Stem Cell Reports, Volume *16*

Supplemental Information

A new platform for high-throughput therapy testing on iPSC-derived lung progenitor cells from cystic fibrosis patients

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SUPPLEMENTAL FIGURES AND LEGENDS

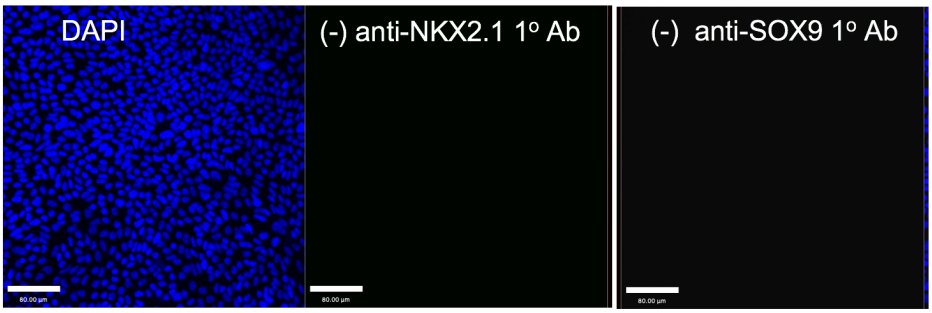

Figure S1: Negative controls for immunofluorescence studies of submerged cultures (Stage 3B of 2015 Wong Protocol), related to Figure 1. Negative controls lack primary antibody recognizing NKX2.1 (middle panel) or SOX9 (right panel)

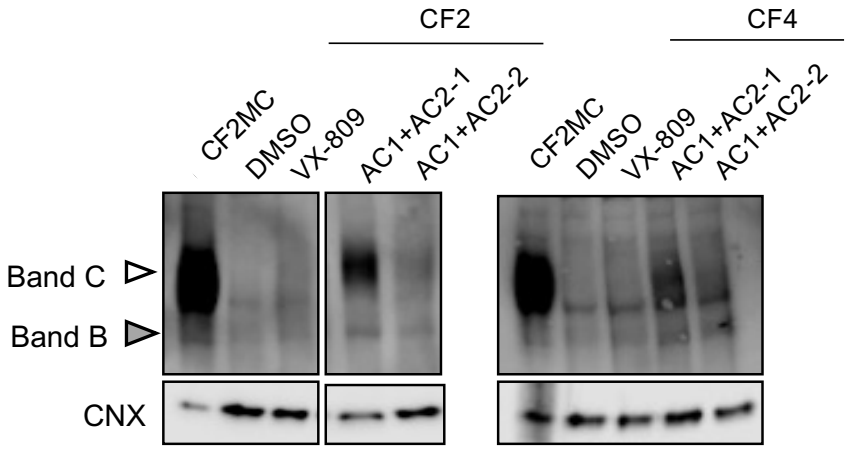

Figure S2: Representative F508del-CFTR protein expression in submerged cultures from 2 donors homozygous for F508del after 48h pre-treatment with DMSO (0.1%), 3μM VX-809, 0.5μM AC1 + 3μM AC2-1 or 0.5μM AC1 + 3μM AC2-2, related to Figure 3. C: mature, complex-glycosylated CFTR; B: immature, core-glycosylated CFTR; CNX, Calnexin as loading control. CF2 edited corresponds to mutation corrected version of CF2. The method for mutation editing is described in (Eckford et al., 2019) and validations of the CF2MC line are listed in **Document S1.**

