Allele-Specific Prevention of Nonsense-Mediated Decay in Cystic Fibrosis Using Homology-Independent Genome Editing

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Nonsense-mediated decay (NMD) is a major pathogenic mechanism underlying a diversity of genetic disorders. Nonsense variants tend to lead to more severe disease phenotypes and are often difficult targets for small molecule therapeutic development as a result of insufficient protein production. The treatment of cystic fibrosis (CF), an autosomal recessive disease caused by mutations in the CFTR gene, exemplifies the challenge of therapeutically addressing nonsense mutations in human disease. Therapeutic development in CF has led to multiple, highly successful protein modulatory interventions, yet no targeted therapies have been approved for nonsense mutations. Here, we have designed a CRISPR-Cas9-based strategy for the targeted prevention of NMD of CFTR transcripts containing the second most common nonsense variant listed in CFTR2, W1282X. By introducing a deletion of the downstream genomic region following the premature stop codon, we demonstrate significantly increased protein expression of this mutant variant. Notably, in combination with protein modulators, genome editing significantly increases the potentiated channel activity of W1282X-CFTR in human bronchial epithelial cells. Furthermore, we show how the outlined approach can be modified to permit allele-specific editing. The described approach can be extended to other late-occurring nonsense mutations in the CFTR gene or applied as a generalized approach for gene-specific prevention of NMD in disorders where a truncated protein product retains full or partial functionality.

INTRODUCTION

Nonsense mutations account for an estimated 11% of the variants underlying inherited disease.1 Though the molecular consequences of a nonsense mutation are varied,2 the predominant outcome of this type of mutation is degradation of the resulting mRNA transcript through a process called nonsense-mediated decay (NMD). Often, this degradation results in the complete absence of protein or residual production of a truncated protein product. If NMD is prevented or escaped, these truncated protein products may result in dominant-negative or gain-of-function phenotypes.3 For an accumulating number of disorders, however, it has been demonstrated that the prevention of NMD can attenuate disease phenotypes.3–5

Cystic fibrosis (CF; MIM: 219700) is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. The CFTR protein is an ion channel that mediates chloride and bicarbonate transport in the epithelial cells of multiple organs including the lungs, pancreas, and intestine.6–8 To date, more than 300 CF-causing mutations have been identified (http://www.genet.sickkids.on.ca/; https://cftr2.org/). The most common mutation, the deletion of phenylalanine at position 508 (F508del-CFTR), induces misfolding of the protein that results in its retention within the endoplasmic reticulum and subsequent degradation by proteasomal pathways. Recently, the combination of two correctors, Tezacaftor (VX-661) and Elekcaftor (VX-445), alongside a channel function potentiator called Ivaftor (VX-770) has been approved by the FDA as TRIKAFTA for patients with one or two F508del-CFTR alleles.9–12 Despite the promising reports that treatment with TRIKAFTA is associated with meaningful improvement in lung function and respiratory-related quality life, no modulator therapies are clinically available for individuals with nonsense mutations in CFTR. The second most common nonsense mutation in CF, W1282X-CFTR (NC_000007.14:g.117642566G > A), is subject to nonsense-mediated mRNA decay and results in residual expression of a truncated protein product.13,14 While it has been shown that CFTR modulators are effective at rescuing the functional expression of W1282X-CFTR in systems of heterologous overexpression, this has not been universally successful in primary cultures of airway epithelia.11,15 Recently it has been demonstrated that a combination of CFTR correctors and a potentiator, alongside a small molecule that inhibits NMD, can rescue...
the functional expression of W1282X-CFTR in patient-derived nasal epithelial cells.16

Through its demonstrated role in maintaining proper gene expression,17,18 NMD underlies a diversity of fundamental biological processes, ranging from the maturation of the immune system to the maintenance of telomeres.19–22 The importance of NMD is highlighted through its broad phylogenetic conservation.23 Accordingly, strategies that rely on global inhibition of NMD may be coupled with a variety of off-target effects with far-reaching phenotypic consequences.

