Tracing oncogene-driven remodelling of the intestinal stem cell niche

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Declaration of Interests
The authors declare no competing interests.

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Abstract

Interactions between tumour cells and the surrounding microenvironment contribute to tumour progression, metastasis and recurrence\(^1\)\(^-\)\(^3\). While mosaic analyses in *Drosophila* have advanced our understanding of such interactions\(^4\)\(^,\)\(^5\), parallel approaches have remained challenging to engineer in vertebrates. Here, we present an oncogene-associated, multicolour, reporter mouse model, the Red2Onco system, that allows differential tracing of mutant and wild-type cells in the same tissue. Applied to the small intestine, we show that oncogene-expressing mutant crypts alter the cellular organization of neighbouring wild-type crypts, driving accelerated clonal drift. Crypts expressing oncogenic KRAS or PI3K secrete BMP ligands that suppress local stem cell activity, while induced changes in PDGF\(_{lo}\) CD81\(^+\) stromal cells by crypts with oncogenic PI3K alter the Wnt signalling environment. Together, these results show how oncogene-driven paracrine remodelling creates a niche environment that is detrimental to the maintenance of wild-type tissue, promoting field transformation dominated by oncogenic clones.

The tumour microenvironment (TME) constitutes a complex ecosystem comprising mutant and wild-type (WT) epithelial cells, as well as endothelial, immune and mesenchymal cells\(^1\)\(^,\)\(^2\). From the earliest phase of tumour initiation, the TME adapts to signals from tumour cells\(^3\). Yet, little is known about how these changes impact on non-malignant cells. To date, much of our understanding has been informed by studies in *Drosophila*, where WT cells are shown to eliminate less “fit” cells through “cell competition”\(^4\)\(^,\)\(^6\). Conversely, tumour cells bearing mutations in genes such as *Myc*, *Ras* or *Apc* can become “super-competitors”, eliminating WT neighbours\(^5\). In mammals, increasing emphasis has been placed on cell competition during tumour development\(^7\), with oncogene-driven changes in both mutant cells and the TME\(^8\) having the potential to influence neighbouring WT cells\(^9\)\(^,\)\(^10\) (Fig. 1a).

Multicolour reporters with oncogenes

Cell labelling strategies have been devised to study fate changes during tumour development\(^11\)\(^,\)\(^12\). While such methods provide insight into factors driving field cancerization, it has been difficult to assess how neighbouring WT cells react to, and influence, tumour growth. To study crosstalk between mutant and WT cells, previous studies have utilized mosaic labelling approaches\(^13\)\(^,\)\(^14\) to study gain- and loss-of-function. However, challenges in their design limit their potential. Here, to circumvent these difficulties, we adapted the multicolour *Confetti* reporter line\(^15\), based on the *Brainbow*-2.1 cassette, to include an additional cDNA cassette following the *tdimer2* red fluorescent protein (RFP) cDNA (Fig. 1b). This inducible Red2Onco system allows co-expression of an oncogene in an RFP+ clone-specific manner. Upon Cre activation, the *R26R-Red2Onco* construct
stochastically recombines to express one of four fluorescent proteins, where only RFP+
clones co-express the oncogenic cDNA (Fig. 1b,c, Supplementary Video1).

To demonstrate the utility of the Red2Onco system, we developed lines for RFP-specific
co-expression of the intracellular domain of Notch1, \( \text{Kras}^{G12D} \) or \( \text{PIK3CA}^{H1047R} \). We
first confirmed that RFP expression overlapped with gene expression (NOTCH1ICD) or
downstream activation (p-ERK for \( \text{Kras}^{G12D} \), p-AKT for \( \text{PIK3CA}^{H1047R} \)) in intestine
(Extended Data Fig. 1a,b). Consistent with their role in driving hyperplasia\(^{16,17} \), we found
that RFP+ cells co-expressing \( \text{Kras}^{G12D} \) or \( \text{PIK3CA}^{H1047R} \) showed increased proliferation
(Extended Data Fig. 1c). Similarly, as expected\(^{18} \), Notch1ICD expression blocked secretory
cell differentiation (Extended Data Fig. 1d,e). The viability of the Red2Onco system was
further confirmed in other organs (Extended Data Fig. 1f,g).

**Mutant intestinal clone expansion**

In mammals, the small intestine is composed of crypts and villi. All absorptive and secretory
lineages are generated by intestinal stem cells (ISCs) that reside at the crypt base. As
ISCs divide, neighbours become displaced from the niche and enter into a differentiation
programme. As a result, ISC-derived clones stochastically expand and contract, leading to
“neutral drift” of clones until they are lost or the crypt becomes monoclonal\(^{19,20} \).

Alongside Wnt, mutations in the RAS-MAPK and PI3K-AKT pathways represent key
drivers of colorectal cancer\(^{11,21,22} \). In contrast to Wnt, activation of RAS signalling does
not lead to obvious changes in tissue architecture, leading to the assumption that mutations
in Wnt signalling may represent the first oncogenic hit. However, the discovery that KRAS
mutations are abundant in ageing human intestinal epithelium\(^{10,23} \), as well as in patients
with Crohn’s disease\(^{24,25} \), questions whether mutations in RAS-MAPK or PI3K-AKT
signalling could also function as drivers of early mutant clone expansion. To investigate the
effect of altered gene expression on clonal competition, we performed lineage tracing with
\textit{Villin-CreERT2,R26R-Red2Onco} mice using a low dosage of tamoxifen (0.2mg/20g body
weight) (Extended Data Fig. 2a-f). At 7 days post-induction, mutant clones already showed
evidence of biased drift (Extended Data Fig. 2g-i). Although both mutant and WT clones
showed a broad distribution of sizes, both the average size and frequency of monoclonal
(fixed) crypts increased more steeply in mutant clones than WT (Extended Data Fig. 2j,k).
These findings echoed results of previous studies based on clonal activation of KRAS using
a conventional reporter construct\(^{26,27} \). Quantitative comparison of isolated, WT (non-red)
clones with the \textit{Confetti} control (Extended Data Fig. 3a-c) indicated that there were no
systemic effects on clone dynamics due to the modified \textit{Confetti} construct.

To resolve the dynamics of mutant clone expansion, we invoked a modelling-based approach
whose validity has been tested previously\(^{19,28} \). Within this framework, crypts are modelled
as a chain of \( N \) “effective” ISCs that line the circumference of the crypt base. ISC loss (at
rate \( \lambda \)) is correlated with the duplication of a neighbour, leading to neutral drift of clone size
(Supplementary Theory). Applied to the \textit{Confetti} control, with an effective stem cell number
for the proximal small intestine of \( N=5,28 \) a quantitative fit to the clone data obtained a
loss-replacement rate of \( \lambda = 0.9/\text{week} \), similar to previous findings (Extended Data Fig. 3a).
Following oncogene expression, mutant cells experience a survival advantage, leading to non-neutral competition. The resulting clone dynamics can be mapped to a refined model in which the loss-replacement rate acquires a bias $0 \leq \delta \leq 1$, with mutant ISCs replacing WT neighbours at rate $\lambda(1 + \delta)$, while WT ISCs replace mutant neighbours at rate $\lambda(1 - \delta)$. Taking $N=5$, the biased drift model predicted accurately the dynamics of clone sizes with $\lambda = 2.4$/week and $\delta = 0.71$ for $\text{Kras}^{G12D}$ (similar to previous estimates\textsuperscript{26,27}), $\lambda = 1.9$/week and $\delta = 0.64$ for $\text{PIK3CA}^{H1047R}$, and $\lambda = 1.1$/week and $\delta = 0.36$ for $\text{Notch1ICD}$ (Extended Data Fig. 3d-f).

Active competition through apoptotic elimination of less fit cells is well-documented in $\text{Drosophila}$ and mouse epiblast\textsuperscript{4}. However, immunodetection of cleaved caspase-3 in crypts of $\text{R26R-Red2Onco}$ mice showed no evidence of increased apoptosis (Extended Data Fig. 3g). By contrast, a 2-hour EdU pulse showed an increased number of EdU+ crypt base columnar cells in mutant clones in all three $\text{Red2Onco}$ models (Extended Data Fig. 3h,i).

**Oncogene-induced effects on neighbours**

To study whether mutant clones influence the fate of WT clones in neighbouring crypts, we performed lineage tracing with Villin-\textit{CreER2};$\text{R26R-Red2Onco}$ mice induced at near-clonal dosage of tamoxifen (2mg/20g body weight), when more than 30% of crypts contained labelled cells. Unexpectedly, at 2 weeks post-induction, we found that the frequency of fixed WT crypts in $\text{Red2-Kras}^{G12D}$ and $\text{Red2-PIK3CA}^{H1047R}$ mice was increased compared to controls (Extended Data Fig. 4a,b), suggesting that mutant cells mediate changes in the dynamics of WT cells. To dissect this phenotype, we used a clonal dosage of tamoxifen (0.2mg/20g body weight) to compare the dynamics of WT clones in crypts either neighbouring (proximate) or remote (>2 crypt diameters) from crypts containing a mutant clone (Fig. 1d). Strikingly, when proximate to mutant crypts, WT clones from $\text{Red2-Kras}^{G12D}$ and $\text{Red2-PIK3CA}^{H1047R}$ mice showed accelerated drift, greatly exceeding that in remote WT crypts or Confetti controls (Fig. 1e,f). These observations were corroborated by clone size quantification, which showed a corresponding increase in average clone size and fixation rate (Extended Data Fig. 4c,d). Further, when segregated according to whether clones were oriented towards (inner) or away (outer) from a neighbouring mutant crypt, the clone size distributions were found to be comparable, suggesting that accelerated dynamics take place within the context of neutral competition (Extended Data Fig. 4e-g).

To make a further quantitative assessment of WT clones in crypts neighbouring mutant crypts we noted that, prior to fixation, neutral clone dynamics are predicted to enter a scaling regime in which the size distribution depends only on the composite “drift rate” $\lambda/N^2$ (Supplementary Theory\textsuperscript{19}). Comparison of the clone size data showed convergence onto the predicted scaling form in both Confetti control and mutant conditions (Fig. 2a). From a fit to the average clone size, we found a drift rate that was 1.9 times larger in clones proximate to mutant crypts compared to Confetti control (Fig. 2b, Extended Data Fig. 4h, Supplementary Theory).

To resolve the origin of accelerated drift, we considered whether the rate of ISC loss/replacement was increased (Extended Data Fig. 4i). However, measurements of the ISC
proliferation rate based on EdU incorporation showed comparable values (Fig. 2c,d), independent of the proximity of WT to mutant crypts, suggesting an unchanged loss-replacement rate. We then estimated the number of ISCs based on Lgr5 expression. When the majority of mutant crypts were already fixed, we found that the number of Lgr5-EGFP+ cells was decreased by 20% in WT crypts neighbouring mutant crypts (Fig. 2e,f). Similar results were found based on Olfm4 expression (Extended Data Fig. 4j). These findings suggest that accelerated clonal drift is associated with a reduction in stem cell number²⁹.

An observed reduction in the physical size of WT crypts neighbouring mutant crypts (Extended Data Fig. 5a-c) questioned whether the change in ISC number is associated with the increase in size of mutant crypts deforming surrounding tissue. However, from measurements of the circularity of WT crypts, we found no evidence for an impact of local mechanical strain (Extended Data Fig. 5d,e). We then reasoned that, if the effect were mediated by paracrine factors from mutant cells, it should scale with the multiplicity of neighbouring mutant crypts (Extended Data Fig. 5f). Consistently, when proximate to multiple mutant crypts, WT clones from Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> mice showed increased rates of clonal drift and fixation alongside a further reduction in ISC number and crypt size (Extended Data Fig. 5g-l).

