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Applications of proteomic technologies for understanding the premature proteolysis of CFTR

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Abstract

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes an ATP-dependent anion channel. Disease-causing mutations can affect channel biogenesis, trafficking or function, and result in reduced ion transport at the apical surface of many tissues. The most common *CFTR* mutation is a deletion of phenylalanine at position 508 ($\Delta F508$), which results in a misfolded protein that is prematurely targeted for degradation. This article focuses on how proteomic approaches have been utilized to explore the mechanisms of premature proteolysis in CF. Additionally, we emphasize the potential for proteomic-based technologies in expanding our understanding of CF pathophysiology and therapeutic approaches.

Keywords

absolute quantification method; *CFTR*; *CFTR* biogenesis; cystic fibrosis; interactomics; proteolysis; proteomics; ubiquitination

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene. *CFTR* encodes a PKA-regulated and ATP-dependent anion channel important in maintaining the hydration of several secretory organs. There are over 1600 known mutations in the *CFTR* gene, and those resulting in the premature proteolysis of CFTR are responsible for the majority of CF disease [201]. $\Delta F508$ CFTR, which is efficiently degraded at the endoplasmic reticulum (ER), is the most common mutation and accounts for approximately 66% of all disease-causing alleles in CF [1]. There are also additional *CFTR* mutations, categorized as class II, that result in the premature proteolysis of CFTR [2]. Class II mutations prevent the trafficking of CFTR to the plasma membrane, leading to decreased chloride and bicarbonate transport across the epithelia of secretory tissues. While several class II CFTR proteins, such as $\Delta F508$, also demonstrate reduced ion transport when rescued to the cell surface [3,4], it may be possible to use CFTR potentiator compounds to enhance the activity of these proteins [5]. Targeting

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the processes that result in the premature degradation of CFTR during biogenesis represents the first step in therapeutic development for class II mutations.

A deeper understanding of the mechanisms responsible for CFTR degradation is necessary for the advancement of therapeutics targeting prevention of premature proteolysis. The examination of CF pathogenesis has benefited greatly from proteomic-based investigations, and we highlight several of these studies in this review. By harnessing the unbiased nature of these methodologies, it is possible to identify molecules that may be unlikely candidates but, nonetheless, of great importance in the regulation of CFTR processing.

CFTR biogenesis & chaperone-assisted folding

CFTR is a 12-pass transmembrane protein, which requires a multitude of accessory proteins to progress through a complex sequence of folding events [6–10]. The domains of CFTR acquire their native conformation cotranslationally at the ribosome, and this process requires the assistance of cellular chaperone/cochaperone machinery [11,12]. Chaperone proteins are intricately involved in optimizing the efficiency of folding by masking hydrophobic patches of a newly synthesized protein [13], and cochaperones assist in this process by regulating chaperone function [14,15]. Studies demonstrate that many chaperones and cochaperones interact with CFTR during the folding process and regulate biogenesis (Table 1). Properly folded CFTR exits the ER, traffics through the Golgi network and reaches the plasma membrane, where it functions as an ATP-dependent anion channel [16,17].

Protein misfolding & proteasomal degradation in CF

A large and diverse group of molecules monitor the cell for damaged or misfolded proteins. It is critical that unsuccessfully folded products are removed from the cell before they can form aggregates and result in cellular toxicity [18]. If a protein is recognized as misfolded, a cascade of events is triggered that results in its specific degradation [19]. This process begins during translation, with the folding status of a nascent protein under the surveillance of ER-associated proteins [20–24]. Chaperones and cochaperones, in addition to promoting productive folding, can also triage misfolded proteins towards the proteolytic pathway [10,25]. The deletion of phenylalanine 508 in CFTR yields a misfolded protein that is predominantly targeted for degradation, with approximately 99% of the protein degraded before it reaches the plasma membrane [26–28]. The inefficiency in successfully folding this large transmembrane protein at the ER is highlighted by the observation that approximately 75% of wild-type CFTR is also degraded before reaching the cell surface [26,29].

One way that proteins are marked for degradation is by the addition of ubiquitin moieties. Ubiquitin monomers are covalently added to proteins, either co- or post-translationally, and polyubiquitin chains are created in a variety of linkages using the seven lysines in ubiquitin (e.g., K6, K11, K27, K29, K33, K48 and K63) [30,31]. K48-linked polyubiquitin chains typically serve as a signal for proteasomal degradation when the polyubiquitin chain contains at least four ubiquitin molecules [32,33]. Other poly-ubiquitin chain linkages, such as K63, can signal non-proteolytic functions, including DNA repair and protein sorting [32–35]. Some proteins contain a single or a small number of lysine residues that are critical for proteasomal degradation [36]. Other proteins require the ubiquitination of a large number of lysine residues or, alternatively, are degraded by the proteasome when all lysines are mutated [37–39].

