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Hallmarks of Therapeutic Management of the Cystic Fibrosis Functional Landscape

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Abstract

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein does not operate in isolation, rather in a dynamic network of interacting components that impact its synthesis, folding, stability, intracellular location and function, referred to herein as the ‘CFTR Functional Landscape (CFFL)’. For the prominent F508del mutation, many of these interactors are deeply connected to a protein fold management system, the proteostasis network (PN). However, CF encompasses an additional 2000 CFTR variants distributed along its entire coding sequence (referred to as CFTR2), and each variant contributes a differential liability to PN management of CFTR and to a protein ‘Social Network’ (SN) that directs the probability of the (patho)physiologic events that impact ion transport in each cell, tissue and patient in health and disease. Recognition of the importance of the PN and SN in driving the unique patient CFFL leading to disease highlights the importance of precision medicine in therapeutic management of disease progression. We take the view herein that it is not CFTR, rather the PN/SN, and their impact on the CFFL, that are the key physiologic forces driving onset and clinical progression of CF. We posit that a deep understanding of each patient’s PN/SN gained by merging genomic, proteomic (mass spectrometry (MS)), and high-content microscopy (HCM) technologies in the context of novel network learning algorithms will lead to a paradigm shift in CF clinical management. This should allow for generation of new classes of patient specific PN/SN directed therapeutics for personalized management of the CFFL in the clinic.

Keywords

cystic fibrosis; protein folding; chaperones; proteostasis; systems biology; bioinformatics; high-content microscopy; high-throughput screening; mass spectrometry; genomics; proteomics; epigenetics; epiproteomics

Introduction: CFTR is not alone

We are all a product of an evolutionary driven mutational program responsible for survival in response to the environment-called natural selection¹. We are, therefore, fundamentally mutant by design. This tells us that all biology is designed to work with variants to drive survival and fitness in the playground of life. While cystic fibrosis (CF) is triggered by variations of the ‘normal’ wild-type CF transmembrane conductance regulator (CFTR) genomic sequence, CF is largely a consequence of a poorly understood cascade of folding and protein interaction challenges events that make the CFTR protein generated by each inherited variant genotype ‘step outside’ its normal functional routine^{2–5}, missteps that drive disease onset and progression (Pankow et. al. (2015), In Press, Nature).

To fully understand CF pathology, solve clinical enigmas (e.g., liabilities associated with each variant and the probability of progression of disease along a particular clinical tract) and, importantly, to efficiently treat the personalized form of the disease found in each individual patient, it is critical to remember that like any protein in the cell, CFTR protein does not operate in isolation. Rather, it works in a dynamic network of components that impact its synthesis, folding, stability, intracellular location and function. These are often unique in their levels and activities in each individual person in response to the inherited genome reflecting past, present and impending future environment(s) for each individual/patient. These interactions comprise what we refer to as the CF ‘Social Network (SN)’. In the past, some of these SN interactors have been referred to as ‘CF modifier genes’, although their functional significance for the most part remains elusive. The SN of wild-type CFTR, the prominent F508del mutation, and the many other 2000 or so variants identified to date contributing to clinical disease (referred to as the ‘CFTR2’ cohort^{6,7}) are deeply connected to an extensive protein fold management system, the Proteostasis Network (PN)^{3,4,8–14}. This PN-coupled SN, herein referred to as the CFTR Functional Landscape (CFFL), is optimized by biology so that a given cell, tissue and individual displays a particular functional genotype to phenotype relationship that plays out in health for wild-type CFTR, or as ‘variations on a theme’ of CF disease for each CFTR variant in each patient. In essence, each CFTR variant can be viewed as an ‘outcast’ that causes the changes in the highly evolved PN and SN interaction strategies that manage the final physiology of ion transport and divergence from healthy tissue, thus ultimately causing disease state. Such PN and SN ‘outcasting’ (i.e., divergence from the norm) triggers the multiplicity of both common and variable changes in interactions resulting in variable CF manifestations and disease progression, which are unique to each patient and that contribute to the current concepts of ‘personalized’ or ‘precision’ care in the clinic. In support of this view, we have recently shown that the F508del CFTR variant will generate a surprisingly large number of new PN and SN interactions that that collectively drive CFFL pathophysiology and, remarkably, can be largely corrected by the appropriate therapeutic management (Pankow et. al. (2015), In Press, Nature).

Getting new therapeutics by understanding the CFFL

It is now recognized that proteins, particularly membrane-spanning proteins such as (normal) CFTR, face major energetic challenges to fold in the context of the lipid bilayer as

well as divergent cytosolic and compartment specific environments^{15,16}. Moreover, most proteins are highly dynamic and conformationally challenged, often being biologically 'disordered' even in the healthy setting. These states are further perturbed by the genotype sequence variants initiating disease in a particular cell, tissue or patient environment. Therefore, any attempt to understand disease from *in vitro* or *in vivo* heterologous cellular models of function that do not normally express this protein will have limited success, albeit potentially targeting evolutionarily conserved features of CFTR functional (partial) responses to the PN and/or SN management. In this view, structural snapshots of '(mis)folded' states derived from biophysical approaches such as X-ray crystallography structures (the presumed holy grail of contemporary biochemistry) and/or computational 'modelling' approaches based on homologous proteins, necessarily provide limited insight into CF therapeutics in the biological setting. Clearly these approaches fail to grasp what is necessarily the more physiologically relevant dynamic PN and SN that contribute to the local physiologic environment of CFTR in each cell, tissue and patient environment in a particular time-frame ranging from early development to aging^{17,18}.