Lastly, we questioned whether mutation-induced changes promote field expansion. Notably, in contrast to Confetti and Red2-NotchICD conditions, the fraction of mutant crypts steadily increased in Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> mice at the expense of WT crypts (Extended Data Fig. 5m,n). We then used the abundance of “cojoined” crypts to estimate the relative frequency of crypt fission and fusion (Extended Data Fig. 5o). Following Bruens et al.³⁰, we found that crypts mutant for Kras<sup>G12D</sup> and PIK3CA<sup>H1047R</sup> showed elevated rates of fission and fusion compared to Confetti control (Extended Data Fig. 5p), suggesting that the deleterious effects of the mutant on neighbouring WT crypts contribute towards field transformation.

### Comparative single-cell analysis

To define the mechanisms that mediate crosstalk, we exploited the design of the Red2Onco system for comparative single-cell (sc) transcriptomics (Fig. 3a). Villin-CreERT2;R26R-Red2Onco mice (Kras<sup>G12D</sup> or PIK3CA<sup>H1047R</sup>) were induced using a mosaic dosage of tamoxifen (4mg/20g body weight). Following dissociation of intestine, individual mutant (RFP+) and WT (YFP+) epithelial cells were sorted by fluorescence activated cell sorting (FACS) and profiled alongside neighbouring stromal tissue (Extended Data Fig. 6a). By combining these results with scRNA-seq data from control Confetti animals, evidence for mutation-driven gene expression changes could be resolved in epithelial, stromal and immune cells (Fig. 3a, Extended Data Fig. 6b, Methods). Epithelial cells from Red2-Kras<sup>G12D</sup>, Red2-PIK3CA<sup>H1047R</sup> and Confetti control were clustered based on marker-gene expression (Fig. 3b, Extended Data Fig. 6c-f, Supplementary Table 1). All major cell types were detected in ratios comparable to previous reports³¹–³⁴. Despite oncogene-induced transcriptional changes, cells from all three conditions overlapped within distinct clusters (Extended Data Fig. 6g, Methods).
To explore the influence of oncogene activation on lineage specification, we examined the proportions of each cell type in Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> mice compared to Confetti control. Notably, in both models, the proportion of mutant Lgr5+ stem cells was significantly decreased, while the proportion of mutant goblet cells was increased (Extended Data Fig. 6h). In Red2-PIK3CA<sup>H1047R</sup> animals, an increase in the proportion of mutant enterocytes was also observed. As with mutant cells, the WT population contained a lower proportion of stem and transit-amplifying (TA) cells in both Red2Onco models (Fig. 3c, Extended Data Fig. 6i), consistent with reduced ISC number (Fig. 2). The reduction in ISC fraction was further confirmed by immunohistochemistry and FACS (Extended Data Fig. 6j-n).

We then questioned whether changes in the fraction of stem and progenitor cells were accompanied by changes in molecular characteristics. We first defined lineage-specific genes as molecular signatures of fate priming using scRNA-seq data from a previous study<sup>32</sup> (Supplementary Table 2). We then calculated the priming score towards differentiation for individual WT (YFP+) and mutant (RFP+) stem and TA cells (Methods). In Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> mice, both WT and mutant stem and TA cells showed an enhanced degree of differentiation towards secretory and absorptive lineages, respectively (Fig. 3d, Extended Data Fig. 7a,b), a result confirmed by marker-based realtime qRT-PCR analysis and immunostaining/in situ hybridization (Extended Data Fig. 7c-j). These results suggested stem cell priming towards differentiation as a potential driver of the reduced stem cell fraction in mutant and neighbouring WT crypts in both Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> mice.

To probe the mechanisms underlying biased differentiation, we then assessed the activities of major signalling pathways<sup>2,35</sup>. Gene set enrichment analysis (GSEA) (Supplementary Table 2, Methods) revealed higher BMP signalling activity in both mutant and WT cells of Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> animals compared to Confetti control, while lower Wnt signalling activity was observed in both mutant and WT cells of Red2-PIK3CA<sup>H1047R</sup> (Fig. 3e, Extended Data Fig. 7k). By contrast, Notch signalling activity was reduced in mutant cells, but not in WT, in both Red2-Kras<sup>G12D</sup> and Red2-PIK3CA<sup>H1047R</sup> animals (Extended Data Fig. 7l,m). Based on these findings, we focused on changes in BMP and Wnt signalling to trace the basis of inter-crypt cellular crosstalk.

**Mutant clones drive niche remodelling**

We then examined direct (epithelial cell-to-cell) and indirect (via mesenchymal or immune cell) routes of communication that could affect BMP and Wnt pathways. Mesenchymal and immune cells were clustered based on expression signatures (Fig. 3f, Extended Data Fig. 8a-c, Supplementary Table 3). Three clusters (labelled STC for “stromal cell”) show marked expression of secreted factors for intestinal stromal cells (Extended Data Fig. 8d).<sup>36,37</sup> Notably, Bmp4 was highly expressed in STC1, whereas STC2 expressed Wnt pathway-modulating factors such as Rspo3, as well as the BMP antagonist, Grem1. We then searched for expression changes in secreted factors (Supplementary Table 4) binding to receptors (Extended Data Fig. 8e,f) that modulate BMP and Wnt pathways. We noticed an increase in the expression of BMP ligands, such as Bmp2 and Bmp7, found mainly in
mutant secretory cells in Red2-Kras$^{G12D}$ and Red2-PIK3CA$^{H1047R}$ mice (Fig. 3g). We also detected an increase in expression of Wnt pathway antagonists, such as Sfrp2 and Sfrp4, as well as a decrease in expression of Wnt pathway agonists, such as Rspo3, in STC2 from Red2-PIK3CA$^{H1047R}$ mice (Fig. 3h).

Changes in ligand expression in mesenchymal cells raised the possibility that the niche environment may become altered following oncogene activation. Following quantification of transcriptional changes in mesenchymal and immune cells (Methods), we found that the STC2 population in Red2-PIK3CA$^{H1047R}$ experienced significant alterations compared to other cell types (Fig. 3i, Extended Data Fig. 8g-l). Additionally, this STC2 population also showed increased levels of genes regulating extracellular matrix, such as Fn1, Mmp2 and collagens (Col6a2 and Col5a2) (Extended Data Fig. 8m). Together, these results showed that epithelial oncogene expression of either mutant KRAS or PI3K induces a BMP-rich signalling environment and, for PI3K, a Wnt-poor environment (Extended Data Fig. 8n).

**Blocking oncogenic niche remodelling**

To validate these findings, we performed in situ hybridizations for Axin2 and Id1, downstream target genes of Wnt and BMP pathways, respectively. As expected from our single-cell analysis, we observed decreased expression of Axin2 in both mutant and neighbouring WT crypts in Red2-PIK3CA$^{H1047R}$, but not Red2-Kras$^{G12D}$ mice (Extended Data Fig. 9a,b). Id1 expression was increased in mutant and neighbouring WT crypts in both Red2Onco models (Extended Data Fig. 9c,d). These results were consistent with realtime qRT-PCR (Extended Data Fig. 9e,f), confirming that Wnt activity is decreased only in Red2-PIK3CA$^{H1047R}$ mice, while BMP activity was increased in both Red2Onco models. These findings were further supported by in vitro organoid assays, where changes in BMP and Wnt signalling mimicked the effects of different niche conditions (Extended Data Fig. 9g-m).

In situ hybridisation, qRT-PCR and organoid assays also confirmed increased expression of Bmp2 in mutant epithelial cells of both Red2Onco models (Fig. 4a,b, Extended Data Fig. 9n), as well as its functionality in vitro (Extended Data Fig. 9o-r).

Recently, PDGFRA$^{lo}$ CD81+ stromal cells were identified as an important source of R-spondin and Gremlin1, a Wnt agonist and BMP antagonist, respectively. We also found that the STC2 population, which showed high Rspo3 expression (Extended Data Fig. 8d), expressed high levels of Greml1 as well as Cd81 compared to other mesenchymal cells and, consistent with McCarthy et al., were located adjacent to the crypt base (Fig. 4c, Extended Data Fig. 10a,b).

Consistent with scRNA-seq analysis (Fig. 3h), in situ hybridisation and realtime qRT-PCR analysis showed higher expression of Sfrp2 and Sfrp4, and lower expression of Rspo3, in STC2 cells in Red2-PIK3CA$^{H1047R}$ mice compared to Red2-Kras$^{G12D}$ mice and Confetti control (Fig. 4c,d, Extended Data Fig. 10b-h). To test whether oncogene-driven changes of STC2 compromises niche function, we co-cultured Lgr5-EGFP+ ISCs with PDGFRA$^{lo}$ CD81- STC1 cells or PDGFRA$^{lo}$ CD81+ STC2 cells isolated from WT and Red2-PIK3CA$^{H1047R}$ mice (Fig. 4e). As expected, we found that the STC2 population from the WT control supported organoid formation in growth factor-deprived conditions (-Nog
Rspo<sub>low</sub>), whereas the STC2 population sorted from Red2<sub>-PIK3CA<sup>H1047R</sup></sub> intestine showed significantly reduced capacity to support organoid formation (Fig. 4f,g).

To further challenge our hypothesis that BMP and Wnt pathways are involved in crosstalk, we utilised inhibitors that block either reception of BMP ligands (LDN193189, BMP type I receptor blocking agent) or secretion of Wnt ligands (LGK974, Porcupine inhibitor)<sup>29</sup>. Administration of LDN193189 and LGK974 to Red2<sub>Onco</sub> mice suppressed BMP and Wnt pathways, respectively (Extended Data Fig. 11a,b). In addition, LDN193189 rescued the rate of clonal drift of WT clones neighbouring mutant crypts, while LGK974 accelerated drift still further (Fig. 4h,i, Extended Data Fig. 11c). Notably, we observed delayed fixation of mutant crypts under LDN193189 treatment, suggesting that unlabelled WT cells in the same crypt compete more efficiently against mutant clones when the BMP effect is abolished (Extended Data Fig. 11d,e). We also confirmed that the phenotypes from both Red2<sub>Onco</sub> systems are largely recapitulated in LSL-<sub>Kras<sup>G12D</sup></sub> and <sub>Pik3ca<sup>H1047R</sup></sub> mice expressing respective oncogenes under the corresponding endogenous locus (Extended Data Fig. 11f-m).

Conclusion

Local cell displacement provides a mechanism to eliminate “loser” cells in epithelial tissues. In this case, direct cell-to-cell competition triggers differentiation/apoptosis of adjacent WT stem cells<sup>39,40</sup>. Our findings show that, through long-range paracrine signals, mutant cells may also affect WT cells in neighbouring domains either directly, or through oncogene-driven alterations of the shared niche environment. Alongside the two oncogenes considered here, mutants with Wnt activation caused by <sub>Apc</sub> loss also show paracrine effects by secreting Wnt inhibitory factors (Extended Data Fig. 12), suggesting that niche remodelling may be a general feature of tumorigenic mutants.

Methods

Mice

All inducible Cre lines (R26R-CreERT2: JAX006965, Villin-CreERT2: JAX020282, Lgr5-EGFP-IRES-CreERT2: JAX008875, Sftpc-CreERT2: JAX028054, Krt5-CreERT2: JAX02915), the R26R-Confetti line (JAX017492), LSL-<sub>Kras<sup>G12D</sup></sub> (JAX008179) line, Apc<sub>fl/fl</sub> line (JAX009045) and Apc<sub>Min</sub> line (JAX002020) were obtained from The Jackson Laboratory. Pik3ca<sub>Lat-H1047R</sub> line was donated by W.A Phillips<sup>41</sup>. Red2<sub>Onco</sub> targeting vectors were generated by gene synthesis. Oncogene sequences were obtained from Addgene (Notch1ICD: Addgene plasmid #15079<sup>42</sup>, <sub>Kras<sup>G12D</sup></sub>: Addgene plasmid #11549<sup>43</sup>, PIK3CA<sup>H1047R</sup>: Addgene plasmid #12524<sup>44</sup>). Mice were created by inserting the Red2<sub>Onco</sub> cassette into the tdimer2 locus in ES cells obtained from R26R-Confetti mice using CRISPR-Cas9 nickase-mediated homologous recombination. Insertion of the oncogenic sequence was confirmed by long-range PCR. Specific genotyping primers were designed outside of the homology arms and were used in combination with primers within the knock-in cassette.
Animal treatments

All experiments were approved by the UK Medical Research Council and University of Cambridge local ethical review committees and conducted according to Home Office project license PPL70/8296. To perform lineage tracing, tamoxifen (Sigma, T5648) dissolved in corn oil was injected intraperitoneally into 8–12-week old mice. As indicated, the dose of tamoxifen used for each experiment was determined based on recombination efficiency. For the clonal analysis, it is crucial that crypts are marked at clonal density so that labelled cells can be identified reliably as the output of an individual labelled cell. Titration experiments indicated this can be achieved by tamoxifen dose of 0.2 mg/20 g mouse body weight. Tamoxifen and chemical inhibitors (LDN193189, BMP type I receptor blocking agent, Selleckchem, S2618; LGK974, Porcupine inhibitor, Cayman Chemical, 14072) were concomitantly administered as indicated (Fig. 4h). LGK974 and LDN193189 were administered every 48 h through oral gavage, in a concentration of 5 mg/kg (LGK974) or 3mg/kg (LDN193189) in a vehicle of 0.5% Tween-80/0.5% methylcellulose. Chemical-treated mice did not show any loss of body weight or physical activity, demonstrating that they had no severe toxic side effects.

