Emergent Properties of Proteostasis in Managing Cystic Fibrosis

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Cystic fibrosis (CF) is a consequence of defective recognition of the multimembrane spanning protein cystic fibrosis conductance transmembrane regulator (CFTR) by the protein homeostasis or proteostasis network (PN) (Hutt and Balch (2010)). Like many variant proteins triggering misfolding diseases, mutant CFTR has a complex folding and membrane trafficking itinerary that is managed by the PN to maintain proteome balance and this balance is disrupted in human disease. The biological pathways dictating the folding and function of CFTR in health and disease are being studied by numerous investigators, providing a unique opportunity to begin to understand and therapeutically address the role of the PN in disease onset, and its progression during aging. We discuss the general concept that therapeutic management of the emergent properties of the PN to control the energetics of CFTR folding biology may provide significant clinical benefit.

CYSTIC FIBROSIS AS A SYSTEMS DISEASE

Cystic fibrosis (CF) is an inherited disorder caused by mutations affecting the function of the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP gated chloride/bicarbonate channel that regulates the hydration of the epithelial surfaces found in multiple tissues including the liver, pancreas, intestine, and lung (Riordan 2008). CF can be viewed as a triad of pathologies (Fig. 1), which, at its root, is a loss-of-function disease in response to uncoupling of the CFTR peptide chain stability from folding pathways defined by the protein homeostasis or proteostasis network (PN) of the cell (Balch et al. 2008; Hutt and Balch 2010) (Fig. 2). The pathophysiological CF triad not only includes a folding defect leading to loss of salt balance, but loss of surface hydration and reduced mucociliary clearance, which trigger a chronic inflammatory response, leading to a multiple organ failure phenotype. It is the triad of these pathological events that is driven by integrated biological networks that are sensitive to the status of the CFTR fold that we hope to regulate through therapeutic management of the PN to, in essence, use normal biology to correct disease biology.
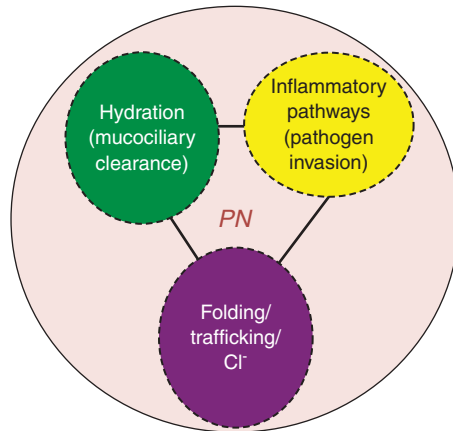
Figure 1. The pathological Cystic Fibrosis triad. Illustrated are the three principle pathologic features observed in the clinic that define the CF disease triad in response to vCFTR. Each feature is likely to be subject to PN management either indirectly through the CFTR fold, or directly by PN components (Fig. 2).

To date >1600 disease-causing mutations have been identified in the CFTR gene (referred to as variant CFTR [vCFTR]). These fall into six classes (I–VI) that represent a breakdown in CFTR folding and function at different stations in its trafficking itinerary through the exocytic and endocytic pathways, and in channel function (Becq 2010) (Fig. 3A). As might be expected, presentation of these different mutations in the clinic is quite diverse, ranging from mild to severe disease pathology, reflecting the location of the mutation in the vCFTR peptide chain sequence, its impact on folding energetics at different steps of its trafficking itinerary, and its indirect or direct effect on channel activity and protein interactions seen in the CFTR network (Wang et al. 2006; Wright et al. 2009). Interestingly, the loss of Phe 508 (ΔF508 vCFTR), a mutation affecting >90% of CF patients, presents and progresses in the clinic with a wide range of pathologies and time frames. This emphasizes the importance of a multiplicity of modifiers (Collaco and Cutting 2008), likely to include as yet many unidentified PN components, that set the stage not only for the initial disease etiology observed at birth, but numerous aging-related changes associated with

loss of vCFTR function leading to the inevitable progressive loss of tissue function (Fig. 1).

The questions we need to address to better understand the CFTR folding/misfolding problem and for generating effective therapeutics to ameliorate disease through modulation of the PN are: (1) Are wild-type (WT) CFTR and vCFTR affected similarly by the composition and activity of the local cellular PN? (2) Do we have suitable models to characterize the role of the PN in disease? (3) What is the maximal level of rescue that we can hope to achieve by manipulating the PN? and finally, (4) Is targeting the CFTR PN sufficient or do we need to develop additional strategies that focus on non-CFTR targets to maximize patient benefit with respect to the CF disease triad (Fig. 1). In other words, can we use the dynamic and emergent properties of the PN to efficiently manifest correction of multiple disease etiologies associated with CF?

