SUPPLEMENTARY MATERIALS AND METHODS

Establishment of human spheroid cultures

Crypt/gland units were isolated and plated in Matrigel as described in the main text. At the end of the isolation procedure, the cell/Matrigel mixture was plated into 2-4 wells of a 24-well tissue culture plate (plating into multiple wells helped to dilute out the effects of cell types that die because their growth is not supported by our media and to ensure line recovery if yeast contamination occurred in a well). Cells were grown in an incubator at 37°C with 5% CO_2.

Initial passages after isolation were performed every 3-5 days at a 1:1-1:2 ratio (Supplementary Figure 2). Generally, passaging was performed every 3 days if >10 spheroids per well were present and every 5 days if there were <10 spheroids per well present. Additionally, spheroids were typically not expanded into more than 2 wells until at least 20 spheroids were present in each well. During the first several passages, the human spheroids were incubated in trypsin-EDTA solution for ~45-60 seconds followed by vigorous pipetting 10-15 times (see below for passaging method). For later passages, spheroids were typically incubated in trypsin-EDTA solution for 60-90 seconds followed by vigorous pipetting 20-30 times. Within the first several passages, the spheroid density typically was sufficient to perform subsequent passages every 3 days at a 1:3-1:4 ratio, with media replenished every other day.

One important difference from our mouse culture system is that the human intestinal spheroids grow better when cultures are maintained as a denser spheroid population (see image in Fig. 1). A second difference was that the addition of two inhibitors to the media was required to maintain the epithelial cell cultures for >3 passages (Supplementary Figure 1): the ROCK inhibitor is required because isolated intestinal epithelial cells are sensitive to anoikis[1], and the TGFBR inhibitor is required to repress TGF-β activity in serum to maximize epithelial cell proliferation[2, 3].

Maintenance of spheroid cultures

Spheroid cultures were maintained in 24-well tissue culture plates, and the medium was replaced every other day. Spheroids were passaged 1:3-1:4 every 3 days. For each passage, the spheroid/Matrigel

was scratched into 500 µL of PBS containing 0.5 mM EDTA (PBS-EDTA) with a 1000 µL pipette and transferred to a 15 mL conical tube. Spheroids were pelleted by centrifugation at 200 *g* for 5 min, and the supernatant was discarded. Spheroids were dissociated by incubating in 200 µL of 0.25% trypsin in PBS-EDTA for 60-90 sec (or 2 min if passaging for Transwell culture) in a 37°C water bath, followed by addition of 500 µL of washing medium and mechanical dissociation by vigorous pipetting (~20-30 times if passaging for maintenance or three-dimensional culture, ~40-50 times if passaging for Transwell culture). An additional 5 mL of washing medium was then added to inactivate the trypsin. Spheroids were pelleted by centrifugation at 200 *g* for 5 min and the supernatant was aspirated, leaving ~200 µL. Spheroids were transferred to a 1.5 mL microfuge tube in 1 mL washing medium and pelleted by centrifugation at 200 *g* for 5 min. The supernatant was carefully removed, and the pellet was re-suspended in Matrigel (15 µL/well). Following polymerization of the Matrigel, 50% L-WRN CM supplemented with 10 µM Y-27632 and 10 µM SB 431542 was added.

Additional notes:

1. The human intestinal spheroids do not survive long exposure to PBS-EDTA or trypsin/PBS-EDTA. Work at a deliberate pace and/or do not work with too many cell lines/wells at once to avoid loss of culture density due to excessive cell death.
2. We prefer a particular trypsin reagent for human intestinal spheroid culture: 10X Trypsin Solution (Sigma T4549) is diluted to 1X with PBS-EDTA, aliquoted and stored at -20°C. For a working stock, single 1X aliquots are thawed and stored at 4°C.
3. Human spheroids grow and perform best in functional assays when they are a small, uniform size. If spheroids become very large, it may be necessary to trypsinize for a longer time period (up to 2 min at 37°C), pipette more vigorously during mechanical dissociation and/or strain through a 40-µm filter after trypsinization.
4. For differentiation experiments, only ROCK inhibitor was included in the media; TGFBR1 inhibitor was not included because it was not required for terminal experiments and because

active TGF-β signaling has been shown to be important for the suppression of proliferation and promotion of differentiation in intestinal epithelial cells[2-4].

Storage and recovery of spheroid cultures

For frozen storage, the spheroid/Matrigel mixture was removed from the well by scratching with a 1000 µL pipette and transferred to a 15 mL conical tube containing 5 mL of washing medium. Cells were pelleted by centrifugation at 200 *g* for 5 min, and the supernatant was removed by aspiration. The cell pellet was re-suspended in freezing medium (primary culture medium plus 10% DMSO; 500 µL/well) and transferred to 1-mL cryotubes (500 µL/cryotube). Cells were frozen slowly overnight at -80°C and then transferred to liquid nitrogen storage. To recover spheroids, frozen stocks were quickly thawed at 37°C, transferred to a 15 mL conical tube containing 5 mL washing medium and pelleted by centrifugation at 200 g for 5 min. The supernatant was removed by aspiration. Spheroids were transferred to a 1.5 mL microfuge tube in 1 mL washing medium, pelleted and re-suspended in Matrigel as described above.