All mice were group housed under specific pathogen-free conditions in individually ventilated cages always with companion mice, and cages were placed under a 12hr light-dark cycle. Food and water were provided ad libitum. Room temperature was maintained at 22 °C ± 1 °C with 30–70% humidity. None of the mice were involved in any previous procedures before the study. Experiments were carried out with male and female animals, except for single-cell transcriptomic analysis, where only females were used. No gender-specific differences were observed.

Intestine preparation

Mice were euthanized by cervical dislocation and the intestine collected by dissection. The small intestine was cut longitudinally and subsequently placed on a piece of cold PBS soaked 3M paper, using forceps to flatten the tissue before fixation in 4% paraformaldehyde at 4 °C O/N (~18 h) with shaking. After fixation the intestine was washed for 3 x 6 h with PBS at 4 °C with shaking.

Tissue clearing and immunofluorescence

CUBIC clearings were performed as previously described45. Briefly, fixed tissue was dissected into small fragments (~2 cm) and transferred into 10 mL CUBIC R1a solution (10% urea, 5% N,N,N’,N’-tetakis(2-hydroxypropyl) ethyl-enediamine, 10% Triton X-100 and 25 mM NaCl in distilled water) in a 15 mL conical tube and incubated for 2 days at 37 °C with shaking. All subsequent incubation steps were then performed at 4 °C on a rotor. The fragments were incubated with blocking and permeabilization solution consisting of 5% DMSO, 0.5% Triton-X-100 and 2% Normal donkey serum (NDS) in PBS O/N. The following day, the solution was replaced with primary antibody for cleaved caspase-3 (1:200; Cell Signaling Technology, 9661), OLFM4 (1:100; Cell Signaling Technology, 39141), p-ERK (1:100; Cell Signaling Technology, 4370), p-AKT (1:100; Cell Signaling Technology, 4060) or KRASG12D (1:100; Cell Signaling Technology, 14429) diluted in blocking solution (1% DMSO, 0.5% Triton-X-100 and 2% NDS in PBS), and the whole
mount was incubated for 3 days. The samples were then washed 6 times with fresh PBS over a 24 h period before incubating with secondary antibody (1:500; donkey anti-rabbit or anti-mouse, Invitrogen) diluted in blocking solution for 3 days. The samples were then washed 6 times with PBS and incubated with 2 μg/mL DAPI in PBS for 24 h. The samples were then transferred into 10 mL CUBIC R2 solution (50% sucrose, 25% urea, 10% 2,20,20’-nitrilotriethanol and 0.1% Triton X-100 in distilled water) in a 15 mL conical tube and incubated for 2 days at room temperature with shaking. To match the refractive index, samples were transferred into an Eppendorf tube containing 1 mL RapiClear 1.52 (Sunjin Lab) and incubated O/N at 4 °C. Samples were then mounted in a 0.25 mm i-spacer (Sunjin Lab) for confocal imaging.

**Proliferation assay**

To measure cell proliferation *in vivo*, 1 mg of 5-ethynyl-2′-deoxyuridine (EdU) (Life Technologies, A10044) was dissolved in 200 μL of PBS and injected into each mouse. Tissues were collected after 2 h and a Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher, C10340) was used to detect EdU+ cells.

**Immunohistochemistry on near-native sections**

Organs were dissected, fixed and embedded in 4% low melt agarose as previously described for generation of 100 μm-thick near-native sections using the LAICA VT 1000S Vibratome. The sections were removed from any remaining agarose using forceps and subsequently transferred to a 12-well plate into wells containing blocking and permeabilization solution (5% DMSO, 0.5% Triton-X-100 and 2% NDS in PBS) to be incubated O/N (~18 h) at 4 °C with shaking. The following day, the blocking and permeabilization solution was replaced with primary antibody for CPA1 (1:200; R&D systems, AF2765), β-catenin (1:200; Santa Cruz, sc-7199), SPC (1:300; Millipore, AB3786), LYZ (1:200; DAKO, A009902-2) or MUC2 (1:200; Abcam, ab90007) diluted in blocking solution (1% DMSO, 0.5% Triton-X-100 and 2% NDS in PBS) and the section incubated for 72 h at 4 °C. Sections were subsequently washed and incubated with secondary antibody (1:500; donkey anti-rabbit or goat AF647, Invitrogen) and DAPI diluted in blocking solution (1% DMSO, 0.5% Triton-X-100 and 2% NDS in PBS) for 48 h at room temperature with shaking. After washing for 3 x 2 h with PBS, sections were carefully transferred from wells to microscope slides using a brush before mounting in RapiClear 1.52 (Sunjin Lab).

**Immunohistochemistry on paraffin sections**

Immunohistochemistry was performed according to standard protocols. Briefly, the intestine was dissected and fixed in 4% PFA O/N at 4 °C before paraffin embedding. Paraffin-embedded sections (5 μm) were rehydrated, and the epitopes were exposed using Tris/ethylenediaminetetraacetic acid (EDTA) buffer. Sections were then incubated in blocking solution (2% donkey or goat serum, 5% DMSO and 0.5% Triton-X-100 in PBS) at room temperature for 2 h. Primary antibody for NOTCH1 (1:100; Abcam, ab52627), β-Catenin (1:50; Sigma, 05-665), or OLFM4 (1:100; Cell Signaling Technology, 39141) was diluted in blocking solution (1% DMSO, 0.5% Triton-X-100 and 2% normal goat serum in PBS) and the sections were incubated in this for 24 h at 4 °C. Sections were subsequently washed and
incubated with secondary antibody (1:500; Goat anti-rabbit HRP, Perkin Elmer) and DAPI diluted in blocking solution (1% DMSO, 0.5% Triton-X-100 and 2% normal goat serum in PBS) for 2 h at room temperature with shaking. The TSA kits, TSA Plus Cyanine 5 System (Perkin Elmer, NEL752001KT) were used for visualization.

**In situ hybridization in cryo sections**

RNA staining was performed with an RNAscope Multiplex Reagent Kit V2 (ACDBio, 323100) following the manufacturer’s protocols. Collected tissues was fixed with 4% PFA, cryoprotected in OCT and sectioned at 16um. Briefly, the sections were pre-treated with Protease IV for 15 min. ACD designed and synthetized probes were used to detect transcripts of interest. Up to two probes were hybridised simultaneously. The RNAscope assay was followed by a regular immunostaining protocol with antibodies for RFP (1:50; SICGEN, AB8181-200), KRAS<sub>G12D</sub> (1:250; Cell Signaling Technology, 14429), p-AKT (1:50; Cell Signaling Technology, 4060) or β-Catenin (1:50; Sigma, 05-665). Sections were subsequently washed and incubated with secondary antibody (1:500; Horse anti-rabbit, Vector Lab or Donkey anti-goat HRP, GeneTex) and mounted with Prolong Gold antifade reagent supplemented with DAPI. Probes for Fabp1 (Cat No. 562831), Axin2 (Cat No. 400331), Id1 (Cat No. 312221), Bmp2 (Cat No. 406661-C2), Grem1 (Cat No. 314741-C3), Sfrp2 (Cat No. 576891-C2), Cd81 (Cat No. 556971), Rsps3 (Cat No. 402011-C3), Wiil (Cat No. 412361-C3), Lgr5 (Cat No. 312171-C2), and Notum (Cat No. 428981) were purchased from ACDBio. The TSA kits, TSA Plus Cyanine 5 (Perkin Elmer, NEL745001KT), TSA Plus Fluorescein (Perkin Elmer, NEL751001KT) and TSA Plus TMR (Perkin Elmer, NEL752001KT) were used for visualization. The multiplexed staining was performed with the help of the Wellcome Trust–Medical Research Council Cambridge Stem Cell Institute Histology facility.

**Single cell sorting for sequencing**

To collect mutant and wild-type cells from neighbouring crypts for single-cell RNA-seq data, we developed a strategy based on mosaic labelling. In this approach, Villin-CreERT2, R26R-Red2Onco mice (Kras<sub>G12D</sub> or PIK3CA<sup>H1047R</sup> mutation) were induced using a high dosage of tamoxifen (4 mg/20 g body weight). The majority of crypts (>56% for Red2-Kras<sub>G12D</sub> and >54% for Red2-PIK3CA<sup>H1047R</sup>) were found to be monoclonal for mutant oncogene expression by 2 weeks post-induction at this level, while almost all of the remaining (wild-type) crypts, 94% for Red2-Kras<sub>G12D</sub> and 92% for Red2-PIK3CA<sup>H1047R</sup>, neighboured mutant crypts. After the dissection of these tissues, intestinal cell dissociation was performed as previously described with a few modifications to deplete villi further and enrich for intestinal crypts.32 Briefly, the proximal half of small intestines were carefully washed with cold PBS and villi were scraped off using a cover slip. The remaining tissue with crypts and lamina propria were then cut into 2 mm fragments and incubated in 10 mL Gentle Cell Dissociation Reagent (STEMCELL technologies) at 4 °C for 30 min. The fragments were then shaken vigorously and the supernatant was collected as the first fraction in a new conical tube. The remaining fragments were further incubated in fresh Gentle Cell Dissociation Reagent and a new fraction was collected every 30 min. The first and second fractions were discarded since they might contain some debris and villi. The third to fifth fractions contained the majority of the intestinal crypts. The crypt-enriched fractions were
then washed with 10 mL of cold PBS and filtered through a 100 μm cell strainer (Falcon). The crypts were then further dissociated into single cells by incubation with TrypLE Express enzyme (GIBCO) at 37 °C for 5 min. The cells were filtered through a 40 μm cell strainer (Falcon). Remaining fragments with lamina propria were further digested with dissociation solution (2.5 mg/mL Liberase TL, Sigma; 10 U/mL DNAse, Promega) at 37 °C for 1 h.

To aid dissociation, we gently mixed tissue pieces by pipetting up and down every 10 min. After 20 min, supernatants were harvested and 1 volume of DMEM containing 10% fetal bovine serum (FBS, Sigma Aldrich) was added while adding 5 mL of fresh dissociation mix to the remaining tissue pieces. This step was repeated 3 times for a total time of 1 h. After completion of the 3 cycles, the remaining intestinal fragments were mechanically disaggregated on a 100 μm mesh using a syringe plunger. The cell suspension was filtered through a 40 μm cell strainer into a 15 mL tube. The tube and filters were washed twice with 1 mL of 2% FBS in PBS and the cell suspension was then centrifuged at 300 g for 5 min. The supernatant was removed and the cell pellets were resuspended in 100 μL of antibody mix (2% FBS; 1:125 CD45 (30-F11)-APC, BD Biosciences; 1:125 EPCAM (G8.8)-PE-Cy7, BioLegend) and incubated for 1 h on ice. Both epithelial and lamina propria cells were then washed with 3 mL of 2% FBS in PBS and filtered once more if clumps were observed. After centrifugation at 300 g for 5 min, the cell pellets were resuspended in 1 mL of 2% FBS and 10 U/mL DNase in PBS for sorting. The MOFLO system (Summit software v5.2, Beckman Coulter) or SH800S Cell Sorter (SH800 software v2.1.5, Sony) was used for cell sorting and data were analysed with FlowJo software (v10.6.2, BD).