THE PN AS A FRAMEWORK FOR CF DISEASE MANAGEMENT

At least a thousand components contribute to the PN (Fig. 2) (Balch et al. 2008; Powers et al. 2009; Hutt and Balch 2010). These include the Hsp/Hsc70 and Hsp90 chaperone systems whose normal activities are regulated by numerous signaling pathways sensing folding and metabolic stress as well as the unfolded protein response (UPR) (Malhotra and Kaufman 2007; Ron and Walter 2007; Lin et al. 2008), the heat-shock response (HSR) (Morimoto 2008; Shamovsky and Nudler 2008), Ca^{2+}-sensing (Burdakov et al. 2005; Petersen et al. 2005), and inflammatory pathways (Medzhitov 2008; Zhang and Kaufman 2008). These are complemented by the components of the ubiquitin-sumoylation proteasome system (UPS) found in the cytosol (Finley 2009), and lysosomal and autophagic degradation pathways that are also sensitive to multiple stress pathways (He and Klionsky 2009). These signaling pathways maintain a healthful proteome by operating as integrated transcriptional/translation/folding systems to control the composition and capacity of the variable PN environments found in
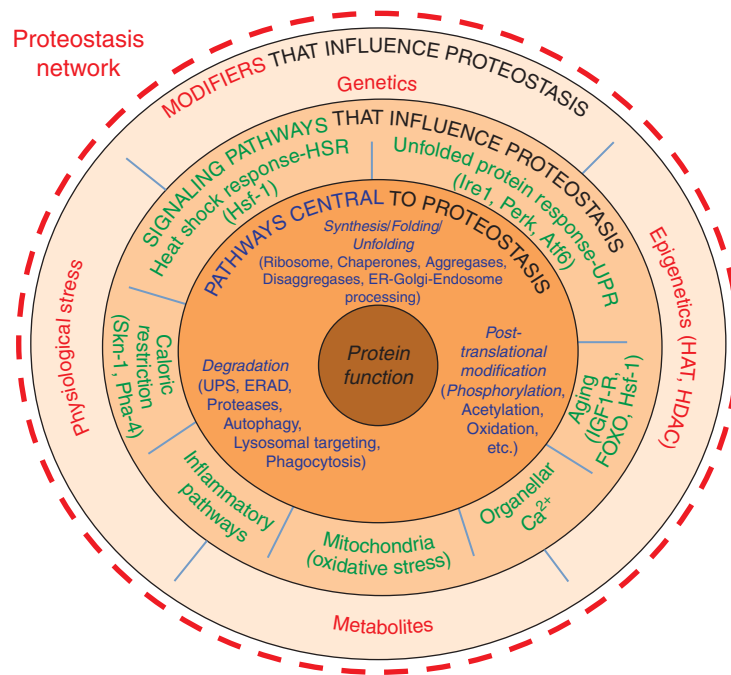
Figure 2. The Proteostasis Network. Shown are the interactions that comprise the PN, responsible for generating and maintaining the protein fold. Components comprising the PN outlined in the first layer (blue font) including the ribosome, Hsc70/Hsp70 and Hsp90 chaperones systems that direct folding, aggregases that promote aggregate formation, disaggregases that disassemble aggregates, as well as pathways that select proteins for degradation [e.g., the ubiquitin-proteasome system (UPS), endoplasmic reticulum (ER)-associated degradation (ERAD) systems, proteases, autophagic pathways, lysosomal/endosomal targeting pathways, and phagocytic pathways, the latter are responsible for the recognition, uptake, and degradation of extracellular proteins]. The second layer shows signaling pathways (green font) that influence the level and activity of components found in the first layer. The third layer (red font) includes genetic and epigenetic pathways, physiologic stressors, and intracellular metabolites that affect the activities defined by the second and first layer. Reproduced with permission from Elsevier Press (Fig. 2 in Powers et al. 2009).

different cell-types. They not only generate the protein fold but they protect it during youth and aging (Morimoto and Cuervo 2009; Powers et al. 2009).

The PN is necessarily ancient and coevolved with the remarkable diversity of peptide chain sequences and folds, reflecting the need to manage diverse patterns of folding energetics (Powers et al. 2009; Prahlad and Morimoto 2009; Hutt and Balch 2010). The PN very likely played an instrumental role in expanding the capacity of polypeptides to function in complex cell and tissue environments, and to perform increasingly precise cellular tasks associated with both cytosolic and membrane compartments found in human biology that ultimately requires complete integration of protein, cell, tissue, and organismal function (Hutt et al. 2009). Given the close coupling between the fold and the activity of the PN, any alterations in the polypeptide chain (e.g., a mutation, cotranslational and posttranslational modifications), or a change in the composition of the PN will lead to a change/breakdown in the folding network potentially triggering human pathologies. Thus, any effort to understand in vivo the impact of a mutation on folding and function will require careful consideration of the interdependence of folding energetics of the peptide chain and the PN in a given cell type. This of course will be tightly linked to the local tissue environment and organismal physiology that
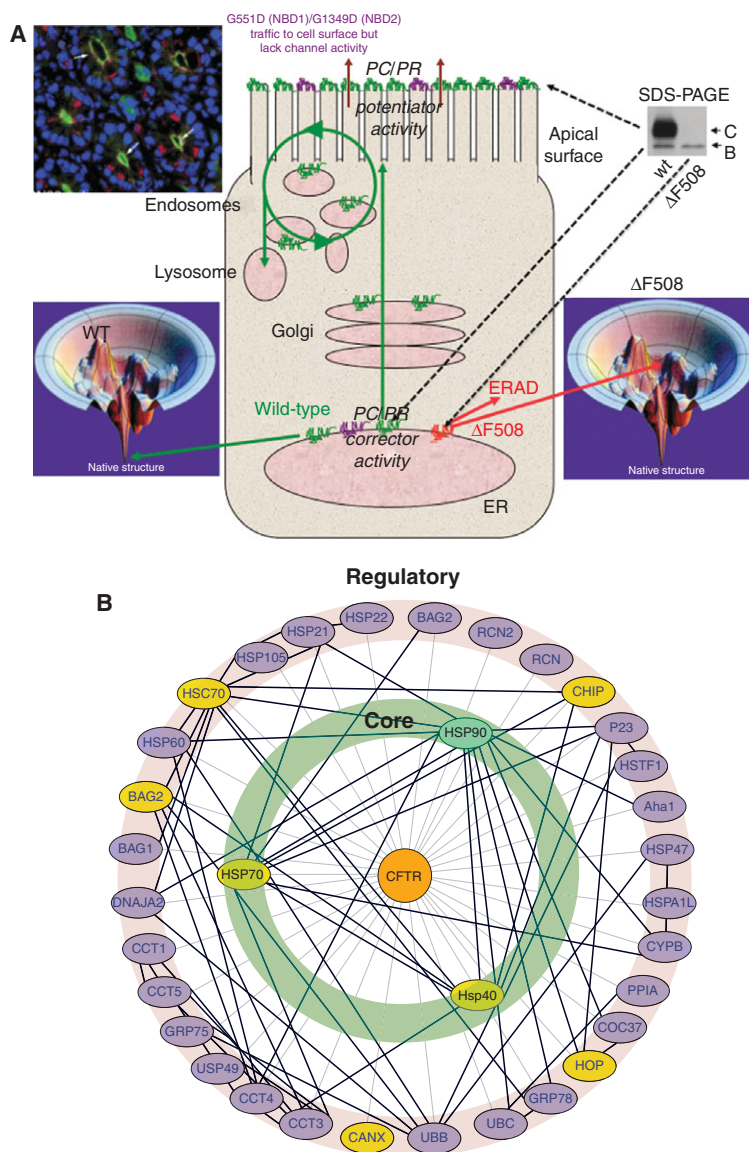
Figure 3. Cystic Fibrosis trafficking. (*A*) Illustrated is the trafficking itinerary of WT and three vCFTR (ΔF508, G551E, G1349D) through the exocytic pathway. Wild-type CFTR folds efficiently as indicated by achieving its native state in the folding landscape (*lower left*) and indicated by the green icon in the endoplasmic reticulum (ER). It is trafficked to the apical surface of lung airway epithelial cells (*upper left*, green) where it has normal channel function. During transport through the Golgi, WT-CFTR is processed from the band B ER associated glycoform to the band C complex glycoform (*upper right*). In contrast, the ΔF508 mutant is unstable in the ER (*lower right*) and as indicated by the red icon in the ER. It is efficiently degraded by ERAD. Typically, the only detectable form of ΔF508 is the band B glycoform restricted to the ER prior to degradation. The G551E and G1349D mutants (purple) are folded and traffic normally to cell surface. However, both have gating defect preventing channel function. The G551E and G1349D vCFTR only require a potentiator to open the channel and restore function. In contrast, ΔF508 requires the addition of a pharmacological chaperone (PC) corrector or alteration of the folding environment by a proteostasis regulator (PR) to restore delivery to the cell surface. (*B*) The vCFTR folding interactome. Genes involved are indicated in capitals. (Wang et al. 2006). Reproduced with permission from Elsevier Press (Fig. 2B in Wang et al. 2006). (*See facing page for legend.*)
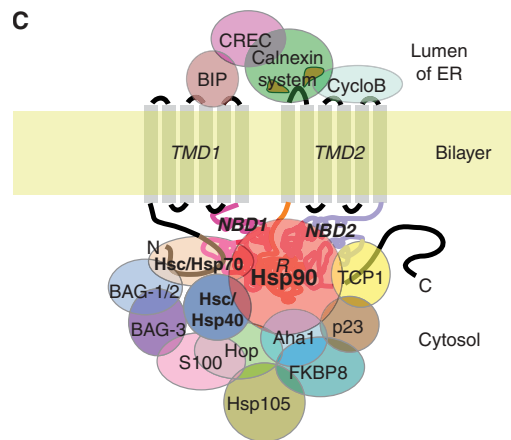
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Figure 3. (*Continued*) (*C*) A key subset of PN factors that are now known to contribute to folding of vCFTR domains facing the cytosolic and the lumenal domain of the ER.

