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SYSTEMS BIOLOGY AND THE NEW OMICS

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What is systems biology?

While Molecular Biology (a science born in the 1960s by the combined efforts of Biochemistry and Genetics from the discovery that the DNA molecule is the support of genetic information) focuses on characterizing the molecular components of living cells, Systems Biology involves predicting how the collective interactions of the cellular components give rise to the physiology and pathology of a given system (1). Since its beginning around 2000, Systems Biology has thus been revolutionizing the biological sciences, by exploring the concept that "*the whole is greater than the sum of the parts*". Advances in Systems Biology have relied very much on the technological developments derived from the Human Genome Project (HGP), the so-called "Omics", that allow the identification of the total content of a given set of molecules (2). Indeed, the Omics approaches have flourished in the biomedical field and cystic fibrosis (CF) is not an exception. The use of genomics, transcriptomics, proteomics (and interactomics), functional genomics, and also metabolomics have contributed significantly to the understanding of human disease mechanisms and to the identification of novel biomarkers for prognosis and diagnosis (3). Herein, we review key studies using these approaches, their major findings, and the challenges ahead.

Transcriptomics

Why study the CF transcriptome?

Transcriptomics is the measurement of all RNA transcripts in a given biological model and is a way of elucidating the contribution of global gene expression to Cell Biology at a systems level (Figure 10.1). It emerged during the 1990s with the development of microarray technology, which allowed for the simultaneous detection of thousands of gene transcripts. The first use of the term "microarray" (a lab-on-a-chip with multiple probes that enable the simultaneous measurement of expression of thousands of genes from a given sample) (4) was followed closely by the first study to use a microarray covering a whole genome, albeit in a single-celled organism, yeast (5). Microarray measurement of human transcriptomes accelerated following publication of the first draft of the human genome (6), which made systematic identification, categorization, and detection of a majority of protein coding human gene transcripts possible for the first time. Pioneering studies of human transcriptomics used the extraordinary power of new bioinformatic techniques which had been developed in tandem to identify subgroups among superficially homogeneous breast cancer tumours based on their gene expression profiles. Such studies demonstrated that tumour classification based on transcriptomics could be more accurate

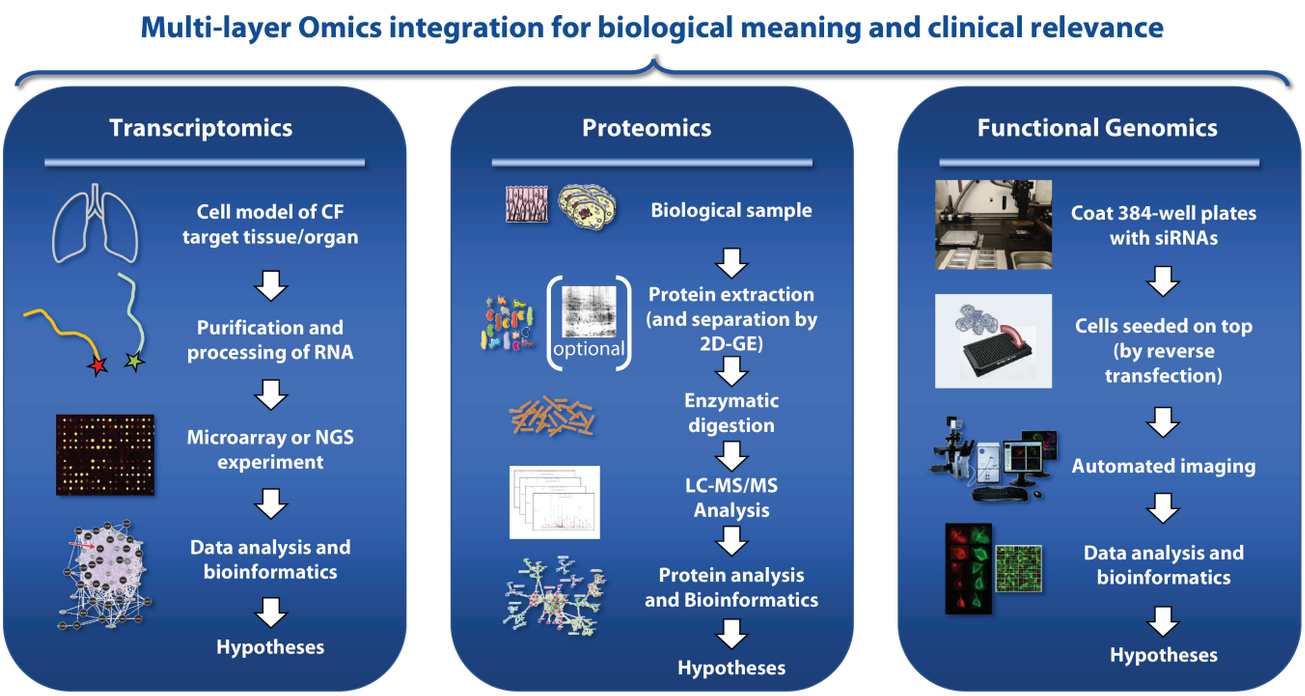

FIGURE 10.1 Prototypical experimental workflows in the transcriptomics, proteomics, and functional genomics of CF.

DOI: 10.1201/9781003262763-10

than classification based on either clinical or histopathological data (7), suggesting the utility of microarrays as a future tool in clinical diagnosis. Based on such promising advances, the application of transcriptomics to the study of other genetic diseases including CF was an enticing prospect. As a heritable disease caused primarily by mutations in a single gene, CF is superficially much simpler than cancer, but it also has much heterogeneity and underlying variation. For example, although more than 2100 different mutations have been reported in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (most presumed to cause CF), individuals homozygous for the most common disease-causing mutation, F508del, can have milder or more severe disease symptoms, and a wide range of outcomes. Such differences might be caused by genetic modifiers, as suggested, for example, by genome-wide association study (GWAS) data (8). There is therefore a need to clarify the hidden interactions underlying CF pathophysiology, which has made transcriptomics a vital tool in understanding CF disease in a Systems Biology context.

How has the CF transcriptome been studied?

A survey of the major findings of CF transcriptomics over the past 20 years reveals much heterogeneity in experimental approaches (see Table 10.1). Individual studies have made use of a variety of human cellular models, and also some animal models which,

TABLE 10.1: Summary of Most Relevant Transcriptomics Studies in the CF Field (in Chronological Order)

Study	Year	Cellular Model/Samples	Aim	Key Findings
Virella-Lowell et al. (11)	2004	CF bronchial epithelial cell line (IB3-1)	To determine CF-specific changes in gene expression in the context of IL-10 expression and *P. aeruginosa* infection	Up-regulation of ubiquitination enzymes and TNF pathway components, and down-regulation of protease inhibitors and protein glycosylation enzymes in CF.
Zabner et al. (16)	2005	Differentiated primary cultures of CF airway epithelia	To determine the impact of CFTR dysfunction on airway epithelial gene expression	CFTR dysfunction found to have little direct impact on airway epithelial gene expression in primary airway epithelial cell cultures used in this study.
Worgall et al. (25)	2005	Alveolar macrophages (AM)	To assess gene expression changes in AM in response to infection with early and late clinical isolates of *P. aeruginosa*, and *B. cepacia*	The majority of gene expression changes associated with inflammatory pathways and signalling systems.
Wright et al. (22)	2006	CF nasal respiratory epithelium	To identify differences in gene expression between DeltaF508 homozygotes with severe vs. mild lung disease, and CF and non-CF control subjects	Differences in CF lung phenotype associated with differential expression of genes involving airway defence, protein ubiquination, and mitochondrial oxidoreductase activity.
Verhaeghe et al. (12)	2007	CF foetal tracheal cell lines (CFT-2)	To investigate the molecular mechanisms underlying intrinsic inflammation in cystic fibrosis airways	Absence of functional CFTR at the plasma membrane leads to intrinsic AP-1 and NF-kappaB activity, resulting in a pro-inflammatory state.
Ribeiro et al. (15)	2009	Differentiated primary cultures of human airway epithelia	To determine how the macrolide azithromycin (AZT) modulates inflammatory responses in airways	AZT treatment led to increased expression of lipid/cholesterol genes and decreased the expression of cell cycle/mitosis genes, but its effects upon inflammatory genes and pathways were variable.
Ogilvie et al. (20)	2011	CF nasal and bronchial epithelium samples	To compare CF-related gene expression in nasal and bronchial epithelium, and determine the utility of nasal epithelium as a pre-screen for lung-directed therapies	CF bronchial and nasal epithelia were found to be transcriptionally distinct, and it was concluded that nasal epithelium is not a good surrogate for the lung. The most enriched pathway in CF bronchial epithelium was inflammatory response.
Hampton et al. (13)	2012	CF bronchial epithelial cells (CFBE41o-)	To determine if the F508-CFTR mutation induces a proinflammatory response in human airway epithelial cells	*P. aeruginosa* elicited a more robust increase in cytokine and chemokine expression in CFBE-wt-CFTR cells than with ΔF508-CFTR, contradicting the assumption that mutant CFTR induces a hyperinflammatory response in human airways.
Clarke et al. (18)	2013	CF nasal epithelial cell samples	To define a robust molecular signature associated with mutant CFTR expression	Up-regulation of genes involved in cell proliferation, and down-regulation of cilia genes were the most notable changes in CF. Small-scale meta-analysis of *in-vivo* studies yielded a molecular signature for CF.
Mayer et al. (10)	2013	CF bronchial epithelial cells (IB3-1) and PBMCs	To identify and validate novel dysfunctional processes or pathways driving the hyperinflammatory phenotype of CF cells	Network analysis of CF cell transcriptional responses to flagellin/IDR-1018 identified dysfunctional autophagy as the target of the peptide via modulation of upstream adenosine monophosphate-activated protein kinase (AMPK)-Akt signalling.

