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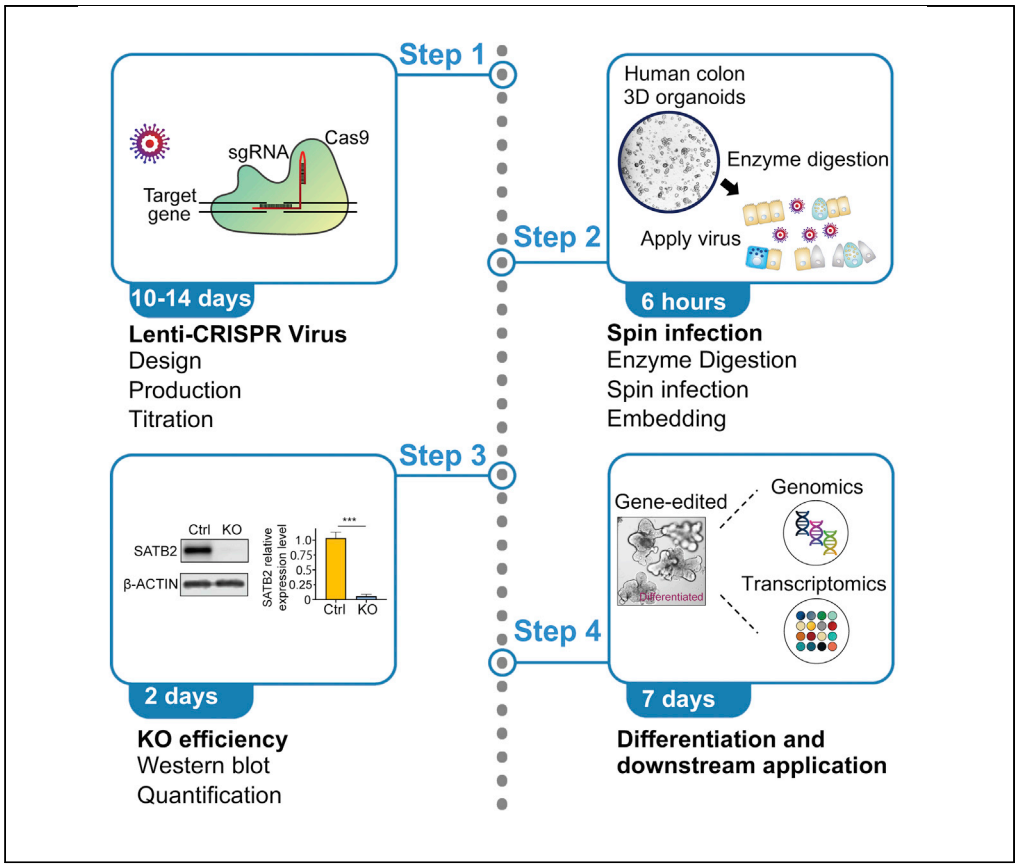
Protocol

Rapid establishment of human colonic organoid knockout lines



Human colonic organoids derived from biopsy or autopsy tissues are a vital tool to study mucosal homeostasis, model colonic diseases, and develop therapeutics. Rapid and reliable generation of knockout organoid lines from multiple donors enables analysis of specific gene functions. Here, we report protocols to produce colonic organoid knockout lines within 1 to 2 weeks using lentiviral delivery of CRISPR-Cas9, achieving knockout efficiency of 90% or greater. These lines are suitable for multi-lineage differentiation and downstream analysis.

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Highlights

Optimized 3D culture of primary human colonic organoids

Protocol for efficient gene knockout with CRISPR-Cas9

Enables multilineage mucosal differentiation

Gu et al., STAR Protocols 3, 101308
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101308

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Rapid establishment of human colonic organoid knockout lines

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https://doi.org/10.1016/j.xpro.2022.101308

SUMMARY

Human colonic organoids derived from biopsy or autopsy tissues are a vital tool to study mucosal homeostasis, model colonic diseases, and develop therapeutics. Rapid and reliable generation of knockout organoid lines from multiple donors enables analysis of specific gene functions. Here, we report protocols to produce colonic organoid knockout lines within 1 to 2 weeks using lentiviral delivery of CRISPR-Cas9, achieving knockout efficiency of 90% or greater. These lines are suitable for multi-lineage differentiation and downstream analysis.

For complete details on the use and execution of this protocol, please refer to Gu et al. (2022).

BEFORE YOU BEGIN

Different methods have been developed and employed to culture human colonic organoids, perform genomic engineering, and differentiate organoids (Dame et al., 2018; Fujii et al., 2018, 2019; Jung et al., 2011; Miao et al., 2020; Sato et al., 2011; Tsai et al., 2018). We evaluated and incorporated components of different protocols, with further optimization, to arrive at this streamlined method. Notably, many protocols use clonal growth to select for mutant lines. Clonal growth of human colonic organoids takes several weeks. Our protocol employs a knockout strategy in bulk cell populations to enable rapid establishment of mutant lines within a week or two. We routinely achieve knockout efficiency of 90% or greater, sufficient for many types of analysis. We note that organoid lines derived from different donors, although morphologically similar, exhibit significant molecular differences. We routinely generate pairs of isogenic control and mutant lines from 4-5 different donors for phenotypic analysis.

Institutional permissions

Human colonic tissues must be obtained in compliance with institutional regulations and guidelines.

General laboratory preparation

1. All protocol steps that involve lentiviral particles must be performed in a BSL2 biosafety lab equipped with a BSL-2 tissue culture hood, humidified incubator, and centrifuges.
2. Prepare 10% Pure Bright® bleach solution for disposal of plasticware and media that come into contact with viral particles.
3. Disinfect the work area each time lentiviral particles are used.
4. Prepare all solutions in the materials and equipment section before sample processing.

Preparation of Lenti-CRISPR virus

Timing: 2 weeks

5. Production of lentiviral particles.
   a. Using SATB2 gene knockout as an example, prepare lentiCRISPRv2-SATB2 plasmids. Human SATB2 sgRNAs designed with the Synthego CRISPR design tool are cloned into a lentiCRISPR v2 vector (Addgene plasmid #52961) using the Zhang lab's cloning protocol. (https://media.addgene.org/data/plasmids/52/52961/52961-attachment_B3xTwla0bkYD.pdf).
   b. LentiCRISPRv2-SATB2 (SATB2-KO) or LentiCRISPRv2 (Ctrl), along with lentiviral second-generation helper plasmids (psPAX2 Addgene #12260 and pMD2.G Addgene #12259), are transfected with lipofectamine 3000 (Thermo Fisher Scientific, L3000015) in HEK293FT cells. Follow the manufacturer's instructions for modification of DNA composition based on plasmid size.

Plasmid	10 cm plate	6 cm plate	6-Well plate
psPAX2	6.97 μg	2.69 μg	1.21 μg
pMD2.G	2.11 μg	0.82 μg	0.37 μg
LentiCRISPRv2	8.26 μg	3.19 μg	1.43 μg

   c. The transfection conditions and procedures should be followed as outlined by the manufacturer's instructions. (https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Flipofectamine3000_protocol.pdf).
   d. Collect the culture medium from the plates and centrifuge for 5 min at 500 × *g* to remove any cells.
   e. Collect the supernatant and filter through a 0.45 μm filter to remove smaller debris.
   f. Concentrate lentivirus with centrifugal ultrafiltration devices (Millipore Sigma, UFC910024) or other suitable methods. We anticipate ~400 μL concentrated virus from approximately 24 mL of medium.
   g. Aliquot concentrated virus at 10–50 μL, depending on usage, per cryogenic tube (Sigma BR114840). Tubes can be stored in a −80°C freezer for up to two years.

Note: Avoid freeze-thaw cycles. Aliquots should be single use.

6. Measure the titer of the virus stock. Measure by median tissue culture infectious dose ($TCID_{50}$). To keep consistency across independent experiment, we titer every batch of new virus in HEK293FT cells.
   a. Plate HEK293FT cells in 96-well plates at 5,000 cells per well in DMEM with 10% FBS for 16 h. (Figure 1A).
   b. Make a serial dilution of the virus, from 10^{-1} to 10^{-8}, in 200 μL media, using 10 μL of virus as shown in the plate diagram below. Select the infected cells with 1 μg/mL puromycin for 3 days and maintain in 0.5 μg/mL puromycin for 1 week.
   c. Count the number of resistant clones. (Figures 1B, a–d).
   d. $TCID_{50}$/mL calculation (Reed–Muench Method).
      i. Calculate Proportionate Distance (PD) between the two dilutions in between 50% resistant: ((% next above 50%)- 50%) / ((% next above 50%) – (% next below 50%)).
      Example calculation for Figure 1B: PD = (70%–50%) / (70%–10%) = 20/60 = 0.333.
      ii. Calculate 50% end point. The log lower number should be selected as the last dilution in which resistance rate is above 50%
      Example calculation for Figure 1B: Log lower = -6.
      iii. Calculate the ID_{50} by taking 10 to the power of the sum of the log lower dilution and the inverse of the PD.

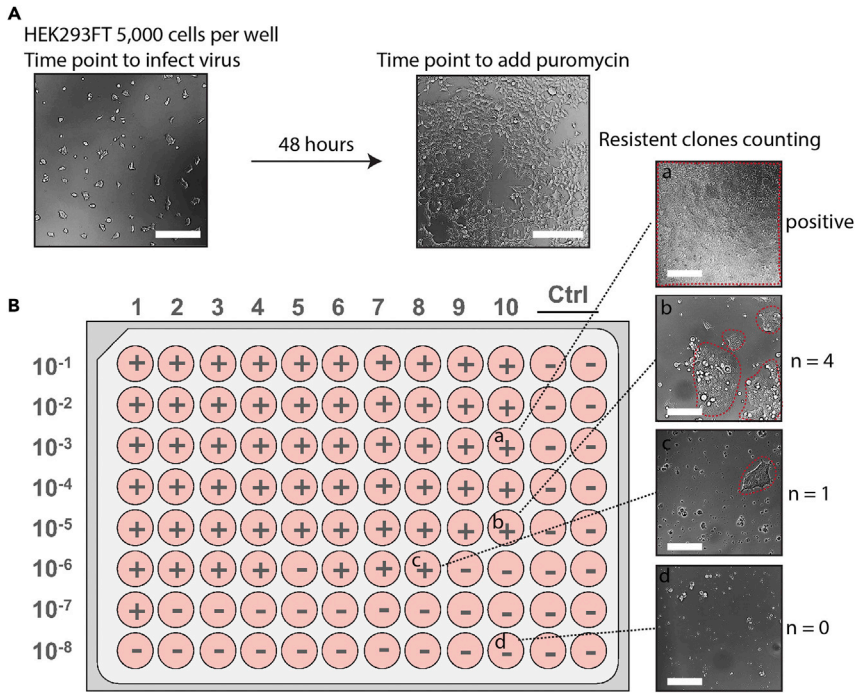

Figure 1. Determine the titer of the lentivirus stock

(A) Images of HEK293FT at the time point of adding virus or puromycin. Scale bars, 100 μm.