will likely trigger compensatory responses to deal with either the fold (Dill et al. 2008) and/or the loss-of-function/gain-of-toxic function phenotype (Morimoto 2006; Morimoto and Cuervo 2009). The challenge will be to restore proteome balance (Hutt and Balch 2010).

To address the folding problem and its relationship to cell biology at a global level, we developed a general model, referred to as FoldEx, that describes, from the point of view of classic Michaelis-Menten kinetics, how the PN can influence the inherent energetics of the polypeptide chain folding kinetics to achieve transport of molecules such as CFTR through the exocytic pathway (Sekijima et al. 2005; Wiseman et al. 2007). This model was recently generalized to integrate folding energetics with the PN in all cellular compartments (Powers et al. 2009). The simple take-home from this general modeling approach (referred to as FoldFx), which provides a rigorous quantitative standard for biological folding, is that the local PN maintains a tight control on the folding energetics encoded by the primary peptide chain sequence to achieve biological function. Mutations such as those found in CF disease, which alter peptide chain folding energetics and its position relative to the inherent PN in the FoldEx and FoldFx models, predict that it

will be necessary to alter PN function through biologic and/or pharmacologic means to restore proteome balance and function (Powers et al. 2009; Hutt and Balch 2010).

CFTR BIOLOGY AND THE PN: WHAT GOES RIGHT AND WHAT GOES WRONG

Diseases such as CF are not only a consequence of a deficiency in trafficking through the exocytic pathway, but in the function and stability of the protein at the cell surface reflecting recycling through the endocytic pathway and/or targeting for degradation by the lysosomal system (Guggino and Stanton 2006; Becq 2010; Okiyoneda et al. 2010) (Fig. 3A). Indeed, the existence of multiple trafficking compartments within the cell suggests that the cell operates in the context of multiple PNs that are unique to each compartment and individually subject to stress signaling regulation (Hutt et al. 2009). Moreover, transmembrane proteins such as CFTR are subject to folding challenges on two sides of the bilayer, the lumenal domain is subject to an unique series of oxidizing PNs found in the various trafficking compartments in which it transiently resides, whereas the cytosolic domain is sensitive to a reducing environment and a completely different set of PN components that remain in common throughout transit (Hutt et al. 2009).

