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Original Article

Amplifiers co-translationally enhance CFTR biosynthesis via PCBP1-mediated regulation of CFTR mRNA

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ABSTRACT

Background: Cystic fibrosis (CF) is a recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. We previously described a first-in-class CFTR modulator that functions as an amplifier to selectively increase CFTR expression and function. The amplifier mechanism is distinct from and complementary to corrector and potentiator classes of CFTR modulators. Here we characterize the mechanism by which amplifiers increase CFTR mRNA, protein, and activity.

Methods: Biochemical studies elucidated the action of amplifiers on CFTR mRNA abundance and translation and defined the role of an amplifier-binding protein that was identified using chemical proteomics.

Results: Amplifiers stabilize CFTR mRNA through a process that requires only the translated sequence of CFTR and involves translational elongation. Amplifiers enrich ER-associated CFTR mRNA and increase its translational efficiency through increasing the fraction of CFTR mRNA associated with polysomes. Pull-downs identified the poly(rC)-binding protein 1 (PCBP1) as directly binding to amplifier. A PCBP1 consensus element was identified within the CFTR open reading frame that binds PCBP1. This sequence proved necessary for amplifier responsiveness.

Conclusions: Small molecule amplifiers co-translationally increase CFTR mRNA stability. They enhance translation through addressing the inherently inefficient membrane targeting of CFTR mRNA. Amplifiers bind directly to PCBP1, show enhanced affinity in the presence of bound RNA, and require a PCBP1 consensus element within CFTR mRNA to elicit translational effects. These modulators represent a promising new and mechanistically novel class of CFTR therapeutic. They may be useful as a monotherapy or in combination with other CFTR modulators.

1. Introduction

Cystic fibrosis (CF) is a life-shortening recessive disorder caused by mutations in both alleles of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [1]. *CFTR* codes for an ion channel that mediates chloride transport across epithelial cell membranes. Mutations resulting in CFTR dysfunction cause chronic obstructive lung disease, intestinal obstruction syndromes, liver dysfunction, exocrine and endocrine pancreatic dysfunction, and male infertility [1,2]. CFTR is a complex, multidomain, membrane-spanning protein that undergoes highly regulated folding and trafficking post-biosynthesis to be functionally mature within epithelial cells [3]. CFTR is co-translationally translocated into the ER through a signal recognition particle (SRP)-dependent signal sequence located in transmembrane helix 1 (TM1) [4,5]. TM1 is inefficient at targeting to the ER membrane, harboring two charged

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residues within the helix, and is reported to direct less than half of nascent CFTR chains to adopt the appropriate topology [4].

The inefficiencies of CFTR folding and trafficking are exacerbated by CF-associated mutations. More than 2000 *CFTR* mutations have been identified, with characterized mutations being assigned to one or more of six major classes based on their impact on CFTR biosynthesis or function [1,3–6]. These classes include mutations that reduce the levels of CFTR mRNA or prevent biosynthesis of CFTR protein, as well as those that encode misfolded, unstable, gating-defective, chloride conductance-defective, or otherwise nonfunctional protein [1–3]. Some singular mutations, such as the deletion of phenylalanine 508 (*F508del*), present in approximately 90% of CF patients, cause defects spanning multiple classes [1].

Therapeutic interventions that reverse or circumvent the general and mutation-exacerbated inefficiencies in CFTR biosynthesis have great potential to provide a clinical benefit. We have previously described small molecule amplifiers, novel first-in-class CFTR modulators that function neither as correctors nor as potentiators, but instead augment the activity of those classes of modulator by providing more CFTR protein substrate [7,8]. The available evidence suggests amplifiers can act independent of the specific CFTR mutation.

Here, we show that amplifiers stabilize CFTR mRNA, require translation to exert their effects, and selectively enhance the translation of *CFTR*. We identify direct binding of amplifier to poly(rC)-binding protein 1 (PCBP1) and show the affinity of amplifier is higher for RNA-bound PCBP1. PCBP1 binds to a consensus sequence present in the open reading frame (ORF) of *CFTR*, and mutation of this sequence abrogates binding as well as the effect of amplifier. Taken together, these results provide new insight into the cellular regulation of CFTR biosynthesis along with mechanistic information on how the amplifier class of CFTR modulators may produce their *in vitro* and clinical benefit.

2. Results

2.1. PTI-CH stabilizes CFTR mRNA and requires only the translated sequence of CFTR

A defining feature of the amplifier class of CFTR modulators is that they increase CFTR mRNA levels in primary human bronchial epithelial (HBE) and human nasal epithelial (HNE) cells as well as in reporter cell lines overexpressing CFTR cDNA [7,8]. To establish whether this previously described activity of the amplifier PTI–CH is conferred through stabilization of CFTR mRNA, we used an approach-to-steady state analysis (see Supplemental Methods) [9,10] to determine whether the decay rate of CFTR mRNA is impacted by incubation with PTI-CH. By using the time to achieve steady-state as captured by incorporation of a labeled nucleotide during synthesis (Fig. S1), one can calculate the message's decay rate, enabling the determination of a half-life for the mRNA. Using this methodology, the half-life of CFTR mRNA was found to be increased approximately 2.6-fold in the presence of PTI-CH compared to the DMSO control (Fig. 1A). This was not a generalized effect, as overall mRNA levels were essentially unchanged between DMSO- and PTI-CH-treated samples (Fig. 1A, lower left panel). Similar results were obtained with an orthogonal approach using an inducible expression system in which transcription of CFTR mRNA was shut-off by addition of doxycycline, wherein PTI-CH increased the half-life of CFTR mRNA by 3-fold (Fig. S2).

To identify the elements of *CFTR* necessary for the response to PTI-CH, constructs were generated that contain only the coding region of *F508del-CFTR*, and as a control, the same vector backbone was generated with the coding region of a mutant form of a topologically similar, related transmembrane protein, the P glycoprotein protein (*G268V-PgP)* (Fig. 1B). We selected this control as we and others have previously shown that G268V-PgP does not respond to CFTR-selective modulators like correctors [11]. or amplifiers [7]. The coding region of *CFTR* was sufficient to confer response to PTI-CH, whereas *PgP* levels were not elevated by PTI-CH.