(B) Representative images (a - d) and results for puromycin-resistant clones. Scale bars, 100 μm.

Example calculation for Figure 1B: log ID_{50} = -6 + 0.333*(-1) = -6.333.
ID_{50}= $10^{-6.333}$.

iv. Calculate TCID 50/mL. Divide ID_{50} by the mL of viral inoculum added to start dilution.
Example below: according to our protocol, viral inoculum amount = 0.01 mL.
TCID 50/mL = 1 / $10^{6.333}$ / 0.01= $10^{8.333}$ =2.15 × 10^{8}.

⚠ CRITICAL: For a more accurate estimation of viral concentration, use virus stock stored at −80°C, NOT freshly concentrated virus. High titer of virus is important for organoids infection. We expect titers of greater than 10^{8} $TCID_{50}$/mL in 293FT cells.

Preparation of human colonic organoids

Timing: 1 week

7. Human colonic organoid culture.
   a. Human colonic organoid culture medium and stock medium compositions are listed in the materials and equipment section.
   b. We use both the TrypLE enzyme treatment and mechanical trituration to fragment human colonic organoids. We note that prolonged TrypLE treatment or excessive mechanical trituration can damage the organoids and reduce their viability. To strike a proper balance for ease of dissociation and tissue preservation, we optimized the Enzyme-trituration step (see step-by-step method details for details).
8. Determination of the appropriate puromycin selection concentration for each colonic organoid line.
   a. Organoid lines from different donors may have different puromycin sensitivity. We use a cell viability assay validated for 3D culture (CellTiter-Glo 3D Cell Viability Assay Kit from Promega #G9682) to determine the optimal concentration for puromycin selection.

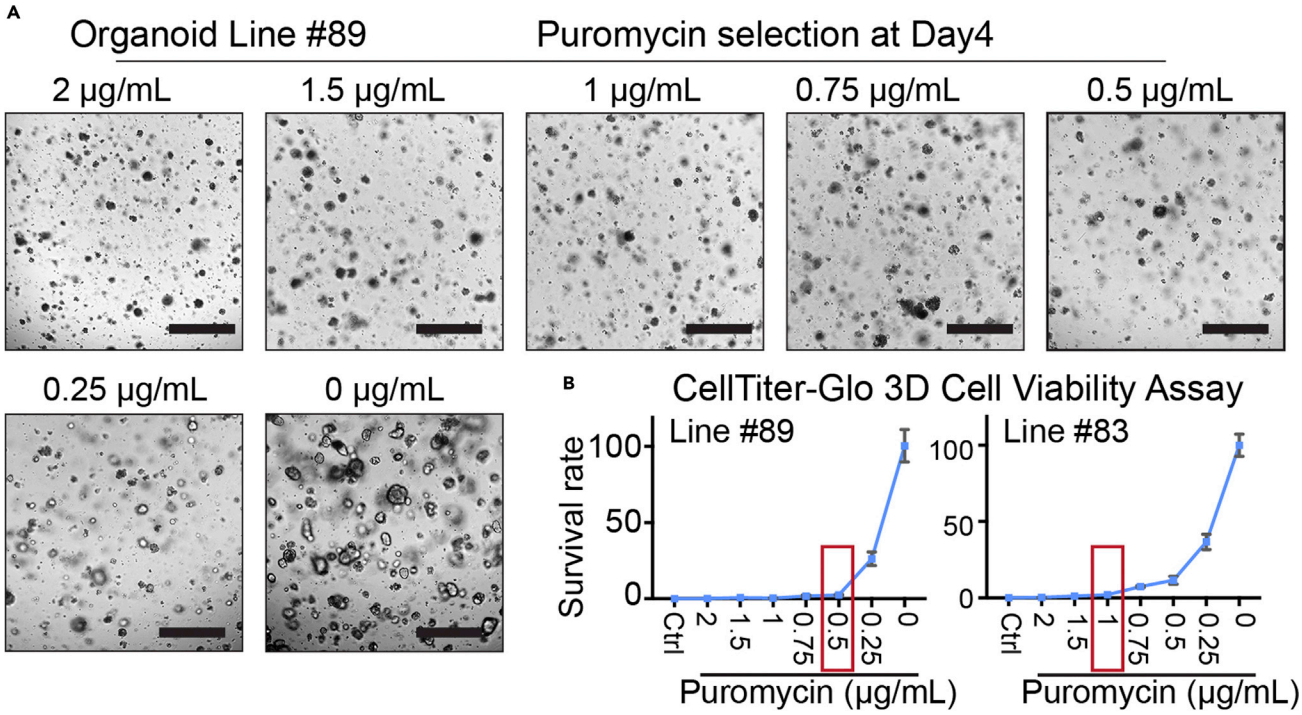

Figure 2. Determination of the appropriate puromycin selection concentration for each colonic organoid line

(A) Images of human colonic organoid line #89 in Matrigel after growing in different puromycin concentrations for 4 days. Scale bars, 200 μm.

(B) Survival curves of Line #83 and #89 measured by CellTiter-Glo 3D cell Viability Assay. Red squares mark the appropriate puromycin concentration for selection. Lines and error bars indicate mean ± S.D.

i. Culture TrypLE enzyme-fragmented human colonic organoids (200–300 small fragments) in 15 μL of Matrigel droplet per well in a 48-well plate for two days.
ii. Change the medium containing different titration (0.25–2 μg/mL) of puromycin and culture for additional 4 days (Figure 2A).
iii. Follow the manufacturer's instructions (for 3D microtissues) for the cell viability assay protocol. https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-3d-cell-viability-assay/?catNum=G9681#protocols.
iv. Record luminescence with the GLOMAX multi+ detection system from Promega.
v. Normalize luminescence of each puromycin titration using the 0 μg/mL control group to calculate the survival rate (Figure 2B). We select the lowest concentration of puromycin that can completely suppress organoid growth.

Alternatives: To estimate the survival rate of organoids after puromycin selection, Matrigel can be dissolved with Cell Recovery solution and stained with Trypan blue.

⚠ CRITICAL: Primocin is needed in primary human colonic organoid culture to suppress growth of microorganisms. Penicillin-Streptomycin is insufficient.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Prep80 (Ascending Colon Organoid)	University of Michigan	Human, 29Y, Female, No active diseases.
Prep83 (Ascending Colon Organoid)	University of Michigan	Human, 45Y, Female, No active diseases.

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Prep87 (Ascending Colon Organoid)	University of Michigan	Human, 21Y, Male, No active diseases.
Prep88 (Ascending Colon Organoid)	University of Michigan	Human, 33Y, Female, No active diseases.
Prep89 (Ascending Colon Organoid)	University of Michigan	Human, 55Y, Male, No active diseases.
HEK293FT	Thermo Fisher Scientific	Cat# R70007
Chemicals, peptides, and recombinant proteins		
TrypLE Express Enzyme	Thermo Fisher Scientific	Cat# 12604021
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific	Cat# 25200072
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# D2650
2-Mercaptoethanol	Sigma-Aldrich	Cat# M7522
4× Laemmli Sample Buffer	Bio-Rad	Cat#1610747
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific	Cat# 89900
Halt™ Protease and Phosphatase Inhibitor Cocktails	Thermo Fisher Scientific	Cat# 78443
10× Tris/Glycine/SDS Buffer	Bio-Rad	Cat# 1610732
10× Tris/Glycine Buffer	Bio-Rad	Cat# 1610734
Blotting Grade Blocker Nonfat Dry Milk	Bio-Rad	Cat# 1706404
Methanol	EMD Millipore	Cat# MX0490-4
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634028
Cell Recovery Solution, Corning	VWR	Cat# 47743-696
Corning Matrigel Matrix Phenol Red Free	VWR	Cat# 47743-722
Recombinant Human EGF Protein, CF	R&D Systems	Cat# 236-EG-01M
CHIR99021,>98%	Sigma-Aldrich	Cat# SML1046
Bovine Serum Albumin (BSA)	VWR	Cat# 10842-692
Primocin	Invivogen	Cat# ant-pm-1
A 83-01	Cayman Chemical	Cat# 9001799
Polybrene	MilliporeSigma	Cat# TR-1003-G
Niacinamide (Nicotinamide)	Sigma-Aldrich	Cat# N5535
Gastrin 1 human	Sigma-Aldrich	Cat# G9020
Y-27632, Dihydrochloride Salt	LC Laboratories	Cat# Y-5301
N-2 supplement (100×)	Thermo Fisher Scientific	Cat# 17502048
B-27 Supplement (50×)	Thermo Fisher Scientific	Cat# 17504044
HEPES	Thermo Fisher Scientific	Cat# 15-630-080
GlutaMAX Supplement	Thermo Fisher Scientific	Cat# 35050061
N-Acetyl-L-Cysteine	Sigma-Aldrich	Cat# A9165
Recombinant Human FGF-basic (FGF-2)	PeproTech	Cat# 100-18B
Recombinant Human IGF-1	BioLegend	Cat# 590908
DAPT	Cayman Chemical	Cat# 13197
SB 202190	Tocris Bioscience	Cat# 1264
Fetal Bovine Serum (FBS)	R&D Systems	Cat# S11150H
Lipofectamine 3000	Thermo Fisher Scientific	Cat# L3000015
Gibco DMEM	Thermo Fisher Scientific	Cat# 11-965-118
Puromycin	Sigma-Aldrich	Cat# P8833
Neomycin (G418)	Sigma-Aldrich	Cat# N6386
Hygromycin B (50 mg/mL)	Thermo Fisher Scientific	Cat# 10687010
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140122
PBS without calcium magnesium	Cytiva	Cat# SH30028
E.Z.N.A Tissue DNA Kit	OMEGA	Cat# D3396
RNeasy Mini Kit	QIAGEN	Cat# 74104
Alcian Blue Stain Kit	Vector Laboratories	Cat# H-3501
Hematoxylin QS	Vector Laboratories	Cat# H-3404
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
Sucrose	Sigma-Aldrich	Cat# S0389
Pure Bright Liquid Bleach	KIK Custom Products	Cat# KIKBLEACH6
Trypan Blue Solution	Thermo Fisher Scientific	Cat# 15250061

