route of administration for vardenafil is associated with an acceptable safety profile. Apart from brief coughing on inspiration, no clinically significant changes in blood pressure or heart rate and no serious adverse events were recorded (Berry et al., 2009). Inhalation drug therapy has several potential advantages over oral and intravenous routes, including rapid onset of pharmacological action, minimized systemic adverse effects and reduced effective drug doses compared to the same drug delivered orally (Berry et al., 2009); this greatly highlights the impact of our work for translational science.

PDE5 INHIBITORS ATTENUATE EXAGGERATED INFLAMMATORY RESPONSES IN CYSTIC FIBROSIS

Another important goal of mutation-specific CF treatment is attenuation of exaggerated lung inflammatory responses (Legssyer et al., 2006; Gavilanes et al., 2009; Meyer et al., 2009). As lung inflammation plays a major role in morbi-mortality in CF, identifying a therapeutic strategy that combines ability to correct the basic ion transport defect and to reduce dysregulated inflammatory responses is very exciting and promising. It has been reported that sildenafil reduces neutrophil lung infiltration in murine airways infected with *P. aeruginosa* (Poschet et al., 2007). In addition, toxicological studies have shown that sildenafil pretreatment attenuates acrolein-triggered airway inflammation associated with mucin overproduction (Wang et al., 2009).

More recently, we have found that vardenafil, selected as a representative PDE5 inhibitor for its longer-lasting CFTR activating effect, modulates the vicious circle of lung inflammation and attenuates the expression of pro-inflammatory cytokines and chemokines and cell infiltrates in the bronchoalveolar lavage (BAL) of CF and wild-type mice (Lubamba et al., 2012b). Intraperitoneal administration of a single pharmacological dose (0.14 mg/kg body weight) of vardenafil is followed by a reducing response in cell infiltrate and in the biosynthesis of several biomarkers of the inflammatory response. Most notably, levels of CCL-2 (chemokine C-C motif ligand), a cytokine playing a key role in the contribution of macrophages in the inflammatory response (Meyer et al., 2009), are significantly reduced in the BAL fluid after vardenafil treatment, particularly in CF animals (**Figure 5**).

The mechanism of action of vardenafil as an anti-inflammatory agent in CF as well as the target-effector cells involved in these responses are under investigation by our group. Altogether, our data indicate that PDE5 inhibitors have a strong therapeutic potential for treating CF. A clinical trial aimed at investigating the safety and efficacy of sildenafil in CF lung disease is currently listed on www.clinicaltrials.gov (NCT00659529).

PERSPECTIVE FUTURE RESEARCH

Beside the clinical application for erectile dysfunction and for pulmonary arterial hypertension, a growing body of research has confirmed putative beneficial effects of PDE5 inhibitors in CF. Recent studies conducted in F508del and in wild-type CFTR expressing *Xenopus laevis* oocytes and human bronchial epithelial cells have indicated that sildenafil acts as a corrector and as a potentiator of the mutant and wild-type protein by distinct cGMP-independent and cGMP-dependent mechanisms respectively (Leier et al., 2012). While in *X. laevis* oocytes, low (1.5 μmol/l) doses were required to rescue F508del-CFTR function and cell membrane localization, suprapharmacological doses roughly 120 times larger than those commonly used for the treatment of erectile dysfunction

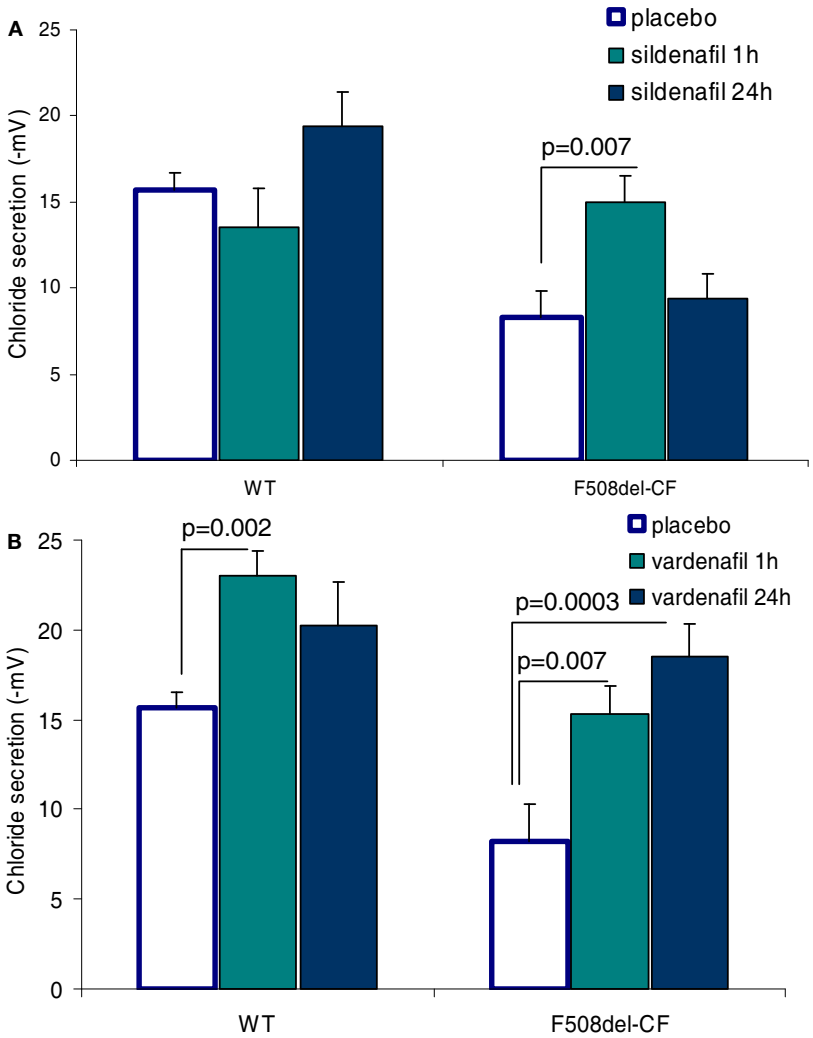

FIGURE 4 | Effect of parenteral (i.p.) administration of sildenafil (A; 0.7 mg/kg body weight) and of vardenafil (B; 0.14 mg/kg body weight) on CFTR-dependent chloride secretion assessed by means of the nasal potential difference (PD) in wild-type (WT) and F508del-CF mice.

Vardenafil stimulates chloride secretion of the wild-type CFTR. The correcting effect of vardenafil lasts at least 24 h. Data are expressed as mean ± SEM of 14–15 placebo treated animals and six animals treated with PDE5 inhibitors (Lubamba et al., 2008).

were needed to achieve the same correcting effects in human bronchial epithelial cells (Leier et al., 2012). In this perspective, adverse drug effects including flushing, headache, and other cardiovascular effects could compromise the potential use of PDE5 inhibitors in CF.

Attempts should therefore be made either to achieve chemical modifications of PDE5 inhibitors with enhanced biochemical potency and selectivity or to allow inhalational therapy of the drugs. A structural analog of sildenafil, KM11060, designated chemically as 7-chloro-4-{4-[4-chlorophenyl)sulfonyl]piperazino}quinoline, has been recently identified as a novel potent corrector of the F508del-CFTR trafficking defect (Robert et al., 2008). F508del-CFTR trafficking was partially restored and maturation of the mutant protein was significantly increased in baby hamster kidney cells treated with low doses for a short duration (10 nM for 24 h or 10 μM for 2 h) of the compound (Robert et al., 2008). Since the morbi-mortality of CF is mostly related with respiratory manifestations and an acceptable safety profile with no serious adverse events was recorded when vardenafil was applied by inhalational route (Berry et al., 2009), topical airway deposition of PDE5 inhibitors (Lubamba et al., 2011) should be considered in future human studies. As a matter of fact, inhalation drug therapy has several potential advantages over oral and intravenous routes, including rapid onset of pharmacological action, minimized systemic adverse effects, and reduced effective drug doses compared to the same drug delivered orally (Dalby and Suman, 2003).

CONCLUSION

Despite great advances in the understanding of the genetics and pathophysiology of the disease, there is still no cure for CF and existing therapies have mainly aimed at alleviating clinical

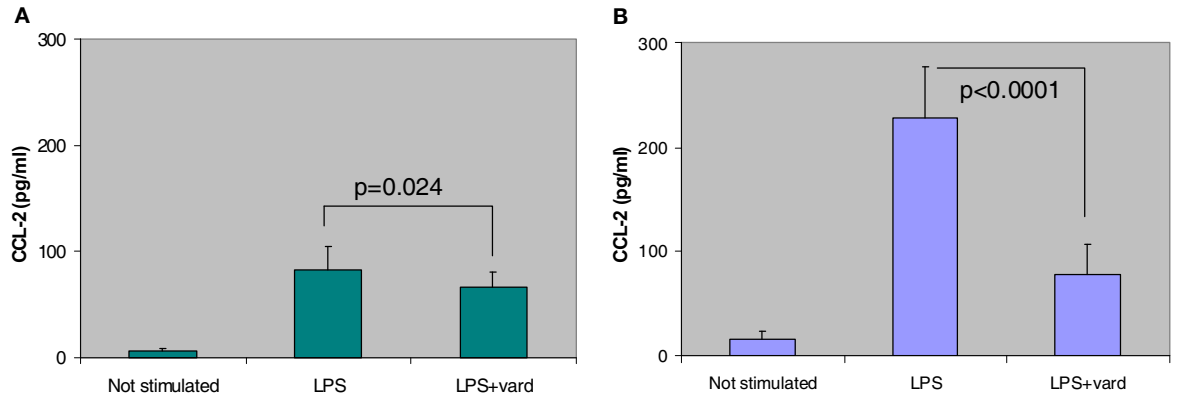

FIGURE 5 | Anti-inflammatory effect of *in vivo* treatment, by i.p. injection, of a single therapeutic dose of vardenafil (vard) to wild-type (A) and F508del-CF (B) mice on the inflammatory response induced by lipopolyssaccharide from *P. aeruginosa* (LPS). Biosynthesis of CCL-2 is significantly reduced in the bronchoalveolar lavage of vardenafil-treated CF and non-CF animals (Lubamba et al., 2012b).

symptoms. Recent experimental evidence has highlighted the potential of PDE5 inhibitors, sildenafil, vardenafil, and taladafil, as therapeutic agents in CF. As the drugs are able to correct the basic transepithelial ion transport abnormalities and to limit exaggerated inflammatory responses related to the presence of F508del-CFTR protein, they can represent promising compounds for fundamental pharmacotherapy in CF. Since the drugs are in clinical use, therapeutic approaches to address F508del-CFTR defects by PDE5 inhibitors can be considered as a "low-hanging fruit" strategy in the drug discovery tree which could speed up their development as CF therapeutics, as compared to other agents that are under investigation only for CF therapy and for which further exploratory studies are needed before being streamed toward clinical testing. In summary, CFTR correction with PDE5 inhibitors is a promising therapeutic approach based on functional correction of F508del-CFTR activity and on a possible anti-inflammatory action in F508del mice. The effects of these compounds on other CF mutation classes remain to be assessed. The routes for administration should also be further explored, and aerosolized delivery of PDE5 inhibitors should be considered.

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Decreasing poly(ADP-ribose) polymerase activity restores ΔF508 CFTR trafficking

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Most cystic fibrosis is caused by mutations in CFTR that prevent its trafficking from the ER to the plasma membrane and is associated with exaggerated inflammation, altered metabolism, and diminished responses to oxidative stress. PARP-1 is activated by oxidative stress and causes energy depletion and cell dysfunction. Inhibition of this enzyme protects against excessive inflammation and recent studies have also implicated it in intracellular protein trafficking. We hypothesized that PARP-1 activity is altered in CF and affects trafficking and function of the most common CF mutant ΔF508 CFTR. Indeed, PARP-1 activity was 2.9-fold higher in CF (ΔF508/ΔF508) human bronchial epithelial primary cells than in non-CF cells, and similar results were obtained by comparing CF vs. non-CF bronchial epithelial cell lines (2.5-fold higher in CFBE41o$^-$ vs. 16HBE14o$^-$, $P < 0.002$). A PARP-1 inhibitor (ABT-888, Veliparib) partially restored CFTR channel activity in CFBE41o$^-$ cells overexpressing ΔF508 CFTR. Similarly, reducing PARP-1 activity by 85% in ileum from transgenic CF mice (*Cftr^{tm1} Eur*) partially rescued ΔF508 CFTR activity to 7% of wild type mouse levels, and similar correction (7.8%) was observed *in vivo* by measuring salivary secretion. Inhibiting PARP-1 with ABT-888 or siRNA partially restored ΔF508 CFTR trafficking in cell lines, and most ΔF508 CFTR was complex glycosylated when heterologously expressed in PARP-1$^{-/-}$ mouse embryonic fibroblasts. Finally, levels of the mature glycoform of CFTR were reduced by peroxynitrite, a strong activator of PARP-1. These results demonstrate that PARP-1 activity is increased in CF, and identify a novel pathway that could be targeted by proteostatic correctors of CFTR trafficking.

Keywords: CF, Cystic fibrosis, ABT-888, PARP-1, oxidative stress, DNA damage PARP-1$^{-/-}$

INTRODUCTION

Cystic fibrosis is the most prevalent inherited disease amongst Caucasians, afflicting ∼70,000 people worldwide (Riordan et al., 1989). The symptoms of cystic fibrosis include progressive respiratory dysfunction due to persistent and repeated cycles of infection and inflammation, and are caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR encodes an ATP-binding cassette (ABC) transporter that functions as a tightly regulated anion channel. Over 1,900 mutations in CFTR have been identified, the most prevalent being an in-frame deletion of Phe at the 508 position (ΔF508; Bobadilla et al., 2002). This mutation, which is present on at least one chromosome in 90% of people with CF, causes the mutant protein to be recognized by the cellular quality control machinery and retained at the ER where it is then degraded (Cheng et al., 1990). ΔF508 also reduces the open probability of mutant channels that reach the plasma membrane shortening their half-life at the cell surface (Lukacs et al., 1994). Although the life expectancy for CF patients has improved in recent years due to improved antibiotics, pancreatic enzyme supplements, and therapeutic regimens, there remains no cure for most people with CF who carry CFTR mutations that cause defective trafficking.

Cystic fibrosis transmembrane conductance regulator mutations cause a myriad of downstream biological changes, and the relationship between these changes and the disease phenotype remains poorly understood. Markers of oxidative stress are elevated in the plasma, presumably due to pulmonary infection (Brown et al., 1996; Collins et al., 1999). CF patients also display increased susceptibility to oxidative-induced DNA damage as measured by urinary excretion of 8-hydroxydeoxyguanosine, and this sensitivity to oxidants may be an inherent property of the disease since it appears to be independent of clinical status (Brown et al., 1995). High intracellular levels of hydrogen peroxide and mitochondrial reactive oxygen species (ROS) have been reported in CFTR-deficient cells (Rottner et al., 2009), and a deficiency in reduced glutathione (GSH) in the respiratory epithelial lining fluid and plasma has been known for some time (Roum et al., 1993). Thus several lines of evidence suggest that CF leads

to redox disturbances, as recently reviewed (Galli et al., 2012). One mechanism used by cells to protect against oxidative DNA damage is PolyADP (Ribose) Polymerase-1 (PARP-1), the most abundant isoform of a family of nuclear enzymes that sense DNA damage and initiate DNA repair. PARP-1 is activated by cell stress and plays an important role during tissue injury (Luo and Kraus, 2005; Pacher and Szabo, 2008). It uses NAD^+ to transfer polymers of ADP-ribose to target proteins at the expense of ATP, a post-translational modification known as poly ADP-(ribosyl)ation (PARylation). PARP-1 function depends on the type, duration and strength of the stress stimuli, and on the proliferative and metabolic state of the cell (Luo and Kraus, 2005), and is intimately tied to nuclear NAD+ metabolism and the broader cellular metabolic profile (Luo and Kraus, 2005).

Under conditions of cell stress the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) becomes hyperactive and depletes cells of NAD^+. This slows glycolysis, reduces electron transport and ATP formation, and may lead to the upregulation of proinflammatory pathways or cell death (Cuzzocrea, 2005; Pacher and Szabo, 2008). Thus, from a pathophysiological standpoint, PARP activation could contribute to disease by driving the cell into an energetic deficit, and also by inducing a state of dysfunction through activation of proinflammatory pathways (Cuzzocrea, 2005). Both these mechanisms have been implicated in CF. We hypothesize that the misfolded mutant CFTR is associated with an increase in PARP activity. Decreasing this activity may restore some functional correction.

In this study we have investigated a possible role of PARP-1 in the regulation of ΔF508 CFTR trafficking and function in CF bronchial epithelial cells. The involvement of PARP-1 in oxidative stress, inflammatory responses, and energy maintenance, and its emerging role as a regulator of intracellular protein trafficking (Abd Elmageed et al., 2011) suggested that it may be an interesting potential target for small molecule correctors in Cystic fibrosis.

MATERIALS AND METHODS

REAGENTS

4-Amino-1,8-naphthalimide (4-AN) and N-(6-Oxo-5,6-dihydro phenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride (PJ34) were obtained from Sigma-Aldrich (Oakville Ontario, Canada). (2-((2R)-2-Methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide dihydrochloride; ABT-888) was purchased from Alexis Biochemicals (Famingdale, NY, USA). VRT-325 was a generous gift from Dr. Robert Bridges (Rosalind Franklin University of Medicine and Science) and the Cystic Fibrosis Foundation Therapeutics Inc. (CFFT). The monoclonal antibody against the R domain of CFTR (clone 23C5) was generated by our lab (Myriam Mirza, Veli-Pekka Määttänen and D. Y. T., unpublished data). M3A7 monoclonal antibody against CFTR was purchased from Chemicon (Billerica, MA, USA). α-tubulin was obtained from Sigma-Aldrich and monoclonal antibody ($IgG2_a$) against PARP-1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PAR was obtained from Trevigen (Gaithersburg, MD, USA). Rabbit anti-hERG antibody was obtained from Calbiochem (Burlington, Ontario, Canada). Peroxynitrite (tetramethylammonium) was obtained from Alexis Biochemicals and prepared in ice cold 0.01 M KOH as per the manufacturer's instructions.

Homozygous Δ508 CFTR mice (*Cftr^{tm1} Eur*; van Doorninck et al., 1995) and wild type littermates controls were used in the *ex vivo* experiments. Breeders and protocols for mouse intestinal assays were kindly provided by B. J. Scholte, M. Wilke, and H. R. de Jonge, Erasmus University Medical Center, Rotterdam, NL. The mice were kept in the animal facility at McGill University and fed a high protein diet (SRM-A, Hope Farms, Woerden, Netherlands) modified to contain pork instead of beef. All procedures followed Canadian Institutes of Health Research (CIHR) regulations and were approved by the faculty Animal Care Committee of McGill University.

CELL CULTURE AND TREATMENTS

HEK293 cells were stably transfected with HA tagged hERG G601S (generous gift of E. Ficker; Wible et al., 2005) and maintained in standard culture conditions.

Stably transfected BHK cells expressing histidine-tagged (His) wt-CFTR or ΔF508 CFTR were maintained in DMEM-F12 media supplemented with 5% FBS and 450 μM methotrexate. 1% L-Glutamax. CFBE41o$^-$ cell lines transduced with TranzVector lentivectors containing ΔF508 CFTR (CFBE41o-ΔF508) and wild type CFTR (CFBE41o$^-$CFTR) cells were kindly provided by J. P. Clancy (Bebok et al., 2005) and were maintained in EMEM (Wisent, St-Bruno, QC, USA) supplemented with 10% FBS and 1% L-Glutamax. For polarized CFBE41o$^-$ monolayers (ΔF508 and wt-CFTR), cells were cultured under liquid/liquid conditions and polarized at the air/liquid interface. Cells were seeded at a density of 2.5×10^5 cells/cm^2 onto 12 mm fibronectin-coated Snapwell inserts (Corning Incorporated). The apical medium was removed after 24 h to establish an air-liquid interface (ALI), and then the cells were cultured for another 6–7 days (Bebok et al., 2005). CFBE41o$^-$ cells were treated with 4-AN (Sigma-Aldrich), PJ34 (Sigma-Aldrich), or ABT-888 (Alexis Biochemicals) for 24 h or as shown and at the indicated concentrations. Low temperature rescue was carried out at 29°C for 24 h or as indicated. DMSO was used as a vehicle at a 1:1000 dilution. Primary Human Bronchial Epithelial cells (HBEs) were isolated from human bronchial tissue by enzyme digestion and cultured in bronchial epithelial growth medium (BEGM; Fulcher et al., 2005) on vitrogen-coated plastic flasks (Vitrogen 100, PureCol, Advanced BioMatrix #5005-B). They were then trypsinized, counted, and cryopreserved or transferred onto collagen VI-coated snapwell culture inserts (Corning, catalog no. 3801) in ALI medium (Fulcher et al., 2005) at a density of 2.5×10^5 cells/insert. During the first 4 days, the ALI medium was changed daily, then apical media was removed and the cells were grown in an ALI for 22 days before use. For the CF HBE cells, the isolation and growth media were complemented with specific antibiotics based on the patient's microbiology report.

IMMUNOBLOTTING AND DENSITOMETRY

BHK cells overexpressing (His) ΔF508 CFTR and wt-CFTR, and CFBE41o$^-$ cells (overexpressing ΔF508 and wt-CFTR) were lysed in RIPA buffer containing protease inhibitors (Roche, Inc.) and 0.8% deoxycholic acid prepared as described (Robert et al., 2008). Briefly, 10 μg (BHK) and 20 μg (CFBE41o$^-$) total protein were separated using 6% (v/v) SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with monoclonal

anti-CFTR antibody (clone 23C5) at a 1:10 dilution overnight at 4°C for CFBE41o$^-$ lysates or 1:1000 dilution for BHK lysates (clone M3A7). Membranes were reprobed for PARP1 with monoclonal antibody against PARP1 at a 1:500 dilution. For immunoblotting against hERG in the HEK293 cells, polyclonal antibody against hERG was used overnight at 4°C at 1:1000 dilution. Membranes were probed with monoclonal anti-tubulin (Sigma-Aldrich) to normalize for protein loading. The relative levels of each CFTR glycoform were estimated by densitometry using the Image J program (http://rsb.info.nih.gov/ij/). The relative amount of band B or band C is calculated as a fraction of tubulin for the respective lane and reported as a fraction of the total (band C/tub + band B/tub). The values reported are expressed as means ± SD ($n = 3$). Data sets were compared by a Student's t-test using SigmaPlot (Systat Software, Inc.).

HALIDE FLUX ASSAY AND VOLTAGE CLAMP STUDIES OF CFBE41o$^-$ CELL MONOLAYERS

Iodide efflux from BHK cells expressing (His) ΔF508 CFTR was assayed using a robotic liquid handling system (BioRobot 800 Qiagen, USA) and Qiagen 4.1 software. Iodide concentration at the end of each sample period was measured using an iodide-sensitive electrode (Orion Research, Inc., Boston, MA, USA) and converted to iodide content released per 1 min interval. Relative iodide efflux rates were calculated by subtracting the baseline from the peak iodide flux (in μmol/min). Data were calculated from at least three independent experiments each with four replicates, and are reported as ±SEM. Short-circuit current (I_{sc}) was measured across monolayers mounted in modified Ussing chambers. CFBE41o$^-$ cells over expressing ΔF508 CFTR and wt-CFTR (250 000) were seeded onto 12-mm fibronectin-coated Snapwell inserts (Corning Incorporated) and the apical medium was removed after 24 h to establish an ALI. CFBE41o$^-$ ΔF508 monolayers were treated on both sides with Opti-MEM medium (no FBS) and one of the following compounds: 0.1% DMSO (negative control), 1 nM ABT-888, 10 μM VRT325. Alternatively, CFBE41o$^-$ΔF508 cells were incubated at 29°C (positive control) for 24 h before being mounted in chambers and voltage clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA, USA). The assay was performed as described previously (Robert et al., 2008).

PARP1$^{-/-}$ AND PARP1$^{+/+}$ MOUSE EMBRYONIC FIBROBLASTS

PARP1$^{-/-}$ and PARP1$^{+/+}$ MEFs were obtained from Françoise Dantzer (CR1, CNRS, University of Strasbourg, France) and maintained in DMEM supplemented with FBS (10%) and gentamicin (1%) at 37°C, 5% CO_2. The cells were transfected with wild type or ΔF508 triple-HA tagged CFTR in pcDNA3.1 plasmid using Fugene according to the manufacturer's guidelines. Briefly, 2×10^5 cells were seeded in a 6-well plate and transfected when at least 50% confluent using 7 μl Fugene: 2 μg DNA. For immunoblotting, 40–50 μg of total protein was loaded into each lane and membranes were probed with monoclonal antibodies against CFTR and tubulin (see above).

PARP ACTIVITY ASSAYS

Approximately 5×10^4/200 μl CFBE41o$^-$ cells were cultured in 96 well plates and treated with ABT-888 at the indicated concentrations for 24 h. Cells were then lysed buffer (supplied in kit) to which 4 mM NaCl, 1% Triton X-100, and 200 μM PMSF are added. Total protein is quantified by the Bradford assay and 25 μg total protein per well was assayed in triplicate. PARP activity was measured in histone-coated strip wells using the High Throughput (HT) Chemiluminescent PARP/Apoptosis Assay (Trevigen) following the manufacturer's procedures. This ELISA measures the incorporation of biotinylated poly(ADP-ribose; PAR) into histone proteins by chemiluminescence after samples are incubated with anti-PAR antibody and then HRP-conjugated secondary antibody (anti-mouse IgG-HRP). Readings were taken using an HT Analyst Criterion Host, and the light output was proportional to PARP-1 activity. PARP activity for each sample is calculated from a standard curve ran in triplicates within each experiment. Results are expressed in mUnits PARP activity/μg of total protein.

In vitro PARP-1 activity was measured HT Universal Chemiluminescent PARP Assay (Trevigen) following the manufacturer's instructions.

Inhibitors are identified when a PARP mediated increase in fluorescence signal indicating the accumulation of NAD^+ (or the decrease in the PARP mediated depletion of NAD^+). In the absence of PARP maximal signal is observed, whereas in its presence minimal signal is observed and% inhibition is calculated: % inhibition = 100 x [NAD remaining] for inhibitor at specific concentration/[NAD remaining] no inhibitor.

Since DMSO inhibits inhibitors were tested by serial dilution in water. Readings were taken automatically by the HT Analyst Criterion Host.

siRNA SILENCING OF *PARP1*

siRNAs (smart pool) were obtained from Thermo Scientific Dharmacon (Lafayette, CO, USA). siRNA was transfected using the NHBE Nucleofector kit (Lonza, Walkersville, USA), program W-001. *siPARP1/2* (200 nM) or scrambled siRNA (200 nM) was used per 10 cm plate of CFBE41o$^-$ cells overexpressing ΔF508 CFTR. Following nucleofection, cells plated in fibronectin-coated 6-well plates in Opti-MEM overnight. The following day the medium was replaced with EMEM supplemented with 1% penicillin/streptomycin, 1% glutamate, and 10% FBS. Cells were lysed and total protein quantified by the Bradford assay. Immunoblots were probed for CFTR and PARP1 to assess trafficking and protein knockdown, respectively, as described above.

EX VIVO ASSAYS OF ABT-888 ON CFTR-DEPENDENT CURRENT

Mice were genotyped by RT-PCR using tail DNA and used for experiments between age 14–17 weeks (24–30 g). Homozygous Δ508 CFTR mice (*Cftr^{tm1} Eur*; van Doorninck et al., 1995) and wild type littermates controls were used in the *ex vivo* experiments. For *ex vivo* experiments, the mucosa from the distal third of the ileum was stripped of muscle and mounted in Ussing chambers as described previously (Robert et al., 2008). Indomethacin (10 μM) was added to both sides to block prostaglandin synthesis and 10 μM amiloride was added apically to inhibit electrogenic Na^+ absorption. CFTR-dependent I_{sc} was measured after 10–15 min equilibration, then 10 μM forskolin and 50 μM genistein

were added (0 h). Both sides were then exposed to 1 nM ABT-888 or vehicle (distilled water) for 4 h, and the I_{sc} response to forskolin + genistein was measured again. Results are expressed as the mean ± SEM of *n* pieces of ileum from 5 wt-CFTR and 5 ΔF508 mice.

SALIVARY SECRETION

The procedure followed those described by Best and Quinton (Best and Quinton, 2005). Homozygous ΔF508 CFTR mice (*Cftr*$^{tm1\ Eur}$; French et al., 1997) and wild type littermates were used at 10–12 weeks, 20–25 g. They were injected intraperitoneally with saline containing ABT-888 (5 mg/kg/day) or vehicle alone (saline) for 2 days. Details of the procedure have been described (Robert et al., 2008). Results are expressed as the mean ± SEM of *N* mice.

RESULTS

ELEVATED PARP-1 ACTIVITY IN HUMAN CF BRONCHIAL EPITHELIAL CELLS

We measured PARP-1 activity in CF and non-CF primary HBEs by using ELISA to determine the rate at which its substrate NAD^+ is assembled into polymers of ADP-Ribose as illustrated in **Figure 1A**. PARP-1 activity was 2.9-fold higher in HBEs from two CF patients when compared with cells from two non-CF subjects (**Figure 1B**, Students *t*-test, $P < 0.05$). Since primary HBEs may vary due to differences in genetic background and perhaps previous infection and inflammation history, PARP-1 activity was also measured in the commonly used CF and non-CF cell lines CFBE41o$^-$ and 16HBE14o$^-$, respectively, which express only endogenous mutant or wild type CFTR at low levels. PARP-1 activity was also higher in the CF cell line compared with the non-CF line (**Figure 1C**, 2.5-fold, $n = 6$, Students' *t*-test, $P = 0.002$), as observed in primary HBEs. PARP-1 activity in CFBE41o$^-$ cells was sensitive to ABT-888 (Veliparib) at 1 nM, decreasing PARP-1 activity ~40% ($P < 0.05$), but not reducing it to wild type levels. ABT-888 is a potent inhibitor of PARP-1 in other cells systems, where it has an $IC_{50} = 5$ nM (Donawho et al., 2007). We treated wild type cells with ABT-888 (1 nM) and found that we could not abolish PARP-1 activity (**Figure 1C**), in agreement with previous evidence that some baseline PARP-1 activity is required for the maintenance of genomic stability (Luo and Kraus, 2005).

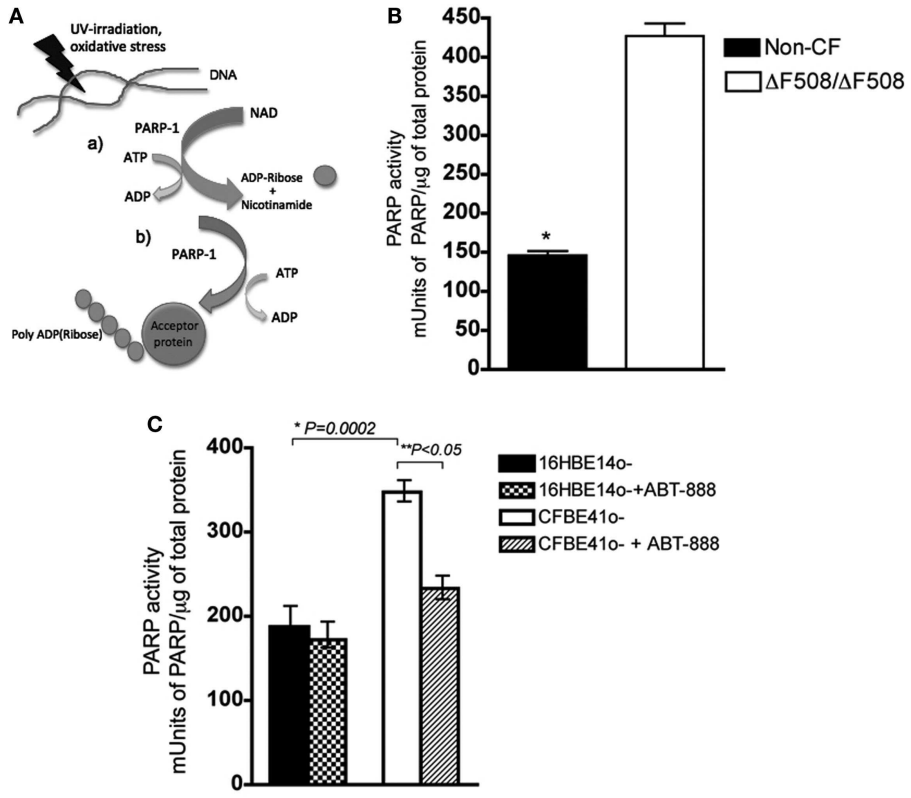

FIGURE 1 | PARP activity is elevated in CF primary and transformed epithelial cells and ABT-888 decreases PARP and activity in CF cells. **(A)** (a) PARP-1 cleaves NAD^+, releasing ADP-Ribose at the expense of ATP (b) PARP-1 synthesizes polymers of ADP-Ribose onto acceptor proteins **(B)** PARP-1 activity was measured in primary lung epithelial cells from two CF patients and non-CF donors. Activity is reported as mUnits of PARP-1/total protein (μg), where 1 unit is defined as the amount of PARP-1 that incorporates 100 pmol poly (ADP-ribose) from NAD^+ into an acid-insoluble form. Bar graph shows the mean ± SD from $n = 3$ measurements per patient, and *denotes a significant difference at a $P < 0.05$ (two-tailed Student's *t*-test). **(C)** PARP-1 activity is higher in the CFBE41o$^-$ (CF) cell line than in the 16HBE14o$^-$ (non-CF) cell line. PARP-1 activity is reduced by ~40% in CFBE cells upon treatment with the PARP-1 inhibitor ABT-888 at the 1 nM (24 h) but ABT-888 has no effect on PARP-1 activity in the non-CF 16HBE14o$^-$. Bar graph represents the mean ± SEM from $n = 5$ with three replicates each. Results are considered significant at $*P = 0.0002$ and $**P < 0.05$, as assessed by the paired, two-tailed Student's *t*-test.

