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Standard Article

Systematic deletion of symmetrical *CFTR* exons reveals new therapeutic targets for exon skipping antisense oligonucleotides

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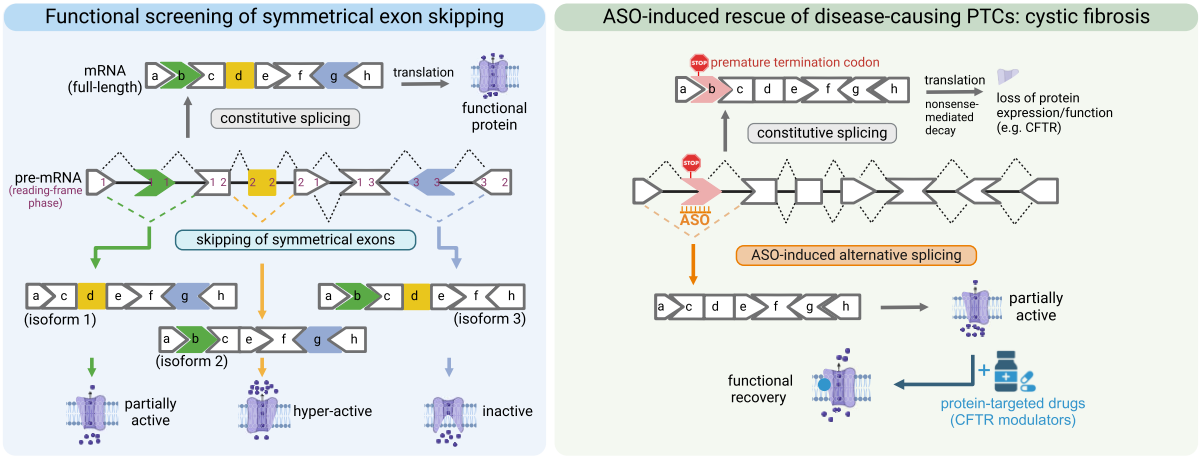
Abstract

There is a major need for therapeutics that treat disease caused by premature termination codons (PTCs). Splice-switching antisense oligonucleotides (ASOs) can be directed to block splicing and cause exon skipping, a process that can be used to effectively remove PTCs from an mRNA. This ASO-induced exon skipping can restore protein coding potential when the exons on either side of the skipped exon are in the same reading frame, or symmetrical. We demonstrate the potential of this approach as a therapeutic using the cystic fibrosis (CF) transmembrane regulator (*CFTR*) gene, which has CF-associated, PTC-causing variants in all 27 of its exons. We functionally screened all CFTR isoforms that can be generated by deletion of symmetrical exons and identify four that are functionally responsive to CFTR modulators. We identified ASOs that induce skipping of these exons and show that they recover CFTR function in airway cells derived from individuals with *CFTR* PTC variants. This study demonstrates that systematic functional analysis of in-frame exon-deleted protein isoforms can successfully identify targets for ASO-based splice-switching therapies, a therapeutic concept that can be broadly applied to any multi-exon protein-coding gene disrupted by PTCs.

Graphical abstract



Introduction

Premature termination codons (PTCs) generated by frameshift and nonsense mutations result in the synthesis of a truncated protein and trigger nonsense-mediated mRNA decay (NMD), largely abolishing functional protein production (1). One therapeutic strategy for PTC-causing disease that has had

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clinical success uses splice-switching antisense oligonucleotides (ASOs) to induce exon skipping to eliminate pathogenic variants and/or reframe the mRNA transcript (2). This approach requires the exon targeted for ASO-induced skipping to be a symmetrical exon, defined as an exon in the same reading frame register as its flanking exons. Thus, when the symmetrical exon is spliced out, or skipped, the open reading frame is preserved. Symmetrical exon skipping is a common natural form of alternative splicing that can generate protein isoforms with altered functions (3). In the case of disease-associated variants in these exons, this mechanism can be harnessed using ASOs to remove PTCs and thereby stabilize the mRNA and recover protein expression. This approach can be therapeutic if the protein that is produced from the ASO-induced spliced transcript is at least partially functional. This strategy is the basis of the FDA-approved splice-switching ASOs targeting PTCs in Duchenne muscular dystrophy (DMD) (2).

To establish targets for exon skipping strategies, an understanding of the function of protein isoforms encoded by mRNAs lacking the skipped exon is required. In principle, rapid functional target identification can be achieved by systematic screening of all possible symmetrical exon deletions within a gene. Here, we demonstrate the utility of this screening approach for investigating treatment strategies for cystic fibrosis (CF), an autosomal recessive disease caused by disruption of the CF transmembrane conductance regulator (*CFTR*) gene, found in over 100,000 people diagnosed across 94 countries (4). *CFTR* encodes a transmembrane anion channel, the loss of which results in thick mucus buildup in multiple organs, most severely affecting the lungs, gut, and pancreas (5). Over 1000 CFTR variants are known to be CF-associated and cause varying degrees of disease severity, based on their effect on expression and protein function (6,7) For some variants, there are effective small-molecule therapeutics that rescue CFTR protein processing and function that are approved to treat people with CF (pwCF). These include potentiator drugs that enhance channel gating and corrector drugs, used in combination with potentiators, that restore processing and trafficking of CFTR to the cell membrane (8–10). The most prevalent treatment, Trikafta, is a combination of the potentiator VX-770 (ivacaftor) and the correctors VX-661 and VX-445 (elexacaftor and tezacafter; i.e. ETI) (9). These small-molecule therapeutics, collectively referred to as highly effective modulator therapeutics (HEMTs), are effective in treating CF caused by more common variants, such as *CFTR-F508del*. However, because these drugs target the CFTR protein, they are not effective in treating CF caused by pathogenic variants that preclude protein production, such as those that produce PTCs. These types of variants account for ∼11% of all CF-causing variants and ∼62% of variants that are not eligible for treatment with current HEMTs (7,11,12).

For one such variant, *CFTR-W1282X*, we and others have reported on an effective ASO approach that corrects CFTR function by inducing the skipping of exon 23, the symmetrical exon that houses the variant (13–15), but additional exon skipping strategies have not been explored. *CFTR* contains many other symmetrical exons that could be similarly eliminated (Figure 1A), and ASO-mediated exon skipping has the potential to treat CF caused by PTCs in these exons (Figure 1B). Here, utilizing a systematic engineered protein screening approach, we identify four CFTR exons, encoding amino acids in the C-terminus of the protein, that can be spliced out and still produce a CFTR isoform with HEMT-responsive function. We show that ASO-induced skipping of these exons can induce the production of these isoforms resulting in therapeutic rescue of CFTR function with HEMTs. These results provide support for a therapeutic path forward for treating CF caused by PTCs in these four exons, which, based on variant expression with F508del, could be as many as ∼1% of pwCF (Supplementary Table S2), a number that will vary depending on population variant frequencies and ETI eligibility. Overall, our findings demonstrate the effectiveness of engineered protein screening to rapidly test for functional isoforms and guide ASO-based therapeutic strategies that can be broadly applied to many genetic diseases.

Materials and methods

Generation of exon deletion expression plasmids

CFTR-Δexon deletion plasmids were constructed from the synthetic *CFTR* high codon adaption index into the pcDNA3.1/Neo(+) vector using the Q5 Site-Directed Mutagenesis Kit (NEB) (Supplementary Table S2) (16). All plasmid deletions were sequenced. Plasmids were reverse transfected into FRT cells with lipofectamine LTX (Thermo Fisher) and OptiMEM (Thermo Fisher). Clonal cell lines were selected with G418 (300 μg/ml) and maintained in media supplemented with G418 (150 μg/ml).

