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Improving Cell Surface Functional Expression of ΔF508 CFTR: A Quest for Therapeutic Targets

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1. Introduction

Cystic fibrosis (CF) is largely a protein misfolding disease. The deletion of a phenylalanine at residue 508 (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR) accounts for 70% of all disease-causing alleles and is present in at least one copy in 90% of CF patients (Kerem et al., 1989). The ΔF508 mutation impairs the conformational maturation of nascent CFTR (Lukacs et al., 1994), and arrests it in an early folding intermediate (Zhang et al., 1998). As a result, the mutant CFTR is retained in the endoplasmic reticulum (ER) (Cheng et al., 1990) in a chaperone-bound state (Yang et al., 1993), The ER-accumulated mutant CFTR fails to efficiently couple to the coatomer complex II (COPII) ER export machinery (Wang et al., 2004), and is degraded by the ubiquitin proteasome system through the ER-associated degradation (ERAD) pathway (Jensen et al., 1995; Ward et al., 1995), leading to loss of CFTR function at the cell surface.

The folding defect of ΔF508 CFTR appears kinetic in nature (Qu et al., 1997). A small fraction of ΔF508 CFTR is able to exit the ER but the escaped mutant protein is not stable at the cell periphery and is rapidly cleared through lysosomal degradation (Lukacs et al., 1993). This second defect further reduces the cell surface localization of this mutant CFTR. Aside from localization defect, the ΔF508 mutation also impairs the channel gating of CFTR, leading to reduced open probability (Dalemans et al., 1991). The threefold defect of ΔF508 CFTR stems from its defective conformation, and impairs the CFTR functional expression at the cell surface, leading to severe clinical phenotype. Given the autosomal recessive inheritance of the disease, improving plasma membrane functional expression of ΔF508 CFTR will benefit the vast majority of CF patients (Gelman & Kopito, 2002).

Numerous research efforts have been made to improve ΔF508 CFTR cell surface functional expression, including elevating its expression, reducing its degradation, enhancing the efficiency of its maturation, increasing its post-ER stability and improving its channel gating. Restoring ΔF508 CFTR conformation will potentially improve its ER folding, its cell surface stability and its channel gating, leading to efficient ΔF508 CFTR rescue. In this chapter, multiple approaches for ΔF508 CFTR rescue will be reviewed, and their advantages as well as limitations will be discussed.

2. Overview of CFTR biogenesis, quality control and exocytic trafficking

CFTR is a member of the ATP-binding cassette (ABC) transporter family, and is composed of two homologous modules each containing a membrane spanning domain (MSD) followed by a nucleotide-binding domain (NBD) (Riordan et al., 1989) (Fig. 1). CFTR is unique in that it has an unstructured regulatory (R) domain inserted between the two homologous modules (Fig. 1). CFTR has two N-linked glycosylation sites on the fourth extracellular loop (Fig. 1). In the ER, the newly synthesized CFTR acquires core-glycosylation at these two sites. Upon transport to the Golgi, the core-glycosylation is processed into the Golgi-specific complex glycosylation, leading to an up-shift in its apparent molecular weight. This difference in processing provides an important means of discriminating the ER-localized, immature CFTR (band B) and the Golgi-processed, mature form (band C). At the steady state, the majority of CFTR is in its mature form. As ΔF508 CFTR is unable to exit the ER, it largely exists in band B. All the major functional domains of CFTR reside on the cytoplasmic side of membrane. Therefore, chaperone-mediated folding events in the cytoplasm play an important role in CFTR maturation and quality control (Yang et al., 1993; Loo et al., 1998; Meacham et al., 1999; Wang et al., 2006).

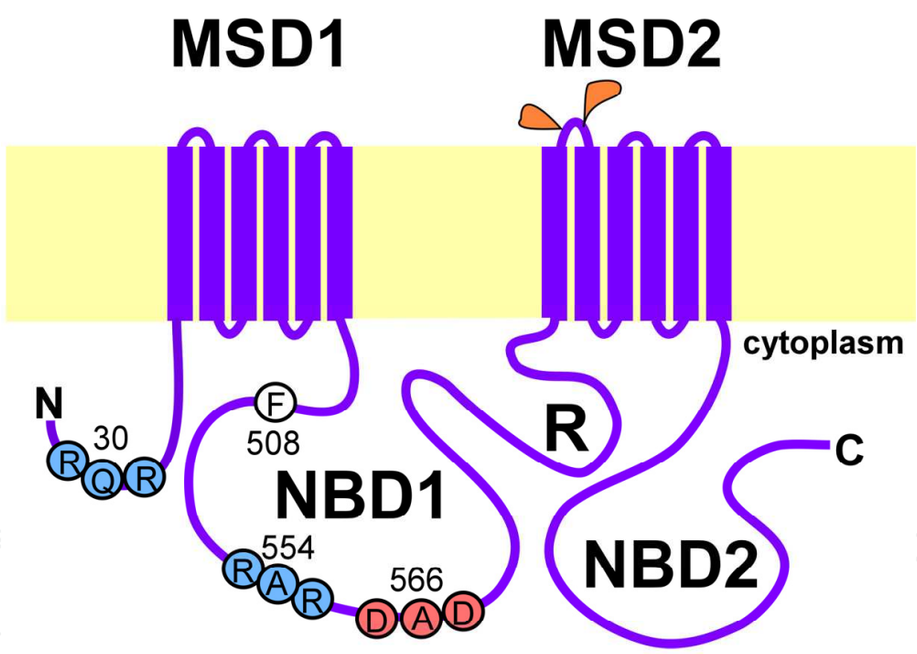


Fig. 1. Domain structure and putative sorting signals of CFTR.

