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Zeinab Homayed, Guillaume Belthier, Tinhinan Lahlou, Laia Bassaganyas, Emmanuelle Sidot, Pierre Giroux, Jennifer Series, Lucile Bansard, François Gerbe, Chenchen Pan, et al.

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# Cancer Cell

## Early dissemination highly enhances liver metastasis development during intestinal tumorigenesis

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<b>Abstract:</b>	<p>The ability of yet benign tumor cells to disseminate from the primary tumor before transforming to their full metastatic potential has recently received increasing attention. So far, the existence of such function of cells at early benign stages of intestinal tumorigenesis could not be demonstrated. Here, we introduced and characterized a genetic mouse model with specific tamoxifen-driven induction of tumorigenesis in intestinal tdTomato expressing cells. This model enabled us, already at the adenoma stage, to detect the sparse but obvious presence of intestinal disseminated cells in the liver. In parallel, an elevated plasma level of TIMP1, most prominently, as well as TNF-<math>\alpha</math>, SDF-1, CXCL2, and M-CSF was detected, a pattern, which was also observed in blood of patients with benign intestinal polyps.</p> <p>In the liver, the presence of early disseminated cells, in synergy with the detected systemic factors, was accompanied by severe infiltration of myeloid cells including macrophages and neutrophils. This enrichment facilitated metastatic growth in this organ when challenged with carcinoma cell inoculation.</p> <p>This study expands the concept of the impact of early disseminated intestinal cells in synergy with circulating factors on the creation of a pre-metastatic niche to promote distant metastases.</p>
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<p><b>Clinical Trials</b></p> <p>Does your manuscript report clinical trial results?</p>	Yes
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Dear Editor,

Based on your wish to discover the whole story during the presubmission process, we would like to submit manuscript entitled "**Early dissemination highly enhances liver metastasis development during intestinal tumorigenesis**" as an original paper to Cancer Cell.

In the classical model of metastasis, the dissemination of tumor cells occurs at late stages of cancer disease. However, recent studies have demonstrated that tumor cells can spread to distant organs at much earlier stages than previously believed. Recently, an elegant computational study from patient samples has strongly suggested that early dissemination could also occur in colorectal cancer. However, despite the prevalence and lethality of colorectal cancer, no functional study on early tumor dissemination had been yet performed.

In this study, we have filled this void by introducing and characterizing an inducible mouse model that enables the tracking of cellular dissemination during the onset of intestinal tumorigenesis.

Using this model, we track rare **intestinal cells disseminating into liver**, which is the main organ for metastasis development in man. This early dissemination induces liver remodeling, including a drastic myeloid cell enrichment. By add back experiments we show that early-disseminated tumor cells alone can remodel the liver when they are injected in mice.

We also show that liver remodeling, induced **by early disseminated cells in synergy with cytokines, greatly facilitates late metastatic cell colonization**

Importantly we confirm these results in various complementary mouse models.

Importantly also, some **myeloid cell-associated cytokines** are more present in the plasma of mice developing adenomas and in **the plasma of patients diagnosed with intestinal polyps**.

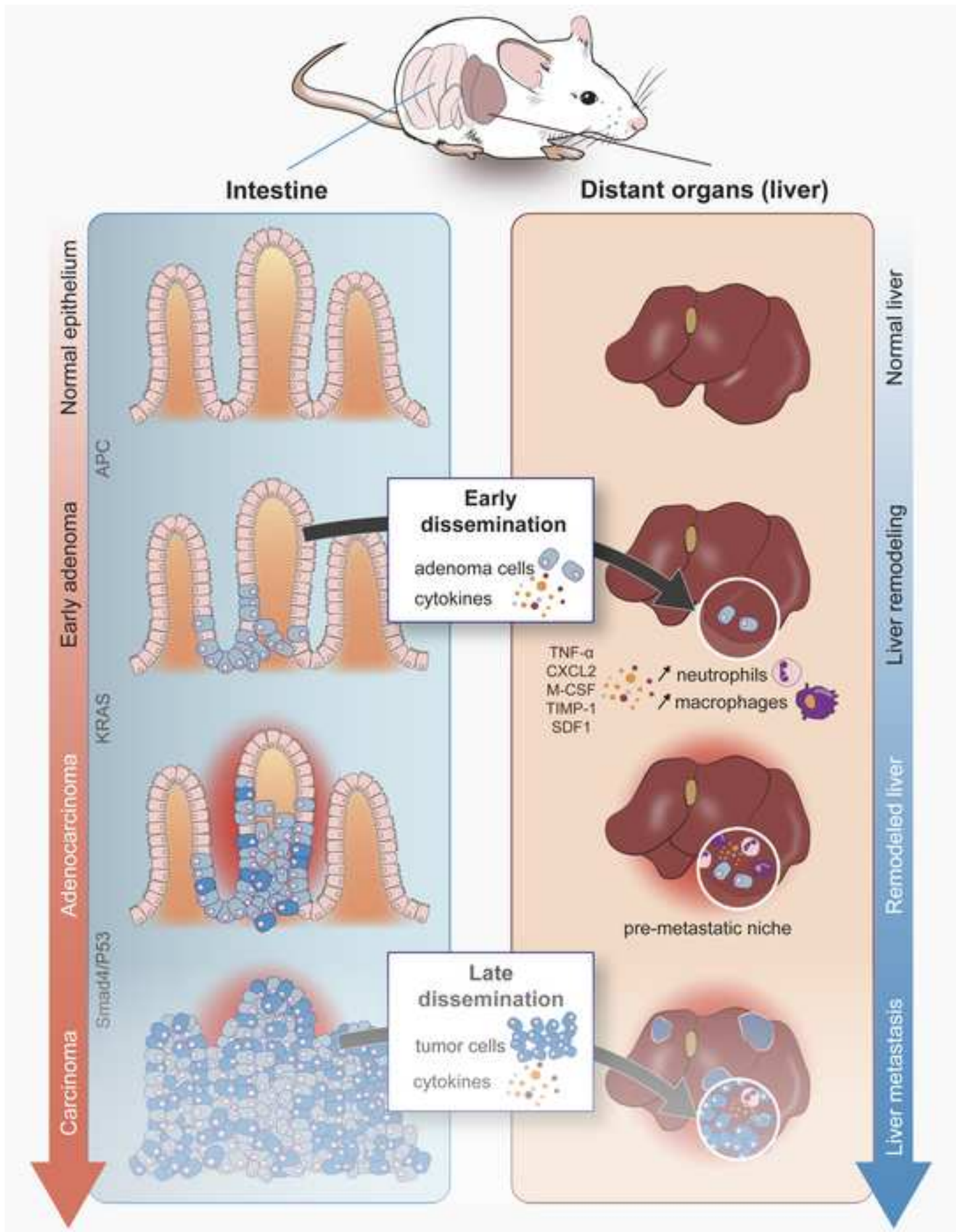
Overall, this is a significant breakthrough in the field of tumor dissemination in colorectal cancer, for which metastasis is invariably the last fatal stage of the disease.

We thank you for your consideration.

Best regards,

Julie Pannequin

A handwritten signature in black ink, consisting of several overlapping loops and a horizontal line, positioned below the name Julie Pannequin.



1 **Early dissemination highly enhances liver metastasis development during intestinal**  
2 **tumorigenesis**

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29 **Keywords**

30 Early dissemination, pre metastatic niche, intestinal tumorigenesis and systemic factors.

31 **Summary**

32 The ability of yet benign tumor cells to disseminate from the primary tumor before transforming  
33 to their full metastatic potential has recently received increasing attention. So far, the existence  
34 of such function of cells at early benign stages of intestinal tumorigenesis could not be  
35 demonstrated. Here, we introduced and characterized a genetic mouse model with specific  
36 tamoxifen-driven induction of tumorigenesis in intestinal tdTomato expressing cells. This model  
37 enabled us, already at the adenoma stage, to detect the sparse but obvious presence of  
38 intestinal disseminated cells in the liver. In parallel, an elevated plasma level of TIMP1, most  
39 prominently, as well as TNF- $\alpha$ , SDF-1, CXCL2, and M-CSF was detected, a pattern, which  
40 was also observed in blood of patients with benign intestinal polyps.

41 In the liver, the presence of early disseminated cells, in synergy with the detected systemic  
42 factors, was accompanied by severe infiltration of myeloid cells including macrophages and  
43 neutrophils. This enrichment facilitated metastatic growth in this organ when challenged with  
44 carcinoma cell inoculation.

45 This study expands the concept of the impact of early disseminated intestinal cells in synergy  
46 with circulating factors on the creation of a pre-metastatic niche to promote distant metastases.

47



## 48 INTRODUCTION

49 According to the World Health Organization (WHO), around 10 million people die each year  
50 from metastatic cancer<sup>1</sup>.

51 Despite a century of studies, the process of tumor dissemination remains poorly described. In  
52 the classical model of metastasis, the dissemination of tumor cells occurs at a late stage of  
53 cancer disease, when the tumor cells have gained their full malignant potential; after the  
54 primary tumor has grown and acquired mutations, the malignant tumor cells can invade the  
55 local tissue, enter the blood or lymphatic vessels, and colonize distant organs.

56 However, there is growing evidence in some cancers that tumor cells spread to distant organs  
57 much earlier than previously believed. For example, early dissemination has been described  
58 in melanoma, pancreas, lung, gastric and breast cancer using elegant mouse models and  
59 validated on patient samples<sup>2-7</sup>.

60 Considering the significant incidence and mortality rates of colorectal cancer (CRC) worldwide,  
61 there has been little functional investigation of early tumor dissemination in this disease.

62 However, circulating epithelial cells have been detected in the blood of some patients that are  
63 prone to colorectal cancer such as those with intestinal polyps<sup>8</sup> and inflammatory bowel  
64 diseases including Crohn's syndrome<sup>9</sup>. Moreover, In 2019, a computational study on  
65 metastatic samples from patients with colorectal cancer proposed that the metastatic seeding  
66 occurs early, when the tumor is not clinically detectable and years before diagnosis and  
67 surgery<sup>10</sup>. Therefore, reaching a comprehensive understanding of the mechanisms underlying  
68 early colorectal cancer (CRC) spread is essential for developing effective therapeutic  
69 strategies to delay or prevent metastasis.

70 To this end, we have generated an inducible transgenic mouse model that enables us to track  
71 early dissemination of intestinal tumorigenesis. This mouse model expresses tdTomato in the  
72 whole intestinal epithelium and spontaneously develops intestinal adenomas due to *Apc*  
73 mutation, which mimics the early stages of the disease.

74 Using this mouse, we provide the first evidence of the early dissemination of intestinal cells  
75 and suggest that, in synergy with systemic factors (TIMP1, TNF- $\alpha$ , SDF-1, CXCL2, and M-  
76 CSF), these cells induce liver remodeling that increases the permissiveness of this organ for  
77 later metastasis-initiating cells.

78 These findings shed light on the complex molecular and cellular mechanisms involved in  
79 metastasis, which begins earlier than previously believed, and highlight a potential role of early-  
80 disseminated tumor cells in driving this process.

## 81 RESULTS

### 82 **Validation of the transgenic mouse model and detection of early intestinal cells in the** 83 **liver**

84 To promote early lesions in the intestine and track potential early dissemination, we generated  
85 an inducible transgenic mouse model, named AVCT, with three elements: *Apc*, which is  
86 considered as the gatekeeper for intestinal tumorigenesis and is mutated in the vast majority  
87 of intestinal tumors<sup>11</sup>, *Villin-CreERT2* where the CreERT2 recombinase is under the control of  
88 the intestine-specific *Villin* promoter and tdTomato. In this model, upon tamoxifen treatment,  
89 the recombinase translocates into the nucleus and thus becomes active, specifically in the  
90 epithelial compartment of the intestine. This drives both the expression of tdTomato in every  
91 recombined epithelial cell of the intestine and, simultaneously, the deletion of exon 14 in only  
92 one allele of the *Apc* gene. Intestinal tdTomato-positive adenoma will then form from rare cells  
93 that underwent a second hit of the WT *Apc* allele (Figure 1A).

94 Heterozygosity of *APC* is key for this study to slow down the process of intestinal tumorigenesis  
95 and recapitulate human pathology. Importantly, control mice for all the experiments, named  
96 VCT, have no *Apc* mutation but still express tdTomato in all intestinal epithelial cells upon  
97 tamoxifen treatment.

98 All the results presented here were obtained 4–5 months after tamoxifen treatment, which is  
99 considered as an early stage of intestinal tumorigenesis. At this age, intestinal adenomas were  
100 identified by a pathologist as high-grade dysplasia, with no observed concurrent carcinoma  
101 lesions. In addition, metastases were never observed at distant organs.

102 The first validation step of this model revealed that tdTomato is indeed expressed throughout  
103 the intestine in AVCT mice and in wild type *Apc* control VCT mice. Equally importantly, after 4-  
104 5 months there were newly formed adenomas in the AVCT mice but, as predicted, not in the  
105 VCT control (Figure 1B). In addition, Figure 1C shows diffuse  $\beta$ -catenin localization in intestinal  
106 adenomas, indicating that the Wnt signaling pathway is activated, as expected for *Apc* mutant  
107 tumors<sup>12</sup>.

108 Having established our cell-traceable intestinal adenoma model, we wanted to know whether  
109 intestinal tdTomato-positive cells could disseminate and be found in distant organs at the  
110 adenoma stages. Since the liver is the main organ of metastasis in colorectal cancer, we have  
111 focused on this organ. Strikingly we observed single cells as well as clusters of a few cells  
112 expressing tdTomato by immunohistochemistry (Figure 1D). In addition, livers were cleared  
113 and stained to visualize early-disseminated intestinal cells in the whole liver. In Figure 1E, the  
114 upper-left panel clearly shows intestinal tdTomato-positive cells sited in the liver of AVCT mice.

115 Figure 1E upper-right and lower panels, show stack areas from different liver regions that  
116 present single or clusters of these disseminated tdTomato-positive cells (in white).

117 To determine whether the early dissemination we had observed was likely a global patho  
118 physiological response to intestinal tumorigenesis, we used an alternative mouse model by  
119 exposing VCT mice to AOM, a carcinogen agent, and DSS, a pro-inflammatory factor  
120 (Supplementary Figure S1A)<sup>13</sup>.

