Cell-type-specific signaling networks in heterocellular organoids

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Despite the widespread adoption of organoids as biomimetic tissue models, methods to comprehensively analyze cell-type-specific post-translational modification (PTM) signaling networks in organoids are absent. Here, we report multivariate single-cell analysis of such networks in organoids and organoid cocultures. Simultaneous analysis by mass cytometry of 28 PTMs in >1 million single cells derived from small intestinal organoids reveals cell-type- and cell-state-specific signaling networks in stem, Paneth, enteroendocrine, tuft and goblet cells, as well as enterocytes. Integrating single-cell PTM analysis with thiol-reactive organoid barcoding in situ (TOBis) enables high-throughput comparison of signaling networks between organoid cultures. Cell-type-specific PTM analysis of colorectal cancer organoid cocultures reveals that shApc, KrasG12D and Trp53R172H cell-autonomously mimic signaling states normally induced by stromal fibroblasts and macrophages. These results demonstrate how standard mass cytometry workflows can be modified to perform high-throughput multivariate cell-type-specific signaling analysis of healthy and cancerous organoids.

Organoids are self-organizing three-dimensional (3D) tissue models comprising stem and differentiated cells. Organoid monocultures typically contain one major cell class (for example, epithelial) and can be cocultured with heterotypic cell types (for example, mesenchymal or immune cell types) to model cell–cell interactions in vitro. When compared with traditional two-dimensional (2D) cell culture, organoids more accurately represent their parental tissue and are emerging as models for studying multicellular diseases such as cancer.

PTM signaling networks underpin fundamental biological phenotypes and are frequently dysregulated in disease. As different cell types have different signaling networks, organoids likely contain cell-type-specific PTM networks that are essential to their biology. Yet, technologies to analyze cell-type-specific PTM networks in organoids and organoid cocultures currently do not exist.

Organoids present several technical challenges over 2D cultures for PTM analysis. Firstly, organoids are embedded in a protein-rich extracellular matrix (ECM) that confounds the application of phosphoproteomic analysis by liquid chromatography–tandem mass spectrometry. Organoids can be removed from ECM before liquid chromatography–tandem mass spectrometry, but as dissociation of live cells alters cell signaling, PTM measurements from dissociated live organoids do not truly represent in situ cellular states. Secondly, as organoids comprise multiple cell types (for example, stem and differentiated cells) and cell states (for example, proliferating, quiescent and apoptotic), bulk phosphoproteomics cannot capture organoid heterogeneity. Although single-cell RNA sequencing can describe organoid cell types, it cannot reveal PTM signaling at the protein level. Finally, low-dimensional methods (for example, fluorescent imaging) cannot capture the complexity of signaling networks comprising multiple PTM nodes. Collectively, to study PTM networks in organoids, we require signaling data that are derived from cells fixed in situ, are cell-type-specific and analyze multiple PTMs simultaneously.

Mass cytometry (MC, also known as cytometry time-of-flight or CyTOF) uses heavy-metal-conjugated antibodies to study >35 proteins in single cells. Although MC is traditionally used for high-dimensional immunophenotyping, it can also detect PTMs in heterocellular systems (for example, peripheral blood mononuclear cells and tissue).

Here, we report the development of a custom multivariate-barcoded MC method to analyze single-cell PTM signaling in organoids and organoids cocultured with stromal and immune cells. This method reveals that intestinal organoids display cell-type-specific signaling networks that are intimately linked with cell state. When applied to colorectal cancer (CRC) organoids cocultured with stromal fibroblasts and macrophages, we discovered that epithelial oncogenic mutations mimic signaling networks normally induced by stromal cells. These results demonstrate that MC can be used for multivariate single-cell analysis of PTM signaling networks in heterocellular organoids.

Results

Single-cell analysis of organoids by MC. To study cell-type- and cell-state-specific signaling in organoids, we developed an MC workflow and applied it to small intestinal organoids. We first pulse live organoids with 125I-iodo-2′-deoxyuridine (125IdU) to identify S-phase cells, fix organoids in Matrigel to preserve cell signaling and stain organoids with 194 cисplatin to label dead epithelia. We then physically and enzymatically dissociate the fixed organoids into single cells before extra- and intracellular heavy-metal antibody staining (Fig. 1a). We performed a comprehensive screen for antibodies that identify intestinal epithelial cell types including stem (LGR5, LRG1, OLFM4), Paneth (Lysozyme), goblet (MUC2, CILA1), enteroendocrine (CHGA, Synaptophysin) and tuft cells (DCAMKL1) and enterocytes (FABP1, Na/K-ATPase). We required that these antibodies bind fixed antigens and are compatible with rare-earth metal conjugation for MC. Cell-type identifica-

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Articles

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18, proliferating often postmitotic (pRB–) or apoptotic (cCaspase3–), whereas differentiated epithelia are.

