

1 **Keystone pathobionts associated with colorectal cancer promote oncogenic reprogramming**

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8

9 **Abstract**

10 *Fusobacterium nucleatum* (Fn) and enterotoxigenic *Bacteroides fragilis* (ETBF) are two
11 pathobionts consistently enriched in the gut microbiomes of patients with colorectal cancer
12 (CRC) compared to healthy counterparts and frequently observed for their direct association
13 within tumors. Although several molecular mechanisms have been identified that directly link
14 these organisms to features of CRC in specific cell types, their specific effects on the epithelium
15 and local immune compartment are not well-understood. To fill this gap, we leveraged single-
16 cell RNA sequencing (scRNA-seq) on wildtype mice and mouse model of CRC. We find that Fn
17 and ETBF exacerbate cancer-like transcriptional phenotypes in transit-amplifying and mature
18 enterocytes in a mouse model of CRC. We also observed increased T cells in the pathobiont-
19 exposed mice, but these pathobiont-specific differences observed in wildtype mice were
20 abrogated in the mouse model of CRC. Although there are similarities in the responses provoked
21 by each organism, we find pathobiont-specific effects in Myc-signaling and fatty acid
22 metabolism. These findings support a role for Fn and ETBF in potentiating tumorigenesis via the
23 induction of a cancer stem cell-like transit-amplifying and enterocyte population and the
24 disruption of CTL cytotoxic function.

25

26 Introduction

27 Colorectal cancer (CRC) is caused by both genetic mutations and aberrant features of the gut
28 microbiome. Specifically, two organisms, *Fusobacterium nucleatum* (Fn) and enterotoxigenic
29 *Bacteroides fragilis* (ETBF), are commonly enriched in the gut microbiomes of CRC patients¹⁻⁷
30 and exacerbate intestinal tumor formation in CRC mouse models^{5,8}. Although a handful of
31 molecular mechanisms have been identified that directly link these organisms with oncogenic
32 pathways, less is known about how they affect distinct cell types within the intestinal
33 compartment.

34
35 Fn was originally identified as an oral pathobiont due to its role in subgingival and periodontal
36 disease^{9,10}, more recent studies find that Fn is associated with a number of cancers, including
37 esophageal cell carcinoma^{11,12}, breast cancer¹³, and most extensively with CRC^{2,7,8,14-17}. Within
38 CRC patients, Fn is spatially enriched in both adenomas and adenocarcinomas^{7,14,16-18}. Fn is
39 often present on CRC tumor tissue and this is linked to its expression of several adhesins,
40 including FadA^{19,20}, and Fap2, the latter of which binds to the sugar residue, Gal-GalNAc^{21,22},
41 overexpressed on CRC tumors²³. In addition to these associations, Fn has been shown to play a
42 causative role in neoplastic transformation, with several recognized mechanisms.

43 *Fusobacterium*-specific effector protein Fap2 interacts with TIGIT (T cell immunoreceptor with
44 immunoglobulin and ITIM domain), a potent mediator of immunosuppression, leading to
45 reduced natural killer cell and cytotoxic T cell mediated cytotoxicity²⁴. Additionally, in *in vitro*
46 and *in vivo* models of CRC, including the commonly used *Apc*^{Min/+} mouse model, Fn protein
47 FadA has been shown to bind to host cells and promote host DNA damage²⁵. This consequently
48 induces beta-catenin and Wnt signaling²⁶ and annexin A1 expression²⁷, which together trigger
49 intestinal cell proliferation^{8,28}.

50
51 Under homeostatic conditions, non-toxigenic *B. fragilis* strains are highly prevalent gut
52 commensals. However, certain *B. fragilis* strains express *B. fragilis* toxin (Bft) and are a
53 common clinicopathological feature in inflammatory bowel disease (IBD)²⁹⁻³¹, diarrheal
54 disease³², and CRC³⁻⁶. ETBF has been shown to play a causal role in murine models of CRC.
55 Specifically, Bft acts as a zinc-dependent metalloprotease that degrades E-cadherin, leading to
56 aberrant signaling by beta-catenin and c-myc, both of which support enterocyte growth and

57 proliferation^{5,33–36}. Furthermore, ETBF exposure elicits robust pro-tumorigenic IL-17 production
58 and Th17 and T regulatory cell responses^{37–40}, further establishing a pro-oncogenic role for this
59 pathobiont.

60

61 To investigate the effects of Fn and ETBF on host intestinal cells, we exposed a mouse model of
62 CRC, as well as wildtype (WT) mice, to these organisms and performed single-cell RNA
63 sequencing (scRNA-seq) on harvested intestinal resections. We utilized an established CRC
64 mouse model that carries a transversion point mutation in one copy of tumor suppressor,
65 *adenomatous polyposis coli (Apc)* (*Apc*^{Min/+}). The biallelic loss of *Apc* is detected in 80-90% of
66 CRC patient cohorts and is an initiating event in sporadic CRC^{41–43}. This mutation predisposes
67 the mice to intestinal tumors and has been previously used to study the effects of both Fn and
68 ETBF on tumor initiation and progression^{8,15,41–44}. Comparing single-cell transcriptional profiles
69 in resections from both WT and *Apc*^{Min/+} mice afforded the opportunity to disentangle the
70 combined effects of genetics and pathobionts on cellular phenotypes without imposing biases
71 upon which cells these organisms most directly affect.

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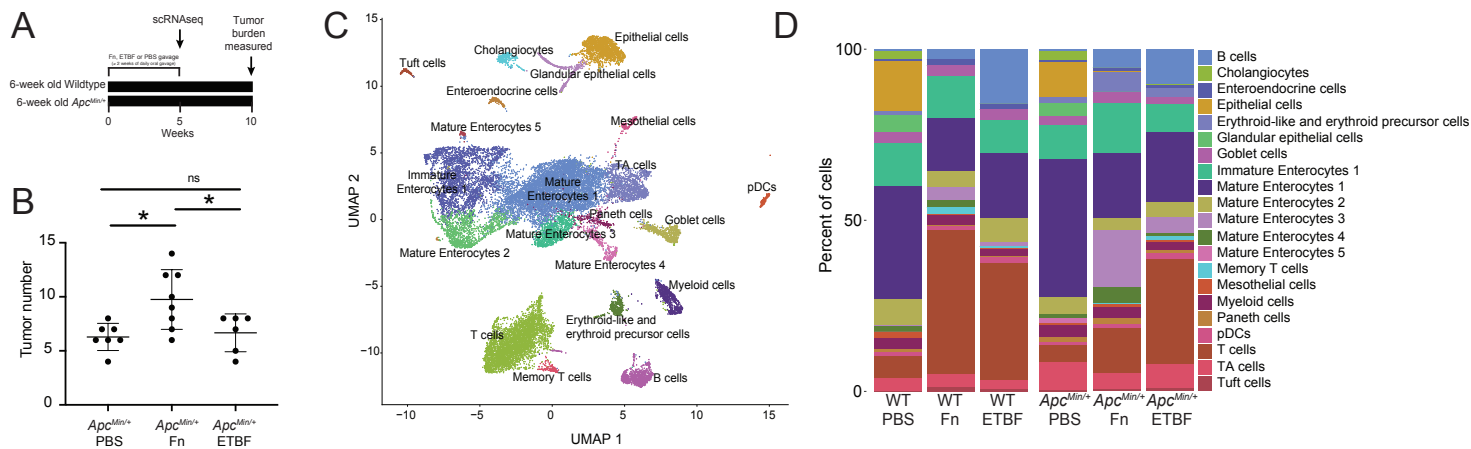
73 **Results**

74

75 **Fn and ETBF alter intestinal cell composition in *Apc*^{Min/+} and wildtype mice**

76 To determine how CRC pathobionts affect the host intestinal microenvironment, we exposed WT
77 and *Apc*^{Min/+} mice to Fn or ETBF. Mice received a daily oral gavage of Fn or ETBF at a
78 concentration of 10⁸ colony forming units (CFUs) to expose intestinal cells to the
79 pathobionts^{8,15,44,45} (**Figure 1A**). Although Fn and ETBF have been reported to reduce survival
80 rates and increase tumor burdens in *Apc*^{Min/+} mice, these effects were limited to mice pre-treated
81 with antibiotics^{8,45–47}. Although antibiotic exposure is associated with increased CRC risk in
82 humans^{48–50}, we chose not to pre-treat mice with antibiotics to avoid introducing confounding
83 effects on host tissue either directly or via altered microbiome composition. Of note, this
84 experimental procedure does deviate from established antibiotic-aided colonization methods and
85 may explain why our downstream findings are different from the literature.^{8,15,44,45} Nonetheless,
86 we observed greater tumor burden 10-weeks after initial pathobiont exposure in the Fn-exposed
87 *Apc*^{Min/+} mice (**Figure 1B**), consistent with previous reports^{8,51}. We were initially surprised that

