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A functional CFTR assay using primary cystic fibrosis intestinal organoids

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We recently established conditions allowing for long-term expansion of epithelial organoids from intestine, recapitulating essential features of the *in vivo* tissue architecture. Here we apply this technology to study primary intestinal organoids of people suffering from cystic fibrosis, a disease caused by mutations in *CFTR*, encoding cystic fibrosis transmembrane conductance regulator. Forskolin induces rapid swelling of organoids derived from healthy controls or wild-type mice, but this effect is strongly reduced in organoids of subjects with cystic fibrosis or in mice carrying the Cftr F508del mutation and is absent in *Cftr*-deficient organoids. This pattern is phenocopied by CFTR-specific inhibitors. Forskolin-induced swelling of *in vitro*–expanded human control and cystic fibrosis organoids corresponds quantitatively with forskolin-induced anion currents in freshly excised *ex vivo* rectal biopsies. Function of the CFTR F508del mutant protein is restored by incubation at low temperature, as well as by CFTR-restoring compounds. This relatively simple and robust assay will facilitate diagnosis, functional studies, drug development and personalized medicine approaches in cystic fibrosis.

CFTR functions as an anion channel and is essential for fluid and electrolyte homeostasis at the epithelial surfaces of many organs, including the lung and intestine. Cystic fibrosis is caused by mutations in the *CFTR* gene[1–3], and disease expression is highly variable between individuals (http://www.cftr2.org/). People with cystic fibrosis have a median life expectancy of approximately 40 years and accumulate viscous mucus in the pulmonary and gastrointestinal tract, which is associated with bacterial infections, aberrant inflammation and malnutrition[4]. Over 1,900 *CFTR* mutations have been identified, but the most dominant mutation (accounting for ~67% of the total mutant alleles worldwide) is a deletion of phenylalanine at position 508 (CFTR F508del) (http://www.genet.sickkids.on.ca/). This causes misfolding, endoplasmic reticulum retention and early degradation of the CFTR protein that prevents its function at the plasma membrane[5]. Other *CFTR* mutations also impair protein folding or production, gating, conductance, splicing or interactions with other proteins[6].

Current therapies for cystic fibrosis are mainly symptomatic and focus on reducing bacterial pressure and inflammation and normalizing nutrient uptake and physical growth. Multiple compounds have been identified recently that target mutation-specific defects of the CFTR protein itself[6,7]. Current clinical trials that target the basic defect aim to (i) induce premature stop codon readthrough, (ii) correct plasma membrane trafficking of CFTR (correctors) and (iii) enhance CFTR gating (potentiators). A phase 3 clinical trial has been successfully completed using the potentiator VX-770 (ivacaftor, Kalydeco) in subjects with the CFTR G551D mutation, demonstrating that mutation-specific drug targeting is feasible[8]. Combination therapy using a corrector (VX-809) and a potentiator (VX-770) is currently being assessed in a phase 2 clinical trial for subjects harboring the CFTR F508del mutation.

Although CFTR-specific drug targeting is promising, the amount of functional CFTR restoration is still limited[9–11]. In addition, subjects show variable responses to these therapies due to undefined mechanisms[8,12–14], suggesting that the ability to predict individual drug responses would facilitate clinical efficacy and drug registration. Together this indicates that development of new compounds and screening of drug efficacy at the level of the individual are urgently needed. Thus far, limited primary cell models are available to screen for compounds that restore the function of mutant CFTR proteins. When such an *in vitro* model can be further expanded to analyze the drug responses of individual subjects, it may improve drug efficacy by selecting drug-responsive subgroups.

Here we demonstrate a rapid and quantitative assay for CFTR function in a mouse and human primary intestinal culture method that was recently developed[15–17]. The culture enables intestinal stem cells to expand into closed organoids containing crypt-like structures

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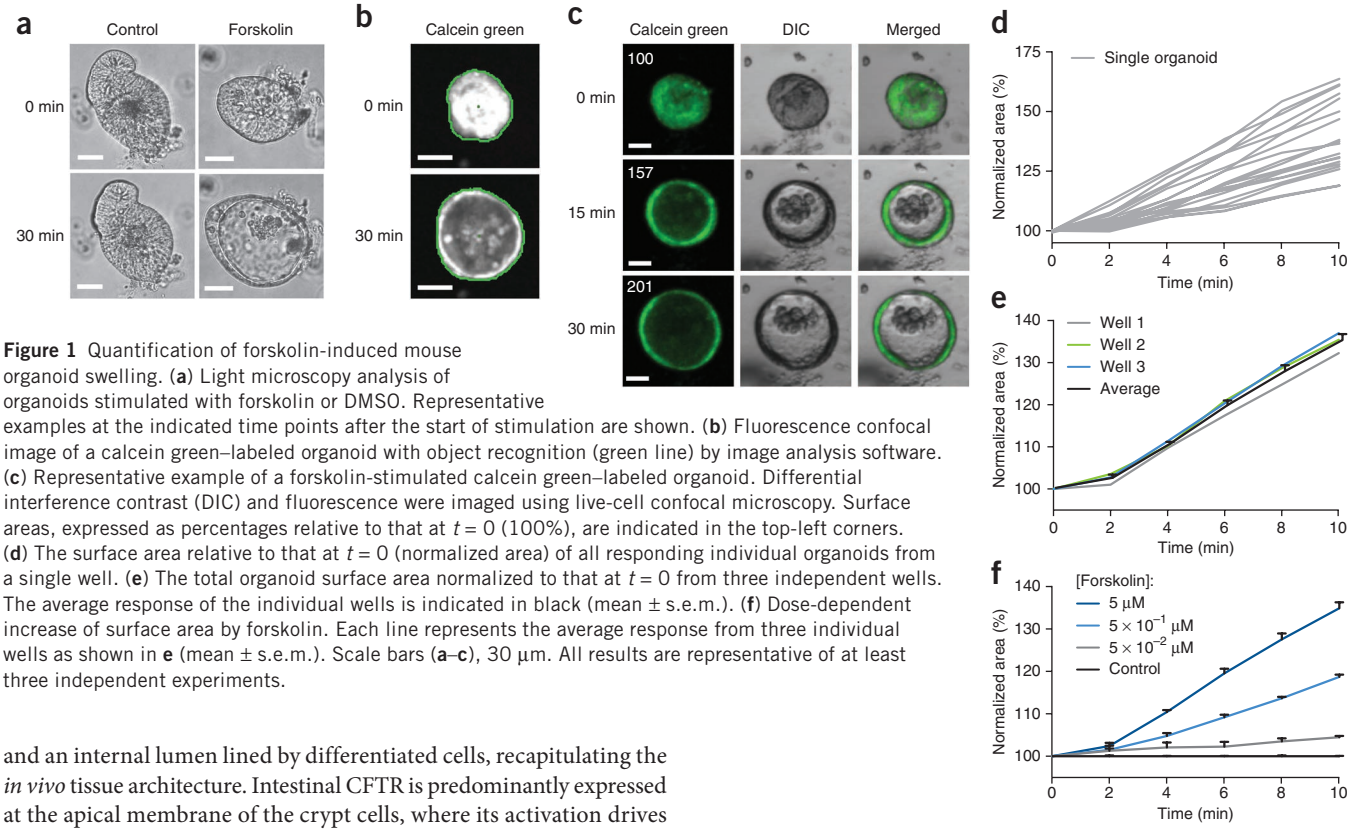

