Cell-Type Specific Signalling Networks in Heterocellular Organoids

Xiao Qin¹, Jahangir Sufi¹$, Petra Vlckova¹$, Pelagia Kyriakidou¹$, Sophie E. Acton², Vivian S. W. Li³, Mark Nitz⁴, Christopher J. Tape¹*

¹ Cell Communication Lab, Department of Oncology, University College London Cancer Institute, London, UK.
² Stromal Immunology Lab, MRC Laboratory for Molecular Cell Biology, University College London, London, UK.
³ Stem Cell and Cancer Biology Lab, The Francis Crick Institute, London, UK.
⁴ Department of Chemistry, University of Toronto, Toronto, Canada.

$These authors contributed equally to this work.
*Correspondence: c.tape@ucl.ac.uk
ABSTRACT
Organoids are powerful biomimetic tissue models. Despite their widespread adoption, methods to analyse cell-type specific post-translational modification (PTM) signalling networks in organoids are absent. Here we report multivariate single-cell analysis of cell-type specific signalling networks in organoids and organoid co-cultures. Simultaneous measurement of 28 PTMs in >1 million single small intestinal organoid cells by mass cytometry reveals cell-type and cell-state specific signalling networks in stem, Paneth, enteroendocrine, tuft, goblet cells, and enterocytes. Integrating single-cell PTM analysis with Thiol-reactive Organoid Barcoding in situ (TOBis) enables high-throughput comparison of signalling networks between organoid cultures. Multivariate cell-type specific PTM analysis of colorectal cancer tumour microenvironment organoids reveals that shApc, Kras$^{G12D}$, and Trp53$^{R172H}$ cell-autonomously mimic signalling 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 signalling analysis of healthy and cancerous organoids.

INTRODUCTION
Organoids are self-organising 3D tissue models comprising stem and differentiated cells. Organoid monocultures typically contain one major cell class (e.g. epithelial) and can be co-cultured with heterotypic cell-types (e.g. mesenchymal or immune cells) to model cell-cell interactions in vitro. When compared with traditional 2D cell culture, organoids more accurately represent their parental tissue and are emerging as powerful models for studying multicellular diseases such as cancer.
Post-translational modification (PTM) signalling networks underpin fundamental biological phenotypes and are frequently dysregulated in disease \(^5\). As different cell-types have different signalling networks \(^6,^7\), organoids likely contain several cell-type specific PTM networks that are essential to their biology. In order to fully utilise biomimetic models of healthy and diseased tissue, we must be able to study PTM signalling networks within organoids. Unfortunately, no technology currently exists to analyse cell-type specific PTM networks in organoids and organoid co-cultures. Organoids present several technical challenges over traditional 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 (LC-MS/MS). Organoids can be removed from ECM prior to LC-MS/MS, but as dissociation of live cells alters cell signalling \(^8\), PTM measurements from dissociated live organoids do not truly represent \textit{in situ} cellular states. Ideally, organoids should be fixed \textit{in situ} to preserve PTM signalling, but LC-MS/MS analysis of heavily cross-linked phosphoproteomes is extremely challenging. Secondly, as organoids comprise multiple cell-types (e.g. stem and differentiated) and cell-states (e.g. proliferating, quiescent, and apoptotic), bulk phosphoproteomics cannot capture the biological heterogeneity present in organoids and organoid co-cultures \(^9\). Although single-cell RNA-sequencing (scRNA-seq) can describe organoid cell-types \(^10\), it cannot measure intracellular PTM signalling at the protein level. Finally, as signalling networks comprise multiple PTM nodes, low-dimensional methods (e.g. fluorescent imaging) cannot capture the complexity of PTM signalling networks \(^9\). Collectively, to study PTM networks in organoids, we
require signalling data that is: 1) cell-type specific, 2) derived from cells fixed in situ, and 3) measures multiple PTMs simultaneously.

Mass cytometry (MC, also known as cytometry time-of-flight (CyTOF)) uses heavy metal-conjugated antibodies to measure >35 proteins in single cells¹¹. Although MC is traditionally used for high-dimensional immunophenotyping, MC can also measure PTMs in heterocellular systems (e.g. peripheral blood mononuclear cells (PBMCs)¹² and tissue³). Given MC’s capacity to measure PTMs in mixtures of fixed cells, we theorised that MC workflows typically applied to immunophenotyping could be modified to study cell-type specific signalling networks in organoids.

Here we report the development of a custom multivariate-barcoded MC method to measure single-cell signalling in epithelial organoids and organoids co-cultured with stromal and immune cells. This method reveals that intestinal organoids display cell-type specific signalling networks that are intimately linked with cell-state. When applied to colorectal cancer (CRC) tumour microenvironment (TME) organoid co-cultures, we discovered that epithelial oncogenic mutations mimic signalling networks normally induced by stromal cells. These results demonstrate how a modified MC method can enable powerful multivariate single-cell analysis of cell-type specific signalling in heterocellular organoids.

RESULTS

Single-Cell Analysis of Organoids by Mass Cytometry

No technology currently exists to study cell-type specific protein signalling networks in organoids. Given its capacity to measure multiple PTMs in single cells, we hypothesised MC could be modified to study cell-type and cell-state specific
signalling in organoids. To test this, we first developed a MC platform to measure single-cell signalling in the classical small intestinal organoid\textsuperscript{13}.

In this method, we first pulse live organoids with \textsuperscript{127}I-5-lodo-2’-deoxyuridine (\textsuperscript{127}IdU) to identify S-phase cells\textsuperscript{14}, fix organoids in Matrigel to preserve cell signalling, and stain organoids with \textsuperscript{194/8}Cisplatin to label dead epithelia\textsuperscript{15}. Using a custom workflow, we then physically and enzymatically dissociate the fixed organoids into single cells prior to extra- and intracellular heavy-metal antibody staining (Fig. 1a). We next performed a comprehensive screen for intestinal epithelial cell-type identification antibodies including stem (LGR5, LRIG1, OLFM4), Paneth (Lysozyme), goblet (MUC2, CLCA1), enteroendocrine (CHGA, Synaptophysin), tuft cells (DCAMKL1), and enterocytes (FABP1, Na/K-ATPase) that bind fixed antigens and are compatible with rare-earth metal conjugation for MC. Cell-type identification antibodies were validated by organoid directed differentiation\textsuperscript{16} (Supplementary Fig. 1) and integrated into a panel of 28 anti-PTM rare-earth metal antibodies spanning multiple core signalling nodes (Supplementary Table 1, 45 parameters (40 antibodies) / cell). When analysed by MC, this method enables the measurement of 28 signalling PTMs across 6 cell-types in >1 million single cells from fixed intestinal organoids (Figs. 1b, c, 2a, and Supplementary Fig. 2a).

Combining \textsuperscript{194/8}Cisplatin and \textsuperscript{127}IdU with canonical cell-cycle markers (e.g. pRB [S807/S811], Cyclin B1, and pHistone H3 [S28]\textsuperscript{14}) allows clear identification of live / dead cells and classification of single organoid cells into cell-cycle stages including G0, G1, S, G2, and M-phase\textsuperscript{17} (Supplementary Fig. 2b). Integrated cell-type and cell-state data from small intestinal organoids confirmed that stem and Paneth cells are largely proliferative (pRB\textsuperscript{*}, cCaspase3 [D175\textsuperscript{*}]), whereas differentiated epithelia are
often post-mitotic (pRB\(^{-}\), IdU\(^{-}\) / pH3\(^{-}\)) or apoptotic (cCaspase3\(^{+}\)) (Fig. 1c). Consistent with the finding that intestinal progenitor cells have permissive chromatin *in vivo*\(^{18}\), proliferating intestinal organoid cells also present H3K4me2 whereas post-mitotic cells do not (Fig. 2a). These results confirmed that a modified MC workflow can provide cell-type and cell-state specific information from millions of single organoid cells.

**Cell-Type and Cell-State Specific Signalling Networks in Intestinal Organoids**

Following cell-type and cell-state identification, we next sought to construct cell-type specific PTM signalling networks in small intestinal organoids. To investigate whether stem, Paneth, enteroendocrine, tuft, goblet cells, and enterocytes employ different PTM signalling networks, we combined Earth Mover’s Distance (EMD)\(^{19, 20}\) and Density Resampled Estimation of Mutual Information (DREMI)\(^{21}\) to build quantitative cell-type specific signalling networks from single-cell organoid PTM data (Fig. 2b and Supplementary Fig. 2). 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) within the network.