(*Continued*)

TABLE 10.1: Summary of Most Relevant Transcriptomics Studies in the CF Field (in Chronological Order) *(continued)*

Study	Year	Cellular Model/Samples	Aim	Key Findings
Stanke et al. (24)	2014	CF intestinal epithelial cell samples	Validation of the EHF gene as a modifier of CF phenotype	In F508del homozygotes carrying two rare EHF alleles, there was differential expression of genes altering protein glycosylation and trafficking, mechanisms enabling targeting of fully functional F508del-CFTR to the apical membrane of epithelial cells. EHF thereby modifies the CF phenotype.
Voisin et al. (14)	2014	Airway epithelial cells expressing F508del-CFTR (CuFi-1)	Evaluation of oxidative stress as a regulator of inflammation in CF	Under oxidative conditions, the transcriptome of CF cells reflects apoptotic transcript modulation.
Chesné et al. (30)	2014	Whole blood samples	Systematic analysis of blood mRNA profiles in CF, pulmonary hypertension, and COPD	Systematic analysis of blood cell transcriptome in patients with chronic respiratory diseases (CRD) identified common and specific signatures relevant to the systemic pathologies. TCF-7 and IL-7R were downregulated whatever the cause of CRD.
O'Neal et al. (29)	2015	CF lymphoblastoid cell lines	Identification of heritable traits associated with CF phenotypic diversity	Genes showing differential expression associated with lung disease severity were enriched for heritable genetic variation and expression quantitative traits.
Zeitlin et al. (23)	2017	CF nasal epithelial cells	To determine the effect of digitoxin on gene expression in epithelial cells of individuals with stable CF	Genes encoding chemokine/cytokine or cell surface receptors in immune cells were decreased in nasal epithelial cells at the higher dose of digitoxin, leading to pathway-mediated reductions in IL-8, IL-6, lung epithelial inflammation, neutrophil recruitment, and mucus hypersecretion.
Kormann et al. (31)	2017	CF whole blood samples	To identify genetic modifiers using RNA-seq based transcriptomic analyses in CF patients with mild and severe lung phenotypes	Global gene expression and enrichment analyses revealed that genes of the type I interferon response and ribosomal stalk proteins are potential modifiers of CF related lung dysfunction.
Polineni et al. (21)	2018	CF nasal mucosal samples	To identify novel CF disease-modifying mechanisms using *in vivo* CF airway epithelial gene expression and GWAS data	Transcriptomic analyses of CF airway epithelia, coupled with genomic (GWAS) analyses, highlight the role of heritable host defence variation in determining the pathophysiology of CF lung disease.
Gong et al. (19)	2019	CF nasal epithelial cell samples	Integration of genetic association and transcriptome data to identify contributing genes and tissues at CF modifier loci	Development of a colocalization framework called Simple Sum (SS) that integrates regulatory and genetic association information, and also contrasts colocalization evidence across tissues or genes.
Levy et al. (26)	2019	PBMCs from healthy donors cultured with plasma samples from CF patients	To determine disease-specific plasma-induced PBMC gene expression profiles from transcriptome data	Identification of distinct molecular signatures among CF patients, potentially enabling personalized prognoses, individualized treatment plans, and monitoring of treatment responses.
Ideozu et al. (27)	2019	CF blood plasma samples	To identify extracellular circulating miRNAs dysregulated in CF	Identification of 11 differentially expressed ECmiRNAs, and validation of their potential targets, including AGO1, DICER1, HMGA1, and MYC, which may be clinically relevant in CF.
Jiang et al. (28)	2019	CF neutrophils before and after therapy	To develop sensitive biomarkers that can be used to monitor pulmonary disease activity in CF	Differential expression of 136 transcripts and 83 isoforms when in neutrophils from CF patients before and after therapy. A machine learning approach was able to successfully separate CF flare samples from those taken from the same patients in convalescence.
Ideozu et al. (38)	2019	PBMCs from healthy donor stimulated with plasma from CF patients	To identify differentially expressed genes and miRNAs that characterize impaired immune responses triggered by CF	151 genes and 41 miRNAs were differentially expressed in CF and 285 genes and 7 miRNAs differed due to CF phenotype.

however, are not discussed here. Most studies focused on representing the target tissue most relevant to CF pathophysiology, namely the airway epithelium. Many studies have used immortalized cell lines derived from human CF tissues, of which many are available (9), including IB3-1, a CF bronchial cell line expressing F508del/W1282X CFTR (10, 11), CFT-2 cells expressing F508del-CFTR from foetal trachea (12), CFBE41o- cells from bronchial epithelium expressing F508del-CFTR (13), and CuFi-1, immortalized human airway epithelial cells expressing F508del-CFTR (14). The use of cell lines has allowed researchers to measure the primary transcriptomic consequences of CFTR deficiency (nearly always represented by the most common mutation in many populations, F508del) in

the absence of bacterial infection and the associated inflammatory spiral of CF lung disease. Other studies have attempted to more closely model the *in-vivo* airway epithelium by using primary cultures of airway epithelial cells (15, 16): such cultures, when induced to polarize by growing at an air-liquid interface (ALI), have been shown to closely approximate the transcriptome of *in-vivo* airway cells (17). However, the most realistic *in-vivo* models used by many studies are native tissues obtained by brushing or curettage from nasal or bronchial epithelium (18–23), or by rectal biopsy (24) of individuals with CF. Such samples are not limited to epithelial cell types, and thereby represent the *in-vivo* transcriptional profiles of intact tissues in all their complexity. Finally, because CF is not just a disease of the airways, studies on the effects of CF on immune function, or attempts to generate systemic biomarkers from transcriptomic data have also used alveolar macrophages (25), peripheral blood mononuclear cells (PBMCs) (10, 26, 27), peripheral blood neutrophils (PBNs) (28), lymphoblastoid cell lines (LCLs) (29) or whole blood samples (27, 30, 31).

Another further variable in studies of the CF transcriptome has been the technology used, with microarrays of various formats gradually being replaced in recent years by RNA sequencing, which allows for novel transcript discovery and has gradually extended the repertoire of detectable transcripts beyond those encoding proteins to include, for example non-coding RNA and micro-RNA transcripts, which may also play an important role in CF (32).

Is there a "Gene signature" in CF?