A rational approach for defining the physiologic source of an unfavourable CFFL stemming from a CF-causing CFTR variant is to understand the biological PN and SN 'disconnections' that are the 'root' cause of the disease³ (Pankow et al. (2015), Nature, In press). Emerging insights suggest that these links are mismanaged through multiple mechanisms, leading to both the loss and/or gain of aberrant protein interactions^{2-4,7,19-21} (Pankow et. al. (2015), In Press, Nature). Protein networks normally work together as a highly coordinated 'team effort' using transient, sequential pathways that are unique to each cell type, organ system and patient-interactions that continue to evolve over a lifespan^{12,22,23}. Moreover, unlike cytosolic proteins, membrane proteins like CFTR do not passively 'sit-still' in one compartment waiting for function 'to arrive at the doorstep'. Instead, they interact with the evolving 'team' that facilitates their synthesis and trafficking through a complex series of dynamic spatial-temporal relationships including trafficking through the exocytic (to the plasma membrane) and endocytic (from the plasma membrane) pathways, each compartment being specifically tuned in a given cell type to adjust CFTR expression/function according to the local PN and SN environments. Such compartmentalization, we now appreciate, can critically expand, constrain and/or collapse CFTR function^{4,9,18,23-25} (Pankow et al. (2015), Nature, In Press). A thorough understanding of these compartmentalized interactions is necessarily required to fully repair the *full* damage caused by the disease. Nevertheless, this is usually considered as a 'burden' by pharmaceutical approaches that want to move quickly towards a therapeutic success on the assumption that a minimal understanding of CFTR and just a few of its PN and/or SN 'friends' and/or 'enemies' will suffice. We posit that if we really want the next generation of CF therapeutic advances to significantly improve both the efficacy and clinical response to team management, it will require a strategic investment in approaches that define the PN and SN components that contribute to CFFL disease mediated by CFTR variants^{2-4,20,26-28} (Pankow et. al. (2015), In Press, Nature).

Getting smart about therapeutics should include a full description of the PN and SN interactions required to maintain the 'normal' CFFL and those that change in response to

each variant CFFL responsible for personalized clinical phenotypes^{2,3} (Pankow et. al. (2015), In Press, Nature). In this view, we need to treat CFTR not as ‘icon’ to be held in awe as it is currently the case, but as just ‘one of the crowd’. Unquestionably, it should still be regarded as a major disease contributor, but whose role in tissue physiology is futile without its many social contacts (the PN and SN) that must be dynamically maintained in evolving environments during disease onset, progression and correction^{2,3} (Pankow et. al. (2015), In Press, Nature). This shift in our perception of CF disease and its therapeutic paradigms, embracing both the PN and SN (Figure 1), we posit presents an unprecedented opportunity to generate a new CFFL framework that will have higher prospects to promote better treatments. These will strike not only at the roots of disease onset, but allow us to control its progression^{28,29}.

Below we describe the efforts ahead that will be required to help us understand the impact of CF from the CFFL perspective managed by the PN and SN connectivity’s. We pose a series of experimental ‘hallmarks’ that we need to achieve to benefit the highly personalized patients’ health- and their unique lifespans (Figure 1). At the first level in this scheme we encounter the proteostasis based ‘PN’ Hallmark (Figure 1). That is, the need to understand the folding problem that is essential to generate and sustain normal (wild-type) and/or CFTR variant function in each cell type. The second level we refer to as the ‘SN’ hallmark’ (Figure 1). The SN Hallmark defines the differential impact of each CFTR variant on the local genome and consequential proteome when compared to normal CFTR^{2,3} (Pankow et. al. (2015), In Press, Nature). It is expected that depending on the specific variant (e.g., a mutation affecting CFTR splicing, folding, traffic and/or activity), the effects on the PN/SN will vary in a fashion likely impacting our understanding of the severity of disease. Likewise, the diverse proteomes found in different cell and tissue types (including splicing, translation, folding, trafficking and signalling factors directing compartmentalization) will affect each CFTR variant differentially, perhaps related to the ‘modifier genes’ concept based on large-scale genomic (GWAS) studies. Accordingly, the PN and SN (Hallmarks 1 and 2) are responsible for the third level, the ‘Functional Liability and Probability’ Hallmarks that ultimately determine cell and tissue (patho)physiology and patient health span. Levels 1–3 are necessarily linked to and driven by the fourth level, the ‘Environment’ Hallmark, that includes both cell autonomous (intrinsic changes in the transcriptome and proteome of the affected cell population), and cell non-autonomous (extracellular) determinants of function (Figure 1). The latter include the impact of the surrounding extracellular tissue dynamics generated by changes, for example, in microbiome/pathogen content of the lung and gut, and/or environmental factors such as cigarette smoke responsible for inflammation and invasion by immunoregulatory cells designed to deal with CFTR imposed folding stress^{21,23}. The fifth level, the ‘Therapeutic’ Hallmark (Figure 1) we posit must embrace the first 4 hallmarks in order to redirect variant-specific PN and SN function(s) back towards a more normal CFFL (Pankow et. al. (2015), In Press, Nature). An understanding of the Hallmarks of CF disease outlined in 1–4 could trigger a generation of CF ‘smart’ therapeutics’ (Hallmark 5) that would impact currently unappreciated, yet likely key players that redirect the PN and SN to assuage mismanaged network connections and, importantly, do so by utilizing evolutionary conserved biological principles that got us to where we are today¹⁸.