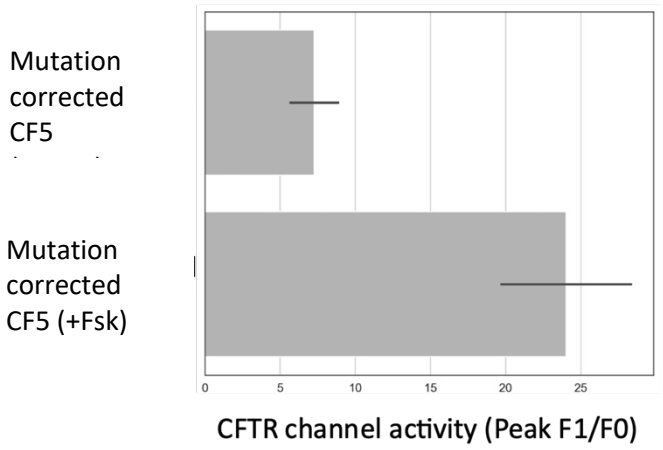

Figure S3: W1282X mutation corrected iPSC line, related to Figure 4. The iPSCs line from a donor, homozygous for W1282X, was corrected on one allele (see **Document S2** for information on the CF5MC line). 10μM Forskolin activated CFTR channel function was conferred with correction in the differentiated to immature lung cultures. CFTR channel activity was measured using the FLiPR assay and the bars represent mean-/+ SD in 4 technical replicates.

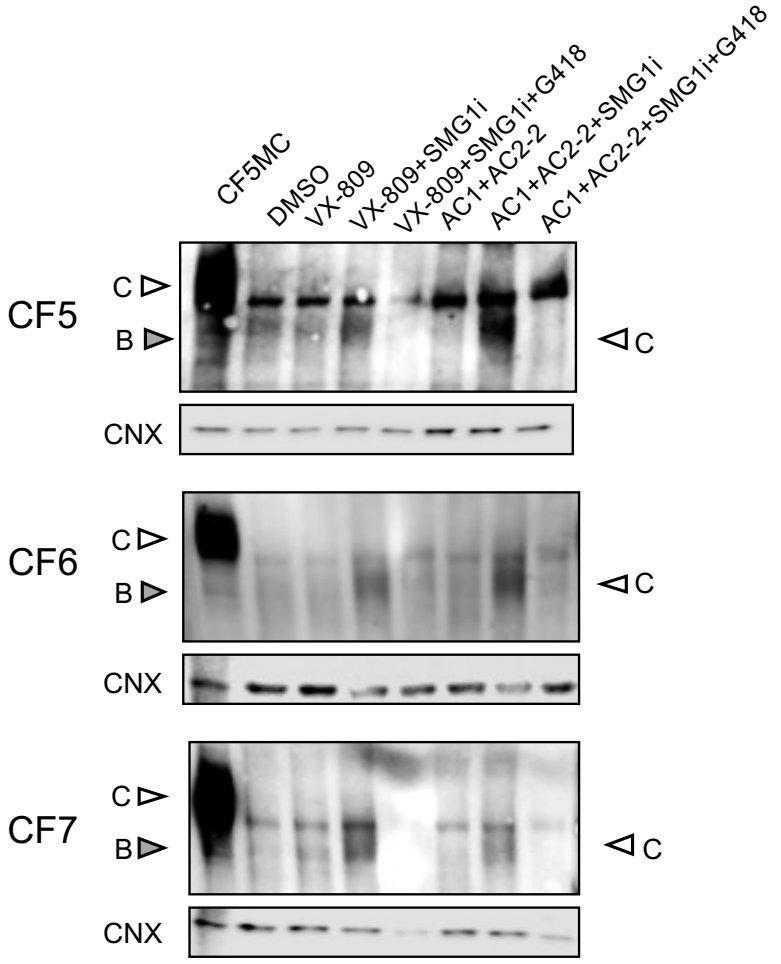

Figure S4: Immunoblotting shows that expression of truncated W1282-CFTR (mature protein = 130 kD protein, open triangle on right) is comparable across donor specific lines after differentiation to lung progenitor (submerged cultures), related to Figure 4. Abundance is enhanced in the presence of SMG1i in all cases. The protein expressed from mutation edited version of CF5, migrates as expected with Band C=170-200 kD and Band B=130 kD.