Presently, gene therapy is being actively explored for the treatment of CF, particularly for those carrying nonsense mutations. Although this approach has shown promising preliminary results in various model systems,24–28 insufficient transgene delivery and expression—largely attributed to loss over cell divisions—have proven to be a challenge in the clinical translation of gene therapy for CF.29,30 Targetable nucleases, most notably the CRISPR-Cas9 system, have become an attractive alternative to traditional gene therapy approaches. The precise correction of disease-causing mutations, however, remains a significant challenge for genome editing technologies. Although several groups have demonstrated mutation correction and restored expression of wild-type (WT) CFTR,31–33 their reliance on the relatively inefficient homology-directed repair (HDR) pathway can be prohibitive in terms of the clinical translation of these strategies. Consequently, many therapeutic applications of genome editing have focused on strategies involving non-homologous end joining (NHEJ),34,35 which tends to occur at a much higher frequency than homology-directed repair.36

In the present study, we describe a CRISPR-Cas9-based genome editing strategy that harnesses NHEJ to prevent NMD in a gene-specific manner. Using a dual-guide approach, we generated an approximately 24-kilobase deletion spanning from exon 23 to exon 27 of the CFTR locus, encompassing the downstream genic region following W1282X-CFTR. While not fully understood, NMD tends to occur in a splicing-dependent manner, triggered by exon-junction complexes remaining downstream of a prematurely-terminated ribosome.37 We hypothesized that this editing strategy would eliminate the formation of exon-junction complexes following the premature stop codon and thus prevent NMD of the edited transcript.38 Using human bronchial epithelial (HBE) cells that are homozygous for the W1282X mutation, we show that the desired deletion can be achieved with high efficiency and that editing results in the restoration of CFTR expression at both the mRNA and protein level. Further, we show that the resulting protein product can be successfully modulated with clinically approved CFTR modulators. To account for the heterogeneity in genotypes across patients with CF, we refined our editing strategy to permit allele-specific editing. Our data demonstrate a novel use case for CRISPR-Cas9 genome editing in gene-specific prevention of NMD, which could be further applied to other genetic diseases caused by nonsense mutations.

**RESULTS**

**CRISPR-Cas9-Mediated Genome Editing Allows for Genomic Truncation of CFTR**

Using CRISPR-Cas9, a genomic deletion can be efficiently generated by simultaneously targeting the region of interest using two flanking guide RNAs.39–42 We hypothesized that removal of the downstream genic region following the mutation site would prevent NMD upon subsequent transcription, thereby stabilizing CFTR expression (Figure 1A). We designed four guide RNAs—two targeting exon 23

![Figure 1. Genome Editing Restores Expression of W1282X-CFTR in Human Bronchial Epithelial Cells](image-url)
following the premature stop codon, and two targeting exon 27, the final exon of CFTR. These guides were designed and selected to minimize potential off-target editing using the CHOPCHOP webtool.43 We transfected each of these guides individually alongside *Streptococcus pyogenes* Cas9 (SpCas9) into HEK293T cells to evaluate editing efficiency. We found that editing efficiencies ranging from 25%–48% (Figure 1B). To introduce the desired deletion, we paired the guides with highest editing efficiency from the two targeted loci. When transfected individually alongside SpCas9 into W1282X-HBE cells, these guides exhibited similar editing activities to those found in the HEK293T experiments (Figure S1A). These guides were co-transfected into an immortalized human bronchial epithelial cell line that was previously gene edited using CRISPR-Cas9 to harbor the W1282X-CFTR variant in homozygosity.44 Using a polymerase chain reaction (PCR)-based assay, we identified a product corresponding to a deletion junction formed across the two cleavage sites in the genomic DNA of the edited cell population (Figure S1B).

**Genome Editing Improves Expression of W1282X-CFTR Protein and mRNA in Human Bronchial Epithelial Cells**

After establishing that the desired deletion was achievable, we sought to evaluate how editing impacted W1282X CFTR expression. In a heterogeneous population of edited cells, we found a 2.4 ± 0.18-fold increase in *CFTR* mRNA expression compared to unedited controls (Figure 1C). Correspondingly, a truncated mRNA transcript was present in the cDNA of the edited W1282X-CFTR HBE cell population that was absent in unedited W1282X-CFTR control HBE cells (Figure S1C). In addition to an increase in *CFTR* mRNA expression, edited cells exhibited modest expression of the truncated CFTR protein, which was absent in unedited cells (Figure 1D). Together, these data demonstrate that the genomic truncation of the W1282X-CFTR variant can successfully prevent NMD.