2.1 CFTR de novo folding in the ER

CFTR is synthesized on the ER membrane. Domain folding occurs largely co-translationally (Kleizen et al., 2005). However, further conformational maturation is necessary to form a fully assembled molecule competent for passing the ER quality control and coupling to COPII for ER export (Zhang et al., 1998; Wang et al., 2004). The F508 residue resides in NBD1 (Fig. 1). The ΔF508-induced misfolding of CFTR starts during translation immediately after the NBD1 emerges from the ribosome (Hoelen et al., 2010). This conformational defect

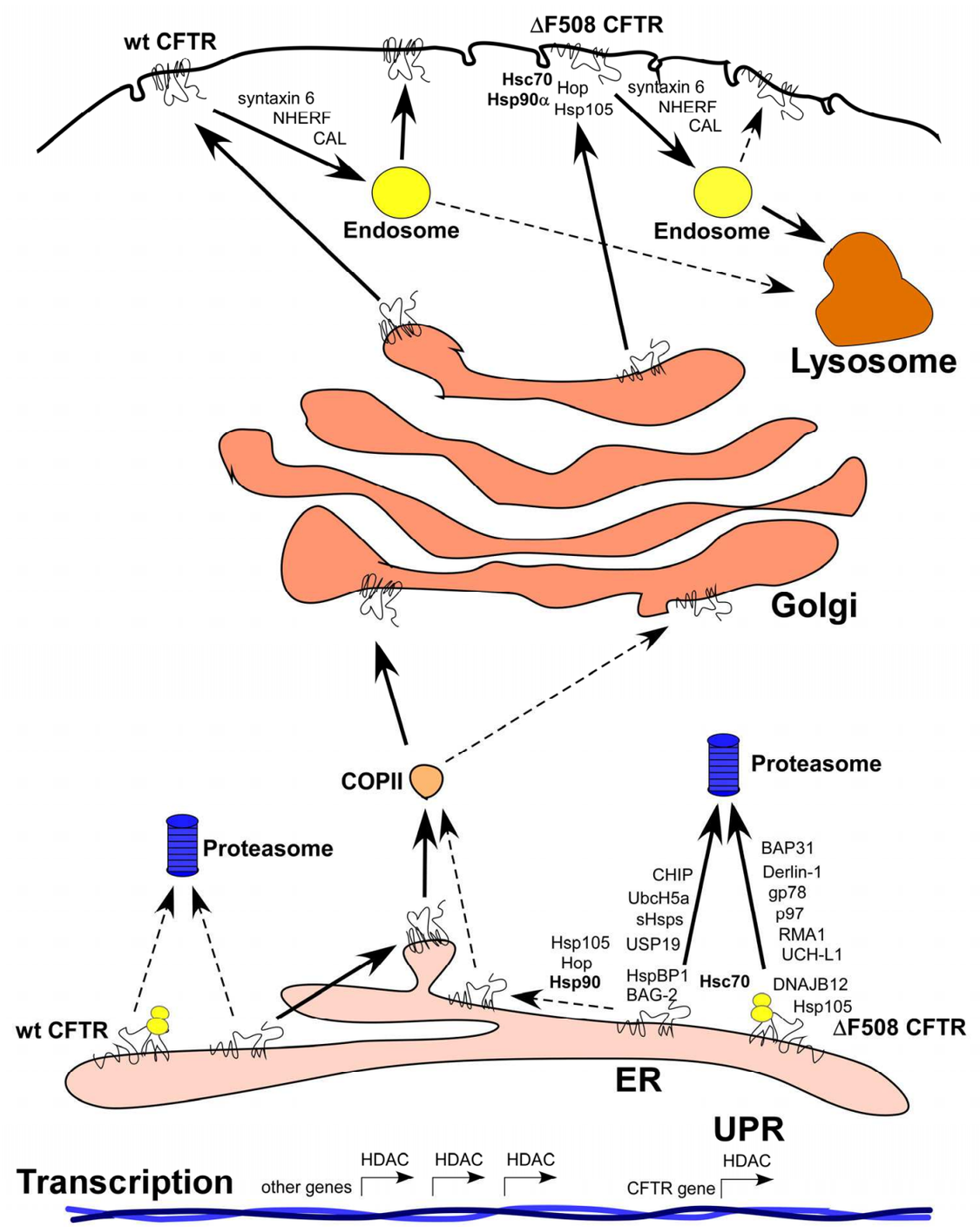
originates in NBD1 but spread throughout the whole molecule through domain-domain interactions, leading to a global conformation defect (Du et al., 2005; Du & Lukacs, 2009; Roy et al., 2010). Restoring wild-type-like global conformation is required for ΔF508 CFTR to pass the quality control and egress from the ER (Roy et al., 2010). Second site mutations in NBD1 have been identified that suppress the ΔF508 processing defect (Teem et al., 1993), and at least some of such suppressing mutations can act co-translationally on the NBD1 misfolding (Hoelen et al., 2010). Therefore, the de novo folding of ΔF508 CFTR at both co-translational and post-translational levels can be targeted for its rescue.

2.2 CFTR quality control in the ER

Newly synthesized CFTR undergo quality control before it can exit the ER. ER quality control starts even before CFTR is fully translated (Fig. 2). A membrane-associated ubiquitin ligase complex containing the E3 RMA1, the E2 Ubc6e and Derlin-1 mediates CFTR co-translational quality control (Sun et al., 2006; Younger et al., 2006). BAP31, an integral membrane protein that associates with Derlin-1 as well as the amino terminus of CFTR, promotes ΔF508 CFTR retrotranslocation from the ER and its subsequent degradation by the cytoplasmic 26S proteasome (B. Wang et al., 2008). P97/valosin-containing protein interacts with gp78/autocrine motility factor receptor in coupling CFTR ubiquitination to its retrotranslocation and proteasome degradation (Carlson et al., 2006; Vij et al., 2006). Interestingly, gp78 was found to cooperate with RMA1 in the ERAD of ΔF508 CFTR (Morito et al., 2008). Moreover, ubiquitin C-terminal hydrolase-L1 (UCH-L1) protects CFTR from co-translational ERAD (Henderson et al., 2010). This co-translational quality control of CFTR appears to be regulated by cytoplasmic Hsc70 as DNAJB12 was recently found to cooperate with Hsc70 and RMA1 in ΔF508 CFTR degradation (Grove et al., 2011). Consistent with this, we found that Hsp105, a nucleotide exchange factor (NEF) for Hsc70, promotes co-translational ERAD of CFTR (Saxena et al., 2011a).

A second ER quality control step occurs largely post-translationally, which is mediated through Hsc70 and cochaperone CHIP (Meacham et al., 2001) (Fig. 2). CHIP functions as a scaffold for the formation of multi-subunit E3 ubiquitin ligase for the post-translational ERAD of CFTR, and such degradation activity is also dependent upon Hsc70, Hdj-2 and the E2 UbcH5a (Younger et al., 2004). Interestingly, HspBP1 and BAG-2, two other NEFs for Hsc70, inhibits the CHIP-mediated post-translational ERAD of CFTR (Alberti et al., 2004; Arndt et al., 2005), suggesting a dual role for Hsc70 in regulating co-translational and post-translational ERAD of CFTR.

