Endogenous murine microbiota member *Faecalibaculum rodentium* and its human homologue protect from intestinal tumour growth

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The microbiota has been shown to promote intestinal tumourigenesis, but a possible anti-tumourigenic effect has also been postulated. Here, we demonstrate that changes in the microbiota and mucus composition are concomitant with tumourigenesis. We identified two anti-tumourigenic strains of the microbiota—*Faecalibaculum rodentium* and its human homologue, *Holdemanella biformis*—that are strongly under-represented during tumourigenesis. Reconstitution of Apc\(^{min/+}\) or azoxymethane- and dextran sulfate sodium-treated mice with an isolate of *F. rodentium* (F. PB1) or its metabolic products reduced tumour growth. Both F. PB1 and *H. biformis* produced short-chain fatty acids that contributed to control protein acetylation and tumour cell proliferation by inhibiting calcineurin and NFATc3 activation in mouse and human settings. We have thus identified endogenous anti-tumourigenic bacterial strains with strong diagnostic, therapeutic and translational potential.

Colorectal cancer (CRC) is a multifactorial disorder influenced by genetic, environmental and lifestyle factors, including the deregulation of the microbiota. A decrease in *Clostridium* and *Bacteroides* and an increase in *Fusobacterium* has been reported in CRC as well as in association with recurrence. The role of bacteria in tumourigenesis has been extensively demonstrated in spontaneously occurring mouse models of tumourigenesis, such as Apc\(^{min/+}\) mice, which carry a mutation in the *adenomatous polyposis coli* (*APC*) gene, which is mutated in more than 80% of sporadic CRCs. Microbiota-derived signals drive ERK phosphorylation and increased stability of the oncogene *Myc*, or trigger the c-Jun/NFKB and STAT3 signalling pathways driving cell proliferation and accumulation of suppressive immune cells within the tumour or the exacerbation of the inflammatory response. Enterotoxigenic *Bacteroides fragilis* and colibactin-producing *Escherichia coli* exert their pro-tumourigenic effect through bacterial toxins. On the other hand, in an inflammation-induced model of colitis-associated CRC, germ-free mice develop significantly more and larger tumours after treatment with azoxymethane (AOM) and dextran sulfate sodium (DSS) compared with specific-pathogen-free mice. After faecal-microbiota transplantation, germ-free mice treated with AOM and DSS develop more tumours than healthy mice if transplanted with faecal microbiota from CRC patients but it is not clear whether this is due to an increase in tumour-promoting bacteria, a decrease in anti-tumourigenic bacteria or both. Furthermore, a diet rich in fibres can protect against tumour development in a microbiota-dependent manner. Thus, although most studies have concentrated on identifying tumour-promoting bacteria, the role and identification of endogenous anti-tumourigenic microbiota remains elusive.

Here, we identified an endogenous strain of the mouse microbiota (*Faecalibaculum rodentium* isolate PB1) and its human counterpart *Holdemanella biformis* belonging to the Erysipelotrichaceae family, which are lost during the early phases of tumourigenesis and block tumour-cell proliferation by reducing activation of nuclear factor of activated T cells, cytoplasmic 3 (NFATc3) and calcineurin.

**Results** *F. rodentium* is under-represented during the early phases of tumourigenesis. We followed the changes in microbiota composition in a longitudinal study in cohorts of Apc\(^{min/+}\) mice and...
**Fig. 1 | F. rodentium** is under-represented during the early phases of tumour development. **a–c,** 16S rRNA gene profiling of the faecal microbiota of WT and Apc\(^{-/-}\) mice at the age of 4, 8 and 12 weeks (n = 8 mice per group). **a,** Shannon diversity index. The box plots show the interquartile range, the horizontal lines show the median values and the whiskers indicate the minimum-to-maximum range. **b,** Genus abundance in WT (inner pie) and Apc\(^{-/-}\) (outer pie) mice. Genera with a relative abundance >1% in at least one of the tested conditions are shown, otherwise they are collapsed into the ‘Other genera’ section. **c,** Relative abundance of the ten-most abundant species in faecal bacterial DNA isolated from eight- (left) and 12-week-old (right) WT and Apc\(^{-/-}\) mice. Abundance is shown as the normalized number of assigned sequences in the 16S rRNA sequencing. **d,e,** Abundance of F. PB1, determined by qPCR, normalized to pan-bacterial primers targeting the 16S rRNA gene (UNI 16S) in bacterial DNA extracted from faeces (**d**, n = 8 mice per group) and mucus from the small intestine (**e**, left) and colon (**e**, right; n = 11 mice per group) of WT and Apc\(^{-/-}\) mice. *P* values were determined using multiple t-tests corrected for multiple comparisons using the Holm–Sidak method to compare F. PB1 abundance between groups at each time point (**d**) or using a two-tailed unpaired Student’s t-test (**e**). Data are from two independent experiments and represented as the mean ± s.e.m.

Among the ten-most abundantly represented taxonomic units of WT mice, we found that only the reads ascribed to *F. rodentium* \(^{13}\) were strongly and significantly under-represented in Apc\(^{-/-}\) mice compared with WT mice (*P* < 0.001). This taxon was not expanded in eight-week-old Apc\(^{-/-}\) mice, coincident with the initiation of tumour development (Fig. 1c and Extended Data Fig. 1c). These data were confirmed by quantitative PCR (qPCR; Fig. 1d). We then isolated and entirely sequenced a strain from WT mice belonging to this taxon and found that it is the only representative of *F. rodentium* in our WT mouse colony (Fig. 1c, not shown). The previously uncharacterized F. PB1 isolate was associated with the mucus of the small and large intestines but was drastically reduced in the mucus from Apc\(^{-/-}\) mice (Fig. 1e). We have thus identified a strain of *F. rodentium* that is normally highly abundant in WT mice and is strongly under-represented in Apc\(^{-/-}\) mice early in tumourigenesis.
**Fig. 2 F. PB1 loss coincides with mucus changes and reduces tumour growth when reintroduced.** a. Mucin expression in the ileal tissue of WT mice and healthy (H) and tumour (T) tissue of ApcMin/+ mice at the age of 8 and 16 weeks was determined using qPCR. The expression levels were normalized to the reference gene Rpl32. Data are from two independent experiments (8 weeks, n = 10 (WT), 8 (H) and 7 (T) mice; 16 weeks, n = 8 (WT) and 9 (H and T) mice). P values were determined using a one-way ANOVA with Bonferroni's post-hoc test to compare expression levels at the same time point. 

b. c. F. PB1 administration experiments in WT and ApcMin/+ mice pretreated with an antibiotic cocktail. b. F. PB1 abundance normalized to pan-bacterial primers targeting the 16S rRNA gene (UNI16S) in bacterial DNA extracted from ileal mucus, determined using qPCR. Data are from two independent experiments (WT, n = 7 (vehicle) and 10 (F. PB1) mice; ApcMin/+ n = 6 (vehicle) and 11 (F. PB1) mice). P values were determined using a two-tailed unpaired Mann–Whitney U-test. c. Representative images of fluorescence in situ hybridization of F. PB1 on the mucosal surface of an ApcMin/+ ileum poly (right) and normal WT (left) ileum. Nuclei have been stained with DAPI (blue). Scale bars, 50 μm; images were obtained at ×40 magnification; n = 3 mice per group. 

d. Four-week-old ApcMin/+ mice were treated with vehicle or F. PB1 until they were 8 weeks old (top). At 12 weeks, tumour multiplicity in the small intestine was assessed (n = 5 mice per group). Significance was determined by multiple t-tests corrected for multiple comparisons using the Holm–Sidak method to compare tumour multiplicity between groups at each time point. e. f. ApcMin/+ mice received vehicle or F. PB1 from week 8 to 12 (e, left). Two independent experiments were performed with consistent results. e. Tumour multiplicity in the small intestine normalized to vehicle-treated ApcMin/+ mice at the age of 12 weeks (right). Data are from two independent experiments (n = 14 mice per group). f. Area (right) and maximum diameter (axis length; left) of ileal dysplastic lesions normalized to the total number of lesions per mouse. Data are from one representative experiment (n = 7 mice per group). The box plots show the interquartile range, the horizontal lines show the median values and the whiskers indicate the minimum-to-maximum range. P values were determined using a two-tailed unpaired Student's t-test (e) and two-tailed unpaired Mann–Whitney U-test (f). a, b, d-f. Data are represented as the mean ± s.e.m. Veh., vehicle.

**F. PB1 loss coincides with mucus changes and protects from tumour development.** The mucus layer serves as a niche for the intestinal microbiota, and changes in its composition may influence the microbiota profile. During tumourigenesis, epithelial cell transformations could lead to modification in the production of mucus—the major components of mucus—resulting in a non-permissive environment for F. PB1. As shown in Fig. 2a, we observed that, similarly to human CRC (hCRC), mucin Muc1 and Muc20 (ref. 15) were overexpressed, whereas Muc5ac was aberrantly expressed in tumours, as it is not normally expressed in the lower gastrointestinal tract16. We found that Muc3 and Muc13 were downregulated; the latter also occurred in the non-tumour region of eight-week-old ApcMin/+ mice, suggesting that this may be an early event in tumourigenesis. We confirmed the downregulation of Muc13 protein in both tumour and non-tumour regions of ApcMin/+ mice compared with WT mice; however, in contrast to the RNA data, Muc1 and Muc20 were downregulated in gut tissues (Extended Data Fig. 2a). This may be due to increased secretion,
as Muc1 was higher in the mucus of Apc\textsuperscript{Min/+} tumours (Extended Data Fig. 2b).

