# Precise targeting of miRNA sites restores CFTR activity in CF bronchial epithelial cells.

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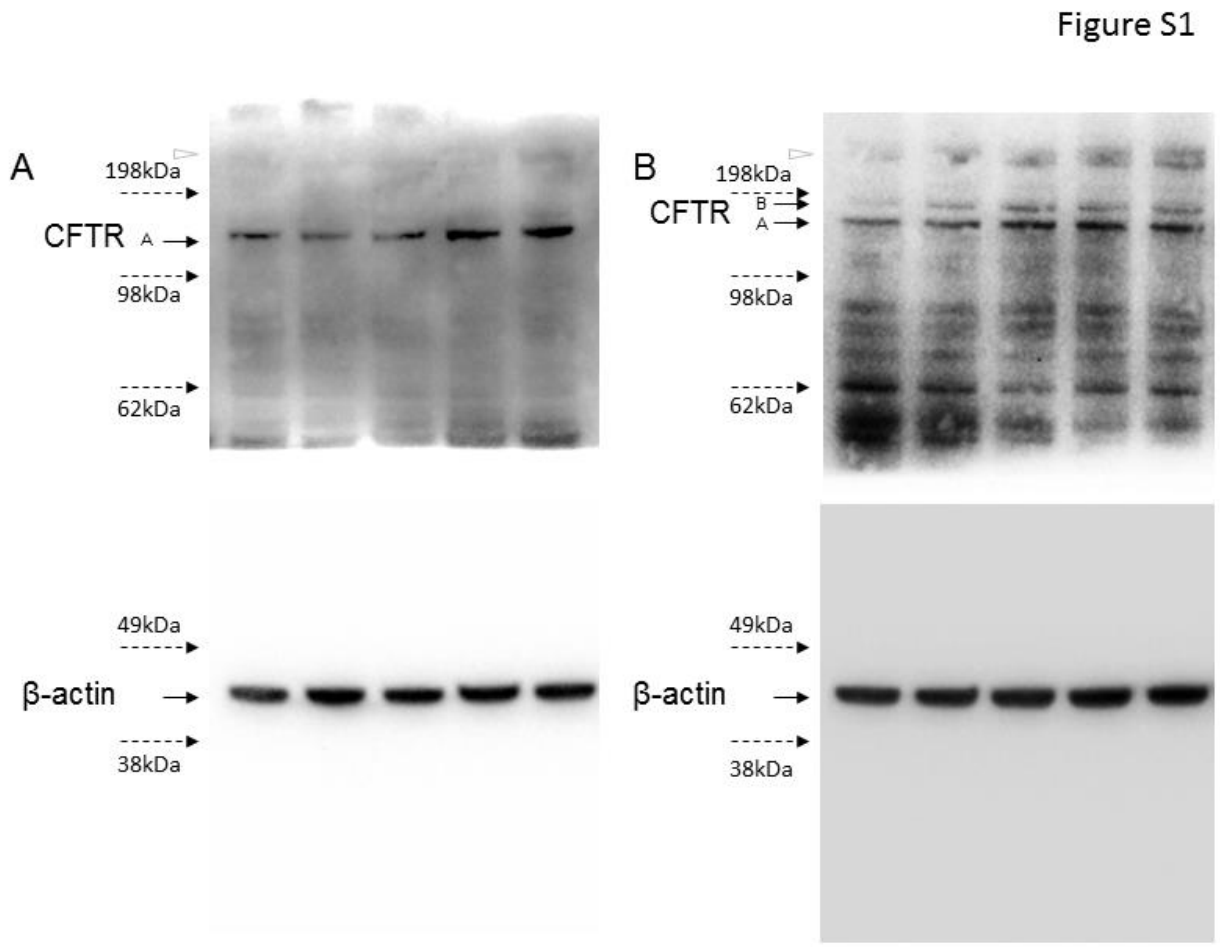
**Supplemental Figures.**

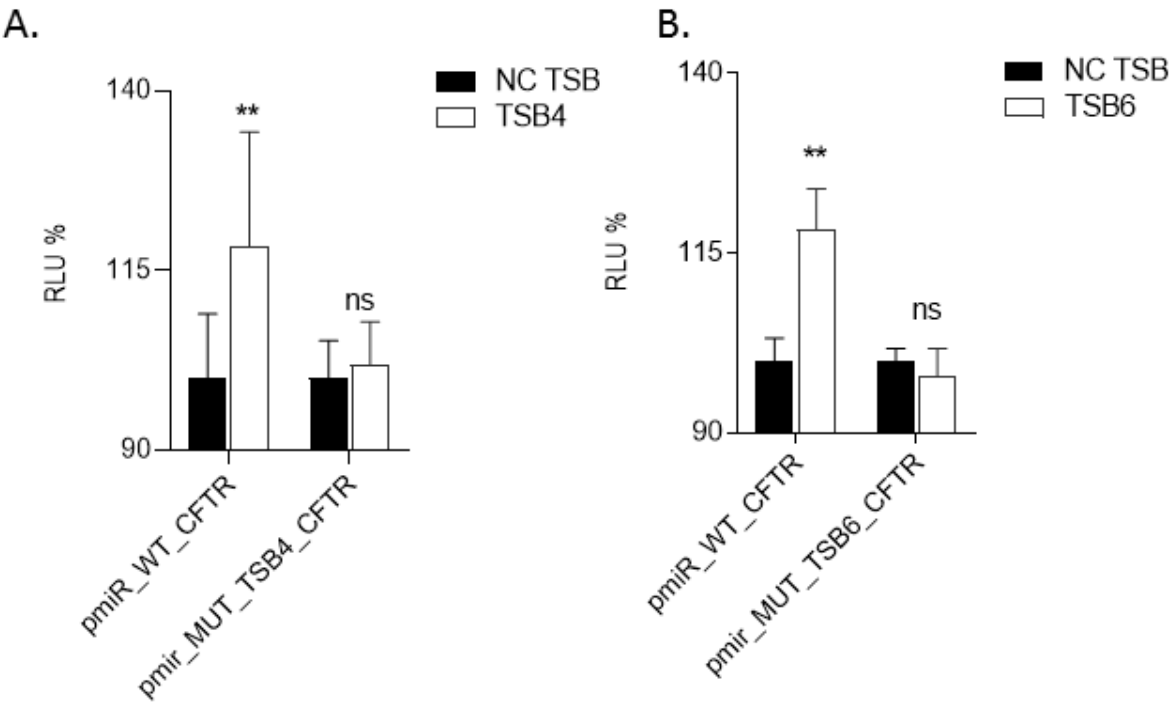
**Supplemental Figure 1. Full size blots of Figure 3.**