Nevertheless, inhibiting CFTR ERAD is not sufficient for ΔF508 CFTR to efficiently exit the ER (Jensen et al., 1995; Pagant et al., 2007). Obviously, another quality control system is responsible for the retention of the foldable pool of ΔF508 CFTR in the ER, the mechanism of which is less clear. We recently showed that the ER exit code and domain conformation both contribute significantly to the exportability of CFTR (Roy et al., 2010). Therefore, chaperone association and/or ER exit code presentation might be two important factors for this last checkpoint of the ER quality control of CFTR. A better understanding of its mechanism will lead to much greater improvement in ΔF508 CFTR maturation.



Shown are the transcription, synthesis, folding and quality control of wild-type and ΔF508 CFTR with a great number of regulators at both the ER and peripheral levels, which are potential molecular targets for ΔF508 CFTR rescue. Complete arrows indicate major pathways, and broken arrows denote minor pathways. Enhancement of some of the minor pathways for ΔF508 CFTR can effectively promote its rescue.

Fig. 2. Regulation of CFTR biogenesis and degradation.

2.3 ER-to-Golgi sorting signals within CFTR

Exit of proteins from the ER requires specific sorting signals on the cytoplasmic side of the ER membrane which is recognizable by the cargo selection complex (Sec23/24) of COPII (Aridor et al., 1998; Nishimura et al., 1999; Miller et al., 2002). A di-acidic ER exit motif (DAD) was identified in the NBD1 of CFTR (Fig. 1), and substitution of the second acidic residue (leading to DAA) abolishes CFTR association with Sec24 and dramatically reduces the export efficiency of CFTR (Wang et al., 2004). The ΔF508 mutation also reduces CFTR association with Sec24 (Wang et al., 2004) but the underlying mechanism might be different from the DAA mutant. Using in situ limited proteolysis to probe the domain conformation of CFTR (Zhang et al., 1998), we showed that the DAA mutant has similar domain conformation as wild-type CFTR despite its inability to efficiently exit the ER (Roy et al., 2010). This is in stark contrast to ΔF508 CFTR which displays global conformational defects including NBD1 (Du et al., 2005; Du & Lukacs, 2009; Roy et al., 2010). Furthermore, DAA CFTR displays lower chaperone association and higher post-ER stability when compared with ΔF508 CFTR (Roy et al., 2010). Therefore, the conformational defects in ΔF508 CFTR contribute significantly to its misprocessing.

ER retention/retrieval signals have been found in the cytoplasmic domains of multiple transmembrane cargo proteins (Nilsson et al., 1989; Zerangue et al., 1999). An RXR ER retention/retrieval signal serves as a quality control check point for the assembly of oligomeric cargo proteins in the ER (Zerangue et al., 1999; Margeta-Mitrovic et al., 2000). The RXR signals are exposed in individual subunits or in incompletely assembled oligomers but are concealed only when the proper assembly of the oligomer is achieved. This mechanism prevents the cell surface expression of improperly assembled cargo molecules. Multiple RXR motifs have been identified in the cytoplasmic domains of CFTR (Fig. 1), and the replacement of key arginine residues results in ΔF508 CFTR rescue, suggesting that the RXR motifs might contribute to the ER retention/retrieval of ΔF508 CFTR (Chang et al., 1999). It is proposed that such RXR motifs are shielded by domain-domain interactions in wild-type CFTR but become exposed when the F508 is deleted (Kim Chiaw et al., 2009). In fact, peptides designed to mimic such a sorting motif were found to functionally rescue ΔF508 CFTR (Kim Chiaw et al., 2009). As the key RXR motif in NBD1 contributes significantly to ΔF508 CFTR global conformation (Qu et al., 1997; Hegedus et al., 2006; Roy et al., 2010), it is unclear if the RXR-mimetics rescue ΔF508 CFTR by influencing ΔF508 CFTR conformation. Determining whether the RXR-mimetics are able to bind to the RXR sorting receptor or whether they block the retention/retrieval of other RXR-containing cargo molecules will provide a definitive answer.

3. Defining ΔF508 conformational defects

F508 resides in NBD1 (Fig. 1), and therefore the deletion of this residue should naturally affect the conformation of NBD1. Early in vitro studies using purified NBD1 revealed a kinetic folding defect in ΔF508 NBD1 (Qu & Thomas, 1996; Qu et al., 1997). However, the crystal structure of ΔF508 NBD1 revealed no major conformational change from the wild-type domain (Lewis et al., 2005). In the mean time, it was found that ΔF508 mutation causes major conformation changes in NBD2 (Du et al., 2005), highlighting the importance of domain-domain interactions in ΔF508 misfolding. This notion was strengthened by the

finding that F508 side chain contributes significantly to NBD1 folding in the context of full-length CFTR (Thibodeau et al., 2005), and by the finding that F508 residue mediates the contact between NBD1 and cytoplasmic loop 4 (CL4) in MSD2 (Serohijos et al., 2008a). Therefore, deletion of F508 triggers a global conformational change in CFTR, leading to misprocessing (Du & Lukacs, 2009).

The apparent lack of a detectable NBD1 conformational change as a result of the ΔF508 mutation remains an enigma as how can the ΔF508 mutation trigger such a profound global conformational change without significantly impacting NBD1 conformation in the first place? The finding that some of the solubilization mutations included in ΔF508 NBD1 for crystallography studies actually rescue the ΔF508 processing defect in the context of full-length CFTR reopened this question (Pissarra et al., 2008). Another twist in our understanding of the impact of F508 deletion on NBD1 conformation came from the finding that the removal of the regulatory insert (RI), a 32-residue segment within NBD1 that is unique to CFTR but not shared by the NBD1's of other ABC transporters, renders ΔF508 NBD1 soluble, dimer-forming and displaying wild-type-like conformation (Atwell et al., 2010). Another study shows that, in the context of full-length CFTR protein, removal of the RI restores maturation, stability and function of ΔF508 CFTR, suggesting that the RI contributes significantly to ΔF508 misfolding in NBD1(Aleksandrov et al., 2010).

