#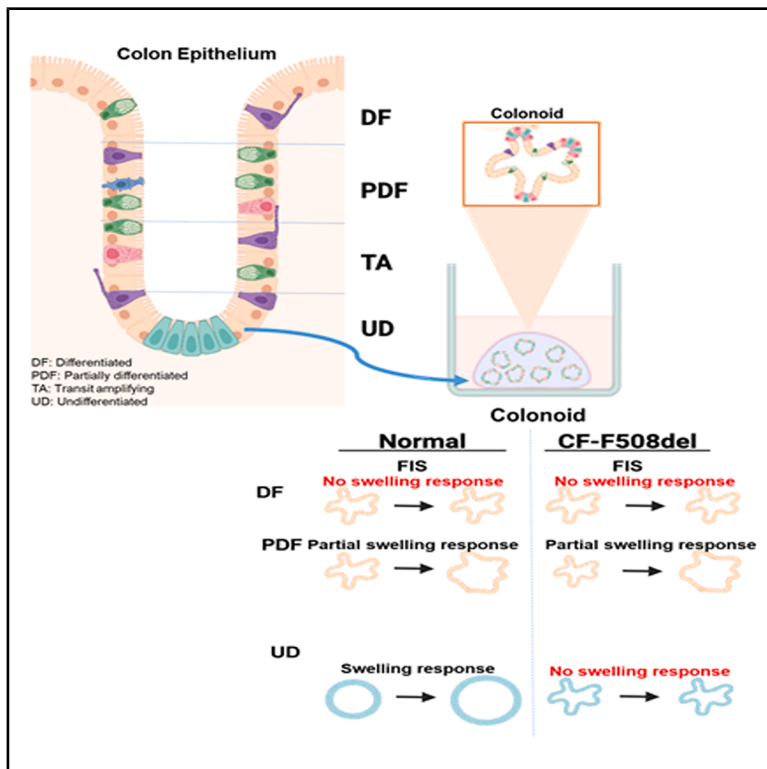 Partially differentiated ileal and distal-colonic human F508del-cystic fibrosis-enteroids secrete fluid in response to forskolin and linaclotide

Graphical abstract

Authors

Yan Rong, Zixin Zhang,
Hugo R. de Jonge, ..., Chung-Ming Tse,
Mark Donowitz, Varsha Singh

Correspondence

vsingh11@jhmi.edu

In brief

Genetics; Molecular biology

Highlights

- CFTR-mediated fluid secretion decreases in differentiated enterocytes

- F508del-CFTR showed active fluid secretion in partially differentiated enterocytes

- Linaclotide-stimulated fluid secretion requires differentiated enterocytes

- Linaclotide and PDEinh enhance the CFTR-modulator response in F508del-CF-enteroids

CellPress

iScience

Article

Partially differentiated ileal and distal-colonic human F508del-cystic fibrosis-enteroids secrete fluid in response to forskolin and linaclotide

Yan Rong,[1] Zixin Zhang,[1] Hugo R. de Jonge,[3,6] Ruxian Lin,[1] Huimin Yu,[1] Rafiq Sarker,[1] Dario Boffelli,[4] Rachel K. Zwick,[5] Ophir D. Klein,[4,5] Chung-Ming Tse,[1] Mark Donowitz,[1,2] and Varsha Singh[1,7,*]

[1]Division of Gastroenterology & Hepatology, Department of Medicine, Baltimore, MD 21205, USA
[2]Department of Cellular and Molecular Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[3]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, P. O. Box 2040, 3000CA Rotterdam, the Netherlands
[4]Department of Pediatrics, Cedars-Sinai Guerin Children's, Los Angeles, CA 94143, USA
[5]Program in Craniofacial Biology and Department of Orofacial Science, University of California, San Francisco, CA 94143, USA
[6]Deceased
[7]Lead contact
*Correspondence: vsingh11@jhmi.edu
https://doi.org/10.1016/j.isci.2025.112453

SUMMARY

Constipation causes significant morbidity in patients with cystic fibrosis (CF). Using CF patient (F508del) derived *ex vivo* ileal and distal colonic/rectal enteroids as a model and the Forskolin Induced Swelling Assay (FIS), we compared CFTR mediated fluid secretion in human enterocytes across the crypt-villus axis. CFTR expression and FIS decreased as enterocytes differentiated from crypt to become partially differentiated and then mature villus cells. While there was no FIS response in undifferentiated (crypt enterocytes) F508del-CF enteroids, partially differentiated F508del-CF enteroids had a swelling response to forskolin (cAMP) and lina-clotide (cGMP), which was ∼48%, and ∼67% of the response in healthy enteroids, respectively, and was pre-vented by a CFTR inhibitor. Also, linaclotide and a general phosphodiesterase (PDE) inhibitor independently enhanced the combined CFTR-modulator-induced FIS response from partially differentiated F508del-CF en-teroids. These findings demonstrate that partially differentiated ileal and distal colonic F508del-CFTR enter-oids can be stimulated to secrete fluid by cAMP and cGMP.

INTRODUCTION

The treatment of patients with cystic fibrosis (CF) with correc-tors/potentiators that target CFTR has led to life prolongation and reduced the REQUIREMENTS for major interventions such as lung transplantation.[1–3] Consequently, additional morbidities associated with CF are becoming more frequently recognized, and attempts to develop solutions have become a high priority. Chronic constipation is a significant complication of CF, which affects ∼47% of patients with CF, despite the appropriate treat-ment of pancreatic insufficiency, as well as concurrent CFTR modulator therapy.[4,5] In patients with CF, impaired anion (chlo-ride (Cl⁻) and bicarbonate (HCO₃) secretory defects reduce fluid secretion across many epithelia. Reduced anion secretion-driven intestinal water secretion, as well as tenacious mucus, are thought to be the major causes of constipation in CF. How-ever, the constipation is contributed to by multiple additional fac-tors that include altered small intestinal or colonic enteric ner-vous system (ENS)/motor function, incompletely corrected malabsorption contributing to bulky stools, colonic inflamma-tion, and changes in the microbiome.[6–8] In addition, the accumu-

lation of thick luminal mucus acts as a physical barrier to the ab-sorption of some nutrients. Current treatments of constipation in patients with CF primarily include laxatives, enemas, and surgi-cal intervention, with no standard of care yet established.[9,10]

Significant advances have been made in determining whether patients with specific CFTR mutations will respond to corrector/potentiator therapy by using an *ex vivo* functional assay based on Forskolin-Induced-Swelling (FIS) in patient-derived intestinal or-ganoids or enteroids.[11–13] These enteroids can be maintained in a crypt-like, undifferentiated state when grown in media contain-ing growth factors Wnt3A, R-spondin, and Noggin, or they can be differentiated into villus-like or colonic surface cells by removal of Wnt3A and R-spondin for 5–6 days.[14–16] In addition, we recently recognized that growth factor removal for 3–4 days resulted in an enterocyte population midway between the crypt-like Cl⁻ secretory cells and the well-differentiated villus or colonic surface enterocytes that take part exclusively in Na⁺ absorptive function.[14] This represents a significant population of enterocytes that express transport proteins involved in NaCl absorption as well as anion secretion, including sodium-hydrogen exchanger-3 (SLC9A3, NHE3), chloride-bicarbonate

exchanger-3 (SLC26A3, DRA), and CFTR. This population is called partially differentiated enterocytes (abbreviated PD). We show in this study that ileal and distal colonic/rectal PD enteroids from the most common CFTR mutant-F508del, exhibit positive, CFTR-dependent fluid secretion in response to forskolin (cAMP) and the cGMP agonist linaclotide, unlike a lack of response in the undifferentiated CF enteroids. We further showed that linaclotide and a general phosphodiesterase inhibitor, theophylline, independently enhanced CFTR-modulator-induced fluid secretion from PD enterocytes. Based on these pre-clinical studies, we suggest that strategies should be developed to include ways to stimulate fluid secretion from PD enterocytes in the treatment of constipation in patients with CF.

RESULTS

Forskolin-induced swelling response is present in partially differentiated enteroids

The FIS response has been used to evaluate CFTR function by quantifying the rates of fluid secretion as rates of enteroid swelling.[11] To define the FIS response in normal distal colonic/rectal (to be referred to as colonic/rectal) enteroids from normal healthy subjects (NHS), we compared fluid secretion in these enteroids grown in Wnt3A, R-spondin, and Noggin (undifferentiated (UD)), and the same enteroids grown in the absence of Wnt3A and R-spondin for 3 days/partially differentiated (PD)) and 6 days/fully differentiated (FD). Enteroid cross-sectional surface area was measured using live-cell microscopy, which showed a rapid expansion of both the lumen and total enteroid surface area in response to forskolin treatment, whereas DMSO-treated enteroids were minimally changed over the 1h study (Figures 1A and 1B). As shown in Figures 1C–1E, a forskolin-stimulated, time-dependent surface area increase occurred in both UD ($55.0 \pm 6.0\%$, $p < 0.05$ vs. DMSO) and PD ($36.0 \pm 4.2\%$ $p < 0.05$ vs. DMSO) NHS colonic/rectal enteroids at 60min, while FD enteroids showed no significant increase in the surface area ($7.0 \pm 4.2\%$) as compared to DMSO control. The FIS response in UD, PD, and FD enteroids correlates with the CFTR protein expression at different days of differentiation[14] (Figures S1A and S1B). To demonstrate that the FIS response was CFTR dependent and to evaluate the contribution of DRA, NHE3, and Calcium-activated Chloride Channels (CaCC), NHS colonic/rectal enteroids were preincubated for 1h with the CFTRinh- Benzopyrimido-pyrrolo-oxazine-dione) (BPO-27) (10μM), DRAinh A250 (10μM), NHE3inh S3226 (20 μM) or a general CaCCinh A01-(15μM).[17–20] Preincubation with BPO-27 significantly reduced FIS in both UD ($10.4 \pm 0.5\%$; $p < 0.05$ vs. Fsk) and PD ($11.5 \pm 0.5\%$; $p < 0.05$ vs. Fsk) NHS enteroids. In contrast, preincubation with the NHE3inh, DRAinh, and CaCCinh did not significantly affect the FIS response in either the UD or PD enteroids (Figures 1C–1E). This indicates no significant contribution of NHE3, DRA, or CaCC to the FIS assays in the normal colon/rectum. Although BPO-27 has been reported in fewer publications compared to another specific CFTR inhibitor, 172, and is known to interact with the CFTR ATP binding site, there is no evidence that BPO-27 has effects beyond inhibiting CFTR. Taken together, these results indicate that the FIS response in the colonic/rectal enteroids is primarily mediated

by CFTR activity, and the magnitude of the swelling response correlates with CFTR expression, both of which decrease with the enterocyte differentiation.

Partially differentiated F508del-cystic fibrosis enteroids have a positive cystic fibrosis transmembrane conductance regulator-dependent forskolin-induced swelling assay response

Previous studies in UD (undifferentiated) enteroids have established that CF enteroids do not respond to FIS unless the CFTR gene is corrected or treated with correctors/potentiators.[21–23] Since CFTR was expressed in PD enteroids, we compared the FIS response in UD, PD, and FD (fully differentiated) NHS (normal healthy subject) and F508del-CF colonic/rectal enteroids. As reported, UD F508del-CF enteroid lacked a FIS response ($6.5 \pm 1.1\%$). Unexpectedly, PD F508del-CF enteroids achieved a positive FIS response ($12.7 \pm 1.3\%$), while FD enteroids had a small non-significant response ($8.7 \pm 1.4\%$) (Figure 2A). The magnitude of the FIS response in PD F508del-CF enteroids was approximately half ($\sim48 \pm 1.6\%$) of the response in PD NHS enteroids (Figure 2B). This response was similar in colonic/rectal enteroids from three F508del-patients with CF tested independently (Figures S2A–S2C). A similar positive FIS response was observed in PD ileal enteroids from a F508del-CF patient (UD:$4.2 \pm 0.6\%$; PD: $12.6 \pm 0.4\%$; FD: $7.2 \pm 0.6\%$) (Figure S3).

Since UD F508del-CF colonic/rectal enteroids were phenotypically different from NHS, enteroids we quantified the initial steady-state lumen area (SLA) of PD enteroids from both groups. This was done by comparing the luminal area to the total enteroid area in each group (Figures S4A and S4B).[11] For the colonic/rectal enteroids, there were no significant differences in the initial SLA between PD NHS and F508del-CF enteroids.

To identify the transport proteins contributing to the FIS response in PD F508del-CF colonic/rectal enteroids, the assay was performed in the presence of CFTRinh-BPO-27 (10μM) and CaCCinh-A01 (15μM). Like the response in PD NHS enteroids, the FIS response from PD F508del-CF enteroids was significantly inhibited by pretreatment with BPO-27, while A01 did not affect the FIS response (Figure 2C). Thus, the positive FIS response in both NHS and F508del-CF PD enteroids is CFTR dependent, and F508del-CFTR secretes fluid in response to forskolin in this population. Due to these qualitatively similar FIS responses in ileal and colonic/rectal F508del-CF enteroids, results from colonic/rectal enteroids were reported for the rest of these studies.