121 Similarly, in our experimental, pathologist has confirmed that the intestine only bears  
122 adenomas (Supplementary Figure S1B), validating that this model also allowed the focus on  
123 very early stages of intestinal tumorigenesis. In the liver of these mice, 3 months post-  
124 treatment, tdTomato-positive intestinal cells could also be detected by immunohistochemistry  
125 at an early stage of tumor development (Supplementary Figure S1C).

### 126 **Early dissemination correlates with myeloid cell enrichment in the liver**

127 AVCT mice never develop liver metastases over the course of their lives. In addition, once  
128 intestinal adenoma cells are injected either subcutaneously or orthotopically in the caecum of  
129 highly immunodeficient mice, these cells never grow into tumors (data not shown), strongly  
130 suggesting that adenoma cells are not tumorigenic. We thus wanted to assess the impact of  
131 early dissemination in the liver. To address this question, we profiled at the single-cell level the  
132 mRNA expression of dissociated liver cells from AVCT and VCT mice 5 months post-tamoxifen  
133 administration. In the AVCT mouse, the UMAP representation shows a significant change in  
134 the liver landscape, particularly an important increase in the myeloid cell population including  
135 macrophages, neutrophils and dendritic cells (DCs) (Figures 2A-C). Differential expression  
136 analysis of AVCT versus VCT myeloid cells and the subsequent gene set enrichment analysis  
137 (GSEA) revealed prominent enrichment in Gene Ontology (GO) terms related to immune  
138 responses, such as activation and migration of myeloid leukocytes, regulation of the  
139 inflammatory response, and regulation of TNF superfamily cytokine production (Figure 2D and  
140 Data S1). Interestingly, high enrichment scores were also detected for non-immune related  
141 terms like 'cell junction organization and the presence of 'collagen-containing extracellular  
142 matrix', suggesting that myeloid cells could induce a matrix remodeling. To further investigate  
143 specific features related to macrophage and neutrophil enrichment in AVCT mouse liver, we  
144 subset all the annotated myeloid cells and re-analyzed them (Figure 2E). There was a clear  
145 accumulation of dendritic cells, recruited macrophages, and neutrophils in AVCT, which was  
146 highly specific, as Kupffer cells remained similar in VCT and AVCT mice (Figure 2F).

147 This re-clustering allowed the distinction of 12 sub-clusters with unique gene expression  
148 patterns, including three different groups of both neutrophils (clusters N1, N4, and N6) and

149 macrophages (clusters M3, M5, and M7), which facilitated the identification of those molecular  
150 phenotypes more associated with AVCT (Figure 2G-I).

151 Overall, the liver of VCT mouse had one main cluster of neutrophils (Cluster N1) and a few  
152 cells of another cluster (Cluster N6), whereas the AVCT mouse liver presented the three  
153 detected clusters N1, N4, and N6, indicating an enrichment and a greater functional diversity  
154 among neutrophil subpopulations in mice bearing intestinal adenomas. Similarly, a common  
155 cluster of macrophages is present in both mouse genotypes (Cluster M7), whereas two  
156 additional differentiated subpopulations were almost exclusive to AVCT (Clusters M3 and M5).  
157 For neutrophils, the top markers of AVCT-specific clusters N4 and N6 exhibit a gene  
158 expression profile similar to that of myeloid-derived suppressor cell-type neutrophils including  
159 expression of *Cebpe*, *Ltf*, *Cd177*, and *Ngp*<sup>14</sup> as presented in the heat map Figure 2 H.

160 In the highly enriched macrophage cluster of AVCT liver, Cluster M3 exhibits significant  
161 enrichment of genes involved in cell proliferation, including *Pclaf*, *Stmn1*, and *Mki67*  
162 (Supplementary Figure S2A). In contrast, Cluster M5 is characterized by enrichment of genes  
163 associated with cell migration, such as *Ahnak*, *Ccr2*, and fibronectin-1 (*Fn1*), and enrichment  
164 of GO terms associated with extracellular matrix (ECM) (Supplementary Figure S2B, C).  
165 Interestingly, *Fn1* has been described for its role in macrophage polarization towards an  
166 immunosuppressive phenotype (M2)<sup>15</sup>. Particularly, this cluster also displays increased  
167 expression of inflammatory chemokines, including *Ccl9* and to a lesser extent *Ccl3*, that are  
168 described for their role in recruiting myeloid progenitors into the pre-metastatic niche<sup>16</sup>. In  
169 addition, CCR2, the receptor of CCL2 is also overexpressed in the M5 cluster. Importantly, the  
170 CCL2-CCR2 signaling axis has been shown to induce the recruitment of other immune cells to  
171 create an immunosuppressive environment. The VCT and AVCT common M7 cluster exhibits  
172 expression of genes involved in antigen presentation and phagocytic functions, including  
173 CD74, H2-Eb1, H2-Aa, and *Axl*, thereby suggesting a putative physiological role for this cluster  
174 (Supplementary Figure S2D).

175 The striking myeloid-derived suppressor cell-like signature found in N4 and N6 neutrophil  
176 clusters specifically in the liver of AVCT mice at adenoma stage of tumor development, along  
177 with the increased abundance of macrophages with an inflammatory profile in the AVCT mouse  
178 liver, prompted us to validate this myeloid cell enrichment at the protein level.

### 179 **Liver remodeling is early induced during intestinal tumorigenesis**

180 We thus employed a multiplexing immunolabelling technology (CyTOF/Hyperion technology)  
181 to validate the myeloid cell enrichment in the liver of AVCT mice. A panel of 12 antibodies was  
182 used to assess the hepatic microenvironmental changes at early stages. Figure 3A shows a  
183 representative region, stained with this panel, where myeloid cells are visualized (including

184 myeloid cells that are labeled with CD11b, macrophages with CD68, neutrophils with MPO,  
185 dendritic cells with cd11c), along with lymphocytes that were labelled with CD3 and vessel wall  
186 and activated stellate cells with  $\alpha$ -sma1. According to cell quantification on 3 different regions  
187 of interest from 3 different mice, only macrophages and neutrophils were significantly enriched  
188 (Figure 3B). We therefore focused our attention to these cell types. Immunohistochemistry  
189 using Iba1 (Figure 3C) and MPO (Figure 3D) antibodies validates macrophage and neutrophil  
190 enrichment on a larger group of mice ( $n \geq 6$ ). Quantification of signal intensities demonstrates  
191 that the myeloid enrichment in the liver of AVCT mice is statistically significant compared to  
192 the liver of VCT mice. In addition, by using CD68 and F4-80 antibodies to stain macrophages,  
193 we show similar results, as presented in Supplementary Figures S3A and S3B, respectively.  
194 We then first performed staining using Iba1 and CD68 antibodies for macrophages as well as  
195 MPO for neutrophils on liver sections from the complementary AOM/DSS-treated mice  
196 (supplementary Figure S3C). Similar results were observed in this model.  
197 To exclude any potential bias effect of the AVCT model (including tamoxifen and exon 14  
198 deletion of one of the *Apc* allele), we also validated myeloid cell enrichment in the liver of  
199 *Apc $\Delta$ 14* and *Apc<sup>1638N</sup>* mice, two distinct mouse models that spontaneously develop intestinal  
200 adenomas<sup>17,18</sup> (Supplementary Figure S3D). Importantly, all these mouse models have an  
201 aberrant activation of the Wnt signaling pathway due to either an APC mutation (for AVCT,  
202 *Apc $\Delta$ 14* and *Apc<sup>1638N</sup>*) or to  $\beta$ -catenin mutation (for the majority of AOM/DSS cases<sup>19</sup>).  
203 Similarly, we observed a slight macrophage enrichment in the liver of mice for which intestinal  
204 tumorigenesis has been induced by a BRAFV600E mutation<sup>20</sup>, suggesting that myeloid cell  
205 enrichment induced by early dissemination could be a global feature of early intestinal  
206 tumorigenesis independent of the initial driver mutation (Supplementary Figure S3E).  
207 Then, because early dissemination has been well described in other cancers, we obtained liver  
208 sections of PanIN stages from KPC (*KrasG12D/+; Trp53 Pdx1-Cre*) mice and  
209 BRAFV600E/*cdkn2a* young mice for pancreatic cancer and melanoma, respectively. In these  
210 livers, we confirmed macrophage and neutrophil enrichment using Iba1 and MPO antibodies,  
211 respectively, demonstrating that early dissemination correlates with myeloid cell enrichment in  
212 several distinct solid tumors (Supplementary Figure S4 A-D).

213

### 214 **Early-intestinal disseminated cells in synergy with circulating cytokines recruit myeloid** 215 **cell in the liver**

216 We showed a clear correlation between the presence of early-disseminated tumor cells and  
217 hepatic enrichment of myeloid cells. Importantly, examination of the distribution of the myeloid  
218 cells revealed that myeloid cells enrichment was observed throughout the tissue (Figure 3).  
219 We therefore sought to investigate the impact of the presence of these intestinal disseminated

220 cells on the transcriptional program of the neighborhood liver cells. GeoMx digital spatial  
221 profiler technology was employed to determine hepatic gene expression in different regions in  
222 the vicinity (presence of tdTomato-positive intestinal cells) or in greater distance (absence of  
223 tdTomato-positive intestinal cells) in AVCT mice, which were compared to areas from VCT  
224 mice as a control (Figure 4A). The Volcano plot in Figure 4B demonstrates that the  
225 transcriptional program is modified in the neighborhood of early-disseminated intestinal cells  
226 in the liver of AVCT mice. Furthermore, gene ontology analysis of the upregulated genes in  
227 the adjacent areas of early intestinal disseminated cells in AVCT revealed the activation of  
228 TNF $\alpha$  signaling via NF-kB. This signaling pathway is a commonly activated pathway when  
229 inflammation is occurring (Figure 4C)<sup>21</sup>. This suggests that early-disseminated intestinal cells  
230 have a local impact on the liver.

231 Since NF-kB is a transcription factor highly involved in the regulation of cytokine expression,  
232 we compared the plasma of AVCT and VCT mice using a cytokine array. Four cytokines out  
233 of a total of 42 were overrepresented in the plasma of AVCT mice (CXCL2, M-CSF, SDF-1,  
234 and TIMP-1). Using ELISA, we quantified the plasma levels of these candidates in addition to  
235 TNF- $\alpha$  and found that they were significantly elevated in AVCT mice relative to VCT mice  
236 (Figure 4D). In fact, SDF-1, known to induce hepatic myeloid cell infiltration when systemically  
237 elevated, belongs to the adenoma-induced set of cytokines. Interestingly, TIMP-1 is the most-  
238 upregulated systemic factor, which prepares a liver-specific pre-metastatic niche that is  
239 composed of broad infiltration of myeloid cells<sup>23</sup>.

240 We next wondered whether early-disseminated intestinal cells, alone in the liver, could directly  
241 induce myeloid cell enrichment. To test this, we injected a small number (1000) of intestinal  
242 adenoma cells in the spleens of WT mice to let these cells rapidly reach the liver<sup>22</sup> (Figure 4E).  
243 We could observe a significant macrophage and neutrophil enrichment compared to PBS-  
244 injected mice (Figure 4F), demonstrating that early-disseminated intestinal cells alone cause  
245 myeloid cell enrichment even in the absence of a primary tumor.

246 Notably, the same pattern of systemic factors, CXCL2, SDF-1, TIMP-1, and TNF- $\alpha$  were also  
247 overrepresented in the plasma of WT mice injected with small number of adenoma cells in their  
248 livers (Figure 4G). This suggests that the increased circulating factor level in the plasma of  
249 AVCT mice is not only due to adenoma secretion but is also a direct consequence of the  
250 presence of early-disseminated tumor cells in the liver. As a proof of concept that injected  
251 adenoma-induced TIMP-1 is sufficient to induce myeloid-recruiting SDF-1 we repeated the  
252 experiment detailed in Figure 4E but in addition, TIMP1 was intraperitoneally administered.  
253 Interestingly, a synergistic effect was observed in mice that have received both intestinal  
254 adenoma cell injection and exogenous TIMP1 (Figure 4H). Figure 4I shows similar results as  
255 expected because a feed-forward loop exists between TIMP1 and SDF-1 secretion as  
256 previously described in the literature<sup>23</sup>.

257 Altogether, these results demonstrate that early intestinal disseminated cells in the liver and  
258 systemic factors act in synergy at adenoma stage to remodel the liver and prepare a pre-  
259 metastatic niche early during tumorigenesis.

260 To highlight the clinical significance of selected cytokines, we measured TNF- $\alpha$ , CXCL-2, M-  
261 CSF, SDF-1, and TIMP-1 levels in the blood of ten patients with intestinal adenomas and ten  
262 healthy individuals, using ELISA. These cytokine candidates were more abundant in the blood  
263 of patients with intestinal adenomas, confirming results obtained in mice (Figure 4J).

#### 264 **Early liver remodeling enhances metastatic cell colonization.**

265 Next, we investigated the functional impact of early liver remodeling, concomitant with early  
266 dissemination, on its metastatic colonization. We recapitulated this process by injecting  
267 syngeneic carcinoma cells (MC38) in the spleen of VCT or AVCT mice (Figure 5A). MC38 cells  
268 are known to be able to colonize the liver after their injection in the spleen of immunocompetent  
269 mice<sup>24</sup>. However, here our intention was to challenge metastasis initiation ability. Therefore,  
270 we injected a moderate number of cells (100,000 cells) and sacrificed the first mice after only  
271 15 days. In these conditions, we detected visible micro-metastases only in the remodeled liver  
272 of AVCT mice (data not shown).

273 When, in the same experiment, mice were sacrificed after 30 days, as expected, metastases  
274 were also detected in the liver of VCT mice. However, there were less metastases in this  
275 control group as compared to the AVCT group. Indeed, more than half of the VCT mice had  
276 no detectable metastasis, whereas AVCT mice presented statistically more liver metastases  
277 (chi-square test; p value = 0.014, Figure 5B). In addition, metastases in the remodeled liver of  
278 AVCT mice were significantly larger than those that developed in the normal liver of VCT mice  
279 (Figure 5C-E).

280 It is noteworthy that similar results were observed in two additional mouse models (*Apc*<sup>Min</sup> and  
281 *Apc*<sup>lox15</sup>) when the murine carcinoma CMT93 cell line injection was used to induce metastasis,  
282 and the metastatic colonization was assessed by luciferase assay (Supplementary figure S. 5  
283 A, B and C, D respectively)

284 Altogether, these results demonstrate that early-disseminated intestinal cells in synergy with  
285 systemic factors induce liver remodeling, which subsequently enhances colonization and  
286 metastasis in the liver.

