Supplementary Methods

Mouse models

All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Animal Welfare Assurance Reference Number no. A3079-01, approved protocol no. 803415 granted to Dr. Lengner) and were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals of the National Research Council of the National Institutes of Health. *Apc*$^{flox/flox}$ mice are from Shibata H. et al. *PMID: 9311916. Lgr5*$^{EGFP\text{-}IRES\text{-}CreERT2}$ (JAX strain 008875), *Kras*$^{LSL\text{-}G12D}$ (JAX strain 008179), C57BL/6J (JAX strain 000664) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Transgenic *Villin-CreER* mice are JAX strain 033019. All animals were maintained on a C57BL/6J genetic background.

Murine organoid culture and genome engineering

Murine colon organoids were cultured following the previously reported protocol by Sato et al., 2009, summarized as follows: The colons from KrasLSL-G12D or C57BL/6J mice were isolated and thoroughly washed with cold D-PBS. Subsequently, the colons were digested using 5 mM EDTA for 20 minutes at 4°C. The released crypts were obtained by squeezing the mucosal layer under a dissection microscope and filtered through a 70-μm cell strainer to remove any tissue fragments. The crypts were then counted and centrifuged at 300 g for 5 minutes. Next, the crypts were embedded in Matrigel and seeded into 24-well plates at a density of approximately 500 crypts in a total volume of 50 μl per well. The basal medium consisting of Advanced DMEM/F12, 10 mM HEPES, 1x Penicillin-Streptomycin, 2 mM GlutaMAX, 1x N2 supplement, 1x B27 supplement, and 1 mM N-acetyl-cysteine was supplemented with 50 ng/ml mEGF and 50% conditioned media derived from L-WRN cells. To prevent cell death by anoikis, 10 μl of Y-27632 (Rho Kinase inhibitor) was added for the first 2 days after seeding. For CRISPR/Cas9 editing, the organoids were cultured in a two-dimensional (2D) format. Matrigel-coated 24-well plates were prepared by diluting Matrigel with D-PBS (1:49) and incubating them for 2 hours at 37°C. Murine colon organoids were gently dissociated into single cells using 1x TrypLE at 37°C for 5 minutes and then seeded into the 2D plates. APK organoids were generated by activating the KrasG12D mutation through Cre-mediated deletion of the stop cassette at the KrasLSL-G12D targeted allele. This was achieved by transfecting a plasmid expressing Cre recombinase along with a pPGK-Puro plasmid and selecting with puromycin. *Apc* and *Trp53* mutations were introduced through CRISPR/Cas9 editing. Specifically, sgRNAs targeting *Apc* and *Trp53* were cloned into the PX330 plasmid and transiently transfected into the organoids. After selection using Nutlin-3 and Noggin, subclones were picked from the engineered bulk organoids, and PCR and Sanger sequencing were employed to confirm the presence of mutations in each subclone. APKS organoids were derived from APK organoids by introducing the *Smad4* mutation using the same CRISPR/Cas9 editing approach. sgRNAs targeting *Smad4* were cloned into the PX330 plasmid, transiently transfected into APK organoids, and selected using TGFβ1. Subclones from the APKS bulk organoids were isolated, and PCR and Sanger sequencing were performed to confirm the presence of the mutations in each subclone. For individual *Apc* mutant (A), *Trp53* mutant (P), and *Apc/Trp53* double mutant (AP) organoids, normal colon organoids were seeded in 2D culture and transiently transfected with sgRNAs targeting *Apc* or *Trp53* or both. Following selection with Noggin and Nutlin-3, subclones were isolated, and PCR amplification and Sanger sequencing were conducted to verify the presence of the mutations in each subclone. For Apc$^{flx/flx}$ organoid experiments, colonic crypts from Villin-CreERT2::Apc$^{fl/fl}$ mice were isolated and cultured using basal medium supplemented with mEGF and 50% conditioned media. The Cre recombinase was activated by adding 0.25 μM (Z)-4-Hydroxytamoxifen to the medium. Genotypes of edited alleles described in supplemental figure 8J-8K.

