F508 Corrector technologies and mechanistic assays available through the CFC.

Overview: This document describes capabilities available through the CFTR Folding Consortium (CFC) and other groups pursuing F508 corrector screens and drug characterization. Included are specific areas of CFC investigator expertise, brief summaries of selected assays relevant to corrector mechanism of action, and new CFC protocols suitable for compound library screening. Academic and commercial laboratories interested in F508del correction are welcome to contact individual CFC members regarding availability of assays, establishment of collaborations, etc.

A. **CF expertise applicable for drug development related to F508del correction.**

- **a.** Aberrant ICL4/NBD1 interaction and NBD1 instability as contributors to the F508del CFTR defect as they apply to small molecule correction and drug synergy.
- **b.** NBD solubilizing and correcting mutations, their impact on F508del CFTR processing/folding, and additivity/synergy with candidate small molecule correctors.
- **c.** Molecular signatures of F508del corrector molecules via the CFTR "roadmap," including a comprehensive profile of drug effects on CFTR cellular biology, protein biochemistry, and function.
- **d.** Establish target and mode of action of corrector compounds.
- **e.** Mediators of F508del surface instability.
- **f.** The role of non-CFTR targets in corrector mechanism of action.
- **g.** Yeast models to analyze CFTR ERAD, epistasis for corrector strategies, and ubiquitin and SUMO modification in the setting of drug treatment.
- **h.** Role of corrector compounds on post-translational modifications of wt or F508del CFTR (e.g. sumoylation, ubiquitination, palmitoylation, phosphorylation, 14-3-3 binding).
- **i.** Mechanisms of co-translational folding of F508del CFTR.
- **j.** Biophysical analysis of F508del CFTR (and domain) folding, protease susceptibility, thermodynamic stability, and effects of corrector molecules on the folded state.
- **k.** Analysis by proteolysis of whether the folded state of purified CFTR and its domains are similar to those in the intact cell.
- **l.** Role of the cellular proteostasis environment on F508del CFTR processing.
- **m.** Purified CFTR domains, domain-specific antibodies, and other molecular tools suitable for studies of F508del CFTR correction.
- **n.** Functional assays of surface localized wt or F508del CFTR in primary human airway epithelial and other cell models.

B. **Summary of selected assays relevant to identifying corrector mechanism of action.**

a. **Assay: F508del conformational stability**
Investigator: Lukacs
The isolated F508del-NBD1 is energetically destabilized by thermal and chemical denaturation. Resistance of F508del-NBD1 containing a single solubilization mutation to denaturation is significantly decreased versus wild type. A subset of corrector molecules that confer conformational stability to the mutant NBD1 should improve CFTR processing. Our assay monitors melting temperature of the isolated F508del-NBD1 using differential scanning fluorimetry with various fluorescent dyes (e.g. Sypro Orange). The assay can identify drugs that interact directly with F508del-NBD1 and rescue the structural defect without confounding effects of the plasma membrane diffusional barrier, cellular metabolism or cell-based side effects of drugs. This type of screen is novel and may produce hits that have highly specific, direct interaction with F508del-CFTR (as pharmacochaperones) but have been missed in cell based HTS.

b. **Assay: Chemical denaturant perturbation**
Investigator: Thomas
The protocol measures resistance to perturbant induced unfolding of NBD1 by determining the extent to which CFTR-NBD1 can partition across a 100kD filter, i.e. the fraction of NBD1 that remains folded following denaturation. An ELISA is used to measure levels of NBD1 after interaction with the size exclusion barrier, and throughput of the assay is moderate to high. Early steps in CFTR folding known to be affected by F508del mutation and corrected by second site suppressors are probed by the CDP *in vitro*. Small molecules that enhance NBD1 folding by this protocol also have been shown to augment processing of full-length F508del CFTR in living cells.

c. **Assay: NBD1 thermal stability**
Investigator: Thomas
Purified NBD1 thermal unfolding is measured by light scattering (or other optical methods) *in vitro*. The assay monitors early steps in CFTR folding known to be 1) affected by the F508del mutation, and 2) corrected by second site suppressor mutations that augment maturational processing of full-length F508del CFTR.