MODULATING PARP-1 ACTIVITY RESTORES CHLORIDE FUNCTION

To examine the influence of PARP-1 on CFTR channel activity, iodide (I^-) efflux was measured from BHK cells overexpressing ΔF508 CFTR after pretreatment with PARP-1 inhibitors for 24 h. Cells were pretreated with inhibitor, loaded by incubation in iodide solution for 1 h, then stimulated acutely with 10 μM forskolin (Fsk) in combination with the potentiator 50 μM genistein (Gst). Pretreating cells with a well characterized corrector VRT325 (10 μM, 24 h) increased the iodide efflux threefold compared to the DMSO vehicle control (**Figures 2A,B**, $P < 0.05$, Student's *t*-test), consistent with a previous report (Loo et al., 2005). Pretreatment with ABT-888 increased iodide efflux (2.7-fold above DMSO; **Figure 2B**, $n = 3$, Students' *t*-test, $P < 0.05$) to levels comparable to VRT325. We also tested other PARP-1 inhibitors with different structures (though all share the same carbonyl group), potencies and mechanisms of action (see **Figure 2C** for structures), and an inactive analog of 4-AN (4-ANin; **Figures 2B,C**). Both PJ34 and 4-AN increased forskolin + genistein stimulated iodide release from BHK cells stably expressing ΔF508 CFTR, suggesting partial rescue of functional channels to the cell surface representing

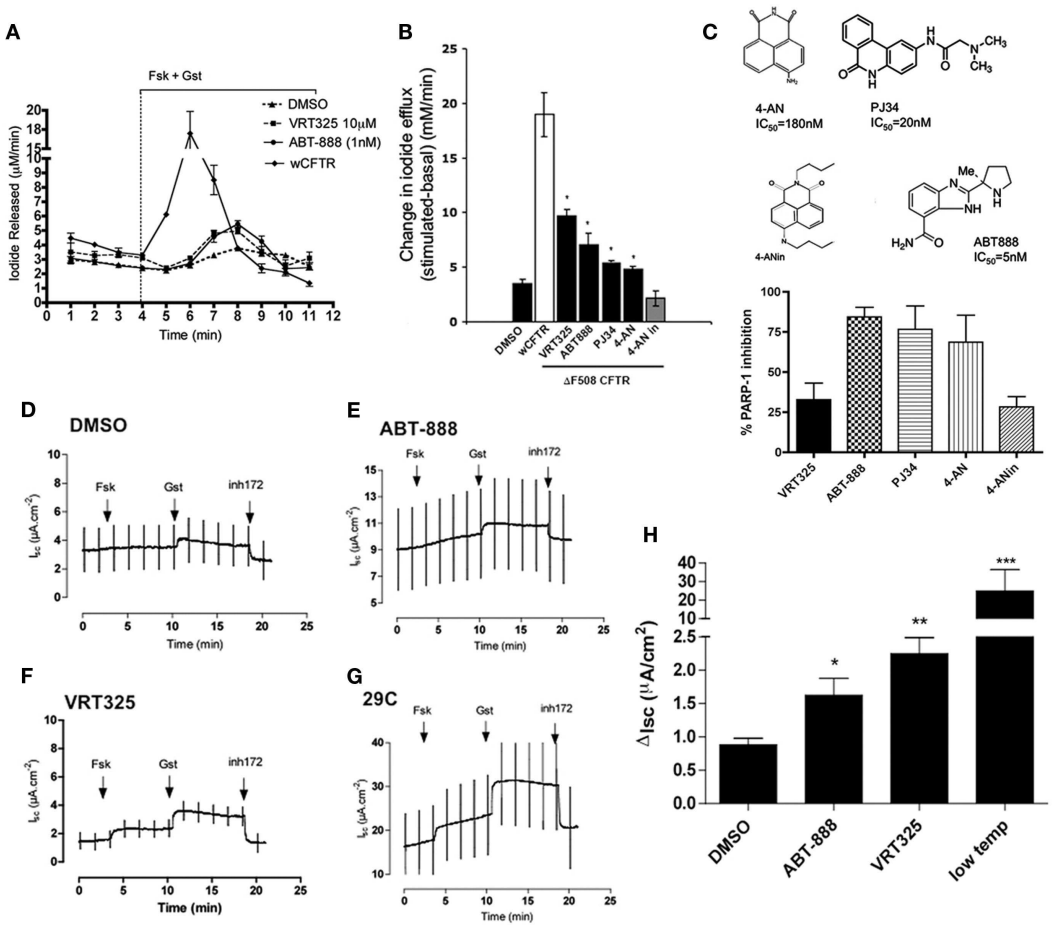

FIGURE 2 | PARP-1 inhibitors partially restore chloride activity in (BHK) CFTR ΔF508 and in polarized CFBE41o⁻ (ΔF508) cells. (A) Iodide efflux trace measuring ΔF508 CFTR function at the plasma membrane in BHK cells treated with ABT-888 (1 nM), DMSO (0.1%), and VRT325 (10 μM) for 24 h. Iodide efflux trace of BHK cells expressing wild type (wt) CFTR is also shown. An arrowhead and bar graph indicate addition and maintenance, respectively, of 10 μM forskolin (Fsk) and 50 μM genistein (Gst). Error bars indicate ±SD ($n = 3$). Note break in axis **(B)** change in iodide efflux upon stimulation with Fsk and Gst defined as the peak iodide efflux after stimulation subtracted from the baseline response summarizing data from all compounds tested; 4-AN (0.1 μM), 4-ANin (0.1 μM), PJ34 (10 μM), ABT-888 (1 μM). VRT-325 (10 μM) and DMSO (0.1%). Error bars indicate ±SEM ($n = 3$). A difference in the means as compared with the DMSO control was tested for statistical significance using paired *t*-tests (*$P < 0.05$). **(C)** Chemical structures of the PARP-1 inhibitors and the inactive 4-AN their published IC_{50} (PARP-1 inhibition) are shown. The extent of PARP-1 inhibition (*in vitro*) was determined for each of the compounds by measuring the NAD^+ remaining in the presence of inhibitor. Results are reported as %inhibition of PARP-1. VRT325, as expected is not a PARP-1 inhibitor and 4-ANin, an inactive analog of 4-AN, is also not an inhibitor. ABT-888 is the most potent PARP-1 inhibitor we tested. The graph summarizes the data from three independent experiments representing the mean ± SEM. **(D–G)** Four panels show polarized CFBE41o⁻ cells stably overexpressing ΔF508 CFTR treated with DMSO (0.1%), ABT-888 (1 nM), VRT325 (10 μM), and low temperature incubation (29°C) for 24 h and current was measured in Ussing chamber. CFTR-mediated currents cause upward deflections because an apical to basolateral current was imposed after permeabilization of the basolateral membrane. **(H)** Bar graph showing the change in I_{sc} (disk) after the addition of 10 μM Fsk and 50 μM Gst, defined as the difference between the sustained current after genistein and baseline arrest before stimulation. Error bars represent mean ± SEM ($n = 4$) at (*$P < 0.05$), (**$P < 0.001$), or (***$P < 0.0001$) relative to DMSO (paired *t*-tests). Note break in *y*-axis.

approximately half of the response obtained with VRT325. The inactive analog (4-ANin) that does not inhibit PARP-1 had no effect (**Figure 2B**). We monitored the *in vitro* PARP-1 activities of the inhibitors tested and found that their relative ability to inhibit PARP-1 correlated with their ability to restore iodide release in (BHK) ΔF508 CFTR (graph, **Figure 2C**) suggesting that their effects are mediated through the inhibition of PARP-1.

These experiments were extended to polarized CFBE41o$^-$ cells that overexpress ΔF508 CFTR. **Figures 2D–G** shows short-circuit current responses to forskolin and genistein and sensitivity to the CFTR inhibitor (CFTRinh-172) after monolayers had been incubated with 1 nM ABT-888 for 24 h. ABT-888 increased the short-circuit current response to forskolin and genistein by almost double compared to that of vehicle controls (**Figures 2D,E**). This was approximately half the rescue elicited by VRT325 (**Figure 2F**), and much less than that produced by low temperature pretreatment (**Figure 2G**) as reported previously for other correctors (Robert et al., 2010). The results under each condition are summarized in **Figure 2H**. Thus, ABT-888 causes partial rescue of the CFTR-dependent short-circuit current response, which is 6.7% of that measured after low temperature incubation and 40% of that induced by the well studied corrector VRT325.

DECREASING PARP-1 ACTIVITY *IN VIVO* RESTORES CHLORIDE ACTIVITY

To determine if PARP-1 inhibitors are effective in other tissues, mouse ileum was mounted in Ussing chambers and treated *ex vivo* with 1 nM ABT-888 for 4 h. Short-circuit current responses to forskolin and genistein were measured using pieces of ileum dissected from ΔF508 CFTR homozygotes and non-CF littermate controls. These were first taken at time 0 (**Figure 3A**), and then measured after 4 h incubation with 1 nM ABT-888 (**Figure 3B**) or saline alone (**Figure 3B**). Incubation with ABT-888 for 4 h increased the response to forskolin + genistein by ∼30% relative to that measured at time 0 (**Figure 3C**, $N = 5$, Students' t-test, $P < 0.05$). Incubation in saline alone for 4 h did not alter the I_{sc} response to forskolin + genistein (**Figure 3C**). The increase observed after this relatively brief (i.e., 4 h) exposure to ABT-888 indicates restoration of ∼7% of the wild type current response. PARP-1 activity in mouse ileum was measured by ELISA immediately after experiments with and without ABT-888. Incubation with ABT-888 for 4 h inhibited 85% of the PARP-1 activity measured in parallel experiments in control saline (**Figure 3D**, $n = 3$, Student's t-test, $P < 0.05$) confirming that it is absorbed rapidly and is effective in native tissue (Muscal et al., 2010).

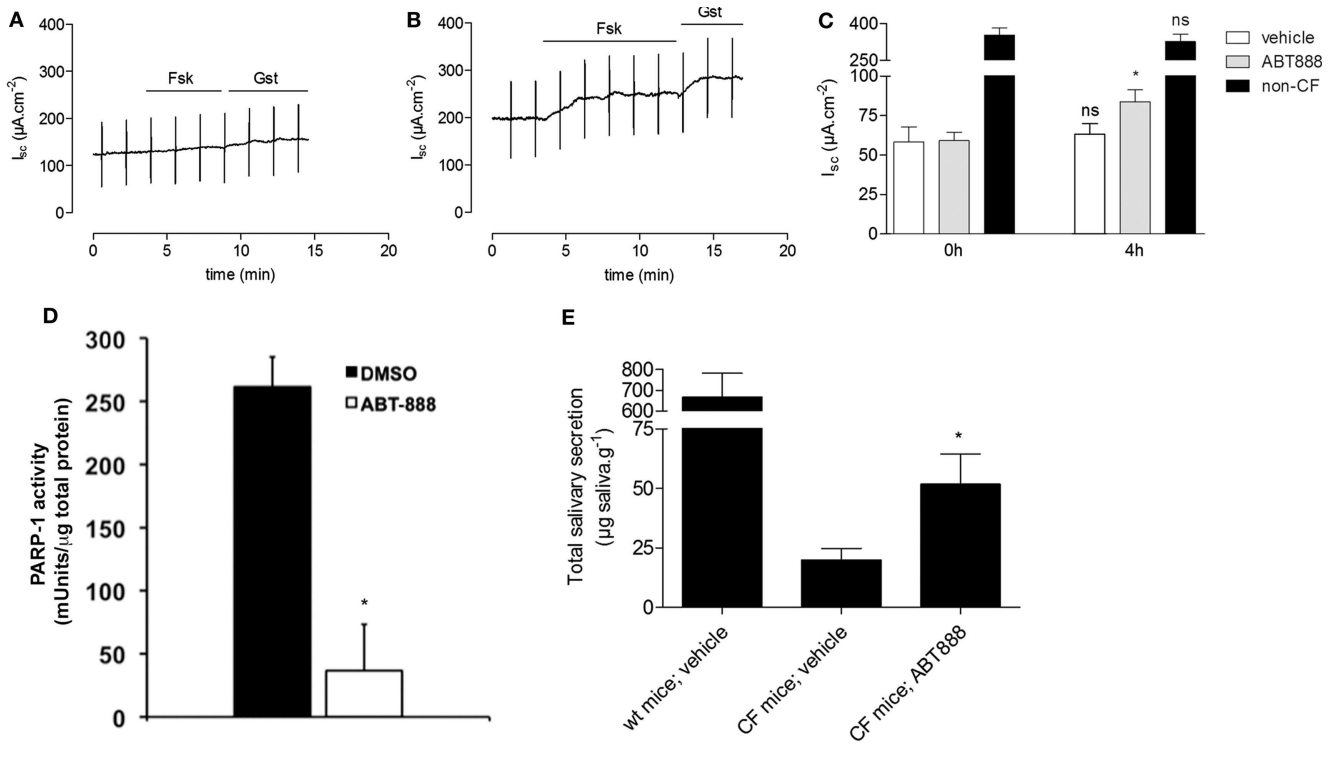

FIGURE 3 | PARP inhibition partially restores the activity of mouse ΔF508 CFTR. (A-B) Representative short-circuit current (I_{sc}) traces from mouse ileum from ΔF508-CFTR homozygous mice and non-CF control littermates. 10 μM forskolin (Fsk) and 50 μM genistein (Gsk) were added before [**(A)**, time 0 h] and after [**(B)**, 4 h] incubation with 1 nM ABT-888. **(C)** Bar graph showing the change in short-circuit current (ΔI_{sc}) after adding Fsk + Gst. I_{sc} responses were measured using 2–3 pieces of ileum from each mouse before (0 h) and after (4 h) pre-treatment with vehicle ($N = 5$ mice), or ABT-888 ($N = 5$ mice). Data are presented as the mean ± SEM; relative to their respective controls at time 0 h, calculated using a paired t-test; $^*P < 0.05$. Note break in axis. **(D)** PARP-1 activity in vehicle-treated ileum ($N = 5$ mice) and ABT-888 treated ileum ($N = 5$ mice) after 4 h incubation. Data are expressed as mean ± SEM, and significance was calculated by paired t-test; $^*P < 0.05$. **(E)** ABT-888 partially restores salivary secretion in mouse salivary glands. Total saliva secreted by homozygous ΔF508 CFTR mice after daily intraperitoneal injection with vehicle (saline; $n = 5$) or ABT-888 ($n = 5$) for 2 days. Means ± SEM; $^*P < 0.05$ by a paired Student's t-test.

Studies were also carried out to assess *in vivo* correction in ΔF508 mice (van Doorninck et al., 1995). ABT-888 (5 mg/kg/day) in saline was given daily by intraperitoneal injection; 3 mg/kg/day dose has been shown previously to inhibit PAR activity *in vivo* (Donawho et al., 2007) and results were compared with saline alone. β-adrenergic stimulated salivary secretion was measured by subcutaneous injection of isoprenaline into the cheek after blocking cholinergic stimulation with atropine (Best and Quinton, 2005). ABT-888 injections increased the saliva secretion response to isoprenaline 2.6 times when compared with untreated CF mice (**Figure 3E**, $P < 0.05$). This corresponds to 7.8% of the response of wild type mice ($51.8 \pm 2.4\,\mu g\,g^{-1}$ vs. $667.5 \pm 4.2\,\mu g\,g^{-1}$; **Figure 3E**).

PARP-1 INHIBITION PROMOTES ΔF508 CFTR MATURATION

Since we observed an improvement in chloride channel activity levels with PARP-1 inhibition, we assume that ΔF508 had matured beyond the ER. To examine this further we monitored the maturation of ΔF508 CFTR by immunoblotting CFBE41o$^-$ cells (overexpressing ΔF508 CFTR) that had been treated with 1 nM ABT-888. After 6 h we observed a band migrating at 175 kDa that may be complex glycosylated ΔF508 CFTR (band C; see **Figure 4A**). We also observed an increase in the core-glycosylated (band B) form of ΔF508 CFTR (**Figure 4A**). We quantified the relative amounts of each band by densitometry and found a significant increase in the fraction of mature CFTR (band C/total) treated with ABT-888 (0.34 ± 0.04) when compared with DMSO (0.20 ± 0.05), **Figure 4B** ($P = 0.02$). We ruled out transcriptional effects of ABT-888 by monitoring the relative abundance of *CFTR* mRNA at 6 and 24 h treatment with ABT-888. When CFBE41o$^-$ cells overexpressing ΔF508 CFTR were treated with ABT-888 (1 nM), real-time PCR revealed no change in CFTR mRNA levels after both 6 and 24 h exposure (**Figure 4C**, $P =$ n.s). To test for specificity of ABT-888, we monitored the maturation of wild type CFTR in CFBE41o$^-$ cells (exogenously expressing wt-CFTR) treated with ABT-888 and two other PARP-1 inhibitors, PJ34 and 4-AN by immunoblotting (**Figure 4D**). However we found no effects on the steady-state levels of the immature or mature glycoforms of wild type CFTR, suggesting that PARP-1 inhibition targets the ΔF508 CFTR mutant specifically. The specificity of PARP-1 inhibition was assessed by comparing the ability of ABT-888 and 4-AN to correct the mutant form of the human ether-à-go-go-related K^+ channel (hERG: hERG G601S) expressed in HEK 293 cells, which is also retained in the ER (**Figure 4E**). PARP-1 inhibition did not improve the processing of the mutant form of hERG (**Figure 4E**) whereas VRT-325 increased the amount of processed hERG G601S (**Figure 4E**). Artemizole and low temperature incubation (29°C) are known to correct G601S and are shown as positive controls (**Figure 4E**). The different selectivities of VRT-325 and the PARP-1 inhibitors also suggest they act through distinct pathways.

To determine if the trafficking correction observed is indeed PARP-1 dependent we examined the effect of silencing PARP-1 in CFBE41o$^-$ cells that overexpress ΔF508 CFTR. siRNA-mediated silencing of PARP-1 (which accounts for 85–90% of the pADPr protein in mammalian cell; Pacher and Szabo, 2008) reduced PARP-1 protein expression as expected (**Figure 4F**), and this was accompanied by the appearance of some complex glycosylated (band C) ΔF508 CFTR, indicating escape from the ER (**Figure 4F**). We quantified the relative amounts of band C and band B by densitometry and found a significant increase in the mature form of CFTR upon silencing of PARP-1 by siRNA (0.35 ± 0.04) relative to si-scramble and/or untransfected (**Figure 4G**) when normalized to tubulin. To extend this to a cell system that is devoid of PARP-1, we carried out experiments with PARP-1$^{-/-}$ mouse embryonic fibroblasts (MEFs) that had been transfected with triple-HA tagged Δ508 CFTR (**Figure 4H**). ΔF508CFTR-3HA was found almost exclusively in the complex glycosylated CFTR (band C) form (**Figure 4H**) confirming that the effect of PARP-1 inhibitors on the trafficking of ΔF508 is mediated by PARP-1 and is not an off-target drug effect. We quantified the relative amounts of immature and mature forms of CFTR normalized against tubulin in PARP-1$^{-/-}$ MEF cells (**Figure 4I**) and found there was no significant difference in the amount of mature CFTR in ΔF508 transfected PARP-1$^{-/-}$ MEFs vs. wCFTR transfected cells (**Figure 4I**, $P =$ n.s.). There was more immature CFTR (band B) in the ΔF508 CFTR transfected cells even when corrected for loading with tubulin (**Figure 4I**, $^*P < 0.05$).

In MEF cells expressing PARP-1 (PARP-1$^{+/+}$) the complex glycosylated (band C) form of CFTR was only detected when wild type CFTR was transfected, whereas ΔF508CFTR was found predominantly in the core-glycosylated (band B) form (**Figures 4H,I**) as expected. Particularly striking is the increase in the expression of CFTR in the absence of PARP-1$^{-/-}$ (**Figure 4I**, $P < 0.05$). We calculated the ratio of band B/C (**Figure 4J**), which we expect to be higher in the ΔF508 CFTR transfected PARP-1$^{+/+}$ cells. While in the PARP-1$^{-/-}$ transfected cells there is no difference between the band B/C ratio in wCFTR and ΔF508 transfected cells (**Figure 4J**, $P =$ n.s.), in the PARP-1$^{+/+}$ cells the band B/C ratio for ΔF508 transfections is five times higher (2.0 ± 0.7 for ΔF508 CFTR vs. 0.41 ± 0.14 for wCFTR, **Figure 4J**, $P < 0.05$).

INCREASING PARP-1 ACTIVATION WITH PEROXYNITRITE INHIBITS THE TRAFFICKING OF WILD TYPE CFTR

Since reducing PARP-1 activity restores mutant CFTR trafficking and function, we hypothesized that increasing PARP-1 activation should reduce trafficking. To test this hypothesis and monitor trafficking decreases, CFBE41o$^-$ cells expressing heterologous wild type CFTR was exposed to the physiologically relevant PARP-1 activator peroxynitrite (Szabo et al., 2007). PARP-1 activity was increased by acute peroxynitrite treatment (3 h) with 100 and 250 μM, reaching a maximal two-fold increase at 250 μM (**Figure 5A**). This inhibition was partially blocked by ABT-888, confirming that the stimulation by peroxynitrite occurs through the activation of PARP-1 (**Figure 5A**).

Treating CFBE41o$^-$ cells overexpressing wild type CFTR with 250 μM peroxynitrite caused a marked reduction in complex glycosylated CFTR (**Figure 5B**), Increasing PARP-1 activity also increased the retention of core-glycosylated CFTR in the ER (**Figure 5B**). There was also some reduction in total CFTR protein expression at 100 and 250 μM. This reduction was apparently not due to apoptosis since cleavage of PARP-1 into 85 and 100 kDa fragments, a hallmark of apoptosis, was not observed (**Figure 5B**). We also observed increased PARP-1 expression in cells treated with 100–250 μM peroxynitrite (compared to untreated wild type

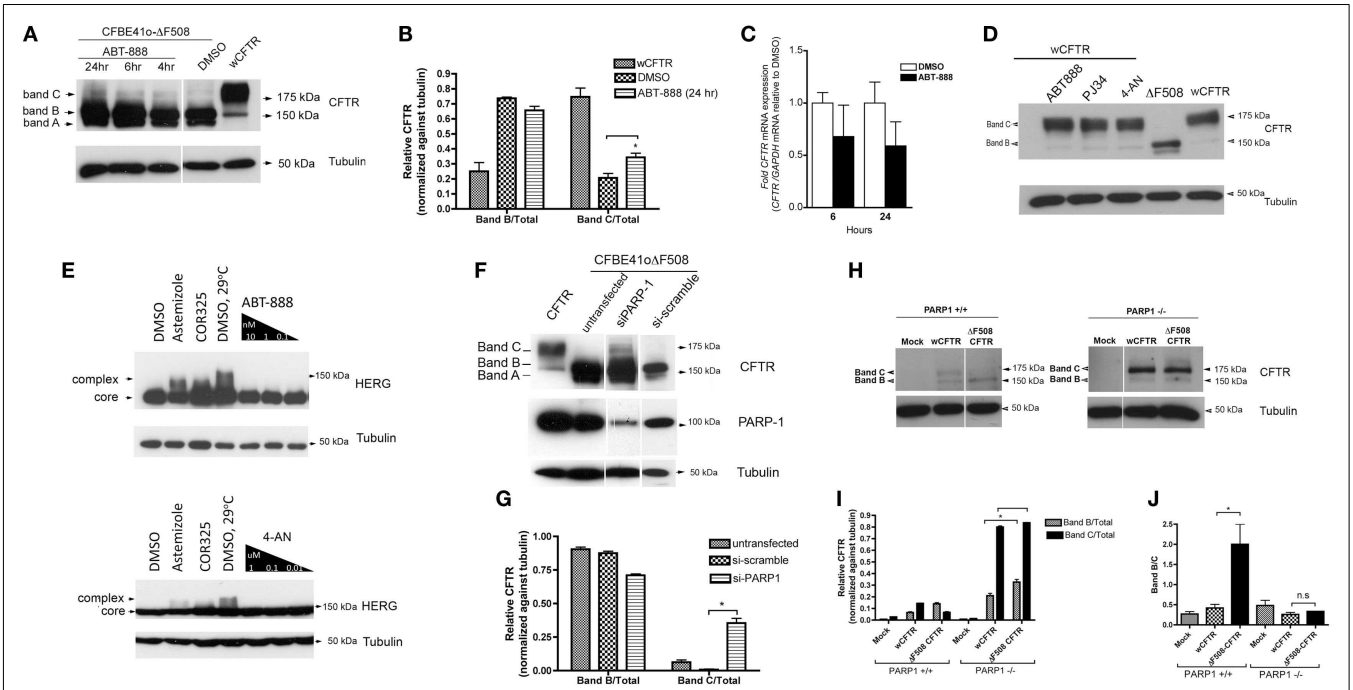

FIGURE 4 | PARP-1 pharmacological inhibition and genetic deletion promotes the trafficking of ΔF508 CFTR in CFBE41o⁻ cells. **(A)** CFBE41o⁻ cells expressing ΔF508 CFTR were treated for the indicated times with 1 nM ABT-888 and immunoblotted against CFTR to monitor increases in complex glycosylation (band C). Note the appearance of band C with ABT-888 treatment and an increase in band B. Band A is also detected (ABT-888 and DMSO). **(B)** Mean ± SD ratios of the densitometry signals for band B/total and band C/total, normalized against tubulin. $^*P < 0.05$ ($n = 3$, *t*-test). **(C)** To rule out transcriptional up-regulation by ABT-888, the relative expression of *CFTR* mRNA in CFBE41o⁻ cells (not overexpressing CFTR) was quantified by qRT-PCR. Although there is a trend toward a decrease in *CFTR* mRNA with ABT-888 treatment at the indicated times it does not reach statistical significance. Results are reported as mean ± SD, $P = n.s$ ($n = 3$, *t*-test). **(D)** PARP-1 inhibitors do not increase the trafficking of wild type CFTR overexpressed in CFBE41o⁻ cells and **(E)** ABT-888 treated HEK 293 cells overexpressing the mutant HERG (G601S) does not increase the trafficking of the ER-retained HERG mutant G601S. We also tested 4-AN at the indicated concentrations in the same system and found no effects suggesting specificity of ABT-888 for the ΔF508-CFTR mutant. **(F)** PARP-1 silencing by siRNA partially restores the trafficking of ΔF508 CFTR in CFBE41o⁻ (overexpressing ΔF508) cells. Immunoblot analysis of CFTR following siRNA-mediated silencing of PARP-1 (48 h). PARP-1 knockdown was monitored by immunoblotting against PARP-1; PARP-1 expression was significantly reduced compared to si-scramble but was not completely abrogated. Note the appearance of band C in siPARP-1 and an increase in band B. **(G)** Mean ± SD ratios of the densitometry signals for band B/total and band C/total, normalized against tubulin. $^*P < 0.05$ ($n = 3$, *t*-test). **(H)** Complete deletion of *PARP-1* restores ΔF508 trafficking. Mouse embryonic fibroblasts lacking PARP-1 (PARP1$^{-/-}$) were transfected with wild type CFTR or ΔF508 CFTR in pCDNA3.1 (and empty plasmid, MOCK). The cells were lysed and analyzed by immunoblotting with anti-CFTR (23C5, 1:20 dilution). Most detectable CFTR migrates at 175 kDa, suggesting the complex glycosylated form of ΔF508 CFTR is predominant in cells lacking PARP-1. When MEF cells containing PARP-1 (PARP1$^{+/+}$) are transfected with same constructs the level of CFTR expression was much lower overall no band C is detected in ΔF508. **(I)** Mean ± SD ratios of the densitometry signals for band B/total and band C/total, normalized against tubulin. $^*P < 0.05$ ($n = 3$, *t*-test) **(J)** band B/C ratios indicate more band B, as expected in the PARP-1$^{+/+}$ cells for ΔF508 CFTR, while in the PARP-1$^{-/-}$ cells there is no difference between the wCFTR and ΔF508 CFTR transfections, suggesting that most of the CFTR is in the mature band C form. $^*P < 0.05$ ($n = 3$, *t*-test).

cells), which reached levels that were comparable to those in CFBE41o⁻ cells overexpressing ΔF508 CFTR (**Figure 5B**).

Overall, our data suggests interdependence between the levels of oxidative stress, PARP activation and CFTR biogenesis. Taken together, we have shown in several CF models, including human primary bronchial epithelial cells (**Figure 1B**) and in CF cells (that do not overexpress ΔF508, **Figure 1C**), that PARP-1 activity is elevated when compared to matched non-CF cells. This suggests oxidative stress caused by a misfolded protein, leading to increased PARP-1 activity.

Decreasing the levels of PARP-1 activity by treatment with PARP-1 inhibitors or the absence of PARP-1, results in a partial restoration of ΔF508 CFTR trafficking and its function.

DISCUSSION

High PARP-1 activation in response to oxidant-mediated DNA damage is an important pathway during tissue injury (Pacher and Szabo, 2008). In this study we considered the modulation of PARP-1 activity by pharmacological inhibition and genetic silencing or deletion and how this affects CFTR function and expression. Physiologically relevant levels of DNA damage and PARP-1 activation have been demonstrated in pulmonary diseases, such as asthma, acute lung injury, and COPD (42–45), however these have not been investigated in CF. In view of the central role it plays in cellular stress responses (Luo and Kraus, 2005) and reports of exaggerated inflammation (Galli et al., 2012), elevated oxidative stress (Galli et al., 2012), and metabolic dysregulation in

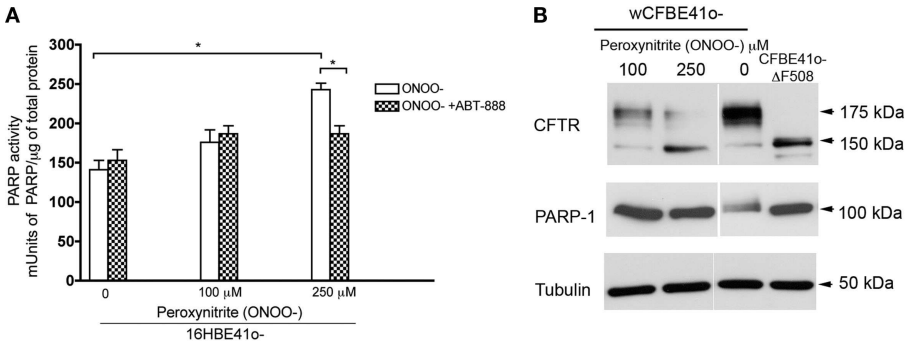

FIGURE 5 | Increasing PARP-1 activity decreases trafficking of wild type CFTR. (A) 16HBE14o$^-$ (non CF) were treated with the indicated doses of peroxynitrite (ONOO$^-$), a potent PARP-1 activator. PARP-1 activity was monitored by ELISA. PARP-1 significantly increased at 250 μM ONOO$^-$ (3 h treatment). Blocking PARP-1 with ABT-888 abolished the effects of peroxynitrite on PARP-1 activation suggesting that the effects we see are PARP-1 mediated. Bar graph represents the mean ± SEM ($n = 3$, with three replicates each). Statistical significance was determined using a paired *t*-test; $^*P < 0.05$. **(B)** To monitor trafficking *decreases*, CFBE41o$^-$ cells stably overexpressing wild type (wt) treated with the indicated concentrations of ONOO$^-$ for 3 h. Lysates from CFBE41o$^-$ cells overexpressing ΔF508 CFTR are also shown. A decrease in trafficking promoted by ONOO$^-$ is observed at 250 μM ONOO$^-$ with a decrease in complex glycosylated CFTR. No cleavage of PARP-1 was observed.

CF patients (Wetmore et al., 2010), we hypothesized that PARP-1 might influence CFTR biology.

We observed higher PARP-1 activity in HBEs derived from patients homozygous for ΔF508 CFTR than in HBEs from non-CF donors, and similarly higher activity in the CFBE41o$^-$ cell line compared to the non-CF line 16HBE14o$^-$. To our knowledge this is the first evidence that PARP-1 is upregulated in CF. Although there may be many differences between the CFBE41o$^-$ and 16HBE14o$^-$ cell lines, e.g., in the functional expression of drug-transporter P-gp assayed by Rhodamine123 (Ehrhardt et al., 2006), a major distinguishing feature is the presence of a misfolded and dysfunctional CFTR channel in CFBE41o$^-$. Further evidence that the difference in PARP-1 activity is due to the presence of ΔF508 CFTR comes from the results with primary cells from patients. Despite the intrinsic variability between different patients, the same pattern was observed, i.e., PARP-1 was higher in cells from both CF patients compared to non-CF subjects. Nevertheless, the relationship between CFTR and PARP-1 activity will need to be extended to a larger cohort in the future.

Since PARP-1 is a DNA damage sensor, one might expect its activity in CF cells to reflect increased DNA damage. Indeed, DNA fragmentation has been reported in intestinal cells from CF patients (Maiuri et al., 1997), and elevated levels of oxidative stress markers and DNA damage have also been reported in CF (Brown et al., 1995, 1996). CF patients present with several abnormalities in oxidative stress, including elevated ROS generation, a constitutive defect in glutathione metabolism, and reduced intake of fat-soluble antioxidant vitamins (Galli et al., 2012), some of which are endogenous or natural PARP-1 inhibitors (Banasik et al., 1992). Aside from nicotinamide, natural occurring inhibitors of PARP-1 include tryptophan-related compounds, purines, unsaturated fatty-acids (including linoleic acid and arachidonic acid), and carotenoids (Banasik et al., 1992) the levels of which are reportedly low in CF patients (Wetmore et al., 2010, Galli et al., 2012).

We observed a correlation between PARP-1 activity and CFTR, consistent with reports that (1) CFTR dysfunction itself can lead to oxidative stress (Bartoszewski et al., 2008; Chen et al., 2008), (2) ROS reduce wild type CFTR protein expression and cAMP-mediated Cl$^-$ secretion in airway epithelia (Bebok et al., 2002), and (3) ER retention of CFTR may contribute to inflammation (Rottner et al., 2009).

PARP-1 activity was modulated by treating CF epithelial cells with the potent PARP-1 inhibitor ABT-888 (Veliparib) at low concentrations, lower than needed to observe maximal inhibition of PARP-1 (maximum inhibition at 1 nM vs. $IC_{50} = 5$ nM). The reason for this extraordinary potency remains unknown, however it was observed in several cell types (**Figures 1–3**). ABT-888 inhibition of PARP-1 activity was variable among different cell types, consistent with previous reports (Virag, 2005). For example, inhibition was stronger in mouse ileum (**Figure 3D**). Although ABT-888's potency as a ΔF508 corrector has been evaluated in recombinant cell lines and model systems it will be important to investigate its action further in primary cells. Based on the previous reports of correctors that work modestly *in vitro* (Pedemonte et al., 2010) not advancing further into pre-clinical or human CF trials for lack of specificity, off-target effects and/or insufficient levels of restoration of trafficking (Pedemonte et al., 2010) highlights the importance of assessing correctability in primary cells. We measured the effects of ABT-888 treatment on ΔF508 CFTR function in native HBEs derived from a single patient by Ussing chamber measurements of chloride activity and while we found a modest increase in activity, this was not statistically significant (data not shown). However, there is a reported large variation in the "correctability" by a single compound in different patients (the short current response of CF primary lung bronchial epithelial cells to VX-809 varied between 3.4–14.9% of non-CF donor lungs; Van Goor et al., 2011). More patients will need to be tested. Additionally, we also predict that there will be variability in the levels of oxidative burden and consequently PARP-1 activation between patients. A larger cohort will have to be tested to address this.

Finally, peroxynitrite activated PARP-1 and reduced the maturation of wild type CFTR (**Figures 5A,B**), and this effect was

blocked by ABT-888, strongly suggesting that peroxynitrite was acting through PARP-1. Although the activation of PARP-1 by peroxynitrite and its involvement in disease is well documented (Pacher and Szabo, 2008), peroxynitrite is a potent oxidant and we cannot exclude other potential mechanisms of action (**Figure 5A**). Previous studies have demonstrated that (1) oxidants affect CFTR function (Rottner et al., 2009), (2) CFTR dysfunction itself may lead to oxidative stress (Chen et al., 2008), (3) oxidative stress suppresses CFTR expression (Cantin et al., 2006; Bartoszewski et al., 2011), and (4) increases in reactive oxygen nitrogen species may decrease wild type CFTR protein expression and cAMP-mediated Cl^- secretion by airway epithelia (Bebok et al., 2002).

ΔF508 CFTR maturation was dramatically altered in PARP-1 knockout cells. Only the mature glycoform was detected in PARP-1 null MEFs (**Figure 4H**). This is consistent with the partial restoration of ΔF508 trafficking in CFBE41o$^-$ cells (**Figures 4A,B**) when PARP-1 activity was inhibited pharmacologically or silenced by RNA interference (**Figures 4F,G**). It has been shown that PARP-1 knockout mice display altered expression of redox-sensitive, AP-1-dependent genes, proinflammatory mediators, and heat shock proteins (including HSP70; Andreone et al., 2003) known to regulate CFTR gene expression, folding or function (McCarthy and Harris, 2005). Moreover, the PARP-1 knockout mouse is also resistant to various models of inflammation, the mechanism of which occurs via deficient NF-κB activation (Schreiber et al., 2006), which requires PARP-1 as a co-activator.

The effects of PARP-1 inhibition seem to be specific for the mutant CFTR form, as we do not observe any improvements in the processing of the wild type CFTR (**Figure 4D**) nor for the mutant form of the human ether-à-go-go-related K^+ channel (hERG: hERG G601S) expressed in HEK 293 cells, which is also retained in the ER (**Figure 4E**).

These results suggest that the effects we observe are ΔF508 CFTR specific and are linked to elevated oxidative stress, which is not elevated in the wild type CFTR cells. We propose that restoring homeostasis through the attenuation of PARP-1 activity increases translation and proteostasis at least partly because oxidative stress is lowered. Although the mechanism of action is yet undetermined, we hypothesize that PARy(lation) of key members of the CFTR folding interactome such as the HSP90 co-chaperone Hop, among others (Gagne et al., 2008), may alter their expression and function promoting folding, and altering interactions with partners, consistent with a rapid response (**Figure 4A**). PARP-1's role in intracellular trafficking is expanding as recently reviewed (Abd Elmageed et al., 2011) highlighting the multiple roles and pleiotropic effects of PARP related pathways.

Achieving therapeutically-relevant trafficking of ΔF508 CFTR *in vivo* may require a combination of two or more corrector drugs, and may also require antioxidant therapies due to underlying defects in the regulation of oxidative stress and inflammation. The present results suggest that addressing the trafficking and oxidative stress problem through manipulation of PARP-1 and related pathways may be a useful approach for restoring homeostasis and should be investigated further in the context of CF therapeutics.

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Ouabain mimics low temperature rescue of F508del-CFTR in cystic fibrosis epithelial cells

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Most cases of cystic fibrosis (CF) are caused by the deletion of a single phenylalanine residue at position 508 of the cystic fibrosis transmembrane conductance regulator (CFTR). The mutant F508del-CFTR is retained in the endoplasmic reticulum and degraded, but can be induced by low temperature incubation (29˚C) to traffic to the plasma membrane where it functions as a chloride channel. Here we show that, cardiac glycosides, at nanomolar concentrations, can partially correct the trafficking of F508del-CFTR in human CF bronchial epithelial cells (CFBE41o-) and in an F508del-CFTR mouse model. Comparison of the transcriptional profiles obtained with polarized CFBE41o-cells after treatment with ouabain and by low temperature has revealed a striking similarity between the two corrector treatments that is not shared with other correctors. In summary, our study shows a novel function of ouabain and its analogs in the regulation of F508del-CFTR trafficking and suggests that compounds that mimic this low temperature correction of trafficking will provide new avenues for the development of therapeutics for CF.

Keywords: cystic fibrosis, CFTR, trafficking, quabain, microarray, connectivity map, hierarchical clustering, CFBE cells

INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a plasma membrane anion channel (Riordan et al., 1989; Anderson et al., 1991; Kartner et al., 1991). The most common CFTR mutation, F508del (Rommens et al., 1989), causes retention of the mutant in the ER and its premature degradation by the proteasome (Cheng et al., 1990; Jensen et al., 1995). Nevertheless, F508del-CFTR can form functional channels having reduced activity (Dalemans et al., 1991), moreover its trafficking is temperature sensitive and can be partially rescued in many cell types by incubation at low temperature (≤29˚C; Denning et al., 1992; Rennolds et al., 2008). It has been estimated that restoring 10–25% of wild-type CFTR (WT-CFTR) activity in patients would alleviate the major symptoms of CF (Pilewski and Frizzell, 1999; Zhang et al., 2009).