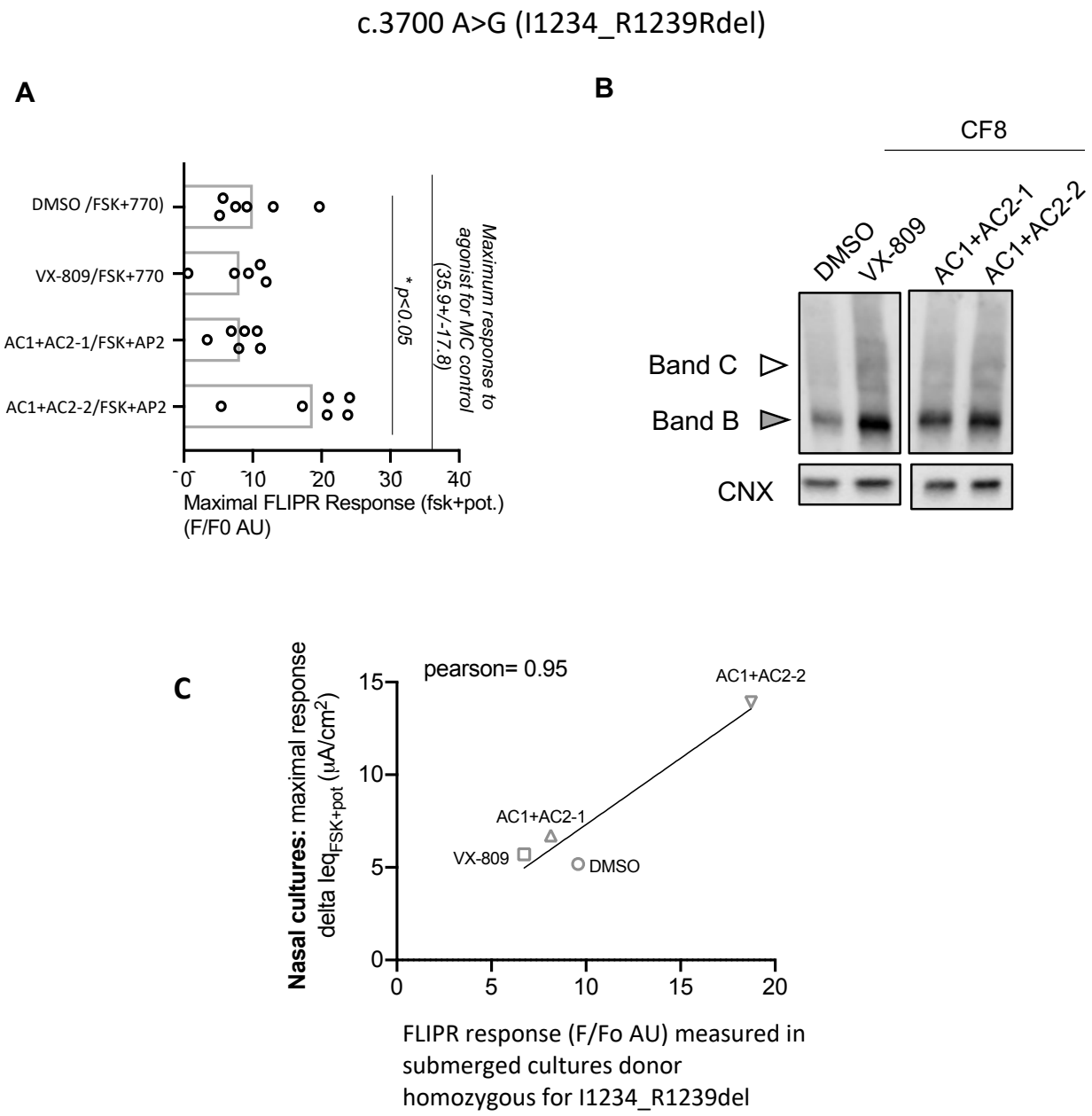

Figure S5: Submerged cultures generated from individual, homozygous for rare mutation (c.3700 A>G), exhibit differential response to modulators that mimics ranking observed in nasal epithelial cultures from the same person, related to Figure 4. (**A)** Submerged cultures from donor=CF8. Bars labeled with pretreatments (24 hours) and magnitude indicates peak FLIPR response after forskolin and potentiator addition. Open symbols derived from single well scans from a single differentiation. Mutation corrected iPSC line for CF8 showed excellent response to forskolin alone as indicated in vertical bar. (**B)** The pretreatment led to modest changes in expression of mature form of CFTR protein in this pilot study (Band C). (**C**) Correlation between best treatment in submerged cultures and primary nasal cultures generated for the same donor, CF8.