Cells and culture conditions

FRT cell lines were cultured in F12 Coon's modification media (Sigma, F6636) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PenStrep). T84 cells were cultured in Dulbecco's modified Eagle medium/Nutrient Mixture F12 (DMEM/F12) media supplemented with 5% FBS and 1% PenStrep. Primary human bronchial epithelial (hBE) cells were acquired from the Compound Screening and Drug Discovery Core at Rosalind Franklin University of Medicine and Science (RFUMS) or the Cystic Fibrosis Foundation and thawed at passage (P) 1 or 2 for analysis. For functional studies, cells were plated on 24-transwell filter plates (0.4 μM pore size, polyester, Corning, catalog #CLS3397). FRT cells were grown in a liquid/liquid interface for 1 week. Primary cells were differentiated in an air/liquid interface for 5 weeks.

ASO treatment

ASOs were 25-mer phosphorodiamidate morpholino oligomers (PMOs) formulated in sterile water (Supplementary Table S3). A nontargeting PMO was used as a negative control, ASO-C (Gene Tools LLC).

T84 cells were transfected with ASOs in DMEM/F12 media supplemented with 5% FBS and 1% PenStrep using Endo-Porter (Gene Tools, 6 μl/ml) for 48 h. Primary hBE cells were transfected using Endo-Porter after differentiation on filter plates as previously described (13) using a 1-h hypo-osmotic shock. The cells were maintained in Dulbecco's phosphate-buffered saline with ASO and Endo-Porter for 4 days until functional analysis.

RNA isolation, RT-PCR and real-time qPCR

RNA was extracted from cells using TRIzol (Thermo Fisher Scientific). Reverse transcription was performed on total RNA

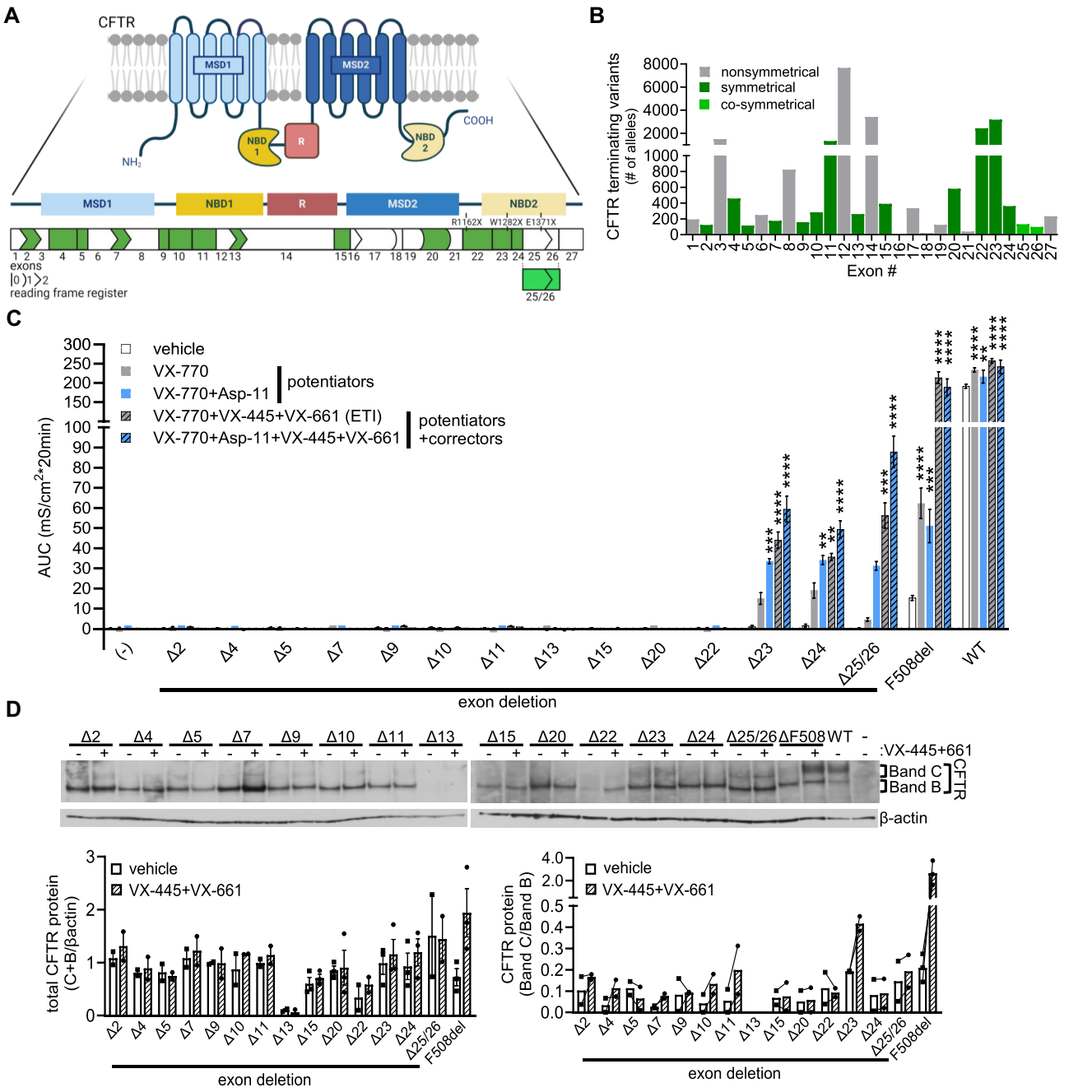

Figure 1. Systematic functional analysis of CFTR isoforms lacking symmetrical exons. (**A**) Schematic of CFTR protein in the cell membrane with protein domains aligned to CFTR exons. Symmetrical exons are shaded green. Co-symmetrical exons 25 and 26 are shaded a light green and depicted below the gene image. (**B**) Quantification of PTCs found in each CFTR exon from pwCF listed in the CFTR2 database (data acquired on 25 September 2024). Green shaded bars represent variants in symmetrical exons. (**C**) Measurement of chloride conductance in cells expressing CFTR or isoforms lacking individual symmetrical exons. Average area under the curve (AUC) was quantified for the forskolin or forskolin + potentiator(s) test periods from conductance traces (Supplementary Figure S1) measured in Fischer rat thyroid (FRT) cells, which lack endogenous CFTR, transfected with empty vector (–), CFTR-F508del, CFTR-WT or each CFTR exon deletion construct. Cells were pretreated with vehicle (dimethyl sulfoxide, DMSO, white bars, first in series) or VX-445 + VX-661 (hashed bars, fourth and fifth in series) for 24 h. Potentiators were added after forskolin addition (VX-770, gray bars, second and fourth in series, or VX-770 + Asp-11, blue bars, third and fifth in series). Error bars are ±SEM (standard error of the mean); replicates are DMSO ($n = 4$), VX-770 ($n = 3$), VX-770 + Asp-11 ($n = 1$), VX-770 + VX-661 + VX-445 ($n = 3$), VX-770 + Asp-11 + VX-661 + VX-445 ($n = 1$) for all, except empty vector ($n = 13, 13, 7, 13, 7$), Δ5 ($n = 5, 3, 2, 2, 2$), Δ13 ($n = 7, 6, 4, 6, 4$), Δ22 ($n = 3, 4, 3, 4, 3$), Δ23 ($n = 8, 9, 8, 9, 8$), Δ24 ($n = 5, 6, 5, 6, 5$), Δ25/26 [$n = 3$ (all)], ΔF508 ($n = 9, 7, 7, 7, 7$) and WT ($n = 16, 16, 7, 16, 7$), respectively; two-way ANOVA with Dunnett's multiple comparison test compared to DMSO treatment within groups; $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (**D**) Immunoblot analysis of CFTR protein, bands C and B, isolated from FRT cells stably transfected with empty vector, CFTR-F508del, CFTR-WT or each CFTR exon deletion construct. Cells were treated with DMSO or VX-445 + VX-661. β-Actin was used as a loading control. (Bottom, left) Total CFTR protein (band C + band B/β-actin) was quantified. (Bottom, right) The ratio of CFTR (band C/band B) isoforms was quantified. Replicates are $n = 2$ and experimental groups are connected by a line.

using the GoScript Reverse Transcription System with an oligo-dT primer (Promega). Splicing was analyzed by radiolabeled polymerase chain reaction (PCR) of resulting cDNA using GoTaq Green (Promega) supplemented with α-^{32}P-deoxycytidine triphosphate (dCTP). Primers for amplification are reported in Supplementary Table S4. Reaction products were run on 6% nondenaturing polyacrylamide gels and quantified using a Typhoon FLA 7000 phosphorimager (GE Healthcare) and ImageJ software.