Cell-based assays for "correctors" of F508del-CFTR trafficking have identified chemically diverse small molecules that correct trafficking with variable efficiency (Pedemonte et al., 2005; Van Goor et al., 2006; Carlile et al., 2007). Some of these correctors are thought to interact directly with CFTR by acting as stabilizing ligands or "pharmacological chaperones" (Loo et al., 2006; Sampson et al., 2011) or on other known cellular targets, e.g., phosphodiesterases (Dormer et al., 2005; Robert et al., 2008) and histone deacetylases (Hutt et al., 2010). However, for the majority of correctors that have been described, neither the target nor the mechanism of action are known (Lukacs and Verkman, 2012). We have previously reported a novel cell-based HTS assay that measures the appearance of HA-tagged F508del-CFTR at the surface of BHK cells (Carlile et al., 2007). Using this assay in a high throughput screen we identified the cardiac glycoside ouabagenin, an aglycone of ouabain, as a moderately potent corrector of F508del-CFTR trafficking. Cardiac glycosides have long been used to treat congestive heart failure and cardiac arrhythmia, and digoxin is still prescribed for atrial fibrillation and atrial flutter (Prassas and Diamandis, 2008). Cardiac glycosides bind to a highly conserved site on human Na^+/K^+-ATPase alpha subunits with a K_d of ∼18 nM (Wang et al., 2001), which is expected to increase several fold in the presence of physiological potassium concentrations. In cardiac myocytes inhibiting the pump increases intracellular sodium and reduces membrane sodium/calcium exchange, leading to elevation of intracellular calcium and increased contractile force (Hoyer et al., 2011). Moreover, clinical studies also suggest that cardiac

Abbreviations: CF, cystic fibrosis; CFBE, cystic fibrosis bronchial epithelial; CFTR, cystic fibrosis transmembrane conductance regulator; CMAP, connectivity MAP; COPII, vesicle coat proteins; ER, endoplasmic reticulum; ES, enrichment score; FDR, false discovery rate; HA, hemagglutinin; HTS, high throughput screening; Na^+/K^+-ATPase, sodium-potassium adenosine triphosphatase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

glycosides inhibit cancer cell proliferation and have potential as novel therapeutic agents against cancer (Newman et al., 2008).

In addition to its action as an inhibitor of Na^+/K^+-ATPase, ouabain has a signaling function at low concentrations (1–10 nM) that is independent of its effect on ion transport (Zhang et al., 2006). Ouabain-bound Na^+/K^+-ATPase can trigger slow calcium oscillations and NF-κB activation, thereby preventing cell death and promoting the proliferation and viability of kidney proximal tubule cells (Li et al., 2006). Interestingly, it has been reported that digitoxin and other cardiac glycosides suppress IL-8-dependent lung inflammation in CF lung epithelial cells (Srivastava et al., 2004). The exact mechanisms by which cardiac glycosides modulate cell proliferation, inflammation, migration, and apoptosis are not known (Aperia, 2007; Prassas and Diamandis, 2008).

Here we describe a novel function for ouabain and its analogs which is linked to its signaling functions. Treatment with low concentrations of ouabain resulted in the functional rescue of F508del-CFTR in human CF bronchial epithelial cells, and also in BHK cells and CF mice that are expected to be less sensitive to ouabain inhibition. Moreover the mechanisms of correction by ouabain and its analogs resemble those of low temperature according to transcriptional profiling and analysis of the Connectivity Map (CMAP) for F508del-CFTR trafficking in polarized parental CFBE41o-cells. Significant connectivity was observed between ouabain and low temperature transcriptional profiles obtained in human CF bronchial epithelial cells and this relationship was confirmed by hierarchical clustering analysis of the expression patterns.

These results reveal a new function for ouabain and its analogs as regulators of F508del-CFTR protein trafficking and indicate that cardiac glycosides act by mimicking low temperature rescue. Transcriptional profiling provides insight into corrector mechanisms, and small molecules that mimic the low temperature signature may be useful in developing therapeutics that correct the trafficking defect in CF.

RESULTS

CARDIAC GLYCOSIDES CORRECT THE TRAFFICKING OF F508del-CFTR TO THE CELL SURFACE

From our initial observation that ouabagenin, can correct F508del-CFTR trafficking in BHK cells (Carlile et al., 2007), we selected a panel of structurally related cardiac glycosides, including ouabain, digoxin, and digitoxin (**Figure 1A**). To confirm that they increase the trafficking of F508del-CFTR to the plasma membrane, we treated BHK cells that express F508del-CFTR-3HA, and measured the appearance of the HA epitope (Carlile et al., 2007). F508del-CFTR-3HA was detectable at the cell surface after 2 h treatment and after 24 h surface expression was increased about 20–30% compared to time 0 h treated cells (**Figure 1B**).

CARDIAC GLYCOSIDES IMPROVE STABILITY AND TRAFFICKING OF F508del-CFTR

The ER-retained glycoform of F508del-CFTR (band B, ∼150 kD) acquires terminal glycosylation (forming band C, ∼175 kD) and traffics through the Golgi. We used immunoblotting to detect the appearance of the mature glycosylated "band C" form of F508del-CFTR in human CF bronchial epithelial cells (CFBE41o-) treated with the individual cardiac glycosides and we evaluated their efficacy in promoting trafficking correction (**Figure 2A**). We compared the amount of band C with that found in cells treated at low temperature (29°C). Treatment with 100 nM ouabain, digitoxin, or digoxin, or 1 μM ouabagenin, increased the steady-state expression of immature (band B) and mature (band C) glycoforms of F508del-CFTR by 2- to 15-fold above vehicle control (**Figure 2B**). We also observed an increase in core-glycosylated F508del-CFTR (band B) upon treatment with the cardiac glycosides (**Figures 2A,B**). There is an overall increase in CFTR protein in the presence of cardiac glycosides, which could result in a "leaky" ER. In order to assess whether the observed increase in trafficking (band C) resulted from ER overload, we calculated the ratio of band C/B (Hutt et al., 2010). We found that all the cardiac glycosides (ouabain, digitoxin, and digoxin) increased the ratio of C/B bands by three- to seven-fold compared with vehicle control (**Figure 2B**) without affecting Na^+/K^+-ATPase protein expression (**Figure 2A**). In **Figure 2C**, we compared our own anti-CFTR antibody (23C5) which we have utilized throughout this whole study to the commercial anti-CFTR antibody (M3A7, from Chemicon). The results show that both antibodies gave the similar results, and our own anti-CFTR antibody can detect CFTR bands using much less cell lysates compared with using commercial antibody. To test if these cardiac glycosides have cytotoxicity on CFBE cells or not, in **Figure 3**, we measured the cell proliferation after 24 h of treatment with each individual cardiac glycoside on CFBE cells, and the results showed that there is no significant cytotoxicity on CFBE cells under 100 nM concentration of cardiac glycosides. Taken together these results show a novel function for ouabain and its analogs in F508del-CFTR folding and trafficking, beyond its well-established role in ion homeostasis.

OUABAIN RESCUES F508del-CFTR FUNCTION

We next investigated if ouabain and its analogs could also rescue F508del-CFTR channel activity. When CFBE/F508del-CFTR cells were pre-treated with 100 nM ouabain, digoxin, digitoxin, or with 1 μM ouabagenin for 24 h, the iodide efflux response evoked by forskolin increased to levels that were 26–32% that of CFBE cells expressing (WT-CFTR; **Figure 4A**).

These results were confirmed by measuring the short circuit current (see Materials and Methods) across polarized CFBE/F508del-CFTR cells that had been pre-treated with 100 nM ouabain for 24 h (**Figures 4BI–V**). A trans-epithelial chloride gradient was imposed and the basolateral membrane was permeabilized using nystatin to ensure that the I_{sc} response was mediated by apical Cl^- conductance (Robert et al., 2008). Representative I_{sc} recordings are shown for WT-CFTR monolayers (**Figure 4BI**) and F508del-CFTR monolayers pre-incubated for 24 h with normal saline at low temperature (29°C; **Figure 4BII**), with DMSO vehicle at 37°C (**Figure 4BIII**), or with 100 nM ouabain at 37°C (**Figure 4BIV**). Ouabain pre-treatment increased the forskolin and genistein-stimulated I_{sc} by ∼1.7-fold compared with controls (**Figures 4BIII–V**, $P < 0.05$). Chloride current was abolished by the CFTR inhibitor $CFTR_{inh}$-172 (Ma et al., 2002; Caci et al., 2008) in each instance, confirming that the stimulated I_{sc} was mediated by CFTR channels. The magnitude of the CFTR-mediated current induced by ouabain ($n = 7$) was 7.5% of that induced by

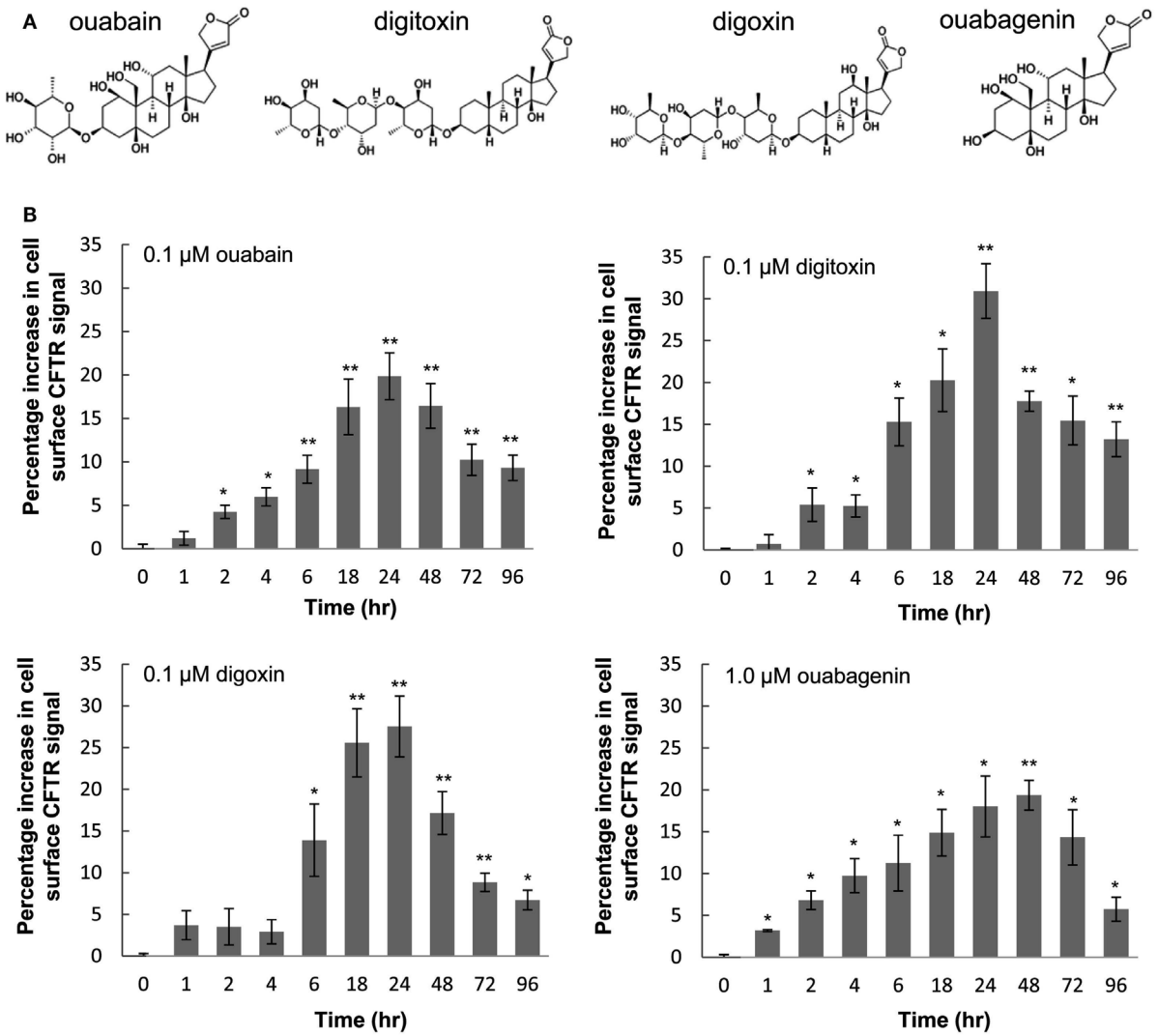


FIGURE 1 | Cardiac glycosides induce trafficking of HA-tagged F508del-CFTR to the cell surface. (A) Chemical structures of cardiac glycosides tested in this study. **(B)** BHK cells expressing F508del-CFTR-3HA were treated with 0.1 μM ouabain, 0.1 μM digitoxin, 0.1 μM digoxin, and 1.0 μM ouabagenin for various time points or with the vehicle control (0.1% DMSO), the cell surface expression of CFTR were monitored by immunofluorescence assay. Data are presented as percentage increase in surface CFTR signal compared to controls (mean ± SD of $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.005$).

low temperature ($n = 6$), which represents 1.4% of the current measured in cells expressing WT-CFTR ($n = 9$; **Figure 4BV**).

The correction of F508del-CFTR trafficking and function by ouabain pre-treatment was further evaluated *in vivo* using a CF mouse salivary secretion assay. The F508del-CFTR trafficking defect can be assayed functionally in the ileum and salivary glands of this CF mouse model (French et al., 1996; Robert et al., 2010). Homozygous F508del-CFTR mice and littermate WT controls received continuous low doses of ouabain (0.01 mg/kg/day) or vehicle for 48 h using a micro-osmotic pump implanted under the skin. Salivary secretion was measured acutely by injection of atropine and then isoprenaline into the cheek. Chronic exposure to low levels of ouabain *in vivo* increased the salivary secretion response by ∼5-fold (**Figure 4C**; $^{*}P < 0.04$, $n = 5$). This value corresponds to ∼8.1% of the secretory response of littermate WT control mice.

In summary, these data provide evidence that ouabain enhances F508del-CFTR trafficking and channel activity *in vitro* in human CF epithelial cells (CFBE41o-) and *in vivo* in F508del-CFTR homozygous CF mice.

OUABAIN REDUCES THE ER CALCIUM STORES IN CFBE CELLS

Retention of misfolded proteins in the endoplasmic reticulum is regulated by chaperone proteins, many of which require $[Ca^{2+}]$ for optimal activity. Although controversial, several studies have shown that $[Ca^{2+}]$ signaling is elevated in CF and that calcium homeostasis in CF airway epithelial cells is disturbed and related to the retention of F508del-CFTR proteins in the ER (Antigny

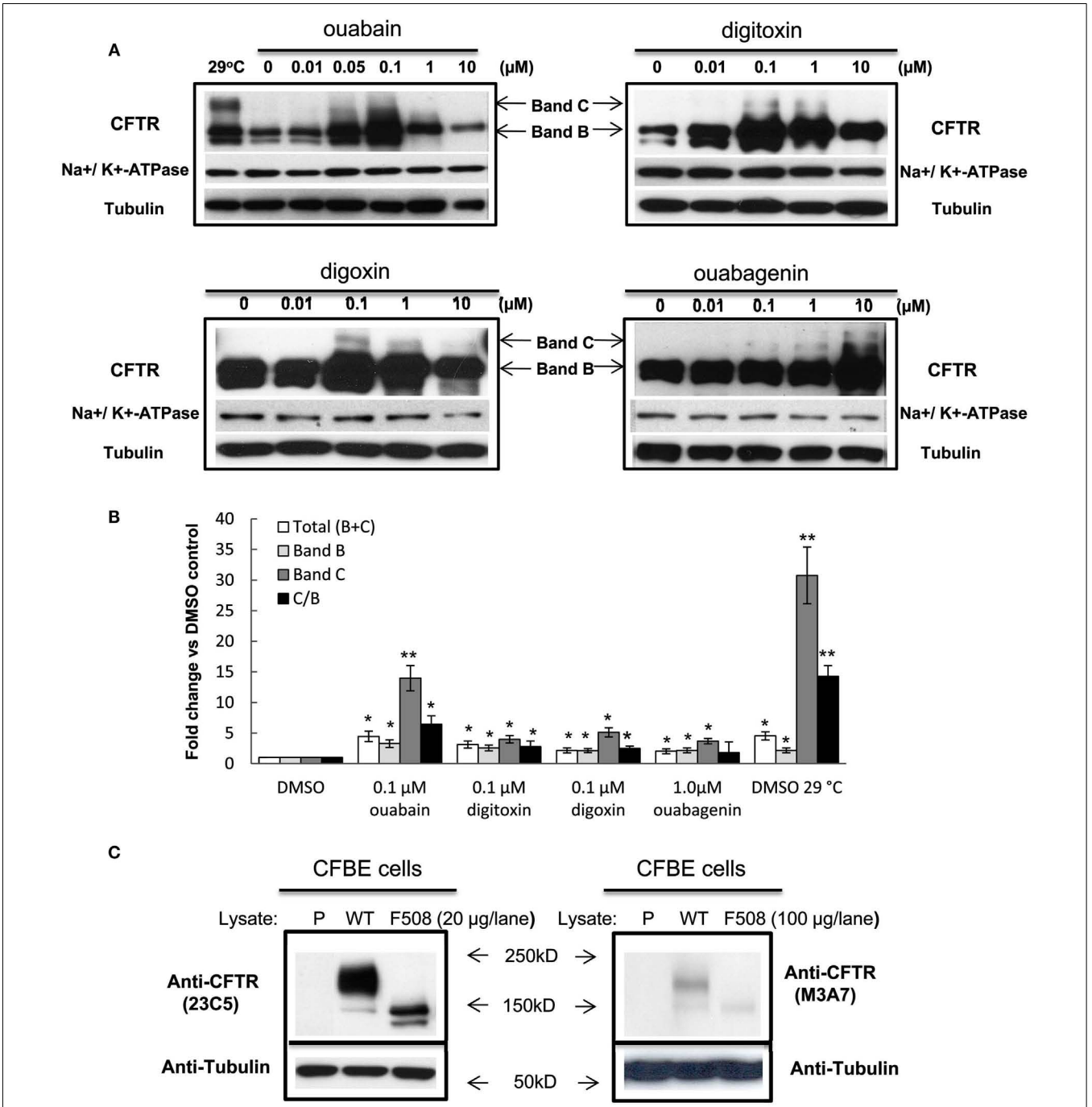


FIGURE 2 | Cardiac glycosides correct trafficking of F508del-CFTR in CF bronchial epithelial cells. (A) CFBE/F508del-CFTR cells were treated with individual concentrations of ouabain, ouabagenin, digoxin, or digitoxin for 48 h and the cell lysates were analyzed by western blotting using anti-CFTR, anti-Na^+/K^+-ATPase alpha1, or anti-tubulin antibodies. CFTR band C and band B are indicated by arrows. Tubulin is shown as a loading control. **(B)** Quantification of the band intensities for **(B)** experiments expressed as fold change vs. DMSO control. Values in the experiments described are presented as means ± SD ($n = 3$). Means were tested for statistical significance using a Student's *t*-test (*$P < 0.05$; **$P < 0.01$). **(C)** The cell lysates from parental CFBE41o- (P), CFBE/WT-CFTR (WT), and CFBE/F508del-CFTR (F508) cells were analyzed using anti-CFTR antibody 23C5 (our own anti-CFTR antibody) or M3A7 (from Chemicon). The molecular weight (kDa, kilodalton) of the markers was shown on the side of the blot.

et al., 2008a,b). As the binding of nanomolar concentrations of ouabain to Na^+/K^+-ATPase α subunits has previously been reported to increase intracellular calcium (Li et al., 2006; Prassas and Diamandis, 2008), we examined the calcium content of the ER stores in WT-CFTR cells and in ouabain treated vs. untreated CFBE/F508del-CFTR cells (see Materials and Methods). As shown

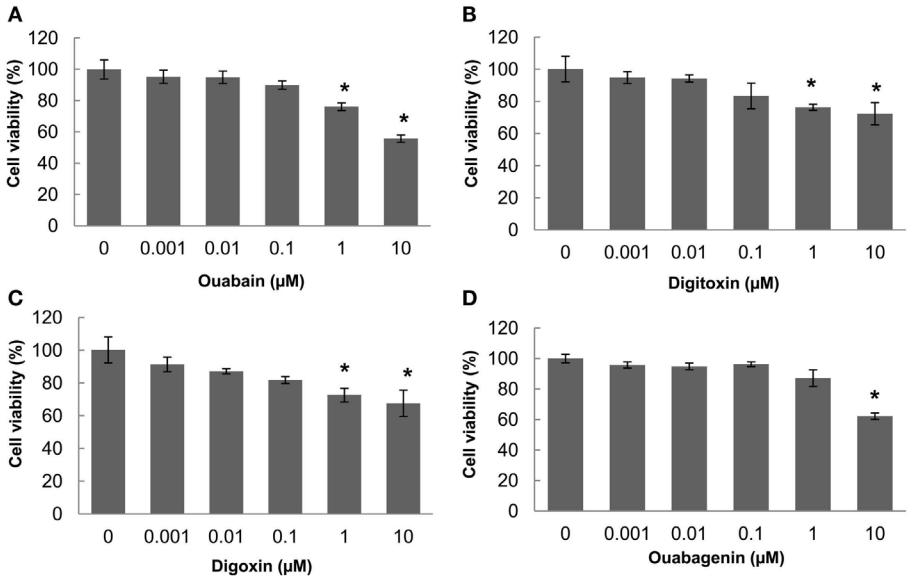


FIGURE 3 | Nanomolar concentrations of cardiac glycosides have no significant cytotoxic effects on CFBE cells at concentrations that correct CFTR trafficking. (A–D) CFBE/F508del-CFTR cells were treated with different concentrations of each cardiac glycoside for 24 h, the cell proliferations were measured using the AlamarBlue assay. The bar graph shows the percentage of the number of viable cells compared with the number of untreated cells, which were assigned a value of 100% (data shown are the mean ± SD of $n = 9$; $^*P < 0.05$).

in **Figure 5**, the cytosolic calcium concentrations in CFBE/WT-CFTR or in CFBE/F508del-CFTR cells are similar before adding thapsigargin. However, after adding thapsigargin, the ER released Ca^{2+} (ER calcium stores) in CFBE/F508del-CFTR cells were about 32% higher than in CFBE/WT-CFTR cells ($^{**}P < 0.008$, $n = 6$), and ouabain treatment reduced ER calcium stores in CFBE/F508del-CFTR cells by ∼47% ($^*P < 0.015$, $n = 6$). Thus, after 24 h of exposure to a low concentration of ouabain, ER calcium stores in CFBE/F508del-CFTR cells were similar to those in CFBE/WT-CFTR cells. And this normalization of ER $[Ca^{2+}]$ in F508del-CFTR cells is also observed by low temperature rescue or by other pharmacological corrections (Antigny et al., 2011).

CONNECTIVITY BETWEEN OUABAIN AND LOW TEMPERATURE TRANSCRIPTIONAL PROFILES SUGGESTS A SIMILAR MODE OF ACTION

Genome wide transcriptional profiling can be used to infer similarities between the mechanisms of action of different compounds. The CMAP is a rich compendium of 6100 genome wide transcriptional profiles from cultured human cells that have been treated with 1309 bioactive small molecules, including ouabain, and other cardiac glycosides. Gene signatures that show positive correlation with reference profiles (instances) in the CMAP share functional similarities and provide clues to the mechanisms of action of the compounds (Lamb et al., 2006).

To explore the mechanism of F508del-CFTR correction by ouabain, we used transcriptional profiling of parental CFBE41o- cells subjected to different treatments. We generated gene expression profiles using two levels of stringency that were set using a False Discovery Rate (FDR) of ≤0.01 and ≤0.05 (Benjamini and Hochberg, 1995) and an absolute fold Change (absFC) >3. The signatures used to query the CMAP (99 probes up- and 208 probes down-regulated) were from polarized parental CFBE41o- cells treated with ouabain for 24 h. As expected we detected ouabain and six other cardiac glycoside reference profiles (instances) in the CMAP with high confidence (**Figure 6A**, P-value = 0, enrichment score (ES) = 0.995; ES, ranging from +1 means correlated; −1 means anti-correlated). We then queried the CMAP with signatures obtained under three well characterized conditions in which F508del-CFTR trafficking is partially corrected: low temperature rescue (29°C) and the corrector compounds VRT-325 (Loo et al., 2006; Varga et al., 2008) and VX-809 (Van Goor et al., 2011). Remarkably, the low temperature signature (FDR ≤ 0.05; 384 probes up- and 329 probes down-regulated), recovered the ouabain and the other six cardiac glycoside instances with very high ESs (**Figure 6B**, P-value = 0, and ES = 0.942). At higher stringency (FDR ≤ 0.01; 81 probes up- and 74 probes down-regulated), the low temperature signature remained tightly correlated with the cardiac glycoside instances, including ouabain (**Figure 6C**, P-value = 0, and ES = 0.972) suggesting that there is a strong similarity. Conversely, when we queried the CMAP with signatures derived from parental CFBE41o- cells treated with VRT-325 and VX-809, which are thought to act as a pharmacological chaperones that directly bind to F508del-CFTR, we found that the 24-h signature for VRT-325 (FDR ≤ 0.05; 20 probes up- and 139 probes down-regulated) was negatively correlated with ouabain instances and VX-809 24 h signature (FDR ≤ 0.05; 97 probes up- and 64 probes down-regulated) null-correlated with ouabain instances (**Figure 6D**).

We next examined a larger number of probes to explore the broader transcriptional response to ouabain and low temperature (31914 probes/41000 Agilent probes, FDR ≤ 0.05; **Figure 7A**) and to confirm this relationship by measuring the similarity of

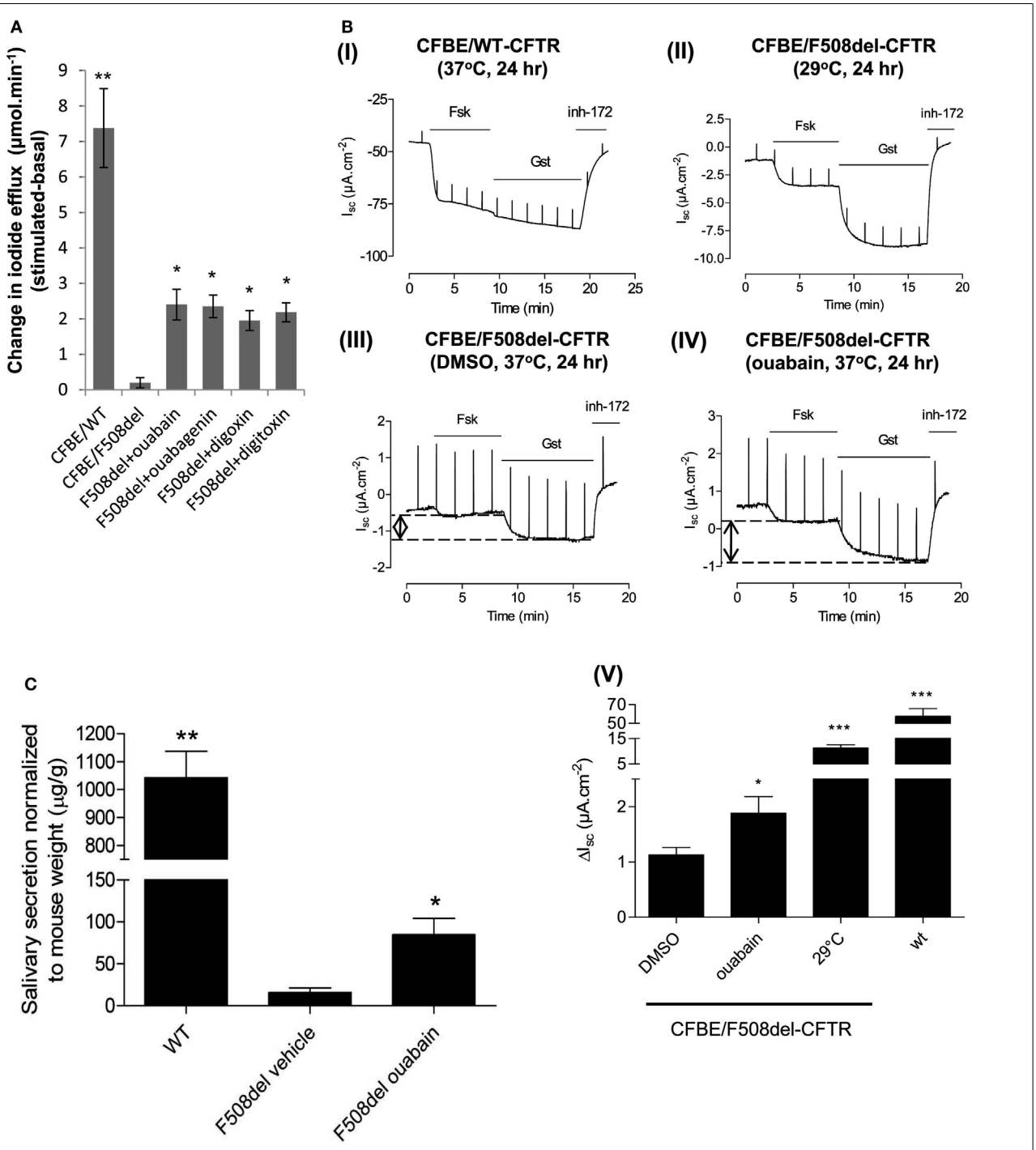


FIGURE 4 | Ouabain rescues F508del-CFTR channel activity in *in vitro* and *in vivo* assays. (A) CFBE/F508del-CFTR cells treated with ouabain, ouabagenin, digoxin, and digitoxin for 24 h, and iodide efflux was monitored. Data shown are the mean ± SD of $n = 4$ ($^*P < 0.03$; $^{**}P < 0.006$). **(B)** CFBE/F508del-CFTR cells were treated with or without 0.1 µM ouabain for 24 h and CFTR channel activity was measured by Ussing chamber assay. The ΔI_{sc} stimulated by ouabain treatment was compared to low temperature incubation (29°C, 24 h) and to CFBE/WT-CFTR. Histogram showing the change in I_{sc} (ΔI_{sc}) after addition of forskolin + genistein, defined as the difference between the sustained phase of the current response after stimulation and the baseline immediately before stimulation. In the bar graph, data, are presented as mean ± SEM as compared to DMSO control [**(I)** $n = 9$ for CFBE/WT-CFTR cells; **(II)** $n = 6$ for 29°C treatment samples; **(III)** $n = 8$ for DMSO control; **(IV)** $n = 7$ for ouabain; **(V)** $^*P < 0.05$; $^{***}P < 0.001$]. **(C)** Salivary secretion in wild-type mice (WT) or F508del-CFTR (F508del) mice treated with vehicle alone or 0.01 mg/kg/day of ouabain for 2 days. Monitored for 30 min following stimulation, results are expressed as the mean ± SEM of $n = 5$ ($^*P < 0.04$; $^{**}P < 0.0004$).

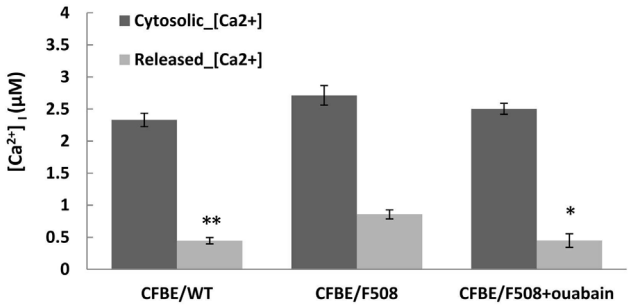


FIGURE 5 | Ouabain reduced the ER calcium stores in CFBE cells. CFBE/WT-CFTR and CFBE/F508del-CFTR cells were treated with, or without-, 100 nM ouabain for 24 h. The cells were then loaded with Fura-2/AM, and peak cytosolic Ca^{2+} concentration was measured as the difference in Fura-2 fluorescence recorded before and after adding 2 μM thapsigargin. Data are mean ± SD of $n = 6$ ($*P < 0.015$; $**P < 0.008$).

the expression patterns. We considered the union of the genes that were differentially expressed in each condition and discarded those that were not changed across the four treatments (ouabain, low temperature, VRT-325, and VX-809; FDR ≤ 0.05) in an unsupervised hierarchical clustering analysis (Eisen et al., 1998). In the output from this type of analysis similar patterns of expression are grouped together. Ouabain and low temperature clustered together based on the correlation coefficient and Euclidean distance measurements suggesting they share a similar mechanism of action, whereas VRT-325 and VX-809 form a distinct group, again suggesting that they share a similar mode of action (**Figure 7B**). To test the hypothesis that ouabain and low temperature rescue operate via a similar mechanism, ouabain treatment of CFBE/F508del-CFTR cells was combined with low temperature incubation in immunoblotting experiments (**Figure 7C**). No increase in F508del-CFTR trafficking was observed with a combination treatment, suggesting these treatments act in a similar manner and are not additive. Conversely, VRT-325, which stabilized the surface pool of F508del-CFTR as well as corr-4a (Varga et al., 2008), were combined to low temperature treatment and they further improved F508del-CFTR maturation in CFBE/F508del-CFTR cells measured by immunoblotting (Jurkuvenaite et al., 2010; Sondo et al., 2011).

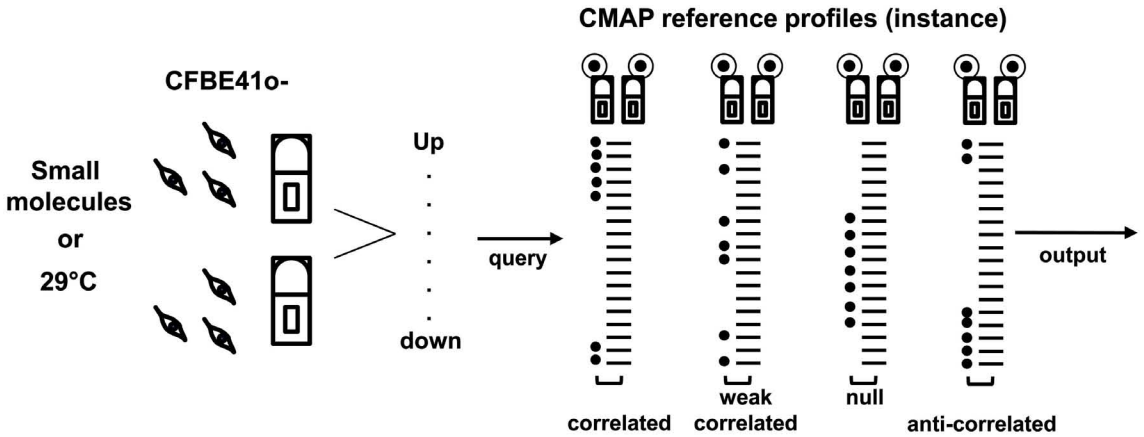
OUABAIN AND LOW TEMPERATURE TREATMENT GENERATE SIMILAR GENE EXPRESSION PROFILES

To gain insight into the molecular processes involved in the stability and trafficking of F508del-CFTR we analyzed the common genes following ouabain and low temperature treatments using the GeneGo Cystic Fibrosis platform (MetaCore™ by GeneGo, Inc.). We obtained 3530 genes in common, 8963 unique genes for ouabain and 687 unique genes for low temperature with FDR ≤ 0.05, and the intersection of ouabain and low temperature transcriptional signatures showed that 84% of the differentially expressed genes at 29°C were also differentially expressed with ouabain treatment (**Figure 8A**, left panel). We performed enrichment analysis to identify functional ontologies in MetaCore with an associated *P*-value (**Figure 8A**, right panel). Enrichment analysis consists of matching gene IDs of possible targets with those in functional ontologies in GeneGo comparison experiments workflow (Shmelkov et al., 2011).

Remarkably, among the common genes shared between ouabain and low temperature treatments we found that the Gene Ontology (GO) processes that were most highly enriched were: Response to Endoplasmic Reticulum Stress ($P = 1.11e^{-11}$), Response to Unfolded Protein ($P = 1.13e^{-11}$), Protein Transport ($P = 1.68e^{-11}$), and the more general Positive Regulation of Biological Processes ($P = 1.61e^{-9}$; **Figure 8A**, right panel). These results suggest that processes associated with the folding and degradation of CFTR were at the interface between the two treatments. To dissect this further we validated a subset of the 3530 common genes by real-time PCR (**Figure 8B**), selecting the ones that were most differentially expressed by both treatments and associated with the most enriched GO processes. After treatment with ouabain and low temperature we observed a decrease in the expression of chaperone genes such as *HSPA8*/Hsc70 and *HSPA1L*/Hsp70 that are involved in protein folding and ER-associated degradation (**Figure 8B**, respectively 60 and 70% reduced by ouabain, 80 and 60% reduced by low temperature; $*P$-value < 0.05). To test if this altered level of mRNA could also be detected at the protein level we measured the expression of Hsc70 and Hsp70 protein by immunoblotting (**Figure 8C**). The expression of Hsc70 and Hsp70 chaperones was decreased and correlated with the appearance of mature F508del-CFTR (glycosylated form, band C), and with increased levels of band B (core-glycosylated form; **Figure 8C**). In contrast, expression of the Unfolded Protein Response (UPR) marker, *HSPA5*/BiP decreased with ouabain treatment but remained unchanged at low temperature, while ER chaperones such as calnexin (tested as a control) were unaltered by ouabain or low temperature (**Figures 8B,C**). We also observed an increase in the *SEC24A* mRNA and protein expression (**Figures 8B,C**) following ouabain treatment. Sec24A (COPII complex subunit) implicated in the binding of CFTR destined to traffic from the ER (Routledge et al., 2010) was up-regulated by ouabain treatment but not at low temperature (**Figure 8C**). At the individual gene level, there were differences in the expression levels between ouabain and low temperature but overall, the striking correlation obtained between the two signatures shows their functional similarities.