Misfolded CFTR, such as $\Delta F508$, is ubiquitinated during assembly in the ER and this serves as a molecular signal for proteasomal degradation [40,41]. CFTR ubiquitination can also occur after retrieval from the plasma membrane, resulting in its lysosomal degradation [42,43]. Ubiquitination involves the sequential involvement of a ubiquitin-activating protein

(E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [44]. Deubiquitinating enzymes also regulate ubiquitination through the cleavage and remodeling of conjugated ubiquitin [45]. Several molecules associated with modulating the ER-associated ubiquitination of CFTR have been described (Table 1). Once ubiquitinated, $\Delta F508$ CFTR is targeted to the proteasome following retrotranslocation, which involves extraction of CFTR from the ER lipid bilayer and presentation to the cytosolic proteasomal machinery (Table 1) [46,47]. The lysine residues that are critical for triggering the ER extraction and proteasomal degradation of CFTR are currently unknown.

Proteomic examination of early proteolysis in CF

Proteomic approaches allow for the global examination of proteins and are a key technology in expanding our understanding of cellular processes and protein functions. Proteomic studies can identify proteins involved in CF pathophysiology and recent advancements in proteomics-based technologies have made it possible to screen for molecules involved in the premature proteolysis of CFTR. In this article, we review proteomic studies that have examined global protein expression in CF. Additionally, we highlight interactomic studies focused on the identification of regulators of CFTR biogenesis and degradation.

Global protein expression analysis in CF

Proteomic techniques have been utilized to analyze various aspects of CFTR biogenesis and trafficking. Expression-based studies have focused on the identification of proteins regulated by misfolded CFTR through the global analysis of identifiable proteins in CF versus non-CF cells. Using 2D gel electrophoresis (2DE), Pollard *et al.* examined protein expression in CF cells compared with wild-type CFTR-corrected matched controls and identified 20 differentially expressed proteins [48]. This steady-state approach was also expanded to analyze global differences in protein synthesis in CF cells by radiolabeling newly synthesized proteins, which identified 31 additional proteins. Of the proteins identified in this study, 30% were associated with NF- κ B signaling, highlighting the importance of this pathway in CF disease. Additional molecules known to be involved in proteasomal degradation (UCH-L1, UBE2N, PSMA3 and PSME2) were also demonstrated to be differentially expressed in the CF proteome.

As an alternative approach for identifying proteins involved in the proteolysis of CFTR, several studies have explored the global effect of $\Delta F508$ CFTR overexpression. Davezac *et al.* examined the role of misfolded CFTR on global protein expression by comparing the effect of wild-type versus $\Delta F508$ CFTR overexpression in HeLa cells [49]. Keratin 8 (K8) and 18 (K18) were observed to be elevated in the $\Delta F508$ CFTR-expressing cells compared with wild-type CFTR controls. Further functional studies demonstrated that reducing K18 expression resulted in increased trafficking of $\Delta F508$ to the plasma membrane. Gomes-Alves *et al.* similarly compared wild-type versus $\Delta F508$ CFTR overexpression *in vitro*, but instead used BHK cells [50]. This study was also expanded to analyze the effect of $\Delta F508$ CFTR(4RK), which lacks the four arginine-framed tripeptide (RXR) motifs of CFTR and partially escapes premature proteolysis. By this approach, 71 proteins were identified as differentially expressed between the cell lines, of which, 35% were known to interact with CFTR or have involvement in the cellular unfolded protein response (UPR) [50]. Gomes-Alves and colleagues demonstrated a similar role for the UPR in the efficient degradation of $\Delta F508$ CFTR by examining the proteome of BHK $\Delta F508$ cells grown at 26°C [51]. $\Delta F508$ CFTR can be rescued to the cell surface by reducing the growth temperature [52], and this study revealed 139 proteins that were altered under low-temperature conditions. Many of the proteins identified are involved in the UPR or repression of cell metabolism, and this suggests involvement of these pathways in low-temperature $\Delta F508$ CFTR rescue [51].