Using in situ limited proteolysis, we identified a definite conformational change within NBD1 as a result of ΔF508 mutation (Roy et al., 2010). The ΔF508 NBD1 conformation, like the conformation of other domains of ΔF508 CFTR, resembles the conformation of an earlier folding intermediate of wild-type CFTR (Zhang et al., 1998; Roy et al., 2010). Furthermore, rescue of ΔF508 CFTR using low temperature or R555K substitution leads to NBD1 as well as global conformational reversion, suggesting that conformational correction is prerequisite for the rescue of the folding and export defects of ΔF508 CFTR (Roy et al., 2010). Using crystallography and hydrogen/deuterium exchange mass spectrometry, Lewis, et al. showed that ΔF508 mutation increases the exposure of the 509-511 loop and increases the dynamics in its vicinity (Lewis et al., 2010). Consistent with the above, a conformational change in ΔF508 NBD1 was observed using a cysteine-labelling technique, and such conformational change is reversed by second site mutations in NBD1 (He et al., 2010). Interestingly, the second site mutations also restore the interactions between NBD1 and its contacting domains (He et al., 2010). Combination of G550E, R553M and R555K suppressor mutations in NBD1 produces a dramatic increase in ΔF508 CFTR processing, and this is accompanied by the enhanced folding of ΔF508 NBD1 both in isolation and in the context of full-length CFTR (Thibodeau et al., 2010). An interesting finding is that while NBD2 is not required for CFTR processing (Pollet et al., 2000), it contributes to ΔF508 CFTR rescue by second site mutations as well as by low temperature (Du & Lukacs, 2009; Cheng et al., 2010). Furthermore, the rescue of ΔF508 CFTR by suppressor mutations requires a continuous full-length CFTR peptide (Cheng et al., 2010), suggesting a role for peptide backbone tension in ΔF508 CFTR rescue (Thibodeau et al., 2005).

Taken together, ΔF508 mutation causes increased exposure of the 509-511 loop in NBD1 and increases its dynamics. These changes not only alter the conformation of NBD1, but through NBD1's interface with CL4 and NBD2, alter the conformation of other domains, leading to global conformational change. Second site mutations within NBD1 can partially correct the

ΔF508 NBD1 conformational defect, which spread to other domains through domain-domain interactions, leading to partial restoration of global conformation as well as processing. Conformation repair is at the heart of ΔF508 correction.

4. Elevating ΔF508 CFTR expression

The severe reduction in ΔF508 CFTR cell surface functional expression results from its defective export, reduced peripheral stability, and subnormal channel gating. Nevertheless, a small fraction of the mutant CFTR can leak from the ER and make its way to cell surface. One simple approach to enhance ΔF508 CFTR cell surface localization is to increase its expression. This can be achieved in cells heterologously expressing ΔF508 CFTR under the control of metallothionein promoter by treatment with sodium butyrate (Cheng et al., 1995). In CF airway epithelial cells, 4-phenylburyrate, a histone deacetylase (HDAC) inhibitor dramatically increases the expression of ΔF508 CFTR at the protein level (Rubenstein et al., 1997). Recently, a group of other HDAC inhibitors including Trichostatin A, suberoylanilide hydroxamic acid (SAHA) and Scriptaid were found to potently increase ΔF508 CFTR transcription in CFBE41o- cells (Hutt et al., 2010).

Interestingly, over-accumulation of ΔF508 CFTR in the ER induces the unfolded protein response (UPR) (Gomes-Alves et al., 2010), and induction of UPR inhibits CFTR endogenous transcription (Rab et al., 2007). The UPR-induced CFTR transcriptional repression is mediated through the transcription factor ATF6, and both DNA methylation and histone deacetylation contribute to this process (Bartoszewski et al., 2008). Therefore, there is a limit to which the transcription of endogenous ΔF508 CFTR can be increased but HDAC inhibitors may potentially alleviate the UPR-induced CFTR transcriptional repression (Fig. 2).

The expression of ΔF508 CFTR can also be regulated at the post-transcriptional level. A recent study revealed that the synonymous codon change of I507 in the ΔF508 allele can cause mRNA misfolding, leading to reduced rate of translation and/or impaired co-translational folding of ΔF508 CFTR (Bartoszewski et al., 2010). Therefore, codon-dependent mRNA folding represents a new mechanism by which ΔF508 CFTR expression can be regulated. Although it is not realistic to change the nucleotide sequence of ΔF508 CFTR in CF patients, identification of this novel mechanism opens up new opportunities for therapeutic intervention at the level of mRNA processing, folding, and stability.

5. Reducing ΔF508 CFTR ERAD

The vast majority of ΔF508 CFTR synthesized in the cells is degraded through the ERAD pathway (Jensen et al., 1995; Ward et al., 1995). Inhibition of ERAD will certainly increase the steady state level of ΔF508 CFTR in the ER and subsequently increase its cell surface localization (Fig. 2). Significant advance in understanding the mechanism of ERAD of ΔF508 CFTR has been achieved during the past 16 years. Hsc70 has been found to regulate both the co-translational and post-translational ERAD of ΔF508 CFTR with two distinct sets of cochaperones (Meacham et al., 2001; Zhang et al., 2001; Alberti et al., 2004; Arndt et al., 2005; Grove et al., 2011; Saxena et al., 2011a). While the functional relationship between the two remains unclear, multiple cochaperones, such as CHIP (Meacham et al., 2001), HspBP1

(Alberti et al., 2004), BAG-2 (Arndt et al., 2005), Hdj-2 (Younger et al., 2004), DNAJB12 (Grove et al., 2011) and Hsp105 (Saxena et al., 2011a) may be targeted for increasing the steady state levels of ΔF508 CFTR. Moreover, 4-phenylbutyrate, which rescues ΔF508 CFTR (Rubenstein et al., 1997), was found to reduce the expression level of Hsc70, subsequently decreases its association with ΔF508 CFTR, and therefore inhibits the ERAD of ΔF508 CFTR (Rubenstein & Zeitlin, 2000). More recently, a soluble sulfogalactosyl ceramide mimic that inhibits the Hsp40-activated Hsc70 ATPase activity, promotes the rescue of ΔF508 CFTR from ERAD (Park et al., 2009). In addition to Hsc70, small heat-shock proteins (sHsps) preferentially associate with ΔF508 CFTR and promote its ERAD (Ahner et al., 2007). It is believed that small heat-shock proteins bind to misfolded ΔF508 CFTR, prevent its aggregation and maintain its solubility during the ERAD (Ahner et al., 2007).

