

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Nasal brushing processing and CRC culture. Human nasal epithelial samples were provided in accordance with consent procedures approved by the Internal Review Board of Regional (Lazio) Reference Center for Cystic Fibrosis, Policlinico Umberto I Hospital, Sapienza University of Rome (Ethics committee ref. 5660 prot 983/19 December 18th 2019). Nasal epithelial cells were obtained through cytology brushing (Doctor Brush, AIESI) of the inferior turbinates from both nostrils, pooled into a single 15-ml conical tube filled with DMEM/F12 (Euroclone) and 5X antibiotics (Penicillin/Streptomycin and Amphotericin B). Samples were repeatedly washed, and recovered cells cultured under the Conditionally Reprogrammed Cell (CRC) methodology, according to our previous protocols (*Sette et al., 2018*). Briefly, epithelial cells were co-cultivated with irradiated (30 gray) murine J2 Swiss 3T3 fibroblasts (Kerafast, Boston, MA, USA) in the presence of 10 μ M Rock inhibitor Y-27632 (Selleck, Munich, Germany), in F medium (3:1 v/v F-12 Nutrient Mixture Ham: DMEM) supplemented with 5% FCS, 0.4 μ g/ml hydrocortisone, 5 μ g/ml insulin, 24 μ g/ml adenine, 8.4 ng/ml cholera toxin, 10 ng/ml EGF. Fibroblasts were cultured in 10% characterized HyClone™ Fetal Bovine Serum (South Logan, Utah, USA) and irradiated when reached 80% confluence. All cells were maintained at 37°C in a humidified incubator, with 5% CO₂. For standard culture conditions (those usually used for primary airway cells), CRC cells were deprived of feeder layer and grown in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza) for 2 weeks before analysis.

Differentiation of CRC in Ali-liquid interface (ALI) culture conditions. To induce differentiation, 1.1×10^5 cells were plated in Corning 3460 inserts, and cultured in CRC complete Medium in both basal and apical chambers until confluence was reached (5-7 days), afterwards medium was replaced with PneumaCult – ALI Medium (Voden) in the basal chamber, leaving the apical chamber empty for 28 days, with medium replacement every other day. Alternatively, ALI differentiation was achieved through culture in standard tissue culture-treated plates as 2D monolayers in ALI medium for 3 weeks.

Growth curve of CRC. To determine cell growth rate of CRC cultures 1×10^5 cells were seeded on feeder layer in complete growth medium and counted by trypan blue exclusion every 4 to 8 days. Proliferation index was calculated as population doublings (PD) using the following formula: $PD = 3.32 \times \log(\text{cell number counted} / \text{cell number plated at day 0})$ (Figure S1).

Clonogenic assay of CRC. CRC cells were plated at single cell density in 96 well plates under CRC conditions. After overnight culture, wells with a single cell were counted and irradiated fibroblasts were added to the wells to support the growth of single cells. Epithelial cell clones rapidly appeared and positive-wells were counted to calculate the percentage of colony forming cells after 5-7 days. Clones exponentially grew for at least 2 weeks proving their extended growth ability. The mean clonogenic efficiency was calculated as mean of three different samples.

CFTR mutational analysis. Genomic DNA was extracted from the CF-CRC by the QIAamp DNA Blood midi kit (Qiagen, Hilden, Germany) and quantified using a fluorimeter (Qubit, Invitrogen, CA, USA). The CFTR (RefSeq NM_000492.4, NG_016465.4) genotype of each preparation of CF-CRC was confirmed by sequencing. The proximal 5'-flanking, all exons and adjacent intronic zones, as well as the 3'-UTR were PCR-amplified and sequenced by a Sanger cycle sequencing protocol (ThermoFisher Scientific, Waltham, MA, USA) in a 96-well format as previously described (*Lucarelli et al., 2006*), using a genetic analyzer (ABI PRISM 3130xl; Applied Biosystems, Foster City, CA, USA). For data analysis, a specific template for SeqScape software version 4.0 (Applied Biosystems) was used (*Ferraguti et al., 2011*). Genotype confirmation was completed by multiplex ligation-dependent probe amplification (SALSA MLPA probemix P091 CFTR, MRC Holland, Amsterdam, The Netherlands).