88 ETBF administration did not result in increased tumor burden, as it does when ETBF is
 89 administered to antibiotic-treated *Apc^{Min/+}* mice^{40,44,45}. ETBF administration, under antibiotic
 90 treated conditions, elicits a robust IL-17 driven inflammatory response that mediates the
 91 recruitment of myeloid cells and ultimately supports tumor cell growth and proliferation in
 92 mice⁵². However, contrary to this pro-tumor phenotype, it is also been shown that ETBF does not
 93 increase the mutations-per-megabase and copy number alterations above that observed in
 94 *Apc^{Min/+}* mice that have been pre-treated with antibiotics⁴⁷. Taken together, without antibiotic-
 95 mediated colonization and the resultant inflammation, macroscopic tumor induction post-ETBF
 96 exposure was likely tempered.
 97



98 **Figure 1. Exposure to CRC-associated pathobionts results in differences in cellular**
 99 **composition and transcriptional profiles.**

100 (A) Depiction of the experiment. (B) Macroscopic tumor burden in *Apc^{Min/+}* mice exposed to Fn
 101 or ETBF sacrificed at 16 weeks of age ($n \geq 6$ *Apc^{Min/+}* mice). Mice were exposed daily to CRC-
 102 associated pathobionts for at least 2 weeks starting at 6-weeks of age. (C) UMAP of
 103 transcriptomic profiles of 24,371 cells from all conditions colored according to their annotations.
 104 (D) A barplot depicting the composition of cells in each experimental condition.

105
 106 We performed scRNA-seq on intestinal tissue from WT and *Apc^{Min/+}* mice after oral dosing of Fn
 107 or ETBF, or phosphate buffered saline (PBS), as a control. Since Fn and ETBF are enriched in
 108 early stages of tumorigenesis (pre-malignant lesions and adenomas) in CRC patients⁵³⁻⁵⁷, we
 109 sacrificed mice at 11-weeks of age corresponding to 5 weeks post-pathobiont exposure or PBS

110 treatment. We transcriptionally profiled 24,371 individual cells, which were clustered into 21
111 different cellular subsets, using Seurat (version 4.1.1)⁵⁸. Cells were annotated with known cell-
112 type specific marker genes^{59,60} and cross-referenced using scMRMA, an automated single-cell
113 annotation algorithm⁶¹ (**Figure 1C**). Cellular compositions across treatment conditions were
114 substantially different, including notable changes across T cells, proliferating enterocyte
115 precursors, and mature enterocytes post-Fn and ETBF exposures (**Figure 1D**).

116

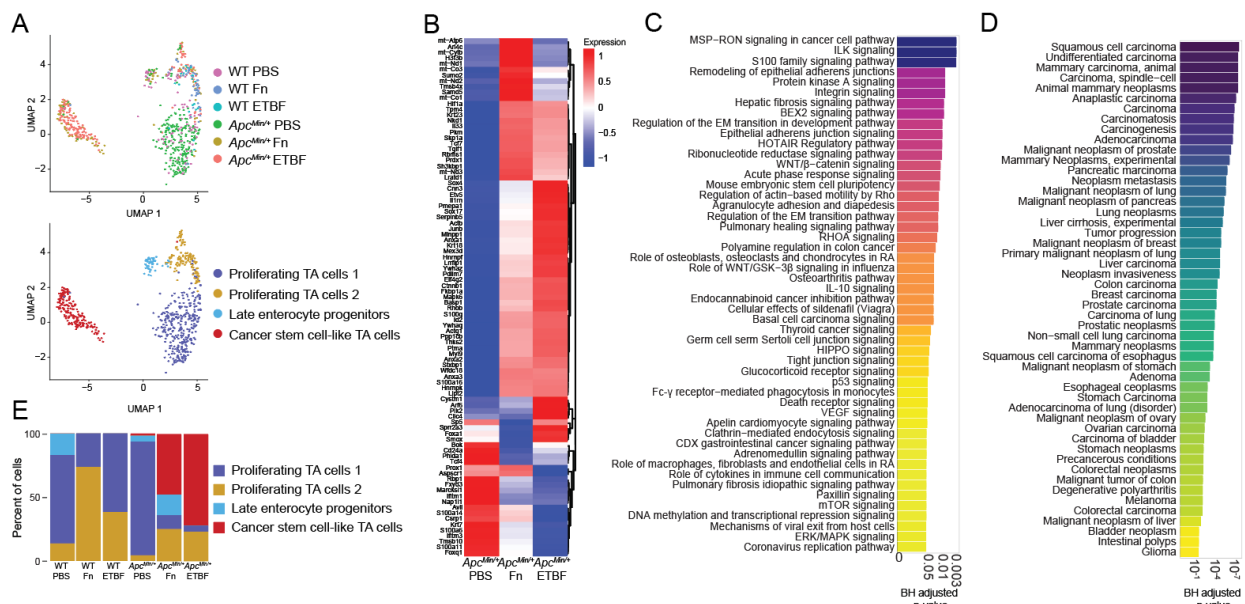
117 **Fn and ETBF promote the outgrowth of cancer stem cell-like transit-amplifying cells and** 118 **cancer-like enterocytes**

119 Transit-amplifying (TA) cells are daughter cells of intestinal stem cells that further differentiate
120 into enterocytes. Due to their high rates of proliferation, they are mutation-prone⁶². Treatment
121 with Fn in co-culture with CRC cell lines has been found to induce the upregulation of stemness
122 associated genes: *CD133*, *CD44*, *Snail1* and *ZEB1*^{63,64}. Similarly, ETBF treatment leads to the
123 increase in stemness in both CRC cell co-cultures and CRC xenograph mouse models, via the
124 upregulation of *JMJD2B*, a histone demethylase⁶⁵. We hypothesized that exposure to Fn and
125 ETBF in *Apc*^{Min/+} mice would exacerbate neoplastic transformation in these cells accordingly.
126 TA cell transcriptomes sub-clustered into four distinct groups, including one that
127 transcriptionally resembles cancer stem cells (CSCs), based similarities in upregulated genes and
128 pathways between the cells we identified and the known phenotypic profile in the literature⁶⁶⁻⁶⁹
129 (**Figure 2A-D**). Using DEG analysis, we identified 91 genes delineating these CSC-like cells
130 from the other TA cell subpopulations (**Figure 2B**). These include upregulated genes that
131 support intestinal cell survival and proliferation, such as *Foxa1*⁷⁰⁻⁷², *Sox4*^{71,73,74}, *Prox1*⁷⁵⁻⁷⁷, and
132 *Ctnnb1*⁷⁸⁻⁸⁰ (Fisher exact test p-values < 0.05, BH-FDR corrected p-values < 0.05, EnrichR).
133 This subpopulation was almost exclusively found in the CRC pathobiont-exposed *Apc*^{Min/+} mice
134 (**Figure 2E**).

135

136 Overall, the CSC-like cells upregulated pro-oncogenic pathways, including integrin and integrin-
137 linked kinase (ILK) signaling, MSP-RON (macrophage-stimulating protein-recepteur d'origine
138 nantais) signaling, and Wnt/ β -catenin signaling, among other pathways relating to stem cell
139 pluripotency and the epithelial-mesenchymal transition (EMT)⁸¹⁻⁸⁶ (**Figure 2C**) (Fisher exact
140 test p-values < 0.05, BH-FDR corrected p-values < 0.05, Ingenuity Platform Analysis (IPA))

