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1 **Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis**

2
3 Nadine Rohwer¹, Sandra Jumpertz^{2,17}, Merve Erdem^{2,17}, Antje Egners², Klaudia T.
4 Warzecha³, Athanassios Fragoulis², Anja A. Kühl⁴, Rafael Kramann⁵, Sabine Neuss⁶, Ines
5 Rudolph¹, Tobias Endermann⁷, Christin Zasada⁸, Ivayla Apostolova⁹, Marco Gerling¹⁰, Stefan
6 Kempa⁸, Russell Hughes¹¹, Claire E. Lewis¹¹, Winfried Brenner⁹, Maciej B. Malinowski^{12,19},
7 Martin Stockmann¹², Lutz Schomburg⁷, William Faller^{13,20}, Owen Sansom¹³, Frank Tacke³,
8 Markus Morkel¹⁴ and Thorsten Cramer^{2,15,16,18}
9

10 ¹Hepatology and Gastroenterology, Charité Universitätsmedizin Berlin, Augustenburger Platz
11 1, 13353 Berlin, Germany; ²Molecular Tumor Biology, Department of General, Visceral- and
12 Transplantation Surgery, RWTH University Hospital, Pauwelsstraße 30, 52074 Aachen,
13 Germany; ³ Medicine III, RWTH University Hospital, Pauwelsstraße 30, 52074 Aachen,
14 Germany; ⁴Research Center Immunosciences, Charité Universitätsmedizin Berlin,
15 Hindenburgdamm 30, 12200 Berlin, Germany; ⁵Medicine II, RWTH University Hospital,
16 Pauwelsstraße 30, 52074 Aachen, Germany; ⁶Pathology, RWTH University Hospital,
17 Pauwelsstraße 30, 52074 Aachen, Germany; ⁷Experimental Endocrinology, Charité
18 Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany; ⁸Max-Delbrück-
19 Centrum, Robert-Rössle-Straße 10, 13125 Berlin, Germany; ⁹Nuclear Medicine, Charité
20 Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany; ¹⁰Biosciences
21 and Nutrition, Karolinska Institutet, Alfred Nobels Allé 8, 17177 Stockholm, Sweden;
22 ¹¹Oncology and Metabolism, University of Sheffield Medical School, Sheffield S10 2RX, UK;
23 ¹²Surgery, Charité Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin,
24 Germany; ¹³Cancer Research UK Beatson Institute, Glasgow G61 1BD, UK; ¹⁴Institute for
25 Pathology, Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany;
26 ¹⁵NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht
27 University, Maastricht, The Netherlands; ¹⁶ESCAM – European Surgery Center Aachen
28 Maastricht, Germany and The Netherlands;
29

30 ¹⁷These authors contributed equally

31

32 ¹⁸Corresponding author

33 ¹⁹Present address: Department for General, Visceral, Vascular, and Pediatric Surgery,

34 Saarland University Hospital, Homburg, Germany

35 ²⁰Present address: The Netherlands Cancer Institute, Amsterdam, The Netherlands

36

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39 Corresponding author:

40 Thorsten Cramer, Molecular Tumor Biology, General, Visceral- and Transplantation Surgery,

41 University Hospital RWTH Aachen, Pauwelsstraße 30, 52074 Aachen, Germany; Phone: +49

42 241 80 36353, Fax: +49 241 80 82068 ; E-mail: tcramer@ukaachen.de

43

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45

46 **Abstract**

47 The hypoxia-inducible transcription factor HIF-1 is appreciated as a promising target for
48 cancer therapy. However, conditional deletion of HIF-1 and HIF-1 target genes in cells of the
49 tumor microenvironment can result in accelerated tumor growth, calling for a detailed
50 characterization of the cellular context to fully comprehend HIF-1's role in tumorigenesis. We
51 dissected cell type-specific functions of HIF-1 for intestinal tumorigenesis by lineage-
52 restricted deletion of the *Hif1a* locus. Intestinal epithelial cell-specific *Hif1a* loss reduced
53 activation of Wnt/ β -catenin, tumor-specific metabolism and inflammation, significantly
54 inhibiting tumor growth. Deletion of *Hif1a* in myeloid cells reduced the expression of
55 fibroblast-activating factors in tumor-associated macrophages resulting in decreased
56 abundance of tumor-associated fibroblasts (TAF) and robustly reduced tumor formation.
57 Interestingly, hypoxia was detectable only sparsely and without spatial association with HIF-
58 1α , arguing for an importance of hypoxia-independent, i.e. non-canonical, HIF-1 stabilization
59 for intestinal tumorigenesis that has not been previously appreciated. This adds a further
60 layer of complexity to the regulation of HIF-1 and suggests that hypoxia and HIF- 1α
61 stabilization can be uncoupled in cancer. Collectively, our data show that HIF-1 is a pivotal
62 pro-tumorigenic factor for intestinal tumor formation, controlling key oncogenic programs in
63 both the epithelial tumor compartment and the tumor microenvironment.

64

65 Introduction

66 Colorectal cancer (CRC) ranks as the fourth most common tumor in the western world,
67 causing a substantial amount of cancer-associated morbidity and mortality [1]. The
68 combination of molecular-targeted drugs with conventional chemotherapeutic agents
69 significantly improved the therapeutic options for CRC patients [2]. However, as also noted
70 frequently for other solid tumors, these new generation treatments rarely result in improved
71 overall outcome, but rather benefit small subgroups of CRC patients [3]. Taken together, an
72 urgent need to identify novel therapy targets exists, demonstrating the necessity for detailed
73 characterization of the molecular CRC pathogenesis.

74
75 The hypoxia-inducible transcription factor 1 (HIF-1) is positively associated with the
76 malignant progression of various tumor entities [4]. While the role of HIF-1 for the
77 pathogenesis of CRC has been addressed by different groups, results are conflicting and the
78 precise function of HIF-1 for intestinal tumorigenesis remains elusive. Stabilization of HIF-1 α ,
79 the regulatory component of HIF-1, in human CRC has been reported via
80 immunohistochemistry, suggesting a pro-tumorigenic function [5-7]. In line with these
81 findings, inhibition of HIF-1 α in human CRC cell lines resulted in reduced xenograft growth
82 [8, 9]. Furthermore, pharmaceutical inhibition of HIF-1 led to diminished growth of
83 autochthonous as well as allograft murine CRC models via reduced angiogenesis and
84 macrophage infiltration [10]. On the other hand, transgenic activation of HIF-1 in intestinal
85 epithelial cells (IEC) remained without effect on tumor formation in murine models of sporadic
86 and colitis-associated colon cancer [11, 12]. Moreover, IEC-specific loss of *Hif1a* did not
87 affect tumor frequency in a chemical model of proximal intestinal tumorigenesis [13]. These
88 results illustrate that the precise role of HIF-1 for intestinal tumorigenesis remains elusive
89 thus far.

90
91 While HIF-1 is widely appreciated as a promising target for cancer therapy, conditional
92 deletion of HIF-1 and HIF-1 target genes in cells of the tumor microenvironment can result in

93 accelerated tumor growth [14-16]. Against this background, a comprehensive analysis of
94 HIF-1's role in tumorigenesis in order to translate the findings into the clinic can only be
95 achieved by considering the cell- and tissue-specific context. Here, we present a detailed
96 deconstruction of HIF-1's role in CRC initiation and progression in IEC- and myeloid cell-
97 specific *Hif1a* knock-out mice [17, 18] using two murine models of intestinal tumor formation:
98 Chemically-induced colon tumors (AOM/DSS (combination of azoxymethane plus dextran
99 sulfate sodium for colitis-associated carcinogenesis) [19]) and the genetic APC^{min} model
100 [20]. This experimental approach enabled us to address the role of HIF-1 in neoplastic
101 epithelial cells as well as in innate immune cells of the tumor microenvironment. We were
102 able to unravel numerous specialized functions of HIF-1. In IECs, HIF-1 serves to induce
103 inflammation, control Wnt/ β -catenin activity and regulate tumor-specific metabolism. In
104 addition, we identified myeloid HIF-1 as essential for the activation of tumor-associated
105 fibroblasts. Of note, intratumoral hypoxia was a rare event, pointing towards an importance of
106 hypoxia-independent, i.e. non-canonical HIF-1 stabilization for intestinal tumorigenesis [21].
107 Collectively, our data show that HIF-1 is a pivotal pro-tumorigenic factor, controlling key
108 oncogenic programs in both the epithelial tumor compartment and the tumor
109 microenvironment.