UMAP distribution of ~1 million single organoid cells analyzed by MC resolves six major intestinal cell types across. (EMD)19,20 and density resampled estimation of mutual information (DREMI)21 to build quantitative cell-type-specific signaling networks from single-cell organoid PTM data (Fig. 2b and Supplementary Fig. 3). In these networks, EMD quantifies PTM intensity (node score) for each organoid cell type relative to the total organoid population and DREMI quantifies PTM–PTM connectivity (edge score).

As canonical WNT signaling is mainly driven by protein interactions, localization and degradation22 rather than by a classical PTM cascade, MC is not well suited to studying the WNT pathway. Despite this limitation, we observe evidence of WNT flux via inhibitors pGSK-3β [S9] and nonphosphorylated β-Catenin in all organoid cell types (Fig. 2a). In contrast, MAPK and PI3K pathways exhibit cell-type specificity. For example, stem cells channel MAPK signaling through pERK1/2 [T202/Y204], p90RSK [T359] and pCREB [S133], but do not connect with pBAD [S112] (Fig. 2a,b). In contrast, differentiated epithelia direct MAPK signaling away from pCREB and towards pBAD when proliferating, and lose all MAPK activity in G0 and apoptosis (Fig. 2a). Despite their strong mitogenic signal-

Cell-type- and cell-state-specific signaling networks in intestinal organoids. Following cell-type and cell-state identification, we next constructed cell-type-specific PTM signaling networks in small intestinal organoids. We combined earth mover’s distance (EMD)19,20 and density resampled estimation of mutual information (DREMI)21 to build quantitative cell-type-specific signaling networks from single-cell organoid PTM data (Fig. 2b and Supplementary Fig. 3). In these networks, EMD quantifies PTM intensity (node score) for each organoid cell type relative to the total organoid population and DREMI quantifies PTM–PTM connectivity (edge score).

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Several PTM signaling events correlate with cell state in intestinal organoids. For example, irrespective of cell type or localization, pP38 MAPK [T180/Y182] and pP120-Catenin [T310] are hyperactivated in M phase (Figs. 1c and 2a). In contrast, TGF-β signaling (via pSMAD2[S465/S467] and SMAD3[S423/S425]) is exclusively active in postmitotic epithelia, consistent with TGF-β’s role in epithelial growth arrest24 (Fig. 2a). To investigate the relationship among cell type, cell state and PTM signaling, we performed principal component analysis (PCA) of PTM-EMDs for each organoid cell type, either proliferating (pRB+) or G0 (pRB–) and in lower crypts (CD44+) or villi (CD44–). Endo., enteroendocrine; Entero., enterocyte; Perm., permissive.

Single-cell organoid multiplexing using thiol-reactive organoid barcoding in situ (TOBis). To study differential signal
transduction in organoid models of healthy and diseased tissue, we need to directly compare PTM networks between different organoid cultures. A major advantage of MC is its ability to perform multiplexed barcoding of experimental variables. However, commercially available palladium-based barcodes cannot bind organoids in situ as they react with Matrigel proteins, meaning that organoids must be removed from Matrigel and dissociated separately before barcoding. To overcome this limitation, we developed a strategy to isotopically barcode organoids while still in Matrigel.

MC barcoding strategies use amine- or thiol-reactive chemistries. We first used fluorescent probes to investigate how these chemistries react with ECM proteins and organoids. Amine-reactive probes (Alexa Fluor 647 NHS ester) bind ECM proteins and thus fail to label organoids in situ (Fig. 3a). In contrast, thiol-reactive probes (Alexa Fluor 647 NHS ester-DOTA-157Gd) bind ECM proteins and can therefore be used to barcode organoids while still in Matrigel. Importantly, as Te and Pt are not typically conjugated to antibodies in MC, TOBis multiplexing does not compromise the number of antigens being measured. Moreover, as barcoding is performed on fixed organoids embedded in Matrigel, TOBis does not require the numerous centrifugation or permeabilization steps used in traditional solution-phase barcoding. This increases organoid sample throughput and single-cell recovery.

Multivariate cell-type-specific signaling analysis of intestinal organoid development. Traditional mass-tag barcoding allows direct comparison of solution-phase cells between experimental conditions. TOBis MC enables PTM signaling networks to be directly compared between solid-phase organoid cultures in a high-throughput manner. To demonstrate this, we applied TOBis to study cell-type-specific epithelial signaling during 7 d of small intestinal organoid development.

