CFTR mRNAs with nonsense codons are degraded by the SMG6-mediated endonucleolytic pathway

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***CFTR* mRNAs with nonsense codons are degraded by the SMG6-mediated
endonucleolytic pathway**

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Abstract

Cystic fibrosis is caused by loss of function mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene resulting in severe lung disease. Nearly 10% of cystic fibrosis patients have at least one *CFTR* allele with a nonsense mutation that generates a nonsense codon in the mRNA. Nonsense mutations can result in significant reduction of gene expression partially due to rapid mRNA degradation through the nonsense-mediated decay (NMD) pathway. It has not been thoroughly investigated which branch of the NMD pathway governs the decay of *CFTR* mRNAs containing nonsense codons. Here we utilized antisense oligonucleotides targeting NMD factors to evaluate the regulation of nonsense codon-containing *CFTR* mRNAs by the NMD pathway. Interestingly, we found that *CFTR* mRNAs with G542X, R1162X, and W1282X nonsense codons require UPF2, UPF3, and exon junction complex proteins for NMD, whereas *CFTR* mRNAs with the Y122X nonsense codon do not. Furthermore, we demonstrated that all evaluated *CFTR* mRNAs harboring nonsense codons were degraded by the SMG6-mediated endonucleolytic pathway rather than the SMG5/SMG7-mediated exonucleolytic pathway. Finally, we found that stabilization of *CFTR* mRNAs by NMD inhibition alone improved functional W1282X protein production, and improved the efficiency of aminoglycoside translational readthrough of *CFTR-Y122X*, *-G542X*, and *-R1162X* mRNAs.

Introduction

Cystic fibrosis (CF) is a monogenetic autosomal recessive disorder caused by loss-of-function mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene¹⁻³. Approximately 10% of the cystic fibrosis population have at least one *CFTR* allele with a nonsense mutation that generates a premature termination codon (nonsense codon) in the mRNA.⁴⁻⁶. Nonsense mutations are often associated with more severe CF phenotypes due to significantly reduced *CFTR* expression as a result of both RNA degradation by the nonsense-mediated decay (NMD) pathway and protein truncation^{7,8}. Efforts in developing therapies for CF caused by nonsense mutations have focused on identifying small molecule readthrough drugs that suppress translation termination at the nonsense codon to produce full-length *CFTR* protein. Currently, no readthrough therapy has been approved for the treatment of CF caused by nonsense mutations. This nonsuccess is in part because of the low abundance of mRNA substrates available for translational readthrough due to degradation by the NMD pathway^{9,10}. Combination therapy will be required to inhibit NMD for the stabilization of mRNAs with nonsense codons while simultaneously promoting translational readthrough. Inhibition of NMD alone may also improve disease phenotypes provided the truncated proteins are functional¹¹.

The NMD pathway is not only an RNA surveillance pathway that degrades mRNAs with nonsense codons, but also a post-transcriptional regulatory mechanism governing approximately 10-20% of the normal transcriptome¹². Therefore, the safety of pharmacological inhibition of the NMD pathway requires thorough investigation. Several lines of evidence suggest that NMD is not a single biochemical pathway in higher eukaryotes, but rather a pathway with several branches^{12,13}. Three branches of the NMD pathway diverging at the stage of PTC recognition have been reported including UPF2-independent, UPF3B-independent, and EJC-independent branches¹²⁻¹⁷.

Each branch of the NMD pathway regulates subsets of endogenous NMD substrates¹². At the step of RNA destruction, several studies reveal that NMD substrate RNAs can be degraded through either SMG6-mediated endonucleolytic degradation or SMG5-SMG7-mediated exonucleolytic degradation pathways¹⁸⁻²⁰. These branch-specific NMD factors could potentially be safer therapeutic targets for diseases caused by nonsense mutations as each of them regulates a subset of endogenous NMD substrates^{12, 13, 21}. For example, we have previously shown ASO-mediated inhibition of branch-specific NMD factor *upf3b* was well tolerated in mice with minimal impacts on the normal transcriptome while alleviating disease phenotypes in a hemophilia mouse model²¹.

However, it is not known which branches of the NMD pathway regulate *CFTR* mRNAs with nonsense codons and if the location of the nonsense codon influences branch-specificity. A better understanding of how the NMD pathway regulates *CFTR* mRNAs containing nonsense codons could provide a path for the development of NMD inhibition therapy for CF caused by nonsense mutations. Here, we sought to use antisense oligonucleotides (ASOs) to identify branch-specific NMD factors whose expression could be reduced to effectively impede NMD and stabilize *CFTR* mRNA expression to alleviate CF disease phenotypes caused by nonsense mutations.