Figure 1 Quantification of forskolin-induced mouse organoid swelling. (**a**) Light microscopy analysis of organoids stimulated with forskolin or DMSO. Representative examples at the indicated time points after the start of stimulation are shown. (**b**) Fluorescence confocal image of a calcein green–labeled organoid with object recognition (green line) by image analysis software. (**c**) Representative example of a forskolin-stimulated calcein green–labeled organoid. Differential interference contrast (DIC) and fluorescence were imaged using live-cell confocal microscopy. Surface areas, expressed as percentages relative to that at $t = 0$ (100%), are indicated in the top-left corners. (**d**) The surface area relative to that at $t = 0$ (normalized area) of all responding individual organoids from a single well. (**e**) The total organoid surface area normalized to that at $t = 0$ from three independent wells. The average response of the individual wells is indicated in black (mean ± s.e.m.). (**f**) Dose-dependent increase of surface area by forskolin. Each line represents the average response from three individual wells as shown in **e** (mean ± s.e.m.). Scale bars (**a**–**c**), 30 µm. All results are representative of at least three independent experiments.

and an internal lumen lined by differentiated cells, recapitulating the *in vivo* tissue architecture. Intestinal CFTR is predominantly expressed at the apical membrane of the crypt cells, where its activation drives the secretion of electrolytes and fluids[18–20]. We found that forskolin[21] induces a rapid swelling of both human healthy control and mouse wild-type organoids that depends completely on CFTR, as demonstrated by analysis of intestinal organoids from *Cftr*^{tm1Cam} knockout (*Cftr*$^{-/-}$) mice or individuals with cystic fibrosis. Forskolin-induced swelling by *in vitro*–expanded rectal organoids is comparable to the forskolin-induced anion currents measured in *ex vivo* human rectal biopsies. Restoration of the function of CFTR F580del mutant protein using low temperature or chemicals was easily detected by organoid-based fluid transport measurements, and responses to CFTR-restoring drugs were variable between organoids derived from different subjects homozygous for F508del. This robust assay is the first functional readout developed in human organoids, to our knowledge, and will facilitate diagnosis, functional studies, drug development and personalized medicine in cystic fibrosis.

RESULTS

Quantification of forskolin-induced organoid swelling

We first assessed whether forskolin, which raises the amount of intracellular cyclic AMP (cAMP) and thereby activates CFTR, could mediate fluid secretion into the lumen of small intestinal organoids derived from wild-type mice. Using live-cell microscopy, we observed a rapid expansion of both the lumen and total organoid surface area after the addition of forskolin, whereas DMSO-treated organoids were unaffected (**Fig. 1a** and **Supplementary Video 1**). Removal of the forskolin reversed the organoid swelling (**Supplementary Fig. 1a**).

We then quantified these responses by automated image analysis. We found excellent cell labeling (>100 times that of the background) using calcein green, a cell-permeable dye that gains fluorescence and is retained within living cells after metabolic conversion. We quantified forskolin-induced swelling (FIS) of organoids using live-cell confocal microscopy and imaging software that calculated the relative increase in the total area of all fluorescent objects per well for each time point after the addition of forskolin (**Fig. 1b**,**c** and **Supplementary Fig. 1b**). The majority of the organoids responded to forskolin stimulation (**Fig. 1d**). Approximately 5–10% of organoids that were either very small or irregularly shaped and nonviable did not respond to forskolin (**Supplementary Fig. 1c**,**d**), but their inclusion did not affect the FIS measurements (**Supplementary Fig. 1e**). A time-dependent surface area increase in three independent wells showed limited variation (**Fig. 1e**) and a dose-dependent relationship with forskolin (**Fig. 1f**). We present the FIS of mouse organoids during the first 10 min, as some wild-type mouse organoids burst and collapsed during longer stimulations (**Supplementary Fig. 2a**–**c**). Together these results show that forskolin-induced organoid swelling can be quantified by automated fluorescent image analysis.

Forskolin-induced swelling of mouse organoids

High levels of *Cftr* mRNA in the organoids supported a role for Cftr in FIS (**Supplementary Fig. 2d**). To demonstrate that FIS is Cftr dependent, we used chemical inhibitors of CFTR[22,23] and *Cftr*$^{-/-}$ (ref. 24) or *Cftr*^{tm1eur} (harboring Cftr F508del) mice[25,26]. Preincubation (2 h) with the CFTR inhibitor $CFTR_{inh}$-172 (ref. 22) or GlyH-101 (ref. 23) reduced FIS by ~90% and ~75%, respectively, and their combination fully prevented FIS (**Fig. 2a**). In addition, FIS was absent in the organoids of *Cftr*$^{-/-}$ mice (**Fig. 2b**,**c** and **Supplementary Video 1**). Calcein green labeling was comparable in wild-type and mutant organoids, indicating that the *Cftr*$^{-/-}$ organoids were viable. Organoids of mice harboring the Cftr F508del mutation displayed low but detectable FIS (**Fig. 2d**,**e** and **Supplementary Video 1**) that was sensitive to $CFTR_{inh}$-172 (**Fig. 2f**), suggesting residual Cftr activity, as has been observed previously[25,26]. Together these data demonstrate that FIS in mouse organoids is completely Cftr dependent.