We posit that by integrating the input revealed by CFFL Hallmarks 1–4 (Figure 1) with therapeutic management Hallmark 5, referred to as the CFFL ‘challenge’ we should be able to develop an information-based platform (Figure 1) that rationalizes the combined use of CF population-based phenotypes properties observed in the clinic (i.e., the natural history of disease progression and response to therapeutics) and ‘personalized genotypes’ emerging from on-going precision-medicine based genomic sequencing efforts. Together, such insights will help promote therapeutics that takes into account interpersonal variability so as to more effectively manage the underlying PN and/or SN defects responsible for disease progression. Such an information database could help explain and ease the burden of health care specialist issues confronting the often diverse and confounding modes of clinical presentation and puzzling variability in therapy responses.

CF Hallmark 1: Managing the fold through the PN

In a perfect world, the ideal approach for variant CFFL resolution to the norm would be to simply replace³⁰, or more recently, reengineer by gene editing the defect found in each CFTR variant in the CF population⁷. While gene editing holds promise, it faces identical problems to those currently plaguing gene replacement therapy^{31,32}—the need to correct the problem at the level of the stem cell niche (that turns over with time), an approach that must be implemented in fully developmentally programmed, but catastrophically failing organ systems that are in the midst of multiple biological crises^{23,33}. We leave the utility of this approach for others to debate³⁴.

To address the PN coupled SN problem in terms that reflect the root cause of disease, i.e., CFFL mismanagement, we first need to understand the impact of the primary coding sequence defect of each CFTR variant at the level of the cellular machinery, referred to as proteostasis^{2–4,21,23,35–45}. The concept of a ‘proteostasis network’ (PN)³⁵ was originally proposed to provide a unifying paradigm to bring together principles of chaperone-mediated protein folding and ubiquitin-proteasome system (UPS) mediated degradation with the role of stress signalling pathways under one ‘umbrella’. The PN coordinates folding, stability and degradation (e.g., the landscape) by maintaining a dynamic functional balance within the entire proteome so as to keep cells/tissues in a physiologically healthy status. As such, the purpose of the PN is to provide support pathways for normal proteome function and, where necessary “first aid” for a damaged proteome^{3,4,8–14,29,46}. Proteostasis is built on ancient and conserved rules that emphasize that there is no such thing as a ‘wild-type’ sequence, but rather a collection of evolving variants that must be continuously managed to optimize function for survival and fitness¹⁸. From an evolutionary perspective, proteostasis pathways have been considerably amplified and specialized to facilitate the expanding complexity of the protein fold found in higher eukaryotes, particularly in response to compartmentalization^{4,18,47–50}, and therefore represent an unparalleled opportunity to ‘use biology to fix biology’, much as the immune system adapts to evolving pathogen challenges, such as *Pseudomonas* species invasion in the lung, to optimize its defensive function.

Proteostasis operates as an ensemble of components consisting of molecular chaperones and proteolytic systems (proteases, the UPS and membrane compartmentalized autophagic and lysosomal pathways), as well as membrane trafficking factors that continuously adjust cargo

and compartment structure-function relationships required for cell and tissue health^{10,51,52}. Molecular chaperones can operate individually or as co-operating chaperone/co-chaperone machines (networks) to acquire and/or maintain the native three-dimensional (3D) conformation of the proteome in response to the environment^{10,51,53,54}. Recent years have seen major advances in our understanding of the basic mechanisms of proteostasis-assisted protein folding dynamics and its impact on the folding 'landscape' or 'energy funnel'^{55–57}, a theoretical biophysical concept that attempts to judge the impact of energetics on protein fold stability and function in health and disease^{4,18,58}.

Work-in-progress for understanding of the role of proteostasis in CF has come largely from studies focussed on the role of PN components in managing the stability and trafficking of the F508del-CFTR variant^{3,4,23,25,36,37,41,59–66}, although our knowledge still remains in its infancy. Known roles for heat shock cognate (Hsc) and heat shock protein (Hsp) (Hsc/p70) and Hsp90 chaperone/co-chaperone folding systems^{3,36,67–69}, small heat shock proteins (sHsps)^{41,61} as well as the UPS^{61,70–73} and the autophagic/lysosomal down-regulation pathways^{25,74–76} likely represent the tip-of-the-iceberg for understanding the CFTR PN management systems supporting function and contributing to disease (Figure 1)^{23,77}.