Antisense technology is a clinically validated drug discovery platform²². ASOs are short synthetic oligonucleotides which bind specifically to their RNA targets through Watson-Crick base pairing to form ASO-RNA heteroduplexes. These DNA-RNA heteroduplexes can be substrates for the ubiquitous endonuclease RNase H, which can lead to the degradation of the target RNA^{22, 23}. We used ASOs to reduce the expression of NMD pathway components so as to

identify branch-specific NMD factors which govern the NMD of *CFTR* mRNAs containing prevalent *CFTR* nonsense codons.

Results

CFF-16HBEge cell lines harboring *CFTR* nonsense alleles have reduced *CFTR* expression and function

We employed the CFF-16HBEge *in vitro* model system to evaluate NMD regulation of *CFTR* mRNAs harboring various nonsense codons²⁴. 16HBE14o- cells were CRISPR-edited to produce CFF-16HBEge lines harboring *CFTR* Y122X, G542X, R1162X, or W1282X nonsense alleles.

We first evaluated *CFTR* expression and function in both 16HBE14o- (parental) and CFF-16HBEge cells. We found that levels of *CFTR* mRNAs from CFF-16HBEge-Y122X, -G542X, -R1162X, and -W1282X cell lines were reduced by 50-80% when compared to levels of wild-type *CFTR* mRNA in parental cells (Fig. 1A). The levels of *CFTR* mRNA in CFF-16HBEge-G542X and -W1282X cells were consistent with previous reports²⁴⁻²⁶. We were unable to detect full-length *CFTR* proteins in any of these cell lines by western blot analysis (Fig. 1B). However, the presence of the partially stable, C-truncated W1282X-*CFTR* protein was detected (Supplementary Fig. 1), as previously characterized^{25, 27-30}. *CFTR* functional evaluation by Ussing chamber assays revealed minimal basal *CFTR* chloride channel function of less than 1 $\mu\text{A}/\text{cm}^2$ in CFF-16HBEge cells containing nonsense alleles, whereas that in the parental cells was 118 $\mu\text{A}/\text{cm}^2$ (Fig. 1C-D). CFF-16HBEge-Y122X cells showed higher *CFTR* mRNA levels of approximately 50% of parental cells and higher *CFTR* function of approximately 1 $\mu\text{A}/\text{cm}^2$ when compared to other CFF-16HBEge cell lines (Fig. 1C-D). Taken together, CFF-16HBEge

cells with CFTR nonsense alleles demonstrated significantly reduced CFTR expression and function.

ASOs mediate efficient NMD factor reduction and NMD pathway inhibition

Efficacious ASOs were generated to reduce the expression of NMD factors SMG1, SMG5, SMG6, SMG7, SMG8, SMG9, UPF1, UPF2, UPF3A, and UPF3B. Cells were treated with unformulated ASOs by free uptake as previously described³¹. RT-qPCR analysis demonstrated efficient reduction of NMD factor mRNAs following ASO treatment, reaching between 87% and 99% target reduction when compared to PBS control (Fig. 2).

The mRNAs encoding NMD factors are themselves targets of NMD and exist under negative feedback regulation by the NMD pathway^{12, 32, 33}. ASO-mediated depletion of NMD factors UPF1, SMG1, SMG6, and UPF2 resulted in robust upregulation of other NMD factors (Fig. 2A-B, E, H), indicative of effective NMD pathway inhibition. ASO-mediated depletion of NMD factors SMG8-9, SMG5, SMG7, and UPF3B resulted in modest inhibition of the NMD pathway (Fig. 2C-D, F-G, J). Finally, depletion of NMD factor UPF3A did not stabilize any evaluated endogenous NMD substrate in a dose-responsive manner (Fig. 2I). Furthermore, ASO-mediated inhibition of NMD factors had no significant effects on wild-type *CFTR* mRNA levels in parental cells (Supplementary Fig. 2).

ASO treatments in CFF-16HBE cells with Y122X, G542X, R1162X, and W1282X nonsense mutations reflected similar levels of NMD factor target reduction and corresponding NMD pathway inhibition as was observed in parental cells for each ensuing experiment.