We next tested whether \textit{F. PB1} was capable of colonizing Apc\textsuperscript{Min/+} mice. We treated WT and Apc\textsuperscript{Min/+} mice with antibiotics to eliminate competing microorganisms and administered \textit{F. PB1} by gavage. As shown in Fig. 2b, \textit{F. PB1} abundance was lower in the ileal mucus of Apc\textsuperscript{Min/+} mice than WT mice at 48 h, suggesting that the Apc\textsuperscript{Min/+} gut is unfavourable for \textit{F. PB1} colonization. This was confirmed by fluorescence-in-situ-hybridization analysis (Fig. 2c).

Thus, to evaluate a possible role of \textit{F. PB1} in tumour protection, we administered it by gavage every other day to ensure appreciable levels throughout the experiment. We treated the Apc\textsuperscript{Min/+} and WT littermates with \textit{F. PB1} either before or during tumour development. We observed that the administration of \textit{F. PB1} from week 4 to 8 did not affect tumour development (Fig. 2d). However, administration of \textit{F. PB1} from week 8 to 12 (that is, when the bacterium was not enriched in Apc\textsuperscript{Min/+} mice) resulted in a clear reduction in the tumour numbers and dimensions (Fig. 2e,f). The reduction in tumour multiplicity observed macroscopically was no longer statistically significant when the number of lesions were microscopically analysed (Extended Data Fig. 2c), indicating that some small lesions were not visible to the eye and that \textit{F. PB1} probably affects tumour growth instead of initiation.

These results suggest that the modification in mucus composition creates an unfavourable environment for \textit{F. PB1} colonization. Administration of \textit{F. PB1} has an antitumour effect only when tumours have already started developing.

\textit{F. PB1} affects tumour cell proliferation without a major impact on adaptive immune cells. The antitumour activity of \textit{F. PB1} may depend on the immune system. We thus assessed the capacity of \textit{F. PB1} to activate an immune response in the absence of confounding microbiota, through monocolonization of germ-free mice. As shown in Extended Data Fig. 3, \textit{F. PB1} did not significantly impact immune-cell development. The number of FoxP3\textsuperscript{+} regulatory T cells in the small intestine lamina propria was unaffected by \textit{F. PB1} and only slightly changed in the large intestine (Extended Data Fig. 3a); in addition, no effects on IL17- or IFNy-producing CD4\textsuperscript{+} T cells were observed at either location (Extended Data Fig. 3b,c). We then analysed the effect of \textit{F. PB1} in Apc\textsuperscript{Min/+} mice. We could not detect any difference in the frequencies of any of the tested CD4\textsuperscript{+} T cell populations (FoxP3\textsuperscript{+} regulatory T, Th1 and Th17 cells) in specific-pathogen-free Apc\textsuperscript{Min/+} mice that were administered \textit{F. PB1}. However, we observed a trend towards a reduction in their absolute numbers (Fig. 3a and Extended Data Fig. 3d,e). We then analysed the innate immune cell components, and found no difference in neutrophil frequencies and counts in the lamina propria of the small intestine, whereas we observed a reduction in all of the mononuclear phagocytes in Apc\textsuperscript{Min/+} mice administered with \textit{F. PB1} (Extended Data Fig. 3f,g).

Consistent with our previous data, we observed a higher, albeit non-significant, frequency of circulating Ly6G\textsuperscript{+}CD11b\textsuperscript{+} neutrophils in 12-week-old Apc\textsuperscript{Min/+} mice compared with their WT littermates\textsuperscript{17} regardless of \textit{F. PB1} treatment. In contrast, treatment with \textit{F. PB1} resulted in the reduction of circulating Ly6G\textsuperscript{+}CD11b\textsuperscript{+} inflammatory monocytes in both WT and Apc\textsuperscript{Min/+} mice (Fig. 3b and Extended Data Fig. 3h). Hence, the reduction of gut inflammatory monocytes may be due to a reduction of circulating monocytes during \textit{F. PB1} treatment.

We then evaluated whether \textit{F. PB1} was acting directly on tumour cell growth. We administered \textit{F. PB1} to eight-week-old mice and then analysed tumour growth two weeks later. Treatment with \textit{F. PB1} induced a reduction in tumour number (macroscopic evaluation; Fig. 3c), tumour size (Fig. 3d), tumour cell proliferation (Fig. 3e,f) and rectal bleeding (Fig. 3g).

We next analysed whether \textit{F. PB1} administration had changed the microbiota composition and its metabolic output. Bacterial DNA was extracted from the faeces of Apc\textsuperscript{Min/+} mice treated with or without \textit{F. PB1} and the 16S ribosomal RNA gene profiling data were analysed. We observed an increase in short-chain fatty acid (SCFA)-producing bacteria in mice treated with \textit{F. PB1}, particularly \textit{Butyricimonas} (butyric acid-producing bacteria\textsuperscript{16}; Extended Data Fig. 4a). As these and the other SCFA-producing bacteria were not reduced in the initial assessment of microbiota in untreated Apc\textsuperscript{Min/+} versus WT mice during tumour development, it is unlikely that they have an impact on tumourigenesis.

It has been reported that Apc\textsuperscript{Min/+} mice with a niacr1 (GPR109A, the receptor for butyrate) gene deletion are more susceptible to tumour development via a mechanism that depends on the microbiota\textsuperscript{16}. We thus evaluated whether \textit{F. PB1} administration may affect the faecal level of SCFAs. We detected an increase in SCFAs (propionate, butyrate and acetate) and a reduction in lactate in the faecal content of mice treated with \textit{F. PB1} at the age of 12 weeks compared with 8 weeks (Fig. 3h). No significant differences in the levels of succinate and isovalerate were observed between treated and untreated mice, whereas a slight increase in valerate was observed in treated mice (Extended Data Fig. 4b).

These results suggest that \textit{F. PB1} affects tumour cell proliferation alone or in cooperation with other bacteria, probably through the release of SCFAs.

\textit{F. PB1} releases SCFAs that have anti-proliferative activities. Butyrate has been described to have histone deacetylase (HDAC) inhibitory activity\textsuperscript{20}, which affects cell proliferation. In contrast to normal epithelial cells, CRC cells do not use butyrate for their growth and its concentration accumulates, acting as an HDAC inhibitor (HDACi)\textsuperscript{21–23}. We carried out a dose-dependent response assay on four different mouse intestinal tumour cell lines (APC, CT26, MC-38 and CMT-93) and found that maximal cell growth inhibition, without cell viability being compromised, occurred at 1–2 mM butyrate and at much higher doses of acetate and propionate (2.5–50 mM). We found that the combination of SCFAs had an additive effect on the inhibition of the proliferation of all tested cell lines (Fig. 4a).

We then assessed whether \textit{F. PB1} itself was capable of synthesizing SCFAs in vitro. \textit{F. PB1} grown in strictly anaerobic conditions was very efficient at producing both butyrate and lactate (Fig. 4b). Interestingly, the concentration of butyrate (1 mM) was very similar to the one able to inhibit cell proliferation as indentified in the dose-dependant response assay (Fig. 4a), whereas that of acetate or propionate was extremely low (250–5,000× lower than the effective concentration on cell lines in vitro). Next, we evaluated whether the \textit{F. PB1} spent medium (SUP), containing SCFAs, had anti-proliferative activity. As shown in Fig. 4c, the addition of SUP drastically inhibited tumour cell proliferation without affecting tumour cell viability (not shown), suggesting that \textit{F. PB1} releases metabolites that interfere with cell proliferation.

A recent report has shown that tumourigenesis in hCRC and Apc\textsuperscript{Min/+} mice is dependent on the calcineurin-inhibited activation of the NFATc3 transcription factor, which is important for cell proliferation\textsuperscript{24}. Because the HDACi panobinostat can induce calcineurin degradation in multiple myeloma cells\textsuperscript{25,26}, we assessed whether the SCFAs produced by \textit{F. PB1} could act as HDAC inhibitors and therefore affect calcineurin and NFATc3 activation. This would explain why the administration of \textit{F. PB1} or its spent medium could inhibit tumour cell proliferation. Treatment with \textit{F. PB1} SUP (Fig. 4d) or the combination of SCFAs (Extended Data Fig. 5a) drastically increased the acetylation of histone H3 (H3K27ac), thereby confirming its HDACi activity. This correlated with the downregulation of calcineurin (PP2B-A) and NFATc3 activation in CRC cell lines (Fig. 4d and Extended Data Fig. 5b).