**Library preparation and sequencing of RNA from single cells**

scRNA-seq libraries were generated using 10X Genomics kits. Since we wished to achieve statistically significant results across the wide range of intestinal cell types, for each biological replicate, cells sorted from gates R5, R6, R7 and R8 (Extended Data Fig. 6a) were pooled in equal ratios (RFP+ epithelial cell: YFP+ epithelial cell: mesenchymal cell: immune cells = 1: 1: 1: 1) and loaded into one channel of a 10X Chromium microfluidics chip to package them into one library. Thus, as a result of this enrichment, the relative proportions of epithelial cells, immune cells and mesenchymal cells are not expected to reflect the in vivo ratios found in the small intestine. In our experiments, 7 biological replicates (2 for the Confetti, 3 for the Red2-Kras<sup>G12D</sup> and 2 for the Red2-PIK3CA<sup>H1047R</sup> models) were used to make 7 libraries in total. The libraries were sequenced on an Illumina HiSeq 4000.

**Intestinal organoid culture and imaging**

Intestinal epithelial organoids were established as previously described<sup>48</sup>. Briefly, we freshly isolated crypts from mouse small intestine and mixed the crypts with 20 μl of Matrigel (Corning). After Matrigel polymerization, the crypts were cultured in ENR medium composed of advanced Dulbecco’s modified Eagle’s medium/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, N2 (Life Technologies), B27 (Life Technologies) and 1 mM N-acetylcysteine (Sigma), 50 ng/ml murine recombinant epidermal growth factor (EGF; Peprotech), R-spondin1 (conditioned medium from 293T-HA-RspoI-Fc cells, 10% final volume), and 100 ng/ml Noggin (Peprotech) for 3 days to generate organoids. Based on the evidences for the oncogene-associated changes in BMP and Wnt...
pathways. We tested two conditions: (1) withdrawal of Noggin from the full culture media (-Nog), which mimics a BMP-rich environment in Red2-Kras<sup>G12D</sup>, and (2) withdrawal of Noggin from R-spondin reduced (10% to 1%) media (-Nog Rspo<sub>low</sub>), which mimics the BMP-rich and Wnt-deprived environment in Red2-PIK3CA<sup>H1047R</sup> (Extended Data Fig. 9h-j). For organoid formation assay (Extended Data Fig. 9l-m), organoids were collected 2 days after treatment and then dissociated into single cell with TrypLE (Thermo Fisher Scientific) for 5 min at 37 °C. The dissociated cells were filtered through a 40 μm cell strainer (Falcon). 2×10<sup>4</sup> collected cells were mixed with 20 μl of Matrigel and seeded in each well of 48-well plate. After 20 min of solidification at 37 °C, 250 μl of WENR medium was added. ENR was supplemented with Wnt3a (conditioned medium from Wnt3a L-cells, 50% final volume), 10 μM Y-27632 (ROCK inhibitor, STEMCELL Technologies) and nicotinamide (Sigma) to make the WENR medium. Organoids were imaged and counted using an EVOS M7000 microscope (EVOS M7000 Revision software v2.0, Thermo Scientific).

To obtain organoids from Red2Onco mice, RFP+ cells were sorted from Villin-CreERT2;Red2Onco mice at 2w post-tamoxifen administration. 1×10<sup>5</sup> sorted cells were pelleted in 20 μl of Matrigel. After solidification at 37 °C, organoids were initially formed and treated with the following conditions: WENR: Wnt3a CM, Egf, Noggin and R-spondin1 CM; ENR: Egf, Noggin, and R-spondin1 CM; -Nog: withdrawal of Noggin from the ENR medium; -Nog Rspo<sub>low</sub>: withdrawal of Noggin and lowered R-spondin1 CM concentration (10% to 1%) from the ENR medium; -Egf + Gefitinib: withdrawal of Egf and addition of Gefitinib (EGFR inhibitor) (Extended Data Fig. 9q). Note that organoids from both mutants are sensitive to growth factor withdrawal while they are resistant to Egf removal.

To produce conditioned medium, 250 μl of WENR medium was added to organoids from Confetti or Red2Onco mice. After 7 days, organoids were supplemented with Noggin-deprived medium (-Nog) to generate conditioned medium. The conditioned medium was harvested after 7 days of culture. The medium was centrifuged at 1000 g for 5 min at 4°C, the supernatant was then filtered through 0.22mm filter (Sartorius). Wild-type organoids were then treated with either conditioned medium from R26R-Confetti or Red2Onco mice for 6 h with or without LDN193189, BMP type I receptor blocking agent (Selleckchem, S2618, 1 μM) to assess the effect of the conditioned medium on activation of BMP signalling.

RNA isolation and quantitative PCR

Isolated intestinal organoids or cells were resuspended in 350 μL of RLT buffer (QIAGEN). Total RNA was isolated using RNaseasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Equivalent quantities of total RNA were reverse-transcribed to synthesize cDNA using SuperScript cDNA synthesis kit (Life Technology). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Thermo). Primer sequences are as follows:

- **Lgr5**: F- CGGGACCTTGAGATTTCCT, R- GATTCGGATCAGCCAGCTAC
- **Olfm4**: F- CGAGACTATCGGATTCGCTATG, R- TTGTAGGCAGCCAGAGGGAG
- **Id1**: F- ATCGCATCTTGTGTCGCTGAG, R- AGTCTCTGGAGGCTGAAAGGT
**In vitro co-culture**

As described above, lamina propria was isolated from mouse small intestine. The cells were resuspended in 100 μL of antibody mix (2% FBS; 1:125 CD45 (30-F11)-APC, BD Biosciences; 1:125 EPCAM (G8.8)-PE-Cy7, BioLegend; 1:125 PDGFRA (CD140a).

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(APA5)-BV421, Biolegend; 1:125 CD81 (Eat-2)-PE, BioLegend) and incubated for 1h on ice. The cells were then washed with 3 mL of 2% FBS in PBS and filtered once more. After centrifugation at 300 g for 5 min, the cell pellets were resuspended in 1 mL of 2% FBS and 10 U/mL DNase in PBS for sorting. 8×10^4 sorted STC1 (CD81- PDGFRA^{lo} EPCAM- CD45-) or STC2 cells (CD81+ PDGFRA^{lo} EPCAM- CD45-) were cultured in 48-well plate in 20 μl of Matrigel together with 3×10^4 EGFP+ ISCs sorted from Lgr5-EGFP-IRESCreERT2 mice. Niche factor-reduced medium (-Nog Rspo_{low}, described above) was supplemented to test supporting function of the mesenchymal cells on organoid formation. Organoids were imaged using an EVOS M7000 microscope (Thermo Scientific).

Confocal imaging and quantification

Mouse small intestine samples were prepared for confocal microscopy as described above. Confocal imaging was performed with a Leica SP5 TCS confocal microscope (LAS software v2.8.0, Leica). The argon laser intensity was set to 20% and Z-step size was set to 2 μm. Laser scanning frequency was set to 400 Hz and the frame average was set to 3. Section images were processed and analysed using ImageJ. To chart the clone dynamics of labelled mutant and wild-type cells, intestinal crypt bottoms were visualised under the confocal microscope and clone sizes were quantified according to their circumferential span at the “+4 row position”, representing the border of the niche domain^{20} (Z-step 8-10; 16-20 μm upward from the crypt base). Clone sizes at the crypt bottom are denoted as eighths of the crypt circumference: 1 indicates that a fraction of 1/8 of the crypt circumference is occupied by a single clone, etc. Clone sizes at the crypt neck are determined as the total circumferential angle spanned by the clone. Statistical analysis of the detailed cellular arrangements of clones at the crypt base confirmed that such measures were representative of ISC clone sizes (Extended Data Fig.2b-e).

To perform reliable and consistent measurements, we set z-stack positions for each crypt by considering the first z-plane showing any epithelial cell as the z=0 position of individual crypts. Consistent with studies based on the original Confetti construct, yellow (EYFP), red (RFP) and cyan (mCFP) labelling was induced in approximately equal proportions, while green (nuclear GFP) cells were observed only infrequently (Extended Data Fig.1b). Note the similar degree of induction between models, which enables comparative clonal analysis using the R26R-Confetti and Red2Onco mice.

The central angle of the clonal circular sector (Extended Data Fig. 2g) was determined by 3 points (P1: clone starting point, Pc: crypt center, P3: clone end point) on the crypt circumference. Only RFP+ clones (wild-type in Confetti mice, mutant in Red2Onco mice) or YFP+ clones (wild-type) were quantified. Wild-type clones in Red2Onco mice were grouped with respect to their proximity to crypts containing mutant clones (Fig. 1d). Wild-type (YFP+) clones distant (3 crypt diameters or more) from crypts containing mutant clones were considered to be “remote” while YFP+ clones neighbouring fixed mutant (RFP+) crypts were considered to be “proximate”. In Extended Data Fig. 4, proximate wild-type clonal data from Fig. 1 were reanalysed and partitioned into two groups, “inner” and “outer”, depending on whether the geometric centre of the clones was positioned in the crypt half nearest to or furthest from the mutant crypt (Extended Data Fig. 4e). For quantification...
of crypt base columnar cells, either the nuclear morphology (Extended Data Fig. 3i,4j) or overlap with Lgr5-GFP was used (Fig. 2d,f). Three-dimensional representations were created using Image J (1.52e). The number of cells per crypt was counted in Z-stacks.

Crypt size and circularity measurement

As indicated in Extended Data Fig. 5a, for analysis of the circularity and area of individual crypts, thresholded images were generated by ImageJ. The regions of interest were then analysed using the “Analyze Particles” function in ImageJ to obtain outlined images and measures of location, size and circularity of individual crypts. Circularity was defined as $4\pi \times \left(\frac{\text{area}}{\text{perimeter}^2}\right)$ so that unity represents a perfect circle and a figure <1 indicates a degree of distortion (Extended Data Fig. 5d,e). For these analyses, the particle size was set to a lower limit of 400 pixels. The size limit corresponds to circular particles of approximately 20 μm in diameter and was chosen to exclude cells in the lamina propria from the analysis.

Intestinal clone dynamics in Red2Onco mice

To address the influence of oncogene expression on the clone dynamics of mutant and wild-type crypts, we use an established statistical modelling approach used previously to study stem cell dynamics of the intestinal crypt under normal and perturbed conditions19,27. In this approach, quantitative features of the clonal data, including the time evolution of the average clone size (scored as fraction of the crypt circumference) and crypt fixation frequency, are fit against a statistical modelling scheme based on a minimal number of adjustable parameters. Best fit parameters are then obtained using a standard least-squares fitting approach. Details of the modelling framework, its justification, and the implementation of the statistical analysis are presented in the Supplementary Theory.

Single-cell RNA-seq data analysis

Data processing for scRNA-seq—The raw sequencing data from the 10X Genomics platform were processed using CellRanger (v2.1.1). CellRanger aligned reads, filtered empty dropouts and counted unique molecular identifiers (UMIs) to generate a count matrix. We used Ensembl GRCm38/mm10 (release 92) appended with the sequences for tdimer2 (RFP) and EYFP as the reference genome for the read alignment. The filtration of empty droplets was also checked with the R package DropletUtils (v1.2.2). To filter out low quality cells, cells with less than 100 genes were removed. In addition, cells with mitochondrial proportions above 15% were also discarded from further analysis. Genes expressed in less than 3 cells were removed. Basic statistics and QC metrics for all samples are included in Supplementary Table 1. EYFP+ cells were considered to be wild-type epithelial cells in the intestinal crypts of Confetti, Red2-Kras$^{G12D}$ and Red2-PIK3CA$^{H1047R}$ animals, whereas EPCAM+ and EYFP- cells were considered to be mutant epithelial cells in the intestinal crypt. UMIs were normalised by a deconvolution method using the R package scran (v1.12.1)49.