DISCUSSION

Cardiac glycosides have been in clinical use for centuries to treat heart failure, and the mechanism of their positive inotropic effect is well characterized. Ouabain and other cardiac glycosides bind Na^+/K^+-ATPase in cardiac myocytes and act by inhibiting its enzymatic activity or down-regulating its expression (Huang et al., 1997; Hoyer et al., 2011). Cardiac glycosides can induce apoptosis and inhibit the growth of cancer cell lines and the pathway to the clinic is expected to be short because the pharmacodynamics and pharmacokinetics of cardiac glycosides are already well-established (Prassas and Diamandis, 2008). Oleandrin, the most promising first generation glycoside-based anticancer drug, is presently in phase I clinical trials to determine the maximum-tolerated dose and evaluate its effect on the pharmacokinetics on chemotherapies administered concurrently (Yang et al., 2009).



Signature abcFC>3	Instance	Enrichment score	Estimated p-value
A	helveticoside	0.996	0
ouabain 24 hr	lanatoside C	0.995	0
fdr ≤ 0.05	digoxin	0.995	0
In CFBE41o-cells	digitoxigenin	0.996	0
	ouabain	**0.995**	**0**
	digoxigenin	0.996	0
	beta-escin	0.535	0.03953
B	helveticoside	0.965	0
Low-Temp 24 hr	beta-escin	0.913	0
fdr ≤ 0.05	digoxin	0.948	0
In CFBE41o- cells	digitoxigenin	0.946	0
	ouabain	**0.942**	**0**
	lanatoside C	0.819	0.00008
	digoxigenin	0.759	0.00194
C ouabain 24 hr fdr ≤ 0.01 In CFBE41o- cells	**ouabain**	**0.994**	**0**
Low-Temp. 24 hr fdr ≤ 0.01 In CFBE41o- cells	**ouabain**	**0.972**	**0**

D Signature fdr ≤ 0.05, abc FC>3	Instance	Enrichment score	Estimated p-value
VRT-325 24 hr	ouabain	-0.485	0.211
VX-809 24 hr	ouabain	0.575	---

FIGURE 6 | Ouabain gene expression signature is highly correlated with low temperature. Cartoon depicting the CMAP concept: pattern-matching algorithms score each established profile for the direction (up- or down-regulated) and strength (fold) of enrichment with the query signature (Lamb et al., 2006). CMAP outputs of 100 nM ouabain (or other small molecules) query signatures obtained in polarized parental CFBE41o-cells treated for 24 h at 37˚C and polarized parental CFBE41o-cells treated for 24 h at 29˚C (Low-Temp.). Probes generated a FDR ≤ 0.05 and an absolute fold change (absFC) >3 were included in the query signatures. Perturbagens are ordered according to their estimated *P*-values (correlated means = 0) and corresponding connectivity scores, ranging from +1 (correlated) to −1 (anti-correlated). **(A)** The genomic changes induced in polarized parental CFBE41o-cells by ouabain 24 h query signature is correlated with ouabain and six cardiac glycosides previously established profiles in CMAP (instances). **(B)** The genomic changes induced in polarized parental CFBE41o-cells by low temperature 24 h specific-signature is highly ranked with ouabain and six cardiac glycoside instances. **(C)** More stringent query signature (FDR ≤ 0.01) increases the ability of low temperature to recover ouabain instance in the CMAP. **(D)** The genomic changes induced in polarized parental CFBE41o-cells by 10 μM VRT-325 and by 1 μM VX-809 query signatures are respectively weakly anti-correlated and null-correlated with the previously established ouabain profile in CMAP.

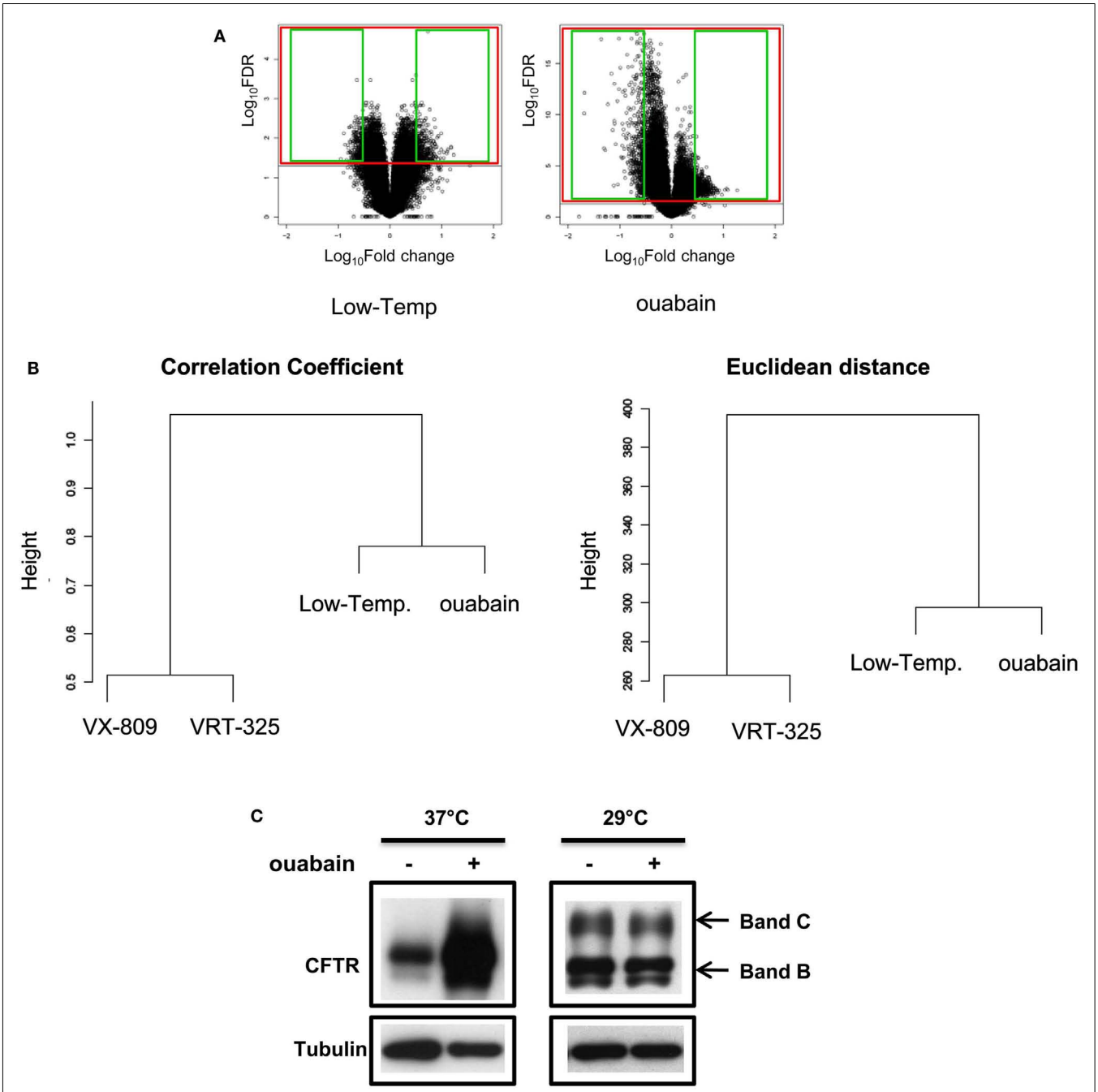


FIGURE 7 | Ouabain and low temperature rescue have a similar mode of action. (A) Volcano plots of ouabain and low temperature gene expression data. Values are presented as the $\log_{10}$ of FDR and fold change. Green squares indicate up- and down-regulated probes for CMAP query signatures for each treatment. Red squares indicate the probes considered for each treatment in the unsupervised hierarchical clustering according to FDR ≤ 0.05. **(B)** Unsupervised hierarchical clustering of ouabain, low temperature, VRT-325 and VX-809 expression patterns (FDR ≤ 0.05) using standard Correlation coefficient and Euclidian distance as similarity measures. The distance between clusters was computed according to complete linkage. **(C)** CFBE/F508del-CFTR cells were treated with or without 0.1 μM ouabain for 48 h at 29 or 37˚C, the cell lysates were analyzed by immunoblotting with anti-CFTR or anti-tubulin antibodies.

In contrast to the apoptotic effects of these drugs on cancer cells, low concentrations of ouabain have also been shown to stimulate the proliferation and inhibit cell death in normal cells (Li et al., 2006). It has been reported that digitoxin and other cardiac glycosides at sub-nanomolar concentrations mimic gene therapy with *CFTR in vitro* and can suppress the hypersecretion of IL-8 by cultured CF airway epithelial cells (Srivastava et al., 2004). More recent data show that digoxin derivates attenuate

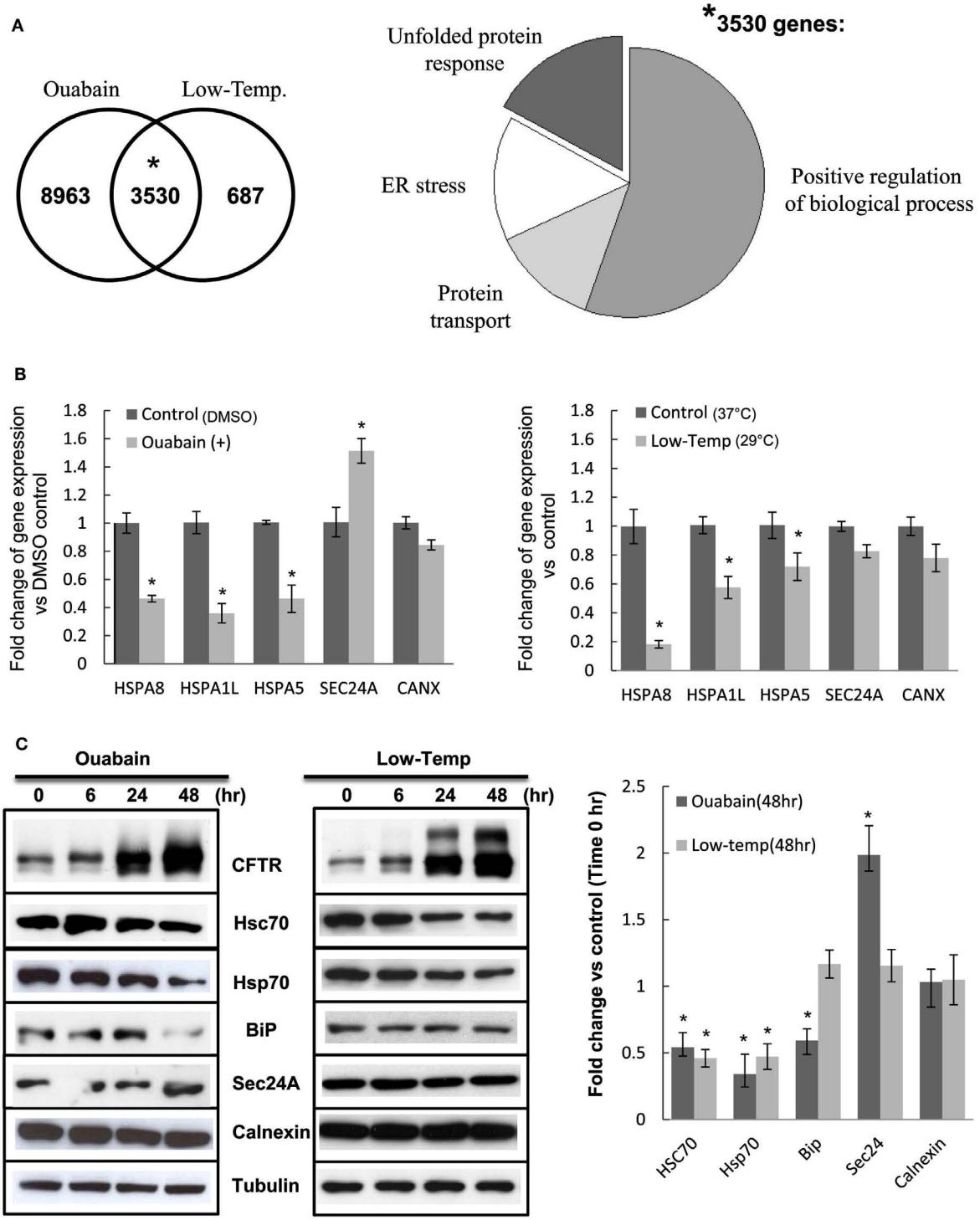


FIGURE 8 | The effects of ouabain and low temperature on ER-related chaperones. (A) Left panel: Venn diagram of the intersection between ouabain and low temperature 24 h signatures according to a FDR ≤ 0.05. The numbers indicate distinct genes. The probability $*P = 0.0001$ was calculated using a hyper-geometric random variable. Right panel: Gene Ontology (GO) cellular processes pie chart of 3530 genes in common between ouabain and low temperature 24 h treatments. **(B)** The total RNA previously extracted for the microarray analysis was tested by real-time PCR using the individual primers for the gene expressions of HSPA8, HSPA1L, HSPA5, SEC24A, and CANX. Data are presented by the fold change of gene expression vs. DMSO control with mean ± SEM of $n = 3$ ($*P < 0.05$) **(C)** CFBE/F508del-CFTR cells were treated with 0.1 μM ouabain or 29°C (Low-Temp.) for 6, 24, or 48 h, then the cell lysates were analyzed by immunoblotting using the individual antibodies. The bar graph shows quantification of the band intensities for blots expressed as fold change vs. Time 0 h control. Values in the experiments described are presented as means ± SD ($n = 3$). Means were tested for statistical significance using a Student's *t*-test ($*P < 0.05$).

inflammatory lymphocyte function and autoimmune diseases (Huh et al., 2011).

Na^+/K^+-ATPase, the target of cardiac glycosides can, in the presence of nanomolar concentrations of ouabain, act as a signal transducer. For instance, it has been reported that ouabain-bound Na^+/K^+-ATPase can, independent of its ion transport function, induce multiple signaling pathways including c-Src and intracellular calcium oscillations (Zhang et al., 2006). Several studies have shown that $[Ca^{2+}]$ is elevated in CF and becomes normalized when the trafficking of F508del-CFTR is corrected by small molecules or low temperature (Norez et al., 2006, 2009; Antigny et al., 2008a). The relationships between CFTR and calcium signaling have recently been reviewed (Antigny et al., 2011), however the role of $[Ca^{2+}]$ in protein biogenesis and trafficking remains incompletely understood. We confirmed the elevation of ER calcium stores in CF cells and showed that ouabain reduces store calcium to normal levels in CFBE cells expressing F508del-CFTR (**Figure 5**).

Here, we demonstrate that nanomolar ouabain increases F508del-CFTR trafficking to the cell surface and partially restores its function in a human CF bronchial epithelial cell line. Ouabain has this effect without causing substantial inhibition of Na^+/K^+-ATPase protein expression or cell viability. Moreover, our *in vivo* results also showed functional rescue of mutant CFTR by ouabain in CF mice and the value corresponds to ∼8.1% of the secretory response of littermate WT control mice and without affecting the mice body weight (control group: starting, 26.36 ± 2.49 g; after 48 h, 26.53 ± 2.63 g, $P > 0.2$; ouabain treated group: starting, 26.98 ± 2.39 g; after 48 h 27.29 ± 2.33 g, $P > 0.2$). Thus our data raise the possibility that cardiac glycosides not only increase total CFTR protein expression, but may also increase the folding yield and trafficking of F508del-CFTR. Ouabain thus joins a growing list of F508del-CFTR corrector compounds that act by modulating proteostasis (Calamini et al., 2012) rather than by acting as pharmacological chaperones that bind to F508del-CFTR (Sampson et al., 2011). Considering the inhibition function of cardiac glycosides on inflammation in cultured CF airway epithelial cells (Srivastava et al., 2004), also associating the long history of cardiac glycosides have being used in clinical treatment, it suggests that ouabain and other cardiac glycosides may have potential therapeutic perspectives for CF patients.

Transcriptional profiling analysis and the CMAP uncovered interesting similarities between very different corrector mechanisms (Lamb et al., 2006). We proved the ability and reliability of the CMAP to connect our ouabain signature and the ouabain reference profiles already present in the CMAP collection, and then found that the resulting transcriptional profile obtained by ouabain treatment resembled that produced by low temperature (29°C) suggesting a similar mechanism of action. The CMAP is a resource that can be used to discover functional connections with a limited number of probes that are up- or down-regulated (≤1000 probes). To better identify the state of the CFBE41o- cells throughout their responses to ouabain, low temperature, VRT-325 and VX-809, we applied an unsupervised hierarchical cluster analysis to obtain a direct measure of similarity of parental CFBE41o-expression patterns. In this approach, a larger number of probes, comparing the ones queried in CMAP, were computed using standard Correlation coefficient and Euclidian distance as measures of similarity. The output groups together genes with similar patterns of expression by a direct measure of similarity and probes which encode for genes that are co-expressed share common mechanisms. We integrated VRT-325 and VX-809 profiles in the clustering analysis not only because both these compounds are thought to bind to F508del-CFTR (Loo et al., 2006; Varga et al., 2008; Van Goor et al., 2011) but also to delineate different "categories" of correctors based on shared mechanisms of action. The addition of this condition enhances our observation by which ouabain and low temperature share a common mechanism and VRT-325 cluster together with VX-809 and we supported our mode of action predictions based on gene expression profiles by combination treatments.

Our study indicates that ouabain and low temperature rescue of F508-CFTR may involve the down-regulation of chaperones (*HSPA8*/Hsc70 and *HSPA1L*/Hsp70), thereby reducing F508del-CFTR degradation; and the up-regulation of COPII components for vesicular export to the Golgi. These two heat shock proteins play important roles in the biosynthesis and degradation of CFTR and it has been shown that a decrease in expression of Hsc70 (in association with the co-chaperone CHIP) results in decreased degradation of F508del-CFTR (Rab et al., 2007; Matsumura et al., 2011). Moreover, we found an increased expression of *SAR1A* (COPII complex subunits; see Supplementary Material) by low temperature incubation. *SAR1A*, together with *SEC24A*, which is up-regulated by ouabain, led to anterograde export of the binding protein to the Golgi (Yoo et al., 2002; Wang et al., 2008). The intersection of ouabain and low temperature transcriptional responses indicates that 84% of the genes that are differentially expressed at 29°C are also differentially expressed with ouabain (**Figure 8A**, left panel). Thus, ouabain may shift the cells to a "permissive" state by mimicking low temperature, thus correcting the F508del-CFTR folding and trafficking defect.

Dissection of the molecular events that underlie rescue by low temperature and mimicking it with a small molecule may be a strategy to identify CF therapeutics. Galietta and colleagues have shown using CFBE41o-cells that low temperature can synergize with correctors such as corr-4a and VRT-325 (Sondo et al., 2011). Therefore our finding suggests that combination treatments that include ouabain may also be synergistic in the treatment of CF.

In summary, our study shows that low concentrations of ouabain can rescue F508del-CFTR by mimicking low temperature rescue in human CF bronchial epithelial cells. Apart from pharmacological chaperones that bind directly to F508del-CFTR the target of and the mechanism of action of most correctors is unknown. Certainly cardiac glycosides are being investigated for use as a cancer therapeutic and for other diseases (Prassas and Diamandis, 2008). We predict that the Na^+/K^+-ATPase or its downstream pathway will be a good place to search for F508del-CFTR correctors.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTIONS

The parental CFBE41o-cell line was originally developed by immortalization of CF (F508del/F508del) bronchial epithelial cells by Dr. D. Gruenert (Kunzelmann et al., 1993). The mutated

protein is expressed at low levels in this cell line, therefore two derivatives were generated by transduction using the TranzVector lentivirus system (Wu et al., 2000) to create CFBE/WT-CFTR and CFBE/F508del-CFTR cell lines in which the CFTR protein can be detected by immunoblots. Those cells were generously provided by Dr. J. P. Clancy (University of Alabama, Birmingham) and cultured in EMEM medium supplemented with 10% FBS. Polarized CFBE41o-cells were cultured initially under liquid–liquid conditions, then allowed to polarize at the air–liquid interface. BHK cells stably expressing F508del-CFTR-3HA were cultured as described previously (Carlile et al., 2007).

THE CELL-BASED TRAFFICKING ASSAY

The surface expression of CFTR was measured as described previously (Carlile et al., 2007). BHK cells stably expressing F508del-CFTR-3HA (bearing a 3HA-epitope tag in the fourth extracellular loop) were treated with cardiac glycosides. Cells were fixed with 4% paraformaldehyde for 15–20 min at 4˚C and incubated with monoclonal anti-HA antibody (Sigma, Canada) solution containing 1% FBS at 4˚C overnight. After washing, the plates were analyzed using a plate reader (Analyst™ HT 96.384, Biosystems, USA; 488 nm excitation, 510 nm emission) to measure background fluorescence, then incubated with anti-mouse IgG antibody conjugated with FITC (Sigma, Canada) at a dilution of 1:100 for 1 h. The cells were washed, then incubated with 100 μl of PBS, and reanalyzed. The mean fluorescence of 12 mock (DMSO) treated wells was used as the background signal and designated 0% cell surface signal. The surface CFTR signal of cells expressing WT-CFTR on the same plate was designated 100%. The compound treated cell fluorescent signal was then given a percentage value relative to these two controls. Control experiments indicated that the vehicle did not affect trafficking when added alone (data not shown).

IMMUNOBLOTTING AND ANTIBODIES

Cells were lysed in RIPA buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 0.08% deoxycholic acid, and lysates were separated by 6% SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with monoclonal anti-tubulin (Sigma), anti-CFTR (monoclonal antibody 23C5, P. Määttänen, M. Mirza, and D. Y. Thomas, unpublished results), anti-CFTR (M3A7, Chemicon), anti-BiP (BD Transduction Laboratories), anti-Hsp70 (Stressgen), rabbit polyclonal anti-calnexin (kindly provided by Dr J. J. Bergeron, McGill University), rabbit anti-Sec24A (Novus Biologicals), and rabbit anti-Hsc70 antibodies (StressMarq). Horseradish peroxidase (HRP)-conjugated secondary antibodies were used and blots were developed using the ECL detection system (Roche, Germany) and exposed to film (Amersham). The films were scanned and analyzed by densitometry using Photoshop (Adobe, Inc.). Quantification of the band intensities for **Figures 2B** and **8C** experiments expressed as fold change vs. DMSO control and normalized by tubulin bands.

CYTOTOXICITY ASSAY

The cytotoxic effects of ouabain or other cardiac glycosides (from Sigma) were determined by using the colorimetric AlamarBlue™ (Biosource, Camarillo, CA, USA) assay, according to the manufacturer's instructions. Briefly, cells were plated in triplicate at a density of 3×10^5 cells/per well in 96-well plates and cultured overnight. Cells were then treated with the different concentrations of ouabain or other cardiac glycosides for 24 h. The medium was removed after 24 h and cells were incubated in fresh medium at 37˚C, 5% CO_2 for 4 h. At the end of the 4-h incubation, 10 μl of AlamarBlue was added to each well and incubated at 37˚C, 5% CO_2 for 18 h. Absorbance was measured at 570 and 600 nm and medium without cells was used as blank. Percent survival was quantified according to the manufacturer's instructions and the untreated sample was set to 100%. Final percent survival was averaged from three triplicates from three independent experiments.

STATISTICS

Values in the experiments described are presented as means ± SD. Means were tested for statistical significance using the Student's *t*-test.

IODIDE EFFLUX ASSAYS

Cystic fibrosis transmembrane conductance regulator channel activity was assayed by measuring iodide efflux with a robotic liquid handing system (BioRobot 8000, Qiagen, USA) using Qiagen 4.1 Software as described previously (Robert et al., 2008). Cells were seeded in 24-well plates allowed to reach 100% confluence, and treated with drug or vehicle for an additional 24 h. Cells were then incubated in iodide loading buffer [136 mM NaI, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM Hepes pH 7.4] for 1 h at 37˚C, then washed with efflux buffer [136 mM $NaNO_3$, 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, and 20 mM Hepes, pH 7.4] and the appearance of I^- was measured after replacing the buffer at 1 min intervals before and during stimulation with 50 μM genistein and 10 μM forskolin using an iodide-sensitive electrode (Orion Research, Inc., Boston, MA, USA). Relative iodide efflux rates were calculated from the difference between the maximal (peak) iodide concentration during stimulation and the minimal iodide concentration before stimulation.

USSING CHAMBER STUDIES

Cystic fibrosis transmembrane conductance regulator channel activity was measured in Ussing chambers as described previously (Robert et al., 2010). Briefly, 2×10^6 cells (CFBE/WT-CFTR or CFBE/F508del-CFTR cells) were seeded onto fibronectin-coated Snapwell 12-mm inserts (Corning Incorporated, Life Sciences, NY, USA) and the apical medium was removed the following day to create an air–liquid interface. Trans-epithelial resistance was monitored using an EVOM epithelial volt ohm meter (World Precision Instruments, Sarasota, FL, USA) and cells were used when the trans-epithelial resistance of the monolayer was 300–400 Ω cm^2. In some experiments, CFBE/F508del-CFTR monolayers were grown at 29˚C or treated with a test compound at 37˚C for 24 h before being mounted in chambers and voltage-clamped using a VCCMC multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA, USA). Apical membrane conductance was functionally isolated by permeabilizing the basolateral membrane with 200 μg/ml nystatin and imposing an apical-to-basolateral Cl^- gradient. The apical bathing solution contained

115 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM mannitol (pH 7.4 with NaOH). The basolateral bathing solution contained 1.2 mM NaCl, 115 mM Na-gluconate, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4 mM $CaCl_2$, 2.4 mM, KH_2PO_4, 1.24 mM K_2HPO_4, 10 mM glucose (pH 7.4 with NaOH). $CaCl_2$ was increased to 4 mM to compensate for its chelation by gluconate. The apical solution contained mannitol instead of glucose to eliminate current mediated by Na^+-glucose cotransporters. Successful permeabilization of the basolateral membrane under these conditions was obvious from the reversal of I_{sc}. Solutions were continuously gassed and stirred with 95% O_2-5% CO_2 and maintained at 37°C. Ag/AgCl reference electrodes were used to measure trans-epithelial voltage and pass current. Pulses (1 mV amplitude, 1 s duration) were imposed every 90 s to monitor resistance. The voltage clamps were connected to a PowerLab/8SP interface (ADInstruments, Colorado Springs, CO, USA) for data collection. Ten micromolars forskolin +50 μM genistein were added to the apical bathing solution to activate CFTR.

SALIVARY SECRETION

The salivary secretion assay was performed as described (Best and Quinton, 2005). Briefly, homozygous Δ508-CFTR mice (*Cftr*^{tm1} *Eur*) and WT mice were 10–12 weeks old and when used weighed 20–25 g. A micro pump (Alzet Model 1003D) was fixed under the skin on the back of mouse to deliver a very low dose of ouabain (0.01 mg/kg/day) or vehicle for 48 h. Mice were anesthetized using ketamine and diazepam and 1 mM atropine was injected subcutaneously into the left cheek to block cholinergic responses. After absorbing any saliva with Whatman filter paper, 100 μM isoprenaline was injected at the same site with 1 mM atropine to induce secretion and saliva was collected on filter paper every 3 min for 30 min. Samples were immediately sealed in a pre-weighed vial and the saliva secretion rate and the total amount were normalized to mouse weight. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and the protocol was approved by the McGill University Animal Care Committee.

ER CALCIUM STORE MEASUREMENTS

Thapsigargin-releasable ER calcium was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca^{2+}-free buffer. In brief, the cells were grown and treated with or without 0.1 μM ouabain for 24 h, then 2×10^6 cells (CFBE/WT-CFTR or CFBE/F508del-CFTR cells) were harvested and washed in Ca^{2+}-free buffer (20 mM HEPES, pH 7.4, 143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 0.1% glucose, 0.1% bovine serum albumin, 250 mM sulfinpyrazone). The cells were resuspended in 200 μl of calcium-free buffer containing 0.02% pluronic acid and subsequently loaded with the cell-permeable fluorescent indicator Fura-2/AM at 3 mM for 30 min at 37°C. After a final wash, the cells were resuspended in Ca^{2+}-free buffer and a 340/380-nm excitation ratio at a 510-nm emission wavelength were obtained using a LS 50B PerkinElmer Life Sciences luminescence spectrophotometer. The fluorescence ratio (340/380) was measured in cells treated with 2 μM thapsigargin and the Fura-2 ratio values converted to $[Ca^{2+}]$ according Grynkiewicz et al. (1985). The peak of thapsigargin-releasable $[Ca^{2+}]_{cyto}$ was calculated as the difference in cytoplasmic calcium measured before and after the addition of 2 μM thapsigargin to cells in Ca^{2+}-free Hanks' buffer.

MICROARRAY ANALYSIS

Polarized parental CFBE41o-cells cultured at the air–liquid interface were used for microarray assays. RNA samples were extracted in 1 ml TRIzol Reagent (Invitrogen, USA), quantificated by spectrophotometry (Nanodrop, USA), and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) $\geq$ of 8 were used for amplification. Total RNA (1 mg) was subjected to two rounds of amplification using the Amino Allyl MessageAMP II aRNA amplification kit (Ambion, Applied Biosystems, USA). The integrity and quantity of the aRNA was revaluated by Nanodrop and Agilent Bioanalyzer, and coupled to Cy3 and Cy5 (Amersham Biosciences, UK). Whole Human Genome 44 K arrays (Agilent Technologies, product G4112A) were used for all experiments. RNA samples (825 ng/each) were subjected to fragmentation followed by 16 h hybridization, washing, and scanning (Agilent Technologies, model G2505B) according to the manufacturer's protocol (manual ID #G4140-90030). Samples were hybridized against Universal Human Reference RNA (Stratagene, ID #740000, La Jolla, CA, USA). Duplicate hybridizations were performed for each sample using reverse-dye labeling. Arrays were washed according to manufacturer's recommendations, scanned using an Agilent dual-laser microarray scanner (Model G2505B), and Cy5/Cy3-signals were quantified using Agilent's Feature Extraction software (v.7.11) with the default parameters.

Microarray quality control reports generated by the Agilent Feature Extraction software were used to detect hybridization artifacts. Probe level raw intensities were processed using R/BioConductor and Limma package (Gentleman et al., 2004). Data were background corrected using "normexp" limma method and normalized in two steps: loess normalization within-array to correct systematic dye-bias and quantile normalization between–arrays to detect systematic non-biological bias. Ratios representing the relative target mRNA intensities compared to Universal Human Reference RNA probe signals were derived from normalized data. To remove "batch effects" across microarray experiments we adjusted the data using the empirical Bayes method available at: http://biosun1.harvard.edu/complab/batch/ (Johnson et al., 2007). PCA plots and Clustering trees of normalized adjusted intensities were drawn for each time-point specific sets of samples to confirm the robustness of the method used.

To find differentially expressed genes (treatment vs. control), a *t*-test was applied for each time-point. For each *P*-value, the Benjamini–Hochberg procedure was used to calculate the FDR (Benjamini and Hochberg, 1995). Genes were considered to be differentially expressed if the corrected FDR ≤ 0.05 (while controlling the expected FDR to no more than 5%). Unsupervised hierarchical clustering was performed on normalized data (FDR ≤ 0.05), with complete linkage and Euclidian and Pearson's correlation distances.

FUNCTIONAL CATEGORY ENRICHMENT ANALYSES

Identification of overrepresented functional categories (pathways and cellular processes) was performed per treatments using the complete set of differentially expressed genes (FDR ≤ 0.05) in the MetaCore™ suit (Version 6.1; GeneGo, Inc., St. Joseph, MI, USA; Nikolsky et al., 2005). The functional analysis were based on MetaCore's proprietary manually curated data base of CF specific contents (Nikolsky et al., 2009).

REAL-TIME PCR

Total RNA was extracted from cells using TRIzol Reagent (Life Technologies, Inc., Burlington, ON, Canada), and the cDNA was synthesized using AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). Real-time PCR was performed using a Stratagene Mx3005PTM system (Stratagene, La Jolla, CA, USA) as follows: 20 μl reaction solution contained 10 μl SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA); 0.4 μl sense and reverse primer (25 ng/μl); 2 μl diluted cDNA; 7.2 μl nuclease-free water. For the cross-validation real-time PCR experiments we used the same total RNA extracted for the microarray assays. The primer sequences were designed according to the GenBank™ accession numbers: GAPDH NM_002046; CFTR NM_000492; HSPA8 NM_006597; HSPA1L NM_005527; HSPA5 NM_005347; SEC24A NM_021982; and CANX NM_001746. The subsequent data analysis was performed using MxPro™ QPCR Software followed by comparative quantification real-time PCR. Gene expression levels were normalized to GAPDH gene expression and compared with untreated control, which was assigned a value of 1.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Pharmacology_of_Ion_Channels_and_Channelopathies/10.3389/fphar.2012.00176/abstract

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Targeting the intracellular environment in cystic fibrosis: restoring autophagy as a novel strategy to circumvent the CFTR defect

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Cystic fibrosis (CF) patients harboring the most common deletion mutation of the CF transmembrane conductance regulator (CFTR), F508del, are poor responders to potentiators of CFTR channel activity which can be used to treat a small subset of CF patients who genetically carry plasma membrane (PM)-resident CFTR mutants. The misfolded F508del-CFTR protein is unstable in the PM even if rescued by pharmacological agents that prevent its intracellular retention and degradation. CF is a conformational disease in which defective CFTR induces an impressive derangement of general proteostasis resulting from disabled autophagy. In this review, we discuss how rescuing Beclin 1 (BECN1), a major player of autophagosome formation, either by means of direct gene transfer or indirectly by administration of proteostasis regulators, could stabilize F508del-CFTR at the PM. We focus on the relationship between the improvement of peripheral proteostasis and CFTR PM stability in F508del-CFTR homozygous bronchial epithelia or mouse lungs. Moreover, this article reviews recent pre-clinical evidence indicating that targeting the intracellular environment surrounding the misfolded mutant CFTR instead of protein itself could constitute an attractive therapeutic option to sensitize patients carrying the F508del-CFTR mutation to the beneficial action of CFTR potentiators on lung inflammation.

Keywords: cystic fibrosis, CFTR, proteostasis regulators, autophagy, BECN1

INTRODUCTION

The proteostasis network ensures intracellular homeostasis in spite of genetic or epigenetic changes in protein conformation, extracellular stress, or aging-associated perturbations (Balch et al., 2008; Hutt et al., 2009; Powers et al., 2009; Gidalevitz et al., 2010; Hutt and Balch, 2010; Roth and Balch, 2011). The accumulation of misfolded/modified proteins due to mutations or due to the aging-related decline of proteostasis contributes to several human conformational diseases including neurodegenerative disorders and type II diabetes (Balch et al., 2008; Powers et al., 2009; Gidalevitz et al., 2010; Roth and Balch, 2011).

Cystic fibrosis (CF), the most common life-threatening genetic disease among Caucasians, constitutes the quintessential example of a "conformational disease" (Balch et al., 2011; Okiyoneda et al., 2011). CF is caused by mutations of the CF transmembrane conductance regulator (CFTR) gene that encodes a cAMP-regulated chloride channel primarily located at the apical membrane of epithelial cells (Quinton, 1999; Welsh et al., 2001; Park et al., 2010). Although more than1800 different mutations have been identified, one single deletion of phenylalanine at position 508 (F508del-CFTR), occurs in about 70–90% of CF patients in Northern Europe and North America (Bobadilla et al., 2002). F508del-CFTR protein can still retain a partial chloride channel activity if rescued at the epithelial surface. However, due to its misfold, F508del-CFTR does not reach the plasma membrane (PM) and is prematurely degraded, thus provoking local inflammation, increased susceptibility to respiratory bacterial infections, and progressive pulmonary and digestive insufficiency (O'Sullivan and Freedman, 2009; Ratjen, 2009).

The birth prevalence of CF is estimated to be one in 3500–4500, with 200-300 new cases each year in Europe. The typical form of CF is diagnosed during early childhood and is characterized by recurrent pulmonary infections, pancreatic insufficiency, and elevated chloride concentrations in sweat. Although CF is a systemic disease, the main cause of death is persistent and untreatable pulmonary *Pseudomonas aeruginosa* infection. Loss of functional

CFTR expression is thought to disturb the balance between fluid secretion and absorption into the epithelial layer, leading to net volume depletion of mucus, increased viscosity, and ineffective bacterial clearance. Bacterial infection in turn induces an increased inflammatory response and signaling, thus fueling a vicious cycle of mucus retention, infection, and inflammation.

Mounting evidences indicate that a constitutive inflammatory condition characterizes CF airways regardless of bacterial exposure. CFTR dysfunction results in constitutive, elevated NF-κB activation resulting in increased production of the pro-inflammatory chemokine, interleukin-8 (Vij et al., 2009; Belcher and Vij, 2010; Bodas and Vij, 2010; Hunter et al., 2010). Moreover, the lack of functional CFTR in macrophages has been reported to increase their responsiveness to inflammatory stimuli via uncontrolled TLR4 signaling (Bruscia et al., 2009, 2011) and to affect their capacity to kill *Pseudomonas aeruginosa* (Di et al., 2006; Deriy et al., 2009; Zhang et al., 2010; Del Porto et al., 2011). These findings support the role of CFTR dysfunction in favoring bronchopulmonary inflammation.

Advances in CF treatment have increased the median predicted survival age from less than 5 years in the 1940s to over 37 years presently (Davis, 2006). In addition to therapeutic approaches that target cellular events downstream of the CFTR defect (Mozzillo et al., 2009; Anderson, 2010; Belcher and Vij, 2010; Ratjen and Grasemann, 2012), other strategies focused on the basic CFTR defect have emerged (Riordan, 2008; Sloane and Rowe, 2010; Amaral, 2011; Lukacs and Verkman, 2012). To date, gene therapy has failed to demonstrate a clinical benefit for CF (Riordan, 2008; Amaral, 2011). Thus, pharmacological strategies aimed at correcting mutation-specific CFTR defects (CFTR-repairing therapies) have gained a prominent role in CF drug discovery.