Short-chain fatty acids are volatile and can be extracted through evaporation. Thus, we compared the effect of the untreated \textit{F. PB1}
Fig. 3 | F. PB1 reduces tumour cell proliferation without a major impact on immune cells. a,b. Wild-type and ApcMin/+ mice were treated with vehicle (Veh.) or F. PB1 from week 8 to 12. a. Flow cytometric analysis of regulatory T (Tregs), Th1 and Th17 cell populations in the lamina propria of the small intestine. FoxP3+ and CD25+ cells were gated on live CD45+CD3+CD4+ cells; Helios+ cells were gated on FoxP3+CD25+ cells (left; n = 12 (WT Veh. and ApcMin/+ F. PB1), 14 (WT F. PB1) and 11 (ApcMin/+ Veh.) mice). IL17+, IFN+ and IL17+IFN+ cells were gated on live CD45+CD3+CD4+ cells (right; n = 9 (WT Veh. and ApcMin/+ Veh.), 11 (WT F. PB1) and 10 (ApcMin/+ F. PB1) mice). b. Flow cytometric analysis of peripheral blood cells. Percentages are relative to the CD45+CD3+ population (n = 13 (WT Veh. and ApcMin/+ F. PB1), 15 (WT F. PB1) and 12 (ApcMin/+ Veh.) mice). c–g. ApcMin/+ mice received vehicle or F. PB1 from week 8 to 10 (c, left). Data are from two independent experiments. c. Tumour multiplicity in the small intestine normalized to vehicle-treated mice received vehicle or F. PB1 from week 8 to 10 (c, left). Data are from two independent experiments. d. Area (right) and maximum diameter (left) of ileal dysplastic lesions (n = 265 (Veh.) and 150 (F. PB1) mice). e. Percentage of CD45+CD3– cells in the polyps of mice treated with vehicle or F. PB1 (n = 4 mice per group). f. Representative images of nuclear Ki67 staining in the polyps of vehicle- or F. PB1-treated mice (right; n = 4 mice per group). Scale bars, 100 μm. g. Bleeding score of vehicle- and F. PB1-treated mice (n = 10 (Veh.) and 11 (F. PB1) mice). h. Faecal concentrations of L-lactate, acetate, propionate and butyrate in WT and ApcMin/+ mice treated with vehicle or F. PB1 from 8 to 12 weeks, detected by ultra-performance liquid chromatography with high-resolution mass spectrometry (UPLC–HR–MS; n = 6 (WT Veh. and WT F. PB1), 11 (ApcMin/+ Veh.) and 10 (ApcMin/+ F. PB1) mice). a–d,g. Data from two independent experiments are represented as the mean ± s.e.m. P values were determined using a one-way ANOVA with Bonferroni’s post-hoc test for multiple comparisons (a), two-tailed unpaired Student’s t-test (b,d,e,g), two-tailed unpaired Mann–Whitney U-test (d) or two-way ANOVA with Bonferroni’s post-hoc test for multiple comparisons (h).
SUP with F. PB1 SUP subjected to evaporation to deplete the SCFAs. Although there was a minor effect of SUP evaporation on the concentration of lactate, the evaporated SUP was strongly impaired in inducing H3 acetylation and NFATc3 downregulation, which suggests that lactate was not involved in this process (Fig. 4a,b and Extended Data Fig. 5c). In contrast, there was very little acetate and propionate in the evaporated SUP, whereas the concentration of butyrate was halved. The residual effect on H3 acetylation and NFATc3 downregulation could thus be due to the left-over butyrate still present in the supernatant after evaporation (Fig. 4f) or to other metabolites that were unaffected by evaporation. Hence, F. PB1 releases metabolites, including SCFAs, that can impact tumour cell proliferation by inhibiting HDACs and thus block NFATc3 and calcineurin activation.

The F. PB1 metabolic products have an anti-proliferative activity in vivo and this is independent of the microbiota. We next evaluated whether the F. PB1 SUP had anti-tumourigenic activity in vivo. The F. PB1 SUP did not statistically affect tumour multiplicity, but the size of the tumour lesions was significantly reduced (Fig. 5a,b and Extended Data Fig. 6a). We then pre-treated 11-week-old ApC<sup>Min/+</sup> mice (that had already developed tumours) with antibiotics to affect the microbiota but not tumour growth, and then administered SUP in the presence of antibiotics. We observed an even greater reduction in the dimensions of the tumour lesions, which indicated that the spent medium had an anti-proliferative activity in vivo that was independent of the microbiota (Fig. 5c,d and Extended Data Fig. 6b). Furthermore, the SUP reduced NFATc3 activation and induced histone H3 acetylation in dysplastic lesions, again in a microbiota-independent fashion (Fig. 5e). These data suggest that the metabolic products of F. PB1 have a direct effect on tumour growth and affect NFATc3 activation.

We then evaluated whether butyrate was sufficient to mediate the anti-proliferative response. We administered 1 mM sodium butyrate (the same concentration found in the SUP of F. PB1) to ApC<sup>Min/+</sup> mice treated with antibiotics to avoid the possibility of butyrate being used up by the indigenous microbiota. As shown in Fig. 5f,g, butyrate had a very similar anti-proliferative activity to F. PB1 SUP, indicating that butyrate is the main effector of F. PB1 activity.
Finally, we showed that the F. PB1 SUP also significantly reduced the dimensions of lesions in a model of inflammation-driven CRC (AOM and DSS), in which tumours preferentially develop in the colon, more closely mirroring the human pathology (Fig. 5h,i and Extended Data Fig. 6c).

Overall, these data show that due to their HDACi activity, the metabolic products of F. PB1—butyrate in particular—control NFATC3 activation, thereby blocking tumour cell proliferation in vivo in Apc<sup>Min<sup>+/+</sup></sup> mice independently of the microbiota. A similar anti-proliferative activity was also observed in an inflammation-driven CRC model.

The anti-proliferative activity of F. PB1 can be exerted by other SCFA-producing bacteria. We then evaluated whether the activity of F. PB1 is shared by other SCFA-producing bacteria. We selected Lactococcus lactis, because it secretes butyrate and some lactate in culture, similarly to F. PB1 (Extended Data Fig. 7a), and has been proposed to have anti-proliferative activities in vitro by an unknown mechanism<sup>13</sup>. We administered L. lactis following a schedule similar to that of F. PB1 but could not detect any anti-tumoural effect (Extended Data Fig. 7b,c). This is probably due to the inability of L. lactis to survive in or colonize the mouse intestine—as we could not detect L. lactis, even in WT mice—independently of the microbiota (Extended Data Fig. 7d). We thus analysed the anti-proliferative activity of the SUP from L. lactis in vivo in antibiotic-treated Apc<sup>Min<sup>+/+</sup></sup> mice. We found that the L. lactis SUP reduced the size of tumour lesions, although not as effectively as the F. PB1 SUP (Extended Data Fig. 7e,f). This difference cannot be due to a reduction in lactate production, as lactate did not impact on tumour cell proliferation in vitro (Extended Data Fig. 7g). The anti-proliferative activity of F. PB1 is therefore not unique to this bacterium and may be common to other bacteria that produce SCFAs, provided that they are able to colonize and/or survive in the intestine long enough to produce butyrate.

*H. biformis* is the equivalent of F. PB1 in humans. We then assessed the relevance of these findings in human CRC. We interrogated a dataset of a shot-gun microbiome analysis carried out in patients with colorectal adenomas<sup>26</sup> and found that, similarly to the mouse, there was a reduction in the abundance of the family of Erysipelotrichaceae in patients with large adenomas compared with healthy individuals, but there was no change in the Shannon diversity index (Fig. 6a). In this family, an undefined genus (*Erysipelotrichaceae non-name*) and a species, *H. biformis*, were strongly under-represented in advanced colon adenomas (Fig. 6b). Interestingly, a high-quality phylogeny of the Erysipelotrichaceae family and the F. PB1 isolate showed that *H. biformis* is the second bacterium phylogenetically closest to F. PB1 among the bacteria that colonize the human gut (phylogenetic distance of 0.273; Fig. 6c).

Similarly to F. PB1, *H. biformis* released SCFAs (Fig. 6d) and the SUP of *H. biformis* inhibited human tumour cell proliferation (HT-29 and Caco-2; Extended Data Fig. 8a). This activity was mediated by the HDAC-inhibitory activity of the SUP, as shown by increased histone acetylation and reduced NFATC3 activation (Fig. 6e and Extended Data Fig. 8b). In vivo, *H. biformis* was unable to survive in or colonize the mouse intestine and we could not observe any anti-proliferative activity in Apc<sup>Min<sup>+/+</sup></sup> mice (Extended Data Fig. 8c–e). However, when we tested the F. PB1 SUP in Apc<sup>Min<sup>+/+</sup></sup> mice, we observed a much higher effect on tumour multiplicity (Fig. 6f,g), suggesting that *H. biformis* may also have some effect on tumour initiation. This remains to be evaluated.

We confirmed our data on human specimens from CRC patients using a technology set up in our laboratory<sup>27</sup>. Tumour specimens from CRC patients were treated with the SCFA mix (acetate:propionate:butyrate, 2:1:1), F. PB1 SUP or *H. biformis* SUP. As shown in Fig. 6h and Extended Data Fig. 9a, the SUPs (F. PB1 and *H. biformis*) as well as the mix of SCFAs induced an increase in H3K27 acetylation and a reduction of NFATC3 protein levels. This correlated with reduced tumour cell proliferation, as shown by lower nuclear Ki67 immunostaining in the presence of either F. PB1 SUP (Extended Data Fig. 9b) or the SCFA combination (Extended Data Fig. 9c). These results suggest that *H. biformis* is the human counterpart of F. PB1.

**Discussion**

The mucus layer has been shown to undergo profound changes during CRC tumourigenes, both in size and in composition due to epithelial cell dedifferentiation<sup>22</sup>. These changes may result in two concomitant and non-mutually exclusive events, which may be responsible for fostering intestinal tumourigenes. On one hand, the increased penetrance or adherence of pro-tumourigenic bacteria may favour immune-cell recruitment and activation, drive tumour cell transformation or Th17 cell activation, thereby contributing to tumour development<sup>29–32</sup>. On the other hand, tumourigenes may be due to the contraction of anti-tumourigenic bacteria that release anti-proliferative metabolites. We focused on the latter and identified a bacterial member of the murine gut microbiota, F. PB1, belonging to the Erysipelotrichaceae family, which is one of the most abundant taxa in the murine gut and is not expanded further in patients with colorectal adenomas<sup>26</sup> and found that, similarly to the mouse, there was a reduction in the abundance of the family of Erysipelotrichaceae in patients with large adenomas compared with healthy individuals, but there was no change in the Shannon diversity index (Fig. 6a). In this family, an undefined genus (*Erysipelotrichaceae non-name*) and a species, *H. biformis*, were strongly under-represented in advanced colon adenomas (Fig. 6b). Interestingly, a high-quality phylogeny of the Erysipelotrichaceae family and the F. PB1 isolate showed that *H. biformis* is the second bacterium phylogenetically closest to F. PB1 among the bacteria that colonize the human gut (phylogenetic distance of 0.273; Fig. 6c).

