## MATERIAL AND METHODS

### Isolation of epithelial crypts from the rabbit caecum

Epithelial crypts were isolated from the caecum of a 18-day-old suckling rabbit raised at the PECTOUL experimental facility (GenPhySE, INRAE, Toulouse, France). The protocol was approved by the local ethics committee (SSA n°115, SSA_2024_006V2). The caecum was isolated after euthanasia and placed in cold PBS (GIBCO, cat#10010-015). The caecum was then opened longitudinally and washed with cold PBS to remove all content. The tissue was minced into 1 $cm^2$ sections before transfer to 5 mL of a pre-warmed (37°C) digestion solution prepared in HBSS without $Ca^{2+}$/$Mg^{2+}$ (ThermoFisher Scientific, cat#14175095) and supplemented with 5 mM EDTA (ThermoFisher Scientific, cat#AM9260G) and 1 mM DTT (Sigma, cat# 10197777001). After incubation (20 minutes at 37°C under slow agitation at 40 g), epithelial crypts were detached by vigorous manual shaking for one minute. The crypt solution was then filtered 100 µm to remove tissue fragments. The filtered crypt solution was centrifuged (300 g, 5 min, 4°C) and the pellet was resuspended in 5 mL cold PBS. Crypts were manually counted and the volume corresponding to approximately 900 crypts was centrifuged (300 g, 5 min, 4°C). The crypt pellet was resuspended in 1 mL freezing solution (80 % DMEM [ThermoFischerScientific, cat#31966047], 10 % fetal bovine serum [ThermoFischerScientific, cat#10270-106], 10 % DMSO [Corning, cat#25-950-CQC], 10 µM Y27632 [StemCell Technologies, cat# 72304]) and transferred in a cryotube, which was placed in a CoolCell™ LX Cell Freezing Container (Corning, cat# 432003) at −80°C for 24 h before long-term storage in liquid nitrogen.

### Culture of rabbit caecal organoids in three dimensions

Cryopreserved rabbit caecum epithelial crypts were thawed at 37°C before centrifugation (300 g, 5 minutes, room temperature). The crypt pellet was resuspended in Matrigel (Corning, cat#354234), plated into a pre-warmed 48-well plate (37°C) (25 µL/well), and left to polymerize for 15 minutes at 37°C. Each well was overlaid with 250 µL of organoid growth medium composed of IntestiCult Organoid Growth Medium (Human) (Stem Cell Technologies, cat# 06010) supplemented with 1% penicillin/streptomycin (PS, Sigma,

cat#P4333) and 100 µg/mL Primocin (InvivoGen, cat#ant-pm-05), at room temperature. The plate was placed in a cell culture incubator (37°C, 5% $CO_2$) and the medium was changed every 2-3 days.

Organoids were passaged 7 days after crypt seeding. After a wash in warm PBS, organoids in Matrigel domes were homogenized in pre-warmed TrypLE (Gibco, cat# 12605-010). After incubation for 5 minutes at 37°C in a $CO_2$ incubator, the Matrigel-cell suspension was homogenized by pipetting and the incubation step at 37°C was repeated once. Digestion was stopped by adding DMEM supplemented with 10% FBS and 1% PS (DMEMc). The contents of each well were pooled into a tube and centrifuged (500 g, 4°C, 5 min). Cells were resuspended in DMEMc and counted with a using a Countess 3 Automated Cell Counter (ThermoFischerScientific, cat#16842556). Organoid cells were resuspended in cold Matrigel:DMEMc (v/v: 2:1), before plating into pre-heated (37°C) 24-well plates (3000 cells/50 µL/well). The plates were left to polymerize for 30 minutes at 37°C. Each well was then overlaid with 500 µL of organoid growth medium at room temperature. The plate was placed in a cell culture incubator (37°C, 5% $CO_2$) and the medium was changed every 2-3 days.

**Culture of cell monolayers derived from the rabbit caecum organoids**

Cell culture inserts for 24-well plates (Corning, cat#353095) were coated with 150 µL of 50 µg/mL type IV collagen derived from human placenta (Sigma, cat# C5533-5MG) at 37°C for 2 hours. After removal of the coating solutions, inserts were dried without the lid under the cell culture cabinet until seeding. Nine days after passaging in 3D, the Matrigel domes with organoids were dissociated by pipetting and transferred into tubes containing 5 mL of DMEMc. The suspension was centrifuged (500 g, 4°C, 5 min), and the supernatant above the Matrigel layer was disrupted by pipetting. Then, organoids were centrifuged (500 g, 4°C, 5 min) and the resulting pellet was resuspended in pre-warmed TrypLE supplemented with 10 µM Y27632 and incubated in a 37°C water bath for 5 minutes before homogenization by pipetting. This cycle of incubation and homogenization was repeated until complete cell dissociation. DMEMc was added to the suspension to stop digestion before centrifugation (500 g, 4°C, 5 minutes). The cell pellet was resuspended in DMEMc and

cells were counted as described before. Cells were resuspended in organoid growth medium supplemented with 20% FBS and 10 µM Y27632 before seeding in collagen IV-coated inserts (2.5 $10^5$ cells/ 200 µL/insert). The same culture medium was also added to the basal side (500 µL). Cells were incubated at 37°C under 5% $CO_2$ atmosphere. Three days after seeding, the culture medium was removed from the basal and apical sides and cell monolayers were washed with PBS. The apical compartment was either submerged in 200 µL warm DMEM supplemented with PS 1% on the apical side (control condition) or remained empty to set up an air-liquid interface (ALI condition). Organoid culture medium supplemented with 20% FBS was added to the basal side (500 µL) in both control and ALI conditions. Four days after seeding, apical (control condition) and basal (control and ALI conditions) media were refreshed. Five days after seeding (i.e. after 48h of culture in control or ALI conditions), the cell monolayers were processed for single cell transcriptomics.