EMD-DREMI analysis revealed cell-type specific PTM signalling networks in small intestinal organoids. As canonical WNT signalling is mainly driven by protein interactions, localisation, and degradation\(^{22}\) – not a classical PTM cascade – MC is not well suited to studying the WNT pathway. Despite this limitation, evidence of WNT flux via inhibited pGSK-3\(\beta\) [S9] and non-phosphorylated \(\beta\)-Catenin is observed in all organoid cell-types (Fig. 2a). In contrast, MAPK and PI3K pathways display unexpected cell-type specificity. For example, stem cells channel MAPK signalling
through pERK1/2 [T202/Y204], pP90RSK [T359], and pCREB [S133], but fail to connect with pBAD [S112] (Fig. 2a, b). On the contrary, differentiated epithelia direct MAPK signalling away from pCREB and towards pBAD when proliferating, and lose all MAPK activity in G0 and apoptosis (Fig. 2a). Despite their strong mitogenic signalling profile, stem cells are unique among proliferating cells in their failure to phosphorylate BAD. This suggests that intestinal stem cells avoid apoptosis independent of the classical BAD-BCL-BAX/BAK axis and may compensate via high MAPK and P38 flux to CREB. Consistent with the observation that PI3K signalling is important for intestinal crypt cells, stem and Paneth cells are enriched for pSRC [Y418] and downstream PI3K effectors such as pPDPK1 [S241], pPKCα [T497], pAKT [T308]/[S473], and p4E-BP1 [T37/T46] (Fig. 2a, b). Despite the presence of the BMP inhibitor Noggin in organoid culture media, Paneth cells display unexpectedly high BMP signalling (via pSMAD1/5 [S463/S465] and SMAD9 [S465/S467]) (Fig. 2a, b). This observation suggests that Paneth cells are either hypersensitive to BMP ligands or can cell-intrinsically activate SMAD1/5/9 (possibly via inhibition of SMAD phosphatases).

Several PTM signalling events correlate with cell-state in intestinal organoids. For example, irrespective of cell-type or location, pP38 MAPK [T180/Y182] and pP120-Catenin [T310] are active in all proliferating cells, and both pAKT [T308] and pMKK4 [S257] are hyperactivated in M-phase (Figs. 1c and 2a). In contrast, TGF-β signalling (via pSMAD2 [S465/S467] and SMAD3 [S423/S425]) is exclusively active in post-mitotic epithelia (Fig. 2a), consistent with TGF-β’s role in epithelial growth-arrest.

To investigate the relationship between cell-type and cell-state in PTM signalling networks, we performed principal component analysis (PCA) of PTM-EMDs for each
organoid cell-type, either proliferating or in G0 (pRB<sup>+/−</sup>), located in lower-crypts or villi (CD44<sup>+/−</sup>). PCA revealed that both cell-state (PC1, 68% variance) and, to a lesser extent, cell-type (PC2, 23% variance) dictate cell-signalling in small intestinal organoids (Fig. 2c and Supplementary Fig. 3). This analysis demonstrates that both cell-type and cell-state are intimately linked with cell-signalling and warns against bulk PTM analysis of organoids where cell-type and cell-state resolution is lost. Collectively, these results confirmed that MC can identify novel cell-type and cell-state specific signalling networks in small intestinal organoids and underscore the importance of single-cell data when studying heterogenous systems such as organoids.

Single-Cell Organoid Multiplexing using Thiol-reactive Organoid Barcoding in situ (TOBis)

We have demonstrated how a modified MC platform can be applied to cell-type and cell-state specific signalling measurement in organoids. However, in order to study differential signal transduction in organoid models of healthy and diseased tissue, we must also be able to directly compare PTM networks between different organoid cultures. In addition to high dimensional single-cell PTM measurements, a major advantage of MC is its ability to perform multiplexed barcoding of experimental variables<sup>25, 26</sup>. Unfortunately, commercially available Palladium-based barcodes cannot bind organoids in situ (Supplementary Fig. 4a) as they react with Matrigel proteins (Supplementary Fig. 4b), meaning that organoids must be removed from Matrigel and dissociated separately before barcoding. Individually removing fixed organoids from Matrigel is a very low-throughput process that limits the scalability of
organoid MC multiplexing. We theorised that if organoids could be barcoded in situ, barcoded organoids could be pooled, dissociated, and processed as a single high-throughput MC sample. To explore this idea, we developed a new strategy to isotopically barcode organoids while still in Matrigel.

MC barcoding strategies can use amine- or thiol-reactive chemistries. We first used fluorescent probes to investigate how each of these chemistries reacts with ECM proteins and organoids. Amine-reactive probes (Alexa Fluor 647 NHS ester) bind ECM proteins (via lysines and N-terminal amines) and thus fail to label organoids in Matrigel. In contrast, thiol-reactive probes (Alexa Fluor 647 C₂ maleimide) bypass ECM proteins and bind exclusively to reduced-cysteines on organoids in situ (Fig. 3a, Supplementary Fig. 4c). We subsequently confirmed that thiol-reactive monoisotopic mass-tagged probes (C₂ maleimide-DOTA-¹⁵⁷Gd) also bind organoids in situ, whereas amine-reactive probes (NHS ester-DOTA-¹⁵⁷Gd) only react ex situ (Fig. 3b). This data confirmed that thiol-reactive chemistries can be used to barcode organoids while still in Matrigel (Fig. 3c). Using this knowledge, we developed a custom 20-plex (6-choose-3, doublet-filtering²⁵, ²⁶) thiol-reactive barcoding strategy based on monoisotopic tellurium maleimide (TeMal) (¹²⁴Te, ¹²⁶Te, ¹²⁸Te, ¹³⁰Te)²⁷ and Cisplatin (¹⁹⁵Pt, ¹⁹⁸Pt)²⁸ that can bypass ECM proteins and bind directly to fixed organoids in situ (Fig. 3d and Supplementary Fig. 4d). This Thiol-reactive Organoid Barcoding in situ (TOBis) approach enables high-throughput multivariate single-cell organoid signalling analysis in a single tube (Fig. 3e).

It is worth noting that as Te and Pt metals are not typically conjugated to antibodies in MC, TOBis multiplexing does not compromise the number of antigens being measured. Moreover, unlike commercially available Pd barcodes (occupying ¹⁰²Pd,
$^{104}Pd$, $^{105}Pd$, $^{106}Pd$, $^{108}Pd$, and $^{110}Pd$ channels), TOB’s barcodes do not clash with the recently developed Cadmium antibody labelling metals ($^{106}Cd$, $^{110}Cd$, $^{111}Cd$, $^{112}Cd$, $^{113}Cd$, $^{114}Cd$, and $^{116}Cd$). Furthermore, as barcoding is performed on fixed organoids embedded within Matrigel, TOB’s does not require the numerous centrifugation or cell membrane permeabilisation steps used in traditional solution-phase barcoding. This greatly increases organoid sample-throughput (Supplementary Fig. 5a—d) and single-cell recovery (Supplementary Fig. 5e—g), thereby facilitating high-throughput organoid MC applications.

Multivariate Cell-Type Specific Signalling Analysis of Intestinal Organoid Development

Traditional mass-tag barcoding allows direct comparison of solution-phase cells (e.g. immune cells) between experimental conditions. When combined with cell-type, cell-state, and PTM probes, TOB’s multiplexing now enables PTM signalling networks to be directly compared between organoid cultures in a high-throughput manner. To demonstrate this, we applied TOB’s to study cell-type specific epithelial signalling during 7 days of small intestinal organoid development (Fig. 4 and Supplementary Table 1, 50 parameters (40 antibodies) / cell).

Analysis of 28 PTMs from ~2 million single organoid cells revealed that after 1 day of culture, organoids seeded as single crypts exist in a ‘recovery’ phase where ~70% cells have entered the cell-cycle ($pRB^+$), but <5% reach S-phase ($IdU^+$) (compared to ~20% in developed organoids) (Fig. 4a). Days 2 and 3 mark a rapid ‘expansion and differentiation’ phase of organoid development where stem, Paneth, goblet cells, and enterocytes activate MAPK, P38, and PI3K pathways – although stem cells again fail
to inhibit BAD (Fig. 4c). By Day 4, intestinal organoids reach a critical ‘divergence’
phase where crypt and villus signalling digress dramatically. While stem and Paneth
cells maintain active MAPK, P38, and PI3K pathways, enterocytes lose major PI3K
(pPDK1, p4E-BP1, pS6 [S235/S236], pAMPKα [T172], pSRC, and pPKCα) and P38
(pP38 MAPK, pMAPKAPK2 [T334], and pCREB) activity (Fig. 4c). As a result, by Days
5 to 7, enterocytes are largely post-mitotic or apoptotic (pRB⁻/ cCaspase3⁺), with
high TGF-β signalling, 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
signalling flux is a core feature of intestinal crypt cells. In contrast, tuft cells display
high TGF-β signalling, low MAPK / P38 / PI3K activity, and low cell-cycle activity
throughout organoid development (Fig. 4c). This implies that irrespective of organoid
age, tuft cells shut down mitotic signalling pathways and terminally exit the cell cycle
once differentiated. Such variations in organoid cell-state (Fig. 4a), cell-type (Fig. 4b),
and PTM activity (Fig. 4c) suggest developmental stage should be carefully
considered when performing organoid experiments. Collectively, this analysis
revealed cell-type specific PTM signalling during intestinal organoid development and
confirmed that TOBis can be used to perform multivariate single-cell signalling
analysis of heterogenous organoid cultures.
Single-Cell Signalling Analysis of CRC TME Organoids

We have demonstrated how a modified MC workflow enables high-throughput comparison of cell-type specific signalling networks in epithelial organoids. Given that MC can theoretically resolve any cell-type, we next expanded this platform to study PTM signalling in heterocellular organoid co-culture models of CRC.