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We confirmed our data on human specimens from CRC patients using a technology set up in our laboratory<sup>27</sup>. Tumour specimens from CRC patients were treated with the SCFA mix (acetate:propionate:butyrate, 2:1:1), F. PB1 SUP or *H. biformis* SUP. As shown in Fig. 6h and Extended Data Fig. 9a, the SUPs (F. PB1 and *H. biformis*) as well as the mix of SCFAs induced an increase in H3K27 acetylation and a reduction of NFATC3 protein levels. This correlated with reduced tumour cell proliferation, as shown by lower nuclear Ki67 immunostaining in the presence of either F. PB1 SUP (Extended Data Fig. 9b) or the SCFA combination (Extended Data Fig. 9c). These results suggest that *H. biformis* is the human counterpart of F. PB1.
during tumourigenesis, presumably due to the different mucus composition. This may explain why its contraction affects tumour development so drastically.

Tumourigenesis in human CRC and ApcMin/+ mice is dependent on the calcineurin-mediated activation of the NFATc3 transcription factor, which drives tumour cell proliferation. NFATc3 is also involved in driving the expression of MUC5ac, which may explain why hCRC and ApcMin/+ mice ectopically express MUC5ac. Calcineurin can be induced to degradation by the HDACi panobinostat. Here, we show that the SUPs from F. PB1 and its human counterpart, H. biformis, act as an HDACi affecting calcineurin and NFATc3 activation, and this results in the inhibition of tumour cell growth independently of the microbiota (Extended Data Fig. 10). In some cases, we also observed an effect of F. PB1 or H. biformis SUP on tumour multiplicity. This could be due to a technical issue as a result of the inability to also detect small tumours but we cannot exclude an effect on tumour initiation.

We found a reduction of lactate in the faeces of mice treated with F. PB1. This was unexpected, as F. PB1 was found to produce large quantities of lactate in vitro, but we cannot anticipate whether it was also produced in vivo. In addition, as lactate is known to be produced and used by tumour cells, the reduction of lactate after F. PB1 administration could be due to either reduced tumour cell proliferation or increased use of lactate by the tumour cells.

![Diagram](image_url)
Fig. 6 | *H. biformis* is the equivalent of F. *PB1* in humans. a, Shannon diversity index (left) and abundance of the family Erysipelotrichaceae (right) in faecal DNA from healthy donors (*n* = 61) and patients with large adenoma (*n* = 15). b, Abundance of the undefined genus *E. noname* and the species *H. biformis*. A two-tailed Wilcoxon rank-sum test comparing the relative abundances of large adenoma (*n* = 61) and patients with large adenoma (*n* = 15) and control (*n* = 61) samples was applied at each taxonomic level. *P* values obtained at the family and genus taxonomic levels were corrected for multiple hypothesis testing using the Benjamin–Hochberg procedure. c, High-quality phylogeny of the Erysipelotrichaceae family and the *F. PB1* isolate. d, Levels of SCFAs in the broth fermented by *H. biformis* (*H. biformis* SUP), determined using UPLC–HR–MS. Data are from *n* = 2 biologically independent experiments. e, Representative western blot from two or three independent experiments performed with consistent results showing the effect of SCFAs MIX, *F. PB1* SUP and *H. biformis* SUP on H3K27 acetylation and NFATc3 expression in human CRC cell lines. Cells that were not treated (NT) or treated with non-fermented medium (Veh.) served as controls. Vinculin was used as a loading control. The densitometric analysis is reported in Extended Data Fig. 8b. f, *g*. Eleven-week-old *Apc<sup>Min</sup>*/<sup>+/</sup>* mice were treated with antibiotics (ABX). After 2 d, mice were treated with vehicle or *H. biformis* SUP for three times (two administrations on the first day and one on the second day) in the presence of ABX. Mice were sacrificed 24 h after the last gavage (*n* = 5 mice per group). g. Tumour multiplicity in the small intestine normalized to vehicle-treated *Apc<sup>Min</sup>*/<sup>+/</sup>* mice. g, Area (right) and maximum diameter (left) of ileal dysplastic lesions normalized to the total number of lesions per mouse. h, Representative western blot from two or three independent experiments performed with consistent results showing the effect of *F. PB1* SUP and *H. biformis* SUP on H3K27 acetylation and NFATc3 expression in ex vivo-treated hCRC samples (bottom). The bar plots (top) show the densitometric quantification of NFATc3 (normalized to vinculin) and H3K27 acetylation (normalized to total H3; *n* = 2 (H. *biformis* SUP) and 3 (F. *PB1* SUP) biologically independent experiments). a, b, e, f, h, Data are represented as the mean±s.e.m. *P* values were evaluated using a two-tailed unpaired Mann–Whitney U-test (a, f and h, right) and two-tailed unpaired Student’s t-test (g, left panel and h). a, g. The box plots show the interquartile range, the horizontal lines show the median values and the whiskers indicate the minimum-to-maximum range.
Alternatively, lactate could be used by other bacterial species for their own growth. The involvement of SCFAs in the observed anti-proliferative activity is demonstrated by the following observations: evaporation to deplete SCFAs led to a strong reduction in the activity of the SUP and the SUP from another butyrate-producing bacterium (*L. lactis*) or butyrate itself can mimic the effect of *F. PB1* at a concentration similar to that found in the SUP. This suggests that the antitumour activity of *F. PB1* is not unique and could be shared with other SCFA-producing bacteria as long as they have the ability to colonize or survive in the gut and produce butyrate locally.

Why does butyrate only inhibit proliferation of tumour cells and not normal epithelial cells? In CRC, tumour cells undergo increased glycolysis rather than mitochondrial oxidative metabolism due to the Warburg effect. Thus, cancer cells do not use butyrate for their growth, the butyrate concentration increases and can act as HDAC inhibitors. Consistent with this, dietary fibres, which promote the growth of butyrate-producing bacteria, affect tumourigenesis in Apc Min/+ mice and Apc−/− mice with a deleted *nica1* are more susceptible to tumour development via a mechanism that depends on the microbiota.

Consistent with data in the literature, we have shown that *H. biformis* is able to produce SCFAs and its spent medium can inhibit tumour cell proliferation. We do not know whether *H. biformis*, similarly to mouse *F. PB1*, is the major bacterium responsible for the antitumour properties in humans or whether other SCFA-producing bacteria are also contracted in humans and may contribute to failure of tumour-growth control. Future studies should aim at addressing this point and assessing whether this species may have a therapeutic potential. As *H. biformis* is reduced in the faeces of patients with large adenomas, it may also be used as a potential biomarker for detecting tumours in their early phase.

Methods

**Bacterial strains.** *F. PB1* was isolated in the Kenya Honda laboratory (RIKEN IMS) from the faecal pellets of 12-week-old WT C57BL/6 littermates of our Apc Min/+ colony as described previously. Briefly, the faeces were suspended in tryptic soy broth, serially diluted and plated on Eggerton–Gagnon (EG) agar plates. Forty-eight colonies were picked and sequenced using pan-bacterial primers targeting the 16S rRNA gene. Similarity to *E. rectituden* was checked against both databases and the sequence retrieved from our metagenomic analysis, resulting in 99.7% and 98% homology, respectively. The *F. PB1* strain was deposed to the German collection of microorganisms DSMZ (type strain no. DSMZ38003). *H. biformis* was purchased from the DSMZ (type strain no. 3989). Both bacteria were cultured in an anaerobic chamber (gas atmosphere N2:CO2:H2 of 80:15:5) in pre-reduced EG broth under anaerobic conditions at 30 °C. The bacterium *Hermesia gottfriedi* strain was purchased from DSMZ (type strain no. 20481) and cultured in pre-reduced MRS broth at 30 °C in static conditions. Bacterial SUP was derived from overnight cultures of the strains in the conditions described above. The medium fermented by the bacteria (SUP) was filtered in 0.25 μm filters and immediately frozen.

**Cell lines.** The mouse (CT26) and human (HT-29 and Caco-2) CRC cell lines were purchased from the American Type Culture Collection. The CMT-93 mouse rectum carcinoma cell line was a gift from D. Artis (Cornell University). The MC-38 mouse carcinoma cell line was a gift from C. Krieg (Zurich University). The APC cell line was derived from Apc−/− small-intestinal adenomas by mechanical disruption. No further authentication method was performed on the purchased cell lines. The loss of heterozygosity (loss of the WT allele and presence of the Min allele) in the APC cell line was checked using a TaqMan assay. CT26 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM l-glutamine. CMT-93 and MC-38 cells were cultured in DMEM medium supplemented with 10% FBS and 2 mM l-glutamine. APC cells were cultured in complete DMEM medium supplemented with insulin-transferrin-selenium-ethanolamine (ITS-X, Gibco) and human EGF (10 pg ml−1). HT-29 cells were cultured in DMEM medium supplemented with 10% FBS and 2 mM l-glutamine. Caco-2 cells were cultured in MEM medium with Earle’s Salt supplemented with 20% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. All of the cell lines were tested to exclude mycoplasma contamination. To evaluate the effect of SCFA (either alone or in combination) and the bacterial SUP, cells were seeded and stimulated after an overnight incubation. The cells were stimulated with sodium acetate (SS636, Sigma-Aldrich), sodium propionate (P5436, Sigma-Aldrich), sodium butyrate (ARK2161, Sigma-Aldrich) or a mix of the three. APC, CT26, MC-38, HT-29 and Caco-2 cells were stimulated with 50 mM sodium acetate, 10 mM sodium propionate, 2 mM sodium butyrate or a mix of 30 mM sodium acetate, 10 mM propionate and 2 mM butyrate. CMT-93 cells were stimulated with 10 mM sodium acetate, 2.5 mM sodium propionate, 1 mM sodium butyrate or a mix of 10 mM sodium acetate, 2.5 mM propionate and 1 mM butyrate. The cells were also stimulated with different concentrations (0.8, 4, 20 mM) of sodium l-lactate (cat. no. 71718, Sigma-Aldrich). Finally, cells were stimulated with the bacterial broth diluted 40% (v/v) in cell culture medium (EG broth) fermented by *F. PB1* or *H. biformis* (SUP) or relative control non-fermented broth (vehicle). In addition, CT26 cells were treated with the fermented broth (also called spent medium) that had been evaporated to remove the SCFAs. The spent medium was evaporated to dryness at 50 °C under reduced pressure (5 mbar). The residue was taken up with water, filtered and diluted 40% (v/v) in cell culture medium.