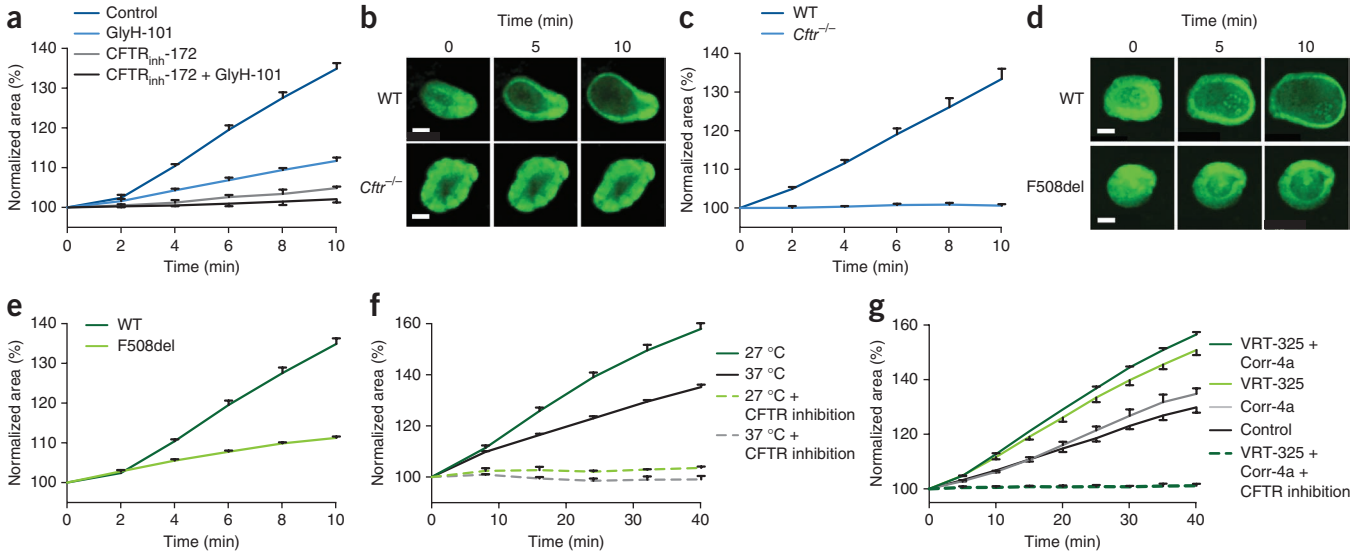

Figure 2 Forskolin-induced swelling of mouse organoids is Cftr dependent. (**a**) Normalized swelling curves of forskolin-stimulated calcein green–labeled organoids preincubated with DMSO, $CFTR_{inh}$-172, GlyH-101 or both $CFTR_{inh}$-172 and GlyH-101 (mean ± s.e.m.). (**b**,**c**) Representative confocal microscopy images (**b**) and quantification of swelling (mean ± s.e.m.) (**c**) of calcein green–labeled *Cftr*$^{-/-}$ organoids and corresponding wild-type organoids in response to forskolin. Scale bars, 50 µm. (**d**,**e**) Similar to **b** and **c** but for F508del Cftr organoids (mean ± s.e.m.). Scale bars, 50 µm. (**f**) Forskolin-induced swelling of calcein green–labeled F508del Cftr organoids cultured for 24 h at 37 °C or 27 °C with or without CFTR inhibition (mean ± s.e.m.). (**g**) Normalized forskolin-induced swelling of F508del Cftr organoids pretreated for 24 h with DMSO, VRT-325, Corr-4a or both correctors with or without CFTR inhibition (mean ± s.e.m.). Note that the timescale in **f** and **g** is larger than elsewhere in the figure. All results are representative of at least three independent experiments.

To show that FIS can detect Cftr correction, we performed temperature-rescue experiments, which is a widely accepted method to increase the function of CFTR F508del mutant protein[27]. Misfolding of F508del CFTR is reduced at 27 °C, leading to enhanced amounts of functional CFTR at the plasma membrane. Indeed, we observed increased FIS after overnight incubation of F508del Cftr organoids at 27 °C (**Fig. 2f**). We next used the chemical correctors VRT-325 (ref. 28) and Corr-4a[29] to restore the function of F508del Cftr (Online Methods). Preincubation (24 h) with VRT-325 enhanced FIS, whereas preincubation with Corr-4a only slightly improved FIS, and the effect of Corr-4a was additive to that of VRT-325 (**Fig. 2g**). CFTR inhibitors prevented FIS after Cftr correction (**Fig. 2f**,**g**). Collectively these results demonstrate that FIS of mouse organoids can reveal functional restoration of F508del Cftr by correction approaches.

Forskolin-induced swelling of human organoids

We next assayed human intestinal organoid cultures. Using western blot analysis, we detected mature CFTR (C band, 170 kDa) and immature CFTR (B band, 130 kDa) in human healthy control organoids, but we detected only the B band in F508del CFTR organoids (**Fig. 3a**). We found no CFTR B or C band in organoids carrying E60X[30] and a previously unreported allele that induces a frame shift in nucleotide-binding domain 2 (NBD2) at residue 1295 (4015delATTT). We further showed the specificity of the CFTR B and C bands using treatment with Endoglycosidase H (Endo H) and PNGase F[5], respectively. CFTR colocalized with apical actin in healthy control organoids but not F508del CFTR organoids (**Fig. 3b**). As observed in mouse organoids, rapid FIS of healthy control organoids was sensitive to chemical inhibition of CFTR (**Fig. 3c**). Human organoids showed somewhat slower kinetics compared to mouse organoids, rarely collapsed and required a longer preincubation time with CFTR inhibitors (3 h) (**Fig. 3c**, **Supplementary Fig. 2c** and **Supplementary Video 2**).

We analyzed FIS in a large number of intestinal organoids derived primarily from the rectum but also from the duodenum, ileum and colon. We observed strong FIS in organoids derived from healthy control subjects (**Fig. 3d** and **Supplementary Fig. 3a**). Rectal organoids derived from individuals compound heterozygous for F508del and A455E[31], a genotype that is associated with mild cystic fibrosis[32], clearly showed reduced amounts of FIS compared to healthy control organoids (**Supplementary Video 2**). Subjects with severe cystic fibrosis genotypes (homozygous for F508del or compound heterozygous for F508del and L927P[33] or G542X[31]) showed much less FIS that was variable between individual subjects (**Fig. 3e** and **Supplementary Video 2**). We observed no FIS in E60X 4015delATTT organoids (**Supplementary Video 2**). Chemical inhibition of CFTR abolished all FIS responses of organoids from subjects with cystic fibrosis (**Supplementary Fig. 3b**).

FIS measurements of *in vitro*–expanded organoids derived from healthy controls or individuals with cystic fibrosis, subdivided into severe and mild genotypes, correlated tightly with forskolin-induced intestinal current measurements (ICMs) performed on rectal suction biopsies[34,35] from which these organoids originated (**Fig. 3f**). Most ICM tracings of biopsies from individuals with cystic fibrosis showed residual forskolin-induced anion currents that corresponded to a quantitatively similar CFTR-dependent forskolin response in the FIS assay (**Supplementary Fig. 4a**–**c**). Together these data indicate that FIS in human organoids can accurately measure CFTR function and show that residual CFTR function in rectal organoids may differ between individuals homozygous for the CFTR F508del mutation.