Dimension reduction and data visualisation—PCA combined with technical noise modelling was applied to the normalised data for dimension reduction, which was implemented by the denoisePCA() function in the R package scran. This denoise PCA does not strictly require explicit feature selection, such as highly variable genes. The data were
then projected using two-dimensional Uniform Manifold Approximation and Projection (UMAP) or t-Distributed Stochastic Neighbor Embedding (t-SNE) with default parameter setting.

The biological replicates for each condition (2 for Confetti, 3 for Red2-Kras$^{G12D}$ and 2 for Red2-PIK3CA$^{H1047R}$ models) overlapped well with each other, implying reproducibility between replicates and providing confidence in the statistical reliability of our comparative analysis. The reproducibility confirmed batch effects between different samples and conditions were negligible and batch effect correction was not necessary for further analysis (Extended Data Fig. 6b).

Data clustering and cluster annotation—Based on expression of marker genes such as Epcam, Vim and Ptprc (Cdh45), all cells were split into three major categories of epithelial cells, mesenchymal cells and immune cells. For each category, we then performed clustering using a graph-based method. First, a shared nearest-neighbor graph was constructed using k nearest-neighbors of each cell (buildSNNGraph function in the R package scran). k was set to 6 for epithelial cells and to 10 for mesenchymal and immune cells. In this graph, two cells were connected by an edge if they shared nearest-neighbours, with the edge weight determined by the highest average rank of the shared neighbours. Then the Walktrap method from the R package igraph (v1.2.4.1) (with steps = 4 as the default option) was used to identify densely connected communities that were considered to be cell clusters.

Cell clusters were annotated based on differentially expressed genes and known marker genes for cell types. To annotate epithelial cells of the intestinal crypts, we referred to marker genes for cell types used in Extended Data Figure 1 of a previous study.32 If a few neighbouring clusters in the dimension reduction spaces shared key expression patterns, they were merged into one cell type manually. Thus, we classified 33 clusters into 8 epithelial cell types (Extended Data Fig. 6c-e). As a result, we confirmed that the fractions of individual cell types in the Confetti control were comparable to those in previous reports.32–34 The fractions were as follows: 29.6% for stem cells; 30.2% for TA cells, 11% for enterocyte progenitors; 5.3% for enterocytes; 3.6% for Paneth cells; 14.7% for goblet cells; 2.4% for tuft cells; 3.2% for enteroendocrine cells. We note that our stringent removal of the villus fraction during sample preparation is likely to explain the smaller fraction of enterocyte lineage cells that are found compared to other reports.32–34. While annotating epithelial, mesenchymal, and immune cells we found that, out of a total of 21,183 cells, 1,400 cells (6.6%) had a very low number of genes, and showed ambiguous and promiscuous expression. We considered them to be pseudo-cells, possibly contaminated with ambient RNA floating in single-cell suspension, and they were removed from further analysis.

Statistical analysis of cell type composition—To have an overview of cell type composition, the fraction of each cell type was calculated by taking the number of a cell type normalized by the total number of cells for each animal. To detect compositional change of cell types, the number of each cell type was modelled as a random count variable using a Poisson process. The rate of detection was modelled using the total number of cells profiled in each condition (RFP+ or YFP+) of an animal as an offset variable, with the condition of each animal (Confetti or Red2Onco) used as a covariate. We fitted the model using the glm...
function in the R package stats (v3.6.0). The p-value for the significance of the effect due to oncogene expression was estimated using a likelihood ratio test on the regression coefficient.

To interpret the fractional change of stem and progenitor cells in Extended Data Fig. 6h, it is important to note that the mutant stem cell fraction may not correlate straightforwardly with the increased division rate observed in Red2-Kras^G12D and Red2-PIK3CA^H1047R conditions (Extended Data Fig. 3h,i). While both mutant and neighbouring WT crypts show a fractional decrease of stem cell number, the absolute number of mutant stem cells in both Red2-Kras^G12D and Red2-PIK3CA^H1047R mice remained comparable to the Confetti and remote WT controls (Extended Data Fig. 6k,l). This implies that although oncogene expression increases the division rate of mutant stem cells, it may result in more rapid production of progenies rather than increasing stem cell number. If oncogene expression drives an increased rate of differentiation, it is more likely that the number of mutant stem cells may be outweighed by an increase in the abundance of their differentiating progenies, leading to a net reduction in the mutant stem cell fraction. It is likely that such changes in differentiation bias are responsible for the significant drop in the fraction of mutant stem cells, as observed in Extended Data Fig. 6h,l,m.

As described above, the compositional changes of immune and mesenchymal cells were analysed as well. The results are displayed in Extended Data Fig. 8g,h except stromal cell 1, 3, B-cell, macrophage 1 and plasma cell that do not show significant fractional change (data not shown).

**Analysis of priming toward differentiation**—To understand the origin of the altered relative proportions of mutant and wild-type epithelial cell types of the Red2Onco mice, we calculated the degree of fate priming of stem cells and their progenitors using the R package FateID (v0.1.9).51 We first identified marker genes for each differentiated sub-lineage, including enterocytes, Paneth cells, goblet cells, Tuft cells and enteroendocrine cells. To this end, we analysed single-cell RNA-seq data generated from small intestinal crypts in a previous study.32 We identified highly expressed genes in each of the differentiated sub-lineages using the findMarkers function from the R package scran (FDR < 0.05). As a result, the numbers of the marker genes for differentiated sub-lineages were as follows: 2,497 genes for enterocyte; 4,941 genes for goblet cells; 2494 genes for Paneth cells; 3,416 genes for Tuft cells; 4660 genes for enteroendocrine cells. Using the marker genes for differentiated sub-lineages, we then calculated the fate bias scores of all epithelial cells for each differentiated sub-lineage using two functions, reclassify and fateBias, of the FateID package with default parameter values. We considered the fate bias scores to be the degree of fate priming. Then the distribution of the priming scores for stem cells and their progenitors in the Red2Onco mice was compared with that from the Confetti control using the Kolmogorov–Smirnov test in GraphPad Prism 8.

**Gene set enrichment analysis (GSEA)—**Based on prior knowledge, we selected three major signalling pathways, Wnt, BMP and Notch, that significantly affect the stem cell and differentiation potential of intestinal stem cells.2,35,52 To define gene sets for the pathways, we curated and referred to previous studies where the pathways of interest were considered to be specifically altered in intestinal crypts. Specifically, as the gene set for the BMP
pathway, we used 293 genes that were highly expressed in BMP4-treated Lgr5+ intestinal organoids\textsuperscript{53}. As the gene set for the Wnt pathway, we used 113 genes (MSigDB id M1428) that were highly expressed in the intestinal crypts of Apc KO mice\textsuperscript{54}. As the gene set for the Notch pathway, we used 315 genes highly expressed in the intestinal crypts of Atoh1 KO mice as compared to DBZ-treated mice\textsuperscript{55}.

Using the manually curated gene sets, we performed gene set enrichment analysis using the R package AUCell (v1.6.1). To identify “active” cells with high enrichment scores, the distribution of the enrichment scores were fitted using the AUCell_exploreThresholds function of the R package AUCell and the cut-off (“Global_k1” value) for the high enrichment scores was selected among those suggested by the AUCell_exploreThresholds function. The fraction of active cells above the cut-off for each pathway is as shown in Fig. 3e and Extended Data Fig. 7m.

See Supplementary Table 2 for the details of the gene sets used in Fig. 3 and Extended Data Fig. 7.

**Estimating degree of transcriptomic change**—To estimate the degree of transcriptomic change for mesenchymal and immune cells, for each cell type we tested the statistical significance of the distance between the cell clusters in the Confetti and Red2Onco mice using two different methods:

In the first method, statistical significance was estimated based on how much larger inter-variability between cell clusters is than intra-variability. To this end, we defined cell-to-cell variability as $1 - \text{(Pearson correlation)}$ for any pair of cells. If two cells from the same model (either the Confetti control or Red2Onco mice) are selected, the cell-to-cell variability was considered to be the intra-variability. On the other hand, if one cell is selected from the Confetti control and the other from a Red2Onco mice to form a pair, the cell-to-cell variability was considered to be the inter-variability. The dispersion and mean of the distributions of the inter-variability and intra-variability was summarised in the form of a t-statistic. To make a null hypothesis distribution for this statistic, we randomly sampled cells for each cell type and formed ‘pseudo-confetti’ and ‘pseudo-Red2’ samples, before calculating the t-statistics for the variability; this was repeated 20,000 times and generated an empirical null hypothesis distribution of the statistics. The significance of larger inter-variability than intra-variability was tested against this null hypothesis distribution, generating a p-value, $P_{\text{VAR}}$.

In the second approach, we used Augur (v1.0.2), a method to rank cell types based on their degree of response to biological perturbations in single-cell data\textsuperscript{56}. Augur employs a machine-learning framework to quantify the separability of perturbed and unperturbed cells within a high-dimensional space of single-cell measurements. To feed our dataset to Augur workflow, the Confetti cells were randomly selected and split into two groups, Confetti set A and B, for each cell type. Then condition-specific AUC values for each cell type were calculated by comparing Confetti set A vs. Red2Onco mice and Confetti set A vs. B using the calculate_auc function with subsample_size=6 and default values for other parameters. To calculate the null distribution of AUCs for each cell type and condition, calculate_auc
function was executed again for *Confetti* set A vs. *Red2Onco* mice and for *Confetti* set A vs. B with subsample_size=6, augur_mode="permute", and default values for other parameters. Using the condition-specific AUCs and their null distributions, the statistical significance of differential prioritization between *Confetti* set A vs. *Red2Onco* mice and *Confetti* set A vs. B was calculated by running calculate_differential_prioritization function with default parameter values. To guarantee robustness of our results, we repeated 50 times the random splitting of the *Confetti* cells followed by the calculation of the statistical significance of the differential prioritisation, and chose the median p-values as the representative ones (P\text{AUGUR}) for each cell type and condition. P\text{VAR} and PAUGUR for each cell type were compared, as shown in Fig. 3i for mesenchymal cells and Extended Data Fig. 8i for immune cells.

**Gene ontology analysis**—Differentially expressed genes in the stromal cell STC2 cluster between *Red2-PIK3CA^{H1047R}* and *Confetti* were identified using the findMarkers function from the R package scran. 377 genes with p-value < 0.05 and absolute value of log2(fold-change) > α (=0.259), where α is the 95th percentile of absolute values of all log2(fold-change), were selected for gene ontology analysis. The gene ontology analysis was performed using DAVID (v6.8)\textsuperscript{57}.

**Statistical analysis**

GraphPad Prism 8 software was used to perform statistical analyses. Unless otherwise specified, statistical significance was determined by applying the Student t test or analysis of variance (ANOVA) to raw values from at least 3 independent experiments. No statistical methods were used to predetermine sample size. No method of randomization was followed and no animals were excluded from this study. The investigators were not blinded to sample allocation during the experiments and assessment of results.

**Sample size, randomization and blinding**

The sample size was chosen based on previous experience in the laboratory and the literature\textsuperscript{15,19}. No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.
Extended Data

Extended Data Figure 1. Red2Onco system: an oncogene-associated multicolour reporter

a, Representative confocal images of mutant clones from sections (Red2-Notch1ICD) or whole mounts (Red2-Kras\textsuperscript{G12D} and Red2-PIK3CA\textsuperscript{H1047R}) of small intestine from Villin-CreERT2;Red2Onco mice at 2w post-tamoxifen administration. Crypt borders are marked with a grey dashed outline.

b, Average clone numbers collected from a single field of image (0.15 mm\textsuperscript{2}) of whole mount small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco mice at 2d post-tamoxifen administration.

c-e, Representative confocal images (left) and quantification (right) of EdU+ proliferating crypt cells (c), LYZ+ Paneth cells (d) and MUC2+ goblet cells (e) from sections of small intestines from Villin-CreERT2;Red2Onco mice at 2w post-tamoxifen administration.