CFTR expression analysis. RNA was extracted from CF-CRC by the RNeasy mini kit (Qiagen, Hilden, Germany). It was reverse transcribed by the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) that includes a mix of oligo(dT) and random hexamers as a priming strategy. Retrotranscription was performed using 1 μg of total RNA in 5.5 μL , 4 μL of 5X iScript reaction mix, 1 U of iScript reverse transcriptase in 1 μL , 9.5 μL of H_2O , in a final volume of 20 μL , according to the manufacturer's instructions. The reactions were

incubated in a PTC 100 thermocycler (Bio-Rad), according to a program that allows the synthesis of the double stranded cDNA: 5' 25 °C, 30' 42 °C and 5' 85 °C.

For sequence analysis of the CFTR cDNA, it was amplified by a protocol that, using an optimized set of primers, produced 6 amplicons spanning the entire CFTR mRNA, as previously described (*Auriche et al., 2010*). The PCR mix was in a final volume of 15 µL containing: 2.5 µL of cDNA mix, 175 µM of each dNTP (Fermentas, Waltham, MA, USA), 1.5 mM MgCl₂, 6 pmol of each primer and 0.5 U GoTaq hot start polymerase with 1X manufacturer's buffer (Promega, Madison, Wisconsin, USA). The PCR step was conducted in a PTC100 thermocycler (Bio-Rad, Hercules, CA, USA) with the following PCR cycle: 2' 95 °C; 35 cycles of 45" 94 °C, 1' 30" 60 °C, 2' 30" 72 °C followed by 7' 72 °C. The amplicons were subsequently analyzed by electrophoresis on a 3% agarose gel, to possibly detect anomalous CFTR mRNAs. All the 6 cDNA amplicons were extracted from agarose and individually sequenced as described above.

For quantitative CFTR expression analysis, starting from the cDNA mix described above, a TaqMan gene expression assay (code 4331182, ID Hs00357011_m1; ThermoFisher Scientific, Waltham, MA, USA) was applied using a specific no-ROX Master Mix (FluoCycle™ II Master Mix for probe, EuroClone, Milan, Italy) according to the manufacturer's instructions. The β-glucuronidase (GUSB) gene was used as housekeeping by the TaqMan assay (code 4331182, ID Hs00939627_m1). Both TaqMan probes were FAM dye-labeled. The final reaction volume was 20 µL, using 1 µL of cDNA mix, 10 µL of 2X no-ROX master mix, 1 µL of specific TaqMan probe assay, 8 µL of H₂O, according to manufacturer instructions. The real time PCR instrument used was the MJ MiniOpticon (Bio-Rad), with the following program: 5' 95 °C and 45 cycles of 15" 95 °C followed by 1' 60 °C. The threshold cycles of both CFTR and GUSB genes were acquired, in triplicate for each sample. The analysis was performed using the ΔCt, calculated as the difference between the average Ct of CFTR and the average Ct of GUSB, and then calculating the value of $2^{-\Delta\Delta C_t}$ referring to H1299 cells.

Flow cytometry and Immunofluorescence. For flow cytometry, 1×10^5 cells were incubated with the following antibodies: FITC-conjugated monoclonal mouse anti-human Epithelial Antigen Clone Ber-EP4 (Dako), FITC-conjugated anti-mouse CD271/LNGFR (Miltenyi Biotech), PE-conjugated monoclonal mouse anti-human TROP-2 (R&D system) or corresponding isotype control antibodies. Staining for integrin α6 was performed with rat

monoclonal anti-integrin $\alpha 6$ Clone GoH3 (Abcam) and secondary goat anti rat Alexa Fluor 647 antibody (Invitrogen). Stained cells were analyzed with FACScan or LSRII flow cytometer (Becton Dickinson). For immunofluorescence, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS and incubated with antibodies to mucin-5B (Santa Cruz, H-300) to assess goblet cells differentiation, with acetylated α -tubulin (Sigma, clone 6-11B-1) for cilia detection. Nuclei were counter-stained with DAPI (Invitrogen). Images were acquired by a FV1000 confocal microscope (Olympus, Tokyo, Japan), using a (Olympus) planapo objective 60x oil A.N. 1.42. Excitation light was obtained by a 408 nm Laser for DAPI, an Argon Ion Laser (488 nm) for Alexa 488, and a Diode Laser HeNe (561 nm) for Alexa 568. DAPI emission was recorded from 415 to 485 nm, FITC emission was recorded from 495 to 550 nm, Alexa 568 from 583 to 628 nm. Images recorded have an optical thickness of 0.5 mm.