Human tissues

Human colorectal cancer and normal colon tissues were obtained previously from patients undergoing elective surgery at Hospital of the University of Pennsylvania with written informed consent under the protocol approved by the University of Pennsylvania Institutional Review Board (Protocol number 827759), as described in doi.org/10.1101/2022.09.13.506996.

Human patient-derived organoid culture

The cancer and normal epithelial tissues were isolated and cultured using previously established protocols (PMID: 21889923). For normal tissue, the mucosal layer was dissected from the whole tissue, followed by incubation with 5 mM EDTA at 4°C for 20 minutes on a roller. The released crypts were collected by spinning down at 300g for 5 minutes after squeezing the mucosal layer firmly against the bottom of the tube. The collected crypts were then seeded with Matrigel into 24-well plates. For cancer tissue, the tissue was digested with collagenase Type IV (Gibco # 17104019, 200 units in 5 ml Dispase) at 37°C for 30 minutes. After digestion, the sample was filtered through a 70 μm cell strainer, and the resulting clumps of cells were seeded with Matrigel into 24-well plates. The culture medium for human organoids consisted of the following components: Advanced DMEM/F12, 10 mM HEPES, 1× Penicillin-Streptomycin (Invitrogen # 15140122), 2 mM GlutaMAX, 1× N2 supplement, 1× B27 supplement, 1 mM N-acetyl-cysteine, 10 nM human gastrin I (Sigma-Aldrich # G9145), 50 ng/ml hEGF (R&D Systems # 236-EG-200), 100 ng/ml Noggin (R&D Systems # 6057-NG-100), 500 nM A83-01 (Tocris # 2939), 10 μM SB202190 (Sigma-Aldrich # S7067), 30 μg/ml human Wnt-3a (ProSpec # CYT-861), 1 mg/ml R-spondin (R&D Systems # 4645-RS-025), and 10 μM Y-27632 (Selleck Chemicals # S1049).

Human iPS-derived colon organoid culture

Wildtype human iPSCs were maintained on plates coated in growth factor reduced Matrigel (Corning) and cultured in StemMACS™ iPS-Brew XF medium (Miltenyi Biotec) supplemented with 1% Pen-Strep (Gibco). Cells were cultured in a 5% CO_2, 5% O_2, and 37ºC incubator and passaged every 4-5 days in small clusters using StemMACS Dissociation reagent (Miltenyi Biotec). Colon organoids were differentiated as previously described with minor modifications. Briefly, cells were washed with PBS, dissociated into single cells using warmed Accutase (STEMCELL Technologies), and seeded at a density of 500,000 cells per well of a matrigel-coated 6-well plate in iPS-Brew XF supplemented with 2 uM Thiazovivin ROCK inhibitor (Cayman Chemical). Definitive endoderm was derived using STEMdiff™ Definitive Endoderm Kit (STEMCELL Technologies) according to the manufacturer's instructions. Following definitive endoderm differentiation, cells were subjected to hindgut differentiation by treatment with 500 ng/ml FGF4 (Peprotech) and 3 µM CHIR99021 in RPMI supplemented with 1x B27 supplement (Gibco), 1x GlutaMAX (Gibco), and 1% Pen-Strep (Gibco). Hindgut medium was refreshed daily for a total of 4 days. From day 8 onward, cells were cultured in colonic differentiation medium containing Advanced DMEM/F12 (Invitrogen) supplemented with 1x B27 supplement (Gibco), 1x GlutaMAX (Gibco), 1% Pen-Strep (Gibco), 3 µM CHIR99021 (Cayman Chemical), 300 nM LDN193189 (Cayman Chemical) and 100 ng/ml EGF (Peprotech). 2D cultures were monitored daily for the next 10-12 days for the emergence of 3D spheroid structures. Where necessary, 3D spheroids were aided in detaching from the monolayer by gentle pipetting. Spheroids were collected and embedded in growth factor reduced Matrigel (Corning) and overlaid with colonic differentiation medium. Organoids were passaged every 10-12 days by mechanical dissociation. Mature organoid cultures were validated by confirming the presence of terminally differentiated colonic cell types by IHC and quantifying the expression of colonic marker genes by qRT-PCR compared to undifferentiated iPSC controls. To generate *APC* and *TP53* knockout (*AP*) CRISPR