To allow comparisons between the fluid secretory response between NHS and F508del-CF enteroids, differences in differentiation and cell types between F508del-CF and NHS enteroids, in UD, PD, and FD colonic/rectal enteroids were investigated. Compared to the NHS, UD F508del-CF enteroids had significantly higher mRNA expression of Leucine-rich repeat-containing G-protein coupled receptor-5 (*LGR5*) and *Ki67,* used as a marker for proliferation, both of which gradually decreased with differentiation in PD and FD enteroids (Figure S4C). The expression of the goblet cell marker *MUC2* mRNA increased with differentiation in PD and FD NHS enteroids, as well as in F508del-CF enteroids. In contrast, the expression of the
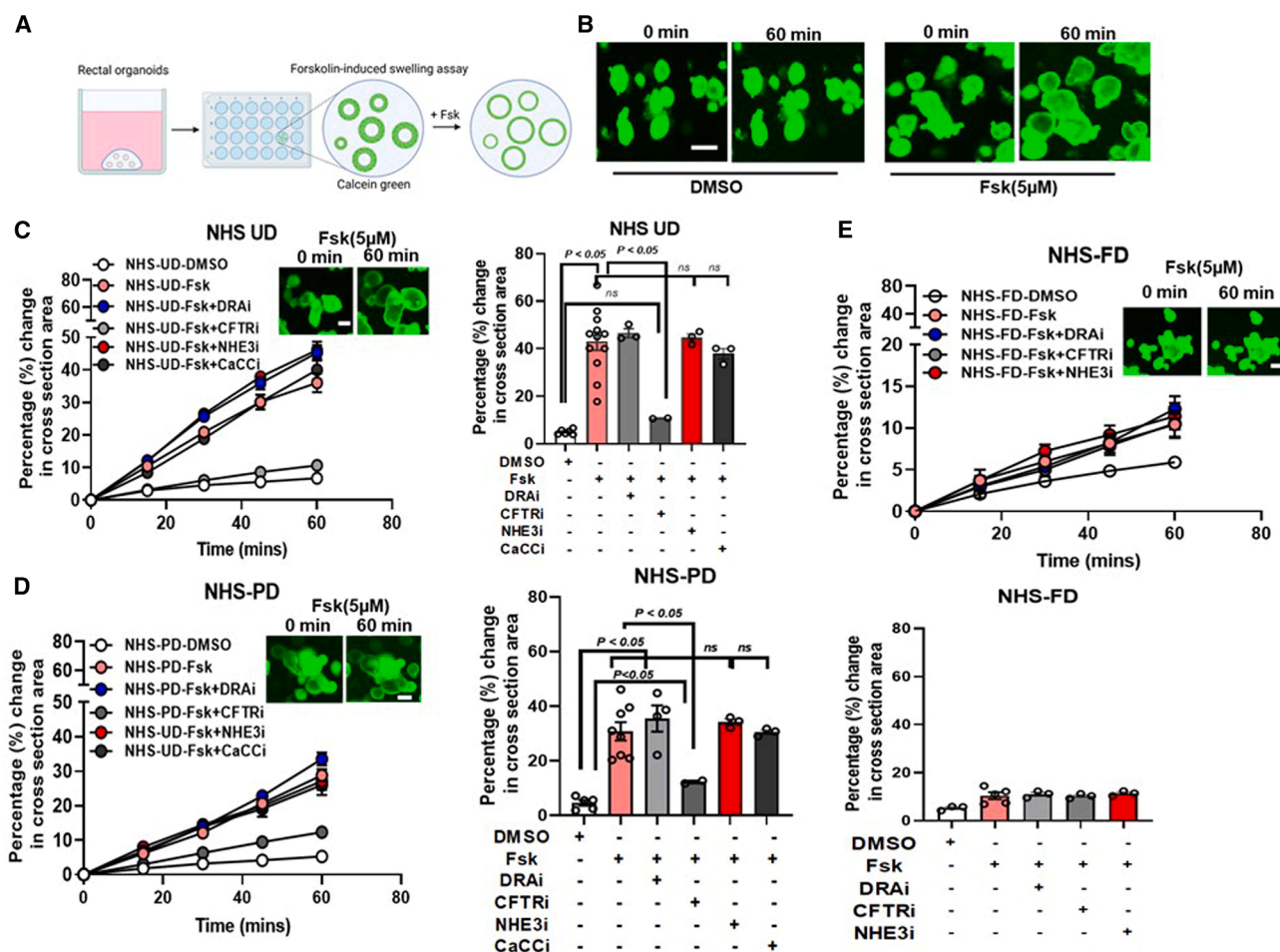
Figure 1. FIS response decreases but is present in partially differentiated enteroids

(A) Graphic illustration showing Forskolin-induced swelling (FIS) assay, and (B) representative fluorescence confocal images of a calcein green AM esters–labeled colonic/rectal enteroids from NHS treated with DMSO (control) or forskolin (Fsk, 5 μM). Images were taken at t = 0, and 60 minutes after stimulation. Scale bar: 10μm. Quantification of FIS in NHS-UD (C), PD (D), and FD (E), alone or pretreated with CFTRinh BPO-27 (10μM), DRAinh A250 (10μM), NHE3inh S3226 (20 μM), or general CaCCinh-A01 (15μM) for 1h. Inset images were taken at t = 0, and 60 min after stimulation. Scale bar: 10μm. Results are expressed as the percentage change in surface area relative to t = 0 (percentage change in cross-section area) measured at 15-min time intervals for 1 h (means ± SD), and the bar graph depicts fluid secretion plots (t = 60 min, means ± SEM, data points represent an average of 3–5 swelling assays in an individual donor, $N = 3$ separate NHS). Analysis of differences was determined with a one-way ANOVA and Bonferroni post hoc test; ns = not significant.

enteroendocrine (EE) marker chromogranin A (*CHGA*) was significantly higher in PD and FD F508del-CF enteroids than in NHS enteroids (Figures S4C and S4D). Thus, F508del-CF enteroids had higher proliferative activity than NHS in the UD condition, and a higher number of CHGA-positive EE cells in FD enteroids, but underwent differentiation that is similar to that in NHS enteroids.

The size of the PD enterocyte population has not been established. To estimate the size of this population, we further analyzed a published single-cell RNAseq dataset of normal human small intestine[24] using the pseudotime description of CFTR, DRA, and NHE3 expressing cells. Pseudotime analysis showed that CFTR expression declined linearly going from stem cells to mature enterocytes; in contrast, the expression of DRA and NHE3 was nearly absent in stem cells, transit amplifying (TA) cells, and enterocyte progenitors, increased in entero-

cytes, and was maximal in mature enterocytes (Figure S5). CFTR was significantly expressed in the area in which the expression of DRA and NHE3 was approaching their maximum levels (pseudotime between 150 and 200). In this interval, the co-expression between these transporters was as follows: CFTR/DRA 88%; CFTR/NHE3 27%; DRA/NHE3 26%; CFTR/DRA/NHE3 25% (Table S1 and Figure S5). Based on the pseudotime analysis, we propose that the cells at the transition zone from enterocytes to mature enterocytes likely represent PD enterocytes. This population represents 16% of villus enterocytes (calculated from cells in Table S1, upper).

Expression of DRA or inhibition of NHE activity does not contribute to fluid secretion in colonic/rectal enteroids

Previous studies in oocytes showed that SLC26 family members, SLC26A6 and SLC26A3, contribute to the cAMP stimulation of
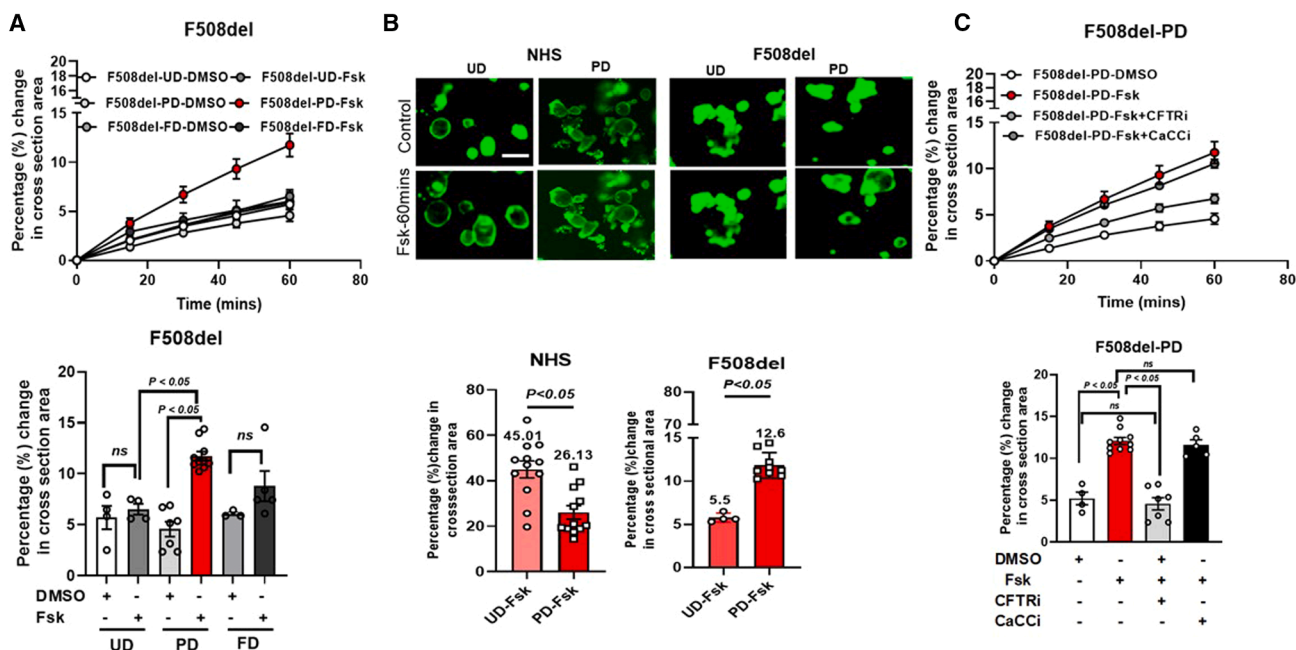
Figure 2. Partially differentiated F508del-CF colonic/rectal enteroids have a CFTR-dependent FIS response

(A) Quantification of FIS showing differences between UD, PD, and FD colonic/rectal enteroids from F508del-CF patients with CF. Results are expressed as the percentage change in surface area relative to t = 0 (percentage change in cross-section area) measured at 15-min time intervals for 1 h (means ± SD), and the bar graph depicts fluid secretion plots (t = 60 min, means ± SEM). Data points represent an average of 5–6 independent assays, each containing 50–100 enteroids from 1 CF patient (above). Combined results from all the 3 patients are shown (later in discussion).

(B) A representative fluorescence confocal images (above) of a calcein green-AM esters–labeled enteroids from NHS and F508del-CF in UD and PD state treated with DMSO (control) or forskolin (Fsk, 5 μM) at t = 0, and 60 min after stimulation. Later in discussion: bar graph of fluid secretion plots (t = 60 min, means ± SEM, data points represent an average of 10–15 independent assays from three NHS and F508del-CF patients with CF). Analysis of differences was determined using a one-way Student's t test. Scale bar: 10 μm.

(C) Quantification of FIS in PD F508del-CF pretreated with CFTRinh BPO-27 (10 μM), or CaCCinh-A01-(15μM) for 1h or alone. Results are analyzed as above and expressed as means ± SEM. An average of 3–12 swelling assays, each containing 50–100 enteroids from colonic/rectal enteroids from 3 separate F508del-patients with CF is shown. Analysis of differences in (A) and (C) was determined with a one-way ANOVA and Bonferroni post hoc test, ns = not significant.

CFTR activity.[25] Since SLC26A3 is expressed in large amounts throughout the human colon and its expression significantly increases with differentiation,[14,26] we investigated whether DRA contributes to the FIS response in partially differentiated (PD) F508del-CF colonic/rectal enteroids. This was evaluated in two ways: 1) by overexpressing DRA in undifferentiated (UD) enteroids; and 2) by knocking down DRA and determining FIS in PD enteroids. To overexpress DRA, a replication-deficient adenovirus containing the coding sequence of human DRA (Flag-DRA) or containing no insert (control) was used to infect NHS and F508del-CF enteroids, followed by performing a FIS assay in the UD state. As shown in Figure 3A, Fsk (5μM) caused a similar FIS response in UD control and Flag-DRA transduced NHS-colonic/rectal enteroids (control: 59.3 ± 2.0%; Flag-DRA: 62.6 ± 0.7%). UD F508del-CF enteroids had no secretory response to Fsk even after being transduced with Flag-DRA. Western blot analysis of enteroids collected post-assay confirmed the expression of Flag-DRA in infected enteroids, while UD enteroids do not express DRA (Figure 3A right).

Using lenti-shRNA against DRA or a scrambled sequence as control, a stable DRA knockdown was made in NHS and F508del-CF colonic/rectal enteroids. Western blot analysis of shRNA-infected cells showed reduced DRA protein expression

by >40% in both NHS and F508del-CF enteroids compared to the control (Figure 3B right). Furthermore, this reduction continued when these enteroids were maintained in a culture medium containing puromycin. These cells were used to measure the FIS response after partial differentiation. As shown in Figure 3B, PD colonic/rectal enteroids from both NHS and F508del-CF with reduced DRA expression had a significant FIS response (NHS: 22.2 ± 1.5%; F508del-CF: 12.0 ± 1.5%). This response was not significantly different than the control-shRNA expressing enteroids, which were 20.2 ± 1.02% for NHS and 11.7 ± 1.5% for F508del-CF (Figure 3B).