Proteomic studies have also been designed to analyze protein expression *in vivo*. Carvalho-Oliveira *et al.* explored protein expression in whole lung extracts from $\Delta F508$ CFTR mice and observed a twofold reduction of lung carbonyl reductase (CBR2) compared with wild-type controls [53]. The use of whole lung homogenates containing cell types that lack CFTR expression may have hindered the detection of additional proteins, including candidates for CFTR proteolysis. Global protein expression in human tissues has been assessed by comparing the protein profile in CF patient samples to non-CF controls. A study by Sloane *et al.* examined sputum from adults and children with CF by 2DE and mass spectrometry (MS) [54]. Sputum samples from CF individuals were found to have elevated myeloperoxidase, α_1 -antitrypsin and IgG fragments. Protein microarrays have also been utilized to globally examine protein expression in CF. Srivastava *et al.* employed an antibody capture microarray to examine the abundance of 507 proteins in CF sera and observed 46 proteins elevated in CF versus control samples, of which, 16 were previously implicated in CF disease pathophysiology or NF- κ B signaling [55]. While these studies highlight the ability to detect differences in protein expression levels between CF and control diseased tissue samples using 2DE/MS and protein microarrays, sputum and sera samples contain an abundance of secreted proteins and inflammatory signaling molecules. These samples offer the opportunity to search for easily accessible biomarkers of disease, but are not enriched in CFTR-expressing cells and, therefore, are not ideal for identifying proteins involved in CFTR processing. The proteomic profiles of epithelial tissues, both bronchial and nasal, from CF individuals have also been investigated, which allows for the direct examination of cell types that are disproportionately affected by CFTR mutations. A study of bronchial tissue from CF and non-CF individuals identified an elevation of the chaperone GRP75, in addition to two other proteins, in the CF samples [56]. Furthermore, an analysis of nasal epithelial cells revealed differential expression of 13 proteins, which included the two chaperones calreticulin and protein disulfide isomerase A3 (GRP58) [57].

These studies highlight the advantages of using a global screen to identify changes in protein expression in CF. Notably, expression-based studies for CF must be undertaken with the caveat that CF is a complex disease that results in many cellular changes that may not be directly related to the presence of misfolded protein. To differentiate protein-expression changes related to misfolded protein versus alterations in chloride transport, experiments can be designed using a *CFTR*-knockout control. Importantly, expression-based 'hits' identified via proteomic techniques must be further explored by complementary biochemical methodologies to elucidate the specific functions in CFTR proteolysis.

Investigating CFTR interactomics

Interactomics is the study of the complex network of cellular protein interactions and the biological significance of those interactions [58]. Interactomic approaches have notable advantages over global proteome profiling, as proteins that interact directly with CFTR are the most likely candidates for regulating its proteolysis. Several studies have utilized interactomic approaches to examine CFTR protein interactions during its biogenesis and degradation. Thelin *et al.* examined the first 25 amino acids of CFTR and explored protein interactions within this region by purification and MS [59]. They discovered that the N-terminal region of CFTR interacts with filamin proteins and this interaction regulates the stability of CFTR at the plasma membrane. In addition, this study identified a disease-causing missense mutation in CFTR (S13F), which reduces binding to filamin and results in excessive lysosomal degradation from the plasma membrane.

The interactome of full-length CFTR has also been examined in several studies. Multidimensional protein identification technology (MudPIT), a shotgun tandem MS (MS/MS) approach [60,61] was utilized to investigate the global interactome of CFTR following its immunopurification [10]. This study identified approximately 170 CFTR binding partners

in the 'CFTR proteome', including three Hsp90 cochaperones; Aha1, p23 and FKBP8. Aha1 protein was further demonstrated to enhance CFTR degradation through the regulation of ATP-dependent folding steps [10,62]. Alternatively, a 2DE approach, followed by MALDI TOF analysis, was used to identify CFTR interactions modulated by treatment with 4-phenylbutyrate [63], a small molecule that enhances the trafficking of $\Delta F508$ CFTR [64]. This study detected a modulation in 16 CFTR protein interactions, of which, 12 were chaperones or cochaperones [65]. The preponderance of chaperones identified in this study suggests that this class of molecules is targeted by 4-phenylbutyrate treatment and may be involved in CFTR rescue, although testing each chaperone individually is necessary to confirm a direct role in CFTR processing.