Cell cycle analysis

Cell cycle analysis was assessed using the Click-iT EdU Cell Proliferation AlexaFluor488 kit (Invitrogen) according to the manufacturer's directions. Spheroids were treated for 2 hours with 10 µM EdU and then dissociated into single cells by trypsinization and vigorous pipetting. A Click-iT staining reaction was performed following fixation and permeabilization. SYTOX AAD (Invitrogen) was used as a DNA content marker. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed with FloJo software.

Histological analysis of human epithelial cell monolayers

For histological analysis (Day 3), cells were fixed in 10% formalin for 10 min, washed 3 times in 70% ethanol and processed for paraffin embedding. Transverse sections (5 µm) were stained with

hematoxylin and eosin (visualized with an Olympus BX51 light microscope with a DP70 digital camera) or used for immunostaining. For immunostaining, sections were de-paraffinized and hydrated, boiled in Unmasking Solution (Vector Laboratories, Burlingame, CA) or Trilogy (Cell Marque, Rocklin, California) for 10-20 min, rinsed in PBS, blocked in 1% bovine serum albumin/0.1% Triton-X100 for 30 minutes and incubated with primary antibody at 4°C overnight. Primary antibodies included rabbit anti-ChgA (1:2000, Abcam, Cambridge, MA), rabbit anti-Muc2 (1:200, Santa Cruz Biotechnology, Inc., Dallas, Texas), goat anti-lysozyme (1:100; Santa Cruz Biotechnology, Inc.), mouse anti-chicken Villin 1 (1:100; AbD Serotec, Raleigh, NC) and rabbit anti-ZO-1 (1:100; Invitrogen). Slides were rinsed 3 times with PBS and incubated for 30-60 min at room temperature with species specific secondary antibodies (1:500; Invitrogen) conjugated to AlexaFluor488 or AlexaFluor546. UEA-1 lectin conjugated to TRITC (1:500, Sigma) was applied during secondary antibody incubation. Slides were washed 3 times in PBS and stained with bis-benzamide (Hoescht 33258, Invitrogen) to visualize nuclei and mounted with Flouromount-G (Southern Biotech). Staining was visualized with either a Zeiss (Oberkochen, Germany) Axiovert 200 microscope with an Axiocam MRM digital camera.

Additional methods for mucus layer analysis

For mucus thickness measurements, medium containing fluorescent beads (FluoSpheres, Invitrogen) was gently added to the apical chamber of each Transwell (1:500 final dilution) and allowed to sediment for 40 min followed by confocal imaging. To disrupt mucus layers, media were aspirated and then monolayers were washed with vigorous pipetting followed by addition of fresh medium containing florescent beads, which were allowed to sediment for 40 min prior to re-imaging.

Preparation of epithelial and bacterial cells for adherence assays

For two-dimensional culture, human intestinal spheroids from ileum or rectum were dissociated and seeded onto 8-well glass chamber slides (Lab-Tek; 1-2 wells of a 24-well plate were used to seed one chamber slide) coated with Matrigel diluted 1:40 in PBS. Spheroids were allowed to recover overnight in

50% L-WRN CM with 10 μM Y-27632. The following day, the medium was replaced with 5% L-WRN CM with 10 μM Y-27632 and changed daily. Prior to the adherence assay (Day 3), the epithelial cells were washed three times with antibiotic-free primary culture media containing only 5% FBS.