Analysis of 28 PTMs from ~2 million single organoid cells revealed that after 1 d of culture, organoids seeded as single crypts exist in a 'recovery' phase where ~70% of cells have entered the cell cycle (pRb+), then the remaining ~30% enter the cell cycle (pRb−), but <5% reach S phase (IdU+) (compared with ~20% in developed organoids). Days 2 and 3 mark a rapid expansion and differentiation phase of organoid development where stem, Paneth and goblet cells, as well as enterocytes, activate MAPK, P38 and PI3K pathways. By day 4, intestinal organoids reach a critical 'divergence' phase where crypt and villus signaling digress. While stem and Paneth cells maintain active MAPK, P38 and P13K pathways,
enterocytes lose major PI3K activity (Fig. 4c). As a result, by days 5–7, enterocytes are largely postmitotic or apoptotic with high TGF-β signaling, whereas stem cells retain mitogenic flux and cell cycle activity (Fig. 4c). Consequently, stem cell number increases while enterocytes become exhausted at the end of intestinal organoid development (Fig. 4a,b). Notably, both stem and Paneth cells continue to display high MAPK, P38 and PI3K activity even at this late stage of organoid culture (Fig. 4c). This suggests that maintaining a stable signaling flux is a core feature of intestinal crypt cells. Such variations in organoid cell state, cell type and PTM activity suggest that developmental stage should be carefully considered when performing organoid experiments.

**Single-cell signaling analysis of tumor microenvironment (TME) organoids.** Given that MC can theoretically resolve any cell type, we next expanded this platform to study PTM signaling in heterocellular organoid coculture models of CRC.

CRC develops through successive oncogenic mutations—frequently resulting in loss of APC activity, hyperactivation of KRAS and perturbation of TP53 (ref. 29). In addition to oncogenic mutations, stromal fibroblasts30,31 and macrophages32 in the TME have quently resulting in loss of APC activity, hyperactivation of KRAS and perturbation of TP53 (ref. 29). In addition to oncogenic mutations, stromal fibroblasts30,31 and macrophages32 in the TME have

To investigate this, we cultured wild-type (WT), shApc (A), shApc and KrasG12D/+ (AK), or shApc, KrasG12D/+ and Trp53R172H−/− (AKP)34,35 colonic epithelial organoids, either alone or with colonic fibroblasts and/or macrophages (Fig. 5a,b and Supplementary Fig. 6). We fixed and TOB barcoded each of these organoid cultures, and performed single-cell signaling analysis in one multivariate MC run (Fig. 5a and Supplementary Table 2). The addition of myeloid (CD68, F4/80) and mesenchymal (Podoplanin) heavy-metal antibodies enabled resolution of epithelial organoids, macrophages and fibroblasts from each barcoded condition (Fig. 5c). This experimental design allowed us to directly compare mutation- and microenvironment-driven cell-type-specific signaling networks in CRC organoid mono- and cocultures.

Oncogenic mutations have a cell-autonomous effect on epithelial signaling. Although APC mutations are known to upregulate WNT signaling33, we found that the loss of APC also activates the P38 pathway, downregulates TGF-β/BMP signaling and activates p120-Catenin in colonic organoids (Fig. 5d). Subsequent oncogenic KrasG12D/+ and Trp53R172H−/− mutations further upregulate not only the classical MAPK pathway, but also major PI3K nodes (Fig. 5d). As a result, AK and AKP organoids display increased epithelial signaling relative to microenvironmental cues from stromal and immune cells is unclear.
PTM-PTM connectivity is regulated largely by genotype, not on PTM regulation to oncogenic mutations (Fig. 5e). In contrast, we found that microenvironmental cues have a comparable impact on cell-intrinsic or the TME (cell-extrinsic) is less clear. Signaling, we discovered polarity in fibroblast and macrophage cell–cell communication. For example, macrophage signaling pathways (MAPK, PI3K and NF-κB) are upregulated by fibroblasts, whereas fibroblast signaling is scarcely altered by macrophages (Supplementary Fig. 8). In contrast, epithelial cells upregulate MAPK and P38 signaling in fibroblasts (Supplementary Fig. 8b), which, in turn, reciprocally activate MAPK and P38 signaling in epithelial cells (Supplementary Fig. 7). These results suggest that colonic fibroblasts regulate intercellular signaling in the colonic microenvironment and should be further investigated as drivers of CRC.