An additional interactomic approach incorporates the use of nondenaturing blue-native (BN) lysis conditions, which allows for a more natural examination of cellular interactions through the maintenance of native protein folding and intact protein complexes. Coupling the BN approach with SDS PAGE or, more recently, with direct examination of complexes by MS/MS, has provided insight into the composition of a variety of membranous complexes [66–71]. BN proteomic approaches are also well suited to studying the CFTR interactome, and the effectiveness of this approach in CF research was exemplified by a study examining native membrane complexes in *CFTR*-knockout mice. In this study, a putative calcium-activated chloride channel, mCICA3, was observed to be downregulated in colon and lung samples of *CFTR*^{-/-} mice, suggesting a reliance on CFTR for membrane localization [72]. Direct investigation of CFTR complexes by BN methodology has not been examined extensively, and this approach may provide additional insight into the CFTR interactome and partners involved in its proteolysis. A recent report established a method for purification of FLAG-tagged CFTR and subsequent separation by BN PAGE [73]. This study revealed that CFTR runs at a single mass of 353 kDa, potentially representing a CFTR homodimer. These conditions can be adapted and optimized to examine the heterogeneous complexes formed at the ER during $\Delta F508$ CFTR biogenesis.

The CFTR interactomic approaches described are effective in the discovery of stable protein interactions; however, it remains possible that more transient interactions are not captured under these conditions. These labile interactions may also play a significant role in CFTR processing, and techniques such as crosslinking and substrate trapping may be used with the current proteomic technologies to identify transient interactions. Crosslinking approaches, coupled to MudPIT analysis, successfully identified transient CFTR interactions that were not captured under standard conditions [10]. Interestingly, many CFTR interactions conversely became undetectable in the presence of a crosslinking agent, possibly as a result of increased background. The effectiveness of a crosslinking strategy was also demonstrated in the identification of global interactions of factor-inhibiting hypoxia-inducible factor (FIH), and in a second study examining the interactions of glycolytic enzymes of *Bacillus subtilis* [74–76]. Importantly, studies using crosslinking are prone to an increase in false-positive results and precautions should be taken when interpreting the experimental results.

Proteomic-based analysis of CFTR ubiquitination

While it is understood that ubiquitination is necessary for the efficient proteasomal targeting of CFTR [40], the specific lysine residues on CFTR that are required for this process have not been elucidated. The large number of CFTR lysine residues accessible to the cytosolic ubiquitination machinery makes the identification of critical ubiquitination residues challenging (Figure 1). Therefore, proteomic techniques are well suited to explore the ubiquitination sites of CFTR.

Ubiquitin modification involves the C-terminal glycine of ubiquitin, which is attached to an ϵ -amine of a lysine residue of the target protein [77]. MS can assist in the identification of

ubiquitinated lysine residues following trypsin digestion, which results in the cleavage of proteins after lysine or arginine residues (unless followed by a proline residue). In the case of a ubiquitinated protein, the modified lysine will no longer be a cleavage site for trypsin, and will maintain the final two glycine residues of ubiquitin (diglycine tag) [78]. This -GG modified peptide fragment can be identified by a mass shift of 114.0428 Da (Figure 2A). This technique has been used in global studies [79–81] and substrate-specific studies [82–84] to identify ubiquitinated residues, and is an ideal approach to identify CFTR ubiquitination sites. Importantly, examining CFTR ubiquitination by this methodology requires the effective purification of CFTR.

Ubiquitin chain linkage is also important in determining the fate of an ubiquitinated protein, and polyubiquitin chains conjugated through different lysine residues can signal multiple functions, including proteasomal degradation [85–87]. Currently, the ubiquitin linkage types conjugated to CFTR and their effects on CFTR biogenesis are unknown. For example, the role of K11 ubiquitin chains in ER-associated degradation has been demonstrated recently [88]; however, the function of this unconventional linkage in CFTR degradation is not defined. Recent advances in the absolute quantification (AQUA) method allow for the quantification of ubiquitination linkage types in mixed samples or conjugated to an individual protein following purification [89]. AQUA of ubiquitin linkages involves spiking in a known amount of heavy isotope-labeled ubiquitin peptides, which can then be distinguished from the ubiquitinated peptides in the sample by MS. The absolute quantity of ubiquitin linkages can be calculated by comparing the ratio of heavy-labeled peptide to peptides derived from the sample of interest (Figure 2B). This technology has successfully quantified ubiquitin linkages on substrates such as cyclin B1 and TRAF1 [83,84]. For CF, there are plenty of potential applications of this technology, including examination of ubiquitin linkages in CF versus non-CF samples on global (total protein ubiquitination) and substrate-specific (CFTR) levels. Additionally, AQUA has applications in identifying changes in CFTR ubiquitination related to modulation of proteolysis, such as following therapeutic interventions that alter CFTR trafficking and degradation. A limitation of AQUA, however, is an inability to determine the ubiquitin chain linkage or length at an individual lysine residue.