**Supplemental Figure 2. Effect of TSBs on mutant luciferase expression plasmids**.

Site-directed mutagenesis was applied to pmir_CFTR_wt vector in order to disrupt the TSB4 and TSB6 MREs. CFBE41o$^-$ cells were co-transfected with (A) wild type (WT) or MUT_TSB4 plasmid and TSB NC/TSB4 or (B) WT or MUT_TSB6 plasmid and TSB NC/TSB6 (100 ng/plasmid, 100 nM TSB, n=3, in triplicate). Luciferase assay was performed at 24 hours after transfection and no significant increase in luciferase activity by TSB4 or TSB6 was observed from plasmids carrying mutated MREs. Data are presented as mean ± SEM, **P≤0.01.

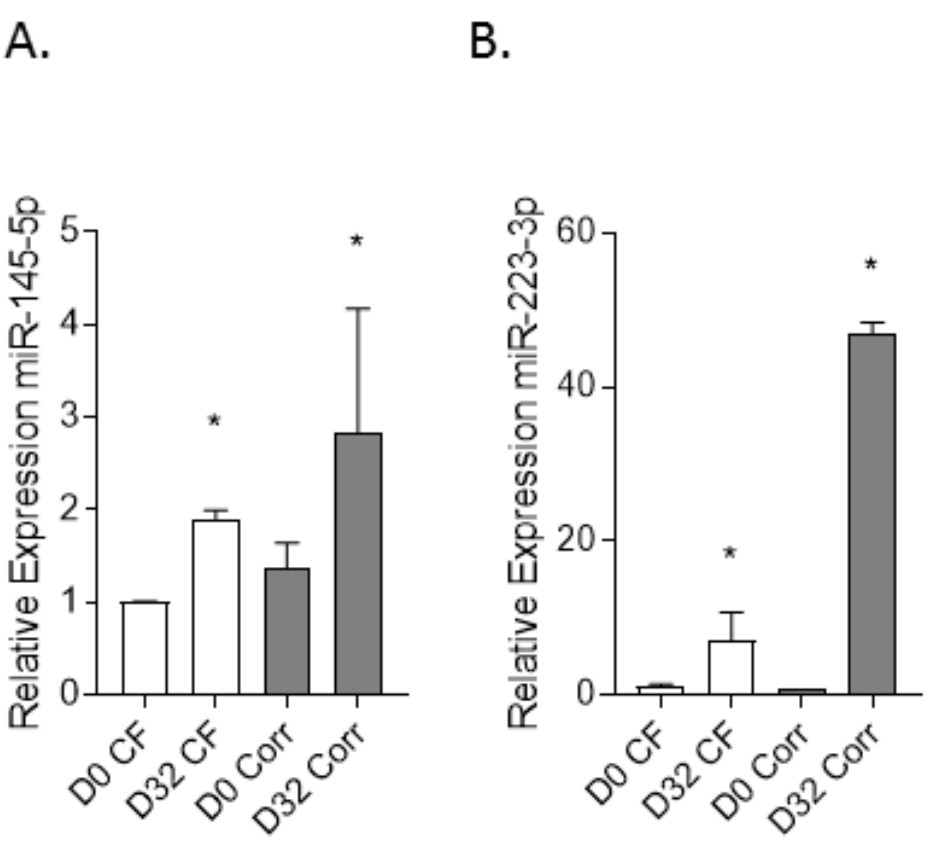
Figure S2



**Supplemental Figure 3. miR-145-5p and mir-223-3p expression is CF and gene-corrected iPSC-derived airway organoids.**

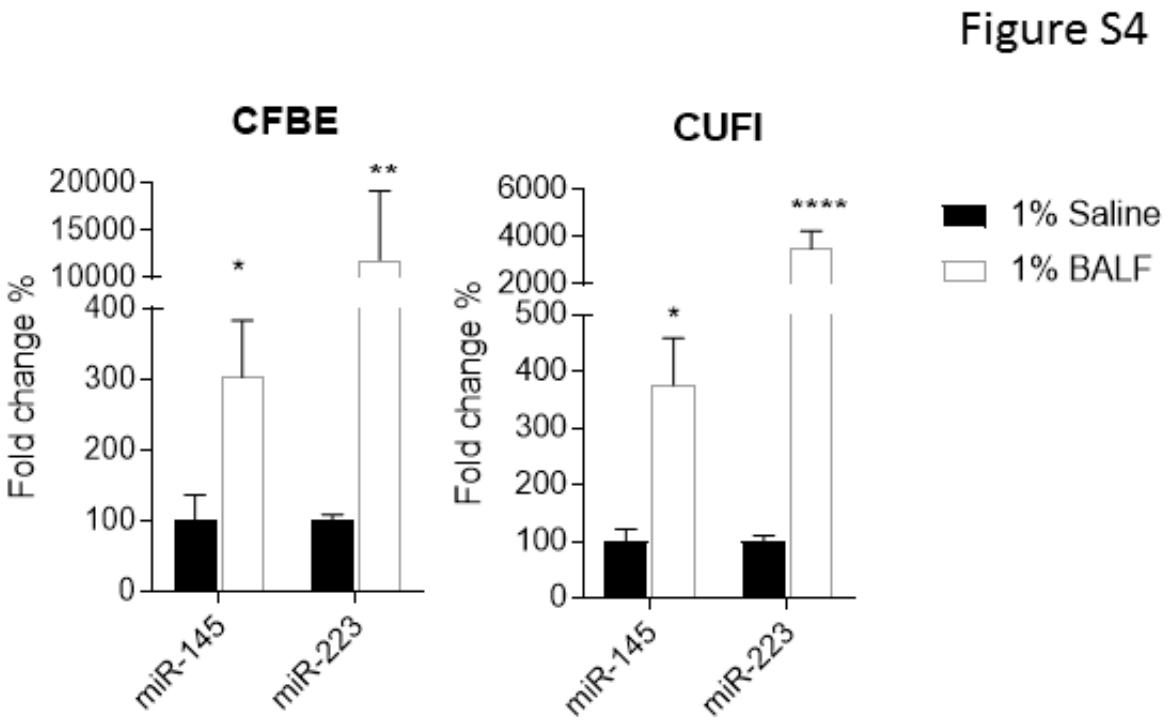
Relative expression levels of (A) miR-145-5p and (B) miR-223-3p in p.Phe508del CFTR homozygous CF (white) iPSCs and CFTR gene-corrected (Corr, grey)) iPSCs was determined by qRT-PCR using individual Taqman assays and normalised to U6snRNA at Day 0 and Day 32 following directed differentiation of iPSCs into lung organoids. *P=0.05 Mann-Whitney v. D0.

Figure S3