**Genome-Edited Human Bronchial Epithelial Cells Produce Functional CFTR Protein after Pharmacological Rescue**

Previous studies suggested that small molecule correctors and potentiators were partially effective in rescuing the channel activity of W1282X-CFTR.15,16,45,46 We sought to determine whether the recently approved drug combination, TRIKAFTA, which targets the F508del mutation, is able to rescue W1282X-CFTR channel activity in homozygous mutant human bronchial epithelial (HBE) cells.16,44 As expected, prior to editing there was no residual forskolin (FkS) activated, i.e., cyclic adenosine monophosphate (AMP)-mediated channel activity conferred by W1282X-CFTR in HBE cells (Figure 2A and 2B). Further, there was no functional rescue of potentiated channel function following pre-treatment with the first-generation corrector, VX-809, or the second-generation corrector combination, VX-661 and VX-445, in the current studies (Figures 2A and 2B). Immunoblotting studies confirmed that W1282X-CFTR protein was truncated and at only 1% of the abundance measured for WT-CFTR in HBE cells (Figures 2C and 2D). There was only a modest increase in the abundance of the truncated protein in the presence of the correctors.
Since deleting the downstream genic region increased protein expression of truncated W1282X-CFTR (Figure 1D), we asked whether modulators could rescue functional expression in the gene-edited HBE cells. While there was no functional response to potentiation with VX-770 alone or with VX-809 pre-treatment with acute addition of VX-770 (Figures 3A and 3B), we did see a significant rescue of potentiated W1282X-CFTR function (70% of the mean forskolin in WT-CFTR HBE cells) after pre-treatment with the novel modulators VX-661 and VX-445. Gene-edited W1282X-CFTR HBE cells demonstrated up to 70% of the mean forskolin response observed in WT-CFTR HBE cells. As shown in Figures 3C and 3D, we achieved approximately 20% of WT-CFTR protein abundance under the best rescue conditions. Altogether, these findings support the use of this editing strategy to generate a partially functional, truncated CFTR mutant that has the potential for channel activity in the presence of channel function modulators.

Allele-Specific Genome Editing of W1282X-CFTR

Most patients harbor the W1282X-CFTR mutation in compound heterozygosity. To account for this, we aimed to modify our strategy to minimize targeting of the other mutant allele. It has been demonstrated that a mismatch in the seed sequence of a guide RNA—the 10–12 nucleotides immediately upstream of the protospacer adjacent motif (PAM) sequence—can significantly reduce or even abolish the editing activity of CRISPR-Cas9.47–49 We strategically designed a guide RNA where the first PAM-proximal nucleotide of the sequence is complementary to the adenine nucleotide underlying the W1282X-CFTR variant (Figure 4A). To assess the precision of this guide RNA, we transfected it in a vector co-expressing SpCas9 into both WT- and W1282X-CFTR HBE cells. We found a significant bias toward alleles harboring the W1282X-CFTR variant compared to alleles that are WT at this locus, with average editing efficiency of 30.25% ± 1.26% and 0%, respectively (Figure 4B). As a control, we designed a reciprocal guide RNA, where the first PAM-proximal nucleotide is complementary to the WT sequence, and found corresponding allelic discrimination with average editing efficiency of 31% ± 1.41% in the context of the mutant allele and 83.5% ± 6.16% in the context of the WT allele (Figure S2).

To generate a deletion, we paired the W1282X-CFTR-specific guide RNA with a guide RNA targeting 108 bases downstream of the native stop codon in the 3' UTR of CFTR (Figure 4C). While this guide RNA is indiscriminate of alleles, we hypothesized that any potential indels formed on the non-targeted allele would have a negligible effect on CFTR expression or function, due to being placed within a non-coding region. To test this, we transfected this guide RNA in a vector co-expressing SpCas9 into WT-CFTR HBE cells and evaluated CFTR protein expression following editing. We found that CFTR expression remained stable, which indicates that this guide is likely benign (Figure S3A). In addition, we found similar results when evaluating CFTR protein expression of WT-CFTR HBE cells after delivery of the W1282X-CFTR targeting guide RNA both individually and combined with the guide targeting the 3' UTR, further supporting the allele-specificity of the designed approach (Figure S3A).
to targeting outside of the coding region of CFTR, we selected the 3' UTR targeting guide as it introduces a novel termination codon 15 bases downstream of the predicted deletion junction. This was necessary as the allele-specific guide RNA generates a double-stranded break immediately before the premature stop codon such that when combined with a guide RNA that targets beyond the coding region of the gene, the result is a removal of both the premature and native stop codon found in the W1282X-CFTR allele. Accordingly, the predicted result of our editing is the production of CFTR protein that is WT up to the 1,281st residue, followed by a novel sequence of four amino acids and a stop codon (Figure 4F).