CRC develops through successive oncogenic mutations – frequently resulting in loss of APC activity, activation of KRAS, and perturbation of TP53. In addition to oncogenic mutations, stromal fibroblasts and macrophages in the TME have also emerged as major drivers of CRC. While the underlying driver mutations of CRC have been well studied, how they dysregulate epithelial signalling relative to microenvironmental cues from stromal and immune cells is unclear.

To investigate this, we cultured wild-type (WT), shApc (A), shApc and Kras\(^{G12D/+}\) (AK), or shApc, Kras\(^{G12D/+}\), and Trp53\(^{R172H/−}\) (AKP) colonic epithelial organoids either alone, with colonic fibroblasts, and/or macrophages (Fig. 5a, b, and Supplementary Fig. 6). Each CRC genotype-microenvironment organoid culture was fixed, TOBis-barcoded, and single-cell signalling analysis was performed in one multivariate MC run (Fig. 5a and Supplementary Table 2, 50 parameters (40 antibodies) / cell). Addition of myeloid (CD68 and F4/80) and mesenchymal (Podoplanin) heavy-metal antibodies enabled clear 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 signalling networks in CRC organoid mono- and co-cultures.

As expected, oncogenic mutations have a large cell-autonomous effect on epithelial signalling. Although APC mutations are well known to upregulate WNT signalling,

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we found that the loss of APC also activates the P38 pathway (pP38 MAPK and
pMAPKAPK2), downregulates TGF-β / BMP signalling (pSMAD2/3 and pSMAD1/5/9),
and activates p120-Catenin in colonic organoids (Fig. 5d). Subsequent oncogenic
Kras$^{G12D/+}$ and Trp53$^{R172H/-}$ mutations further cell-autonomously upregulate not only the
classical MAPK pathway, but also major PI3K nodes (pPDK1, pAKT, pS6, and p4E-BP1) (Fig. 5d). As a result, AK and AKP organoids display increased stem / 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 signalling. However,
to what extent this is driven by oncogenic mutations (cell-intrinsic) or the TME (cell-
extrinsic) is less clear. To investigate this, we directly compared mutation- and
microenvironment-driven signalling in CRC organoids. To our surprise, we found that
microenvironmental cues have a comparable impact on PTM regulation to oncogenic
mutations (Fig. 5e). In contrast, while mutations and stromal cells can both drive
epithelial PTM activity, PTM-PTM connectivity is regulated largely by genotype, not
microenvironment (Fig. 5f). This observation suggests that oncogenic mutations
fundamentally re-wire signalling networks, whereas stromal cells regulate acute
signalling flux. We also found that stromal cells further upregulate the PI3K pathway
(pS6, p4E-BP1, and pAKT) in CRC organoids that already contain Kras$^{G12D}$ and
Trp53$^{R172H}$ mutations (Fig. 5d and Supplementary Fig. 7). Microenvironmental hyper-
activation of the epithelial PI3K pathway may contribute towards the poor prognosis
of CRC patients with highly stromal tumours.$^{30, 31}$
In addition to mutation- and microenvironment-driven epithelial signalling, we
discovered previously unreported polarity in fibroblast and macrophage cell-cell
communication. For example, macrophage signalling pathways (MAPK, PI3K, and NF-κB) are heavily upregulated by fibroblasts (Supplementary Fig. 8a, c, e), whereas fibroblast signalling is scarcely altered by macrophages (Supplementary Fig. 8b, d, f). In contrast, epithelial cells upregulate MAPK and P38 signalling in fibroblasts, which in turn, reciprocally activate MAPK and P38 signalling in epithelial cells (Supplementary Fig. 7 and 8b). These results suggest that colonic fibroblasts are major regulators of intercellular signalling in the colonic microenvironment and should be further investigated as drivers of CRC.

Oncogenic Mutations Mimic Stromal Signalling Networks

Cell-type specific PCA of EMD-PTMs suggested that mutation- and microenvironment-driven signalling in colonic organoids are related (Fig. 5e). To further investigate the parity between genotypic and microenvironmental regulation of epithelial signalling, we overlaid single-cell MC data from WT, A, AK, and AKP organoids onto a fixed-node microenvironmental Scaffold map\textsuperscript{36} constructed from WT colonic organoids alone or co-cultured with colonic fibroblasts and/or macrophages (Fig. 6a and Supplementary Fig. 9a). This unsupervised analysis confirmed that Apc, Kras, and Trp53 oncogenic mutations mimic the signalling profile of WT organoids in the presence of stromal cells. Inverted organoid genotype Scaffold maps also expose a striking similarity between mutation- and microenvironment-driven signalling (Supplementary Fig. 9b). Direct comparison of organoid PTMs revealed that both PI3K / PKC (pPDPK1, pPKCa, pAKT, p4E-BP1, pS6, pSRC, pP120-Catenin, and pAMPKα) and P38 / MAPK (pP38 MAPK, pMAPKAPK2, pP90RSK, pCREB, and pBAD) nodes are analogously upregulated by
oncogenic mutations and microenvironmental cues (Figs. 5d and 6b). Activation of these pathways by either oncogenic mutations or stromal cells correlates with decreased apoptosis and increased mitogenic cell-state in colonic organoids (Fig. 5d).

Taken together, multivariate cell-type specific PTM analysis of organoid co-cultures elucidated several fundamental processes in CRC: 1) oncogenic mutations re-structure signalling networks in cancer cells, whereas microenvironmental cues drive acute signalling flux, 2) stromal cells hyper-activate PI3K signalling in colonic epithelial cells that already carry Kras and Trp53 mutations, and 3) oncogenic mutations cell-autonomously mimic an epithelial signalling state normally induced by stromal cells. These results collectively confirmed that TOBis-multiplexed MC enables discoveries of novel cell-type specific signalling relationships between different cell-types in organoid models of the tumour microenvironment.

**DISCUSSION**

Organoids are heterocellular systems that comprise multiple cell-types and cell-states. Cell-type specific PTM signalling networks regulate major biological processes and are frequently dysregulated in disease. As a result, understanding cell-type specific signalling networks is fundamental to the utility of organoids and organoid co-cultures. Existing bulk PTM technologies (e.g. LC-MS/MS and anti-phospho antibody arrays) cannot describe cell-type or cell-state specific signalling relationships and therefore limit our understanding of organoid biology. While scRNA-seq can characterise cell-type specific transcription, it cannot measure protein-level signal transduction which ultimately drives biological phenotypes. To
overcome these challenges, we demonstrated how a modified MC workflow that combines monoisotopic cell-type, cell-state, and PTM probes can be used to study cell-type specific signalling networks in organoids. This method uncovered novel cell-type specific signalling in intestinal epithelia and revealed an intimate relationship between cell-signalling and cell-state in organoids. We showed how Thiol-reactive Organoid Barcoding in situ (TOBis) enables high-throughput comparison of signalling networks across different organoid mono- and co-cultures. Application of this technology to CRC TME organoid co-cultures revealed that oncogenic mutations mimic stromal signalling cues and demonstrated how highly mutated CRC cells can be further dysregulated by fibroblasts and macrophages.

While this study has focused on intestinal organoids, we expect this method to be fully compatible with organoids derived from other tissues (e.g. brain, liver, pancreas, kidney etc.). Cell-type identification probes for each tissue should be carefully validated, but otherwise the TOBis multiplexing and PTM analysis framework we report should be compatible with all organoid models (including those grown in defined hydrogels\textsuperscript{37}). Moreover, our extension of MC to study colonic fibroblasts and macrophages implies that PTM signalling can be measured in any cell-type co-cultured with organoids (e.g. PBMCs co-cultured with organoids\textsuperscript{3} and air-liquid interface tumour microenvironment organoids\textsuperscript{38}).