Early studies of the CF transcriptome focused on describing lists of differentially expressed (DE) genes and identifying functionally related gene clusters or perturbed cellular pathways associated with a lack of functional CFTR expression (Table 10.1). In IB3-1 cells expressing F508del/W1282X *CFTR*, genes encoding ubiquitin enzymes and TNF pathway components were found to be upregulated compared to cells expressing wt-CFTR, while genes for protease inhibitors and protein glycosylation enzymes were found to be down-regulated (11). Another study in CFT-2 foetal tracheal cells described upregulation of genes activated by NF-κB and AP-1 and concluded that gene expression in the CF cells reflected an "intrinsic proinflammatory state" (12). A study in another cell line, CuFi cells polarized on filters and grown at the ALI found upregulation of genes involved in inflammation, regulation of signal transduction and cellular survival, and downregulation of ribosomal metabolism, chromatin modification, and translational elongation genes (14). In human native tissues, which more closely reflect the *in-vivo* cellular environment, genes involved in protein ubiquitination, mitochondrial oxidoreductase activity, and lipid metabolism were found to be upregulated and airway defence and protein metabolism genes were downregulated in nasal epithelium from individuals with severe CF (but excluding those with symptoms of sinus or pulmonary exacerbation in order to minimize the potential influence of active local infections) (22), while in bronchial epithelial brushings from young individuals with CF, gene expression associated with an inflammatory response was detected (20). In a further study on native nasal epithelial cells, genes involved in regulation of cell proliferation and encoding ESR-1 targets were found to be upregulated, while genes encoding ciliary proteins were found to be downregulated (18).

Many of the studies described here differed in their basic conclusions or produced what seemed to be almost exclusive lists of differentially expressed genes. Some groups have therefore performed meta-analyses of independent CF microarray studies, by reanalysing published data sets uniformly and applying less stringent statistical cut-offs to produce longer gene lists suitable for downstream analysis of enrichment of pathways or functional categories such as Gene Ontology (GO) terms. Using this "exploratory" method of reanalysis, one meta-analysis of four datasets evaluating the effect of the F508del-CFTR mutation on human airway epithelial cell gene expression identified a consistent downregulation of the MHC class I antigen presentation pathway (33), and failed to identify consistent upregulation of inflammatory genes in CF cells. A meta-analysis of differentially expressed genes from human native nasal and bronchial cells proposed an "*in-vivo*" gene signature for CF airway tissues composed of 30 interconnected genes involved in processes such as inflammation, wounding, defence, and calcium binding (18). A more complex study by the same group included a variety of diseases and conditions along with studies of CF in a large-scale comparison of differential gene expression. Strong functional components of DE gene lists in CF related to injury and de-differentiation were identified, and many DE genes were common to CF and other inflammatory lung diseases and conditions, including chronic obstructive pulmonary disease (COPD) and smoking (34). This suggested that certain functionally significant components of CF-related gene expression profiles are shared with other inflammatory lung conditions and more general associated processes, although the specific association of mutant CFTR with a defect in differentiation has been supported by other studies (35). Another study detecting gene signatures in whole blood mRNAs from individuals with CF, pulmonary hypertension (PAH), or COPD, found a signature common to CF and PAH, and two signatures exclusive to each disorder (30). The CF signature confirmed the stimulation of the innate immune response during CF, but many of the individual genes from the signature were shared with DE gene lists found in PAH and COPD.

Several of the above-mentioned studies uncovered inflammatory gene expression signatures associated with CF cells. Such data have been used to investigate a longstanding controversy surrounding the potential existence of intrinsic proinflammatory gene expression in cells triggered by mutant CFTR, for which several alternative explanations have been proposed (36). Some transcriptomic studies of CF in cell lines have detected an increase in inflammatory gene expression which could not possibly be the result of responses to bacterial infection (11, 12, 14). However, other studies did not detect such a profile, or even found the opposite (13), leading to the conclusion that responses to CFTR deficiency may be dependent upon the specific cellular model or other experimental factors like the external milieu. Further data have located much of the exaggerated inflammatory responses to bacterial infection seen in mice to CD3+ lymphocytes (37), where CFTR is also expressed (albeit at very low levels), suggesting that the CF hyperinflammatory gene expression is at least partially located in cells circulating outside the epithelium. Two studies measuring transcriptomes of PBMCs from healthy individuals treated with blood plasma from individuals with CF of varying severity demonstrated that different gene expression profiles can be elicited in circulating immune cells depending on the grade of CF disease severity (26, 38).

Identifying CF disease modifiers

CF transcriptomics have been instrumental in the identification of gene modifiers of CF disease phenotypes including severity of symptoms arising from several affected organs. Differences in gene expression profiles between nasal epithelium from individuals with mild and severe CF were described in an early microarray study (22), where specific candidate gene modifiers of CF involved in airway defence and inflammation were identified, and significant upregulation of genes involved in protein ubiquitination and mitochondrial

oxidoreductase activity distinguished severe from mild samples. In another powerful study, 754 LCLs from individuals homozygous for F508del-CFTR were classified according to three CF phenotypes (severity, age of onset of persistent *Pseudomonas aeruginosa* infection, and meconium ileus) and subjected to global gene expression analysis. One novel gene modifier of CF severity was identified (*LPAR6*), and expression of genes in several functional pathways was associated with each phenotype under study, demonstrating that transcriptomic data alone was sufficient to identify CF disease modifiers (29). This was also the case for a study of peripheral blood leukocyte transcriptomes from 32 individuals expressing F508del-CFTR and classified as having milder or more severe CF (31). Here, gene expression data alone was used to identify genes of the type I interferon response and several ribosomal stalk proteins as potential modifiers of CF disease severity.

A fuller and more precise understanding of the potential functional significance of such data may require integration of other sources of data, however, as in a study (24) which stratified transcriptomes of rectal biopsies from CF individuals expressing F508del-CFTR by genotyping *EHF*, a transcription factor gene identified in a genome-wide association study (GWAS) as a modifier of CF severity (39). Rare *EHF* alleles were associated with upregulation of genes involved in protein glycosylation and membrane protein trafficking that were related to modification of F508del-CFTR activity, rescuing some residual function of the mutant protein, and this association was found in individuals with milder CF disease phenotypes. Two other studies, both measuring gene expression in nasal epithelium, have also integrated transcriptomic and GWAS datasets to identify modifiers of CF disease severity. In the first, it was shown that heritable traits linked to increased expression of non-*CFTR* genes, particularly those regulating inflammatory responses to environmental *stimuli*, play a key role in CF lung disease severity (21). In the second, transcriptome data were integrated with GWAS data in a large-scale study to determine whether a novel *meconium ileus* modifier *locus* impacts gene expression in a tissue- or organ-specific manner (19). As research in CF focuses more on implementing personalized medicine, identification of the contributing organ and understanding of tissue specificity for gene modifiers, as exemplified by the latter study, will enhance the utility of such approaches.

Biomarkers for disease or treatment monitoring

One of the most promising potential applications of transcriptomics in CF research is the development of biomarkers to monitor disease progression or responses to therapy. The effect of an anti-inflammatory peptide, IDR-1018, on gene expression in airway cells and PBMCs (10) was used to identify dysfunctional autophagy as the target of the peptide which modulated the CF-related inflammation. In another study, gene expression in nasal epithelium of individuals with CF following administration of digitoxin led to suppression of mRNAs encoding chemokine/cytokine or cell surface receptors in immune cells, leading to pathway-mediated reductions in IL-8, IL-6, lung epithelial inflammation, neutrophil recruitment, and mucus hypersecretion (23). In another biomarker study, RNA from circulating neutrophils was sequenced before and after therapy for CF pulmonary exacerbations (CFPE), and the results demonstrated the potential of machine-learning approaches to classify disease states and developing sensitive biomarkers which might be used to monitor pulmonary disease in CF (28). Finally, a recent study identified differential expression of extracellular circulating microRNAs in CF which may be used in the future as valid biomarkers of CF disease with functionally relevant targets (27).

Knowing how distinct mutations in the *CFTR* gene alter the transcriptome has provided important insights into compensatory mechanisms in diseased cells, which has led in turn to identification of key processes in disease progression. Such information has been vital in understanding how CFTR deficiency affects cell biology, both as a primary defect and in the context of CF disease. Furthermore, the data provided by transcriptomics have been used to identify biomarkers of responses to bacterial infection or treatment, and to pinpoint targets for future treatment strategies. However, the true benefits of a Systems Biology approach depend upon the integration of transcriptomics with other approaches including proteomics, which can provide complementary data from another point of view, thereby broadening our overall understanding of CF as a dynamic system.