Real-time quantitative PCR (qPCR) was performed with PrimeTime Gene Expression Master Mix and PrimeTime qPCR probe assay kits for *CFTR* transcripts normalized to *HPRT1* (IDT; Supplementary Table S4). All reactions were analyzed in triplicate. Real-time PCR was performed on an Applied Biosystems (ABI) ViiA 7 Real-Time PCR System and analyzed by the ΔΔCT method.

Protein isolation and immunoblot analysis

Cell lysates were prepared using NP-40 lysis buffer (1% Igepal, 150 mM NaCl, 50 mM Tris–HCl, pH 7.6) supplemented with 1× protease inhibitor cocktail (Sigma–Aldrich, cat #11836170001). Protein concentration was measured using a Coomassie (Bradford) protein assay (Thermo Fisher, cat #23200). Cell lysates were prepared with 4× NuPage LDS sample buffer (Invitrogen) for a final concentration of 1–2 mg/ml, incubated at 37°C for 5 min and loaded into a 4–12% Bis-Tris gel (Criterion) for sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation in 1× MOPS buffer. Samples were transferred in 1× CAPS buffer to polyvinylidene difluoride membranes. Protein was detected using primary anti-CFTR antibodies 570 or 596 (Riordan Lab UNC, Cystic Fibrosis Foundation, diluted 1:1000) and anti-β-actin (C4, Santa Cruz Biotechnology, diluted 1:2000). Secondary anti-mouse poly-HRP was added for chemiluminescent detection with HRP substrate (CFTR:Femto, β-actin:Classico; Immobulin, Sigma). Signal was quantified with ImageJ software.

Automated conductance/equivalent current assay

FRT cell lines and primary hBE cells were treated with VX-445 + VX-661 (1 μM + 3 μM for hBE cells or 3 μM + 3.5 μM for FRT cells; Selleckchem) or vehicle (0.1% or 0.2% DMSO) at 37°C for 24 h. Cells were switched from culture media to HEPES-buffered (pH 7.4) F12 Coon's modification running media (Sigma, F6636) apically and basolaterally and allowed to equilibrate for 1 h at 37°C without CO_2. For functional measurements, the transepithelial voltage (V_t) and resistance (R_t) measurements were recorded at 37°C with a 24-channel TECC robotic system (EP Design, Belgium) as previously described (13,17). Benzamil (6 μM), forskolin (10 μM), VX-770 (1 μM) with or without ASP-11 (20 μM), and Inh-172 (20 μM) or bumetanide (20 μM) additions were added sequentially. Conductance (G_t) was calculated by the reciprocal of the recorded R_t ($G_t = 1/R_t$) and plotted as conductance traces (Supplementary Figure S1). Equivalent currents from hBE donors were assessed similarly and calculated using Ohm's law ($I_{eq} = V_t/R_t$). AUC measurements of forskolin, forskolin + VX-770 or forskolin + VX-770 + ASP-11 were done using a one-third trapezoidal rule for each test period using Microsoft Excel. Independent experiments (n) included at least two technical replicates (wells) that were averaged and used as one biological replicate.

Immunofluorescence imaging

FRT cell lines grown on high-precision glass 12 mm circle coverslips (No. 1.5H) (Paul Marlenfeld GmbH) were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice. Subsequently, the cells were incubated in 3% bovine serum albumin for 20 min. Cells were incubated with the monoclonal anti-CFTR antibody (570, Riordan Lab UNC, 1:500) and rabbit polyclonal anti-Claudin antibody (1:100, Zymed) for 1 h. Cells were then incubated with goat anti-mouse secondary antibody conjugated with Alexa 488 and goat anti-rabbit secondary antibody conjugated with Alexa 594 (Invitrogen, 1:1000) for 1 h and mounted using ProLong Gold antifade mounting reagent with DAPI (Life Technologies). Cells were observed on a Zeiss Elyra structured illumination microscope using a 63×/1.40 NA objective using z-90 nm sectioning. In the axial direction, channel alignment was performed using 100 nm tetraspeck beads. Final images were prepared using Zeiss Zen software.

Statistics

Statistical analyses were performed using GraphPad Prism 10.2.3 or Microsoft Excel. Specific statistical tests used in each experiment are reported in the figure legends.

Results

Systematic deletion of *CFTR* symmetrical exons reveals functional protein isoforms

CFTR has 13 symmetrical exons that can be removed without disrupting the protein open reading frame. Additionally, the reading frame is maintained when exons 25 and 26 are eliminated together (Figure 1A and Supplementary Figure S2) (18). We and others have previously shown that ASO-induced skipping of exon 23 results in a partially functional CFTR protein when co-treated with established HEMTs (13–15). To identify additional pathogenic variants that could potentially be treated using this therapeutic approach, we analyzed CFTR protein lacking each symmetrical exon. For this, we created a series of *CFTR* plasmids designed to express high levels of CFTR with individual deletions of each of the 13 exons and both exons 25 and 26 together (CFTR-Δex) and analyzed *CFTR* expression and function after stable transfection into FRT cells, which do not express endogenous *CFTR*. CFTR-Δex isoform expression was confirmed by reverse transcription PCR (RT-PCR) and immunoblot analysis (Figure 1D and Supplementary Figure S3A).

FRT CFTR-Δex cell lines were grown to confluence on Transwell filter plates and cAMP-activated conductance across the plasma membrane was measured following forskolin stimulation to assess isoform activity. We also screened for responsiveness to HEMTs including ETI and the novel potentiator Asp-11, which has been shown to work in synergy with VX-770 to further increase the function of some CFTR protein isoforms, especially those that disrupt the second nucleic binding domain (NBD2) of CFTR (19,20). None of the expressed CFTR isoforms had measurable conductance in the FRT cells in the absence of HEMTs (white bars, Figure 1C and Supplementary Figure S1). Treatment with VX-770 alone (gray bars, Figure 1C) did not result in measurable conductance; however, co-potentiation with Asp-11 in

combination with VX-770 (blue bars, Figure 1C) resulted in significant forskolin-induced conductance from CFTR-Δ23 and CFTR-Δ24 compared to vehicle-treated cells. Cells expressing CFTR-Δ23, CFTR-Δ24 and CFTR-Δ25/26 had significant conductance following ETI treatment (gray hashed bars, Figure 1C) and all three deletion isoforms responded most robustly when treated with a combination of Asp-11 and ETI (blue hashed bars, Figure 1C).

Immunoblot analysis of the protein isoforms with and without corrector treatment was performed to measure CFTR protein expression, as assessed by the levels of fully glycosylated CFTR band C and core glycosylated band B (Figure 1D). All cells produced measurable band C and band B CFTR except for CFTR-Δ13. With the exception of Δ13, differences in conductance activity likely were not due to differing expression, as there was not a strong correlation between protein expression and conductance among the isoforms (Supplementary Figure S3B). Analysis of protein processing showed an increase in band C protein after ETI treatment in comparison to band B for all isoforms except CFTR-Δ5, CFTR-Δ9, CFTR-Δ15, CFTR-Δ20 and CFTR-Δ22, indicative of recovery of proper CFTR folding and trafficking (Figure 1D). These results provide evidence that this activity was specific to modulator effects on the isoforms. Additionally, immunofluorescence analysis of cells expressing CFTR-Δex23, 24 and 25/26 treated with ETI suggested some appropriate localization of the expressed proteins to the cell membrane (Supplementary Figure S4). Taken together, these findings indicate that CFTR isoforms lacking exons 23, 24, or 25 and 26 together can transport chloride across the membrane when treated with HEMTs, making them candidates for combined HEMT and ASO-mediated exon skipping therapies.