All evaluated *CFTR* mRNAs with nonsense codons are regulated by UPF1 and SMG1

To validate *CFTR* mRNAs in CFF-16HBEges are NMD targets, we treated these cells with ASOs targeting core NMD factors UPF1 and SMG1. We found that *UPF1*- or *SMG1*-ASO treatment led to stabilization of all evaluated *CFTR* mRNAs with nonsense codons, which indicate these transcripts are bona fide NMD targets (Fig. 3). UPF1 depletion resulted in 1.5- to 2.5-fold upregulation of *CFTR* mRNAs with nonsense codons (Fig. 3). Interestingly, SMG1 depletion resulted in robust stabilization of all *CFTR* mRNAs with nonsense codons which reached levels comparable to wild-type *CFTR* mRNA levels in parental cells (Fig. 3). However, ASOs targeting SMG1 regulatory cofactors *SMG8* or *SMG9*, or both in combination, did not stabilize any evaluated *CFTR* mRNAs with nonsense codons (Fig. 3).

***CFTR* mRNAs with nonsense codons G542X, R1162X, and W1282X, but not Y122X, are recognized by UPF2 and UPF3**

mRNAs with nonsense codons can be recognized by different branches of the NMD pathway^{12, 14-17}. We used ASO-mediated depletion of NMD branch-specific factors to examine which NMD branch recognizes *CFTR* mRNAs with differing nonsense codons. CFF-16HBEge cells were treated with ASOs targeting *UPF2*, *UPF3A*, or *UPF3B* followed by analysis of NMD factor target reduction and *CFTR* mRNA upregulation by RT-qPCR. We observed that *UPF2*-ASO treatment stabilized *CFTR-G542X*, *-R1162X*, and *-W1282X* mRNAs to the levels that were approximately 50% of wild-type *CFTR* mRNA levels observed in parental cells; however, *CFTR-Y122X* mRNA was not stabilized by *UPF2*-ASO (Fig. 4A-D). Furthermore, we observed depletion of either *UPF3A* or *UPF3B* did not significantly stabilize any *CFTR* mRNAs containing nonsense codons in CFF-16HBEge cells. However combination treatment of *UPF3A*- and *UPF3B*-ASOs stabilized *CFTR-G542X*, *-R1162X*, and *-W1282X* mRNA, but not *CFTR-Y122X* mRNA, in a dose-responsive manner when compared to the PBS control (Fig. 4A-D).

These results suggest that *CFTR* mRNAs with nonsense codons G542X, R1162X, and W1282X, but not Y122X, are recognized by NMD factors UPF2 and UPF3, and that UPF3A and UPF3B paralogs are functionally redundant in regulating *CFTR* transcripts.

One function for UPF2 and UPF3 proteins is to bridge UPF1 to the EJC to stimulate UPF1 helicase activity³⁴. Therefore, we evaluated if the EJC is required for NMD of *CFTR* mRNAs harboring nonsense codons by using ASO that depletes the EJC core component *eIF4A3* (Supplementary Fig. 3). In CFF-16HBEg cells treated with *eIF4A3*-ASO, *CFTR* mRNAs with nonsense codon W1282X, but not Y122X, were stabilized (Fig. 4E-F). Taken together, these results suggest that unlike other *CFTR* mRNAs with nonsense codons, the degradation of *CFTR*-Y122X mRNA is not regulated by UPF2, UPF3 or EJC proteins.

All evaluated *CFTR* mRNAs with nonsense codons are degraded by the SMG6-mediated endonucleolytic decay pathway

After nonsense codon recognition, destruction of *CFTR* mRNAs could be arbitrated by either the SMG6-mediated endonucleolytic, SMG5/SMG7-mediated exonucleolytic, or a combination of both decay pathways^{18, 19, 35}. We found that the *SMG6*-ASO significantly stabilized all evaluated *CFTR* mRNAs harboring nonsense codons to levels comparable to wild-type *CFTR* mRNA levels in parental cells (Fig. 5A-D). Maximal upregulation of *CFTR* mRNAs with nonsense codons was achieved when ASO-mediated *SMG6* mRNA reduction was approximately 90% (Fig. 5A-D). In contrast, *SMG5*-, or *SMG7*-ASO alone or in combination did not stabilize any evaluated *CFTR* mRNAs with nonsense codons despite achieving more than 95% target reduction (Fig. 5A-D).

We next confirmed SMG6-dependent degradation of *CFTR* mRNAs using primary human bronchial epithelial cells homozygous for the W1282X nonsense mutation (hBE W1282X/W1282X). Efficient ASO-mediated depletion of *SMG6* mRNA by approximately 90% in hBE W1282X/W1282X cells resulted in a significant upregulation of *CFTR* mRNA by 3.4-fold when compared to PBS control (Figure 5E, Supplementary Fig. 4). Evaluation of *SMG5*-, or *SMG7*-ASO alone or in combination did not significantly stabilize *CFTR* mRNAs in hBE W1282X/W1282X cells (Fig. 5E). Collectively, these data suggest that *CFTR* mRNAs harboring nonsense codons are predominately degraded via the SMG6-mediated endonucleolytic pathway rather than the SMG5/SMG7-mediated exonucleolytic pathway.