In addition to standard single-cell organoid signalling experiments, the new barcoding technology reported here holds substantial promise for organoid screening. While drug screens of patient-derived organoid (PDO) monocultures have shown great potential\textsuperscript{39,40}, their reliance on bulk viability measurements (e.g. CellTiter-Blue) implies that they cannot be used to evaluate drugs targeting stromal and/or
immune cells or provide any mechanistic understanding of drug performance and/or resistance. In contrast, TOBis-multiplexed MC characterises cell-type specific signalling networks, cell-cycle states, and apoptotic readouts across all cell-types in PDO and PDO co-cultures, making it a powerful tool for MC drug / CRISPR screen and evaluation of biological therapies. Given its ability to resolve multiple cell-types, TOBis MC would be particularly powerful for evaluating biological therapies against solid tumours where cell-type specificity is essential for resolving drug (e.g. CAR T-cell) and target (organoid) phenotypes. Future development of TOBis barcodes using additional TeMal (×7 possible) and Cisplatin (×4 possible) isotopologs will greatly 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 signalling networks in organoid monocultures and uncover novel cell-cell signalling relationships in organoid co-cultures. Given the widespread adoption of organoids as biomimetic models of healthy and diseased tissue, we propose cell-type specific PTM analysis as a powerful technology for multivariate organoid phenotyping.
METHODS

Organoid Culture

Intestinal organoids were generated as describe by Sato et al.\textsuperscript{13}. Briefly, the small intestine of 8- to 12-week-old Lgr5-EGFP-ires-CreERT2 mice was dissected, opened longitudinally, and cut into 2- to 5-mm segments. Tissue fragments were washed with ice-cold PBS and incubated with 2 mM EDTA (Sigma 03690) in PBS (Thermo 10010056) for 1 hr at 4 °C. After removal of EDTA, tissue fragments were washed vigorously in cold PBS to release the crypts. Supernatant fractions from the washes were collected and centrifuged at 1,200 rpm for 5 mins. Cells were washed with 15 mL advanced DMEM/F-12 (Thermo 12634010), passed through a 70 µm cell strainer (Fisher 11597522) to enrich for intestinal crypts, and centrifuged at 600 rpm for 2 mins. The cell pellet was resuspended in Growth Factor Reduced Matrigel (Corning 354230) and cultured at 37 °C in the presence of 5% CO\textsubscript{2}.

Small intestinal organoids were maintained in advanced DMEM/F-12 (Thermo 12634010) supplemented with 2 mM L-Glutamine (Thermo 25030081), 1 mM N-Acetyl-L-Cysteine (Sigma A9165), 10 mM HEPES (Sigma H3375), 1× B-27 Supplement (Thermo 17504044), 1× N-2 Supplement (Thermo 17502048), 50 ng/mL murine EGF (mEGF, Thermo PMG8041), 50 ng/mL murine Noggin (mNoggin, Peprotech 250-38), 500 ng/mL murine R-Spondin-1 (mR-spondin-1, Peprotech 315-32), and 1× HyClone\textsuperscript{TM} Penicillin Streptomycin Solution (Fisher SV30010).

Murine colorectal cancer (CRC) organoids carrying oncogenic mutations (\textit{shApc} (A), \textit{shApc} and \textit{Kras}\textsuperscript{G12D/+} (AK), or \textit{shApc}, \textit{Kras}\textsuperscript{G12D/+}, and \textit{Trp53}\textsuperscript{R172H/-} (AKP))\textsuperscript{34,35} were a kind gift from Prof. Lukas Dow (Cornell University) and are described in Dow & O’Rourke et al., Cell, 2015\textsuperscript{34} and O’Rourke et al., Nature Biotechnology, 2017\textsuperscript{35}. Colonic
organoids were maintained in advanced DMEM/F-12 (Thermo 12634010) supplemented with 2 mM L-Glutamine (Thermo 25030081), 1 mM N-Acetyl-L-Cysteine (Sigma A9165), 10 mM HEPES (Sigma H3375), 1× B-27 Supplement (Thermo 17504044), 1× N-2 Supplement (Thermo 17502048), 100 ng/mL murine WNT-3a (mWNT-3a, Peprotech 315-20), 50 ng/mL murine EGF (mEGF, Thermo PMG8041), 50 ng/mL murine Noggin (mNoggin, Peprotech 250-38), 500 ng/mL murine R-Spondin-1 (mR-spondin-1, Peprotech 315-32), 10 mM Nicotinamide (Sigma N0636), and 1× HyClone™ Penicillin Streptomycin Solution (Fisher SV30010).

For passaging, small intestinal organoids were retrieved from Matrigel using ice-cold PBS and broken up mechanically by passing through a 23G, 5/8'' needle (Terumo AN-2316R). Colonic organoids were dissociated mechanically by pipetting or enzymatically with TrypLE™ Express Enzyme (Thermo 12604013). Organoid fragments were collected using a benchtop centrifuge, washed with ice-cold PBS, and reseeded in fresh Matrigel. The passage was performed every 4 to 7 days at a ratio of 1:2 or 1:3.

Pre-Treatment and Fixation of Organoids for Mass Cytometry

$^{127}$I-iodo-2',deoxyuridine ($^{127}$IdU) (Fluidigm 201127) was added directly to organoid culture media to a final concentration of 25 μM and incubated at 37 °C for 30 mins before fixation to identify cells in S-phase$^{14}$. 5 mins before fixation, protease and phosphatase inhibitors (Sigma P8340 / Sigma 4906845001) were added to organoid cultures to preserve cell signalling during fixation$^8$. As dissociation of live tissue alters cellular states$^{41}$ (including PTMs$^9$), all organoids were fixed in 4% PFA (Thermo J19943K2) for 60 mins at 37 °C to preserve cell-signalling. (Note: during method
optimisation, we also trialled alternative fixatives such as Glutaraldehyde (0.2%, 1%, 2.5%), Ethanol (5%, 10%), and Glyoxal (pH 4.0, pH 5.0), but concluded that 4% PFA was optimal. 1.6% PFA can also be used but we encourage users to test a range of PFA for their specific antibody panel.) Following fixation, organoids were washed ×2 with PBS and incubated in 250 nM ¹⁹⁴/⁸Cisplatin (Fluidigm 201194 / 8) in PBS for 10 mins on a rocker to stain dead cells¹⁵. During optimisation we found this condition yields strong ¹⁹⁴/⁸Pt staining with a wide dynamic range suitable for efficient dead cell removal in silico. Organoids were then washed ×2 with PBS to remove residual Cisplatin. Fixed organoids were subsequently dissociated for single-cell analysis immediately or stored at 4 °C.

**Single-Cell Dissociation of Fixed Organoids**

After organoids were fixed and stained with Cisplatin, the final wash was removed and a solution of fresh 0.5 mg/mL Dispase II (Thermo 17105041), 0.2 mg/mL Collagenase IV (Thermo 17104019), and 0.2 mg/mL DNase I (Sigma DN25) in PBS was added to the organoids. (During optimisation we found Dispase II is essential for disrupting epithelial cell-cell contacts, Collagenase IV improves Matrigel degradation, and DNase I digests extracellular genomic DNA from dead organoid cells to reduce sample viscosity. We encourage users to test alternative dissociation enzymes for the specific cellular composition of their experimental system.) Organoid droplets were then scraped from the well, pooled, and the enzyme / organoid solution was transferred to a gentleMACS C-Tube (Miltenyi 130-096-334). Fixed organoids were dissociated into a single-cell suspension using the gentleMACS Octo Dissociator (with Heaters) (Miltenyi 130-096-427) at 37 °C for 50 mins using a custom program.
Following dissociation, C-Tubes were centrifuged at 800 $\times g$ for 1 min to collect cells from blades and all liquid was transferred to a fresh polypropylene FACS tube (Corning 352063). Single organoid cells were then washed $\times 2$ in Cell Staining Buffer (CSB) (Fluidigm 201068) (5 mins, 800 $\times g$) to remove enzymes / cellular debris and 35 $\mu m$ filtered (Fisher 10585801) (70 $\mu m$ (Fisher 11597522) when the culture contains fibroblasts) to remove residual clumps.

**Heavy-Metal Antibody Conjugation and Panel Design**

All antibodies were custom conjugated with rare-earth / heavy metals using X8 polymers and monoisotopic metals from Fluidigm (Fluidigm 201300). Non-Fluidigm metals / nitrates were also used: $^{89}$Y (Sigma 217239), $^{113}$In (Trace Sciences), $^{115}$In (Trace Sciences), $^{157}$Gd (Trace Sciences), and $^{209}$Bi (Sigma 254150). Antibody panels (Supplementary Tables 1 and 2) were carefully designed and titrated in accordance with known monoisotopic impurities and antigen abundance to ensure minimal cross-channel contamination.