CFTR restoration in cystic fibrosis organoids

We next assessed functional restoration of F508del CFTR in human organoids using either low temperature or the chemical correctors

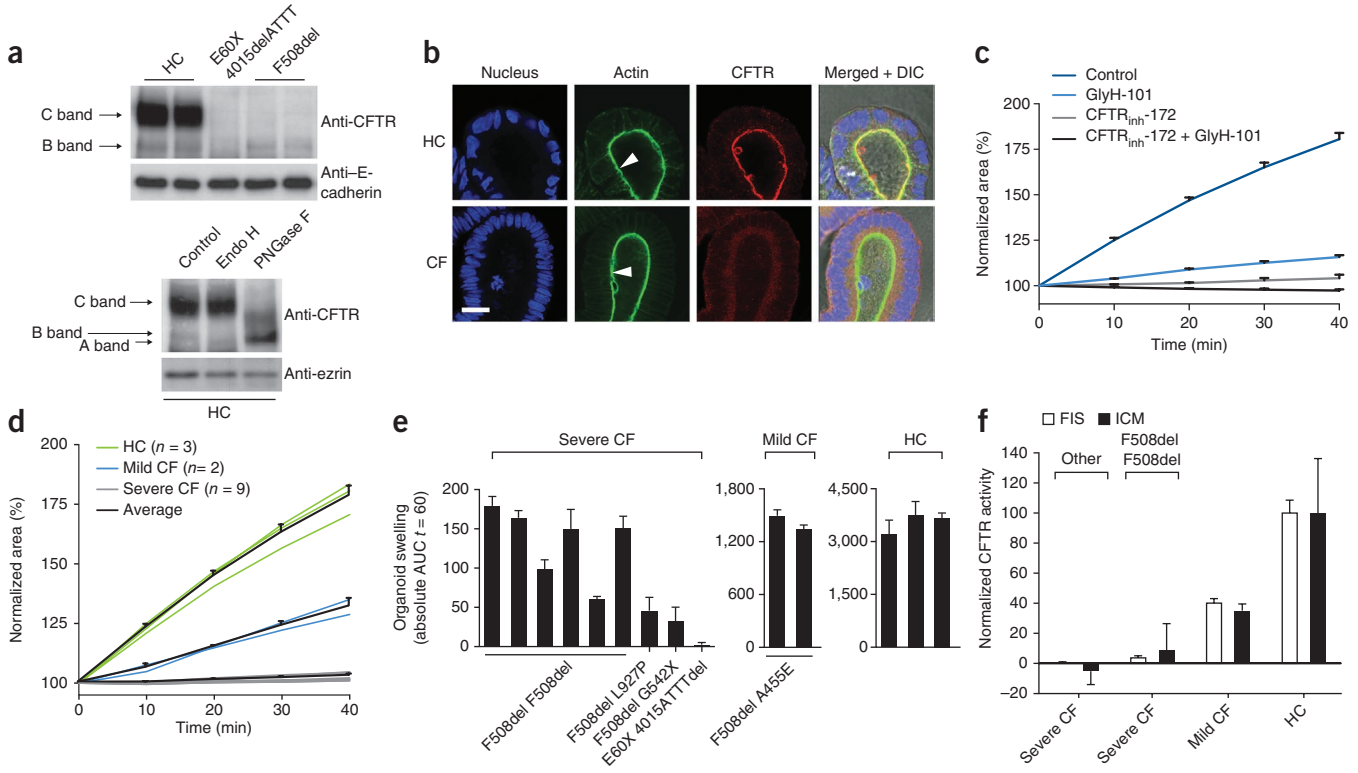

Figure 3 Forskolin-induced swelling in human organoids is CFTR dependent. (**a**) Western blot analysis of CFTR and E-cadherin (loading control) expression in human rectal healthy control (HC; $n = 2$), E60X 4015delATTT ($n = 1$) and homozygous F508del CFTR ($n = 2$) organoids (top) and CFTR and ezrin (loading control) expression in whole-cell lysates of human rectal organoids that were either not treated (control) or treated with the deglycosylation enzyme Endo H or PNGase F (bottom). (**b**) CFTR detection by the monoclonal antibody to CFTR M3A7 in a healthy control and F508del CFTR organoid co-stained with phalloidin-FITC (actin) and DAPI (nucleus). DIC and fluorescence were imaged using confocal microscopy. Scale bars, 20 µm. CF, cystic fibrosis. The arrowheads indicate the apical membrane. (**c**) Quantification of forskolin-induced healthy control organoid swelling after preincubation with DMSO, $CFTR_{inh}$-172, GlyH-101 or both $CFTR_{inh}$-172 and GlyH-101 (mean ± s.e.m.). (**d**) Forskolin-induced swelling of rectal organoids derived from three individual healthy controls, two individuals with a mild cystic fibrosis genotype (F508del A455E) and nine individuals with a severe cystic fibrosis genotype (one individual with E60X 4015ATTTdel, one with F508del G542X, one with F508del L927P and six with F508del F508del). The average swelling in the different groups is indicated in black (mean ± s.e.m.). (**e**) FIS responses of organoids from healthy controls or subjects with cystic fibrosis expressed as the absolute area under the curve (AUC) calculated from the time periods shown in **d** (baseline = 100%, $t = 60$ min). Each bar represents AUC values averaged from at least three independent experiments per individual (mean ± s.e.m.). (**f**) Comparison of CFTR activity measured by FIS of organoids from healthy controls and subjects with cystic fibrosis or by ICMs of the corresponding rectal biopsies. ICM bars of the different indicated groups represent forskolin-induced CFTR-dependent cumulative chloride secretion (µA cm^{-2}) relative to the average healthy control response (set at 100%). FIS bars represent FIS expressed as the AUC averaged from at least three independent experiments per individual as shown in **f** relative to the average healthy control response (100%). (Healthy controls, $n = 3$; mild cystic fibrosis, $n = 2$; severe cystic fibrosis (F508del F508del), $n = 5$; severe cystic fibrosis (other; E60X 4015ATTTdel and F508del G542X), $n = 2$; mean ± s.d.). All results are representative of at least three independent experiments. ICMs were performed on four rectal biopsies.