tumoroids, gRNA sequences targeting exon 8 of *APC* (5-GGCAACTTCTGGTAATGGTC-3) or exon 10 of *TP53* (5-GAATGAGGCCTTGGAACTCA-3) were cloned into the PX330 vector (Addgene plasmid #42230). Organoids were removed from Matrigel using Dispase II (Gibco), dissociated into single cells using warmed TrypLE Express (Gibco), and resuspended in colonic differentiation medium without antibiotic. CRISPR vectors were transfected into cells using Lipofectamine Stem (Invitrogen) according to the manufacturer's instruction. Organoid transfection plates were then centrifuged at 600 g for 1 hr at 32ºC and incubated in a 37ºC incubator for an additional 3-4 hrs to allow transfection. Organoid cells were collected, centrifuged, replated in serial dilutions of growth factor reduced Matrigel, overlaid with colonic differentiation medium supplemented with 2 uM Thiazovivin (Cayman Chemical) and allowed to recover for 48 hours. Two days after transfection, organoid medium was replaced with AP selection medium (colonic differentiation medium lacking CHIR99021 and supplemented with 5 uM Nutlin-3 (Cayman Chemical) to select for organoids with loss of *APC* and *TP53* function, respectively). Selection medium was refreshed every other day for 10-12 days. After 12 days, individual *APC* and *TP53* inactivating mutations were confirmed by Sanger sequencing. Genotypes of edited alleles are found in Supplemental figure 8J-8K.

Lentiviral infection

The viral vectors were prepared using the ZymoPURE II™ Plasmid Maxiprep kit (Zymo Research # D4202) and subsequently transfected into HEK293T cells along with packaging plasmids (psPAX2 plasmid #12260 and pMD2.G plasmid #12259). After 48 and 72 hours, the viral supernatant was collected and filtered through a 0.45 μm filter. To concentrate the virus, the supernatant was then combined with Lenti-X™ Concentrator (Clontech # 631231) and incubated for 3 hours at 4°C following manufacture's instruction. The viral particles were resuspended in 10% of their original volume. The organoids were dissociated into single cells and seeded onto 2D plates. They were then infected with lentivirus along with 1 μg/ml polybrene (Sigma-Aldrich # TR-1003-G). Three days post-infection, the organoids were selected by treating them with the appropriate selection antibiotics: Blasticidin (20 μg/ml) and puromycin (10 μg/ml).

Quantitative RT-PCR

The samples were lysed using TRIzol (Thermo Fisher Scientific # 15596026) to isolate total RNA. For cDNA synthesis, 1 μg of total RNA was used with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific # K1622) following the manufacturer's instructions. Quantitative PCR reactions were performed using SYBR Green (Applied Biosystems # 4367659) under standard conditions on the QuantiStudio 7 Flex Real-Time PCR system (Applied Biosystems). The data obtained were analyzed using QuantStudio RT-PCR software. Custom primers were validated using standard SYBR Green qRT-PCR, followed by melting curve analysis. The data were then normalized to the housekeeping gene *Gapdh*. The primer sequences used to assess the mRNA expression levels of mouse *Notum*, human *NOTUM*, *Ascl2*, *Axin2*, *Ccnd1*, and *Lgr5* are listed in Supplementary Table 3.

Immunohistochemistry and immunofluorescence staining

Tumors obtained from mice were rinsed with cold DPBS, fixed in 4% PFA (Electron Microscopy Sciences # 15710), embedded in paraffin, and sectioned. The Haematoxylin & Eosin staining was performed at the Morphology Core of the UPenn NIDDK P30 Center for Molecular Studies in Digestive and Liver Diseases. For immunostaining, antigen retrieval was conducted by heating the slides in 0.01 M Tris-EDTA (pH 9.0) using a pressure cooker. Immunohistochemistry was performed using the ABC peroxidase method (Vector Laboratories # PK-4001) on the sections.

For immunofluorescence staining, the sections were first blocked with 10% donkey serum in PBS for 1 hour, followed by overnight incubation with primary antibodies at the appropriate dilution at 4 ºC. The next day, the sections were incubated with Cy2- or Cy3-conjugated fluorescent secondary antibodies (Jackson Laboratory) and counterstained with DAPI using mounting media (Vector Laboratories). The following antibodies were used: Ki67 (Abcam # ab15580, 1:500), E-cadherin (CST # 3195T, 1:200), and Notum (Abcam # ab106448, 1:500).