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intestine from Villin-CreERT2; R26R-Confetti or Red2Onco mice at 4w post-tamoxifen administration.

f, Representative confocal images of 100 μm-thick sections or whole mounts of tissues from adult R26R-CreERT2; Red2Onco mice (skin and stomach corpus), SftpC-CreERT2; Red2Onco mice (lung) or Krt5-CreERT2; Red2Onco mice (oesophagus) at 1w, 2w and 4w post-tamoxifen administration. The white dashed line indicates the epithelial lining. β-catenin stained as a cell membrane marker. SPC marks alveolar type II cells in lung.

g, Representative confocal images of sectioned mouse embryonic pancreas tissue from the R26R-CreERT2; Red2-Kras\textsuperscript{G12D} model at embryonic day 18.5, 6d post-tamoxifen administration. Magnified panel to the right shows an example of acinar cell expansion in developing pancreas. CPA1 marks acinar cells.

Conf: R26R-Confetti; R2N1: Red2-Notch1ICD; R2KR: Red2-Kras\textsuperscript{G12D}; R2P3: Red2-PIK3CA\textsuperscript{H1047R}. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) was determined by one-way ANOVA with Games-Howell’s multiple comparisons test (b,c) or unpaired two-tailed t-test (d,e) from biological replicates. Data are means ±SD (b) or means ± SEM (c,d,e). Exact P values are presented in Source Data. Scale bar: 50 μm (a,c,d,e,f) or 200 μm (g).
Extended Data Figure 2. Oncogenes drive non-neutral clone expansion in the mouse intestinal crypt

a, Schematic illustration of clonal events within the Red2Onco system (left panel) and representative tile scan images (right panel). Images are representative of tissues quantified in (h-k). Arrow: WT crypts; Arrowhead: fixed mutant crypts.

b, Representative confocal images of the base and neck of crypts. Images are representative of tissues quantified in (e).
**c-e**, Graph demonstrating strong correlation between clone size at the base and neck of a crypt from *Villin-CreERT2;R26R-Confetti* (2w post-tamoxifen administration) or *Red2Onco* mice (1w post-tamoxifen administration).

**f**, Average number of clones per 100 crypts.

**g**, Schematic illustration of mutant (RFP+) and WT (YFP+) clones in crypts, remote from each other.

**h**, Representative confocal images at 4d, 1w, 2w and 3w post-tamoxifen administration. Images are representative of tissues quantified in (**i-k**). Red2-Wild-type: remote YFP+ clones; Red2-Mutant: RFP+ clones.

**i**, Heatmaps indicate the relative clone fractions of the indicated sizes (columns) at various time points post-induction (rows). Black dots denote mean ± SEM.

**j,k**, Average clone size (**j**) and percentage of monoclonal crypts (**k**) at different time points post-tamoxifen administration. Asterisk for statistical significance omitted in graphs (**j,k**) for better visualization.

Confocal images of small intestine from *Villin-CreERT2;R26R-Confetti* (**a,b,h**) or *Red2Onco* mice (**a,h**). Crypt borders are marked with a white dashed outline (**a,b**). Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) was determined by unpaired two-tailed Pearson’s correlation test (**c-e**), one-way ANOVA with Games-Howell’s multiple comparisons test (**j**) or unpaired two-tailed t-test (**k**) from biological replicates. Data are means ±SD (**f**) or means ± SEM (**j,k**). Exact P values are presented in Source Data. Scale bar: 100 μm (**a,b**) or 50 μm (**h**).
Extended Data Figure 3. Biophysical modelling of mutant clone expansion

**a,d,** Contour plots showing mean-square differences of clone size distribution between neutral drift model and YFP clone data from Confetti (left), WT crypts remote from mutant crypts in R2KR (middle) and R2P3 (right) in (a); and between biased drift model and RFP mutant (MT) clone data from R2KR (left), R2P3 (middle) and R2N1 (right) in (d). Plots: scan of loss/replacement rate $\lambda$ vs. time-delay between injection and induction in (a); and drift bias $\delta$ with time-delay of 0.29w (R2KR), 0w (R2P3), and 0.43w (R2N1) in (d). Blue lines in (d): constraint $\lambda (1 - \delta) = \lambda_{WT}$, where $\lambda_{WT} =$ loss/replacement rate inferred from
Confetti (Supplementary Theory). Analysis in (a) and (d) based on data in (c) and (f), respectively.

b,e, Average clone size (effective stem cell number) in (b) and (e) from (a) and (d), respectively. Points show data, lines show model prediction at optimal parameter values. In each case, total effective stem cell number $N=5$, so that an average clone size of, e.g., 2 corresponds to circumferential angle of $360° \times 2/5$.

c,f, Distribution of clone sizes in (c) and (f) for models from (a) and (d), respectively. Points show data, lines show model prediction at optimal parameter values.

g, Representative confocal images of cleaved Caspase-3+ apoptotic cells. A single cleaved Caspase-3+ apoptotic cell in villus tip indicated by white arrow as positive control.

h,i, Representative confocal images (h) and quantification (i) of EdU+ proliferating crypt base columnar cells.

Whole mount of small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco at 1w (g) or 2w (h,i) post-tamoxifen administration. Conf: R26R-Confetti; R2N1: Red2-Notch1ICD, R2KR: Red2-Kras$^{G12D}$, R2P3: Red2-PIK3CA$^{H1047R}$. Significance (*$P<0.05$; **$P<0.01$; ***$P<0.001$) determined by unpaired two-tailed $t$-test (i) from biological replicates. Data are means±SEM (b,c,e,f,i). Exact $P$ values in Source Data. Scale bar: 50μm (g) or 25μm (h).
Extended Data Figure 4. Mutant crypts perturb clonal dynamics of WT cells in neighbouring crypts

a, Representative confocal images of tissues quantified in (b). Fixed (monoclonal) WT crypts are indicated by white arrows.

b, Percentage of monoclonal WT small intestinal crypts.

c,d, Average clone size (c) and percentage of monoclonal crypts (d) of remote and proximate WT (YFP+) clones at different time points after tamoxifen administration.

e, Schematic illustration for proximate WT clones in relation to fixed mutant crypts.
f, Heatmaps indicate the relative clone fractions of the indicated sizes (columns) at various time points post-induction (rows). Black dots denote mean ± SEM.

g, Average clone size of proximate (inner and outer) WT (YFP+) clones at different time points post-tamoxifen administration.

h, Average clone size $\langle \theta \rangle / 360^\circ$ of WT (YFP+) clones in crypts neighbouring fixed mutant crypts as a function of time $t$ post-induction. Points show mean ± SEM. Blue line shows a fit to the square root dependence predicted by the neutral drift model (Supplementary Theory). Orange line shows the 95% confidence interval.

i, Schematic illustration of factors affecting rate of clonal drift (Supplementary Theory).

j, Representative images (left panel) and quantification (right panel) of OLFM4+ ISCs. Arrow: proximate WT crypts; Arrowhead: fixed mutant crypts. Crypt borders are marked with grey dashed outlines.

Confocal images of whole mount small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco mice (a), and Lgr5-EGFP-IRES-CreERT2;Red2Onco mice (j) at 2w post-tamoxifen administration. Significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) was determined by one-way ANOVA with Games-Howell’s multiple comparisons test (c,g) and unpaired two-tailed t-test (b,d,j) from biological replicates. Data are means ±SEM (b,c,d,f,g,h) or means ±SD (j). Exact $P$ values are presented in Source Data. Asterisk for statistical significance omitted in graphs (c,d,g) for better visualization. Scale bar: 50 μm (a,j).
Extended Data Figure 5. Reduced effective stem cell number leads to accelerated drift dynamics

a, Original image (left panel) was thresholded (upper-right) and outlined (lower-right) to measure crypt size and circularity. Image representative of tissues quantified in (b-e).

b,c,d,e, Scatter and violin plots display size (b,c) and circularity (d,e) of WT crypts vs. distance from nearest fixed mutant (RFP+) crypt.

f,g, Illustration (f) and confocal images (g) of clones representative of tissues quantified in (h,i).

h, Heatmaps indicate relative clone fractions of given sizes. Black dots denote mean±SEM.
i, Percentage of monoclonal crypts of proximate WT (YFP+) clones. 

j,k, Confocal images (j) and quantification (k) of EGFP+ (Lgr5+) ISCs. Images representative of tissues quantified in (k,l). White dashed line: EGFP+ cells in WT crypts.

l, Violin plots display size of WT crypts in relation to multiplicity of neighbouring mutant crypts. n number for each group is shown.

m,n, Representative confocal images of Red2Onco intestine (m) and fractions of WT crypts from single field (0.15mm²) (n).

o, Illustration (left) and representative images (right) of crypt fission and fusion event in ‘8-shaped crypts’30. Images representative of tissues quantified in (p).

p, Percentage of crypts undergoing crypt fission (upper) or fusion (lower). Whole mount of small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco mice (a-i,o,p,m), and Lgr5-EGFP-IRES-CreERT2;Red2Onco mice (j,k) at indicated time-points. Proximate WT crypts and fixed mutant crypts indicated by white arrows and arrowheads, respectively (g,j). Crypt borders marked by dashed grey outlines (g,j,o). In (b,d), blue shaded area and red dashed line indicate 95% confidence interval of R26R-Confetti controls and average distance between the centre of fixed mutant crypt and proximate WT crypts, respectively. Significance (*P<0.05; **P<0.01; ***P<0.0001) determined by unpaired two-tailed t-test (c,e,i,k,l,p). Data are means±SD (i,k,n) or means±SEM (h,p). Exact P values presented in Source Data. Scale bar: 50μm (a,g,j,m,o).
Extended Data Figure 6. Oncogene-driven signalling changes

a, FACS sorting strategy to isolate cells from Confetti and Red2Onco. R1: live; R2: singlet; R3: mesenchymal/immune (EPCAM-); R4: epithelial (EPCAM+); R5: mutant-epithelial (RFP+); R6: WT epithelial (YFP+); R7: immune (CD45+); R8: mesenchymal (CD45-).

b, Box and whisker plots showing distributions of Pearson correlation coefficients in averaged log2-transformed normalised UMIs for cell types across all pairs of mice from same (white) and between different (grey) conditions.
c, UMAP of epithelial cells detected by Louvain. k: k nearest-neighbour value.
d, UMAPs showing distribution of averaged expression of marker genes. Colour bars: averaged log2-transformed normalised UMIs. Upper-left from Fig. 3b.
e, Heatmap representing marker expression for epithelial cells. Coloured panel (left) groups marker genes (right) for cell types. Colour bar: auto-scaled log2-transformed normalised UMIs.
f, Heatmaps representing differential gene expression for epithelial cells in Red2Onco compared to Confetti. Parentheses: number of differentially expressed genes. Colour bar: log2(fold-change). (Supplementary Table 1.)
g, UMAPs showing distributions of mutant (RFP+) and WT (YFP+) epithelial cells for Confetti and Red2Onco.
h,i, Fractions of mutant (h) and WT (i) epithelial cells in Red2Onco and Confetti. See Fig. 3c for other WT.
j,k,l, Confocal images (j) of EGFP+ cells, representative of tissues quantified for stem cell number (k) and fraction (l). In (j), white arrows indicate WT crypts proximate to mutant (MT) crypts. White-dashed lines mark crypts. Scale bar: 25μm.
m,n, FACS plots (m) and quantification (n) of EGFP + stem cell fractions from R5 or R6 (a). Small intestine from Lgr5-EGFP-IRES-CreERT2;Red2Onco at 2w post-induction (clonal dosage (0.2mg/20g body-weight) for (j,k,l), mosaic dosage (4mg/20g body-weight) for (m,n)). Significance (*P<0.05; **P<0.01; ***P<0.0001; statistically not significant (n.s.) P>0.05) determined by two-sided Kolmogorov–Smirnov test (b), two-sided likelihood ratio test (h,i) and one-way ANOVA with Games-Howell’s multiple comparisons test (k,l,n). Data mean±SEM (h,i,k,l) or mean±SD (n). Exact P values in Source Data.
Extended Data Figure 7. Mutant crypt induces primed differentiation

**a,b,** Priming scores of stem (SC) and TA cells of mutant (a) and WT (b) crypts toward secretory and enterocyte lineages in Red2Onco and Confetti. Thin (25th and 75th percentile) and thick (50th percentile) black dotted lines. Green and black asterisk: higher and lower in Red2Onco compared to Confetti, respectively.