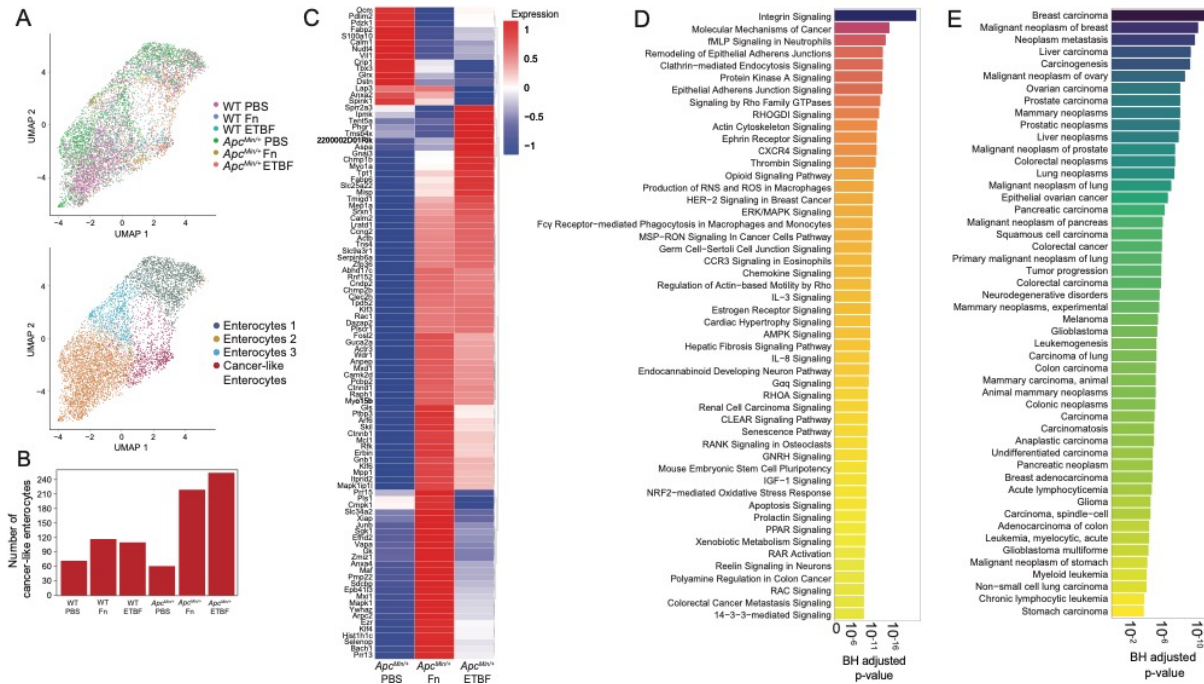
141 canonical pathway analysis, the gene list used as the input for IPA was the result of a comparison
 142 (Wilcoxon test) between CSC-like cell clusters and the other three TA cell clusters). There were
 143 few significant differentially enriched pathways between these CSC-like TA cells specific to
 144 each pathobiont exposure, although Myc-targeting was comparably elevated in cells derived
 145 from *Apc^{Min/+}* mice exposed to ETBF (**Supplemental Figure 1**). As for Fn-exposed CSC-like
 146 cell population, fatty acid metabolism was enriched compared to those exposed to ETBF, a
 147 finding which is supported by *in vitro* experiments linking this phenotype to enhanced self-
 148 renewal(**Supplemental Figure 1**)⁶³. The top 50 most significant human gene-disease annotations
 149 for the DEGs in the CSC-like TA cell population are all cancers, including several related to the
 150 colon (**Figure 2D**) (Fisher exact test p-values < 0.05, BH-FDR corrected p-values < 0.05,
 151 DisGeNET). These colon-specific gene-disease annotations were unique to the CSC-like TA
 152 cells (**Supplemental Figure 2**). However, a second cluster of TA cells (proliferating TA cells 2)
 153 had similar gene-disease associations to the CSC-like TA cells, albeit different DEGs and
 154 enriched pathways. Interestingly, this cluster comprised predominantly cells from wildtype mice
 155 exposed to each of the pathobionts (**Figure 2E, Supplemental Figure 3**). These data suggest
 156 that exposure to CRC-associated pathobionts promotes the induction of cancer-stem cell-like
 157 cells within the *Apc^{Min/+}* mice that possess transcriptomic hallmarks of human cancer stem cells.
 158



159
 160 **Figure 2. TA cells from *Apc^{Min/+}* mice adopt cancer stem-cell like phenotypes after exposure**
 161 **to CRC-associated pathobionts.**

162 (A) UMAP of transcriptomic profiles of TA cells according to experimental condition (top) and
163 subclusters (bottom) (n=682). (B) A heatmap displaying all 91 upregulated genes for the CSC-
164 like cell cluster (compared to the other TA populations) for each genotype-treatment, ($\log_2(\text{fold-}$
165 $\text{change}) \geq 0.25$ (Wilcox test), Bonferroni-corrected p-value < 0.05 , Seurat), plotted as average
166 expression values. (C) A barplot depicting the top 50 IPA Canonical Pathways genesets for the
167 cancer stem cell-like cell population, based on corrected p-values (BH-FDR-corrected p-value $<$
168 0.05 , IPA). (D) A barplot depicting the top 50 genesets according to DisGeNET (y-axis) for the
169 CSC-like cell population, plotted in descending according to corrected p-values (Fisher exact
170 test, BH-FDR corrected p-values < 0.05 , EnrichR). (E) A barplot depicting the percent
171 composition of the cell populations per genotype and treatment.

172
173 Mature enterocytes, derived from TA cells, are directly exposed to the microbiome and make up
174 the vast majority of the cells within CRC tumors^{68,87}. Both Fn and ETBF treatment increases
175 tumor burden due to the outgrowth of transformed enterocytes in certain mouse models and drive
176 rapid proliferation of epithelial cell lines in co-culture experiments^{8,16,26,31,88,89}. Within the mature
177 enterocyte cell population, we performed unsupervised clustering on cellular transcriptional
178 profiles, resulting in four groups (**Figure 3A**). One group was noticeably enriched for cells
179 derived from *Apc*^{Min/+} mice exposed to Fn and ETBF and displayed a unique cancer-associated
180 profile (**Figure 3B, Supplemental Figure 4**). Within this subset, 693 genes are differentially
181 upregulated compared to the other three enterocyte sub-clusters, including the Wnt signaling
182 mediator *Ctnnb1*, canonical cancer markers *STAT3* and *HIF1 α* , and *Klf3*, *Klf4*, *Klf5* and *Klf6*, all
183 of which exhibit tumor suppressive properties in many cancers, including CRC^{80,90-92} (**Figure**
184 **3C, Supplemental Figure 4**). When compared to all other mature enterocyte sub-populations,
185 the DEGs for this subset were enriched for genesets involved in PI3K/AKT/mTOR signaling,
186 p53 signaling and apoptotic pathways (**Figure 3D**) (Fisher exact test p-values < 0.05 , BH-FDR
187 corrected p-values < 0.05 , EnrichR). Analysis using the IPA platform was consistent with
188 DisGeNET, showing a significant enrichment of disease and functional annotations associated
189 with tumorigenesis (**Supplemental Figure 4**). We did not observe any significant differences in
190 this sub-population that was specific to either Fn or ETBF (data not shown). Overall, these data
191 suggest that this mature enterocyte population from pathobiont-exposed *Apc*^{Min/+} mice adopts a
192 cancer-like phenotype, like that observed in TA cells from the same mice.



193
 194 **Figure 3. Mature enterocytes from pathobiont-treated *Apc*^{Min/+} mice display cancer-like**
 195 **phenotypes.**

196 (A) UMAP of transcriptomic profiles of 6,719 enterocyte populations colored by experimental
 197 condition(top) and by sub-clusters (bottom). (B) A barplot displaying the number of cells within
 198 each sub-cluster, according to experimental condition. (C) A clustered heatmap displaying the
 199 top 100 upregulated genes (log₂(fold change) ≥ 0.25 (Wilcoxon test, BH-FDR corrected p-values
 200 < 0.05, Seurat), plotted as average expression values (Seurat) for the cancer-like enterocytes
 201 compared to all other enterocyte populations. (D)A barplot depicting the top 50 IPA Canonical
 202 Pathways genesets (y-axis) based on corrected p-values (Fisher exact test, BH-FDR corrected p-
 203 values < 0.05, IPA) for the cancer-like enterocytes. (E) A barplot depicting the top 50 genesets
 204 according to DisGeNET for the cancer-like enterocyte population, plotted in descending
 205 according to corrected p-values (Fisher exact test, BH-FDR corrected p-values < 0.05, EnrichR).

206
 207 Together, these results support a model in which these pathobionts can influence cancer-
 208 associated signaling cascades, CRC initiation via CSC-like cell population induction and CRC
 209 progression by cancer-like enterocyte enrichment within the context of *Apc*^{Min/+} mouse model.
 210 Supporting our work, a recent study investigating the interplay between Fn and human CRC
 211 tumors found that epithelial cell population with a high Fn burden upregulated Myc, mTORC1