Annexin V/PI staining assay

APKS organoids were seeded into 24-well plates and treated with ABC99 (500 nM/mL) or SB202190 (10 μM/mL) or a combination of both for 2 days, with DMSO used as the vehicle control. To detect apoptosis, Dead Cell Apoptosis Kits (Thermo Scientific #V13245) were utilized, and the analysis was conducted following the manufacturer's instructions. The fluorescence of the cells was measured using flow cytometry, with the fluorescence emission at 530 nm and 575 nm measured using 488 nm excitation.

Co-immunoprecipitation assays

Apc mutant (A) and *Apc/Trp53* double mutant (AP) organoids were seeded with Matrigel and cultured for 3 days. The organoids were then treated with either DMSO or ABC99 (500 nM/ml) for 1 hour. Subsequently, the cells were harvested using 1x TrypLE. After washing with D-PBS, the cell pellet was lysed using NP40 Cell Lysis Buffer (Thermo Fisher Scientific #FNN0021) supplemented with a protease/phosphatase inhibitor cocktail (CST #5872S). The lysates were cleared by centrifugation at 12,000 rpm for 15 minutes. The resulting supernatant was transferred to a new tube, and the pellet containing cell debris and non-soluble fractions was discarded (50 μl of the supernatant was saved as Input). Dynabeads™ Protein A (Thermo Fisher Scientific #10008D) were resuspended and 50 μl was transferred to a tube for each sample. The beads were then incubated with 4 μg of anti-GPC1 (Proteintech #16700-1-AP, 1:1,000) or anti-GPC4 (Proteintech #13048-1-AP, 1:1,000) or anti-Rabbit IgG (CST #3900) for 3 hours at room temperature. After washing with 200 μl of PBS with Tween-20 (0.01%), the samples were incubated with the beads overnight at 4°C on a rocking platform. The beads were washed in lysis buffer and resuspended in 50 μl of 2x SDS gel loading buffer, followed by incubation at 70°C for 10 minutes. The proteins were then separated on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblotting using anti-IGF1Rβ (CST #3027S, 1:1,000) or anti-TGFBR1 (BOSTER #PB10101, 1:1,000) antibodies. The signals were detected using TidyBlot reagent (BIO-RAD #STAR209PA), visualized with the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific #34577), and imaged using the Bio-Rad Chemidoc TMMP imaging system.

Western blot analysis

To analyze protein expression, the 3D organoids were harvested using Cell Recovery Solution. The released organoids were washed with D-PBS, pelleted, and then lysed using 1x RIPA Buffer (CST #9806) supplemented with a protease/phosphatase inhibitor cocktail (CST #5872S). The lysed organoids were disrupted through sonication and spun down at 14,000 g for 15 minutes. The resulting supernatant was transferred to a clean tube, and the protein concentration was determined using the Bradford assay (Bio-Rad). The cell lysates were mixed with an appropriate volume of 4x loading buffer and incubated at 98°C for 10 minutes. Equal amounts of protein were loaded into each lane of an 8-12% SDS-PAGE gel. The membranes were blocked with 5% milk for 1 hour at room temperature and then incubated with the indicated primary antibodies diluted in 5% milk overnight at 4°C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies for 1 hour. Western blotting analyses were performed using the following

primary antibodies: anti-NOTUM (Abcam #ab106448, 1:1,000), anti-β-ACTIN (Abcam #ab6276, 1:5,000), anti-GPC1 (Proteintech #16700-1-AP, 1:1,000), anti-GPC4 (Proteintech #13048-1-AP, 1:1,000), anti-AKT (CST #4691, 1:1,000), anti-P-AKT (CST #4060, 1:1,000), anti-P38 (Proteintech #4064-1-AP, CST, 1:1,000), anti-phospho P38 (CST #4511, 1:1,000), anti-PS6 (CST #4858, 1:1,000), anti-p-SMAD3 (CST #9520, 1:1,000), anti-SMAD3 (CST #9523, 1:1,000), anti-SMAD4 (CST #46535, 1:1,000), anti-IGFR1β (CST #3027S, 1:1,000), and anti-TGFBR1 (BOSTER #PB10101, 1:1,000). The signals were detected using HRP-conjugated secondary anti-rabbit antibody (CST #7074S, 1:2,000) and anti-mouse antibody (CST #7076S, 1:5,000), visualized with the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific # 34577), and imaged using the Bio-Rad Chemidoc TMMP imaging system.