Proteomics

What is proteomics?

Proteomics has been applied to the large-scale study of the proteome, a term coined in 1994 that blends the words "protein" and "genome" to identify the full set of proteins that are present (or synthesized) by a certain cell type or organism under a specific set of conditions at a given time. Introduction of two-dimensional gel electrophoresis (2D-GE) in the 1970s was the first step in the advent of proteomics, due to its ability to resolve complex mixtures of proteins, but most of the advances in the identification and characterization of proteomes have relied on the development of mass spectrometry (MS) technologies and also on bioinformatics, without forgetting protein fractionation techniques.

Robust proteomics studies typically rely on three major steps: (1) a method that allows the fractionation of complex protein mixtures; (2) the use of a mass spectrometer that is able to provide data that can be used to identify individual proteins; and (3) bioinformatics tools to analyse and organize/assemble the MS data. Fractionation of complex protein mixtures has been classically done with the use of 2D-GE, but advances in MS instrumentation have allowed gel-free approaches — especially due to the use of tandem approaches that couple liquid chromatography (LC) to MS. Development of novel instruments has been the strong driving force in the field allowing assessment of not only protein presence but also their quantification, localization, post-translational modifications, isoforms and molecular interactions. However, and when compared to genomics and transcriptomics that have propelled medicine due to the availability of single-molecule DNA sequencing, proteomics has lagged behind because MS does not truly sequence a protein but rather classifies it. Nevertheless, it has significantly contributed to understanding molecular mechanisms underlying normal and disease phenotypes and to identification of critical diagnostic and prognostic biomarkers.

The most widely used approach for protein identification is termed "bottom-up" proteomics that uses proteolytic digestion of proteins prior to their analysis by MS (Figure 10.1). This approach can achieve high-resolution separation but gives a low percentage coverage of the protein sequence, losing significant information about post-translational modifications and isoforms resulting from alternative splicing. In contrast, top-down proteomics does not involve protein fragmentation but is more dependent on expensive instrumentation and operator skills besides not having been yet achieved on a large-scale — for this reason, this approach is not suited for the analysis of complex protein mixtures.

Proteomics has mainly been used in the CF field in two types of studies (Table 10.2): those assessing the full proteome of cells

TABLE 10.2: Summary of Most Relevant Proteomics Studies in the CF Field (in Chronological Order)

Study	Year	Cellular Model/Samples	Aim	Key Findings
Davezac et al. (40)	2004	HeLa cells stably transfected with wt- or F508del-CFTR	Global proteome assessment of proteins differentially expressed in wt- vs F508del condition	Keratins 8 and 18 are differentially expressed in F508del vs. WT suggesting a role in the traffic of CFTR.
Pollard et al. (43)	2005	IB3-1 cells	Proteomic profiling of lung epithelial cells to identify high abundance proteins	High abundance proteins identified include sets of proteins associated with inflammation, including the classical NF-κB pathway.
Roxo-Rosa et al. (51)	2006	Nasal cells from individuals homozygous for F508del and non-CF controls	Comparative proteomics profiling to identify potential biomarkers from differentially expressed proteins	Differentially expressed proteins in nasal cells from individuals homozygous for F508del include proteins related to chronic inflammation and others involved in oxidative stress.
Singh et al. (94)	2006	IB3-1 cells	Assessment of total proteome changes in response to 4-PBA treatment	Treatment with 4-PBA induces changes in the levels of proteins such as chaperones, proteins involved in cellular defense, protein biosynthesis, trafficking, and ion transport.
Wang et al. (54)	2006	BHK cells stably transfected with wt- or F508del-CFTR	Assessment of CFTR interactome comparing wt- and F508del-CFTR	F508del-CFTR fails to achieve a folded conformation in the context of the dynamics of the chaperone folding environment.
Singh et al. (46)	2008	IB3-1 cells	Assessment of CFTR interacting proteins in response to 4-PBA treatment	Treatment with 4-PBA increases the association of CFTR with a set of ER-associated degradation chaperones.
Gomes-Alves et al. (47)	2009	BHK cells stably transfected with wt- or F508del-CFTR	Assessment of total proteome related to the expression of wt- or F508del-CFTR and in response to low temperature treatment	Unfolded protein response induction and repression of specific subsets of metabolism are the major processes elicited by low temperature treatment that may generate a favourable environment for F508del-CFTR rescue.
Gomes-Alves et al. (95)	2009	Serum and nasal epithelial cells from individuals with CF and non-CF controls	Assessment of protein profiles for biomarker identification	Identification of protein signatures that may serve as diagnostic/prognostic markers for CF.
Gomes-Alves et al. (50)	2010	BHK cells stably transfected with wt-, F508del- or F508del-4RK-CFTR	Total proteome changes in response to rescue of F508del-CFTR by second site mutations	Overexpression of F508del-4RK-CFTR modulates expression of several CFTR interactors, including ER stress and unfolded protein response proteins.
Coppinger et al. (55)	2012	HEK293 cells stably transfected with wt- or F508del-CFTR	Quantification of the CFTR-interacting chaperome in wt- or F508del-CFTR expressing cells	F508del-CFTR is restricted to a chaperone-bound folding intermediate, and this contributes to its loss of trafficking and increased targeting for degradation.
Teng et al. (67)	2012	HeLa cells stably transfected with wt- or G551D-CFTR	Assessment of CFTR interactome for G551D-CFTR	Caliumenin is an interactor of G551D-CFTR probably by regulating a specific maturation and trafficking pathway.
Pankow et al. (56)	2015	CFBE41o- and 16BHE14o- cells	Interactome of wt- and F508del-CFTR and response to low temperature and VX-809 treatment	F508del-CFTR has a specific interactome that is extensively remodelled upon low temperature — or small molecular weight compound-rescue — and this remodelling is crucial for the rescue.
Reilly et al. (57)	2017	CFBE41o- and 16BHE14o- cells	Interactome of wt- and F508del-CFTR	Cells expressing F508del-CFTR have upregulated mTOR activity and inhibition of this pathway increases CFTR stability.
Braccia et al. (53)	2018	CFBE41o- cells and primary human bronchial epithelial cells	SWATH-MS label-free proteomics to assess global proteome changes associated with CF	Analysis in patient derived materials revealed over 150 proteins whose expression is altered — including known CFTR interactors but also protein not previously known to be related to CF.
Puglia et al. (58)	2018	CFBE41o- and 16BHE14o- cells	Interactome of wt- and F508del-CFTR	Proteins differentially associated with F508del- vs. wt-CFTR identify ezrin, Hdp70 and endoplasmin as central hubs in CFTR homeostasis.

(*Continued*)

TABLE 10.2: Summary of Most Relevant Proteomics Studies in the CF Field (in Chronological Order) (*Continued*)

Study	Year	Cellular Model/Samples	Aim	Key Findings
Canato et al. (59)	2018	CFBE14o- cells stably transduced with wt-, F508del- or F508del-4RK-CFTR	Interactome changes involved in F508del-CFTR ER escape upon AFT abrogation	Upon AFT abrogation, the interactome of F508del-CFTR is changed. Modulation of these differential interactors — in particular KIFC1 — is able to rescue the mutant's trafficking defect.
Hutt et al. (68)	2018	CFBE41o- cells stably transduced with CFTR bearing 5 different mutations	Comparative interactome of CFTR expressing common mutations	Comparative analysis of proteins interacting with CFTR bearing different variants allows identification of protein interactions contributing to the basic defects associated with each of them, particularly F508del- and G551D-CFTR.
Santos et al. (60)	2019	CFBE41o- cells stably transduced with CFTR bearing ERQC related variants	Comparative interactome of CFTR variants related to ER exit and retention	Comparative analysis reveals that the folding status of CFTR is much more relevant in defining its interactome than its cellular localization.
Matos et al. (61)	2019	CFBE14o- cells stably transduced with wt- or F508del-CFTR	Differential PM interactome of wt- and rescued F508del-CFTR	Comparative analysis between membrane specific interactomes identifies calpain 1 as a critical interactor that regulates specifically rescued F508del-CFTR.
Santos et al. (66)	2020	CFBE14o- cells stably transduced with wt-CFTR	CFTR Interactome under activation of EPAC1	Activation of EPAC1 by cAMP prompts its association with CFTR through recruitment of cytoskeleton-associated proteins.
Loureiro et al. (64)	2020	CFBE14o- cells stably transduced with wt-CFTR	CFTR Interactome under phosphorylation by SYK	CFTR phosphorylation by SYK recruits interacting proteins that regulate CFTR membrane stability.
McDonald et al. (69)	2022	HEK cells stably transfected with CFTR bearing F508del and other class II mutations	Interactome changes associated with trafficking mutations and response to VX-809	Comparative analysis of the interactome of CFTR bearing different variants that cause ER retention identifies protein degradation proteins as critical players for rescue by VX-809.