Partially differentiated colonic/rectal enteroids express a significant amount of NHE3, and these cells are also a major site for NHE2 expression.[27] Inhibition of NHE3 results in enhanced fluid accumulation into the intestinal lumen due to the loss of the Na$^+$ absorptive function of enterocytes.[24,28,29] We hypothesized that the FIS response from PD F508del-CF enteroids could be due to cAMP (Fsk) induced inhibition of brush border NHE3 activity. Therefore, we measured the FIS in PD enteroids pretreated (1h) with the specific NHE3 inhibitor, 20μM S3226 (3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-*N*-isopropylidene-2-methyl-acrylamide dihydro-chloride), or with CFTRinh BPO-27 (10μM).[19,30] Pretreatment with 2 μM dimethyl
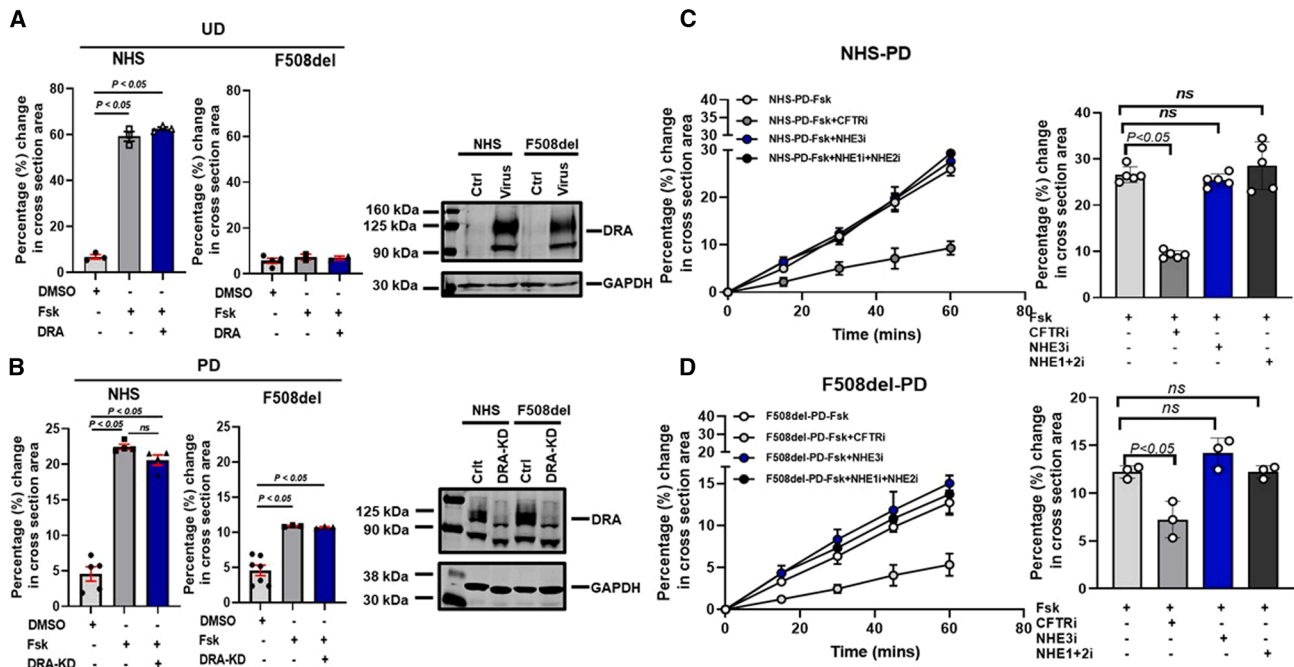
Figure 3. Expression of DRA or inhibition of NHEs does not affect fluid secretion in NHS and F508del-CF colonic/rectal enteroids

Bar graph shows the quantitation of FIS response in colonic/rectal enteroids from (A) UD NHS and F508del-CF overexpressing control or DRA-adenovirus with representative Western blot (right) of enteroids collected post-assay show the overexpression of Flag-DRA, and (B) PD NHS and F508del-CF enteroids overexpressing control or DRA-shRNA, with representative Western blot (right) showing KD of DRA in DRA-shRNA (DRA-KD) expressing enteroids. Data are means ± SEM. Quantification of FIS in PD colonic/rectal enteroids from (C) NHS and (D) F508del-CF pretreated with BPO-27 (10μM), dimethyl amiloride (2μM), or S3226 (20μM). Results are expressed as the percentage change in the cross-sectional (surface) area relative to t = 0, measured at 15-min time intervals for 1 h (means ± SD), and the bar graph depicts fluid secretion plots (t = 60 min, means ± SEM). Data points represent an average of 3–5 independent assays, each containing 50–100 enteroids from one NHS or F508del CF patient. Combined results from $N = 3$ independent NHS and F508del-patients with CF are shown. Analysis of differences in (A), (B), (C), and (D) was determined with a one-way ANOVA and Bonferroni's post hoc test, ns = not significant.

amiloride (DMA) was used to inhibit NHE2 and NHE1 activities, to ensure the specificity of the contribution of NHE3. As shown in Figures 3C and 3D, the FIS response in NHS and F508del-CF colonic/rectal enteroids was not affected by exposure to DMA or S3226, compared to the FIS in their absence, while the CFTR inhibitor reduced the FIS response as in Figures 1 and 2. Overall, these results indicate that the FIS response in colonic/rectal enteroids does not depend upon the expression of DRA, NHE3, NHE2, or NHE1 activities.

Linaclotide stimulates fluid secretion from partially differentiated colonic/rectal enteroids

Since PD F508del-CF colonic/rectal enteroids achieved a partial fluid secretion in response to cAMP (FSK) (Figures 2A and 2B), we investigated the effect of another second messenger, cGMP, on fluid secretion from PD enteroids. For this, we used the guanylate cyclase-C (GC-C) agonist linaclotide. Linaclotide stimulates the production and accumulation of intracellular cGMP, which acts as a second messenger to enhance fluid secretion in the intestine.[31,32] The effect of linaclotide on CFTR regulation, particularly in human colonic/rectal samples, and the pathways associated with these effects have not been reported. The linaclotide (10μM) effects on the swelling response in UD, PD, and FD NHS, and F508del-CF colonic/rectal enteroids

were determined over 2hrs. As shown in Figures 4A and 4B, linaclotide did not cause a swelling response in UD NHS (4.5 ± 2.1%) or F508del-CF (5.7 ± 1.4%) enteroids. In PD and FD NHS enteroids, linaclotide alone (in the absence of forskolin) caused a time-dependent fluid secretion (PD: 18.3 ± 1.3%; FD: 13 ± 1.1%). The linaclotide-induced swelling from PD NHS enteroids was significantly higher than that from FD enteroids ($p < 0.05$ vs. PD). Similarly, PD F508del-CF enteroids showed a significant swelling response to linaclotide (12.8 ± 0.5%). Linaclotide did not cause a statistically significant swelling response in FD F508del-CF enteroids, compared to the untreated conditions (9.4 ± 0.4% vs. 13.9 ± 2.2%; p = ns) (Figure 4B). The swelling response to linaclotide in PD F508del-CF enteroids was ∼67% of the linaclotide response in PD NHS enteroids (Figures 4A and 4B).

Linaclotide is known to employ the cGMP/cyclic-GMP-dependent kinase II (cGKII) pathway to elicit intestinal fluid secretion. To confirm that the essential signaling machinery is present, in addition to the presence of GCC (Figures S4C and S4D), we examined the cGKII expression in UD, PD, and FD colonic/rectal enteroids from NHS and F508del-patients with CF. As a positive control, lysate from Caco-2/bbe cells transduced with adenovirus containing the coding sequence for rat cGKII was used.[33] A 70kDa cGKII protein band was detected in lysates from Caco2-bbe-cGKII cells, but not from either NHS or F508del-CF
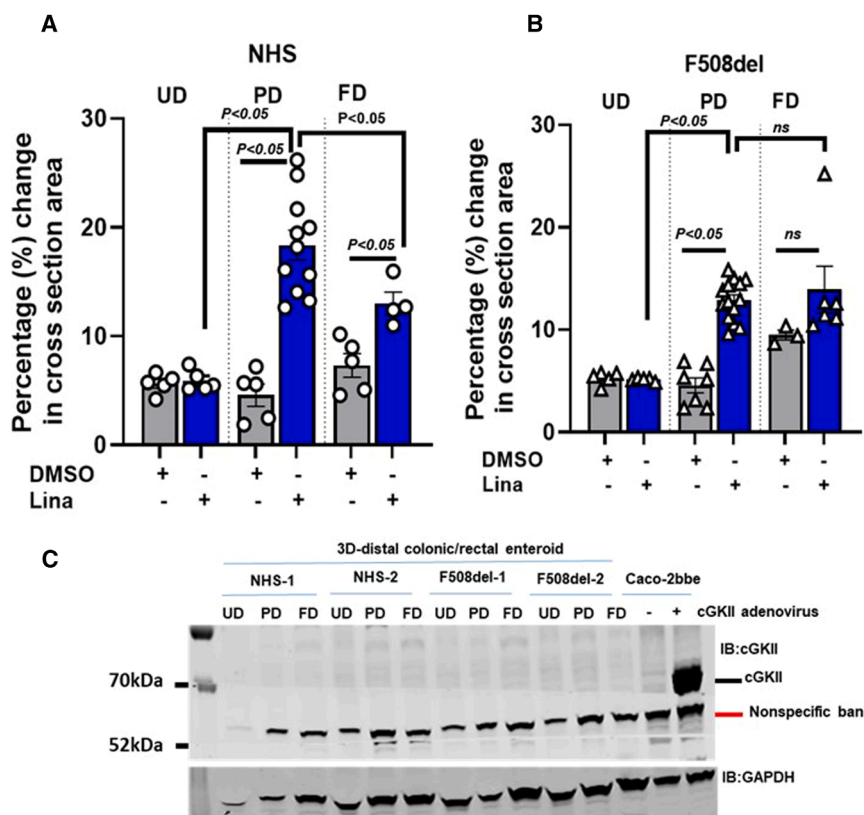
Figure 4. Linaclotide stimulates fluid secretion from partially differentiated colonic/rectal enteroids

Quantification of linaclotide (10μM) effect on swelling response in UD, PD, and FD colonic/rectal enteroids from (A) NHS and (B) F508del-patients with CF measured over a 2h period. A time-dependent increase in organoid surface area normalized to that at t = 0 was calculated. Results are represented as a bar graph depicting fluid secretion plots (t = 120 min, means ± SEM). Data points represent an independent experiment consisting of 50–100 enteroids, $N = 3$ separate NHS or F508del-CF. Analysis of differences was determined with a one-way ANOVA and Bonferroni post hoc test, ns = not significant. (C) Representative Western blot from SDS-PAGE analysis of lysates prepared from UD, PD, and FD colonic/rectal enteroids from 2 separate NHS and F508del-patients with CF and lysates from Caco2-bbe cells expressing control or cGKII-adenovirus as a positive control.

enteroids, while a lower-sized non-specific band was present in lysates from NHS and F508del-CF enteroids as well as in Caco-2 cells not transduced with cGKII (Figure 4C). Overall, these results suggest that linaclotide stimulates fluid secretion from PD distal colonic/rectal enteroids that do not express significant amounts of cGKII.

Linaclotide-stimulated fluid secretion in colonic/rectal enteroids is partially CFTR- and NHE3-dependent and involves additional transport processes

To determine the contribution of CFTR to linaclotide-induced fluid secretion, PD NHS and F508del-CF enteroids were preincubated with BPO-27 (10μM) for 1h before the linaclotide-induced swelling assay. Preincubation with BPO-27 partially reduced the linaclotide effect by ∼33% (18.8 ± 1.4; 12.4 ± 0.7) in NHS and ∼16% (13 ± 0.4; 11 ± 0.7) in F508del-CF PD enteroids (Figures 5A and 5B). This indicates that while CFTR contributes to linaclotide-mediated fluid secretion from PD NHS and F508del-CF distal colonic/rectal enterocytes, an additional transport protein(s) is involved. Previous reports suggest that linaclotide-induced gut fluidity is caused by the inhibition of NHE3 activity.[34] The way NHE3 inhibition could contribute to the linaclotide-induced swelling response would be by reducing the rate of fluid absorption from the enteroids in response to the cGMP increase. Thus, to understand the role of NHE3 in the linaclotide response from PD enteroids, we pretreated the enteroids with NHE3inh S3226 (20 μM) and then measured linaclotide-induced swelling. We observed a partial decrease in linaclotide

response in the presence of S3226 in PD NHS as well as in F508del-CF distal colonic/rectal enteroids (NHS:14.4 ± 1.1%; F508del-CF:10.6 ± 0.7%) (Figure 5C). We also investigated whether a combination of S3226 (20 μM) + BPO-27 (10μM) would further inhibit the linaclotide-induced swelling response. The pretreated combination of S3226 + BPO-27 further blocked the effect of linaclotide, but there was a significant residual linaclotide swelling response in both NHS (10.5 ± 2.0%) and F508del-CF (8.8 ± 1.03%) enteroids. This suggests the involvement of an additional transport protein(s) beyond NHE3 and CFTR in the linaclotide-induced swelling from PD enteroids in both NHS and F508del-CF distal colonic/rectal enteroids.