Generation of vectors and sgRNA cloning

To generate gene-specific sgRNA vectors, several plasmids were utilized. These include pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene #42230), LentiCRISPR v2 expression vector (Addgene #52961), LentiCas9-Blast (Addgene #52962), and LRG (Addgene #65656). These plasmids express sgRNA and/or Cas9 nuclease. The pLKO_TRC005 (RNAi Consortium) plasmid was employed for shRNA delivery. pUltra (Addgene #24129) was used for cDNA expression. For sgRNA cloning, pX330 was digested with BbsI (NEB #R3539S) and ligated with BbsI-compatible annealed sgRNA oligos targeting exon 16 in *Apc* (target sequence GTAATGCATGTGGAACTTTGTGG, PAM sequence underlined), or exon 4 in *Trp53* (target sequence CCCTCCGAGTGTCAGGAGCTCC, PAM sequence underlined), or exon 2 in *Smad4* (target sequence GATGTGTCATAGACAAGGTGGG, PAM sequence underlined). Lenti-CRISPR v2 and LRG were digested with BsmBI (NEB #R0739S) and ligated with BsmBI-compatible annealed sgRNA oligos targeting exon 2 in *Notum* (target sequence AGGAAGACCTGGAGGCGCCGTGG, PAM sequence underlined), or exon 1 in *Gpc1* (target sequence CGCTGGTCGTCTGCGCCCGCGGG, PAM sequence underlined), or exon 1 in *Gpc4* (target sequence CGACGTCTCTACGTGTCCAAAGG, PAM sequence underlined), or exon 2 in *Gpc6* (target sequence CCGAGCCTTCACGTCCGCCGAGG, PAM sequence underlined). For shRNA targeting *Notum*, pLKO_TRC005 was digested with NdeI (NEB #R0111S) and EcoRI (NEB #R3101), and then ligated with shNotum oligo targeting exon 10 (target sequence GCCAGTGGCTATACATCCAGA). To achieve Notum overexpression, mouse *Notum* was cloned from colon organoid cDNA using the primers Notum-F (ATATCTAGAATGGGAGGAGAGGTGCGCGTGCT) and Notum-R (CGCGGATCCCTAGTTCCCATTACTCAGCATCCCTA). The PCR product and pUltra were digested with XbaI (NEB #R0145S) and BamHI (NEB #R3136T), followed by ligation and sequence validation.

Standard mutation nomenclature

The standard mutation nomenclature, as recommended by the HGVS (http:// http://varnomen.hgvs.org) was used to assign nucleotide numbers and name identified mutations from sequencing data. In this nomenclature, nucleotide numbering is based on the translation initiation codon, with the A of the ATG designated as position 1. Frameshift mutations resulting in a shifted reading frame are indicated by the position of the stop codon at position X in the altered frame.

Endoscope-guided orthotopic transplantation

Male C57BL/6J mice, approximately 8 weeks old, obtained from the Jackson Laboratory, were used for orthotopic transplantation experiments. Syngeneic luciferase-expressing organoids, matching the sex of the recipient mice, were isolated from Matrigel using Cell Recovery Solution