d. **Assay: High content microscopy, CFTR surface localization**
Investigators: Sorscher, Chung
This cell-based imaging assay can be used to identify, quantify, and validate small molecule correctors of F508del-CFTR. The approach utilizes an Evotec Opera™ automated confocal microscopy system and tracks surface localization of F508del-CFTR via two-dimensional imaging in permeabilized cells labeled with antibodies that recognize internal CFTR epitopes. High content imaging of total F508del-CFTR may also identify new compounds that increase the overall F508del CFTR pool available for folding correction. The HTS platform is being adapted for use with primary human airway epithelium, a model that correlates well with *in vivo* efficacy of CFTR modulators.

e. **Assay: Cell surface density of F508del using 3HA- or HRP-tagged CFTR**
Investigator: Lukacs
These protocols detect CFTR cell surface density using either immunoperoxidase or direct enzymatic assay. The former requires incubation steps to ensure primary (HA) and secondary (HRP) antibody binding. A CFTR-HRP fusion eliminates antibody incubations by incorporating HRP in an extracellular CFTR loop and can be used to biochemically quantify levels of CFTR in the plasma membrane (independent of anion transport mechanisms and/or PKA activation). The assay is a) robust, b) sensitive, c) allows adjustment of CFTR transcriptional activation using the tetracycline-induction system and d) uses CFBE cells, which closely resemble the *in vivo* target tissue.

f. **Assay: ASL volume measurements in primary HBE**
Investigators: Myerburg, Pilewski and Frizzell
This approach to ASL volume measurement can serve as a primary or secondary screen for F508del CFTR correctors. The assay is presently run using Transwell filters (diameter of 6 mm) that support growth of well-differentiated primary human bronchial epithelia (HBE). HBEs are most likely to predict clinical efficacy, and ASL represents a critical outcome measure that tracks mucociliary clearance. This type of screening assay has not been used previously by either commercial or academic groups.

g. **Assay: ADP-Glo Hsp90-cochaperone HTS Assay**
Investigator: Balch
This is a luminescent based ADP detection assay. The principle is to measure activity by quantifying the amount of ADP produced during a specific kinase reaction.
The test has significance, since it allows investigation of cellular kinase activity in the setting of F508del corrective treatment.

h. **Assay: Small molecule impact on CFTR susceptibility to ER associated degradation.**
Investigator: Skach, Brodsky
This assay determines whether corrector molecules, by modifying the CFTR fold, alter susceptibility of WT or F508del CFTR to ERAD under conditions where synthesis, ubiquitination and degradation can be tracked independently. Membranes containing *in vitro* synthesized CFTR are isolated and incubated in a reconstituted ERAD system to determine whether a particular corrector molecule promotes CFTR folding in such a way as to protect it from ERQC machinery. The system allows CFTR synthesis and the timing of compound addition to be precisely controlled. If CFTR is stabilized, we can also determine whether the corrector stimulates folding co-translationally, posttranslationally, or during the degradation reaction. (The approach helps distinguish direct effects on CFTR from alterations of the cellular quality control/degradation machinery.) Positive results would support a more detailed analysis of degradation machinery, such as p97, chaperones or co-chaperones, or proteasome subunits.

Wild type and F508del-CFTR susceptibility to ERAD and maturation are also measured in the presence or absence of small molecules by a metabolic labeling/pulse-chase analysis. This assay can be performed in the presence of proteasome inhibitors and under conditions (i.e., low temperature) in which F508del-CFTR is rescued to uncover synergistic effects on CFTR rescue. Pulse-chase analyses are performed in HEK293 cells after transient overnight transfection with desired CFTR-expression constructs.

i. **Assay: Small molecule effects on chaperone interaction.**
Investigator: Skach
In vitro-synthesized CFTR readily associates with Hsp 70, and inhibiting Hsp70 binding protects CFTR from degradation. Once folded, Hsp70 interactions are diminished. This protocol determines whether correctors act directly or indirectly to influence CFTR interactions with the Hsp70 axis by quantifying the amount of Hsp70 bound to newly synthesized CFTR in the presence and absence of the small molecule. We then assess whether Hsp70 release (and/or timing of release) by corrector compounds occurs co- or post-translationally by adding the corrector during (or after) translation. Similar methods are used to investigate the role of co-chaperones such as Hsp40 or CHIP during folding and CFTR stabilization.

