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Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis

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Abstract

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

Introduction

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

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

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

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

Results

Epithelial *Hif1a* controls intestinal tumor growth on multiple levels

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

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

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

Hif1a in myeloid cells regulates intestinal tumor formation independent of inflammation

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

Hif1a-deficient tumor-associated macrophages migrate and function normally

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

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

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

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

the stroma of APC^{min} and AOM+DSS-induced adenomas (Figure 5A). Strikingly, deletion of *Hif1a* in myeloid cells resulted in greatly reduced numbers of TAFs in both tumor models (Figure 5A). This result pointed towards a crucial role of *Hif1a* in myeloid cells for TAF development. Interestingly, the importance of macrophages for fibroblast activation in the context of wound healing is well established [42, 43]. To analyze this further, we focussed on alternatively activated macrophages (AAM) given their central role in the control of organ fibrosis [42]. Expression of various pro-fibrotic genes in AAMs was readily detectable, but loss of *Hif1a* remained without greater effect (Figure S5A). To achieve experimental data that more closely resemble the *in vivo* situation, we analyzed pro-fibrotic factor expression in tumor-associated macrophages (TAM) directly isolated from intestinal adenomas. This approach indeed unravelled a central role of *Hif1a* in TAMs for the expression of various fibroblast-activating factors, e.g. COX-2, IGF-1, IL-1β and granulin (Figure 5B). In our analyses, the expression of TGF-β1, the archetypical pro-fibrotic factor, was constantly lower in *Hif1a*-null TAMs, but did not reach statistical significance (Figure 5B). As the activation of TGF-β1 is a complex and highly regulated process [44], we hypothesized that HIF-1α is of importance in this setting. Indeed, HIF-1α-deficient AAMs displayed reduced levels of bioactive TGF-β in the supernatant (Figure 5C), suggesting a functional importance of HIF-1α for TGF-β activity elicited by TAMs. Next, we addressed the role of *Hif1a* for macrophage-mediated fibroblast proliferation [42, 45]. While conditioned medium from AAM enhanced survival of primary murine intestinal fibroblasts (MIFs), no difference between WT and *Hif1a*-deficient macrophages was detectable (Figure S5B). Finally, we sought to address the secretion of pro-tumorigenic cytokines, another tumor-promoting function of TAFs [46]. Stimulation of primary MIFs with conditioned medium from AAM induced gene expression of IL-6, HGF and epiregulin (*Ereg*), factors with established tumor-promoting activity in the intestine [47, 48]. Of note, this effect was significantly reduced upon deletion of *Hif1a* in macrophages (Figure 5D).

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

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

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

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

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

Discussion

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

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

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-1α
318 [27, 29]. Complementing the published work, we were able to show that HIF-1α is essential
319 for DSS-induced gene expression of *Muc1* and *3* in murine intestinal eptithelial cells. In
320 contrast, our results do not support a functional relevance of HIF-1α 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-1α 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-1α 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

relevance of HIF-1 for the control of gene expression of pro-fibrotic factors (e.g. TGF-β1, endothelin-1, fibronectin-1 and COX-2 [71-74]) in different cell types, *Hif1a* deletion in AAMs remained without effect on mRNA expression of a comprehensive set of pro-fibrotic factors in our experimental setup. To address the importance of the tissue context, we isolated tumor-associated macrophages (TAMs) from intestinal adenomas. Of note, in these cells the expression of various pivotal pro-fibrotic genes was indeed regulated by *Hif1a*. These results not only underscore the significance of *Hif1a* for TAM-mediated activation of TAFs, but again illustrate the importance of experimental conditions that more closely resemble the tissue context.

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

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

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

Materials and methods

Animals and Mouse models

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).