ERAD components such as RMA1 (Younger et al., 2006), gp78 (Morito et al., 2008), Derlin-1 (Sun et al., 2006; Younger et al., 2006), BAP31(B. Wang et al., 2008) and p97 (Carlson et al., 2006; Vij et al., 2006) can also be targeted. Although not essential for ERAD of CFTR (Carlson et al., 2006), interference of p97 expression in CF airway epithelial cells increases the steady state levels of ΔF508 CFTR in bands B and C, and enhances the CFTR-mediated chloride conductance across the plasma membrane (Vij et al., 2006). Interestingly, this effect is accompanied by reduction in interleukin-8 level which might alleviate the CF-associated airway inflammation (Vij et al., 2006). Other regulators of the p97-gp78 complex have been identified, which also influence the steady state level of ΔF508 CFTR (Nagahama et al., 2009; Ballar et al., 2010). Recently, ubiquitin-specific protease 19 (USP19), an ER-localized, membrane-anchored deubiquitinating enzyme, was shown to rescue ΔF508 CFTR from ERAD (Hassink et al., 2009), suggesting that deubiquitinating enzymes are another class of viable targets for rescuing ΔF508 CFTR (Fig. 2).

6. Enhancing ΔF508 CFTR maturation

Despite its obvious importance in rescuing ΔF508 CFTR, relatively little is known concerning how to improve the maturation of ΔF508 CFTR in the ER. The major reason is that ΔF508 CFTR hardly matures if at all at physiological temperature. However, at reduced temperature, ΔF508 CFTR does achieve conformational maturation much more efficiently, leading to greatly enhanced functional expression at the cell surface (Denning et al., 1992). Interestingly, such a temperature-sensitive phenotype is cell-dependent, suggesting that cellular machinery plays an essential role in the process (X. Wang et al., 2008). We found that the increased conformational stability provided by low temperature combines with chaperone actions in facilitating ΔF508 CFTR maturation at reduced temperature (Roy et al., 2010). Therefore, the temperature-dependent maturation of ΔF508 CFTR serves as an excellent model system in understanding the role of the cellular chaperone machinery in the forward folding of ΔF508 CFTR.

Mild heat shock greatly potentiates the temperature rescue of ΔF508 CFTR, and this is dependent upon transcription, suggesting that the upregulation of heat inducible chaperones promotes ΔF508 CFTR maturation (X. Wang et al., 2008). Using a series of chaperone- or cochaperone-deficient cell lines, we demonstrate that an Hsp70-Hsp90 chaperone network operates on the cytoplasmic face of the ER membrane facilitating the maturation of ΔF508 CFTR at reduced temperature. Cochaperone Hop, which physically

and functionally links Hsp70 and Hsp90 through its multiple tetratricopeptide repeat (TPR) domains, is essential for the temperature-dependent maturation of ΔF508 CFTR, and Hsp105 is an integral player in the system (Saxena et al., 2011b). We also found that Hsc70, Hsp90β, Hop, Hsp105 and Hdj-2 are functionally linked during the temperature rescue of ΔF508 CFTR. Depletion of Hsp90β, Hop or Hsp105 also reciprocally reduces some or all of other chaperone components (Saxena et al., 2011b). It is highly likely that these folding components, and perhaps other yet unidentified chaperones or cochaperones, form a functionally organized chaperone network on the cytoplasmic side of the ER membrane, facilitating the conformational maturation of ΔF508 CFTR at reduced temperature. Given a clear role for Hsp90 in wild-type CFTR maturation at physiological temperature (Loo et al., 1998), we believe such a cytoplasmic chaperone network functions in the cell under physiological conditions. While its effect on ΔF508 CFTR maturation is more pronounced at reduced temperature, it should also impact ΔF508 CFTR maturation at the physiological temperature. Consistent with this prediction, overexpressing Hsp105 promotes ΔF508 CFTR processing at both the reduced and physiological temperatures (Saxena et al., 2011a). An in-depth analysis of this process will reveal novel molecular targets that promote the maturation of ΔF508 CFTR (Fig. 2).

Another approach to enhance ΔF508 CFTR maturation is through transcomplementation (Cormet-Boyaka et al., 2004). Such rescue requires co-expression of a sizeable segment of CFTR that contains wild-type sequence corresponding to the region where F508 is located. Such transcomplementation does not result in changes in Hsc70 association but is believed to improve ΔF508 CFTR forward folding through intra- and/or inter-molecular domain-domain interactions. A related but distinct approach to promote ΔF508 CFTR maturation is to co-express a fragment of ΔF508 CFTR containing NBD1 and R domains (Sun et al., 2008). This mutant fragment of CFTR can actually sequester key chaperone components from the endogenous ΔF508 CFTR and lead to its rescue. Moreover, co-expressing an N-terminal truncated CFTR mutant (Δ264) can not only transcomplement ΔF508 CFTR but also dramatically increases the protein expression levels of both wild-type and ΔF508 CFTR (Cebotaru et al., 2008). As the Δ264 mutant CFTR associates with VCP and HDAC6, two components involved in retrotranslocation of proteins from the ER, and is more efficiently degraded by the proteasome than ΔF508 CFTR, high level expression of this mutant may interfere with ΔF508 CFTR ERAD and hence increase its steady state level. Taken together, co-expression of CFTR fragments might rescue ΔF508 CFTR by improving its folding, helping it escape ER quality control and protecting it from ERAD. As these fragments have much lower molecular weight than full-length CFTR, they can be used as potential agents for CF gene therapy.

7. Increasing ΔF508 CFTR peripheral stability

The ΔF508 CFTR has reduced conformational stability in post-ER compartments and therefore turns over rapidly at the cell periphery (Lukacs et al., 1993; Sharma et al., 2001; Sharma et al., 2004). Increasing ΔF508 CFTR half-life at cell periphery is an important strategy for effective rescue of ΔF508 CFTR. CAL, a Golgi-associated, PDZ domain-containing protein that binds to the C-terminus of CFTR, reduces the half-life of CFTR at the cell surface (Cheng et al., 2002). RNA interference of endogenous CAL in CF airway

epithelial cells increases plasma membrane expression of ΔF508 CFTR and enhances transepithelial chloride current (Wolde et al., 2007). The Na^+/H^+ exchanger regulatory factor (NHERF), a subplasma membrane PDZ domain protein, competes with CAL in associating with CFTR and promotes its plasma membrane localization (Cheng et al., 2002). Knockdown of NHERF1 promotes the degradation of temperature-rescued ΔF508 CFTR at the cell surface of human airway epithelial cells (Kwon et al., 2007). Expression of dominant-negative dynamin 2 mutant K44A increases CFTR cell surface expression, and counteracts the effect of CAL overexpression on CFTR cell surface stability (Cheng et al., 2004). SNARE protein syntaxin 6 binds to CAL and reduces CFTR cell surface stability in a CAL-dependent manner (Cheng et al., 2010). Therefore, CAL and its functional partners are viable molecular targets for increasing cell surface stability of ΔF508 CFTR (Fig. 2).