An understanding of the PN pathways impacting the stability and function of each CFTR variant to achieve a more fundamental understanding of the druggable PN and SN will require systematic application of emergent mass spectrometry (MS) approaches^{3,68} (Pankow et. al. (2015), In Press, Nature). MS offers an unprecedented opportunity to rigorously quantitate the cellular proteome (the protein composition of the cell) and their interactions in similar vein to that of sequencing/microarray efforts that allow us to elaborate the genomic, transcriptomic and translasome (ribosome profiling) environments of each cell type in health and disease^{9,78–81}. Whereas genomics, transcriptomics and translasome offer 'birds-eye' views of differences in healthy and disease states, MS offers an opportunity to study something far more important, the composition of the basal protein PN and SN states that directly or indirectly differentially influence normal and/or CFTR variant function defined by local, proteomic environments^{82–88}. Furthermore, MS technologies have the important capability to absolutely and precisely quantify cellular protein levels and protein-protein interactions (Figure 1, Hallmarks 1 and 2) of the PN and SN as shown recently for F508del-CFTR^{2,3} (Pankow et. al. (2015), In Press, Nature). For example, we have shown F508del is captured in a 'chaperone trap'^{3,89}, a state proposed to trigger CF disease reflected by the altered CFFL. The chaperone trap concept supersedes the more simplistic and presiding view that it is the variant that triggers disease, emphasizing the primary importance of the CFFL and the PN coupled SN to understand disease and its progression in the clinic. In fact, it is the *interaction* of each CFTR variant with its PN/SN that causes the diseased state.

In addition to identifying the proteome and interaction networks, MS can quantitatively determine the 'epi'-proteomic modifications (similar to the 'epi'-genetic modifications that influence genome function) including phosphorylation, glycosylation, methylation, acetylation, sumoylation and ubiquitination, among others, all adducts that significantly contribute to the CFFL of each CFTR variant responsible for cell and tissue (patho)physiology^{90–93} (Pankow et. al. (2015), In Press, Nature). MS, particularly the new quantitative, multiplexed technologies such as provided by tandem-mass-tag (TMT) MS

(TMT-MS) approaches, among others⁹⁴, allow us to perform hypothesis-driven experiments to develop and understand clinical endpoints found in the inherited CFTR variants, their response to the daily ‘burden’ of environmental factors (including pathogens, pollutants, and diet), and of course, responses to therapeutics across the entire timeline of disease progression. MS sampling of either single and/or multiple conditions or time-points provides ‘deltas’ that allow us to, in an unbiased manner, interrogate, for example, the specific PN/SN-driven CFTR variant changes, potentially leading to the identification of candidate (e.g., physiologic, bioremediation and/or ‘rehabilitation’) biomarkers to drive therapeutics^{2,3,89,95–99} (Pankow et. al. (2015), In Press, Nature).

CF Hallmark 2: Making connections-the CF social interaction network (SN)

It is important to acknowledge that in the epithelial cell and in the context of a tissue the role of CFTR is more than just that of the isolated ion channel reconstituted in its purified form or as subdomain fragments in the test tube, or even as an intact channel in reconstituted lipid bilayers. CFTR is by nature a social beast. It is a hub in a community effort of the SN involving complex cell and tissue physiologies (Figure 1) (Pankow et. al. (2015), In Press, Nature) whose connections in different tissue environments become crucial to understand from genomic, proteomic and functional perspectives. Out of their physiological context, many biochemical/biophysical properties of CFTR have reduced value, particularly in CF disease, given the extensive contribution of the PN and the corresponding SN in the management of cell health³⁵ (Pankow et. al. (2015), In Press, Nature).

In addition to the proteostasis management ‘cloud’ surrounding CFTR in the living cell¹⁸, CFTR has in addition been reported to regulate a significant number of other SN components including those connecting epithelial ion channels/ transporters, such as the epithelial Na⁺ channel (ENaC), several SLC26 transporters (namely A6, A8 and A9 transporting both chloride and bicarbonate), the voltage-gated potassium channel (KvLQT1), the aquaporin 3 (AQP3) water channel and the calcium-activated chloride channel Anoctamin 1 (Ano1/TMEM16A), among others^{100–109}. These interactions are sensitive to multiple signalling pathways¹¹⁰ that likely impact both normal and perhaps differentially, CFFL. Moreover, CFTR interacts with and is regulated by, in unknown ways, the cytoskeleton⁹⁵, numerous folding and trafficking components^{2–4} (Pankow et. al. (2015), In Press, Nature), and plays a number of additional functions promoting liquid movement across the surface epithelium and airway dehydration^{111,112}, mucus secretion^{109,113}; fluid secretion by submucosal glands¹¹⁴, prevention of mucosa acidification^{115,116}, exocytosis/ endocytosis²⁵, overall lung homeostasis¹¹⁷, mucosal immunodeficiency¹¹⁸, CFTR-related inflammation¹¹⁹; and CFTR-dependent epithelial cell differentiation^{120,121} as evident in the wild-type and F508del CFFLs (Pankow et. al. (2015), In Press, Nature). Thus, CFTR in health and, likely, in disease where protective/bypass pathways may become activated to minimize the impact of unfavourable CFFL on human pathophysiology²³ become paramount to understand. Understanding such social contacts, likely unique for each CFTR variant and cell type, would provide a more rational basis for development and application of personalized clinical management profiles to effectively improve patient health span.