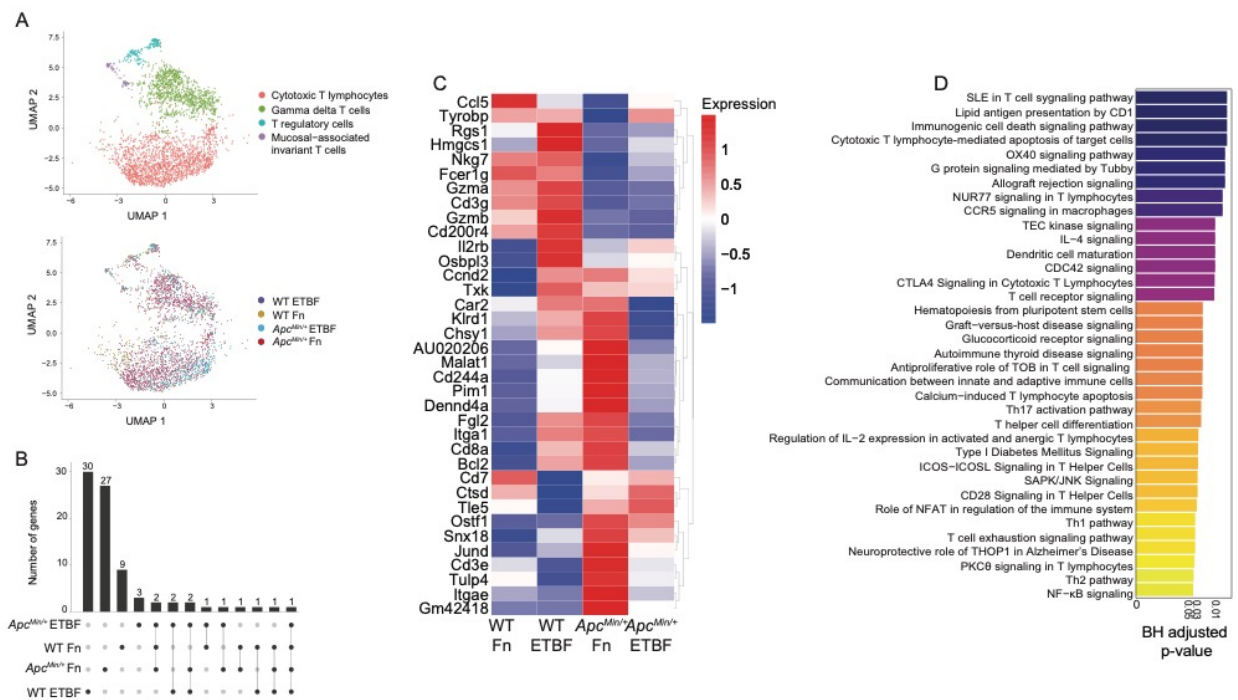
212 and PI3K-AKT-mTOR signaling pathways. This important finding suggests that the enrichment
213 of cell growth and proliferation signaling programs are a specific deleterious outcome elicited by
214 Fn and in our study, ETBF as well.⁷

215

216 **Pathobionts elicit similar effects in both-specific effects on cytotoxic T cells are abrogated**
217 **in *Apc^{Min/+}* mice**

218 T cells are critical for tumor immunosurveillance^{93,94}. However, the colorectal tumor
219 microenvironment drives T cells, including potent anti-cancer CD8⁺ cytotoxic T lymphocytes
220 (CTLs), towards immunosuppressive, senescent, and exhausted states^{95–97}. In addition, CRC
221 pathobionts Fn and ETBF exhibit profound T cell modulatory effects. In previous studies using
222 *Apc^{Min/+}* mice, ETBF exposure led to enhanced T cell differentiation skewing towards Th17 cells
223 and away from CTLs, albeit this effect was indirect, mediated through the recruitment and
224 activation of myeloid derived-suppressor cells (MDSCs)^{40,98}. Similarly, Fn triggers the expansion
225 of MDSCs in *Apc^{Min/+}* mice, although without any effect on T cell populations⁸. However, in
226 humans, Fn abundance within the tumor is inversely correlated with tumor-specific T cell
227 abundances⁹⁹, and in cell culture, Fn directly binds human T cells and inhibits their function,
228 potentially via interactions between TIGIT and Fn adhesin, Fap2^{24,100}. Nevertheless, we did not
229 observe specific changes involving TIGIT engagement because mouse TIGIT does not bind to
230 Fap2²⁴. To define the T cell subsets in our single-cell dataset, we characterized 3,101 T cells.
231 The cells were partitioned using marker genes, yielding 4 subclusters: CTLs, $\gamma\delta$ T cells, T
232 regulatory cells, and mucosal-associated invariant T cells (**Figure 4A**). We focused on
233 characterizing the CTL population, based on previous observations, and because they possess the
234 cytotoxic function essential to the ablation of tumor growth. We also investigated whether
235 microbe-specific transcriptional changes occurred in the myeloid cell compartments and
236 although the myeloid cell counts were considerably low, proinflammatory macrophages derived
237 from the Fn-treated *Apc^{Min/+}* mouse were enriched for positive regulation of SMAD signaling
238 and epithelial-to-mesenchymal transition compared with those from the WT mouse, though
239 pathways did not pass the Bonferroni-correction threshold. (**Supplemental Figure 5**) (Fisher's
240 exact test p-values < 0.05, Bonferroni-corrected p-values < 0.05, EnrichR). The numbers of
241 CTLs isolated from the PBS control animals were also low and were therefore removed from
242 downstream analyses. Of the genes that define the CTL cluster, made up of cells from

243 pathobiont-exposed mice, we observed that genes central to CTL function, including the
 244 cytolytic granule constituents, *Gzma* and *Gzmb*^{101,102}, and to a lesser extent *Cxcr6*^{103–105}, a
 245 chemokine receptor (not shown), are upregulated in the WT pathobiont-exposed mice, but not
 246 the *Apc*^{Min/+} pathobiont-exposed mice (**Figure 4B and C**). These results suggest that the *Apc*^{Min/+}
 247 background, possibly due to tumor-mediated immunosuppression, can mollify cytolytic CTL
 248 responses that are observed in wild-type post-pathobiont exposed counterparts.
 249



250
 251
 252 **Figure 4. Pathobionts elicit similar effects in both-specific effects on cytotoxic T cells are**
 253 **abrogated in *Apc*^{Min/+} mice.** (A) UMAP of transcriptomic profiles of 3,101 T cell populations
 254 colored by sub-cluster (top) and by experimental condition (bottom). (B) Upset plot depicting the
 255 differentially expressed genes that each CTL population ($\log_2(\text{fold-change}) \geq 0.25$, Wilcoxon
 256 test, BH-FDR-corrected p -value < 0.05) based on sample, the set size is the total number of
 257 genes expressed and the intersection size the number of genes that are shared by dataset, an
 258 individual sample alone indicating that the genes are only expressed by the cells in that dataset
 259 and lines representing shared genes. (C) A heatmap displaying the top 36 upregulated genes
 260 ($\log_2(\text{fold-change}) \geq 0.25$, Wilcoxon test, BH-FDR-corrected p -value < 0.05), plotted as average
 261 expression values (Seurat) for the cytotoxic T lymphocytes across each dataset. (D) Barplot

262 depicting the top 36 IPA Canonical Pathways genesets (y-axis) based on corrected p-values
263 (Fisher exact test, BH-FDR corrected p-values < 0.05, IPA). The gene list used as input for
264 canonical pathway analysis were the genes upregulated by ETBF-exposed WT CTLs, when
265 compared to ETBF-exposed *Apc^{Min/+}* CTLs.

266

267 To better understand how the *Apc^{Min/+}* model affects CTLs post-ETBF exposure, we compared
268 the transcriptional profiles from ETBF-exposed *Apc^{Min/+}* with ETBF-exposed WT mice. We
269 found WT ETBF-exposed CTLs upregulated genesets involved in cytotoxic T
270 lymphocyte-mediated apoptosis of target cells, T cell receptor signaling and OX40 signaling
271 pathway^{106–108}, suggesting that ETBF treatment under normal conditions elicits a robust CTL
272 response, and that this is suppressed in the *Apc^{Min/+}* mice (Fisher's exact test p-values < 0.05,
273 Bonferroni-corrected p-values < 0.05, IPA canonical pathway analysis) (**Figure 4D**). These
274 results further support a model where CRC pathobionts induce T-cell dependent immunogenicity
275 that is largely abrogated when tumors are present.

276

277 Discussion

278 Recent cancer pathophysiology studies have shown that the gut microbiota can play a significant
279 role in tumor initiation, progression, or both^{109–112}. Within CRC patients' gut microbiomes,
280 organisms such as Fn and ETBF act as pathobionts, because of their ability to induce host
281 inflammation, DNA damage, and cell proliferation^{109–111}. These bacteria are thought to initiate
282 the formation of carcinogenic bacterial biofilms and antagonize host immunity by tempering
283 anti-tumor immunity^{14,15,24}. Despite a growing body of evidence supporting the role of bacteria in
284 CRC tumor burden and patient survival^{109–111}, much of the work uncovering the mechanisms
285 underpinning this phenomena have been restricted to experiments using cell culture or on
286 specific cell types isolated from mouse models.

287

288 The scRNA-seq data presented here suggests that there are cell-specific and pathobiont-specific
289 effects evident in immune and epithelial tissue. Our analysis reveals that Fn and ETBF can
290 provoke a CSC-like transcriptional profile in TA cells. These CSC-like TA cells bridge
291 pathophysiological observations with specific cellular responses, including, but not limited to,
292 known stemness genes. Moreover, mature enterocytes, which appear to be susceptible to

293 neoplastic transformation, are an emergent feature of *Apc*^{Min/+} intestinal cell profiles post-Fn and
294 ETBF exposure. CTLs, on the other hand, displayed transcriptomes evident of reduced cytotoxic
295 capacity in pathobiont-exposed *Apc*^{Min/+} mice, when compared to their pathobiont-exposed
296 wildtype counterparts. By directly comparing Fn- and ETBF-exposed mice, we observed
297 consistent features invoked by both pathobionts in TA, enterocyte and CTL populations. These
298 results suggest that pathobiont exposure can foster an environment conducive to the outgrowth of
299 tumorigenic intestinal cell populations.