**c,** qPCR of lineage markers (Lgr5: ISC; Clca1: Goblet cell; Fabp1, Alpi: Enterocyte; Mki67: proliferation) using sorted RFP+ or YFP+ cells from Villin-CreERT2;R26R-Confetti or Red2Onco at 2w post-tamoxifen administration.

**d,e,** Confocal images (d) and quantification (e) of MUC2+ goblet cells.

**f,h-j,** Images (f) from RNA in situ hybridization of enterocyte marker Fabp1 and quantification in remote WT (Remote_R2KR, Remote_R2P3) (h), proximate WT (Prox_R2KR, Prox_R2P3) (i) and mutant crypts (MT_R2KR or MT_R2P3) (j) along crypt axis. In (f), Fabp1+ cells in lower crypts (below +8) marked by white arrow.

**g,** Illustration of cellular localisation along crypt axis. Position 0: crypt base cell.

**k,l,** UMAPs showing distributions of enrichment scores for BMP (k left), Wnt (k right), and Notch (l) pathways in epithelial cells of Red2Onco and Confetti. Colour bars: enrichment scores.

**m,** Fractions of “active” cells with high enrichment scores for Notch pathway in mutant (MT) and WT epithelial cells from Red2Onco and Confetti.
Small intestine sections from Villin-CreERT2;R26R-Confetti or Red2Onco at 2w post-tamoxifen administration (d,f). WT and mutant crypts marked with white and grey dashed outline, respectively (d,f). Remote WT in crypts separated by >3 crypt diameters from mutant crypts. Proximate WT in crypts neighbouring fixed mutant crypts. Significance (*P<0.05; **P<0.01; ***P<0.0001) determined by two-sided Kolmogorov–Smirnov test (a,b), unpaired two-tailed t-test (c,e,h,i,j) and two-sided likelihood ratio test (m). Data are mean±SEM (c,e,m) or mean±SD (h-j). Exact P values in Source Data. Scale bar: 50 μm (d,f).
Extended Data Figure 8. Mutation-induced environmental changes

**a**, t-SNE representing immune cells from *Confetti* and *Red2Onco*. 

**b,c**, Heatmaps representing differential expression (DE) patterns for mesenchymal (b) and immune (c) cells from *Red2Onco* and *Confetti*: top 300 genes or less (FDR<0.05, pairwise t-test). Colour bar: averaged Z-scores of log₂-transformed normalized UMIs.

**d**, Secretion factor expression in stromal clusters for *Confetti*. In (d,f), dot size: percentage of cells expressing gene; colour: average expression.

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e, UMAPs showing expression of Bmpr1a and Fzd7 in epithelial cells. Colour bar: log2-transformed normalized UMIs. Inset from Fig. 3b.
f, Dot plots showing expression of receptors upstream of BMP and Wnt pathways for epithelial cells.
g,h, Fractions of mesenchymal (g) and immune (h) cells in Red2Onco and Confetti. Data: mean±SEM. p-values from two-sided likelihood ratio test: *, p<0.05; **, p<0.01; n.s., statistically not significant (p>0.05).
i, Degree of transcriptomic change for immune cells estimated by cell-to-cell variability, p-value (PVAR), or separability of perturbed and unperturbed cells, p-value (PAUGUR). Dotted lines: − log10(0.01). Dot colour: cell types; Dot shape: Red2Onco.
j, Enriched biological processes from gene ontology (GO) analysis of DE genes in STC2 of Red2-PIK3CAH1047R relative to Confetti. p-value from one-sided Fisher exact test. Dotted line: − log10(0.05).
k,l, Heatmaps representing DE genes and their numbers (parenthesis) for mesenchymal (k), and immune (l) cells in Red2Onco compared to Confetti. Colour bar: log2(fold-change).
(Supplementary Table 3.)
m, Volcano plot representing DE genes in STC2 of Red2-PIK3CAH1047R relative to Confetti. p-value from two-sided pairwise t-test. Red dots: genes for biological processes of (j). Vertical (log2(fold-change)=0.259) and horizontal (−log10(0.05)) dotted lines.
n, Model of direct and indirect crosstalk between mutant and WT crypts in Red2Onco. ENC:endothelial cell; GLC:glial cell; IC:intestitial cell of Cajal; MF:myofibroblast; STC1,2,3:stromal cell 1,2,3; BC:B-cell; DC:dendritic cell; Mono:monocyte; MP1,2:macrophage 1,2; PLC:plasma cell; TC:T-cell. Exact P values in Source Data.
Extended Data Figure 9. Mutant clones secrete functional BMP ligands

a–d, Representative in situ hybridization images and quantification of Axin2 (a and b) and Id1 (c and d) on sections of small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco mice at 2w post-tamoxifen administration. Arrowheads: fixed mutant crypts. Crypts are marked with dashed outline.

e,f, qPCR analysis of Axin2 (e), and Id1 (f).

g, Experimental setup for (h)–(j).
**h,** Bright-field images of intestinal organoids after 2 days of treatment. The number and size of crypt-like budding structures are reduced in treated organoids.

**i,** qPCR analysis of lineage markers.

**j,** Representative images of Lgr5-EGFP organoids show that the number of Lgr5+ cells decreases following treatments.

**k,** Experimental setup for (**l,m**).

**l,m,** Bright-field images (**l**) and quantification (**m**) of intestinal organoids after 6 days of culture in WENR medium.

**n,** qPCR analysis of BMP ligands (*Bmp2* and *Bmp7*).

**o,** Experimental setup for (**p**).

**p,** qPCR analysis of *Id1* using WT organoids after the CM treatment.

**q,** Bright-field images of intestinal organoids from *Villin-CreERT2;R26R-Confetti* or *Red2Onco* mice at 1 month post-tamoxifen administration. Insets show RFP expression of the mutant organoids.

**r,** qPCR analysis of WT and mutant organoids cultured in ENR medium.

In (**e,f,n**), Sorted RFP+ or YFP+ cells from *Villin-CreERT2;R26R-Confetti* or *Red2Onco* mice at 2w post-tamoxifen administration (4mg/20g body weight, mosaic dosage) were analysed. MT: mutant crypts; Prox: proximate WT crypts. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) was determined by one-way ANOVA with Games-Howell’s multiple comparisons test (**b,d**) and unpaired two-tailed t-test (**e,f,i,m,p,r**). Quantification graphs show data from 3 independent experiments (**i,m,p,r**). Data are presented as mean ± SD (**b,d,i,m,p**) or mean ± SEM (**e,f,n,r**). Exact *P* values are presented in Source Data. Scale bar: 50 μm (**a,c,h**), 100 μm (**j,q**) and 500 (m).
Extended Data Figure 10. Mutant clones drive niche stromal remodelling

a, Heatmap showing marker gene expression for STC2 among mesenchymal cells. Colour bar: averaged Z-scores of log$_2$-transformed normalized UMIs over all cells within a cell type in Confetti.

b,c, Representative multiplexed in situ hybridization images (b) and quantification (c) of Sfrp2 in Grem1+ cells on small intestine sections from Villin-CreERT2;R26R-Confetti or Red2Onco at 2w post-tamoxifen administration. Fixed mutant crypts indicated by white arrowheads. Crypts marked with grey dashed outlines. Grem1+ STC2 cells indicated by white arrow.

d, Heatmap showing expression of marker genes and secreted factors in STC1,2 from Red2Onco and Confetti. Colour bar: averaged Z-scores of log$_2$-transformed normalized UMIs over all cells within a cell type and condition.

e, Projection of Pdgfra expression (middle) onto UMAP from Fig. 3f (Left) for comparison. Projection of Cd81 expression onto Pdgfra$^{lo}$ cell clusters (STC1,2) (right). Colour bar: log$_2$-transformed normalized UMIs.

f, Sorting strategy to isolate STC2 from intestinal mesenchymal cells by FACS. R1: non-immune cells (CD45-). R2: mesenchymal cells (EPCAM-). R3: PDGFRA$^{lo}$ population.

g, qPCR of STC2 marker (Cd81, Grem1), STC1 marker (Frzb) and secreted Wnt modulators (Rspo3, Sfrp2, Sfrp4) using sorted (CD45-,EPCAM-,PDGFRA$^{lo}$,CD81-) cells (STC1) or (CD45-,EPCAM-,PDGFRA$^{lo}$,CD81+) cells (STC2) from Villin-CreERT2;R26R-Confetti or Red2Onco mice at 2w post-tamoxifen administration.

h, qPCR of Telocyte markers (Pdgfra, FoxI1) using sorted STC1,2 and (PDGFRA$^{hi}$) Telocytes.

ENC: endothelial cell; GLC: glial cell; IC: intestinal cell of Cajal; MF: myofibroblast; STC1,2,3: stromal cell 1,2,3. Significance (*$P<0.05$; **$P<0.01$; ***$P<0.0001$) determined by one-way ANOVA with Games-Howell’s multiple comparisons test (c) and unpaired two-tailed t-test (g,h). Data presented as mean±SD (c,g) or mean±SEM (h). Exact $P$ values in Source Data. Scale bar: 25 μm (b).
Extended Data Figure 11. Functional validation of oncogene-driven niche remodelling

a,b, qPCR analysis of \textit{Id1} (a), \textit{Axin2} and \textit{Lgr5} (b) after administration of indicated inhibitor.

c, Fraction of monoclonal WT (YFP+) crypts remote from (Remote), or proximate to (Prox) mutant crypts in \textit{Red2Onco} mice.

d, Heatmaps indicate the relative clone fractions of the indicated sizes. Black dots denote mean ± SEM.

e, Fraction of monoclonal (RFP+) mutant crypts in \textit{Red2Onco} mice.
f,g, Representative confocal images (f) and quantification (g) of EGFP+ (Lgr5+) ISCs. Images are representative of tissues quantified in (g). Arrow: proximate WT crypts; Arrowhead: fixed mutant crypts.

h, Representative confocal images of whole mount small intestine. Images are representative of tissues quantified in (i). Arrowhead: fixed mutant crypts.

i, Violin plots of proximate WT crypt size.

j,k, RNA in situ hybridization (j) and quantification (k) of Bmp2. Arrowhead: fixed mutant crypts. MT: mutant crypts; Prox: proximate WT crypts.

l,m, Representative multiplexed in situ hybridization images (l) and quantification (m) of Rspo3 in Cd81+ cells. White arrow: Cd81 positive STC2 cells; Arrowhead: fixed mutant crypts.