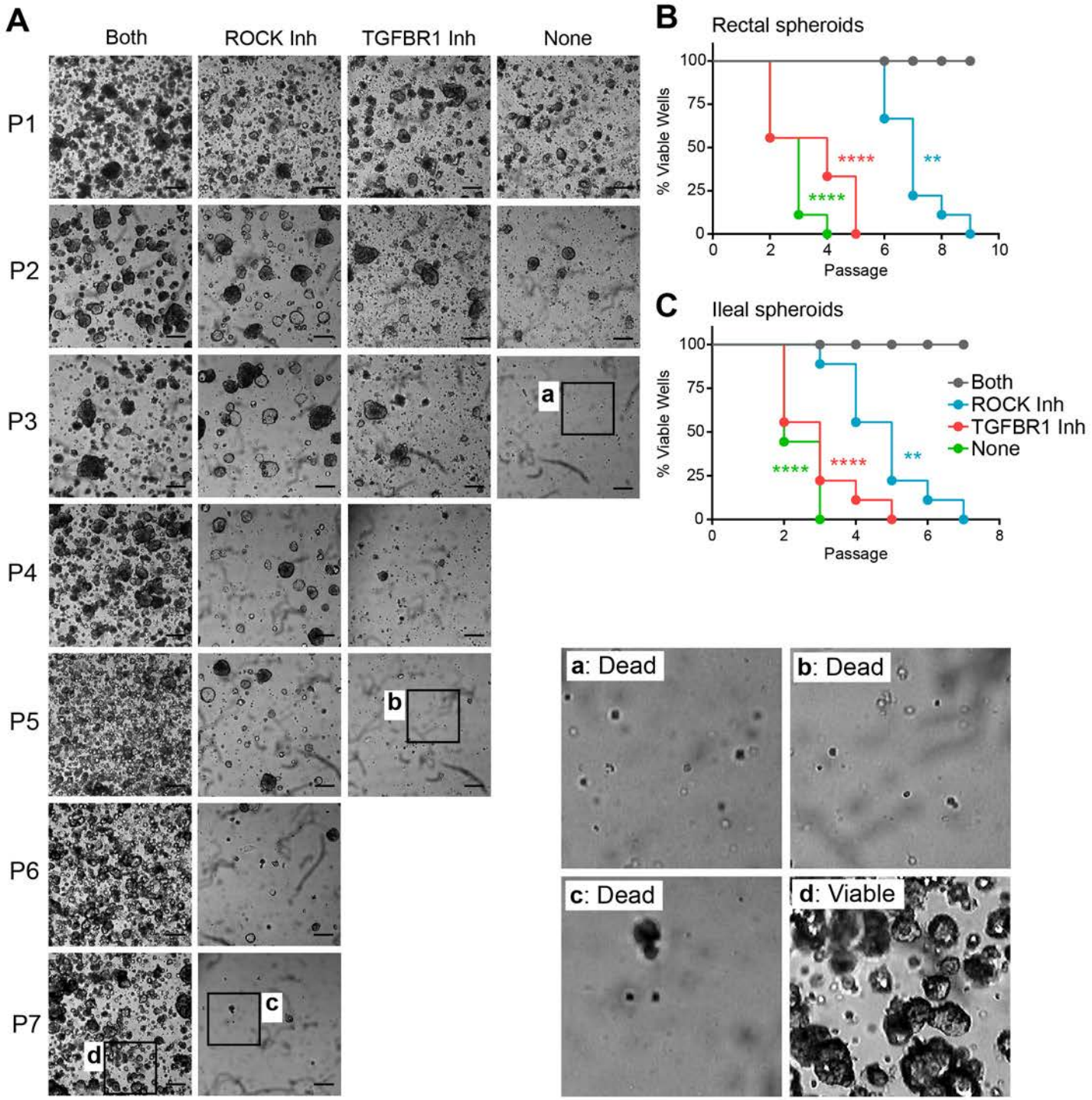
To prepare the bacterial stocks, *E. coli* strains were streaked from frozen stocks to MacConkey agar and grown overnight at 37^{o}C. A single colony was used to inoculate 5 ml of LB broth and incubated overnight at 37°C without agitation. Cultures were pelleted and re-suspended in antibiotic-free DMEM with FCS and 0.5% D-mannose to a concentration of ~10^{9} colony forming units/mL, confirmed by OD_{600} and bacterial titration.

Supplemental statistical analyses

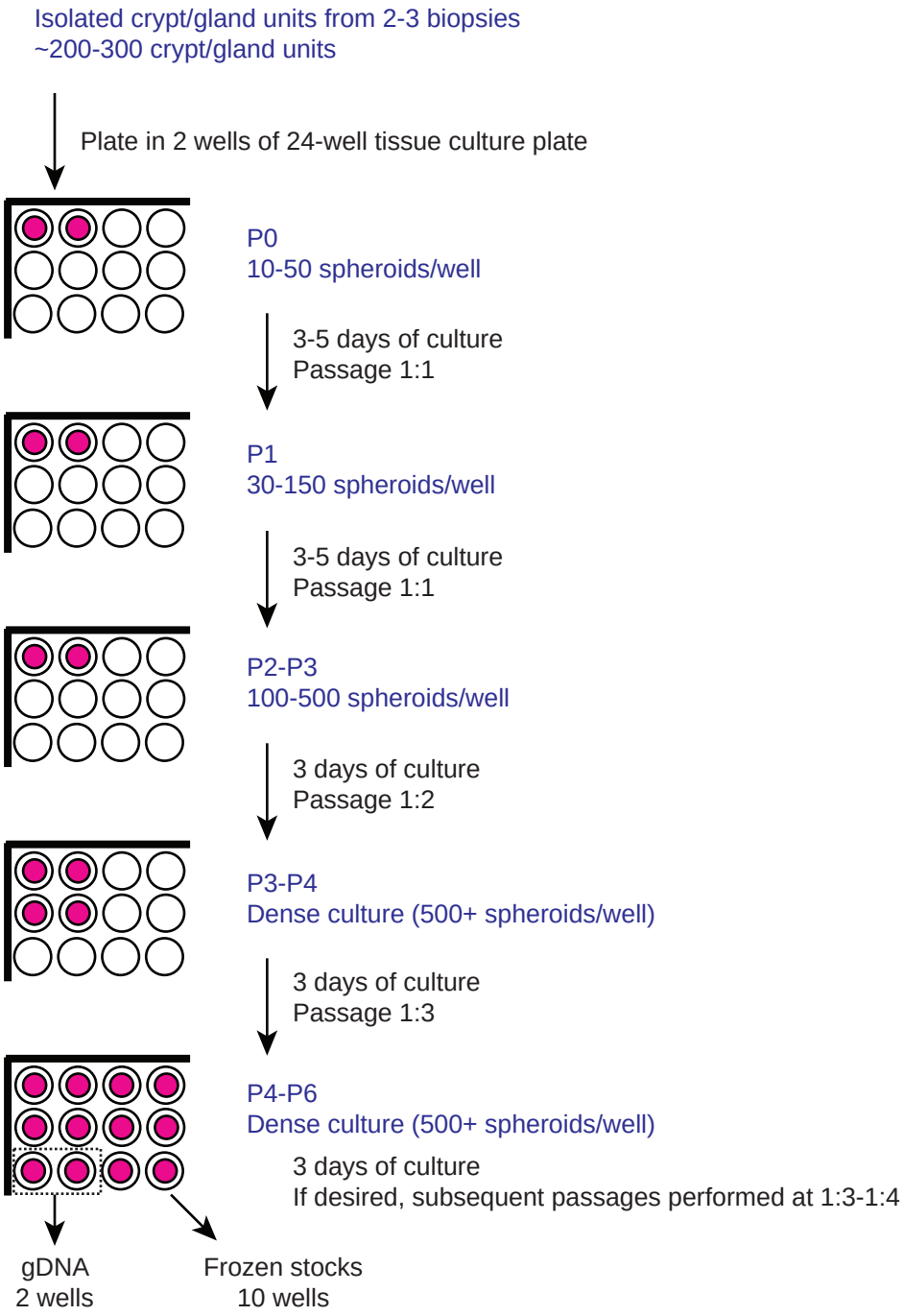
Statistical analyses were performed with Prism GraphPad v6 software. For analysis of the survival curve serial passaging experiment (percent well viability), a log-rank test was used to determine statistical significance. For all other analyses, a Student's t test or 1-way ANOVA followed by a Dunnett's multiple comparisons or Tukey post test was used. $P<0.05$ was considered to be significant.