110

111 **Results**

112 **Epithelial *Hif1a* controls intestinal tumor growth on multiple levels**

113 IEC-specific loss of *Hif1a* (termed Hif1a^{IEC}) was established by breeding *villin-cre* mice [22]
114 with animals harbouring homozygously floxed *Hif1a* alleles [18]. Conditional *Hif1a* deletion
115 was characterized on various levels and found to be highly efficient (Figure S1A). Tumor size
116 in both the AOM/DSS and APC^{min} model was significantly decreased in Hif1a^{IEC} mice (Figure
117 1A). While proliferation was not different between the genotypes (data not shown), Hif1a^{IEC}
118 adenomas displayed significant differences regarding the number of apoptotic tumor cells
119 (Figure S1B) and CD31-positive endothelial cells as well as *Vegfa* expression (Figure S1C),
120 pointing towards a functional importance of HIF-1 α in tumor cells for resistance towards

121 apoptosis and angiogenesis. To further analyze the mechanisms underlying the reduced
122 tumor size in *Hif1a*^{IEC} mice, we first characterized the activity of the Wnt/ β -catenin pathway,
123 the central oncogenic driver of CRC. As can be seen in figure 1B-D, the expression of
124 selected β -catenin target genes is significantly reduced in adenomas of *Hif1a*^{IEC} mice. Of
125 note, nuclear translocation of β -catenin was not affected by the *Hif1a* KO (Figure S1D).
126 Furthermore, the expression of TCF-1 and LEF-1, two pivotal binding partners for β -catenin
127 that have been characterized as HIF-1 targets by the group of Celeste Simon in murine
128 embryonic stem and teratocarcinoma cells [23] was independent of HIF-1 in murine intestinal
129 adenomas (Figure S1E). While these results strongly suggest a functional role of HIF-1 for
130 Wnt/ β -catenin activity in murine intestinal tumorigenesis, the underlying molecular
131 mechanisms remain elusive at this point. Next, we addressed the role of *Hif1a* for tumor-
132 specific glucose metabolism as metabolic reprogramming represents an emerging hallmark
133 of cancer and several glycolytic enzymes are transcriptionally controlled by HIF-1 [24]. Mass
134 spectrometry-based analysis of intratumoral glucose levels, routing of ¹³C-labelled glucose
135 as well as *in vivo* imaging by PET/CT revealed diminished uptake and metabolization into
136 lactate of glucose in tumors of *Hif1a*^{IEC} mice (Figure 1E, F). Finally, we performed a
137 comprehensive analysis of inflammatory activity as chronic inflammation is pro-tumorigenic in
138 CRC [25]. *Hif1a*^{IEC} mice displayed reduced loss of body weight (Figure 2A) and colitis activity
139 after DSS challenge (Figure 2B). Intestinal gene expression and protein secretion of pro-
140 inflammatory factors were significantly lower in DSS-treated *Hif1a*^{IEC} mice (Figure 2C, D). We
141 included a characterization of mucins as these evolutionary preserved glycoproteins are of
142 crucial importance innate immune responses in the gut [26]. As *Muc1*, 2 and 3 can be
143 regulated by HIF-1 [27-29], we investigated their gene expression patterns and found no
144 difference under basal conditions. In contrast, *Muc1* and 3 were strongly upregulated by DSS
145 in a HIF-1 α -dependent manner (Figure S2A). Quantification of the inflammatory infiltrate via
146 immunohistochemistry did not show significantly reduced numbers of macrophages,
147 granulocytes, T cells and Tregs after DSS in the colon of *Hif1a*^{IEC} animals (Figure S2B). This
148 at first surprising result is in line with published findings demonstrating that clinical and

149 biochemical inflammation markers do not necessarily overlap with the cellular infiltrate in the
150 DSS model [30]. Experiments with small intestinal organoids were conducted to further
151 address the specific contribution of IECs in this setting. After assuring efficient *Hif1a* deletion
152 (Figure S2C), organoids were subjected to different pro-inflammatory stimuli. These
153 experiments nicely confirmed that the pro-inflammatory response of IECs critically depends
154 on *Hif1a* (Figure 2E). Taken together, these data point towards a complex role for *Hif1a* in
155 IECs during intestinal tumor formation, comprising resistance to apoptosis, angiogenesis,
156 Wnt/ β -catenin activity, metabolic reprogramming and inflammation.

157

158 ***Hif1a* in myeloid cells regulates intestinal tumor formation independent of** 159 **inflammation**

160 Macrophages in the tumor microenvironment exert a number of tumor-supporting functions
161 and are positively associated with the malignant phenotype [31]. We and others have shown
162 that *Hif1a* is of pivotal importance for various aspects of macrophage function [17, 32].
163 Against this background, we sought to characterize the role of *Hif1a* in macrophages for
164 intestinal tumor formation. We found that myeloid cell-specific loss of *Hif1a* (termed *Hif1a*^{MC})
165 resulted in a highly significant reduction of both tumor number and size (Fig. 3A). In order to
166 identify the underlying mechanisms, we first analyzed the inflammatory response. Rather
167 unexpectedly, various assays of inflammation (e.g. determination of weight loss (Fig. 3B),
168 disease activity index (Fig. 3C)) as well as gene expression and protein secretion of
169 established pro-inflammatory markers (Fig. 3D-F)) failed to show a significant difference
170 between wildtype and *Hif1a*^{MC} mice.

171

172 ***Hif1a*-deficient tumor-associated macrophages migrate and function normally**

173 Next, we determined intratumoral macrophage numbers via immunohistochemistry (IHC).
174 While macrophage abundance was clearly greater in adenomas compared to surrounding
175 normal mucosa (not shown), no difference was detectable between the genotypes (Figure
176 4A). Intestinal leukocyte subsets were subsequently analyzed in more detail by flow

177 cytometry. Tumor-bearing mice displayed a prominent increase in CD11c⁺ macrophages and
178 CD11b⁺ dendritic cells, while CD11c⁻ macrophages were reduced. The changes in myeloid
179 subsets were comparable between wildtype and *Hif1a*^{MC} mice, except for a slightly more
180 pronounced reduction of CD11c⁻ intestinal macrophages in tumor-bearing *Hif1a*^{MC} mice
181 (Figure 4B). Intestinal lymphoid cell populations did not differ significantly between wildtype
182 and *Hif1a*^{MC} mice (Figure 4B). Notably, tumor-bearing animals showed typical alterations of
183 extraintestinal myeloid cells [33], including increase of monocytes in blood and bone marrow
184 and accumulation of Gr1⁺ CD11b⁺ myeloid-derived suppressor cells (MDSC) in bone
185 marrow, but not spleen (Figure S3). Extraintestinal myeloid cells did not differ between
186 wildtype and *Hif1a*^{MC} mice. Next, we decided to analyze the direct tumor-supporting action of
187 macrophages as these cells secrete numerous pro-tumorigenic factors [34]. To this end,
188 spheroids of APC^{min} adenomas were stimulated with conditioned medium (CM) from primary
189 murine macrophages. While we were able to detect a significant growth-promoting effect of
190 macrophage CM, the loss of *Hif1a* did not affect the outcome (Figure 4C). As macrophages
191 represent pivotal modulators of stem-like/progenitor cells [35], which are critical drivers of
192 colonic carcinogenesis [36], we decided to quantify these cells in our experimental setting.
193 Visualization of *Lgr5* and *Prox1*, two established progenitor/stem-cell markers in intestinal
194 tumors [37, 38], demonstrated robust presence of these cells in AOM/DSS adenomas, albeit
195 without differences between the genotypes (Figure S4A). We took into consideration that
196 other cells of myeloid origin might underlie the reduced tumor formation in *Hif1a*^{MC} mice.
197 While mast cells can influence intestinal tumorigenesis under certain experimental conditions
198 [39], no difference in intratumoral abundance of this cell type was noted, arguing against a
199 functional importance of mast cells in our setting (Figure S4B).

200

201 ***Hif1a* in myeloid cells is essential for the activation of tumor-associated fibroblasts**

202 Myeloid cells in the tumor microenvironment are known to interact with various cell types,
203 including tumor-associated fibroblasts (TAF). These cells are abundant in human CRC and
204 significantly impact on disease progression [40, 41]. TAFs were readily detectable via IHC in

205 the stroma of APC^{min} and AOM+DSS-induced adenomas (Figure 5A). Strikingly, deletion of
206 *Hif1a* in myeloid cells resulted in greatly reduced numbers of TAFs in both tumor models
207 (Figure 5A). This result pointed towards a crucial role of *Hif1a* in myeloid cells for TAF
208 development. Interestingly, the importance of macrophages for fibroblast activation in the
209 context of wound healing is well established [42, 43]. To analyze this further, we focussed on
210 alternatively activated macrophages (AAM) given their central role in the control of organ
211 fibrosis [42]. Expression of various pro-fibrotic genes in AAMs was readily detectable, but
212 loss of *Hif1a* remained without greater effect (Figure S5A). To achieve experimental data that
213 more closely resemble the *in vivo* situation, we analyzed pro-fibrotic factor expression in
214 tumor-associated macrophages (TAM) directly isolated from intestinal adenomas. This
215 approach indeed unravelled a central role of *Hif1a* in TAMs for the expression of various
216 fibroblast-activating factors, e.g. COX-2, IGF-1, IL-1 β and granulins (Figure 5B). In our
217 analyses, the expression of TGF- β 1, the archetypical pro-fibrotic factor, was constantly lower
218 in *Hif1a*-null TAMs, but did not reach statistical significance (Figure 5B). As the activation of
219 TGF- β 1 is a complex and highly regulated process [44], we hypothesized that HIF-1 α is of
220 importance in this setting. Indeed, HIF-1 α -deficient AAMs displayed reduced levels of
221 bioactive TGF- β in the supernatant (Figure 5C), suggesting a functional importance of HIF-
222 1 α for TGF- β activity elicited by TAMs. Next, we addressed the role of *Hif1a* for macrophage-
223 mediated fibroblast proliferation [42, 45]. While conditioned medium from AAM enhanced
224 survival of primary murine intestinal fibroblasts (MIFs), no difference between WT and *Hif1a*-
225 deficient macrophages was detectable (Figure S5B). Finally, we sought to address the
226 secretion of pro-tumorigenic cytokines, another tumor-promoting function of TAFs [46].
227 Stimulation of primary MIFs with conditioned medium from AAM induced gene expression of
228 IL-6, HGF and epiregulin (*Ereg*), factors with established tumor-promoting activity in the
229 intestine [47, 48]. Of note, this effect was significantly reduced upon deletion of *Hif1a* in
230 macrophages (Figure 5D).