F508del-cystic fibrosis treatment with combined cystic fibrosis correctors (elexacaftor+tezacaftor) and a potentiator (ivacaftor) partially increases cystic fibrosis transmembrane conductance regulator-mediated fluid secretion from undifferentiated and partially differentiated colonic/rectal enteroids

Since CFTR correctors and a potentiator increase the activity of F508del-CFTR, the response of these CF drugs in undifferentiated (UD) and partially differentiated (PD) F508del-CF colonic/rectal enteroids was determined. For these studies, the enteroids were exposed to CF correctors: elexacaftor+tezacaftor 3μM each for 24h and the potentiator: ivacaftor 5μM for 1h. This is the combination of corrector and potentiator approved by the FDA as triple therapy in Trikafta.[35,36] We refer to this combination as combined CFTR-modulators. Undifferentiated and PD F508del-CF enteroids were pretreated with elexacaftor+tezacaftor 3μM each for 24h and with ivacaftor for an hour, and FIS was measured and compared with DMSO-treated control enteroids; in addition, the effects of DRAinh A250 (10μM) and CFTRinh BPO-27 (10μM) were also determined. As shown in
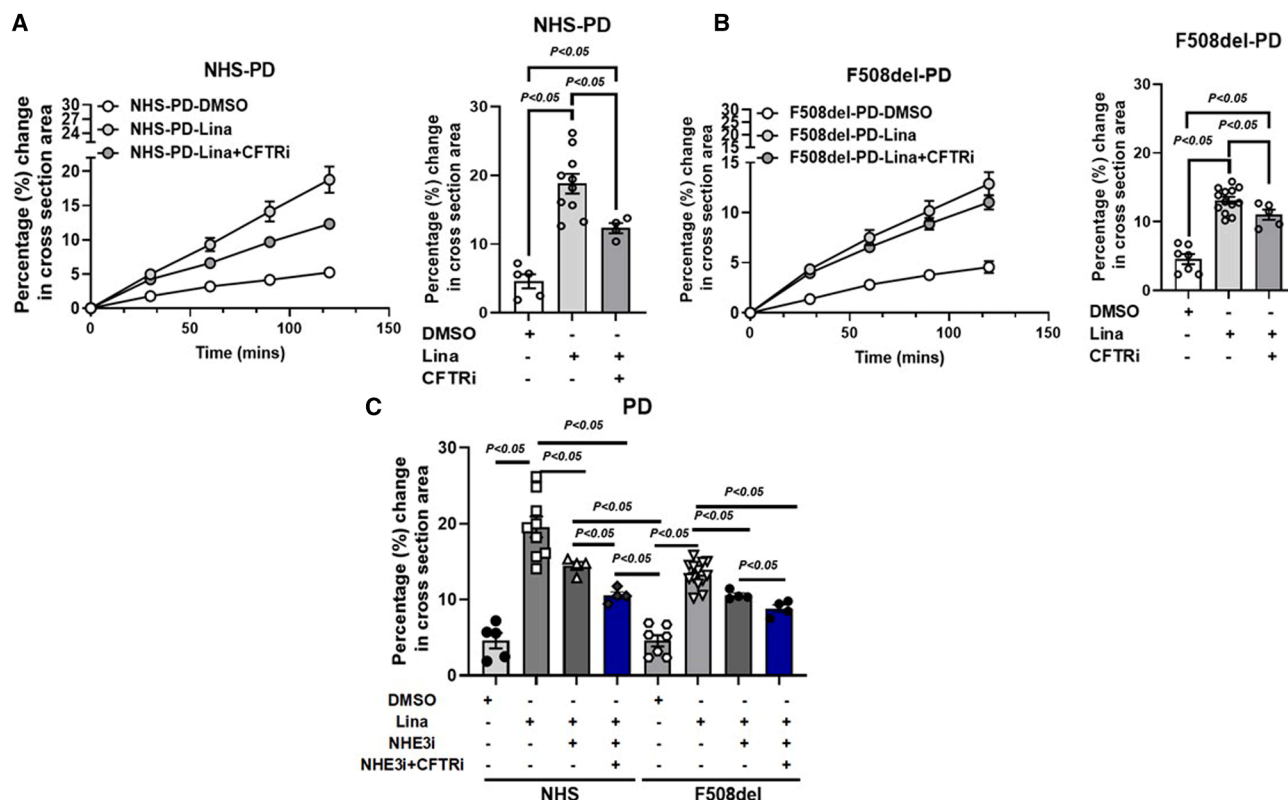
Figure 5. Linaclotide-stimulated fluid secretion in colonic/rectal enteroids was partially blocked by CFTRinh-BPO, and NHE3inh S3226

The role of CFTR in Linaclotide (10μM)-induced swelling was measured by pretreating PD enteroids from (B) NHS and (D) F508del-patients with CF with BPO-27 (10μM). A time-dependent increase in enteroids surface area normalized to that at t = 0 was measured at 15-min time intervals for 2 h (means ± SD). Bar graph depicting fluid secretion plots (t = 120 min, means ± SEM). Data points represent an independent experiment consisting of 50–100 enteroids, N = 3 separate NHS and F508del-CF. Data points represent an independent experiment consisting of 50–100 enteroids, N = 3 separate NHS and F508del-CF. Analysis of differences in (A) and (B) was determined with a one-way ANOVA and Bonferroni post hoc test.

(C) Linaclotide-induced swelling in PD NHS (left) and F508del-CF distal colonic/rectal enteroids (right) was performed alone or after pretreatment with S3226 (20 μM) or S3226 (20 μM) + BPO-27 (10μM) for 1h. An increase in enteroid surface area in response to Linaclotide (10μM) normalized to that at t = 0 was analyzed as above and expressed as a bar graph depicting fluid secretion plots (t = 120 min, means ± SEM). Data points represent an average of 2–3 independent assays in enteroids from NHS and F508del-patients with CF, N = 3 separate NHS and F508del-CF. p value was determined by 1-way ANOVA with Bonferroni's adjustment for multiple comparisons.

Figure 6A, UD F508del-CF enteroids had a positive FIS response only after treatment with combined CFTR-modulators (27.1 ± 2.0%). This FIS response was less than the response in UD NHS enteroids performed in the absence of a combined CFTR-modulator (43 ± 3.8%, Figure 1C). The Fsk response in combined CFTR-modulator treated F508del-CF enteroids was reversed by pretreatment with the CFTRinh (8.1 ± 1.0%) but not by the DRAinh (Figure 6A). A similar assay was performed in PD F508del-CF enteroids. The PD F508del-CF enteroids without CF correcting drugs had a FIS response of 11.9 ± 0.4%; when treated with the combined CFTR-modulators, the FIS response was enhanced to 18.1 ± 1.5%, $p < 0.05$. As in UD enteroids, significant FIS response was lost with pretreatment with the CFTR inhibitor but not with the DRAinh (Figure 6B). Together, these results suggest that the correctors/potentiators that make up Trikafta increase CFTR function from UD and PD F508del-CFTR enteroids. F508del-CFTR is partially activated by the cAMP in PD enteroids, and the addition of combined CFTR-modulators can further enhance this activity.

A non-selective phosphodiesterase inhibitor, theophylline, and linaclotide independently enhance the response of the combined CFTR-modulators on fluid secretion in F508del-CF colonic/rectal enteroids.

The effect of the combined CFTR-modulators on the FIS response from undifferentiated (UD) and partially differentiated (PD) distal colonic/rectal F508del-CF enteroids was lower than the FIS response in untreated NHS enteroids. To maximize the effect of the combined CFTR-modulators, we investigated the effect of two pharmacological compounds: i) a non-selective phosphodiesterase (PDE) inhibitor, theophylline, and ii) linaclotide, on the combined CFTR-modulators' effect on fluid secretion. Phosphodiesterases (PDEs) are a group of enzymes that catalyze the hydrolysis of phosphodiester bonds of the second messengers, cAMP, and cGMP, thus reducing the cyclic nucleotide levels.[37,38] Inhibition of PDEs has been used to stimulate CFTR activity and to treat patients with asthma and chronic obstructive pulmonary disease.[39] Colonic/rectal enteroids were pretreated with theophylline (100μM-1h), which was present

A

F508del UD

- ○ F508del-UD-DMSO
- ○ F508del-UD-Fsk
- ○ F508del-UD-Fsk+CF_modulator
- ◐ F508del-UD-Fsk+CF_modulator+DRAi
- ● F508del-UD-Fsk+CF_modulator+CFTRi

F508del UD

DMSO	+	-	-	-	-
Fsk	-	+	+	+	+
CF modulator	-	-	+	+	+
DRAi	-	-	-	+	-
CFTRi	-	-	-	-	+

B

F508del-PD

- ○ F508del-PD-DMSO
- ○ F508del-PD-Fsk
- ○ F508del-PD-Fsk+CF_modulator
- ◐ F508del-PD-Fsk+CF_modulator+DRAi
- ● F508del-PD-Fsk+CF_modulator+CFTRi

F508del-PD

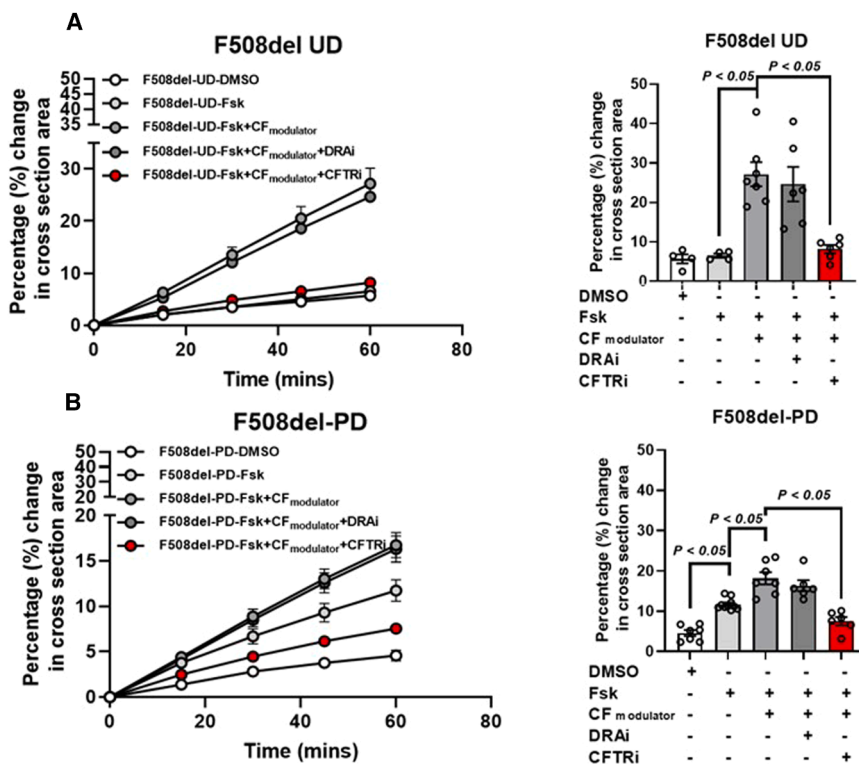| DMSO | + | - | - | - | - |
Fsk	-	+	+	+	+
CF modulator	-	-	+	+	+
DRAi	-	-	-	+	-
CFTRi	-	-	-	-	+

Figure 6. F508del-CF correction with combined CFTR-modulators enhances CFTR-mediated fluid secretion from UD and PD colonic/rectal enteroids

FIS in (A) UD and (B) PD F508del-CF colonic/rectal enteroids under basal or in response to combined CFTR-modulators (tezacaftor:VX-661+elexacaftor:VX-445 3µM each for 24h followed by ivacaftor:VX-770 (5µM) for 1h) pretreated with vehicle or BPO-27 (10µM) or A250 (10µM). Untreated or combined CFTR-modulators-treated colonic/rectal enteroids were stimulated with Fsk (5µM), CFTR$_{inh}$ BPO-27, and DRA$_{inh}$ A250. Results are expressed as the percentage change in surface area relative to t = 0 measured at 15-min time intervals for 1 h (means ± SD), and the bar graph depicts fluid secretion plots (t = 60 min, means ± SEM). An average of 3–12 swelling assays, each containing 50–100 enteroids from distal colonic/rectal enteroids from N = 3 separate F508del-patients with CF is shown. Analysis of differences was determined with a one-way ANOVA and Bonferroni post hoc test.

throughout the assay period. As shown in Figure 7A, the FIS response in UD NHS enteroids was significantly enhanced due to PDEinh (Fsk:45 ± 3.7; Fsk+PDEinh: Fsk:65 ± 2.1; $p < 0.05$). In UD F508del-CF enteroids, theophylline exposure did not cause a FIS response, unless CF enteroids were exposed to the combined CFTR-modulators (24.5 ± 2.0%), which allowed theophylline to significantly enhance the fluid secretion (37.6 ± 1.1%; $p < 0.05$) (Figure 7B). Similarly, in PD F508del-CF enteroids the FIS response was not significantly affected by theophylline treatment (13.3 ± 5.1%; 11.9 ± 5.1%; NS); however, the combined CFTR-modulators increased FIS was significantly enhanced by theophylline treatment (16.3 ± 1.4%; 22.4 ± 2.1%; $p < 0.05$) (Figure 7B). Colonic/rectal enteroids from all three F508del-patients with CF had a similar theophylline-related enhancement of the combined CFTR-modulators stimulated secretion (Figure S6). Of note, F508del-CF colonic/rectal enteroids had significantly higher mRNA expression of PDE (PDE4) than NHS (Figure S4C).