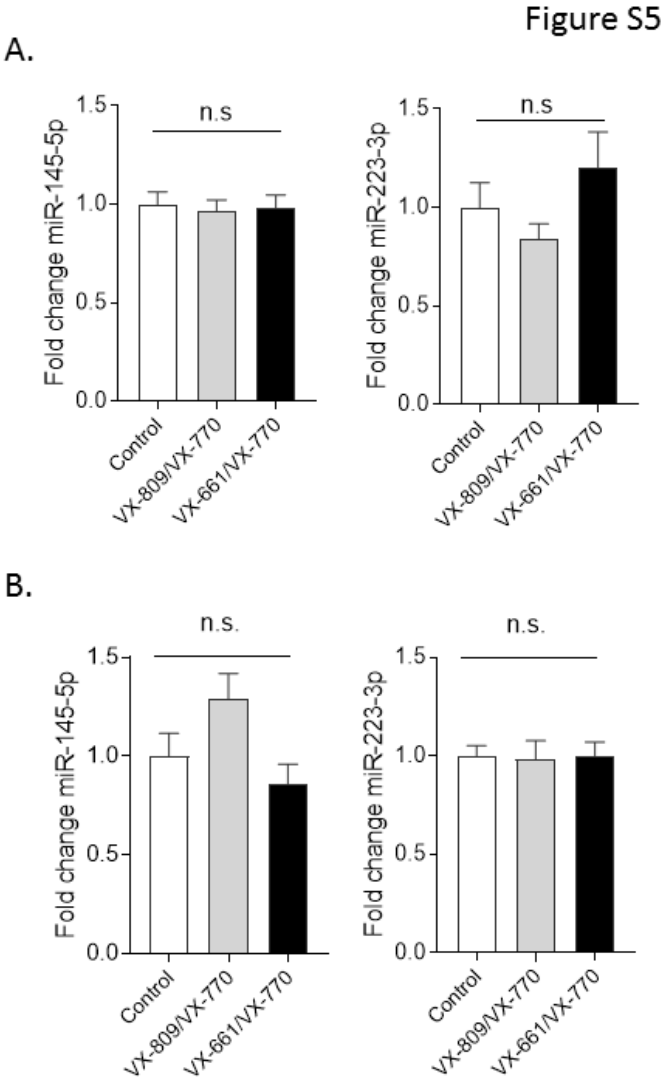
**Supplemental Figure 4. Effect of CF BALF on miR-145-5p and miR-223-3p in CF cell lines.**

CFBE41o- and 16HBE14o- cells were treated with saline or CF BALF as indicated for 20 h and relative expression levels, shown as fold change (%), of miR-145-5p and miR-223-3p were determined by qRT-PCR using individual TaqMan assays and normalised to U6snRNA (n=2 in triplicate for each cell line)**.** Data are presented as mean ± SEM, *P=0.02, **P=0.0003 and ****P<0·0001.



**Supplemental Figure 5. Effect of CFTR modulators on miR-145-5p and miR-223-3p in CF BEC lines.**

(A) CFBE41o- and (B) Cufi-1 cells were seeded overnight before stimulation with 5 uM VX-770/VX-809 (ivacaftor/lumacaftor) or 5 uM VX-770/VX-661 (ivacaftor/tezacaftor) for 48 hr. RNA was extracted, reverse transcribed to cDNA and used for quantification of miR-145-5p, miR-223p and U6 snRNA by qPCR. miRNA expression was normalized to U6 expression and fold change calculated using the $2^{(-\Delta\Delta Ct)}$ method. Results shown are the mean ± SEM of at least 3 independent experiments. Data were analysed for statistical significance by comparing the delta Ct values between groups using Student's t-test.