## 287 DISCUSSION

288 In this work, we used various complementary mouse models to demonstrate that intestinal  
289 tumors spread at an adenoma stage during tumorigenesis. Moreover, we confirmed that the  
290 early spread of intestinal cells, along with the secretion of some factors, is associated with an  
291 enrichment of neutrophils and macrophages in the liver, as well as matrix remodeling. Finally,  
292 we demonstrated that the liver remodeling that occurs at an early stage promotes the later  
293 colonization of this organ and metastatic development.

294 It was previously believed that tumor dissemination only occurred during the later, malignant,  
295 stages of tumor development. However, some disseminated tumor cells detected in patients  
296 with melanoma, pancreatic, and breast cancer harbored a lower mutation burden than primary  
297 tumors, suggesting that dissemination may occur during the early stages of tumor  
298 evolution<sup>2,25,26</sup>. In breast cancer for example, consecutive papers have decoded cell signaling  
299 associated with early dissemination by proving that a sub-population of Her2<sup>+</sup>p-p38<sup>lo</sup>p-  
300 Atf2<sup>lo</sup>Twist1<sup>hi</sup>E-cad<sup>lo</sup> cancer cells spread early to target organs by activating a Wnt-dependent  
301 epithelial-mesenchymal transition (EMT)-like dissemination program<sup>27,28</sup>.

302 At late stages of tumor development, the premetastatic niche has been well described in the  
303 literature by several groups<sup>29-31</sup>. They have shown that the pre-metastatic niche can be  
304 prepared through a complex interplay between circulating factors secreted by primary  
305 advanced tumors, the immune system, and local stromal components. Indeed, many  
306 cytokines, such as TIMP-1 or SDF-1<sup>23</sup>, are first released by tumor cells and then secreted by  
307 myeloid and stromal cells, creating a vicious circle that prepares the soil for disseminated cells  
308 to seed and colonize distant organs. Other components such as exosomes, miRNA, cell-free  
309 circulating DNA, and lncRNA have also been proposed to be part of this process<sup>32,33</sup>.

310 However, our novel findings presented here reveal that the preparation of pre-metastatic  
311 niches takes place at adenoma stage of intestinal tumorigenesis. Moreover, we demonstrate  
312 that early-disseminated cells, in synergy with systemic factors, exert an impact on the hepatic  
313 microenvironment, promoting metastasis development.

314 In recent years, tumor dormancy has emerged as a promising process to target. Indeed,  
315 disseminated tumor cells can infiltrate, enter into cell cycle arrest in distant organs and can  
316 remain dormant for varying amounts of time. The present work diverges from this theory  
317 because early-disseminated tumor cells have a significant impact on hepatic subpopulations,  
318 which strongly suggests that they secrete factors and respond to extracellular signals and thus  
319 are probably not dormant. Nevertheless, the fact that AVCT mice do not develop liver  
320 metastases, even after one year, strongly suggests that the early-disseminated tumor cells  
321 observed in the liver of 4–5-month-old animals are not cycling.



322 To further decipher the mechanisms involved in early dissemination, aging will be an important  
323 parameter to consider. In the present work, it is important to note that the microenvironment in  
324 young mice used in our study may not fully reflect the human aged pathology. In fact, recent  
325 research has shown that fibroblasts in aging mice may play a critical role in regulating tumor  
326 cell proliferation inducing a phenotype switching from a dormant to a proliferative state through  
327 Wnt5a secretion<sup>34</sup>.

328 Early dissemination includes primary tumor cell departure to reach distant organs as well as  
329 circulating factors secreted by either adenomas or recruited immune and stromal cells.

330 In this work, we have focused our analysis on systemic factors that are differentially  
331 represented in the plasma of AVCT mice in comparison with VCT mice. This comparative  
332 analysis was performed first with a cytokine array, and the top candidates were quantified by  
333 ELISA. Four cytokines were selected; namely TIMP1, M-CSF, SDF-1, and CXCL2, as well as  
334 TNF- $\alpha$ , that are all associated with macrophage and neutrophil recruitment in cancer of various  
335 organs<sup>22,36-42</sup> and could, thus, explain the myeloid cell enrichment observed in the liver of AVCT  
336 mice.

337 For example, TIMP-1 has been found to correlate with poor prognosis in several cancers,  
338 including colorectal cancer, where it has been associated with liver metastasis and tumor  
339 relapses upon comparing its plasmatic level in patients with different stages of CRC. In the  
340 same study, authors nicely showed that TIMP-1 helps prepare the premetastatic niche, notably  
341 through neutrophil recruitment thanks to the SDF-1/CXCR4 axis. However, they did not  
342 quantify the level of TIMP-1 in the plasma of either healthy donors or patients with intestinal  
343 adenomas, whereas we show that this increase is very early during intestinal tumorigenesis  
344 both in mice and humans. CXCL2 is primarily produced by neutrophils and binds to CXCR2,  
345 which in turn couples with Gai to stimulate neutrophils and thus initiate a vicious circle<sup>42,43</sup>.  
346 Then, CCL2 produced by tumor cells, is an important chemokine for the recruitment and  
347 retention of macrophages, which could partially explain macrophage enrichment in the liver of  
348 AVCT mice. Finally, TNF- $\alpha$  activates NF- $\kappa$ B, a well-known transcription factor regulating the  
349 expression of many cytokines, including TIMP1, M-CSF, SDF-1, and CXCL2<sup>21,35,36,44</sup>.

350 Interestingly, the immunosuppressive phenotype highlighted by single-cell RNA sequencing  
351 has been nicely illustrated through intravital live imaging. Indeed, in mouse liver exhibiting early  
352 dissemination, metastatic cells were completely ignored by neutrophils and macrophages  
353 (supplementary video S1), in contrast to control mice where myeloid cells attacked metastatic  
354 cells (supplementary video S2 and S3).

355 In conclusion, our present work advances the understanding of tumor dissemination in the  
356 context of intestinal tumorigenesis. Using different elegant mouse models, we have

357 demonstrated that this process starts at adenoma stage. Furthermore, our findings suggest  
358 that early disseminated cells, in synergy with systemic factors, trigger significant changes in  
359 the liver microenvironment. These changes create a permissive microenvironment, forming  
360 the early pre-metastatic niche and promoting the colonization of metastatic cells. Our study  
361 has significant implications not only for fundamental cancer research but also for clinical  
362 practice, as we have identified systemic factors (TNF- $\alpha$ , TIMP1, M-CSF, SDF-1, and CXCL2)  
363 that are overrepresented in the blood of patients with benign intestinal adenomas, and could  
364 be used as potential biomarkers.

365

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### 379 **Author contributions**

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381 Z.W., C.B., and J.P.; Investigation, Z.H, G.B, T.L., J.S., C.B., L.B., C.P, M.N., C.B., P.G., and  
382 A.T. Formal Analysis; L.B., Z.H, G.B, Z.W., C.B., and J.P Writing – Original Draft, Z.H, and  
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385 Supervision, J.P.

### 386 **Declaration of interests**

387 Authors declare no conflict of interest

388

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390

391

392 **Figure legends:**

393

394 **Figure 1: Generation and validation of the transgenic mouse model and early detection**  
395 **of intestinal cells in the liver**

396 (A) Schematic representation of the VCT and AVCT mouse models employed in the study.  
397 Tamoxifen administration in these models induces tdTomato expression in intestinal epithelial  
398 cells. In AVCT mice, tamoxifen additionally induces a mutation in one APC allele. This  
399 gatekeeper of intestinal tumorigenesis is then subject to a second hit in rare cells that will  
400 initiate adenoma formation.

401 (B) Immunohistochemistry on intestinal swiss roll sections demonstrates a high recombination  
402 efficiency of tamoxifen treatment in the AVCT mouse and control VCT mice. The resulting  
403 expression of tdTomato all intestinal epithelial cells is revealed by RFP antibody staining.  
404 Adenomas are visible in the AVCT mice (zoom in). Black crosses indicate intestinal adenomas.

405 (C) Immunofluorescence on an adenoma of AVCT mouse adenoma, using anti RFP (red) and  
406 anti  $\beta$ -catenin (green) antibodies, shows that adenomas express tdTomato and have  
407 abnormally high Wnt signaling pathway activity, illustrated by cytoplasmic/nuclear diffused  $\beta$ -  
408 catenin accumulation compared to adjacent normal tissue.

409 (D) Immunohistochemistry using RFP antibody on liver sections from 6 different AVCT mice,  
410 sacrificed between 2-5 months post tamoxifen, reveals the presence of rare tdTomato positive  
411 early-disseminated tumor cells (black arrows).

412 (E) Tissue clearing of the liver using vDisco and its imaging using Miltenyi Ultramicroscope  
413 Blaze. Reconstitution of a whole 3D liver lobe stained with NanoTag FluoTag-Q anti-RFP (in  
414 red) and Propidium Iodide (in turquoise) to label cell nuclei (Upper panel, left). Raw images of  
415 different areas of the livers presenting early-disseminated tumor cells (red arrows, NanoTag  
416 FluoTag-Q anti-RFP in white) (upper panel, right and lower panel)

417

418 **Figure 2: Early dissemination correlates with myeloid cell enrichment in the liver of**  
419 **AVCT mice**

420 (A) Uniform manifold approximation and projection (UMAP) integrated analysis of AVCT and  
421 VCT liver cells labeled according to their classification by ScType

422 (B) UMAP cells labeled according to their classification by ScType and splitted by condition  
423 AVCT vs VCT

424 (C) The subpopulation composition of each animal.

425 (D) Selected Gene Ontology gene-sets enriched in AVCT myeloid cell populations compared  
426 with VCT. NES, normalized enrichment score

427 (E) UMAP plot displaying the integrated re-analysis of myeloid cell populations, colored  
428 according to Seurat re-clustering.

429 (F, G) UMAP splitting AVCT/VCT of myeloid cells, labelled according to Sctype annotation (G),  
430 and highlighting the specific neutrophil and macrophages subclusters.

431 (H, I) Heatmap of the top 15 differentially expressed markers defining each neutrophil (H) and  
432 macrophage (I) subpopulation.

433

434 **Figure 3: Liver remodeling is early induced during intestinal tumorigenesis**

435 (A) Representative Cytof/hyperion image of liver sections from VCT mice (left) and AVCT mice  
436 (right) after induction reveals a strong dysregulation in different immune populations;  
437 macrophages are labeled with CD68 in yellow and cd11b in white; neutrophils are labelled with  
438 MPO in red; dendritic cells with CD11c in magenta and vessel walls and activated stellate cells  
439 with a-sma1 in green.

440 (B) Number of cells expressing macrophage marker (CD68); myeloid cells (CD11b). Neutrophil  
441 marker (MPO) and dendritic cells (CD11c) per region of interest (ROI). Columns, mean; Bars.  
442 SD; Students t test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

443 (C) Immunohistochemistry using Iba1 antibody to detect macrophages on liver sections from  
444 VCT mice (left panel) and AVCT intestinal adenoma bearing mice (middle panel). Right panel  
445 shows signal intensity quantification. Violin plot Students t test  $n > 14$  \*\*\*\* p value  $< 0.0001$

446 (D) Immunohistochemistry using MPO antibody to detect macrophages on liver sections from  
447 VCT mice (left panel) and AVCT intestinal adenoma bearing mice (middle panel). Right panel  
448 shows signal intensity quantification. Violin plot Students t test  $n > 6$ , \*\*\*\* p value  $< 0.0001$

449

450 **Figure 4: Early-intestinal disseminated cells in synergy with circulating cytokines**  
451 **recruit myeloid cell in the liver**

452 (A) Examples of chosen regions of interest, for spatial transcriptomic analysis, on liver sections,  
453 from VCT and AVCT mice, containing or not intestinal early-disseminated tumor cells  
454 expressing tdTomato.

455 (B) Volcano plot depicting transcriptional differences highlighted by spatial transcriptomic  
456 analysis on liver sections from AVCT mice containing or not intestinal early-disseminated tumor  
457 cells.

458 (C) Heatmap showing TNF- $\alpha$  signaling activation on regions of interest containing intestinal  
459 early-disseminated tumor cells expressing tdTomato on AVCT mouse liver sections.

460 (D) Cytokine (TIMP1, SDF-1, M-CSF, TNF- $\alpha$  and CXCL2) level fold change, in the plasma of  
461 AVCT mice, compared to VCT mice, assessed by ELISA. Columns, mean; Bars. SD; Students  
462 t test. \*\*\*,  $P < .001$ ; \*\*\*\*,  $P < .0001$

463 (E) Schematic description of adenoma cells injected in the spleen of WT mice to assess the  
464 impact, of early-disseminated tumor cells alone, on myeloid cell enrichment in the liver. IS:  
465 Intrasplenic injection

466 (F) Immunohistochemistry using F4-80 antibody (top panel) to detect macrophages and MPO  
467 antibody (bottom panel) on liver sections from WT mice injected with PBS or adenoma cells in  
468 their spleen. Right panel shows signal intensity quantification of each marker. Columns, mean;  
469 Bars. SD; Students t test F4-80: n=5 \* p value= 0.0307; MPO n=3 \* p value= 0.0334

470 (G) Cytokine (TIMP1, SDF-1, M-CSF, TNF- $\alpha$  and CXCL2) level fold change, assessed by  
471 ELISA, in the plasma of WT mice, injected with adenoma cells into their spleen, compared to  
472 WT mice injected with PBS Columns, mean; Bars. SD; Students t test. \*\*,  $P<0.01$  \*\*\*,  $P<0.001$ ;  
473 \*\*\*\*,  $P<0.0001$

474 (H) Timp1 plasmatic level assessed by ELISA in PBS intrasplenically injected mice with PBS  
475 followed by PBS treatment (IS PBS+ PBS); in PBS intrasplenically injected mice with PBS  
476 followed by Timp1 treatment (IS PBS+ Timp1); in intrasplenically injected mice with adenoma  
477 cells followed by PBS treatment (IS adenoma cells+ PBS) and in intrasplenically injected mice  
478 with adenoma cells followed by Timp1 treatment (IS adenoma cells+ Timp1). Columns, mean;  
479 Bars. SD; Students t test. \*\*\*,  $P<0.001$ ; \*\*\*\*,  $P<0.0001$ .