**Bacterial profiling of intestinal microbiota.** DNA from faecal pellets and mucus scraped from the small intestine and colon was extracted using a QIAamp DNA isolation kit (MP) following a published protocol. The V5–V6 hypervariable regions of the bacterial 16S rRNA genes were amplified and processed with a modified version of the Nextera protocol. The metagenomic libraries obtained were sequenced using MiSeq Illumina platform with a 2 × 250 paired-end approach. Metagenomic amplicons were analysed by applying the BioMas pipeline. First, the paired-end reads were merged into consensus sequences using PEAR and subsequently dereplicated applying USEARCH, maintaining the information about the total number of reads supporting each consensus sequence. Second, the paired-end reads that remained non-overlapping were only considered for statistical analysis if both read ends were ≥50 bp long and they had a quality trimming (Phred quality cut-off of ≥25). Third, both the merged sequences and the unmerged reads were matched against the Ribosomal Database Project database (release 11.2) using Bowtie2 (ref. 5). The mapping data were filtered according to two parameters: identity percentage and query coverage (≥70%). In particular, sequences that yielded an identity percentage ≥97% were classified to species level and those with identity ≥90% and <97% were classified at a higher taxonomic rank. Finally, all mapped reads fulfilling the settled filters were taxonomically annotated using the Tango tool. The assigned genera were filtered—only those for which at least five reads per samples were considered present. The read counts were normalized using an approach similar to the RPMK (reads per kilo-base per million read) normalized counts approach (total normalized reads at the rank level ≥1,000,000). The statistical differences between the faecal microbiota at the genus and species level of WT and Apc−/− mice were calculated using the DESeq2 R package. Taxa specifically associated with one of the analysed conditions were identified using the linear discriminant analysis effect size (LDFse).

**Mice.** This study employed both male and female mice from the C57BL/6J and C57BL/6NIH strains. Six-week-old C57BL/6J mice were purchased from Haelan Laboratories. C57BL/6J/ApcMin/+ mice (referred to as Apc Min/+ , ref. 40) were maintained as an inbred strain in our animal facility. For experiments where Apc Min/+ mice were employed, WT littermates born from the same mothers of experimental mice were used as controls. All of the mice were maintained in micro-isolation cages in a pathogen-free animal facility. Germ-free ICR male mice were maintained in the isolators at RIKEN IMS. Experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health. On the basis of our experience with animal models and according to animal-welfare policy (directive 2010/63/EU), which strongly suggests the use of a limited number of animals, we estimated that two experiments with n = 5 mice per group would allow us to reach statistical significance. Animals were randomly allocated to each treatment group. The different treatment groups were processed identically and animals in the different treatment groups were exposed to the same environment. The investigators were not blinded during experimental mice allocation and outcome assessment. For the macroscopic analysis of the tumour lesions, we first calculated the average of tumour numbers in the vehicle group; the number of tumours of an individual mouse was regarded as a percentage of this average.

**Histological evaluation.** Formalin-fixed and paraffin-embedded swiss rolls of colon and small intestine were sectioned to thicknesses of 3–4 μm and the sections were stained with haematoxylin and eosin. For histopathological examination and scoring, the haematoxylin and eosin slides were evaluated by an expert pathologist. The extent of inflammatory changes was defined according to the score proposed.
any chronic inflammatory bowel disease, patients with hereditary syndrome (such as familial adenomatous polyposis and hereditary non-polyposis colorectal cancer) and use of antibiotics in the previous four weeks. All patients gave informed written consent and the institutional review board approved the study by ECOG's ethical committee. Human biological samples were sourced ethically and their research use was in accordance with the terms of the informed consent provided. Case selection was therefore independent and blinded to baseline characteristics, treatments received, clinical outcome and molecular characterization to reduce any potential self-selection bias.

Administration of F. PB1 to germ-free mice, and F. PB1, H. biformis and L. lactis to ApcMin/+ mice. In experiments of gnotobiotic colonization, five germ-free ICR male mice were administered 250 μl F. PB1 culture (optical density (OD600=0.6; corresponding to about 5 × 10^6 c.f.u. μl^-1) orally and the abundance of small-intestinal cells was added after 4 weeks to evaluate the effect of exogenous F. PB1 administration, WT and ApcMin/+ mice were administered frozen bacterial stocks equivalent to 250 μl of culture at the logarithmic growth phase orally 3× a week, according to different schedules (see Figs. 2a-c and 3c). The mice were sacrificed at the age of 8, 10 or 12 weeks and the tumor multiplicity in the small intestine and colon was assessed. Neutrophil and inflammatory monocyte abundances in circulating blood and regulatory T, Th1 and Th17 cell abundances in the small intestinal and colonic lamina propria were assessed. The bleeding score was assigned as follows: 0, negative to Hemoccult (Beckman Coulter); 1, positive to Hemoccult and 2, gross bleeding. To evaluate the effect of exogenous H. biformis and L. lactis administration, ApcMin/+ mice were administered frozen bacterial stocks orally 3× a week from the age of 8–10 weeks. In the monoclonization experiments, WT and ApcMin/+ mice were treated with or without antibiotic cocktail (1 g l^-1 ampicillin, 1 g l^-1 neomycin and 0.5 g l^-1 vancomycin in their drinking water and 2 mg metronidazole administered by oral gavage every 2 d) for 7 d and challenged with vehicle, F. PB1 or L.lactis for 3 d in a row. The mice were sacrificed after 48 h and the bacterial abundance was validated in the faeces, and ileal and colonic mucus using a qPCR assay with specific primers (Supplementary Table 1).

Spent medium administration to ApcMin/+ mice or C57BL6/J WT mice treated with AOM and DSS. Eight-week-old C57BL6/J mice were treated with AOM (10 mg kg^-1 body weight) by intraperitoneal injection. After 3 d, they received DSS 1.5% (w/v) in their drinking water for 7 d. The mice were allowed to recover for 14 d. This schedule was repeated for two cycles. During the second recovery (from week 12 to 14), the mice were treated with EG broth fermented by F. PB1 (F. PB1 SUP) or relative control non-fermented broth (vehicle) by oral gavage (200 μl per mouse) 3× per week for two weeks and the tumour lesions were analysed. Culture broths fermented with F. PB1, H. biformis or L. lactis (F. PB1 SUP, H. biformis SUP or L. lactis SUP) or not (vehicle) were administered by oral gavage 3× per week for two weeks to eight-week-old ApcMin/+ mice.

Vehicle (EG for F. PB1 or H. biformis; MRS broth for L. lactis), SUP and butyrate (1 μmol l^-1) were also administered by oral gavage (200 μl per mouse) 3× in a row (twice on day 1) in combination with the antibiotic cocktail (1 g l^-1 ampicillin, 1 g l^-1 neomycin and 0.5 g l^-1 vancomycin in their drinking water and 2 mg metronidazole administered by oral gavage every 2 d) to 11-week-old ApcMin/+ mice pretreated with the antibiotic cocktail for 2 d. In these experiments, the mice were sacrificed 24 h after the last gavage.

Quantification of faecal and spent-medium SCFAs. The SCFAs in the faecal and SUP (F. PB1, H. biformis and L. lactis) samples were quantified as previously described^20, with a few modifications. In detail, 100 mg faeces or 200 μl SUP was resuspended in 2 ml of 0.010% HCOOH and vortexed for 1 min. The suspension was centrifuged at 1,000g for 2 min at 4°C and the supernatant was recovered. The residue was extracted again as described above. The supernatants were combined and the volume was adjusted to 5 ml with a solution of 0.001% HCOOH in water. All extracts were stored at −20°C. Before UPLC–HR-MS analysis, the samples were diluted 1:100 in 0.001% HCOOH and centrifuged at 15 000g for 5 min. The UPLC–HR-MS analysis was carried out using an Exactrap Orbitrap MS with an HESI-II probe for electrospray ionization (Thermo Scientific). The ion source and interface conditions were as follows: spray voltage, −3.0 kV; sheath gas flow rate, 35 ml min^-1; auxiliary gas flow rate, 10 ml min^-1; temperature, 120°C; and capillary temperature, 320°C. A 1.8-mm HSS T3 column (150 × 2.1 mm; Waters) was used for separation at a flow rate of 0.2 ml min^-1. The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH3OECH2CN (1:1; v/v solvent B). A 5 μl aliquot of the sample was separated by UPLC using the following elution gradient: 0% B for 4 min, 0–15% B over 6 min, 15–20% B over 5 min, 20% B for 13 min, followed by a return to the initial conditions in 1 min. The column and samples were thermostated at 30°C. The UPLC was equipped with full-scan mass spectrometry in the range 50–130 m/z. The resolution was set at 50 K, the AGC target was 1×10^6 and the maximum ion-injection time was 100 ms. The ion with a m/z of 91.0038, corresponding to the HCOOH dimer (2M−H)−, was used as the lock mass. The mass tolerance was 2 ppm. The mass spectrometry data were processed using the Xcalibur software (Thermo Scientific). Analytical grade...
SCFAs were used as standards (Sigma-Aldrich). Five-point external calibration curves were adopted to quantify the pyruvic, lactic, succinic, acetic, propionic, butyric, isobutyric, valeric and isovaleric acids in the faecal samples. The SCFA concentrations were expressed in millimoles per 100 g of wet faeces.