Supplemental Tables

Table S1

Small molecules	Concentration
VX-809	3 μM
AC-1	0.5 μM
AC2-2	3 μM
SMG1i	0.5 μM
G418	200 μg/mL

Table S2

Primer Sequences	
CFTR	Fwd: 5'- CTATGACCCGGATAACAAGGAGG-3'
CFTR	Rev: 5'- CAAAAATGGCTGGGTGTAGGA-3'
GADPH	Fwd: 5'- CTGGGCTACACTGAGCACC -3'
GADPH	Rev: 5'- AAGTGGTCGTTGAGGGCAATG -3'

Supplemental Experimental Procedures

Differentiation to submerged lung progenitor culture:

Human iPSCs were obtained from the Cystic Fibrosis Individualized Therapy (CFIT) program (Eckford et al., 2019). The submerged lung progenitor cultures were generated from iPSCs as previously described (Wong et al., 2015). Human iPSCs were grown on six-well plates (Corning) coated with Matrigel (Corning) and maintained with mTeSR media(Stem Cell Technologies). Cultures were expanded weekly with Gentle Cell Dissociation Buffer (GCDR, Stem Cell Technologies) at 70-90% confluency at a 1:10 ratio. For definitive endoderm (DE) induction, single-cell suspensions were generated from five-minute GCDR incubation at 37°C followed by scraping and gentle trituration. Cells were plated onto six-well plates in media supplemented with 10 μM Y27632 compound (Stem Cell Technologies) for 24 hours. DE cultures were generated using the StemDiff Definitive Endoderm Kit (Stem Cell Technologies) as per manufacturer's protocol for 5 days. To differentiate anterior foregut endoderm (AFE) culture, cells were treated with differentiation basal medium (KnockOut DMEM, 10% KnockOut serum replacement, 1% penicillin-streptomycin, 2mM Glutamax, 0.15 mM monothioglycerol, and 1 mM non-essential amino acid) supplemented with FGF2 (500 ng/mL) and SHH (50 ng/mL) for 24 hours. On the second day of AFE differentiation, cells were dissociated into single-cell suspensions and plated onto type IV collagen coated (60 ug/mL, Sigma) 96-well plates at a density of 25,000 cells per well. The media was supplemented with 10 μM Y27632 compound for 24 hours and was changed every 48 hours for an additional three days. For directed differentiation to lung progenitor cells, cultures were overlayed with differentiation basal medium supplemented

with FGF7 (50 ng/mL), FGF10 (50 ng/mL) and BMP4 (5 ng/mL) for 5 days, and then FGF7 (10 ng/mL), FGF10 (10 ng/mL) and FGF18 (10 ng/mL) for 5 days.

Apical Chloride Conductance (ACC) Assay for CFTR function:
The ACC assay was used to assess CFTR mediated changes in membrane depolarization using methods as previously described (Ahmadi et al., 2017; Erwood et al., 2020). In summary, iPSC derived- submerged lung cultures were incubated with zero sodium, chloride and bicarbonate buffer (NMDG 150 mM, Gluconic acid lactone 150 mM, Potassium Gluconate 3 mM, Hepes 10 mM, pH 7.42, 300 mOsm) containing 0.5 mg/ml of FLiPR® dye for 30 mins at 37°C. Wt-CFTR function was measured after acute addition of Fsk (10 µM) or 0.01% DMSO control. Cells were chronically rescued with corrector compounds for 24 hours. Post drug rescue, F508del-CFTR function was measured after acute addition of Fsk (10 µM) and VX-770 (1 µM) or AP-2 (1.5 µM). CFTR functional recordings were measured using the FLIPR® Tetra High-throughput Cellular Screening System (Molecular Devices), which allowed for simultaneous image acquisition of the entire 96 well plate. Images were first collected to establish baseline readings over 5 mins at 30 second intervals. Forskolin (-/+Potentiators) were then added to stimulate CFTR mediated anion efflux. Post drug addition, CFTR mediated fluorescence changes were monitored and images were collected at 15 second intervals for 70 frames. CFTR channel activity was terminated with addition of CFTRInh172 (10 µM) and fluorescence changes were monitored at 30 second intervals for another 25 frames.