**Supplemental Tables**

**Supplemental Table 1. Data for TSB design by Exiqon**

| TSB | Position | Seed[a] | ddG | Target sequence on CFTR 3'UTR (seed underlined + 15 bp flanking) | miRNA name | miRNA sequence |
|---|---|---|---|---|---|---|
| 1 | 983 | 8:0:1 | -13.46 | ataggttgatggtggtatgttttcaggctagatgtatg | has-miR-494-3p | UGAAACAUACACGGGAAACCUC |
| 2 | 1474 | 8:0:0 | -10.45 | tgccttctcaactccaaactgactcttaagaagactgc | hsa-miR-223-3p | UGUCAGUUUGUCAAAUACCCCA |
| 3 | 1055 | 8:1:0 | -7.52 | gacacactgaagaagcaccaatcatgaattagttttat | hsa-miR-509-3p | UGAUUGGUACGUCUGUGGGUAG |
| 4 | 166 | 8:1:1 | -6.78 | taaggggaattgaggacactgatatgggtcttgataaa | hsa-miR-223-3p | UGUCAGUUUGUCAAAUACCCCA |
| 5 | 1490 | 8:1:1 | -6.63 | aactgactcttaagaagactgcattatatttattactg | hsa-miR-145-5p | GUCCAGUUUUCCCAGGAAUCCCU |
| 6 | 298 | 8:1:1 | -6.50 | cttgccatgtgctagtaattggaaaggcagctctaaat | hsa-miR-145-5p | GUCCAGUUUUCCCAGGAAUCCCU |
| 7 | 1269 | 8:0:1 | -6.48 | tgaaatattatgttaaaactgggacaggggagaaccta | hsa-miR-145-5p | GUCCAGUUUUCCCAGGAAUCCCU |
| 8 | 1223 | 8:1:1 | -6.03 | atttttatatttgaaatattgacttttatggcactag | hsa-miR-223-3p | UGUCAGUUUGUCAAAUACCCCA |
| 9 | 1506 | 8:1:0 | -2.91 | gactgcattatatttattactgtaagaaaatatcactt | hsa-miR-101-3p | UACAGUACUGUGAUAACUGAA |

[a]MiRNA recognition elements (MREs) for the lead microRNAs were predicted using the PITA algorithm (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) which identified multiple sites for each miRNA. For each site, the position within the 3'UTR, the type of seed and the ΔΔG values are provided. The "X:Y:Z" notation in the "seed" column represents the size of the seed (X), the number of mismatches (Y) and the number of G:U wobble pairs (Z). For example, 8:0:1 means that the seed is an 8-mer, has no mismatches but contains a single G:U wobble. ΔΔG = ΔG(duplex)– ΔG(open) where ΔG(duplex) is the hybridization energy of the miRNA to the binding site and ΔG(open) is the energy required to open the local RNA secondary structure around the binding site. The more negative the ΔΔG the stronger the expected binding of a miRNA to that site. TSBs that targeted the lead MRE sites with a ΔΔG cut-off of from -13.46 to -6.06 were designed. The miR-101-3p at position 1506 with a ΔΔG of only -2.61 was included since Viart *et al.* already demonstrated that blocking that site can enhance CFTR levels.

**Supplemental Table 2**. **Aerosol characteristics of PLGA-TSB4 and PLGA-TSB6.**

| Formulation | VMD (microns) | Fine Particle Fraction less than 5 microns (%) | Fine Particle Fraction less than 3.3 microns (%) | Aerosol Output Rate (ml/min) |
|---|---|---|---|---|
| 0.9 % saline (buffer) | 4.06 ± 0.01 | 63.12 ± 1.21 | 39.59 ± 0.07 | 0.42 ± 0.01 |
| PGLA + 4 (1mg/mL) | 4.41 ± 0.01 | 58.97 ± 2.87 | 34.35 ± 1.27 | 0.51 ± 0.01 |
| PGLA + 6 (1mg/mL) | 4.35 ± 0.03 | 59.23 ± 2.01 | 35.50 ± 1.81 | 0.48 ± 0.00 |
| | | | | |

0.9 % saline was used as a base formulation buffer. VMD = Volumetric Median Diameter of the aerosol droplets generated using the Aerogen Solo nebuliser (Aerogen Ltd, Galway, Ireland) by each of the formulations. Fine Particle Fraction (%) = the percentage of droplets measured with a VMD below 5 and 3 microns respectively. These are predictors of the respirable potential of an aerosol. Aerosol Output Rate = the liquid volume of therapeutic aerosolised per minute. This is used as an indicator of how long a potential aerosol therapy may take.

**Supplemental Table 3. Primers names and sequences employed in this study.**

| Name | Sequence |
|---|---|
| **Cloning primers** | |
| CFTR_clon_F | aacgagctcgctagcctcgagagagcagcataaatgttgac |
| CFTR_clon_R | caggtcgactctagactcgagttcacacaaatgtatggatt |
| **Mutagenesis primers** | |
| CFTR_MUT_TSB4 | ggtaaggggaattgaggacac**ACT**tatgggtcttgataaatggct |
| CFTR_MUT_TSB6 | tcctgaaaacccttgccatgtgctagtaat**CTT**aaaggcagctctaaatg |
| **ASO-PCR primers** | |
| ASO_wt_TSB4 | gggaattgaggacac**TGA** |
| ASO_mut_TSB4 | gggaattgaggacac**ACT** |
| ASO_wt_TSB6 | cttgccatgtgctagtaat**TGG** |
| ASO_mut_TSB6 | cttgccatgtgctagtaat**CTT** |
| **Sequencing primers** | |
| pmir_seq_F | gtggtgttgtgttcgtggac |
| pmir_seq_R | cagccaactcagcttccttt |

Mutagenesis primers: the mutant nucleotides are reported in capital letter, bold. ASO-PCR primers: Allele-Specific Oligonucleotide primers (wild type and mutant nucleotides in capital letter, bold) were designed to screen mutant from non-mutant colonies after mutagenesis. ASO-forward primers were used in combination with pmir_seq_R. Sequencing primers: pmir_seq_F and pmir_seq_R primers were designed on the plasmid sequence and they were employed for post-cloning screening and sequencing check.