Cytoplasmic chaperone Hsc70 was shown to mediate the uncoating of clathrin-coated vesicles (Schmid & Rothman, 1985; Chappell et al., 1986) and hence regulates the peripheral trafficking of membrane bound cargo proteins such as CFTR. Recently, a more direct role for cytoplasmic Hsp70-Hsp90 chaperone network in regulating ΔF508 CFTR peripheral quality control was revealed, where Hsc70, Hsp90α, Hop and other chaperone components collaborate with the ubiquitin system in promoting the cell surface degradation of this mutant CFTR (Okiyoneda et al., 2010). This finding uncovers a great number of new potential chaperone targets for regulating cell surface stability of ΔF508 CFTR. However, as the cytoplasmic Hsp70-Hsp90 chaperone network also facilitates the maturation of CFTR in the ER (Loo et al., 1998; Meacham et al., 1999; Wang et al., 2006), a critical balance must be maintained between the two seemingly opposing effects of the Hsp70-Hsp90 chaperone network at the ER and the peripheral levels in order to effectively rescue ΔF508 CFTR (Fig. 2).

Of particular interest, we found that Hsp105 is involved in both processes. At the ER level, Hsp105 facilitates the Hsp70-Hsp90-mediated maturation of ΔF508 CFTR at reduced temperature (Saxena et al., 2011b). At the peripheral level, Hsp105 preferentially associates with the rescued ΔF508 CFTR, and stabilizes it in post-ER compartments (Saxena et al., 2011a). It is currently unclear whether Hsp105 functionally relates to Hsc70, Hop and Hsp90α at the cell periphery or it act on its own. While Hsp105 acts in the same direction as the cytoplasmic Hsp70-Hsp90 network at the ER level, it acts in opposite direction to Hsc70, Hop and Hsp90α at the cell periphery. Understanding these aspects is critical to the effective enhancement of ΔF508 CFTR cell surface functional expression by modulating cytoplasmic chaperone machinery.

8. Improving ΔF508 CFTR channel gating: Potentiator or corrector?

Although the primary defect in ΔF508 CFTR is impaired export (Cheng et al., 1990), it has aberrant channel gating as reflected in reduced channel open probability (Dalemans et al., 1991). Correcting such a defect will also improve the overall cell surface functional expression of ΔF508 CFTR. The G551D substitution in CFTR, a mutation causing severe CF, does not impact its export to plasma membrane but primarily impairs its channel opening (Tsui, 1995; Li et al., 1996). VX-770, a small molecule potentiator (improving channel gating) developed for G551D CFTR by the Vertex Pharmaceuticals Inc., also increases the channel open probability of ΔF508 CFTR (Van Goor et al., 2009). Interestingly, small molecule compound VRT-532

display both corrector (improving maturation) and potentiator activities for ΔF508 CFTR by binding directly to the mutant protein (Wellhauser et al., 2009). Recently, a fragment of a phenylglycine-type potentiator was successfully linked to a fragment of a bithiazole corrector to form a "hybrid" potentiator-corrector molecule, the cleavage of which by intestinal enzymes is able to release separate potentiator and corrector for ΔF508 rescue in vivo (Mills et al., 2010). Using high-throughput screen, multiple small molecules with independent potentiator and corrector activities for ΔF508 CFTR were also identified (Phuan et al., 2011). Using the above approaches, more efficient rescue of ΔF508 CFTR can be achieved.

9. Conformational repair: One stone and three birds

Given that the root cause of CF in the majority of patients lies in the conformational defects of ΔF508 CFTR, repairing its conformational defects will potentially lead to improved export, stability (both in the ER and at the cell periphery) and channel gating. Effective development of novel approaches in conformational repair relies on a thorough understanding of the conformational defects of ΔF508 CFTR and their correction. Given that F508 residue resides in NBD1, NBD1 is a central domain for the understanding of ΔF508 conformational repair. In addition, as domain-domain interactions within CFTR play an important role in altering or maintaining CFTR global conformation (Du et al., 2005; Du & Lukacs, 2009), key interfaces between different domains are also important in CFTR conformational repair (Serohijos et al., 2008a).

An excellent attempt was made early on in screening for suppressor mutations in NBD1 which restores the export of ΔF508 CFTR (Teem et al., 1993). This was made possible by swapping a portion of CFTR NBD1 into yeast *STE6* gene encoding an ABC transporter that delivers α-factor out of the cell which is necessary for mating. When ΔF508 mutation is included into the *STE6*-CFTR chimera, the yeast fails to transport α-factor. Using this system, second site mutations within the CFTR NBD1 portion were identified that rescue ΔF508 CFTR (Teem et al., 1993; Teem et al., 1996). Interestingly, R555K, one of such ΔF508 suppressor mutations, causes a global conformational reversion in ΔF508 CFTR, leading to increased export and enhanced post-ER stability (Roy et al., 2010). R555K, when combined with other rescue subsitutions, improves ΔF508 CFTR conformation and processing (Chang et al., 1999; Hegedus et al., 2006), and significantly increases the open probability of ΔF508 CFTR (Roxo-Rosa et al., 2006). These data support the notion that conformational repair is a highly effective approach for enhancing ΔF508 CFTR cell surface functional expression, ameliorating all three facets of ΔF508 defect.

Another approach for designing NBD1 conformational repair employs molecular dynamics simulation. Molecular dynamics has the advantage over structural biology in that it reveals information on folding kinetics and dynamics. Using this approach, key differences in the distribution of meta-stable intermediates have been identified between wild-type and ΔF508 NBD1, and additional rescue mutations can be designed (Serohijos et al., 2008b). These rescue mutations, if validated experimentally, will significantly advance our understanding of NBD1 folding both alone and in the context of full-length CFTR.