Flow cytometry. Peripheral blood was sampled in heparin and the red blood cells were lysed. The samples were stained with anti-CD45.2, -CD3, -Ly6C, -Ly6G and -CD11b antibodies. Neutrophils were defined as Ly6G+C-CD11b+ and inflammatory monocytes as Ly6C+C-CD11b+ in the CD45-CD3- population. Small intestinal and colonic lamina propria lymphocytes were isolated by incubating intestinal chucks in PBS, 5% FCS, 1.5 mM EDTA and 1 mM dithiothreitol at 37 °C for 15 min to remove the epithelial cells. The lamina propria cells were mechanically isolated in RPMI medium containing 5% FCS using a GentleMACS dissociator. The cells were permeabilized using a FoxP3 intracellular staining kit (BioLegend) and stained with anti-CD45, -CD3, -CD4, -CD25, -FoxP3 and -Helios antibodies. For Th1 and Th17 detection, the lamina propria cells were incubated for 4h with phorbol myristate acetate (50 ng ml−1; Sigma-Aldrich), ionomycin (500 ng ml−1; Sigma-Aldrich) and Golgostop (BD Biosciences). The cells were then stained with anti-CD45.2, -CD3, -IL17 PE and -IFNγ antibodies. For mononuclear phagocytes identification, the lamina propria cells were stained with anti-CD45.2, -CD11b, -F4/80, -CD11c, -Ly6G and -Ly6C antibodies. Dead cells were excluded with the Fixable Viability Stain510 (BD Biosciences). Samples were acquired using FACSCantoII and Fortessa (BD Biosciences) and analysed with FlowJo (Treestar).

See Supplementary Table 3 for detailed information.

Western blotting. For western blot analysis, cells and tissue samples were lysed using RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors (Complete Mini, EDTA-free; Roche), and tyrosine protein-, acid- and alkaline-phosphatase inhibitors (Phosphatase inhibitor cocktail 2, Sigma-Aldrich) 4h after stimulation. The lysates were centrifuged at 13,000 g for 10 min and the supernatants were collected. The cell lysates were treated with a Bradford assay (Bio-Rad) and equal amounts of the proteins were run on a 12% PAGE gel, followed by western blotting. After 30 min at room temperature in blocking solution—5% milk or 5% BSA in Tris-buffered saline and Tween 20 (TBST; 10 mM Tris pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20)—the membranes were probed with primary antibodies in 5% milk or 5% BSA in TBST overnight at 4 °C, washed in TBST and incubated for 1h at room temperature with secondary antibodies: goat anti-rabbit–horseradish peroxidase (HRP; 1:1,000; cat. no. 1070-6515, Santa Cruz Biotechnology), mouse anti-muc20 (1:1,000; PA5-50238, Thermofisher). Visualization was carried out using Grubbs’ test and excluded from the analysis. Statistical significance between two groups was determined using two-tailed unpaired Student’s t-test, multiple t-tests corrected for multiple comparisons using the Holm–Sidak method, the Mann–Whitney U-test or Wilcoxon’s rank-sum test, whereas the comparison of multiple groups was carried out using the Kruskal–Wallis test followed by Dunn’s test and one- or two-way ANOVA, followed by Bonferroni’s or Tukey’s post-hoc test. The Benjamini–Hochberg procedure was used to adjust P values for multiple testing. The data displayed normal variance. A probability value of *P<0.05 was considered significant. All statistics and reproducibility information and sample sizes in the figure are legends. The sample size was chosen by taking the means of the target values between the experimental group and the control group, the standard error and the statistical analysis used into consideration. For the animal studies, sample size was defined on the basis of past experience with the models. The minimum number of animals necessary to achieve the scientific objectives was used for ethical reasons. Animals were allocated randomly to each treatment group. Different treatment groups were processed identically and animals in different treatment groups were exposed to the same environment. In the immunohistochemistry and immunofluorescence analyses, the investigators were unaware of the experimental groups.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability Source Data for the figures and Extended Data figures are provided in the online version of the paper. Raw sequencing data and metadata associated to samples are available online at the NCBI under the accession number PRJNA64752.

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Author contributions

E.Z. and C.P. ideated, performed and analysed all of the experiments. T.S. and F.S. helped with the execution of experiments. A.B., I.S. and S.Guglietta helped with the execution of the mouse experiments. R.F., M.M. and G.Pesole performed the 16S RNA metagenomic analyses. L.M. and W.V. designed and carried out the histological analyses. C.P. helped with the execution of experiments. A.B., I.S. and S.Guglietta helped with the execution of experiments. G.F. is a recipient of a grant from the Italian Ministry of Health (grant no. 2016.00913.CT).
performed the confocal analyses. J.T. executed the metabolomic analyses. B.O. assisted in
the execution of the in vitro experiments. K.A. and K.H. isolated F. PB1 and carried out
the germ-free experiments. S.A. and S.Guglielmetti set up F. PB1 growth and supernatant
production. S.C. set up H. biformis and L. lactis growth and supernatant production. G.F.
performed the FACS analyses. F.A. and N.S. performed the phylogenetic analysis and
human CRC dataset interrogation. G.Penna contributed with ideas and interpretation of
results. M.R. ideated the study, coordinated the work and wrote the manuscript.

**Competing interests**
The authors declare no competing interests.

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Extended Data Fig. 1 | Faecal microbiota diversity upon tumour progression in Apc\textsuperscript{Min/+} mice. a, Chao1 and Simpson indexes of the faecal microbiota of WT and Apc\textsuperscript{Min/+} mice; n = 8 mice/group. Box plots show the interquartile range, median value and whiskers min to max. Data from two independent experiments.