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
CellTiter-Glo 3D Cell Viability Assay Kit	Promega	Cat# G9682
Antibodies		
Rabbit monoclonal anti SATB2 (WB 1:1,000)	Abcam	Cat# Ab92446 RRID: AB_10563678
Mouse monoclonal anti β-ACTIN (WB 1:5,000)	Sigma-Aldrich	Cat# A5441 RRID: AB_476744
Rabbit polyclonal anti MUC2 (IHC 1:500)	Invitrogen	Cat# PA5-79702 RRID: AB_2746817
Anti-mouse IgG, HRP-linked (WB 1:10,000)	Cell Signaling Technology	Cat# 7076S RRID: AB_330924
Anti-Rabbit IgG, HRP-linked (WB 1:10,000)	Cell Signaling Technology	Cat# 7074S RRID: AB_2099233
Goat anti-Rabbit IgG ImmPRESS™ Secondary (Ready for use)	Vector Laboratories	Cat# MP-7451 RRID: AB_2631198
Software and algorithms		
GraphPad Prism 9		https://www.graphpad.com v9.1.2 (225) RRID: SCR_002798
ImageJ		https://imagej.nih.gov/ij/v1.51 (100) RRID: SCR_003070
Adobe Photoshop and Illustrator		https://www.adobe.com RRID: SCR_014199 RRID: SCR_010279
Recombinant DNA		
psPAX2	Gift from Didier Trono	Addgene #12260
LentiCRISPRv2	Sanjana et al., 2014	Addgene #52961
pMD2.G	Gift from Didier Trono	Addgene #12259
Others		
Falcon Cell Strainer 70 µm	Corning	Cat# 352350
Tissue-Tek Cryomold	SAKURA	Cat# 4565
Mini-PROTEAN TGX Stain-Free Precast Gels	Bio-Rad	Cat# 4568126
Non-Tissue Culture Treated Plate, 24 wells	Corning	Cat# 351147
Syringes with BD Luer-Lok® Tip, 30 mL, sterile	BD	Cat# 302832
Syringe Filters with Acrylic Housing, 0.45 µm, Polyethersulfone, Sterile	VWR	Cat# 28145-505
Acrodisc Syringe Filters, 0.2 µm, Sterile,	Pall Corporation	Cat# 4612
Nalgene; Rapid-Flow; Sterile Disposable Filter Units with PES	Thermo Fisher Scientific	Cat# 566-0020
Seal-Rite 1.5 mL microcentrifuge tube, natural, sterile	USA Scientific	Cat# 1615-5510
EPPENDORF TUBE 5.0 mL	USA Scientific	Cat# 4011-9401
Centrifuge Tubes with Flat Caps, 15 mL	VWR	Cat# 10026-076
Centrifuge Tubes with Flat Caps, 50 mL	VWR	Cat# 10026-078
Multiwell Cell Culture Plates, 6 wells	VWR	Cat# 10062-892
Multiwell Cell Culture Plates, 12 wells	VWR	Cat# 10062-894
Multiwell Cell Culture Plates, 48 wells	VWR	Cat# 10062-898
Tissue Culture Plates 96 wells	USA Scientific	Cat# CC7682-7596
TipOne RPT 10 µL XL	USA Scientific	Cat# 1180-3810
TipOne RPT 200 µL graduated	USA Scientific	Cat# 1180-8810
TipOne RPT 1,000 µL XL graduated	USA Scientific	Cat# 1182-1830
Cryogenic tube	Sigma-Aldrich	Cat# BR114840
Centrifugal ultrafiltration devices	MilliporeSigma	Cat# UFC910024
Serological pipettes, 10 Ml	VWR	Cat# 89130-898
Biological safety cabinet	The Baker Company	Cat# SG403
Water bath 180 Series, Model 2831	Thermo Scientific	Cat# 51221073
CO2 tissue incubators, Series 3	Thermo Scientific	Cat# 4110
Inverted contrasting tissue culture microscope Ts2	Nikon	Cat# 136772
Fluorescence microscopy Ti2	Nikon	Cat# TI2-D-PD
Swing rotor centrifuge	Eppendorf	Cat# 5811T

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GLOMAX multi+ detection system	Promega	Cat# E8032
Fume hood	Air master system CORP	Cat# HD08018
NanoDrop 2000 Spectrophotometer	Thermo Scientific	Cat# T993
MINI-100 Orbital Genie Shaker	Scientific industries	Cat# SI-MI100
Digital Heat-block	VWR	Cat# 12621-084
Mini-Protean® Tetra Cell, 2-Gel System	Bio-Rad	Cat# 1658005

MATERIALS AND EQUIPMENT

Tissue culture materials

10% Pure Bright® bleach solution

Take 100 mL of Pure Bright® liquid bleach and add 900 mL of tap water to make 10% bleach solution. Make fresh 10% bleach solution before each experiment.

Matrigel

Store Matrigel in −20°C. Thaw a 10 mL bottle of Matrigel at 4°C 16–20 h. Aliquot 0.5 mL or 1 mL per tube and store at −20°C. Pre-thaw an aliquot at 4°C 1 h before each experiment.

L-WRN conditioned medium

The L-WRN cell line was originally made in Dr. Thaddeus Stappenbeck's Lab (VanDussen et al., 2019). Follow the steps below for collecting WRN conditioned medium.

- Prewarm 25 mL of basic medium in a 50 mL tube in a 37°C water bath.
- Thaw one cryotube of L- WRN cells (1×10^7) in a 37°C water bath.
- Transfer the cells to prewarmed basic medium immediately after the ice disappears. The cells are very fragile. Excessive shaking and pipetting should be avoided.
- Transfer the basic medium containing the cells to a 175-cm^2 cell culture plate.
- Incubate the cells 16–20 h in a cell culture incubator.
- Change the medium to 25 mL of fresh prewarmed basic medium.
- Culture the cells to 100% confluency. This should take about two days.
- Wash the cells with 10 mL of 18°C–25°C PBS by rocking the plate back and forth several times. Aspirate the PBS.
- Add 2 mL of 0.25% trypsin/EDTA to the plate. Tap the plate several times to ensure the plate has been completely covered.
- Incubate the plate for 3 min in a cell culture incubator.
- Add 23 mL of basic medium supplemented with 500 μg/mL G418 and 500 μg/mL hygromycin, making a total volume of 25 mL in each plate.
- Pipette the cell suspension several times to ensure that the cells are equally distributed.
- Distribute the 25 mL cell suspension into five 175-cm^2 plates, 5 mL per plate. Add 20 mL of basic medium with G418 and hygromycin to each plate. The total volume in each plate should be 25 mL.
- Culture the cells to confluency. This takes approximately 2–3 days.
- Wash the cells with 10 mL of 18°C–25°C PBS by rocking the plate back and forth several times. Aspirate the PBS.
- Add 2 mL of 0.25% trypsin/EDTA per plate. Tap each plate several times to ensure the solution covers the plate entirely.
- Incubate the plates for 3–5 min at 37°C.
- Add 18 mL of basic medium to make a total volume of 20 mL.
- Pipette the cell suspension several times to ensure that the cells are equally distributed.
- Transfer 100 mL of medium containing the cells into two 50 mL tubes.
- Centrifuge the 50 mL tubes at 300 × *g* for 5 min.
- Discard the supernatant and resuspend the cell pellet in 25 mL of basic medium.

- Add 24 mL of basic medium to 25 175-cm^2 plates. Then, add 1 mL of the cell suspension to each plate. At the conclusion, there will be 25 plates, each with 25 mL of medium.
- Incubate the plates for 2–3 days until the cells reach overconfluency and have cell aggregates coming off the bottom of the plate and into the medium.
- Aspirate the medium from each plate.
- Rinse the cells with 5 mL (per plate) of primary culture medium. Rock the plate back and forth to cover the entire surface area. Aspirate the primary culture medium.
- Add 25 mL of primary culture medium per plate.
- Incubate the plates for 24 h at 37°C.
- Collect the medium from the plates and transfer to 50 mL tubes. Two plates worth of medium can be pooled into one 50 mL tube. Add 25 mL of fresh primary culture medium to the plates and place them back into the incubator. This step should only be done with two plates at a time to avoid drying out the cells.
- Centrifuge the 50 mL tubes containing the conditioned medium at 2,000 × *g* for 5 min and decant the supernatant through a 0.2 μm filter into a large bottle. Filter ~625 mL of conditioned medium, which can be stored at 4°C during the collection period.
- Repeat steps 28–30 every 24 h, collecting the second, third, and fourth rounds of conditioned medium.
- Mix each of the four collected conditioned medium rounds at equal proportion.
- Aliquot the mixed media at volumes of 25 mL or 12.5 mL, depending on usage, into 50 mL tubes. The tubes can be stored at −80°C for up to 18 months. Avoid freeze-thaw cycles. Aliquots should be used only once.

Human EGF (500 μg/mL)

Centrifuge the tube before opening. Reconstitute the 1 mg vial in 2 mL of sterilized Milli-Q water, aliquot 20 μL or 40 μL per tube and store at −80°C for up to one year. Avoid freeze-thaw cycles. Can keep in 4°C for up to one week.

CHIR99201 (20 mM)

Centrifuge the tube before opening. Reconstitute the 5 mg vial by adding 537.5 μL of DMSO to make a 20 mM stock. Prepare 15 μL aliquots and store at −20°C for one year. Avoid more than two freeze-thaw cycles.