expressing wt-CFTR versus mutant samples (either cell lines or patient-derived materials) and those assessing interactomes (i.e., the full set of proteins interacting with a bait protein, usually CFTR). Here we cover the most relevant studies in the field and how they contributed to the elucidation of disease mechanisms and, later, also of rescuing mechanisms.

Assessing total proteome and its relationship with CFTR mutations

Similarly to transcriptome studies, characterization of the proteome (i.e., the full set of proteins expressed by a cell or tissue) associated with CFTR mutants was first described for F508del-CFTR, in comparison to wt-CFTR. The first reported studies analysed the total proteome in HeLa cells stably overexpressing either wt- or F508del-CFTR cDNA using 2D-GE and MS. Such experiments identified two keratins — keratin 8 (K8) and 18 (K18) — with increased expression in cells expressing F508del-CFTR with a possible role in CFTR trafficking. Interestingly, down regulation of K18 with siRNA enabled rescue of functional protein to the PM (40). Later studies have confirmed that a similar role is observed for K8, that contrarily to K18, can bind directly to CFTR NBD1 (41).

Similar studies were also carried out in the more relevant cell model IB3-1, which is a compound heterozygote bronchial epithelial cell line derived from an individual with CF with the genotype F508del/W1282X (42). With the aim of obtaining what was called a "composite atlas of the high abundance CF lung epithelial proteome", the authors identified several sets of proteins associated with inflammation, namely, members of the NF-κB family such as p65 (RelA) and RelB (43). The same cellular model was used to assess how 4-phenylbutyrate (4-PBA) affects the proteome of IB3-1 cells. Used to treat urea cycle disorders (44), 4-PBA is an oral butyrate derivative which, at one point, was also proposed as a chemical chaperone in CF (45). The study identified a change in several proteins, including chaperones and proteins involved in cellular defence, protein biosynthesis, trafficking activity, and ion transport (46).

Other authors have performed similar analysis in heterologous systems, namely BHK cells expressing either wt- or F508del-CFTR. This work analysed the impact of low temperature (known to promote some rescue of F508del-CFTR) upon the proteome of cells expressing F508del-CFTR and identified the unfolded protein response (UPR) induction and some cell-metabolism repression as the major response pathways that may generate a favourable cellular environment to promote F508del-CFTR rescue. Interestingly, deregulation of RACK1, which can affect CFTR stability at the PM, was also partially repaired after low temperature treatment (47). Further clues to how F508del-CFTR could be rescued resulted from studies with BHK cells expressing F508del-CFTR in cis with 4 "revertant" mutations, called F508del-4RK-CFTR. Such arginine-to-lysine substitutions abrogate ER retention motifs known as arginine framed tripeptides and allow the mutant protein leave the ER (48, 49). These studies identified a particular cellular environment orchestrated by the unfolded

protein response (UPR). Indeed, several UPR components (such as GRP94, PDI, GRP75) were found to be overexpressed in F508del-4RK-CFTR cells by comparison to F508del-CFTR, thus suggesting the possible involvement of UPR to the rescue of the mutant protein by the 4RK substitutions (50).

Aside from the use in cell lines, with or without CFTR overexpression, total proteome studies were also carried out in samples from individuals with CF. Comparative proteomic profiling was performed in nasal cells from individuals homozygous for F508del and compared to non-CF controls (51). The study, performed using a classical approach in which 65 selected spots were excised from 2D-GE and identified by MS, revealed a set of differentially expressed proteins including proteins related to chronic inflammation and others involved in oxidative stress injury. Interesting, alterations were also detected in cytoskeleton proteins, which agrees with many other studies found in the literature (reviewed recently in [52]), and for some mitochondrial proteins, which is properly related to the description of oxidative stress as implicated in the aetiology of CF (51).

More recent studies revisited analysis of total proteome using label free approaches for protein quantification, such as sequential window acquisition of all theoretical mass spectra (SWATH-MS) proteomics, the state-of-the-art methodology for such an analysis (53). After realizing that this tool fails to detect several proteins which are central to the biology of CF, the Armirotti group used CFBE41o- cells — a popular model in the field — to improve the applicability of SWATH-MS proteomics in CF. They then used a purpose-built extended ion library to assess the total proteome in primary cultures of human bronchial epithelial cells (HBE) obtained from individuals homozygous for F508del and from non-CF controls. They identified more than 150 proteins dysregulated in CF, including some which were previously known as CFTR regulators or involved in CF pathophysiology but also some previously unknown as players in CF (53).

Interactomics: Using proteomics to identify CFTR-interacting proteins

Simultaneously to the progress being made in identifying global patterns for protein expression linked to the expression of wt- and mutant CFTR, proteomics has also been used to identify protein-protein interactions (PPIs) of CFTR at a global level, i.e., interactomics — thus collecting extra information when compared to studies assessing whether one or a few proteins interact with CFTR.

The first study of this kind used BHK cell lines overexpressing wt- or F508del-CFTR and CFTR immunoprecipitation using conditions that pull-down PPIs which were identified with an approach called multidimensional protein identification (MudPIT). The authors thus obtained two "CFTR interactomes" revealing increased interaction of chaperones, in particular Hsp90 co-chaperones, with F508del- versus wt-CFTR. Interestingly, partial silencing of one of these co-chaperones — the ATPase regulator Aha-1 — was able to promote cell surface rescue of F508del-CFTR. This study suggested that the failure of F508del-CFTR to achieve a proper folded conformation in response to the chaperone folding environment is a major player in the pathophysiology of CF, namely, for F508del and other class II mutants and provides a general framework for correction of misfolding diseases (54). The same group later showed, using a different heterologous cell system, that F508del-CFTR folding leads to a stalled intermediate in stoichiometric association with chaperone PPIs such as Hsp70, Hsp90, and co-chaperone Hsp40, a so-called "chaperone trap", which could be released by incubation at low temperature (55).

Interactomic studies were also performed to understand responses to pharmacological chaperones, as in a study using IB3-1 cells treated with 4-phenyl butyrate (4-PBA). CFTR immunoprecipitation followed by subcellular fractionation identified a subset of endoplasmic reticulum (ER)-associated degradation chaperones (GRP94, HSP84, GRP78, GRP75, and GRP58) that were modulated with 4-PBA treatment and were associated with the immature form of ER-resident CFTR, also known as "band B". This was the first report that chemical rescue of mutant CFTR (albeit modest) alters its specific PPIs and that chaperones are the most relevant players with differential association with wt- and mutant CFTR, namely, those in class II (46).

More recent studies have focused on the identification of CFTR interactome in relevant cell models such as CFBE41o- cells — either comparing the parental line with the wt-CFTR expressing counterpart 16HBE14o- or using the overexpressing models, stably transduced with wt- or F508del-CFTR.