The still partially functional F508del-CFTR protein can be rescued at the PM by means of experimental low thermal conditions (Denning et al., 1992), as well as by so-called correctors, which are molecules that avoid the intracellular retention and degradation of F508del-CFTR protein (Pedemonte et al., 2005; Verkman et al., 2006; Verkman and Galietta, 2009), as extensively reviewed by Molinski et al. and Pedemonte et al. in other chapters of this Special Topic. A number of CFTR corrector molecules have been identified by high-through put screening (Galietta et al., 2001; Pedemonte et al., 2005; Van Goor et al., 2006, 2011). Several CFTR correctors have proved their efficacy in rescuing F508del-CFTR *in vitro*. However, their efficacy in ameliorating the CF lung phenotype, either in pre-clinical models or in CF patients, has not yet established. A recent clinical trial with the most promising CFTR corrector, VX-809 (Van Goor et al., 2011), in F508del-CFTR homozygous patients demonstrated modest dose-dependent reductions in sweat chloride (Clancy et al., 2012). However, beyond this laboratory parameter, no improvement in lung function or CF complications was reported (Clancy et al., 2012; Elborn, 2012).

The pool of F508del-CFTR molecules that can reach the PM after treatment with currently available corrector molecules is unstable. This instability can be explained by carboxyl-terminus heat shock cognate 70 (HSP70)–interacting protein (CHIP)-mediated Ubiquitination of F508del-CFTR (Okiyoneda et al., 2010), followed by redirection of the protein from endosomal recycling toward lysosomal delivery and subsequent degradation (Sharma et al., 2004; Okiyoneda et al., 2010). This seminal observation of Lukacs' group can explain why CF patients carrying the misfolded F508del-CFTR respond poorly to molecules that increase the activity of CFTR channel (CFTR potentiator) (Davis, 2011; Ramsey et al., 2011). Indeed, the rescued F508del-CFTR is no longer available at the PM for the action of CFTR potentiators. Therefore, combining CFTR correctors and potentiators may be a suitable approach for F508del-CFTR patients, provided that the corrector molecules are effective in increasing F508del-CFTR PM stability after rescue. Currently, phase II clinical studies evaluating the combination of VX-809 and the potentiator VX-770 in CF patients that express F508del-CFTR are underway (Elborn, 2012).

Restoration of a functional proteostasis network by the administration of proteostasis regulators (PRs) has emerged as a novel approach to correct protein misfolding in conformational diseases (Mu et al., 2008; Powers et al., 2009; Gidalevitz et al., 2010; Balch et al., 2011). Therefore, strategies aiming at manipulating peripheral proteostasis could represent a promising area of research in CF drug discovery. Understanding the mechanisms underlying the derangement of proteostasis consequent to defective CFTR function could help improving the search of new drug candidates for CF patients carrying F508del-CFTR mutants.

THREE TO TANGO IN CYSTIC FIBROSIS: CFTR, TRANSGLUTAMINASE 2, AND AUTOPHAGY

DEFECTIVE CFTR FUNCTION PERTURBS THE POST-TRANSLATIONAL NETWORK OF CF EPITHELIAL CELLS

An impressive derangement of cellular homeostasis takes place in CF airways. Tissue transglutaminase (TG2) is upregulated in CF epithelial cells at the transcriptional and even more at the post-transcriptional levels (Maiuri et al., 2008). TG2 is a versatile multifunctional protein that changes its function depending on external and internal signals (Nurminskaya and Belkin, 2012). In the presence of high Ca^{2+} levels, TG2 works as a crosslinking enzyme, catalyzing several post-translational modifications of target proteins. At low Ca^{2+} concentrations, TG2 may function as a G-protein or as a protein disulfide isomerase, thus contributing to the functionality of mitochondrial respiratory chain complexes (Nurminskaya and Belkin, 2012). Increased levels of TG2 are observed in several human pathologies including neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases, as well as in chronic inflammatory conditions (Taylor et al., 2003; Malorni et al., 2008; Iismaa et al., 2009; Mastrobernardino and Piacentini, 2010). Most proteins involved in the pathogenesis of neurodegenerative diseases, as huntingtin, ataxin1, tau, and alpha-synuclein, were reported to be TG2 substrates (Mastrobernardino and Piacentini, 2010). Increased TG2 expression has also been reported for glioblastomas, malignant melanomas, and pancreatic ductal adenocarcinomas. Moreover, TG2 expression is often associated with an increased metastatic activity or acquisition of drug-resistance (Antonyak et al., 2004; Karin and Greten, 2005; Kim et al., 2006; Satpathy et al., 2007).

In CF airway epithelial cells, TG2 undergoes small ubiquitin like-modifier (SUMO)ylation (Luciani et al., 2009), a post-translational modification that affects the stability and functions of proteins. SUMOylation is a key player of the post-translational network as it regulates transcription, nuclear translocation, stress

responses, and chromatin structure. Moreover, it influences intracellular localization and stability of modified proteins (Geiss-Friedlander and Melchior, 2007; Meulmeester and Melchior, 2008; Tempè et al., 2008). SUMOylation is accomplished by an enzymatic cascade that involves E3 ligases which orchestrate SUMO-modifications in response to stress. We discovered that the protein inhibitor of activated STAT (PIAS)y, which is induced by reactive oxygen species (ROS) and participates in the SUMOylation of NF-κB essential modulator (NEMO) upon genotoxic stress (Mabb and Wuerzberger-Davis, 2006), is upregulated in CF epithelia in response to oxidative stress and then mediates SUMOylation of TG2 (Luciani et al., 2009). Indeed, TG2 contains three SUMO acceptor sites (consensus sequence: ψ_kxE) in its sequence. SUMOylation of lysines is incompatible with the Ubiquitination of these residues (Muller and Hoege, 2001). Thus, TG2 SUMOylation ultimately results in the inhibition of TG2 ubiquitination, thereby preventing its proteasomal degradation. This sustains high intracellular TG2 protein levels, coupled to prolonged TG2 enzyme activation as the result of the elevated Ca_i^{2+} content. Indeed, emerging evidence support the role of elevated intracellular calcium concentration in mediating the signaling events that impair homeostasis in CF epithelia, as reviewed by Antigny et al. (2011a) in another chapter of this Special Topic. Although the mechanisms underlying the disturbed calcium homeostasis observed in CF remain incompletely understood, recent studies suggest that impaired calcium signaling may be the result of either increased agonist-mediated activation of G-protein-coupled receptors or abnormal regulation of calcium storage compartments (Egan et al., 2002, 2004; Ribeiro et al., 2005a,b; Norez et al., 2006a,b; Martino et al., 2009). Moreover, the abnormal Ca^{2+} response observed in CF cells depends on the presence of CFTR at the cell surface and this reciprocal regulation of CFTR and Ca^{2+} channels has been described in the literature (Antigny et al., 2011b).

Therefore, in CF, increased intracellular levels of ROS, induced by defective CFTR function, lead to the upregulation of the SUMO E3-ligase PIASy, which facilitates TG2 SUMOylation, persistent high TG2 protein levels, and sustained TG2 activation as the result of "permissive" elevated Ca^{2+} levels. The presence of high TG2 levels might in turn sustain ROS, as it is known that TG2 may stimulate the activity of the mitochondrial respiratory chains (Malorni et al., 2008). Remarkably, inhibiting CFTR, either by gene silencing or by means of pharmacological inhibitors, recapitulates these post-translational modifications of TG2 through upregulating ROS levels in cell lines expressing wild-type (wt)-CFTR (Luciani et al., 2009).

These post-translational changes of TG2 protein, induced by defective CFTR, may have functional implications in epithelial homeostasis. Sustained TG2 activation leads to crosslinking, increased ubiquitination, and functional sequestration of the TG2 substrates peroxisome proliferator-activated receptor (PPAR)γ and IκBα (Daynes and Jones, 2002; Kim et al., 2006; Maiuri et al., 2008). Indeed, the anti-inflammatory molecule PPARγ undergoes SUMOylation in response to its agonists, thus interacting with the N-CoR-histone deacetylase (HDAC) 3 co-repressor complex to maintain a repressor condition (Pascual et al., 2005). TG2-mediated ubiquitination of PPARγ inhibits its SUMOylation and interaction with N-CoR. Similarly, crosslinking and ubiquitination of IκBα inhibits IκBα SUMOylation and favors NF-κB activation and nuclear translocation (Luciani et al., 2009). Both events were reported to favor inflammation in CF airways. Therefore, TG2 can function as a rheostat of the post-translational network in response to CF-associated oxidative stress. TG2 SUMOylation with sustained TG2 activation switches off the post-translational regulatory mechanisms and perturbs the intracellular environment (**Figure 1**).

TG2-mediated protein ubiquitination and crosslinking may lead to protein aggregation and proteasome overload, thus favoring aggresome formation (Muma, 2007; Dohm et al., 2008). Misfolded or post-translationally modified proteins that cannot be degraded by the proteasome machinery can be stocked in the cytoplasm in the form of aggresomes (Kawaguchi et al., 2003; Kirkin et al., 2009). Accordingly, ubiquitylated PPARγ and IκBα aggregates are sequestered within histone-deacetylase (HDAC)6^+/vimentin$^+$ intracellular aggresomes in CF epithelial cells (**Figure 2**).

Therefore, proteostasis of F508del-CFTR epithelia is affected by a combination of genetic defect (resulting from the misfolded CFTR protein) and post-translational alterations (through the ROS/TG2 axis).

TG2 is localized in multiple cellular compartments including cell surface and extracellular matrix. Besides its crosslinking activity on ECM substrates, extracellular TG2 is also endowed with PDI, or GTPase functions (Nurminskaya and Belkin, 2012). However, the potential relevance of the extracellular TG2 in CF is still unknown.

DEFECTIVE CFTR DISABLES AUTOPHAGY

Given the overproduction of ROS together with the endoplasmic reticulum (ER) stress induced by the mutant CFTR, one would expect an activation of autophagy in F508del-CFTR homozygous epithelial cells. Autophagy is pivotal in promoting cellular clearance of protein aggregates and removal of ROS sources, such as damaged mitochondria (Mizushima et al., 2008; Kirkin et al., 2009; Korolchuk et al., 2009; Kroemer et al., 2010; Moreau et al., 2010). Surprisingly, however, human and mouse CF airways exhibit a pronounced defect in autophagy, as indicated by reduced autophagosome formation, and the accumulation of sequestosome 1 (SQSTM1), a major autophagic substrate also known as p62. This occurs in spite of the normal expression of major autophagy genes (Luciani et al., 2010, 2011). A defective autophagic response to bacterial infection has also been reported in murine CF macrophages. Reduced autophagosome formation in CF macrophages promotes *Burkholderia cenocepacia* survival and hypersecretion of IL-1β (Abdulrahman et al., 2011).

Autophagy results in the lysosomal degradation of cytoplasmic organelles or cytosolic components after their sequestration in two-membraned vesicles (Kroemer et al., 2010; Yang and Klionsky, 2010; Codogno et al., 2011; Mizushima et al., 2011). In the last few years, autophagy has emerged not just as a simply degradative process, but also as a cellular mechanism essential for the maintenance of cellular homeostasis and of the energetic balance (Kroemer et al., 2010). Thus, disabled autophagy is associated with and is relevant to several human diseases including cancer, viral infection, neurodegenerative diseases, respiratory pathologies, and

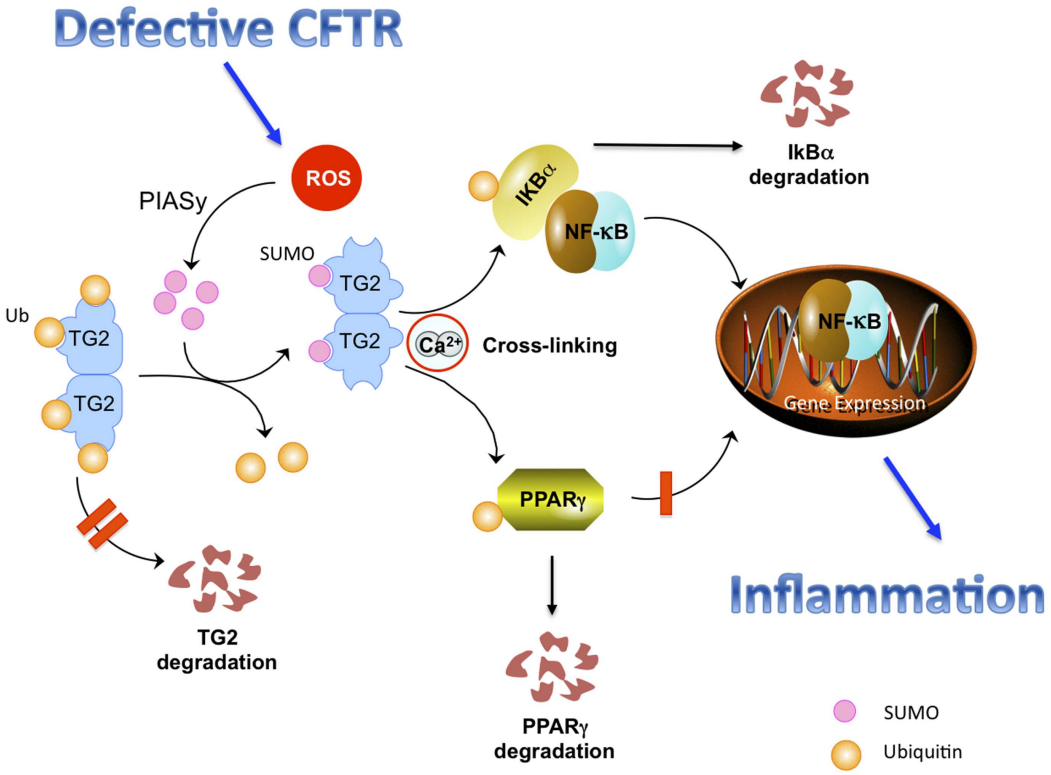

FIGURE 1 | Defective CFTR-induced perturbation of the post-translational network in CF epithelial cells. Defective CFTR leads to increased levels of reactive oxygen species (ROS) that increase the levels of the SUMO E3-ligase PIASy, causing TG2 SUMOylation, that, in turn, inhibits TG2 ubiquitination, and avoids its proteosomal degradation, thus sustaining increased TG2 protein levels. Sustained TG2 activation mediates crosslinking of PPARγ and IκBα, which undergo ubiquitination and proteasome degradation. This inhibits nuclear translocation of PPARγ and favors nuclear translocation of NF-κB, stimulating inflammation.

chronic inflammatory disease (Levine et al., 2011; Rubinsztein et al., 2011; Sridhar et al., 2012; Patel et al., 2013).

Through which mechanisms is autophagy inhibited in CF? We have demonstrated that the inhibition of autophagy in CF epithelial cells is part of the complex perturbation of the post-translational network consequent to defective CFTR function. Disabled autophagy in CF epithelial cells is a consequence of TG2-mediated crosslinking and functional sequestration of BECN1, a major player of autophagosome formation, which exhibits target sites (QP, QxxP) for crosslinking by TG2 (Luciani et al., 2010).

BECN1 is a haploinsufficient tumor suppressor protein that is essential for autophagy (Sinha and Levine, 2008; He and Levine, 2010; Maiuri et al., 2010). Accumulating evidence indicate that BECN1 dissociates from Bcl-2 during stress conditions, such as starvation, thus promoting autophagy (Pattingre et al., 2005; Maiuri et al., 2007, 2010; Axe et al., 2008; Hayashi-Nishino et al., 2009). Subsequently, BECN1 interacts with the class III phosphatidyl-inositol 3 kinase (PI3K), human vacuolar protein sorting (hVps)34 (Matsunaga et al., 2009; Zhong et al., 2009), facilitating its activation. The ER-associated class III PI3K activity is crucial for the initiation of autophagosome formation (Axe et al., 2008; Hayashi-Nishino et al., 2009).

Reduced BECN1/Bcl-2 interaction upon starvation is observed in CF cells, suggesting an intracellular environment favorable to autophagy induction. Moreover, BECN1 interacts with the essential components of the PI3K complex IIIhVps34, hVps15, Ambra1, as well as with Atg14L, a BECN1 interactor that diverts hVps/Class III PI3K into an autophagic role (Liang et al., 2008; Matsunaga et al., 2009). However, in CF epithelial cells, the BECN1 interactome is dislodged away from the ER as a consequence of BECN1 crosslinking and is sequestered within HDAC6^{+} aggresomes. This impairs autophagosome formation in CF cells (Luciani et al., 2010).

Autophagy deficient CF cells accumulate SQSTM1 (p62), an ubiquitin-binding (and LC3-binding) protein (Bjørkøy et al., 2005; Kirkin et al., 2009; Mathew et al., 2009; Duran et al., 2011) that is selectively degraded by autophagy. Autophagy upregulation has been reported as a compensatory response to proteasome inhibition, thus revealing a crosstalk between the proteasome-based and the autophagy-based degradation pathways (Komatsu et al., 2007; Kirkin et al., 2009; Korolchuk et al., 2009; Lamark and Johansen, 2010). SQSTM1 accumulation resulting from autophagy inhibition contributes to proteasome overload and favors aggresome formation, while disabled autophagy inhibits the clearance of such protein aggregates. Altogether, the combined inhibition of protein and aggresome turnover may also influence the fate of misfolded CFTR. Indeed, the enforced expression of F508del-CFTR in CF epithelial cell

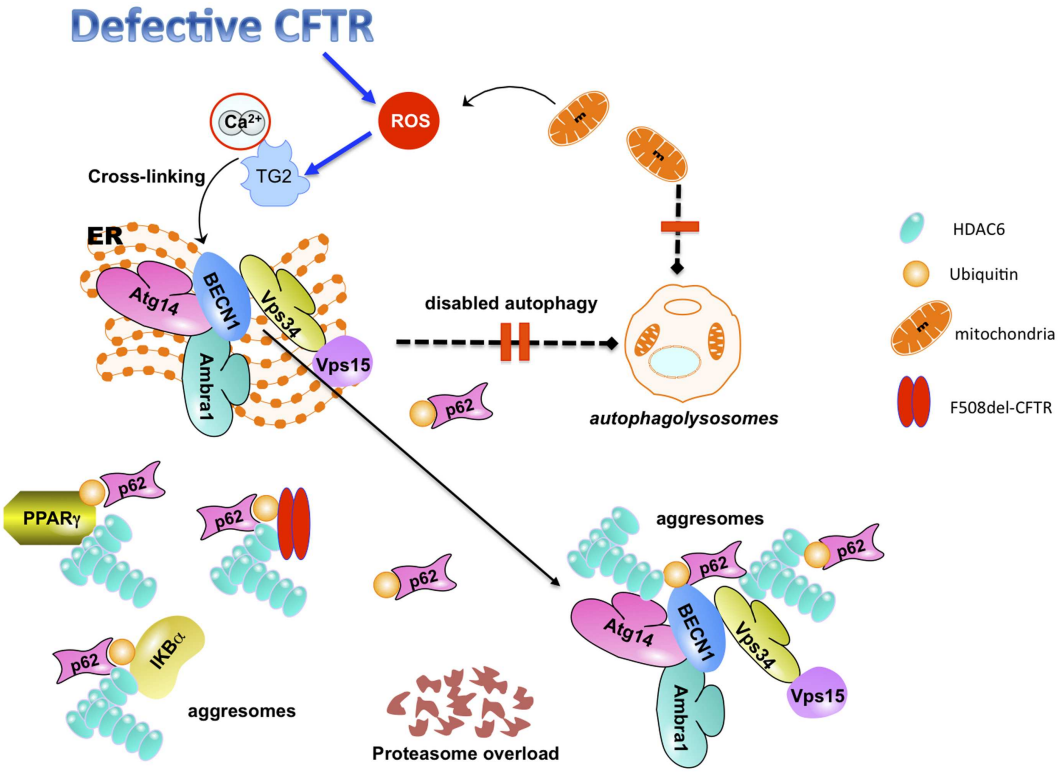

FIGURE 2 | TG2-mediated inhibition of autophagy in CF epithelial cells. Defective CFTR-mediated TG2 activation leads to BECN1 crosslinking and displaces BECN1 interactome away from the endoplasmic reticulum (ER). This mislocalization inhibits autophagosome formation, disables autophagy, and induces accumulation of SQSTM1 (p62). SQSTM1 accumulation leads to proteasome overload and favors sequestration of cross-linked TG2 substrates (PPARγ, IκBα, BECN1) within HDAC6$^+$ aggresomes. The combined inhibition of protein and aggresome turnover may also favor the accumulation of F508del-CFTR (together with SQSTM1) within HDAC6$^+$/ubiquitin$^+$ intracellular aggregates. Defective autophagy inhibits the clearance of damaged mitochondria that contribute to the generation of pro-inflammatory ROS.

lines favors the accumulation of misfolded CFTR (together with SQSTM1) within HDAC6$^+$/ubiquitin$^+$ intracellular aggregates (Luciani et al., 2010).

We suggest that this cascade of events can generate a vicious feed-forward loop, as it impairs the clearance of damaged mitochondria, thus increasing ROS generation that in turn enhances TG2 activation and BECN1 sequestration, further sustaining airway inflammation (**Figure 2**).

RESTORING PROTEOSTASIS AMELIORATES LUNG INFLAMMATION THROUGH RESCUING AUTOPHAGY IN CF

Inhibiting TG2 activity by cystamine (and its reduced form cysteamine) or targeting ROS can reduce inflammation in F508del-CFTR airways, both *in vivo* in F508del-CFTR homozygous mice (*Cftr*^{F508del} mice) and in *ex vivo* using explanted human polyp biopsies from CF patients (Raia et al., 2005; Luciani et al., 2010). The effects of cystamine on airway inflammation are mediated by its ability to rescue BECN1 and autophagy, as either BECN1 depletion used *in vitro* or administration of the PI3K complex III inhibitor 3-methyl-adenine (3-MA) used *in vivo* in *Cftr*^{F508del} mice, abrogated these beneficial effects of cystamine. Either enforced BECN1 overexpression or SQSTM1 depletion *in vivo* recapitulated the effects of cystamine in ameliorating lung inflammation in *Cftr*^{F508del} mice (Luciani et al., 2010, 2012). Similarly, cysteamine has already been successfully used in mouse models of Huntington's disease to improve disease-related phenotype (Karpuj et al., 2002).

Importantly, we demonstrated that amelioration of lung inflammation in *Cftr*^{F508del} mice secondary to cystamine treatment persists up to 10 days beyond cystamine withdrawal, unless the rescue of BECN1 and autophagy are inhibited by the administration of 3-MA during washout (Luciani et al., 2012). These data suggest the provocative hypothesis that, once the cellular environment has been re-directed toward a physiological status, a driving force is re-established within the cell, so as to prolong these beneficial effects. Could this "newly re-established" player be the functional CFTR itself at the epithelial surface? Indeed, manipulating proteostasis might actually improve the function of misfolded proteins (Balch et al., 2008; Roth and Balch, 2011).

TARGETING AUTOPHAGY AS A NEW STRATEGY TO ENABLE THE ACTION OF CFTR POTENTIATORS ON F508del-CFTR

F508del-CFTR rescued at the PM by means of corrector strategies is rapidly dismissed and re-directed to lysosomes for degradation (Sharma et al., 2004; Okiyoneda et al., 2010, 2011; Lukacs

and Verkman, 2012). Accordingly, the biochemical half-life of PM F508del-CFTR is lower than 4 h (Lukacs et al., 1993; Heda et al., 2001). Therefore, F508del-CFTR is no longer present at the PM and cannot interact with CFTR potentiators after rescue.

Recently, we have reported that overexpression of BECN1, administration of cystamine, or depletion of SQSTM1 by RNA interference, can favor the trafficking of F508del-CFTR protein to the epithelial cell surface *in vitro* in CF epithelial cell lines (CFBE41o- or IB3-1, carrying F508del/F508del or F508del/W1282X CFTR, respectively), *ex vivo* in nasal polyp biopsies from CF patients, and *in vivo* in *Cftr*^{F508del} mice. Interestingly, these treatments can restore a functional CFTR in CF cell lines and in primary brushed nasal epithelial cells from F508del-CFTR homozygous patients (Luciani et al., 2012).

Therefore, PR-based strategies in CF (as administration of cystamine or genetic restoration of BECN1) may have a dual effect, as they reduce lung inflammation while rescuing a functional mutant CFTR to the epithelial surface.

TARGETING AUTOPHAGY IMPROVES F508del-CFTR PM STABILITY IN AIRWAY CF EPITHELIA WELL BEYOND DRUG WASHOUT

In addition to its ability to rescue F508del-CFTR, cystamine is effective in delaying the disposal of PM resident F508del-CFTR protein and generates permissive conditions to prolong F508del-CFTR PM residence well beyond cystamine washout. These effects are mediated by the ability to restore BECN1 and autophagy, as both BECN1 depletion and 3-MA abrogate the beneficial effects of cystamine. PM resident mutant F508del-CFTR is still functional after cystamine withdrawal, as it retains the ability to respond to forskolin added together with CFTR potentiators (as genistein or VX-532 or VX-770) well beyond the washout period. This prolonged function of F508del-CFTR was observed in CF cell lines, as well as in primary brushed nasal epithelial cells from F508del-CFTR homozygous patients. Moreover, cystamine sustains F508del-CFTR re-location at the lung epithelial surface after 10 days following washout *in vivo* in *Cftr*^{F508del} mice, unless that cystamine was combined with 3-MA (Luciani et al., 2012).

These effects of cystamine can explain how the anti-inflammatory effects as a result of the restoration of autophagy (either via pharmacological intervention, as cystamine, or by the enforced expression of BECN1) persist well beyond its withdrawal in *Cftr*^{F508del} mice. They probably rely on CFTR itself. Indeed, the pro-autophagic effects of cystamine persist after 10 days following cystamine withdrawal unless CFTR was depleted during washout, suggesting that these anti-inflammatory effects are mediated by the ability to sustain a functional CFTR at the cell surface (Luciani et al., 2012). Therefore, sustained CFTR function at the PM can interrupt the cascade of ROS generation, TG2 activation, BECN1 sequestration, and autophagy inhibition, and ultimately reduce lung inflammation.

These findings could also explain how cystamine, which is not an autophagy inducer, is highly effective in restoring autophagy within a "*CF environment*." The fact that cystamine can regulate peripheral proteostasis is also supported by the observation that cystamine (but not CFTR correctors as VX-325 or Corr-4a) is effective in sustaining PM stability of F508del-CFTR even if it has previously been rescued at the PM by low temperature.

TARGETING AUTOPHAGY ENABLES THE BENEFICIAL ACTION OF POTENTIATORS ON F508del-CFTR

These findings indicate that PRs may be used to rescue and stabilize F508del-CFTR at the PM of CF epithelial cells. In principle, this strategy could lower the ER quality control (QC) threshold of all misfolded proteins, thus interfering with the QC fidelity. However, besides its effects on F508del-CFTR rescue at the PM, cystamine can also delay the disposal of PM resident F508del-CFTR protein. The evidence discussed in this review supports that defective CFTR suppresses autophagy within the CF epithelial environment (though the ROS/TG2 pathway), and, conversely, that rescuing autophagy can restore a functional CFTR at the PM. Altogether, these insights suggest the existence of a vicious cycle in which defective CFTR functions destabilizes the CFTR protein and that can be interrupted by cystamine.

Considering these factors, one would expect that sustaining PM residence of F508del-CFTR by PRs, could allow potentiators to improve Cl^- transport though PM resident CFTR molecules. Indeed, our recent data indicate that, if proteostasis has been previously restored by cystamine, potentiators can become effective in sustaining the anti-inflammatory effects of cystamine *in vivo* in *Cftr*^{F508del} mice. Importantly, genistein, which has no effects on its own in *Cftr*^{F508del} mice, synergistically interacts with cystamine to reduce lung inflammation triggered by the challenge with lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*. The same effects are observed if proteostasis has been previously re-established in *Cftr*^{F508del} mice by means of genetic manipulations, such as lentiviral expression of BECN1 or shRNA-mediated depletion of SQSTM1 (Luciani et al., 2012).

These observations indicate that pharmacological measures that ameliorate the cellular environment in which mutant CFTR traffics, instead of specifically targeting the misfolded protein itself, can result in improved disease outcome.

PERSPECTIVES

CFTR-REPAIRING STRATEGIES AND ANTI-INFLAMMATORY THERAPIES: TWO SIDES OF THE SAME COIN?

The data discussed in this review suggest that different approaches could be envisaged to control CF lung inflammation. Some therapies focus on conventional or emerging anti-inflammatory molecules, downstream of CFTR. Others, as cystamine and other yet-to-be-developed PRs, rely on the rescue and stabilization of functional CFTR at the PM. Apparently, cystamine can interrupt the dangerous cycle leading to lung inflammation, thus opening a new scenario in the search of the most appropriate CFTR-repairing strategy.

Searching the appropriate CFTR corrector is a challenging issue in drug development. An ideal drug candidate for the treatment of F508del-CFTR patients should not only aim at rescuing trafficking of mutant CFTR, be it through the conventional Golgi-mediated exocytic pathway (Ward et al., 1995; Quinton, 1999; Amaral, 2004, 2011) or the unconventional GRASP-dependent secretory pathway (Gee et al., 2011), but also at sustaining the rescued mutant CFTR at the PM, to allow the combined action of potentiators on PM resident F508del-CFTR. Ideally, one single molecule should be endowed with all these properties to minimize undesirable effects. Our recent observations suggest that F508del-CFTR patients could

be sequentially treated with two single pharmacological agents, first with cystamine and then with CFTR potentiators.

The findings discussed in this review also highlight the importance of testing F508del-CFTR correctors for their capacity to exert a prolonged control of lung inflammation in pre-clinical models, before initiating clinical trials. So far, our strategy has been successfully tested in nasal polyp biopsies from F508del-CFTR homozygous patients as well as *in vivo* in F508del-CFTR homozygous mice (Luciani et al., 2012). At this stage, clinical trials on CF patients are justified.

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Class 1 CF mutations

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Since the discovery of the gene that causes Cystic Fibrosis, our knowledge of how mutations in this gene cause the varied pathophysiological manifestations of this disease has increased substantially. This knowledge has led to the possibility of new therapeutic approaches aimed at the basic defect. Class I mutations of CFTR include premature termination codons (PTCs) or stop codons. In the last 10 years there has been a concerted international effort to utilize the concept of read-through of the stop codon producing full length functioning CFTR protein. This author considers that this approach will result in clinical trials in CF patients carrying these mutations.

Class I mutations include PTCs or nonsense codons. A nonsense mutation is a single point alteration in DNA that results in the inappropriate presence of a UAA, UAG, or UGA stop codon in the protein-coding region of the corresponding messenger RNA (mRNA) transcript. Such a stop codon causes premature cessation of translation, with protein truncation leading to loss of function and consequent disease. Nonsense mutations are responsible for about 10% of cystic fibrosis cases worldwide. However, in Israel, nonsense mutations are the cause of cystic fibrosis in most patients (Kerem et al., 1997). As such mutations produce little functional CFTR, these patients usually have a phenotype of CF with exocrine pancreatic insufficiency.

The increased understanding of ribosomal function, the process of translation, and small molecules that change the interaction between the ribosome and mRNA have led to the identification of several agents that are capable of suppressing PTCs. This has resulted in a novel strategy to treat CF and other genetic disorders caused by PTCs by restoring full length protein.

Aminoglycoside antibiotics were the first drugs demonstrated to suppress PTCs in disease-causing mutations, allowing the translation of full length proteins (Hermann, 2007). Aminoglycosides are antibacterial agents, their mode of action is interfering with normal translation via binding to the bacteria 16S rRNA. There is reduced discrimination between cognate and near-cognate tRNA hence reducing translational fidelity. Eventually, there is accumulation of truncated and non-functioning proteins resulting in bacterial cell death.

Gorini and Kataja (1964) demonstrated that aminoglycosides may suppress PTCs and lead to full length translation in *E. coli.* Aminoglycosides may also bind to human 18S rRNA subunit reducing discrimination of near-cognate tRNAs. This interaction is less stable than in bacteria but may be sufficient to lead to an insertion of a near-cognate aminoacyl-tRNA into the ribosomal A site that is subsequently incorporated into the polypeptide chain.

Howard et al. (1996) described PTC suppression by the synthetic aminoglycoside geneticin (G418) to restore function in HeLa cells expressing nonsense codons in 1996. This pivotal work was extended to four nonsense mutations of cftr who were expressed by the human airway cell line IB3-1.In this study, the commonly used aminoglycoside, gentamicin, was incubated with these cells and full length protein was produced (Bedwell et al., 1997).

ANIMAL MODELS

Two mouse models have been developed that contain PTCs including the mdx mouse model of Duchenne Muscular Dystrophy and the G542X-hCFTR mouse which is a transgenic model of CF. Barton-Davis et al. (1999) reported suppression of PTC in the dystrophin gene of the mdx mouse by gentamicin. Intra-peritoneal injection of gentamicin restored the full length dystrophin protein in both skeletal and cardiac muscle. Similar studies in the G542X-hCFTR mouse model with gentamicin injections caused full length functional CFTR protein in intestinal tissues. There was also a tendency to increased survival in these mice (Du et al., 2002).

CLINICAL TRIALS

The preclinical studies mentioned above have led to a number of clinical trials designed to test both proof of principle and efficacy in patients with genetic diseases caused by PTCs. As stated earlier, about 60% of CF patient in Israel carry PTCs or Class I mutations. An initial open label pilot study showed a significant improvement of Nasal Potential Difference measurements (NPD) after the instillation of gentamicin nose drops (Wilschanski et al., 2001). This was followed by a double-blind, placebo-controlled study on 24 patients which included NPD measurement and membrane localization by immuno-fluorescent staining utilizing an anti-body directed against the C-terminus of CFTR (Wilschanski et al., 2003). These studies utilized nasal gentamicin administered for 2 weeks which resulted in significant improvements of basal PD and chloride secretion representing CFTR function in the treatment arms compared with placebo. Together with this immuno-fluorescent staining was positive in the treatment group. These results were specific for patients with Class I mutations with no effect in the control group of patients homozygous for the Delta F508 mutation. In both studies, the vast majority of patients with PTCs expressed at least one copy of the W1282X CFTR mutation which is highly prevalent in CF patients of Ashkenazi Jewish descent. In a study performed in the USA, intravenous administration of gentamicin administered for 1 week also resulted in NPD improvement representing CFTR function in four out of five patients with Class I CF mutations (Clancy et al., 2001). Sermet-Gaudilus et al. (2007) reported similar results following 15 days of systemic gentamicin treatment in six out of nine CF patients carrying the Y122X mutation. In all these studies there was a variability of response with some patients not responding to gentamicin. Linde et al. showed that this NMD variability may be related to nonsense-mediated mRNA decay (NMD) – the major machinery evolved to protect against

harmful products of nonsense mutations. This is a post-transcriptional translation-dependent surveillance mechanism that prevents the synthesis of proteins carrying PTCs. NMD has been shown to degrade transcripts carrying disease-causing nonsense or frameshift mutations. It is the efficiency of NMD which affect the level of transcripts carrying PTCs, which govern the response to read-through treatment. Response to gentamicin was found only in patients with a higher level of transcripts (Linde et al., 2007). Down regulation of NMD in cells carrying the W1282X mutation increased the level of CFTR nonsense transcripts and enhanced the CFTR chloride channel activity in response to gentamicin. This may have a critical clinical correlation in the read-through of PTCs in various diseases. However, the inconvenience of parenteral administration and the potential for serious toxic effects preclude long-term systemic use of gentamicin for suppression of nonsense mutations.

Recently a novel agent PTC124 or Ataluren was developed through an extensive high throughput screening program using a luciferase based system (Welch et al., 2007). The molecule is a 1,2,4-oxadiazole benzoic acid and is reported to interact with mammalian ribosomes in a manner distinct from aminoglycosides. Ataluren does not have antibiotic activity and is orally bioavailable. Studies in myocytes isolated from the *mdx* mouse defined target doses and exposures to rescue dystrophin function. After treatment with, full length dystrophin was localized in skeletal and cardiac tissue. In the G542X-hCFTR mouse oral and intraperitoneal administration led to detectable full length CFTR localization at the apical cell membrane of intestinal glandular cells by immuno-fluorescent staining together with improved chloride conductance as assayed by trans-epithelial ion transport (Du et al., 2008). Correction of CFTR chloride transport was incomplete. Less than 30% of the short-circuit current that was observed in wild-type mice occurred in the CF mice. This suggests that potential clinical benefit would only need partial restoration of protein function.

Phase I studies in healthy volunteers established the initial safety profile for Ataluren, and defined dosing regimens to achieve target trough plasma concentrations (of 2–10 μg/mL) that are known to be active in preclinical models.