A83-01 (10 mM)

Centrifuge the tube before opening. Reconstitute the 10 mg vial by adding 2.37 mL of DMSO to make a 10 mM stock. Prepare some 6 μL aliquots and store at −20°C for one year. Store large volume aliquots (200 μL) at −80°C for 3 years. Avoid more than two freeze-thaw cycles.

Niacinamide (2.5 M)

Weigh out 15.265 g niacinamide powder and dissolve in Milli-Q water to a final volume of 50 mL. Filter with 0.2 μm syringe filters. Aliquot 400 μL per tube and store at −20°C for one year.

Human Gastrin1 (100 μM)

Centrifuge the tube before opening. Reconstitute the 0.5 mg vial in 2.38 mL sterilized PBS, aliquot 10 μL per tube and store at −20°C for up to one year. Store big volume aliquots (200 μL) at −80°C for 3 years. Avoid freeze-thaw cycles. Can keep in 4°C for up to one week.

B-27 and N-2 supplement

Store B-27 and N-2 supplement in −20°C. Thaw one bottle at 4°C. Aliquot 0.5 mL of N-2 and 1 mL of B-27 per tube and store in −20°C. Pre-thaw aliquots 10 min before each experiment at 4°C.

N-acetyl-L-cysteine (0.5 M)

Weight out 4.08 g N-Acetyl-L-Cysteine powder and dissolve in Milli-Q water to a final volume of 50 mL. Filter with 0.2 μm syringe filters. Aliquot 200 μL per tube and store at −20°C for one year.

Y-27632 (20 mM)
Resuspend 10 mg of Y-27632 in 1.56 mL of PBS to make a 20 mM stock. Aliquot 25 μL per tube and store at −20°C for one year. Avoid freeze-thaw cycles. Can keep in 4°C for one week.

FGF-2 (250 μg/mL)
Centrifuge the tube before opening. Reconstitute the 0.25 mg vial in 1.0 mL of sterilized PBS. Prepare 10 μL aliquots and store at −80°C for two years.

IGF-1 (500 μg/mL)
Centrifuge the tube before opening. Reconstitute the 1 mg vial in 2.0 mL of sterilized PBS. Prepare 10 μL aliquots and store at −80°C for two years.

SB 202190 (30 mM)
Centrifuge the tube before opening. Reconstitute the 10 mg vial by adding 1.0 mL of DMSO to make a 30 mM stock. Prepare 20 μL aliquots and store at −20°C for one year. Avoid freeze-thaw cycles.

Puromycin (1 mg/mL)
Resuspend 25 mg of puromycin in 2.5 mL of sterilized Milli-Q water to make a 10 mg/mL stock and store at −80°C for three years. Further dilution is needed to make 1 mg/mL stock at −20°C. Prepare 50 μL aliquots and store at −20°C for one year. Avoid freeze-thaw cycles. Can keep in 4°C for two weeks.

G418 (250 mg/mL)
Resuspend 5 g of neomycin in 20 mL of Milli-Q water to make a 250 mg/mL stock. Filter with 0.2 μm syringe filters. Aliquot 200 μL per tube and store at −20°C for two years.

0.1% bovine serum albumin (BSA)
To prepare 0.1% BSA in PBS, dissolve 0.5 g of BSA in 500 mL of 1× PBS. Decant the supernatant through a 0.2 μm filter into a large bottle and store at 4°C for one year.

DAPT (10 mM)
Centrifuge the tube before opening. Reconstitute the 5 mg vial by adding 1.16 mL of DMSO to make a 10 mM stock. Prepare 20 μL aliquots and store at −20°C for one year. Avoid freeze-thaw cycles.

Western blot materials

4× sample loading buffer
Take 900 μL of 4× Laemmli Sample Buffer and add 100 μL 2-Mercaptoethanol in a fume hood to make 4× sample loading buffer.

TBST (Tris Buffered Saline with 0.1%Tween-20)
Take 100 mL of 10× Tris Buffered Saline (TBS, Corning) and add 900 mL Milli-Q water to make 1× TBS. 10% Tween-20: Weight 5 g Tween-20 in a 50 mL tube and add 1× TBS to make a total volume of 50 mL. Add 10 mL 10% Tween-20 to 990 mL 1× TBS to make 1× TBST. Store at 18°C–25°C for 2 months.

Sample running buffer
Take 100 mL of 10× Tris/Glycine/SDS buffer and add 900 mL Milli-Q water to make 1× sample running buffer. Prepare fresh buffer for each use.

Sample transfer buffer
To make 1 L of sample transfer buffer add 100 mL 10× Tris/Glycine buffer to 700 mL Milli-Q water and 200 mL methanol solution. Prepare the buffer on the experimental day. Keep it at 4°C for a minimum of one hour before use. Discard unused buffer at the end of the day.

Immunohistochemistry materials

4% paraformaldehyde (4% PFA)

All processes involving 4% PFA solution must be operated in a fume hood and any materials must be disposed of properly.

Preheat a water bath to 65°C, weigh out 20 g PFA powder add 450 mL Milli-Q water with 0.5 mL of 10 N NaOH. Incubate in the 65°C water bath until all the powder has dissolved. This usually takes ~ 30 min. Mix well every 5 min. Allow to cool to 18°C–25°C, while adding 25 mL of 20× DPBS and adjusting the final pH value to 7.4. Finally, adjust the total volume to 500 mL by addition of Milli-Q water. Prepare 50 mL aliquots and store at −20°C for one year. Thaw to 4°C before using. Can be stored at 4°C for one week.

30% sucrose solution

To prepare 30% sucrose solution, dissolve 150 g of sucrose in 350 mL of Milli-Q water, add 25 mL 20× DPBS, and adjust to total volume of 500 mL. Decant the supernatant through a 0.2 μm filter into a large bottle and store at 4°C for one year. Alternatively, it can be used immediately and stored at 4°C for up to one month.

Tris Buffered Saline with 0.1%Tween-20 (TBST)

Reagent	Final concentration	Amount
10× Tris Buffered Saline	1×	100 mL
Tween-20	0.1%	1 mL
MilliQ	n/a	Adjust to 1,000 mL
Total	**n/a**	**1,000 mL**

Store at 18°C–25°C for 2 months.

Sample transfer buffer

Reagent	Final concentration	Amount
10× Tris/Glycine	1×	100 mL
Methanol solution	20%	200 mL
MilliQ	n/a	700 mL
Total	**n/a**	**1,000 mL**

Keep it at 4°C for a minimum of one hour before use. Discard unused buffer at the end of the day.

4% Paraformaldehyde (4% PFA)

Reagent	Final concentration	Amount
PFA power	4%	20 g
NaOH (10 N)	n/a (pH value determinate)	0.5 mL
20× DPBS	1×	25 mL
MilliQ	n/a	Adjust to 500 mL
Total	**n/a**	**500 mL**

Storage conditions: −20°C for up to 6 months or 4°C for up to 1 week.

30% Sucrose solution

Reagent	Final concentration	Amount
Sucrose	30%	150 g
20× DPBS	1×	25 mL
MilliQ	n/a	Adjust to 500 mL
Total	**n/a**	**500 mL**

Decant the supernatant through a 0.2 μm filter into a large bottle and store at 4°C for one year. Alternatively, it can be used immediately and stored at 4°C for up to one month.

Basic medium		
Reagent	Final concentration	Amount
Gibco DMEM	n/a	440 mL
GlutaMAX Supplement (200 mM)	2 mM	5 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
FBS	10%	50 mL
Total	**n/a**	**500 mL**
Storage conditions: 4°C for up to 6 months.		

Primary culture medium		
Reagent	Final concentration	Amount
Advanced DMEM/F-12	n/a	488 mL
GlutaMAX Supplement (200 mM)	2 mM	6 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	6 mL
FBS	10%	100 mL
Total	**n/a**	**500 mL**
Storage conditions: 4°C for up to 6 months.		

Human Intestine 3D Organoid Culture Medium (HCM)		
Reagent	Final concentration	Amount
L-WRN Condition Medium	50%	25 mL
GlutaMAX Supplement (200 mM)	2 mM	0.5 mL
HEPES (1 M)	10 mM	0.5 mL
Primocin (50 mg/mL)	100 μg/mL	100 μL
N-2 supplement (100×)	1×	0.5 mL
B-27 supplement (50×)	1×	1 mL
N-acety-L-cysteine (500 mM)	1 mM	100 μL
EGF (500 μg/mL)	50 ng/mL	10 μL
CHIR99021 (20 mM)	2.5 μM	6.25 μL
A 83-01 (10 mM)	500 nM	2.5 μL
Y-27632 # (20 mM)	10 μM	25 μL
Gastrin-1 (100 μM)	10 nM	5 μL
Nicotinamide (2.5 M)	10 mM	200 μL
Advanced DMEM/F-12	n/a	22 mL
SB202190 (30 mM)	100 μM	16.7 μL
Total	**n/a**	**50 mL**
Storage conditions: 4°C for up to 1 week.		

⚠ CRITICAL: Warm medium before adding SB202190 during the last step. Avoid adding SB202190 in cold medium, which will lead to precipitation. To avoid anoikis, supplement culture medium with Y-27632 for the first passage.

Human Intestine 3D Organoid Differentiation Medium (HDM)		
Reagent	Final concentration	Amount
L-WRN Condition Medium	25%	12.5 mL
GlutaMAX Supplement (200 mM)	2 mM	0.5 mL
HEPES (1 M)	10 mM	0.5 mL
Primocin (50 mg/mL)	100 μg/mL	100 μL
B-27 supplement (50×)	1×	1 mL
N-acety-L-cysteine (500 mM)	1 mM	100 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
A 83-01 (1 μM)	500 nM	2.5 μL
Y-27632	100 μM	25 μL
Gastrin-1 (100 μM)	10 nM	5 μL
FGF-2 (250 μg/mL)	50 ng/mL	10 μL
IGF-1 (500 μg/mL)	100 ng/mL	10 μL
Advanced DMEM/F-12	n/a	35.2 mL
Total	**n/a**	**50 mL**

Storage conditions: 4°C for up to 1 week.

Embedding material		
Reagent	Final concentration	Amount
HCM	25%	100 μL
Matrigel	75%	300 μL
Total	n/a	400 μL

Storage conditions: 4°C to be used immediately.