For the linaclotide response, the fluid secretion was measured in the combined CFTR-modulators treated UD and PD colonic/rectal enteroids from F508del-patients with CF in response to Fsk alone or with linaclotide (10µM) over a 2h period. As shown in Figure 7C, UD and PD F508del-CF colonic/rectal enteroids treated with the combined CFTR-modulators had significant fluid secretion in response to Fsk (UD:26.3 ± 2.9%; PD:19.2 ± 1.3). Linaclotide further enhanced this fluid secretion but only in PD F508del-CF enteroids (Fsk +combined CFTR-modulators:19.2 ± 1.3; Fsk+combined CFTR-modulators+linaclotide: PD:27.7 ± 2.5%, $p < 0.05$). Linaclotide enhanced the fluid response in PD enteroids in each

of the three F508del-patients with CF (Figure S7). These results suggest that the inhibition of PDE and the addition of linaclotide should be considered for testing as a strategy to enhance the fluid

secretory effect of the combined CFTR-modulators in F508del patients with CF.

DISCUSSION

Constipation in patients with CF is multifactorial. However, given that the primary defect in CF relates to reduced anion secretion, an accepted driver of stool water amount, most emphasis in approaching ways to deal with CF patient constipation has been to improve the reduced anion secretion. Formal clinical trials of treatment for constipation in patients with CF are limited. In a study of 438 patients with CF on some combinations of elexacaftor/tezacaftor/ivcaftor, for up to 6 months, there were no clinically significant changes in bowel characteristics, symptoms related to chronic constipation, or constipation-related quality of life assessments.[40] Despite no evidence demonstrating efficacy, current approaches to treat CF constipation include the use of any of a multitude of laxatives, including lubiprostone, linaclotide, as well as osmotic agents, and take advantage of the demonstration that CFTR correctors plus potentiators enhance CFTR-mediated anion secretion in most patients with CF.[35,41] Based on our findings, we suggest that an effective strategy for enhancing the treatment of chronic constipation in patients with CF is to utilize a combination of agonists, which alone or together, enhance the increased fluid secretion caused by the correctors and potentiators, while also targeting the enterocyte populations studied in this research.

Using human enteroids as a preclinical model, we showed that CFTR is the major contributor to the fluid secretion in the distal colonic/rectal enterocytes, and its expression and secretory
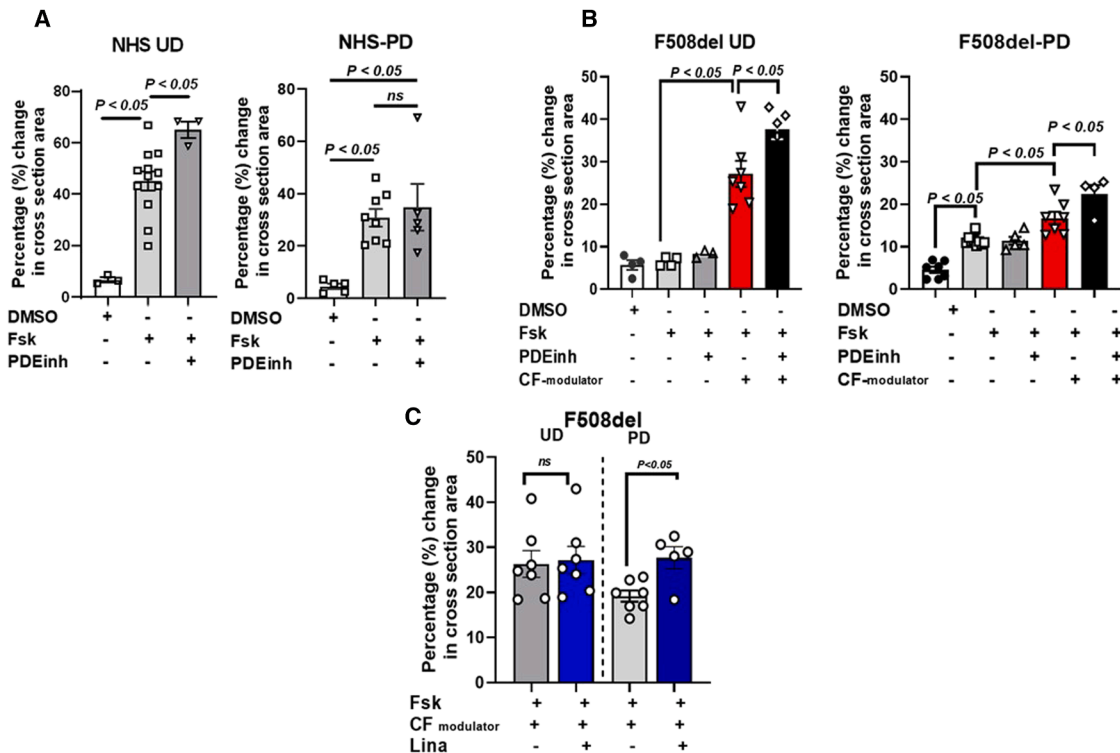
Figure 7. Theophylline and linaclotide independently enhance the combined CFTR-modulators response from F508del-CF colonic/rectal enteroid

(A) Effect of theophylline (100μM-1h) on FIS (5μM) response in UD, and PD enteroid from NHS.

(B) Effect of Theophylline (100μM-1h) or combined CFTR-modulators (tezacaftor +elexacaftor (3μM) for 24h; ivacaftor (5μM) for 1h) and in combination on FIS (5μM) in UD, and PD F508del-CF enteroid. Changes in surface area relative to t = 0 were calculated, and the bar graph depicts fluid secretion plots (t = 60 min, means ± SEM). Data points represent an independent experiment consisting of 50–100 enteroids, n = 3 separate NHS.

(C) Quantitation of Linaclotide effect on FIS response in combined CFTR-modulators treated UD and PD F508del-CF enteroids. Enteroids were pretreated with combined CFTR-modulators as above and were used to determine FIS in the presence of DMSO or Linaclotide (10μM). Results were analyzed as above and expressed as means ± SEM of fluid secretion plot at t = 120 min. Data points represent an average of 2–3 independent assays in enteroids from NHS and F508del-patients with CF, n = 3 separate NHS and F508del-CF. p value by 1-way ANOVA with Bonferroni's adjustment for multiple comparisons.

activity decrease as the enterocytes move from crypts to become differentiated villus/surface cells. In addition, we previously showed that as the crypt cells differentiate, they are called partially differentiated enterocytes, which express transport proteins and have characteristics of both the crypt and mature villus.[14] Here we demonstrate that in the small intestine, these cells express CFTR together with DRA and often with NHE3. This population of cells secretes fluid in response to forskolin (cAMP), although in smaller amounts compared to crypt cells. The percentage of the PD enterocyte population in the human small intestine and colon has not yet been established. The previously reported scRNA-seq analysis of the human small intestine has suggested that there is a significant population of villus enterocytes expressing DRA and CFTR (Table S1). This result is supported by the reanalysis of a recent human ileal single-cell-RNAseq report, including privileged information provided by *Magness* et al.*,* that divided ileal enterocytes into early (~UD), intermediate (~PD), and late (~FD) populations.[42] This result showed that a significant number of the intermediate enterocyte populations express DRA, CFTR, and NHE3. Nonetheless, the quantitative contribution of the PD enterocytes awaits single-

cell proteomic analysis. Importantly, NHE3, DRA, and CFTR, when present in the same cells (as in PD enterocytes), interact dynamically, supporting that there appears to be unique regulation, particularly of DRA in this population.[14,43]

We used the FIS assay developed by Dekkers et al. to evaluate the quantitative contribution of the PD enterocytes in comparison with the UD (crypt) and FD (villus) enterocyte populations.[44] Using human colonic/rectal enteroids, we showed that F508del-CF UD enteroids had a positive FIS response only following treatment with the combined CFTR-modulators and with the non-selective phosphodiesterase inhibitor theophylline. In contrast, PD F508del-CF enteroids had a positive FIS response to increased cAMP alone, which was further enhanced by theophylline, and by the combined CFTR-modulators, and there was increased secretion when the combined CFTR-modulators were studied together with theophylline. Additionally, PD F508del-CF enteroids secreted fluid in response to linaclotide, and there was a larger effect of the combined CFTR-modulators in the presence of linaclotide. Thus, this PD enterocyte population appears to represent a potentially important, previously unrecognized drug target for increasing intestinal (ileum and distal

colon/rectal) fluid secretion in patients with CF. The additivity of the effect of theophylline to the FIS assay and the correctors +potentiator response suggests that the assay is not saturating under the conditions of the study and that multiple agonists used together may be useful in the treatment of CF constipation.

Linaclotide is emerging as a promising candidate for constipation in patients with CF. However, no clinical studies of its efficacy have been reported on this patient population. Moreover, linaclotide use in treating otherwise healthy patients with chronic constipation is not effective in as many as 50% of patients.[45,46] As previously established, linaclotide causes its effects by binding to guanylate cyclase C (GCC) and by increasing cGMP. Of note, intestinal GCC expression varies widely based on species differences; there is significant mRNA expression in mouse colonic crypts, but as demonstrated in this study, not in human colonic crypts. Moreover, it was reported in the initial molecular identification of cGKII that rat transverse colon, distal colon, and rectum have very little cGKII, an observation shown here to be true in human colonic/rectal enteroids.[47] In this context, it is surprising that linaclotide caused fluid secretion in PD enteroids in both NHS and F508del-CF colonic/rectal enteroids, which lacked cGKII even though they did express GCC (Figure S3C). While not tested in this study, high concentrations of cGMP have been reported to activate CFTR via PKA in Xenopus oocytes that lack cGKII.[48,49]

The complex signaling for linaclotide is further indicated by reported studies of effects in (a) rat proximal colon, and (b) mouse jejunum, both of which express cGKII. In rat proximal colon, the linaclotide-stimulated Cl^- secretion was partially inhibited by the cGKII inhibitor H8, but no studies probed what mediated the residual component, although suggestions were made regarding the potential involvement of the cGMP activation of PKA or inhibition of a phosphodiesterase inhibitor.[50,51] To our knowledge, ours is the first report showing that linaclotide-induced fluid secretion occurs in the human distal colon/rectum and that this occurs specifically in the PD enterocyte populations. Of all the transport proteins investigated in the swelling assays of this study, CFTR contributed solely or partially to the swelling response, and NHE3 was the only other protein identified that contributed to the linaclotide-induced secretion. Importantly, a part of the linaclotide swelling response appeared to be independent of NHE3 plus CFTR, indicating the involvement of additional transport protein(s).

Our studies using normal human and F508del-CF enteroids add to the preclinical studies of ways to increase intestinal fluid secretion, which has been primarily carried out in mouse and rat intestines. The recognition that PD enterocytes represent a previously unrecognized enterocyte population with a unique distribution of transporters involved in Na^+ and Cl^- transport and with the unique acute regulation offers the opportunity to extend preclinical studies to understand ways to alter intestinal transport in patients with CF to increase intestinal fluid secretion. While the percentage of the PD enterocyte population remains under evaluation, the magnitude of the FIS response in PD F508del-CF colonic/rectal enteroids with combined CFTR-modulators was ~60% of that in UD F508del enteroids, even in the face of reduced CFTR expression. The mechanism for the CFTR-dependent fluid secretion from PD F508del-CF enteroids is not known, but it does not appear to involve an increased brush-border expression of CFTR since the C band was not increased with forskolin stimulation in the PD compared to the UD F508del-CF enteroids. We have not yet evaluated other potential mechanisms, such as changes in the open probability of the CFTR channel. A recent study utilizing artificially modified human enteroids demonstrated the significant role of BEST4/CA7$^+$ cells in maintaining electrolyte and fluid homeostasis in response to bacterial diarrheal toxins.[52] Further studies using single-cell RNAseq and proteomics from UD and PD F508del-CF distal colonic/rectal enterocytes are needed to pinpoint specific cell types and signaling molecules that are distinct to the PD population. Importantly, given that constipation in patients with CF is multifactorial, clinical studies in patients treated with combined CFTR-modulators such as Trikafta combined with additional agents (cAMP, and cGMP agonists, and PDEinh) to treat constipation will be required to define the most helpful regimens, which may vary between individuals as well as based on the nature of the CF mutation.

Limitations of the study

The size of the PD population in the human small intestine and colon has not yet been adequately defined. Single-cell RNAseq analysis of the human colon has not been reported as yet in parallel to the studies in the human small intestine shown in Figure S5 and reported in detail.[53] Evidence for the potential role of the PD population in *in vivo* studies, while suggestive of a role for this population, is not definitive. Consistent with this contribution is that the measurement of active anion secretion in the duodenum and colon of F508del mice demonstrated a small Cl^- dependent active anion secretion ($5\mu mol.cm^-h^-$).[54] Moreover, the multiple previous studies using human enteroids to evaluate CF-related CFTR function used Wnt, R-Spondin exposed, undifferentiated enteroids, and thus the role of partially differentiated cell population was never studied.

However, the presence of a partially differentiated colonic enterocyte population is supported by the similar maturation pattern of enterocytes from the small intestine and colon, with a similar although slightly longer half-life of the colonic enterocytes to approximately 5–7 days during which the crypt cells mature into surface enterocytes.[55] Importantly, while approximately two-thirds of normal human proximal colon in the Turner Laboratory Human Transport Atlas shows both CFTR and DRA in colonocytes in the upper crypt and/or surface cells, the cells expressing DRA with and without CFTR were intermingled. Thus, as in the past when we used undifferentiated vs. fully differentiated human enteroids grown by altering the presence of growth factors (Wnt, R-spondin) to model and allow study separately of the intestinal crypt and villus,[56] we support that we are similarly able to use the altered presence of growth factors to study separately undifferentiated, fully differentiated and partially differentiated colonocytes.