WT CFTR is localized to the apical surface of epithelial cells where it regulates water and salt (Na^+ and Cl^-) homeostasis to control the fluidity of the extracellular mucus layer critical for the function of multiple tissues including lung, intestine, and pancreas (Riordan 2008) (Fig. 3A). The 1480 amino acid polypeptide chain of CFTR is composed of two membrane-spanning domains (MSD1 and MSD2), each containing six transmembrane helices (TM), two nucleotide-binding domains (NBD1 and NBD2), and a cytoplasmic regulatory (R) domain. CFTR is synthesized by membrane-bound polyribosomes. Its nascent MSDs are imbedded in the bilayer of the endoplasmic reticulum (ER) where it acquires two N-linked glycans as part of the first step in the exocytic pathway (Fig. 3A). Despite its ER association,

CFTR folding requires extensive support from the cytosolic PN (Fig. 3B) to mediate proper folding of the cytosolic NBD1, R, and NBD2 domains with only small lumenal oriented loops connecting MSDs 1 and 2 contributing to the fold (Fig. 3C). The latter appear to be sensitive to the luminal chaperone systems. On proper folding, export from the ER to downstream compartments is managed by the COPII machinery (Wang et al. 2004; Wang et al. 2008), a critical rate-limiting event in disease (Wang et al. 2004). Following delivery to the Golgi compartment (Fig. 3A), the N-linked glycans undergo complex glycosylation preceding transfer to the cell surface where it is subject to regulation by endocytic pathways. Channel activity is regulated by a complex play of interactions involving kinases, phosphatases, and other binding partners that couple chloride transport with Na^{2+} transport (Guggino and Stanton 2006; Amaral and Kunzelmann 2007; Becq 2010).

We now appreciate that folding and maintenance of this multidomain protein as a functional chloride channel uses a sophisticated PN pathway (Fig. 3b,c) to promote not only proper domain assembly, but also interdomain assembly (Ollero et al. 2006; Wang et al. 2006; Wright et al. 2009). Molecular modeling approaches and cross-linking studies have shown that the NBD1 domain, synthesized immediately after MSD1, contacts the cytoplasmic loop (CL) 1 (MSD1) and 4 (MSD2) whereas NBD2, synthesized after MSD2, contacts CL2 (MSD1) and 3 (MSD2), interactions requiring substantial PN protection given the 5–10 minute time span required to synthesize full-length CFTR in vivo (He et al. 2008; Mornon et al. 2008; Rosser et al. 2008; Serohijos et al. 2008). Interestingly, in heterologous expression systems, even wild-type (WT) CFTR folding in the ER compartment is a very inefficient process with >70% targeted for degradation by ER-associated degradation (ERAD) pathways, possibly reflecting differences in PN pathways. Whether this is true for endogenous expression in the context of the native PN found in human tissues remains controversial. One possibility is that cells present in an organismal tissue environment contain a different PN pool dictated by cell

nonautonomous signaling pathways. In contrast, >95% of ΔF508 is retained in the ER in most cell/tissue types and targeted for degradation by ERAD, although some cell types are clearly more permissive for export of ΔF508 than others, particularly heterologous cell culture expression systems, suggesting that the local PN strongly influences early folding events.

PN components facilitating folding of the cytosolic NBD1 and NBD2 are now recognized to include the Hsp40/Hsc-Hsp70 (Meacham et al. 1999; Meacham et al. 2001; Younger et al. 2004; Younger et al. 2006; Morito et al. 2008; Rosser et al. 2008; Grove et al. 2009) and Hsp90 chaperone systems (Loo et al. 1998; Fuller and Cuthbert 2000; Wang et al. 2006; Sun et al. 2008; Koulov et al. 2010). Calnexin, a membrane-anchored ER-associated chaperone, has been shown to interact with the immature forms of both WT and ΔF508 vCFTR (Pind et al. 1994), although its exact role in folding remains controversial (Zhang et al. 2001; Okiyoneda et al. 2002; Amaral 2004; Okiyoneda et al. 2004; Norez et al. 2006; Okiyoneda et al. 2008). In several studies, ΔF508 is found to be associated more strongly with Hsp40/Hsc-Hsp70 complexes than with WT, although these studies did not distinguish immature and mature pools in the case of WT. The peripheral (plasma membrane) reduce temperature rescued ΔF508 vCFTR pool is now appreciated to bind Hsc-Hsp70 suggesting that the PN influences CFTR folding and stability throughout its cellular itinerary (Hutt and Balch 2010; Okiyoneda et al. 2010). Moreover, the ΔF508 vCFTR-calnexin complex was found to have a longer half-life (Pind et al. 1994). One possibility that remains to be examined is that the misfolded ΔF508 vCFTR may be trapped in PN complex(es). Perhaps being unable to escape from a folding intermediate state, ΔF508 is targeted for degradation by the proteasome through the UPS pathways, potentially involving Hsp27 (Qu et al. 1997; Zhang et al. 2001; Youker et al. 2004; Sun et al. 2006; Wang et al. 2006; Zhang et al. 2006; Ahner et al. 2007; Vembar and Brodsky 2008). Recent studies suggest that it is the interaction of the PN

component CHIP with Hsc-Hsp70 (Meacham et al. 2001) through the activity of the E3 ligase RMA1 (Grove et al. 2009) that promotes degradation. Intriguingly, siRNA silencing of the Hsp90 ATPase activator co-chaperone Aha1 (Fig. 3C) in HEK293 cells and in primary airway cells homozygous for ΔF508 resulted in stabilization, trafficking, and restoration of channel activity at the cell surface (Wang et al. 2006) (W. Balch and R. Frizzell, unpubl.). What is now very clear is that the primary defect in CF disease is an early PN-mediated folding event in the ER (Fig. 3b,c; see below Fig. 5) that requires further maintenance activity of the PN to prevent ERAD and targeting of cell surface rescued variant to the lysosomal pathway (Hutt and Balch 2010; Okiyoneda et al. 2010). Thus, the answer to the first question posed above is that WT and ΔF508 do interact with the PN differentially and that modulation of the PN function could contribute significantly to the treatment of CF by achieving proteome balance (Hutt et al. 2009; Hutt et al. 2010).