300

301 The effects on TA cells, enterocytes and cytotoxic T lymphocytes that we observe were each
302 affected by the underlying genetic background of the CRC mouse model we used. The
303 *Apc*^{Min/+} mouse model recapitulates a relevant mutation in human CRC (80-90% of all sporadic
304 CRC cases) and is therefore the most widely utilized mouse model for CRC. However, there are
305 some notable differences between this model's pathophysiology versus that which is observed in
306 humans. For instance, the primary site of tumorigenesis in the *Apc*^{Min/+} mouse is the small
307 intestine, rather than the colon¹¹³. Examining the effects of CRC-associated pathobionts in
308 additional mouse models of CRC, including those that exhibit greater colonic tumor burden (*e.g.*
309 mice carrying inducible mutations in *Apc*, *Kras*, and *p53* specific to the colon, such as those
310 driven by Villin or Cdx2)¹¹⁴ could further enhance our understanding of colon-specific
311 tumorigenesis mediated by Fn and ETBF. Notwithstanding these alternatives, the *Apc*^{Min/+} model
312 affords the ability to elucidate microbe-specific transcriptional responses in a system free of
313 numerous cancer drivers and in a model within which these organisms have shown to affect
314 tumorigenesis.

315

316 This study demonstrates the effects of repeat exposure to CRC pathobionts. There are several
317 limitations of our experimental design. First, we did not use antibiotics nor germ-free mice, as
318 we wanted to maintain the native murine microbiome. This came with the caveat that without
319 antibiotics, Fn and ETBF colonization is not robust. We tracked colonization through the study
320 using qPCR with Fn and ETBF specific probes and found that Fn and ETBF engraftment was
321 often below the limit of detection (data not shown). Our results highlight the cellular effects of
322 short-term repeat exposure on intestinal tissue. These results support the hit-and-run
323 carcinogenesis model¹¹⁵⁻¹¹⁸, whereby CRC pathobionts exposure is transient but the pro-tumor

324 effects elicited pathobionts manifest by experimental endpoints. Additionally, we were interested
325 in providing a detailed single-cell characterization of both epithelial subtypes and immune cells
326 from both small intestine and colon. For that reason, we pooled and sequenced cells from both
327 anatomical sites. By doing this, we were able to capture epithelial cell heterogeneity, including
328 the detection and characterization of cancer stem cell-like transit-amplifying cells and cancer-
329 like enterocytes. While this method of single-cell preparation reduced our ability to capture
330 immune cells and other lower abundance cell types such as Paneth and enteroendocrine cells in
331 particular, we avoided examining transcriptional changes induced by cell enrichment methods¹¹⁹.

332

333 Transient exposure, rather than colonization, may have tempered the pro-tumorigenic effects of
334 ETBF (Figure 1B), and possibly Fn, via niche exclusion and/or colonization resistance^{120–122}.
335 Moreover, transient exposure and lack of antibiotic use could limit the pathobiont's access to
336 many of the cell populations traditionally associated with their pathogenic inflammatory etiology
337 such T cells and macrophages, which largely are in the lamina propria, and spatial distance from
338 direct interactions with Fn and ETBF, and their pathogen-associated molecular patterns^{123,124}.
339 Nonetheless, we still find that transient exposure to Fn and ETBF in the *Apc*^{Min/+} model triggers
340 transcriptional programs that support the outgrowth of CSC-like cells and cancer-like
341 enterocytes. Similar short-term exposures to ETBF induces robust cytotoxic T cell responses in
342 wildtype mice. Taken together, this suggests that Fn and ETBF pro-tumor effects could be more
343 robust than previous thought.

344

345 Fn and ETBF are known for their ability to trigger distinct tumor promoting mechanisms. Fn
346 adhesin FadA modulates aberrant Wnt signaling via E-cadherin and β -catenin in enterocytes^{26,27}.
347 ETBF possesses a DNA damaging toxin, Bft, and induces Myc signaling in enterocytes and an
348 inflammatory immune cascade largely mediated by Th17 cells and IL-17^{34,35,38}. One of our
349 study's important findings is that Fn and ETBF, despite their unique tumorigenic proclivities,
350 mostly overlap mechanistically as evidenced by the similar cancer-associated transcriptional
351 programs evoked in enterocyte and enterocyte pre-cursors (Table 1). This suggests that both
352 organisms have common CRC initiating and/or supporting characteristics that affect similar cell
353 types. These findings were enabled by the significant number of enterocytes sequenced across
354 our murine intestinal samples. Herein lies a key shortcoming as well, which does not represent

355 common biology. By probing thousands of enterocytes, other rarer cell types were found in
356 smaller numbers. For this reason, comparative analyses between Fn and ETBF treatments across
357 almost all other cell types, including across both *Apc*^{Min/+} and wild-type mice, were
358 underpowered, and we could not delineate statistically significant differences (BH corrected p-
359 value < 0.05). Nevertheless, our findings still represent an important step in delineating
360 enterocyte and TA cell-specific transcriptomic changes post CRC pathobiont exposure and
361 warrants future investigations delving into larger swath of intestinal cells in depth.

362
363 Although Fn and ETBF are perhaps the most well-known CRC-associated pathobionts, a fuller
364 picture of CRC initiation and progression likely involves other key microbial players. For
365 example, *pks*⁺ *E. coli* is an *E. coli* strain that produces colibactin, a genotoxin that cause double
366 strand breaks in the intestinal cells' DNA also has the ability to transform cells^{125–127}. The
367 development of polymicrobial biofilms is another emergent feature of CRC. Biofilms are
368 significantly enriched in right sided colon adenomas (precancerous lesion) versus adjacent
369 healthy tissue and have been causally linked with CRC in mouse models^{14,15,128}. Additionally,
370 other oral pathobionts beyond Fn, such as *Parvimonas micra*, *Peptostreptococcus stomatis*,
371 *Peptostreptococcus anaerobius* and *Gemella morbillorum*, are commonly enriched in patients
372 with CRC^{111,129,130}. Experimentally, *P. anaerobius* and *P. micra* having been shown to play a
373 causal role in oncogenesis in azoxymethane and *Apc*^{Min/+} mouse models, respectively^{131,132}.
374 Pertaining to these organisms, major questions in the field remained about how these oral
375 microbes, in concert with gut pathobionts, seed biofilms and, if so, whether the biofilms promote
376 tumorigenesis in the colon^{126,133–136}. Performing similarly designed scRNA-seq experiments
377 using additional organisms and eventually consortia will likely be invaluable in delineating the
378 modulatory effects gut bacteria have on CRC tumor initiation and development.

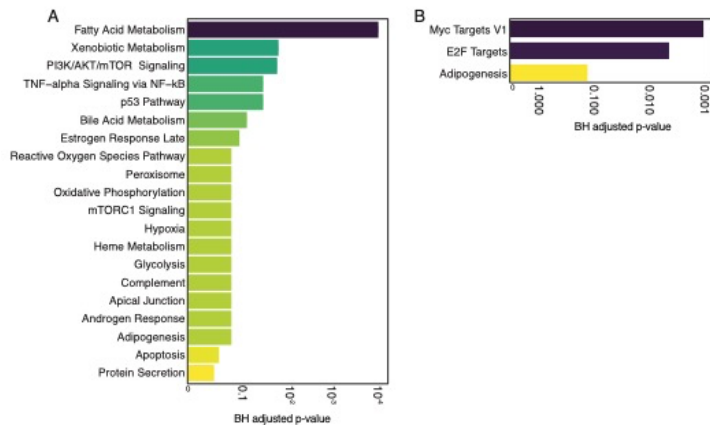
379
380 Tumor-specific microbiomes, biofilm formation, and microbiome dysbiosis are all implicated in
381 CRC progression. Using scRNA-seq, we were able to reconstruct cell type-specific effects that
382 occur post-pathobiont exposure. However, recently developed approaches that enable combined
383 host transcriptomics with microbiome species mapping^{137,138} will provide additional spatial
384 contextualization, directly associating specific gut microbiota with cell-specific transcriptional
385 changes occurring within the tumor microenvironment. Studying the effects of Fn, ETBF and

386 other pathobionts *in vivo*, using unbiased approaches like these offer the promise of identifying
387 marker genes that may be used to enhance cancer diagnostics and therapeutics.