VRT-325, Corr-4a, C8 (http://cftrfolding.org) and VX-809 (ref. 36) and the potentiator VX-770 (ref. 9). Incubation of CFTR F508del homozygous organoids at low temperature (27 °C) increased FIS (**Fig. 4a**). We next established dose-response curves for a single treatment of VX-809 (preincubated for 24 h) or VX-770 (added simultaneously with forskolin) in organoids from six subjects homozygous for CFTR F508del (**Fig. 4b**) and measured half-maximal effective concentration (EC_{50}) values of 135 ± 40 nM (mean ± s.d.) and 161 ± 39 nM for the two treatments, respectively. These dose responses are within the ranges reported in human bronchial epithelial cells[9,36]. VX-809 combined with VX-770 induced increased FIS, which was abolished by chemical CFTR inhibition (**Fig. 4c**). We next assayed FIS after 24 h of preincubation with multiple correctors in F508del homozygous organoids. All correctors increased FIS, albeit with different efficacies (**Fig. 4d–f**, **Supplementary Video 3** and **Supplementary Fig. 5a**). We observed that VRT-325 and Corr-4a or C8 and Corr-4a synergistically increased FIS, which was in contrast to the additive effect of VRT-325 and Corr-4a in mouse organoids (**Fig. 2g**). Restoration of the function of F508del CFTR by temperature and chemicals were all sensitive to CFTR inhibitors (**Fig. 4a**,**c–f**). These data indicate that FIS can reliably measure correction or potentiation of F508del CFTR.

We next studied FIS responses to a panel of CFTR-restoring drugs in rectal organoids derived from nine individuals harboring various severe *CFTR* mutations, including six F508del homozygous organoids. We observed different amounts of drug-induced FIS between various F508del homozygous organoids (**Fig. 5a–c** and **Supplementary Fig. 5b**). FIS was variable between organoids after incubation with single drugs, and the distribution of high and low responders was unique for a restoration approach (**Fig. 5a–c**). The CF5 organoid seems to be a low responder to any corrector or to VX-770 but showed an exceptionally small response to VRT-325. The CF3 and CF5 organoids showed similar responses to VX-809 but differed in their responses to

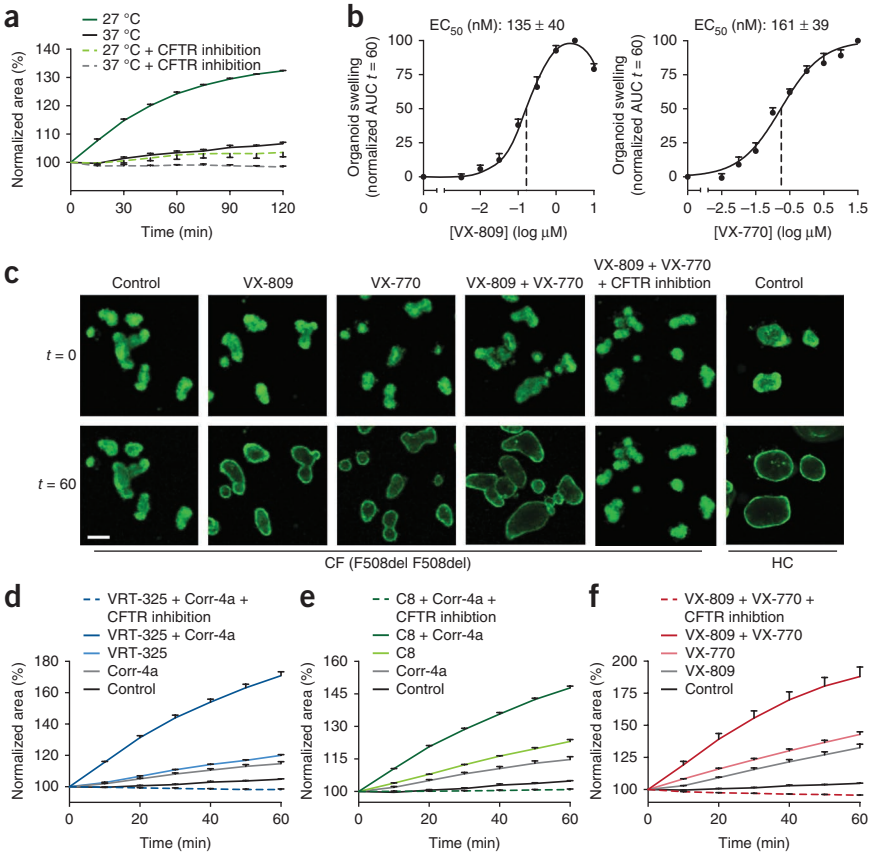

Figure 4 Chemical CFTR correction in human rectal organoids from subjects with cystic fibrosis. (**a**) Normalized swelling of forskolin-induced calcein green–labeled F508del CFTR organoids cultured for 24 h at 37 °C or 27 °C with or without CFTR inhibition (mean ± s.e.m.). (**b**) EC_{50} values of F508del organoids for VX-809 or VX-770. The lines represent FIS expressed as the AUC (baseline 100%, $t = 60$ min) calculated from the time periods shown in **d**–**f** relative to DMSO-treated (0%) and VX-809-treated (log(0.5) μM) or VX-770-treated (log(1.5) μM) (100%) organoids ($n = 6$ F508del homozygous organoids; mean ± s.e.m.). (**c**) Representative confocal microscopy images of calcein green–labeled healthy control and F508del CFTR organoids in response to forskolin after pharmacological restoration of CFTR. Scale bars, 100 μm. (**d**–**f**) Time lapse analyses of normalized forskolin-induced swelling of F508del CFTR organoids pretreated for 24 h with DMSO, VRT-325 (10 μM), Corr-4a (10 μM) or both correctors with or without CFTR inhibition (**d**), with DMSO, C8 (10 μM), Corr-4a (10 μM) or both correctors with or without CFTR inhibition (**e**) or stimulated with the corrector VX-809 (24 h pretreatment; 3 μM), the potentiator VX-770 (simultaneously with forskolin; 3 μM) or a combined compound treatment with or without CFTR inhibition (**f**) (mean ± s.e.m.).

C8. The measured FIS over the expected FIS (additive values of single treatment; **Supplementary Fig. 6**) indicated stronger synergy between VRT-325 and Corr-4a than between C8 and Corr-4a and was relatively constant between most organoids. All F508del compound heterozygous organoids also responded to correction, but no correction or potentiation was observed in E60X 4015delATTT organoids (**Fig. 5a**–**c**). We next compared the efficacy of approaches to restore the function of F508del CFTR by comparing the amount of FIS to that in mock-treated F508del A455E organoids or healthy control organoids (**Fig. 5d**). This comparison indicated that VX-809 is the most potent corrector and combined treatment with VX-809 and VX-770 induced FIS beyond the amounts in F508del A455E organoids, reaching ~60% of the FIS in healthy controls. Together these results demonstrate that the potency of CFTR-targeting compounds to restore CFTR function varies widely between organoids from individuals with cystic fibrosis, including CFTR F508del homozygous organoids.