SUPPLEMENTAL REFERENCES

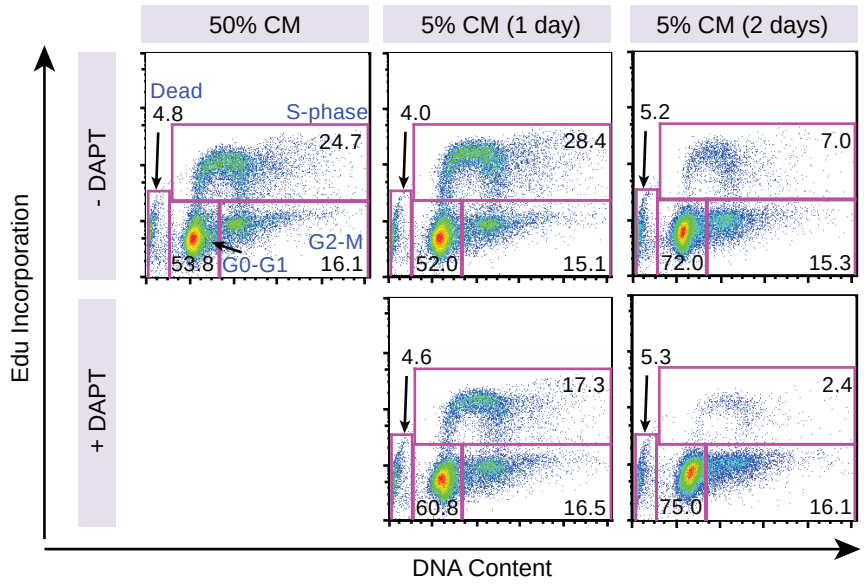
1. Grossmann J, Maxson JM, Whitacre CM, et al. New isolation technique to study apoptosis in human intestinal epithelial cells. Am J Pathol 1998;153:53-62.
2. Bach SP, Renehan AG, Potten CS. Stem cells: the intestinal stem cell as a paradigm. Carcinogenesis 2000;21:469-76.
3. Moses HL, Yang EY, Pietenpol JA. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 1990;63:245-7.
4. Miyoshi H, Ajima R, Luo CT, et al. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. Science 2012;338:108-13.