388

389 Supplemental Figures

390



391

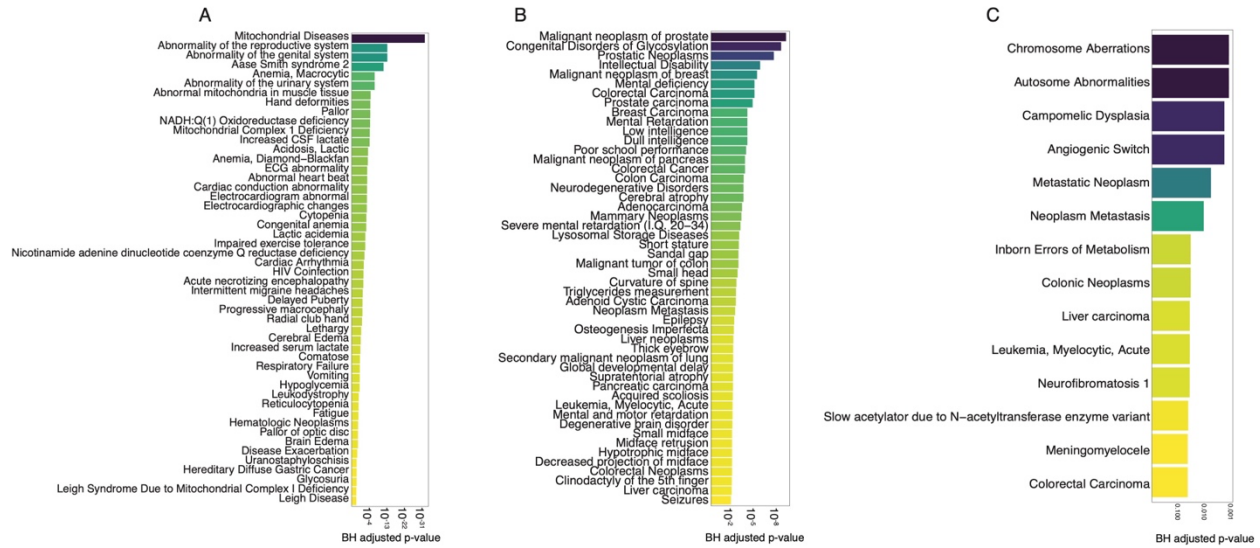
392

393 **Supplemental Figure 1. CSC-like TA cells from Fn- and ETBF-exposed *Apc^{Min/+}* mice**
394 **differed in key pathways.**

395 (A) Top 20 differentially enriched pathways (MSigDB Hallmarks 2020) represented in the
396 transcriptomes of cells from CSC-like TA cells from the Fn-exposed *Apc^{Min/+}* mouse as
397 compared to the PBS-treated *Apc^{Min/+}* mouse. (n= 175 cells, Fisher exact test, BH-FDR-corrected
398 p-values < 0.05, EnrichR) (B) Top 3 differentially enriched pathways (MSigDB Hallmarks 2020)
399 represented in the transcriptomes of cells from CSC-like TA cells from the ETBF-exposed
400 *Apc^{Min/+}* mouse as compared to the PBS-treated *Apc^{Min/+}* mouse. (n= 175 cells, Fisher exact test,
401 BH-FDR-corrected p-values < 0.05, EnrichR). Supplemental Figure 1 complements Figure 2.

402

403



404

405

406 **Supplemental Figure 2. Cancer-specific gene-disease associations with DEGs identified in**

407 **TA cells were specific to those from pathobiont-exposed *Apc*^{Min/+} mice.**

408 (A) A barplot depicting the top 50 genesets according to DisGeNET (y-axis) for the proliferating

409 TA cells (1), plotted in descending according to corrected p-values (x-axis, Fisher exact test, BH-

410 FDR corrected p-values < 0.05, EnrichR). (B) A barplot depicting the top 50 genesets according

411 to DisGeNET (y-axis) for the proliferating TA cells (2), plotted in descending according to

412 corrected p-values (Fisher exact test, BH-FDR-corrected p-values < 0.05, EnrichR). (C) A

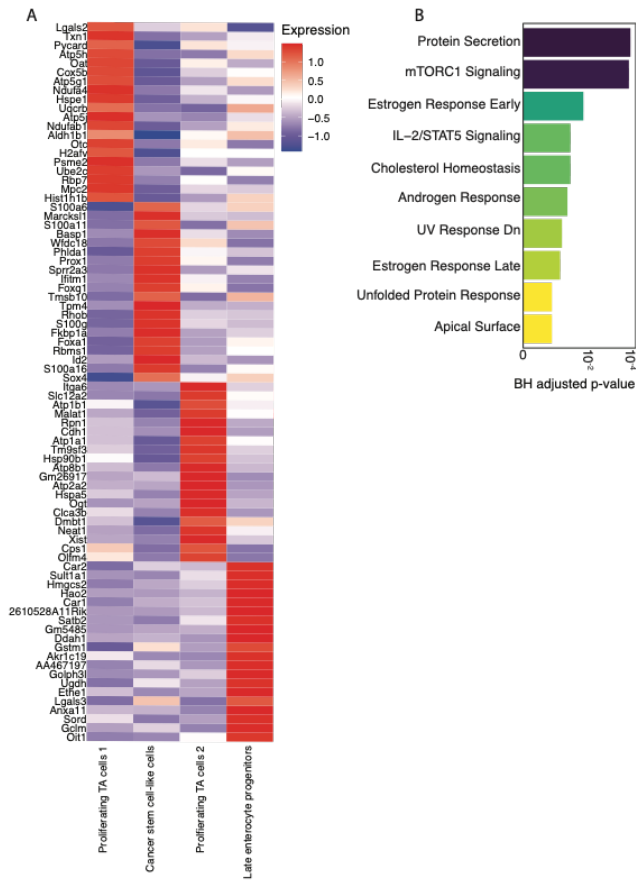
413 barplot depicting the top 14 genesets according to DisGeNET (y-axis) for the late enterocyte

414 progenitors, plotted in descending according to corrected p-values (Fisher exact test, BH-FDR

415 corrected p-values < 0.05, EnrichR). Supplemental Figure 2 complements Figure 2.