231

232 **Myeloid-mediated activation of TAF precursor cells depends on *Hif1a***

233 TAFs can originate from different cellular sources, amongst others pericytes, mesenchymal
234 stems cells (MSCs) and fibrocytes [46]. We sought to investigate if macrophages are able to
235 polarize these cell types into myofibroblasts and whether *Hif1a* is important in this setting.
236 Gli1-positive pericytes, marked by tdTomato expression [49], as well as MSCs readily
237 polarized into myofibroblasts after addition of macrophage conditioned medium (CM, Figure
238 6A and B). Intriguingly, HIF-1 α was of central importance in this setting as the effect on
239 pericytes was completely and that on MSCs partially abolished upon *Hif1a* deletion.
240 Fibrocytes are of myeloid origin, display features of monocytes as well as fibroblasts and
241 contribute to fibrosis in various organs and tumors [50, 51]. We took advantage of an
242 established protocol to generate fibrocytes *ex vivo* from splenic monocytes [52]. Of note,
243 *Hif1a*-deficient monocytes displayed greatly reduced capacity for fibrocyte production
244 (Figure 6C). Furthermore, the expression of various tumor-supporting factors in fibrocytes
245 was found to be regulated by *Hif1a* (Figure 6D). Taken together, these results point to a
246 hitherto unknown function of *Hif1a* for the activation of TAF precursor cells of different origin.

247

248 **Non-canonical stabilization of HIF-1 α predominates in murine intestinal tumors**

249 The luminal cell layer of the colon is characterized by physiological hypoxia [53]. Nuclear
250 HIF-1 α protein is readily detectable in luminal enterocytes (Figure S6A), suggesting hypoxia-
251 induced HIF-1 α protein stabilization in the gut under physiological conditions. Against this
252 background, we sought to determine the relevance of hypoxia for HIF-1 α protein stabilization
253 in murine intestinal tumors. Intriguingly, hypoxic areas were detected only sporadically in
254 AOM+DSS and APC^{min} adenomas (Figure 7A and B). This pattern of hypoxia was clearly not
255 able to explain the pervasive stabilization of HIF-1 α protein in both tumor types. Of note,
256 hypoxia-independent means of HIF-1 α protein stabilization have been identified and are
257 gradually receiving more attention [21, 24]. As activation of various oncogenes has been
258 shown to result in hypoxia-independent HIF-1 stabilization [24], we decided to investigate the
259 role of oncogenes and tumor suppressor genes characteristic for colon cancer [54]. To mimic
260 the molecular events that instigate tumor formation in the APC^{min} model, mice with inducible

261 *Apc* deletion were analyzed [55]. Of note, acute *Apc* loss resulted in enhanced HIF-1 α
262 protein stability (Figure 7D). To analyze the role of oncogene activation for HIF-1 α
263 stabilization, we took advantage of transgenic mice with doxycycline-inducible, epithelial-
264 specific expression of oncogenes with relevance for CRC pathogenesis [56, 57]. The
265 strongest effect was noted for PIK3CA^{H1047R}, the activation of which resulted in robust HIF-1 α
266 protein stabilization of the entire epithelial cell lining (Figure 7E). Induction of oncogenic
267 KRAS^{G12V} or degradation-resistant β -catenin resulted in localized HIF-1 α stabilization in
268 luminal epithelial cells (Figure 7F,G). These results point towards a functional role of key
269 tumor suppressors and oncogenes regulating the Wnt/ β -catenin, phosphatidylinositol-3-
270 kinase and mitogen-activated protein kinase cascades for non-canonical HIF-1 α stabilization
271 during intestinal tumor formation and progression.

272

273 **Discussion**

274 Here, we addressed the functional importance of HIF-1 for colon cancer in a cell type-specific
275 manner, using transgenic mice harbouring either an intestinal epithelial cell- (*Hif1a*^{IEC}) or a
276 myeloid cell-specific (*Hif1a*^{MC}) *Hif1a* deletion [17, 18, 22]. We found that *Hif1a* plays multiple
277 non-redundant roles in these two key cell types of intestinal tumors (summarized in Figure
278 S8). Our finding of reduced tumor growth in *Hif1a*^{IEC} mice is in line with earlier reports
279 showing *Hif1a*-dependent growth of human CRC xenografts [8, 9]. Also in line with our
280 results, the group of Celeste Simon reported that application of a chemical HIF inhibitor
281 decreased tumor formation in mice bearing AOM+DSS adenomas and s.c. CT26 allografts
282 [10]. Of note, transgenic overexpression of oxygen-stable HIF-1 α in intestinal epithelial cells
283 did not further accelerate the formation of AOM+DSS and APC^{min} tumors [11]. Deletion of
284 *Hif1a* in IECs did not affect tumor size in a chemical model of proximal colon cancer [13],
285 arguing for the importance of cell type- and location-specific factors that need to be further
286 investigated.

287

288 In our experiments, *Hif1a*^{IEC} mice displayed lower levels of pro-inflammatory cytokines in
289 acute DSS-induced colitis, suggesting an activating function of HIF-1 in IECs for intestinal
290 inflammation. This result contrasts with earlier reports from various independent groups.
291 Karhausen *et al.* noted enhanced activity in hapten-induced intestinal inflammation upon
292 conditional loss of *Hif1a* in IECs [53] and identified reduced barrier integrity in mutant mice as
293 the underlying mechanism. Against this background, we determined intestinal barrier function
294 in our mice and could not find a difference between WT and *Hif1a*^{IEC} mice (Figure S7). Later,
295 Shah *et al.* reported no effect of IEC-specific *Hif1a* loss on the severity of acute DSS-induced
296 colitis [58]. It is well established that the susceptibility to experimentally induced colitis is
297 genetically determined and differs substantially between inbred strains of mice [59].
298 Karhausen *et al.* and Shah *et al.* used mice with a mixed genetic background while our mice
299 were >99% C57Bl6/J, potentially explaining the different results. Additional support for a
300 protective role of HIF-1 during intestinal inflammation came from two simultaneously
301 published seminal reports by the groups of Sean Colgan and Cormac Taylor showing that
302 inhibitors of prolyl hydroxylases (PHDs), a group of enzymes crucial for HIF degradation,
303 protect against murine colitis [60, 61]. In our opinion, different explanations are possible
304 regarding the conflict with our data, e.g. a functional importance of other PHD targets, e.g.
305 NF- κ B, and hydroxylase-independent functions of PHD inhibitors. Furthermore, systemically
306 administered PHD inhibitors target every cell they encounter while in our genetic model HIF-
307 1 was exclusively deleted in IECs, precluding a direct comparison of the different
308 experimental approaches. Regarding *Hif1a* in myeloid cells, we did not observe significant
309 changes in DSS-induced intestinal inflammation and this is well in line with data from a
310 Korean group [62]. On the contrary, Sandra Winning, Joachim Fandrey and colleagues
311 reported protective roles for *Hif1a* in myeloid [63] and dendritic cells [64] upon DSS
312 challenge. Of note, Kim *et al.* could not detect a functional importance of *Hif1a* in myeloid
313 cells for AOM+DSS-induced tumor formation, clearly contrasting with our data [62]. The
314 exact mechanisms for these opposing results remain elusive at this time.

315

316 Our analysis of mucin gene expression confirms earlier reports showing induction of mucin
317 gene expression by DSS [65] and the notion that *Muc1* and *3* are direct targets of HIF-1 α
318 [27, 29]. Complementing the published work, we were able to show that HIF-1 α is essential
319 for DSS-induced gene expression of *Muc1* and *3* in murine intestinal epithelial cells. In
320 contrast, our results do not support a functional relevance of HIF-1 α in IECs for basal
321 expression of these mucin isoforms. Taken together, these data argue for a causal role of
322 HIF-1-induced *Muc1* and *3* for the inflammatory response to DSS, a notion well in line with
323 the established importance of mucins for innate immunity [66].

324

325 Seminal work in the laboratory of Randall Johnson had shown that HIF-1 α is essential for
326 myeloid cell-mediated inflammation and that *Hif1a*-deficient macrophages fail to migrate
327 towards pro-inflammatory cues [17]. Our results contrast with this observation as we did not
328 detect reduced macrophage abundance in tumors of *Hif1a*^{MC} mice. However, our data are
329 well in line with a later study from the Johnson lab demonstrating no effect of the myeloid
330 cell-specific *Hif1a* KO on the abundance of F4/80-positive macrophages in a murine breast
331 cancer model [67]. The precise reason(s) for this discrepancy must remain elusive at this
332 time, but context- and model-specific factors could well play a significant role. It is
333 conceivable that infiltration of macrophages into tumors is not as sensitive to *Hif1a* deletion
334 as tissue infiltration in the context of inflammation. In addition, it is possible that proliferation
335 of local macrophages compensates for reduced macrophage infiltration into KO adenomas
336 [68]. This would result in a situation where the findings by Cramer *et al.* [17] and Doedens *et*
337 *al.* [67] would be well in line with our current data.

338

339 Our findings support a central role of HIF-1 α in myeloid cells for the activation of fibroblasts
340 in the stroma of intestinal tumors. In the context of wound healing and organ fibrosis, the
341 importance of macrophages, especially that of alternatively activated macrophages (AAM),
342 for fibroblast activation is well established [69, 70]. AAMs are of special significance as they
343 express various fibroblast-activating factors [42]. While earlier reports showed a functional

344 relevance of HIF-1 for the control of gene expression of pro-fibrotic factors (e.g. TGF- β 1,
345 endothelin-1, fibronectin-1 and COX-2 [71-74]) in different cell types, *Hif1a* deletion in AAMs
346 remained without effect on mRNA expression of a comprehensive set of pro-fibrotic factors in
347 our experimental setup. To address the importance of the tissue context, we isolated tumor-
348 associated macrophages (TAMs) from intestinal adenomas. Of note, in these cells the
349 expression of various pivotal pro-fibrotic genes was indeed regulated by *Hif1a*. These results
350 not only underscore the significance of *Hif1a* for TAM-mediated activation of TAFs, but again
351 illustrate the importance of experimental conditions that more closely resemble the tissue
352 context.