Despite the importance of PN and SN efforts to date ^{122–125}, we still largely lack a systematic, quantitative and dynamic understanding of their role in management of with normal CFTR and CF-causing variants in different cell and tissue types, in different patients, and in response to aging and the extracellular environment (including pathogen altered microbiomes). CFBE41o-lung cell lines expressing the F508del variant have recently been characterized from an MS perspective (Pankow et. al. (2015), In Press, Nature) given their general use by the field as a common platform to study CF pathophysiology and its response to therapeutics. Such cell-based, overexpression models can be useful to detect potential underlying general principles that could be operational within complex proteome networks in the patient, particularly given the very low abundance of CFTR expression in human primary tissue when expressed under control of endogenous promoters. Because CFBE41o-cells clearly do not recapitulate human tissue environments, it will also be essential to explore the role of the PN and SN for different CFR variants in, for example, tissue-derived primary human bronchial epithelial (hBE) cells obtained from CF patients and grown on transwell environments ¹⁹, in organoid cultures derived from rectal and lung biopsy ¹²⁶, and/or in tissue explants.

To overcome the low abundance expression of CFTR in these primary tissue applications, the use of rapidly evolving advanced and sensitive multiplexed TMT-MS approaches will help to provide improved statistical confidence of datasets. Multiplex technologies will also allow us to interrogate the common and/or divergent features of the same or different CFTR variant interactions derived from different patients under rigorously controlled analytic conditions. As such, PN coupled SNs could provide explanations for phenotypic differences in patients with the same CFTR variant and why current therapeutics have highly variable responses among different patients with the same CFTR genotypes. CFFLs resulting from such studies, if appropriately statistically powered, could point towards broad cross-sectional therapeutic approaches. Moreover, interventions tailored to a particular PN coupled SN ‘theratype’⁷ could lead to ‘tailored’, i.e., personalized therapeutics, so as to effectively improve management of clinical disease by acting at its root cause⁵.

CF Hallmark 3: Defining CF functional liabilities and probabilities

To understand mechanisms that extend beyond the protein-protein interactions dictated by PNs and SNs (Hallmarks 1 and 2), we need to address the physiological consequences of such interactions—the CF functional liabilities and probabilities contributing to the CFFL¹²⁷ (Figure 1). Liabilities are the disease phenotypes contributed by each CFTR variant in a given cell, tissue and patient; probabilities encompass the impact of each CFTR variant on disease onset and progression in response to the environment. Among the powerful tools available to assay such processes are those that use a ‘functional genomic’ approach in the physiological context of intact living cells. These include quantitative high-content microscopy (HCM) and related imaging techniques ^{128,129} that measure normal and CFTR variant response to intervention with potential biological (gene) and/or chemical perturbants. Such studies can add considerable insight to genomic and proteomic efforts to help localize the role of PN and SN components in the context of complex compartmentalized trafficking pathways characteristic of the individual CFTR variants using both biased and unbiased high-content screening (HCS) methods ^{123,130}. For example, one study ¹²⁵ took a candidate

gene (biased) approach and overexpressed ~450 cDNAs fused to the halide-sensitive YFP (yellow fluorescent protein) marker in order to identify factors promoting rescue of F508del-CFTR to the plasma membrane in non-epithelial HEK 293 cells. Among the 9 top hits from this screen, one (STAT1, Signal Transducer and Activator of Transcription 1) could rescue F508del-CFTR function. Taking another candidate gene approach¹²⁴ focussed on ~750 kinases and associated signalling proteins reflecting the intense role of phosphorylation in CFTR function, 20 novel suppressors of F508del maturation were identified, notably the signalling pathways triggered by fibroblast growth factor receptor 1 (FGFR1). The authors inhibited FGFR1 in intestinal organoids derived F508del/F508del mice with the FGFR1 antagonist SU5402¹³¹ and observed rescue of F508del-CFTR. In a third candidate gene approach, the histone deacetylase HDAC7 was found to play a central role in rescue of F508del that could be mitigated by the HDACi SAHA⁹³ (Pankow et. al. (2015), In Press, Nature).

In contrast, to the above candidate change approaches, an unbiased high-content siRNA screen¹²³ focussed on finding new drug targets for CF by identifying genes that are able to down-regulate the activity of ENaC, which is excessive in CF and the current target of 'bypass' therapeutic approaches¹³². This study used an automated live-cell assay with human lung epithelial cells¹³³, which were exposed to ~17,000 different small interfering RNAs, designed to reduce the function of >6,000 different genes in the genome by HCM. Among the more than 700 genes that enhanced the function of ENaC reflecting its PN/SN environment, diacylglycerol kinase isoform *iota* (DGK ι) emerged as a promising drug target for CF. Indeed, chemical inhibition of DGK ι led to normalization of both sodium and fluid absorption in CF airways to non-CF levels in primary human lung cells from CF patients. This is an excellent example of 'using biology to fix biology'. Indeed, by manipulating DGK ι , ENaC activity and dehydration were brought to *physiological* levels, whereas *blocking* ENaC would 'flood' the airways with water, thus going from CF to another pathological condition, i.e., pulmonary edema.