We co-delivered these two guide RNAs alongside SpCas9 into W1282X-CFTR HBE cells to assess their impact on CFTR expression.
By PCR, we were able to detect the formation of a deletion junction in genomic DNA extracted 72 h post-transfection. Using a three primer PCR assay, we quantified the proportion of DNA containing the desired deletion relative to unedited DNA, finding similar amplification intensity of each product (Figure S4A). The introduction of the intended stop codon requires perfect joining of the two double-stranded breaks. We subcloned the amplified deletion junction and found the predicted editing in only 5% of clones screened (2/39 clones). Instead, we found a heterogeneous population of deletion junctions with indels of varied length. In each of the editing outcomes, however, a novel stop codon was reached within 1–36 amino acids of the 1,281st residue, with the majority of editing outcomes (30/39 clones) producing a stop codon within four amino acids.

Next, we analyzed the effect of our editing strategy on CFTR expression at the level of mRNA and protein. We extracted protein and mRNA from both edited (W1282X-CFTR edited) and unedited (W1282X-CFTR) HBE cells, 5 days post-confluence. The expression of CFTR mRNA in a heterogeneous population of edited cells was increased 2.34 ± 0.26-fold when compared to unedited controls (Figure 4D). Again, we found the expression of a truncated CFTR mRNA product (Figure S4B) and truncated CFTR protein product in the edited W1282X-CFTR cells that was absent in W1282X-CFTR control cell population. (Figure 4E).

Finally, we asked whether the CFTR protein expression rescued by allele-specific editing could be modulated by second-generation modulator compounds similar to our non-specific editing experiments. As shown in Figures 5A and 5B, we measured residual function of the truncated CFTR product after editing in an allele-specific manner that was not further potentiated by VX-770. After the addition of the combination of VX-661 and [R]-VX-445, we observed rescued potentiated channel function (60% of the mean forskolin in WT-CFTR HBE cells) in W1282X-CFTR HBE cells after allele-specific editing. We also found that the corrector modulators enhanced the relative expression of truncated CFTR in allele-specific edited W1282X-CFTR HBE cells (Figures 5C and 5D).

**DISCUSSION**

Significant advancements have been made in the therapeutic development for CF, culminating in a wide variety of highly effective small molecule therapies. These therapies, which have largely focused on the predominant F508del-CFTR variant, are inapplicable to patients harboring nonsense mutations in CFTR. For one such nonsense mutation, W1282X-CFTR, it has been demonstrated that if NMD can be prevented, either through heterologous expression or through the pharmacological inhibition of NMD, the truncated protein product can be effectively modulated using existing CFTR modulators.
In our previous experience, the steady-state levels of W1282X protein, endogenously expressed in epithelial cell lines and in primary tissues, are negligible. This reduced expression can be reversed by treatment with nonsense mediated decay inhibitors, pointing to the negative impact of this pathway. Recently, we tested three NMD small molecule inhibitors with different mechanisms of action in W1282X-CFTR HBE cells.16 We compared the relative efficacy of a SMG-1 kinase inhibitor (SMG1), 5-azacytidine (Vidaza, that acts via the MYC-dependent pathway), and NMD-14 that targets the SMG7 protein and disrupts SMG7-UPF1 interactions. Interestingly, we observed that modulation of NMD by inhibition of SMG-1 protein was the most effective in rescuing the truncated W1282X-CFTR channel function after pre-treatment with CFTR correctors. The clinical utility of such NMD inhibitors, however, is uncertain given the potential for toxic off-target effects. It is well-established that for CFTR, the final domain of this multi-domain protein, is not required for its folding and assembly. Truncation mutants lacking the second nucleotide binding domain are properly processed and trafficked to the surface of cells in heterologous expression systems.14–16 These truncation mutants, however, do require pharmacological modulators for channel function activation. These observations prompted the development of the therapeutic strategy described in this paper.

We designed a homology-independent genome editing strategy that successfully suppresses NMD of the second most common disease-causing nonsense mutation in CF. The magnitude of functional rescue reported here is similar to what we found previously with the most effective NMD inhibitors.16 Our approach generates a truncated CFTR transcript that escapes NMD and produces a protein product that can be therapeutically modulated with existing small molecule compounds designed for the more common F508del-CFTR variant.