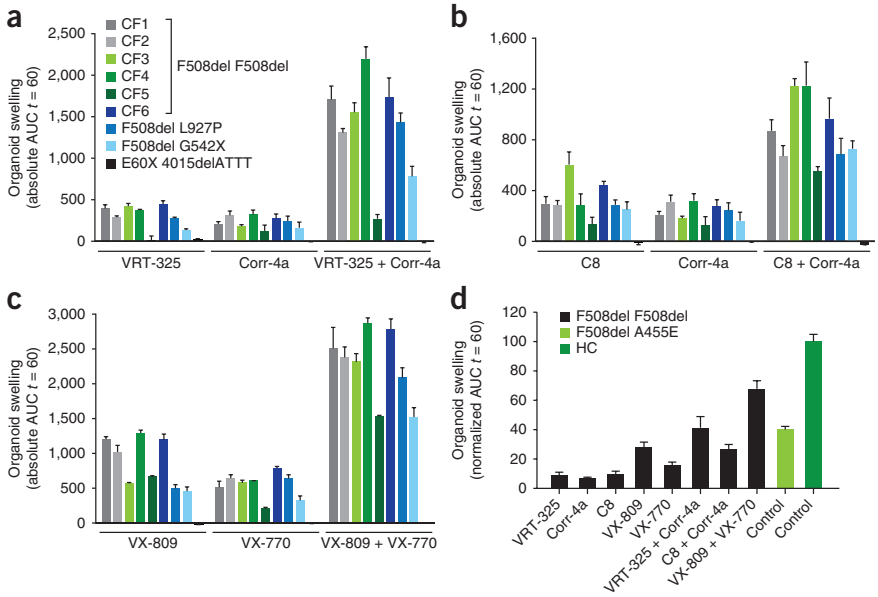

Figure 5 Differential FIS between organoids from subjects with cystic fibrosis after chemical CFTR restoration. (**a**–**c**) Quantification of FIS in organoids derived from nine individuals with cystic fibrosis pretreated for 24 h with VRT-325 (10 μM), Corr-4a (10 μM) or both correctors (**a**), with C8 (10 μM), Corr-4a (10 μM) or both correctors (**b**) or stimulated with VX-809 (24 h pretreatment; 3 μM), VX-770 (simultaneously with forskolin; 3 μM) or both compounds (**c**). The bars correspond to the bars shown for the 'severe CF' group in **Figure 3e**. Each bar represents FIS expressed as the AUC calculated from the time periods shown in **Figure 4d–f** (baseline = 100%, $t = 60$ min) corrected for the FIS of DMSO-treated organoids and averaged from at least three independent experiments performed at weekly intervals (mean ± s.e.m.). (**d**) Average FIS responses of compound-treated F508del F508del organoids ($n = 6$ from **a**–**c**) and DMSO-treated F508del A455E organoids ($n = 2$) relative to the average FIS of DMSO-treated healthy control organoids ($n = 3$) expressed as the AUC calculated from the time periods shown in **Figure 4d–f** (baseline = 100%, $t = 60$ min; mean ± s.e.m.).

DISCUSSION

Our results collectively indicate that FIS of mouse and human intestinal organoids is CFTR dependent. Rapid FIS probably results from the near-physiological characteristics of intestinal organoids. Previous data have indicated that forskolin can increase luminal expansion in organoid-like structures grown from renal MDCK cells, colonic LIM1863 cell lines or mouse intestinal spheroids[20,37,38], but the larger amplitude and rate of the FIS probably results from higher CFTR expression levels in the primary tissue culture model used here.

Fluid transport measured by FIS in rectal organoids correlated to the ICMs performed on the corresponding rectal suction biopsies. The FIS assay can therefore be a valuable supplement to the electrical measurements of CFTR function currently carried out in cystic fibrosis treatment and research centers and may complement ICM data. Using ICMs and FIS, we found that most subjects harboring CFTR F508del showed some residual CFTR function, suggesting that F508del CFTR is expressed at low levels at the apical surface[39,40]. This is also supported by the induction of FIS by the potentiator VX-770 in the absence of correctors, as was previously reported for human bronchial epithelial cells[9]. Clinical data also support low apical expression of F508del CFTR in subjects homozygous for F508del[41].

The paired FIS and ICM allows for the comparison of fluid secretion rates and ion fluxes as measured by ICM. On the basis of the geometry of organoids during FIS and the assumptions that the average organoid lumen is a sphere and the average swelling is similar in all three dimensions and is linear during an experiment, we calculated an initial fluid secretion rate of 26 ± 23 µl (mean ± s.d.) h^{-1} cm^{-2} in healthy control organoids (corresponding to an estimated current of 1.0×10^2 µA cm^{-2} on the basis of isotonic chloride secretion). When we assumed isotonic chloride secretion during ICM, we estimated that the measured currents would correspond to an approximate fluid secretion rate of 12 µl h^{-1} cm^{-2}. This rate largely exceeds the values reported previously for cysts from MDCK cells[42] and airway epithelium[43].

We demonstrate that FIS can measure functional restoration of CFTR by drugs. Notably, we observed that drug responses between organoids derived from subjects with cystic fibrosis are variable, even between CFTR F508del homozygous organoids. This raises the possibility that this *in vitro* assay may predict *in vivo* drug responsiveness of individual subjects. An ideal therapeutic model for cystic fibrosis would be to screen the effectiveness of available CFTR-restoring drugs directly after diagnosis to optimize treatment at the personal level before disease onset. Personalized medicine approaches may also facilitate the development and approval of drugs to which only subgroups of subjects respond and limit the economic risks associated with drug research. Furthermore, FIS of organoids can be used for approval of drugs in subjects that are genotypically mismatched with drugs that have been validated for a specific *CFTR* genotype. Interim phase 2 results of a current trial published on websites of the North American Cystic Fibrosis Foundation (http://www.cff.org/) and Vertex (http://www.vrtx.com/) indicate that drug responses to VX-809 and VX-770 together or to VX-770 alone[14] are highly variable between subjects harboring CFTR F508del. However, the predictive potential of organoid-based measurements of CFTR function for *in vivo* drug responsiveness remains to be established.