### Oncogenic mutations mimic stromal signaling networks

To further investigate the parity between genotypic and microenvironmental regulation of epithelial signaling, we overlaid single-cell MC data from WT, A, AK and AKP organoids onto a fixed-node macroenvironmental Scaffold map (Fig. 6a and Supplementary Fig. 9). This unsupervised analysis confirmed that Apc, Kras and Trp53 oncogenic mutations mimic the signaling profile of WT organoids in the presence of stromal cells. Inverted organoid genotype Scaffold maps also expose a similarity between mutation- and microenvironment-driven signaling (Supplementary Fig. 7).

| stem and progenitor cell-type markers LRIG1 and CD44, decreased apoptosis and increased mitogenic cell state relative to WT and A organoids (Fig. 5d). Both oncogenes and stromal cells can dysregulate cancer cell signaling. However, to what extent this is driven by oncogenic mutations (cell-intrinsic) or the TME (cell-extrinsic) is less clear. We found that microenvironmental cues have a comparable impact on PTM regulation to oncogenic mutations (Fig. 5e). In contrast, PTM–PTM connectivity is regulated largely by genotype, not microenvironment (Fig. 5f). This observation suggests that oncogenic mutations fundamentally rewire signaling networks, whereas stromal cells regulate acute signaling flux. Stromal cells further upregulate the PI3K pathway in CRC organoids that already contain KrasG12D and Trp53R172H mutations (Fig. 5d and Supplementary Fig. 7). Microenvironmental hyperactivation of the epithelial PI3K pathway may contribute towards the poor prognosis of patients with CRC with highly stromal tumors.

### In addition to mutation- and microenvironment-driven epithelial signaling, we discovered polarity in fibroblast and macrophage cell–cell communication. For example, macrophage signaling pathways

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Fig. 6 | Oncogenic mutations mimic stromal signaling networks. a, Scaffold maps constructed from WT organoids either alone or cocultured with colonic fibroblasts and/or macrophages. b, PTM-EMDs for PI3K/PKC and P38/MAPK signaling nodes in colonic organoids following genotypic and microenvironmental regulation.

Direct comparison of organoid PTMs revealed that both PI3K/PKC and P38/MAPK nodes are analogously upregulated by oncogenic mutations and microenvironmental cues (Figs. 5d and 6b).

Taken together, TOBis-multiplexed cell-type-specific PTM analysis of organoid cocultures elucidated several fundamental processes in CRC: (1) oncogenic mutations restructure signaling networks in cancer cells, whereas microenvironmental cues drive acute signaling flux; (2) stromal cells hyper-activate PI3K signaling in colonic epithelial cells that already carry Kras and Trp53 mutations; and (3) oncogenic mutations cell-autonomously mimic an epithelial signaling state normally induced by stromal cells.

Discussion

Organoids are heterocellular systems comprising multiple cell types and cell states. Cell-type-specific PTM signaling networks regulate major biological processes and are frequently dysregulated in disease. As a result, understanding cell-type-specific signaling networks is fundamental to the utility of organoids and organoid cocultures. We demonstrated how a modified MC workflow that combines monospecific cell type, cell type and PTM probes can be used to study cell-type-specific signaling networks in organoids. TOBis enables high-throughput comparison of signaling networks across different organoid mono- and cocultures.

While this study has focused on intestinal organoids, we expect this method to be fully compatible with organoids derived from other tissues. Cell-type identification probes for each tissue should be carefully validated, but otherwise the TOBis multiplexing and PTM analysis framework should be compatible with all organoid models (including those grown in defined hydrogels\(^3\)). Moreover, our extension of MC to study colonic fibroblasts and macrophages implies that PTM signaling can be measured in any cell type cocultured with organoids (for example, peripheral blood mononuclear cells cocultured with organoids\(^1\) and air–liquid interface TME organoids\(^8\)).

TOBis MC holds substantial promise for organoid screening. While drug screens of patient-derived organoid monocultures have shown great potential\(^3\,4\), their reliance on bulk viability measurements means that they cannot be used to evaluate drugs targeting stromal and immune cells or provide any mechanistic understanding of drug performance and resistance. In contrast, a TOBis-multiplexed MC screen will provide cell-type-specific signaling networks, cell cycle states and apoptotic readouts across all cell types in patient-derived organoid cocultures. These features make TOBis MC a powerful tool for evaluating drug and CRISPR perturbations and anti-organoid biological therapies (for example, CAR T cells). Future development of TOBis using additional TeMal and cisplatin isotopologs will expand organoid multiplexing capacity and advance this technology to high-throughput organoid screening applications.

In summary, this study demonstrates how a modified MC platform can reveal cell-type-specific signaling networks in organoid monocultures and uncover cell–cell signaling relationships in...
organoid cocultures. Given the widespread adoption of organoids as biomimetic models of healthy and diseased tissue, we propose cell-type-specific PTM MC analysis as a powerful technology for multivariate organoid phenotyping.

**Online content**
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Organoid culture. Intestinal organoids were generated as described by Sato et al.13. Organoid culture.