b, Tumour multiplicity in the small intestine of Apc\textsuperscript{Min/+} mice assessed at different time points; weeks 6, 28 n = 5; weeks 8, 26 n = 8; weeks 10 n = 9; weeks 12, 16, 24 n = 3; weeks 14 n = 10; weeks 20 n = 33; weeks 22, 30 n = 4 mice/group. c, Normalized read counts for differentially represented species in WT and Apc\textsuperscript{Min/+} mice shown as heatmaps. The counts were log transformed and used to define the colour gradient of the heatmap; n = 8 mice/group. Data from two independent experiments. a–c, Data are represented as means ± s.e.m.
Extended Data Fig. 2 | Mucin production is altered in the ileum of Apc\textsuperscript{Min/+} mice. a, Representative Western blots of two independent experiments with consistent results showing the abundance of Muc1, Muc13 and Muc20 in ileal extracts from WT (healthy tissues, H) and healthy (H) and tumour (T) tissues of Apc\textsuperscript{Min/+} mice analysed at 11 weeks of age; Right: densitometric quantification (WT \(n = 3\); Apc\textsuperscript{Min/+} \(n = 4\) biologically independent samples). b, Representative confocal images of WT and Apc\textsuperscript{Min/+} mice ileum intestinal tissues stained with Muc1 (red) and DAPI (blue); scale bars, 50 \(\mu\)m. Mean fluorescence intensity of MUC1 was measured to quantify the expression of the protein in the intestinal of Apc\textsuperscript{Min/+} and WT mice (WT \(n = 7\), Apc\textsuperscript{Min/+} \(n = 5\) biologically independent samples). c, Apc\textsuperscript{Min/+} mice were treated with vehicle (\(n = 10\) mice/group) or F. PB1 (\(n = 11\) mice/group) from week 8 to 10. Number of microscopic lesions, shown as % relative to vehicle, was evaluated on H&E slides by an expert pathologist. a–c, Data from two independent experiments and represented as means \(\pm\) s.e.m. \(P\) values were determined by one-way ANOVA using Tukey post-test (a), two-tailed unpaired Mann Whitney U test (b), or two-tailed unpaired Student’s t-test (c).
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | F. PB1 does not have a major impact on immune cells. **a-c,** Flow cytometry analyses of the small intestinal and colonic lamina propria of germ-free ICR mice monocolonized with F. PB1 (GF + F. PB1 n = 10 mice/group) for the presence of T reg (**a**), Th17 (**b**) and Th1 (**c**) cells. Germ-free (GF, n = 9 mice/group) and SPF (n = 7 mice/group) mice were used as controls. **d-g,** WT and Apc<sup>Min/+</sup> mice treated with vehicle (Veh) or F. PB1 from week 8 to 12. **d,** Flow cytometric analysis of T reg, Th1 and Th17 cell populations in the small intestinal lamina propria. FoxP3<sup>+</sup> cells are gated on the live CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> cells; Helios<sup>+</sup> is gated on the FoxP3+CD25<sup>+</sup> cells (WT Veh, Apc<sup>Min/+</sup> F. PB1 n = 12; WT F. PB1 n = 14; Apc<sup>Min/+</sup> Veh n = 11 mice/group). IL17+, IFNγ+ and IL17+ IFNγ+ cells are gated on the live CD45+CD3+CD4+ cells (WT Veh, Apc<sup>Min/+</sup> Veh n = 9; WT F. PB1 n = 11; Apc<sup>Min/+</sup> F. PB1 n = 10 mice/group). Data shown as % of CD45<sup>+</sup> cells (**d**) or as absolute number/whole tissue (**e**). **f-g,** Flow cytometric analysis of mononuclear phagocytes (CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages, CD11c<sup>+</sup>CD11b<sup>+</sup> dendritic cells and Ly6C<sup>+</sup>CD11b<sup>+</sup> inflammatory monocytes) and neutrophils (Ly6G<sup>+</sup>CD11b<sup>+</sup>) in the small intestinal lamina propria (WT Veh n = 5; WT F. PB1, Apc<sup>Min/+</sup> F. PB1 n = 6; Apc<sup>Min/+</sup> Veh n = 4). Data shown as percentages relative to the CD45<sup>+</sup>CD3<sup>+</sup> population (**f**) or as absolute number/whole tissue (**g**). **h,** Flow cytometric analysis of peripheral blood cells. Data shown as absolute number/ml blood (WT Veh, Apc<sup>Min/+</sup> F. PB1 n = 13; WT F. PB1 n = 15; Apc<sup>Min/+</sup> Veh n = 12 mice/group) P values were determined by one-way ANOVA with Bonferroni post-test (**a-d**), Kruskal-Wallis with Dunn post test (**e**), two-tailed unpaired Mann-Whitney U test (**f-g, h**) or two-tailed unpaired Student’s t-test (**h, Ly6G<sup>+</sup>CD11b<sup>+</sup>). **a-h,** Data are represented as means ± s.e.m.
Extended Data Fig. 4 | F. PB1 administration alters microbiota and its metabolic profile. a, LEfSe comparison results between the microbiota of Vehicle and F. PB1 treated mice at 12 weeks with the highest linear discriminant analysis LDA score (log10 ≥ 2.0); Veh, n = 8; F. PB1 n = 14 mice/group. b, Faecal concentrations of succinate, isovalerate and valerate in WT and ApcMin/+ mice treated with vehicle (Veh) or F. PB1 as detected by UPLC–HR–MS; WT Veh, WT F. PB1 n = 6; ApcMin/+ Veh n = 11; ApcMin/+ F. PB1 n = 10 mice/group. Data from two independent experiments and represented as means ± s.e.m. P values were determined by two-way ANOVA with Bonferroni post-test.
Extended Data Fig. 5 | SCFAs and F. PB1 spent medium (SUP) increase histone H3 acetylation and reduce NFATc3-calcineurin pathway in vitro on mouse intestinal tumour cell lines. a, Representative WB showing H3K27 acetylation, PP2B-A and NFATc3 in cell lines treated (+) or not (-) with a mix of SCFAs. Three independent experiments were performed with consistent results. b, Densitometric quantification of WB in Fig. 4d showing NFATc3 and PP2B-A (normalized to actin) and H3K27 acetylation (normalized to vinculin). Two or three independent experiments were performed with consistent results (n = 2 or n = 3 biologically independent experiments). Data are represented as means ± s.e.m. and P values were determined by two-tailed unpaired Student’s t-test. c, Densitometric quantification of WB in Fig. 4e showing NFATc3 (normalized to vinculin) and H3K27 acetylation (normalized to H3 tot). To calculate the protein expression induced by SUP evap as a percentage, the densitometric value of SUP was assumed to be 100%. Data from two independent WB (n = 2 biologically independent experiments).
Extended Data Fig. 6 | Effect of F. PB1 spent medium (SUP) in tumourigenesis. a–c, Representative histologies from ileal and colon sections of Apc<sup>Min</sup>+/− mice (a, Veh n = 13; SUP n = 11 mice/group; b, Veh n = 6; SUP n = 7 mice/group) and AOM and DSS treated WT mice (c, Veh n = 5; SUP n = 6 mice/group) treated with vehicle or F. PB1 SUP. Sections stained for H&E from FFPE blocks of swiss roll of small (a, 5X magnification, scale bars 500 μm; b, 3X magnification, scale bars 750 μm) and large (c, 2X magnification, scale bars 900 μm) bowel and treated as indicated by labels. Slides were scanned by Aperio Scanscope and digital images were obtained. Dysplastic lesions were selected and measured. In the lower right high power insert, a detail of dysplastic glands (200X magnification, blue scale bars 100 μm).
Extended Data Fig. 7 | L. lactis does not colonize the mouse gut but its spent medium (SUP) has anti-tumourigenic effect in vivo. **a**, Quantification of SCFAs in SUP of F. PB1 and L. lactis by UPLC–HR–MS. Data from 2 or 6 independent experiments (L lactis SUP n = 2; F. PB1 SUP n = 6 biologically independent experiments). **b,c**, Apc\textsuperscript{Min/+} mice received vehicle (Veh, n = 7 mice/group), F. PB1 or L. lactis (n = 8 mice/group) from week 8 to 10. **b**, Tumour multiplicity in the small intestine normalized to vehicle-treated Apc\textsuperscript{Min/+} mice. **c**, Area and maximum diameter (axis length) of ileal dysplastic lesions normalized to the total number of lesions per mouse. Box plots show the interquartile range, median value and whiskers min to max. **d**, qPCR of L. lactis abundance normalized to panbacterial primers targeting the 16S rRNA gene (UNI 16S) in bacterial DNA extracted from faeces (both at time 0 and 48h after last gavage) and mucus from the ileum and colon of WT and Apc\textsuperscript{Min/+} mice pretreated or not with antibiotics (ABX) and then monocolonized with L. lactis (n = 4 mice/group). **e,f**, 11 weeks old Apc\textsuperscript{Min/+} mice treated with broths not fermented (Veh) or fermented by L. lactis (SUP) in the presence of ABX (n = 6 mice/group). **e**, Tumour multiplicity in the small intestine normalized to vehicle-treated Apc\textsuperscript{Min/+} mice. **f**, Area and maximum diameter of ileal dysplastic lesions normalized to the total number lesions per mouse. Box plots show the interquartile range, median value and whiskers min to max. **g**, Cell proliferation assay on mouse CRC cell lines treated or not with sodium lactate at different concentrations for 48h. t0 is the signal from cells at the time of stimulation. Two independent experiments were performed with consistent results. Data presented as means ± s.d. of a representative experiment (n = 6 biologically independent samples). P values were determined by two-tailed unpaired Mann-Whitney U test (e,f), one-way ANOVA with Bonferroni post-test (b,g) or Kruskal-Wallis test with Dunn post-test (c,d). Data are presented as means ± s.e.m. in a-f.
Extended Data Fig. 8 | *H. biformis* does not colonize the mouse gut but its spent medium (SUP) increases histone H3 acetylation, reduces NFATc3 activation and proliferation in vitro on human colorectal cancer cell lines. a, Cell proliferation assay on human CRC cell lines treated or not (NT) with broth fermented by *F. PB1* (*F. PB1 SUP*) or by *H. biformis* (*H. biformis SUP*) and with a mix of SCFAs as a control. t0 is the signal from cells at the time of stimulation. Two independent experiments were performed with consistent results. Data are presented as means ± s.d. of a representative experiment (*n* = 6 biologically independent samples). b, Densitometric quantification of WB in Fig. 6e showing NFATc3 and H3K27 acetylation (normalized to vinculin). Two or three independent experiments were performed with consistent results (*n* = 2 or *n* = 3 biologically independent experiments). c-e, *ApcMmin*−/− mice received vehicle (Veh) or *H. biformis* from week 8 to 10 (*n* = 5 mice/group). c, Tumor multiplicity in the small intestine normalized to vehicle-treated *ApcMmin*−/− mice. d, Area and maximum diameter (axis length) of ileal dysplastic lesions normalized to the total number of lesions per mouse. Box plots show the interquartile range, median value and whiskers min to max. e, qPCR of *H. biformis* abundance normalized to panbacterial primers targeting the 16S rRNA gene (UNI 16S) in bacterial DNA extracted from faeces and mucus from the ileum. *P* values were determined by one-way ANOVA using Bonferroni (a) or Tukey (b, NFATc3 HT29 and H3K27 Caco2) post-test, by two-tailed unpaired Student’s *t*-test (b, H3K27 HT29) or by two-tailed Mann-Whitney U test (c-e). Data are represented as means ± s.e.m. in b-e.
Extended Data Fig. 9 | Treatments with SCFAs or *F. PB1* spent medium (SUP) increase histone H3 acetylation, reduce NFATc3 activation and proliferation in human CRC samples. **a***-***c**, Human colon tumour samples (hCRC) incubated *ex vivo* with broth fermented with *F. PB1* (SUP) or non-fermented (Veh); with medium alone (NT) or with SCFAs MIX. **a**, Representative immunohistochemistry of one out of three independent experiments of H3K27 acetylation and NFATc3 expression. Scale bars, 50 μm. **b**,**c**, Representative images of ileal dysplastic lesions stained with Ki67. Scale bars, 50 μm. Three independent experiments were performed with consistent results. Dot plots show the percentage of Ki67 positive cells relative to vehicle (**b**) or to NT (**c**). Data from three independent experiments (**b**, n = 7; **c**, n = 5 biologically independent samples), are represented as means ± s.e.m. and P values were determined by two-tailed unpaired Mann-Whitney U test.
Extended Data Fig. 10 | Model. Upon intestinal tumourigenesis selectively some bacterial species do not expand, due to different mucus composition or to competition with other bacteria and their corresponding metabolites. If these bacterial species are reintroduced, they induce the release of SCFAs. F. PB1 or H. biformis metabolic products act as HDAC inhibitors in the adenomas inducing an increase in acetylation and downmodulation of the calcineurin-NFATc3 pathway, which is involved in cell proliferation.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- FACS Diva (BD, v6.2 and v8.0.1), Leica Application Suite X (v3.5.5.19976), Real-Time PCR System Software (Applied Biosystems, v.1.0), Aperio ImageScope Software (Leica Biosystems v12.3.5.3048), Xcalibur v4.0, PEAR (v0.9.6), Usearch (v6.0.307), Bowtie2 (v2.3.4.1), Tango (v1.0)

Data analysis
- Prism (GraphPad software, v6.01b), Excel (Microsoft Office, v17.7.7), FlowJo (FlowJo, v10.5.3), Fiji (ImageJ) v2.0.0-rc-69/1.52n), PhyloPhAn2 (v0.40), curatedMetagenomicData (v1.14.1), MetaPhlAn2 (v2.7.8), DESeq2 (v3.2), Vegan (v2.5.6), LEfSe (v1.0.7), ggplot2 (v3.2.1)

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

**Sample size**

Sample size was chosen taking into consideration the means of the target values between the experimental group and the control group, the standard error and the statistical analysis used. For animal studies, sample size was defined on the basis of past experience with the models, to detect differences of 20% or greater between the groups (10% significance level and 80% power). For ethical reasons, the minimum number of animals necessary to achieve the scientific objectives was used.