480 (I) SDF-1 plasmatic level assessed by ELISA in PBS intrasplenically injected mice with PBS  
481 followed by PBS treatment (IS PBS+ PBS); in PBS intrasplenically injected mice with PBS  
482 followed by Timp1 treatment (IS PBS+ Timp1); in intrasplenically injected mice with adenoma  
483 cells followed by PBS treatment (IS adenoma cells+ PBS) and in intrasplenically injected mice  
484 with adenoma cells followed by Timp1 treatment (IS adenoma cells+ Timp1) Columns, mean;  
485 Bars. SD; Students t test. \*\*\*,  $P<0.001$ ; \*\*\*\*,  $P<0.0001$ .

486 (J) Cytokine (TIMP1, SDF-1, M-CSF, TNF- $\alpha$  and CXCL2) level fold change, in the plasma of  
487 patients bearing intestinal adenomas, compared to the plasma of healthy donors, assessed by  
488 ELISA. Columns, mean; Bars. SD; Students t test. \*,  $P<0.05$ ; \*\*,  $P<0.01$ , \*\*\*,  $P<0.001$ ;  
489 \*\*\*\*,  $P<0.0001$ .

490

#### 491 **Figure 5: Early liver remodeling enhances metastatic cell colonization**

492 (A) Schematic description of 100 000 MC38 carcinoma cells injected in the spleen of VCT or  
493 AVCT (13 and 10 mice respectively) to assess the impact of liver remodeling on the success  
494 of late metastatic cell colonization.

495 (B) Number of VCT and AVCT mice developing no tumor, or less or more than 10 liver  
496 metastases following MC38 cell injection in the spleen.

497 (C) Mean of liver metastasis, with a size lower than 10 mm<sup>2</sup> in VCT and AVCT mice 30 days  
498 after MC38 cell injection in the spleen. Columns, mean; Bars. SEM; Students t test. \*;  $P<0.05$

499 (D) Representative photos of liver with metastases from VCT and AVCT mice 30 days following  
500 MC38 cell injection in the spleen

501 (E) Representative hematoxylin and eosin staining of liver sections with metastases from VCT  
502 and AVCT mice 30 days following MC38 cell injection in the spleen

503

504

## 505 **STAR Methods**

### 506 **Transgenic mouse models**

507 All mice were housed in a SPF environment. All animal experiments were approved by the  
508 French Agriculture and Forestry Ministry (APAFIS number 20720- 2019051314421302).

### 509 **Generating the AVCT mouse model**

510 To create the *Apc*, *Villin*, *Cre*, *Tomato* mouse used in this paper we used *Apc*<sup>lox/lox</sup> and  
511 *Villin*<sup>CreERT2</sup> transgenic (AV) that have been previously described<sup>17</sup>. These *Apc*<sup>lox/lox</sup>; *Villin*<sup>CreERT2</sup>  
512 bigenic mice were crossed with Ai14 reporter mice<sup>45</sup> (JAX:007908) where a lox-P flanked stop  
513 cassette prevents the expression of the red fluorescent protein, tdTomato. 6-8 weeks old  
514 transgenic descendent mice *Apc*<sup>lox/WT</sup>; *Villin*<sup>CreERT2</sup>; Ai14<sup>KI/WT</sup> were put on a tamoxifen diet  
515 (0.5g/kg tamoxifen+5% saccharose) SAFE®E8220 for 2 weeks to induce recombination in all  
516 intestinal epithelial cells. The percentage of tdTomato-positive intestinal crypts was analyzed  
517 using immunohistochemistry on whole intestinal swiss rolls (n=4), reporting recombination of  
518 ~90%. Female and male mice were used in all the experiments, and they were all of mixed  
519 genetic backgrounds (C57BL/6J and 129S6). Livers from these mice were harvested 4-5  
520 months post-tamoxifen administration.

521 Other mouse models employed in this study

522 *Apc*<sup>Min/+</sup> mice (Jackson Laboratories) and *Apc*<sup>15Lox/+</sup>; *villinCre* were maintained on C57BL/6  
523 background. *Apc*<sup>15Lox/+</sup>; *VillinCre* were established by breeding *Apc*<sup>15Lox/Lox</sup> (provided by Dr.  
524 Riccardo Fodde, Netherland) with *VillinCre* (provided by Dr. Averil Ma, USCF). ACTB-EGFP  
525 mice and c-fms-EGFP transgenic mouse lines were maintained in-house and crossed with  
526 *Apc*<sup>Min/+</sup> to get *Apc*<sup>Min/+</sup>/ACTB-EGFP/cfms-EGFP mice for intravital imaging. All mouse  
527 experiments were approved by the Institutional Animal Care and Use Committee of University  
528 of California, San Francisco.

529 The *Apc*<sup>Δ14</sup> mouse model was previously described<sup>17</sup>. Mice were sacrificed at the age of 3  
530 months for further investigations.

531 Livers from *Apc*<sup>1638N</sup> mutant mice (previously described in<sup>18,46</sup>) were kindly provided by S. Fre  
532 lab .

533 FFPE liver sections from BRAFV600E *VillinCre* were kindly provided by Owen Sansom. The  
534 mouse model was previously described<sup>20</sup>.

535 FFPE liver sections from KPC mice, sacrificed at PANIN stage, were kindly given by Dr  
536 Guillermet-Guibert in CRCT in Toulouse; the mouse model was previously described<sup>47</sup>.

537 FFPE liver sections from a melanoma mouse model were kindly given by Dr. Le Cam in IRCM  
538 in Montpellier. Briefly in the mouse model (*Braf*<sup>tm1Cpri</sup>; *Cdkn2a*<sup>tm1Rdp</sup>; *Rosa26*<sup>Tomato</sup>; Tg(*Tyr*-  
539 cre/ERT2), melanomas appears in an average between 6 and 9 months after TAM application  
540 on animals harboring the *Cdkn2a*<sub>null</sub> allele at the homorozygote stage. Liver sections were  
541 obtained from animals sacrificed 7 months post TAM where no signs of liver metastases were  
542 observed.

### 543 **Chemically induced colorectal cancer**

544 The AOM/DSS colon carcinogenesis model is largely described<sup>48</sup>. *Villin*<sup>CreERT2</sup>/Ai14<sup>KI/WT</sup> mice  
545 were first put on a tamoxifen diet (0.5g/kg tamoxifen+5% saccharose) SAFE®E8220 for 2

546 weeks, to induce the tdTomato expression in all intestinal epithelial cells. 2-3-month-old mice  
547 received a single intraperitoneal injection of Azoxymethane (AOM) (10mg/kg body weight, 250  
548 ng/mouse) (Sigma #A5486) followed by dextran sodium sulfate (DSS) administration (2% in  
549 drinking water) 1 week after the AOM injection for five consecutive days. 2 additional cycles of  
550 DSS were administered with a 1-week break (Fig. Supp. 1). Mice were monitored daily for body  
551 weight change and health status. Mice were sacrificed 2-3 months after the end of the protocol.

## 552 **Adenoma culture**

553  $APC^{lox/WT}/Villin^{CreERT2}/Ai14^{KI/WT}$  mice aged 4 months were sacrificed, and intestinal adenomas  
554 were micro-dissected using forceps and micro-dissection scissors. Adenomas were  
555 dissociated using a mouse tumor dissociation kit (Miltenyi) following manufacturer's protocol.  
556 Dissociated adenoma cells were passed through a 70 $\mu$ m cell strainer, centrifuged, suspended  
557 in DMEM-F12 (Gibco), and plated in 50% Matrigel drops in 12-well plates. After Matrigel  
558 polymerization, the drop was covered with DMEM-F12 medium supplemented with EGF, N2,  
559 and glutamine. This culture medium ensured the expansion of transformed cells only.

## 560 **Cell Culture.**

561 Murine colon cancer cell line (MC38) was purchased from ATCC. CMT93 mouse rectum  
562 cancer cell line (provided by Dr. Fabrice Pierre, Unité Mixte de Recherche 1089, Toulouse,  
563 France) express an mCherry fluorescent tag and luciferase reporter (CMT93-mCh-Luc). These  
564 cell lines were cultured in DMEM (Gibco) containing 10% FCS and 1% penicillin/streptomycin  
565 at 37°C under 5% CO<sub>2</sub>.

## 566 **Sample preparation**

567 Livers from  $Apc^{lox/WT}/Villin^{CreERT2}/Ai14^{KI/WT}$  and  $Villin^{CreERT2}/Ai14^{KI/WT}$  mice were harvested at 5  
568 months post-tamoxifen administration. Dissected livers were digested using a cocktail of  
569 Collagenase I (100mg/g of liver weight), Hyaluronidase (100mg/g), and DNase (1.25 mg/g) in  
570 RPMI medium at 37°C under agitation for 45 minutes. Digested cells were passed through a  
571 70 $\mu$ m cell strainer and centrifuged to remove the enzymatic solution. Red blood cells were  
572 removed using red blood lysis buffer (Miltenyi) and dead cells were removed using a debris  
573 removal kit (Miltenyi) according to manufacturer's protocol. Live cells were sorted using sytox  
574 green dye on a FACSAriaII (BD). Sorted cells were directly processed into the scRNA  
575 sequencing experiment.

## 576 **Single-cell RNA sequencing**

### 577 ***Demultiplexing, quality control and analysis***

578 Image analyses and base calling were performed using the NovaSeq Control Software and  
579 the Real-Time Analysis component (Illumina). Demultiplexing was performed using the 10X  
580 Genomics software Cellranger mkfastq (v6.1.1), a wrapper of Illumina's software bcl2fastq  
581 (v2.20)]. The quality of the raw data was assessed using FastQC (v0.11.9) from the Babraham  
582 Institute and the Illumina software SAV (Sequencing Analysis Viewer). FastqScreen (v0.15.0)  
583 was used to estimate the potential level of contamination.

584 Cell suspensions were loaded on a Chromium controller (10x Genomics, Pleasanton, CA,  
585 USA) to generate single-cell Gel Beads-in-Emulsion (GEMs). Single-cell RNAseq libraries

586 were prepared using a Chromium Single cell 3'RNA Gel Bead and Library Kit V3.1 (P/N  
587 10000268, 1000120, PN-1000215 10x Genomics). GEM-RT was performed in a C1000 Touch  
588 Thermal cycler with 96-Deep Well Reaction Module (Bio-Rad; P/N 1851197): 53°C for 45 min,  
589 85°C for 5 min; held at 4°C. After RT, GEMs were broken and the single-strand cDNA was  
590 cleaned up with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific; P/N 37002D).  
591 cDNA was amplified using the C1000 Touch Thermal cycler with 96-DeepWell Reaction  
592 Module: 98°C for 3 min; 12 cycles of 98°C for 15 s, 63°C for 20 s, and 72°C for 1 min; 72°C for  
593 1 min; held at 4°C. The amplified cDNA product was cleaned up with SPRIselect beads (SPRI  
594 P/N B23318). Dual Indexed sequencing libraries were constructed with SPRIselect following  
595 these steps: (1) Fragmentation end repair and A-tailing and size selection; (2) adapter ligation  
596 and cleanup; (3) sample index PCR and size selection. The barcode sequencing libraries were  
597 quantified by quantitative PCR (KAPA Biosystems Library Quantification Kit for Illumina  
598 platforms P/N KK4824). Sequencing libraries were loaded at 200 pM on an Illumina  
599 NovaSeq6000 using the following read length: 28 bp Read1, 10 bp I7 Index, 10 bp I5 Index,  
600 90 bp Read2.

## 601 **Data analysis:**

602 Alignment, gene expression quantification, and statistical analysis were performed using the  
603 Cell Ranger Single Cell Software Suite from 10x Genomics, Inc., against their pre-built Mus  
604 Musculus's GRCm39 reference genome. To discard ambient RNA falsely identified as cells,  
605 the Cell Ranger count was run a second time with the option `-force-cells` to force the number  
606 of cells to detect. 'Cell Ranger aggr' was then used to combine each sample result into one  
607 single analysis. Data processing and visualization were performed with Seurat package  
608 v4.3.0<sup>49,50</sup>. A total of 2,446 cells with >30,000 counts and from 500 to 6,000 expressed genes  
609 were considered for VCT, whereas 2,569 cells with >55,000 counts and from 500 to 7,000  
610 genes were considered for AVCT. Raw unique molecular identifier counts were log-normalized  
611 and the top 2,000 highly variable genes were identified using the *FindVariableFeatures*  
612 function. To account for biological and technical batch differences between animals, the Seurat  
613 anchor-based integration workflow was followed<sup>51</sup>. After integration, gene expression was  
614 scaled and principal component analysis was performed using the *RunPCA* function  
615 considering 20 principal components. Next, the *RunUMAP*, *FindNeighbors* and *FindClusters*  
616 functions were applied, based on the first 10 principal components, with a cluster resolution  
617 set to 0.5. The number of components was determined based on the elbow plot. Cell type  
618 assignment was automatically performed using ScType<sup>52</sup> with a customized version of the cell  
619 marker database (Data S2). Briefly, we selected and manually curated the top-scored  
620 differentially expressed genes identified per cell type in the total mouse liver cell atlas provided  
621 by Guilliams M et al. Table S1<sup>49</sup>, excepting the Hepatic Stem and Progenitor Cells (HSPCs).

## 622 *Analysis of Myeloid Cells*

623 The *FindMarkers* function using Wilcoxon rank sum test was then used to identify differentially  
624 expressed genes between VCT and AVCT for all the annotated myeloid cell populations (i.e.,  
625 dendritic cells, recruited Macrophages, Kupffer Cells and Neutrophils) from raw counts, using  
626 thresholds of genes detected in >20% of cells, showing >0.25 log<sub>2</sub>-transformed fold-change  
627 and adjusted p-value <0.05. Gene Set Enrichment Analysis was performed using the dedicated  
628 function from ClusterProfileR<sup>53</sup>, ranking genes by the log<sub>2</sub>-transformed average fold-change  
629 and using Gene Ontology gene sets obtained from the msigdb package (v7.5.1)<sup>54</sup>. The  
630 significance cutoff was set at 5% Benjamini-Hochberg (BH) FDR.



631 Non-myeloid cells were then removed, and the dataset was normalized, integrated and  
632 analyzed again following the same procedure described above. In this case, the  
633 *FindAllMarkers* function was used to identify differentially expressed genes between all the re-  
634 defined clusters of myeloid cells, considering 'only positives' markers and thresholds of genes  
635 detected in >15% of cells and showing >0.15 log<sub>2</sub>-transformed fold change. Due to the reduced  
636 number of cells and to obtain a more confident approximation of the functional diversity of  
637 these subclusters, gene set enrichment analysis only included markers with an adjusted p-  
638 value <0.01. Additionally, incorporated the REACTOME gene set, and the significance cutoff  
639 of the enriched pathways was set at 1% (BH) FDR for neutrophils and 0.1% for macrophages.