Fixation. As dissociation of live tissue alters cellular states41 including PTMs8, all organoids were fixed in 4% paraformaldehyde (PFA) (Thermo J19943K2) for specific antibody panel. Following fixation, organoids were washed twice with PBS supplemented with 2 mM EDTA (Thermo 28906) for 10 min, washed in CSB and incubated in DNA Intercalator (Fluidigm 201309) for 10 min. Fixed single organoid cells (1–5 × 106) were resuspended in 5 ml of TrypLE Express Enzyme and incubated at 37 °C for 40 min on a rocker to stain dead cells15. During optimization we found that this condition yields high recovery, and treated with combinations of 3 μM CHIR99021 (GSK-3β inhibitor) (Cambridge Bioscience SM39), 2 μM IWP-2 (PORCN inhibitor) (Cambridge Bioscience 13033), 1 mM valproic acid (HDAC inhibitor) (Cambridge Bioscience SM39) and 10 μM DAPT (γ-Secretase inhibitor) (Cambridge Bioscience SM15) to directly target organoid differentiation pathways in specific cell types as described by Yin et al. (Supplementary Fig. 1). Directed-differentiated organoids were analyzed by IF and MC as described in "MC analysis of single organoid cells" and "Immunofluorescence (IF) staining of organoids."

Small intestinal organoid directed differentiation. Small intestinal organoids were seeded and cultured in complete organoid medium for 24 h to allow organoid recovery, and treated with combinations of 3 μM CHIR99021 (GSK-3β inhibitor) (Cambridge Bioscience SM39), 2 μM IWP-2 (PORCN inhibitor) (Cambridge Bioscience 13033), 1 mM valproic acid (HDAC inhibitor) (Cambridge Bioscience SM39) and 10 μM DAPT (γ-Secretase inhibitor) (Cambridge Bioscience SM15) to directly target organoid differentiation pathways in specific cell types as described by Yin et al. (Supplementary Fig. 1). Directed-differentiated organoids were analyzed by IF and MC as described in "MC analysis of single organoid cells" and "Immunofluorescence (IF) staining of organoids."

Single-cell dissociation of murine intestinal crypts. The small intestine of 8–12-week-old Lgr5-EGFP-tres-CreERT2 mice was dissected and intestinal crypts were isolated as described above (see Organoid culture section). The crypts were resuspended in 5 ml of TrypLE Express Enzyme and incubated at 37 °C for 45 min, and mixed every 10 min to avoid cells clumping. The cells were centrifuged at 1,200 r.p.m. for 5 min, resuspended in 5 ml of 4% PFA and fixed at 37 °C for 1 h. For flow cytometry, cells were washed once with PBS, 15 μM filtered twice to remove residual clumps and stored at 4 °C before MC analysis.

Amine- versus thiol-reactive in situ organoid probes. To investigate alternative probe chemistries for in situ organoid barcoding, fixed small intestinal organoids were stained with either 50 nM Alexa Fluor 647 NHS ester (Thermo A20006) or 50 nM Alexa Fluor 647 C6 maleimide (Thermo A20347) for 1 h while still in Matrigel. Organoid probe intensities were visualized by confocal microscopy using identical settings for each probe. To confirm IF observations using MC, fixed small intestinal organoids were stained with either 200 nM NHS-DOTA (Macrocyclics B-280) or 200 nM maleimide-DOTA (Macrocyclics B-272) coupled to 10 μM Te6 (Tables 1 and 2) for 1 h in situ on Matrigel or ex situ on microfluidic chips. Organoids were then dissociated into single cells and analyzed by MC.

TOBIs. Fixed organoids were washed twice in PBS and stained in situ with 10 nM cisplatin (196Pt, 198Pt) (a kind gift from Olga Ornatsky, Fluidigm) and 1–3 μM Te6 (10 μM Te6, 10 μM Te6, 10 μM Te6) barcodes for 1 h on a rocker (barcoding matrices in

Metals from Fluidigm (Fluidigm 201300). Non-Fluidigm metals or nitrates were also used: 198Pt (Sigma 217239), 199In (Trace Sciences), 199In (Trace Sciences), 195Gd (Trace Sciences) and 209Bi (Sigma 254150). Antibody panels (Supplementary Tables 1 and 2) were carefully designed and titrated in accord with known monoisotopic impurities48 and antigen abundance to ensure minimal cross-channel contamination.