**Data exclusions**

Grubb’s test was applied to exclude outliers.

**Replication**

To verify the reproducibility of the experimental findings, all the experiments were performed at least two/three times each (reported in the figure legends), giving us similar results.

**Randomization**

Animals were allocated randomly to each treatment group. Different treatment groups were processed identically, and animals in different treatment groups were exposed to the same environment.

**Blinding**

In IHC and IF analyses, the investigators were unaware of the experimental groups.

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### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies            |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology         |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChIP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- FACS antibodies: CD16/32 – unconjugated (clone93, 14-0161-82, Ebiosciences, 1:50); CD45.2-V450 (clone 104, 560697, BD biosciences, 1:200); CD3-PE-Cy7 (clone 145-2C11, 552774, BD biosciences, 1:200); CD25-APC-Cy7 (clone PC61, 100226, Biolegend, 1:200); Foxp3-PE (clone FJK-16-15, 12-5773-82, Ebiosciences, 1:100); Helios-Alexa-fluor647 (clone 2F6, 51-9883-82, Ebiosciences, 1:100); IL17-PE (clone TC11-18H10, 559502, BD biosciences, 1:100); IFNγ-APC (clone XM61.2.5, 554113, BD biosciences, 1:100); Ly6G-APC (clone 1A8, 560599, BD biosciences, 1:200); Ly6C-APC-Cy7 (clone 1A8, 127624, Biolegend, 1:200); Ly6C-FITC (clone AL-21, 553104, BD biosciences, 1:200); F4/80-APC (clone BM8, 17-4801-82, Ebiosciences, 1:200); CD11b-PerCP-Cy5.5 (clone N418, 117339, Biolegend, 1:200); Fixable viability stain510 (564406, BD Biosciences, 1:1000). For flow gating we used fluorescence minus one (FMO) controls.

- Western blot antibodies: anti-PP2B-A (cat. n°sc-9070, clone H-209, Santa Cruz Biotechnology, 1:1000); anti-NFATc3 polyclonal (clone M-75, cat. n°sc-38321, lot H2010, Santa Cruz Biotechnology, 1:1000); anti-NFATc3 monoclonal (clone F-1, cat. n°sc-8405, lot D1117, Santa Cruz Biotechnology, 1:1000); anti-Histone H3 acetyl K27 (cat. n°ab4729, lot. GR18322-1, abcam, 1:1000); anti-Histone H3 (cat. n°ab1791, abcam, 1:1000); anti-actin (clone AC-40, cat. n°A4700, Sigma, 1:1000); anti-vinculin (clone HVIN-1, cat. n°V93264, Sigma, 1:10000); anti-AAc1 (clone F-19, cat. n°sc-6826, lot. K0512, Santa Cruz Biotechnology, 1:200); anti-Muc13 (cat. n°ab1245654, lot. GR119772-30, abcam, 1:1000); anti-Muc20 (cat. n° PA5-50238, lot. SK247788, Thermofisher, 1:1000); secondary antibodies goat anti-rabbit-HRP (cat. n°170-6515, Bio-Rad, 1:10000); goat anti-mouse-HRP (cat. n°170-6516, Bio-Rad, 1:10000); rabbit anti-goat (cat. n°PO449, lot. 20036798, DAKO, 1:2000).

- Immunofluorescence: primary antibody anti-Muc1 (Rabbit polyclonal antibody, clone aa 474-630, cat. n°5-C343984, lot. 72576, LifeSpan Bioscience, 1:100); secondary antibody donkey anti-rabbit-cy3 (cat. n° 711165153, lot. 130437, Jackson Immuno...
Immunohistochemistry: anti-Ki67 antibody (ab15580, abcam, 1:200); anti-NFATc3 polyclonal (clone M-7, cat. n°sc-8321, lot H2010, Santa Cruz Biotechnology, 1:200); anti-NFATc3 monoclonal (clone F-1, cat. n°sc-8405, lot D1117, Santa Cruz Biotechnology, 1:200); anti Histone H3 acetyl K27 (cat. n°ab4729, lot. GR183922-1, abcam, 1:500 for human and 1:800 for mouse tissues).

Validation
For FACS analysis, after neutralization of unspecific binding with αCD16/CD32 (clone 93, EBioscence), single-cell suspensions were stained with specific mAb (primary antibodies directly conjugated) to assess the phenotype. To validate IHC antibodies, positive and negative controls were incubated with and without primary antibody. Reactivity of primary antibodies for WB and IF was verified adding a positive control in each experiment. Specificity of secondary antibody for IF was verified using only secondary antibody as negative control.

### Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
CT26, HT-29 and Caco-2 cell lines were purchased from the American Type Culture Collection (ATCC). CMT-93 cell line is a kind gift of Dr. David Artis (Cornell University, NY, USA). MC-38 cell line is a kind gift of Dr Carsten Krieg (Zurich University, Switzerland). APC cell line was derived from ApcMin/+ small intestinal adenomas by mechanical disruption.

Authentication
Cell lines were purchased from the American Type Culture Collection (ATCC) and no other authentication method was performed. In APC cell line, derived from ApcMin/+ small intestinal adenomas, the loss of heterozygosity (loss of the WT allele and presence of the Min allele) was checked by TaqMan assay.

Mycoplasma contamination
All cell lines were tested to exclude mycoplasma contamination.

Commonly misidentified lines
No commonly misidentified cell lines were used.

### Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
This study employed both male and female mice (Mus musculus) of the following strains as model organisms: C57BL6/J; C57BL6/J-ApcMin/J; Germ-free ICR. 6 weeks old C57BL6/J mice were purchased from Harlan Laboratories. C57BL6/J457 ApcMin/J (referred to as ApcMin/+ were maintained as inbred strain in our animal facility. For experiments where ApcMin/+ were employed, wild type littersmates born from the same mothers of experimental mice were used as controls. All mice were maintained in microisolator cages in specific pathogen-free (SPF) animal facility. Germ-free ICR male mice were maintained in the isolators at RIKEN IMS (Yokohama, Japan).

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from the field.

Ethics oversight
Experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and approved by the Italian Ministry of Health (n. 139/15).

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

### Human research participants

Policy information about studies involving human research participants

Population characteristics
Colonial human specimens were obtained from patients diagnosed with colon cancer and undergoing surgery at IEO. Inclusion criteria were newly diagnosed CRC patients (stage: 1 to III), aged between 35-70 years old, performance status 0-1 based on the Eastern Cooperative Oncology Group (E 948 COG). Exclusion criteria were a personal history of malabsorption syndrome or any chronic inflammatory bowel disease, or subjects with hereditary syndrome (such as FAP HNPCC) and use of antibiotics in the previous four weeks.

Recruitment
All patients given written informed consent and were enrolled in institutional protocol approved by IEO’s ethical committee. Human biological samples were sourced ethically and their research use was in accord with the terms of the informed consent provided. Case selection was therefore independent and blinded to baseline characteristics, treatments received, clinical outcome and molecular characterization to reduce any potential self-selection bias.

Ethics oversight
The protocol was approved by the IEO’s ethical committee.

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

Plots

Confirm that:
- The axis labels state the marker and fluorochome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Peripheral blood was sampled in heparin and red blood cells were lysed. Small intestinal and colonic lamina propria (LP) lymphocytes were isolated incubating intestinal chunks PBS 5% FCS 1.5 mM EDTA 1mM DTT at 37°C for 15 min to remove epithelial cells. LP cells were mechanically isolated in RPMI 5% FCS with GentleMACS dissociator.

Instrument
FACSCanto II and Fortessa (BD Bioscience)

Software
FlowJo (Treestar, v10.5.3)

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Fig. 3a and Extended Data 3a-e: gate on SSC-A/FSC-A, gate on FSC-A/FCS-H for singlets, gate on CD45+ viability dye-, gate on CD3+CD4+ Tregs gated as FoxP3+ CD25+. Within FoxP3+ CD25+ gated Helios+ events. For Th1/Th17 after gating on CD3+ CD4+ quadrant gate on IL17 and IFNγ. Gate on SSC-A/FSC-A, gate on FSC-A/FCS-H for singlets, gate on CD45+CD3- population. Neutrophils were gated as CD11b+ Ly6G+, inflammatory monocytes were gated as CD11b+Ly6Chi. Extended Data 3f,g: gate on SSC-A/FSC-A, gate on FSC-A/FCS-H for singlets, gate on CD45+ viability dye-CD3- population. Neutrophils were gated as CD11b+Ly6G+. Within the NOT(neutrophils)NOT(macrophages) events we gated the macrophages as CD11b+F4/80+. Within the NOT(neutrophils)NOT(macrophages) NOT(lymphocytes)/ NOT(endothelial cells) events we gated the myeloid dendritic cell population as CD11c+CD11b+ and the Inflammatory monocytes population as CD11c-CD11b+Ly6Chi.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.