For this study, the F508del-CFTR mutant was chosen because this accounts for about 70% of CFTR mutations worldwide and is present in approximately 85% of patients with CF.[57,58] Future studies are needed to understand if the PD enterocyte population has a similar active role in mutations in the other classes of defective CFTRs, which would indicate that the proposed

pharmacological approach might be more generally useful for treating the constipation of patients with CF.

Given the limited sample size and the lack of data regarding the age and gender of patients with CF, we are unable to determine whether there are any differences in the response from the PD enterocyte population between male and female patients with CF.

Although enteroids demonstrate various cell types from their native tissue, they still exclusively contain epithelial cells. Thus, co-culture studies of intestinal enteroids with cell types such as ENS, endothelial, and immune cells are needed to examine the contribution of these cells to fluid secretion by interacting with epithelial cells. As of now, the co-culture systems remain relatively simple, incorporating only one or a few cell types, but failing to simulate the complexity of the intact intestine.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Varsha Singh (vsingh11@jhmi.edu).

Materials availability
Enteroids generated in this study are available upon request as per Johns Hopkins University policy.

Data and code availability
- Data: Previously published single-cell sequencing data[53] analyzed here are available under accession code GSE201859.
- Code: This article does not report the original code or source code.
- Additional information: Any additional information required to reanalyze the data, the original immunofluorescence images, and western blots reported in this article is available upon reasonable request through the lead contact.

AUTHOR CONTRIBUTIONS

Conceptualization, V.S; methodology, Y.R., Z.Z., R.R., R.S., and M.T.; resources, H.R., H.Y.; investigation, D.V., R.Z., and O.K.; writing-original draft, V.S.; writing-review and editing, V.S and M.D.; funding acquisition, V.S and M.D.; supervision, V.S and M.D.

DECLARATION OF INTERESTS

The Authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Intestinal enteroid cultures from human biopsies
  - Forskolin-induced swelling of enteroids
  - RNA isolation and quantitative real-time PCR
  - Adenoviral Flag-DRA, and cGKII infection
  - DRA knockdown in distal colonic/rectal enteroids
  - Immunofluorescence staining and confocal image analysis
  - Immunoblotting
  - Single-cell RNA sequencing analysis of normal human small intestine
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2025.112453.

REFERENCES

1. Balfour-Lynn, I.M., and King, J.A. (2022). CFTR modulator therapies - Effect on life expectancy in people with cystic fibrosis. Paediatr. Respir. Rev. 42, 3–8. https://doi.org/10.1016/j.prrv.2020.05.002.

2. Lopez, A., Daly, C., Vega-Hernandez, G., MacGregor, G., and Rubin, J.L. (2023). Elexacaftor/tezacaftor/ivacaftor projected survival and long-term health outcomes in people with cystic fibrosis homozygous for F508del. J. Cyst. Fibros. 22, 607–614. https://doi.org/10.1016/j.jcf.2023.02.004.

3. Pullen, L.C. (2020). A new future for patients with cystic fibrosis. Am. J. Transplant. 20, 1213–1214. https://doi.org/10.1111/ajt.15893.

4. Abraham, J.M., and Taylor, C.J. (2017). Cystic Fibrosis & disorders of the large intestine: DIOS, constipation, and colorectal cancer. J. Cyst. Fibros. 16, S40–S49. https://doi.org/10.1016/j.jcf.2017.06.013.

5. Stefano, M.A., Poderoso, R.E., Mainz, J.G., Ribeiro, J.D., Ribeiro, A.F., and Lomazi, E.A. (2020). Prevalence of constipation in cystic fibrosis patients: a systematic review of observational studies. J. Pediatr. 96, 686–692. https://doi.org/10.1016/j.jped.2020.03.004.

6. Tuggle, K.L., Birket, S.E., Cui, X., Hong, J., Warren, J., Reid, L., Chambers, A., Ji, D., Gamber, K., Chu, K.K., et al. (2014). Characterization of defects in ion transport and tissue development in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout rats. PLoS One 9, e91253. https://doi.org/10.1371/journal.pone.0091253.

7. Veeze, H.J., Sinaasappel, M., Bijman, J., Bouquet, J., and de Jonge, H.R. (1991). Ion transport abnormalities in rectal suction biopsies from children with cystic fibrosis. Gastroenterology 101, 398–403. https://doi.org/10.1016/0016-5085(91)90017-f.

8. Pier, G., Prince, A., and Cantin, A. (2011). Cystic fibrosis: an-ion transport issue? Nat. Med. 17, 166–167. https://doi.org/10.1038/nm0211-166.

9. Raia, V., Fuiano, L., and Staiano, A. (2010). The ESPGHAN Cystic Fibrosis Working Group: defining DIOS and constipation in cystic fibrosis with a multicenter study on the incidence, characteristics, and treatment of DIOS. J. Pediatr. Gastroenterol. Nutr. 50, 8. https://doi.org/10.1097/MPG.0b013e3181a6da59.

10. van der Doef, H.P.J., Kokke, F.T.M., Beek, F.J.A., Woestenenk, J.W., Froeling, S.P., and Houwen, R.H.J. (2010). Constipation in pediatric cystic fibrosis patients: an underestimated medical condition. J. Cyst. Fibros. 9, 59–63. https://doi.org/10.1016/j.jcf.2009.11.003.

11. Dekkers, J.F., Berkers, G., Kruisselbrink, E., Vonk, A., de Jonge, H.R., Janssens, H.M., Bronsveld, I., van de Graaf, E.A., Nieuwenhuis, E.E.S., Houwen, R.H.J., et al. (2016). Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. Sci. Transl. Med. 8, 344ra84. https://doi.org/10.1126/scitranslmed.aad8278.

12. Crawford, D.K., Mullenders, J., Pott, J., Boj, S.F., Landskroner-Eiger, S., and Goddeeris, M.M. (2021). Targeting G542X CFTR nonsense alleles with ELX-02 restores CFTR function in human-derived intestinal organoids. J. Cyst. Fibros. *20*, 436–442. https://doi.org/10.1016/j.jcf.2021.01.009.

13. Aalbers, B.L., Brunsveld, J.E., van der Ent, C.K., van den Eijnden, J.C., Beekman, J.M., and Heijerman, H.G.M. (2022). Forskolin induced swelling (FIS) assay in intestinal organoids to guide eligibility for compassionate use treatment in a CF patient with a rare genotype. J. Cyst. Fibros. *21*, 254–257. https://doi.org/10.1016/j.jcf.2022.01.008.

14. Donowitz, M., Sarker, R., Lin, R., McNamara, G., Tse, C.M., and Singh, V. (2022). Identification of Intestinal NaCl Absorptive-Anion Secretory Cells: Potential Functional Significance. Front. Physiol. *13*, 892112. https://doi.org/10.3389/fphys.2022.892112.

15. Noel, G., Baetz, N.W., Staab, J.F., Donowitz, M., Kovbasnjuk, O., Pasetti, M.F., and Zachos, N.C. (2017). A primary human macrophage-enteroid co-culture model to investigate mucosal gut physiology and host-pathogen interactions. Sci. Rep. *7*, 45270. https://doi.org/10.1038/srep45270.

16. Tse, C.M., Zhang, Z., Lin, R., Sarker, R., Donowitz, M., and Singh, V. (2023). The Air-Liquid Interface Reorganizes Membrane Lipids and Enhances the Recruitment of Slc26a3 to Lipid-Rich Domains in Human Colonoid Monolayers. Int. J. Mol. Sci. *24*, 8273. https://doi.org/10.3390/ijms24098273.

17. Cil, O., Phuan, P.W., Gillespie, A.M., Lee, S., Tradtrantip, L., Yin, J., Tse, M., Zachos, N.C., Lin, R., Donowitz, M., and Verkman, A.S. (2017). Benzopyrimido-pyrrolo-oxazine-dione CFTR inhibitor (R)-BPO-27 for antisecretory therapy of diarrheas caused by bacterial enterotoxins. Faseb J *31*, 751–760. https://doi.org/10.1096/fj.201600891R.

18. Haggie, P.M., Cil, O., Lee, S., Tan, J.A., Rivera, A.A., Phuan, P.W., and Verkman, A.S. (2018). SLC26A3 inhibitor identified in small molecule screen blocks colonic fluid absorption and reduces constipation. JCI Insight *3*, e121370. https://doi.org/10.1172/jci.insight.121370.

19. Schwark, J.R., Jansen, H.W., Lang, H.J., Krick, W., Burckhardt, G., and Hropot, M. (1998). S3226, a novel inhibitor of Na+/H+ exchanger subtype 3 in various cell types. Pflugers Arch. *436*, 797–800. https://doi.org/10.1007/s004240050704.

20. Shi, S., Guo, S., Chen, Y., Sun, F., Pang, C., Ma, B., Qu, C., and An, H. (2020). Molecular mechanism of CaCC(inh)-A01 inhibiting TMEM16A channel. Arch. Biochem. Biophys. *695*, 108650. https://doi.org/10.1016/j.abb.2020.108650.

21. Ramalho, A.S., Furstova, E., Vonk, A.M., Ferrante, M., Verfaillie, C., Dupont, L., Boon, M., Proesmans, M., Beekman, J.M., Sarouk, I., et al. (2021). Correction of CFTR function in intestinal organoids to guide treatment of cystic fibrosis. Eur. Respir. J. *57*, 1902426. https://doi.org/10.1183/13993003.02426-2019.

22. Boj, S.F., Vonk, A.M., Statia, M., Su, J., Vries, R.R.G., Beekman, J.M., and Clevers, H. (2017). Forskolin-induced Swelling in Intestinal Organoids: An In Vitro Assay for Assessing Drug Response in Cystic Fibrosis Patients. J. Vis. Exp. *120*, 55159. https://doi.org/10.3791/55159.

23. Berkers, G., van Mourik, P., Vonk, A.M., Kruisselbrink, E., Dekkers, J.F., de Winter-de Groot, K.M., Arets, H.G.M., Marck-van der Wilt, R.E.P., Dijkema, J.S., Vanderschuren, M.M., et al. (2019). Rectal Organoids Enable Personalized Treatment of Cystic Fibrosis. Cell Rep. *26*, 1701–1708. https://doi.org/10.1016/j.celrep.2019.01.068.

24. Subramanya, S.B., Rajendran, V.M., Srinivasan, P., Nanda Kumar, N.S., Ramakrishna, B.S., and Binder, H.J. (2007). Differential regulation of cholera toxin-inhibited Na-H exchange isoforms by butyrate in rat ileum. Am. J. Physiol. Gastrointest. Liver Physiol. *293*, G857–G863. https://doi.org/10.1152/ajpgi.00462.2006.

25. Ko, S.B.H., Zeng, W., Dorwart, M.R., Luo, X., Kim, K.H., Millen, L., Goto, H., Naruse, S., Soyombo, A., Thomas, P.J., and Muallem, S. (2004). Gating of CFTR by the STAS domain of SLC26 transporters. Nat. Cell Biol. *6*, 343–350. https://doi.org/10.1038/ncb1115.

26. Tse, C.M., Yin, J., Singh, V., Sarker, R., Lin, R., Verkman, A.S., Turner, J.R., and Donowitz, M. (2019). cAMP Stimulates SLC26A3 Activity in Human Colon by a CFTR-Dependent Mechanism That Does Not Require CFTR Activity. Cell. Mol. Gastroenterol. Hepatol. *7*, 641–653. https://doi.org/10.1016/j.jcmgh.2019.01.002.

27. Salari, A., Zhou, K., Nikolovska, K., Seidler, U., and Amiri, M. (2023). Human Colonoid-Myofibroblast Coculture for Study of Apical Na(+)/H(+) Exchangers of the Lower Cryptal Neck Region. Int. J. Mol. Sci. *24*, 4266. https://doi.org/10.3390/ijms24054266.

28. Janecke, A.R., Heinz-Erian, P., Yin, J., Petersen, B.S., Franke, A., Lechner, S., Fuchs, I., Melancon, S., Uhlig, H.H., Travis, S., et al. (2015). Reduced sodium/proton exchanger NHE3 activity causes congenital sodium diarrhea. Hum. Mol. Genet. *24*, 6614–6623. https://doi.org/10.1093/hmg/ddv367.

29. Clayburgh, D.R., Musch, M.W., Leitges, M., Fu, Y.X., and Turner, J.R. (2006). Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. J. Clin. Investig. *116*, 2682–2694. https://doi.org/10.1172/JCI29218.

30. Kim, Y., Anderson, M.O., Park, J., Lee, M.G., Namkung, W., and Verkman, A.S. (2015). Benzopyrimido-pyrrolo-oxazine-dione (R)-BPO-27 Inhibits CFTR Chloride Channel Gating by Competition with ATP. Mol. Pharmacol. *88*, 689–696. https://doi.org/10.1124/mol.115.098368.

31. Busby, R.W., Bryant, A.P., Bartolini, W.P., Cordero, E.A., Hannig, G., Kessler, M.M., Mahajan-Miklos, S., Pierce, C.M., Solinga, R.M., Sun, L.J., et al. (2010). Linaclotide, through activation of guanylate cyclase C, acts locally in the gastrointestinal tract to elicit enhanced intestinal secretion and transit. Eur. J. Pharmacol. *649*, 328–335. https://doi.org/10.1016/j.ejphar.2010.09.019.