Expanding on previous studies showing that amplifiers appear to work independently of a particular CF-causing mutation in CFTR [7,8], we next examined the ability of amplifier to increase endogenous CFTR mRNA levels in primary HBE cells derived from donors with different *CFTR* mutations and genotypes. Independent of the mutation or genotype for *CFTR*, PTI-CH conferred a similar ~1.5-fold increase in CFTR mRNA levels (Fig. 1C).

Because the translated sequence of *CFTR* is the only portion required to confer responsiveness to amplifier, we sought to establish whether translation itself was necessary for the action of PTI-CH. CFTR mRNA levels were measured over time in the presence of the translational elongation inhibitor cycloheximide. Blocking translational elongation at the time of amplifier addition prevented the PTI-CH-mediated increase in CFTR mRNA levels in *F508del*/*F508del* HBE cells (Fig. 1D). Pre-treatment with PTI-CH for 20 h prior to cycloheximide addition led to an increase in CFTR mRNA abundance of 2.5-fold (Fig. 1E). However, this increase in CFTR mRNA was not maintained over a subsequent 8 h if cells were treated with cycloheximide (Fig. 1E). These combined results reveal that the stabilization of CFTR mRNA by PTI-CH involves a mechanism dependent on translational elongation.

2.2. Amplifiers enhance the membrane-localized and polysome-associated fractions of CFTR mRNA

Co-translationally translocated membrane and secretory proteins such as CFTR are synthesized by ribosomes localized to the rough endoplasmic reticulum (ER) [12–14]. To further elucidate the selectivity for CFTR and the context in which amplifier co-translationally stabilizes CFTR mRNA, we examined the subcellular fraction of CFTR mRNA that responds to amplifier. *F508del*-CFBE cells were lysed with mild detergent to separate cytosolic mRNA from that bound to the ER, and RNA was purified from these fractions. Quantitative RT-PCR (qPCR) showed that incubation with PTI-CH led to a preferential increase in CFTR mRNA in the ER fraction (isolation of which was confirmed by immunoblotting for the ER-specific Grp78/BiP) of approximately 4-fold relative to vehicle treated cells (Fig. 2A), approximately double that of the cytoplasmic fraction. Control mRNAs did show enrichment for the appropriate fraction based on the encoded protein's subcellular localization (see Fig. S3). However, mRNAs for the ER-localized chaperone (Grp78/BiP), the transmembrane protein PgP, the cytoplasmic actin, and the ribosomal RNA (18S) did not respond to amplifier (Fig. 2A). This enhanced enrichment in CFTR mRNA in the ER-containing membrane fraction is consistent with actively translating CFTR mRNA being the predominant species upon which amplifier acts.

Targeting of translating ribosomes to the ER membrane for co-translational translocation of proteins is mediated by the SRP complex, which recognizes signal sequences encoded within the nascent polypeptide of its substrate proteins [5]. The membrane targeting of CFTR has been shown to be inefficient [4]. The primary signal for membrane targeting is the charge-containing TM1, which is inefficient at delivering CFTR to the membrane. A secondary membrane-targeting signal in the form of TM2 is more efficient than TM1 however, it acts post-translationally [4]. To understand whether amplifier works on this process in order to exert an increase in the ER-association of CFTR mRNA, we used site-directed mutagenesis to introduce TM1 and TM2 mutations (Fig. 2B) reported to enhance and reduce membrane targeting of CFTR, respectively [4]. Mutation of the two charged E92 and K95 residues in TM1 to alanine increased its hydropho-