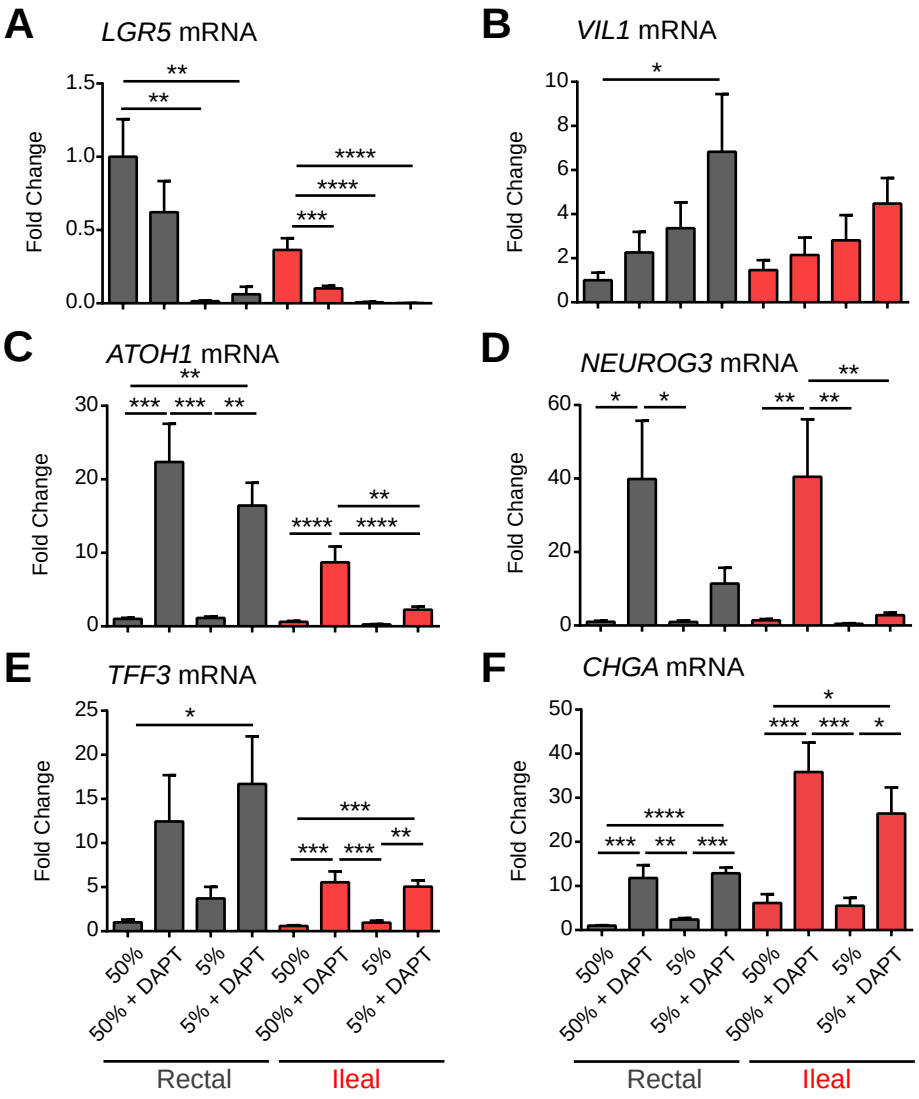
Supplementary Figure 1. Addition of ROCK and TGFBR inhibitors to the 50% L-WRN CM is required for long-term passage of spheroid lines. (A-C) Human rectal (n = 3 lines) and ileal (n = 3 lines) spheroids were cultured in 50% L-WRN CM plus Y-27632 (ROCK Inh), SB 431542 (TGFBR1 Inh), both inhibitors (Both) or without inhibitors (None). Spheroids were serially passaged 1:3 every 3 days with images taken just prior to each passage. (A) Images for one representative experiment with the relative passage number (P1-P7) shown on the left. (B, C) Summaries of serial passage experiments showing percent well viability at each passage for rectal (B) and ileal (C) cultures (n = 3 experiments per line). **, P<0.01; ***, P<0.001; ****, P<0.0001 by log-rank test compared to "Both": χ^2 = 10.72, P = 0.0011 (B, ROCK); χ^2 = 17.95, P<0.0001 (B, TGRBR1); χ^2 = 18.48, P<0.0001 (B, None); χ^2 = 10.21, P = 0.0014 (C, ROCK); χ^2 = 18.14, P<0.0001 (C, TGFBR1); χ^2 = 17.41, P<0.0001 (C, None). Inh, inhibitor. Bars, 200 μm.



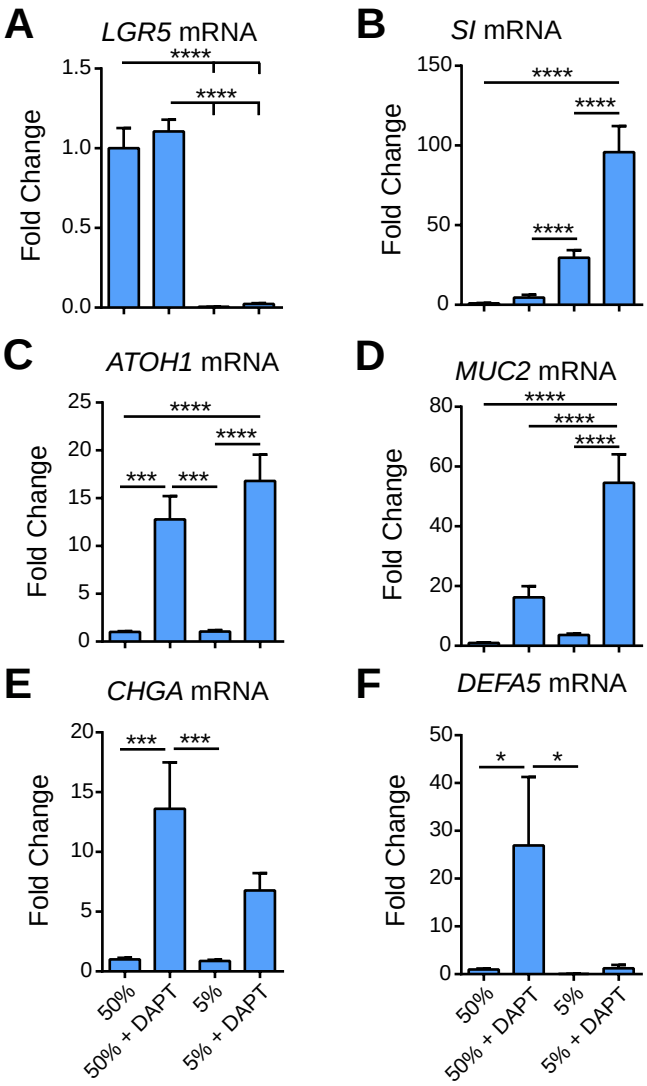
Supplementary Figure 2. Work flow for establishing gastrointestinal epithelial cell cultures from endoscopic biopsies. Isolated crypt/gland units were embedded in Matrigel and plated into 2-wells of a 24-well tissue culture plate (occupied wells denoted by pink circles). Generally, a culture could be expanded to 12 densely seeded wells within 4-6 passages (15-25 days). The typical passage scheme and approximate number of spheroids per well is provided. gDNA, genomic DNA.