STEP-BY-STEP METHOD DETAILS

Lentiviral spin infection of human colonic organoids

Timing: 6 h

The goal of this protocol is to maximize CRISPR knockout efficiency in human colonic organoids. The protocol optimizes virus spin infection of human colonic organoids. For the experiment outline below, the scale is for a 12-well plate.

1. Before you begin:
   a. Pre-cool centrifuge to 4°C.
   b. Pre-coat plasticware with sterile 0.1% BSA. Add sufficient 0.1% BSA to coat 1.5 mL, 5 mL, 15 mL, and 50 mL tubes at RT for 20 min. Remove BSA solution completely before use. Prepare a 15 mL tube with 5 mL of 0.1% BSA to be used for coating pipettes by pipetting up and down twice before using.
   c. Pre-warm a plate centrifuge to 37°C. Performing spin infection at lower temperatures may reduce transduction rate.

2. Organoid TrypLE enzyme fragment preparation.
   a. Human colonic organoids grown for 5–7 days after passage are ready for virus infection, as shown in Figure 3A. We typically plate 25–50 organoids per 10 μL Matrigel and passage the organoids at a 1:5 ratio.
   b. Completely remove the culture medium and add 1 mL of cold cell recovery solution per well of a 12-well plate. Cell recovery solution should be kept at 4°C.
   c. Incubate the plate on ice for 30 min to dissolve the Matrigel. Use a BSA-coated 1 mL pipette to transfer 2 wells worth of organoids to a 15 mL BSA-coated tube. Centrifuge the tube at 4°C 300 × *g* for 3 min.

Note: A clear cell pellet without residual Matrigel should be observed. If there is still Matrigel, discard the supernatant, add 1 mL of fresh cold cell recovery solution and mix, incubate for 10 min at 4°C, then centrifuge at 4°C 300 × *g* for 3 min. Repeat the incubation and centrifugation if any Matrigel is still present until a clearly defined cell pellet is observable.

d. Discard the supernatant and resuspend the pellet in 0.3 mL TrypLE with 10 μM Y-27632 by tapping the bottom of tube. Incubate at 37°C in a tissue incubator for 3 min.
e. Pipette the organoids up and down vigorously, at least 10 times, using a BSA-coated TipOne RPT 1,000 μL XL graduated pipette. Be careful to avoid introducing bubbles. After the pipetting, the organoids should be broken into small fragments as shown in the upper part of Figure 3B.
f. Inactivate the enzyme digestion by adding 10 mL of 18°C–25°C 10% FBS in advanced DMEM/F12 medium.
g. Pass the solution through a 70 μm filter into a 50 mL BSA-coated tube. Single cells and small fragments of organoids (<70 μm) should now be present in the medium as shown in the lower part of Figure 3B.
h. Remove10 μL of the cell suspension and dilute it with 10 μL of trypan blue solution.
i. Calculate the living cell numbers as shown in Figure 3C per volume.
j. Add approximately 2×10^5 cells per 1.5 mL Eppendorf tube and centrifuge at 4°C 300 × *g* for 10 min.

3. Virus infection. Prepare 10% Pure Bright® bleach solution for the disposal of plasticware that comes into contact with viral particles and media from this step onward.
   a. Prepare a mixture of virus and polybrene in a non-tissue culture coated 24-well plate. Add 20 μL of Lenti-virus with approximately 1×10^8 viral particles and 2 μL of 1 mg/mL polybrene per well of a 24 well dish.
   b. Discard the supernatant from step 2h and resuspend the pellet in 180 μL of HCM medium. Add the suspension into one well containing virus and polybrene.
   c. Centrifuge the 24-well plate at 37°C 1,000 × *g* for 30 min.
   d. Cells are located at the bottom of the plate as shown in Figure 3D. Carefully take out the plate without disturbing the cells and gently add 300 μL of prewarmed HCM to make a total volume of 500 μL.
   e. Incubate at 37°C in a tissue incubator for 4 h.
   f. Use a BSA-coated 1 mL pipette to transfer the cells into a 1.5 mL BSA-coated tube. Centrifuge at 4°C 300 × *g* for 5 min.
   g. Discard the supernatant and resuspend the pellet in 400 μL embedding material. Mix well and place 10–15 μL per droplet into one well of a 6-well plate, up to 35–40 droplets total per well (Figure 3E).
   h. Place the plate in a 37°C tissue incubator for 10 s.
   i. Invert the plate and incubate at 37°C in the tissue incubator for another 10 min.
   j. Add 2 mL of prewarmed HCM per well. Put the plate back into the incubator.

Note: We use 75% Matrigel for organoid droplets, which does not interfere with droplet formation and reduces cost.

⚠ CRITICAL: Coating tubes and pipettes with 0.1% BSA will significantly reduce organoid loss during the handling steps due to organoid adherence to plastic surfaces.

Passaging and stocking lentiviral-infected organoids

Timing: 1 week

4. Puromycin selection.
   a. After 3 days of culture as Matrigel drops, as shown in Figure 3F, change HCM to media containing 1 μg/mL puromycin.
   b. Culture for 3 days.
   c. Replace media with fresh HCM containing freshly added 1 μg/mL puromycin.
   d. Culture for additional 2 days, as shown in Figure 3G.

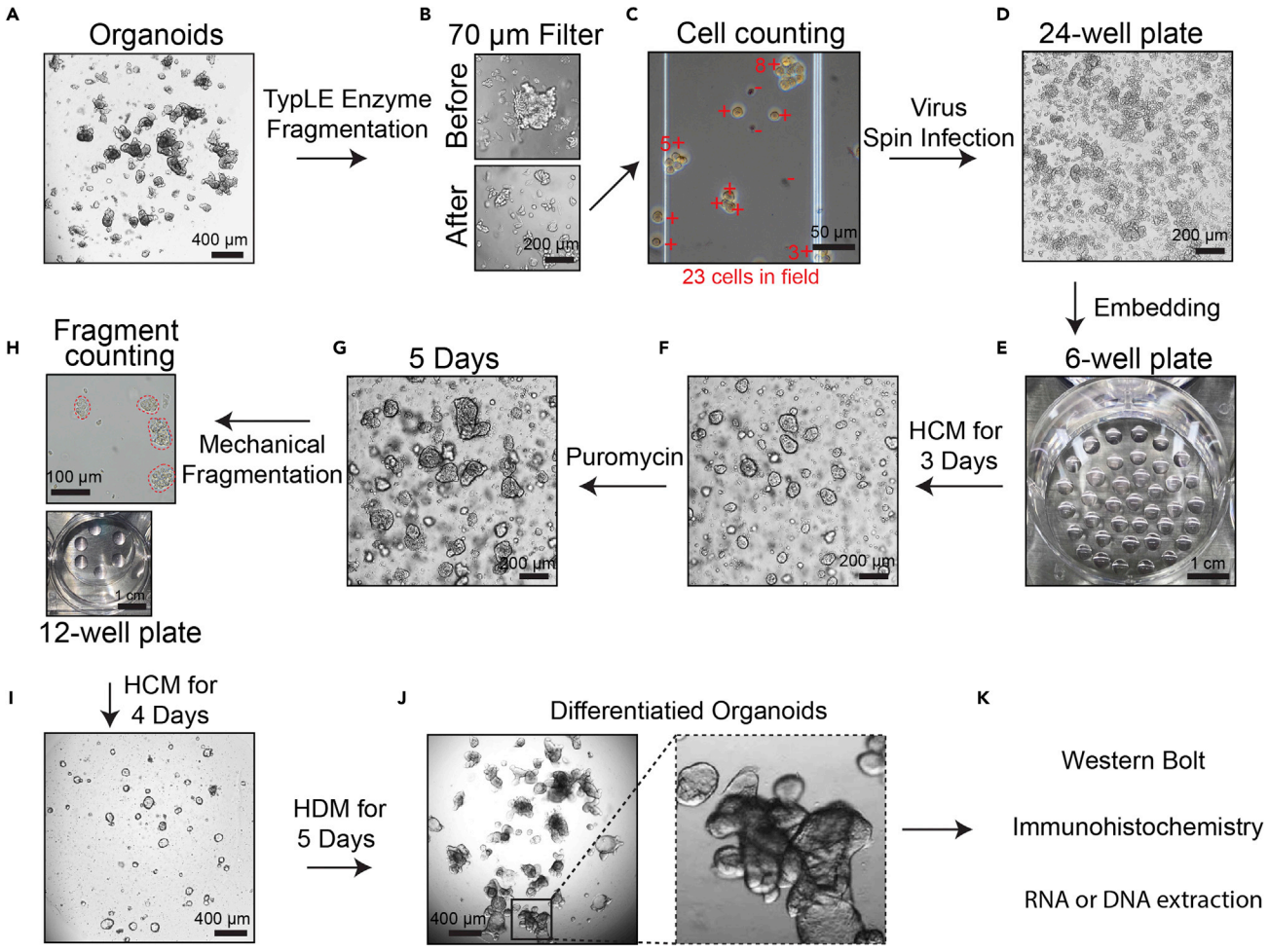

Figure 3. Lentiviral spin infection of human colonic organoids and subsequent growth and differentiation

(A) Organoids in Matrigel ready for infection.

(B) TyrpLE-digested fragments filtering through a 70 μm filter to remove big fragments.

(C) An example of counting organoid fragments before lentiviral infection. Live fragments are labeled with (+) signs.; debris or dead cells labeled with (-).

(D) Organoid fragments after lentiviral spin infection in a 24-well plate.

(E) Matrigel drops with embedded organoid fragments in one well of a 6-well plate.

(F) Image of infected organoids after 3-day growth in HCM medium.

(G) Image of infected organoids after 5 days of puromycin selection.

(H) Organoids are fragmented and counted as shown in the upper panel. Fragments are re-embedded in Matrigel in a 12-well plate as shown in the lower panel.

(I) Image of nascent organoids in Matrigel one day after embedding.

(J and K) Organoids after differentiation in HDM for 5 days and ready for assays.

5. Stocking and expanding organoids.
   a. Remove the culture medium completely and add 2.6 mL of cold cell recovery solution per well to the 6-well plate.
   b. Incubate for 30 min on ice to dissolve Matrigel.
   c. Gently transfer half of the organoids in the cell recovery solution (1.5 mL) using a BSA-coated 1 mL pipette and centrifuge at 4°C 300 × *g* for 3 min.
   d. Discard the supernatant and resuspend in 600 μL cell stock solution (10% DMSO + 90% FBS). Freeze 300 μL per cryotube for future expansion and studies.