A robust study comparing CFBE41o- and 16HBE14o- cells used deep proteomics analysis methods and identified more than 600 individual high-confidence CFTR PPIs and a F508del-CFTR-specific interactome which was extensively remodelled upon rescue — either chemically with VX-809 or physically with low temperature — providing further insight into CF disease mechanisms for the most common mutation (56). Similar studies by other labs identified an upregulation of mTOR activity, whose inhibition could be used to increase CFTR stability and expression, probably through an impact on Bcl-2-associated athanogene 3 (BAG3), a regulator of autophagy (57). Another study using the same cellular models highlighted pathways in which ezrin, Hsp70, endoplasmin, and lamin A/C were identified as PPIs and central hubs in CFTR homeostasis and some proteins such as serpin H1, prelamin A/C, protein-SET, and cystatin-B were for the first time associated with CF, identifying potential targets for F508del-CFTR stabilization and/or rescue (58).

Identification of CFTR PPIs has also been used to get further insight into the ER quality control (QC) that discriminates between folded and misfolded CFTR. Differential interactions of F508del-CFTR versus F508del-4RK-CFTR (which escapes ER retention due to the abrogation of the retention signals) identified kinesin family member C1 (KIFC1) as a stronger interactor with F508del-CFTR. Furthermore, decreasing KIFC1 levels or activity stabilizes the immature form of F508del-CFTR by reducing its degradation (59). Another study assessing the interactions of an ER-retained variant to which the diacidic-export signal was removed, showed that the folding status of CFTR is determinant over trafficking ability to define its global interactome (60).

Interactome studies have also been used to characterize different processes that regulate CFTR plasma membrane (PM) stability. A study focusing specifically on differential PM interactions comparing wt- and low temperature or VX-809 rescued F508del (rF508del)-CFTR, identified calpain 1 (CAPN1) as an exclusive rF508del interactor that prevents active ezrin recruitment, impairs anchoring to actin, and reduces its PM stability. These results suggest that modulation of CAPN1 is a possible strategy to rescue the PM stability defect of rF508del-CFTR (61). Another study focusing on spleen tyrosinase kinase (SYK), previously shown to phosphorylate CFTR regulating its PM levels (62, 63), identified the adaptor protein SHC1, which recognizes tyrosine-phosphorylated CFTR and promotes its internalization, thus highlighting another possibility to improve membrane

stability (64). A third study explored in depth the mechanisms through which activation of the exchange protein directly activated by cAMP1 (EPAC1) increase CFTR PM levels (65). Using PPI profiling and bioinformatic analysis to identify proteins that interact with CFTR under EPAC1 activation, an enrichment in cytoskeleton-related proteins was identified — in particular the capping protein CAPZA2 and the inverted formin INF2, a positive and a negative regulator respectively, for the increase of CFTR at the PM after EPAC1 activation (66).

Interactomes have also been applied to the elucidation of disease mechanisms for mutations other than F508del. Comparing CFTR interactomes using CFTR immunoprecipitation, 2D-GE and MS identification in HeLa cells expressing either wt- or G551D-CFTR identified calumenin as a stronger interactor of the mutant protein (67). Another study focused on different disease-causing mutations (G85E, F508del, G551D, R560T, and N1303K) and identified a number of PPIs contributing to the basic defects associated with such mutants. Treatment with the modulators VX-809 and VX-770 significantly altered interactions of F508del- and G551D-CFTR. The comparative analysis performed among multiple CF-causing mutations established a methodological approach for the use of proteomics in understanding the impact of variation in CF disease (68).

Finally, a recent study used interactomics of CFTR expressed in HEK cells to characterize the impact of VX-809 in different class II (i.e., trafficking) mutants. Comparison between hyper-responsive (P67L and L206W) and low-responsive (F508del and G85E) mutants identified differential associations with proteasomal and autophagy degradation machinery, highlighting proteostasis characteristics of mutant-specific responses to modulators (69).

Table 10.1 summarizes some of the most relevant studies that used proteomics to unravel CF disease mechanisms, to identify novel therapeutic targets and to understand the mechanism of action (MoA) for different rescuing strategies.

Functional genomics

What is functional genomics?

Functional genomics can be briefly described as the assignment of functional annotations to genes/proteins. In other words, it is the study of how the functions of individual genes/proteins interplay in cells to yield a given phenotype. In the context of a disease such as CF, functional genomics refers to the description of the functions whose dysregulation contributes to pathogenesis, ultimately to be targeted — through activation or inhibition — to restore homeostasis, i.e., the healthy state. Accordingly, functional genomics addresses dynamic gene expression in time and space to establish genotype-phenotype connections at "Omics" level. Although some authors employ "functional genomics" as a catch-all term for genomics, transcriptomics, proteomics and metabolomics, herein, we approach functional genomics in the sense of "phenomics", i.e., the systematic establishment of genotype-phenotype relationships at genome-wide scale through the integration of multidimensional data on a cell- or organism-wide level (70).

Experimental approaches

One way of identifying gene/protein function is through reverse genetic screens. This strategy involves modulating gene expression and quantitatively describing the outcoming phenotype using a pre-validated assay (Figure 10.1). Most phenotypic screens are based on RNA interference (RNAi), a process where the presence of double-stranded (ds) or small interfering RNA (siRNA) in cells causes the catalytic degradation of complementary mRNA with the consequent decrease in protein translation (gene knock-down). Provided that complementarity rules are observed and that all gene transcripts are considered, RNAi perturbation reagents can be designed for any gene. The most common RNAi reagents are siRNAs, which are 21-base pair (bp) RNA molecules, usually available as duplexes. siRNAs do not spontaneously permeate cell membranes, requiring transfection reagents (*e.g.,* cationic lipids) to become available at the cytoplasm. The first genome-wide siRNA screens were performed in the early 2000s (71). Most CF-related screens are not genome-wide, with researchers opting instead for libraries targeting functionally related genes or the subset of genes whose proteins have minimally known functions: the "druggable genome". Gene silencing dependent on siRNAs is transient and partial, but the typical siRNA *in-vivo* longevity of ~5 days (except for the most long-lived mRNA molecules) is sufficient to examine their contribution. To circumvent these limitations, short hairpin RNA (shRNA) genes can be stably incorporated into the genome through lentiviral transfection, resulting in effective gene knock-out. More recently, nuclease-based gene perturbation (zinc finger nucleases, TALENs, and CRISPR-Cas9) have become available, but their application in CF has so far been related to mutagenesis, rather than reverse genetic screening. Conversely, cDNA libraries enable gene overexpression studies, which may also be effective, except for most expressed genes. Most CF-related studies so far have relied on arrayed libraries, where only one gene is perturbed at a time.

Large-scale gene perturbation studies rely on assay miniaturization, sample handling automation and high-throughput (HT) data acquisition and processing. Two kinds of readouts are possible: spectroscopic measurements acquired on a plate reader (high-throughput screening, HTS) and image-based features (high-content screening, HCS). Both approaches enable genomic-level screening to describe the contribution of each individual gene to a given CF-related process. But these platforms also enable bottom-up drug discovery projects: by studying CF model systems and identifying genes or proteins whose inhibition restores the control phenotype, these genes may be categorized as CF drug targets. Accordingly, inhibitors of those proteins can be designed, thus overcoming many of the shortcomings in traditional large-scale molecule screening, where the drug target is often unknown. Notwithstanding, model system limitations and modifier gene variants in CF individuals must always be considered when assessing the biomedical relevance of functional genomics studies.

CFTR targeting screens

Given that CFTR gene mutations are the primary cause of CF, several functional genomics projects have been based on assays monitoring mutant CFTR properties as a function of gene perturbations (see Table 10.3). The most common is the halide-sensitive yellow fluorescent protein (HS-YFP) quenching assay. This assay uses iodide as a surrogate for the natural CFTR permeants, i.e., chloride (Cl^-) or bicarbonate (HCO_3^-) and reports CFTR ion transport activity through rate of fluorescence decay triggered by iodide influx into cells in the presence of CFTR activators (*e.g.,* forskolin, IBMX) and potentiators (*e.g.,* ivacaftor or genistein). This assay, which has been improved over the past 20 years, has been extensively used for the discovery of novel CFTR modulators involving libraries of approved or investigational compounds as well as natural products.