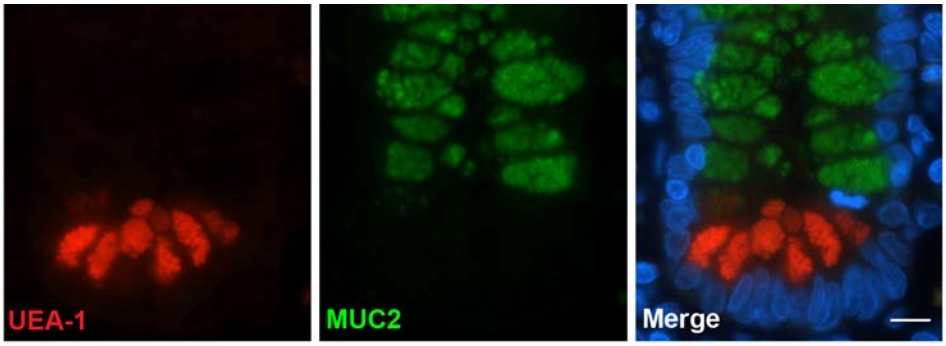
Supplementary Figure 3. Reduced L-WRN CM concentration results in cell cycle exit. Representative dot plots from one cell cycle analysis experiment (rectal line). For these experiments, spheroids were passaged (Day 0) and allowed to recover overnight in 50% L-WRN CM. Media was replaced daily, with some cultures changed to 5% or 5% L-WRN CM plus 5 µM DAPT for the indicated number of days. On Day 3, the spheroids were treated with EdU for 2 hr, dissociated, stained and sorted by flow cytometry based on EdU incorporation and SYTOX AAD (DNA content marker). Fluorescence-activated cell sorting (FACS) was performed with a FACSCalibur (BD Biosciences), and data were analyzed with FloJo software. For each sample, 30,000 events were collected.



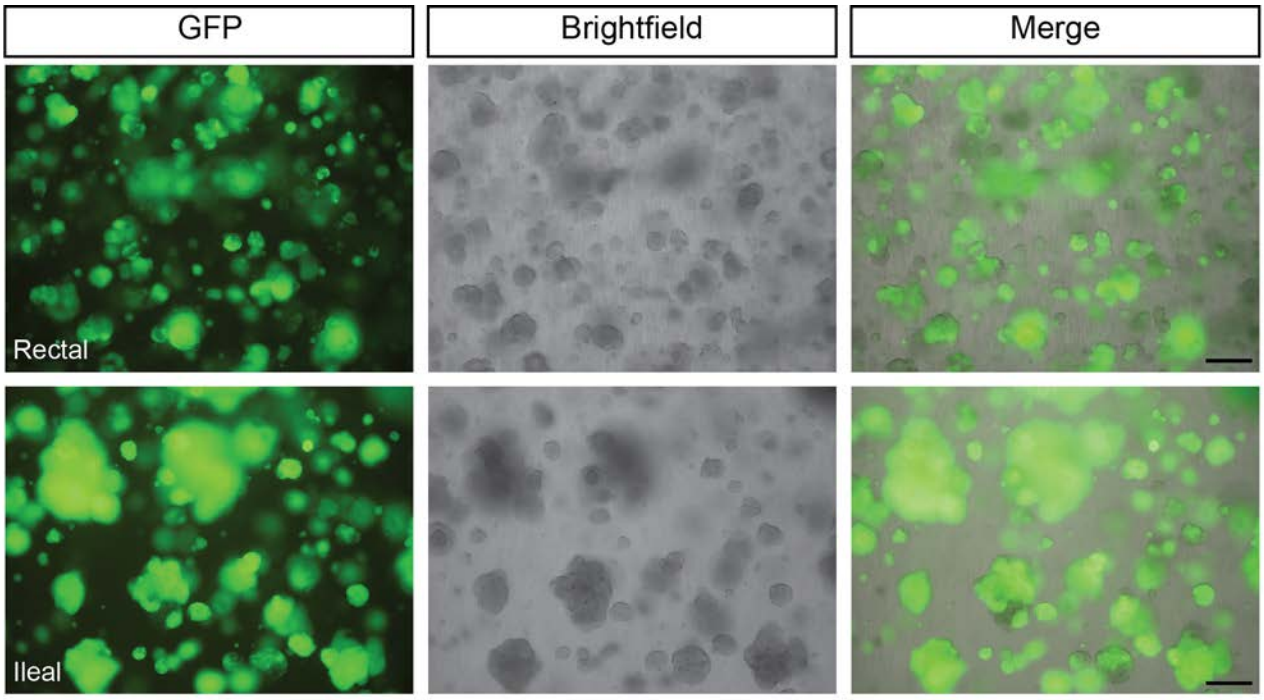
Supplementary Figure 4. Rectal and ileal spheroids retain a site-specific program of differentiation. (A-F) Human rectal (n = 3 lines) and ileal (n = 3 lines) spheroids were passaged (Day 0) and cultured in 50% L-WRN CM overnight. The following day (Day 1), medium was replaced to either 50% L-WRN CM, 50% L-WRN CM + 5 µM DAPT, 5% L-WRN CM or 5% L-WRN CM + 5 µM DAPT (all with ROCK inhibitor). Media were replaced daily, and cell lysates were collected for RNA extraction on Day 3. Gene expression levels of *LGR5* (A), *VIL1* (B), *ATOH1* (C), *NEUROG3* (D), *TFF3* (E) and *CHGA* (F) were analyzed by qRT-PCR. Data are presented as fold change relative to rectal 50% L-WRN CM (mean ± s.e.m.; n = 3 per line). *, P <0.05; **, P <0.01; ***, P <0.001; ****, P <0.0001 by 1-way ANOVA followed by a Tukey's post-test compared to 50% L-WRN CM of the same region: F = 7.86, P = 0.0005 (A, Rect); F = 17.28, P <0.0001 (A, Ile); F = 2.73, P = 0.0599 (B, Rect); F = 1.94, P = 0.1429 (B, Ile); F = 12.82, P <0.0001 (C, Rect); F = 12.73, P <0.0001 (C, Ile); F = 6.62, P = 0.0013 (D, Rect); F = 11.83, P <0.0001 (D, Ile); F = 3.70, P = 0.0215 (E, Rect); F = 13.21, P <0.0001 (E, Ile); F = 14.57, P <0.0001 (F, Rect); F = 10.60, P <0.0001 (F, Ile).