(Corning #354253), incubated on ice for 30 minutes. Cell clusters equivalent to 2 × 10^4 cells for each mouse were resuspended in cold D-PBS with 0.5% BSA and 10% Matrigel. The transplantation of organoids into the colon mucosa of recipient mice was performed using optical colonoscopy. This was achieved by utilizing a custom injection needle (Hamilton Inc., 33 gauge, small Hub RN NDL, 12 inches long, point 4, 45-degree bevel, catalog #7803-05), a 100 μl syringe (Hamilton Inc., part number 7656-01), a transfer needle (Hamilton Inc., part number 7770-02), and a Urethroscope (part number 8626.431). A total of 70 μl of the cell suspension was delivered to the colon mucosa, with one injection performed per mouse. Colonoscopy was conducted 2-8 weeks following organoid transplantation to evaluate tumor development. Tumor size was quantified *in situ* by photographing the tumors and measuring the percentage of lumen occlusion caused by the tumor. Tumor sizes were measured in Adobe Photoshop relative to the luminal diameter, and the percent tumor occlusion of the lumen was recorded. The change in percent tumor occlusion relative to the baseline measurement was calculated every two weeks and averaged within the two groups. Tumors were assessed at the experimental endpoint. IVIS imaging was utilized to monitor tumor development once a week during treatment with ABC99 (10 mg/kg) or Oil until the experimental endpoint at 8 weeks post-implantation.

Subcutaneous organoid implantation

Male C57/B6J mice, 8 weeks old, were obtained from the Jackson Laboratory and housed in a specific pathogen-free environment. Syngeneic mouse APKS tumoroids infected with sgNotum virus or sgControl were isolated from Matrigel using Cell Recovery Solution. The cell clusters, equivalent to 1 × 10^5 cells for each mouse, were then suspended in 200 μl of cold D-PBS with 0.5% BSA and 10% Matrigel. The cell suspension was injected into the subcutaneous space of the loose skin between the shoulder blades of the mice. After a period of 4 weeks following transplantation, the tumors were isolated, and their weights were measured.

Adenoma modeling in *Lgr5^{CreER}::Apc$^{flx/flx}$* mice

Lgr5$^{EGFP\text{-}IRES\text{-}creERT2}$::Apc$^{fl/fl}$ mice were administered five daily intraperitoneal (IP) injections of 1 mg tamoxifen (Sigma-Aldrich, T5648). After 20 days, the small molecule NOTUM inhibitor ABC99 (10 mg/kg) or vehicle control corn oil was given by IP injection for the duration of the experiment-four additional weeks.

Clonal organoid formation/seeding efficiency assays

To obtain single cells from organoids in 3D, TrypLE™ Express Enzyme (1X) (Thermo Fisher #12605) was used to digest the organoids for 5 minutes in a 37°C water bath. The cell numbers were determined using a hemocytometer, and single viable cells were then seeded in 24-well plates with Matrigel at a density of 500 cells per well. Each cell type was seeded in triplicate.

Four days after seeding, brightfield images of each well were captured using a Leica DMi8 microscope (Leica, Germany). The images were manually counted using Photoshop software to determine the number of organoids formed. Seeding efficiencies were calculated as the percentage of single cells that successfully formed an organoid.

Organoid competition assay

The co-culture experiments were performed using two different cell types: mouse *Apc$^{\Delta}$* and *APKS^{Hypo}*. For the *Apc$^{\Delta}$* co-culture, *Apc$^{\Delta}$* organoids were transfected with either the pUltra-Notum-GFP or pUltra-GFP plasmid to achieve overexpression of NOTUM. Following transfection, GFP+ cells were sorted and 2000 green cells (*Apc$^{\Delta}$* pUltra-Notum-GFP or *Apc$^{\Delta}$*-pUltra-GFP) were combined with 2000 *Apc$^{\Delta}$* cells. The cell mixture was then seeded in Matrigel for subsequent

culture. After 4 days of incubation, either an EdU assay or an organoid forming assay was conducted. For the *APKS^{Hypo}* co-culture, *APKS^{Hypo}* cells infected with the pCDH-EF1a-eFFly-mCherry plasmid were transfected with either the pUltra-Notum-GFP or pUltra-GFP plasmid to induce NOTUM overexpression. GFP+ cells were subsequently sorted, and 2000 green cells (*APKS^{Hypo}*-pUltra-Notum-GFP or *APKS^{Hypo}* -pUltra-GFP) were combined with 2000 red fluorescence cells (*APKS^{Hypo}*). The cell mixture was then seeded in Matrigel for subsequent culture. Similarly, after 4 days of incubation, either an EdU assay or an organoid forming assay was performed.