Robust HCS platforms are now in place to determine the PNs and SNs of normal and F508del-CFTR¹³⁰ and therefore in principle applicable to all CFTR variants. In general, analysis of SN physiologic dynamics¹³⁴ holds great promise for deepening our understanding of the multiple variables contributing to disease progression in the lung, as a current primary focus, but also the co-morbidities such as CF-related diabetes¹³⁵, pancreatitis¹³⁶, bone disease¹³⁷, or defective spermatogenesis¹³⁸ that co-evolve along the patient aging timeline in response to increasing proteostasis challenges in managing not just CFTR but the collapsing proteome^{10,22,35,78,79,139-141}.

CF Hallmark 4: Embracing the CFTR lifestyle-the Environment

The environment counts whether you are young or old, whether you are studying the impact of CFFL in the lung, intestine and/or pancreas, and/or whether you are facing an extracellular challenge such as occurs in exacerbations in response to, for example, pathogen load and/or cigarette smoke. What are the metrics that we need to develop to help us advance in our understanding of the CFFL in variant disease states and their response(s) to the environment? While a genomic (the inherited genome, epigenetic marks and the

transcriptome, traslasome) and proteomic (MS) reads will provide rigorous bottom-up views of the cell-autonomous cellular environment influencing the first 3 Hallmarks outlined above and in Figure 1, the goal of the CFTR2 effort⁷ is more of a top-down perspective to provide the necessary personalized or ‘precision’ medicine (<http://www.nih.gov/precisionmedicine>) baseline for calibrating clinical onset and progression, and the impact of therapeutics^{5,7} for each CFTR variant. In a simplistic form, this has led to the ‘theratype’ concept where a given variant can be classified according to a particular response to existing therapeutics and/or ‘small molecule’ drug-like compounds currently in development. However, there is considerable room for improvement, expansion and refinement of our understanding of meaning of a ‘theratype’. For example, contributions to understanding and redefining the theratype concept may result from the global OMIC analyses that include genome wide association studies (GWAS), RNAseq, CHiPseq, epigenetic chromatin mark monitoring, traslasome, where genes with potential ‘altered’ expression and/or function can be defined by bioinformatic (meta-analyses) of multiple studies in healthy and disease-associated states¹⁴². In these approaches, patterns and/or mechanisms underlying disease phenotypes reflecting known gene functions (e.g., inflammation, defence response, etc.) can be inferred (Pankow et. al. (2015), In Press, Nature) to provide a new definition for clustering and targeting variant theratypes. Many challenges remain. Indeed, recent comprehensive reads of whole-genome differences from thousands of ‘normal’ individuals reveal an unappreciated diversity in SNPs, epigenetic marks^{143–145}, and links between regulatory DNA and target genes^{146–151} that now confound our interpretation of past genomic studies such as GWAS. Indeed, while genomic studies do start to fill an intellectual void in our understanding of the potential variables contributing to disease onset and progression, such results are largely correlative¹⁵², and have limited utility to define a causal relationship reflecting the impact of particular gene product on CF pathophysiology¹⁵³.

A more definitive and absolute quantitative approach to assess the impact of the environment on the CFFL is to use MS to generate, for example, CF whole-cell proteomic and/or interaction signatures or HCM to define PNs and SNs and thereof disease intensity and progression (Pankow et. al. (2015), In Press, Nature). It is now possible to perform quantitative whole-cell proteome analysis using microgram quantities of biopsied samples that could give us unbiased reads of the protein content and CFTR interactions in the cell and, potentially the requisite PN and SN pathways (Figure 1, Hallmarks 1 and 2) contributing to CFTR variant functional liabilities and probabilities (Figure 1, Hallmark 3) in response to the development, the changing environment and aging. Moreover, MS can also define direct responses to therapeutic intervention, by tapping into the very events we hope to correct at the level of proteome function^{154,155} (Pankow et. al. (2015), In Press, Nature). Thus, knowledge of changes in each CFTR variant’s PN and SN and their corresponding CFFLs could provide a means to (1) identify unanticipated ‘debilitating’ and ‘rehabilitation’ targets, (2) identify specific phenotypic characteristics of CFTR variants, namely those requiring the same type of restoration efforts distinct from the current more conventional classification system based on metrics of protein folding (band B stability in the ER, band C/B ratios reflecting trafficking through exocytic and endocytic pathways), and function such as ion channel properties^{6,7} and, (3) differentiate between environmental

factors contributing to a specific tissue pathophysiology and more general physiological perturbations common to all organ environments.