**Mass Cytometry Analysis of Single Organoid Cells**

1–5 $\times 10^6$ fixed single organoid cells were blocked in CSB and stained with organoid-specific extracellular rare-earth metal antibody cocktails (Supplementary Tables 1 and 2) for 30 mins. Cells were then washed $\times 2$ with CSB (5 mins, 800 $\times g$) and permeabilised in 0.1% Triton X-100 (Sigma T8787) in PBS for 30 mins. Cells were washed $\times 2$ in CSB and further permeabilised with ice-cold 50% methanol (Fisher 10675112) for 10 mins on ice. (Note: during method optimisation we found dual 0.1% Triton X-100 and 50% Methanol provides the best all-round permeabilisation for a
broad range of anti-PTM antibodies.) Permeabilised cells were then washed ×2 in CSB and stained with intracellular rare-earth metal antibody cocktails (Supplementary Tables 1 and 2) for 30 mins. Stained cells were washed ×2 in CSB, fixed in fresh 1.6% formaldehyde (Thermo 28906) for 10 mins, washed in CSB, and incubated in DNA Intercalator (Fluidigm 201192A) overnight at 4 °C. The following day, cells were washed ×2 in CSB, resuspended in Maxpar Water (Fluidigm 201069) containing 20% (v/v) EQ Beads (Fluidigm 201078) and 2 mM EDTA at ~0.5 × 10^6 cells / mL. Cells were then 35 μm filtered (Fisher 10585801) (70 μm (Fisher 11597522) when the culture contains fibroblasts) and immediately analysed using a Helios Mass Cytometer (Fluidigm) (100 – 300 events / sec). Files were normalised against EQ beads, de-barcoded into each experimental condition (when required), and uploaded to the Cytobank platform (http://www.cytobank.org/).

**Immunofluorescence (IF) Staining of Organoids**

Intestinal organoids were cultured in 8-well μ-Slides (ibidi 80826). After culture medium was removed, cells were washed with PBS and fixed with 4% PFA (Thermo J19943K2) for 30 mins at 4 °C. Cells were washed twice with PBS and permeabilised with 0.2% Triton™ X-100 (Sigma T8787) in PBS for 30 mins at room temperature. Cells were washed again, incubated with PBS containing 1% BSA (CST #9998) and 0.3% Triton™ X-100 for 30 mins, followed by incubation with primary antibodies diluted in 1% BSA / 0.3% Triton™ X-100 / PBS overnight at 4 °C. Cells were washed with PBS, stained with secondary antibodies and 4',6-Diamidino-2-Phenylindole (DAPI) (Thermo D1306), with or without Alexa Fluor™ 488 / 568 Phalloidin (Thermo A12379 / A12380) for 1 hr at room temperature, away from light. Cells were washed
with PBS and mounted with Fluoromount-G™ mounting medium (Thermo 00-4958-02). Samples were imaged with a Zeiss LSM880 confocal microscope and images were analysed using FIJì. EdU staining was performed using the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit (Thermo C10640) following the manufacture’s protocol.

**Small Intestinal Organoid Directed Differentiation**

Small intestinal organoids were seeded and cultured in complete organoid medium (described above) for 24 hrs to allow organoid recovery, and treated with combinations of 3 μM CHIR99021 (GSK-3β inhibitor) (Cambridge Bioscience SM13), 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) for 3 days to direct organoid differentiation towards specific cell-types as described in Yin et al.16 (Supplementary Fig. 1). Directed-differentiated organoids were analysed by IF and MC as described above.

**Single-Cell Dissociation of Murine Intestinal Crypts**

The small intestine of 8- to 12-week-old Lgr5-EGFP-ires-CreERT2 mice was dissected and intestinal crypts were isolated as described above (see ‘Organoid Culture’). The crypts were resuspended in 5 mL of TrypLE™ Express Enzyme and incubated at 37 °C for 45 mins, mixed every 10 mins to avoid cells clumping. The cells were centrifuged at 1,200 rpm for 5 mins, resuspended in 5 mL of 4% PFA, and fixed at 37 °C for 1 hr. Fixed cells were washed once with PBS, 35 μm filtered twice to remove residual clumps, and stored at 4 °C prior to 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 C₂ maleimide (Thermo A20347) for 1 hr while still in Matrigel. Organoid probe intensities were visualised by confocal microscopy using identical settings for each probe (as described above). 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 $^{157}$Gd (Trace Sciences) for 1 hr *in situ* (in Matrigel) or *ex situ* (removed from Matrigel). Organoids were then dissociated into single cells and analysed by MC (as described above).

Thiol-reactive Organoid Barcoding *in situ* (TOBis)

Fixed organoids were washed ×2 in PBS and stained *in situ* with 10 nM Cisplatin ($^{196}$Pt, $^{198}$Pt)$^{28}$ (a kind gift from Dr. Olga Ornatsky, Fluidigm) and 1—3 μM TeMal ($^{124}$Te, $^{126}$Te, $^{128}$Te, $^{130}$Te)$^{27}$ barcodes for 1 hr on a rocker (barcoding matrices in Supplementary Tables 3 and 4). (Note: organoids can also be barcoded overnight at 4 °C.) The lower concentration of Cisplatin used for TOBis (10 nM) relative to dead cell stains (250 nM) is to ensure $^{196}$Pt and $^{198}$Pt signal intensities align with $^{124}$Te, $^{126}$Te, and $^{130}$Te signals. TOBis barcoding can be performed in 6-well (3 mL barcodes), 12-well (2 mL barcodes), 24-well (1 mL barcodes), 48-well (500 μL barcodes), and 96-well (200 μL barcodes) plates. In our experience up to 10,000 cells / μL Matrigel (representing confluent intestinal organoid cultures) can be efficiently barcoded, but we suggest optimising barcode concentrations for alternative model systems.
Organoids were washed ×3 in CSB containing 1 mM L-Glutathione (Sigma G6529) for 5 mins on a rocker to quench unbound barcodes (Supplementary Fig. 5h) and ×1 in PBS prior to pooled-dissociation (described above).

To directly compare TOBis and Maxpar (Fluidigm 201060) performance for in situ barcoding, 20 wells of established small intestinal organoids in the 12-well culture format (3 × 30 μL Matrigel droplets / well) were fixed as described above and washed ×2 with PBS. TOBis Barcode 1—20 was mixed respectively with Maxpar Barcode 1—20 in PBS and added to each well. The cells were incubated at room temperature for 60 mins, washed ×3 in CSB containing 1 mM L-Glutathione (Sigma G6529) for 5 mins on a rocker to quench unbound barcodes, dissociated into single cells, and analysed by MC.

Maxpar in situ versus ex situ Organoid Barcoding Comparison

Established small intestinal organoids were fixed in 4% PFA and washed ×2 with PBS. Maxpar barcodes were resuspended in Barcode Perm Buffer as per the manufacturer’s protocol (Fluidigm 201060). For in situ barcoding, cells were incubated in Barcode Perm Buffer for 10 mins followed by barcoding solution incubation at room temperature for 60 mins. The cells were washed ×2 with CSB to quench unbound barcodes and proceeded to dissociation as described above. For ex situ barcoding, cells were dissociated and barcoded according to the manufacturer’s protocol. The in situ and ex situ samples were pooled into a single tube and analysed by MC. Cells were de-barcoded and single-cell counts were analysed in GraphPad Prism 7 (two-tailed unpaired t-test).
To demonstrate the reactivity of Maxpar barcodes to Matrigel (hence its incompatibility with organoid barcoding *in situ*), empty Matrigel droplets were seeded in 12-well plates (3 × 30 μL droplets / well), fixed with 4% PFA, washed ×2 with PBS, and incubated with PBS, Maxpar barcode #20 (\(^{106}\text{Pd}, ^{108}\text{Pd}, \text{and} ^{110}\text{Pd}\) resuspended in PBS or CSB for 60 mins at room temperature. The Matrigel droplets were then washed ×3 with CSB, ×3 with PBS, and dissolved in ice-cold Maxpar Water (Fluidigm 201069). Matrigel concentration was measured by BCA assay (Thermo 23225) and all samples were diluted to a protein concentration of 250 μg/mL prior to analysis on solution mode using a Helios Mass Cytometer (time per reading = 1 sec, settling time = 10 msec). The dual counts of \(^{110}\text{Pd}\) were measured and analysed in GraphPad Prism 7 (two-tailed unpaired *t*-test).

**TOBis versus Maxpar Cell-Recovery Comparison**

During optimisation we observed that organoids barcoded using the Maxpar Cell-ID™ 20-Plex Pd Barcoding Kit (Fluidigm 201060) had much smaller cell pellets than those processed by TOBis. We hypothesised this cell-loss was due to the increased dissociation and centrifugation steps required for Maxpar barcoding when compared to TOBis. To investigate this, we directly compared both TOBis and Maxpar barcoding recoveries across a range of organoid seeding densities. Established small intestinal organoids (4-day-culture) were removed from Matrigel and reseeded in 24-well plates (1 × 50 μL Matrigel droplet / well) across 6 serial dilutions (100—3.125%) in duplicate to generate a dynamic range of organoid cell numbers. After recovering for 12 hrs in complete organoid culture medium, organoids were fixed in 4% PFA and washed in PBS (see above). One replicate of the organoids
was barcoded *in situ* using TOBis (described above) and the other replicate was individually dissociated and barcoded *ex situ* using the Maxpar kit following the manufacturer’s instructions. Cells were stained with rare-earth metal antibodies and analysed by MC. Cells were de-barcoded and single-cell counts were analysed in GraphPad Prism 7 (two-tailed ratio-paired *t*-test).