640

#### 641 **Spatial transcriptomic analysis:**

642 The Technology Access Program (TAP) from Nanostring Technology in Seattle, USA, was  
643 used to run GeoMx digital spatial profiler on liver FFPE sections. Briefly, 3 FFPE liver sections  
644 from different AVCT mice and 2 FFPE sections from different VCT mice were stained with  
645 morphological markers: PanCK, iba1, TdTomato (for early-disseminated tumor cells) and DAPI  
646 along with oligo-conjugated antibodies. Custom regions of interest were assigned either 'close  
647 to' or 'far from' early-disseminated tumor cells, and randomly selected regions were picked  
648 from VCT sections. From these regions, barcodes were cleaved using UV light in the GeoMX  
649 digital spatial profiler instrument, then hybridized, and finally counted using the Ncounter  
650 system. A remote access to the data, allowed the analysis of the data along with quality control  
651 assessment, normalization, and differential expression gene evaluation between the different  
652 regions of interest. Differential gene expression was evaluated by Linear Mixed Model (LMM)  
653 test with BH correction. Volcano plot was constructed using VolcaNoseR web page<sup>55</sup>.

#### 654 **Immunolabelling**

655 Tissues were fixed using 10% buffered formalin overnight at room temperature and embedded  
656 into paraffin using Spin Tissue Processor STP 120. 4 µm sections of FFPE tissue were used  
657 for immunolabeling. Sections were first dewaxed by heating them to 56°C, immersed in five  
658 serial xylene baths, and then dehydrated in serial ethanol baths. Antigens were unmasked  
659 using either boiling citrate pH 6.2 or boiling tris-base pH 9 for 20 minutes. Non-specific sites  
660 and permeabilization were done using a blocking buffer (TBS containing 5% Donkey serum,  
661 0.2% Triton X-100) for at least 30 minutes. Sections were then incubated with primary  
662 antibodies (diluted in blocking buffer) overnight at 4°C. The next day, slides were washed and  
663 then incubated with the secondary antibody at room temperature for 1 hour.

664 For immunofluorescence staining, fluorescent secondary antibodies (donkey Fluor 488 anti  
665 mouse; donkey 547 Fluor anti-rabbit (from Jackson Immuno Research) were used, and nuclei  
666 were stained with DAPI. Slides were mounted using fluoromount G and were observed using  
667 an epifluorescent microscope (Zeiss Axiomager Z1)

668 For immunohistochemistry staining, the endogenous peroxidase activity was quenched using  
669 hydrogen peroxide/methanol 1.5% for 20 minutes at room temperature before applying the  
670 primary antibody. The secondary antibodies we used are coupled to peroxidase (Histofine).  
671 Staining development was done using FastDAB followed by hematoxylin counterstaining.  
672 Slides were then mounted using Pertex and then scanned with a NanoZoomer 2.0 HT  
673 (Hamamatsu)

674

## 675 **CyTOF/Hyperion assay**

### 676 *Antibodies and Metal Conjugation*

677 Antibodies were conjugated using the Maxpar® X8 Antibody Labeling Kit according to the  
678 manufacturer's instructions (PRD002 Rev 14, Fluidigm, Standard Biotools).

### 679 *Imaging mass cytometry*

680 4µm FFPE tissue sections were deparaffinized and rehydrated and subsequently submitted to  
681 heat-induced epitope/antigen retrieval for 30 minutes at 96 °C using the Dako Target Retrieval  
682 Solution at pH 9 (S236784-2, Agilent technologies) in water.

683 Unspecific antibody staining was blocked with Superblock™ (37515, ThermofisherScientific)  
684 at room temperature (RT) for 45 min, and then with FcR Blocking Reagent (130-092-575,  
685 Miltenyi) at RT for 1h. Slides were washed in PBS/0.2% Triton X-100 (PBS-T) and incubated  
686 with a metal-tagged antibody cocktail at 4°C overnight. After washes in PBS-T, nuclei were  
687 stained with iridium (1:400 in PBS; Fluidigm, Standard Biotools) for 30min at RT, washed in  
688 PBS and then in distilled water for 5min and dried at RT for 30 min.

689 Pre-selected regions of interest were acquired with the Hyperion Imaging System (Fluidigm,  
690 Standard Biotools) by a UV laser ablation at 200Hz. Data were exported as MCD files and  
691 visualized using the Fluidigm MCD™ viewer 1.0.560.6.

692 Cell segmentation was obtained using the ImcSegmentationPipeline<sup>56</sup>. Briefly, MCD files were  
693 first converted to TIFF format. Then pixel classification was done with Ilastik v1.3.3<sup>57</sup> and  
694 applied to images with CellProfiler v4.1.3<sup>58</sup> software to generate the segmentation mask and  
695 the corresponding single-cell file (.fcs) using the R package flowCore version 2.6.0.

## 696 **Intrasplenic injection**

697 Amplified culture adenoma cells (as previously described above) were dissociated using gentle  
698 cell dissociation reagent and labeled with green dead cell stain for cell sorting. Green negative  
699 cells were sorted in a BD FACSAria II. 1000 cells were resuspended in 50 µl of PBS and  
700 injected into the spleen of C57BL6J female mice aged 6-8 weeks.

701 Murine colon cancer cell line (MC38) was used to establish a model of liver metastasis.  
702 Intrasplenic injection was done as previously described<sup>22</sup>. Briefly, 100,000 live cells were  
703 resuspended in 50ul of PBS and injected into the spleen of VCT and AVCT mice 5 months  
704 post-tamoxifen administration. 5 minutes after the injection, spleens were removed and mice  
705 were then sacrificed 1 month after the injection.

## 706 **Timp1 administration**

707 Timp1 recombinant protein was generously provided by Achim Kruger. Mice were treated  
708 every second day by intraperitoneal injection of 4,93 ug recombinant Timp1 protein per  
709 injection.

## 710 **Splenic Injection for in vivo liver metastasis experiments (IVIS)**

711 Mice were anesthetized with isoflurane (Henry Schein) and small incisions were made on the  
712 skin and peritoneum of left lateral flank to exteriorize spleen. 2x10<sup>6</sup> CMT93-mCh-Luc cells in

713 50  $\mu$ l Dulbecco's PBS (D-PBS) were then injected into spleen, followed by closure of incisions  
714 with sutures and surgical staples. Mice were monitored after surgery. Bioluminescence  
715 imaging was performed using an IVIS Spectrum and image radiance values were normalized  
716 using Living Image (Caliper LifeScience). Living Image Software (PerkinElmer, Waltham, MA,  
717 USA) was used to analyze the signals in manually selected regions of interest. Data were  
718 expressed as photons per second per square centimeter per steradian ( $p/s/cm^2/sr$ ).

719

## 720 **ELISA**

721 For mouse plasma, samples were diluted to one-third. ELISA for Timp-1 and SDF-1 (from R&D  
722 systems); M-CSF, CXCL2 and TNF-  $\alpha$  (from Preprotech 900-M54) was performed according  
723 to manufacturer's protocol

724 For human plasma, samples were diluted to one-third and ELISA for Timp-1(Preprotech 900-  
725 M438), M-CSF (R&D systems DMC00B), CXCL2 (Preprotech 900-M120), SDF-1 Preprotech  
726 900-M92) and TNF-  $\alpha$  (DTA00D) was performed according to manufacturer's protocol.

727

## 728 **Tissue clearing**

729 Perfusion-fixed mice were processed for active whole-body vDISCO labelling as described  
730 previously<sup>59</sup>. Briefly after decolorization, decalcification and permeabilization, mice were  
731 perfused for 6d with 250ml of the same permeabilization solution containing 35  $\mu$ l of  
732 nano booster, (NanoTag FluoTag-Q) 1:7,000 in dilution and 290 $\mu$ l of Propidium Iodide (PI, to  
733 label cell nuclei). Mice were then incubated for an additional 2 days with the same labeling  
734 solution refreshed with an extra 5 $\mu$ l of nano booster. Finally, the mice were cleared according  
735 to the vDISCO protocol. The cleared livers of the animals were imaged using Miltenyi  
736 Ultramicroscope Blaze with a 4x objective.

## 737 **Intravital Imaging**

738 The intravital spinning-disk confocal microscopy, surgical procedures, and anesthesia of mice  
739 were done as described previously<sup>60</sup>. Briefly, a microlensed spinning-disk confocal scan head  
740 (CSU-10b, Yokogawa Corporation; modified by Solamere Technology Group) was coupled to  
741 a motorized, inverted fluorescence microscope (Zeiss Axiovert 200M inverted fluorescence  
742 microscope; Carl Zeiss) and four-color imaging achieved using an argon laser for 488-nm  
743 excitation, a krypton laser for 568 and 647 excitation or solid-state 561-nm and 640-nm lasers,  
744 a 405-nm solid-state laser, and selective emission filters. Images were collected with an  
745 intensified charge-coupled device camera (XR-Mega-10EX S-30; Stanford Photonics). Live,  
746 long-term, multi-position imaging using a robotic stage was performed with 10 $\times$ Fluar 0.5 NA or  
747 20 $\times$  Fluar 0.75 NA lens air objectives, using the Micro-Manager software. Mice were initially  
748 anesthetized with 4% isoflurane (at 21% oxygen, balance nitrogen; Summit Anesthesia  
749 Solutions), and surgery was performed with 2.5% (vol/vol) isoflurane. Before surgery, mice  
750 received retro-orbitally 7  $\mu$ g of Alexa Fluor 647-conjugated Ly-6G antibody. The ventral surface  
751 of the mouse was prepared for surgery with isopropyl alcohol or betadine, a ventral midline  
752 incision made with sterilized scissors, the liver was surgically exposed and a glass microscope  
753 slide glued below the left lobe of the liver. Then,  $2 \times 10^6$  CMT93-mCh-Luc cells in 50  $\mu$ l  
754 Dulbecco's PBS (D-PBS) were injected into the spleen. The glass slide was rotated to expose  
755 the inner surface of the liver, and the mouse was transferred to the microscopic stage. During  
756 imaging, isoflurane was reduced to the lowest concentration at which the mouse did not react  
757 to pain, typically 0.8–1.0%. We used an oximeter probe (MouseOx, Starr Life Sciences) to  
758 monitor and display the heart rate, the arterial oxygen saturation of the blood, and the

759 distension of blood vessels caused by the pulse and the breathing, adjusting the anesthesia  
760 levels appropriately to the individual mouse. During the imaging procedure, isoflurane was  
761 delivered in a humidified mix of nitrogen and oxygen [at least 21% (vol/vol)], with oxygen  
762 adjusted to achieve >95% oxygen saturation of arterial blood as measured with an oximeter  
763 probe. Mice were covered with a recirculating heated water blanket during imaging (Gaymar).  
764 Time-lapse videos were assembled using Bitplane Imaris (v6.3–7.6 for Windows 64x),  
765 adjusting brightness and contrast for clarity, as well as applying smoothing filters to reduce  
766 noise.  
767

768 **Patient samples:**

769 Blood from patients with intestinal adenomas was obtained within the clinical trial  
770 NCT05648240 and patients all gave their consent.  
771

772

773

774 **Supplementary Video S1-3**

775 Videos from intravital live imaging on the liver of *Apc<sup>Min/+</sup>/ACTB-ECFP/cfms-EGFP* mouse (S1)  
776 and *APC WT/ACTB-ECFP/cfms-EGFP* mice (S2-3) 15 minutes post intrasplenic injection of  
777 CMT93 expressing mcherry. Macrophages are visualized in green, CMT93 tumor cells in red,  
778 hepatocytes in blue and neutrophils in white. For further details refer to method section.

779

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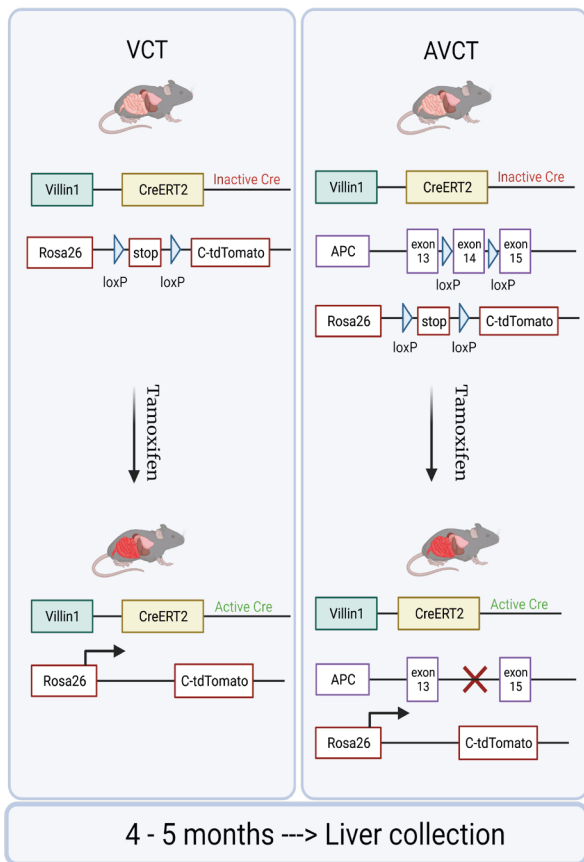


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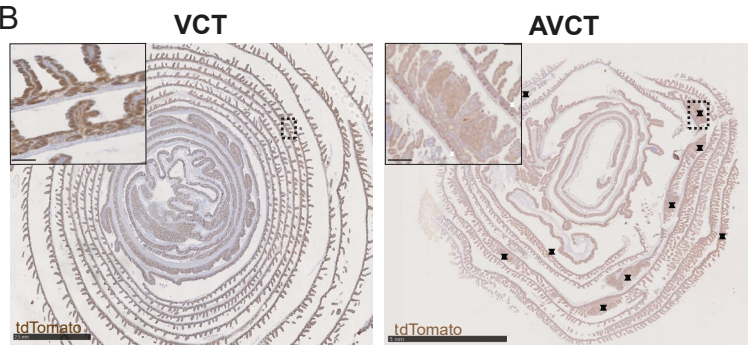
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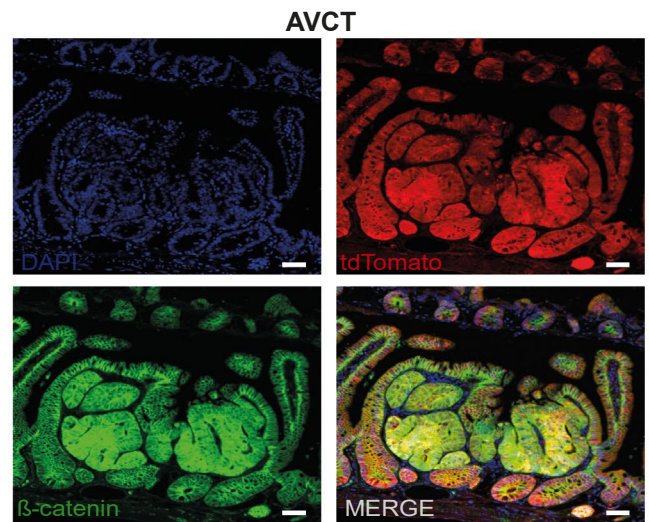
A