NMD inhibition improves translational readthrough therapy

Translational readthrough of nonsense codons, although it occurs at low frequency, can result in full-length protein that can improve disease phenotypes³⁶⁻⁴⁰. Therefore, we sought to evaluate if upregulation of *CFTR* mRNAs with nonsense codons induced by depletion of NMD factors can result in meaningful increases of full-length CFTR protein expression and function for phenotype rescue in CFF-16HBEge cells.

CFF-16HBEge cells were cultured at liquid-liquid interface on Transwell inserts and were treated with either *SMG1*- or *SMG6*-ASOs for 6 days. Although efficient depletion of SMG1 or SMG6 was achieved at both the mRNA and protein levels, the stabilization of *CFTR* mRNAs did not lead to detectable increases of full-length CFTR protein in CFF-16HBEge cells (Figure 6; Supplementary Fig. 5-8). We also could not detect increases of truncated CFTR proteins or CFTR functional improvement in CFF-16HBEge-Y122X, -G542X or -R1162X cells when compared to PBS control (Fig. 6A-F, 7A-F). Interestingly, in CFF-16HBEge-W1282X cells both *SMG1*- and *SMG6*-ASO treatments significantly increased the level of C-truncated

CFTR protein by nearly 35-fold when compared to PBS control (Figure 6G-H), which resulted in increases of CFTR function between 2.6-fold and 4.2-fold, respectively (Fig. 7G-H).

We then tested if the upregulation of *CFTR* nonsense codon-containing mRNAs, which were induced by NMD inhibition, could improve the outcome of translational readthrough therapy. Cells were treated with aminoglycoside geneticin (G418) during the final 2 days of ASO treatment. We found that the combinations of *SMG1*- or *SMG6*-ASO with G418 increased full-length CFTR protein production in CFF-16HBEge-Y122X, -G542X, and -R1162X cells (Fig. 6A-F). Consistent with the observed protein upregulation, *SMG1*- or *SMG6*-ASO improved CFTR function by 1.6 to 2-fold when compared to G418 treatment alone in CFF-16HBEge cells containing Y122X, G542X, and R1162X nonsense mutations (Fig. 6A-F, 7A-F). Interestingly, unlike other *CFTR* nonsense mutations, full-length W1282X CFTR protein production could not be detected by western blot following ASO treatments with or without G418 (Fig. 6G-H), which is consistent with previous reports^{24, 27, 28}. However, the combination of *SMG1*- or *SMG6*-ASO with G418 resulted in an enhancement of W1282X CFTR function by nearly 2.7-fold and 3.4-fold, respectively, when compared to G418 treatment alone (Fig. 7G-H). Collectively, these data suggest NMD inhibition can significantly improve the outcome of translational readthrough therapy and lead to CFTR protein and function upregulation.

Discussion

In this study, we used ASOs to reduce the expression of each NMD pathway component to determine which NMD branch regulates the degradation of *CFTR* mRNAs containing nonsense codons. We confirmed that the core NMD factors UPF1 and SMG1 regulate all evaluated *CFTR* mRNAs with nonsense codons (Fig. 3). Interestingly, *SMG1*-ASO triggered a

more robust upregulation of *CFTR* mRNAs than *UPF1*-ASO, suggesting SMG1 could be the rate-limiting factor for degradation of *CFTR* nonsense-codon containing mRNAs in CFF-16HBEge cells. However, ASO-mediated reduction of SMG1 regulating factors SMG8 and SMG9 alone or in combination did not stabilize *CFTR* mRNAs in CFF-16HBEge cells. We and others have reported that SMG8 or SMG9 depletion have limited impacts on NMD of β -*GLOBIN* mRNA when compared to SMG1 depletion in either MHT or Hela cells ^{21, 41}. However, another study found SMG8 depletion was sufficient for stabilizing nonsense codon-containing *COL6A2* mRNA in Ullrich disease fibroblasts ¹¹. NMD pathway regulation of NMD substrates can be subject to cell type- and tissue-specific contexts, which could explain why SMG8 and SMG9 do not regulate degradation of *CFTR* mRNAs in CFF-16HBEge cells but are essential for the destruction of transcripts harboring nonsense codons in other cell types ^{11, 12}.

We next evaluated if the recognition of *CFTR* nonsense codon-containing mRNAs requires UPF2, UPF3, or EJC factors by ASO-mediated reduction of these branch-specific NMD factors. We found that recognition of *CFTR-W1282X* mRNAs were regulated by UPF2, UPF3 paralogs, and the EJC factor eIF4A3. Conversely, levels of *CFTR* mRNAs with the Y122X nonsense codon were not affected by ASOs targeting *UPF2*, *UPF3* paralogs, or *eIF4A3*; suggesting that these proteins are not required for the recognition of *CFTR-Y122X* mRNA for NMD.