To investigate organoid cell-type recovery between Maxpar and TOBis barcoding, equal cell numbers across replicates of each strategy were identically gated in UMAP space (see below) and the percentages of stem, Paneth, enteroendocrine, tuft, goblet cells, and enterocytes were calculated. Cell-type percentages were analysed in GraphPad Prism 7 (linear regression of correlation).

**Small Intestinal Organoid Time-Course**

Intestinal organoids were retrieved from Matrigel using ice-cold PBS, broken up mechanically by sequentially passing through a 23G, 5/8” needle (Terumo AN-2316R) 6 times and a 26G, 1/2” needle (HSW, 4710004512) 3 times to obtain a uniform cell suspension. Organoid fragments were 70 µm filtered twice and centrifuged at 200 ×g for 5 mins. The cell pellet enriched with single crypts was washed with cold PBS, collected using a benchtop centrifuge, and resuspended in Matrigel prior to seeding. The cell ratio seeded for Days 1—7 was 30: 9: 6: 5: 4: 3: 3 to ensure comparable organoid recovery and density from each time point. At each time point, the organoids were incubated with 25 µM $^{127}$IdU, protease / phosphatase inhibitors, and fixed in 4% PFA for 60 mins 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 samples were stained with 250 nM $^{194}$Cisplatin, TOBis barcoded (described above)
(Supplementary Table 3), stained, and analysed in one MC experiment (Supplementary Table 1, 50 parameters (40 antibodies) / cell).

**Colonic Fibroblast Isolation, Immortalisation, and Cell Culture**

Colonic fibroblasts were isolated as described by Khalil et al. Freshly dissected murine (C57BL/6, 6- to 8-week-old) colon tissue was flushed with ice-cold PBS, cut open, washed again in PBS, and incubated in 5 mM EDTA / PBS at 250 rpm, 37 °C for 15 mins. This process was repeated for a total of ×5 EDTA / PBS washes. Washed colon tissue was transferred to a fresh tube of sterile DMEM (Thermo 41966052) supplemented with 1 mg/mL Dispase II (Thermo 17105041) and 1 mg/mL Collagenase D (Sigma 11088858001). The colon / enzyme solution was incubated at 250 rpm, 37 °C for 30—60 mins (until the tissue started to look ‘stringy’). Digested colon tissue was then centrifuged at 200 ×g, 4 °C for 5 mins. Supernatant was discarded and the pellet was resuspended in 10 mL ACK Lysing Buffer (Thermo A1049201). Cells were centrifuged at 200 ×g, 4 °C for 5 mins, and the pellet was resuspended in DMEM + 10% FBS (Thermo 10082147). Cells were 100 μm filtered (Miltenyi 130-098-463) into a T75 flask and incubated at 5% CO₂, 37 °C. After 3 hrs, cells were washed ×2 with PBS to remove debris. Adhered cells were cultured with DMEM + 10% FBS + 1× Insulin-Transferrin-Selenium (ITS-G) (Thermo 41400045). After 1 week of culture, fibroblasts were observed to proliferate, while other cell-types (e.g. epithelial cells and leukocytes) senesce and/or die. Colonic fibroblasts were immortalised using pBABE-HPV-E6 retrovirus produced in Phoenix-ECO cells (a kind gift from Prof. 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 co-culture visualisation. Immortalised colonic fibroblasts were cultured in DMEM + 10% FBS + 1× ITS-G at 5% CO₂, 37 °C. Cells were checked for mycoplasma infection monthly using the MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza LT07-701) and remained negative throughout this project. IF staining confirmed that colonic fibroblasts were positive for intestinal mesenchymal markers such as Vimentin (D21H3, CST), Podoplanin (PDPN) (8.1.1, BioLegend), PDGFRα (APA5, Abcam), FOXL1 (ab95286, Abcam), and GLI-1 (C-1, Santa Cruz) in both 2D and 3D cultures.

**Primary Macrophage Isolation and Cell Culture**

Freshly dissected murine (C57BL/6, 10- to 12-week-old females) femurs and tibias (Charles River Laboratories) were flushed ×5 with 10 mL RPMI 1640 Medium (Thermo 11875093) + 10% FBS (Thermo 10082147). Cells were centrifuged at 300 ×g for 5 mins, resuspended in RPMI + 10% FBS, 40 μm filtered (Fisher 11587522), and centrifuged at 300 ×g for 5 mins. Supernatant was discarded and the pellet was resuspended in 2 mL ACK Lysing Buffer (Thermo A1049201) for 5 mins at room temperature. Monocytes were washed in PBS, centrifuged at 300 ×g for 5 mins, resuspended in 1 mL 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 media (LCCM) before experiments. IF staining confirmed that the cells were positive for intestinal macrophages markers such as CD45 (30-F11, BioLegend), CD68 (FA-11, BioLegend), CD11b (M1/70, BioLegend), F4/80 (BM8, BioLegend), and CX3CR1 (SA011F11, BioLegend) in both 2D and 3D cultures.
Heterocellular CRC Tumour Microenvironment (TME) Organoid Culture

Wild-type (WT) murine colonic organoids and CRC organoids carrying oncogenic mutations A, AK, and AKP were a kind gift from Prof. 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 — WENR) for 8 hrs prior to the experiment. Colonic fibroblasts were cultured in DMEM supplemented with reduced FBS (2%) and 1× ITS-G for 24 hrs before the experiment. Primary bone marrows were differentiated into macrophages using RPMI + 10% FBS + 25% LCCM for 7 days before the experiment.

To establish the CRC TME culture, organoids were passaged at a ratio of ~1:2.5; colonic fibroblasts were seeded at 6,000 cells / μL, 5,000 cells / μL and 4,000 cells / μL Matrigel for monoculture, 2-way co-cultures, and 3-way co-cultures respectively; primary macrophages were seeded at 9,000 cells / μL, 8,000 cells / μL and 7,000 cells / μL Matrigel for monoculture, 2-way co-cultures, and 3-way co-cultures respectively. Organoids, fibroblasts, and macrophages were mixed in Matrigel before seeding at 3 x 30 μL droplets per well in a 12-well plate. Each microenvironment culture was maintained in WENR-free advanced DMEM F/12 (Thermo 12634010) supplemented with 2 mM L-Glutamine (Thermo 25030081), 1 mM N-Acetyl-L-Cysteine (Sigma A9165), 10 mM HEPES (Sigma H3375), 1× B-27 Supplement (Thermo 17504044), 1× N-2 Supplement (Thermo 17502048), 1× Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A) (Thermo 51300044), and 1× HyClone™ Penicillin Streptomycin Solution (Fisher SV30010) for 48 hrs. All cultures were incubated with 25 μM ¹²⁷IdU, protease / phosphatase inhibitors, and fixed in 4% PFA for 60 mins at 37 °C (as described above). Dead cells were stained with 250 nM ¹⁹⁴Cisplatin as
described above. Organoids were barcoded using TOBis (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) / cell). Single organoid, fibroblast, and macrophage cells were analysed by MC (as described above).

**Single-Cell Signalling Data Analysis**

All single cells were gated for Gaussian parameters (Event length, Centre, Residual, and Width values), DNA^{high} (^{191}Ir and ^{193}Ir), and Cisplatin^{low} (^{94/8}Pt). For small intestinal organoids and colonic organoids, intact epithelial cells were gated with EpCAM^{+} / Pan-CK^{+} and CEACAM1^{+} / Pan-CK^{+} respectively. Intact colonic fibroblasts were gated with RFP^{+} / PDPN^{+}, and primary macrophages were gated with CD68^{+} / F4/80^{+}. Removal of cells stained positive for mutually exclusive cell-type / cell-state markers was performed as part of data pre-processing procedures (gating strategies incorporated in the publicly deposited datasets). Cells were then clustered and visualised in UMAP (Uniform Manifold Approximation and Projection)\textsuperscript{48} space and gated for cell-type and cell-state makers before proceeding to PTM analysis (Supplementary Fig. 2a—c).

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 visualise high-dimensional MC datasets in two-dimensional space, where cell gating was performed to identify cell populations or to remove residual outliers when required. All data was arcsinh.
transformed with a cofactor of 5. All parameters used to generate UMAPs are listed in Supplementary Table 5.