CF THERAPEUTICS AND THE PN

It should now be apparent that achieving correction of CF through the PN will be a multifaceted problem. Indeed, an extensive therapeutic pipeline has been developed by the CF foundation (http://www.cff.org/research/DrugDevelopmentPipeline/) to address this challenge. Although this pipeline has been instrumental in moving the median survival age from 5–37 years old, it is largely a consequence of endpoint disease management at clinical centers using therapeutics that include digestive enzyme supplements, hypertonic NaCl inhalation protocols that facilitate increased lung function and mucociliary clearance, and, importantly, antibiotics that prevent recurrent bacterial infections that is the primary cause of premature death of CF patients (Proesmans et al. 2008; Frerichs and Smyth 2009; Jones and Helm 2009; Verkman and Galietta 2009; Becq 2010; Belcher and Vij 2010; Kreindler 2010; Zemanick et al. 2010). While no drugs to date are known to directly target the basic folding problem in disease, the quest for such

drugs is in full force (Amaral and Kunzelmann 2007; Verkman and Galietta 2009; Becq 2010; Belcher and Vij 2010) with the goal of correcting the defects leading to the pathological triad (Fig. 1). Indeed, a large number of scaffolds have been identified that weakly influence the trafficking defect and/or channel activity. A number of these modulators can be found in the Cystic Fibrosis Foundation Therapeutics (CFFT) modulator library and are freely available to established and new investigators interested in pursuing CF.

Restoration of function through folding can occur through several mechanisms. One possibility is direct energetic stabilization of the fold through the use of therapeutics generally referred to as pharmacological chaperones (PCs) (Loo et al. 2008) (Fig. 4). "Corrector" PCs are thought to directly bind to the misfolded protein and provide additional stability as has been shown for G-protein coupled receptors (GPCRs) (Conn et al. 2007), Gaucher's (Yu et al. 2007; Mu et al. 2008), and transthyretin (TTR) mutants (Tojo et al. 2006; Hurshman Babbes et al. 2008). For example, compounds C3 and C4 from the CFFT modulator library have been suggested to mediate their activity through direct interaction with the CFTR protein (Loo and Clarke 2007; Wang et al. 2007; Loo et al. 2008; Grove et al. 2009). An alternative group of PCs used in the CF field are referred to as potentiators. CF potentiators promote surface channel activity but do not promote folding and/or trafficking of the nascent polypeptide. Importantly, the potentiator drug Vertex 770 (http://www.cff.org/research/ClinicalResearch/FAQs/VX-770/) (Caputo et al. 2009; Van Goor et al. 2009; Wellhauser et al. 2009; Moran 2010) has recently completed a Phase II clinical trial with the G551D mutant (Fig. 3A), which is trafficked from the ER normally, cell surface localized but lacks channel activity. Treatment of patients leads to a significant increase in nasal potential difference, sweat chloride, and lung function, all hallmarks of restoration of lung function. Vertex 770 is thought to bind CFTR and favor channel open probability facilitating increased chloride flux (Riordan 2008), therefore artificially altering functionality, as do agonists
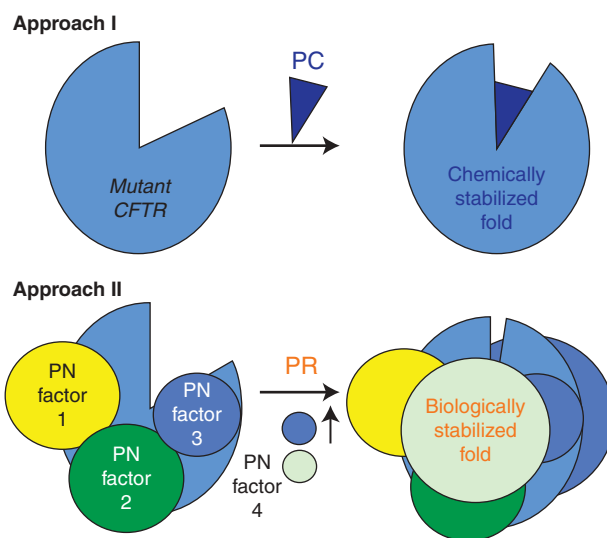
Approach I

Approach II

Figure 4. PCs and PRs. Illustrated are two approaches to target the folding problem in CF. PC is a pharmacological chaperone that can be a corrector or a potentiator by binding directly to the misfolded protein and energetically stabilizing the fold; PR is a proteostasis regulator that alters the level or content of PN factors (colored balls) that would improve the vCFTR fold.

and antagonists for GPCRs (Ulloa-Aguirre and Conn 2009).

In contrast to PCs, which may target specific steps in CFTR folding, a more encompassing approach may be achieved by using proteostasis regulators (PRs) (Balch et al. 2008) (Fig. 4), that is, molecules that adjust the composition and/or concentration of the PN (Fig. 2) in a manner that favor ΔF508 vCFTR folding. PRs could be, in principle, similar to factors that stimulate stress responses leading to changes in PN composition of the cell that alleviate challenging misfolding problems. A PR that affects the cytosolic PN governing the folding of ΔF508 vCFTR could provide a more stabilizing environment, not only for ER export, but also for function at the cell surface. It is also possible that a combination of the various classes of PCs (correctors, potentiators) and PRs may synergize, as has been observed for Gaucher's disease in which rapid degradation in the ER could be reduced by PR modulation of the PN, allowing sufficient protein to be produced to engage a PC corrector leading to an additional level of energetic stabilization either early or late in the trafficking pathway and rescue of the disease

phenotype (Mu et al. 2008). Very recently, a potential PR (cystamine) that triggers autophagy pathways has been suggested as a potential mechanism to re-establish trafficking ΔF508 (Luciani et al. 2010). A lack of demonstration of functional channel activity at the surface limits the impact of such observations.

To date, the mechanism of action (MOA) of compounds identified from cell-based, high throughput screens (HTSs) designed to detect recovery of CFTR function at the cell surface are largely unknown. A case in point is Vertex 809, a compound jointly developed by the CFFT and Vertex. Vertex 809, unlike Vertex 770, is considered a corrector of the nascent polypeptide fold as it promotes exit from the ER (http://www.cff.org/treatments/Pipeline/). Thus, it could function either as a PC corrector or as a PR (Fig. 4). In a recent Phase II clinical trial with homozygous ΔF508 patients, 809 had a modest, but encouraging capacity to correct nasal potential difference (http://www.vrtx.com/current-projects/drug-candidates/VX-809.html), although no effect on lung function was observed in the treatment regimens used to test potential efficacy. Given the modest

Cite this article as *Cold Spring Harb Perspect Biol* 2011;3:a004499

improvement in channel function, the next study will proceed as a dual drug therapy by combining Vertex 809 with Vertex 770 (http://www.vrtx.com/current-projects/drug-candidates/vx-770.html), thereby providing a mechanism to further activate the channel function.