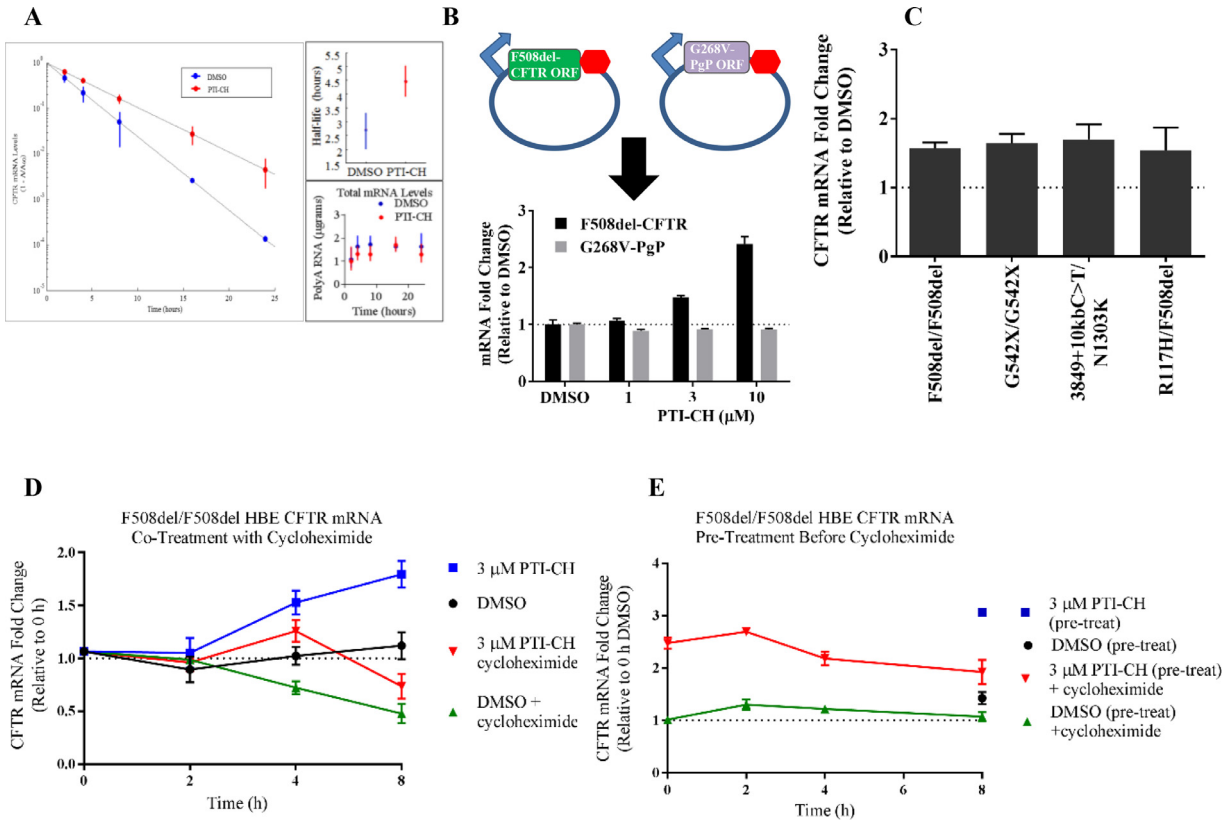


Fig. 1. Amplifiers Selectively Increase CFTR mRNA Stability, Requiring Only the Translated Sequence of CFTR mRNA, and Act Through a Translation-Dependent Mechanism. (A) The approach-to-steady-state was determined using EU-labeled RNAs from CFBE F508del cells in the presence and absence of PTI-CH, and the decay rate was calculated as described in Supplementary Methods to provide the decay curves shown in the large panel and the mRNA half-life shown in the upper right panel. (B) The open-reading frames of either *F508del-CFTR* or *G268V-PgP* were constructed in the same vector backbone, and their response to 24 h PTI-CH incubation at the indicated concentrations was measured by quantifying CFTR or PgP mRNA using qRT-PCR. (C) Primary HBE cells derived from donors with the indicated genotypes/mutations were assessed for their CFTR mRNA response to the PTI-CH amplifier by qRT-PCR. (D) Co-incubation of PTI-CH with cycloheximide in F508del/F508del HBE cells was followed by isolation of RNA and quantitation of CFTR mRNA at the given time points by qRT-PCR. Error bars represent the SEM of $n = 3$–6 biological replicates. (E) F508del/F508del HBE cells were preincubated with PTI-CH for 20 h prior to cycloheximide addition and collection of time points after that addition to isolate RNA and quantify CFTR mRNA levels. Error bars represent the SEM of $n = 3$–6 biological replicates.

bicity and strongly reduced the amplifier response (Fig. 2C). In contrast, mutation of TM2 residues (E116K/G126D) reported to reduce membrane incorporation of CFTR trended towards enhancing the amplifier response. Combination of the four mutations (E92A/K95A/E116K/G126D) showed the TM1 mutations to be epistatic to the TM2 mutations in terms of amplifier response, being indistinguishable from the TM1 mutations alone.

To further establish that translating CFTR mRNA is the target of the amplifier mechanism as implicated in the experiments described above using CFTR cDNA-overexpressing cell lines, we next measured the effect of amplifier on the association of endogenous CFTR mRNA with polysomes in primary HBE cells. For these experiments, a more advanced (in terms of pharmacokinetic, i.e., drug-like properties) analog of PTI-CH, PTI-CV, was employed. This analog was also used for the chemical proteomics experiments (see below). PTI-CV and PTI-CH are highly similar molecules with equivalent *in vitro* potency and efficacy towards both CFTR mRNA and chloride transport (Fig. S4). We used a discontinuous sucrose gradient approach to collapse the diverse polysome ensemble into a single fraction [15]. (Fig. 2D). In lysates incubated with vehicle alone, the distribution of CFTR mRNA has its highest proportion (approximately 67%) in association with monosomes, while only 11% is found in polysomes, a distribution closely paralleling that of the housekeeping gene *GAPDH* (70% and 10%, respectively) (Fig. 2E). While incubation with amplifier had little effect on the distribution of GAPDH mRNA, the CFTR mRNA re-distributed from the monosome fraction (44%) to the higher-order polysome fraction (36%), which mimics the mRNA distributions of the more highly expressed housekeeping protein ACTB in both vehicle-alone and amplifier-treated lysates (36% and 41%, 31% and 42%). We next sought to identify the cellular proteins mediating the ability of amplifier to improve *CFTR* translation.

2.3. Chemical proteomics approach identifies PCBP1 interaction with amplifier

To identify cellular proteins that interact with the amplifier small molecules, we covalently conjugated PTI-CV to beads. To identify proteins that might mediate the amplifier effect on CFTR mRNA, lysates were made from two tissue sources that show CFTR increases in response to amplifier, mouse fetal liver and primary HBE cells. In both cases, lysates were incubated for 16–20 h with the PTI-CV-conjugated beads. Following washing and elution, the eluted proteins were identified by mass spectroscopic analysis. Specificity for PTI-CV was assessed by quantitative comparison to two control pulldown conditions: (1) pulldown with beads conjugated to PTI-CV in the presence of 100-fold molar excess of free competitor, where lysates were pre-incubated with non-conjugated PTI-CV and (2) pulldown with unconjugated beads.