Notably, we demonstrate that the protein product generated is effectively modulated using the drug combination comprising TRIKAFTA.11,50 This stands as the first demonstration of the potential therapeutic benefit of TRIKAFTA specifically for the W1282X-CFTR variant. In its final iteration, our approach was modified such that targeting was specific to alleles carrying the W1282X-CFTR mutation. This allele-specific approach can be applied to patients who harbor the W1282X-CFTR mutation in heterozygosity while leaving the non-targeted allele intact (i.e., N1303K/W1282X).

Although our results and those of previous publications show that the truncated W1282X-CFTR can reach the cell surface and mediate chloride conductance after treatment with CFTR modulators, the stability of the truncated protein at the apical membrane of epithelia will be compromised. The carboxyl CFTR terminus (1479–1480) is responsible for binding to several PSD-95/Dlg/ZO-1 (PDZ) domain-containing proteins including NHERF-1 (Na+/H+ exchanger regulatory factor isoform 1), NHERF-2, CAP-70 (CFTR-associated protein), and CAL (CFTR associated ligand).24 It has been demonstrated that overexpression of NHERF1 stabilizes the most common CFTR mutation, F508del, at the apical membrane.25 Furthermore, the CFTR corrector VX-809 stabilizes F508del-CFTR at the cell surface by increasing the interaction between F508del-CFTR and NHERF1.36–38 As recently suggested by Lukacs and colleagues, however, steady-state abundance of mutant CFTR that lacks its carboxy-terminal PDZ motif on the apical membrane may be maintained, at least in part, by efficient transcytosis from the basolateral membrane. This group has shown that mutant CFTR lacking six residues from its carboxy terminus exhibits transcytosis to the apical membrane and this does not require PDZ-dependent interactions.39

Here, we chose to truncate the CFTR gene at the site of the premature termination codon (PTC), with the truncation encompassing the entire downstream genetic region. It has been documented, however, that the size of deletion is highly correlated with the efficiency of deletion.60 Although we show that the editing efficiency achieved is sufficient for significant rescue of expression, there are optimizations to the editing strategy that could further increase deletion efficiency and that are applicable to both W1282X-CFTR and other pathogenic PTCs. For example, sensitivity to NMD is correlated with distance from the PTC to the nearest downstream exon junction complex, with insensitivity being documented at distances greater than 50 nucleotides.61–63 Accordingly, a deletion that joins the PTC-containing exon to the next following exon greater than 50 nucleotides in length could reduce or eliminate NMD. If applied to W1282X-CFTR, this would require removing 10 kilobases, which may occur at a higher frequency than the presented 24-kilobase deletion. This approach, however, would need to be validated on a case-by-case basis, as multiple exceptions to the 50-nucleotide NMD boundary have been documented,64–69 and there may be a tradeoff between deletion efficiency and residual NMD activity.70 CRISPR-Cas9-mediated exon skipping71 is another viable homology-independent genome-editing approach that could be applied to the W1282X-CFTR variant, as exclusion of the mutation-containing exon preserves the proper reading frame. The stability and drug responsiveness of the resulting protein product, however, would need to be verified, though it is likely that this protein product could be similarly modulated with some combination of existing therapeutic compounds.

To further translate this strategy, varied optimizations will be required with respect to delivery. The most common vehicle being explored for the delivery of genome-editing components is the adeno-associated viral (AAV) vector.72 The large size of SpCas9, however, poses a challenge given the limited packaging capacity of AAV vectors.73 This has recently been addressed by the development of various split-SpCas9 systems that allow for packaging of the enzyme across multiple AAV particles for in vivo reconstitution.74–76 Alternatively, the editing strategy could be translated to one of the more compact Cas9 orthologs, such as Staphylococcus aureus, Campylobacter jejuni, or Streptococcus thermophilus, all of which have been demonstrated in vivo using AAV-based delivery.77–79

While demonstrated with the W1282X-CFTR variant, this strategy could be extended to any nonsense or frameshift mutation occurring downstream of the 1,2814 residue, such as Y1307X-, Q1412X-, or...
S1455X-CFTR. It is possible that a larger C-terminal truncation could be similarly modulated, further expanding the number of CFTR nonsense mutations targetable using this approach. Further, our strategy could serve as a generalized approach to the prevention of NMD in genetic diseases where a truncated protein product of the causative gene retains full or partial functionality.\textsuperscript{3,5,80}