416

417



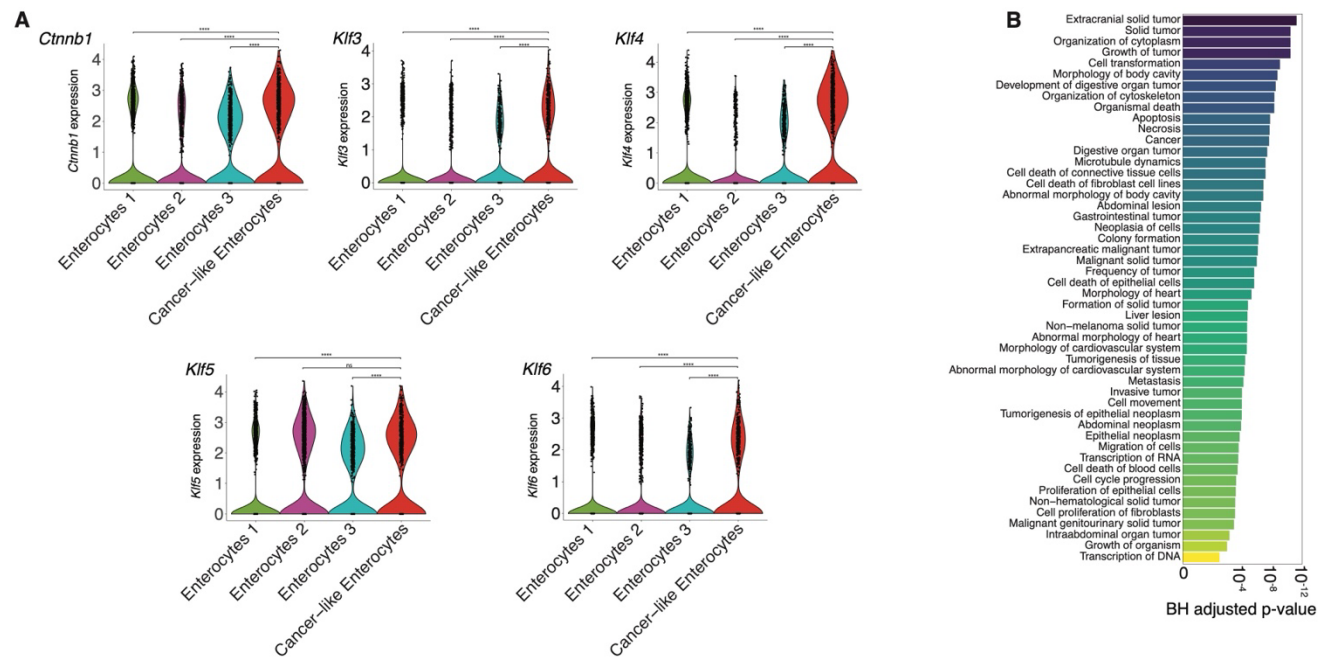
418

419

420 **Supplemental Figure 3. Proliferating TA cells 2, similar to CSC-like TA cells in notable**
 421 **disease associations, diverge at the gene and pathway levels.** (A) The TA cells depicted here
 422 are the 4 subclusters of the complete TA cell population and are an aggregate from all mouse
 423 samples (*Apc^{Min/+}* mice treated with PBS, Fn or ETBF and wild type mice treated with PBS, Fn
 424 or ETBF. A heatmap displaying the top 20 upregulated genes for each TA cluster, $\log_2(\text{fold-}$
 425 $\text{change}) \geq 0.25$ (Wilcox test), corrected p-value < 0.05 (Bonferroni correction), Seurat), plotted
 426 as average expression values (Seurat). (B) Differentially enriched pathways represented in the
 427 transcriptomes of proliferating TA cells 2 compared with other TA cell populations. Barplot
 428 depicting the top 10 genesets according to the Molecular Signatures Database Hallmark 2020
 429 (MSigDB Hallmarks 2020) for the cancer-like cell population, plotted in descending according
 430 to corrected p-values (Fisher exact test, BH-FDR corrected p-values < 0.05 , EnrichR).

431 Supplemental Figure 3 complements Figure 2 and Supplemental Figure 2.

432



433

434

435 **Supplemental Figure 4. Transcriptome profiles of cancer-like enterocytes were enriched in**

436 **cancer-like genes and pathways. (A) Violin plots displaying selected CRC-associated genes**

437 **and their expression levels across 4 enterocyte clusters ($\log_2(\text{fold-change}) \geq 0.25$, Wilcoxon test,**

438 **Bonferroni-corrected p-value < 0.05). (B) Barplot depicting the top 50 IPA Diseases and**

439 **Functions annotations based on corrected p-values (Fisher exact test, BH-FDR corrected p-**

440 **values < 0.05,) for the cancer-like enterocyte subpopulation. Statistical comparisons were**

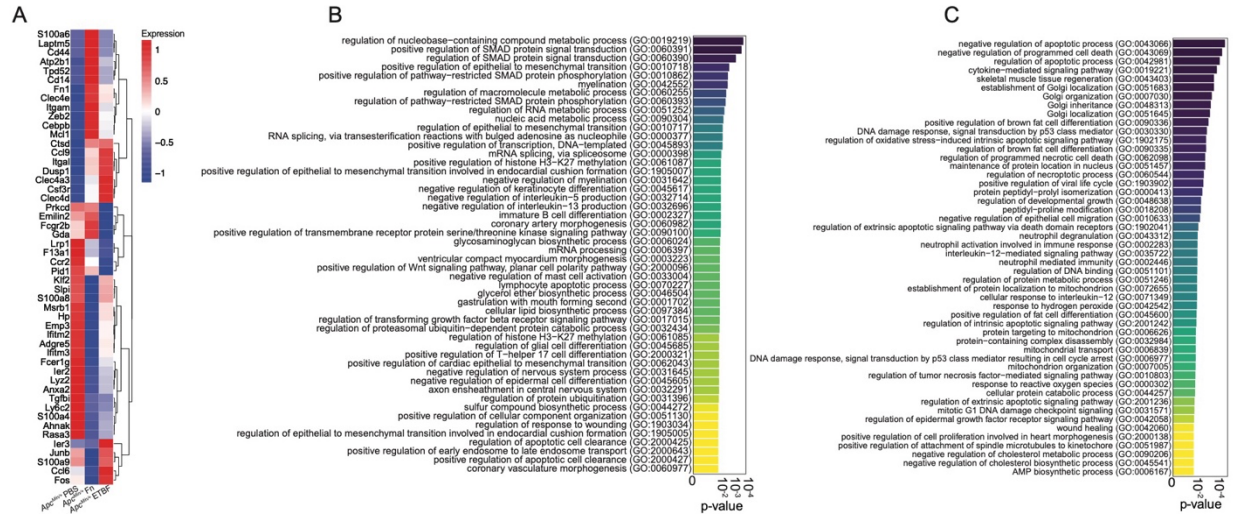
441 **performed using a pairwise Wilcoxon test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = p**

442 **≤ 0.0001), comparing the cancer-like enterocyte population to all other mature enterocyte**

443 **clusters (see Supplemental Figure 4A). Supplemental Figure 4 complements Figure 3.**

444

445



446

447

448 **Supplemental Figure 5. Proinflammatory macrophages derived from the Fn-exposed**

449 ***Apc*^{Min/+} mouse upregulate pathways associated with TGF-β/SMAD signaling and**

450 **epithelial-to-mesenchymal transition. (A) A heatmap displaying the top 50 upregulated genes**

451 **defining the proinflammatory macrophage population compared across each dataset (\log_2 (fold-**

452 **change) ≥ 0.25 , Wilcoxon Rank Sum test, p-value < 0.05 (unadjusted), Seurat), plotted as**

453 **average expression values. (B) Barplot depicting the top 50 enriched genesets according to the**

454 **Gene Ontology Biological Processes 2021 (GOBP21) for proinflammatory macrophages derived**

455 **from Fn-exposed *Apc*^{Min/+} mice when compared to PBS control *Apc*^{Min/+} mice, plotted in**

456 **descending according to p-values (Fisher exact test p-values < 0.05 , unadjusted, EnrichR). (C)**

457 **Barplot depicting the top 50 enriched genesets according to the Gene Ontology Biological**

458 **Processes 2021 (GOBP21) for proinflammatory macrophages derived from Fn-exposed *Apc*^{Min/+}**

459 **mice when compared to ETBF exposed *Apc*^{Min/+} mice, plotted in descending according to p-**

460 **values (Fisher exact test p-values < 0.05 , unadjusted, EnrichR).**

461

462 **Materials and Methods**

463

464 **Ethical considerations**

465 This study conformed to the National Institutes of Health guidelines on the care and use of

466 laboratory animals. Mouse studies were performed following procedures approved by the

467 Institutional Animal Care and Use Committee at Cornell University (Protocol ID #2016-0088)

468

469 **Bacterial Strains and Culturing**

470 *Fusobacterium nucleatum* subsp. *nucleatum* strain VPI 4355 [1612A] (ATCC 25586) was
471 purchased from American Type Culture Collection (ATCC). *Bacteroides fragilis* (Veillon and
472 Zuber) Castellani and Chalmers strain 2-078382-3 (ATCC 43858) (ETBF) was purchased from
473 American Type Culture Collection (ATCC). Fn and ETBF were grown anaerobically at 37°C on
474 Bacto™ Brain Heart Infusion Broth (BD, Sparks, MD) supplemented with 0.01% Hemin in 1M
475 NaOH, 0.1% Resazurin (25 mg/100ml distilled water), 10% NaHCO₃ in distilled water, and agar
476 if bacteria were plated. Bacteria were grown overnight and diluted to 10⁸ colony forming units
477 (CFU), the amount needed for oral gavage.

478

479 **Mice**

480 All mice (C57BL/6-*Apc*^{Min/+}/J and C57BL/6-Wild type) were maintained at the barrier mouse
481 facility at Weill Hall at Cornell University. *Apc*^{Min/+} and wild-type mice were initially ordered
482 from Jackson Laboratory and then bred in the barrier facility. The *Apc*^{Min/+} mice used in these
483 experiments have a chemically induced transversion point mutation (a T to an A) at nucleotide
484 2549. This results in a stop codon at codon 850, truncating the APC protein. Experimental and
485 breeding mice were provided with *ad libitum* access to autoclaved water and rodent chow
486 (autoclavable Teklad global 14% protein rodent maintenance diet #2014-S; Envigo). To avoid
487 cage effects on the microbiota, mice were housed individually at the time of initial Fn or ETBF
488 exposure. To monitor for infectious agents such as helminths, sentinel mice were used during the
489 duration of the experiment in the mouse facility to ensure that results following perturbation with
490 Fn and ETBF were a result of specific bacteria and not confounding agents. Every week, food
491 intake and animal weight were recorded, and mice were placed in clean cages with freshly
492 autoclaved chow and water weekly. Mice were handled under inside a biosafety cabinet with
493 frequent glove changes and disinfection between mice during stool collection and monitoring of
494 body weight. Stool was collected weekly throughout the course of all experiments. Bacterial oral
495 gavage experiments were performed every day for a period of at least 14 consecutive days for
496 ETBF, and up 35 days for Fn^{8,25,45}, beginning at 6 weeks of age. Bacteria were fed at a
497 concentration of 10⁸ CFU per day. Sham treatment consisted of sterile Ca²⁺ and Mg²⁺ free
498 phosphate buffered saline gavaged daily for the entirety of the experiment. Single-cell RNA

499 experiments concluded when the mice were 11 weeks old and tumor burden experiments
500 concluded when mice were 16 weeks old.