Immunoblot and analysis of pharmacologic rescue of mature CFTR protein. For immunoblotting studies, 20 μ g of total lysate proteins from each sample were resolved on 3–8% polyacrylamide gel electrophoresis NuPAGE Tris-Acetate (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes. The following primary antibodies were used: mouse monoclonal antibody CFTR-596 (CFTR Antibody Distribution Program (Cystic Fibrosis Foundation, UNC-Chapel Hill), mouse monoclonal FOXJ1 clone 2A5 (eBioscience™), rabbit polyclonal CK14 (Biolegend), mouse monoclonal nucleolin C-23 (Santa Cruz), mouse monoclonal DM1A alpha Tubulin (Novus Biologicals) and mouse monoclonal β -actin (Sigma-Aldrich) antibodies. Peroxidase-conjugated secondary antibodies were purchased from Amersham™.

For evaluation of the ability of drugs to rescue CFTR protein maturation CF-CRC cells were plated in 6 well plates (2×10^5 cells/well) and cultured in ALI medium for 3 weeks (medium replaced every other day) to allow cell differentiation and expression of higher levels of CFTR protein. For the last 48 hours cells were exposed to drugs (Lumacaftor 5 μ M, Tezacaftor 20 μ M, Elexacaftor 3 μ M or their combinations) before cell lysis and lysates processed as described above. Quantification of immunoblot bands intensity were performed through Image lab software (Chemidoc XRS+, Biorad). To quantify CFTR maturation, the relative amount of CFTR band-C protein was normalized to nucleolin or tubulin measured in the identical protein sample, and these levels were used for subsequent calculations.

CRC-derived organoid generation and forskolin-induced swelling assay. Cells were suspended at 50000 cells/100 μ L in Growth Factor-Reduced matrigel (Corning), vigorously but carefully pipetting to generate a single-cell suspension while avoiding the generation of air bubbles. This mixture was seeded in 100 μ L aliquots into 24 well plates, creating a spherical “drop” of matrigel. The plates were incubated at 37 °C and 5% CO₂ for 30 min, to allow matrigel setting. CRC medium was added to the wells to cover the matrigel drop. After 3-4 days cells were shifted in PneumaCult–ALI Medium until mature 3D structure was formed (typically after 21 days, with the presence of a lumen and a slightly thickened spheroid wall, suggesting a pseudostratified epithelium with motile cilia (Movie 1 and Movie 2), replacing medium every other day. For functional assays, organoids were pre-treated with VX809 (Lumacaftor), VX661 (Tezacaftor), VX445 (Elexacaftor) or their combinations (all drugs purchased from Selleck Chemicals), for 48 hours, at the same doses as described above. Spheroid images were captured (10X magnification) using Time-lapse imaging station (Olympus, Tokyo, Japan), at time 0 and after 2 days of subsequent stimulation with 5 μ M VX770 (Ivacaftor) and 20 μ M Forskolin (Selleck Chemicals), to monitor and assess spheroid swelling (n = 10 spheroids per condition for all experiments described were analyzed). Images were analyzed by manually delineating the area of each spheroid using ImageJ software. Staff performing the analysis were blinded to mutation or condition of each image. Spheroid area (basal and after stimulation) percent change was calculated for each individual spheroid.

Analysis of CFTR activity in Fluid Re-adsorption assay. After differentiation of CRC in ALI-culture conditions (as described above) cells were left untreated or treated with drugs or drug combinations (in triplicate) for 2 days (in the basolateral chamber). Medium with drugs was replenished in the basolateral chambers and cells were washed 3 times (apical chamber) with 0.5 ml PBS with Ca⁺⁺/Mg⁺⁺, then 200 μ l of FSK-containing PBS were added and overlaid with 300 μ L mineral oil to avoid evaporation. Plates were put back into incubator for 2 days to allow liquid reabsorption in the epithelium. Liquid (PBS plus oil) was collected, separated through brief centrifugation and PBS was measured to determine the amount of volume re-adsorbed in each sample. Volume of liquid re-adsorbed (ΔV) was calculated as follows: initial volume of PBS - residual volume measured) / (membrane area (1.12 cm²) x 48 hours and expressed as μ l / (cm² x hr).