j. **Assay: Small molecule effects on NBD1 folding.**
Investigator: Skach
A FRET-based assay now enables monitoring of nascent polypeptide folding during synthesis to determine whether corrector compounds directly affect NBD1 folding. This assay allows us to test whether compounds interact specifically with ribosome-bound folding intermediates. Importantly, experimental conditions mimic actual intermediates in the *de novo* folding pathway and do not require denaturant or full-length domains. In addition, the protocol determines whether corrector molecules influence early folding events and/or act at later steps in the pathway that are disrupted by F508del. This approach provides a new way to assess whether correctors act directly on NBD1 and may allow us to discriminate the specific stage of folding during which this takes place.

k. **Assay: Small molecule effects on CFTR ubiquitination and SUMOylation *in vitro***
Investigator: Brodsky
Proteins such as F508del-CFTR are targeted for ER associated degradation (ERAD). Once ERAD substrates become ubiquitinated, they pass a "point of no return" such that improved folding cannot rescue the protein from degradation. We propose that some small molecules that rescue F508del-CFTR trafficking may also alter protein ubiquitination. Recent data indicate that protein SUMOylation represents another critical determinant during ER protein quality control. This modification may also be affected by small molecule correction. We have developed quantitative *in vitro* assays by which CFTR ubiquitination and SUMOylation can be assessed. In brief, ER microsomes are prepared from yeast expressing CFTR, and a reaction is assembled that contains ^{125}I-labeled ubiquitin or SUMO-1, an ATP regenerating system, and concentrated cytosol. After the reaction is quenched, the modified CFTR is precipitated and phosphorimaging used to measure the degree of protein ubiquitination or SUMOylation. Corrector molecules are titrated using these *in vitro* assays, and effects compared with positive controls. In the event that an agent alters protein ubiquitination or SUMOylation, we also measure apparent K_Is for the interaction.

l. **Assay: Identifying timing of corrector action on F508del**
Investigator: Braakman
We conduct radioactive pulse-chase studies in intact cells of choice and add and remove corrector compounds at different times during the incubations (before the pulse, after the pulse, up to or from a range of chase times). We then analyze rescue from the ER (appearance of band C) and conformational rescue of CFTR domains by

immunoprecipitation of limited proteolytic fragments with a range of antibodies that specify domains (e.g. MSDs and NBDs).
We will establish whether the drug target is cellular when the compound acts by preincubation only of cells, or whether the target is CFTR, when the compound's presence during or after CFTR synthesis is a requirement for rescue. This can be confirmed with *in vitro* translations of CFTR.

m. **Assay: Identifying CFTR subdomain targets for small molecule correctors**
Investigator: Braakman
Radioactive pulse-chase experiments in intact cells with wt CFTR, F508del, or other patient mutants in the presence or absence of small molecules (see above) will be followed by limited proteolysis and immunoprecipitation of various domain fragments. This way we identified the domain target for CF106951 (also termed "corrector 18"). C-terminally truncated constructs (which mimic nascent chains) and/or *in vitro* translations are used to confirm the findings.
If a cross-linkable variant is available for the corrector compound, we can identify its target using i*n vitro* translation in the presence of the cross-linkable compound, followed by limited proteolysis and IP with a range of antibodies to detect the subdomain to which corrector has bound. An alternative approach is pulse-chase in intact cells followed by permeabilization to wash out the cytosol, prior to addition of a cross-linkable corrector (otherwise similar to the *in vitro* translation).

n. **Assay: Analysis by proteolysis of whether the folded state of purified CFTR and its domains are similar to those in the intact cell.**
Investigator: Braakman
Working with purified full-length CFTR or its domains (or other targets) always leaves the question as to whether protein conformation is native. We compare the conformation of purified protein with that of *in vitro* translated or in-cell expressed radioactively labeled protein using limited proteolysis.