Pause point: After puromycin selection, the organoids can be frozen until needed for processing the next step.

e. Pipette the other half of the organoids into small fragments by pipetting up and down vigorously at least 20 times using a BSA-coated TipOne RPT 1,000 μL XL graduated pipette. Be careful to avoid introducing bubbles. Watch under a microscope and pipette until no large pieces of organoids remain. The average size of organoid fragments should be less than 100 μm in diameter.
f. Count the number of organoid fragments. Figure 3H, upper).
g. Transfer to a BSA-coated 5 mL tube and centrifuge at 4°C 300 × *g* for 5 min.
h. Discard the supernatant. Suspend the pellets in embedding material with ~20 fragments per one droplet of 10 μL. Add 6 droplets per well to a 12-well plate (Figure 3H, lower).
i. Organoids appear after one day in culture (Figure 3I).
j. Culture for additional 3–4 days in HCM with 0.5 μg/mL puromycin.

Note: In steps 5f and 5h, the organoid fragment number may vary due to puromycin selection and fragmentation. We usually get a total of 250–1,000 fragments.

Take 500 fragments for example, resuspend in 250 μL embedding material and embed 4 wells in a 12-well plate.

⚠ CRITICAL: Embedding the organoids at low density as indicated ensures even growth.

CRISPR knockout efficiency validation

Timing: 2 days

6. Collect organoids for western blot.
   a. Continue from step 5j. Remove the culture medium completely and add 1 mL of cold cell recovery solution to one well of a 12-well plate.
   b. Using a BSA-coated 1 mL pipette, gently break up the Matrigel droplets and transfer to a BSA-coated 1.5 mL tube.
   c. Incubate the tube on ice for 30 min to dissolve Matrigel.
   d. Centrifuge the tube at 4°C and 300 × *g* for 3 min.
   e. Discard the supernatant. Wash the pellet with 1 mL of cold cell recovery solution on ice for 5 min.
   f. Centrifuge the tube at 4°C and 300 × *g* for 3 min.
   g. Discard the supernatant. Lyse the cell pellets in 100 μL RIPA lysis and extraction buffer with 1 μL protease and phosphatase inhibitor cocktails at 4°C for 30 min.
   h. Centrifuge the tube at 4°C and 15,000 × *g* for 10 min.
   i. Collect the supernatant from the spin for the BCA protein assay by following the protocol below: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf.
   j. Adjust the total protein concentration of each Ctrl and KO group to 1.25 μg/μL.
   k. Thoroughly mix 20 μL of 4× sample loading buffer with 60 μL of the samples in new 1.5 mL tubes by pipetting. The resulting protein concentration will be 1 μg/μL. Incubate the tubes on a digital heat-block at 95°C for 10 min.
   l. Prepare sample transfer buffer as specified in the materials section while waiting for the samples to heat. Place the buffer into a 4°C refrigerator for at least one hour, allowing it to cool before using.
   m. After the 10 min incubation on the heat-block, centrifuge the sample tubes at 15,000 × *g* at 18°C–25°C for 5 min.

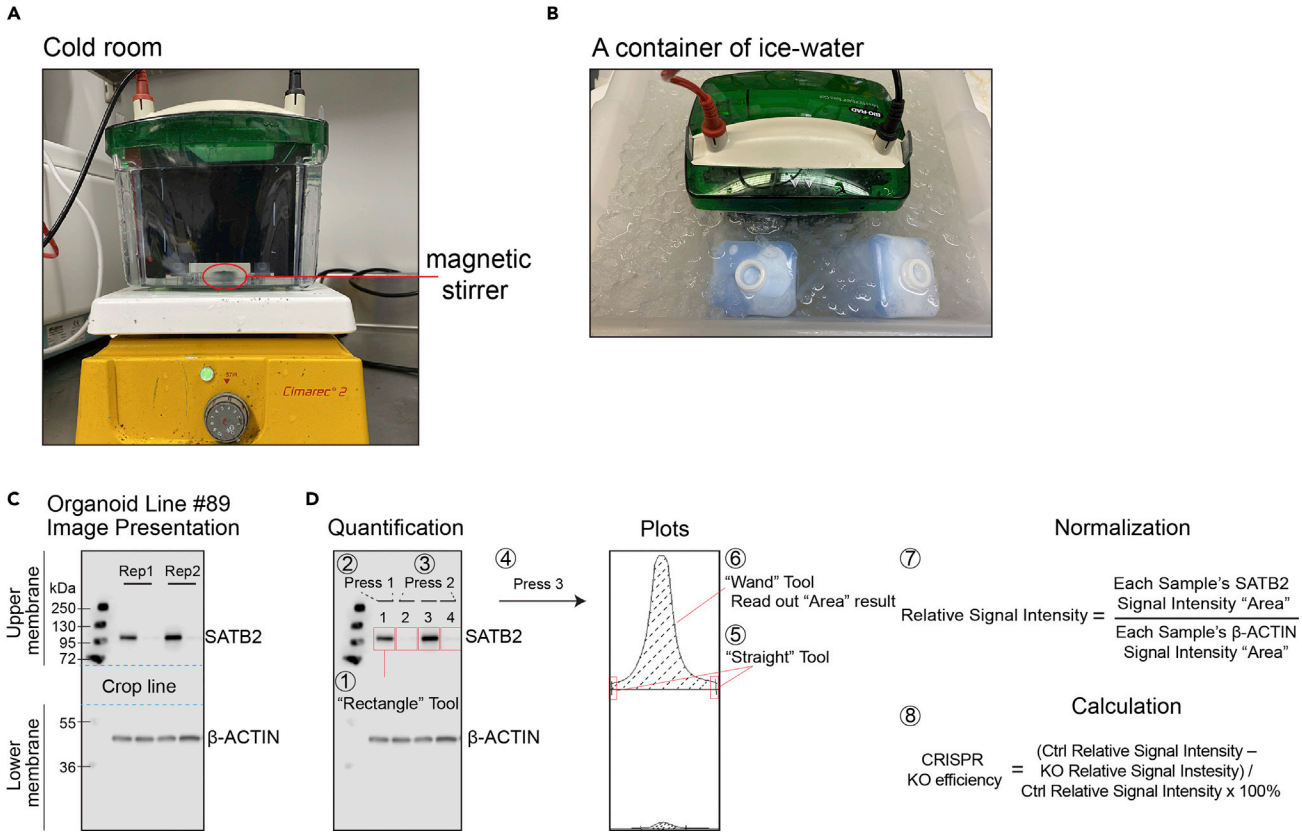

Figure 4. CRISPR KO efficiency determination

(A) An image shows how to use the magnetic stirrer when performing the transfer in a cold room.

(B) An image shows how to use a container of ice-water for the transfer step.

(C) Two independent lentiviral infection experiments for human colonic organoid line #89. Western blot analysis for both the CRISPR target protein SATB2 and control β-ACTIN. Membrane was cropped into two parts along the crop line as shown in the picture.

(D) Detailed steps for calculating the CRISPR KO efficiency using ImageJ software.

n. Load 10 μg (10 μL) per lane per sample and 3 μL of protein ladder in one lane in a precast SDS polyacrylamide gel.

o. Run the gel with sample running buffer in a Mini-Protean® Tetra Cell, 2-Gel System, at 180 V with constant voltage for 30–50 min, until the dye reaches the bottom the gel.

p. Remove the gel from the cassette and soak the gel in 1 L of 4°C sample transfer buffer.

q. Cut the PVDF membrane to a similar size as the gel. Assemble the Bio-Rad electroblotting cassette and place the electrodes in the blotting unit, according to the manufacturer's instructions.

r. Transfer the protein from the gel to the membrane at 200 mA with a constant current in a cold room. Use a magnetic stirrer (Figure 4A) to help equally distribute heat and keep the transfer buffers homogeneous. Alternatively, the transfer can be done in a container of ice-water with a magnetic stirrer (Figure 4B).

s. Remove the PVDF membrane from the blotting cassette and mark the orientation of membrane with a pencil. Rinse the membrane with Milli-Q water.

t. Block any non-specific binding of the PVDF membrane with 20 mL of blocking buffer (5% nonfat dried milk in TBST) for 1 h at 18°C–25°C. Shake the membrane with the blocking buffer on the orbital shaker at a low-speed setting.

u. Incubate the PVDF membrane with primary antibodies diluted in 3% BSA in TBST at dilutions appropriate for the specific protein and 1:5,000 for Anti-β-ACTIN at 4°C 16–20 h. Shake the membrane incubating in the primary antibodies on the orbital shaker at a low-speed setting.

v. Remove the unbound primary antibodies by washing with 20 mL of TBST 3 times for 5 min each on the orbital shaker at a high-speed setting.
w. Incubate the membrane with secondary horseradish peroxidase (HRP) conjugated antibodies diluted in blocking buffer at a 1:10,000 dilution at 18°C–25°C for 1 h on the orbital shaker at a low-speed setting.
x. Wash off the unbound secondary antibody with 20 mL of TBST for 5 min at 18°C–25°C on the orbital shaker at a high-speed setting. Repeat this wash 4 additional times.
y. Crop the PVDF membrane just below the 72 kDa marker line into two membranes. The higher molecular weight membrane (upper), for example, is for SATB2 detection, and the lower molecular weight membrane (lower) is for β-ACTIN detection (Figure 3A).
z. Place both the upper and lower membranes with the protein ladder side up on plastic wrap. For the upper membrane, add 2 mL of enhanced chemiluminescent substrate (Pico from Thermo Fisher) on top of it. For the lower membrane, dilute the substrate with TBST at a 1:5 ratio before adding it on top. Incubate the membranes with substrate for 2 min at 18°C–25°C.
aa. Put the two membranes back to their original directions and record the signal with a Li-COR C-Digit, according to the manufacturer's instructions (Figure 4C).