32. Bryant, A.P., Busby, R.W., Bartolini, W.P., Cordero, E.A., Hannig, G., Kessler, M.M., Pierce, C.M., Solinga, R.M., Tobin, J.V., Mahajan-Miklos, S., et al. (2010). Linaclotide is a potent and selective guanylate cyclase C agonist that elicits pharmacological effects locally in the gastrointestinal tract. Life Sci. *86*, 760–765. https://doi.org/10.1016/j.lfs.2010.03.015.

33. Chen, T., Kocinsky, H.S., Cha, B., Murtazina, R., Yang, J., Tse, C.M., Singh, V., Cole, R., Aronson, P.S., de Jonge, H., et al. (2015). Cyclic GMP kinase II (cGKII) inhibits NHE3 by altering its trafficking and phosphorylating NHE3 at three required sites: identification of a multifunctional phosphorylation site. J. Biol. Chem. *290*, 1952–1965. https://doi.org/10.1074/jbc.M114.590174.

34. McHugh, D.R., Cotton, C.U., Moss, F.J., Vitko, M., Valerio, D.M., Kelley, T.J., Hao, S., Jafri, A., Drumm, M.L., Boron, W.F., et al. (2018). Linaclotide improves gastrointestinal transit in cystic fibrosis mice by inhibiting sodium/hydrogen exchanger 3. Am. J. Physiol. Gastrointest. Liver Physiol. *315*, G868–G878. https://doi.org/10.1152/ajpgi.00261.2017.

35. Middleton, P.G., Mall, M.A., Dřevínek, P., Lands, L.C., McKone, E.F., Polineni, D., Ramsey, B.W., Taylor-Cousar, J.L., Tullis, E., Vermeulen, F., et al. (2019). Elexacaftor-Tezacaftor-Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. N. Engl. J. Med. *381*, 1809–1819. https://doi.org/10.1056/NEJMoa1908639.

36. Ridley, K., and Condren, M. (2020). Elexacaftor-Tezacaftor-Ivacaftor: The First Triple-Combination Cystic Fibrosis Transmembrane Conductance Regulator Modulating Therapy. J. Pediatr. Pharmacol. Ther. *25*, 192–197. https://doi.org/10.5863/1551-6776-25.3.192.

37. Levy, I., Horvath, A., Azevedo, M., de Alexandre, R.B., and Stratakis, C.A. (2011). Phosphodiesterase function and endocrine cells: links to human disease and roles in tumor development and treatment. Curr. Opin. Pharmacol. *11*, 689–697. https://doi.org/10.1016/j.coph.2011.10.003.

38. Yuan, F., Ren, H., Tan, W., Wang, Y., and Luo, H. (2022). Effect of phosphodiesterase-4 inhibitor rolipram on colonic hypermotility in water avoidance stress rat model. Neuro Gastroenterol. Motil. *34*, e14317. https://doi.org/10.1111/nmo.14317.

39. Blanchard, E., Zlock, L., Lao, A., Mika, D., Namkung, W., Xie, M., Scheitrum, C., Gruenert, D.C., Verkman, A.S., Finkbeiner, W.E., et al. (2014). Anchored PDE4 regulates chloride conductance in wild-type and

DeltaF508-CFTR human airway epithelia. Faseb J *28*, 791–801. https://doi.org/10.1096/fj.13-240861.

40. Schwarzenberg, S.J., Vu, P.T., Skalland, M., Hoffman, L.R., Pope, C., Gelfond, D., Narkewicz, M.R., Nichols, D.P., Heltshe, S.L., Donaldson, S.H., et al. (2023). Elexacaftor/tezacaftor/ivacaftor and gastrointestinal outcomes in cystic fibrosis: Report of promise-GI. J. Cyst. Fibros. *22*, 282–289. https://doi.org/10.1016/j.jcf.2022.10.003.

41. O'Brien, C.E., Anderson, P.J., and Stowe, C.D. (2011). Lubiprostone for constipation in adults with cystic fibrosis: a pilot study. Ann. Pharmacother. *45*, 1061–1066. https://doi.org/10.1345/aph.1Q219.

42. Burclaff, J., Bliton, R.J., Breau, K.A., Ok, M.T., Gomez-Martinez, I., Ranek, J.S., Bhatt, A.P., Purvis, J.E., Woosley, J.T., and Magness, S.T. (2022). A Proximal-to-Distal Survey of Healthy Adult Human Small Intestine and Colon Epithelium by Single-Cell Transcriptomics. Cell. Mol. Gastroenterol. Hepatol. *13*, 1554–1589. https://doi.org/10.1016/j.jcmgh.2022.02.007.

43. Sarker, R., Lin, R., Singh, V., Donowitz, M., and Tse, C.M. (2023). SLC26A3 (DRA) is stimulated in a synergistic, intracellular Ca(2+)-dependent manner by cAMP and ATP in intestinal epithelial cells. Am. J. Physiol. Cell Physiol. *324*, C1263–C1273. https://doi.org/10.1152/ajpcell.00523.2022.

44. Dekkers, J.F., Wiegerinck, C.L., de Jonge, H.R., Bronsveld, I., Janssens, H.M., de Winter-de Groot, K.M., Brandsma, A.M., de Jong, N.W.M., Bijvelds, M.J.C., Scholte, B.J., et al. (2013). A functional CFTR assay using primary cystic fibrosis intestinal organoids. Nat. Med. *19*, 939–945. https://doi.org/10.1038/nm.3201.

45. Nelson, A.D., Camilleri, M., Chirapongsathorn, S., Vijayvargiya, P., Valentin, N., Shin, A., Erwin, P.J., Wang, Z., and Murad, M.H. (2017). Comparison of efficacy of pharmacological treatments for chronic idiopathic constipation: a systematic review and network meta-analysis. Gut *66*, 1611–1622. https://doi.org/10.1136/gutjnl-2016-311835.

46. Weinberg, D.S., Lin, J.E., Foster, N.R., Della'Zanna, G., Umar, A., Seisler, D., Kraft, W.K., Kastenberg, D.M., Katz, L.C., Limburg, P.J., and Waldman, S.A. (2017). Bioactivity of Oral Linaclotide in Human Colorectum for Cancer Chemoprevention. Cancer Prev. Res. *10*, 345–354. https://doi.org/10.1158/1940-6207.CAPR-16-0286.

47. Jarchau, T., Häusler, C., Markert, T., Pöhler, D., Vanderkerckhove, J., De Jonge, H.R., Lohmann, S.M., and Walter, U. (1994). Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. Proc. Natl. Acad. Sci. USA *91*, 9426–9430. https://doi.org/10.1073/pnas.91.20.9426.

48. Stahl, K., Stahl, M., de Jonge, H.R., and Forrest, J.N., Jr. (2015). ANP and CNP activate CFTR expressed in Xenopus laevis oocytes by direct activation of PKA. J. Recept. Signal Transduct. Res. *35*, 493–504. https://doi.org/10.3109/10799893.2015.1015738.

49. Ahsan, M.K., Tchernychev, B., Kessler, M.M., Solinga, R.M., Arthur, D., Linde, C.I., Silos-Santiago, I., Hannig, G., and Ameen, N.A. (2017). Linaclotide activates guanylate cyclase-C/cGMP/protein kinase-II-dependent trafficking of CFTR in the intestine. Physiol. Rep. *5*, e13299. https://doi.org/10.14814/phy2.13299.

50. Vaandrager, A.B., Bot, A.G., and De Jonge, H.R. (1997). Guanosine 3′,5′-cyclic monophosphate-dependent protein kinase II mediates heat-stable enterotoxin-provoked chloride secretion in rat intestine. Gastroenterology *112*, 437–443. https://doi.org/10.1053/gast.1997.v112.pm9024297.

51. Vaandrager, A.B., Bot, A.G., Ruth, P., Pfeifer, A., Hofmann, F., and De Jonge, H.R. (2000). Differential role of cyclic GMP-dependent protein ki-

nase II in ion transport in murine small intestine and colon. Gastroenterology *118*, 108–114. https://doi.org/10.1016/s0016-5085(00)70419-7.

52. Wang, D., Spoelstra, W.K., Lin, L., Akkerman, N., Krueger, D., Dayton, T., van Zon, J.S., Tans, S.J., van Es, J.H., and Clevers, H. (2025). Interferon-responsive intestinal BEST4/CA7(+) cells are targets of bacterial diarrheal toxins. Cell Stem Cell *32*, 598–612.e5. https://doi.org/10.1016/j.stem.2025.02.003.

53. Zwick, R.K., Kasparek, P., Palikuqi, B., Viragova, S., Weichselbaum, L., McGinnis, C.S., McKinley, K.L., Rathnayake, A., Vaka, D., Nguyen, V., et al. (2024). Epithelial zonation along the mouse and human small intestine defines five discrete metabolic domains. Nat. Cell Biol. *26*, 250–262. https://doi.org/10.1038/s41556-023-01337-z.

54. Xiao, F., Li, J., Singh, A.K., Riederer, B., Wang, J., Sultan, A., Park, H., Lee, M.G., Lamprecht, G., Scholte, B.J., et al. (2012). Rescue of epithelial HCO3- secretion in murine intestine by apical membrane expression of the cystic fibrosis transmembrane conductance regulator mutant F508del. J. Physiol. *590*, 5317–5334. https://doi.org/10.1113/jphysiol.2012.232124.

55. Kim, S.J., Cheung, S., and Hellerstein, M.K. (2004). Isolation of nuclei from label-retaining cells and measurement of their turnover rates in rat colon. Am. J. Physiol. Cell Physiol. *286*, C1464–C1473. https://doi.org/10.1152/ajpcell.00139.2003.

56. Yin, J., Tse, C.M., Avula, L.R., Singh, V., Foulke-Abel, J., de Jonge, H.R., and Donowitz, M. (2018). Molecular Basis and Differentiation-Associated Alterations of Anion Secretion in Human Duodenal Enteroid Monolayers. Cell. Mol. Gastroenterol. Hepatol. *5*, 591–609. https://doi.org/10.1016/j.jcmgh.2018.02.002.

57. Bobadilla, J.L., Macek, M., Jr., Fine, J.P., and Farrell, P.M. (2002). Cystic fibrosis: a worldwide analysis of CFTR mutations–correlation with incidence data and application to screening. Hum. Mutat. *19*, 575–606. https://doi.org/10.1002/humu.10041.

58. Lopes-Pacheco, M. (2019). CFTR Modulators: The Changing Face of Cystic Fibrosis in the Era of Precision Medicine. Front. Pharmacol. *10*, 1662. https://doi.org/10.3389/fphar.2019.01662.

59. Singh, V., Johnson, K., Yin, J., Lee, S., Lin, R., Yu, H., In, J., Foulke-Abel, J., Zachos, N.C., Donowitz, M., and Rong, Y. (2022). Chronic Inflammation in Ulcerative Colitis Causes Long-Term Changes in Goblet Cell Function. Cell. Mol. Gastroenterol. Hepatol. *13*, 219–232. https://doi.org/10.1016/j.jcmgh.2021.08.010.

60. Vaandrager, A.B., Tilly, B.C., Smolenski, A., Schneider-Rasp, S., Bot, A.G., Edixhoven, M., Scholte, B.J., Jarchau, T., Walter, U., Lohmann, S.M., et al. (1997). cGMP stimulation of cystic fibrosis transmembrane conductance regulator Cl- channels co-expressed with cGMP-dependent protein kinase type II but not type Ibeta. J. Biol. Chem. *272*, 4195–4200. https://doi.org/10.1074/jbc.272.7.4195.

61. Vaandrager, A.B., Smolenski, A., Tilly, B.C., Houtsmuller, A.B., Ehlert, E.M., Bot, A.G., Edixhoven, M., Boomaars, W.E., Lohmann, S.M., and de Jonge, H.R. (1998). Membrane targeting of cGMP-dependent protein kinase is required for cystic fibrosis transmembrane conductance regulator Cl- channel activation. Proc. Natl. Acad. Sci. USA *95*, 1466–1471. https://doi.org/10.1073/pnas.95.4.1466.