Splice-switching ASOs induce skipping of CFTR symmetrical exons

We next tested splice-switching ASOs that target each symmetrical exon for skipping. A series of PMO ASOs for each exon were designed to base pair with the canonical acceptor and donor splice sites or putative exonic splicing enhancer sequences and further selected for their predicted binding affinity (Figure 2A). Additionally, because a common variant in exon 25 (E1371X) is found near the donor splice site, we identified an ASO sequence that aligned along the variant site (ASO-25EX) to assess the potential for allele specificity. ASOs were tested for their ability to induce exon skipping of endogenous *CFTR-WT* pre-mRNA by transfection into T84 cells. RT-PCR analysis of RNA isolated after 48 h of incubation identified at least one ASO that was effective at skipping each target exon, except exon 26 where combination with exon 25 skipping ASOs was necessary (Figure 2B and Supplementary Figure S5A and B).

Functional screen of the CFTR-Δex clones indicated that only isoforms lacking exon 23, 24 or 25/26 are responsive to HEMT; thus, we focused on ASOs targeting these exons. An ASO that base pairs to the 5′ splice site region of exon 23 (ASO-23A) induces skipping of exon 23, but also causes splicing to a cryptic splice site that results in an out-of-frame CFTR mRNA. Additional treatment with an ASO that base pairs at the cryptic splice site (ASO-23B) eliminates cryptic splicing and greatly enhances skipping of exon 23 in a dose-dependent manner (Figure 2C) (13). Likewise, a robust dose-dependent increase in exon 24 was observed following treatment with a single ASO, ASO-24-3 (Figure 2D). Treatment with ASO-25-2 or 25EX, alone or in combination with ASO-26-4 (ASO-25EX + 26), resulted in the skipping of exon 25 or 26 individually or both exons 25 and 26 together (Δ25/26) (Figure 2E and Supplementary Figure S5B). Though dual skipping of exons 25 and 26 was not induced at a high level, higher dosing of ASO-25EX + 26 provided some improvement (Figure 2E). Because elimination of exon 25 alone results in a frameshift that creates a PTC in the penultimate exon 26, which may escape NMD because it is within 50 nucleotides of the last exon–exon junction, ASO treatment could provide sufficient splice modulation to increase CFTR mRNA and protein for further rescue with HEMTs (21).

Functional CFTR isoforms generated by ASO-induced symmetrical exon skipping in cells derived from CF patient airways

We recently reported that treatment with ASOs that induce exon 23 skipping can rescue CFTR function in cells homozygous for *CFTR-W1282X* (13). This rescue required treatment with ETI. Here, we tested whether compound heterozygous cells expressing only a single copy of *CFTR-W1282X* along with another variant that is associated with minimally functional CFTR could produce sufficient CFTR-Δex23 to provide a functional benefit in genotypes that are not currently approved for HEMTs. We also tested co-potentiation with Asp-11, to assess whether greater functional rescue could be achieved by modulator optimization. CFTR function was assessed via equivalent current analysis in hBE cells isolated from pwCF.

Cells from donors expressing two copies of *CFTR-W1282X* or with one copy and another nonsense variant, *CFTR-R553X* or *CFTR-R1162X*, were treated with vehicle, ASO-C, or ASO-23AB with or without Asp-11 and ETI. In all donors, treatment with ASO-23AB resulted in a significant increase in chloride currents over the control ASO when treated with ETI, and co-treatment with Asp-11 resulted in an even greater ASO-induced rescue of CFTR function (Figure 3A). In donor cells with *CFTR-W1282X* and *CFTR-F508del*, ASO-23AB treatment had little effect on the rescue already achieved by ETI alone, similar to previous results (13). Interestingly in these cells, ASO-23AB and co-potentiator treatment with VX-770 and Asp-11 together resulted in significant rescue of CFTR function, but Asp-11 addition with ETI lowered functional recovery (Figure 3A and Supplementary Figure S6A). This effect on ETI-dependent function was also observed in cells homozygous for *CFTR-F508del*, where ASO-23AB treatment had no effect on modulator rescue (Supplementary Figure S6A). Co-potentiation alone provided a small but statistically significant rescue in cells from the $CFTR^{W1282X/R553X}$ donor (Figure 3A). These results suggest that HEMT optimization could both provide significant rescue for minimal function variants and further improve the ASO-induced CFTR-Δex23 isoform function. The results also caution that effects on the second CFTR allele should be considered, as differential modulator effects may occur.

We have reported that ASO-induced exon skipping could have partial allele specificity for *CFTR-W1282X* due to the elimination of splicing factor binding sites (13). Similarly here, ASO treatment induced ∼80% exon 23 skipping in cells homozygous for *CFTR-W1282X*, compared to compound