o. **Assay: Impact of small molecule correctors during target knockdown**
Investigator: Frizzell
For this protocol, we have developed methods to knock-down a number of CFTR binding partners that influence F508del CFTR processing. These include 1) Derlin-1 & Derlin-2 (some isoform selectivity exists between WT and mutant); 2) RMA1 (acts in concert with Derlin and knockdown elicits a 3-fold increase in F508del Band C); 3), Csp (acts late in quality control and knockdown increases CFTR 2-4 fold; 4), Hdj-1 and Hdj-2 (knockdown of the latter gives near-WT levels when combined with low temperature); 5) Aha1 (displacement from CFTR produces a two-fold increase in Band B; 6) Hsp27 (knockdown gives a 4-fold increase in F508del Band B); and 7) CHIP (knockdown produced a 2-fold increase in F508del Band C). Conditions that are additive or synergistic with corrector molecules of interest suggest action at a distinct site. Target knockdown that obviates the action of a corrector molecule can serve as evidence for a similar cellular mechanism. In the latter case, we also attempt to verify findings by co-immunoprecipitation, anticipating a reduction in CFTR-target interaction. Additional protein knockdowns that contribute to F508del rescue are under evaluation by the CFC, and can be tested in combination with corrector molecules in a similar fashion.

p. **Assay: Do corrector molecules interact with ER to Golgi to plasma membrane trafficking of F508del-CFTR?**
Investigator: Guggino
Our laboratory has shown previously that the trafficking of CFTR from Golgi to the plasma membrane is regulated by three proteins, the PDZ domain containing protein, CAL, the small molecular weight GTPase, TC10, and the SNARE protein, STX6. Coordinated function of these proteins regulates steady state levels of immature Band B F508del-CFTR which resides in the ER, and repression of CAL by RNAi can redirect F508del-CFTR to the cell surface. Assays available in our laboratory are used to determine whether knockdown of CAL, TC10 or STX6 enhance the rescue observed following small molecule correction. The goal here is to determine whether 1) these CFTR binding partners represent a rate-limiting step during F508del CFTR processing, and 2) corrector agents work by a mechanism independent of the CAL pathway.

q. **Assay: Investigating cAMP dependent activation of F508del CFTR following correction.**
Investigator: Sorscher
Several laboratories have shown that low-temperature or C4a corrected F508del CFTR does not respond to forskolin or other cAMP-dependent perturbations in the CFBE cell line. This is in contrast to wild type CFTR in the same cell model, which is strongly activated by forskolin. We attribute this finding to defects in F508del CFTR folding, and have designed protocols to determine whether small molecules mediating F508del CFTR correction confer a normal (wild type) response to cAMP in the CFBE model. Genistein serves as a positive control (robust activation of low temperature or C4 corrected F508del CFTR in CFBE). The assay provides a macroscopic test of whether corrector molecules confer a better-folded conformation of mutant CFTR at the cell surface.

r. **Assay: Quantifying ASL depth, ciliary beat frequency, and mucus transport after corrector treatment of F508del primary airway epithelial cells.**
Investigators: Sorscher, Rowe
Spectral domain-optical coherence tomography (SD-OCT) monitors differences in airway surface liquid (ASL) and periciliary liquid (PCL) depth, cilia beat frequency, and mucus transport in wild-type versus CF respiratory epithelia. In this protocol, we treat primary airway cells with corrector agents in order to establish whether a particular compound improves F508del function. Additional exploratory endpoints (e.g. optical mucus density) can also be quantified. The method provides an important tool for ranking corrector compound analogues as a means of drug optimization. An SD-OCT device is also being developed for *in vivo* (bronchoscopic) measures of airway epithelial function, so that studies of this type can be conducted in human subjects in the near future.

s. **Assay: Dependence of proinflammatory mediators on F508del correction.**
Investigator: Pollard
Clinically, there is a close association between CFTR mutations such as F508del and G551D, and a massive proinflammatory phenotype in the CF airway. CF airway inflammation is primarily dominated by CF neutrophils, which have responded to high levels of the chemokine interleukin-8 (IL-8), being produced by CF airway epithelial cells. Cultured CF airway epithelia cells produce increased IL-8, and these levels are reduced when cells are rescued pharmacologically or by gene transfer with wildtype CFTR. In support of drug discovery that rescues both CFTR trafficking and IL-8

expression, our laboratory has developed a platform for quantitative, high throughput assay of IL-8 (Z-Factor > 0.95) on the electrochemiluminescence MesoScale platform. The system is also multiplexed to permit measurement of other cytokines and chemokines, and can be used to test novel corrector molecules for their effects on IL-8 (and other cytokine) release by epithelial cells.