**Supplemental Methods:**

**Cell culture and treatments**

Non-CF (16HBE14o$^-$) and CF (CFBE41o$^-$, genotype p.Phe508del/p.Phe508del) human bronchial epithelial cells were kindly donated by D. Gruenert (California Pacific Medical Centre Research Institute, San Francisco, CA, USA) and maintained in MEM+Glutamax (Gibco) with 10% fetal calf serum (FCS; Gibco) and 1% penicillin–streptomycin (Pen-Strep; Gibco). Non-CF (Nuli-1) and CF (Cufi-1, genotype p.Phe508del/p.Phe508del) human bronchial epithelial cells were received as a gift from Prof Zabner (University of Iowa, USA) and grown in Bronchial Epithelium Basal Medium (BEBM, Lonza) supplemented with BEGM SingleQuots Kit (Lonza, containing epidermal growth factor, hydrocortisone, bovine pituitary extract, transferrin, bovine insulin, triiodothyronine, epinephrine, retinoic acid), gentamicin (Gibco, 10 μg/ml), and amphotericin B (Gibco, 1.25 μg/ml). In some experiments, CFBE41o$^-$ and Cufi-1 cells were treated for 20 hours with 1% CF bronchoalveolar lavage fluid (BALF) or 48 hours with CFTR modulators, i.e. VX-770 ivacaftor (5 μM) in combination with VX-809 lumacaftor (5 μM) or VX-661 tezacaftor (5 μM), all dissolved in DMSO, alone or in combinations with TSBs, or with DMSO as negative control (0·1% in cell culture medium). Primary human CF bronchial epithelial cells (passage one, CF519 and CF609) were also purchased and grown in hAEC medium (Epithelix) for proof-of-principle experiments on the effect of TSB4 and TSB6 (naked or encapsulated in PLGA NPs) on CFTR protein expression. All the cell lines were maintained in a humidified incubator at 37°C in 5% $CO_2$.

Primary human bronchial epithelial cells were isolated from the proximal airways of six CF (all p.Phe508del/p.Phe508del, two males and four females, mean age 29.25±1.12 years) and six non-CF patients (five males and one female, mean age 41.60±8.93 years), each undergoing lung transplant for end stage lung diseases or from organ donor lungs not suitable for human use. Ethics committee approval (University of Pittsburgh IRB Exempt Protocol #13090311and #15090205) and informed consent were granted for all samples. Bronchial epithelial cells (passage one) were cultured on human placental collagen-coated Transwell (0.33 $cm^2$, 0.4-μm pore size) permeable supports at a density of $\sim 2 \times 10^5$ cells/$cm^2$ in bronchial epithelial growth medium at air-liquid interface for 4–5 weeks until they reached complete differentiation. RNA was then extracted with TriReagent (Sigma) according to the manufacturer's instructions and its purity and concentration were measured with Nanodrop 8000 (ThermoFisher Scientific).

Induced pluripotent stem cell (iPSC)-derived lung organoids were generated from a CFTR mutant iPSC line of genotype p.Phe508del/p.Phe508del**.** In brief, the CF iPSC line (clone RC2 202 generated from an individual donor with cystic fibrosis) [1] and its zinc-finger nuclease gene-edited subclones engineered to carry one corrected CFTR allele (p.Phe508del/wild type), were obtained from the iPSC Core of the CReM of Boston University and Boston Medical Center [2]. These syngeneic iPSC lines were differentiated towards lung epithelium using an optimized version of a published serum-free, co-culture free directed differentiation protocol. The major stages of the protocol include (i) definitive endoderm induction, (ii) generating anterior foregut endoderm through TGF-β and BMP4 inhibition, (iii) NKX2-1 induction with media supplemented with Chir, FGF10, KGF, BMP4 and Retinoic Acid. Employing a CD47hi/CD26lo sort strategy iPSC-derived lung progenitors were sorted to purity on day 15. These cells were re-plated in 12-well plates at a concentration of 1,000 cells per microliter in 50μL of growth-factor

reduced Matrigel in a "proximalizing" composed of FGF2, FGF10, dexamethasone, cAMP and IBMX. After 7-10 days each Matrigel droplet typically contains thousands of small well-defined organoids.

**miRNA qRT-PCR**

RNA from CF and non-CF cell lines (n=6 for each cell line) or adult primary cells (n=6 each) was purified by using Tri-Reagent (Sigma Aldrich) according to the manufacturer's instructions and its purity and concentration were measured using a Nanodrop 8000 (ThermoFisher Scientific). The TaqMan® MicroRNA Reverse Transcription Kit (ThermoFisher Scientific) was employed for reverse transcription using stem-loop specific miRNA primers starting from 100 ng of total RNA. miRNA expression was measured using a pre-designed TaqMan MicroRNA assay (ThermoFisher Scientific) on the LC480 LightCycler (Roche) according to manufacturer's instructions. miRNA relative to U6 snRNA was determined using the $2^{(-\Delta\Delta Ct)}$ method.

**miRNA Profiling**

*Patient cohort* - Six paediatric p.Phe508del *CFTR* homozygous CF patients and six paediatric non-CF patients were recruited. Both cohorts were sex-matched (all male) and age matched (mean age CF: 3±2.45 years, non-CF: 3.33±1·51 years). Bronchial brushing samples were taken from CF patients during routine disease-specific clinical bronchoscopy, while brushings from non-CF patients were taken during bronchoscopy for diagnostic purposes. Ethics committee approval (Crumlin Hospital) and informed consent were granted for all samples.