Of the PTI-CV-binding proteins with peptides identified (Table S3), 5 were found from both tissues using this strategy, and four of these overlapping proteins were confirmed by immunoblotting in independent pulldown experiments (Fig. 3A,B and Fig. S5). Intriguingly, two of the PTI-CV binding proteins, PCBP1 and PCBP2, are

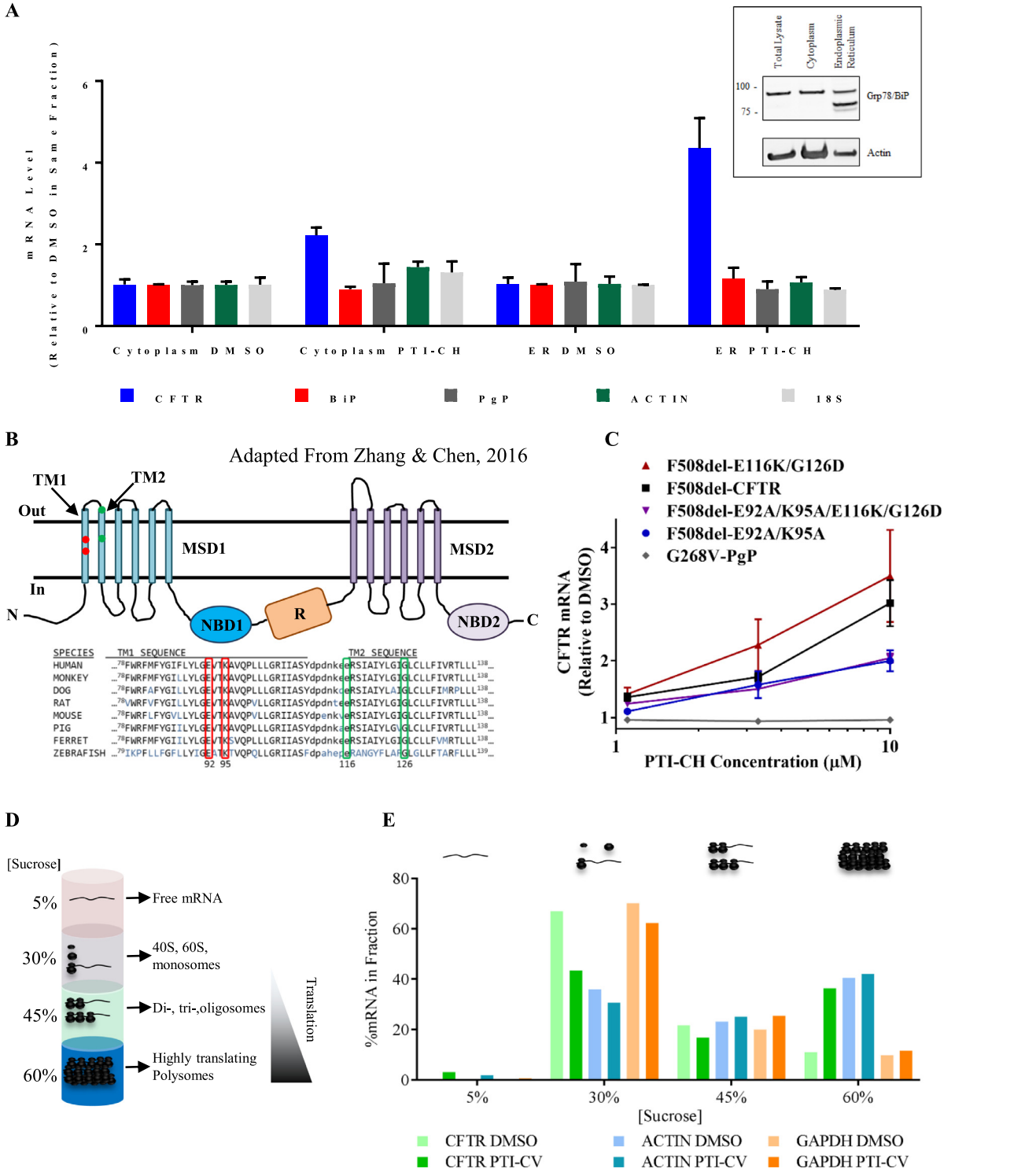


Fig. 2. Amplifiers enrich CFTR mRNA association with the ER membrane, are influenced by membrane targeting efficiency, and increase CFTR mRNA association with polysomes. (A) CFBE F508del cells were incubated with PTI-CH or DMSO for 24 h and then subjected to subcellular fractionation into cytoplasmic and ER fractions followed by mRNA isolation and quantitation by qRT-PCR. (B) Domain structure of CFTR, highlighting the conserved TM1 and TM2 helices that direct CFTR to the ER membrane. (C) Mutations in CFTR TM1 and TM2 previously shown to modulate membrane–targeting efficiency [4] were introduced by site-directed mutagenesis and the constructs were transfected into HEK 293 cells and tested for their CFTR mRNA response to increasing concentrations of PTI-CH. (D) Diagram of the fractionation of RNA over a discontinuous sucrose gradient [15] to separate and concentrate actively translating polysome fractions. (E) Lysates from HBE cells treated with DMSO or PTI-CV were fractionated using a discontinuous sucrose gradient and the proportion of the indicated mRNAs were determined by qRT-PCR in the fractions corresponding to the sedimentation species shown in panel D.