7. Quantification of CRISPR knockout efficiency.
   a. Use Image J to measure the intensity of the signal. The results are denoted as "Area." For each western blot, bands are analyzed following the steps shown in Figure 3B.
      i. The western blot image is a gray-scale profile. Open the image using Image J.
      ii. Use the "Rectangle" tool to draw an appropriately sized rectangle around the first lane (Figure 4D).
      iii. Press 1 (command plus 1 for mac) to select the rectangle box as the first sample.
      iv. Drag the box to the next blot lane and press 2 (command plus 2 for mac). Repeat this step until all samples are marked. Image J will automatically align the rectangles on the same vertical axis as the first sample.
      v. Press 3 (command plus 3 for mac) to generate a profile plot of each lane.
      vi. To enclose the peak, use the "Straight" tool to draw a line, forming a closed peak area around each sample. The straight line also can be used as a judgment of background noise. Our results show negligible background.
      vii. Using the "Wand" tool, click inside the peak and measure the peak "area" for each sample.
      viii. Repeat the measurement two more times, starting over from the initial image. Average the signal intensity of the three measurements.
      ix. Do the same for the β-ACTIN blot.
   b. Normalize the experimental sample (SATB2 as an example here) signal intensity to β-ACTIN to get relative signal intensity.
   c. Calculate target CRISPR knockout efficiency using the following formula: CRISPR KO efficiency = (Ctrl relative signal intensity – KO relative signal intensity) / Ctrl relative signal intensity × 100% (Figure 4D).

Note: If you work with a gene that does not have validated antibodies, you can use a DNA mismatch detection assay with T7 endonuclease1. A detailed method is described in (Sentmanat et al., 2018).

Organoid differentiation and downstream analysis with immunohistochemistry, RNA and DNA analysis

Timing: 1 week

8. Organoid differentiation protocol.

a. Continue from step 5j. Organoids are differentiated in HDM media (without puromycin, nicotinamide, SB202190, CHIR9902, and EGF, with 25% L-WRN conditioned medium, and addition of FGF2- and IGF-1). The concentrations of each chemical are listed in the materials and equipment section.
b. Change differentiation medium every two days.
c. Differentiate the organoids for 5–6 days (Figure 3J). There will be no obvious morphological changes after differentiation, although one might observe increased branching structures. Our analysis of lineage markers indicate that growing for 5–6 days is sufficient for both absorptive and secretory cells to differentiate.
d. Differentiated organoids are ready for analyses including immunohistochemistry and RNA or DNA analysis (Figure 3K).

9. Organoid preparation for immunohistochemistry.
a. Remove culture medium completely and add 1 mL of cold cell recovery solution per one well of a 12-well plate.
b. Incubate for 30 min on ice to dissolve Matrigel.
c. Using a BSA-coated 1 mL pipette, gently transfer the differentiated organoids to a BSA-coated 5 mL tube. The 1 mL pipette tip needs to be cut to a ~4 mm bore width before use.
d. Centrifuge at 4°C 100 × *g* for 3 min.
e. Discard the supernatant. Add 1.4 mL 4% PFA and fix at 4°C for 1 h on a nutator.
f. Centrifuge at 4°C 300 × *g* for 3 min.
g. Wash twice with DPBS.
h. Add 30% sucrose solution and let sit at 4°C for at least 2 h or 16–20 h.
i. Centrifuge down organoids at 4°C 100 × *g* for 3 min, discard supernatant, leaving ~50 μL 30% sucrose solution (Figure 5A).
j. Add roughly 450 μL O.C.T. compound to a biopsy Tissue-Tek Cryomold.
k. Cut a 1 mL pipette tip to a 2–3 mm bore width.
l. Transfer the organoids in 30% sucrose solution to the top of the O.C.T. compound in the mold (Figure 5B).
m. Mix O.C.T. with organoids and 30% sucrose by swirling with forceps.
n. Centrifuge with a swing rotor centrifuge (Figure 5C) at 4°C 100 × *g* for 3 min.
o. The organoids should distribute evenly at the bottom of mold (Figure 5D) and are ready for freezing and cryosection.

10. RNA extraction.
a. Remove culture medium completely and wash with DPBS.
b. For RNA extraction, add 600 μL RLT lysis buffer (Qiagen) with 1% 2-mercaptoethanol directly per one well of a 12-well plate.
c. Gently shake until Matrigel and organoids are dissolved. This usually takes 3–5 min at 18°C–25°C.
d. Follow the manufacturer's instructions for the RNeasy Mini Kit from Qiagen, the handbook can be downloaded at https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en

Note: For one well of a 24-well plate or 48-well plate scale, we use the RNeasy Micro Kit from Qiagen. You need to remove Matrigel with cell recovery solution before adding lysis buffer if the volume of Matrigel exceeds 100 μL (Mini Kit) or 40 μL (Micro Kit). The yield of RNA will decrease dramatically in the presence of excess Matrigel.

11. DNA extraction.
a. Remove culture medium completely and add 1 mL of cold cell recovery solution per one well of a 12-well plate.
b. Incubate for 30 min on ice to dissolve Matrigel.
c. Using a BSA-coated 1 mL pipette, gently transfer the differentiated organoids to a BSA-coated 1.5 mL tube.

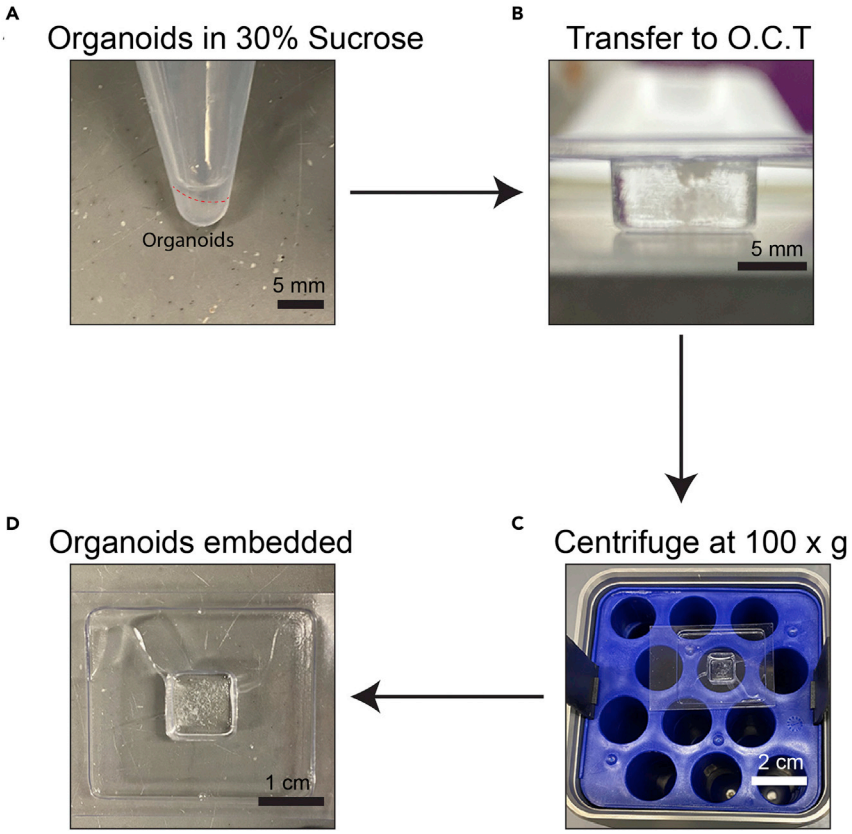

Figure 5. Organoid fixation and embedding for histology and immunohistochemistry

(A) Organoids fixed in 4% PFA were subsequently treated with 30% sucrose solution. Image shows organoids with ~50 μL sucrose ready for transfer to O.C.T compound.

(B) Organoids in O.C.T compound.

(C and D) Centrifuge at 100 × *g* with a swing rotor centrifuge to pull organoid to the bottom of the embedding mold as shown in (D).

d. Centrifuge at 4°C 100 × *g* for 3 min.

e. Discard the supernatant. Follow the manufacturer's instructions of E.Z.N.A Tissue DNA Kit from OMEGA. The protocol could be downloaded at https://www.omegabiotek.com/product/genomic-dna-kit-e-z-n-a-tissue-dna-kit/?cn-reloaded=1.

⚠ CRITICAL: The standard differentiation medium (HDM) favors differentiation of the absorptive lineage, i.e., colonocytes, whereas secretory goblet cells are few and appear immature (Figure 6). We have found that supplementing HDM with 5 μM DAPT in step 8a can significantly enhance goblet cell differentiation (Figure 6), to levels resembling human colonic mucosa in vivo.

EXPECTED OUTCOMES

We expect ready establishment of human colonic knockout lines with this protocol. By routinely testing 3 separate gRNAs per gene, we have been able to identify at least one guide RNA to yield a knockout efficiency of 90% or higher ((Gu et al., 2022) and unpublished observations). Control and mutant organoids can be efficiently differentiated towards absorptive or secretory lineages and analyzed by protein, RNA, or DNA assays. We note that colonic organoids derived from different donors exhibit substantial inherent molecular differences. For phenotypic analysis of any given gene, we recommend generating 4–5 pairs of isogenic control and mutant organoid lines from different donors.

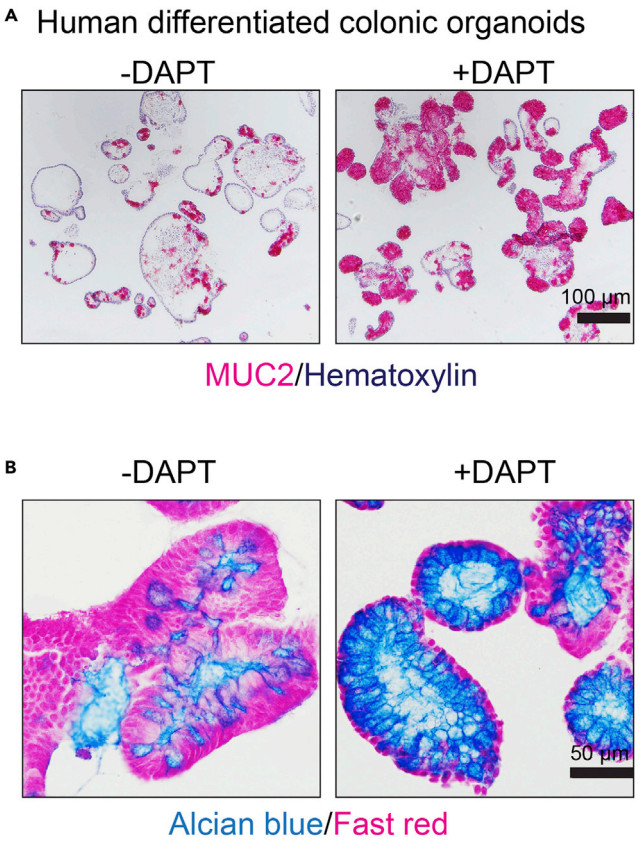

Figure 6. DAPT promotes goblet cell differentiation

(A) Representative images of differentiated organoid with or without DAPT stained with anti-MUC2 antibody (Red) and Hematoxylin nuclear stain (blue).