501

502 **Tumor burden enumeration**

503 For tumor enumeration, *Apc^{Min/+}* mice were euthanized at 16 weeks of age, and colons and small
504 intestines were excised. Macroscopic tumors were counted from both anatomical sites. The
505 tumor counts were plotted using Prism (version 8.2.1). For statistical analysis, Mann-Whitney
506 two-tailed tests were used to compare treatment groups using Prism. Each groups had an $n \geq 6$
507 mice.

508

509 **Single cell dissociation from fresh mouse colons and small intestines**

510 This protocol was adapted from Haber et al 2017⁵⁹. To generated single-cell suspensions,
511 *Apc^{Min/+}* and wild type mice were euthanized at 11 weeks of age, colons and small intestines
512 were excised, rinsed with ice cold sterile 1X Ca^{2+} and Mg^{2+} free PBS (Gibco, 14190144) and
513 flushed of fecal contents using a blunt 1.5-inch 22G needle filled with ice cold sterile 1X Ca^{2+}
514 and Mg^{2+} free PBS (Gibco, 14190144). The tissue was opened longitudinally and sliced into
515 small fragments roughly 1 cm in length. The tissue was incubated in RPMI supplemented with
516 L-glutamine (Corning, 45000-396), 1 mM EDTA (Neta Scientific, QB-A611-E177-10), and 10%
517 FBS (Avantor, 97068-085) for 90 minutes, shaking every 30 minutes. The tissue was then
518 incubated at 37°C for 15 minutes and continuously shaken. The supernatant was passed through
519 a 100 μm cell strainer and held on ice until loading the cells on 10X Chromium. The remaining
520 tissue was resuspended in RPMI (Corning, 45000-396) supplemented with 20% FBS (Avantor,
521 97068-085), 0.1 mg/ml DNase I (Thermo Scientific, 90083), and 0.5 mg/ml collagenase A
522 (Millipore Sigma, 10103586001) and incubated at 37°C on a shaker for 30 minutes. The tissue
523 was then gently mechanically dissociated using a rubber plunger of a syringe. The tissue and the
524 dissociated contents were passed through a 100 μm cell strainer. The single cell suspension was
525 then pelleted via centrifugation (400 x g for 10 minutes at 4°C). The cell suspension was
526 resuspended in 1X Ca^{2+} and Mg^{2+} free PBS (Gibco, 14190144) containing 0.04% weight/volume
527 BSA (VWR, 97061-420) and combined with earlier collected fraction and placed on ice. Sample
528 viability was determined before loading the cells on 10X Chromium using the Countess II

529 Automated Cell Counter (ThermoFisher). The desired number of transcriptomes from viable
530 cells for each sample was 5000-6000 cells per sample.

531

532 **Single-cell RNA sequencing library preparation**

533 5000-6000 viable ($\geq 70\%$ alive) cells per sample (from colon and small intestine tissues) were
534 targeted on the 10X Genomics Controller using one lane per mouse/sample for Gel Beads in
535 Emulsion (GEM). Cells from the small intestine and colon were pooled together before GEM
536 creation. Briefly, cells were separated into GEMs along with beads coated in oligos that capture
537 mRNAs using a poly-dT sequences. This was followed by cell lysis and barcoded reverse
538 transcription of mRNA, followed by amplification, and enzymatic fragmentation and 5' adaptor
539 and sample index attachment. Single-cell libraries were generated using the Chromium Next
540 GEM Single Cell 3' Library Construction V3 Kit (10X Genomics) and were then sequenced on
541 an Illumina NextSeq 2000 run with the 100 bp P2 kit for all samples. Sequencing data were
542 aligned to the mouse reference, mm10 (Ensembl 84) reference genome using the Cell Ranger
543 5.0.1 pipeline (10X Genomics).

544

545 **Single-cell RNAseq data processing and visualization**

546 The output of Cell Ranger is a cell-by-gene unique molecular identifier (UMI) expression matrix
547 for each sample. The expression matrices for each sample are loaded into the Seurat R package
548 (Seurat version 4.1.1, R version 4.1.0 and 4.2.0). The standard Seurat dataset processing
549 workflow was followed. In brief, cells with less than 200 genes, more than 2,500 genes, and
550 more than 35% mitochondrial genes are filtered out. After filtering, the remaining cells were
551 normalized by the total expression, multiplied by the default scale factor (10,000), and log
552 transformed. We then used default Seurat functions to identify highly variable genes with one
553 parameter modification. FindVariableFeatures' nfeature parameter was set to 3,000 instead of
554 2,000 (default). Next, we scaled the data to regress out variation from mitochondrial genes. We
555 performed principal component analysis (PCA) on the scaled data with variable genes. The top
556 20 principal components were used for downstream analysis, including dimensionality reduction
557 steps including clustering cells to identify cell populations (clusters). We implemented Uniform
558 Manifold Approximation and Projection for dimensional reduction using the top 20 PCs and
559 visualized.

560

561 **Marker-gene identification and cell-type annotation**

562 To define cell types for each cluster, we used Seurat's FindAllMarkers with the following
563 parameters: a minimum percent expression value of 25%, log₂fold change threshold of 0.25 and
564 a corrected p-value < 0.05 (Bonferroni correction). We looked only at transcripts that were
565 upregulated. We analyzed canonical markers and assigned cell annotations accordingly (see
566 Supplemental Table 1). We cross-referenced our cell type annotations with gene lists defined in
567 Haber et al.⁵⁹ and Moor et al.⁶⁰ We cross-reference the cell type assignments with a single cell
568 annotation algorithm, scMRMA in R as well.⁶¹

569

570 **Reclustering, visualization, and analysis of transit-amplifying cells, mature enterocyte (1) 571 and T cell populations**

572 We used the 682 TA cells, 6,719 mature enterocytes (1), and 3,101 T cells and re-clustered them
573 using Seurat. Marker genes for each subclusters were identified using a minimum percent
574 expression value of 25%, log₂fold change threshold of 0.25 and a corrected p-value < 0.05
575 (Bonferroni correction) in Seurat. Cell types were assigned based on the expression of these
576 marker genes. Cell clusters expressing marker genes from multiple unrelated cell types
577 (doublets) were removed from analysis. All sub-clustering analysis was carried out with 20
578 principal components and similar resolution parameters; TA cells and T cells were analyzed with
579 a resolution of 0.4 and mature enterocytes (1) with a resolution of 0.3 in Seurat. The marker gene
580 list used to classify cell subtypes can be found in Supplemental Table 1. Cell populations were
581 visualized using Uniform Manifold Approximation and Projection in Seurat. Cell were
582 enumerated, whether as percent of sample or absolute count, using the dittoSeq's (version 1.8.1)
583 bar plot visualization function.

584

585 **Differential gene expression and geneset enrichment analysis**

586 Differentially gene expression was carried out using Seurat's FindAllMarkers and FindMarkers
587 functions with the following cutoffs: log₂(fold change) ≥ 0.25 (Wilcox test), corrected p-value <
588 0.05 (Bonferroni correction) and a minimum percent expression value of either the default, 10%,
589 or 25% for certain other analyses. For these analyses, only upregulated genes were used. We
590 visualized DEGs using the Seurat's DoHeatmap and dittoHeatmap (dittoSeq) for heatmaps,

591 dittoPlot(dittoSeq) for violin plots and UpSetR (version 1.4.0) for upset plots. For statistics
592 associated with violin plots (Supplemental Figure 4), we performed a two-sample Wilcoxon test,
593 comparing each normal enterocyte cluster against the cancer-like enterocyte cluster using the
594 `stat_compare_means` function in `ggpubr` (version 0.5.0). For gene set enrichment analysis, the
595 gene list used as input were generated as detailed above using FindMarkers (Seurat). A suite of
596 tools and databases were implement for these analysis and are as follows: Ingenuity Pathway
597 Analysis (IPA, Qiagen) including canonical pathway and disease and function analysis,
598 DisGeNET (version 7.0) via Enrichr^{139,140}, and MSigDB Hallmarks 2020 via EnrichR.¹⁴⁰

599

600 **Acknowledgements**

601 We thank the de Vlaminck lab for helpful conversations. This work was supported in part by a
602 grant from the National Institutes of Health (1R33CA235302-01A1). J.J. was funded by a
603 fellowship from the Center for Vertebrate Genomics at Cornell University. I.L.B. was funded by
604 the National Institutes of Health (1DP2HL141007), a Pew Foundation Research Fellowship and
605 a Packard Foundation Fellowship.

606

607 **Data Availability**

608 Single-cell RNA-seq data are being deposited at NCBI GEO and will be made publicly available
609 upon publication.

610

611 **References**

612

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