t. **Assay: Phosphorylation pathways relevant to F508del processing.**
Investigator: Pollard
Functional rescue of F508del CFTR in epithelial cells from both CF lung and CF pancreas can be achieved by activation of a signaling pathway initiated by Phosphatidylinositol 3 Kinase (PI3K). The rescue mechanism involves a series of intervening kinases, including PI3K-dependent Kinase 1 (PDK1) and Serum-glucocorticoid Dependent Kinase -1 (SGK1), which terminate on the E3ligase NEDD4-2. Phosphorylated NEDD4-2 is unable to bind and ubiquitinylate mutant CFTR, thus freeing the mutant protein from proteosomal destruction. Rescue can also be obtained by using siRNA to knock down NEDD4-2. The intervening kinases represent drugable targets which avoid using PI3K as an initial driver for rescue. This assay tests F508del corrector molecules for effects on the relevant kinase enzymes, with endpoints that include (i) plasma membrane C-Band, identified by impermeant biotin labeling on a Western blot; (ii) activation of chloride conductance by cAMP, using the MQAE assay; and (iii) reduction of IL-8 production using a sensitive and quantitative electrochemilumenescence ELISA (see **s**, above).

u. **Assay: F508del-CFTR peripheral membrane trafficking.**
Investigator: Lukacs
Our laboratory has developed assays to determine the cell surface turnover, internalization, recycling and ubiquitination of the F508del-CFTR in HeLa cells. Corrector agents and solvent effects can be compared with regard to each of these membrane trafficking steps. Positive results prompt an investigation of corrector molecule effects upon wash out, including steady-state ubiquitination of the F508del-CFTR in post-ER compartments. Cell surface turnover, endocytosis and recycling efficiency are determined by an anti-HA Ab binding assay with immunoperoxidase detection (see also above). Overcoming the folding defect of the F508del-CFTR should be associated with normalized peripheral membrane trafficking. Partial suppression of the folding defect would likely be reflected by a trafficking phenotype with intermediate features between temperature rescued F508del and the wt CFTR channel protein

v. **Assay: FAP-CFTRs report cell surface protein and intracellular trafficking.**
Investigator: Frizzell
Assays to detect CFTR at the cell surface, including the correction of F508del CFTR, involve multiple wash steps and frequently rely on signals that saturate rapidly. We recently developed tools and cell lines that report the correction of mutant CFTR trafficking to the cell surface and have validated the assay using CFFT panel correctors. FAP tags activate fluorogen signals 15-20,000-fold, offering a large dynamic range and excellent signal-noise properties. FAP-CFTR can be assayed using fluorogens that are cell impermeant (e.g. for mutant correction) or permeant (e.g. to document expression or for intracellular studies). The utility for detection of F508del correction has been demonstrated in the 96-well format, with excellent Z-score, and using FACS, and on this basis, we have applied for an NIH R03 from the Molecular Libraries Screening Center

Network. We have also used this assay to track the itinerary of CFTR endocytosed from the cell surface to define the compartments through which CFTR traverses, and where it pauses. This represents a new and simple assay of F508del CFTR that may permit the discovery of more efficacious drugs.

C. New CFC assays suitable for compound library screening

- **a.** ASL depth (R. Frizzell, M. Myerberg)
- **b.** Surface CFTR (G. Lukacs)
- **c.** FAP-CFTR (R. Frizzell)
- **d.** High content microscopy (E. Sorscher)
- **e.** Ubiquitination blockade (J. Brodsky)
- **f.** NBD1 folding (P. Thomas)
- **g.** Inflammatory and other mediator release (H. Pollard)
- **h.** YFP and siRNA assays for F508del correction (B. Balch)
- **i.** Folding and proteolysis (I. Braakman)
- **j.** CFTR ELISA (biochemical protocol) (P. Thomas)
- **k.** ASL depth by SD-OCT (E. Sorscher)
- **l.** FAP-CFTRs (Frizzell and others)
- **m.** Human Hsp70 and Hdj2 (J. Brodsky)
- **n.** Yeast expression systems for CFTR and F508del CFTR (J. Brodsky)

D. **Functional assays of surface localized wt or F508del CFTR in primary human airway epithelial cells (from Chantest)**. Interested parties are invited to contact Dr. C. Penland at the CFF for studies of mechanistic or therapeutic significance using primary airway cell models available from Chantest.

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