MATERIALS AND METHODS

Cell Culture and Transfection

HEK293T cells were purchased from ATCC and were cultured in Dulbecco’s modified Eagle’s medium (Wisent) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Pen-Strep; Gibco). 16HBE cells (a generous gift from Dr. D.C. Gruenert, University of California, San Francisco, CA, USA) and CFF-16HBEge W1282X-CFTR cells (from Cystic Fibrosis Foundation) were cultured in Eagle’s minimum essential medium (Wisent) supplemented with 10% fetal bovine serum and 1% Pen-Strep (Gibco). We seeded 500,000 cells for transfection in 2 mL of media in six-well plates using Lipofectamine 3000 (Thermo Fisher Scientific). Cells were transfected with 3,000 ng of a Cas9 and guide RNA co-expression vector, pSpCas9(BB)-2A-Puro (PX459) V2.0, which was a gift from Feng Zhang (Addgene plasmid #62989). To enrich for transfected cells, we subject 24 h post-transfection cells to 0.9 μg/mL or 2 μg/mL of puromycin, for HBE cells and HEK293T cells, respectively, for 72 h.

Compound Description

VX-809, VX-661, and VX-770 were provided by Selleck Chemicals; [R]-VX-445 (N-(1,3-dimethylpyrazol-4-yl)sulfonyl-6-[3-(3,3,3-trifluoro-2,2-dimethyl-propoxy)pyrazol-1-yl]-2-[(4R)-2,2,4-trimethyl-pyrrrolidin-1-yl]pyridine-3-carboxamide) and [S]-VX-445 (N-(1,3-dimethylpyrazol-4-yl)sulfonyl-6-[3-(3,3,3-trifluoro-2,2-dimethyl-propoxy)pyrazol-1-yl]-2-[(4S)-2,2,4′-trimethylpyrrrolidin-1-yl]pyridine-3-carboxamide) were provided by MedChemExpress. For these studies we employed the R enantiomer of VX-445 provided by MedCHEMEXPRESS. Comparative studies of the enantiomers showed similar activity using the fluorescein plate reader (FLIPR) assay in W1282X-CFTR HBE cells (maximal activation: R enantiomer 103.4 ± 1.144 and S enantiomer 105 ± 0.7141).

Genomic DNA Isolation and PCR

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer’s protocol. PCR was performed using DreamTaq Polymerase (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Estimation of Genome Editing

PCR amplification from the genomic DNA of a bulk population of edited cells centered on the predicted editing site was performed. These amplicons were PCR purified using QiAquick PCR Purification Kit following the manufacturer’s protocol (QIAGEN) and Sanger sequenced using an internal primer. To test guide efficiency, we used the Sanger sequencing files from unedited and edited cells as an input into the online sequence trace decomposition software Synthego Performance Analysis, ICE Analysis (2019, v2.0. Synthego).\textsuperscript{81} To assess the editing outcomes found after allele-specific editing, we performed a PCR to amplify the deletion junction formed between the two double-stranded break points. This amplicon was then purified and sub-cloned using the pGEM-T Easy Vector System (Promega). The cloned plasmid was extracted from 40 unique clones and sequenced with a primer complementary to the vector backbone. To assess editing activity in the WT HBE cell populations (represented in Figure S3), we digested PCR products using the T7 Endonuclease 1 Enzyme (New England Biolabs) following the manufacturer’s instructions. This was necessary as sequencing-based methods were infeasible for the 3′ UTR-targeted region due to long adenine tracts interfering with Sanger sequencing. To estimate frequency of the deletion product in genomic DNA from a bulk edited population, we used a three-primer PCR strategy. Primer pairs were designed such that the combination of three primers would result in amplification of both unedited DNA and DNA spanning a deletion junction. Each primer pair utilizes the same forward primer and distinct amplification is derived from the reverse primer design. Choice of reverse primers was based on maintaining similar DNA amplicon size between targets and matching melting temperatures to the universal forward primer.