62. Moor, A.E., Harnik, Y., Ben-Moshe, S., Massasa, E.E., Rozenberg, M., Eilam, R., Bahar Halpern, K., and Itzkovitz, S. (2018). Spatial Reconstruction of Single Enterocytes Uncovers Broad Zonation along the Intestinal Villus Axis. Cell *175*, 1156–1167.e15. https://doi.org/10.1016/j.cell.2018.08.063.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DRA	Santa Cruz	SC-376187
cGKII	Santa Cruz	SC-393126
GAPDH	Sigma-Aldrich	G8795
CFTR	660, 217, 596	CF consortium
CHGA	Immunostar	20085
GUCY2C	LSBio	LS-C498735
MUC2	Santa Cruz	sc7314
E-cadherin	BD Biosciences	610182
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Life Technologies	A11008
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Life Technologies	A11003
Bacterial and virus strains		
pLKO-1-DRA	Addgene	8453
Chemicals, peptides, and recombinant proteins		
Y-27632	Selleck	S1049
EGF Recombinant Mouse Protein	Invitrogen	PMG8043
CHIR99021	Selleck	S1263
N-2 Supplement	Invitrogen	17502-048
B-27 Supplement	Invitrogen	17504-044
HEPES	Invitrogen	15630-080
GlutaMAX	Invitrogen	35050-061
CHIR99021	Selleck	S1263
Triton-X-100	Sigma-Aldrich	93443
Matrigel	Corning	356231
Paraformaldehyde	Electron Microscopy Sciences	15710
Hoechst 33342	Invitrogen	H3570
Organoid Harvesting Solution	R&D System	3700-100-01
Acetylcysteine	Sigma-Aldrich	A7250
Human epidermal growth factor	ThermoFisher	A42555
Gastrin	AnaSpec	AS-64149
A83-01	Tocris	TB2939-RMU
SB202190	MilliporeSigma	152121-30-7
Primocin	InvivoGen	ant-pm-05
Deposited data		
Sequencing data analyzed here are available under the accession code.		GSE201859
Experimental models: Cell lines		
Caco-2bbe	ATCC	
Experimental models: Organisms/strains		
Model	Genotype	Gender
Distal colon/rectal organoid	F508del mutation	NA
Distal colon/rectal organoid	F508del mutation	NA
Distal colon/rectal organoid	F508del mutation	NA

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ileal organoid	F508del mutation	NA
Distal colon/rectal organoid	Normal	Female
Distal colon/rectal organoid	Normal	Male
Distal colon/rectal organoid	Normal	Male
Ileal organoid	Normal	Male
Oligonucleotides		
LGR5	ORIGENE	HP207145
Ki67	ORIGENE	HP206104
MUC2	ORIGENE	HP206138
CHGA	ORIGENE	HP205193
DRA	ORIGENE	HP200096
SI	ORIGENE	HP203589
PDE4	ORIGENE	HP233178
18S rRNA	ORIGENE	HP220445
GUCY2C	ORIGENE	HP208194
Software and algorithms		
Olympus		Olympus, Tokyo, Japan)
Fiji		ImageJ-2020
Prism 6 and 8	GraphPad	https://www.graphpad.com/scientific-software/prism/
MACRO		ImageJ[22]
Other		
Wnt3A Conditioned Medium (Atcc Crl-2647)	ATCC	Crl-2647
Spondin1 Conditioned Medium	Gift From Calvin Kuo lab, Stanford University	
Noggin Conditioned Medium	Gift From Gijs van den Brink Tytgat Institute for Liver and Intestinal Research	

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Colon lines	Known Ailment	Gender	Ethnicity	Age	Origin of biopsies
Patient 1	F508del mutation	NA	NA	NA	Distal colon/Rectum
Patient 2	F508del mutation	NA	NA	NA	Distal colon/Rectum
Patient 3	F508del mutation	NA	NA	NA	Distal colon/Rectum
Patient 4	F508del mutation	NA	NA	NA	Ileum
Healthy subject 1	normal	Female	White	66	Distal colon/Rectum
Healthy subject 2	normal	Male	Asian	52	Distal colon/Rectum
Healthy subject 3	normal	Male	White	56	Distal colon/Rectum
Healthy subject 4	normal	Male	Asian	52	Ileum
NA, not available					

METHOD DETAILS

Intestinal enteroid cultures from human biopsies

One ileal and three distal colon/rectal enteroids lines each from separate homozygous F508del-CF patients (adult) were obtained from Hugo R. de Jonge from the Biobank of Enteroids at the Hubrecht Foundation for Organoid Technology (MEC-2012-317). Normal human ileal and distal colonic/rectal enteroids lines established from 3 separate normal healthy subjects (NHS) including both male and female subjects were provided by the Hopkins GI Core Center. All experimental protocols were approved by the Johns Hopkins University Institutional Review Board (NA_00038329) and the Hubrecht Institute Human Research Review Board. Enteroid cultures

were established from ileal and colonic biopsies obtained after endoscopic screening colonoscopies and upper endoscopies in normal subjects with no pathology found. Informed consent for tissue collection, generation, storage, and use of the enteroids was obtained from all participating patients. Enteroids were established and maintained as we have described.[15,59] Briefly, enteroids generated from isolated intestinal crypts were maintained as cysts embedded in Matrigel (Corning, USA) in 24-well plates and cultured in Wnt3A, R-spondin, and Noggin-containing growth media, as we have described.[15] The medium was refreshed every 2–3 d, and enteroids were passaged 1:3 every 7–10 d. Enteroids from passages 1–30 were used for these studies.

Homozygous F508del-CF and site-matched enteroids from separate NHS were studied in undifferentiated (UD), 3d-differentiated (PD), and 6d-differentiated (FD) states.

Experiments were repeated at least 3-4 times and performed on each enteroid line.

Human cell lines used in this study include Caco-2bbe, which was acquired from ATCC. The cell line was authenticated by short tandem repeat (STR) profiling at ATCC to confirm cell line identity. Cells were tested negative for mycoplasma contamination.

Forskolin-induced swelling of enteroids
Human enteroids from a 6-8-day-old culture were seeded in a flat-bottom 24-well culture plate in 10 μl Matrigel commonly containing 20–200 enteroids and 500 μl culture media. For studies on undifferentiated (UD) enteroids, the assays were performed 1 day after seeding. For partially (PD) and fully differentiated (FD) enteroids, the growth media were replaced with a differentiation media (without Wnt-3A and R-spondin) for 3 or 6 days respectively before the assay was performed. On the day of assay, growth or DF media were removed and enteroids were incubated for 60 min with 500 μl DMEM (without growth factors) containing 10 μM calcein green (Invitrogen). For optimal inhibition of transport proteins, enteroids were preincubated with CFTR$_{inh}$-BPO (10 μM), DRAinh A250 (10μM), and CaCCinh-A01(15μM) (all gifts from Alan Verkman), dimethyl amiloride (2 μM) (Sigma-Aldrich, St. Louis, MO) for NHE1+2 inhibition, NHE3inh S3226 (20μM) (gift from Sinofi -Aventis). High-resolution spheroid images were captured using a Keyence BZ-X700 fluorescence microscope. Three wells were used to study one condition, and up to 60 wells were analyzed per experiment. For treatment with CFTR correctors, enteroids were preincubated for 24 h with tezacaftor (VX-661), and elexacaftor (VX-445) (3 μM each) (both from Selleck Chemicals LLC). For treatment with a CFTR potentiator, 5 μM VX-770 (Selleck Chemicals LLC) was added an hour before forskolin addition. The concentrations used were arbitrarily chosen since direct addition to the enteroids was assumed different from systemically administered drugs in patients.

Organoid swelling was measured as previously described.[11,22,44] Enteroids were fluorescently labeled with calcein green and the total enteroid surface area per well over time was monitored by a Keyence BZ-X700 fluorescence microscope. Agonist-stimulated organoid swelling was calculated as described using an organoid swelling analysis macro for ImageJ.[22] Briefly, to quantify swelling, total calcein-green surface areas were selected for each time point and expressed as a percentage increase with respect to the initial time point (T=0mins, set at 100%). The relative area increase was expressed per 15-minute time intervals and measurements were generated for each condition (T=0 to 60 min for Fsk or 120 min for linaclotide because the rate of secretion for linaclotide was slower than with cAMP). The area under the curve (AUC) (T=60 or 120 min, baseline 100%) was calculated using Prism.

RNA isolation and quantitative real-time PCR
Total RNA was extracted from 3D cultures using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 to 2 μg of RNA using SuperScript VILO Master Mix (Life Technologies). Quantitative real-time PCR was performed using Power SYBR Green Master Mix (Life Technologies) on a QuantStudio 12K Flex real-time PCR system (Applied Biosystems, Foster City, CA). Each sample was run in triplicate, and 5 ng RNA-equivalent complementary DNA was used for each reaction. Commercially available primer pairs from OriGene Technologies (Rockville, MD) were used: *LGR5*: HP207145; *Ki67*: HP206104; *MUC2*: HP206138; *CHGA*: HP205193; *DRA*: HP200096; *SI*: HP203589; *PDE4:* HP233178; *18S rRNA*: HP220445. The relative fold changes in mRNA levels of genes were determined by using the $2^{-\Delta\Delta CT}$ method with human 18S ribosomal, RNA simultaneously studied and used as the internal control for normalization and shown as fold change compared with the NHS-UD control.

Adenoviral Flag-DRA, and cGKII infection
Triple Flag-tagged human DRA was cloned into the adenoviral shuttle vector ADLOX.HTM under the control of a cytomegalovirus promoter. The preparation of adeno-Flag-DRA and cGKII was reported previously.[33,60,61] To infect with adenovirus (Adeno-Flag-DRA, or cGKII) enteroids were collected after melting the Matrigel and were mechanically dissociated through pipetting (30-50x). Enteroids were washed with basal media and centrifuged at 4°C, 900 x g for 5 min. The supernatant was discarded and the pellet was resuspended in enteroid growth media (500μl) combined with 10μl of the adenoviral solution with Y-27632 and CHIR99021(10μM) in one well of a 24-well plate. The plate was centrifuged at 32 °C, 600 x g, for 1 hr followed by incubation in a tissue culture incubator at 37°C at 5% CO_2 for 6 hr. Adeno-infected enteroids were collected and centrifuged at 4°C, 900 x g for 5 min, and plated with Matrigel. The plate was incubated at 37 °C for 10 min to allow the Matrigel to polymerize, then a total of 500 μL of growth media with Y-27632 and CHIR99021 (10μM each) was added to the well. For the study, the Y-27632 and CHIR99021 containing growth media were replaced with UD or DF media as required for the assay.

DRA knockdown in distal colonic/rectal enteroids

Enteroids were prepared as above and the adenoviral solution was replaced with a lentiviral solution containing 10mg/ml polybrene with 5μl control or DRA-shRNA + Y-27632 and CHIR99021(10μM each) in growth media (500μl). The shRNA-infected enteroids were collected and centrifuged at 4°C, 900 x g for 5 min, plated with Matrigel, and treated as above. Three days after the infection, enteroid media were replaced with growth media containing puromycin (0.2μg/ml) to maintain stable expression of the shRNAs.

Immunofluorescence staining and confocal image analysis

Immunostaining of 3D enteroids was done in suspension. Briefly, recovered enteroids were fixed in 4% paraformaldehyde in 10 mmol/L phosphate buffer (pH 7.4) for 30 minutes at 4°C and then washed 2× with phosphate-buffered saline. Enteroids were permeabilized and stained in phosphate-buffered saline with 2% bovine serum albumin, 15% fetal bovine serum 0.25% Triton X-100, and 1% saponin for 60 minutes (all Sigma-Aldrich St. Louis, MO, USA), followed by overnight incubation with antibodies. Primary antibodies used: mouse anti-MUC2 (sc7314, Santa Cruz Biotechnology, Dallas, TX), anti-E-cadherin (Clone 36, BD Biosciences) anti-GUCY2C (LS-C498735, LSBio, Lynnwood, WA), anti-ChgA (20085, Immunostar). All primary antibodies were diluted at 1:100 and incubated overnight. Stained cells were then washed 3 times for 10 min each with PBS followed by incubation with Alexa-488/568 conjugated Goat anti-Rabbit and anti-mouse antibodies respectively (Molecular Probes/Invitrogen, USA) diluted 1:500 in PBS. Hoechst (Vector Laboratories, USA) was used at a 1:1000 dilution in PBS for nuclear/DNA labeling. After incubation, cells were washed 3 times for 10 min each and mounted in ProLong Gold (Vector Laboratories, USA) overnight at 4°C. Images were collected using ×20 objective on FV3000 confocal microscope (Olympus, Tokyo, Japan) with Olympus and ImageJ software (NIH).

Immunoblotting

For protein detection, 3D enteroids from NHS or F508del-CF patients were harvested in phosphate-buffered saline after melting the Matrigel and rinsed 2 times with phosphate-buffered saline. Cell lysate preparation and Western blot were performed using validated antibodies for DRA (SC-376187; mouse monoclonal, 1:500; Santa Cruz, Dallas, TX), CFTR (CF660, CF217 from CF consortium), and anti-CFTR mAb 596 (nucleotide-binding domain 2 [NBD2] 1204–1211 aa) (Cystic Fibrosis Foundation, https://www.cff.org), diluted 1:500 in TBST 2% (w/v) skim milk cGKII (SC-393126; mouse monoclonal, 1:500; Santa Cruz, Dallas, TX) and glyceraldehyde-3-phosphate dehydrogenase (G8795; mouse monoclonal, 1:5000; Sigma-Aldrich). Protein bands were visualized and quantitated using an Odyssey system and Image Studio software (LI-COR Biosciences, Lincoln, NE).

Single-cell RNA sequencing analysis of normal human small intestine

The entire small intestine from two deceased but otherwise, normal people were obtained from the research-consent deceased organ donor program obtained through an IRB-approved research protocol with donor Network West in collaboration with the UCSF Viable Tissue Acquisition Lab Core. The studies with this tissue were described previously that include single-cell RNA-sequencing of the entire small intestine divided along the horizontal axis into 30 segments, which for the current report were incorporated into a single analysis consisting of the entire small intestine.[53] As reported, villus zonation scoring used MATLAB version 2018b to annotate the enterocytes according to their position along the crypt-villus axis.[62] In addition, villus domains were assigned using landmark genes to separate villus enterocytes into 6 zones.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical testing based on the raw data was assessed using Prism statistical software version 6 and 8 (GraphPad). Significance was considered $p < 0.05$ using Student's t-tests or One-way ANOVA with Bonferroni post hoc test. All experiments were performed on all colonoid lines from healthy subjects and CF F508del-CF patients. Some experiments were also repeated on 1 ileal enteroid line from a healthy subject and a F508del-CF patient. N refers to the number of independent replicates performed.