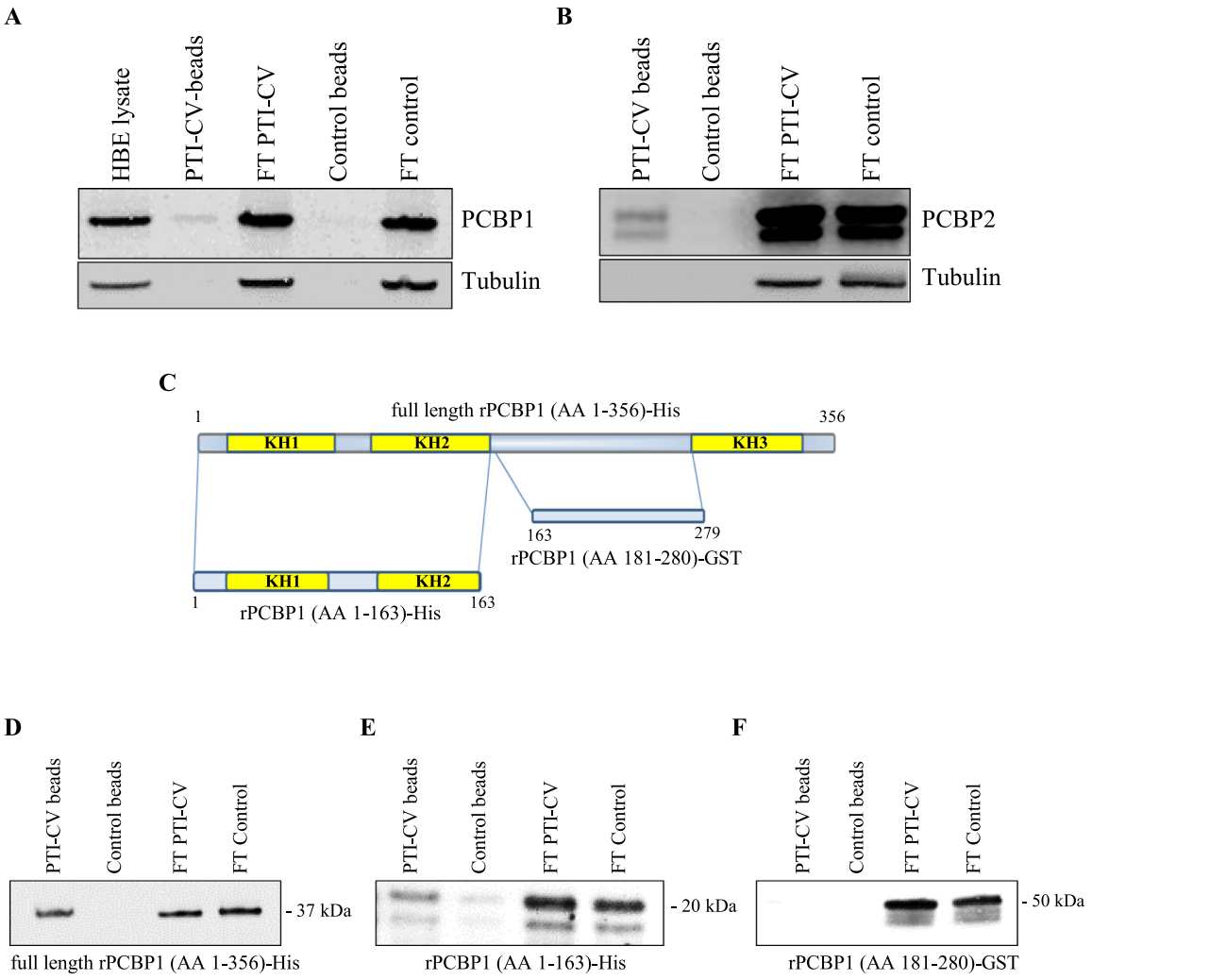


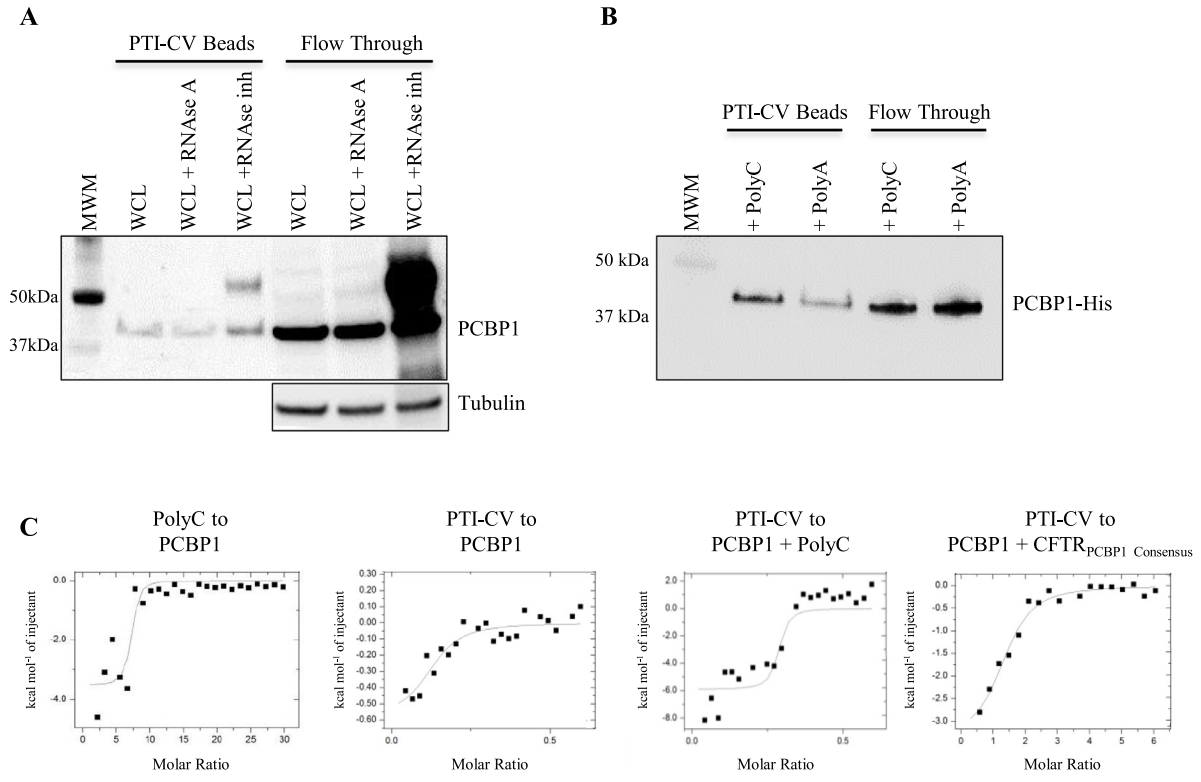
Fig. 3. Amplifier Binds to PCBP1 and PCBP2 in HBE Cell Lysates and the Direct Interaction with PCBP1 is Enhanced by RNA. Pulldowns of F508del/F508del HBE lysates with amplifier-conjugated or control beads were probed in immunoblots of bound and flow-through fractions with antibodies detecting: (A) endogenous PCBP1 and (B) endogenous PCBP2. (C) Schematic showing PCBP1 domain structure indicating the amino acid locations of the three RNA-binding KH domains. (D–F) Recombinant protein or domains were incubated with amplifier-conjugated or control beads and immunoblots of bound and flow-through fractions from the pulldowns probed to detect: (D) recombinant full-length PCBP1 by probing with anti-His-tag, (E) Recombinant N-terminal 163 amino acids containing KH1 and KH2 domains by probing with anti-His tag and, (F) a recombinant GST-fused linker region between KH2 and KH3 domains by probing with anti-GST.