B

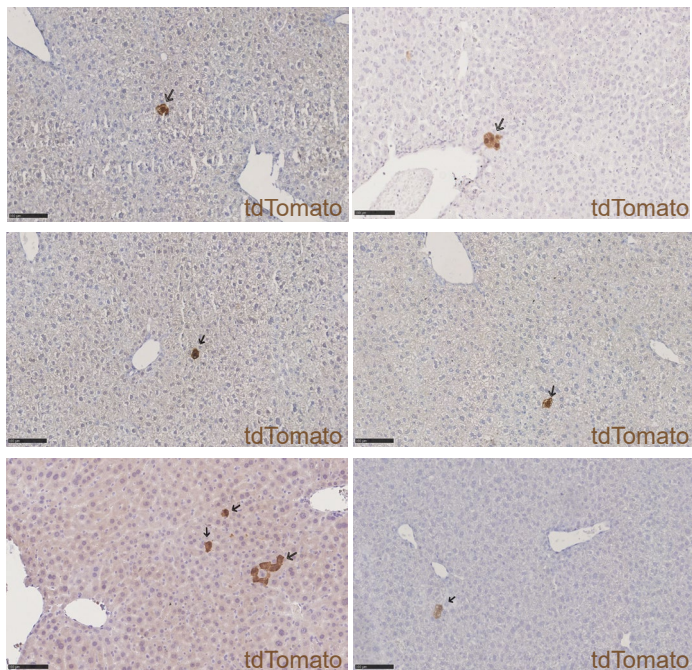


C



D

Liver of AVCT mice



E

Liver of AVCT mice

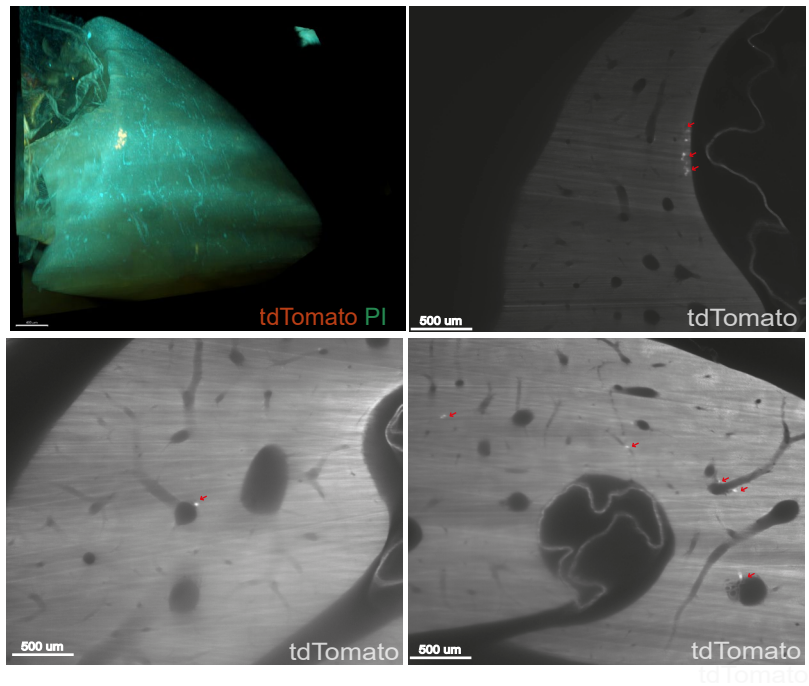


Figure 1

Figure 2

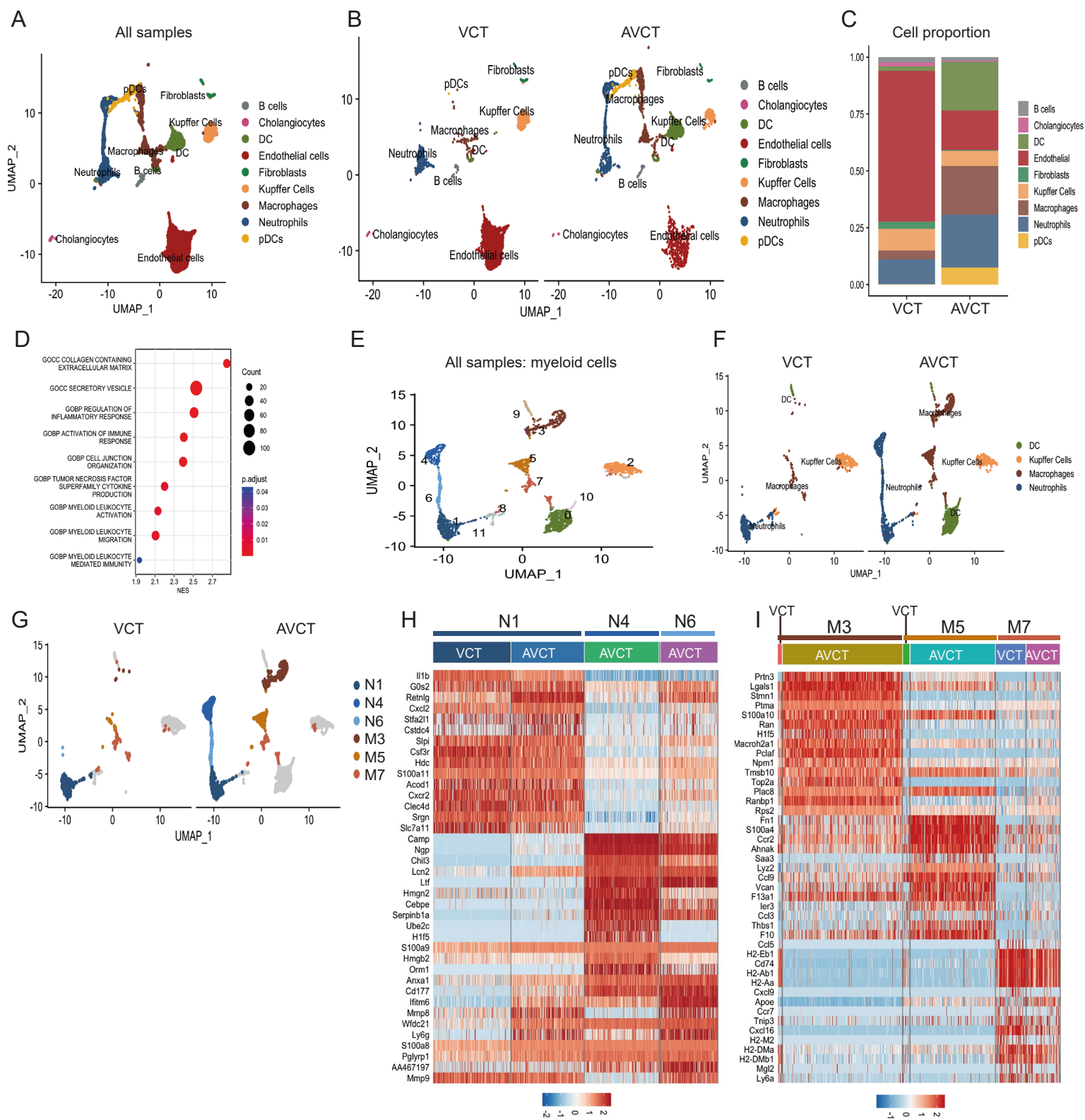


Figure 2

Figure 3

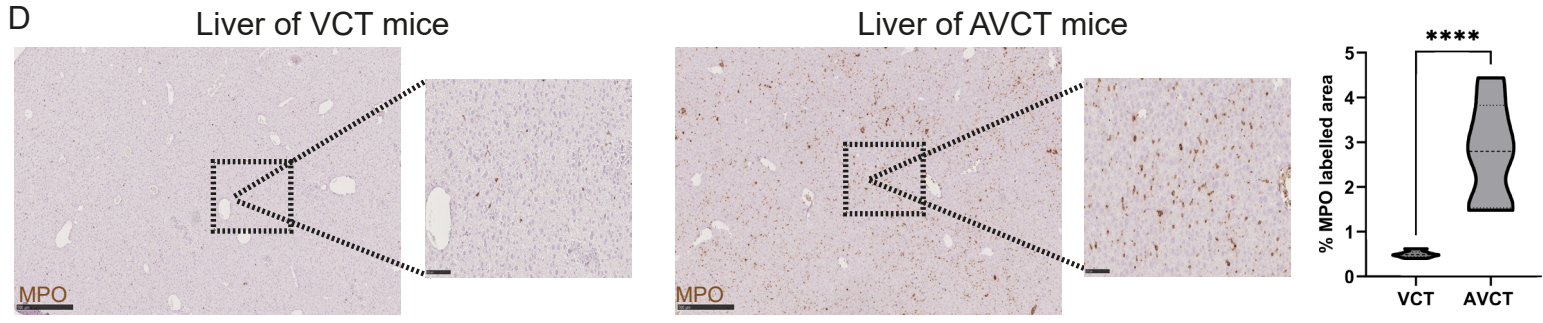
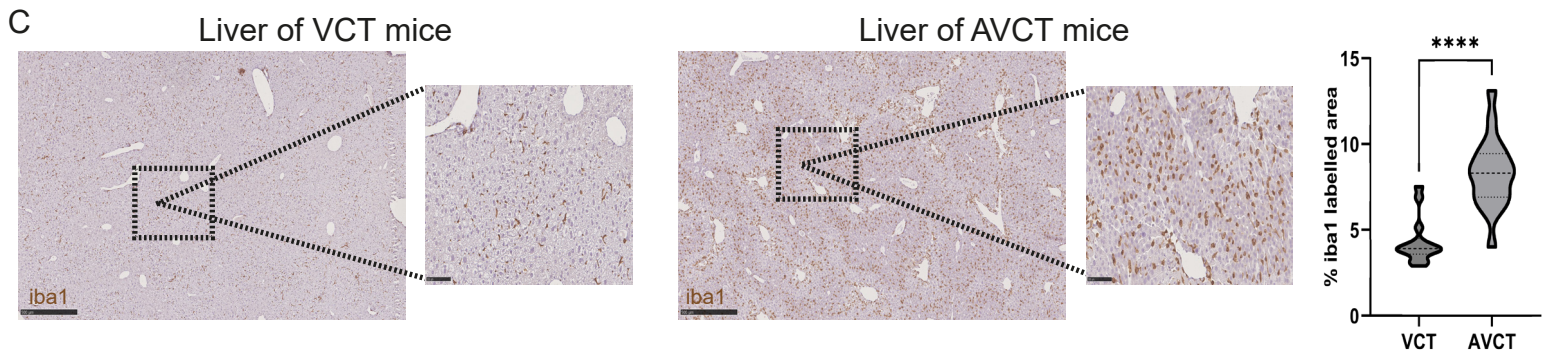
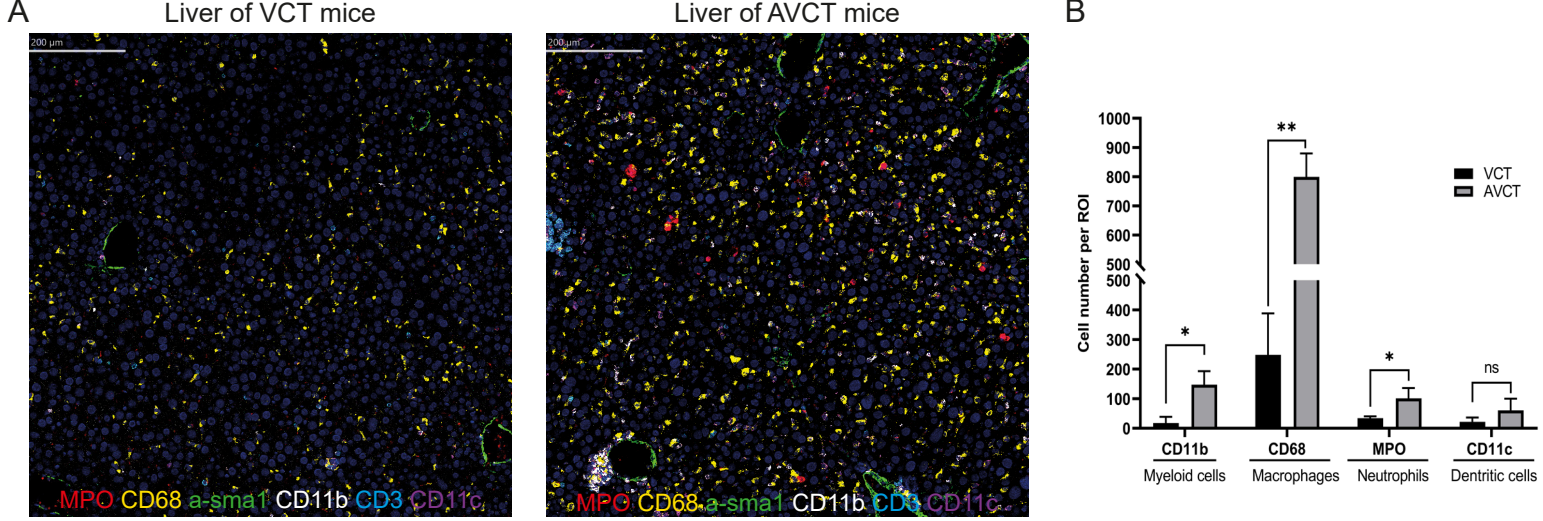