Individual drug responses may be predicted currently using *ex vivo* rectal biopsies[44] or primary airway tissue culture models[45]. Compared with these techniques, organoid cultures seem superior in generating large data sets from individual subjects. CFTR function analysis in organoid cultures is relatively easy, fast and robust. The organoids autodifferentiate into tissue-recapitulating structures in 96-well plates, allowing for the measurement of up to 80 organoids per well and up to 96 conditions per experiment. In this format, dose-response curves measured in triplicate for multiple drugs per individual can be easily generated at multiple culture time points, as we demonstrate here.

Using our image analysis approach, higher throughput approaches to identify new compounds that restore CFTR function may be developed. When we compare the drug responses in organoids with the limited clinical data that have been published in subjects homozygous for CFTR F508del[13,14] (http://www.cff.org/), only the combination of VX-809 and VX-770 has been reported to improve lung function in approximately 50% of these individuals. This combination induces approximately 1.5-fold more FIS in CFTR F508del homozygous organoids as compared to untreated F508del A455E organoids and up to 60% of the FIS seen in healthy controls. It is not uncommon that treatment effects in *in vitro* models are superior to the effects measured *in vivo*, but the correction in the FIS assay also exceeds the correction in cultured human bronchial epithelium by approximately twofold[9,36]. This might indicate that tissue-specific factors may control corrector efficacy. It is also probable that FIS rates are underestimated in healthy control organoids when CFTR expression is no longer rate limiting for FIS beyond a particular threshold by, for example, basolateral ion transport. These data suggest that new CFTR-restoring drugs may have clinical impact when FIS reaches up to ~60% of the FIS in wild-type organoids.

Two important aspects of organoid cultures render this technology highly suitable for follow-up studies. First, organoids can be greatly expanded while maintaining intact stem cell compartments during long-term culture (over 40 passages)[16]. The generation of large numbers of cells will aid cell biological and biochemical studies of CFTR-dependent cellular alterations and is a prerequisite for high-throughput screens. Second, primary cell banks from individuals with cystic fibrosis can be generated by storage of organoids in liquid nitrogen. These can be used to identify and study cellular factors associated with clinical phenotypes and would allow for the analysis of newly developed drugs at the individual level using materials that have been acquired previously.

In addition, this assay may be suitable for the development of drugs to treat secretory diarrhea, a life- threatening condition that results from CFTR hyperactivation by pathogenic toxins such as cholera toxin[46] (**Supplementary Fig. 7**), and for electrolyte homeostasis studies in general.

We describe a quick and robust assay for the quantification of CFTR function using a primary intestinal culture model that recapitulates essential features of the *in vivo* tissue architecture. This relatively simple assay will facilitate diagnosis, functional studies, drug development and personalized medicine approaches in cystic fibrosis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.F.D. performed and designed experiments, interpreted results and wrote the manuscript. C.L.W. performed experiments. H.R.d.J. performed ICMs and interpreted data and reviewers' comments. I.B. isolated rectal biopsies and performed ICMs. H.M.J. included subjects with cystic fibrosis. K.M.d.W.-d.G. included subjects with cystic fibrosis. A.M.B. performed western blot analyses. N.W.M.d.J. performed CFTR mRNA analyses. M.J.C.B. provided mouse Cftr knockout materials. B.J.S. provided mouse Cftr F508del materials. E.E.S.N. interpreted data and reviewers' comments. S.v.d.B. generated reagents for human and mouse organoid cultures. H.C. funded organoid media and interpreted data and reviewers' comments. C.K.v.d.E. obtained funding, interpreted data and reviewers' comments and included subjects with cystic fibrosis. S.M. included healthy control subjects and interpreted data and reviewers' comments. J.M.B. obtained funding, designed experiments, interpreted experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. *Cftr*^{tm1Cam} knockout (*Cftr*$^{-/-}$) mice[24] were backcrossed with FVB mice, and *Cftr*^{tm1eur} (Cftr F508del)[25,26] were backcrossed with C57BL/6 (F12) mice. Congenic FVB *Cftr*$^{-/-}$ mice (male) or C57BL/6 Cftr F508del mice (male and female) were used with their wild-type littermates. The mice were maintained in an environmentally controlled facility at Erasmus MC Rotterdam, and the animal studies were approved by the Erasmus MC ethical committee.

Human material. Approval for this study was obtained by the ethics committees of the University Medical Centre Utrecht and Erasmus MC Rotterdam. Informed consent was obtained from all subjects participating in the study. Rectal organoids from healthy controls and subjects with cystic fibrosis were generated from four rectal suction biopsies after ICMs obtained (i) during standard cystic fibrosis care (one individual each harboring E60X 4015ATTTdel, F508del G542X or F508del L927P and five individuals harboring F508del F508del), (ii) for diagnostic purposes (one healthy control) or (iii) during voluntary participation in studies approved by the University Medical Center Utrecht and Erasmus MC ethics committees (two healthy controls and one individual harboring F508del F508del). Material from an individual homozygous for F508del CFTR and a healthy control was derived from proximal ileum rest sections after surgery for meconium ileus. Four duodenal biopsies were obtained from two subjects with cystic fibrosis by flexible gastroduodenoscopy to generate F508del F508del and F508del exon17del organoids. The same procedure was used to obtain four biopsies from two subjects with suspected celiac disease. The biopsies were macroscopically and pathologically normal and used to generate healthy control organoids.

Crypt isolation and organoid culture from mouse intestine. Mouse organoids were generated from isolated small intestinal crypts and maintained in culture as described previously[15]. R-spondin 1 (Rspo1)-conditioned medium (stably transfected Rspo-1 HEK293T cells were kindly provided by C.J. Kuo, Department of Medicine, Stanford, CA) was used instead of recombinant Rspo-1 and was added to the culture medium at a 1:10 dilution. *Cftr*$^{-/-}$ and Cftr F508del organoids were obtained from proximal and distal small intestinal segments, respectively. Organoids from passages 1–10 were used for confocal imaging.