Implementation of proteomics in the discovery & analysis of therapeutic interventions

Proteomic technologies have a substantial number of potential applications in the discovery and analysis of novel therapeutic approaches for CF treatment. Several categories of therapeutic approaches that have garnered significant attention in CF research include the discovery of chemical inhibitors and small-molecule correctors, modulation of chaperone function, regulation of the ubiquitin–proteasome system, siRNA-mediated gene silencing, and gene therapy. The following section will discuss the relevance of proteomics in the identification of target molecules and the analysis of therapeutic approaches, in the context of these categories.

Chemical inhibitors & small molecules

Small-molecule screens have identified compounds, such as corr-4a, that reduce CFTR proteolysis or enhance CFTR trafficking [90,91]. These studies have focused on an ability to rescue CFTR to the cell surface and have not explored the possibility that these molecules may change the expression of other proteins. Proteomics affords an opportunity to analyze large-scale changes to total protein signatures following treatment with CFTR correctors. For example, 2DE was utilized to examine the effect of treating CF cells with the small molecule 4-phenylbutyrate, which rescues $\Delta F508$ CFTR to the cell surface [63]. Many of

the 85 proteins that were shown to be modulated by this chemical belong to the chaperone family, supporting a chaperone-mediated mechanism of CFTR rescue. Corrector molecules may not directly interact with CFTR, and identifying changes in global protein expression or interactions following treatment with novel CFTR correctors may provide insight into the molecular mechanism of action. Additionally, potential negative off-target effects associated with altered expression of other proteins modulated by these compounds may be revealed.

Recent studies suggest that modifying large-scale gene-expression profiles with small molecules represents a viable therapeutic approach for CF. A study by Hutt *et al.* describes the rescue of $\Delta F508$ CFTR using histone deacetylase (HDAC) inhibitors [92]. This study revealed that inhibition of HDAC7 can rescue $\Delta F508$ CFTR to the cell surface by modulating a variety of genes that participate in CFTR degradation. Harmon *et al.* describe a study of CF-null mice, which exhibit altered expression of genes involved in lipid metabolism [93]. They observed that treating CFTR-null mice with synthetic PPAR- γ ligand partially returned gene-expression patterns to that of wild-type mice and reduced disease severity. While the effect was not due to altered proteolysis, as CFTR is not expressed in this mouse strain, this study provides additional support for therapeutic approaches that broadly alter gene-expression profiles. While these studies have utilized microarrays to predict protein expression, a direct correlation does not always exist between transcript and protein [94], and the use of complimentary proteomic approaches is warranted in the characterization of these compounds.

Chaperones

Chaperones and cochaperones are intricately involved in CFTR processing and can promote both successful folding and premature degradation (Table 1). Modulating chaperone expression and activity has been demonstrated to rescue $\Delta F508$ CFTR to the cell surface [95,96]. Importantly, the client proteome for an individual chaperone is often large and therapeutic approaches directed at targeting chaperones could have significant non-CFTR-related effects. For example, proteomic analysis of the Hsp90 interactome identified 61 binding partners, many of which were altered by treatment with the Hsp90 inhibitor geldanamycin [97]. The off-target consequences associated with altering global protein folding in CF can also be examined with proteomic techniques.

ER-associated degradation/ubiquitination machinery/proteasome

A majority of CFTR degradation is ubiquitin dependent via proteasome-mediated degradation, and interfering with this process may provide a viable therapeutic strategy. Proteasome inhibitors, for example, have been used in cell models to successfully rescue misfolded $\Delta F508$ CFTR [25,40,98]. In CFTR overexpression models, however, complete proteasome inhibition can result in aggresome formation, which may have undesirable consequences on cell survival [99,100]. Additionally, proteasome inhibition may significantly modulate non-CFTR protein levels. The effectiveness of proteomics in examining the global effects of treatment with inhibitors of proteolysis was demonstrated in a study analyzing the effect of proteasome inhibitors on leukemic cells, and in a second study examining proteasome inhibitor-mediated modulation of protein sumoylation [101,102]. Similar proteomic approaches can be utilized when exploring the usefulness of proteasome inhibitors to rescue misfolded CFTR.