paralogs that share 86% sequence identity. PCBP1 contains no introns and is thought to have arisen by retrotransposition of PCBP2 mRNA [16]. PCBP1 and PCBP2 are reported to promote RNA stabilization [17]. RNA degradation [18], translational enhancement [19] and translational repression [20] depending on the target mRNA and location of the PCBP binding consensus site within it. Knockouts of either PCBP1 or PCBP2 result in embryonic lethality [21]. Importantly, PCBP1 protein is comparably expressed in all three of the cellular model systems used in this study to characterize the effect of amplifier on CFTR mRNA (Fig. S6).

PCBP1 and PCBP2 bind to cytosine (C)-rich consensus sequences via their three RNA-binding, K-homology (KH) domains [22]. We sought to determine what PCBP1 region or domains were important for the interaction with PTI-CV. The domain structure of full length PCBP1 as well as the recombinant fragments tested are shown in Fig. 3C. We detected direct binding of recombinant PCBP1 to PTI-CV (Fig. 3D). A recombinant fragment of PCBP1 that consists predominantly of the two most amino-terminal KH domains also bound to PTI-CV (Fig. 3E). A recombinant fragment of the region between the second and third KH domains did not show detectable binding to PTI-CV (Fig. 3F). Based on these observations, it appears the N-terminal region of PCBP1, principally composed of the first two RNA-binding KH domains is at least one site of amplifier binding. We thus investigated whether RNA influences PTI-CV binding to PCBP1.

2.4. The presence of RNA enhances the affinity of PTI-CV for PCBP1

PCBP1 preferentially recognizes C-rich regions in RNA [22]. Endogenous PCBP1 binding to PTI-CV appears to be enhanced in primary HBE lysates by the presence of the RNase inhibitor RNasin® when compared to untreated and RNase A-treated lysates (Fig. 4A). Similarly, pre-incubation of recombinant PCBP1 with synthetic poly-cytosine (polyC) RNA enhanced the amount of PCBP1 binding to PTI-CV in a pulldown experiment over the levels of binding seen when the pre-incubation was with poly-adenosine (polyA) RNA (Fig. 4B). The influence of RNA on the interaction between PTI-CV and PCBP1 was quantitatively explored using isothermal titration calorimetry (ITC). Pre-incubation of a saturating amount of polyC with recombinant PCBP1 increased the affinity of PTI-CV for PCBP1 (Fig. 4C). Free PCBP1 is bound by PTI-CV with a micromolar dissociation constant, while in the presence of polyC RNA, the interaction of the amplifier with the protein has a K_D of 47 nM. Control experiments showed no detectable interaction between free polyC



Sample cell	Kd(M)	ΔH (cal.mol)	ΔS (cal/moldeg)
PolyC (750 μM) vs. PCBP1 (5 μM)	1.8 e-7	-3513	19
PTI-CV (150 μM) vs. PCBP1 (5 μM)	1.0 e-6	-585.8	25.5
PTI-CV (150 μM) vs. PCBP1(5 μM) + PolyC (50 μM)	4.7 e-8	-5900	13.7
PTI-CV (150 μM) vs. PCBP1(5 μM) + CFTR Consensus (50 μM)	8.3 e-7	-3407	16.4

Fig. 4. The Direct Interaction of Amplifier with PCBP1 is Enhanced by RNA. (A) F508del/F508del HBE lysates were made in the absence of, or in the presence of RNAse A to degrade RNA, or RNASein to protect cellular RNA, and pulldowns with amplifier-conjugated beads were performed, followed by immunoblotting to detect endogenous PCBP1. (B) Recombinant PCBP1 was pre-incubated with either polyC or polyA RNA oligomers and then subjected to pulldown with amplifier-conjugated beads followed by immunoblotting with antibodies against PCBP1. (C) Isothermal titration calorimetry showing binding of polyC RNA oligomers to recombinant PCBP1, amplifier PTI-CV to PCBP1, and PTI-CV to a complex of pre-incubated polyC RNA and PCBP1 or a complex of pre-incubated $CFTR_{PCBP1\ Consensus}$ RNA oligo and PCBP1.

RNA and PTI-CV (data not shown). These results suggest that the interaction of amplifier with PCBP1 is enhanced by RNA binding, and the possibility that binding of PCBP1 to CFTR mRNA may be required for the amplifier effect.

Indeed, PCBP1 has been reported to regulate CFTR mRNA levels in mouse oocytes [23]. We generated an RNA sequence from the CFTR ORF containing a consensus site for PCBP1 binding [24]. (described below) that upon pre-incubation with PCBP1 resulted in the interaction of PTI-CV with the PCBP1-CFTR consensus showing high nanomolar binding, with a binding enthalpy that is nearly six fold that of PTI-CV binding to free PCBP1 (Fig. 4C). We next explored whether the consensus was important for the CFTR increase due to amplifier in a cellular context.

2.5. PCBP1 affects CFTR levels and directly binds a minimal consensus sequence in the CFTR *ORF that mediates amplifier responsiveness in cells*

We attempted to generate a PCBP1 knockout line to explore the effect on CFTR and the amplifier response, but no viable clones were identified, presumably because of the essential nature of PCBP1. We therefore used siRNA to knock down PCBP1 levels (to 20% of control) and saw reductions in both basal and amplifier induced CFTR mRNA levels (Fig. S7). The effect was likely incomplete because of residual levels of PCBP1, compensatory responses from PCBP2 or other proteins, or both.