High resolution crystal structure of full-length CFTR is currently unavailable. However, the crystal structures of multiple ABC transporters including the p-glycoprotein have been

solved (Locher et al., 2002; Dawson & Locher, 2006; Aller et al., 2009). Attempts to use these structures as bases for modeling full-length CFTR have provided new insights into the role of F508 residue in domain-domain interactions (Jordan et al., 2008; Loo et al., 2008; Serohijos et al., 2008a; Mornon et al., 2009). These studies, when backed up by biochemical analyses, are an excellent start point to probe ΔF508 global conformational defects and their repair.

10. Large-scale target identification for the rescue of ΔF508 CFTR

Aside from the above mechanism-based identification of therapeutic targets for ΔF508 rescue, several large-scale target identification regimes have been quite successful. The functional follow-up of these studies has yielded and will yield many novel molecular targets.

The first such attempt was to use proteomics to identify CFTR-interacting proteins between wild-type and ΔF508 CFTR (Wang et al., 2006), which revealed, among others, an ER-associated chaperone network facilitating CFTR biogenesis and quality control. In an attempt to gain information on the potential mechanism of ΔF508 chemical rescue by 4-phenylbutyrate, a pharmacoproteomic approach was used to identify changes in protein expression in CF airway epithelial cells in response to 4-phenylbutyrate treatment (Singh et al., 2006). This approach was then followed by a comparison of ΔF508 CFTR-interacting proteins between the chemically rescued (by 4-phenylbutyrate) and genetically repaired (by introducing wild-type CFTR) CF airway epithelial cells (Singh et al., 2008). Protein targets involved in the ERAD, protein folding and inflammatory response have been identified, and proteins that were modulated in the ER as well as on the plasma membrane have been isolated (Singh et al., 2008).

Recently, a high-throughput functional screen was designed to identify proteins that promote the rescue of ΔF508 CFTR (Trzcinska-Daneluti et al., 2009). In this study, 450 different proteins were fused to a chloride-senstive yellow fluorescent protein and were expressed in a ΔF508 CFTR-expressing stable cell line. The cells were screened for their ability to rescue the ΔF508 functional defect at the plasma membrane. Several proteins that are known to rescue ΔF508 CFTR as well as novel target proteins have been identified. Further functional characterization will reveal their usefulness as potential therapeutic targets.

Another excellent approach worth noting is the use of functional small interfering RNA screen to identify proteins that are involved in peripheral quality control of ΔF508 CFTR (Okiyoneda et al., 2010). This approach took advantage of a well developed cell surface ELISA assay measuring CFTR plasma membrane localization, where three HA-tags have been engineered in an extracellular domain of CFTR. The siRNAs targeting a great number of ubiquitin E3 ligases, ESCRT proteins, E2 enzymes and chaperone/cochaperones were introduced into the above cells, and the plasma membrane stability of the rescued ΔF508 CFTR-3HA was quantified. This study led to the identification of an Hsp70-Hsp90 chaperone network facilitating the peripheral quality control of ΔF508 CFTR. Functional followup of these chaperone proteins will not only reveal critical mechanistic information but also uncover yet unidentified molecular targets.

11. Small molecule modulators for ΔF508 CFTR

One of the major strategies for developing effective therapeutics for CF is to identify small molecule compounds that can improve ΔF508 CFTR cell surface functional expression. Using cell-based functional assay for CFTR-mediated chloride conductance combined with high-throughput screening of small molecule compound libraries, multiple CFTR modulators have been identified, affecting ΔF508 CFTR trafficking and/or channel function (Van Goor et al., 2006; Verkman et al., 2006). Once promising scafolds have been identified, structural optimization can be performed to enhance their biological activities, pharmacokinetics, and safety. In fact several of the above compounds are currently in clinical trial for treating CF.

While the functional screening as mentioned above has the benefit of identifying small molecule compounds that improve the aggregate endpoint readout on ΔF508 CFTR cell surface functional expression, the mechanisms by which these compounds do so are unknown. The compounds can either bind directly to CFTR to affect its folding and/or channel gating, or they can bind to other cellular proteins that regulate CFTR biogenesis, cell surface protein-protein interactions, or its degradation. Understanding these mechanistic aspects of a specific CFTR modulator will lead to the design and identification of additional molecular targets and CFTR modulators. This is especially important as only a limited number of efficacious CFTR modulators have been identified through the functional screen. In order to obtain an FDA-approved drug for CF, more of such compounds are desparately needed to feed into the CF drug discovery pipeline.

Recently, new screening strategies have been designed to improve the variety of workable lead compounds. These compounds might not have been identified during the functional screen because they do not provide the above-the-threshold functional readouts. However, if they have special properties that can enhance certain key aspects of ΔF508 CFTR rescue, such compounds can be further engineered or optimized to produce a much greater efficacy in terms of functional rescue of ΔF508 CFTR. A new strategy has been developed where small molecule compound libraries were screened by their ability to improve the plasma membrane localization of ΔF508 CFTR (Carlile et al., 2007).

A conformation-based virtual screen for ΔF508 CFTR modulators represents one step further as it aims at the core defect of ΔF508 CFTR (i.e. abberrant conformation). Recently, one attempt was made by the EPIX Pharmaceuticals Ltd to identify small molecule correctors for ΔF508 CFTR (Kalid et al., 2010). In this study, a total of three potential small molecule binding cavities were identified at a number of domain-domain interfaces of CFTR, and small molecule compounds were screened in silico for their ability to bind to these cavities. The initial hits derived from the virtual screen were then subjected to functional screen, which yielded a ten-fold increase in hit rate as compared to conventional screen regimes.