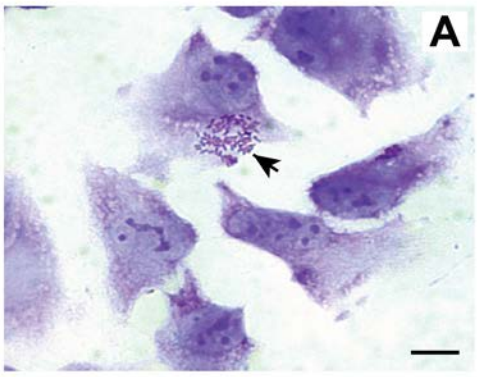
Supplementary Figure 5. Differentiation of human duodenal spheroid epithelial cells. (A-F) Human duodenal spheroids (n = 3 lines) were passaged (Day 0) and cultured in 50% L-WRN CM overnight. The following day (Day 1), medium was replaced to either 50% L-WRN CM, 50% L-WRN CM + 5 µM DAPT, 5% L-WRN CM or 5% L-WRN CM + 5 µM DAPT (all with ROCK inhibitor). Media were replaced daily, and cell lysates were collected for RNA extraction on Day 3. Gene expression levels of *LGR5* (A), *SI* (B), *ATOH1* (C), *MUC2* (D), *CHGA* (E) and *DEFA5* (F) were analyzed by qRT-PCR. Data are presented as fold change relative to 50% L-WRN CM (mean ± s.e.m.; n = 3 per line). *, *P* <0.05; ***, *P* <0.001; ****, *P* <0.0001 by 1-way ANOVA followed by a Tukey's post-test compared to 50% L-WRN CM condition: F = 62.82, *P* <0.0001 (A); F = 27.97, *P* <0.0001 (B); F = 22.09, *P* <0.0001 (C); F = 25.65, *P* <0.0001 (D); F = 9.46, *P* = 0.0001 (E); F = 3.64, *P* = 0.0239 (F).



Supplementary Figure 6. UEA-1 lectin localizes to human Paneth cells. Formalin-fixed paraffin-embedded tissue from the human small intestine was immunostained for UEA-1 lectin (red) and MUC2 (green). A single, representative crypt base is shown. Bar, 10 μm.



Supplementary Figure 7. Generation of GFP-expressing human intestinal spheroid lines via lentivirus infection for mucus layer analysis. Rectal and ileal human intestinal spheroids were infected with a lentiviral construct that contained a non-specific, scrambled shRNA and a GFP expression cassette. Very high infection efficiency was observed, with nearly 100% of the epithelial cells in both the rectal and ileal lines expressing the GFP marker within 3 days after infection (data not shown). The high infection efficiency resulted in the persistence of mostly pure GFP-expressing cell populations for several passages without the requirement for selection of positive clones (images are from the fourth passage post-infection). Bar, 200 μm.



Supplementary Figure 8. Sparse adherence of E. coli O157:H7 strain 86-24 to HeLa cells. HeLa cells (A) were incubated with enterohemorrhagic *E. coli* O157:H7 strain 86-24 for 1 hr followed by several washes and staining with crystal violet to visualize adherent bacteria (dark purple). Sparse adherence was noted for the O157:H7 strain with HeLa cells; a single bacterial colony was observed in a single field (arrow). Other fields of view had no adherent cells. Bar, 20 μm.

Supplementary Table 1 List of established epithelial cell lines from regions of the gastrointestinal tract.