Our group reported a phase II clinical trial of PTC124 in 23 patients with cystic fibrosis (Kerem et al., 2008). This open label study included two consecutive 28-day cycles, each of 14 days of treatment followed by 14 days of washout. In the first cycle, patients received daily postprandial doses of 4, 4, and 8 mg/kg. The doses were increased in the second cycle to 10, 10, and 20 mg/kg. Convincing changes in NPD were observed in more than half the patients in the first cycle. Interestingly, this effect was seen in only about a third of the patients in the second cycle. Coupled to this finding, modest but statistically significant improvements in lung function and bodyweight were observed after the first cycle which, in general, persisted to the end of the second cycle. Following this study, 19 of these patients were enrolled in a 12 week open label extension study. NPD improvements were reported over time in both the higher and lower dose treatment groups including four patients who did not respond to PTC124 in the 2 week study. This was accompanied by modest improvements in pulmonary function and a significant reduction in quantitative cough assessment (Wilschanski et al., 2011; **Figure 1**). A similar phase 2 study was performed on adults in the United States which did not reach statistical significance

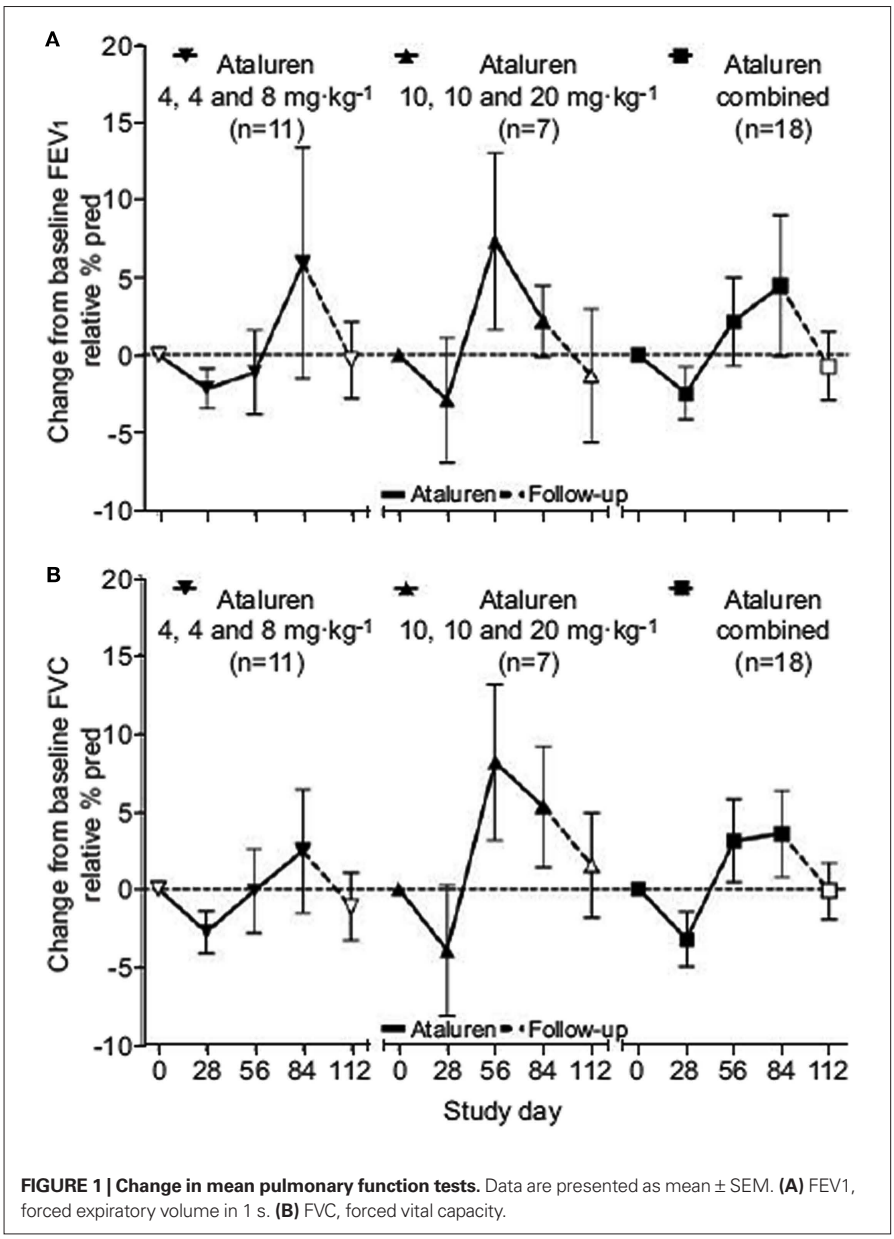

FIGURE 1 | Change in mean pulmonary function tests. Data are presented as mean ± SEM. **(A)** FEV1, forced expiratory volume in 1 s. **(B)** FVC, forced vital capacity.

in nasal potential difference measurements. This may be due to the multitude of sites performing the trial each having relatively few patients and the different mutations carried by the patients.

A similar phase 2a study was carried out in children in France and Belgium. Twenty-two children aged 6–18 years of age completed a dose-ranging crossover study. There was significant improvement in NPD and nasal epithelial CFTR protein by immunofluorescence (Sermet-Gaudelus et al., 2010).

The development of agents that suppress premature stop codons, such as Ataluren, is not without theoretical risk, because there are at least two potential concerns about its mode of action. First, Ataluren might lead to erroneous suppression of native stop codons, and second, Ataluren might disrupt NMD. Encouragingly, Ataluren seems to be remarkably selective for premature, rather than native, stop codons, and it seems to restrict its action to those ribosomes that are involved in productive translation of proteins rather than those that are involved in NMD. These preclinical findings were supported by the observation that *CFTR* mRNA levels are largely unaffected by Ataluren treatment (unlike after gentamicin administration).

Suppression of PTCs with small molecules is emerging as a rational approach to treat a variety of genetic disorders including CF. Following these positive findings, a multinational Phase 3 placebo-controlled efficacy trials is currently underway. These studies provide hope that a treatment strategy could be applied to the basic defect rather than downstream manifestations of the disease.

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Pharmacological correctors of mutant CFTR mistrafficking

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The lack of phenylalanine 508 (ΔF508 mutation) in the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) Cl^- channel represents the most frequent cause of CF, a genetic disease affecting multiple organs such as lung, pancreas, and liver. ΔF508 causes instability and misfolding of CFTR protein leading to early degradation in the endoplasmic reticulum and accelerated removal from the plasma membrane. Pharmacological correctors of mutant CFTR protein have been identified by high-throughput screening of large chemical libraries, by *in silico* docking of virtual compounds on CFTR structure models, or by using compounds that affect the whole proteome (e.g., histone deacetylase inhibitors) or a single CFTR-interacting protein. The presence of multiple defects of the CFTR protein caused by the ΔF508 mutation and the redundancy of quality control mechanisms detecting ΔF508-CFTR as a defective protein impose a ceiling to the maximal effect that a single compound (corrector) may obtain. Therefore, treatment of patients with the most frequent CF mutation may require the optimized combination of two drugs having additive or synergic effects.

Keywords: cystic fibrosis, CFTR, trafficking defect, drug discovery, chloride channel

INTRODUCTION

Cystic fibrosis (CF), one of the most common inherited diseases (∼1/3000 in Caucasian populations), is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes for a cAMP-regulated chloride channel expressed at the apical surface of epithelial cells in the airways, intestine, pancreas, and other organs. Defective Cl^- secretion, arising from CFTR mutations, causes a multi-organ disease. In the airways, impaired mucociliary clearance favors recurrent bacterial infection and severe lung damage.

The CFTR protein is composed of five distinct domains: two membrane-spanning domains (MSD1 and MSD2), each having six segments that completely cross the phospholipid bilayer and contribute to the formation of the hydrophilic channel through which anions are transported; two nucleotide-binding domains (NBD1 and NBD2) that are exposed to the cytosol and participate in ATP binding and hydrolysis; a regulatory domain (R) whose phosphorylation regulates channel gating (Riordan, 2005).

The most frequent mutation among CF patients is the ΔF508 mutation, affecting a phenylalanine residue residing in NBD1. Its frequency varies geographically, ranging from about 50% in southern Europe to 70–90% in northern Europe and North America (Bobadilla et al., 2002). Because of its high frequency and severity it has a high priority as a therapeutic target.

Just a few years after the discovery in 1989 of the CF causative gene, the ΔF508 mutation was found to affect the expression and function of the CFTR protein in different ways (Riordan, 2008). The most severe defect consists of a strongly decreased ability to mature and to traffic from the endoplasmic reticulum (ER) to the plasma membrane (PM). The mutant protein is detected by cell quality control (QC) mechanisms as being defective and is degraded by the ubiquitin/proteasome system (Younger et al., 2006; Riordan, 2008). However, it was also found that degradation of the ΔF508-CFTR protein can be reversed by incubating cells at low temperature or with high concentrations of chemical chaperons such as glycerol (Denning et al., 1992; Sato et al., 1996). These experiments demonstrated that the trafficking defect associated with the ΔF508 mutation is correctable, proof of concept for the development of pharmacotherapy using small molecules that correct the basic defect.

However, rescue by low temperature or overexpression also revealed that ΔF508 causes additional defects. First, electrophysiological experiments, particularly patch-clamp recordings, showed that channel activity is significantly reduced by the mutation (Dalemans et al., 1991; Haws et al., 1996). Despite a strong elevation in cytosolic cAMP (CFTR is physiologically activated by cAMP-dependent phosphorylation), open channel probability was approximately one-third of the wild-type protein (Haws et al., 1996). Second, the ΔF508-CFTR protein has a reduced half-time in the PM due to accelerated internalization and degradation (Lukacs et al., 1993; Riordan, 2008).

This type of information evidenced the difficulty in rescuing ΔF508-CFTR expression and function because of the possible requirement of multiple drugs to address the different defects. In particular, it was found that maneuvers that were able to improve trafficking did not affect the channel gating defect and vice versa. Therefore, pharmacotherapy of ΔF508 probably has to be based on the combination of two different types of drugs, generically named *corrector* and *potentiator*, in order to address the trafficking and gating defects respectively (Verkman and Galietta, 2009).

The search for CFTR potentiators has been particularly successful. Campaigns of high-throughput screening and other approaches have identified a plethora of active compounds (Verkman et al., 2006; Verkman and Galietta, 2009). Notably, CFTR

potentiators not only increase the activity of ΔF508-CFTR but also of other CFTR mutants with even more severe gating defects. One of these potentiators, VX-770, identified by Vertex Pharmaceuticals (Van Goor et al., 2009) has been particularly successful in clinical trials in patients with G551D (Ramsey et al., 2011), a mutation characterized by very low channel activity but with normal protein trafficking. The drug (named Kalydeco) has been recently approved by the FDA to treat G551D patients.

The search for CFTR correctors has been more difficult and less successful compared to that for potentiators. However, the good results obtained with VX-770 demonstrates that pharmacotherapy of the basic defect in CF is feasible. This represents a formidable driving force for academic laboratories and industry involved in the search of ΔF508 correctors. In the following sections we will summarize the results obtained so far using different approaches and define possible strategies for the future.

HIGH-THROUGHPUT SCREENING FOR ΔF508 CORRECTORS

In the absence of indications about specific drug targets to rescue ΔF508-CFTR, the most promising and straightforward approach was the screening of large small molecule libraries using functional or biochemical assays. The rationale for this type of approach was that the rescue of the mutant protein from the ER, resulting in increased targeting to the PM (**Figure 1**), could be measured as an increase in CFTR-dependent anion transport or by directly detecting the CFTR protein on the cell surface with an antibody.

VERKMAN PROJECT

The first report on the identification of ΔF508- CFTR correctors by screening a very large collection (150,000) of small molecules was published in 2005 by Verkman and collaborators (Pedemonte et al., 2005). The screening assay utilized Fischer rat thyroid (FRT) epithelial cells co-expressing ΔF508-CFTR and the yellow fluorescent protein (YFP) halide indicator YFP-H148Q/I152L in a 96-well microplate format. FRT cells were first used by Sheppard et al. (1994) to study CFTR function. Subsequently, we found that FRT cells are highly useful to identify CFTR pharmacological modulators (Galietta et al., 2001; Zegarra-Moran et al., 2002). First, untransfected FRT cells have negligible levels of anion transport. Therefore, the activity of mutant CFTR after stable expression is not contaminated by endogenous Cl^- channels. Second, FRT cells strongly attach to the cell culture support thus resisting all procedures required by high-throughput screening (e.g., cell washings and compound addition). Finally, FRT cells are suitable for a series of electrophysiological assays such as short-circuit current and patch-clamp recordings.

To identify correctors, FRT cells were incubated for 24 h with compounds, washed, and then stimulated acutely with a cocktail of a cAMP agonist plus genistein as a potentiator. CFTR activity in the cell membrane was calculated from the rate of YFP fluorescence quenching caused by extracellular addition and therefore influx of iodide. The study led to the identification of five classes of ΔF508-CFTR correctors (Pedemonte et al., 2005). In particular, two classes of molecules appeared as the most interesting. Class 4 correctors act by improving folding efficiency and by stabilizing immature (core-glycosylated) ΔF508 protein. It is reasonable to assume that the target of class 4 correctors resides in the ER QC system. Instead, class 2 correctors increase the residency time of the mutant protein in the PM, suggesting that the mechanism of action involves the peripheral QC system that targets ΔF508-CFTR toward lysosome-mediated degradation. However, only class 4 correctors, in particular corr-4a, showed efficacy on primary bronchial epithelial cells (Pedemonte et al., 2005). The extent of rescue in these cells was relatively small, with maximal CFTR activity being only 8% of that measured in non-CF cells. Further studies on class 2 compounds identified a particular set, aminoarylthiazoles (AATs), with an interesting dual activity. These compounds improve ΔF508 trafficking as well as channel gating thus reducing the requirement of a potentiator (**Figure 1**). Interestingly, the effect of AATs on gating was not that of a classical potentiator since it required several hours of treatment. Despite being effective in several cell lines expressing ΔF508, AATs did not reach a significant activity in primary bronchial epithelial cells (Pedemonte et al., 2011).

VERTEX COMPOUNDS

In addition to the potentiator VX-770, Vertex Pharmaceuticals has also obtained significant results in the discovery of correctors. The company screened a library of 164,000 chemically diverse drug-like compounds using a cell-based assay of membrane potential on NIH-3T3 cells expressing ΔF508-CFTR (Van Goor et al., 2006). The assay reports ΔF508-CFTR activity as a cAMP-stimulated depolarization in the presence of a Cl^- gradient. Screening identified 13 structurally distinct scaffolds with corrector activity, six of which were also active on FRT cells with ΔF508-CFTR. The mechanistic data obtained on the quinazolinone class (i.e., VRT-325) suggest that the compounds act primarily or initially at the level of the ER to facilitate the folding and export of ΔF508-CFTR (Van Goor et al., 2006). More important, the subsequent round of optimization of one of the hits from the primary screening led to the investigational drug VX-809 (Van Goor et al., 2011). This compound appeared to be particularly effective in primary cultures of bronchial epithelial cells from ΔF508 CF patients. In combination with the potentiator VX-770, the corrector elicited a 25% rescue. The efficacy shown *in vitro*, plus the safety and tolerability *in vivo*, have allowed the advancement of VX-809 into clinical trials. However, the efficacy of the drug *in vivo* in ΔF508 patients (Clancy et al., 2012) is significantly lower than that of the potentiator VX-770 in G551D patients (Ramsey et al., 2011). For example, the lowering of chloride concentration in sweat, a good indicator of CFTR activity *in vivo*, was 48 mM for VX-770 in G551D patients and 8 mM for VX-809 in ΔF508 patients. In contrast to the potentiator, the corrector did not improve respiratory function or CFTR activity measured by nasal potential recordings (Clancy et al., 2012). These results highlight the particular difficulty in correcting the trafficking defect of the ΔF508 mutation with respect to the gating defect of G551D.

SILDENAFIL ANALOGS AND RDR1

Researchers at The McGill University identified novel CFTR correctors from a library of 42,000 compounds, by means of a biochemical high-throughput assay in a 96-well microplate format

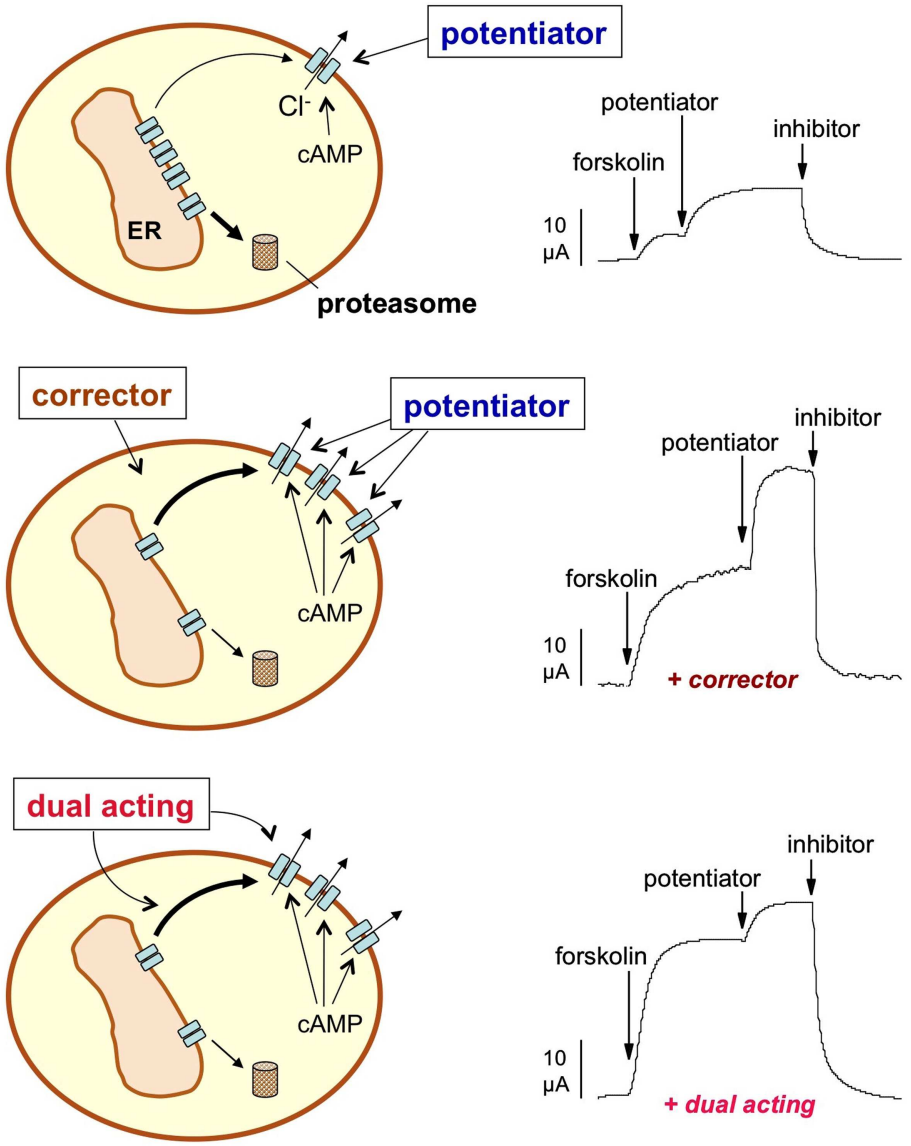

FIGURE 1 | Pharmacological rescue of ΔF508-CFTR. The activity of ΔF508-CFTR in the plasma membrane (PM) may be increased by long-term treatment with a corrector, a small molecule that rescues ΔF508-CFTR from the endoplasmic reticulum (ER) and/or increases the half-time of the protein in the PM. The effect of a corrector can be evaluated at the functional level by various technologies such as by directly measuring Cl^- currents with electrophysiological techniques (e.g., by short-circuit current recordings shown in the Figure). CFTR activity is first triggered with a cAMP-elevating agent (forskolin) and then further increased with a potentiator, a compound that corrects the intrinsic channel gating defect caused by ΔF508. Finally, a CFTR inhibitor is used to measure the total Cl^- current dependent on CFTR. Incubation with a corrector enhances the total current (middle trace) due to the increase in the number of CFTR channels in the PM. A dual-acting compound (bottom trace) not only increases the total current but also the fraction of the current that is elicited cAMP alone thus minimizing the requirement for a potentiator.

(Robert et al., 2008). Screening was performed using BHK cells, which stably express ΔF508-CFTR bearing three tandem hemagglutinin (HA) epitope tags in the fourth extracellular loop after amino acid 901. The appearance of ΔF508-CFTR or wild-type CFTR at the cell surface was monitored in a plate reader with an anti-HA antibody and a fluorescent secondary antibody. The study led to the identification of different compounds, in particular the approved drug sildenafil, along with several structural analogs with improved potency, having activity as ΔF508-CFTR correctors. Later on, the same group developed a new assay, based on differential scanning fluorimetry, to identify pharmacological chaperones of ΔF508-CFTR, i.e., compounds that bind and act directly on the mutated NBD1 domain of ΔF508-CFTR. The hits derived from the previous cell-based screen for CFTR correctors were tested by the authors, which identified one compound, the phenylhydrazone RDR1, able to bind to and thermally stabilize purified murine ΔF508-NBD1 *in vitro* (Sampson et al., 2011).

MPB COMPOUNDS

A small-scale screening for CFTR activators performed by Becq et al. (1999) using iodide efflux experiments resulted in the description of a class of tricyclic compounds called benzo[c]quinoliziniums or MPB compounds. The compounds MPB-07 and MPB-27 appeared as selective activators of wild-type CFTR in different cell systems. Subsequently, synthesis of new derivatives identified MPB-91 as a potent activator of G551D-CFTR (Derand et al., 2001). Soon after, by studying the ΔF508-CFTR activity and the trafficking by immunofluorescence in freshly isolated native airway epithelial cells from CF patients, the authors realized that treatment of cells with MPB-07 caused dramatic relocation of ΔF508-CFTR to the apical region such that the majority of CF cells showed a pattern similar to that of non-CF cells (Dormer et al., 2001). Further studies demonstrated that benzo[c]quinoliziniums selectively inhibit degradation of the ΔF508 protein, by protecting a proteolytic cleavage site by direct binding to the first cytoplasmic domain of ΔF508-CFTR, thus resulting in increased ΔF508-CFTR trafficking (Stratford et al., 2003).

STRUCTURE-BASED CORRECTOR DESIGN

Although high-resolution structural information on full-length CFTR protein is still missing, studies on the structure of CFTR NBD1 and homologous ABC transporters has provided insights into the three dimensional architecture of CFTR (Lewis et al., 2004, 2005; Rosenberg et al., 2011; Lukacs and Verkman, 2012). In native CFTR, NBD1 interfaces with the cytoplasmic loops 4 (CL4) and 1 (CL1) in MSD2 and MSD1, while NBD2 associates with CL2 and CL3 of MSD1 and MSD2 respectively (Lukacs and Verkman, 2012). These interfaces not only transmit the ATP-dependent conformational changes occurring in NBDs to MSDs during channel gating, but also play a crucial role in CFTR biogenesis (Lukacs and Verkman, 2012). Indeed, ΔF508 mutation destabilizes the conformation of MSD1, MSD2, and NBD2, by impairing the assembly of the interface between NBD1 and MSD2/MSD1, resulting in protein misfolding (Lukacs and Verkman, 2012).

EPIX PROJECT

Starting from the structural information available for CFTR and other ABC proteins, researchers at Epix Pharmaceuticals performed an *in silico* structure-based screening for ΔF508 correctors utilizing homology models of CFTR (Kalid et al., 2010). After modeling the intracellular region of CFTR, they identified three cavities at inter-domain interfaces: (1) the interface between the two NBDs; (2) the interface between NBD1 and CL4, in the region of the F508 deletion; (3) the multi-domain interface between NBD1 and 2 and CL1, 2, and 4. The working hypothesis was that compounds binding at these interfaces may improve the stability of the protein, potentially affecting the folding yield or surface stability. *In silico* structure-based screening of a focused library of ∼100,000 compounds (extracted from the EPIX in-house database containing ∼4-million unique compounds) highlighted 496 candidate compounds that were tested in functional assays. The study resulted in the identification of 15 novel compounds of diverse chemotypes, active as ΔF508 folding correctors. Interestingly, all the binding sites subjected to screening yielded CFTR potentiators as well as correctors. In addition, several of the chemical series were found to harbor the potential for both types of activities, with small chemical modifications independently modulating the activity as corrector or potentiator. Notably, the study also led to the identification of several compounds with a dual corrector-potentiator activity (dual-acting). According to the authors, this could be due to the fact that they used a CFTR model representing the conducting state of the channel. Stabilizing this state by direct binding of small molecules may increase the open probability of the channel (potentiation), improve the stability of the protein (potentially affecting the folding yield or surface stability of the protein, i.e., correction), or both.

HYPOTHESIS-DRIVEN SEARCH FOR ΔF508 CORRECTORS

The mechanisms of action of correctors have not been clarified, and it is not known whether they interact directly with CFTR (i.e., acting as pharmacological chaperones) or with other intracellular proteins (**Figure 2**). However, considering the discrepancies between their effects on heterologous expression systems versus native epithelial cells from CF patients (Pedemonte et al., 2010), it is reasonable to conclude that many correctors do not interact directly with ΔF508-CFTR to favor its folding and stabilization. If that were the case, one would expect an activity that is independent of cell background. Rather, it is probable that many correctors modulate QC mechanisms responsible for mutant CFTR detection and degradation (i.e., they act as "proteostasis regulators"; see Calamini et al., 2011).

Proteostasis regulators (**Figure 2**) are considered interesting therapeutic agents to treat genetic diseases with protein misfolding defects. Indeed, loss of proteostatic control has been implicated in aging and in multiple disorders of protein misfolding, in which the chronic expression and accumulation of misfolded, oxidized, and aggregated proteins leads to cellular dysfunction. There is increasing evidence that misfolded proteins expressed in diseases of protein conformation are not efficiently counterbalanced by a compensatory induction of cellular stress responses such as the heat shock response and the unfolded protein response (Calamini et al., 2011). Enhancing the activity or increasing the expression of molecular chaperones through genetic techniques or pharmacological manipulation has been shown to restore proteostasis in several disease models (Calamini et al., 2011).

MODULATION OF HISTONE ACETYLATION

Histone acetyl transferases (HATs) and deacetylases (HDACs) are enzymes that mediate post-translational acetylation and deacetylation reactions, respectively, of histones, transcription factors, and other cytosolic factors, leading to modulation of transcriptional events during development and in response to environmental changes (Hutt et al., 2010). Researchers at the Scripps Institute (La Jolla, CA, USA), headed by William Balch, evaluated the effect of knocking down single HDACs to address their specific roles in human health and disease (Hutt et al., 2010). The study demonstrated that HDAC7 suppression by siRNA-mediated silencing or with the HDAC inhibitor SAHA, resulted in a substantial increase in stabilization, trafficking, and activity of ΔF508 cell surface chloride channel activity. The authors proposed that the mechanism by which HDAC inhibition may ameliorate CF and possibly other misfolding diseases involves the capacity to create an intracellular

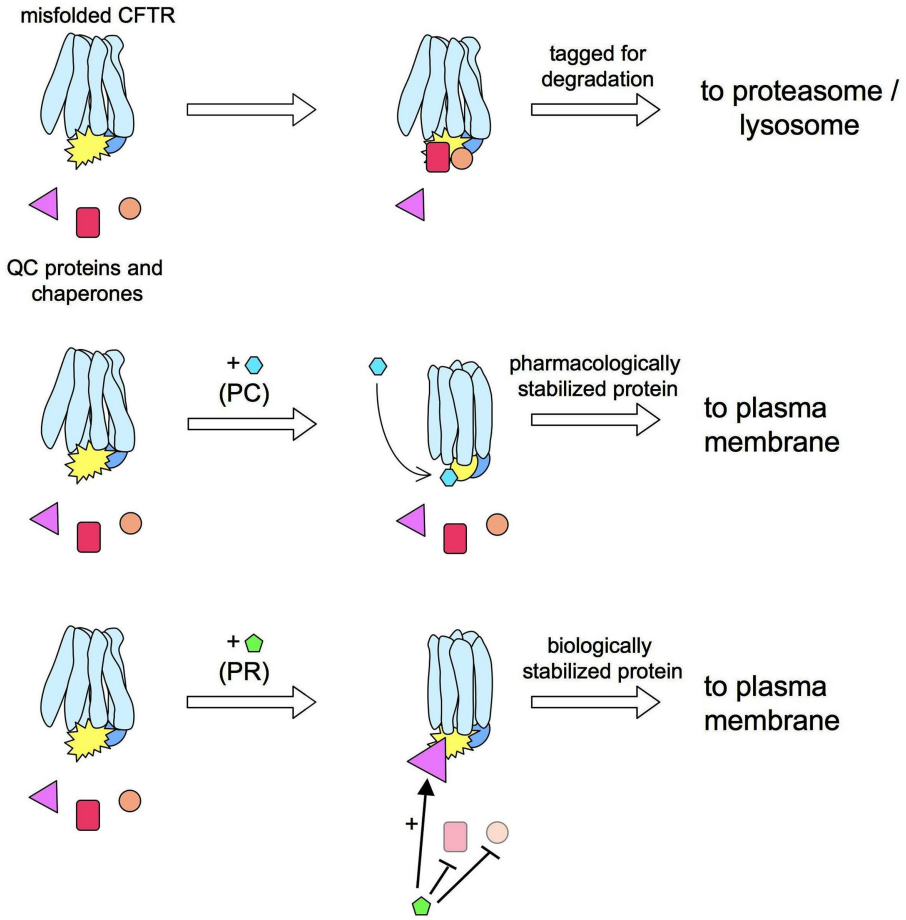

FIGURE 2 | Pharmacological chaperones vs. proteostasis regulators. ΔF508-CFTR rescue may be obtained by a pharmacological chaperone (PC) that interacts directly with the mutant protein. For example, a PC may increase the CFTR stability by improving the interaction between CFTR domains. This effect would prevent the detection of ΔF508-CFTR by quality control (QC) proteins thus allowing more protein in the plasma membrane (PM). An alternative approach for ΔF508-CFTR is the use of a proteostasis regulator (PR). These compounds act by globally changing the proteome, or a more restricted group of proteins, to create an environment more benign toward mutant CFTR.

environment that is more benign toward misfolded proteins (Hutt et al., 2010). The efficacy of SAHA in primary airway epithelial cells from ΔF508 patients has not been confirmed in other studies (Sondo et al., 2011; Van Goor et al., 2011). This may indicate that the net balance of effects induced by HDAC inhibitors may be significantly affected by experimental conditions.

MODULATION OF ER CALCIUM PUMPS

Experimental evidence suggests that inhibitors of ER calcium pumps correct the ΔF508 trafficking defect through partial inhibition of the interaction between ΔF508-CFTR and calnexin, a ER lectin-like protein that binds monoglucosylated oligosaccharides (Norez et al., 2006a). On this basis, Becq and colleagues hypothesized that by inhibiting the deglucosylation of ΔF508 protein in the ER, glucosidase inhibitors may prevent the interaction of ΔF508-CFTR with calnexin and hence its entry into the degradation pathway (Norez et al., 2006b). To verify this hypothesis, the authors tested two compounds that inhibit ER α-1,2-glucosidase, miglustat (an *N*-alkylated imino sugar also called *N*-butyldeoxynojirimycin), and castanospermine, as well as an inactive imino sugar analog (*N*-butyldeoxygalactonojirimycin). The study demonstrated that miglustat rescues ΔF508-CFTR in human and mice epithelial cells and prevents the interaction of ΔF508-CFTR with calnexin in the ER, suggesting that inhibition of deglucosylation of nascent proteins may be the molecular mechanism of the compound's effect (Norez et al., 2006b).

LESSONS FROM BIOCHEMISTRY AND CELL BIOLOGY

The improved knowledge over the last few years of the molecular mechanisms involved in CFTR biosynthesis, trafficking, and degradation is helping us understand the consequences of the ΔF508 mutation and the suitability of these mechanisms as therapeutic targets (Lukacs and Verkman, 2012). First of all, it is clear that ΔF508-CFTR is scrutinized by multiple quality control checkpoints both at the level of the ER and the PM. In particular, nascent ΔF508-CFTR is marked early on for degradation in the ER by the ubiquitin ligase RMA1 in combination with Derlin-1 (Younger

et al., 2006). At a later stage, when mutant CFTR is fully synthesized, other proteins, such as the ubiquitin ligase CHIP, intervene (Younger et al., 2006). Interestingly, a siRNA-based small scale screening has revealed that CHIP and other proteins involved in ER-associated degradation of CFTR are also important in peripheral QC and affect the half-time of mutant CFTR in the PM (Okiyoneda et al., 2010). Therefore, there is a redundancy of mechanisms responsible for the detection of ΔF508-CFTR as a mutant protein. Another aspect of ΔF508-CFTR is the possibility of trafficking to the PM by an unconventional route. Under particular conditions, such as incubation of cells at low temperature or blockade of ER-to-Golgi transport, ΔF508-CFTR may reach the cell surface in a Golgi-independent way (Gee et al., 2011). The plethora of QC and trafficking mechanisms associated with ΔF508-CFTR explains the different observations reported in various studies. For example, it has been repeatedly reported that the combination of small molecules has additive or synergic effects on ΔF508-CFTR rescue (Pedemonte et al., 2011). This kind of effect may also be obtained by combining a corrector with the silencing of a QC protein. For example, treatment with corr-4a plus silencing of RMA1 led to a 13-fold increase in ΔF508-CFTR maturation (Grove et al., 2009). The additive/synergic effects of drug combinations clearly point to different mechanisms of action. In fact, it was found that corr-4a affects a step downstream of RMA1 (Grove et al., 2009). Another consequence of QC redundancy is the sensitivity to cell background. It is reasonable to hypothesize that the relevance of some mechanisms may change from one cell type to another as we have recently demonstrated (Pedemonte et al., 2011). This has important practical implications: a corrector found by screening in a given cell line may not be effective in another cell type and, particularly, in primary airway epithelial cells.

Different studies indicate that the instability of ΔF508-CFTR arises from two main characteristics: the intrinsic instability of NBD1 and the defective docking of NBD1 to CL4. These results point to two separate defects, both of which need to be corrected. This requirement has been recently demonstrated in two independent studies (Mendoza et al., 2012; Rabeh et al., 2012). High levels of ΔF508 rescue have been obtained only when suppressing mutations have been introduced both in NBD1 and in CL4. The first type of mutation, such as I539T or R555K, increases the stability of NBD1. The second type of mutation, namely R1070W, improves the interaction of NBD1 with CL4 by providing an aromatic group that compensates for the lack of F508.

PERSPECTIVES

In conclusion, the increasing knowledge on ΔF508 is indicating that it may not be possible to fully correct the trafficking defect with a single compound. Several *in vitro* studies point out that a large rescue may be obtained only with a combination of correctors. More effective correctors may be identified by high-throughput screening of compounds with novel and unexplored structure, by exploiting the increasing information available on CFTR structure, or by taking advantage of the identification of important proteins of the CFTR interactome. In this respect, genome-wide siRNA screening could be very useful to identify novel proteins with a high relevance for CFTR QC, trafficking, and regulation. The possible need for two correctors to treat ΔF508 represents a problematic scenario in terms of drug development and clinical testing. However, the concept of drug combination in CF (e.g., a corrector plus a potentiator) is already accepted. The use of two correctors instead of one potentiator and a corrector may be justified if both compounds together elicit a high level of CFTR function. In the near future, novel and effective treatments for the CF basic defect are expected. These advances also represent an important proof of concept and a paradigm for other genetic diseases.

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Functional rescue of F508del-CFTR using small molecule correctors

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High-throughput screens for small molecules that are effective in "correcting" the functional expression of F508del-CFTR have yielded several promising hits. Two such compounds are currently in clinical trial. Despite this success, it is clear that further advances will be required in order to restore 50% or greater of wild-type CFTR function to the airways of patients harboring the F508del-CFTR protein. Progress will be enhanced by our better understanding of the molecular and cellular defects caused by the F508del mutation, present in 90% of CF patients. The goal of this chapter is to review the current understanding of defects caused by F508del in the CFTR protein and in CFTR-mediated interactions important for its biosynthesis, trafficking, channel function, and stability at the cell surface. Finally, we will discuss the gaps in our knowledge regarding the mechanism of action of existing correctors, the unmet need to discover compounds which restore proper CFTR structure and function in CF affected tissues and new strategies for therapy development.

Keywords: F508del-CFTR folding, trafficking, conformational stability, intra-molecular defects, small molecule correctors, drug discovery

MOLECULAR DEFECT CAUSED BY F508del IN CFTR

The major Cystic Fibrosis mutation, F508del, causes multiple defects in the CFTR protein, leading to its impaired assembly during synthesis and reduced post-translational stability. Recently, it has been argued that a single small molecule compound may be unable to "correct" the conformational maturation, channel activity, and unfolding of the full-length mutant protein at the cell surface, given the existence of multiple intra- and inter-domain defects.

On the basis of biophysical studies of the isolated first nucleotide binding domain (NBD1) bearing the F508del mutation, together with biochemical studies of the full-length mutant protein, it has become clear that the deletion of F508 induces multiple structural defects in CFTR (Du et al., 2005; Serohijos et al., 2008; Du and Lukacs, 2009; Thibodeau et al., 2010; Yu et al., 2011; Aleksandrov et al., 2012; Mendoza et al., 2012; Rabeh et al., 2012). The absence of F508 in NBD1 leads to kinetic and thermal instability of the isolated domain (Protasevich et al., 2010; Wang et al., 2010a). Biochemical studies of the full-length protein in cell membranes have revealed that F508del-NBD1, in the amino terminal half of the protein, fails to mediate appropriate interactions with the carboxy terminal half of the protein (Du et al., 2005; Du and Lukacs, 2009). Both the intra-domain (NBD1) and the intra-molecular (CFTR) defects will be discussed in the following paragraphs. Furthermore, we will discuss recent evidence supporting the idea that both intra-domain and intra-molecular aberrations will need to be corrected in order to restore near Wt biosynthesis and post-translational stability to F508del-CFTR.