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

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

Flow cytometric analyses of murine leukocyte populations

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

Statistical analysis

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

independent experiments and expressed as means + SEM. Before statistical analysis, data were checked for normal distribution (Shapirow Wilk test) and comparable variance (F-test for equality of variances for data with normal distribution). Comparisons between two groups of normally distributed data with equal variances were performed using the unpaired two-sided Student`s *t* test. Differences were considered statistically significant at $p<0.05$. Sample size, statistical tests and *p* values are indicated in the figure legends. The asterisks in the graphs indicate statistically significant changes with *p* values: * $p < 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Statistical analysis was done using Prism 4.0 software (GraphPad Software, San Diego, California, USA).

Author Contributions

Conceptualization, N.R., M.M. and T.C.; Methodology, N.R., M.E., S.J., A.E., K.T.W., A.A.K., 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, Investigation, and Visualization, N.R., M.E., S.J., A.E., A.A.K., R.K., S.N., I.R., M.G., C.Z., S.K., M.B.M., M.M. and T.C.; Writing, N.R., F.T., M.M. and T.C.; Supervision, Resources, 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.; Project Administration, T.C.

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775 **Figure Legends**

776 **Figure 1. HIF-1α 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.

790
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

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

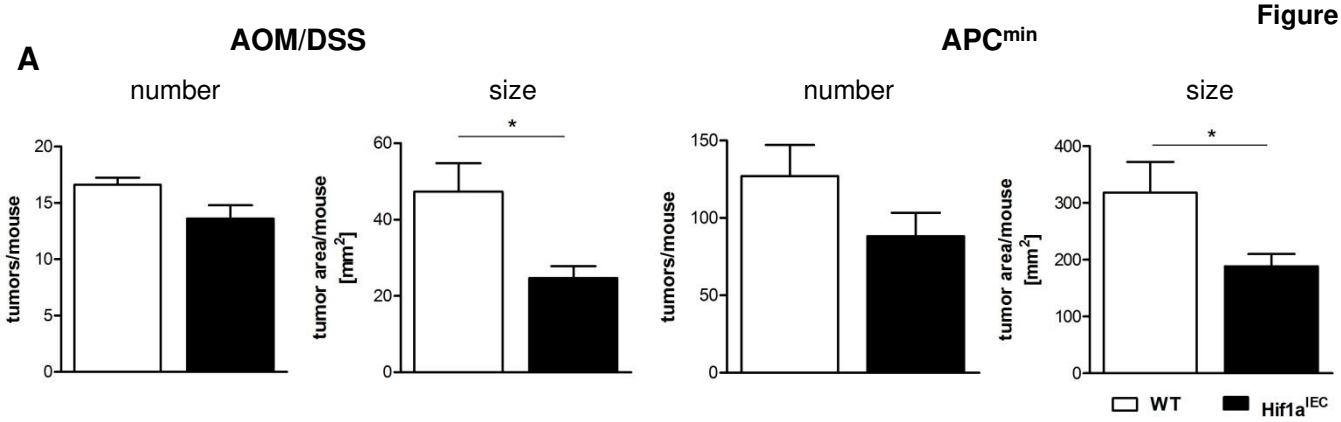
Figure 4. Tumoral abundance of macrophages and stimulation of adenoma growth *ex vivo* is not affected by loss of *Hif1a*. (**A**) Representative F4/80 stainings of intestinal sections obtained from AOM/DSS-treated (above) and APC^{min} (below) WT and Hif1a^{MC} mice. Right, quantification of F4/80-positive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

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

Figure 6. Myeloid cell-mediated activation of TAF precursor cells depends on *Hif1a*. (**A**) Representative images and quantification of $Gli1^{+}$ MSC isolated from bone-chips of bigenic $Gli1CreER^{t2}$;tdTomato mice and cultured with supernatants of CAM or AAM from either wildtype or $Hif1a^{MC}$ mice and stained for alpha smooth muscle actin (αSMA) indicating myofibroblast differentiation (*n* = 3 per group). (**B**) Mesenchymal stem cells were cultured in collagen gels for 14 days either in stem cell expansion medium (SCEM, control) or in a mixture of SCEM and supernatants of M0, CAM or AAM (*n* = 3 per group). Collagen gel areas were measured using the ImageJ software. (**C**) Representative images of fibrocytes differentiated in vitro from splenic monocytes from WT and and $Hif1a^{MC}$ mice. Magnification 25x (upper panel) and 100x (lower panel). (**D**) Relative mRNA expression of selected pro-tumorigenic factors in differentiated fibrocytes from WT and and $Hif1a^{MC}$ mice (*n* = 3 biological replicates with technical duplicates). Data are represented as mean + SEM. *p<0.05 by unpaired two-sided Student's t test.