Therapeutics focused on CFTR degradation can potentially be tailored to have greater specificity by targeting E3 ubiquitin ligases or deubiquitinating enzymes, which have greater substrate specificity. Several molecules involved in the ubiquitination pathway have been implicated in CFTR processing, including Rma1, CHIP, SCF^{Fbx2} and UCH-L1 [23,25,103,104]. Still, many of these molecules have not been characterized fully, and other

proteins may be altered by modulating the activities of these enzymes. For this reason, the identification of all substrates for these enzymes is of importance. Proteomics can provide an effective strategy for substrate identification, as was demonstrated in studies of the E3 ligases Rsp5, Nedd-4 and Nedd-2 [105–107].

siRNA

Large-scale siRNA screens can be utilized to identify regulators of CFTR proteolysis. One such reverse proteomic approach utilizing a siRNA screen was employed by Trzcinska-Daneluti *et al.* to identify proteins that regulate $\Delta F508$ CFTR trafficking. This screen examined the effect of 446 proteins on CFTR conductance and identified 13 proteins that increased chloride transport by more than 25% [108]. Similar approaches can be utilized to examine the effect of additional proteins on CFTR biogenesis.

siRNA technologies also have potential therapeutic applications and recent studies utilizing this method of targeted inhibition have been promising [109–112]. The effectiveness of siRNA therapeutics, however, is limited by their targeting specificity, both for cell type and mRNA target. Studies have demonstrated that siRNA can have undesirable off-target effects, which are related to nonspecific knockdown and stimulation of innate immunity by the dsRNA molecules [113–115]. The specificity of siRNA-mediated gene knockdown and potential proinflammatory consequences can be assessed by proteomic methodologies.

Gene therapy

As CF is a monogenic disorder, gene therapy is considered a viable therapeutic approach. However, gene therapy has confronted significant challenges related to packaging the large *CFTR* gene, targeting the appropriate cells, host immune response and stability of transgene expression [116–121]. Proteomic approaches are well suited to analyze the global effect of a transgene in both cellular and animal models, and this was demonstrated by *in vitro* studies analyzing the effect of adenoassociated viral transduction of CF cells with wild-type *CFTR* [48,65]. Proteomic techniques can provide a key resource in analyzing future gene therapy approaches for CF.

Expert commentary

A majority of CF disease is caused by class II mutations of *CFTR*, which result in misfolding and premature degradation of the protein. Preventing premature degradation of CFTR, and alternatively promoting its trafficking to the cell surface, is a rational approach to CF therapy. While previous studies have successfully revealed proteins and pathways involved in CFTR folding and degradation, our understanding of these processes remains incomplete. Continued proteomic investigations can provide a pivotal resource in advancing our understanding of CFTR proteolysis through large-scale protein-identification approaches. The unbiased nature of proteomic methodologies enables the identification of molecules that may not be likely candidates but are, nonetheless, of great importance in regulating CFTR biogenesis. Once identified, these targets can be tested by alternative techniques to determine a precise role in CFTR folding and degradation.

Expression-based studies in CF have identified numerous molecules that may be involved in disease pathogenesis. Many of these investigations, however, have relied on an ability to resolve proteins by 2DE. More sensitive MS-based quantification approaches, such as isotope coded affinity tags (ICAT; cysteine labeling) [122,123], isobaric tag for relative and absolute quantification (iTRAQ; amine labeling) [124] and stable-isotope dimethyl labeling (reductive amination) [125], provide alternative methods for detecting relative abundance of proteins. For example, CF and non-CF protein samples would be modified *in vitro* by one of

these methods, such that the derived peptides could be differentiated by MS/MS. Utilization of these techniques can potentially identify critical mediators of CFTR degradation that are undetectable by gel-based approaches.

A misfolded protein, such as $\Delta F508$ CFTR, must be degraded to prevent cellular toxicity; however, rapid degradation may preclude sufficient time for protein folding. Therefore, a delicate balance between these two processes exists in the cell. Efforts to promote the folding or reduce the degradation of CFTR hold potential to rescue the misfolding defect and allow CFTR to escape the ER. Many molecules that participate in these processes have been identified, but the effects of these proteins are not straightforward (Figure 3). There exists a dichotomy between pro- and anti-degradative effects on CFTR for several molecules, such as the chaperone Hsc70. Protein interactions that regulate the folding and proteasomal targeting of CFTR can occur during different stages of protein synthesis and this temporal separation may contribute to the opposing effects. Attempting to separate the different phases of CFTR synthesis may enhance our understanding of the mechanisms guiding folding and degradation. For example, efforts focused on isolating ribosome-associated CFTR could reveal molecules that regulate the co-translational folding and ubiquitination.