Abbreviations: ABC, ATP binding cassette; Aha1, activator of Hsp90 ATPase; ASM, acid-sphingomyelinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, C-terminal Hsp70 interacting protein; COP, coat complex; CoPos, corrector-potentiator compounds; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FRET, fluorescence resonance energy transfer; F508del, deletion of phenylalanine at position 508; GRASP, Golgi reassembly stacking proteins; Hdj-2, human DnaJ 2; Hsp, heat shock protein; HTS, high-throughput screening; ICL, intracellular loop; LPS, lipopolysaccharide; MSD, membrane spanning domain; NMR, nuclear magnetic resonance; NHERF1, Na^+/H^+ exchanger regulatory factor isoform 1; NBD, nucleotide binding domain; Pgp, P-glycoprotein; PKA, protein kinase A; PM, plasma membrane; R domain, regulatory domain; TM, transmembrane; Ubc, ubiquitin-conjugating; Wt, wild-type; YFP, yellow fluorescent protein.

NBD1: INTRA-DOMAIN DEFECTS CONFERRED BY F508del

The crystal structures of human NBD1 (Wt and F508del) were generated using proteins bearing second site mutations, introduced to confer stable protein fragments suitable for such structural studies (Lewis et al., 2004, 2005, 2010). As a result, these models lack information regarding the relative thermodynamic instability of the mutant protein. However, the crystal structures do provide a structural template with which to compare CFTR nucleotide binding domains (NBDs) with the NBDs of other ABC family members. As for other family members, NBD1 of CFTR possess a central, core F1-ATPase like subdomain, comprised of two non-contiguous sequences, i.e., the amino terminal region: G451-P499 and the carboxy terminal region (including D565-Q637). The NBD1 of CFTR also possesses an α-helical subdomain (495–565), conserved amongst other members of the ABC superfamily of transporters. F508 resides in this α-helical

subdomain. Unique features of CFTR-NBD1 include disordered or flexible regions that are either missing or exhibit multiple orientations in the crystal structure. These unique regions include the "regulatory insertion" (RI: 405–436), the "structurally diverse region" (SDR: 536–550), and the "regulatory extension" (RE: 656–673). This latter region is now considered to comprise the amino terminal region of the phosphorylated, regulatory region, called the "R domain." As shown in **Figure 1**, the amino and carboxy terminal residues of NBD1 are close to one another, underscoring the complexity of its folding involving the formation of specific subdomain interactions.

Biophysical studies of F508del-NBD1 in solution (some lacking the so-called, second site "stabilizing" mutations) revealed inherent alterations in kinetic and thermal stability (Protasevich et al., 2010; Wang et al., 2010a). In isothermal denaturation studies, Hunt and colleagues showed that NBD1 unfolding is strongly influenced by F508del and that unfolding is delayed by the "stabilizing" mutations utilized in the crystal studies (Wang et al., 2010a). Similarly, Brouillette and colleagues showed that F508del also influenced temperature dependent unfolding of NBD1 (Protasevich et al., 2010). In both cases, Mg-ATP binding delays unfolding of the Wt but not the mutant NBD1. The FRET-based folding studies by the Skatch group showed that while F508del mutation does not impair ATP binding, it does impair ATP-dependent interactions between the two non-contiguous regions (amino and carboxy terminal regions) the core F1-ATPase domain. These studies highlight the potential consequences of F508del on the canonical (ABC protein) subdomain interactions in NBD1.

Other biophysical studies highlight the potential consequences of F508del on the interactions mediated by the non-conserved or unique regions of CFTR. NMR studies of F508del-NBD1 (bearing certain stabilizing mutations) revealed alterations in the phosphorylation-regulated affinity of flexible regions (specifically the RI region) with the core of the NBD1 domain (Kanelis et al., 2010). Interestingly, this intra-domain interaction is strengthened in F508del, leading the authors of this work to speculate that F508del causes allosteric changes in NBD1 affecting not only intra-domain interactions but possibly, also preventing critical intra-molecular interactions as well.

As previously mentioned, second site "stabilizing mutations" protect the isolated F508del-NBD1 from unfolding in denaturation studies. The first stabilizing mutations were identified in the ABC conserved, canonical subdomains, and cluster in the α-helical subdomain (G550R, R553Q, R555K), in the γ switch (F494N), and ATP binding core subdomain (Q637R). These findings support the claim that interactions between these canonical subdomains are perturbed by F508del and more importantly, that these regions could constitute targets for pharmacological intervention. More recently, it was determined that substitution of residues in the (SDR: 536–550) of human F508del-NBD1 to residues found in avian F508del-NBD1 led to a profound increase in biosynthetic maturation of the full-length protein (Aleksandrov et al., 2012). These findings together with previous studies of the RI region (Aleksandrov et al., 2010), prompt the speculation that these dynamic, disordered regions of F508del-CFTR are important in mediating intra-molecular as well as intra-domain folding.

INTRA-MOLECULAR DEFECTS CONFERRED IN CFTR BY F508del

It has been shown that intra-domain defects caused by F508del in NBD1 lead to defects in assembly of the full-length protein and to defects in post-translational stability. It is well known that defective assembly of F508del-CFTR is detectable as the marked reduction in the conversion of core glycosylated F508del-CFTR to complex glycosylated protein and decreased functional expression on the cell surface (Cheng et al., 1990). This hallmark biochemical profile (i.e., reduced complex glycosylated protein on a Western blot) reports the ER retention of the mutant protein. Lukacs and colleagues were the first to probe the conformational defects of the mutant protein using limited proteolysis (Du et al., 2005; Du and Lukacs, 2009). Protease resistance is known to provide insight into the conformational compactness of proteins folded in cells and the protease resistance of F508del-CFTR was shown to be significantly reduced relative to the Wt-CFTR protein. Protease digest patterns, analyzed by SDS-PAGE and probed using domain specific antibodies, revealed that the protease resistance of NBD2 was particularly reduced in the context of the full-length mutant protein, relative to the full-length Wt-CFTR. These were the first data to reveal the possible consequences of a misfolded F508del-NBD1 on assembly with the second half of the CFTR protein during translation.

Misassembly of the full-length F508del-CFTR protein likely occurs at several intra-molecular junctures as there are multiple loci at which NBD1 directly interacts with domains in the second half of the full-length protein. The identification of a pivotal juncture was guided by molecular models of the full-length CFTR protein generated using the crystal structure of the bacterial ABC transporter, Sav1866 as a template (**Figure 1**). In the models of CFTR based on Sav1866 protein, NBD1 interacts with NBD2 and with MSD2. NBD1 interacts with MSD2 via the coupling helix presented by the long helical extension known as intracellular loop 4 (ICL4; **Figure 1B**; Serohijos et al., 2008; Mornon et al., 2009; Dalton et al., 2012). The consequence of F508del in disrupting the NBD1: NBD2 interface is still under investigation. To date, interventions aimed at disrupting or enhancing this interaction do not appear to affect biosynthesis and processing of F508del-CFTR (Thibodeau et al., 2010). These findings suggest that, even if F508del-CFTR is shown to perturb this interface, this would not have a significant effect on CFTR folding. On the other hand, interventions aimed at modifying the interaction between the surface on NBD1 lacking F508 and the coupling helix presented by ICL4 significantly enhance the biosynthesis and processing of F508del-CFTR (Mendoza et al., 2012; Rabeh et al., 2012).

Disease-causing mutations in the coupling helix of ICL4 that cause ER retention have been described (L1065P, R1066C, and G1069R), supporting the idea that this region mediates important interactions during folding (Mendoza et al., 2012). Substitution of the arginine at position 1070 with tryptophan (R1070W) in the context of the Wt-CFTR, introduces a bulky group on the face of the coupling helix that interacts with NBD1 and like the substitutions above, this leads to misprocessing. Further support for the hypothesis that this helical segment conferred by ICL4, interacts with the NBD1 surface containing F508 in the full-length protein came from chemical cross-linking studies of engineered interfacial cysteine pairs. Importantly, deletion of F508, impairs chemical

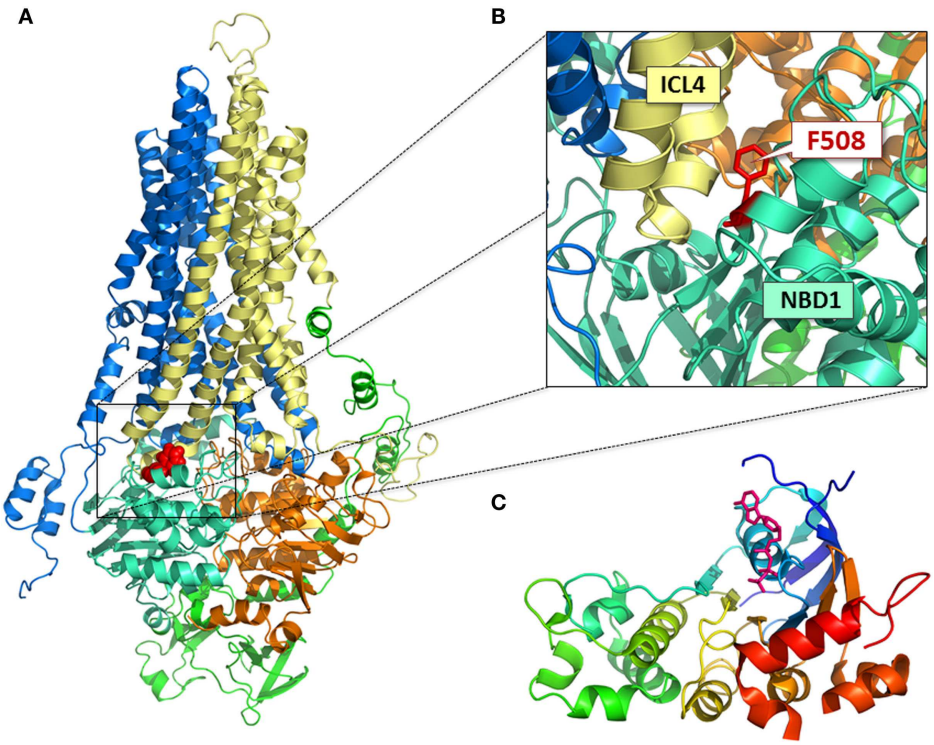

FIGURE 1 | Structural models of CFTR. (A) Full-length homology model of CFTR (Mornon et al., 2009); MSD1, blue; MSD2, yellow; NBD1, cyan; NBD2, orange; R domain, green; F508, red; **(B)** position of F508 at the ICL4:NBD1 interface; **(C)** crystal structure of NBD1 (Lewis et al., 2010); ATP, pink.

cross-linking of the same cysteine pairs in the full-length protein, supporting the idea that this intra-molecular interaction is perturbed in the full-length mutant protein.

Introduction of R1070W or V510D in the F508del-CFTR protein partially corrects folding of the full-length protein, highlighting the idea that even in the absence of F508, assembly of the CFTR can be partially restored through structural changes at key loci in the protein (Thibodeau et al., 2010; Mendoza et al., 2012). Similarly, the second site mutations, previously discussed with regard to their efficacy in stabilizing the isolated F508del-NBD1, i.e., the second site mutations in the ABC conserved core ATP binding subdomains (G550E, R553Q, and R555K) also promote improved processing of the full-length F508del-CFTR. Similarly, second site mutations in unique, flexible regions of NBD1 (i.e., I539T) partially correct the processing defect in F508del-CFTR.

Recent studies by the Lukacs (Rabeh et al., 2012) and the Thomas (Mendoza et al., 2012) groups tested the idea that correction of the thermodynamic and kinetic defects in F508del-NBD1 by second site "stabilizing" mutations may be sufficient to restore proper assembly of the full-length mutant protein. Employing biophysical methods, including circular dichroism, dynamic light scattering, and fluorescence, both groups confirmed that the introduction of "stabilizing mutations" residing in the ABC α-helical subdomain (G550E, R553M, R555K) and the structural diverse region (I539T), fully corrects defects in kinetic and thermal stability of the isolated F508del-NBD1 domain. However, these second site mutations failed to restore folding of the full-length mutant protein to greater than 15% of the Wt-CFTR protein. However, in combination with R1070W, a mutation that reconstitutes a more Wt-like ICL4: NBD1 interface, the NBD1-"stabilizing" mutants mediate full correction and near normal processing. Hence, these authors argue that pharmaceutical interventions which "correct" the thermodynamic instability of NBD1 alone will lack therapeutic efficacy. However, the results of the studies by Riordan and colleagues appear to dispute this view. This group found that compound mutations in the SDR of F508del-NBD1 or deletion of the entire RI region were sufficient to restore Wt folding to the full-length mutant protein in the absence of stabilizing mutations at the ICL4: NBD1 interface (Aleksandrov et al., 2012).

Clearly, there is still much to learn regarding intra-domain and intra-molecular interactions vital for proper folding and assembly of CFTR. The field would benefit greatly from biophysical studies which directly probe the intrinsic determinants for folding or unfolding of the full-length CFTR protein and the major mutant. To date, the only assay for folding of the full-length protein is assessment of the acquisition of complex glycosylation and this readout reflects a complex series of events, with a significant number of these processes being mediated by proteins other than CFTR.

F508del-CFTR IN THE CELL

DEFECTIVE INTERACTION WITH THE CHAPERONE AND ER QUALITY CONTROL MACHINERY

CFTR folding is modified by cellular chaperones of the ER which specifically and transiently bind to immature CFTR to prevent aggregation and facilitate efficient folding (Meacham et al., 1999;

Wang et al., 2006a; Rosser et al., 2008). These include heat shock protein (Hsp) 70 and its co-chaperone human DnaJ 2 (Hdj-2) which form the cytosolic chaperone complex (Meacham et al., 1999), Hsp90 and its co-chaperone activator of Hsp90 ATPase (Aha1; Wang et al., 2006a), and calnexin (Rosser et al., 2008). Hdj-2/Hsp70 is localized at the cytosolic face of the ER in which Hdj-2 binds to Hsp70 to activate the ATPase activity of the chaperone and binds to specific proteins for folding through its farnesyl tail (Meacham et al., 1999). This complex facilitates both co- and post-translational folding of native CFTR and stabilizes NBD1 as well as its interaction with the R domain (Meacham et al., 1999). The formation and stabilization of the NBD1-R domain interaction then reduces binding and releases the protein from the complex (Meacham et al., 1999). Hsp90 is localized in the cytosol with Aha1 binding to conduct similar roles as Hdj-2 with Hsp70 (Wang et al., 2006a). The mechanism of CFTR folding facilitated by the Aha1/Hsp90 complex remains unknown, however this complex has been shown to be essential for CFTR folding and stability (Wang et al., 2006a). Calnexin, with its lectin domain localized in the ER lumen, also binds to CFTR at two glycosylation sites in extracellular loop 4 of MSD2 (Rosser et al., 2008). The binding of calnexin to CFTR at those sites stabilize MSD2, and facilitates the formation and stabilization of the interaction between MSD2 and MSD1 (Rosser et al., 2008).

The F508del mutation results in altered interactions of CFTR with its cellular chaperones (Meacham et al., 1999; Wang et al., 2006a; Rosser et al., 2008). It has been shown that the Hdj-2/Hsp70 complex interacts with F508del-CFTR approximately twice as much as that of Wt-CFTR (Meacham et al., 1999). This prevents the formation of the NBD1-R domain interaction as the increased residency with the cytosolic chaperone complex buries those sites necessary for that interaction and consequently the folding of the full-length protein (Meacham et al., 1999). In addition, the folding energy required for F508del-CFTR to achieve its native folded conformation far exceeded the capacity of Hsp90 to facilitate its proper folding which emphasizes a major difficulty in correcting the defect of F508del-CFTR (Wang et al., 2006a). There were also more Hsp90 co-chaperones such as Aha1 in the F508del-CFTR interactome which correlates as greater association of the complex with the misfolded protein (Wang et al., 2006a). This increased association blocked the folding pathway of the mutant protein as reduction of Aha1 resulted folding and stability rather than degradation (Wang et al., 2006a). Calnexin was found to interact more with F508del- than Wt-CFTR which also leads to ER retention of the mutant protein (Okiyoneda et al., 2004; Farinha and Amaral, 2005).

The cytosolic chaperone complex can also target the protein for degradation through the ER-associated degradation (ERAD) pathway when correction of misfolding is energetically unfavorable (Younger et al., 2004). ERAD is activated upon the formation the E3 complex by binding of co-chaperones Hdj-2 (Meacham et al., 1999), cytosolic U-box protein CHIP (Meacham et al., 2001), and E2 UbcH5 (Younger et al., 2004) to Hsc or Hsp70 (Zhang et al., 2001). The E3 complex can differentiate between native and misfolded CFTR and ubiquitinates the misfolded protein post-translationally (Younger et al., 2006). The other known ER interacting proteins, including RING domain protein (RMA1/RNF5), E2 ubiquitin-conjugating enzyme (Ubc6e), and the transmembrane quality control factor Derlin-1 further ubiquitinates the misfolded protein (Younger et al., 2006). This complex can detect the aberrant protein at early stages of co-translational folding (i.e., during translation of NBD1, the site of the mutation; Rosser et al., 2008). The ubiquitinated protein will then be targeted to the proteasome for degradation (Younger et al., 2006).

ALTERED TRAFFICKING AND SURFACE STABILITY EXHIBITED BY F508del-CFTR

Normally Wt-CFTR is core glycosylated in the ER and traffics to the Golgi, where it is complex glycosylated (**Figure 2**). On a Western blot, it runs as two distinct bands: the lower one at around 150 kDa, referred to as Band B (the core glycosylated form) and the heavier Band C (the complex glycosylated form, around 170 kDa). Conventionally, mature CFTR on the cell surface is mostly complex glycosylated (Xie et al., 1996). With the F508del-CFTR mutation, there is a folding defect in the protein which prevents its trafficking from ER to Golgi, represented as a single Band B running at around 150 kDa, and an absence of Band C (Cheng et al., 1990; Qu and Thomas, 1996).

The trafficking of CFTR requires the optimal presence of many chaperones and co-chaperones. Hsps and co-chaperones like Hdj-2 (Meacham et al., 1999), play an important role in folding and trafficking. Also, the coat complex II (COPII) is required for trafficking from the ER. The interaction of a di-acidic ER exit motif within CFTR with COPII is essential for exit from the ER. This interaction is not required for ERAD (Wang et al., 2004). Trafficking of CFTR occurs from the ER to ER-Golgi-intermediate compartment to Golgi. A COPI machinery is described for anterograde and retrograde trafficking between the stacks of Golgi complexes (Yu et al., 2007). Sub-populations of COPI vesicles are described to perform the function of anterograde as well as retrograde transport (Malsam et al., 2005). Prevention of COPI recruitment to the membrane traps Wt-CFTR in the ER. Additionally, prevention of COPI dissociation from the membrane has the same effect. Complete depletion of β-COPI results in trapping of CFTR in the pre-Golgi compartment. The interaction of COPI with CFTR occurs through an RXR motif (i.e., $R^{553}AR^{555}$) present on CFTR. F508del-CFTRdoes not traffic to the plasma membrane, but deletion of the RXR motif rescues this phenotype (Zerangue et al., 1999; Kim Chiaw et al., 2009). Thus, the interaction of COPI with F508del-CFTR leads to retrograde transport, back to ER, while COPI interaction with Wt-CFTR leads to anterograde transport. With respect to the cell lines used, the trafficking of CFTR can be COPI dependent or independent. It is COPI dependent in HEK293, HeLa cells, and human epithelial cell lines like HT-29, while COPI independent in BHK and CHO cell lines (Rennolds et al., 2008).

The anterograde transport of CFTR is regulated in part by competitive binding of CFTR with14-3-3 regulatory proteins and COPI. It is considered that increased COPI binding results in retrograde transport, while increased 14-3-3 binding results in anterograde transport. 14-3-3 binds to the RXR motif of CFTR, independent of the phosphorylation status (Liang et al., 2012). An increase in phosphorylated CFTR enhances binding of 14-3-3 with CFTR, and decreases binding of COPI with CFTR. From

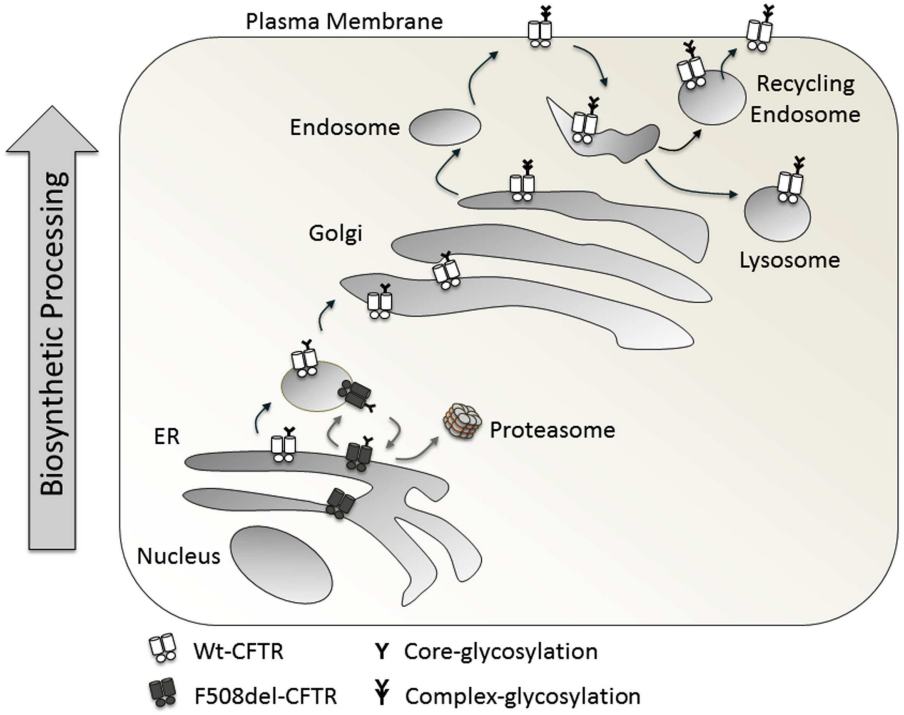

FIGURE 2 | Biosynthetic processing of Wt-CFTR and F508del-CFTR.

the perspective of protein biogenesis, cAMP/protein kinase A (PKA) stimulation by forskolin increases CFTR steady-state levels. Additionally, over-expression of 14-3-3 β and ε increases CFTR steady-state levels, thus 14-3-3 seems to protect CFTR from degradation (Liang et al., 2012). Furthermore, considering that proper folding of the protein is important for trafficking, the molecular chaperones, and co-chaperones are therefore important for proper trafficking of CFTR.

Cytoskeleton

Despite attempts at correcting the defects of F508del-CFTR, the misfolded protein in cell-based systems continues to result in regulatory and gating channel activity defects (Hwang et al., 1997) which are not apparent in purified protein systems (Li et al., 1993). This major discrepancy indicates the significance of the cellular environment, specifically the intracellular organization, which is absent in purified protein systems, as an essential factor in the regulation and function of the mutant protein (Monterisi et al., 2012). The proteins involved in the intracellular cytoskeletal organization include the Na^+/H^+ exchanger regulatory factor isoform protein NHERF2, as well as NHERF1, ezrin, and F-actin which form a complex known as NHERF1-ezrin-actin (Guerra et al., 2005). NHERF proteins are members of the PSD-95/Disk-large/ZO-1 (PDZ) domain protein family which contain two PDZ domains and an ezrin/radixin/moesin (ERM) domain (Hall et al., 1998; Wang et al., 1998). These cytoskeletal proteins interact through their PDZ domains, and bind to the carboxy terminal PDZ motif of CFTR (Hall et al., 1998; Wang et al., 1998). NHERF2 interacts with lysophosphatidic acid 2 (LPA_2) and CFTR to form the CFTR-NHERF2-LPA_2 complex which is essential for compartmentalization of cAMP levels, and consequently the regulation of CFTR channel activity (Zhang et al., 2011). NHERF2 is also essential in regulating LPA-mediated phospholipase C-β3 (PLC-β3; Zhang et al., 2011). NHERF1 interacts with the PKA anchoring protein, ezrin, through its ERM domain (Dransfield et al., 1997). Ezrin, in addition to anchoring PKA, connects to the intracellular cytoskeleton by binding with F-actin (Sun et al., 2000). The importance of NHERF1 for the localization and activity of endogenous CFTR has been shown by several groups, such that in human bronchial epithelial (HBE) cells overexpressing NHERF1 the expression levels and activity of CFTR was significantly enhanced (Guerra et al., 2005; Favia et al., 2010; Monterisi et al., 2012). Using HBE cells, previous studies have disrupted these interactions using site-directed mutagenesis, and found that the NHERF1-ezrin-actin complex is essential for stabilizing CFTR by facilitating anterograde trafficking and anchoring to the apical membrane (Moyer et al., 2000; Guerra et al., 2005; Monterisi et al., 2012). Other studies have also claimed that this complex is critical for efficient regulation of CFTR activity and necessary for the localization of sufficient levels of cAMP as well as PKA activity in the appropriate subcortical or membrane compartment (Monterisi et al., 2012).

Based on fluorescence studies, F508del-CFTR has exhibited altered localization in CF bronchial epithelial (CFBE) cells (Guerra et al., 2005; Favia et al., 2010; Monterisi et al., 2012). The cellular localization of NHERF1 differs between CFBE and HBE cells, such that it is expressed in the cytosol and mainly at the cell surface in HBE cells, whereas it was expressed in the cytosol yet absent

from the cell surface in CFBE cells (Guerra et al., 2005). The actin cytoskeleton of CFBE cells was also found to be disordered compared to HBE cells (Favia et al., 2010). The differential localization of NHERF1 and the disorganization of the cytoskeleton accounts for the differential localization between F508del-CFTR and Wt-CFTR (Favia et al., 2010). The regulation of F508del-CFTR channel activity was also compromised in CFBE cells (Monterisi et al., 2012). The efficient regulation of CFTR requires cAMP levels and PKA activity to be localized in the subcortical compartment (Monterisi et al., 2012). However, there were significantly higher levels of cAMP and PKA in the cytosol than in the subcortical compartment of CFBE cells compared to HBE cells (Monterisi et al., 2012). Subsequently, it was reported that the disorganization of the actin cytoskeleton of CFBE cells caused these defects, as cAMP could freely diffuse from the subcortical to the cytosolic compartment (Monterisi et al., 2012). Furthermore, since CFTR expression at the cell surface is dependent on the formation of the CFTR-NHERF1-ezrin-actin complex, the disorganization of the intracellular cytoskeleton, resulting from the aforementioned regulatory defects, significantly reduced expression of F508del-CFTR at the apical plasma membrane and led to retention in the ER (Monterisi et al., 2012).

GRASP pathway

There is recent evidence that F508del mutant protein can be rescued to the cell surface through an unconventional pathway, referred to as GRASP (Golgi reassembly stacking proteins) dependent secretory pathway. Conventionally the complex glycosylated form of CFTR is considered to be present on the cell surface. But this unconventional Golgi-independent pathway can allow for surface expression of the core glycosylated form of CFTR. The transgenic GRASP55 expression in F508del-CFTR homozygous mouse, could rescue the mutant protein to the plasma membrane and was functional as noted by short-circuit currents using mice colon (Gee et al., 2011). As the mutant F508del-CFTR does have some chloride channel activity, activation of this pathway in the patients having the F508del-CFTR mutation can lead to surface expression of the mutant channel and thereby help in improving function.

Lipid rafts

Lipid rafts, small membrane domains which are rich in sphingophospholipids and cholesterol, have been implicated to play a role in CF pathology, although the mechanism is controversial. Ceramide is also a key constituent of the lipid raft, and like cholesterol and cholesterol ester, its subcellular distribution is thought to be modified in CF affected tissues (Gentzsch et al., 2007). The PDZ-interacting domain of CFTR is responsible for its localization to lipid rafts within the apical membrane, and facilitates formation of a signaling complex with receptors (Dudez et al., 2008). One such complex with CFTR includes: Tumor necrosis factor receptor 1 (TNFR1) and c-Src, and this complex is thought to play a key role in regulating TNF-α mediated cytokine signaling within the epithelial cell. In CF affected epithelia, ceramide levels and ceramide mediated signaling through lipid raft-localized TNF-α receptors is thought to increase (Dudez et al., 2008; Bodas et al., 2011). The mechanisms underlying the change in ceramide metabolism remain unknown. The proposed role of CFTR in ceramide metabolism via the regulation of endosomal pH has been challenged (Grassmé et al., 2003; Barriere et al., 2009; Haggie and Verkman, 2009). Optimal plasma membrane ceramide concentrations are also regulated by acid-sphingomyelinase (ASM). The balance between ASM and acid ceramidase is essential to maintain optimal ceramide on the cell surface in normal tissues (Teichgräber et al., 2008). CF mice with partial genetic ASM deficiency ($Cftr^{-/-}/Smpd1^{+/-}$) display reduced inflammation and reduced susceptibility to pseudomonal infection (Grassmé et al., 2008; Teichgräber et al., 2008; Kitatani et al., 2009; Becker et al., 2010; Grassmé et al., 2010). Taken together, this suggests a role for ceramide and lipid raft mediated signaling in CF associated inflammation and pathogenesis in CF mice (Wojewodka et al., 2011). However further studies are required to resolve the current controversies regarding the underlying mechanisms.

Recycling and peripheral quality control

The newly formed CFTR has to pass through various quality control check-points at the ER and periphery (post-Golgi). Normally at the periphery, Wt-CFTR undergoes recycling from the plasma membrane to early endosomes and back to the cell surface. Mutant F508del-CFTR is misfolded and is susceptible to ubiquitination, re-routing it from recycling to multivesicular bodies and lysosomal degradation (Sharma et al., 2004). The cellular half-life of Wt-CFTR is greater than 24 h, while that of F508del-CFTR is around 7 h. However, the biochemical half-life of plasma membrane Wt-CFTR is greater than 48 h and that of F508del-CFTR is <4 h (Lukacs et al., 1993; Heda et al., 2001). Thus, even if the mutant F508del-CFTR is rescued by temperature to the plasma membrane, it is less stable, suggesting the role of peripheral quality control in removing the misfolded mutant protein. Molecules involved in clearing the mutant CFTR from the plasma membrane were identified through screens, which revealed the role of chaperones, enzymes, and other molecules involved in ubiquitination like CHIP, Hsc70, and Hsp90. This indicates that similar molecules might be involved in ERAD and post-ER clearance of mutant protein from the plasma membrane (Okiyoneda et al., 2010). The peripheral quality control therefore serves as a check-point for the amount of misfolded protein expressed on the plasma membrane (Wolins et al., 1997).

MECHANISM OF ACTION OF EXISTING CORRECTOR COMPOUNDS

Efforts in identification of correctors using HTS approaches have been very fruitful, and there is much we can learn from each CFTR corrector molecule. Hundreds of compounds classified as CFTR correctors have been identified in literature to date (Pedemonte et al., 2005; Van Goor et al., 2006; Carlile et al., 2007; Kalid et al., 2010; Lin et al., 2010; Van Goor et al., 2011). Most of these molecules are deemed unsuitable for clinical use namely due to low efficacy, cell type specificity, and/or toxicity profiles. However, these compounds provide precedent as a useful scientific tool to probe how an ideal corrector may affect CFTR, and a potential scaffold for future drug designs. An understanding of the mechanism of action and binding site of previous generation CFTR correctors would be a leap forward toward the rational drug design

of CFTR correctors. To date, no corrector mechanism of action has been entirely resolved, and no clear corrector binding site has been defined. However, through the admirable efforts of many groups, we are now aware of some key features of previous generation correctors.

CORR-4a

Corr-4a is a bisaminomethylbithiazole derivative identified in a high-throughput screen for CFTR correctors by Pedemonte et al. (2005; **Figure 3**). Corr-4a has a mild correction effect of the F508del-CFTR (effective in the low μM range), and a nearly complete correction effect on the rare mutant V232D-CFTR (Caldwell et al., 2011). In an effort to understand the mechanism of Corr-4a, Cyr and colleagues examined Corr-4a efficacy in cells with inactivated RMA1 and CHIP ubiquitin ligases. They discovered that Corr-4a affects biogenic intermediates after MSD2 synthesis, and likely only corrects defects in F508del-CFTR which are not recognized by RMA1 E3 ubiquitin ligase (Grove et al., 2009). Additionally, Loo et al. (2009) have demonstrated that treatment with Corr-4a can lead to the partial restoration of inter-domain interactions between MSD1 and MSD2 when F508del-CFTR is expressed in two halves. To date, there is no evidence that Corr-4a acts directly on CFTR. Gene expression profiling studies show that Corr-4a treatment does not significantly alter the cell's transcriptome, suggesting the effect is fairly specific to CFTR (Sondo et al., 2011), though Corr-4a mediated correction effects have been reported for folding mutants of Pgp and hERG (Van Goor et al., 2011). Since the year of its identification, Corr-4a has proven that pharmacological rescue of CFTR is a viable therapeutic strategy. Corr-4a itself may have low efficacy and an uncertain target, but it has fueled the search for next generation correctors.

VRT-325

VRT-325, a quinazoline, is a well studied CFTR corrector molecule (**Figure 3**). It was identified in a HTS designed by Vertex Pharmaceuticals with the support of the Cystic Fibrosis Foundation (Bethesda, USA). VRT-325 is generally effective in cell systems in the 1–10-μM range (Van Goor et al., 2006). Studies by Bear and colleagues have demonstrated that VRT-325 binds directly to purified, reconstituted CFTR, and modifies its ATPase activity. VRT-325 is the first pharmacological chaperone which has been demonstrated to bind directly to CFTR, offering much promise for the future of CFTR correctors as drugs (Kim Chiaw et al., 2010). Despite this finding, VRT-325 has been shown to improve the trafficking of other membrane proteins, including Pgp and hERG folding mutants (Van Goor et al., 2011). Like Corr-4a, VRT-325 does not have a major effect on the cell's transcriptome, so it is likely producing a protein specific response (Sondo et al., 2011). Many efforts have been put forth to elucidate the mechanism of this correction effect. Limited proteolysis revealed an improvement in the stability of NBD1, but not the second half in the presence of VRT-325 (Yu et al., 2011). The F508del mutation is known to destabilize NBD1, thus restoring its stability is critical step toward restoring F508del-CFTR trafficking and function. Like Corr-4a, VRT-325 has been reported to restore inter-domain interactions at the membrane spanning domains based on co-expressing CFTR in two halves, though this effect was not detectable via limited protease sensitivity of the full-length protein (Loo et al., 2009; Yu et al., 2011). Interestingly, despite its positive effect on CFTR trafficking, in high concentrations VRT-325 (25 μM) was shown to inhibit CFTR-mediated ion flux due to a decrease in ATP-dependent conformational dynamics (Kim Chiaw et al., 2010). This well studied corrector has demonstrated that a small molecule can directly interact with F508del-CFTR, and is capable of partially correcting its intrinsic folding by stabilization of individual domains and inter-domain interfaces.

RDR1

RDR1 is a CFTR corrector compound first identified in a HTS using isolated NBD1 by Carlile et al. (2007; **Figure 3**). RDR1 has a mild correction effect compared to equal concentrations of VRT-325, approximately half functional correction at 10 μM (Sampson et al., 2011). Despite its lower efficacy, it represents an interesting scaffold for corrector compounds because it binds directly to isolated NBD1, and is a mild potentiator of CFTR channel activity (Sampson et al., 2011). Differential scanning fluorometry was used to demonstrate that RDR1 improves the thermostability of F508-NBD1. Owing to its direct binding to and stabilization of NBD1, RDR1 is a valuable compound after which future correctors can be designed.

VX-809

The latest CFTR corrector advancing though clinical trials is VX-809, a novel compound discovered in a HTS by Vertex Pharmaceuticals (**Figure 3**; Van Goor et al., 2011). Little is known about the mechanism behind VX-809 correction of F508del-CFTR, but current insights are promising. At nanomolar concentrations VX-809 is a highly specific corrector, capable of correcting F508del-CFTR, but not other misfolded membrane proteins (Pgp and hERG mutants). VX-809 confers significant resistance to proteolysis of full-length F508del-CFTR and NBD2 fragments, suggesting an improved overall fold (Van Goor et al., 2011). Further work will be required to understand the molecular interactions which lead to VX-809's effective and specific F508del-CFTR correction. As a compound only recently made available to the academic community, mechanistic insight is still lacking, however there is much motivation to understand this compound and the potential for more efficacious, and clinically relevant correctors.

DRUG DISCOVERY

As previously described, several structural and functional aberrations require correction in order to restore activity of F508del-CFTR. These structural defects involve intra- and inter-domain interfaces, and subsequently constitute multiple therapeutic targets. Repair of these interfaces can potentially be achieved using pharmacological chaperones (i.e., small molecules) which repair each aberrant site. However, a more desirable therapy would be to identify a single small molecule that intrinsically repairs multiple defects. This would avoid any adverse drug interactions a regimen of multiple therapeutic drugs could incur. It is not enough to repair NBD1 of F508del-CFTR since it has been shown that improved stability of this domain is not sufficient to produce a globally stable protein (Rabeh et al., 2012). Therefore, targeting full-length F508del-CFTR is necessary to restore the biosynthesis, stability, and activity back to wild-type levels.