Organoid proliferation assays

For EdU assays, organoids or tumoroids with different genetic mutations were infected with overexpression, knockdown, or control viruses for two days. Subsequently, they were digested into single cells using TrypLE™ and seeded into 24-well plates at a density of 3,000 cells per well, with each cell type seeded in triplicate. The organoids were cultured for 4 days, with media changes every other day. After the culture period, 10 μM EdU (Thermo Fisher #E10187) was added to the culture media and incubated for 2 hours. EdU staining was performed using the Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher #10634) following the manufacturer's protocol. The stained cells were analyzed using an LSR Fortessa flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (BD Biosciences).

For MTT assays, organoids or tumoroids with different genetic mutations were infected with overexpression, knockdown, or control viruses for two days. Subsequently, they were digested and seeded into 96-well plates at a density of 1,000 cells per well, with each cell type seeded in 12 wells. Cell proliferation assays were performed using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assays at specified time points. The absorbance of the samples was measured at 560 nm. All data were expressed as the mean from at least three independent biological experiments, with each condition having n=3 replicates per experiment.

scRNA-seq library preparation

The normal mouse colon was opened and cut longitudinally. It was then minced into small pieces and incubated with 1 U/ml Dispase (STEMCELL Technologies # 07923) supplemented with 0.05 mg/ml Liberase (Sigma-Aldrich # 05401127001) for 1 hour in a 37°C water bath. For tumors obtained from Apc^{Min} mice, AOM/DSS mice, or mice with tumors generated by orthotopic injection of APKS tumoroids, the tumors were isolated and digested using Dispase (1 U/ml) with Liberase (0.05 mg/ml). The sample suspensions were shaken every ten minutes and then passed through 70 μm cell strainers. The cells were washed with 0.04% BSA-DPBS and then stained with Cell Multiplexing Oligo (10X Genomics # 1000261) and incubated for 5 minutes at room temperature. Following washing as per the 10x Genomics protocol, the cells were resuspended in 0.04% BSA-DPBS with DAPI, and live single cells were purified using FACS (BD FACS Aria B). Library preparation was carried out using the 10x Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) following the manufacturer's instructions (10x Genomics). Approximately 5000-10000 cells were partitioned into Gel Beads in Emulsion (GEMs) with cell lysis and barcoded by reverse transcription of mRNA into cDNA. This was followed by amplification, enzymatic fragmentation, and attachment of 5' adaptors and sample indices. The sample libraries were subsequently subjected to sequencing on the NovaSeq 6000 platform from Illumina, aiming for a targeting depth of 50,000 reads per cell.

scRNA-seq data processing

Sequencing reads for the human and mouse samples were first pre-processed with 10x Genomics Cell Ranger pipeline and aligned to the GRCh38 reference and GRCm38 (mm10) reference genome respectively. An initial filtering was performed on the raw gene-barcode matrix output by the Cell Ranger cellranger count function, removing barcodes that have less than 1000 transcripts (quantified by unique molecular identifier (UMI)) and 500 expressed genes ("expressed" means that there is at least 1 transcript from the gene in the cell). Barcodes that pass this filter were considered as cells and were fed into downstream analysis. For samples multiplexed using the TotalSeq-B protocol, cells were demultiplexed by performing Louvain clustering on the UMAP generated with the hashtag count matrix. Gene-barcode UMI count matrix combined from all datasets was size-factor corrected and log transformed to produce a normalized gene expression matrix. We used the VisCello package to generate a series of PCAs and UMAPs for different cell subsets. The processing pipeline was described previously in Zhu et al (Q. Zhu, J. I. Murray, K. Tan, J. Kim, qinzhu/VisCello: VisCello v1.0.0 2019; https://zenodo.org/record/3262313). Briefly, we applied an "informative feature (IFF) selection" procedure to select genes that have high gini coefficient which indicates the "inequality" (therefore specificity) of the gene's expression across clusters. Principal component analysis (PCA) was then performed on the IFF-cell matrix, and the top PCs were used as features for the UMAP algorithm. UMAP was computed using the *umap* function in the uwot R package, with "cosine" distance metric, 30 nearest neighbors, and the rest of the parameters as default. Louvain clustering was run on the k-nearest neighbor graph (k = 20) constructed from cell embeddings on the UMAP.