CF Hallmark 5: Therapeutic intervention

Current high-throughput screening (HTS) efforts attempt to target CFFL by small molecules that correct channel function in heterologous cell-based culture models through use of chemically diverse libraries of compounds involving unbiased, ‘shot-gun’ approaches. Through these efforts, significant advances have been made in the identification of putative CFTR-folding modulators (pharmacological chaperones) including the ‘correctors’ VX-661 and VX-809/Lumacaftor and ‘potentiators’ such as VX-770/Ivacaftor^{5,156–158} that either alone and/or in combination hold promise for treating CF. The FDA approval of Ivacaftor for multiple G551D like phenotypic variants including (G178R, S549N, S549R, G551S, G1244E, S1251N, S1255P and G1349D)^{159,160}, found at the cell surface with gating defects^{161,162}, and the FDA-approval of a combination of Lumacaftor and Ivacaftor for treatment of F508del¹⁵⁶ are examples of successful application of these technologies. Yet, we are left with many challenges in terms of disease management given, for example, the modest impact of Lumacaftor-Ivacaftor combination on mitigating F508del disease phenotype¹⁵⁶ or Ivacaftor improving lung function in patients over 18 yrs with Arg117His variant but not in children¹⁶³. Even if operating directly^{27,58,158,164–166}, such pharmacological chaperones may likely only perturb a transient state of the variant CFTR folds that contribute to the dysfunctional CFFL. Moreover, the ability of both ‘caftors’ to correct multiple CFTR variants suggests that they likely operate indirectly through the activity or influence of the PN or the SN. From a chemical perspective, of course, they are not perfect in design. Moreover, not unlike the chemical perturbation that arises from amino acid substitutions in the polypeptide chain found in inherited disease, such compounds will also harbour physiologically perturbing side-effects and associated liabilities/probabilities to correction^{167,168}. Because the above cell-based HTS approaches largely operate in a knowledge vacuum, by ignoring many of the variables raised in Hallmarks 1–4 (Figure 1), the consequence is that we bring into the clinic often sub-optimal molecules with modest efficacy that take will years to optimize through biased and perhaps ill-suited structure-activity relationship (SAR)¹⁵⁶ efforts, followed by trial and error with diverse patient CFFL phenotypes who are already in a stressed state not necessarily even considered during drug development. Put in another way, we try to smooth the edges of the square peg to fit it into a round hole.

An alternative to generic heterologous cell-based HTS assays is to directly stabilize *in vitro* CFTR variant affected domains, such as the NBD1 domain harbouring F508del either as an isolated fragment, or as reconstituted purified protein in artificial lipid bilayers^{164,169}. The goal here, for example, is to identify small molecules that chemically perturb activity, for example, based on a reporter assay that measure a change in activity (such as ATPase for NBD1 domain or chloride flux for full-length CFTR in the bilayer), or a change in structure, such as thermal denaturation/renaturation kinetics of purified NBD1, as a surrogate metric of biological impact. The *in vitro* approach, while conceptually simplistic in design, has numerous obstacles to success particularly with the realization that we are not necessarily targeting a ‘fixed’ folded structure *in vivo*, but a structure that is intrinsically

conformationally mobile in response to the variable PN and SN in different cell compartments, and in multiple cell and tissue specific environments that can often be difficult to access in the patient. Given these variables, it is no surprise that most clinical candidates obtained through HTS or such targeted approaches fail in about 50% of Phase II trials¹⁷⁰ and 66% of Phase III trials¹⁷¹. Such high rates of failure stem from an inadequate understanding of how disease is managed by the PN and SN physiology responsible for individual variability of the human population¹⁷².

Thus, it now becomes imperative to understand the status of a therapeutic agent as it relates to normal and variant CFFL as highlighted in Hallmarks 1–4 to generate a more comprehensive understanding of therapeutic management (Figure 1). Here, chemical therapeutic management of CF should be viewed as a multiplexed problem that ultimately must realign the CFFL biology as a ‘collective’ or ‘community’ effort to effectively mitigate function in disease^{4,5,21} (Pankow et. al. (2015), In Press, Nature). Rehabilitation of, not confinement into, a specific targeted ‘drugged’ state, is a potential key to improved therapeutics. This conclusion is reinforced by recent observations that it will likely take multiple ‘corrector’ therapeutics to achieve a reasonable level of folding, stability and function to recover the normal CFFL physiology to have an impact on clinical disease management^{28,168,173–177} (Pankow et. al. (2015), In Press, Nature). By increasing our knowledge of the impact of the PN and/or common and specialized SN components contributing to the activity of each CFTR variant, we anticipate discovery of high-profile drug candidates that could mitigate CFTR metastability and/or initiate repair of variants^{10,18,51–53,78,178,179}. For example, molecules contributing to this novel chemical space are beginning to emerge in CF as well as other disease venues through a deeper understanding, in particular, of the PN^{23,78,180–186}.

We now posit that therapeutic correction of the fundamental CFFL problem driving CF disease should not be through direct binding to CFTR variants—rather, mutant CFTRs should be left ‘free to manoeuvre’ through their many structure-function relationships by adjusting the activity of the maladapted CFFL management pathways shown recently²³ and/or by nudging their activities towards more functional PN and SN sensitive CFFL states (Pankow et. al. (2015), In Press, Nature). A concern frequently raised for PN therapeutics (and potentially applicable to ‘SN therapies’) is that the lack of ‘specificity’ for the defective target such as a variant CFTR may bring about many possible adverse effects considering the centrality of the PN or an SN component in maintaining the functional proteome. In contrast, as shown in the HDACi SAHA study⁹³ and, more recently, for the F508del correction network using proteomics to validate all changes in the CFFL (Pankow et al (2015) Nature, In press), the real goal of PN (and likely SN therapy) is to modulate expression only slightly. As the PN operates as a ‘team’ to impact the function of the defective protein in when out of balance with its partners, a correctional adjustment for a *specific variant CFFL* can be significant in response to appropriate PN adjustor. All other proteins harboring ‘normal’ landscapes will unlikely be affected, as they are already ‘wild-type’ and likely robust to further PN modification as we have previously suggested¹⁵. In fact, the PN (and potentially SN therapies) are likely to have lower side effects than any other drug currently in use when used in a calibrated approach to adjust the needs of the

aberrant CFFL to the local environment-as nature would perform over time during self-managed stress responses that occur on a daily basis^{23,187}. In other words, the hero/heroine (the therapeutic) is only as good as the value of the supporting cast found on the set. Unfortunately, the supporting cast essential to ‘make it so’ is often only poorly acknowledged, if at all, at the end of current CF therapeutic scripts. This needs to change.