REFERENCES

- Auriche C., Di Domenico E.G., Pierandrei S., Lucarelli M., Castellani S., Conese M., Melani R., Zegarra-Moran O., Ascenzioni F. CFTR expression and activity from the human CFTR locus in BAC vectors, with regulatory regions, isolated by a single step procedure. *Gene Therapy* (2010) 17(11):1341-1354.
- Ferraguti G., Pierandrei S., Bruno S.M., Ceci F., Strom R., Lucarelli M. A template for mutational data analysis of the CFTR gene. *Clinical Chemistry and Laboratory Medicine* (2011) 49(9):1447-1451.
- Lucarelli M., Narzi L., Piergentili R., Ferraguti G., Grandoni F., Quattrucci S., Strom R. A 96-well formatted method for exon and exon/intron boundary full sequencing of the CFTR gene. *Analytical Biochemistry* (2006) 353(2):226–235.
- Sette G., Salvati V., Giordani I., Pillozzi E., Quacquareni D., Duranti E., De Nicola F., Pallocca M., Fanciulli M., Falchi M., Pallini R., De Maria R., Eramo A. Conditionally reprogrammed cells (CRC) methodology does not allow the in vitro expansion of patient-derived primary and metastatic lung cancer cells. *International Journal of Cancer*, (2018) 143(1):88-99.

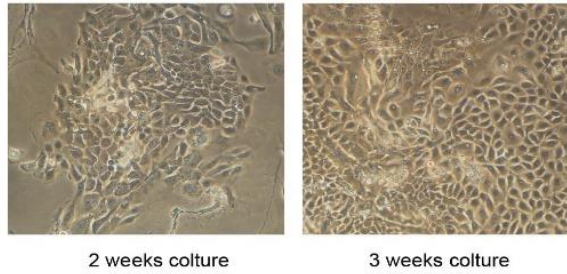
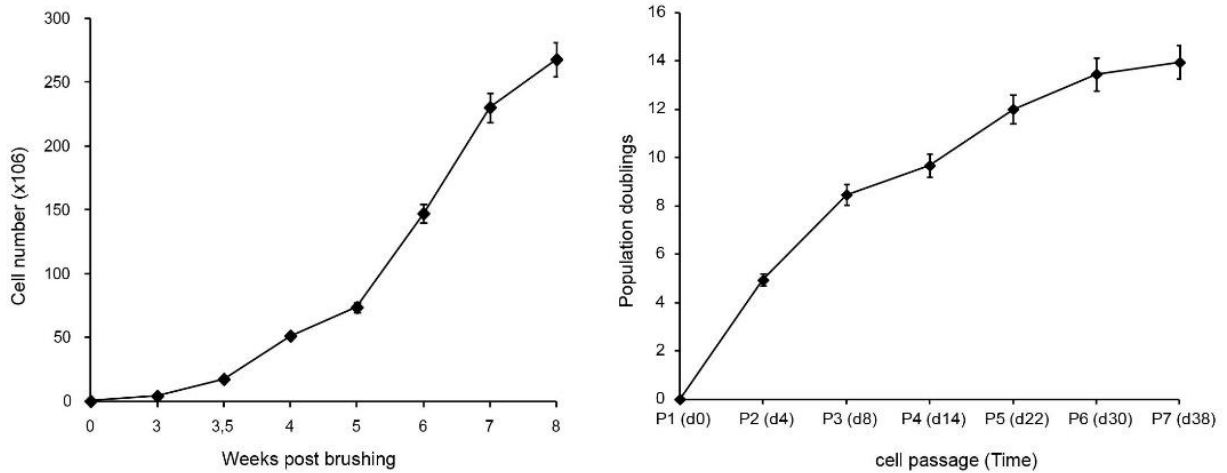
A**B**

Figure S1. Cells obtained from nasal brushing of CF patients are able to extensively proliferate under CRC culture conditions, generating long-term cultures of CF-CRC-AESC. **A)** Images show that these cells grow as epithelial-like adherent clones (2 weeks) and form homogeneous monolayers (3 weeks). **B)** Growth curve (left) and population doublings (right) showing CRC methodology ability to generate huge amounts of cells with prolonged growth potential and active proliferation rates.