Earth Mover’s Distance (EMD) was computed with the Python package scprep49 (https://github.com/KrishnaswamyLab/scprep) using default parameters. EMD scores were signed by the difference of the median intensity of a given parameter between the population of interest relative to the denominator (specified below): positive for up-regulation or negative for down-regulation. Cell populations were manually gated and exported from Cytobank, with all channels to be analysed arcsinh transformed (cofactor = 5). For single-time-point small intestinal organoids (Fig. 2b and Supplementary Fig. 3), EMD was calculated between each cell-type / state and the entire epithelial cell population. For the small intestinal organoid 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, 6b and Supplementary Figs. 7, 8a—d), EMD was calculated between each cell-type in each condition and the combined population of all cell-types across all conditions.

$k$-Nearest Neighbours Density Resampled Estimation of Mutual Information ($k$NN-DREMI)$^{21}$ was computed with the Python package scprep49 using default parameters. Cell populations were manually gated in UMAP space and exported from Cytobank, with all the channels to be analysed arcsinh transformed (cofactor = 5). For small intestinal organoids (Fig. 2b and Supplementary Fig. 3), $k$NN-DREMI scores among 28 PTMs (Supplementary Table 1) were calculated, which yielded a total of 756 PTM-PTM combinations for each cell-type / cell-state. Similarly, $k$NN-DREMI scores for 756 PTM-PTM pairs across 28 PTMs (Supplementary Table 2) were computed for
each cell-type in the CRC TME model (Fig. 5f, Supplementary Figs. 7, 8a, b, e, and f). Heatmaps were generated using the R package RColorBrewer (https://cran.r-project.org/web/packages/RColorBrewer/) based on EMD and DREMI calculations. Signalling maps were compiled in OmniGraffle Professional from the heatmaps with the nodes (PTMs) coloured by EMD scores and edges (PTM-PTM pairs) by DREMI scores.

Principal Component Analysis (PCA) was performed on z-score normalised EMD (Fig. 2c, 5e, and Supplementary Fig. 8c, d) or DREMI (Fig. 5f and Supplementary Fig. 8e, f) scores using the Scikit-Learn package PCA estimator in Python (https://scikit-learn.org/stable/modules/generated/sklearn.decomposition.PCA.html) with default parameters. All measurements used for PCA are listed in Supplementary Table 5.

Force-directed Scaffold Maps\textsuperscript{36} were constructed using the R package Scaffold (https://github.com/nolanlab/scaffold) with parameters specified below. Landmark populations were manually gated and exported from Cytobank with all data arcsinh transformed (cofactor = 5). For small intestinal organoid directed differentiation (Supplementary Fig. 1), pRB \([S807/S811]\) cells were used for the Scaffold analysis. UMAP-gated stem, Paneth, enteroendocrine, goblet, tuft cells, and enterocytes from untreated organoids were used as landmark nodes, and the untreated sample was used as the reference dataset. All measurements including cell-type / cell-state markers and PTMs were used to generate the Scaffold maps (Supplementary Table 5). For the CRC TME model (Fig. 6a and Supplementary Fig. 9), epithelial cells from each condition were clustered by the measurements of all cell-type / cell-state markers and PTMs (Supplementary Table 5). Microenvironment-focused Scaffold maps (Fig. 6a and Supplementary Fig. 9a) were generated using epithelial cells from
WT organoid monoculture and WT organoids co-cultured with macrophages and/or fibroblasts as landmark nodes, and epithelial cells from the WT organoid / macrophage / fibroblast co-culture sample as the reference dataset. For the genotype-focused Scaffold maps (Supplementary Fig. 9b), epithelial cells from monocultures of WT, A, AK, AKP organoids were used as landmark nodes, and the WT sample was used as the reference dataset. Selected cell-type and cell-state markers were used to generate the Scaffold maps (Supplementary Table 5).

Data Availability
All raw data, processed data, and working illustrations are available as a Community Cytobank project (https://community.cytobank.org/cytobank/experiments#project-id=1271).

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AUTHOR CONTRIBUTIONS

X.Q. Designed the study, performed organoid and MC experiments, analysed the data, and wrote the paper.

J.S. Developed TOBis, designed rare-earth metal antibody panels, performed MC analysis, and analysed data.

P.V. Isolated and characterised colonic fibroblasts, macrophages, cultured organoids, and analysed data.

P.K. Performed UMAP, EMD, DREMI, and PCA data analysis.

M.N. Developed TeMal barcodes.

S.A. Provided murine monocytes and intestines for fibroblast isolation.

V.L. Provided murine small intestines for organoid isolation.

C.T. Designed the study, analysed the data, and wrote the paper.

COMPETING INTERESTS

M.N. has pending intellectual property on the use of tellurium reagents for mass cytometry applications which has been licensed to Fluidigm Corporation.
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Figure 1 – Cell-Type and Cell-State Identification of Single Organoid Cells by Mass Cytometry.

a) Experimental workflow. Live organoids are pulsed with $^{127}\text{IdU}$ to label S-phase cells, treated with protease / phosphatase inhibitors, fixed with PFA to preserve post translational modification (PTM) signals, and stained with $^{194/8}\text{Cisplatin}$ to label dead cells. Fixed organoids are then dissociated into single cells, stained with rare-earth metal-conjugated antibodies, and analysed by single-cell mass cytometry (MC). The resulting dataset contains integrated cell-type, cell-state, and PTM signalling information.

b) Confocal immunofluorescence (IF) of small intestinal organoids stained with rare-earth metal-conjugated MC antibodies highlighting individual cell-type and cell-state markers (red), F-Actin (white), and DAPI (blue), scale bars = 50 μm. Each image is representative of at least five organoids in independent IF experiments. (See Supplementary Fig. 1 for antibody validation via directed differentiation.)

c) UMAP (Uniform Manifold Approximation and Projection) distribution of 1 million single organoid cells analysed by MC resolves six major intestinal cell-types across proliferating, S-phase, M-phase, and apoptotic cell-states. Colours represent normalised local parameter intensity. (See Supplementary Fig. 2 for cell-type and cell-state classification.)

Figure 2 – Cell-Type and Cell-State Specific Signalling Analysis of Intestinal Organoids.
a) UMAP distributions of PTMs across 1 million single organoid cells analysed by MC. Cell-type and cell-state UMAP guide is shown top left (see Supplementary Fig. 2 for cell-type and cell-state classification). Combining cell-type, cell-state, and PTM measurements enables cell-type specific analysis of intestinal organoid signalling. Colours represent normalised local parameter intensity.

b) Cell-type specific PTM signalling networks in small intestinal organoids, with nodes coloured by PTM-EMD (Earth Mover’s Distance) scores quantifying PTM intensity (relative to all organoid cells) and edges coloured by DREMI (Density Resampled Estimation of Mutual Information) scores quantifying PTM-PTM connectivity. Small intestinal organoids display cell-type specific signalling networks.

c) Principal Component Analysis (PCA) of PTM-EMDs for all organoid cell-types, either proliferating (pRB⁺) / G0 (pRB⁻) or in lower-crypts (CD44⁺) / villi (CD44⁻). Organoid cell signalling is dictated by cell-state (PC 1) and cell-type (PC 2). (See Supplementary Fig. 3 for complete EMD-DREMI signalling maps.)

Figure 3 – Thiol-reactive Organoid Barcoding in situ (TOBis) for Single-Cell Organoid Multiplexing.

a) Confocal IF of fixed GFP⁺ small intestinal organoids stained with either amine-reactive fluorescent probe Alexa Fluor 647 NHS ester or thiol-reactive fluorescent probe Alexa Fluor 647 C₂ maleimide while still in Matrigel, scale bars = 50 μm. Each IF image is representative of three independent experiments. Amine-reactive probes bind diffusely to Matrigel with very poor binding to organoids, whereas thiol-reactive probes bypass Matrigel and bind directly to organoids (see Supplementary Fig. 4c for Matrigel background staining).
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b) Small intestinal organoids stained with either amine-reactive NHS ester-DOTA-$^{157}$Gd or thiol-reactive C$_2$ maleimide-DOTA-$^{157}$Gd in situ (still in Matrigel) or ex situ (removed from Matrigel) and analysed by MC. While both probes bind organoid cells ex situ, only thiol-reactive C$_2$ maleimide-DOTA-$^{157}$Gd bind organoids in situ. Data is representative of three independent experiments.

c) Model of amine- and thiol-reactive barcodes in organoid culture.

d) Thiol-reactive tellurium maleimide (TeMal) ($^{124}$Te, $^{126}$Te, $^{128}$Te, $^{130}$Te) and Cisplatin ($^{196}$Pt, $^{198}$Pt) isotopologs combined to form a 20-plex (6-choose-3) doublet-filtering organoid barcoding strategy.

e) Thiol-reactive Organoid Barcoding in situ (TOBis) workflow. When combined with cell-type, cell-state, and PTM probes, TOBis allows organoids to be barcoded while still in Matrigel and rapidly processed as a single sample. (See Supplementary Fig. 5 for additional details.)