Patient	Tissue Region	Patient Age (years)	Diagnosis* (Non-IBD/CD/UC)
1	**Rectum**	**52**	**Non-IBD**
2	**Neo-terminal ileum**	**39**	**UC**
2	Ileal pouch	39	UC
3	**Neo-terminal ileum**	**29**	**CD**
4	**Rectum**	**25**	**CD**
5	**Rectum**	**62**	**Non-IBD**
6	Rectum	60	Non-IBD
7	Left colon	23	UC
7	Right colon	23	UC
8	Rectum	37	CD
9	Rectum	55	Non-IBD
10	Rectum	42	Non-IBD
11	Rectum	76	Non-IBD
11	Right colon	76	Non-IBD
12	Stomach (body)	58	Non-IBD
12	Stomach (fundus)	58	Non-IBD
13	Right colon	61	CD
14	Rectum	44	CD
15	Rectum	57	CD
16	Rectum	47	Non-IBD
16	**Terminal ileum**	**47**	**Non-IBD**
17	Rectum	71	CD

17	Right colon	71	CD
18	Rectum	19	UC
18	Terminal ileum	19	UC
19	Rectum	47	UC
19	Esophagus (distal)	47	UC
20	Rectum	49	Non-IBD
21	Left colon	56	UC
22	**Duodenum**	**35**	**CD**
23	**Duodenum**	**54**	**Non-IBD**
24	**Duodenum**	**69**	**Non-IBD**
25	Rectum	40	Non-IBD
25	**Terminal ileum**	**40**	**Non-IBD**
26	Terminal ileum	44	UC
27	Rectum	60	Non-IBD
28	Rectum	28	UC
28	Terminal ileum	28	UC
29	Rectum	57	Non-IBD
29	Terminal ileum	57	Non-IBD
30	Rectum	51	Non-IBD
30	Terminal ileum	51	Non-IBD
31	Terminal ileum	69	Non-IBD
32	Rectum	34	UC
32	Terminal ileum	34	UC
33	**Terminal ileum**	**50**	**Non-IBD**
34	**Rectum**	**34**	**Non-IBD**
34	Terminal ileum	34	Non-IBD

35	Rectum	31	UC
35	Terminal ileum	31	UC
36	Terminal ileum	23	CD
37	Terminal ileum	29	CD
38	Rectum	30	CD
38	Terminal ileum	30	CD
39	Terminal ileum	33	CD
40	Rectum	56	UC
40	Terminal ileum	56	UC
41	Terminal ileum	37	CD
42	Rectum	32	Non-IBD
42	Terminal ileum	32	Non-IBD
43	Terminal ileum	25	CD
44	Terminal ileum	60	Non-IBD
45	Terminal ileum	50	Non-IBD
46	Terminal ileum	29	CD
47	Terminal ileum	23	CD

Bold indicates lines used to generate the data presented in this study.

*All cell lines from IBD patients were established from regions of non-active inflammatory disease.

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

n.a., not available.

Supplementary Table 2 Oligonucleotide sequences used for quantitative reverse transcriptase PCR.

Gene	*Forward Primer (5' to 3')*	*Reverse Primer (5' to 3')*
ATOH1	GCAAAAGAATTTGTCTCCTTCTCT	ATCCGAGTCACTGTAATGGGAAT
AXIN2	CATGACGGACAGCAGTGTAGA	AACTCCAGCTTCAGCTTTTCC
CA1	ACAACGATAACCGATCAGTGC	CTTTGCAGAATTCCAGTGAGC
CHGA	AGAATTTACTGAAGGAGCTCCAAG	TCCTCTCTTTTCTCCATAACATCC
DEFA5	AGGAAATGGACTCTCTGCTCTTAG	TTGCACTGCTTTGGTTTCTATCTA
GAPDH	GACCTGCCGTCTAGAAAAACC	GCTGTAGCCAAATTCGTTGTC
KI67	AAGAGAGTGTCTATCAGCCGAAGT	GTGGCCTGTACTAAATTGACTGTG
LGR5	CCTTCATAAGAAAGATGCTGGAAT	GTTTAATGGGGGAAATGTACAGAG
LYZ	GGTTACAACACACGAGCTACAAAC	AGTTACACTCCACAACCTTGAACA
MUC2	AGGATCTGAAGAAGTGTGTCACTG	TAATGGAACAGATGTTGAAGTGCT
MYC	GGCACTTTGCACTGGAACTTA	GCAGTAGAAATACGGCTGCAC
NEUROG3	CCCATTCTCTCTTCTTTTCTCCTT	GTTGAGGCGTCATCCTTTCTAC
OLFM4	TTCTCCTAGCCCTTCTGTTCTTCC	TTCCAAGCGTTCCACTCTGTCC
SI	CTGCATTTGAAAGAGGACAGC	ACTCTGCTGTGGAAGTCCTGA
SLC10A2	GGGTTACTCCCTGGGGTTTC	CCATGACATTTCTTGTATGCCACA
TFF3	AGCTCTGCTGAGGAGTACGTG	ACAGAAAAGCTGAGATGAACAGTG
TRPV6	GACAGGAGACGGGACCTCTA	TTGGCAGCTAGAAGGAGAGGA
VIL1	ACTACTGGATTGGCCAGGACT	TGGACGTCATAGGAGTTGGTC
VIM	CAGGCTCAGATTCAGGAACAG	CAGAGAGGTCAGCAAACTTGG
GAPDH (DNA assay)	TGACAACGAATTTGGCTACAGC	TGATGGTACATGACAAGGTGC
malB (DNA assay)	AACAGTGTTTCCAGACTACCG	TTCCCAGTCATTCTGTTGTGC