*Purification of bronchial epithelial cells from bronchial brushings* - The bronchial brushes containing sample were immediately transferred to ice-cold Collection medium (EMEM (Gibco, USA) completed with 10 U/ml penicillin-streptomycin, 2.5 µg/ml Amphotericin B, 5 µg/ml gentamycin (Gibco, *USA*) and 80 µg/ml tobramycin (Calbiotech, UK)). Samples were processed on the same day. While kept on ice, tissue and mucus were visibly dislodged from the brushes into the medium. With brushes held suspended in their respective tubes, the tubes were centrifuged at 240 g for 10 minutes at 4°C. Supernatant was subsequently removed and the pellets were resuspended in 3 ml ice-cold completed Bronchial Epithelial Growth Medium (BEGM; Lonza, Switzerland). Tissue was pelleted by centrifugation at 240 g for 5 minutes at 4°C and after removing the supernatant, the pellet was resuspended in 200 µl BEGM.

Blood cells were depleted by combining the EasySep Human CD45 and Human Glycophorin A immunomagnetic depletion kits (#18259 and #18352; Stemcell Technologies, Canada). To the suspension, 6 µl CD45 antibody and 15 µl GlyA antibody from these kits were added and thoroughly mixed by pipetting. The suspension was incubated on ice for 15 minutes after which 27 µl of magnetic particles were added and mixed. The mixture was incubated on the bench for 10 minutes after which it was made up to 2·5 ml with BEGM at room temperature. Magnetic particles were purified with the EasySep magnet for 10 minutes and the supernatant was decanted to a new tube. This was centrifuged at 240 g for 5 minutes at 4°C and the supernatant was removed. To the remaining pellet, 700 µl QIAzol Lysis Reagent (Qiagen, Germany) and after vortexing the lysate was stored at -80°C.

*Cytology* - Mixed 10 ml cell suspension from two bronchial brushes was split into two aliquots, which were centrifuged at 125 g for 5 minutes at 4°C. Supernatant was decanted and pellets were resuspended in 200 µl BEGM and transferred to round-bottom tubes on ice. RNA was isolated from one sample, while the other sample was simply made up to 2.5

ml with BEGM. 1 ml of unpurified sample and 2.5 ml of purified sample were subsequently transferred to slides by cytospin. Slides were fixed and stained with Hema 'Gurr' rapid staining set (VWR, USA) to aid in the differentiation between haematopoietic cells and epithelial cells. Slides were analysed by microscopy. Additional microscopic analysis was performed on unfixed, purified live samples stained with trypan blue.

*RNA isolation* - Isolation of total RNA, including miRNAs, from the purified BECs was performed with the miRNeasy Micro kit (Qiagen, Switzerland). Samples were eluted in 14 µl RNase-free water.

*High-throughput miRNA expression analysis* - BEC RNA samples were used for high-throughput miRNA expression analysis. RNA concentrations were measured using the Qubit 3.0 fluorimeter (ThermoFisher Scientific, USA) and the Qubit RNA HS Assay kit (ThermoFisher Scientific, USA). 754 miRNAs were reverse transcribed and pre-amplified as per manufacturer's protocol using the Megaplex™ Primer Pools, Human Pools Set v3.0 (ThermoFisher Scientific, USA) combined with the TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher Scientific, USA) and the TaqMan PreAmp Master Mix (ThermoFisher Scientific, USA) respectively.

High throughput quantitative real-time PCR (qPCR) was performed with the TaqMan OpenArray Human MicroRNA Panels (ThermoFisher Scientific, USA) as per manufacturer's protocol on the QuantStudio 12K Flex system (ThermoFisher Scientific, USA). GEO identifier: GSE128861.

*Openarray data and statistical analysis* - OpenArray profiling data were analysed using the ExpressionSuite Software (Life Technologies). Differential expression analysis was performed by applying a Student's *t*-test to the normalized Ct values between the two groups and the *p*-values were adjusted for multiple testing by controlling the false discovery rate (FDR). A miRNA was considered to be differentially expressed if the adjusted *p*-value was $\leq 0.05$.

**RNA sequencing**

*Cell culture* - Immortalized human bronchial epithelial cell models of CFBE41o- stably expressing wild type (WT)-CFTR or F508del-CFTR, respectively, were cultured in Minimum Essential Media (MEM) containing 10% Fetal bovine serum (FBS), 50µg/mL streptomycin, 50U/mL Penicillin, 2mM L-Glutamine, 0.55 µg/mL Puromycin (2·2 µg/mL Puromycin for CFBE41o- cells). Cells were seeded on 24 mm inserts (Corning) coated with 10% collagen (PureCol, Type I Bovine Collagen Solution, Advanced BioMatrix) at 1 $x10^6$ and cultured in air liquid interface (ALI) for 7-8 days.

*RNA Isolation* – WT-CFBE41o- and F508del-CFBE41o- cells cultured in ALI in six separate experiments from different cultures were collected in microcentrifuge tubes using phosphate buffer saline. Total RNA was isolated from cell lysate using the Quick-RNA Miniprep Kit (Zymo Research, R1054) according to the manufacturer's instructions, with additional on-column DNase treatment with the RNase-free DNase Set (Zymo Research) to remove contaminating genomic DNA for downstream applications.

*Small RNA library preparation, data analysis, and miRNA profiling* - The starting material, 100ng of total RNA was used with the QIAseqTM miRNA Library Kit (Qiagen, Maryland, USA) following manufacturer's instruction. The RNA quantity and quality was assessed by Nanodrop, Qubit 2.0 Fluorimeter and Agilent Bioanalyzer Tapestation 2200. In short, 3' and 5' adapters were ligated to total input RNA. Reverse transcription, followed by PCR, was used to create cDNA constructs. This process selectively enriches those fragments that have adapter molecules on both