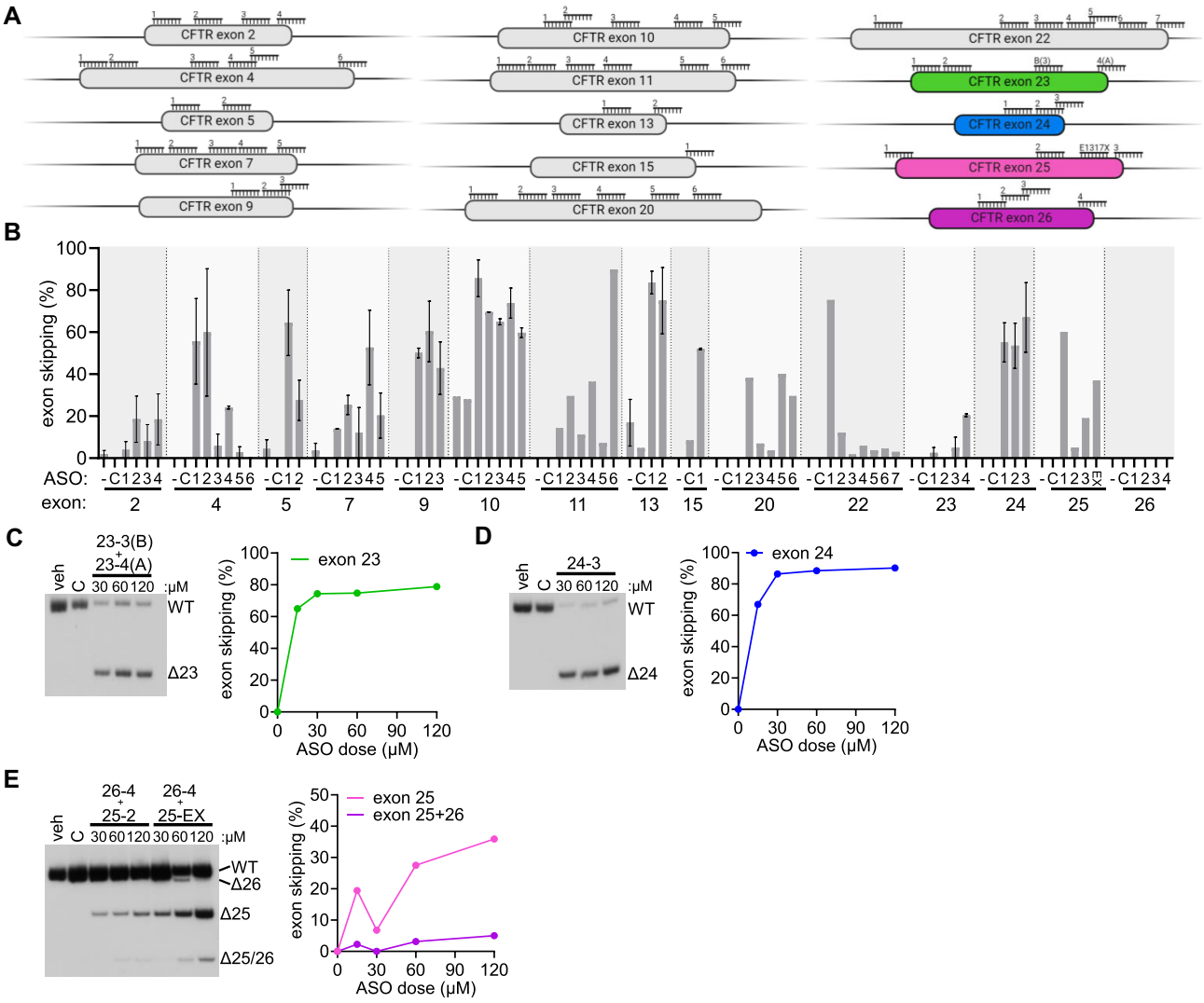

Figure 2. ASOs induce skipping of symmetrical exons. (**A**) Schematic of ASOs aligned to each *CFTR* exon targeted for skipping. (**B**) RT-PCR quantification of exon skipping [Δ/(full-length + cryptic + Δ)] × 100 for each ASO (15 μM). (**C–E**) (Left) RT-PCR analysis of RNA isolated from T84 cells transfected with vehicle (veh), control ASO (C, 120 μM), or ASO-23A + ASO-23B (**C**), ASO-24-3 (**D**) or ASO-26-4 + ASO-25-2 or ASO-25EX (**E**) at indicated doses. (Right) The percent of transcripts with exon skipping was plotted in relation to ASO dose. Primer pairs within exons flanking each exon targeted for skipping were used for analysis (Supplementary Table S3).

heterozygous cells where ASO treatment induced only ∼30–60% exon skipping (Figure 3B), and in cells homozygous for *CFTR-F508del*, ASO-23AB treatment induced <20% skipping of the wild-type exon 23 (Supplementary Figure S6B). Taken together, these results suggest that exon 23 containing the W1282X sequence is more susceptible to ASO-23AB-induced skipping than the wild-type sequence and that ASO-induced exon 23 skipping could be therapeutic for pwCF expressing at least one copy of *CFTR-W1282X*.

Skipping of exon 24 also results in a CFTR protein that is functional when treated with HEMTs (Figure 1). Thus, we evaluated the effect of ASO-induced exon 24 skipping on CFTR function in patient donor cells. Unfortunately, airway cells from a donor with a PTC in exon 24 were not available. Instead, we tested ASO-24-3 in hBE cells derived from a person with the *CFTR-N1303K* variant, a CF-associated missense variant in exon 24 that results in a protein with defective biogenesis and gating abnormalities. This variant has shown some response to HEMTs but is not currently FDA approved for HEMT treatment (19,22–23). Given the activity of CFTR-Δ24 expressed in FRT cells (Figure 1), we hypothesized that CFTR-Δ24 would have greater activity than *CFTR-N1303K* and, because of this enhanced activity, ASO-induced skipping of exon 24 would result in elevated currents in the donor cells. Compound heterozygous hBE cells expressing *CFTR-N1303K* and *CFTR-F508del* were transfected with ASO-24-3 and treated with ETI and Asp-11. Compared to the control ASO, there was little effect of ASO-24-3 treatment on ETI rescue of CFTR activity and Asp-11 had no additional effect (Figure 3C). This result suggests that ASO-induced skipping of exon 24 will not provide a therapeutic benefit over ETI to individuals with *CFTR-N1303K* and *CFTR-F508del*. However, ASO-24-3 and co-potentiator treatment did result in a small but statistically significant rescue compared to ASO with VX-770 alone, and co-potentiation alone had no effect (Figure 3C) suggesting that, despite inducing only modest (∼20%)