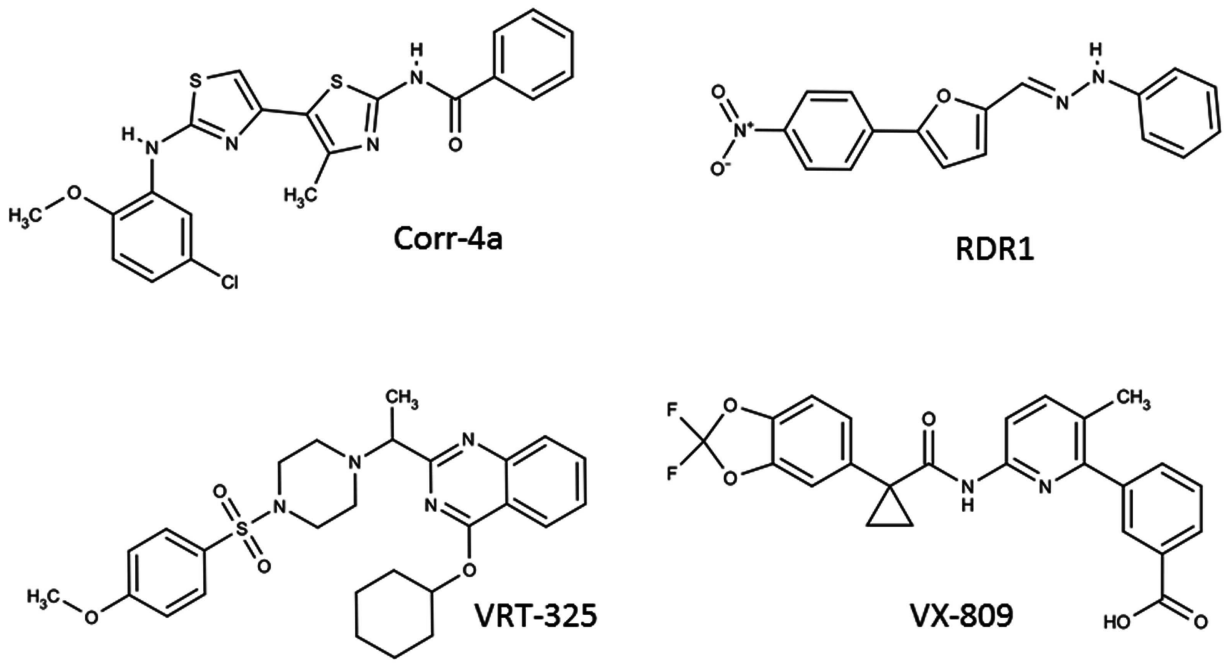

FIGURE 3 | F508del-CFTR corrector compounds.

Although targeting F508del-CFTR directly is desirable, therapeutics which promote trafficking, repress degradation, and increase synthesis of this mutant protein via chaperones and co-chaperones could also be useful. However, this approach would likely have non-specific and toxic effects, since these chaperones are necessary for proper folding of many other proteins. Additionally, it has been shown that even after low temperature rescue, the peripheral protein quality control machinery removes structurally compromised F508del-CFTR from the plasma membrane, and thus could be another target for therapeutics (Lukacs et al., 1993; Heda et al., 2001). Likewise, removal of F508del-CFTR from the cell surface is ubiquitination-dependent and involves several E3 ubiquitin ligases (e.g., CHIP, gp78) which could also be targeted in order to rescue F508del-CFTR (Meacham et al., 2001; Morito et al., 2008).

Interestingly, many solubilizing mutations which enhance the biosynthesis of F508del-CFTR have been identified, and could provide insight into functional interfaces which need to be repaired for restoration of functional activity. Drugs that mimic the structural consequences of these stabilizing mutations could be of therapeutic use. For example, a peptide containing a diarginine (RXR)-based ER retention motif was found to compete with the aberrantly exposed $R^{553}AR^{555}$ within NBD1 of F508del-CFTR, subsequently preventing its ER retention and promoting anterograde trafficking to the cell surface (Kim Chiaw et al., 2009). By targeting intracellular pathways which compete with F508del-CFTR biosynthesis, enhanced expression of this major mutant can be functionally rescued to the cell surface.

Another potential route of functional rescue could involve targeting *F508del-CFTR* mRNA. RNA as a drug target has been shown to improve the outcome of type 1 muscular dystrophy *in vitro* (Parkesh et al., 2011; Childs-Disney et al., 2012). Likewise, Bartoszewski et al. (2010) showed that the trinucleotide deletion causing F508del, which is found in the majority of patients with CF (i.e., the out-of-frame *CTT* deletion between amino acids Ile^{507} and Phe^{508}) and rendering a synonymous single nucleotide polymorphism at Ile^{507}, caused instability of *F508del-CFTR* mRNA due to the enhanced size of hairpin loops relative to wild-type CFTR mRNA. These larger hairpins increased the rate of degradation, and resulted in less mRNA being retained in the cell for translation. In this same study, the authors generated F508del by deleting the trinucleotide corresponding to amino acid Phe^{508} directly (i.e., *TTTdel*) and showed that although this same deletion causes F508del, the RNA primary sequence differed from (*CTTdel*)*-F508del-CFTR* and was sufficient to retain wild-type mRNA loop secondary structure. An abundance of *(TTTdel)-F508del-CFTR* was present at physiological temperature relative to *(CTTdel)-F508del-CFTR*, and allowed for enhanced low temperature rescue at the protein level. This demonstrates the fragility of the naturally occurring *(CTTdel)-F508del-CFTR* mRNA, in addition to the well documented instability at the protein level. Thus, if the loop structure of the naturally occurring F508del-CFTR mRNA could be induced to mimic that of wild-type CFTR (or even *TTTdel*) with therapeutics, sufficient transcript would be available for translation, even though the underlying mutation remains. This approach could enhance the half-life of the misfolded mRNA, increase the synthesis of nascent F508del-CFTR, and establish a novel pool of therapeutic targets which could then be corrected with small molecule protein correctors. Although this approach could improve downstream protein synthesis of F508del-CFTR, it

would not directly address the underlying protein folding defects which cause disease.

Although not a small molecule therapeutic, *CFTR* gene therapy, in which the wild-type *CFTR* gene is introduced into the target tissues (e.g., lung, gut), could be another potential approach to treat CF. This delivery method has been under investigation as a CF therapy for over 20 years, and although it may seem straightforward in principle, gene transfer into the lungs has proven to be a problematic endeavor (Griesenbach and Alton, 2012). Gene therapy involves the introduction of foreign DNA using liposomal or viral vectors, and as a result, each approach has had poor clinical outcomes, having issues with low transfer efficiency and immunoreactivity, respectively (Cao et al., 2011). Therefore, a current approach involves pluripotent stem cell therapy using humanamniotic mesenchymal stem cells which are reprogrammed into the required cell type (e.g., bronchial epithelial cells) and which contain wild-type *CFTR* (Paracchini et al., 2012). This method could allow for functional tissue regeneration by means of topical and systemic administration of stem cells, with the goal of replacing dysfunctional tissues containing F508del-CFTR. However, this approach is still in the investigational stage, and favorable experimental results are needed to allow further pursuit at the clinical level.

IDENTIFICATION OF SMALL MOLECULE CORRECTORS

There are many chemical libraries which have been compiled by academics and pharmaceutical companies alike in the past few decades, and it is likely that within these libraries an F508del-CFTR corrector or pro-corrector (requiring structural optimization) exists. Therefore, these small molecules need to be included in HTS assays which investigate their ability to functionally correct F508del-CFTR. Three approaches which are used to identify and validate small molecule correctors include:

(1) *In silico* tools to identify putative binding sites for corrector compounds
(2) *In vitro* techniques using purified CFTR protein to identify and validate correctors
(3) Cell-based assays to validate functional correction and investigate mechanism of action of identified small molecules

The choice of chemical compounds to use in HTS, as well as methodologies to investigate and validate novel small molecule correctors will be discussed in detail below.

Compound libraries

Compound libraries used in HTS approaches will depend on what is available to the investigator. Most approaches use in house compounds, while others rationally design compounds based on the binding site of the target receptor. The size of the library is an important factor, since the larger the screen the more statistically likely that true positive and thus biological hits will be found. In HTS approaches used to find F508del-CFTR correctors, libraries comprised of thousands to hundreds of thousands of chemical compounds are typically used (Pedemonte et al., 2005; Van Goor et al., 2006; Robert et al., 2010). Structural diversity of compounds in each library is usually large and will subsequently enhance the quality and breadth of the screen, since the likelihood of finding efficacious, specific, and non-toxic correctors *in vivo* will come from identification of drugs which target F508del-CFTR itself, yet do not interfere with normal channel activity.

It is interesting to note that previous corrector screens have used chemical libraries of 2,000–164,000 compounds, and typically the hit rate is ∼0.01–0.03% (Lin et al., 2010). This low yield suggests that larger libraries would be more successful. Furthermore, successful compounds found from HTS must be drug-like, and be able to have therapeutic properties once administered to patients. Thus, any compounds which do not abide by Lipinski's Rule of Five need to be discarded or optimized at the outset of a screen (Lipinski et al., 2001). Molecules which could become a drug or pro-drug, are retained and tested for corrector activity. Hits from such HTS must then be validated using more rigorous assays of biological activity, usually involving purified CFTR protein. Such leads are then derivatized, optimized, and subjected to further validation.

Lin et al. (2010) used a library containing >3,000 FDA-approved drugs to search for small molecule correctors and potentiators in cell-based assays, and ∼40 chemicals with F508del-CFTR corrector activity were identified. Their choice to screen previously approved drugs is advantageous, since it would streamline application from bench to bedside, saving many years it would normally take to become approved for human indications. Additionally, since CF is a disease in which few therapeutic interventions exist, the Orphan Drug Act allows the approval process to be facilitated, reaching market much sooner than other drugs at the same stage of development (Thorat et al., 2012).

Although not directly addressed by all HTS approaches for correctors, it has been known for many years that F508del-CFTR activity suffers from a channel gating defect (Dalemans et al., 1991). Thus, the consequence of F508del requires more than just a small molecule for trafficking, and so a drug must have potentiator activity as well. Ideally, a small molecule will have both corrector and potentiator activity in order to repair both defects. Thus, corrector-potentiator compounds are needed; one such class of compounds that has shown this activity includes cyanoquinolines (Knapp et al., 2012). Dual screens which address the folding and gating defects would be advantageous in the discovery of a single therapeutic compound.

One approach which is less resource intensive than *in vitro* and *in vivo* studies, yet has had successful applications in identifying bioactive small molecules is that of *in silico* drug discovery (Varady et al., 2003; Klebe et al., 2004; Evers et al., 2005). *In silico* compound libraries can include naturally occurring molecules from flora and fauna, chemicals from *de novo* synthesis, those which do not physically exist but have been computationally designed, and more importantly small molecules which have been rationally designed from protein structures. Since compound structures can be easily modified *in silico*, this approach can be a powerful tool for finding novel therapeutics which satisfy allosteric and electrostatic requirements of the receptor (i.e., F508del-CFTR) binding site(s).

In silico approaches: virtual screening and rational drug design

In silico methods are advantageous since they can identify compounds that bind or "dock" directly to F508del-CFTR, something

that cannot be initially confirmed in cell-based assays. Molecular docking has proved useful in the discovery of α1A adrenergic receptor and dopamine D3 receptor antagonists, which is relevant to cardiovascular disease and Parkinson's disease, respectively (Varady et al., 2003; Evers et al., 2005). In these two cases, the small molecules were found using virtual screening and structure-based rational design from the atomic detail of putative binding sites. Interestingly, many of these designer drugs are inhibitors of their protein targets. Therefore, it may be difficult to design F508del-CFTR correctors which does not inhibit but instead enhance expression and activity.

Virtual screening can be used to identify novel correctors of F508del-CFTR with higher throughput than can be achieved using a cell-based approached (hundreds of millions as opposed to hundreds of thousands). Due to this large volume, and keeping the hit rate constant (based on previous studies), a larger number of correctors will statistically be found. Indeed, reported hit rates for virtual screening are ∼10-fold higher than that for *in vitro* HTS (∼3–5% compared with ∼0.3%; Van Goor et al., 2006; Carlile et al., 2007; Kalid et al., 2010). However, it must be noted that there are currently no crystal structures of CFTR in the presence or absence of small molecules (such structures are desirable for virtual screening), and CFTR structures previously used are homology models based on the related bacterial ABC transporter, Sav1866 (Serohijos et al., 2008; Mornon et al., 2009). Importantly, this type of screening approach is advantageous due to the speed and cost of utility (it is rapid and inexpensive), although a major limitation is that any positive hits need to be confirmed *in vitro*. Furthermore, virtual screening typically uses static or rigid protein structures for docking of small molecules, and so another caveat is that it does not take into account the dynamic nature of proteins.

The purpose of virtual screening is to discover novel scaffolds of small molecule modulators of CFTR activity. This could in turn identify novel therapeutic binding sites within F508del-CFTR which can then be validated *in vitro* and further optimized using quantitative structure-activity relationship studies to create a more efficacious corrector. As such, a study by Kalid et al. (2010) identified several *in silico* correctors which docked to intra-molecular interfaces (e.g., NBD1:NBD2, NBD1:ICL4) within F508del-CFTR, and which were then subsequently validated *in vitro*. This further supports the notion that multiple defects and thus therapeutic targets exist within the mutant protein, and suggests that current Sav1866-based homology models of CFTR must have some degree of accuracy (Serohijos et al., 2008; Mornon et al., 2009).

In vitro: NBD1 binding assays and techniques using purified protein

Small molecule correctors can also be identified *in vitro*, via binding assays using isolated domains of F508del-CFTR and assays using purified full-length mutant protein. The small molecule RDR1 was found to enhance the thermostability of F508del-NBD1, suggesting that this compound binds directly to improve the folding of this isolated domain (Sampson et al., 2011). From this, RDR1 was extended to the full-length protein in cell surface expression assays, and was also found to improve folding of full-length F508del-CFTR. These studies suggest that this compound repairs an intra-molecular interface involving NBD1 (e.g., NBD1:ICL4, NBD1:NBD2), and facilitates proper folding and subsequent trafficking to the cell membrane, a characteristic of a corrector. However, it is uncertain if this compound is able to potentiate the activity of F508del-CFTR in addition to its corrective properties.

Likewise, purified full-length F508del-CFTR has been used to investigate the effects of small molecule correctors and potentiators. This approach is ideal since it eliminates chaperones as potential targets, and instead identifies F508del-CFTR as the therapeutic receptor. This could allow for faster identification of F508del-CFTR-specific drugs which will not have off target effects and/or toxicities. Although the mechanism of action of known correctors is not well characterized, there have been several studies which suggest that these small molecules bind directly to F508del-CFTR; although at which site within the protein structure is not well understood. For example, Kim Chiaw et al. (2010) demonstrated that VRT-325 binds directly to inhibit the ATPase activity of purified and reconstituted full-length F508del-CFTR, while Yu et al. (2011) showed that VRT-325 decreased the protease susceptibility of F508del-NBD1 in HEK cells, suggesting that this small molecule binds directly to NBD1 or an interface involving NBD1.

VALIDATION OF BIOACTIVE COMPOUNDS: FUNCTIONAL ANALYSIS OF F508del-CFTR USING CELL-BASED SYSTEMS

Cell surface expression and iodide efflux assays

Lead candidate compounds from *in silico* screening, as well as *in vitro* binding and functional assays using purified protein need to be validated in cell-based assays, in order to elucidate the mechanism of action and further improve activity by optimization of chemical structure. One such approach is to use cells overexpressing F508del-CFTR containing a hemagglutinin tag in the fourth extracellular loop, which can be monitored using cell surface immunofluorescence. In this assay, F508del-CFTR which has been "rescued" using correctors will have a hemagglutinin tag exposed to the extracellular matrix, and can subsequently be detected using antibodies. This assay has a reported hit rate of ∼0.06–0.8%, and can be adapted to work with most cell types (Carlile et al., 2007; Robert et al., 2008). However, further validation using biochemical tools is required to assess the mechanism of action of putative direct binding correctors.

Additionally, cell surface expression of F508del-CFTR can be monitored using cells co-expressing a yellow fluorescent protein (YFP) variant which is sensitive to halides (Galietta et al., 2001; Pedemonte et al., 2011b). The fluorescence of this YFP variant is quenched in the presence of chloride or iodide, and can be used to detect CFTR activity and thus cell surface expression. In brief, these F508del-CFTR/YFP expressing cells are put into a solution containing halides, and after addition of forskolin (a CFTR activator) the amount and rate of fluorescence quenching via halide influx is proportional to the amount of functional F508del-CFTR at the cell surface. This method has identified several correctors; however, the mechanism of action of these compounds is poorly understood (Pedemonte et al., 2011b).

Although assays using fluorescence dequenching have also been used to detect corrector activity, they have previously not been amenable to HTS approaches due to the cost and lack of sensitivity. The halide-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl) quinolinium is routinely used for this purpose, such

that cells expressing F508del-CFTR are loaded with both fluorophore and halide, and after an incubation period, forskolin is added to activate channel activity and the amount of cell surface protein is assessed by means of halide efflux (and an increase in fluorescence; Jayaraman et al., 1999; Mansoura et al., 1999). Academic laboratories have been using this method for many years to assess the activity of CFTR mutants and small molecules on a low throughput scale, however perhaps now this technique can be scaled up to HTS for novel corrector-potentiator compounds since the cost and sensitivity of halide-sensitive fluorescence quenchers and dequenchers has improved in recent years.

Iodide efflux assays using an iodide sensitive electrode have also been one of the main functional assays for assessing F508del-CFTR activity after small molecule "rescue." In these studies, cells overexpressing F508del-CFTR are loaded with iodide, and after an iodide gradient is establish, forskolin is added to activate CFTR and allow for iodide efflux, which is proportional to the amount of functional protein on the cell surface (Yu et al., 2011). This assay is very sensitive, being able to detect iodide in the nanomolar – micromolar range. However, although these experiments are suitable for investigation of putative mechanisms of action of small molecule correctors and potentiators, this approach is not suitable for HTS assays in its current state, due to the cost of each iodide sensitive probe (multiple probes are required for HTS) as well as the need for calibration prior to each measurement.

REQUIREMENTS FOR THE DESIGN OF AN IDEAL CORRECTOR

CORRECTOR BINDING SITE

Little high resolution structural information, other than crystal structures of isolated NBD1, has been published on CFTR (Lewis et al., 2004, 2005). Molecular models based on prokaryotic ABC transporters have been developed to attempt to define the structural features of the protein that allow transduction of the ATP binding and hydrolysis signals in the cytosolic NBDs to increased probability of opening and closing of the conduction pathway through the helical domains that span the membrane (Serohijos et al., 2008; Mornon et al., 2009; Dalton et al., 2012). These models have been instructive in suggesting interactions between NBD1 and NBD2, between the NBDs and the TM domains via the ICLs, and recently a model of the unique regulatory R region and its possible interaction with the remainder of the protein. Ford et al. (2011) have created low resolution structures of CFTR that appear to confirm the close interaction of the TM domains with the NBDs, as predicted from the molecular models (**Figure 1**).

As described above, the NBDs interact with the TM domains via lengthy helical segments that extend from the TM segments into the cytosol (**Figures 1A,B**). Shorter helical segments at the foot of the long extensions have been termed "coupling helices" and sit parallel to the NBD surface. There is crossover between sets of helices in the TM regions such that both TM segments in the first and second half of the protein interact via coupling helices to both NBD domains. The coupling helices may interact with hydrophobic patches on the NBD surface and act as signal transduction platforms to aid in the transfer of information from ATP binding-hydrolysis to channel opening and closing. In fact, the closeness of the F508 residue on the surface of NBD1 to the coupling helix ICL4 suggests that partial disruption of this interaction platform may in part be responsible for the structural and functional consequences of F508del.

As described in the models by the Callebaut group (Mornon et al., 2009), there appears to be subtle but significant movement of domains relative to each other in the channel closed-open transition. The NBDs slide relative to one another upon ATP binding-hydrolysis at the catalytic site and the coupling helices may provide a pivot point, allowing a twisting along the helical extension and TM helices. Kirk and coworkers suggest that changes in the orientation of the long helical segments are important for channel gating and might occur during the ATP binding-hydrolysis cycle (Wang et al., 2010b).

Of particular difficulty in modeling is the unique and intrinsically disordered R domain, which has no structural analog among prokaryotic ABC transporters used to generate CFTR models. The R domain is a highly charged 241 residue linker region possessing multiple phosphorylation sites for PKA and other kinases that joins the two halves of the protein (Tabcharani et al., 1991; Chappe et al., 2005; Kongsuphol et al., 2009). The R domain appears to be a disordered region that is sufficiently flexible to undergo dynamic interactions with other CFTR domains to regulate function. Although the domain is disordered, it contains regions with the propensity to form α-helices in the non-phosphorylated state and upon PKA phosphorylation, this propensity is reduced (Ostedgaard et al., 2000; Baker et al., 2007). The isolated R domain becomes less compact with phosphorylation, and interactions with multiple domains appear to be modulated (Chappe et al., 2005; Baker et al., 2007; Hegedus et al., 2008). The regulatory nature of the R domain suggests it may be a prime binding site for correctors and potentiators whereby the inter-domain interactions are modulated, either strengthening or weakening interactions and inducing altered structural conformations in distant regions of the protein. Modulator interaction at the R domain may correct the domain–domain interactions disrupted through deletion of F508.

Cross-linking experiments have shed some light on conformational maturation in mutant proteins and changes induced by binding of small molecules, and suggest dynamic changes in some regions of the protein. Clarke and co-workers have used a variety of corrector molecules including VRT-325, Corr-4a, and others along with chemical cross-linking and sensitivity to glycosidases to show that small molecule correctors rescue folding mutants such as F508del-CFTR (Wang et al., 2006b, 2007a,b; Loo et al., 2009). They have shown that folding of the TM domains occurs in the absence of the NBDs when treated with corrector VRT-325, indicating that the binding site for this molecule is not in the NBD domains and that direct binding can induce folding of the TMDs. They have also shown that addition of multiple corrector compounds increases the amount of rescued protein, suggesting that these molecules bind to diverse sites in the protein rather than a single corrector site to promote CFTR maturation, likely by multiple mechanisms.

It seems reasonable that corrector binding sites are located at domain–domain interface regions that are critical for signal transduction, and the corrector molecules function by promoting

Wt structure and stability at these interfaces to allow the protein to escape the quality control machinery of the ER.

WHAT'S WRONG WITH FIRST GENERATION CORRECTORS?

At least some small molecules, such as butyrate and glycerol, as well as incubation at low temperatures (Denning et al., 1992), can promote increased CFTR trafficking to the cell surface, but these treatments are highly non-specific for CFTR and otherwise not suitable for use in patients. At least some first generation CFTR correctors interact directly with CFTR to promote its rescue (Loo et al., 2009; Wellhauser et al., 2009; Kim Chiaw et al., 2010) rather than exerting their effect non-specifically by increasing total protein expression and lowering fidelity of ER quality control mechanisms. Corrector compounds could work by modifying aberrant interactions of F508del-CFTR with chaperone proteins or degradation pathways (Wang et al., 2006a; Younger et al., 2006).

As described above, most first generation correctors, such as VRT-325, appear to stabilize only some F508del-CFTR domains and domain:domain interactions, and they rescue only a fraction of the protein trafficking to the cell surface (Loo et al., 2005, 2006; Van Goor et al., 2006; Wang et al., 2007b,c; Kim Chiaw et al., 2010). VRT-325, for example, has been estimated to rescue F508del-CFTR to just ∼15% of the maturation efficiency of Wt-CFTR at a concentration of 6.7 μM (Van Goor et al., 2006). CFTR has sufficient affinity for this molecule that it can be used at low micromolar concentrations, promoting maturation of both F508del-CFTR and other processing mutants, and results in some CFTR activity at the cell surface, which are important characteristics of a suitable CFTR corrector molecule for use in patients. However the molecule is not CFTR-specific, rescuing misprocessed Pgp mutants as well as CFTR, meaning it would be expected to have significant off target effects (Wang et al., 2007c).

Most current generation CFTR correctors, such as VX-809 do not potentiate CFTR activity at the cell surface (Van Goor et al., 2011), or indeed many such as VRT-325 partially inhibit channel activity (Kim Chiaw et al., 2010). VRT-325 significantly lowers the ATP hydrolytic function of CFTR at as low as 10 μM concentrations, and at 25 μM reduces the apparent affinity of CFTR for ATP by ∼10-fold, as determined by ATPase activity measurements on purified protein. VRT-325 appears to stabilize NBD1 as it is capable of restoring compactness in this domain, but has no effect on the stability of the C-terminal half of the protein (Yu et al., 2011). It is likely that a small molecule that increases the stability of the NBD domain, but does not bind at this location, will have aberrant and rigid domain–domain interactions with at least the first TM domain of the protein whereby the interactions are not properly modulated by ATP binding-hydrolysis. An ideal CFTR corrector should promote full CFTR maturation without sacrificing regulation and channel function.

VRT-532 is a dual-acting molecule that possesses both weak corrector and robust potentiator activity for mutant CFTR that is not specific to the F508del genotype (Wang et al., 2006b; Wellhauser et al., 2009). However its corrector activity is too low to produce sufficient increases in F508del-CFTR trafficking for meaningful rescue of the protein to the cell surface. This molecule, and others (Mills et al., 2009; Pedemonte et al., 2011a; Phuan et al., 2011; Knapp et al., 2012; Leier et al., 2012), suggest that a multi-acting compound that is capable of both corrector and potentiator roles is possible.

F508del-CFTR that reaches the cell surface has a short half-life at that location (Lukacs et al., 1993; Heda et al., 2001). Many first generation correctors likely do little to stabilize rescued F508del-CFTR at the cell surface. Loss of surface expressed protein is likely to have a major effect on observed channel function at the cell surface over time. Gentzsch et al. (2004) clearly demonstrated that turnover of F508del-CFTR at the cell surface is significantly higher than Wt-CFTR turnover. Under conditions where F508del-CFTR is first rescued by low temperature incubation (27°C), nearly all protein is lost from the cell surface within 4 h incubation at 37°C, while ∼40% of surface expressed Wt-CFTR remains. Collawn and co-workers showed that Corr-4a significantly enhances the stability of rescued F508del-CFTR at the cell surface, up to 12 h at 37°C in surface biotinylation experiments and activity measurements (Varga et al., 2008). While they showed that low temperature treatment reduces proteasomal function, Corr-4a treatment at 37°C may directly inhibit the E1–E3 ubiquitination pathway, as well as reducing endocytosis (Varga et al., 2008). Ussing chamber studies showed that Corr-4a increases cAMP-mediated F508del-CFTR activity by >60% after 6 h incubation at 37°C in the presence of the corrector. Corr-4a treatment alone is therefore not sufficient to produce maximal CFTR activity, however addition of a potentiator molecule can increase the activity of the protein. The off target effects due to the use of a small molecule such as Corr-4a that may alter the ubiquitination pathway and endocytic cell surface protein recycling would preclude such a molecule from being used to treat patients (Varga et al., 2008).

Young et al. (2009) showed that when dynamin-associated removal of Wt-CFTR and temperature-rescued F508del-CFTR from PM is inhibited by the dynamin inhibitor "dynasore," significantly more CFTR remains at the cell surface, indicating that dynamin-associated processes are important for the cell surface stability of rescued protein. When dynasore is used in conjunction with correction by Corr-4a, significantly more cell surface CFTR is present than either treatment alone, suggesting that multiple mechanisms can be combined to improve CFTR restoration. Stability of surface expression indicates an important mechanism by which CFTR must be corrected to produce sustained robust restoration of CFTR activity.

CHARACTERISTICS OF AN IDEAL CORRECTOR

We submit that a corrector will need to repair at least three major defects in CFTR that results from the F508del mutation in order to be maximally effective in correcting the F508del-CFTR phenotype clinically. Namely (1) it must efficiently rescue F508del-CFTR trafficking to the cell surface, ideally to >50% of Wt levels, as heterozygous individuals do not suffer the symptoms of CF. This may involve improving the folding of the protein and/or aiding in escaping ER quality control mechanisms by other means, (2) it must act to increase the compromised activity of F508del-CFTR channels to near Wt levels while retaining phosphorylation-dependent regulation of its activity, and (3) it should increase the stability of the mutant protein on the cell surface to near Wt levels

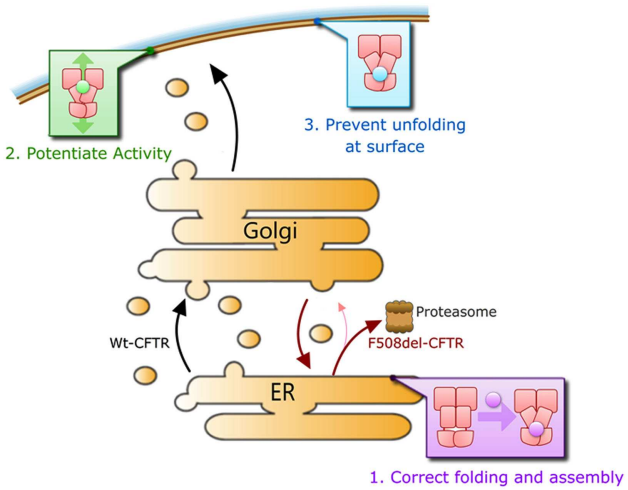

FIGURE 4 | Overview of molecular defects requiring repair for functional rescue of F508del-CFTR.

of residence time (**Figure 4**). A defect in any one of these steps would lead to disease.

Potentiation of the small amounts of F508del-CFTR that naturally reach the cell surface is insufficient to have a measurable improvement in patient clinical outcomes. In F508del-CFTR homozygous individuals, when treated over 16 weeks with the pure CFTR potentiator VX-770 (Ivacaftor; Kalydeco), there was no change in measures of the disease (Flume et al., 2012). This is in contrast to the dramatic improvement of G551D-CFTR patients upon treatment with VX-770 (Ramsey et al., 2011). The G551D-CFTR protein traffics normally to the cell surface and is thought to have a typical Wt residence time, but lacks any CFTR channel function. VX-770 increases the channel open probability of normally trafficked Wt-, G551D-, and F508del-CFTR at the cell surface (Van Goor et al., 2009). Biosynthetic rescue of sufficient amounts of CFTR and normal cell surface residence time are clearly critical to patient clinical response.

As described in other chapters of this Special Topic, correction of significant amounts of F508del-CFTR to the cell surface can be mediated by the pure corrector VX-809 (Van Goor et al., 2011). When patients were treated with this molecule alone in clinical trials, there was little improvement in clinical outcomes over the course of the trial (Clancy et al., 2011). This may be due to a lack of significant function of rescued F508del-CFTR, as VX-809 is a pure corrector molecule (Van Goor et al., 2011). Recent reports suggest that dual treatment of patients with the corrector VX-809 and the potent potentiator VX-770 produces at least some improvement in patient outcomes over the course of the trial (Vertex Pharmaceuticals, 2012), though this improvement does not appear to be to near Wt levels. Full peer-reviewed results of this study are eagerly awaited. It is unclear if VX-809-rescued F508del-CFTR has sufficient residence time at the cell surface. A lack of full Wt-CFTR response under conditions of treatment with both the corrector VX-809 and the potentiator VX-770 suggests the need to address the remaining defect of cell surface residence time in biosynthetically rescued F508del-CFTR.

In our view, correctors should be highly specific for CFTR to avoid off target effects and work via direct binding to the protein to restore proper Wt-like folding. They must correct both inter- and intra-domain folding defects induced by deletion of F508 in NBD1. This would permit proper biosynthetic processing, including typical post-translational modification to that observed in Wt-CFTR, which should result in normal trafficking to the cell surface and proper activity at that location. An F508del-CFTR molecule that has undergone folding close to the conformation seen in Wt would be expected to possess high levels of properly regulated channel activity, and would not be recognized as aberrant protein that is prematurely removed from the cell surface. If a small molecule is not CFTR-specific, not only would it have potentially toxic effects on other proteins and systems that could render them unsuitable for sustained patient use, they could be rapidly removed from the cell via the activity of the Pgp drug pump (Loo et al., 2012).

The term corrector efficacy "ceiling" has been used to describe a theoretical maximal amount of correction that may be afforded to F508del-CFTR, and the concern is whether interventions may be sufficient to restore the activity of the protein to a level that mitigates the most severe clinical symptoms of the disease (Mendoza et al., 2012; Rabeh et al., 2012). It appears that both the folding of NBD1 and its interaction with the remainder of the protein via ICL4 are severely altered when F508 is deleted, resulting in more than one defect that must be corrected. Evidence suggests that correctors that focus on repair of a single one of these defects will be only weakly effective in correcting disease (Mendoza et al., 2012; Rabeh et al., 2012). Perhaps multiple corrector molecules will be required to correct each individual folding defect arising from this mutation. In support of this concept, certain secondary site mutations on the F508del background suppress the F508del mutation (Thibodeau et al., 2010), and other secondary site mutations in conjunction with corrector treatment result in much higher levels of biosynthetic rescue than corrector alone (Yu et al., 2011). There may indeed be a small molecule that can correct these multiple defects, or we may actually reach a ceiling beyond which we cannot further correct CFTR biosynthetic trafficking to the cell surface. In our view the best way to overcome any corrector efficacy ceiling would be to develop a compound that rescues folding as much as possible and simultaneously promotes maximal surface stability of the rescued protein, while it maximizes the regulated channel activity of that surface-targeted protein. The combined effect may be sufficient to overcome CF symptoms. Estimates vary regarding how much CFTR must be rescued (Noone et al., 2000; McKone et al., 2003; Pedemonte et al., 2005) to give normal function, and this would certainly be influenced by the levels of activity of that protein and its residence time at the cell surface, however as heterozygotes are unaffected by disease, a total of 50% restoration of CFTR activity mediated by an aggregate correction-potentiation-surface stabilization mechanism seems to be a desirable target.

Vertex Pharmaceuticals is taking a strategy whereby patients would be treated with two molecules: a pure corrector such as VX-809 to target the protein to the cell surface, and a VX-770, a pure potentiator to increase the activity of the deficient protein at the cell surface. While this strategy appears to be showing promise

clinically (Vertex Pharmaceuticals, 2012), we feel the approach is not ideal. Treatment with two molecules gives rise to possible drug interactions and potential increased toxicity issues. It remains to be seen whether VX-809 or VX-770 are sufficient to promote Wt levels of membrane surface stability. Indeed patients may be required to take a third treatment that enhances CFTR cell surface residence time, while not adversely interacting with either of the other two drugs or binding to their binding sites on the protein.

The development of a combined corrector-potentiator-membrane stabilizer molecule would: (1) result in combined repair of all of these defects, which would surpass a "ceiling" for each individual component and greatly improve overall clinical outcomes, (2) reduce drug interactions and toxicity for a combined single treatment versus administration of two to three separate drugs, (3) allow targeting of the single molecule to one target, which would be enhanced over attempts to target three separate drugs to nearby targets on the same protein, and (4) reduce development costs for a single drug versus producing three separate drugs.

SECOND GENERATION SCREENS: BETTER SCREENS WILL FIND BETTER COMPOUNDS

Cell-based screens have been the most successful approach thus far to identify and develop small molecules for the treatment of CFTR mutations (Van Goor et al., 2006, 2009, 2011). These models however typically employ over-expression systems of non-patient derived cells with an endpoint changes in anion conductance or membrane potential as the readout. These systems are anticipated to be highly selective for non-specific compounds, such as molecules that increase protein expression, decrease ER quality control, or even molecules that have direct effects on competing ion channels. Temperature rescuing mutant CFTR followed by acute treatment has been used to identify potentiators while longer treatments with small molecules followed by activity measurements are used to discover corrector molecules. These screens have been effective in identifying potentiators and weak correctors of CFTR trafficking, primarily first generation molecules that may at least partially inhibit CFTR function. Identification of more advanced, second generation small molecules that correct trafficking significantly, potentiate function and maintain cell surface residency will require new methods of screening.

There is significant patient-to-patient variability in disease severity and clinical progression of CF which is not accounted for solely by the associated CFTR genotypic background (Hamosh and Corey, 1993; Li et al., 2011). Gene modifiers are thought to contribute to patient-to-patient variability in disease severity (Wright et al., 2011), and would be anticipated to result in varied response to treatment by small molecules. This variability needs to be taken into account when developing treatments for disease. One can anticipate the future use of patient derived stem cells to produce differentiated lung or other organ cells to test the efficacy of various treatments in a particular patient background before applying the most effective to the patient. Currently, patient derived differentiated cells sourced from explant lung tissue following transplantation are a valuable tool to test the efficacy of small molecules in different genetic backgrounds.

To identify the most effective small molecules with features of correctors, potentiators and small molecules that improve the cell surface stability of mutant CFTR, new approaches will be needed to combine screens for each of these functions. The most clinically useful small molecules will bind directly to mutant CFTR and thus new methods of screening should monitor for direct binding of small molecules to the protein. The subset of small molecules from a library that bind to CFTR with high affinity could then be screened for molecules that correct, potentiate, and enhance surface stability of CFTR in more traditional assays, including evaluation with patient derived differentiated cell systems, where molecules that primarily target quality control machinery or other ion channels would already be selected against in the initial screen.

SUMMARY AND FUTURE OUTLOOK

F508del-CFTR is the most common cystic fibrosis causing mutation, leading to protein misfolding and aberrant trafficking from the ER to Golgi, resulting in a lack of functional expression on the cell surface. As a therapeutic approach, several small molecule correctors have been shown to repair structural defects by binding specifically to F508del-CFTR to improve folding and assembly, and enhance trafficking and expression on the plasma membrane. In addition to these characteristics, such compounds must also stabilize the mutant protein on the cell surface by preventing its unfolding, and further potentiate channel activity. Drug discovery efforts have identified few promising corrector compounds, such as VX-809, which facilitate correction of F508del-CFTR conformation, thereby increasing forward trafficking of the mutant protein; recent clinical trials have also had encouraging results with these therapies. Future therapeutic approaches may require a combination of drugs to repair the aforementioned defects in order to achieve significant clinical outcomes. Alternatively, an ideal pharmacological intervention would involve a single therapeutic small molecule which can correct the structural and functional defects simultaneously.

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