860 **Figure 7. Non-canonical stabilization of HIF-1α in murine intestine.** (**A**)
861 Immunofluorescent analysis of hypoxyprobe (red) and HIF-1α (green) in AOM/DSS-induced
862 (left) and APC^{min} (right) adenomas. (**B**) Analysis of relative number of cells in adenomas
863 expressing either hypoxyprobe (HP) or HIF-1α. (**C**-**G**) Immunohistochemical staining of HIF-
864 1α 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.

Figure 1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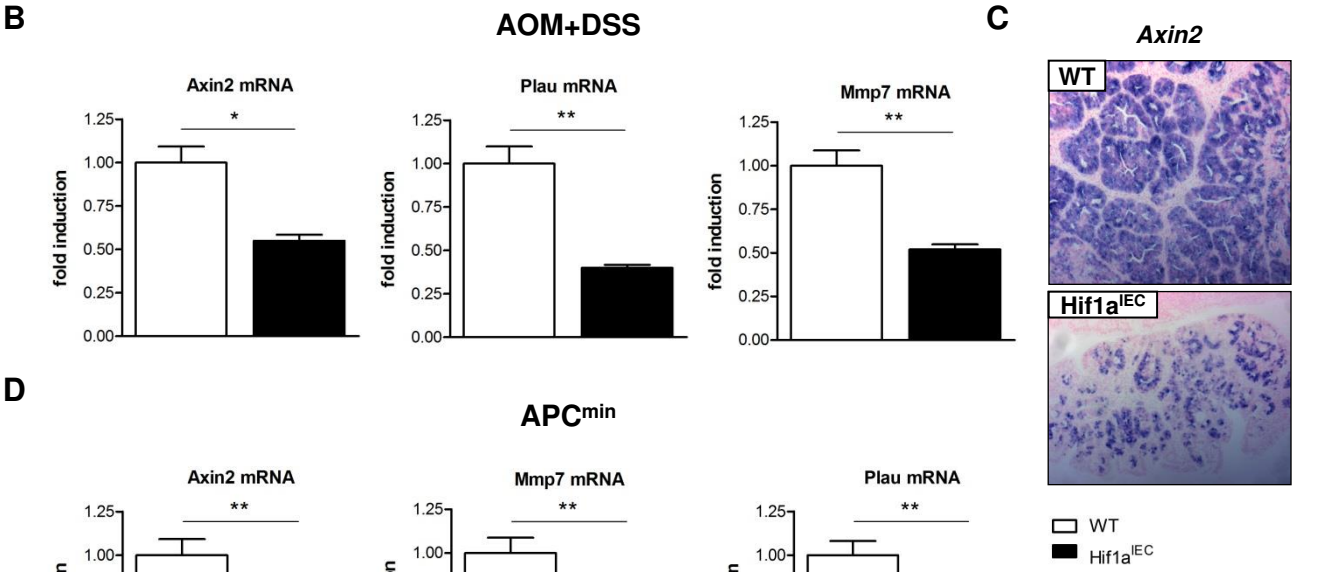
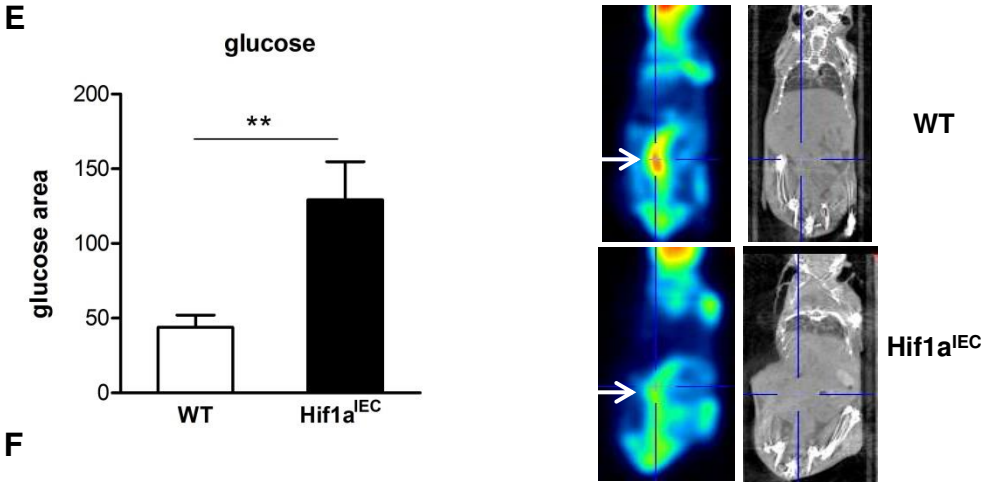
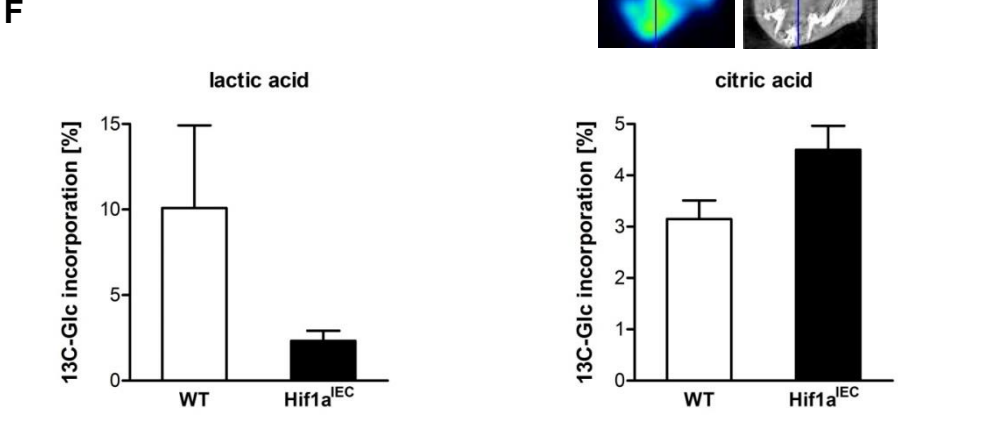
tumors/mouse
tumor area/mouse [mm2]
WT
Hif1aIEC