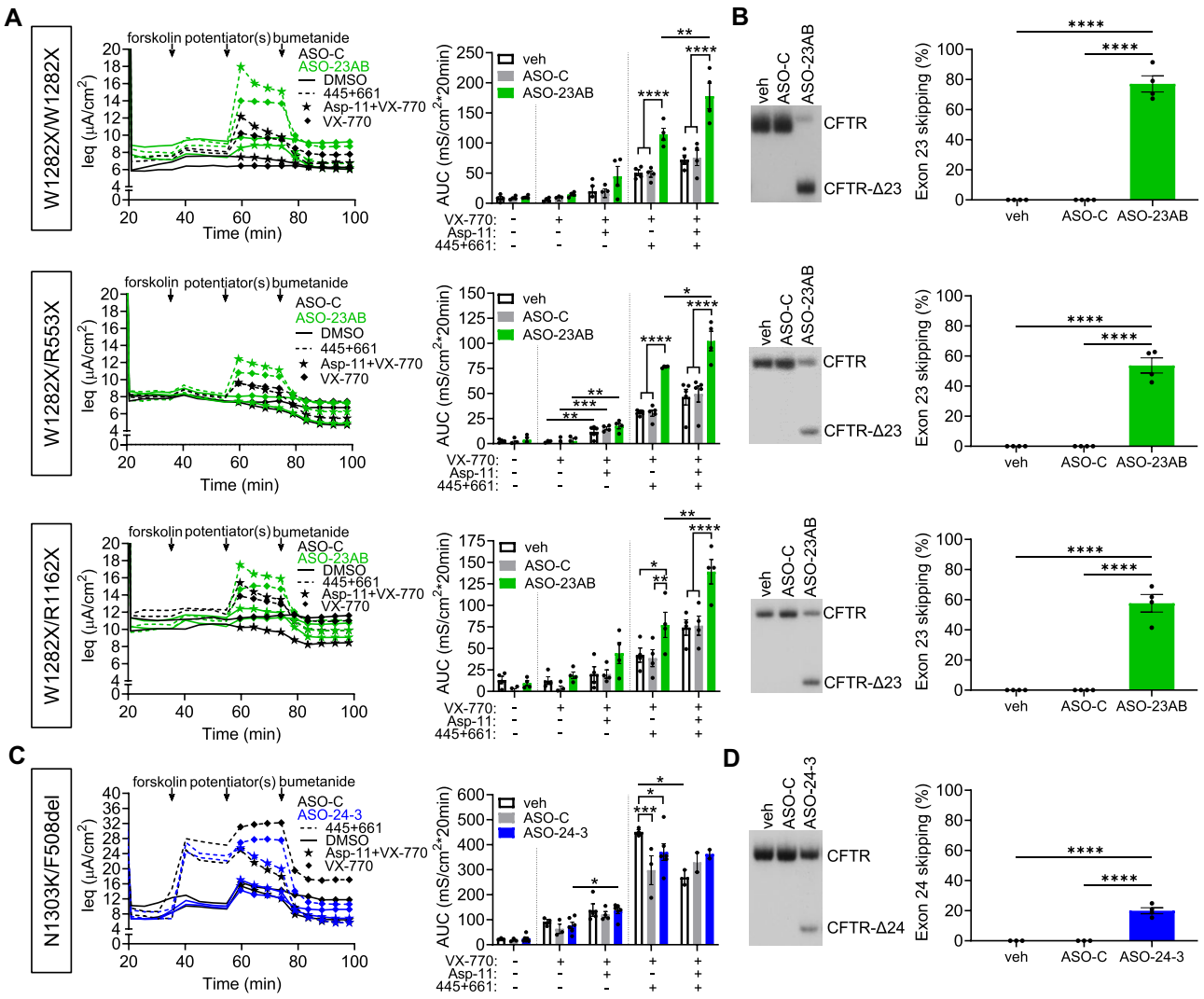

Figure 3. ASO treatment induces exon skipping and rescues CFTR function in primary hBE cells isolated from pwCF when used with HEMTs. (**A**) (Left) Equivalent current (I_{eq}) traces of primary hBE cells from CF donors expressing *CFTR-W1282X*. The genotype of each donor is indicated on the left. Cells were transfected with ASO-C (80 μM) or ASO-23AB (40 μM each) and treated with vehicle (DMSO, solid lines) or VX-445 + VX-661 (dashed lines) for 24 h before functional analysis. Forskolin, potentiator [VX-770 (diamond) or VX-770 + Asp-11 (star)] and bumetanide additions are indicated. (Right) Average AUC of the current traces was quantified for the forskolin or forskolin + potentiator(s) test periods for each treatment group. Error bars are ±SEM; $n = 4$, except $n = 5$ for controls in R553X heterozygous cells; two-way ANOVA with Tukey's multiple comparison test within groups to assess ASO effects or Šídák's multiple comparison test between groups to assess ASP-11 effects; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (**B**) (Left) RT-PCR analysis of exon 23 splicing in hBE cells indicated to the left. (Right) Quantification of the percent of mRNA with exon 23 skipping for each treated donor. Error bars are ±SEM; $n = 4$; one-way ANOVA with Tukey's multiple comparison test; $^{****}P < 0.0001$. (**C**) (Left) Equivalent current (I_{eq}) traces of primary hBE cells from a CF donor compound heterozygous for *CFTR-N1303K* and *CFTR-F508del*. Cells were transfected with ASO-C (40–160 μM) or ASO-24-3 (40–160 μM) and treated as in panel (A). (Right) Average AUC of the current trace was quantified for the forskolin or forskolin + potentiator(s) test periods for each treatment group. Error bars are ±SEM; $n = 3$, except $n = 6$ for ASO-24-3 and $n = 2$ for ETI + Asp-11; two-way ANOVA with Tukey's multiple comparison test within groups to assess ASO effects or Šídák's multiple comparison test between groups to assess ASP-11 effects; $^{*}P < 0.05$ and $^{***}P < 0.001$. (**D**) (Left) RT-PCR analysis of exon 24 splicing in hBE cells. (Right) Quantification of exon 24 skipping. Error bars are ±SEM; $n = 3$ (control) and $n = 4$ (ASO-24-3); one-way ANOVA with Tukey's multiple comparison test; $^{****}P < 0.0001$.

exon 24 skipping (Figure 3D), the ASO-induced CFTR-Δ24 isoform is more responsive to co-potentiation than CFTR-N1303K. Further optimization of ASO-induced skipping may result in greater activity, particularly in the context of other CF variants that are not responsive to ETI. Nonetheless, *CFTR-N1303K* is an imperfect model for exon skipping therapies as there is no evidence of mRNA degradation. Further testing in donor cells with an exon 24 PTC, as well as optimization of exon 24 skipping ASOs, is needed to assess the potential of this approach for CF caused by exon 24 PTCs.

A potential functional CFTR isoform generated by ASO-induced multi-exon skipping

Similar to CFTR-Δ23 and CFTR-Δ24, CFTR-Δ25/26 retains conductance when treated with ETI and is responsive to co-potentiation with Asp-11 (Figure 1C). ASO-25EX alone induces robust exon 25 skipping (Figure 2B). Though not an in-frame exon, skipping of exon 25 could be therapeutic if the particular downstream PTC in the penultimate exon does not activate NMD, which has been shown to be the case for several PTC variants at the 3′ distal region of CFTR,

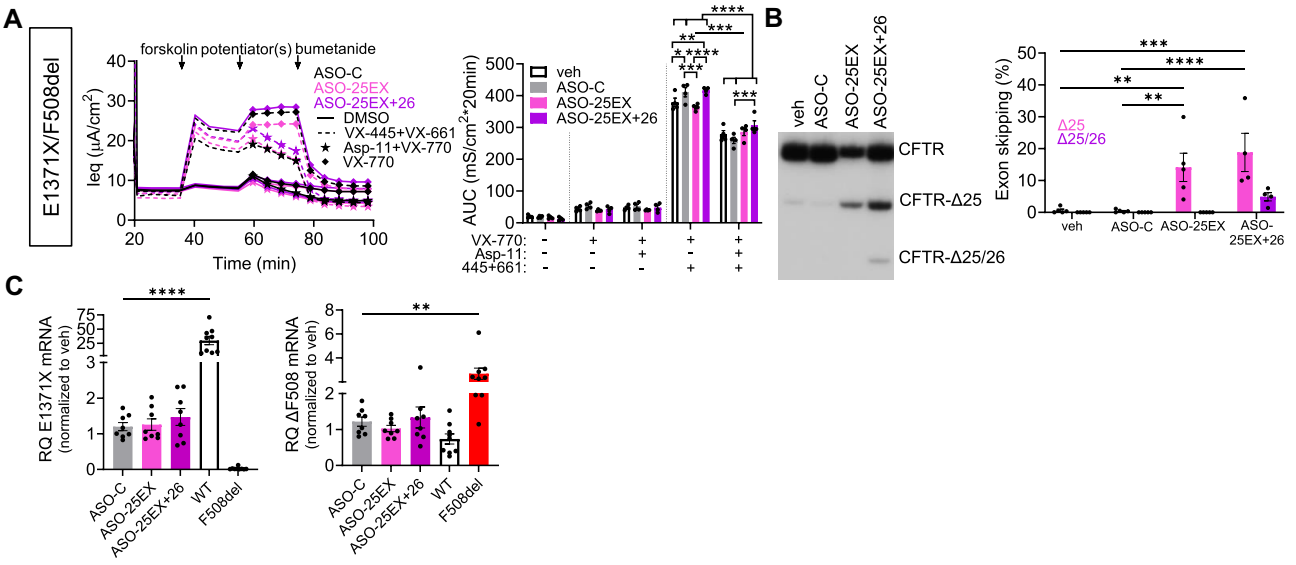

Figure 4. An ASO cocktail induces multi-exon skipping of CFTR exons 25 and 26 in primary hBE cells isolated from pwCF. (**A**) (Left) Equivalent current (I_{eq}) traces of primary hBE cells from a CF donor compound heterozygous for *CFTR-F508del* and *CFTR-E1371X*. Cells were transfected with ASO-C (40–240 μM), ASO-25 (40–120 μM) or ASO-25 + ASO-26 (40–120 μM each) and treated with vehicle (DMSO, solid lines) or VX-445 + VX-661 (dashed lines) for 24 h. Forskolin, potentiator [VX-770 (diamond) or VX-770 + ASP-11 (star)] and bumetanide additions are indicated. (Right) Average AUC of the current traces was quantified for the forskolin or forskolin + potentiator(s) test periods for each treatment group. Error bars are ±SEM; $n = 4$; two-way ANOVA with Tukey's multiple comparison test within groups to assess ASO effects or Šídák's multiple comparison test between groups to assess ASP-11 effects; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (**B**) (Left) RT-PCR analysis of exon 25 or exon 25/26 splicing in hBE cells from panel (A). (Right) Quantification of exon 25 or 25/26 skipping. Error bars are ±SEM; $n = 5$; two-way ANOVA with Tukey's multiple comparison test; $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (**C**) RT-qPCR analyses of total (left) *CFTR-E1371X* mRNA (exons 11–12) or (right) *CFTR-F508del* mRNA (exons 11–12) from treated hBE cells compared to RNA from a non-CF or homozygous CFTR-F508del donor. Error bars are ±SEM; $n = 8$, except $n = 9$ for WT and F508del; one-way ANOVA with Dunnet's multiple comparison test to ASO-C; $^{**}P < 0.01$ and $^{****}P < 0.0001$.