ends. PCR was performed with primers that anneal to the ends of the adapters. Quality was examined using Agilent Bioanalyzer Tapestation 2200 and Qubit 2.0 Fluorimeter with miRNA library pre sequencing QC protocol mentioned in the QIAseqTM miRNA Library Kit. The cDNA libraries were pooled at a final concentration of 2.5pM. Cluster generation and 75 bp single read single-indexed sequencing was performed on Illumina NextSeq 500. miRNA sequencing data were analyzed using GeneGlobe Data Analysis bioinformatics pipeline from Qiagen. The raw QIAseq miRNA sequencing files were uploaded to GeneGlobe Data Analysis Center (Qiagen) for quality control, alignment to the human reference genome (GRCh38) and expression quantification. Briefly, 3'adapter and low quality bases were trimmed off from reads using cutadapt. Reads with less than 16bp insert sequences or with less than 10bp UMI sequences were discarded. The remaining reads were collapsed to UMI counts and aligned to miRBase (release v21) mature and hairpin databases sequentially using Bowtie v1·2. All reads assigned to a particular miRNA or piRNA ID were counted, and the associated UMIs were clustered to count unique molecules. The primary analysis output file was downloaded from GeneGlobe. EdgeR package was used for the differential analysis based on UMI counts for miRNA. miRNAs were filtered from the analysis if their count per million (as computed by edgeR's cpm function) were not greater than 2 in at least half of the samples. The trimmed mean of M-values normalization method implemented in edgeR was used for normalization. Volcano plot was generated by ggplot2 to visualize the differential expression of miRNAs in CFBE41o- cells and 16HBE14o- cells at baseline. Expression of miRNAs with binding sites in CFTR-3'UTR conserved or poorly conserved among mammals (TargetScanHuman Release 7.2) were shown in red and black, respectively, as opposed to non-conserved miRNAs (grey). $P < 0.05$ corresponding to $-\text{Log}10 > 1.3$ was considered significant. N=6/group from different cultures. GEO identifier: GSE128912.

**Plasmid construction and dual-luciferase assay**

The full length human *CFTR* 3'UTR luciferase plasmid was amplified using Q5 High-Fidelity DNA Polymerase (NEB) and inserted into *Xho*I-digested pmirGLO vector (Promega) using the CloneEZ Cloning Kit (Genscript). Plasmids were isolated from bacterial cultures with the Plasmid Midi Kit (Qiagen). In order to prove the specificity of TSB4 and TSB6 for *CFTR* mRNA sequence, mutagenesis reactions were performed to disrupt their binding sites within the 3'UTR region. Starting from pmir_CFTR_wt, QuikChange II Site-Directed Mutagenesis Kit (Agilent) was used to insert site-specific mutations whose presence was subsequently checked by screening with allele-specific oligonucleotide PCR (ASO-PCR) and sequencing. Two mutant plasmids were generated and they are now on referred as "pmir_CFTR_mut_TSB4" and "pmir_CFTR_mut_TSB6". The sequence of cloning, mutagenesis and sequencing primers is reported in Supplemental Table 3.

In luciferase assay experiments, CFBE41o$^-$ cells were seeded in a 96-wells plate at a final density of 10,000 cells/well and incubated for 24 hours. Cells were then co-transfected with 100 ng of pmir_CFTR_wt and 100 nM of each TSB. In some experiments, cells were transfected with 100 ng of pmir_CFTR_wt/mut_TSB4/mut_TSB6 and 100 nM of non-targeting (NC) TSB/TSB4/TSB6. Transfection mixes were prepared in Optimem reduced-serum media (Gibco) using Genejuice and Ribojuice (Novagen) as transfection reagents for plasmid DNA and TSBs, respectively. Luciferase activity was assessed at 24 hours after transfection using Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions. RLU (relative luciferase units) expressed as mean value of the firefly

luciferase/*Renilla* luciferase ratio of three independent experiments performed in triplicate were used for statistical analyses.

**CFTR gene and protein expression analysis**

For *CFTR* mRNA expression, CFBE41o- cells were seeded in a 24-wells plate at a final density of 80000 cells/well and after 24 hours they were transfected with 100 nM of NC TSB/TSB4/TSB6/TSB7/TSB8 in Optimem reduced-serum media and Ribojuice transfection reagent. At 48 hours post-transfection, total RNA was extracted using TriReagent, and equal quantities were reverse transcribed into cDNA using miScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. qRT-PCR was performed on the Roche LC480 LightCycler. Primers for *CFTR* (forward 5'-CAAGGAGGAACGCTCTATCG-3', reverse 5'-AGAACACGGCTTGACAGCTT-3') and *RPLP0* (forward 5- GGCAGCATCTACAACCCTGA-3', reverse 5'-AACATTGCGGACACCCTCC-3') were obtained from IDT (Leuven, Belgium). Expression of *CFTR* relative to *RPLP0* was determined using the $2^{(-\Delta\Delta Ct)}$ method.

For CFTR protein expression, CFBE41o$^{-}$, Cufi-1 or primary CF BECs (Epithelix) cells were seeded in a 6-wells plate at a final density of 250000 cells/well and after 24 hours they were transfected with 100 nM of NC TSB/TSB4/TSB6/TSB7/TSB8 in Optimem reduced-serum media and Ribojuice transfection reagent. In some experiments, primary bronchial epithelial cells were transfected with TSB4 or TSB6 encapsulated in PLGA NPs at a final concentration of 100 nM. At 72 hours, total protein was extracted RIPA buffer (supplemented with Halt Protease inhibitor Cocktail, Cat # 1862209, Thermo Scientific, and 0.5M EDTA solution, Cat # 1861274, Thermo Scientific) and quantified with BCA Protein Assay Kit (Thermo Scientific). Equal volumes of whole-cell lysates from were separated on 4-12% Bis-Tris acrylamide gels (Thermo Fisher Scientific), transferred to polyvinylidene difluoride membranes (Roche), and probed with mouse anti-CFTR (1:400, R&D Systems catalog no. MAB25031) or anti-actin antibodies (1:25000, Millipore catalog no. MAB1501). Anti-mouse IgG, HRP-linked antibody (1:2500, Cell Signaling, catalog no 7076S) was used as a secondary antibody for one hour at RT for both CFTR and β-actin antibodies. Detection was achieved using Immobilon Western Chemiluminescent HRP Substrate (Millipore, catalog no WBKLS0100) and membranes were analysed by densitometry using the ImageLab software. For quantitative analysis, the signal intensity of each band was normalized with actin densitometry values. Epifluorescence microscopy was used to visualise transfection of fl-TSB4 and fl-TSB6 into primary CF BECs.