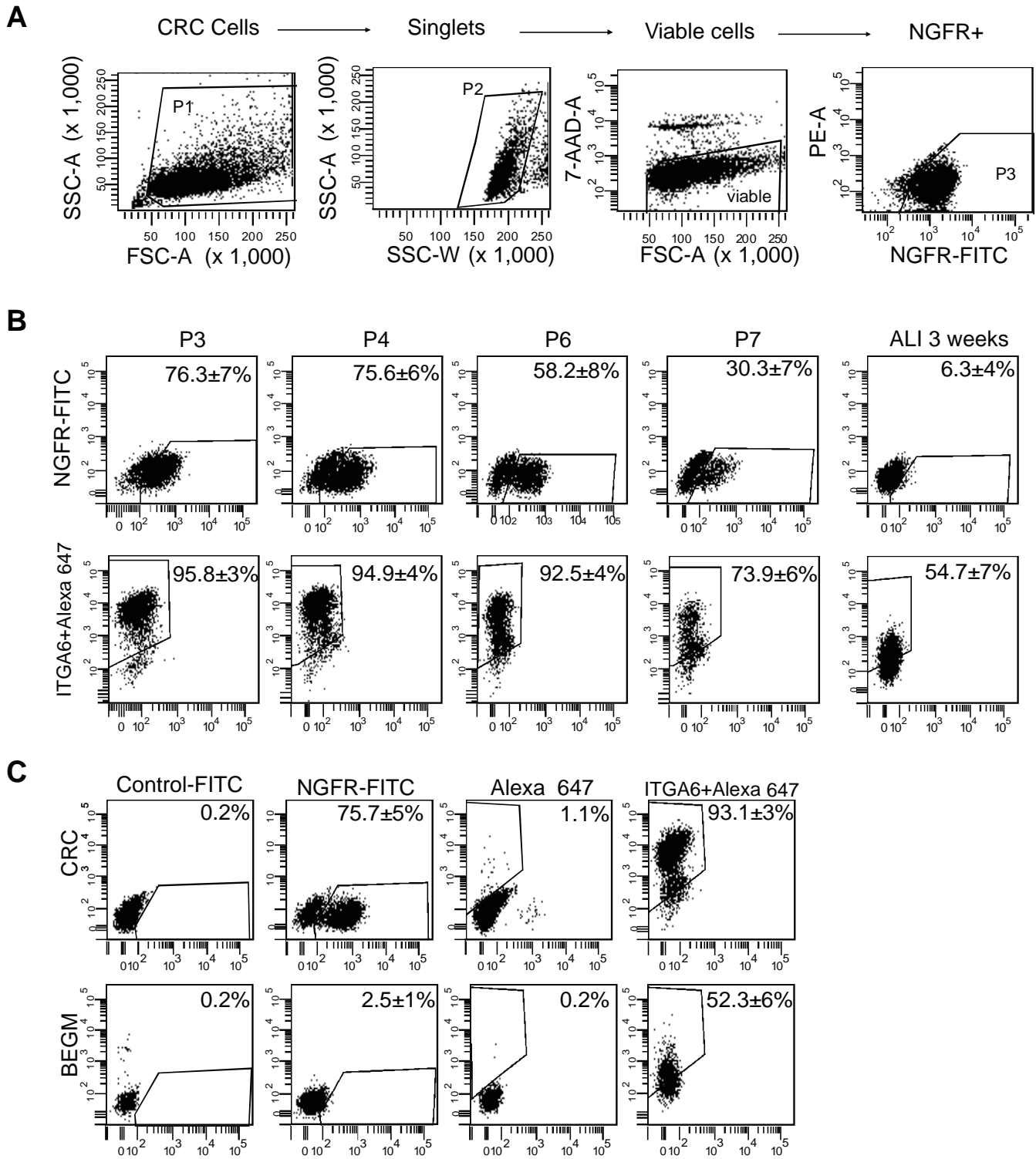


Figure S2. A) Example of the complete gating strategy used for flow cytometry to evaluate cell viability and cell doublets for the acquisition and analysis of single viable cells. B) Maintenance of basal stem cell-associated markers NGFR and ITGA6 during prolonged passaging in vitro. Flow cytometry analysis of passage 3 (P3) to passage 7 (P7) and ALI differentiated cells (3 weeks) is reported. C) Flow cytometric comparison of basal stem cell-

associated marker expression (NGFR, ITGA6) in CF-CRC cultures (upper panels) and standard growth condition (BEGM) cultures (lower panels).