In general, ongoing efforts to identify CF therapeutics through biological analyses of PN function (e.g., Wang et al. 2006; Morito et al. 2008) strongly support the possibility that compounds that alter the folding energetics and/or PN biology of vCFTR have high potential for generating a more wild-type-like function, and hence, could have significant benefit in the clinic.

MODEL SYSTEMS TO IDENTIFY PN THERAPEUTICS

To study CF, we need both cell and animal model systems that accurately reproduce the operation of the PN in health and disease. With a strong focus on restoration of lung function, both primary WT and homozygous ΔF508 (ΔF/ΔF) human bronchial epithelial (HBEs) (ΔF/ΔF-HBEs) cells can be obtained from patient lungs during transplant and grown as epithelial monolayers on transwell dishes to assess trafficking, channel activity, and surface hydration at the air-liquid-surface (ALS) interface that lead to restoration of mucociliary function. These are routinely used by the CF community. At this juncture, human ΔF/ΔF-HBEs are the gold standard for judging the efficacy of CF therapeutics coming through HTS pipeline for patient clinical trials. However, these are a challenging resource to generate and subject to the availability of tissue from CF patient lung transplants, although significant progress has been made in generating frozen stocks. It is important to note that HBEs derived from patients have very large differences in their channel activity and ability to respond to PCs and likely PRs, suggesting both the health of the lung at transplant and patient genetic and epigenetic (environmental) background has a major impact on the activity of the PN in maintaining vCFTR functionality.

Given the above limitation, the field has used a range of cell types including heterologous expression systems and a wide range of immortalized, tissue specific cell lines (lung, pancreas, and intestine) that can be readily maintained in culture. The key point is that each cell line and animal model (see below) likely reflect different PNs and therefore have limitations relative to providing insight into the pathophysiology of human disease. In most cases, the choice of the cell line used to define the role of the PN in CF disease is dictated by its availability, behavior (e.g., growth rate, formation of polarized monolayers, transfection efficiency, expression level of the exogenous gene, etc.), and strength of the endpoint assay that varies from biochemical assessment of trafficking patterns using SDS-PAGE to channel activity using Ussing chambers, fluorescence reporters and imaging, and patch-clamp recordings. In particular, use of polarized epithelial cells such as the lung CFBE41o- cell line expressing ΔF508 will likely be more physiologically relevant in terms of PN interactions than heterologous expression systems using fibroblast-derived cell lines. In this regard, the CFTR Folding Consortium (CFC) (http://www.cftrfolding.org/) works with a series of model systems and has developed over 25 different assays that measure different features of CFTR trafficking and channel function. The CFC functions as a community resource to leverage this wide range of assays to generate the CFC "RoadMap" that attempts to measure the impact of a particular therapeutic on WT and vCFTR trafficking and function, with the goal of providing critical insight into MOA of newly discovered therapeutics.

To understand how studies in a particular cell line can impact our understanding of the PN in human disease, one can simply look at the half-life of the ER-restricted form of human ΔF508 vCFTR in a variety of cell lines. In general, ΔF508 half-life was found to be on the order of 1 h in Fischer rat thyroid (FRT), baby hamster kidney (BHK), monkey kidney (COS), and CFBE41o-human airway cell. In yeast, degradation is particularly notable and fast—even when WT CFTR fails to escape the ER,

suggesting a complete mismatch of CFTR folding requirements with the evolutionarily emergent properties of the yeast PN. Interestingly, and in contrast to the rather uniform short half-life of ΔF508 vCFTR in the ER in a variety of cell lines, the mature form of ΔF508 vCFTR that has trafficked to the cell surface showed a half-life of approximately 4 h in CHO cells compared to 1 h in CFBE41o- cells. It also showed altered endocytic recycling properties when comparing polarized and nonpolarized cell lines. This represents a significant difference in post-ER protein stability, possibly reflecting a difference in the PN environment and peripheral folding maintenance by the PN in these two cell types (Fig. 5) (Hutt and Balch 2010; Okiyoneda

et al. 2010). Moreover, a recent comparison of 13 potentiators showed that three different mutants, including ΔF508 (NBD1, ER degraded, Fig. 3A), G551D (NBD1, cell surface localized, Fig. 3A), and G1349D (NBD2, cell surface localized) (Pedemonte et al. 2010), expressed in FRT cells or the human alveolar epithelial cells A549 responded to 10 of the 13 compounds tested, supporting the conclusion that these potentiators may be binding to vCFTR directly and their binding is not influenced by cellular environment (Pedemonte et al. 2010). Interestingly, when they examined the effect of known correctors on the aforementioned cells lines as well as on ΔF/ΔF-HBEs, they found that all but one of the correctors from the
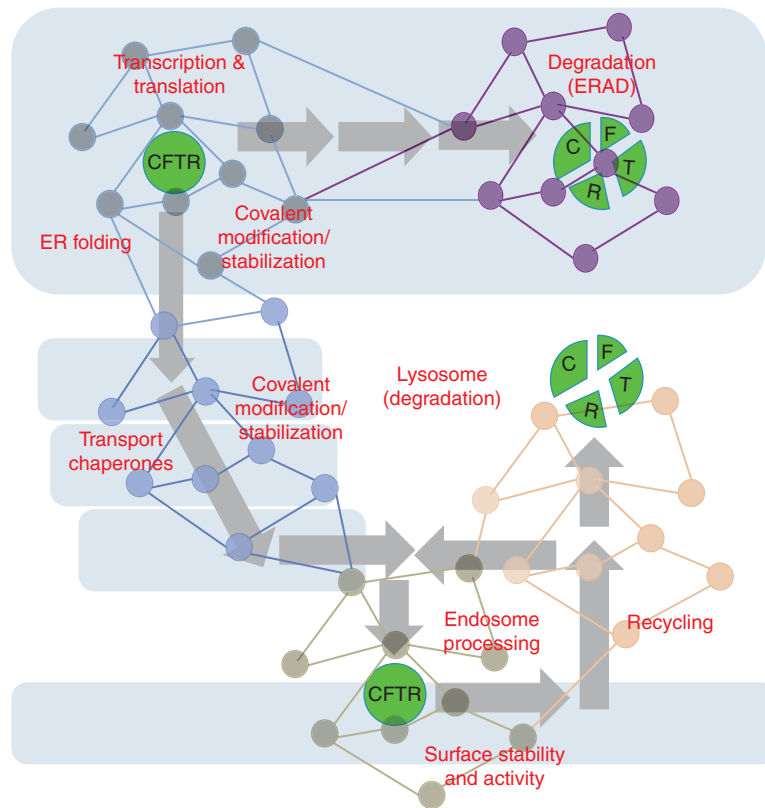