353

354 TAFs were for the longest time unequivocally considered to support tumor growth and
355 progression [46]. This concept was recently challenged by reports from three independent
356 groups, showing accelerated tumor progression upon either genetic ablation of α SMA-
357 positive TAFs or attenuated stroma formation in murine pancreatic ductal adenocarcinoma
358 [75, 76]. At first glance, this observation contrasts with our data arguing for a tumor-
359 supporting role of TAFs. Besides cancer type- and context-specific factors, the different
360 experimental approaches are strong candidates for potential explanations. The group of
361 Raghu Kalluri used an elegantly designed genetic model resulting in direct ablation of TAFs.
362 Our experimental setup, on the other hand, targeted TAFs indirectly via deletion of *Hif1a* in
363 myeloid cells. The studies by Ozdemir *et al.* and Rhim *et al.* point towards previously
364 unappreciated tumor-inhibiting functions of TAFs. How pro- and anti-tumor aspects of TAFs
365 are regulated is an intriguing question. While our data suggest that myeloid *Hif1a* impacts
366 mainly on tumor-supporting aspects of TAFs, future work has to address the eligibility of HIF-
367 1 inhibitors in the setting of a stroma-targeting cancer therapy.

368

369 Our study highlights that HIF-1 α can be stabilized by multiple means in the colon cancer
370 context. We show that loss of APC, as well as oncogenic activation of PIK3CA or KRAS in
371 intestinal epithelial cells is followed by HIF-1 α stabilization. Our findings give credence to the

372 concept of non-canonical HIF-1 α stabilization that was recently coined by Amato Giaccia and
373 colleagues [21]. It is important to note that we cannot exclude a role for hypoxia downstream
374 of the genetic changes and that this study has not determined the exact molecular link(s)
375 relaying oncogenic events to HIF-1 α stabilization. However, our data add a further layer of
376 complexity to HIF-1 α regulation, and suggest that hypoxia and HIF-1 α stabilization can be
377 uncoupled in cancer.

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Materials and methods

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Animals and Mouse models

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In all experiments, male and female mice were divided randomly into homogeneous groups according to their weight, age and sex (VillinCre/*Hif1a*^{loxP/loxP}, VillinCre/*Hif1a*^{loxP/loxP}/APC^{+/-min}, LysMCre/*Hif1a*^{loxP/loxP} and LysMCre/*Hif1a*^{loxP/loxP}/APC^{+/-min}, all on a C57BL/6J background). For the AOM/DSS model, 6-8 week-old mice were injected intraperitoneally with 10 mg/kg AOM (azoxymethane; Sigma-Aldrich, Germany) followed by three cycles of 2% dextran sodium sulfate (DSS; MP Biomedicals, Germany) in drinking water for five days and normal drinking water for 16 days. Mice were sacrificed 8 weeks after AOM injection. Tumors were counted and measured under a dissecting microscope by a blinded investigator and colonic normal and tumor tissue snap frozen in liquid nitrogen for *ex vivo* analysis. In the acute DSS model, 10-12 week-old mice received 2% DSS in the drinking water for 6 days and were sacrificed on the last day of DSS administration. In order to assess DSS colitis activity, body weight, stool consistency and the presence of occult or gross blood were determined and a scoring system was applied [77]. Spheroids of APC^{+/-min} mice were cultured as described [78]. Oncogene-inducible mice have been described before [56, 57]. For histology, small intestines and colons were removed, flushed with PBS, fixed in 10% neutral buffered formalin at 4°C overnight and paraffin-embedded. Experimentation and transgenic animal generation was approved by authorities in Berlin (Landesamt für Gesundheit und Soziales: G0004/07, G0185/09, G0143/14).

402 Isolation and stimulation of bone marrow-derived macrophages (BMDM)

403 Bone marrow was collected from tibiae, femurs and humeri of 8-12 weeks old wildtype and
404 Hif1a^{MC} mice. After flushing out the marrow, red blood cells were lysed with ACK buffer and
405 cells were seeded on plastic plates in RPMI supplemented with 10% FBS, 100 U
406 penicillin/ml, 100 µg/ml streptomycin. Next day, non-attached cells were collected and
407 cultured in RPMI supplemented with 20% FBS and 30% L929-conditioned medium for one
408 week. Differentiated BMDMs were stimulated for 48 h with LPS (100 ng/ml, Sigma Aldrich)
409 and γ-IFN (20 ng/ml) for classically activated (CAM) and with IL-4 (20 ng/ml, both from
410 eBioscience) for alternatively activated macrophages (AAM). For RNA isolation, cells were
411 harvested using TRIzol (Invitrogen) from M0 (non-polarized), CAM and AAM. To collect
412 conditioned media from polarized macrophages, cells were extensively washed with PBS 48
413 h after stimulation and fresh media was added for an additional 24 h.

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415 Flow cytometric analyses of murine leukocyte populations

416 Small intestine was rinsed thoroughly and incubated in buffer containing EDTA to remove the
417 mucus. Cells were minced and digested with type IV collagenase (Cellsystems). Blood was
418 taken from the right ventricle. Small intestine, blood, bone marrow, and spleen cells were
419 subjected to red blood cell lysis using Pharm Lyse (BD Biosciences, San Jose, USA). Flow
420 cytometric analysis was done as described in detail before [79]. Antibodies were purchased
421 from eBioscience (F4/80, #25-4801-82, CD3e, #25-0031-82, CD4, #17-0042-82 and CD8a,
422 #25-0081-82), BD Biosciences (CD45, #557659, CD11b, #550993, Ly6G, #551460, CD19,
423 #551001, CD107a, #553793, NK1.1, #553165, Gr1, #552093 and I-A^b, #553552) and
424 Biolegend (CD11c, #117312 and CD103, #121420).

425

426 Statistical analysis

427 Sample sizes were determined according to our experience in previous experiments and no
428 statistical methods were used for predetermination. All experimental samples were included
429 in the final analyses. Unless indicated otherwise, all data were representative of at least two

430 independent experiments and expressed as means + SEM. Before statistical analysis, data
431 were checked for normal distribution (Shapiro Wilk test) and comparable variance (F-test
432 for equality of variances for data with normal distribution). Comparisons between two groups
433 of normally distributed data with equal variances were performed using the unpaired two-
434 sided Student's *t* test. Differences were considered statistically significant at $p < 0.05$. Sample
435 size, statistical tests and p values are indicated in the figure legends. The asterisks in the
436 graphs indicate statistically significant changes with p values: * $p < 0.05$, ** $p \leq 0.01$ and ***
437 $p \leq 0.001$. Statistical analysis was done using Prism 4.0 software (GraphPad Software, San
438 Diego, California, USA).

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440

441 **Author Contributions**

442 Conceptualization, N.R., M.M. and T.C.; Methodology, N.R., M.E., S.J., A.E., K.T.W., A.A.K.,
443 A.F., S.N., M.G., I.R., T.E., C.Z., S.K., R.H., M.B.M., W.F. and M.M.; Formal Analysis,
444 Investigation, and Visualization, N.R., M.E., S.J., A.E., A.A.K., R.K., S.N., I.R., M.G., C.Z.,
445 S.K., M.B.M., M.M. and T.C.; Writing, N.R., F.T., M.M. and T.C.; Supervision, Resources,
446 and Funding Acquisition, R.K., S.K., C.E.L., W.B., M.S., L.S., O.S., F.T., M.M. and T.C.;
447 Project Administration, T.C.

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775 **Figure Legends**

776 **Figure 1. HIF-1 α in IECs controls intestinal tumor formation, Wnt/ β -catenin activity and**
 777 **glucose metabolism. (A)** Tumor number and size of WT and Hif1a^{IEC} mice in the AOM/DSS
 778 (left, $n = 7$ per group) and APC^{min} model (right, $n = 7$ per group). **(B)** Relative mRNA
 779 expression of Wnt/ β -catenin target genes in colon tumors of WT and Hif1a^{IEC} mice following
 780 AOM/DSS treatment ($n = 3$ biological replicates with technical duplicates). **(C)** RNA *in-situ*
 781 hybridization of the Wnt/ β -catenin target gene *Axin2* in AOM/DSS adenomas of WT and
 782 Hif1a^{IEC} mice. **(D)** Relative mRNA expression of Wnt/ β -catenin target genes in WT and
 783 Hif1a^{IEC} APC^{min} tumors ($n = 3$ biological replicates with technical duplicates). **(E)** Glucose
 784 levels (left) and FDG-PET/CT analysis (right) of AOM/DSS-induced colon tumors (white
 785 arrow) of WT and Hif1a^{IEC} mice ($n = 7$ per group). **(F)** Metabolization of glucose into lactate
 786 (left, $n = 4$ per group) and citrate (right, $n = 13$ per group) in colon tumors of WT and Hif1a^{IEC}
 787 mice following AOM/DSS treatment determined by stable isotope-resolved metabolomics.
 788 Data are represented as mean + SEM. * $p < 0.05$; ** $p < 0.01$ by unpaired two-sided Student's t
 789 test.

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791

792 **Figure 2. IEC-specific deletion of *Hif1a* reduces intestinal inflammation. (A)** Body weight
 793 change of WT and Hif1a^{IEC} mice following AOM/DSS treatment ($n = 14$ per group), **(B)**
 794 disease activity index ($n = 5$ per group), **(C)** relative mRNA expression of inflammatory genes
 795 in colon tissues ($n = 3$ biological replicates with technical duplicates) and **(D)** secretion of
 796 cytokines by colonic explants of WT and Hif1a^{IEC} mice on day 6 of acute DSS treatment ($n =$
 797 5 per group). **(E)** Pro-inflammatory cytokine mRNA expression in small intestinal organoids
 798 from WT and Hif1a^{IEC} mice. Shown are mean and standard deviation of technical duplicates
 799 from one representative experiment (no statistical analysis performed). Unless indicated
 800 otherwise, data are represented as mean + SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired
 801 two-sided Student's t test.