A recent report using a HEK293 *CFTR* expression minigene (EMG) system showed *CFTR* mRNAs with Y122X can evade NMD, as *CFTR-Y122X* mRNA levels were similar to wild-type *CFTR* mRNA. The EMG system contains the entire coding region of *CFTR* and the introns flanking exon 4, which possesses the Y122X nonsense codon ⁴². Here, we provide evidence *CFTR-Y122X* mRNA is a bona fide NMD substrate, as depletion of NMD factors

UPF1, *SMG1*, and *SMG6* results in mRNA stabilization in CFF-16HBEge-Y122X cells. However, the level of *Y122X* mRNA is higher when compared to other *CFTR* nonsense codon containing mRNAs in CFF-16HBEge cell lines (Fig. 1), suggesting *Y122X* mRNA may partially evade NMD. It has been reported that translation reinitiation events occurring downstream of a nonsense codon could protect mRNAs from NMD, presumably due to the removal of the EJC-UPF2-UPF3 complex⁴³. Previous studies have found that methionine codons M150, M152, M156, and M265 can mediate alternate translation initiation events resulting in the production of N-truncated CFTR protein products^{44, 45}. It is possible that translation reinitiation events downstream of the Y122X nonsense codon partially protect *CFTR Y122X* mRNA from NMD degradation. In support of this hypothesis, we observed that the depletion of UPF2, UPF3 paralogs, or EJC factor eIF4A3 did not stabilize *CFTR-Y122X* mRNA in CFF-16HBEge-Y122X cells. Collectively, our results suggest that *CFTR Y122X* mRNA is degraded by the NMD pathway independent of UPF2, UPF3 paralogs, and EJC factor eIF4A3. One advantage of the CFF-16HBEge cell model system is that the *CFTR Y122X* nonsense mutation exists within the native genomic context which could more closely reflect the endogenous situation. However, further investigation using primary patient cells containing the *CFTR Y122X* nonsense mutation is required.

After nonsense codon recognition and tagging by SMG1-mediated phosphorylation of UPF1, destruction of NMD substrates can occur through endonucleolytic cleavage by SMG6 or deadenylation and decapping executed by the SMG5/SMG7 heterodimer^{35, 46, 47}. Several lines of evidence suggest significant redundancy exists between the endo- and exonucleolytic decay routes as transcriptome-wide analysis found overlapping NMD substrate specificity¹⁸. Interestingly, however, we found ASO-mediated depletion of *SMG6*, but not *SMG5* or *SMG7*,

significantly upregulated all evaluated *CFTR* mRNAs regardless of the nonsense codon location in the transcript. Recent reports provide evidence that some nonsense codon-containing NMD substrates can be preferentially routed through SMG6-dependent endonucleolytic decay rather than the SMG5/7-dependent exonucleolytic decay pathway¹⁹. This report supports our observations as turnover of *CFTR* mRNAs containing nonsense codons are predominately arbitrated through the SMG6 endonucleolytic route, but not the SMG5/7-dependent decay route. ASO-mediated depletion of *SMG6* stabilized *CFTR* mRNAs with nonsense codons to a similar degree as the depletion of core NMD factors *UPF1* and *SMG1*, suggesting branch-specific inhibition of SMG6 could serve as a pan NMD inhibitor for *CFTR* mRNAs with various nonsense codons.

We confirmed that CFF-16HBEge-W1282X cells were highly responsive to NMD inhibition by *SMG1*-ASO as we have previously reported²⁵, raising the possibility that NMD inhibition alone may be sufficient to improve cystic fibrosis disease phenotypes caused by the W1282X nonsense mutation. Interestingly, when combining both NMD inhibition and translational readthrough approaches, we observed augmented W1282X CFTR functional parameters when compared to corresponding levels of protein upregulation (Fig. 6-7). The functional improvement detected by the Ussing chamber assay could be attributed to the combination of both the upregulated C-terminal truncated protein and diminutive amounts of full-length protein induced by G418. Even though we are unable to detect full-length CFTR protein expression following G418 treatment by western blot, minute amounts of full-length CFTR protein contribute a significant portion of the observed functional improvement.