MC analysis of single organoid cells. Fixed single organoid cells (1–5 × 106) were blocked in organoid-specific extracellular rare-earth metal antibody cocktails (Supplementary Tables 1 and 2) for 30 min. Cells were then washed twice with CSB (5 min, 800g) and permeabilized in 0.1% Triton X-100 (Sigma T8787) in PBS for 30 min. Cells were washed twice in CSB and further permeabilized with ice-cold 30% methanol (Fisher 10675112) for 10 min on ice. (Note: during method optimization we found that dual 0.1% Triton X-100 and 30% methanol immersion provides the best all-round permeabilization for the organoid PTM antibodies.) Permeabilized cells were then washed twice in CSB and stained with intracellular rare-earth metal antibody cocktails (Supplementary Tables 1 and 2) for 30 min. Stained cells were washed twice in CSB, fixed in fresh 1.6% formaldehyde (Thermo 28906) for 10 min, washed in CSB and incubated in DNA Intercalator (Fluidigm 201192A) overnight at 4 °C. The following day, cells were washed twice in CSB and resuspended in Maxpar Water (Fluidigm 201069) containing 20% (v/v) EQ beads46 (Fluidigm 201078) and 2 mM EDTA at ~0.5 × 106 cells per ml. Cells were then 35 μM filtered (Fisher 10585801) (70-μm Fisher 11957522) when the culture contains fibroblasts) and immediately analyzed using a Helios Mass Cytometer (Fluidigm) (100–300 events per second). Files were normalized against EQ beads, de-barcoded42 into each experimental condition (when required) and uploaded to the CYTObank platform (http://www.cytobank.org/).
Supplementary Tables 3 and 4). (Note, organoids can also be barcoded overnight at 4 °C.) The lower concentration of cisplatin used for TOB (10 nM) relative to dead cell stains (250 nM) is to ensure that 110Pd and 111In signals intensities align with 110Pd, 10B, and 10C signals across a ratio of organoids (as performed in 6–7.5 ml of barcodes), 12–well (2 ml of barcodes), 24–well (1 ml of barcodes), 48–well (500 µl of barcodes) and 96–well (200 µl of barcodes) plates. In our experience, up to 10,000 cells per µl of Matrigel (representing confluent intestinal organoid cultures) can be efficiently barcoded, but we suggest optimizing barcoding concentrations for alternative model systems. Organoids were washed twice in PBS and resuspended in Matrigel before seeding. The cell pellet enriched with single crypts was washed with cold PBS, collected using a benchtop centrifuge and resuspended in Matrigel before seeding. The cell ratio spaced for 1 day, 2, 3, 4, 5, 6 and 7 was 9:3:6:5:4:3:3, respectively, to ensure comparable organoid recovery and density from each time point.

At each time point, the organoids were incubated with 25 µM 111In and protease/phosphatase inhibitors, and fixed in 4% PFA for 60 min at 37 °C as described above. Organoids were washed with PBS and stored at 4°C until samples from all time points were collected. All cells were barcoded with 110Pd, 111In, TOB1 barcoded (Supplementary Table 3), stained and analyzed in one MC experiment (Supplementary Table 1; 50 parameters (40 antibodies) per cell).

Colonial fibroblast isolation, immortalization and cell culture. Colonial fibroblasts were isolated by Khalid et al. [36]. Freshly dissected murine (C57BL/6, 6–8-week-old) colon tissue was flushed with ice-cold PBS, cut open, washed again in PBS and incubated in 5 ml EDTA/PBS at 250 rpm, 37 °C for 15 min. This process was repeated for a total of five EDTA/PBS washes. Washed colon tissue was transferred to a fresh tube of sterile DMEM (Thermo 41966052) supplemented with 1 mM 110Pd (Suppl Fig. 17) and 1 mg/ml Penicillin/Streptomycin (Sigma P5722) incubated at 250 rpm, 37 °C for 30–60 min (until the tissue started to look ‘stringy’). Digested colon tissue was then centrifuged at 200 g, 4 °C for 5 min. Supernatant was discarded and the pellet was resuspended in 10 ml of ACK Lysing Buffer (Thermo A1049201). Cells were centrifuged at 200 g, 4 °C for 5 min, and the pellet was resuspended in DMEM + 10% FBS (Hyclone, I1001) and 10 nM 194cisplatin as described above. Organoids were barcoded in Phoenix–ECO cells (a kind gift from Erik Sahai, The Francis Crick Institute, London) and stably transfected with RFP using the pCMV-DsRed-Express plasmid with Lipofectamine 3000 (Thermo L3000001) to aid coculture visualization. Immortalized colonial fibroblasts were cultured in DMEM + 10% FBS + 1x Insulin–Transferrin–Selenium (ITS)–G (Thermo 4140045). After 1 week of culture, fibroblasts were observed to proliferate, while other cell types (e.g., macrophages, neutrophils) were not. Colonial fibroblasts were immortalized using pBABE-HPV-E6 retrovirus produced in Phoenix–ECO cells (a kind gift from Erik Sahai, The Francis Crick Institute, London) and stably transfected with RFP using the pCMV-DsRed-Express plasmid with Lipofectamine 3000 (Thermo L3000001) to aid coculture visualization. Immortalized colonial fibroblasts were cultured in DMEM + 10% FBS + 1x ITS–G at 37 °C. Cell wells were incubated in the MycoAlert PLUS Mycoplasma Detection Kit (Lonza LT07-701) and remained negative throughout this project. IF staining confirmed that colonial fibroblasts were positive for intestinal mesenchymal markers such as CD45 (30-F11, Biologic), PD68 (PA5-12B5, Affymetrix), CD68 (FA-11, Biologic), CD11b (M1/70, Biologic), F4/80 (BM8, Biologic) and CX3CR1 (SA011F11, Biologic) in both 2D and 3D cultures.