Proteomic experiments currently utilize several model systems, including cell culture, animal models and human samples. Animal models are a promising avenue for CF research and novel model systems, such as the CF pig and ferret, have been developed in recent years, which may hold important clues regarding CFTR processing [126,127]. Indeed, it has been demonstrated that there are species-specific differences in CFTR processing, but whether this is due to intrinsic differences in CFTR or results from altered cellular interactions is currently unknown [128]. Additionally, animal models have potential usefulness in the development and testing of therapeutics. Human samples, obtained from individuals with CF, can also be analyzed by proteomic techniques. These studies provide a unique opportunity to analyze the *in vivo* conditions that contribute to CFTR processing and disease pathogenesis. Analysis of human samples is limited by the amount of tissue obtained, but previous proteomic studies have demonstrated the importance of these samples in the discovery of signaling pathways altered in CF. Coupled with the continued development of novel proteomic tools, studies using patient-derived samples hold great promise for future investigations.

The process of ubiquitinating CFTR represents a large gap in our understanding of CFTR degradation. While the study of CFTR ubiquitination is no small feat, owing to the complexity of CFTR, the importance of this modification in proteolysis dictates the necessity to expand our understanding of this pathway. Elucidating the critical lysine residues and the types of ubiquitin chains conjugated to CFTR, by MS and AQUA, can provide mechanistic insight into the biogenesis and degradation of CFTR. Moreover, global changes in ubiquitination may be associated with protein misfolding in CF, and proteomic techniques can aid in exploring this possibility.

Proteomic technologies are rapidly advancing, and novel methodologies for the isolation and analysis of CFTR will evolve. Many of the technological advances will occur in fields outside CF research, and collaborative efforts will be essential in adapting these techniques to explore CFTR folding and degradation. Additionally, understanding the mechanisms influencing other protein-misfolding diseases, such as Alzheimer's and Parkinson's diseases, is of great importance. Applying this knowledge to the CFTR framework holds the potential to transform our understanding of the complexities of premature proteolysis in CF.

Five-year view

There remain significant challenges ahead in the understanding of premature proteolysis in CF. The last 5 years in CF research have provided many advances in the identification of molecules involved in CFTR biogenesis and degradation, and we envision the next 5 years will yield additional mechanistic insight into this process. Proteomic technologies, both current and novel, will likely play a central role in expanding our understanding of these molecules.

While the list of CFTR binding partners is continually growing, it is far from complete, and the systematic application of proteomic approaches to explore the CFTR interactome in relevant human cell types is of great importance. The most obvious candidates for modulators of CFTR degradation are those that interact directly with the protein during its biogenesis, highlighting the necessity to increase our understanding of CFTR interactions. Further work focusing on the CFTR interactions at the ER, by subcellular fractionation, may enhance the ability to reveal protein interactions that are important in CFTR degradation. Additionally, the development of BN approaches and techniques to probe transient CFTR interactions can provide further insight into the CFTR interactome and reveal proteins involved in the proteolytic process.

Proteomic tools provide an excellent resource for the development of therapeutic interventions designed to reduce or bypass the premature proteolysis in CF. First, we envision the use of proteomics in the elucidation of therapeutic mechanisms of action. Furthermore, the analysis of global cellular responses to novel treatments will assist in the avoidance of toxic therapeutic side effects. In the next 5 years, we anticipate that proteomics will play a critical role in advancing our understanding of many aspects of the premature proteolysis in CF – from biological mechanisms to therapeutic approaches.