Figure 3

Figure 4

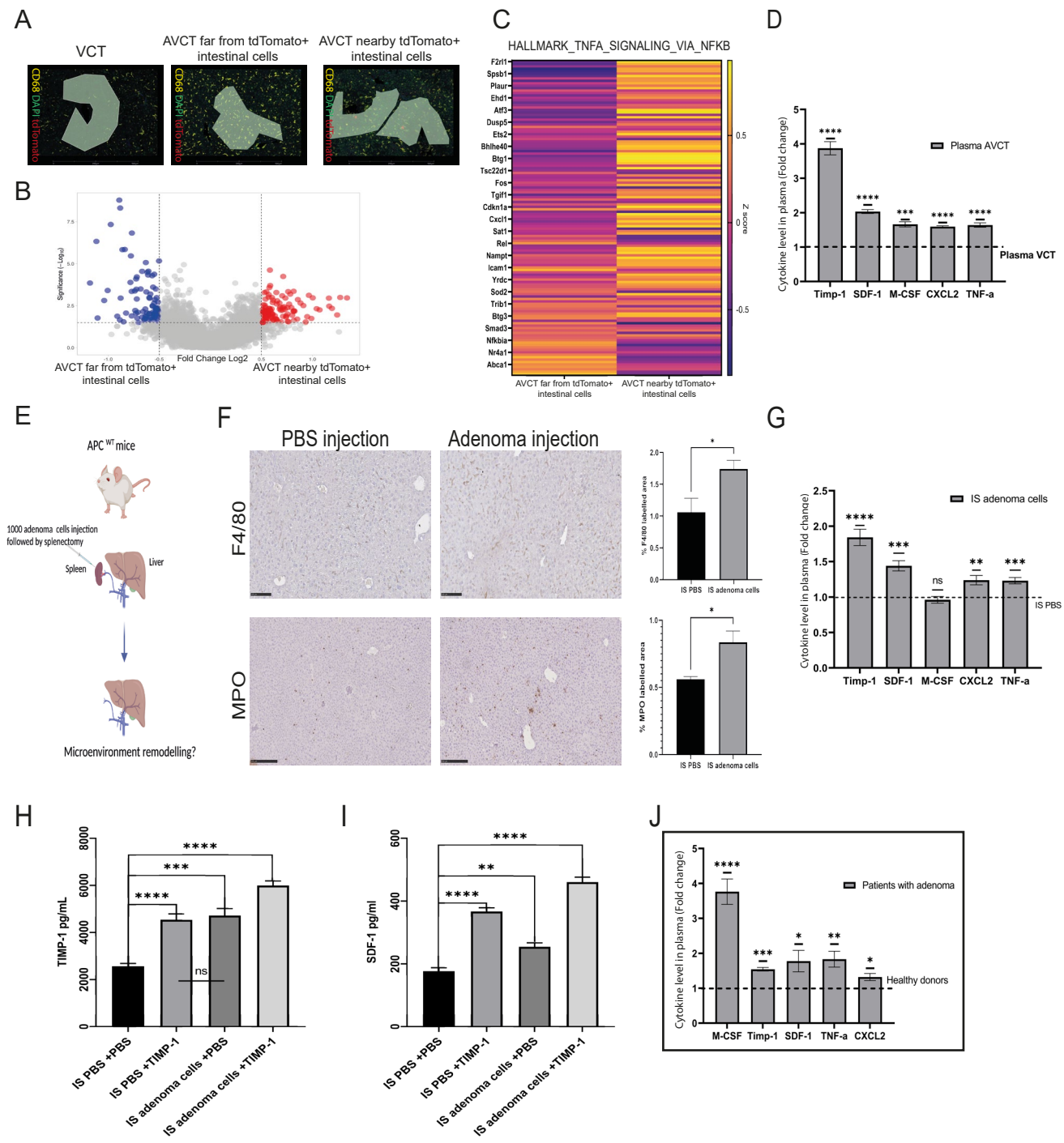


Figure 4

Figure 5

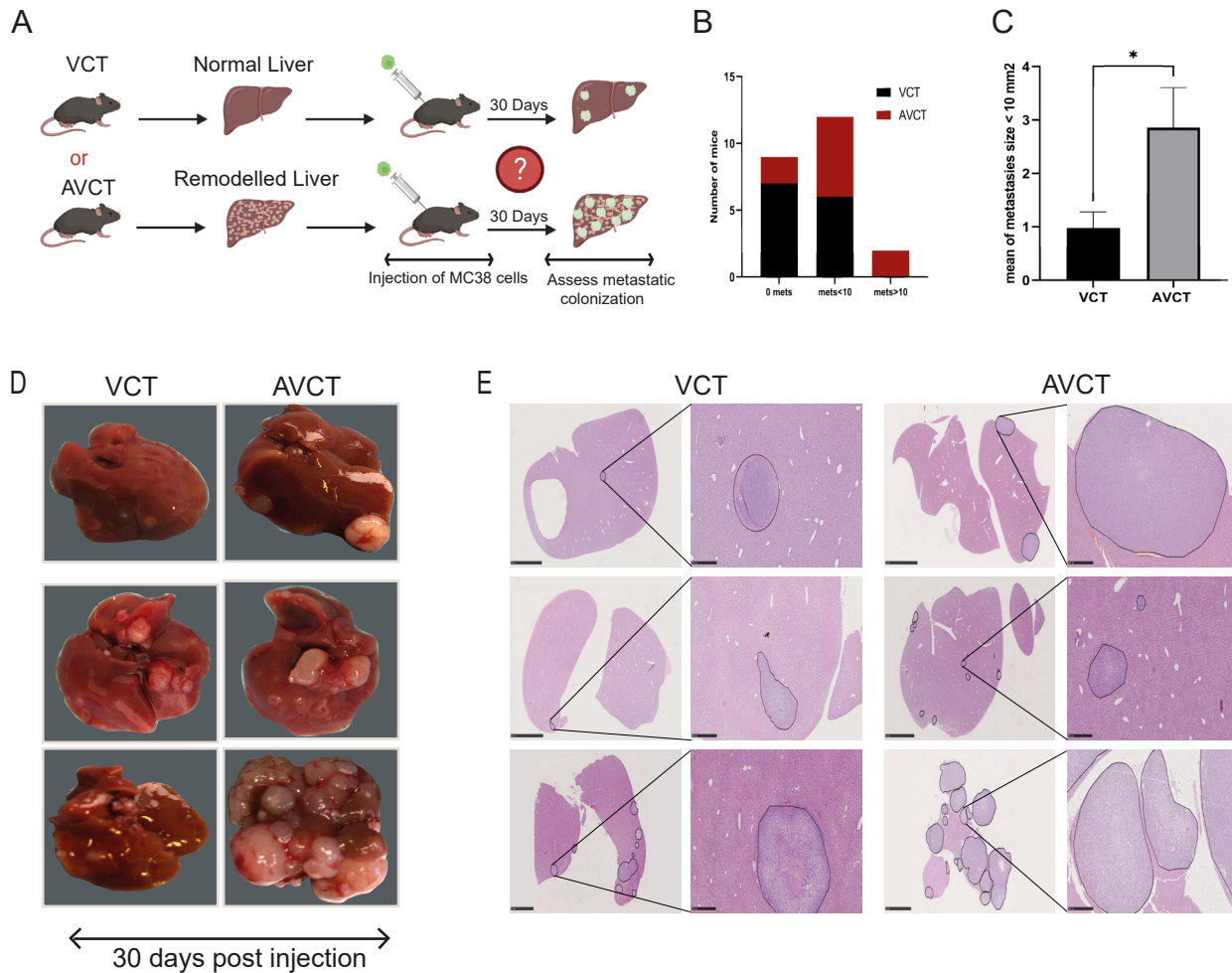
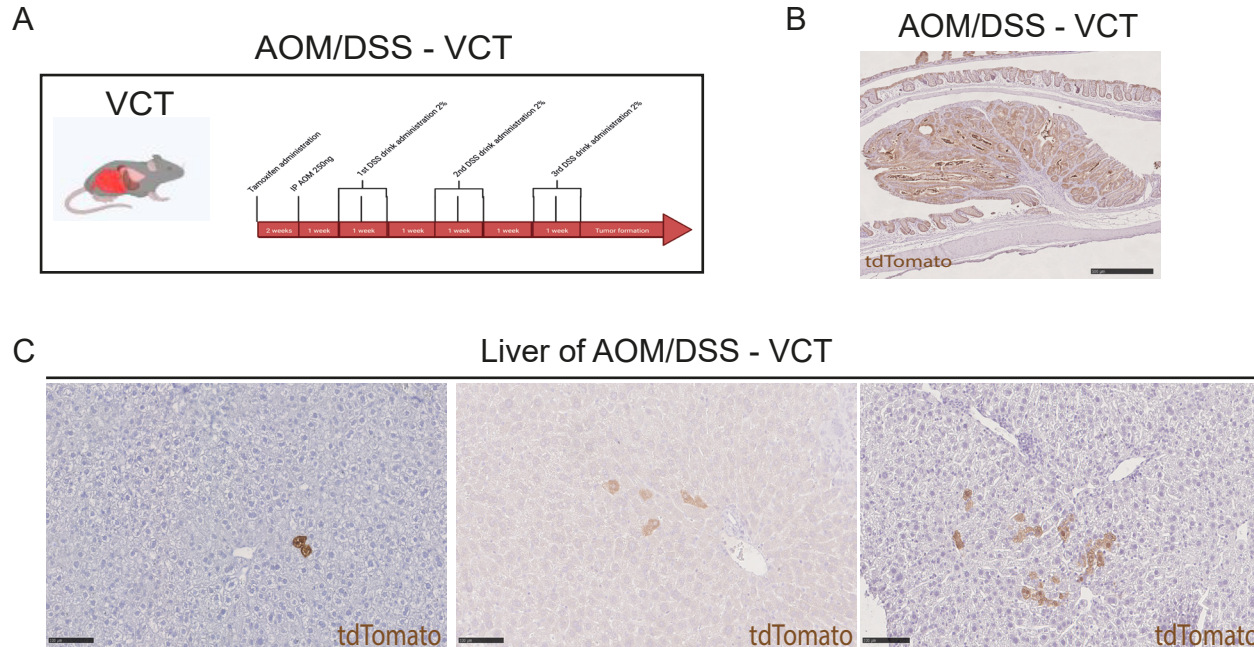
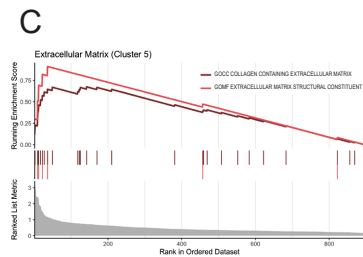
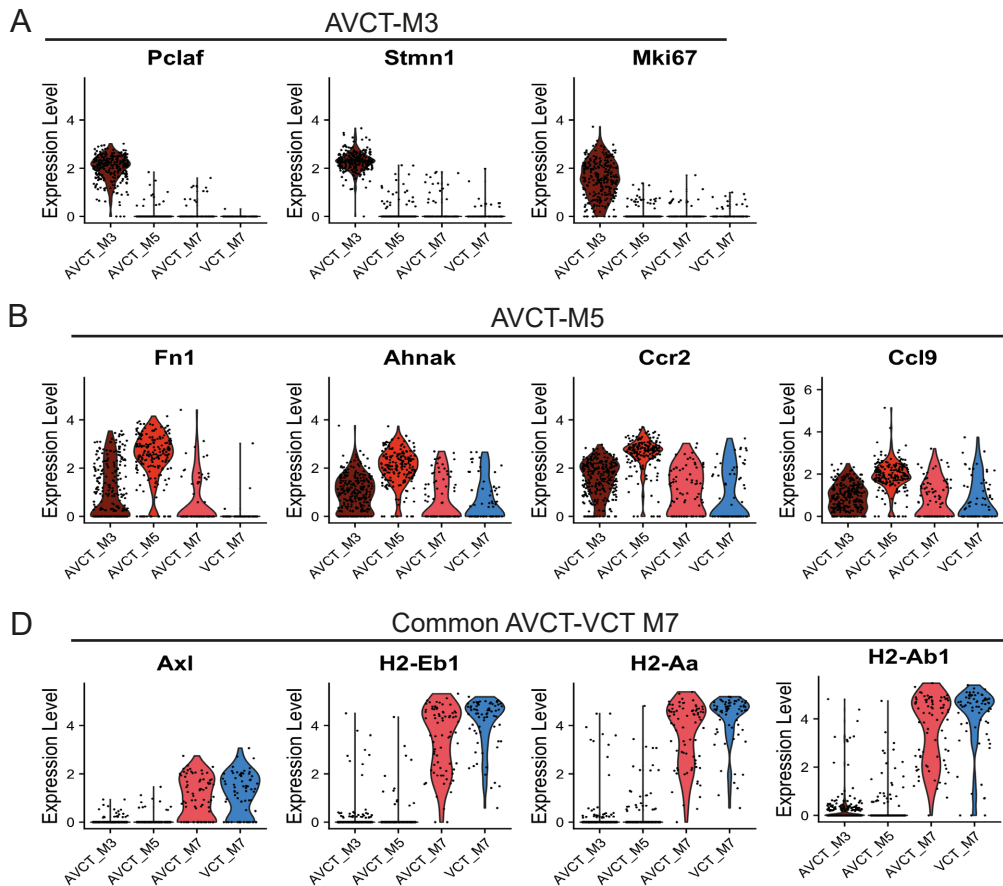