802

803 **Figure 3. *Hif1a* in myeloid cells controls intestinal tumor formation without affecting**
 804 **inflammation. (A)** Tumor number and size in WT and *Hif1a*^{MC} mice after AOM/DSS
 805 administration (left, (*n* = 4 per group) and in the APC^{Min} model (right, *n* = 5 per group). **(B)**
 806 Body weight change of WT (*n* = 7) and *Hif1a*^{MC} (*n* = 8) mice following AOM/DSS treatment.
 807 **(C)** Disease activity index (*n* = 5 per group), **(D)** Relative mRNA expression of inflammatory
 808 genes in colon tissues (*n* = 3 biological replicates with technical duplicates) and **(E)** Secretion
 809 of cytokines by colonic explants of WT and *Hif1a*^{MC} mice on day 6 of acute DSS treatment (*n*
 810 = 5 per group). **(F)** Relative mRNA expression of inflammatory genes in intestinal tissues of
 811 WT and *Hif1a*^{MC} control (*n* = 3 biological replicates with technical duplicates) and APC^{min}
 812 mice (*n* = 4 biological replicates with technical duplicates). Data are represented as mean +
 813 SEM. **p*<0.05; ***p*<0.01 by unpaired two-sided Student's *t* test.

814

815

816 **Figure 4. Tumoral abundance of macrophages and stimulation of adenoma growth *ex***
 817 ***vivo* is not affected by loss of *Hif1a*.** **(A)** Representative F4/80 stainings of intestinal
 818 sections obtained from AOM/DSS-treated (above) and APC^{min} (below) WT and *Hif1a*^{MC} mice.
 819 Right, quantification of F4/80-positive cells (*n* = 6 per group). **(B)** Total leukocytes were
 820 isolated from small intestines of wildtype and *Hif1a*^{MC} mice remaining either untreated or
 821 bearing APC^{min} adenomas and analyzed by flow cytometry (*n* = 4 per group). Relative
 822 numbers of different subtypes are shown. Flow cytometric analyses of the CD11c-
 823 macrophage subset (I-Ab+, CD11c-, CD11b+, CD103-, F4/80+), the CD11c+ macrophage
 824 subset (I-Ab+, CD11c+, CD11b+, CD103-, F4/80+) and the CD11b+ dendritic cell subset (I-
 825 Ab+, CD11c+, CD11b+, CD103+, F4/80-) were performed. Lymphoid leukocytes of the small
 826 intestine were subdivided into T cells (CD4+, CD8+) and NK cells (CD3-, NK1.1+). **(C)** Effect
 827 of macrophage conditioned media (CM) on spheroid formation from APC^{min} adenomas. Left,
 828 representative image of spheroids after stimulation. Quantification of spheroid number
 829 (middle) and diameter (right) after stimulation with conditioned media from WT and *Hif1a*-KO
 830 macrophages (*n* = 2 per group). Data in **A** and **B** are represented as mean + SEM. **p*<0.05
 831 by unpaired two-sided Student's *t* test. **C** right shows mean ± SD.

832 **Figure 5. *Hif1a* in myeloid cells is essential for activation and pro-tumorigenic gene**
 833 **expression of intestinal fibroblasts. (A)** Immunohistochemical analysis of myofibroblast
 834 markers α SMA and FSP-1 in intestinal sections from AOM/DSS-treated (above) and APC^{min}
 835 (below) WT and Hif1a^{MC} mice. **(B)** Relative mRNA expression of pro-fibrotic genes in tumor-
 836 associated macrophages isolated from APC^{min} adenomas from WT ($n = 2$ biological
 837 replicates with technical duplicates) and Hif1a^{MC} ($n = 4$ biological replicates with technical
 838 duplicates) mice. **(C)** Determination of bioactive TGF- β in the supernatant of alternatively
 839 activated WT and *Hif1a*-KO macrophages ($n = 3$ per group). **(D)** Relative mRNA expression
 840 of pro-tumorigenic genes in primary murine intestinal fibroblasts stimulated with conditioned
 841 media from WT and *Hif1a*-KO macrophages ($n = 3$ biological replicates with technical
 842 duplicates). Data are represented as mean + SEM. * $p < 0.05$, ** $p < 0.01$ by unpaired two-sided
 843 Student's t test.