including exon 26 (24). The combination of ASO-26-4 with ASO-25EX (ASO-25EX + 26) results in modest skipping of both exons 25 and 26 (Figure 2E). To test these ASOs further, hBE cells that are compound heterozygous for *CFTR-E1371X*, a PTC in exon 25, and *CFTR-F508del* were treated with ASO-25EX alone or ASO-25EX + 26 and assessed for CFTR activity in the presence or absence of ETI and Asp-11. ASO treatment had little effect on activity without ETI treatment (Figure 4A). With ETI, ASO-25EX + 26 showed no additional rescue and ASO-25EX resulted in slightly lower function compared to the control ASO. As seen before in cells expressing *CFTR-F508del*, Asp-11 treatment reduced ETI response but did show a significant increase in function in combination with ASO-25EX + 26 treatment compared to control treated cells, which may suggest that Asp-11 has some specificity for CFTR-Δ25/26, which could lead to elevated function in genotypes heterozygous with variants other than *CFTR-F508del* (Figure 4A). ASO-25EX treatment resulted in ∼10–20% exon 25 skipping alone or in combination with ASO-26-4 and ASO-25EX + 26 induced a low level of skipping of both exons 25 and 26 (Figure 4B). Allele-specific qPCR analysis indicated no change in *CFTR-E1371X* mRNA expression with following ASO treatment (Figure 4C). The low level of ASO-induced skipping makes it difficult to assess whether ASO treatment may effectively stabilize mRNA produced from *CFTR-E1371X*, via the removal of the PTC in the ASO-induced CFTR25/26 isoform. However, the modest effect on CFTR function with ETI and Asp-11 treatment, despite low levels of skipping, suggests that further optimization of ASO-induced exon 25 and 26 skipping could be beneficial to people with PTCs in exon 25 or 26.

Inactive CFTR produced from ASO-induced exon 22 skipping confirms functional limits of exon-deleted isoforms

Systematic screening of protein isoforms engineered by ASO-induced exon skipping revealed that CFTR C-terminal isoforms lacking amino acids encoded by exons 23, 24, or 25 and 26, but not exon 22 or any other upstream exon, are functional as chloride channels when treated with ETI (Figure 1). To delineate more clearly the limits of an exon skipping approach to exons encoding the C-terminus of CFTR, we tested ASO-induced skipping of exon 22 using ASO-22-1 in hBE cells derived from two pwCF expressing *CFTR-R1162X* (compound heterozygous with *W1282X*) or *CFTR-c.3658delC* (p.K1177SfsX15) (compound heterozygous with *F508del*). In the *CFTR-c.3658delC* cells, ASO-22-1 treatment did not improve activity relative to that already achieved by ETI treatment alone and Asp-11 had no additional effects (Figure 5A). In the *CFTR-R1162X* cells, ETI and Asp-11 treatment significantly improved CFTR function in control treated cells, likely due to the effect on *CFTR-W1282X*, but treatment with ASO-22-1 eliminated this response (Figure 5A). Lack of functional recovery was not due to insufficient skipping of exon 22 as treatment with ASO-22-1 resulted in ∼30% exon 22 skipping in *CFTR-3659delC* cells and ∼70% in *CFTR-R1162X* cells (Figure 5B). These results, along with the conductance activity measured from CFTR-Δ22 in FRT cells, suggest that ASO-induced skipping of exon 22 does not result in an active CFTR isoform and that the protein domains encoded by exons 1–22 are likely essential for functional activity and rescue with HEMTs. These results define the boundaries of any potential exon skipping therapy for CF as sequences

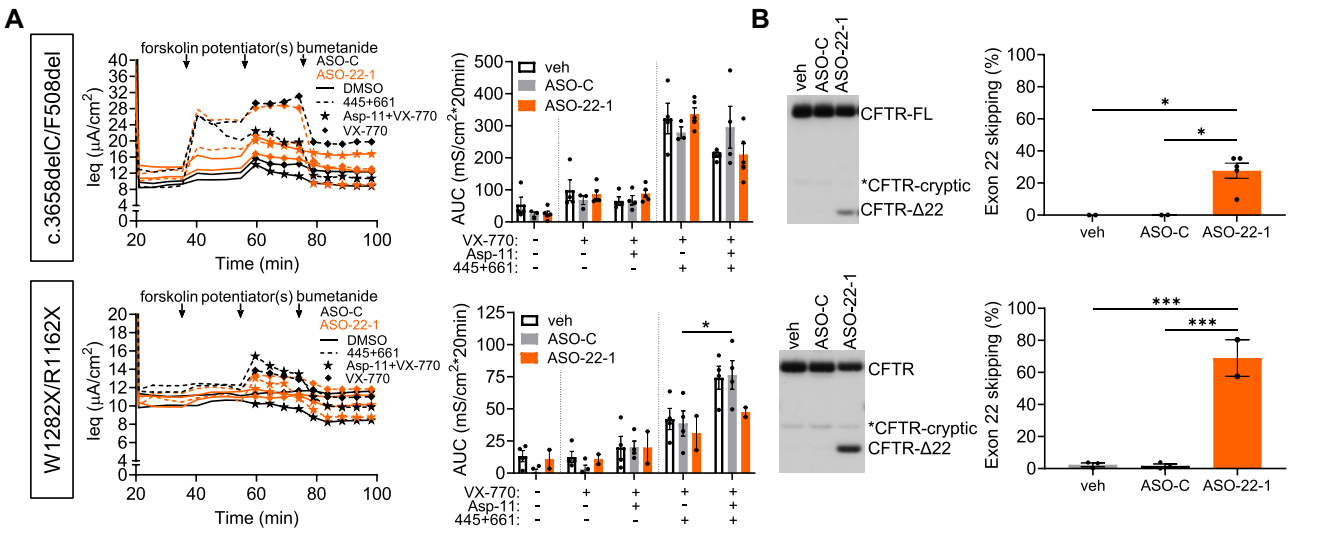

Figure 5. ASO treatment induces exon 22 skipping but does not rescue CFTR function in primary hBE cells isolated from a CF individual expressing PTCs. (**A**) (Left) Equivalent current (I_{eq}) traces of primary hBE cells from CF donors expressing at least one nonsense variant in exon 22. The genotype of each donor is indicated at the left. Cells were transfected with ASO-C (40–160 μM) or ASO-22-1 (40–160 μM) and treated with vehicle (DMSO, solid lines) or VX-445 + VX-661 (dashed lines) for 24 h. Forskolin, potentiator [VX-770 (diamond) or VX-770 + Asp-11 (star)] and bumetanide additions are indicated. (Right) Average AUC of the current traces was quantified for the forskolin or forskolin + potentiator(s) test periods for each treatment group. Error bars are ±SEM; $n = 4$, except $n = 5$ for c.3658delC ASO-22-1; two-way ANOVA with Tukey's multiple comparison test within groups to assess ASO effects or Šídák's multiple comparison test between groups to assess ASP-11 effects; $^{*}P < 0.05$. (**B**) (Left) RT-PCR analysis of exon 22 splicing from cells in panel (A). (Right) Quantification of exon 22 skipping. Error bars are ±SEM; $n = 5$ for c.3658delC cells and $n = 2$ for R1162X cells; one-way ANOVA with Tukey's multiple comparison test; $^{*}P < 0.05$ and $^{***}P < 0.001$.

downstream of exon 22 and suggest that truncation or deletions after CFTR exon 22 could provide a therapeutic benefit for all variants effecting expression in CFTR exons 23–27.

Discussion

Identifying targets of ASO-mediated exon skipping that can restore functional potential to an inactivated protein is relevant to therapeutic development for diseases, such as CF, which are often caused by pathogenic variants that result in PTCs. The current study systematically tests each skippable (i.e. symmetrical) exon of the CFTR gene for its functional dispensability and identifies four exons at the 3′ end of the transcript that are viable targets for restoring function by ASO-induced exon skipping. Further testing in primary cells from pwCF that have PTCs in these exons validated ASO-induced rescue of CFTR function in combination with HEMTs. In total, this approach could be potentially applied to treat an estimated 1.2% of pwCF (Supplementary Table S1). This general approach can be used to screen for and validate spliced isoforms from other disease-associated genes to identify potential ASO targeting strategies.