**Organoid cell monolayer dissociation and multiplexing**

Cells dissociation was performed on n=4 cell culture inserts per condition (control or ALI). Cell monolayers were washed in PBS before adding pre-warmed TrypLE supplemented with 10 µM Y27632 in the apical (200 µL) and basolateral (500 µL) compartments. Cells were incubated at 37°C for 20 minutes with homogenization every 5 minutes by pipetting. The dissociated cells were transferred into 1.5 mL tubes containing 1 mL of cold DMEMc to stop digestion and centrifuged (500 g, 5 min, 4°C). The pellet was resuspended in 100 µL of cold PBS containing 10% FBS and 10 µM Y27632 before counting. Approximately $10^5$ cells/insert were resuspended in 1 mL with cold PBS containing 10% FBS and 10 µM Y27632 before centrifugation (300 g, 5 min, 4°C). The supernatant was removed and 100 µL of a single Cell Multiplexing Oligo (CMO) solution from the 3’ CellPlex Kit Set A (10X Genomics, cat#1000261) were added to each tube (1 CMO/insert). The cells were homogenized with the CMO solution by pipetting 15 times before incubation at room temperature for 5 min. Subsequently, 1.4 mL of cold PBS containing 10% FBS and 10 µM Y27632 was added before mixing by pipetting and centrifugation (300 g, 5 min, 4°C). The supernatant was discarded and cells were resuspended in 100 µL of cold PBS containing 10% FBS and 10

µM Y27632 before pooling cells derived from inserts treated in the same condition (control or ALI) in equal proportions (1:1:1:1, n=4 inserts pooled per conditions).

**Cell sorting and library construction**

For viability assessment, cells were centrifuged (300 g, 5 min, 4°C) and resuspended in 1 mL of PBS supplemented with 10 µM Y27632 and 1 µL of LIVE/DEAD™ Fixable Violet Dead Cell Stain (ThermoFisher Scientific, cat#L34963). After incubation (4°C, 30 minutes, in the dark), cells were centrifuged (300 g, 5 min, 4°C) and the pellet was washed twice with FACS buffer (3% FBS, 2 mM EDTA, 10 µM Y27632 in PBS) before filtering (40 µm). Approximately $10^5$ live single-cells were sorted by using a BD Influx cell sorter instrument with a 100 µm nozzle, under 20 psi at the I2MC Cytometry and Cell sorting TRI platform (Toulouse, France). Cells were centrifuged (300 g, 10 min, 4°C), resuspended in PBS and manually counted.

For each sample (control or ALI), 50 000 cells were used for encapsulation into droplets using Chromium NextGEM Single Cell 3′ Kit v3.1 (10X Genomics, cat#PN-1000268) with Feature Barcoding technology for Cell Multiplexing, according to manufacturer's protocol (10x Genomics CG000388 Rev C user guide). Briefly, after generation of Gel bead-in-EMulsions (GEMs) using Chromium Next GEM Chip G, GEMs were reverse transcribed in a C1000 Touch Thermal Cycler (BioRad) programmed at 53°C for 45 min, 85°C for 5 min, and held at 4°C to produce barcoded cDNA from polyA mRNA and barcoded DNA from the CMO. Then, single-cell droplets were broken and cDNA was isolated and cleaned with Cleanup Mix containing DynaBeads (ThermoFisher Scientific). cDNA was then amplified by PCR with a C1000 Touch Thermal Cycler programmed at 98°C for 3 min, 11 cycles of (98°C for 15 s, 63°C for 20 s, 72°C for 1 min), 72°C for 1 min, and held at 4°C. cDNA derived from mRNA or CMO were separated thanks to differential clean up with SPRIselect beads (Beckman Coulter, cat#B23317). 3' Gene Expression Library was constructed with approximately 80-90 ng of amplified cDNA, which was fragmented, end-repaired, A-tailed, index adaptor ligated, and cleaned with SPRIselect beads in between steps. Post-ligation product was

amplified and indexed with a C1000 Touch Thermal Cycler programmed at 98°C for 45 s, 13 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 20 s), 72°C for 1 min, and held at 4°C. Cell Multiplexing Libraries were constructed and indexed by PCR too, using similar parameters : 98°C for 45 s, 6 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 20 s), 72°C for 1 min, and held at 4°C. The sequencing-ready libraries were cleaned up with SPRIselect beads. Libraries were pooled following the recommendations, and loaded with 1% PhiX on two S1 lanes of the NovaSeq 6000 instrument (Illumina) using the NovaSeq 6000 S1 Reagent Kit v1.5 (100 cycles), and the following sequencing parameters: 28 bp read 1 – 10 bp index 1 (i7) – 10 bp index 1 (i5) – 88 bp read 2.

**scRNA-seq pre-processing, filtering, normalization and clustering**

Cell Ranger software (version 7.1.0, 10x Genomics) was used to align and quantify the raw sequencing data using the rabbit reference genome (GCF_009806435.1_UM_NZW_1.0). A custom reference file was generated using the mkgtf command with the parameters '--attribute=gene_biotype:protein_coding' and '--attribute=gene_biotype:lncRNA', followed by the mkref command with default parameters. The multiplexed data was then analyzed using the multi pipeline, run with default parameters. Demultiplexed fastq files were generated from the bam files produced by the multi pipeline using bamtofastq (version 1.4.1, 10x Genomics) with the parameter '--reads-per-fastq=1000000000'.