**Forskolin-induced swelling (FIS) assay.**

Organoids were treated with TSB plus Lipofectamine for 6 hours and the suspension was washed in DMEM and replated in 3D Matrigel. At 'T0' (before FIS assay, T24h) the treatments were added to wells 1) DMSO +Forskolin, 2) 3mM VX809+ 3mM VX770 + Forskolin, or 3) 3mM VX 661+3mM VX770 +Forskolin. An automated high definition microscope (Keyance) in Z stack mode was used to capture baseline and interval phase and fluorescent images of each experimental well, thereby measuring the combined surface area of all organoids per well at baseline (T0) and 24 hours after FIS. The total organoid surface area of each well was reported as "normalised area" (T0 normalized area=100%). The percentage average increase in total organoid surface area after FIS was then calculated.

**MQAE assay for measurement of intracellular chloride**

CFBE41o- cells were seeded in a black 96-wells plate with a clear flat bottom at a final density of 10000 cells/well and incubated for 24 hours. Cells were then transfected with 100 nM of NC TSB/TSB4/TSB6 in Optimem reduced-serum media and Ribojuice transfection reagent. At 48 hours post-transfection, cells were loaded with 1 mM fluorescent membrane permeable chloride indicator N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) in phenol red–free MEM at 37°C, 5% CO2. After 6 h, fluorescence was measured using a Wallac Victor$^2$ plate reader (Perkin Elmer, excitation and emission wavelengths of 355 and 460 nm, respectively) in 0.1-s intervals for 1 min to determine the baseline of each experiment. The cells were then washed and maintained in Forskolin (FSK, 20 μM) dissolved in MQAE-free, phenol red–free MEM + 1% FCS to activate CFTR channel. After treatment with FSK, fluorescence was measured for a further 10 min in 2-s intervals and background-corrected fluorescence values were used for statistical analyses.

**YFP assay for CFTR functional analyses**

CFTR channel activity was assessed in transiently transfected CF bronchial epithelial cells with the use of the halide-sensitive yellow fluorescent protein (YFP)-H148Q/I152L. The plasmid encoding YFP-H148Q/I152L protein (now on referred as "YFP plasmid") was kindly donated by Dr Nicoletta Pedemonte from Gaslini Institute (Genova). CFBE41o- and Cufi-1 cells were seeded in a black 96-wells plate with a clear flat bottom at a final density of 10000 cells/well and incubated for 24 hours. Cells were then co-transfected with 150 ng of YFP plasmid and 100 nM of NC TSB/TSB4/TSB6 prepared in Optimem reduced-serum media and Genejuice or Ribojuice transfection reagent, respectively. In some experiments, cells were also treated with CFTR modulators for 48 hours, as described above. At 48 hours post-transfection, cells were washed once with PBS and incubated for 30 min with 60 μL of FSK (20 μM) in phenol-red free MEM + 1% FCS. The fluorescent signal was measured on a Wallac Victor$^2$ plate reader (Perkin Elmer, excitation and emission wavelengths of 490 and 535 nm, respectively) before and after addition of 165 μL of an iodide-enriched PBS (i.e. containing 137 mM of NaI instead of NaCl). For each well, background-corrected fluorescence values were normalised for the initial value before addition of $I^-$ and used for statistical analyses.

**High Content Screening**

*Fixation, immunofluorescence and fluorescence staining* - For High Content Screening (HCS) experiments CFBE41o- cells were seeded in a glass bottom 96-well plate at a final density of 10,000 cells/well and after 24 hours were transfected with 100 nM of TSB4 or TSB6 encapsulated in rhodamine-conjugated PLGA in Optimem reduced-serum media. At 4 hours post-transfection the cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT) and washed with PBS. Prior to imaging cells were permeabilised with 0.5% saponin for 30 min at RT, followed by three washes with PBS. Then, cells were incubated with a primary antibody against lysosomal associated membrane protein-2 (LAMP-2; 1 μg/ml final concentration, Cat. No. ab25631, Abcam) for 1 hour at RT, followed by PBS washes and incubation for 1 hour at RT with an anti-mouse IgG specific secondary antibody (10 μg/ml, Cat. No. A-11001, Thermo Fisher) in a PBS solution containing 200 μg/μl Hoechst 33342 (ImmunochemistryTechnologies,

Bloomington, USA) and 2 units/ml Alexa-Fluor Phalloidin (Molecular Probes, Thermo Fisher, Cat. No. A22287) to counterstain nuclei and actin respectively.

Automated confocal image acquisition and analysis - HCS confocal images were acquired on an Opera Phenix High Content Screening System (Perkin Elmer, Massachusetts, USA) using 20x/1.0 NA water immersion objectives with effective XY resolution of 0.66 μm. Images were analysed using the Columbus image data storage and analysis system v.2.8.2.1205 (Perkin Elmer, Massachusetts, USA). Basic flatfield correction was applied on images. Cell segmentation was obtained using Hoechst and LAMP-2 channels. Cells in the borders of the images were excluded. Nanoparticle objects were detected and segmented in the population of cells using 'Find Spots' building block. Method B was applied to detect spots, using a high value of Detection Sensitivity (0.85) as the signal of rhodamine in the cytoplasm of cells appeared to be low. To quantify Nanoparticle uptake by cells, the number of Spots in cells was normalised to the number of cells.

**Supplemental References.**

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