(B) Representative images of differentiated organoids with or without DAPT stained with Alcian blue (blue) and fast red (red).

LIMITATIONS

We tested both constitutive CAS9 and inducible CAS9 in our knockout system and found that constitutive CAS9 expression is needed for high knockout efficiency. We have not fully examined the off-target rate of constitutive CAS9 in our system but by employing the latest gRNA design tools, the off-target rate is expected to be low. In addition, control organoids with constitutive CAS9 expression showed no difference from wild-type organoids in morphology, growth, or differentiation over multiple passages.

We routinely achieved knockout efficiency of 90% or greater and were able to maintain the high knockout rate over many generations by continued selection with low dose puromycin. The advantage of our approach is the relative ease and speed of generating knockout lines, but it does not yield 100% knockout efficiency. If desired, one can use clonal growth to derive clonal lines with uniform knockout (Kawasaki et al., 2020; Matano et al., 2015). Each clonal line can be analyzed to determine the specific genomic disruption that disabled the gene. Aside from the longer time required to establish clonal lines, one potential caveat is that different clonal lines derived from the same donor may have inherent differences - careful molecular and functional evaluation would be needed to confirm similarity to each other and to the parental line.

TROUBLESHOOTING

Problem 1

Slow growth of colonic organoids (Figure 7). Primary cause is dated or incorrect culture medium. Occasionally, some colonic organoid lines have a slow growth rate. (step 4).

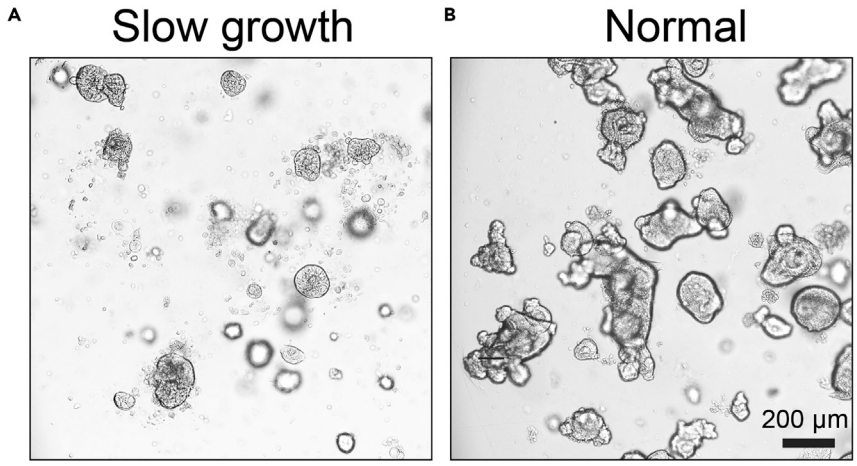

Figure 7. Organoids with normal or low growth after 7-day culture in Matrigel
(A) Organoids with low growth.
(B) Organoids with normal growth.

Potential solution

- Check the medium components and make fresh medium.
- Change medium every 2–3 days.

Problem 2

Viral titer is low. Potential causes include poor 293FT cell quality, low plasmid purity, or poor transfection rate. (before you begin).

Potential solution

- Make sure the 293FT cells are in good condition (no tight clusters, cells look healthy, Figure 8).
- Check plasmid quality (260/280 ratio greater than 1.8). Plasmid concentration ideally greater than 1 mg/mL in Milli-Q water.
- Test transfection reagents with a fluorescence reporter (GFP or RFP) plasmid.

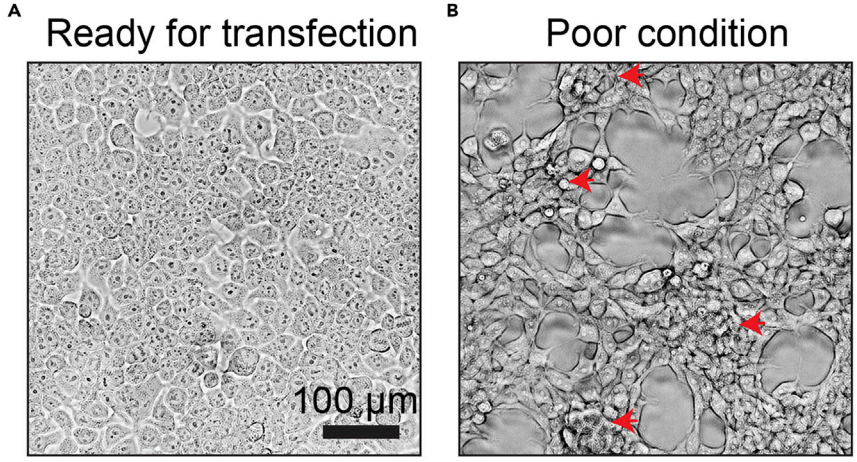

Figure 8. 293FT cell condition
(A) Representative bright-field image of healthy 293FT cells ready for transfection at 95%–100% confluence.
(B) Representative bright-field image of 293FT cells with poor condition. The blue arrow shows compact cell clusters of uneven cell sizes although the culture is not confluent.

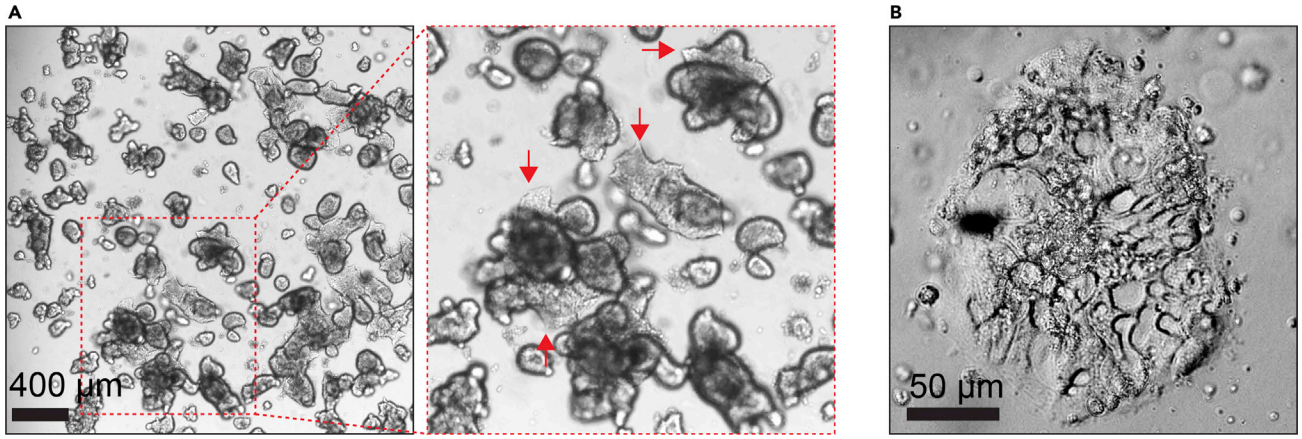

Figure 9. Organoid attachment to bottom of plate

(A) Bright-field images of human colonic organoids at high density in which attachment to the plate surface often occurs (attached organoids growing on 2D surface indicated by red arrows).

(B) Organoids attached to plate often begin to degenerate after a few days.

Problem 3

Knockout efficiency is low (less than 85%). Primary cause is CRISPR design and low viral infection rate. (step 7).

Potential solution

- Test additional CRISPR gRNAs. We routinely test 3 gRNAs per gene, which nearly always yields at least one gRNA with >85% knockout efficiency.
- Use Lentivirus at appropriate titer as outlined in the protocol.

Problem 4

Organoid attachment to the bottom of plate (Figure 9). Primary cause is high organoid density and slow gelling during the embedding process. (step 5).

Potential solution

- Embed about 20 fragments in a droplet of 10 μL Matrigel. Do not embed too many in one droplet as shown in Figure 8A. For an experiment involving downstream applications, we suggest about 20 organoids in a droplet of 10 μL of Matrigel for best results.
- Invert the plate during Matrigel drop formation step.
- Use 100% Matrigel instead of 75%.
- Pre-warm plate for 10 min in 37°C tissue incubator. Perform Matrigel plating on warmed flat shipping packs (Fisher #353153).

Problem 5

Goblet cell differentiation is incomplete or there is excessive cell death. (step 8).

Potential solution

DAPT usually should not induce apoptosis between 1 μM to 10 μM. Should you observe excessive cell death or incomplete goblet difference with particular lines we recommend optimizing the DAPT concentration in the 1–10 μM range.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Qiao Zhou] (jqz4001@med.cornell.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not include any new data or codes that are not presented in the published article.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Health (R01 DK125817) and the Tri-institutional Stem Cell Initiative to Q.Z. We thank members of the Zhou lab for help and support. We thank the Michigan Medicine Translational Tissue Modeling Laboratory for providing colon organoid lines. The Translational Tissue Modeling Laboratory is a University of Michigan funded initiative (Center for Gastrointestinal Research, Office of the Dean, Comprehensive Cancer Center, Departments of Pathology, Pharmacology, and Internal Medicine) with support by the Endowment for Basic Sciences. This work was supported by the Intestinal Stem Cell Consortium (U01DK103141 to J.R.S.), a collaborative research project funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the National Institute of Allergy and Infectious Diseases (NIAID).

AUTHOR CONTRIBUTIONS

W.G. performed the experiments, wrote the manuscript, and designed the figures. J.L.C. assisted W.G. in some of the experiments, drew the graphical abstract, and edited the manuscript. J.R.S and M.K.D. provided human colonic organoids and technical suggestions. Q.Z. oversaw the work and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The University of Michigan, J.R.S., and M.K.D. are designated licensors to MilliporeSigma of colon organoid lines, prep 80, 83, 87, 88, and 89.

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