Primary macrophage isolation and cell culture. Freshly dissected murine (C57BL/6, 12–12-week-old females) femurs and tibias (Charles River Laboratories) were flushed five times with 10 ml of RPMI 1640 Medium (Thermo 11875165) and 10% FBS (Thermo 10082147). Cells were centrifuged (300 g, 4 °C for 5 min), resuspended in RPMI + 10% FBS, 40 µm filtered (Fisher 11587522) and centrifuged at 400 g for 5 min. Supernatant was discarded and the pellet was resuspended in 2 ml of ACK Lysing Buffer (Thermo A1049201) for 5 min at room temperature. Monocytes were washed in PBS, centrifuged at 400 g for 5 min, resuspended in 1 ml of Recovery Cell Culture Freezing Medium (Thermo 12648010) and stored in liquid nitrogen until use. Bone marrow–derived macrophages were expanded and activated in RPMI + 10% FBS + 25% L929–cell conditioned medium before experiments. IF staining confirmed that the cells were positive for intestinal macrophage markers such as CD45 (30-F11, Biologic), CD68 (FA-11, Biologic), CD11b (M1/70, Biologic), F4/80 (BM8, Biologic) and CX3CR1 (SA011F11, Biologic) in both 2D and 3D cultures.

Heterocellular CRC TME organoid culture. WT murine colon organoids and CRC organoids carrying oncogenic mutations A, AK and AKP were a kind gift from Lukas Dow (Cornell University) and cultured as described above. Following expansion in complete media, organoids were cultured in the absence of exogenous growth factors (mEGF, mNoggin, mR-Spondin-1 and mWNT-3a (WNR)) for 8 h before the experiment. Colonial fibroblasts were cultured in DMEM supplemented with reduced FBS (2%) and 1x ITS–G for 24 h before the experiment. Primary bone marrows were differentiated into macrophages using RPMI + 10% FBS + 25% L929–cell conditioned medium for 7 days before the experiment. To establish the TME culture, organoids were cocultured with primary macrophages at a ratio of ~1:2.5; colonial fibroblasts were seeded at 6,000 cells per ml, 5,000 cells per µl and 4,000 cells per µl Matrigel for monoculture, two-way cocultures and three-way cocultures, respectively; and primary macrophages were seeded at 9,000 cells per ml, 8,000 cells per µl and 7,000 cells per µl Matrigel for monoculture, two-way cocultures and three-way cocultures, respectively. Organoids, fibroblasts and macrophages were mixed in Matrigel at 250 nM 111In as described above. Organoids were barcoded using TOB1s (Supplementary Table 4), pooled into a single tube, dissociated into
single cells, 70-μm filtered and stained for MC analysis (Supplementary Table 2; 50 parameters (40 antibodies) per cell). Single organoid, fibroblast and macrophage cells were analyzed by MC.

**Single-cell signaling data analysis.** All single cells were gated for Gaussian parameters (event length, center, residual and width values), DNA45 (45Ig and 45IIc) and cisplatin42 (42Pt). For small intestinal organoids and colonic organoids, intact epithelial cells were gated with EpCAM+/Pan-CCK41 and CEACAM1+/Pan-CCK41, respectively. Intact colonic fibroblasts were gated with RFP+/PDCA125, and primary macrophages were gated with CD68+/F4/8040. Removal of cells stained positively for mutually exclusive cell-type and cell-state markers was performed as part of data preprocessing procedures (gating strategies incorporated in the publicly deposited datasets). Cells were then clustered and visualized in UMAP45 space and gated for cell-type and cell-state makers before proceeding to PTM signaling analysis (Supplementary Fig. 2).

UMAP analysis was performed with the Python package umap (https://umap-learn.readthedocs.io/en/latest) using default parameters unless otherwise specified (Supplementary Table 5). UMAP was used to visualize high-dimensional MC datasets in 2D space, where cell gating was performed to identify cell populations or to remove residual outliers when required. All data were arcsinh transformed with a cofactor of 5.