Figure 5. Management of vCFTR functions by the PN. A hypothetical view of the activity of subsets of factors present in the PN that function at the indicated step of the vCFTR folding and trafficking itinerary. Adjustments to one or more nodes through the activity of PRs cannot only restore CFTR function, but re-establish connectivity to the biological network of the cell supporting normal physiology, thus alleviating the pathologic CF triad contributing to onset and progression of disease (Fig. 1).

CFFT modulator library were effective in only one cell line, however, C3 and C4, shown to act directly on vCFTR, were effective in all three cell lines (Pedemonte et al. 2010). Thus, correction by a PC or PR appears very sensitive to the PN, emphasizing the importance of model systems to accurately recapitulate the human tissue environment.

To address the organismal respond to therapeutics, a number of mouse models have been generated to better understand the physiological complications associated with the intact diseased tissue. Strikingly, all of the CF mice models fail to recapitulate respiratory defects associated with human CF and therefore fail to manifest inflammatory markers. Moreover, they lack susceptibility to bacterial infection and do not recapitulate pancreatic and hepatobiliary phenotypes found in the human condition (Scholte et al. 2004). However, they do have defects in nasal potential and develop prominent intestinal obstruction, resulting in peritonitis and nutritional insufficiency typically seen in CF patients. A number of reasons could account for the observed differences between mouse and human pathophysiology. They include variations in airway biology and/or the expression of alternative, cAMP-stimulated, nonCFTR chloride channels in the murine lung. In addition to these physiological and cellular differences, the amino acid sequence of the mouse CFTR shows only 88% similarilty and 78% identity to human CFTR suggesting possible differences in the structure-function relationship of these proteins with their respective handling by the evolutionary and environmental triggered emergent properties of the mouse and human PNs (Liu et al. 2007).

The recent development of the CF pig model is a very exciting addition to the CF field in that the pig phenocopies human lung and intestinal disease (Rogers et al. 2008; Stoltz et al. 2010; Wine 2010). In addition to striking similarities in porcine and human lung physiology, a sequence alignment between pCFTR and hCFTR reveals that the two proteins share 92% identity, suggesting that the proteins may have similar folding properties that would require a similar level of involvement of conserved PN components for folding. In fact, single channel recordings of the two proteins revealed very similar unitary channel conductance (6.7 pS for hCFTR vs. 8.3 pS pCFTR) as well as identical open probability ($P_o = 0.39$) (Rogers et al. 2008). Additionally, it was found that, in isolated airway epithelia from porcine trachea, pCFTR showed a similar amiloride sensitive sodium current to that seen in human airway epithelial cells. A close examination of trafficking of WT and mutant ΔF508 CFTR in human cells revealed that although both WT pCFTR and hCFTR are similarly delivered to the cell surface, the ΔF508 mutant of pCFTR shows a significant level of escape from the typical ER retention resulting in significant localization to the apical membrane. In addition, the pΔF508-CFTR showed a higher P_o and residual basal current as compared to the same mutation in hCFTR (Rogers et al. 2008; Stoltz et al. 2010). These differences could in part be attributed to the sequence differences between porcine and human CFTR. In general, the pig lung recapitulates the CF-triad pathology (Fig. 1). However, differences may also exist between the PN of the porcine and human lung cells, which could contribute to onset and intensity of pathophysiology. In general, current evidence suggests that the CF pig may provide a good model of CF lung disease allowing for a better understanding of the emergent properties of the PN in disease (Stoltz et al. 2010; Wine 2010). It will likely serve as a good experimental model for the testing of novel therapeutics aimed at treating the basic defect in the human disease.

In summary, the answer to question 2 posed above (What is the value of models in assessing PN function in restoration of vCFTR?) is that although the primary defect observed in humans (e.g., failure to fold and exit the ER) is preserved in all models tested, PN pathways that promote restoration of function may be more cell type and species specific, reflecting the composition and regulation of the PN that must integrate the vCFTR fold with many other aspects of cellular function found in different cell and organismal states.

CHARACTERIZATION OF NEW TARGETS AFFECTING RESTORATION OF TISSUE FUNCTION

Although phenotypic rescue of disease through the PN by use of small molecules that affect CFTR folding is an important goal of the CF drug development pipeline, alternative strategies are now evident including directly targeting Na^{++}-channel regulation that is defective in the absence of CFTR (Boucher 2007; Donaldson and Boucher 2007; Lazarowski and Boucher 2009), up-regulation of alternative Ca^{2+}-activated Cl^- channels (CaCC) to replace vCFTR loss, development of anti-inflammatory strategies to counter the hyperactive inflammatory state found in response to the absence of WT CFTR and in the diseased CF lung, and/or provision of mucolytic agents to improve clearance and reduced pathogenesis (e.g., the CF triad (Fig. 1) (Proesmans et al. 2008; Frerichs and Smyth 2009; Jones and Helm 2009; Verkman and Galietta 2009; Becq 2010; Belcher and Vij 2010; Kreindler 2010; Zemanick et al. 2010).