An alternative to the above high-throughput screening approach is to explore the possibility of using FDA-approved drugs for other conditions or other small molecule compounds that are safe for human use for rescuing ΔF508 CFTR. Sodium 4-phenylbutyrate is approved for clinical use in patients with urea cycle disorders. 4-Phenylbutyrate, like sodium butyrate, is also a transcriptional regulator that inhibits HDAC (Jung, 2001). 4-Phenylbutyrate was shown to rescue ΔF508 CFTR through a number of mechanisms including biosynthesis, folding and

transport (Rubenstein et al., 1997; Rubenstein & Zeitlin, 2000; Choo-Kang & Zeitlin, 2001; Wright et al., 2004; Singh et al., 2006). More recently, SAHA (Vorinostat), an HDAC inhibitor approved by FDA for the treatment of cutaneous T cell lymphoma through epigenetic pathways (Monneret, 2007), was shown to restore cell surface functional expression of ΔF508 CFTR to 28% of wild-type level (Hutt et al., 2010). Doxorubicin (Adriamycin), a cancer chemotherapy agent, increases cell surface functional expression of ΔF508 CFTR through increasing its folding, promoting its chaperone dissociation and inhibiting its ubiquitination (Maitra et al., 2001; Maitra & Hamilton, 2007). Sildenafil (Viagra) was also shown to promote ΔF508 CFTR apical trafficking by unknown mechanism (Dormer et al., 2005). S-Nitrosoglutathione (GSNO), an endogenous bronchodilator (Gaston et al., 1993), was found to increase the expression and maturation of ΔF508 CFTR in airway epithelial cells (Zaman et al., 2006). Interestingly, GSNO was recently found to function at least in part through inhibiting Hop expression (Marozkina et al., 2010), suggesting that small molecules compound can promote ΔF508 CFTR rescue through modulating chaperone machinery.

12. Chaperone environment: A critical but complex part of the equation

Cellular chaperone machinery plays an important role in the synthesis, maturation, quality control of CFTR (Fig. 2). Due to misfolding, ΔF508 CFTR has more extensive association with molecular chaperones (Yang et al., 1993; Jiang et al., 1998; Meacham et al., 1999; Wang et al., 2006; Sun et al., 2008; Roy et al., 2010). Therefore, the impact of chaperone machinery on ΔF508 CFTR is greater than on wild-type CFTR. This notion is further underscored by the recent finding that cytoplasmic Hsp70-Hsp90 chaperone network promotes the peripheral quality control of ΔF508 CFTR (Okiyoneda et al., 2010). Modulating chaperone environment can not only impact the quality control of ΔF508 CFTR at either the ER or the peripheral level but also can dramatically influence its maturation (Loo et al., 1998; Zhang et al., 2002; Saxena et al., 2007; Saxena et al., 2011b).

Heat shock response is a transcriptional program by which cells upregulate the expression of an array of genes including those encoding molecular chaperones to cope with the massive need for protein folding and degradation as a result of elevated temperature or toxic agents (Morimoto et al., 1990). Therefore, conditions or agents that induce heat shock response will up-regulate the cellular chaperone machinery to enhance folding and ERAD of ΔF508 CFTR. Consistent with this finding, mild heat shock dramatically potentiates the temperature-rescue of ΔF508 CFTR (X. Wang et al., 2008). Another cellular response that up-regulate the cellular chaperone machinery is the unfolded protein response (UPR) (Sidrauski et al., 1998). This is particularly relevant to ΔF508 CFTR as over accumulation of this mutant protein in the ER induces such a response, leading to downregulation of CFTR endogenous transcription (Rab et al., 2007; Bartoszewski et al., 2008). Aside from the above two, the inherent variation in the cellular chaperone machinery among different tissues or cell types will also significantly affect the cell surface functional expression of ΔF508 CFTR (Varga et al., 2004; X. Wang et al., 2008; Rowe et al., 2010). Therefore, understanding the functional organization of the chaperone machinery in airway epithelial cells is highly relevant to the development of effective rescue strategies for ΔF508 CFTR.

Certain chemicals such as celastrol can globally influence the cellular chaperone machinery through inducing the heat shock response (Westerheide et al., 2004). Other epigentic

modulators can also influence the expression of mutliple molecular chaperones (Wright et al., 2004; Hutt et al., 2010). Interfering with ER lumenal chaperone activities by depleting the ER calcium stores promotes the escape of ΔF508 CFTR from the ER quality control and enhances its cell surface expression (Egan et al., 2002). Certain small molecule compounds directly modulate the expression or activity of molecular chaperones (Jiang et al., 1998; Loo et al., 1998; Marozkina et al., 2010). Furthermore, small molecule compound can act as chemical chaperones to stabilize the conformation of ΔF508 CFTR, enhancing its cell surface functional expression (Brown et al., 1996; Fischer et al., 2001).

13. Conclusion

The ΔF508 mutation is present in over 90% of CF patients. This mutation impairs the conformational maturation of CFTR leading to defective export, reduced stability and abberrant channel gating. Improving the cell surface functional expression of this mutant CFTR will benefit the vast majority of CF patients. While many approaches can be taken toward this goal, conformational rescue is the most effective, postitively impacting all three molecular defects of ΔF508 CFTR. The ΔF508 CFTR molecule is the most important target for the development of therapeutics. A clear understanding of its biogenesis, quality control and conformation is fundamental. In the cell, the synthesis, folding, quality control, trafficking and degradation of CFTR is dependent upon its interactions with multiple cellular machineries (Fig. 2). Such interactions provide additional opportunites for therapeutic interventions. The cellular protein homeostasis as regulated by the chapeorone machinery provides an important chemical environment for ΔF508 CFTR. Such an enironment is regulated by multiple cellular responses or epigenetic modulators. Understanding the relationship between such cellular environment and ΔF508 CFTR cell surface functional expression will provide additional molecular targets for intervention.

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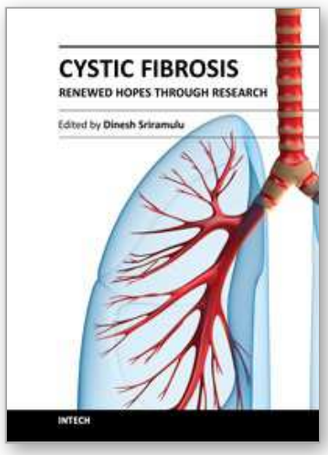

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Living healthy is all one wants, but the genetics behind creation of every human is different. As a curse or human agony, some are born with congenital defects in their menu of the genome. Just one has to live with that! The complexity of cystic fibrosis condition, which is rather a slow-killer, affects various organ systems of the human body complicating further with secondary infections. That's what makes the disease so puzzling for which scientists around the world are trying to understand better and to find a cure. Though they narrowed down to a single target gene, the tentacles of the disease reach many unknown corners of the human body. Decades of scientific research in the field of chronic illnesses like this one surely increased the level of life expectancy. This book is the compilation of interesting chapters contributed by eminent interdisciplinary scientists around the world trying to make the life of cystic fibrosis patients better.

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