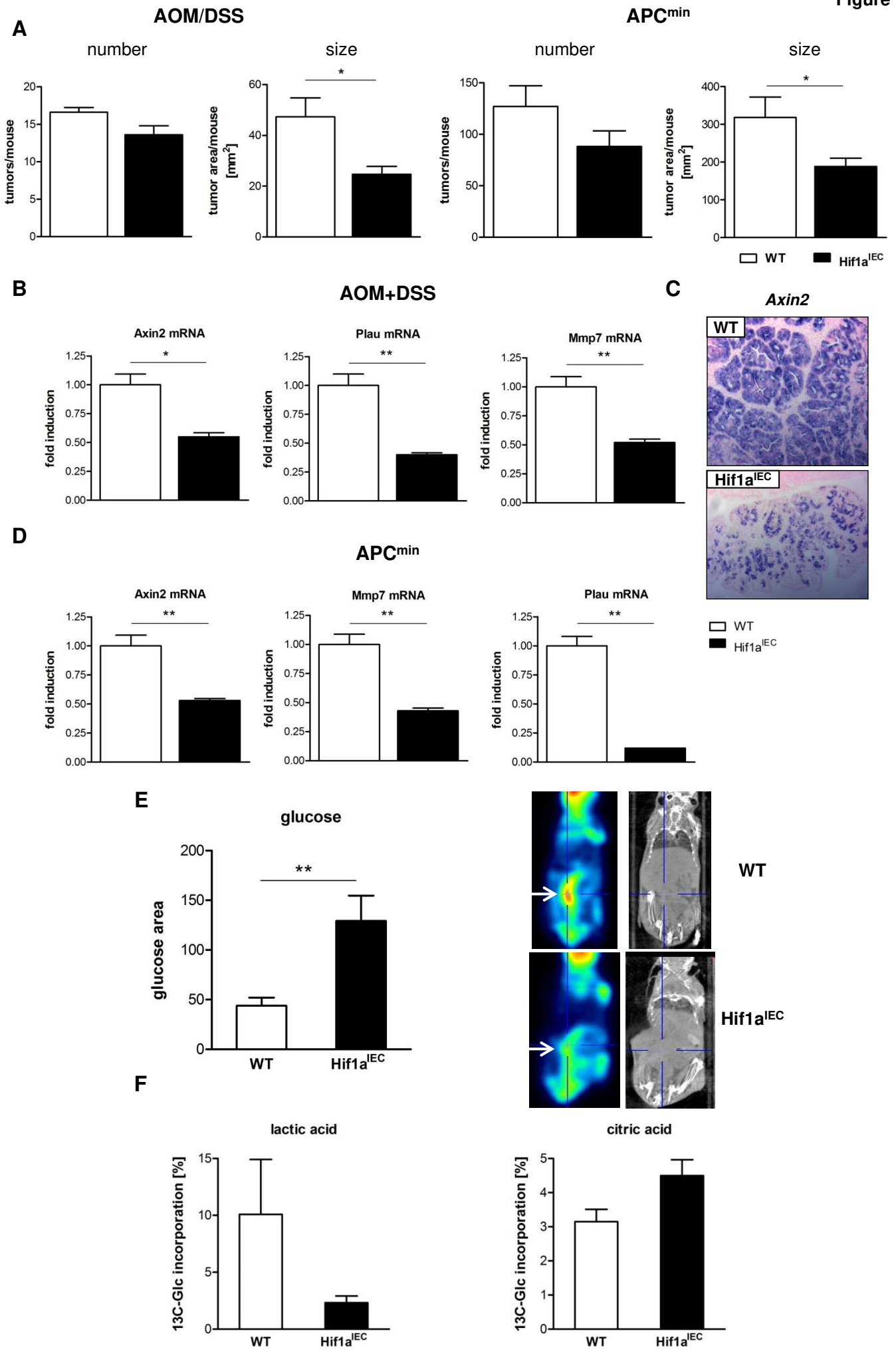
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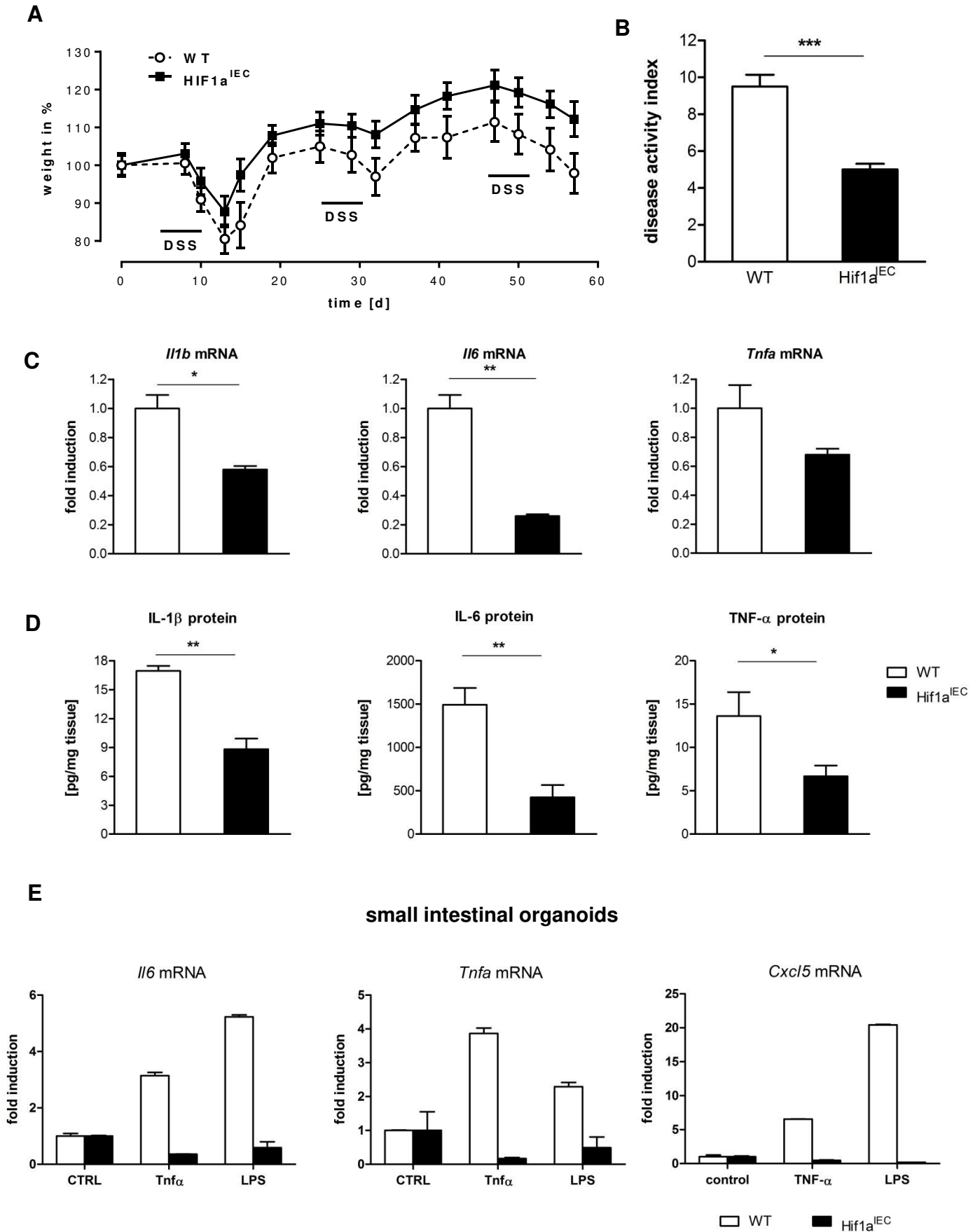
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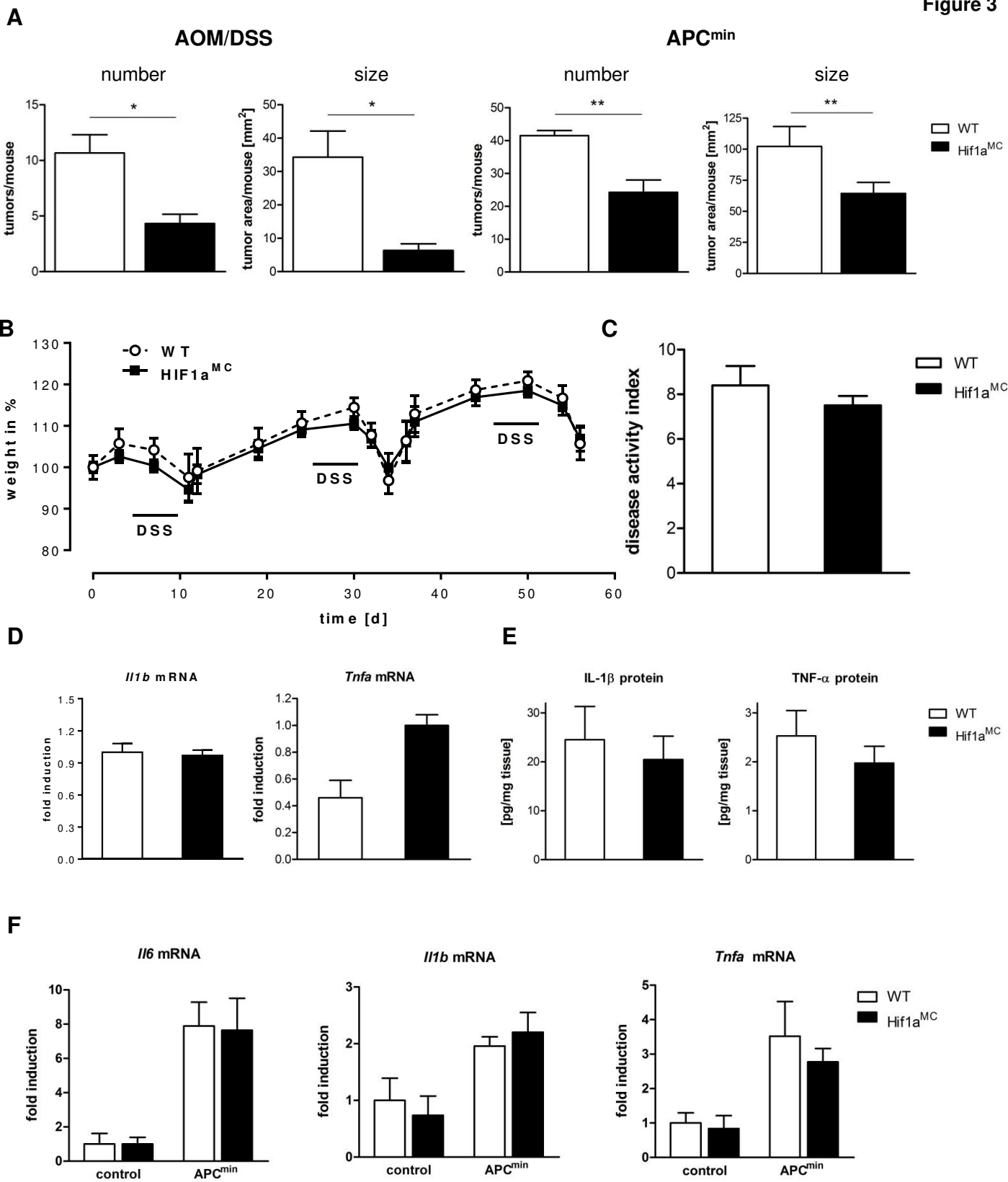
846 **Figure 6. Myeloid cell-mediated activation of TAF precursor cells depends on *Hif1a*.**
 847 **(A)** Representative images and quantification of Gli1⁺ MSC isolated from bone-chips of
 848 bigenic Gli1CreER¹²;tdTomato mice and cultured with supernatants of CAM or AAM from
 849 either wildtype or Hif1a^{MC} mice and stained for alpha smooth muscle actin (α SMA) indicating
 850 myofibroblast differentiation ($n = 3$ per group). **(B)** Mesenchymal stem cells were cultured in
 851 collagen gels for 14 days either in stem cell expansion medium (SCEM, control) or in a
 852 mixture of SCEM and supernatants of M0, CAM or AAM ($n = 3$ per group). Collagen gel
 853 areas were measured using the ImageJ software. **(C)** Representative images of fibrocytes
 854 differentiated in vitro from splenic monocytes from WT and Hif1a^{MC} mice. Magnification
 855 25x (upper panel) and 100x (lower panel). **(D)** Relative mRNA expression of selected pro-
 856 tumorigenic factors in differentiated fibrocytes from WT and Hif1a^{MC} mice ($n = 3$
 857 biological replicates with technical duplicates). Data are represented as mean + SEM.
 858 * $p < 0.05$ by unpaired two-sided Student's t test.

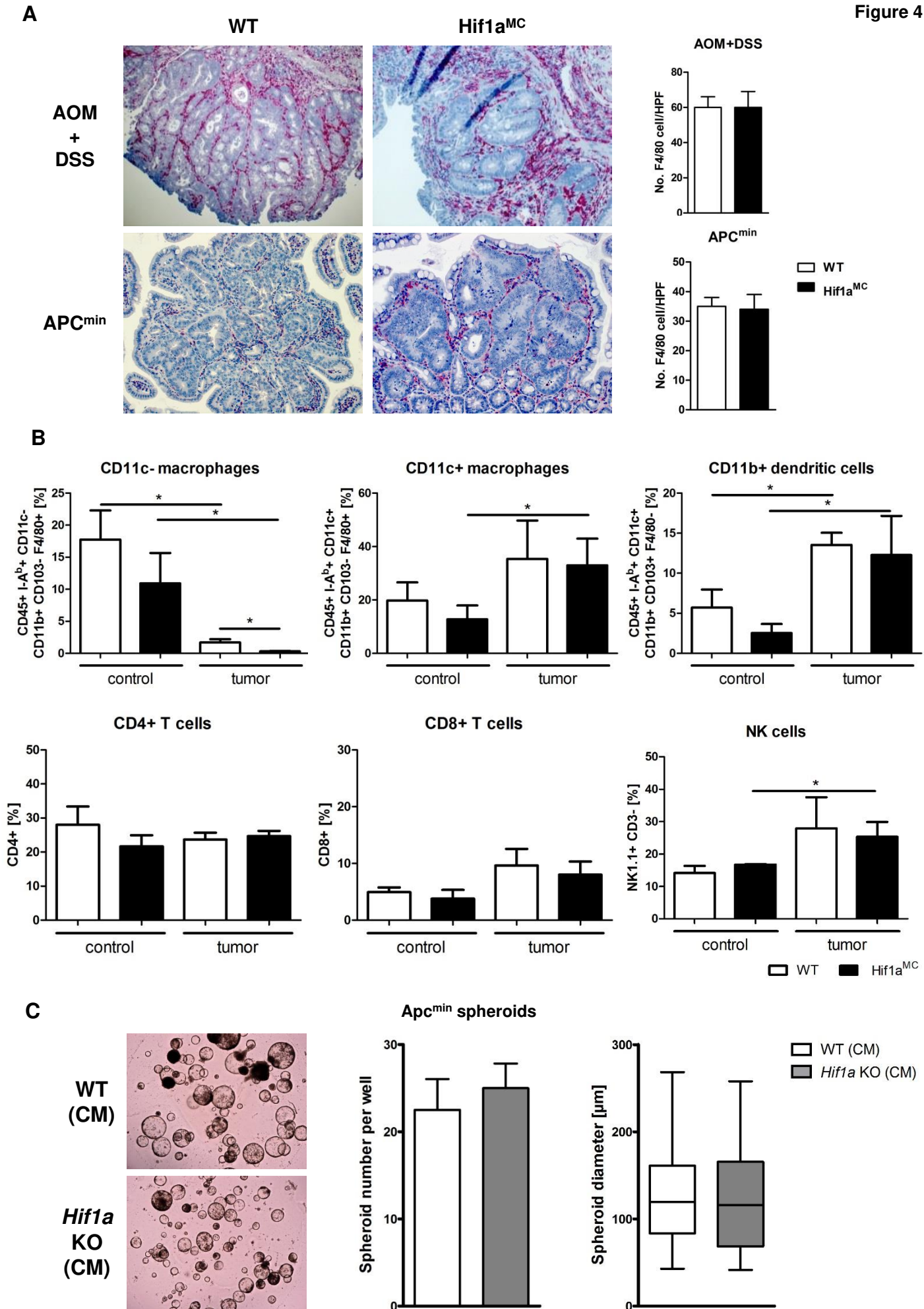
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860 **Figure 7. Non-canonical stabilization of HIF-1 α in murine intestine. (A)**
861 Immunofluorescent analysis of hypoxypromoter (red) and HIF-1 α (green) in AOM/DSS-induced
862 (left) and APC^{min} (right) adenomas. **(B)** Analysis of relative number of cells in adenomas
863 expressing either hypoxypromoter (HP) or HIF-1 α . **(C-G)** Immunohistochemical staining of HIF-
864 1 α in intestinal sections from mice with inducible expression of firefly luciferase **(C, FLUC)**,
865 PIK3CA^{H1047R} **(E)**, KRAS^{G12V} **(F)**, stabilized β -catenin **(G)** or inducible loss of APC **(D)**. Data in
866 B show mean + SD, 4 adenomas from 2 mice per model were evaluated. **p<0.001 by
867 unpaired two-sided Student's t test.