Although each of the above therapeutic approaches would be expected to provide end-point benefit, known drugs do not solve the folding problem associated with loss of CFTR-misfolding, loss of trafficking, and degradation in the ER. Thus, an important goal is to understand the MOA of drugs obtained from cell-based screens that could function through PN modulators that would solve this problem. In this regard, an extensive effort has been devoted to the development of a CF "interaction" network through data-mining efforts provided by GeneGo (CF MetaMiner [CF]) (Wright et al. 2009) that provides a carefully annotated, systems level view of components contributing to CFTR function and correction of disease etiology. Moreover, in addition to studies on the WT and ΔF508-CFTR interactome (Wang et al. 2006), a series of bioinformatic studies have analyzed both the genomic and whole cell proteomic responses of human tissue to disease (Lipecka et al. 2006; Ollero et al. 2006; Pollard et al. 2006; Singh et al. 2006; Singh et al. 2008). Together, these studies illuminate an extensive network of interactions that will need to be

restored to obtain full correction of the disease phenotype (Fig. 5). All of these pathways are heavily impacted by the PN (Powers et al. 2009).

In contrast to bioinformatic approaches based on the current extensive CF literature (e.g., GeneGo), genomic approaches (e.g., microarrays) report indirectly on CFTR function by monitoring cellular mRNA pools. They report on changes in stress response pathways reflecting PN modulation in response to disease and correction. In contrast, proteomic studies based on mass spectrometry report the level of recovery of known interactors in response to disease and correction of the fold (Wang et al. 2006) (W. Balch and J. Yates, unpubl.). The CFC (http://www.cftrfolding.org/) has used both genomic and proteomic data as well as the GeneGo MetaMiner database to highlight a series of gene products, referred to as the "top 10" that include a number of PN components, that are likely candidates for corrective action in CF disease (W. Skach and R. Frizzell, pers. comm.). The ability to identify key players involved in restoration of CFTR function emphasizes the importance of further studies that seek to better understand the individual contribution of these proteins in CFTR folding, trafficking, and function, and the development of reagents that directly target these players to influence restoration of vCFTR function (Fig. 5). For example, screens against key regulators of the UPR, heat shock, and other PN regulatory pathways may yield a wealth of therapeutics that manipulate the PN in a fashion that is favorable for correction of vCFTR. Moreover, these compounds could differentially affect the various disease causing mutants depending on the folding step that is disrupted and the local PN capacity (Powers et al. 2009; Hutt and Balch 2010). In this regard, correction through the PN could provide a personalized approach to correction of CF disease (Becq 2010). The value of targeting the PN is that therapeutically corrective environments would target the unique energetics of the fold influenced by different mutations and the local folding environment of a patient.

The above studies directly address question three posed earlier: Do we need to focus on

additional targets/pathways to generate maximum therapeutic benefit? The answer to this question is a definitive yes, even if only to provide further endpoint benefit to the pathophysiology observed in the clinic.

EMERGENT PROPERTIES OF THE PN AS A GUIDE FOR RESCUE OF MISFOLDING DISEASE

An overview of the concepts critical for the development of PN-based therapeutics as a corrective solution for CF and the experimental approaches that will ultimately impact these efforts has been outlined above. In principle, correcting the vCFTR fold could significantly restore the underlying defect (e.g., yield a WT interactome) that could result in "self-correction" of the pathology triad (Fig. 5) typically observed in disease in response to a gain of "normal" channel function. This again focuses on question 3: Are there limits to what we can correct? At the moment there is not a definitive answer. At one level, a combination of at least two PN therapeutics, one that targets the primary defect, folding, and export from the ER, and a second that targets cell surface stability and/or channel gating would be predicted to have significant benefit and would serve as a starting point. This hypothesis is being tested in the combined Vertex 809/Vertex 770 phase II clinical trial mentioned above, although in the absence of an understanding of the role of 809 in correction, Vertex 770 may not be the best approach (http://www.vrtx.com/current-projects/drug-candidates/VX-809.html). Until we more fully understand the role of PN biology in CFTR and vCFTR function, we may have to move more aggressively to assemble the various operating parts of the PN to understand what a truly effective therapeutic strategy needs to be for correction.

Given the above, it is anticipated that PN-based therapeutics would work most effectively when started early and would, in principle, require administration throughout life to maintain function. However, the vast majority of CF patients already have seriously compromised organ systems, particularly the lung, given its continuous exposure to pathogens. How much corrective activity would be required from PN modulators to achieve efficacy in late stage disease remains an open question. Indeed, until we more completely understand the PN-based folding problem, we will continue to require additional strategies that focus on non-CFTR targets to maximize patient benefit (question 4 posed above). At the minimum, the hope would be that therapeutics that augment the function of the PN would arrest disease progression so that the disease could be more effectively managed through the plethora of therapeutics that target endpoint pathophysiology (Fig. 1) that are now available in the CF pipeline (http://www.cff.org/research/Drug-DevelopmentPipeline/). Interestingly, it is well known that epithelial cells in most organ systems routinely undergo turnover, particularly in response to damage as occurs in the CF airway; thus, there remains the potential for correction through epigenetic mechanisms that may teach the cell to more effectively manage the ΔF508 pathophysiological phenotype (Hutt et al. 2010). Moreover, it is now recognized that worm and mouse PN environments can be readily manipulated to protect against folding disorders and aging through the modulation of aging, diet, and oxidative pathways (Cohen et al. 2006; Cohen et al. 2009; Douglas and Dillin 2010; Mair et al. 2009; Panowski and Dillin 2009). It is therefore not unreasonable to suggest that similar approaches may be applicable to CF and a broad range of folding disorders.

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