TABLE 10.3: Summary of Most Relevant Functional Genomics Studies in the CF Field (in Chronological Order)

Study	Year	Cellular Model/Samples	Aim	Key Findings
Carlile et al. (76)	2007	BHK cells expressing 3HA-tagged F508del-CFTR	Identification of CFTR correctors among 2000 drug-like compounds	Identification or re-discovery of five strong (dacthal, glycyrrhizic acid, chloramphenicol, carboplatin), six medium (*e.g.* sildenafil) and 18 weak F508del-CFTR correctors. Biochemical and functional validation of sildenafil (20 μM) and carboplatin (0.1-10 μM).
Trzcinska-Daneluti et al. (72)	2009	HEK293 MSR GripTite cells stably expressing F508del-CFTR and co-expressing HS-YFP fusion proteins	Identification of proteins that correct F508del-CFTR upon overexpression	Identification of known and novel proteins correcting F508del-CFTR function by overexpression: STAT1, ET-1, HSPA4, SAKS1, AP2M1, LGALS3, TFG, Cav2, PAP/REG3α, and others.
Okiyoneda et al. (91)	2010	HeLa and IB3 cells expressing 3HA-tagged CFTR	Identification of the ubiquitination mechanism of low temperature rescued F508del-CFTR	Unfolded regions of PM-located F508del-CFTR interact with chaperones (Hsc70, DNAJA1, Hsp90), which promote ubiquitination (through CHIP-UbcH5c), endocytosis and lysosomal degradation.
Trzcinska-Daneluti et al. (80)	2012	HEK293 MSR GripTite cells stably expressing wt-/F508del-CFTR and transiently expressing HS-YFP	Identification of kinase inhibitors that correct F508del-CFTR	F508del-CFTR is rescued by inhibitors of receptor Tyr kinases (SU5402, SU6668), Ras/Raf/MEK/ERK or p38 pathways (*e.g.* [5Z]-7-oxozeaenol) or GS-3β (GSK-3β Inhibitor II and Kenpaullone).
Almaça et al. (88)	2013	A549 cells conditionally expressing wt- or F508del-CFTR	Identification of ENaC traffic and function regulators	160 confirmed ENaC activator genes. Characterization of two original regulators: DGKι (PI signalling) and CNTFR (mTOR signalling).
Botelho et al. (81)	2015	CFBE41o- cells conditionally expressing mCherry-Flag-CFTR (wt and F508del-CFTR variants)	Identification of CFTR traffic regulator proteins and drug-like molecules	High-content CFTR traffic screening platform. F508del-CFTR traffic is regulated by silencing *COPB1* (traffic rescue) or *OR2AG1* (traffic inhibition).
Tomati et al. (74)	2018	CFBE41o- cells stably expressing F508del-CFTR and HS-YFP	Identification of F508del-CFTR activity regulators	Identification of 37 proteins whose silencing rescued F508del-CFTR activity (FAU, UBE2I, UBA52, MLLT6, UBA2, CHD4, PLXNA1, TRIM24 and others). FAU interacts with F508del-CFTR and promotes its degradation.
Lérias et al. (89)	2018	CFBE41o- cells conditionally expressing 3HA-TMEM16A-eGFP	Identification of TMEM16A traffic regulators	High-content TMEM16A traffic screening platform. TMEM16A traffic and function are regulated by silencing *COPB1* (enhancement) or *ESYT1* (inhibition).
Perkins et al. (79)	2018	HEK293 cells stably expressing FAP-CFTR (wt- and F508del-CFTR variants)	Identification of kinases that rescue and stabilize F508del-CFTR at the cell surface	Identification of at least 32 kinases whose inhibition rescues F508del-CFTR surface expression. Flow cytometry-based validation of siRNA- or compound-based inhibition of CAMKK1 (STO-609) and RAF1 (GW 5074).
Mutolo et al. (73)	2018	Calu-3 cells	Identification of transcriptional regulators that reduce wt-CFTR mRNA levels	Identification of 37 factors that upon depletion elevated CFTR mRNA levels more than two-fold. Depletion of KLF5 or EHF improved CFTR synthesis and function.
Hutt et al. (75)	2018	CFBE41o- cells stably expressing F508del-CFTR and HS-YFP	Identification of proteostasis network pathways and components affecting F508del-CFTR folding and function	siRNA-mediated silencing of eIF3a decreased the translation of CFTR variants, leading to increased CFTR stability, trafficking, and function at the cell surface.
Checa et al. (92)	2021	HEK293, HCT116 and U2OS cells	Identification of oxidative stress resistance genes	Resistance of CF submucosal gland cells to oxidative stress increases by silencing genes involved in alternative splicing, cell communication, motility, remodeling, DNA repair, and PI3K/AKT/mTOR signalling, as well as by treatment with everolimus, doxazosin, or fostamatinib.
Lim et al. (84)	2022	CFBE41o- cells conditionally expressing mCherry-Flag-CFTR (wt and F508del-CFTR variants)	Validation of PM-specific CFTR interactors	PM-specific interactome of wt- and F508del-CFTR (447 proteins), including ST6GALNAC1, FKBP6, CAPZB, VAPA, XAGE3, and FGL2. Characterization of FGL2 as a functional interactor.
Pinto et al. (90)	2022	CFBE41o- cells conditionally expressing 3HA-TMEM16A-eGFP	Identification of TMEM16A traffic regulators	20 validated TMEM16A regulator genes. Silencing of the G-protein coupled receptors ADRA2C and CXCR3 increased TMEM16A-mediated chloride secretion. Overexpression had opposite effects.
Botelho et al. (85)	2022	CFBE41o- cells conditionally expressing mCherry-Flag-CFTR (several CFTR variants)	Identification of CFTR traffic and function regulator proteins	Identification of the kinases DGKG, GRK5, LRRK1, STYK1, and TPK1 as potential novel CF drug targets. Pharmacological inhibition of GRK5 rescues F508del-CFTR traffic and function.

The first application of the HS-YFP assay to functional genomics occurred in 2009 at the Rotin lab in Toronto (72) through gain-of-function experiments performed on HEK293 MSR GripTite cells expressing F508del-CFTR. The authors overexpressed 446 HS-YFP fusion proteins belonging to several classes: chaperones, trafficking proteins, established or putative CFTR interactors, proteins involved in the ubiquitin system, signalling proteins, and randomly chosen proteins. Using a high-content (image-based) analysis, the authors identified two hit sets: 13 "top hits" (effects >25%) and 21 "second-tier hits" (effects of 20–25%). Both sets showed a larger rescue than the one obtained with corrector 4a, one of the first molecules shown to correct — albeit in a modest and unspecific manner — the folding and traffic defects characteristic of F508del-CFTR. These hits included proteins involved in protein maturation, processing and trafficking, and signal transduction among other processes. Special emphasis was placed on two hits: STAT1, due to previous evidence that this protein is inhibited in CF epithelial cells; and HspA4, whose phenotype could be recapitulated with the HspA4 stimulator Velcade (Bortezamib). The authors also screened for inhibitors of low-temperature rescued F508del-CFTR, for applications in chronic diarrhoea, and identified the de-ubiquitination enzymes USP47 and UCHL5 as hits.

All other CFTR-based screens were siRNA-based (loss-of-function) assays, with four studies reported in 2018 (see Table 10.3). The Harris lab (73) screened an siRNA library targeting 1528 transcription factors (TFs) and respective regulatory proteins in Calu-3 cells, identifying 38 TFs which, when depleted, increased wt-CFTR mRNA levels in Calu-3 cells by >2-fold. The strongest increases were observed by knocking down KLF5 (6-fold), EHF and BRD8 (~3-fold), the first two having established roles in the airway epithelium. The repressive nature of BRD8, KLF5, and ING2 was validated in primary HBE cells. siRNA depletion of the strongest hit (KLF5) in Calu-3 cells was found to significantly increase basal Cl^- channel activity using the fluorescent Cl^--sensor MQAE.