Figure 5



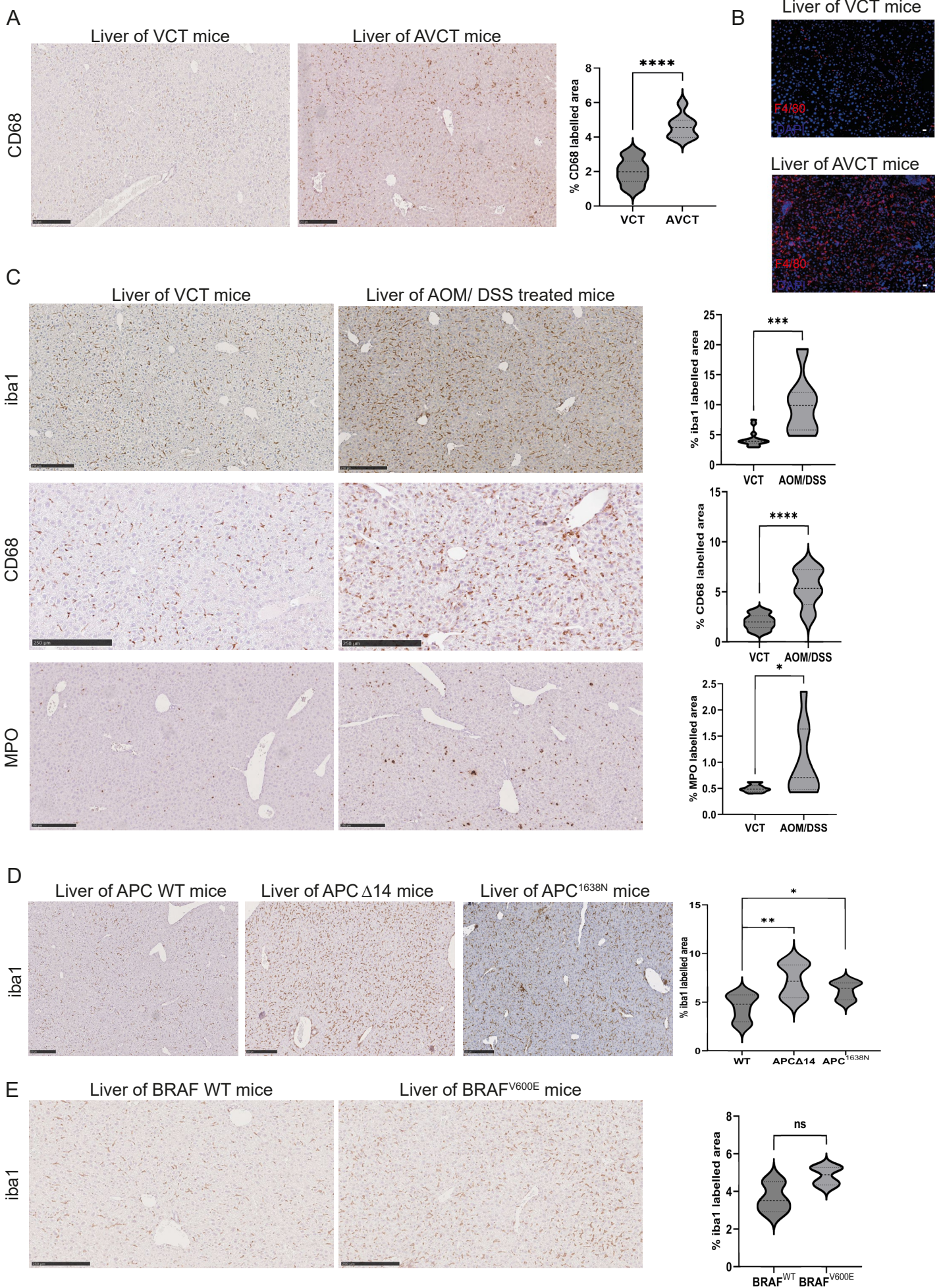
### Supplementary Figure S1: **A complementary mouse model for tracking early CRC dissemination**

- (A) Schematic description of AOM/DSS protocol applied to tamoxifen-treated VCT mice to induce intestinal tumorigenesis.
- (B) Immunohistochemistry using RFP antibody to detect dTomato positive cells on a colonic adenoma and showing a homogenous tdTomato staining validating the mouse model.
- (C) Immunohistochemistry using RFP antibody to detect dTomato positive cells on liver sections from 3 different mice with colonic adenomas reveals the presence of rare intestinal tdTomato early-disseminated tumor cells



Supplementary Figure S2: **Characterization of macrophages subpopulations in the liver of VCT and AVCT mice**  
 Violin plots showing the distribution of expression level of the indicated genes across clusters for macrophages cluster M3; M5 and M7 (A, B and D respectively) (see the result part for details)  
 (C) GSEA plot displaying the enrichment score “ES” of extracellular matrix related GO terms in cluster M5 (P adjust value of GOMF and GOCC 0.0003 and 0.00084 respectively; Enrichment Score of GOMF and GOCC 0.9113 and 0.67426 respectively)





Supplementary Figure S3

Supplementary Figure S3: **Myeloid cell enrichment in complementary mouse models of early intestinal tumorigenesis**

(A) Immunohistochemistry using CD68 antibody to detect macrophages on liver sections from VCT mice (left panel) and AVCT intestinal adenoma bearing mice (middle panel). Right panel shows signal intensity quantification.

CD68: n>8, \*\*\*\*p value < 0.0001

(B) Representative image of immunofluorescence using F4-80 antibody to detect macrophages on liver sections from VCT mice (upper panel) and AVCT intestinal adenoma bearing mice (lower panel).

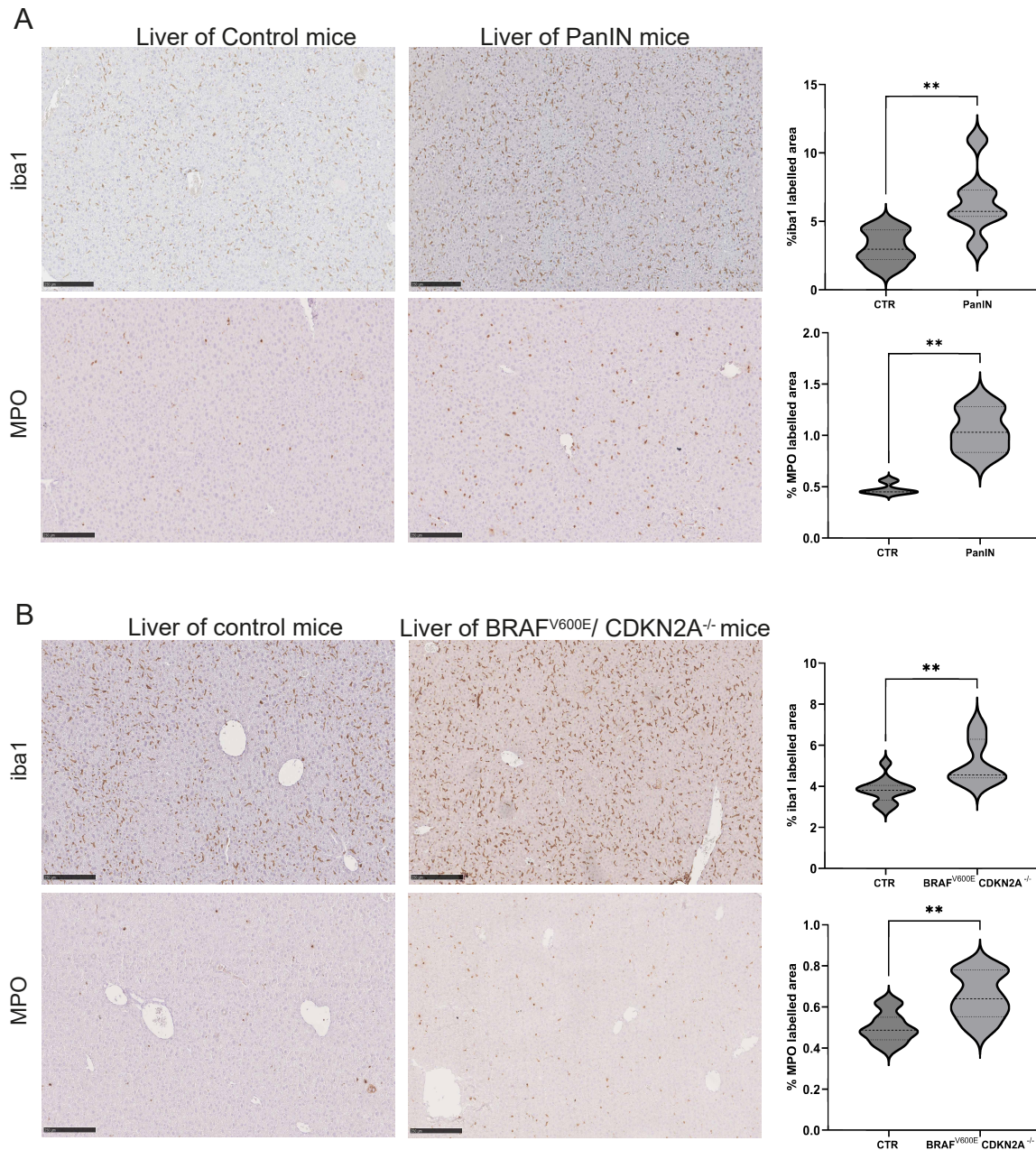
(C) Immunohistochemistry using CD68 and Iba1 antibodies (top and middle panel respectively) to detect macrophages and MPO antibody (bottom panel) on liver sections from AOM and tamoxifen-treated VCT mice bearing colonic adenomas. Right panel shows signal intensity quantifications

Iba1: n>7, \*\*\* p value = 0.0007 CD68: n>5 \*\*\*\* p value < 0.0001 MPO: n>10 \* p value= 0.0235

(D) Immunohistochemistry using Iba1 antibody to stain macrophages on liver sections from APC WT mice (left panel), APCD14 mice (middle panel) and APC1638N mice (right panel). Far right panel shows signal intensity quantifications n=7, \*\* p value =0.0061 \* p value =0.0231

(E) Immunohistochemistry using Iba1 antibody to detect macrophages on liver sections from BRAF WT mice (left panel) and BRAFV600E intestinal adenoma bearing mice (middle panel).

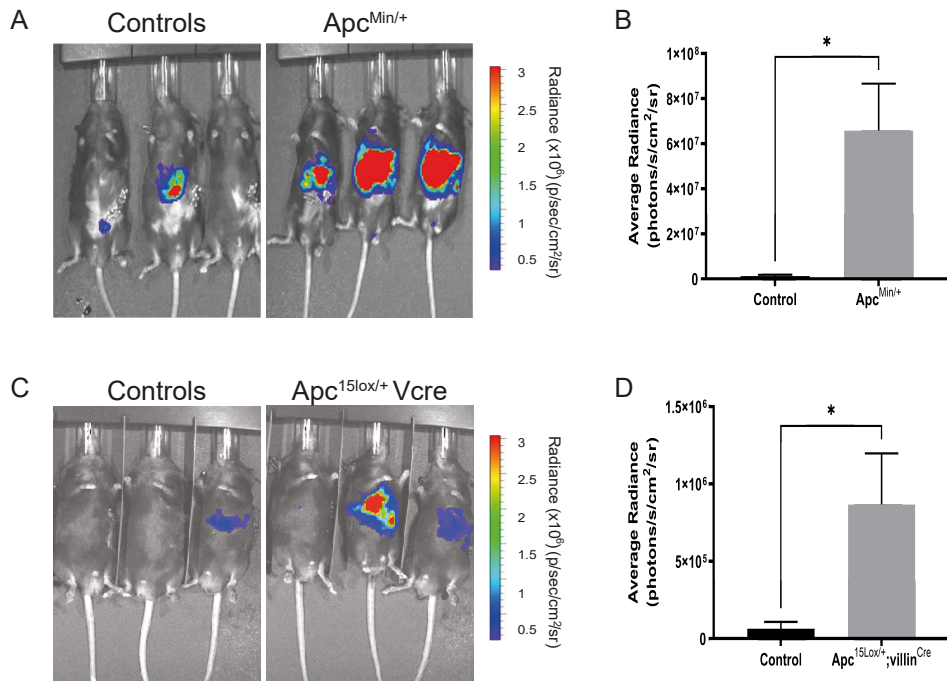
Right panel shows signal intensity quantifications. N=3, p value = 0.0594



Supplementary Figure S4: **Myeloid cell enrichment in early stages of pancreatic and melanoma mouse models**

(A) Immunohistochemistry using Iba1 antibody (top panel) to detect macrophages and MPO antibody (bottom panel) on liver sections from KPC mice at the PanIN stage (for pancreas cancer). Right panel shows signal intensity quantifications Iba1: n>7, \*\* p value = 0.0018; MPO n>3 \*\* p value= 0.0039

(B) Immunohistochemistry using Iba1 antibody (top panel) to detect macrophages and MPO antibody (bottom panel) on liver of BRAFV600/CDKN2A<sup>-/-</sup> mice. Right panel shows signal intensity quantifications Iba1: n>6, \*\* p value = 0.0036 MPO: n>6, \*\* p value = 0.0043



**Supplementary Figure S5: *Apc*-mutated mice are more susceptible to develop liver metastasis than WT mice**

All animals received intrasplenic injection of  $2 \times 10^6$  CMT93-Luc-mCherry cells and bioluminescent signal was acquired by using IVIS invivo imaging system twice a week for 2 weeks.

(A,B) On the left, representative image of bioluminescent signals in the liver at 2 weeks in WT versus  $Apc^{Min/+}$  mice.

On the right, data were shown with the mean radiance  $\pm$  SEMs of 6 WT and 7  $Apc^{Min/+}$  mice.

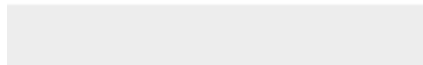
(C,D) On the left, representative image of bioluminescent signals in the liver at 2 weeks in WT versus  $Apc^{15Lox/+};Vcre$  mice.

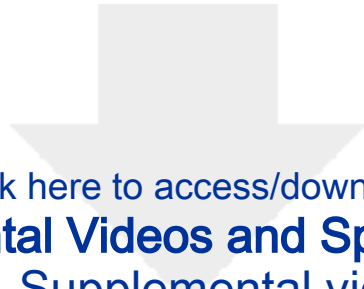
On the right, data were shown with the mean radiance  $\pm$  SEMs of 10 WT and 11  $Apc^{15Lox/+};Vcre$  mice.



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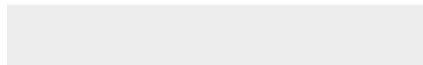
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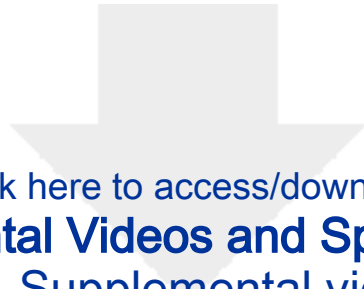




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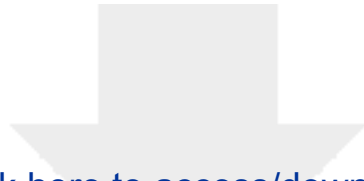




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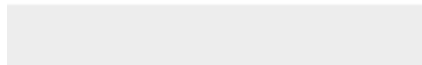
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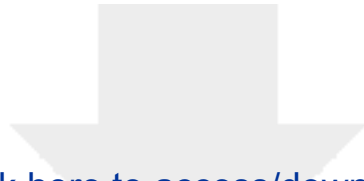


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