We and others have previously demonstrated the potential of ASO-induced exon skipping for treating CF caused by *CFTR-W1282X* (13–15). Additional testing of this approach in patient cells expressing genotypes not currently eligible for HEMTs provides strong evidence that combining ASO treatment with HEMTs could provide therapeutic benefit to these patients and others who have PTCs in exon 23 (Figure 3). Our testing of additional exon deletion isoforms in comparison to the CFTR-Δex23 protein product demonstrates that CFTR-Δex24 and CFTR-Δex25/26 have comparable levels of function and HEMT response (Figure 1), providing evidence that this strategy could be efficacious as a treatment for people with PTCs in C-terminal exons. Our subsequent screening identifies ASOs that induce skipping of these exons (Figure 2).

Further ASO design and testing in CF patient cells is needed to advance these approaches. However, screening is limited by a lack of patient cells with required genotypes needed for functional testing, limiting our analysis to cells with less-than-ideal CFTR genotypes for definitively evaluating therapeutic benefit. Additional work involving hBE cells, gene-edited immortalized cell lines or induced pluripotent stem cell models with rare PTC variants is needed to help demonstrate the functional utility of ASO-induced exon skipping for exons 24, 25 and 26. As such, no additional benefit over ETI was observed with ASO-mediated exon 24 skipping in patient cells expressing *CFTR-N1303K* with *CFTR-F508del* (Figure 3). However, because *CFTR-N1303K* has not been shown to compromise CFTR expression, it is a suboptimal target for this strategy. Furthermore, the tested donor cells' co-expression with *CFTR-F508del* makes it difficult to assess ASO + HEMT efficacy against the *CFTR-F508del* ETI response. Significant ASO-specific rescue was seen with co-potentiation treatment alone, suggesting that modulated CFTR-Δ24 has some benefit. However, further testing in cells from pwCF with PTCs in exon 24 and other undruggable genotypes is needed. Regarding exon 25/26 skipping, responses from patient cells expressing *CFTR-E1371X* revealed a significant response from ASO-25EX + 26 compared to ASO-C-treated cells with Asp-11 addition to ETI. However, again, ASO-specific functional rescue of *CFTR-E1371X* directly was difficult to assess with *CFTR-F508del*. These results suggest that ASO treatment could be promising for the treatment of CF caused by PTCs in these exons (Figure 4). Further optimization of ASOs is needed to identify those most effective at inducing exon skipping. Several machine learning tools are available and in development

to help predict effects on splicing that could assist with ASO target selection (25).

In addition to ASOs, a number of other therapeutic strategies are being explored to improve stability and translation of mRNA with PTCs, though currently none have been successfully implemented in patients. Several approaches to block NMD and/or increase translational readthrough of PTCs to recover full-length protein expression have been tested with limited success so far (26–30). Gene-specific, ASO-mediated suppression of NMD offers a more tailored approach that may overcome these issues but is limited because of the potential need for multiple ASOs to block all of the downstream exon junction complexes, which trigger NMD (31,32). Suppressor tRNA/ACE-tRNA as well as editing and CFTR replacement strategies are also being explored as a means to target specific PTCs in CF and other diseases. Delivery of the therapeutic molecules and off-target issues are a concern for all of these approaches, including ASOs (33–40). Off-target effects of ASOs must also be carefully evaluated during the drug development process but have been less problematic in the case of splice blocking approaches that require not only base pairing but also targeting of regulatory features for activity (41).

ASOs may offer advantages over other strategies as a validated therapeutic platform, proven largely safe in humans. In-frame exon skipping strategies utilizing ASOs have been successfully applied to address PTCs in DMD (2) and explored in other disease models (42–48). ASO delivery to the respiratory system, one of the primary targets for CF therapeutics, has been demonstrated clinically for asthma and other inflammatory lung conditions (49) and most recently as an antiviral approach for SARS-CoV-2 (50,51). Naked ASOs have been successfully delivered to the lung and enter multiple cell types, including CFTR expressing epithelial cells (52,53,55), though questions about whether the targeted cell types are relevant for CF treatment remain (54). ASOs have long-lasting effects in primary hBE cells isolated from pwCF (17), and could have similar durability *in vivo* considering that ASO activity for other indications can last weeks to months in terminally differentiated cells (55,56). Lung epithelia may turn over relatively slowly, with estimates of 6–18 months (57). Aerosolization of ASOs as a delivery modality has been demonstrated and targeted *CFTR* transcripts in both a CF-like lung disease model in mice and non-human primates as well as pwCF (58–61). Though significant studies are ongoing in the clinic, ASOs for CF have not yet advanced beyond clinical trials (62). Despite promising *in vitro* studies, cell models do not encompass all factors involved with drug delivery, especially complicated CF phenotypic barriers in the lung, and *in vivo* delivery remains a significant hurdle for ASO drug development for CF.

In addition to therapeutic discovery, exploring the effects of exon deletions can provide information on protein function that may inform and facilitate future drug discovery and development. Systematic screening of CFTR exon deletion isoforms reveals that the C-terminal exons are not essential for the fundamental function of CFTR when HEMTs are applied, a result confirmed by ASO testing in CF airway cells. Previous studies have shown that proteins with C-terminal truncations up to amino acid 1217 retain partial surface expression and function and are responsive to HEMTs (63–66). The domains encoded by exons 23, 24 and 25/26 encompass parts of NBD2 (NBD2 = 1203–1440, Δ23 = Δ1240–1291, Δ24 = Δ1292–1321 and Δ2526 = Δ1321–1413, Supplementary Figure S2), and the retained function of these exon deletions, but not exons upstream, is consistent with these reports (Figure 1C). CFTR-Δ23 and CFTR-Δ24 also retain critical sites in NBD2 for ATP binding and NBD dimerization (67), which are eliminated in CFTR-Δ25/26. Similar conductance levels between the isoforms (Figure 1C) suggest that, if this region is critical for dimerization, NBD dimerization is not critical for proper CFTR function with HEMT treatment. Furthermore, removal of PTCs in these exons via exon skipping allows for translation to the C-terminus, which includes amino acids important for stability, gating and localization (66,68–70). Overall, our functional data show that the deletion of exon 23, 24 or 25/26 results in CFTR protein responsive to HEMT rescue and suggest that ASO-mediated exon skipping of CFTR after exon 22 could be a therapeutic pathway. In addition, these results provide evidence that pwCF with splicing mutations that eliminate exons 23–26 are good candidates for ETI treatment if not currently approved for its use.

Co-potentiation with Asp-11, previously shown to increase the activity of other variants that are unresponsive to ETI, further enhanced CFTR isoform function (Figures 1 and 3–5) (19,20). This result suggests that new modulator discovery focused on enhancing function from CFTR deletion isoforms could provide additional benefit to ASO-induced rescue of expression, especially considering that the binding sites of the modulators are eliminated by some deletions. For example, the deletion of exon 20 eliminates a potential VX-770 binding pocket and, though not responsive to VX-770, might be responsive to alternative potentiators (71). Class I correctors bind in the first transmembrane domain and could be affected by several of the deletions, including exon 9 that has been previously explored for exon skipping and showed no response to the class 1 corrector VX-809 (72,73). Elimination of exon 9 and additional exons within the membrane spanning domains eliminated VX-661 and VX-445 rescue of protein processing (Figure 1D). The differing binding sites of VX-445 and VX-661 improve the efficacy of ETI and similar approaches to modulator design may be beneficial for each deletion construct (74).

Taken together, our results inform CF drug development and have important implications for treating other diseases caused by variants that introduce PTCs. Treating such variants by eliminating the exons they reside in and increasing productive protein expression could be a broadly applied therapeutic strategy. Our investigation into the effects of symmetrical exon removal on CFTR expression and function provides a roadmap for the systematic evaluation of symmetrical exon deletion, revealing additional ASO therapeutic targets for CF drug development and instructing on a therapeutic strategy for discovering treatment approaches for other diseases.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary data

Supplementary Data are available at NARMME Online.

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Conflict of interest statement

None declared.

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