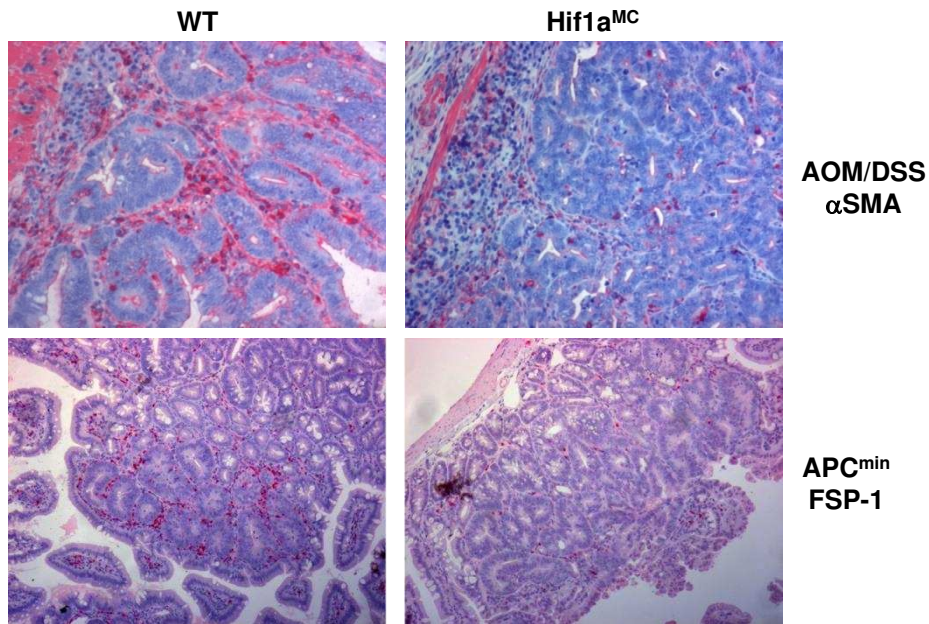




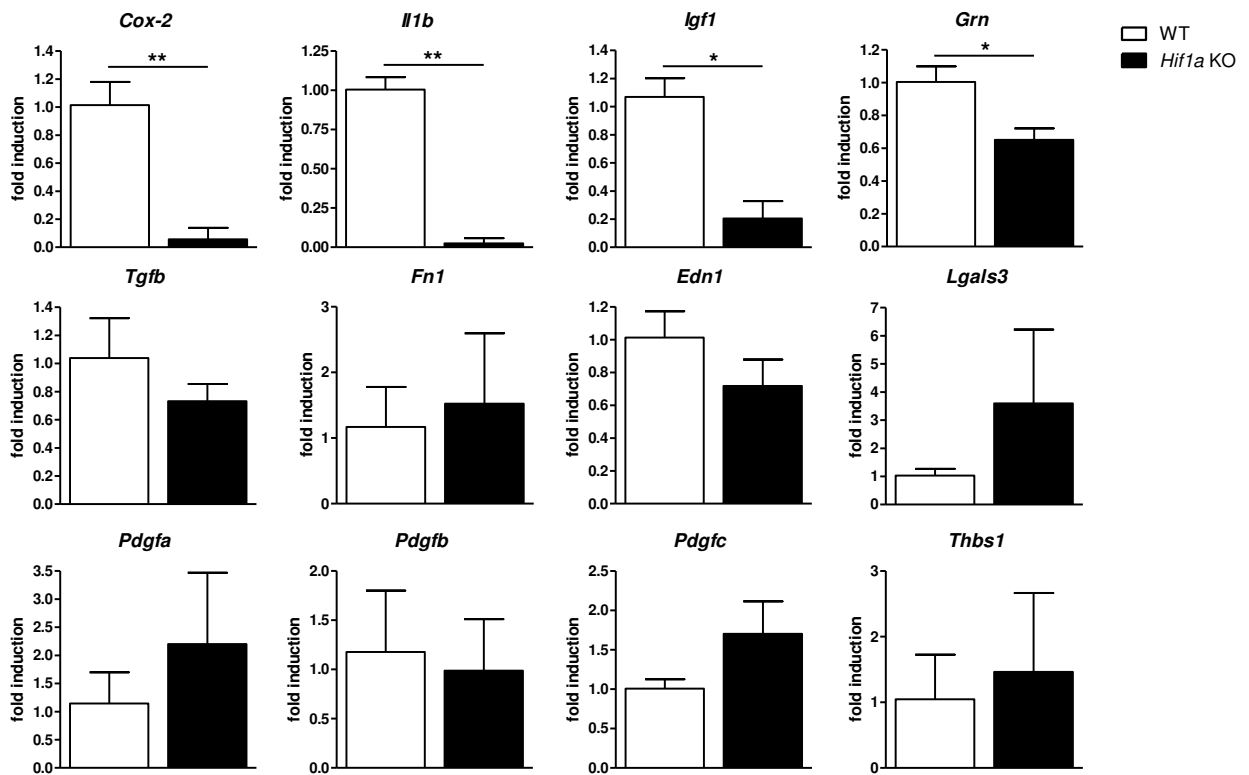




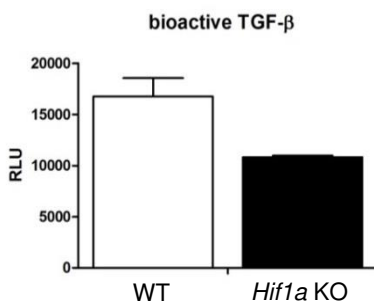
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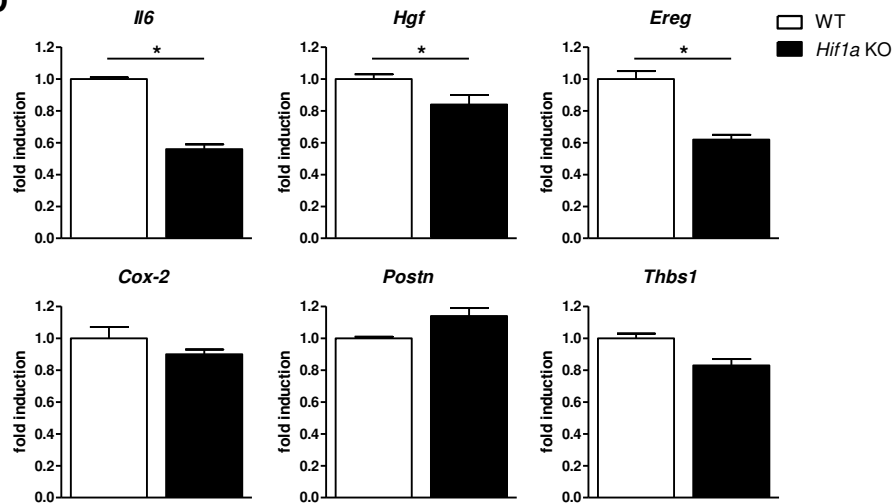
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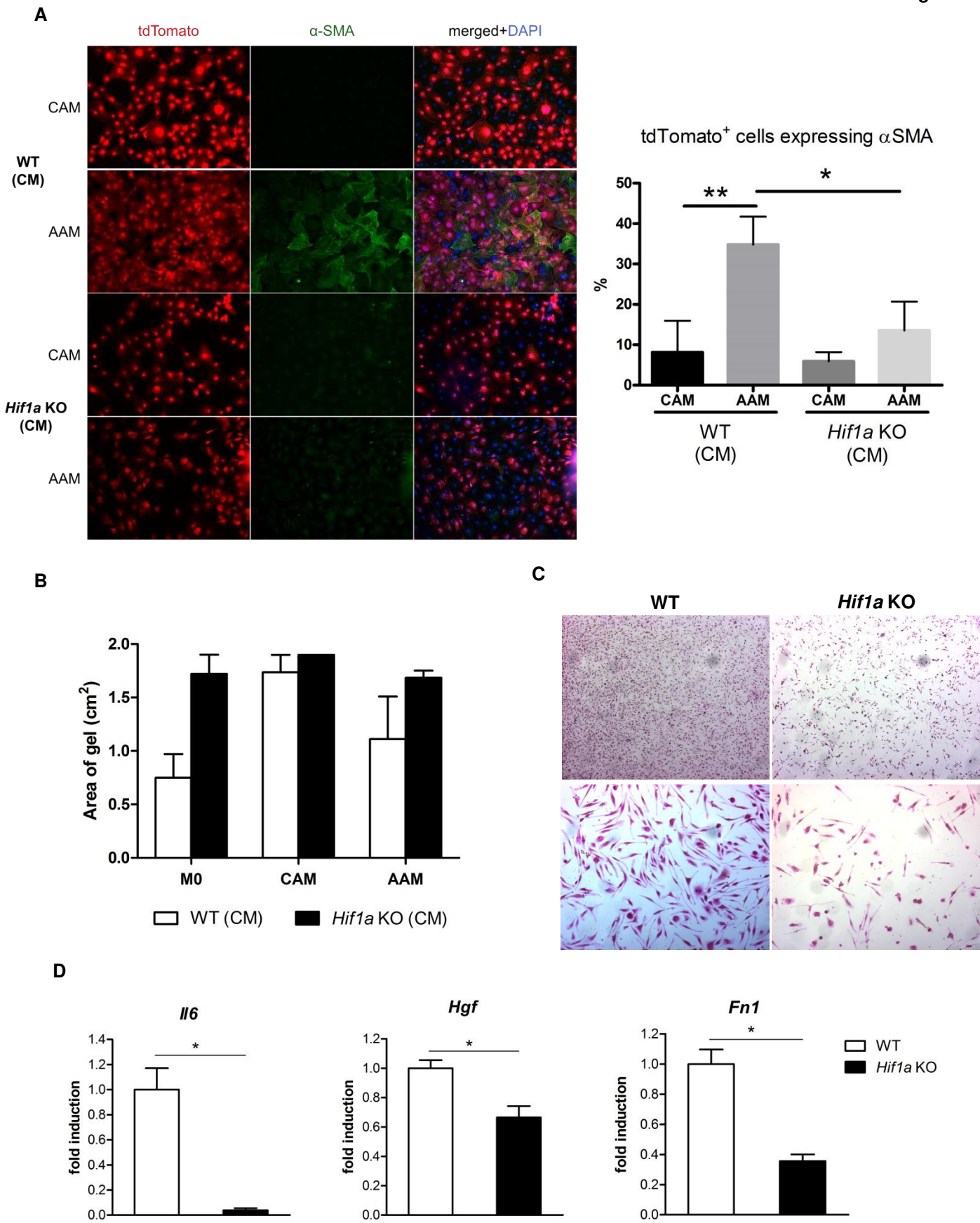