Crypt isolation and organoid culture from human biopsies. Crypt isolation and culture of human intestinal cells has been described previously[16]. In short, biopsies were washed with cold complete chelation solution and incubated with 10 mM EDTA for 30 (small intestine) or 60 (rectum) min at 4 °C. The supernatant was harvested, and EDTA was washed away. Crypts were isolated by centrifugation and embedded in Matrigel (growth factor reduced, phenol free; BD bioscience) and seeded (50–200 crypts per 50 µl Matrigel per well) in 24-well plates. The Matrigel was polymerized for 10 min at 37 °C and immersed in complete culture medium consisting of advanced DMEM/F12 supplemented with penicillin and streptomycin, 10 mM HEPES, Glutamax, N2, B27 (all from Invitrogen), 1 µM *N*-acetylcysteine (Sigma) and the following growth factors: 50 ng ml^{-1} mouse epidermal growth factor (mEGF), 50% Wnt3a-conditioned medium (WCM) and 10% noggin-conditioned medium (NCM), 20% Rspo1-conditioned medium, 10 µM nicotinamide (Sigma), 10 nM gastrin (Sigma), 500 nM A83-01 (Tocris) and 10 µM SB202190 (Sigma). The medium was refreshed every 2–3 d, and organoids were passaged 1:4 every 7–10 d. Organoids from passages 1–10 were used for confocal live-cell imaging. For production of WCM and NCM, Wnt3a-producing L Cells (ATCC, CRL-264) were selected for high-expressing subclones, and human full-length noggin was stably transfected into HEK293T cells, respectively. Amounts and activity of the expressed factors in each batch were assessed using dot blots and luciferase reporter plasmids (TOPflash and FOPflash; Millipore) as described previously[47,48].

Stimulation assays. Human or mouse organoids from a 7-day-old culture were seeded in a flat-bottom 96-well culture plate (Nunc) in 5 µl Matrigel commonly containing 20–80 organoids and 100 µl culture medium. One day after seeding, organoids were incubated for 60 min with 100 µl standard culture medium containing 10 µM calcein green (Invitrogen). For optimal CFTR inhibition, organoids were preincubated for 2 (mouse) or 3 h (human) with 50 µM $CFTR_{inh}$-172, 50 µM GlyH-101 or their combined treatment (both from Cystic Fibrosis Foundation Therapeutics, Inc). After calcein green treatment (with or without CFTR inhibition), 5 µM forskolin was added, and organoids were directly analyzed by confocal live-cell microscopy (LSM710, Zeiss, ×5 objective). Three wells were used to study one condition, and up to 60 wells were analyzed per experiment. For CFTR correction, organoids were preincubated for 24 h with 10 µM VRT-325, 10 µM Corr-4a, 10 µM C8 (all from Cystic Fibrosis Foundation Therapeutics, Inc), 3 µM VX-809 (Selleck Chemicals LLC, Houston, USA) or combinations thereof, as indicated. For CFTR potentiation, 3 µM VX-770 (Selleck Chemicals LLC) was added simultaneously with forskolin. Dilutions of VX-809 and VX-770 were used as indicated in **Figure 4b**.

Quantification of organoid surface area. Forskolin-stimulated organoid swelling was automatically quantified using Volocity imaging software (Improvision). The total organoid area (*xy* plane) increase relative to that at $t = 0$ of forskolin treatment was calculated and averaged from three individual wells per condition. The AUC was calculated using Graphpad Prism.

Statistical analyses. A Kolmogorov-Smirnov test was used to test whether the ICM and FIS data were normally distributed. A paired Student's *t* test was used to compare FIS with or without preselection of responding organoids (**Supplementary Fig. 1e**). A Spearman's rank correlation test was used to correlate ICM measurements with organoid swelling (**Supplementary Fig. 4c**). $P < 0.05$ was considered statistically significant. All data were analyzed in SPSS statistics version 20.0 for Windows.

RNA isolation and quantitative PCR. RNA was isolated with the RNeasy minikit (Qiagen) and quantified by optical density from human duodenal organoids that were cultured for >12 weeks. cDNA was synthesized from 1 µg of RNA by performing RT-PCR (Invitrogen). From mouse small intestinal organoids that were cultured for >6 weeks, RNA was isolated using TRIzol (Invitrogen) and quantified by optical density. cDNA was generated from 500 ng using the iScript cDNA synthesis kit (Bio-Rad). mRNA levels of human *CFTR* and mouse *Cftr* were determined by quantitative real-time RT-PCR with the SYBR Green method (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) or β2m mRNA abundance was used to measure cDNA input.

Western blot analysis. For CFTR protein detection, organoids from healthy controls or subjects with cystic fibrosis were lysed in Laemmli buffer supplemented with complete protease inhibitor tablets (Roche). Lysates were analyzed by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% milk protein in TBST (0.3% Tween, 10 mM Tris, pH 8, and 150 mM NaCl in H_2O) and probed overnight at 4 °C with a combination of the mouse monoclonal CFTR-specific antibodies 450, 570 and 596 (1:5,000, Cystic Fibrosis Folding consortium) followed by incubation with goat mouse-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000, Jackson ImmunoResearch, 115-035-146) and enhanced chemiluminescence (ECL) development. For CFTR deglycosylation, healthy control organoids were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% triton) supplemented with complete protease inhibitor tablets (Roche) and incubated with PNGase F and Endo H for 3 h at 33 °C (both from New England BioLabs).

Immunocytochemistry. Complete organoids from a 5-day-old culture were incubated with methanol (Sigma) for 10 min at −20 °C. Organoids were probed with the mouse monoclonal CFTR-specific antibody M3A7 (1:25, Abcam, AB4067) for 16 h at 4 °C followed by simultaneous incubation of Alexa Fluor 649–conjugated secondary antibodies (1:500, Jackson ImmunoResearch, 115-495-205) and phalloidin-FITC for 1 h at 4 °C (1:200, Sigma). Organoids were embedded in Mowiol containing DAPI (1:10,000) and analyzed by confocal microscopy as described previously[49].

ICM. Trans-epithelial chloride secretion in human rectal suction biopsies (four per subject) was measured as described previously[34] using a recent amendment (repetitive prewashing)[35], which better accentuates forskolin-induced

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anion current responses by reducing basal cAMP levels. In short, the biopsies were collected in PBS on ice and directly mounted in adapted micro-Ussing chambers (aperture. 1.13 or 1.77 mm^2). After equilibration, the following compounds were added in a standardized order to the mucosal (M) or serosal (S) side of the tissue: amiloride (0.01 mM, M), to inhibit amiloride-sensitive electrogenic Na^+ absorption; carbachol (0.1 mM, S), to initiate cholinergic Ca^{2+}-linked and protein kinase C–linked Cl^- secretion; DIDS (0.2 mM, M), to inhibit DIDS-sensitive, non-CFTR Cl^- channels such as the Ca^{2+}-dependent Cl^- channels; histamine (0.5 mM, S), to reactivate the Ca^{2+}-dependent secretory pathway and measure the DIDS-insensitive component of Ca^{2+}-dependent Cl^- secretion; and forskolin (0.01 mM, S), to fully activate CFTR-mediated anion secretion. Crude short circuit current values (μA) were converted to μA cm^{-2} on the basis of the surface area of the aperture.

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