EMD was computed with the Python package scprep (ref. 13) using default parameters. EMD scores were signed by the difference of the median intensity of a given parameter between the populations to the denominator (space below) relative to the numerator (space above) for up-regulation or negative for down-regulation. Cell populations were manually gated and exported from Cytobank, with all channels to be analyzed arcsinh transformed (cofactor = 5). For single-time-point small intestinal organoids (Fig. 2b and Supplementary Fig. 3), EMD was calculated between each cell type and state and the entire epithelial cell population. For the small intestinal immune-time-course experiment (Fig. 4c), EMD was calculated between each cell type from each time point against the combined population of all epithelial cells across all time points. For the CRC TME model (Figs. 5d, e and 6b and Supplementary Figs. 7 and 8a–d), EMD was calculated between each cell type in each condition and the combined population of all cell types across all conditions.

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**Author contributions**

X.Q. designed the study, performed organoid and MC experiments, analyzed the data and wrote the paper. J.S. developed TOB, designed rare-earth metal antibody panels, performed MC analysis and analyzed data. P.K. performed UMAP, DREMI and PCA data analysis. M.N. developed TeMal barcodes. S.E.A. provided murine monococytes and intestines for fibroblast isolation. V.S.W.L. provided murine small intestines for organoid isolation. C.J.T. designed the study, analyzed the data and wrote the paper.

**Competition interests**

M.N. has pending intellectual property on the use of tellurium reagents for mass cytometry applications which has been licensed to Fluidigm Corporation.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41592-020-0737-8.

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**Peer review information**

Nina Vogt and Nicole Rusk were the primary editors on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | Mass cytometry data in this study was collected with a Helios mass cytometer using the Fluidigm CyTOF Software (Version 6.7).

Data analysis | Data analysis was performed with Cytobank (Version 7.2.0) and publicly available R and python packages. Specifically:

Python 3.6 with packages:
- umap 0.3.9
- scprep 0.12.2
- scikit-learn 0.21.1

R 3.5.2 with packages:
- scaffold 0.1
- ggplot2 3.0.0
- RColorBrewer 1.1-2

Statistical tests were performed using Graphpad Prism (Version 7.0).
Immunofluorescence staining images were processed using Fiji (Version 2.0.0).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
No sample size calculation was performed as this is a method development and proof-of-concept project. Sample size was determined based on the experience and expertise of the investigators.

Data exclusions
All cells were gated for Gaussian discrimination parameters [Event length, Centre, Residual, and Width values] as recommended by Fluidigm to remove non-events such as debris and doublets. The cell gating strategy was developed for this study and described in Supplementary Figure 2.

Replication
Multiple cohorts of wild-type mice were used to derive organoids in this project, yielding comparable results and thereby validated the robustness of our methods. Details on technical/biological replications of experiments are included in relevant figure legends.

Randomization
Animals were randomly chosen for inclusion in this study.

Blinding
No conditions presented in this study required blinding. All mice used were wild-type colonies maintained under standard housing conditions.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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Methods

n/a Involved in the study
☐ ChiP-seq
☐ Flow cytometry
☐ MRI-based neuroimaging

Antibodies

Antibodies used
All antibody information, including clone name and supplier, is provided in Supplementary Tables 1 and 2.

Validation
Cell-type identification antibodies are validated as demonstrated in Supplementary Figures 1 and 2.
PTM antibodies selected for this study are widely used by the mass cytometry community and well-validated. Antibody panels were carefully designed and titrated in accordance with known monoisotopic impurities and antigen abundance to ensure minimal cross-channel contamination.
## Eukaryotic cell lines

Policy information about [cell lines](#)

| Cell line source(s) | - Murine colorectal cancer organoids carrying oncogenic mutations (shApc [A], shApc and KrasG12D/+ [AK], or shApc, KrasG12D/+, and Trp53R172H/- [AKP]) were a kind gift from Prof. Lukas Dow (Cornell University).
| | - Phoenix-ECO cells used for retrovirus production were a kind gift from Prof. Erik Sahai (The Francis Crick Institute). |

**Authentication**

Cell lines used in this study have not been authenticated during the development of the project.

**Mycoplasma contamination**

Cells were checked for mycoplasma infection monthly using the MycoAlertTM PLUS Mycoplasma Detection Kit [Lonza LT07-701] and remained negative throughout this project.

**Commonly misidentified lines (See [ITAG register](#))**

No commonly misidentified cell lines were used in this study.

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## Animals and other organisms

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| Laboratory animals | - Murine small intestinal organoids were derived from 8- to 12-week-old Lgr5-EGFP-ires-CreERT2 mice provided by Dr. Vivian Li (The Francis Crick Institute).
| | - Murine bone marrow-derived macrophages were isolated from 10- to 12-week-old C57BL/6 mice provided by Dr. Sophie Acton (University College London).
| | - Murine colonic fibroblasts were isolated and immortalised as described in online Methods. |

| Wild animals | This study did not involve the use of wild animals. |

| Field-collected samples | This study did not involve the use of field-collected samples. |

| Ethics oversight | All animal work carried out was approved by local ethical review and licensed by the UK Home Office. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.