Opting for a more physiologically relevant cell model, the Pedemonte lab developed a CFBE41o- cell line, representing the bronchial epithelium of a CF individual, co-expressing F508del-CFTR and HS-YFP, which was used to screen an siRNA library targeting a "druggable genome" composed of 6650 genes using the HS-YFP assay (74). The screen resulted in 37 confirmed hits. The most effective targets were UBA52, CHD4, TRIM24, UBA2, UBE2I, and FAU, most of which were additive to VX-809 and validated by Western blot quantification. The most relevant hit was FAU, a poorly characterized protein consisting of the ubiquitin-like protein FUBI fused to the ribosomal protein S30. FAU silencing caused a 65% increase in F508del-CFTR PM trafficking, increased F508del-CFTR half-life in cell lines and increased CFTR-mediated Cl^- currents in primary cultures of HBE cells derived from an individual with CF F508del-homozygous. No interplay with the alternative Cl^- ion channel, TMEM16A was detected.

The Balch lab analysed the role of the community of proteins interacting with CFTR and involved in its biogenesis, stability, degradation, trafficking, and function (globally referred as "proteostasis network", PN) in the functional repression of F508del-CFTR in CFBE41o- cells (75). Using an siRNA library targeting 456 genes (including translational machinery, cytosolic and ER luminal chaperones, degradative enzymes, and post-translational regulatory proteins), the authors identified 367 PN components whose silencing significantly rescued F508del-CFTR function. Of these, only 73 were CFTR interactors. Hits were analysed in terms of a shortest-path connection to CFTR (Network-Augmented Genomic Analysis), leading to the selection and validation of eIF3a silencing as an effective manoeuvre to correct fundamental F508del-CFTR defects.

Several groups have developed screening models which monitor CFTR cell surface availability, regardless of the gating status. PM-specific CFTR labelling is hindered by the small extracellularly exposed surface and lack of suitable antibodies. The solution has been the incorporation of genetically-encoded tags, such as the triple haemagglutinin (3HA) (76) or horseradish peroxidase (HRP) (77) at the fourth extracellular loop (ECL4), which does not impact CFTR folding or activity. Alternatively, several authors (*e.g.,* [78]) performed N-terminal fusions of extracellularly facing fluorogen-activating-protein (FAP), a protein with sub-nanomolar affinity for malachite green-based fluorogens, which become intensely fluorescent in the bound state. This model was used by the Bruchez lab to identify genes whose inhibition restores F508del-CFTR cell surface abundance (79). The screen of a kinome siRNA library (715 target kinases) showed that repression of CAMKK1 or RAF1 with siRNA or inhibitors promoted PM expression of F508del-CFTR to levels beyond those achieved by VX-809. The strategy of prioritizing kinases due to their potential as drug targets had already been employed by the Rotin lab, which screened a library containing 231 clinical or preclinical kinase inhibitors (80). Functional F508del-CFTR rescue was observed when inhibiting receptor tyrosine kinases (RTKs, such as FGFRs, VEGFR, and PDGFR), members of the Ras/Raf/MEK/ERK or p38 pathways and GSK-3β.

Some of the most advanced models were introduced by the Amaral lab, combining the relevant CFBE41o- cell line with a double-tagged CFTR traffic reporter, which can report on total CFTR expression (N-terminal mCherry) as well as PM localization (Flag tagged ICL4) in a HCS platform (81). Expression of the mCherry-Flag-F508del-CFTR reporter is inducible, enabling CFTR synthesis in the absence of siRNA-targeted genes. Several variants were constructed by Amaral and Farinha, including wt- and CFTR traffic mutants, F508del genetic revertants (82) as well as reporters expressed in A549 cells (83). Research with the F508del-CFTR traffic reporter has so far highlighted COPB1 and OR2AG1 as CFTR traffic regulators (81) and validated interatomic data supporting the interaction of the fibrinogen-related superfamily serine protease FGL2 with wt- and F508del-CFTR as leading to increased activity (84). Recently, a druggable genome siRNA screen (targeting 9128 genes) using this system identified five kinases as potential novel CF drug targets: DGKG, GRK5, LRRK1, STYK1, and TPK1 (85). Pharmacological inhibition of the β-2 adrenergic signalling regulator GRK5 (86) resulted in functional F508del-CFTR rescue, pointing to preclinical applications.

Screens targeting other ion channels

Besides enlightening CFTR regulatory pathways, functional genomics has also provided knowledge on other ion channels (Table 10.3). The epithelial sodium (Na^+) channel (ENaC) has a well-established role in the CF pathogenesis cascade, as it exacerbates airway dehydration by driving excessive Na^+ absorption in the respiratory epithelium when CF-causing CFTR mutations exist (87). The Amaral lab developed an assay which uses the FLIPR membrane potential (FMP) fluorescent dye in combination with the specific ENaC inhibitor amiloride to monitor ENaC activity by HCS (83). This assay was used to screen an siRNA library targeting 6409 druggable genes, kinases and secretion

regulators (88). The study described 738 genes which activate and 887 genes which inhibit ENaC. Placing an emphasis on therapeutically desirable targets, the authors noted that the phosphatidylinositol (PI) pathway was the most enriched in the primary screen, validated 132 ENaC activator genes, and classified them into known ENaC processes and pathways. Two novel ENaC regulators emerged: (1) DGKι, a key enzyme in the PI pathway believed to activate ENaC by regenerating the pool of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the inner PM leaflet, whose inhibition with the chemical blocker DGK_{inh} normalized ENaC activity; and (2) CNTFR, which couples to the mTOR pathway.

Among alternative (i.e., non-CFTR) Cl^- channels that can compensate for the absence of functional CFTR, TMEM16A/ANO1 is the most well-studied and most promising. The Amaral lab developed a HCS assay traffic analogous to the double-tagged CFTR reporter system to monitor TMEM16A (3HA-TMEM16A-eGFP [89]). A pilot assay uncovered the COP-I component COPB1 and the PIP_2- and calcium (Ca^{2+})-dependent ER-PM tethering factor ESYT1 as negative and positive regulators of TMEM16A traffic, respectively. This platform was recently used to screen a custom siRNA library targeting ~700 genes that inhibit F508del-CFTR PM expression, interact with CFTR, or regulate protein trafficking (90). This study validated 20 siRNAs which enhanced TMEM16A traffic, including those targeting the G-protein coupled receptors (GPCRs) ADRA2C and CXCR3, which were suggested as potential targets for pharmacological mutation-agnostic restoration of transepithelial Cl^- secretion in CF.

Functional genomics towards characterization of other CF features

Additional studies focused on other features (Table 10.3). The Lukacs lab used CFTR constructs modified with an extracellular 3HA-tag in combination with siRNA screens to examine the peripheral quality control mediators which are responsible for folding surveillance, ubiquitination, recycling, and degradation of F508del-CFTR rescued to the cell surface, stressing their relevance for the maintenance of cell homeostasis (91). Two studies from 2021 addressed the contribution of oxidative stress in CF, a relevant theme as chronic lung inflammation in individuals with CF establishes the conditions whereby reactive oxygen species contribute to destruction of the airway epithelium. A genome-wide siRNA screen found 167 siRNAs targeting 444 genes able to confer oxidative stress resistance in CF mucosal glands (92). These hit genes were involved in alternative splicing, cell communication, motility and remodelling, DNA repair, and the PI3K/AKT/mTOR signalling pathway. Another study characterized the oxidative stress response regulator BACH1 as a modifier of the chromatin architecture at the CFTR *locus* (93). This gene had been identified in a CFTR-centred functional screen (73).

Concluding remarks

Knowing how distinct CFTR gene mutations alter the transcriptome and proteome has provided important insights into compensatory mechanisms in diseased cells, which has led in turn to identification of key processes in disease progression. Furthermore, as research in CF focuses more on implementing personalized medicine, such data have been increasingly used to identify biomarkers of disease and of treatment efficacy. In parallel, functional genomics studies have focused on the global identification of modifier genes that critically affect the life cycle of CFTR from mRNA to protein function, not only to further understand CF physiopathology but also identify potential novel drug targets that specifically rescue the cellular defect inherent to a given mutation.

However, the true benefits of a Systems Biology approach depend upon the integration of all Omics, so as to provide "holistic" data that can: (1) thoroughly describe CF physiopathology; (2) make predictions of how this condition evolves as a dynamic system; and most importantly, (3) understand how we may "perturb" the system to avoid the diseased state.

Accordingly, designing approaches that succeed in integrating available (and novel) data from these different "Omics" is the next challenge that needs to be overcome to extract even more biological significance and clinical impact out of these large datasets.

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