To establish whether PCBP1 interacts with CFTR mRNA we first looked to identify a candidate region within the ORF that would be recognized by PCBP1. Indeed, a C-rich sequence starting from base-pair 2876 contains a consensus previously reported to be bound by PCBP1 [24]. We therefore generated a fluorescently labeled 25-mer RNA oligonucleotide of this CFTR ORF sequence in order to biochemically test for interaction with PCBP1. As controls, oligonucleotides with two of the C's in the consensus synonymously mutated, and three arbitrarily selected oligonucleotides from other sites in the CFTR coding sequence were also generated (Fig. 5A). The oligonucleotides were incubated with recombinant PCBP1 (rPCBP1) and evaluated for binding by electrophoretic mobility shift analysis (EMSA).

The $CFTR_{PCBP1consensus}$ oligonucleotide has a high affinity for rPCBP1, in that free oligonucleotide was undetectable by electrophoretic analysis. Mutation of two of the cytosines in the con-

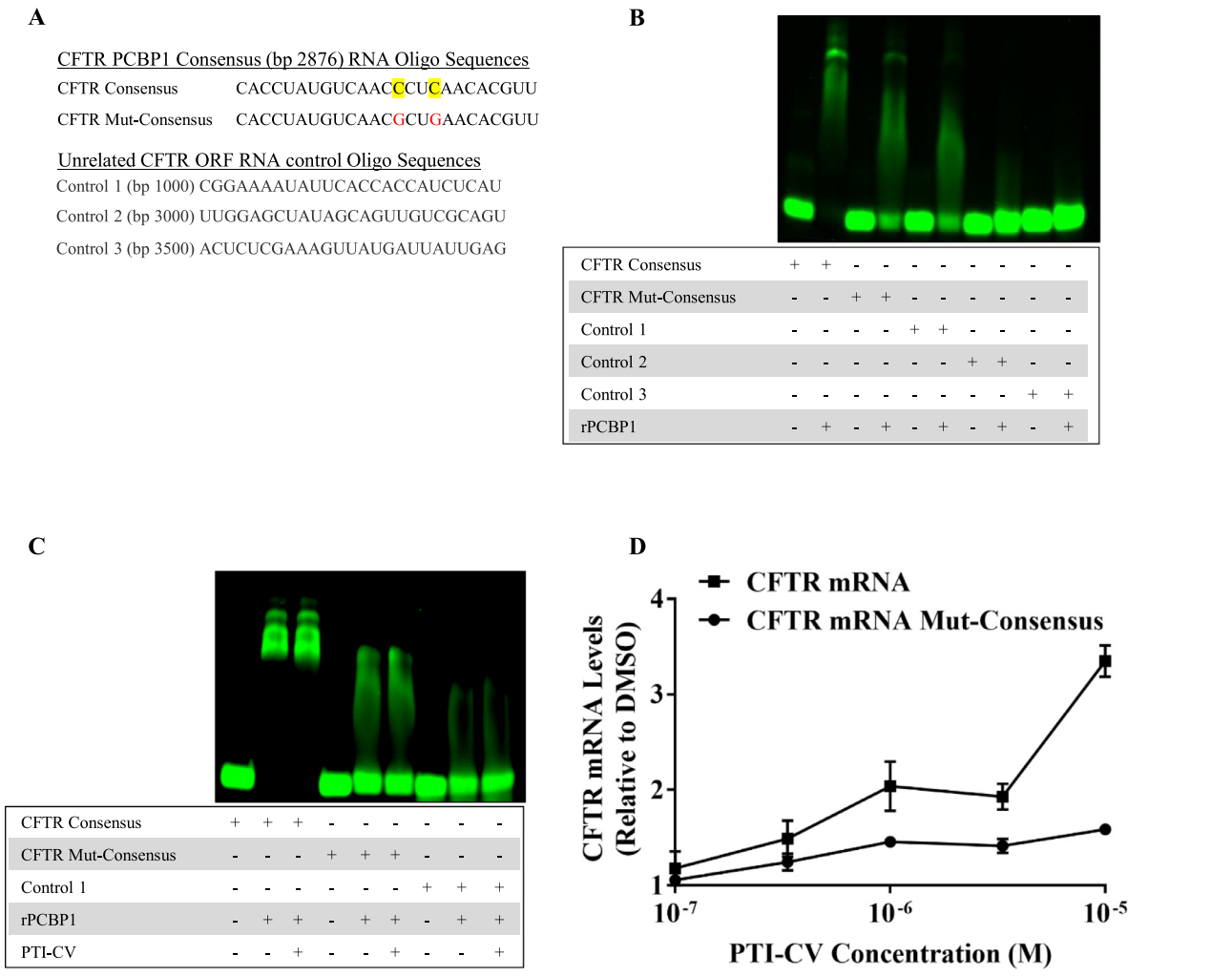


Fig. 5. A PCBP1 Consensus Binding Site from the CFTR ORF Binds to PCBP1 and When Mutated, Abrogates the Amplifier Response. (A) List of RNA oligomer sequences derived from the CFTR ORF used in the studies in panels B and C. Numbering is from the start codon to the first base-pair of the oligo. (B) EMSA of CFTR-derived oligos with recombinant PCBP1 protein (rPCBP1). (C) EMSA in the presence of PTI-CV amplifier for recombinant PCBP1 and CFTR-derived oligos. (D) HEK 293 cells were transfected with constructs expressing the CFTR ORF or a site-directed mutant of the CFTR ORF that had synonymous mutations in the consensus site (CFTR Mut-Consensus in panel A) and the response to amplifier of CFTR mRNA was assessed at the indicated concentrations by qRT-PCR. Symbols represent means, and error bars are SEM of 3 biological replicates.

sensus ($CFTR_{PCBP1mutated}$) resulted in greatly diminished binding (Fig. 5B). Furthermore, higher molecular weight species of complex, potentially indicative of complexes containing multiple PCBP1 and/or oligonucleotide molecules, were exclusively observed with $CFTR_{PCBP1consensus}$. Of the three control sequences derived from different regions throughout the CFTR coding sequence, only one shifted upon PCBP1 addition, with no detectable higher-order complexes such as those observed with $CFTR_{PCBP1consensus}$. Interestingly, this control sequence contained a pair of dinucleotide cytosines spaced by a single intervening nucleotide, partially mimicking the minimal consensus site.