Real-time Quantitative PCR:
As previously described (Cao et al., 2020), total mRNA was extracted with RNeasy® Plus Micro Kit, following enclosed instructions. After measuring the spectrophotometric quality of extracted RNA through 260/280 ratios of 2.0 and 260/230 ratios of 1.8-2.2, mRNA samples used to reverse transcribe 1 µg of cDNA using iScript™ cDNA Synthesis Kit. Quantitative real-time PCR was performed with PowerUP SYBR Green Mastermix Master Mix on ViiA7 (Applied Biosystems). Gene expression is normalized to house-keeping gene GAPDH and expressed relative to control human tissue RNA extracts (2^ΔΔCT). A total run of 40 cycles. Cycle threshold (CT) values above 38 were considered “not expressed”. The primers used for amplification are described in the following table.

RNA Sequencing and Analysis: RNA samples were extracted using methods described previously (Di Paola et al., 2017; Laselva et al., 2020e). RNA samples with an RNA integrity number (RIN) greater than 8.5 was submitted to The Centre of Applied Genomics (TCAG) at SickKids for bulk RNA sequencing. In brief, RNA libraries were generated using NEB Ultra II Directional mRNA with an average of 69,954,038 reads from each library on the Illumina HiSeq 2500 platform using high-throughput V4 flowcells.
Post sequencing quality control was performed using open-source software FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trim galore (F.) was used to remove low quality sequences and trim adapters. Paired-end reads were aligned to the human reference genome (hg38) using STAR (version 2.7.1a) (Dobin et al., 2013). The resulting bam files containing aligned sequences were subsequently processed using SAMtools (Li et al., 2009), and raw counts generated with featureCounts (Liao et al., 2014) were used for downstream analysis (transcripts per gene). R package DESeq2 (v.1.24.0) (Love et al., 2014) was used to calculate size factors for each sample and perform regularized-logarithm rlog transformation of read counts. The 100 genes with the highest variance in expression across all samples were subjected to principal component analysis (PCA).

Immunofluorescence: Samples were fixed in 4% paraformaldehyde and then washed three times with PBS, 5 minutes per wash at room temperature. Cell permeabilization was performed using 0.05% TritonX-100 followed by three PBS washes. Samples were blocked using 5% bovine serum albumin for 1 hour and incubated with primary antibody against TTF1 (NKX2-1) (Abcam, AB76013), SOX9 (Abcam, AB76997), and ZO-1(Invitrogen, ZO1-1A12) overnight at 4 degrees. After removal of primary antibody, samples were washed 3 times with PBS, 5 minutes per wash and incubated with secondary antibodies (Invitrogen, A32744, A32733, and A-11001) plus nuclear marker DAPI for 1 hour. Samples were then washed 3 times with PBS, 5 mins per wash at room temperature. Images were acquired on the SP8/STED confocal microscope (Leica).

Western blotting: Samples were collected in ice cold PBS and pelleted through centrifugation at 4°C (500g for 7 mins). Post centrifugation, the cell pellet was re-suspended in 200uL of modified radioimmunoprecipitation assay butter (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS and 0.1% (v/v) Triton X-100) containing a protease inhibitor cocktail for 10 min. After centrifugation at 13,000 rpm for 5 min, the soluble fractions were analyzed by SDS-PAGE on 6% Tris-Glycine gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated in 5% milk and CFTR bands were detected using the mAb 596. Calnexin (CNX) was used as a loading control and detected using a Calnexin-specific rAb (1:5000). The blots were developed with using the Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE, USA) in a linear rage of exposure (1-20 min). Relative levels of CFTR protein were quantitated by densitometry of immunoblots using ImageStudioLite (LI-COR Biosciences, Lincoln, NE, USA) (Chin et al., 2019; Laselva et al., 2019).

Supplemental References:

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