Key issues

- Mutations in cystic fibrosis transmembrane conductance regulator (*CFTR*) that result in premature degradation are responsible for the majority of cystic fibrosis (CF). $\Delta F508$ accounts for 66% of all disease-causing alleles and results in a terminally misfolded protein that is efficiently degraded.
- The use of proteomic techniques for global expression analysis in CF samples, both *in vitro* and *in vivo*, can reveal proteins that may be regulated by the misfolded protein. This methodology has identified several key pathways that demonstrate altered expression in CF.
- Interactomic studies have revealed key proteins involved in the proteasomal degradation of CFTR. Further studies, specifically those focused on the endoplasmic reticulum localized interactome and transient interactions, can identify novel proteins in this process.
- The ubiquitination of CFTR remains a poorly defined aspect of CFTR proteolysis. The ubiquitinated lysine residues of CFTR can be identified by mass spectrometry, and the types and relative amounts of ubiquitin linkages conjugated to CFTR can be explored by absolute quantification techniques. This approach can also be expanded to investigate global (non-CFTR) changes in ubiquitination in CF samples.
- Proteomic technologies are well suited to identify and examine novel therapeutic strategies. Significant efforts have focused on small-molecule correctors of CFTR, chaperones, the ubiquitin–proteasome pathway, siRNA

gene knockdown and gene therapy. Each of these approaches can benefit from proteomic techniques and technologies.

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Figure 1. Cystic fibrosis transmembrane conductance regulator (CFTR) contains 87 lysine residues accessible to the cytoplasmic ubiquitination machinery

The lysine residues in the cytoplasmic domains of CFTR are identified. Domain boundaries are derived from Lewis *et al.* [148] and the CF Mutation Database [201].

Figure 2.

(A) The ubiquitination of cystic fibrosis transmembrane conductance regulator (CFTR) can be analyzed by trypsin digestion and mass spectrometry. A ubiquitinated lysine (in this case K503 of CFTR) will not be cleaved by trypsin and will retain a di-glycine tag from the conjugated ubiquitin. K503 is highlighted in green and Phe508 is highlighted in red. (B) Ubiquitin linkages can be quantified by absolute quantification (AQUA) technology. Peptides derived from polyubiquitin chains of different linkages (in this case K48 and K11) can be compared with a known amount of heavy-isotope-labeled peptides to quantify the amount of each linkage type. The relative and absolute amounts of each ubiquitin linkage type conjugated to purified CFTR can be calculated by AQUA.

Figure 3. Proteins that control the folding, ubiquitination and retro-translocation of cystic fibrosis transmembrane conductance regulator (CFTR) direct its premature proteolysis, and several of these molecules are illustrated

These proteins are depicted temporally to reflect regulation of different phases of CFTR biogenesis. Proteins colored blue indicate enhancers of CFTR proteolysis, and proteins colored purple represent inhibitors. Note that several proteins have opposing effects on CFTR degradation at different stages of folding. The figure was constructed based on the references listed in Table 1.

Table 1

Proteins implicated in the premature proteolysis of cystic fibrosis transmembrane conductance regulator.

Class	Protein	Subcellular Compartment	Positive/negative regulation	Ref.
Chaperones	Hsp70	Cytosol	+/-	[27,95,129,130]
	Hsc70	Cytosol	+/-	[11,131]
	Hsp90	Cytosol	-	[132]
	Calnexin	ER transmembrane	+/-	[22,96,133,134]
	α A-crystallin	Cytosol	+	[135]
Cochaperones	Aha1	Cytosol	+	[10]
	HspBP1	Cytosol	-	[136]
	BAG-2	Cytosol	-	[137]
	Csp	ER associated	-	[138]
	Hdj-1	Cytosol	-	[130]
	Hdj-2	Cytosol	-	[11,139]
Ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s)	UbcH5a (E2) and CHIP (E3)	Cytosolic	+	[25]
	Ubc6e (E2) and RNF5/Rma1 (E3)	ER associated	+	[23]
	SCF ^{Fbx2} (E3)	Cytosolic	+	[104]
	gp78 (E3)	ER associated	+	[140]
E3 inhibitors	SVIP	ER anchored	-	[141]
Deubiquitinating enzymes	UCH-L1	ER associated	-	[103]
	Usp19	ER tail anchored	-	[142]
Retrotranslocation	VCP/p97	Cytosol/ER associated	+	[98]
	Derlin-1	ER transmembrane	+	[24]
	Sec61p	ER transmembrane	+	[143]
	Sar1p/CopII	ER associated	+	[144]
	26S proteasome	Cytosolic	+	[40]
	UBXD1	Cytosolic	-	[145]
	Ufd1	Cytosolic	+	[146]
	BAP31	ER transmembrane	+	[147]
Carbohydrate-binding proteins	EDEM	ER lumen	+	[146]

Proteins that regulate CFTR biogenesis, and the class of molecules to which they belong, are listed. The subcellular localization and effect of each protein on CFTR proteolysis, whether positive (+: enhances degradation) or negative (-: reduces degradation), is also indicated.

ER: Endoplasmic reticulum.