Figure 4 – Cell-Type Specific Signalling During Intestinal Organoid Development.

a) Time-course confocal IF of intestinal organoid development illustrating S-phase (EdU*, magenta) and apoptotic (cCaspase 3 [D175]*, green) cells, scale bars = 50 μm. Images are representative of at least five organoids in independent time-course and IF experiments. Each time point was barcoded by TOBis, pooled into a single sample, and analysed by MC. Cell-density UMAP distributions of 2 million single organoid cells reveal changes in cell-type and cell-state during organoid development.

b) Cell-type composition of small intestinal organoids during development. Stem cells accumulate at the expense of enterocytes during organoid culture.
c) Cell-type specific PTMs and cell-states of stem, Paneth, enteroendocrine, tuft, goblet cells, and enterocytes during intestinal organoid development. Cell-state analysis shows the proportions of apoptotic, G0-, G1-, S-, G2-, and M-phase cells. Irrespective of time point, stem, Paneth, and enteroendocrine cells are stably mitotic, whereas tuft, goblet cells, and enterocytes are frequently post-mitotic. Stem, Paneth, enteroendocrine, and tuft cells display stable signalling over time, whereas goblet cell- and enterocyte-signalling diverge from Day 4.

**Figure 5 – Single-Cell Signalling Analysis of Colorectal Cancer (CRC) Tumour Microenvironment Organoids.**

a) Experimental design. CRC organoid genotypes (wild-type (WT), shApc (A), shApc and Kras<sup>G12D/+</sup> (AK), shApc, Kras<sup>G12D/+</sup>, and Trp53<sup>R172H–</sup> (AKP)) were cultured in the presence or absence of colonic fibroblasts and/or macrophages (without exogenous growth factors). Each condition was TOBis-barcoded, pooled into a single sample, and analysed by MC (28 PTMs / cell).

b) Confocal IF of a WT colonic organoid (Pan-CK, green) co-cultured with colonic fibroblasts (RFP, red), and macrophages (CD45, grey) (TOBis 4), scale bar = 50 μm. Image is representative of five independent co-culture and IF experiments.

c) UMAP distribution of the colonic microenvironment model resolves single epithelial cells (green), fibroblasts (red), and macrophages (grey) (TOBis 4).

d) PTMs, progenitor cell-types, and cell-states of colonic epithelial organoids across all genotype / microenvironment combinations. The grey and red shades in the microenvironmental conditions represent macrophages and fibroblasts respectively.
Qin et al. (See Supplementary Figs. 7 and 8 for complete EMD-DREMI signalling maps of organoids, macrophages, and colonic fibroblasts.)

**e)** PCA of 28 PTM-EMDs for colonic epithelial organoids across all genotype / microenvironment combinations. CRC organoids with AK / AKP mutations mimic the signalling flux driven by colonic fibroblasts. (See Supplementary Fig. 8c, d for PTM-EMD PCAs for macrophages and colonic fibroblasts.)

**f)** PCA of 756 PTM-DREMI s for colonic epithelial organoids across all genotype / microenvironment combinations. Epithelial signalling connectivity is regulated by genotype rather than microenvironment. (See Supplementary Fig. 8e, f for PTM-DREMI PCAs for macrophages and colonic fibroblasts.)

**Figure 6 – Oncogenic Mutations Mimic Stromal Signalling Networks.**

**a)** Scaffold maps constructed from WT organoids either alone or co-cultured with colonic fibroblasts and/or macrophages. Unsupervised distribution of A, AK, and AKP colonic organoids revealed that oncogenic mutations mimic signalling profiles driven by stromal fibroblasts and macrophages. (See Supplementary Fig. 9a for all genotype / microenvironment combinations and Supplementary Fig. 9b for mutation-driven Scaffold maps.)

**b)** PTM-EMDs for PI3K / PKC and P38 / MAPK signalling nodes in colonic organoids following genotypic and microenvironmental regulation. Cell-type specific PTM analysis demonstrates oncogenic mutations and microenvironmental cues upregulate analogous signalling nodes in epithelial colonic organoids.
Figure 1

a

Organoid Fixation → Single-Cell Dissociation → Rare-Earth Metal Abs → Mass Cytometry → Single-Cell Protein Data → Single-Cell Organoid Signalling

b

Epithelial Stem Tuft Goblet

Cell-Type

Lower-Crypt Paneth Enteroendocrine Enterocyte

Proliferating S-Phase M-Phase Apoptotic

Cell-State

pRB [S807/S811] EdU Histone H3 [S28] Caspase 3 [D175]

pRB [S807/S811] EdU Histone H3 [S28] Caspase 3 [D175]

Relative Marker Intensity UMAP1 UMAP2

127I EdU 162 Dy 173 Yb 171 Yb 175 Lu 144 Nd 127 I 89 Y 142 Nd

c

Epithelial Stem Tuft Goblet

Cell-Type

Lower-Crypt Paneth Enteroendocrine Enterocyte

Proliferating S-Phase M-Phase Apoptotic

Cell-State

pRB [S807/S811] EdU Histone H3 [S28] Caspase 3 [D175]

pRB [S807/S811] EdU Histone H3 [S28] Caspase 3 [D175]
Figure 2

(a) 1 Million Small Intestinal Organoid Cells

- Figure 2a: Visualization of 1 Million Small Intestinal Organoid Cells, highlighting various cellular types such as Enteroocyte, Goblet, Paneth, and Enteroendocrine. The diagram also highlights different sections like Proliferating (pRB) and Lower-Crypt (CD44).

(b) Total Organoid: 55% pRB*, 8% cC3*

- Total Organoid: 55% pRB*, 8% cC3*
  - Tuft: 40% pRB*, 10% cC3*
  - Goblet: 43% pRB*, 11% cC3*
  - Enteroendocrine: 72% pRB*, 5% cC3*
  - Stem: 100% pRB*, 0% cC3*
  - Paneth: 74% pRB*, 1% cC3*

(c) Cell-State

- Cell-State analysis showing different cell types and states, with a focus on pRB, pRB, cC3, and other markers.

Legend for PTMs:
- EMD: Protein
- DREAM (Enhanced Modular Annotation)
- Protein
- PTM x PTM
- PTM x PTM
- PTM x PTM

Key:
- Orange: Up-Regulated in Cell-Type
- Green: Down-Regulated in Cell-Type
- Gray: N.G. (No significant change)

Color Scale:
- 1.0: Red
- 0.8: Orange
- 0.6: Yellow
- 0.4: Green
- 0.2: Blue
- 1.2: Dark Blue

Connections:
- Weak: 0.1 to 0.3
- Strong: 0.6 to 0.75
**Figure 3**

**a**

- NHS ester-Alexa 647 (Amine Reactive)
- GFP
- C₂ maleimide-Alexa 647 (Thiol Reactive)

**b**

- Organoids in situ
- Organoids ex situ

**c**

- Amine Barcodes Bind Matrigel
- Thiol Barcodes Bind Organoids

**d**

- TeMal
- Thiol Reactive
- Cisplatin

**e**

- Thiol Organoid Barcoding in situ (TOBis)
- High-Throughput Organoid Processing
- Debarcode Single Cells

- Organoid 1 → TOBis 1
- Organoid 2 → TOBis 2
- Organoid 20 → TOBis 20

- Mass Cytometry
- Rare-Earth Metal Abs
- Pooled Dissociation

- 20-plex TOBis (6-choose-3)
Figure 4

(a) Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7
---|---|---|---|---|---|---
TOB1 ↓ | TOB2 ↓ | TOB3 ↓ | TOB4 ↓ | TOB5 ↓ | TOB6 ↓ | TOB7 ↓

Phase: Recovery | Expansion + Differentiation | Divergence | Stem Survival + Enterocyte Exhaustion

(b) Day: 1 2 3 4 5 6 7

<table>
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<tr>
<th>Cell-State</th>
<th>Stem</th>
<th>Paneth</th>
<th>Enteroendo.</th>
<th>Tuft</th>
<th>Goblet</th>
<th>Enterocyte</th>
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(c) % Cell-Type

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<th>pMEK1/2</th>
<th>pS6</th>
<th>pAKT</th>
<th>pNF-κB</th>
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<td>Jnk</td>
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<td>PDK</td>
<td>p53</td>
<td>MAPKAPK2</td>
<td>MAPKAPK2</td>
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Divergent Signalling

Stable Signalling

Mitotic

Post-Mitotic

EMD

Up-Reg. in Cell-Type

Down-Reg. in Cell-Type

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