Our results advance the understanding of how the NMD pathway regulates CFTR mRNAs containing nonsense codons and provides direction for the development of therapeutic

NMD inhibitors for cystic fibrosis caused by nonsense mutations. Our findings suggest inhibition of the branch-specific NMD factor SMG6 is a promising therapeutic target for the stabilization of *CFTR* mRNAs harboring prevalent nonsense codons. Further work is warranted to evaluate the safety of NMD pathway modulation in the lung and develop highly efficacious translational readthrough compounds needed to achieve the required therapeutic index for prevalent *CFTR* nonsense mutations.

Materials and Methods

Antisense oligonucleotide preparation

Antisense oligonucleotides (ASOs) used in this study were 16-mer phosphorothioate oligonucleotides that contain constrained ethyl (cEt) modifications at positions 1-3 and 14-16, which flank a central 10-nucleotide deoxy gap. Oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (PerkinElmer Life and Analytical Sciences, Richmond, CA) and purified as previously described³¹. Lyophilized ASOs are prepared for use by reconstitution in Dulbecco's buffered saline (Thermo Fisher Scientific, Carlsbad, CA) followed by filter sterilization using a 0.2- μ m filter and quantification by UV spectrometry.

Cell culture and ASO treatment

16HBE14o- cells, an immortalized human bronchial epithelial cell line⁴⁸, were genome-edited to produce CFF-16HBE cell lines harboring *CFTR* Y122X, G542X, R1162X, or W1282X nonsense mutations as previously described²⁴. *CFTR* nonsense mutations in CFF-16HBE cells exist within the endogenous *CFTR* allele and are expressed by the native *CFTR* promoter. CFF-16HBE cells were cultured in MEM containing 10% fetal bovine serum (R&D

Systems, Minneapolis, MN), 100 U/ml penicillin/ 100 U/ml streptomycin (Thermo Fisher Scientific, Carlsbad, CA), and Glutamax (Thermo Fisher Scientific, Carlsbad, CA) until 90% confluent. Cells were then seeded into collagen I-coated 96-well plates at a concentration of 10,000 cells/well and cultured until 90% confluent prior to ASO treatments. ASOs were diluted into fresh cell culture medium, which was subsequently added to the cells for treatment. Cells were treated for 6 days with replenishment of ASO-containing medium at day 3.

Primary human bronchial epithelial cells (hBEs) homozygous for the W1282X nonsense mutation were provided by the Cystic Fibrosis Foundation and cultured as previously described with minor modifications ⁴⁹. Briefly, hBE cells were thawed and cultured in Pneumacult EX expansion media (STEMCELL Technologies, Vancouver, Canada) in tissue culture flasks pre-coated with NIH3T3-conditioned medium. hBE cells were then seeded onto 0.4 μ m pore size polycarbonate Transwell inserts (Corning, Corning, NY, #3381) pre-coated with NIH3T3-conditioned medium. hBE cells were then differentiated for more than 28 days at air-liquid interface in Pneumacult ALI medium (STEMCELL Technologies, Vancouver, Canada) with fresh medium replenishment every 3 days. hBE cells were treated with ASOs by free uptake from the basolateral compartment for a total of 6 days.

RNA extraction and RT-qPCR

Cultured CFF-16HBE cells and hBE cells were lysed following ASO treatments and total RNA was prepared using the PureLink Total RNA Purification Kit as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). The RT-qPCR analysis was performed with QuantStudio 6 and 7 Flex real-time PCR systems (Applied Biosystems, Foster City, CA) using AgPath-ID One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA). RT-qPCR results were normalized to quantified total RNA using the Quant-iT Ribogreen RNA reagent

(Molecular Probes, Eugene, OR). TaqMan primer-probe set sequences are listed in additional file 1.

CFTR channel function analysis in CFF-16HBE cells

CFF-16HBE cells were seeded onto collagen IV (Sigma-Aldrich, Cat# C7521, St. Louis, MO) pre-coated Snapwell inserts (Corning, Corning, NY; 12-mm filter diameter with 0.4- μ m pore size) for 24 h at a density of 4.52×10^5 cells/cm². Cells were treated with ASOs at a final concentration of 1 μ M by free uptake at liquid-liquid interface for a total of 6 days with treatment media replenished at day 3, with or without 100 μ M geneticin (G418) (Thermo Fisher Scientific, Carlsbad, CA) added for the final 48 h. Snapwell inserts were then mounted into Ussing chambers and short circuit current (Isc) measurements were recorded with the VCC MC8 multichannel voltage-current clamp amplifier (Physiologic Instruments, San Diego, CA). Cells were bathed in a basolateral to apical chloride ion gradient. Composition for basolateral buffer was 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1.8mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM D-(+)-glucose (Sigma-Aldrich, St. Louis, MO), and apical buffer composition was 137 mM sodium-gluconate, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM D-(+)-glucose (Sigma-Aldrich, St. Louis, MO). All buffers were adjusted to physiological pH 7.4. Isc and epithelial resistance were calculated using Acquire & Analyze software (Physiologic Instruments, San Diego, CA). To assess CFTR-mediated current, the following compounds were added sequentially: 7 μ M benzamil (Selleckchem, Houston, TX) to the apical side, 20 μ M forskolin (Sigma-Aldrich, St. Louis, MO) to apical and basolateral sides, 1 μ M VX-770 (Selleckchem, Houston, TX) to the apical side, and 20 μ M CFTR inhibitor 172 (Selleckchem, Houston, TX) to the apical side. The current (Isc) mediated by CFTR was calculated as the difference (Δ Isc) from stabilized current achieved after forskolin addition or VX-770 addition