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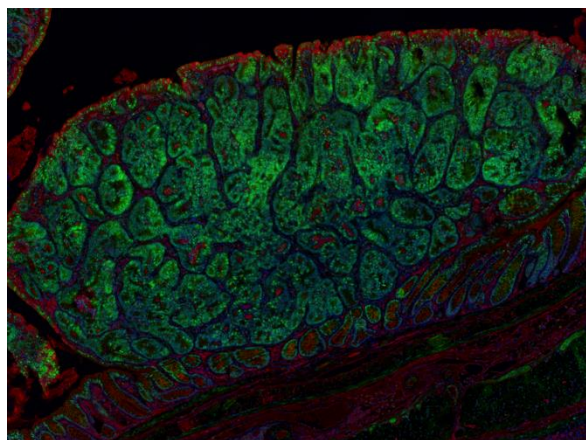
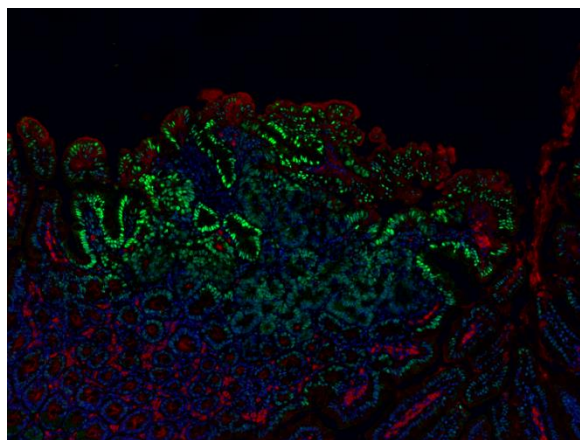


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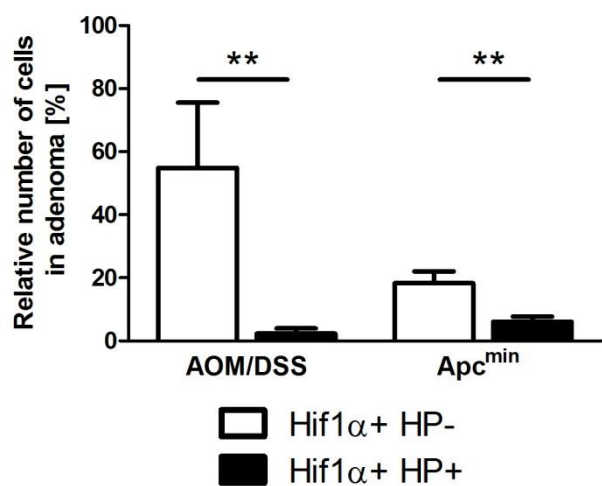




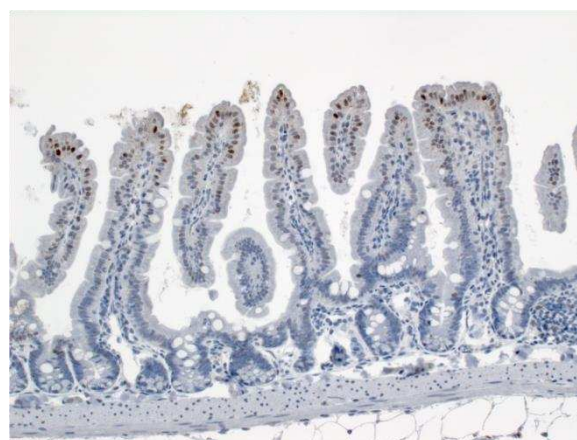
A

hypoxyprobe / HIF-1 α AOM
+
DSSAPC^{min}

B

HIF-1 α

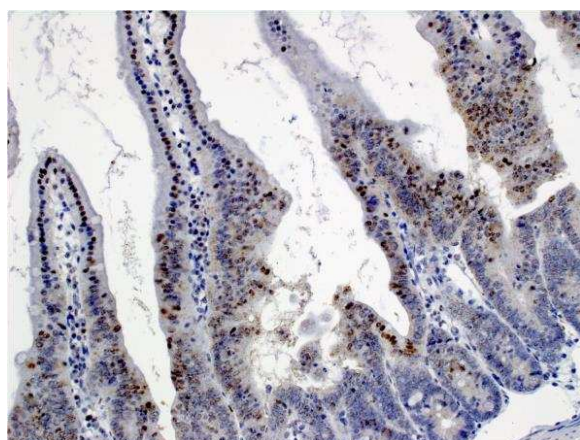
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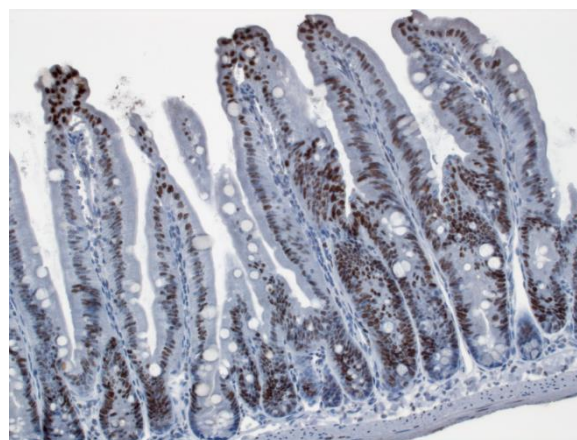
FLUC

D

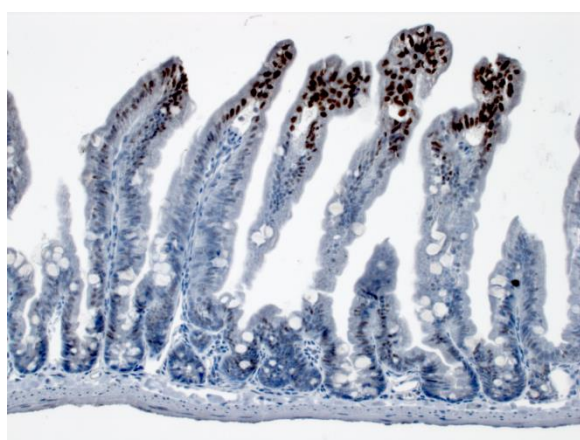
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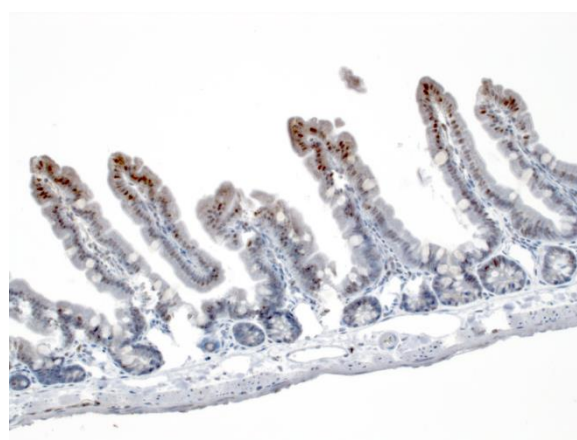
E

PIK3CA
(H1047R)

F

KRAS
(G12V)

G

CTNNB1
(stab)