CFTR Channel Function in CFF-16HBEge W1282X-CFTR Cells

16HBE14o- cells, previously genome-edited to produce the homozygous CFF-16HBEge W1282X-CFTR cell line, were obtained from the Cystic Fibrosis Foundation.\textsuperscript{44} Cells were grown at 37°C for 5 days post-confluence, submerged on 96-well black-well, clear-bottom culture plates (Costar) in EMEM media (Wisent BioProducts) with 10% fetal bovine serum (Wisent BioProducts) and 1% Pen-Strep (Wisent BioProducts). 24 h before the assay, cells were treated with DMSO, 3 μM VX-809 (Selleck Chemicals), and 10 μM VX-661 (Selleck Chemicals) + [R]-VX-445 (MedChemExpress). Cells were then loaded with blue, membrane potential dye dissolved in chloride-free buffer (150 mM NMDG-glucosone, 3 mM potassium glutonate, 10 mM HEPES, pH 7.30, 300 mosm) for 30 min. The plate was then read in a FLIPR Tetra (Molecular Devices) at 37°C (excitation: 530 nm, emission: 560 nm). CFTR was stimulated with 10 μM forskolin (Sigma-Aldrich) and either 1 μM VX-770 (Selleck Chemicals) or DMSO. The assay was terminated with 10 μM CFTIRinh172 (Cystic Fibrosis Foundation Therapeutics). Changes in membrane potential were normalized to the point before the addition of agonist and to the DMSO control response.\textsuperscript{82,83}

Protein Isolation and Western Blot Analysis

For all protein assays, total protein lysate was extracted 5 days after cellular confluency, where cells were trypsinized from their wells, pelleted, and washed three times with 1× PBS (GIBCO). Protein was isolated by resuspension in 150 μL of a one-to-one solution of radioimmunoprecipitation assay (RIPA) homogenizing buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1-mM EDTA) and RIPA double-detergent buffer (2% deoxycholate, 2% NP-40, 2% Triton X-100 in RIPA homogenizing buffer) supplemented with a protease-inhibitor cocktail (Roche). Cells were subsequently incubated on ice for 30 min, then centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was collected and stored at −80°C. Whole protein
concentration was measured using Pierce BCA Protein Assay Kit according to the manufacturer’s protocol (Thermo Fisher Scientific). SDS-PAGE separation was completed by running 10 μg of total protein on a NuPAGE 3%–8% Tris-Acetate gel (Thermo Fisher Scientific) or 6% Tris-Glycine gel (Life Technologies) as previously described.9,10 Next, proteins were transferred to a nitrocellulose membrane using the iBlot 2 transfer apparatus (Thermo Fisher Scientific). A 5% milk solution in 1 × TBST was used for blocking for 1 h at room temperature. The membrane was then incubated with the CFTR primary antibody (University of North Carolina Chapel Hill: Anti-CFTR 596) at 4°C overnight. Primary antibody solution was removed, and the membrane was washed three times with 1 × TBST. This was followed by a 1-h incubation at room temperature with horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG; Abcam: ab6721). For protein measurements after functional analysis, CNX was used as a loading control (Sigma: C4731). For all other protein measurements, beta-actin (ACTB) was used as a loading control (Santa Cruz: SC-47778). After three washes with 1 × TBST, signal detection was achieved using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) according to the manufacturer’s protocol or with ECL (Amer sham) using the Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE, USA) in a linear range of exposure (2–5 min).10 Relative levels of CFTR protein were quantitated by densitometry of immunoblots using ImageStudioLite (LI-COR Biosciences, Lincoln, NE, USA).87

RNA Isolation and Quantitative PCR

Assays of CFTR mRNA expression were performed 5 days after cellular confluence. Cells were harvested, washed three times with 1 × PBS, and pelleted, then total RNA was extracted using the RNeasy Mini Kit following the manufacturer’s protocol (QIAGEN). Next, 1,000 ng of mRNA was reverse transcribed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer’s protocol. Quantitative PCR utilizing Fast SYBR Green Master Mix (QIAGEN) was performed on a Step One Plus Real Time PCR machine (Applied Biosystems). Primers specific to endogenous GAPDH were used as an internal control. Differential expression between edited and unedited samples was analyzed using the ΔΔCt method.

Statistical Analyses

All graphs were plotted as the mean, with error bars indicating standard deviation or the standard error of the mean. Differences between groups were assessed by two-tailed unpaired t test with Welch’s correction. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental material can be found online at https://doi.org/10.1016/j.omtm.2020.05.002.

AUTHOR CONTRIBUTIONS

S.E., O.L., C.E.B., and E.A.I. conceptualized this research and designed the experiments. S.E., O.L., T.M.I.B., R.A.B., and A.H.R. performed the experiments. S.E. and O.L. analyzed experimental results. S.E. and O.L. wrote the manuscript. S.E., O.L., C.E.B., and E.A.I. edited the final manuscript. All authors approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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