following the baseline achieved by addition of CFTR inhibitor 172 (CFTRinh-172)-mediated current stabilization.

Immunoblotting

CFTR and NMD factor protein levels were measured by western blot analysis. Cell protein lysate was collected with IP Lysis Buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Carlsbad, CA). Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA). A total of 40-50 µg of protein was separated by gel electrophoresis using a 3-8% Criterion XT Tris-acetate protein gel and transferred to a 0.2-µm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were probed with mouse IgG2β anti-human CFTR (UNC596; UNC CFTR Antibody Distribution Program, Chapel Hill, NC) followed by goat anti-mouse HRP-conjugated secondary antibody (Thermo Fisher Scientific, Carlsbad, CA) and detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Carlsbad, CA). Membranes were then probed with anti-Na⁺/K⁺-ATPase (SCBT, #sc-21712, Dallas, TX) followed by detection with ECL Prime detection reagent (GE Healthcare; Chicago, IL). Signals following probing for anti-β-actin (Cell Signaling Technologies, #3700, Danvers, MA), anti-SMG6 (Abcam, #ab87539, Cambridge, UK), and anti-SMG1 (Bethyl Laboratories, #A300-393A, Montgomery, TX) antibodies were assessed using the Odyssey infrared system (Li-COR, Lincoln, NE) following incubation with IRDye secondary antibodies (Li-COR, Lincoln, NE). Protein expression was quantified using either ImageJ or Li-COR Odyssey software. Only the mature, fully-glycosylated C band of CFTR protein was quantified.

Statistical analysis

GraphPad Prism version 7 software (GrapPad Prism, Inc., San Diego, CA) was used to perform statistical analyses. Data are presented as mean $\pm$ SEM and are representative of at least two independent experiments performed in triplicate. Differences between two groups were compared using the unpaired *t* test. Statistical analyses between three or more groups ANOVA was performed followed by indicated multiple comparison tests. $p < 0.05$ was considered statistically significant.

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Author Contributions

E.S, L.H, and M.K performed experiments. E.S, L.H, M.K, M.M, A.R, B.M, and S.G helped conceptualize and design experiments. All authors helped write and review the manuscript.

Competing Interests

Edward Sanderlin, Melissa Keenen, Alexey Revenko, Shuling Guo, Lulu Huang, and Brett Monia are employees of Ionis Pharmaceuticals. Martin Mense is an employee of the Cystic Fibrosis Foundation.

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532 Figure 1

Figure 1: Characterization of the CFF-16HBEge in vitro model system. CFF-16HBEge cells harboring *CFTR* Y122X, G542X, R1162X, and W1282X nonsense mutations have significantly reduced *CFTR* expression and function when compared to parental cells. (A) RT-qPCR analysis of *CFTR* mRNA in parental and CFF-16HBEge cells with nonsense mutations. *CFTR* mRNA levels were normalized to total RNA levels. The wild-type (WT) *CFTR* level in parental cells was set to 1 and is indicated by the dotted blue line. (B) Representative western blot image of *CFTR* protein expression in parental and CFF-16HBEge cells. Na⁺/K⁺-ATPase (NKA) was used as a loading control. (C) Representative short circuit current (I_{sc}) traces of parental and CFF-16HBEge cells obtained using the Ussing Chamber assay. 7 μM benzamil was added to establish baseline current followed by the sequential addition of 20 μM forskolin, 1 μM VX-770, and 20 μM *CFTR* inhibitor-172 (*CFTR*inh-172) at times indicated by arrowheads. (D) ΔI_{sc} of *CFTR*-attributed current in parental and CFF-16HBEge cells. Data are presented as mean ± SEM. Statistical significance was analyzed by ANOVA followed by Dunnett's multiple-comparison test (***) $p < 0.001$, **** $p < 0.0001$).