The CFFL Challenge

We now suggest an urgent need to support the Hallmarks outlined in Figure 1 as the clock is ticking on human suffering. This will allow us to identify and characterize key PN components (Hallmark 1) mediating SN connectivity (Hallmark 2) that drives biology of CFTR variant functional liability and probability (Hallmark 3) and its many responses to the environment (Hallmark 4) to achieve therapeutic efficacy (Hallmark 5), in what we refer to as the ‘CFFL Challenge’ (Figure 1). Such an integrated understanding of CF disease states provide us with a refreshing legacy for the upcoming generation of CF patients and will allow us for the first time to systematically use this knowledge base to more rationally understand and drive drug discovery. Such an approach will give us for the first time a deep sense of the personalized, yet societally stratified, clinical genotype-to-phenotype-to-theratype disease relationships. They should pinpoint corresponding corrective measures that will be likely necessary to leverage our way to ‘smart’ disease management in the clinic (Pankow et. al. (2015), In Press, Nature). Indeed, the theratypes⁷ theme that now highlights our current appreciation of the differential CFTR variant impact on clinical progression as (individualized ‘patient codes’), is likely only the tip-of-the-iceberg of new thinking that will be required to help us define, redefine and reassess effective disease management in the context of the Hallmarks outlined in Figure 1. This more ‘global’ view of CF as ‘learning’ exercise on how to manage the CFFL unique to each patient (Figure 1) should allow us to assemble a more comprehensive view of CF biology from nature’s perspective, and the natural selection processes that got us to where we are today, for better or worse, that require redirection. The sooner we start support of the CFFL Challenge (Figure 1), the sooner we will get there.

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Abbreviations

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFTR2	CFFL, cystic fibrosis functional landscape
SN	social network
ER	endoplasmic reticulum

MS	mass spectrometry
HCM	high-content microscopy
HTS	high-throughput screening
PN	proteostasis network
UPS	ubiquitin-proteasome system

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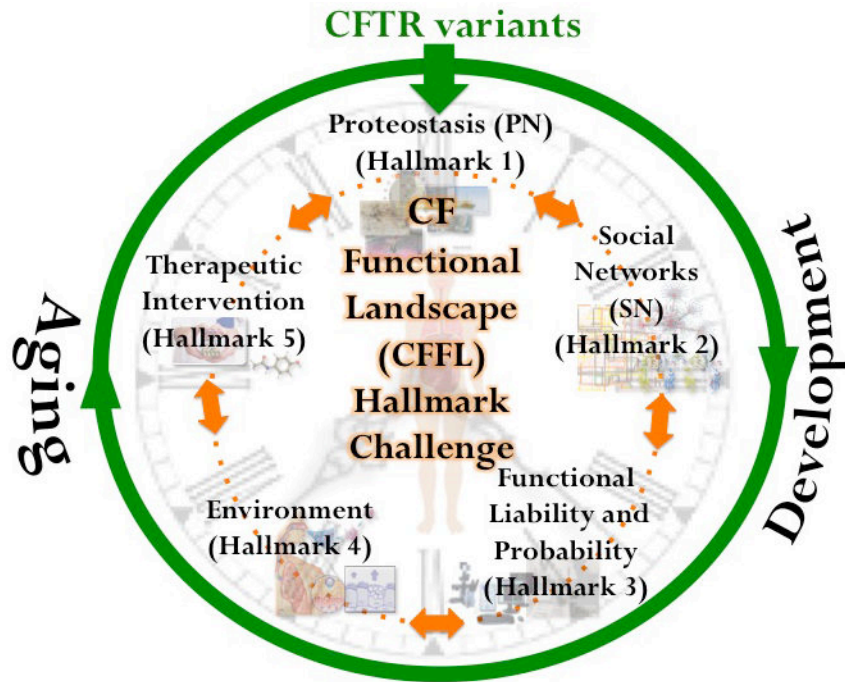


Figure 1. The CF(TR) Functional Landscape (CFFL) Hallmark Challenge

The proteostasis network (PN)-coupled social network (SN-) of CFTR (Hallmarks 2 and 3), constitute the CFTR Functional Landscape (CFFL) which is optimized by biology so that a given cell, tissue and individual displays a particular functional genotype to phenotype relationship that occurs as healthy for wild-type CFTR, or as ‘variations on a theme’ in CF disease for each CFTR variant in each patient, providing an associated ‘Functional Liability and Probability’ (Hallmark 3) and its many responses to the environment (Hallmark 4) to achieve therapeutic efficacy (Hallmark 5), in what we refer to as the ‘CFFL Challenge’ Environment.