Whole mount (f-i) and sections (j-m) of small intestine from Lgr5-EGFP-IRES-CreERT2 control (L5), Lgr5-EGFP-IRES-CreERT2;LSL-KrasG12D (enKR) or Pik3caLat-H1047R (enP3) mice at 2w post-tamoxifen administration. In (c,d,e), Graphs show data collected at 2w after concomitant administration of indicated drug and tamoxifen. Crypt borders are marked by dashed outlines (f,h,j,l). In (f,h,j,l), White (f,h) or Red (j,l): immunostaining for mutant KRASG12D in enKR, or p-AKT in enP3. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) was determined by one-way ANOVA with Games-Howell’s multiple comparisons test (k,m) and unpaired two-tailed t-test (c,e,g,i). Data are presented as mean ± SD (a,b,g,k,m) or mean ± SEM (c,e). Exact P values are presented in Source Data. Scale bar: 50 μm (f,h,j) and 25 μm (l).
Extended Data Figure 12. *Apc* mutation induces reduction of stem cells in the neighbouring wild-type crypts

**a,b,** Representative confocal images of small intestine from Villin-CreERT2; *Apc* 

\textit{f/f} mice at at 2 weeks (2w) post-tamoxifen administration (**a**) and *Apc* 

\textit{Min/+} mice at 12 weeks of age (**b**). Images are representative of 2 independent experiments. OLFM4 staining shows a reduced number of stem cells in wild-type crypts neighbouring mutant crypts. *Apc* mutant foci (Villin-CreERT2; *Apc* 

\textit{f/f}) or polyp (*Apc* 

\textit{Min/+}) are indicated by grey dashed outlines. Crypt borders are marked by white dashed outlines. Scale bar: 50 μm.
c, Bright-field images of intestinal organoids after 7 days of culture in ENR medium. Images are representative of 3 independent experiments. Note that organoids from Villin-CreERT2; Apc<sup>fl/fl</sup> mice form spheroids under ENR media condition. Scale bar: 500 μm.

d, qPCR analysis of Wnt target gene (Axin2) and secreted Wnt inhibitory factors (Dkk2, Wif1 and Notum) following Apc deletion. Data are presented as mean ± SD. N = 3 independent experiments. Quantification graphs show data from 3 independent experiments. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) was determined by unpaired two-tailed t-test.

e,f, Representative multiplexed in situ hybridization images of Axin2 and Wif1 (e), and Lgr5 and Notum (f) on sections of small intestine from Villin-CreERT2; Apc<sup>fl/fl</sup> mice at 2w post-tamoxifen administration. Images are representative of 2 independent experiments. Axin2 (e) and Lgr5 (f) staining shows a reduced number of stem cells in wild-type crypts neighbouring Apc mutant crypts. Apc mutant foci (Villin-CreERT2; Apc<sup>fl/fl</sup>) are indicated by grey dashed outlines. Crypt borders are marked by white dashed outlines. Scale bar: 50 μm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

The single-cell RNA-seq data generated for this study has been deposited in ArrayExpress under E-MTAB-8656. The reference genome sequence was downloaded from the Ensembl (http://www.ensembl.org/Mus_musculus) and used for alignment of the single-cell RNA-seq data. To evaluate stem cell priming, single-cell RNA-seq data was obtained from the Single Cell Portal (https://portals.broadinstitute.org/single_cell/study/small-intestinal-epithelium) and used to define gene sets for differentiated sub-lineages of epithelial cells. The lists of marker genes used to annotate types of epithelial, mesenchymal, and immune cells in Fig. 3b, f and Extended Data Fig. 6d, 8a,b,c are given in Supplementary Tables 1 and 3. Gene sets used in Fig. 3d,e, and Extended Data Fig. 7a,b,k,l,m are provided in Supplementary Table 2. Source data associated with Figs. 1-4 and Extended Data Figs. 1-12 are provided with the paper.
Code Availability

The statistical analysis of the clone fate data, based on a fit to the established modelling scheme, was performed using a FORTRAN (G95 compiler) code developed for this study. The single-cell RNA-seq data was analysed using publicly available R packages.

The codes and data used for clonal analysis and scRNA-seq data analysis have been deposited in GitHub (available at https://github.com/BenSimonsLab/Yum_Nature_2021).

References


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Figure 1. Red2Onco system: an oncogene-associated multicolour reporter

**a**, Schematic showing possible routes for crosstalk between mutant and neighbouring wild-type (WT) cells.

**b**, Red2Onco knock-in strategy. The 2A peptide sequence and oncogene cDNA (Kras\(^{G12D}\), PIK3CA\(^{H1047R}\) or Notch1ICD) were cloned in-frame downstream of the RFP cDNA in the R26R-Confetti cassette\(^{15}\), which encodes four fluorescent proteins.

**c**, Representative images from sections (left and upper-right panel) or whole mounts (lower-right panel) of small intestine from Villin-CreERT2;Red2-Kras\(^{G12D}\) mice at 2 days (d) or
2 weeks (w) post-tamoxifen administration. Images are representative of 3 independent experiments. White dashed line indicates mucosal lining. Crypt fission and fusion events are visible in lower-right image, and indicated by white arrow and arrowhead, respectively.
d, Schematic illustration of a WT (YFP+) clone in proximity to a fixed (monoclonal) mutant (RFP+) crypt. Clone sizes quantified as defined in Extended Data Fig. 2g.
e, Representative confocal images of WT (YFP+) clones remote from, or proximate to, fixed mutant (RFP+) crypts in whole mount small intestine from Villin-CreERT2;R26R-Confetti or Red2Onco mice at 1w, 2w and 3w post-tamoxifen administration. Images are representative of tissues quantified in Fig. 1f.
f, Heatmaps indicate relative clone fractions of indicated sizes (columns) at various time points post-induction (rows). Black dots denote mean±SEM. N = 6 mice per group and time point. (103,80,169) clones scored for Conf, (93,129,178) for Remote WT R2KR, (106,94,187) for Remote WT R2P3, (90,99,241) for Proximate WT R2KR, and (64,137,88) for Proximate WT R2P3 at (1w,2w,3w) post-induction, respectively. Remote WT: WT clones located in crypts separated by >2 crypt diameters from mutant crypts. Proximate WT: WT clones located in crypts neighbouring fixed mutant crypts. R26R-Confetti and remote WT control data from Extended Data Fig. 2i are reproduced for comparison in heat maps (f). Scale bar: 200μm (c-side views), or 50μm (c-bottom view,e).
Figure 2. Reduced effective stem cell number leads to accelerated drift of WT clones in crypts neighbouring mutant crypts

a. Cumulative size distribution of WT (YFP+) clones in crypts neighbouring crypts monoclonal for given mutant together with Confetti control plot against the angular clone size, $\theta$, rescaled by the average, $\langle \theta \rangle$. Points show data from 2 time-points and dashed line denotes scaling function, $\exp[-\pi (\theta / \langle \theta \rangle)^2/4] \times 100\%$, predicted by neutral drift model (see main text and Supplementary Theory).

b. Corresponding average clone size $\langle \theta \rangle/360^\circ$ as a function of time post-induction, scaled by effective drift rates, $x = t\lambda / N^2$ obtained from a fit to predicted square root dependence (dashed line) (Supplementary Theory). In (a,b), experimental data as in Fig. 1f and, for R2N1-prox, $N=6$ mice per group and time-point, and (87,128) clones were scored at (14d,21d) postinduction.

c-d. Representative confocal images (c) and quantification (d) of EdU+ proliferating Lgr5+ stem cells in WT crypts neighbouring mutant crypts. Proliferating stem cells in WT crypts are outlined with a white dashed line. $N = 5$ mice per group. For each mouse, 100 crypts analysed.
Representative confocal images (e) and quantification (f) of Lgr5-eGFP+ stem cells. EGFP+ cells in WT crypts are outlined with a white dashed line. N = 5 mice per group. For each mouse, 100 crypts analysed. Whole mount small intestine were imaged from Lgr5-EGFP-IRES-CreERT2;Red2Onco mice at 2w post-tamoxifen administration (c,e). Proximate WT crypts and fixed mutant crypts are indicated by white arrows and arrowheads, respectively (c,e). Crypt borders are marked by dashed grey outlines (c,e). Conf: R26R-Confetti; R2N1: Red2-Notch iiCD; R2KR: Red2-KrasG12D; R2P3: Red2-PIK3CAH1047R. Significance (*P < 0.05; **P < 0.01; ***P < 0.001) determined by unpaired two-tailed t-test (d,f). Data are means±SEM (b) or means ±SD (d,f) from biological replicates. Exact P values presented in Source Data. Scale bar: 50μm.
Figure 3. Comparative single-cell analysis identifies oncogene-driven niche changes

**a**, Schematic of comparative single-cell analysis. Mutant (MT) and WT epithelial, mesenchymal and immune cells isolated from small intestine. By comparing profiles across models, effect of oncogene expression on MT and WT epithelial cells and surrounding environment can be resolved.

**b**, UMAP showing clustering of epithelial cells based on marker expression: (Stem) cells, (TA) cells, enterocyte progenitors (EP), enterocyte (Ent), enteroendocrine cells (EEC), (Goblet) cells, (Paneth) cells and (Tuft) cells.

**c**, Fractions of WT epithelial cell types in Red2Onco models and Confetti control.

**d**, Priming scores of WT (YFP+) stem and TA cells toward enterocyte lineages in Red2Onco and Confetti. 25th and 75th percentiles denoted by thin black dotted lines; 50th percentile by thick black dotted line. Green asterisk: higher in Red2Onco compared to Confetti.

**e**, Fractions of “active” cells with high enrichment scores for BMP (left) and Wnt (right) pathways in MT and WT epithelial cells from Red2Onco and Confetti.

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f, t-SNE representing mesenchymal cell clusters. ENC: endothelial cell; GLC: glial cell; IC: interstitial cell of Cajal; MF: myofibroblast; STC1,2,3: stromal cell 1,2,3.

g,h, Dot plots showing expression of secreted factors known to modulate BMP and Wnt signalling in MT epithelial cells from Red2Onco and WT cells from Confetti (g) and in mesenchymal cells (h). Dot size denotes percentage of cells expressing given gene, colour denotes average expression across all cells of that type.

i, Degree of transcriptomic change for each mesenchymal cell type in Red2Onco (Methods). Conf: R26R-Confetti; R2KR: Red2-KrasG12D; R2P3: Red2-PIK3CAH1047R. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) determined by two-sided likelihood ratio test (c,e) and two-sided Kolmogorov–Smirnov test (d). Data presented as mean±SEM (c,e) from biological replicates (n=2 for Conf, R2P3; n=3 for R2KR). Exact P values presented in Source Data.
Figure 4. Functional validation of oncogene-driven niche remodelling

a, b, Representative in situ hybridisation images (a) and quantification (b) of Bmp2. WT and mutant crypts marked with white and grey dashed outline, respectively. MT: mutant crypts; Prox: proximate WT crypts. For each group, 50 crypts analysed from N=3 mice.

c, d, Representative multiplexed in situ hybridisation images (c) and quantification (d) of Rsop3 in Cd81+ cells. Crypts marked with grey dashed outlines. For each group, 50 crypt pairs analysed from N=3 mice.

e, Sorted Lgr5+ cells were cultured with either PDGFRA^{lo}CD81- STC1 cells or PDGFRA^{lo}CD81+ STC2 cells from Confetti or Red2-PIK3CA^{H1047R} intestine.
Representative bright-field images (f) and quantification (g) of intestinal organoids formed after 4d of co-culturing. N=3 independent experiments. MesConf, MesR2P3: mesenchymal cells from Confetti, Red2-PIK3CA\textsuperscript{H1047R} mice.

Representative confocal images (h) and heatmap distribution of clone fractions (i) of whole mount small intestine from Villin-Cre\textsuperscript{ERT2};R26R-Confetti or Red2Onco mice at 2w post-induction. Specific drug (LDN193189: BMP type I receptor blocking agent; LGK974: Porcupine inhibitor) or vehicle was administered following the dosing regimen (top right). Fixed WT crypts indicated by white arrows. Black dots denote mean±SEM. N=3 mice per group. 212 clones scored for Conf+Veh, (85,78,96) clones for Remote WT R2KR in (Veh,LDN, LGK) conditions, (91,90,113) for Remote WT R2P3, (130,108,75) for Proximate WT R2KR, (132,81,65) for Proximate WT R2P3.

Sections of small intestine from Villin-Cre\textsuperscript{ERT2};R26R-Confetti or Red2Onco mice at 2w post-induction (a,c). Fixed mutant crypts indicated by white arrowheads (a,c). Conf: R26R-Confetti; R2KR: Red2-Kras\textsuperscript{G12D}; R2P3: Red2-PIK3CA\textsuperscript{H1047R}. Significance (*P < 0.05; **P < 0.01; ***P < 0.0001) determined by one-way ANOVA with Games-Howell’s multiple comparisons test (b,d) and unpaired two-tailed t-test (g) from biological replicates. Data are means±SD (b,d,g). Exact P values presented in Source Data. Scale bar: 50μm (a,h), 25μm (c) or 500μm (f).