To investigate whether amplifier, which shows an improved affinity for PCBP1 binding in the presence of RNA, had any effect on the binding of the $CFTR_{PCBP1consensus}$ by PCBP1, we performed EMSA in the presence of PTI-CV (Fig. 5C). PTI-CV did not show any obvious effect on the interaction of PCBP1 with $CFTR_{PCBP1consensus}$, nor with $CFTR_{PCBP1mutated}$, nor the control oligonucleotide derived from nucleotides 1000–1025 of CFTR. As the electrophoretic mobility of the complexes was not impacted by amplifier, there is not a clear influence of amplifier on the PCBP1-CFTR mRNA complex in this experiment. We next examined whether the same mutations in the $CFTR_{PCBP1consensus}$ that disrupted PCBP1 binding would impact the effect of amplifier on CFTR mRNA in a cellular context.

To explore whether the PCBP1consensus mediates the action of amplifier on CFTR mRNA, we introduced the synonymous mutations from $CFTR_{PCBP1mutated}$ (see Fig. 5A) into full length CFTR cDNA and compared the resulting construct, CFTR mRNA Mut-Consensus, to CFTR mRNA in terms of their response to amplifier. HEK 293 cells were transiently transfected with one of the other constructs, and incubated with increasing concentrations of PTI-CV. After 24 h, RNA was isolated and the levels of CFTR mRNA determined by qRT-PCR. Whereas amplifier robustly increased the steady state levels of CFTR mRNA, C-G mutation of two sites within the PCBP1consensus abrogated the response to amplifier (Fig. 5D).

3. Discussion

In an earlier study [7]. we reported the results of a phenotypic high-throughput screen that identified a novel pharmacological class of small molecules that rescue CFTR function, referred to as amplifiers. The amplifier CFTR modulators possess novel characteristics relative to other modulator classes in that they stabi-

lize CFTR mRNA and increase the amount of expressed protein. They are thus complementary to other modulator classes, providing more substrate protein for the downstream actions of correctors and potentiators on CFTR.

Amplifiers are selective for CFTR. Neither a related ABC transporter nor other control genes showed responses to amplifier for total, ER-associated, or translating (as represented by polysome-bound fraction) mRNA abundance. The amplifier class of modulator does not depend on CFTR elements outside of its open reading frame. Indeed, translational elongation is critical for amplifier activity, consistent with its action early in CFTR biosynthesis, at a point in its life-cycle when protein and mRNA are still interdependent. Amplifiers work independently from CF-causing mutations, providing similar increases in CFTR mRNA levels across genotypes and mutations in primary HBE cells.

Engineered mutation of a pair of amino acids in the TM1 signal sequence previously shown to improve membrane targeting of CFTR [4] greatly reduced amplifier responsiveness. This result is consistent with amplifier acting co-translationally in modulating the inherent inefficiency of the process of CFTR translocation into the ER membrane. The consequence of increasing the hydrophobicity of TM1 is improved membrane insertion [4], thus circumventing the deficit in the CFTR life-cycle for which amplifier provides its beneficial effect by improving CFTR mRNA translation, a co-translocational event. In contrast, the mutations in TM2 used here were shown to reduce membrane insertion [4], and as such would increase the CFTR mRNA's reliance on TM1 to be target to the ER. We therefore sought to identify the cellular mediators of such a novel mechanism of CFTR regulation.

Chemical proteomics allowed the identification of PCBP1 as a direct binding target of the amplifier small molecule CFTR modulator. A region with two of the domains of PCBP1 important for binding to mRNA also appear to be involved in binding to amplifier, and the presence of RNA profoundly increased the affinity between amplifier and PCBP1. PCBP1 recognizes C-rich consensus sites in its targets. A known PCBP1 target mRNA, ALOX15B, is lower in CF bronchoalveolar lavage [25] showed an increase in response to amplifier (Fig. S8). We found that the translated sequence of CFTR also contains a consensus binding site for PCBP1, and we show in this study that this consensus is necessary for the increase in CFTR mRNA abundance in response to the amplifier. Of note, there are three CF-causing mutations listed in CFTR2 (www.CFTR2.org) that affect the $CFTR_{PCBP1consensus}$ sequence and thus may impact PCBP1 recognition of this sequence. These mutations are listed in Table S2. In particular, one mutation deletes 15 of the 25 nucleotides of $CFTR_{PCBP1consensus}$ and it would be interesting to study whether this allele would be refractory to amplifier in cells from people with CF who have this mutation.

We propose amplifiers as a novel class of CFTR modulating small molecules that provide increased expression of CFTR by a novel mechanism that involves promoting its translation. The features of amplifier as a CFTR modulator bestow the potential to provide therapeutic benefit as a stand-alone drug for CFTR mutants with a sufficient degree of residual function. Amplifiers also offer an orthogonal approach with the possibility of enhancing the efficacy of current CF modulator therapeutics. The description of the PCBP1-mediated regulation of CFTR described herein as revealed by the mechanism of amplifier offers insight into the cellular regulation of CFTR biosynthesis. Further studies will elucidate the full potential of this regulation as a target for next-generation CF therapeutics yet to be developed.

4. Methods

Methods Sections 4.1–4.12 are described in the Supplementary material appendix.

Declaration of competing interest

The authors are employees or scientific advisors of Proteostasis Therapeutics, Inc. and conducted this research as part of the company's drug discovery efforts.

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Supplementary materials

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