Figure 2

Figure 2: Treatment of parental cells with ASOs targeting NMD pathway components resulted in efficient target reduction and NMD pathway inhibition. RT-qPCR analysis for NMD factor mRNA levels in parental cells treated with ASOs targeting (A) *UPF1*, (B) *SMG1*, (C) *SMG8*, (D) *SMG9*, (E) *SMG6*, (F) *SMG5*, (G) *SMG7*, (H) *UPF2*, (I) *UPF3A*, and (J) *UPF3B*. NMD factor mRNA levels were normalized to total RNA levels. PBS control levels were set to 1 and are indicated by dotted blue lines. Data are presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by Dunnett's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

597 Figure 3

Figure 3: *CFTR* mRNAs containing nonsense codons are regulated by UPF1 and SMG1 but not SMG8 or SMG9. RT-qPCR analysis of (A) *CFTR-Y122X*, (B) *CFTR-G542X*, (C) *CFTR-R1162X*, and (D) *CFTR-W1282X* mRNAs treated with ASOs targeting *UPF1*, *SMG1*, *SMG8*, and *SMG9*. *CFTR* mRNA levels were normalized to total RNA levels. *CFTR* mRNA levels in PBS-treated CFF-16HBEge cells were set to 1 as indicated by blue dotted lines. Wild-type (WT) *CFTR* mRNA levels in parental cells are indicated by orange dotted lines. Data are presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by Dunnett's multiple-comparison test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 4

Figure 4: *CFTR* mRNAs with nonsense codons G542X, R1162X, and W1282X, but not Y122X, are recognized by UPF2 and UPF3 paralogs. RT-qPCR analysis of CFF-16HBEge cells harboring A) Y122X, B) G542X, C), R1162X, or D) W1282X *CFTR* nonsense alleles treated with ASOs targeting *UPF2*, *UPF3A*, and *UPF3B*. CFF-16HBEge cells harboring E) Y122X or F) W1282X *CFTR* nonsense alleles treated with ASO targeting *eIF4A3*. *CFTR* mRNA expression was normalized to total RNA levels and PBS control levels were set to 1 as indicated by blue dotted lines. Wild-type (WT) *CFTR* mRNA levels from parental cells are indicated by orange dotted lines. Data are presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by Dunnett's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

650 Figure 5

651

652

Figure 5: *CFTR* mRNA with nonsense codons are degraded through the SMG6-mediated endonucleolytic decay pathway. RT-qPCR analysis of CFF-16HBEge cells harboring A) Y122X, B) G542X, C) R1162X, or D) W1282X *CFTR* nonsense mutations were treated with ASO targeting *SMG6*, *SMG5*, and *SMG7*. (E) RT-qPCR analysis of *CFTR* mRNA levels in hBE cells homozygous for the W1282X nonsense mutation following treatment with ASOs targeting *SMG6*, *SMG5*, and *SMG7*. *CFTR* mRNA levels were normalized to total RNA levels in CFF-16HBEge and hBE cells. PBS control levels for *CFTR* mRNA expression were set to 1 as indicated by blue dotted lines. Wild-type (WT) *CFTR* mRNA levels from parental cells are indicated by orange dotted lines. Data are presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by Dunnett's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

Figure 6

Figure 6: *CFTR* mRNA stabilization resulting from ASOs targeting *SMG1* or *SMG6* improves translational readthrough outcomes and elevate *CFTR* protein expression in CFF-16HBEge cells. Representative western blot images (left) and quantification (right) of (A-B) Y122X, (C-D) G542X, (E-F) R1162X, and (G-H) W1282X *CFTR* protein expression in CFF-16HBEge cells following indicated treatment conditions. Complex-glycosylated band C of *CFTR* was evaluated for western blot quantification. Na^+/K^+ -ATPase (NKA) was used as a loading control. Data are presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by Sidak's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

710 Figure 7

711 **Figure 7:** ASOs targeting *SMG1* or *SMG6* improve translational readthrough outcomes and
712 upregulate CFTR function in CFF-16HBEge cells. Representative Isc traces (left) and Δ Isc
713 (right) were obtained from Ussing chamber assays of CFF-16HBEge cells containing (A-B)
714 Y122X, (C-D) G542X, (E-F) R1162X, or (G-H) W1282X *CFTR* nonsense mutations. For the
715 Ussing chamber assay, 7 μ M benzamil was added to establish baseline currents followed by the
716 sequential addition of 20 μ M forskolin, 1 μ M VX-770, and 20 μ M CFTR inhibitor-172
717 (CFTRinh-172) at times indicated by arrowheads to determine CFTR function. Data are
718 presented as mean $\pm$ SEM. Statistical significance was analyzed by ANOVA followed by
719 Dunnett's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

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