B
AOM+DSS
Axin2 mRNA
Plau mRNA
Mmp7 mRNA
fold induction
C
Axin2
WT
Hif1aIEC
WT
Hif1aIEC

D
APCmin
Axin2 mRNA
Mmp7 mRNA
Plau mRNA
fold induction
WT
Hif1aIEC


E
glucose
glucose area
WT
Hif1aIEC
WT
Hif1aIEC


F
lactic acid
citric acid
13C-Glc incorporation [%]
13C-Glc incorporation [%]
WT
Hif1aIEC
WT
Hif1aIEC

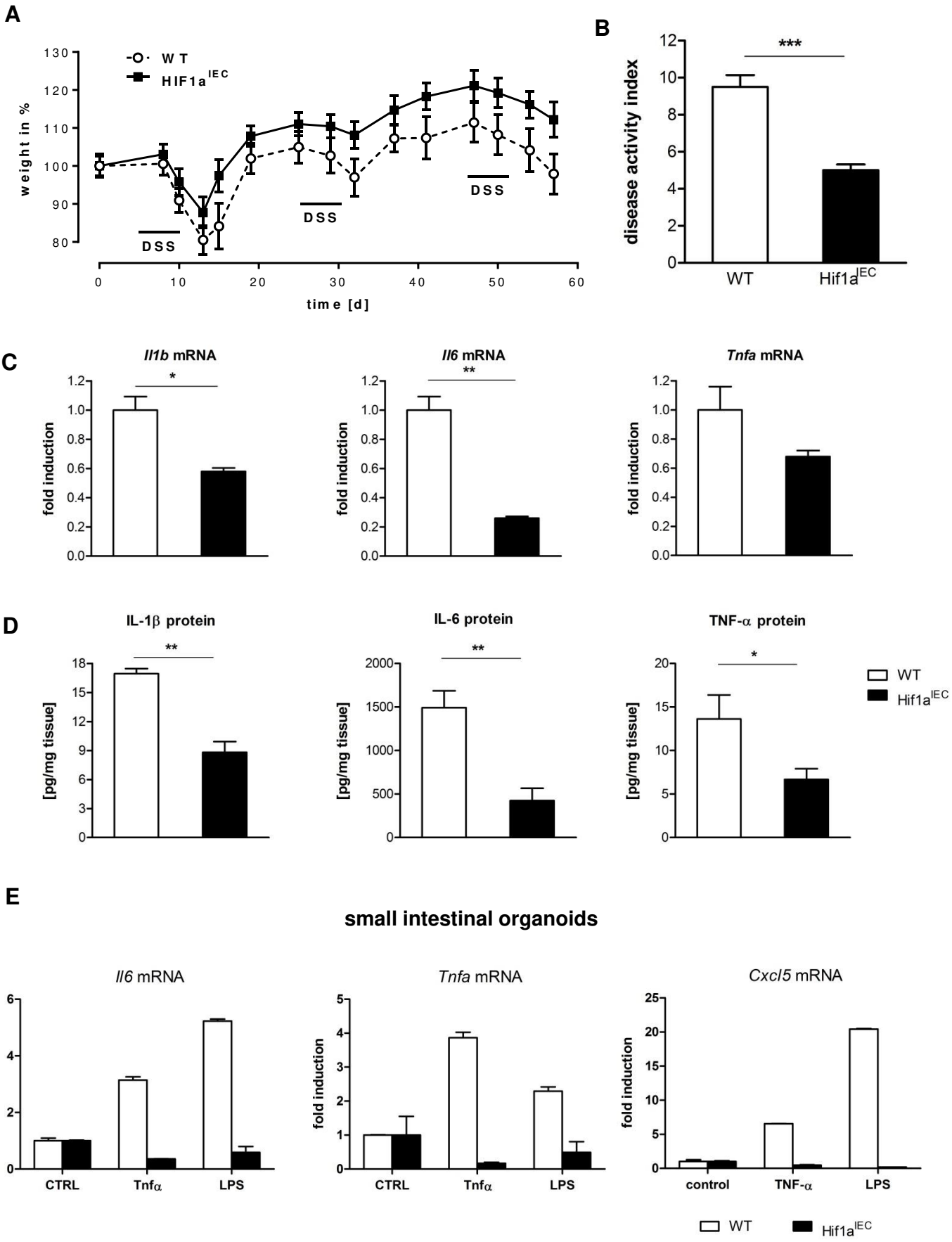

A
WT
HIF1a IEC
weight in %
time [d]
DSS
DSS
DSS
B
disease activity index
***
WT
Hif1a IEC
C
Il1b mRNA
Il6 mRNA
Tnfa mRNA
fold induction
*
**
D
IL-1β protein
IL-6 protein
TNF-α protein
[pg/mg tissue]
WT
Hif1a IEC
E
small intestinal organoids
Il6 mRNA
Tnfa mRNA
Cxcl5 mRNA
fold induction
CTRL
Tnfα
LPS
control
TNF-α
WT
Hif1a IEC

Figure 3

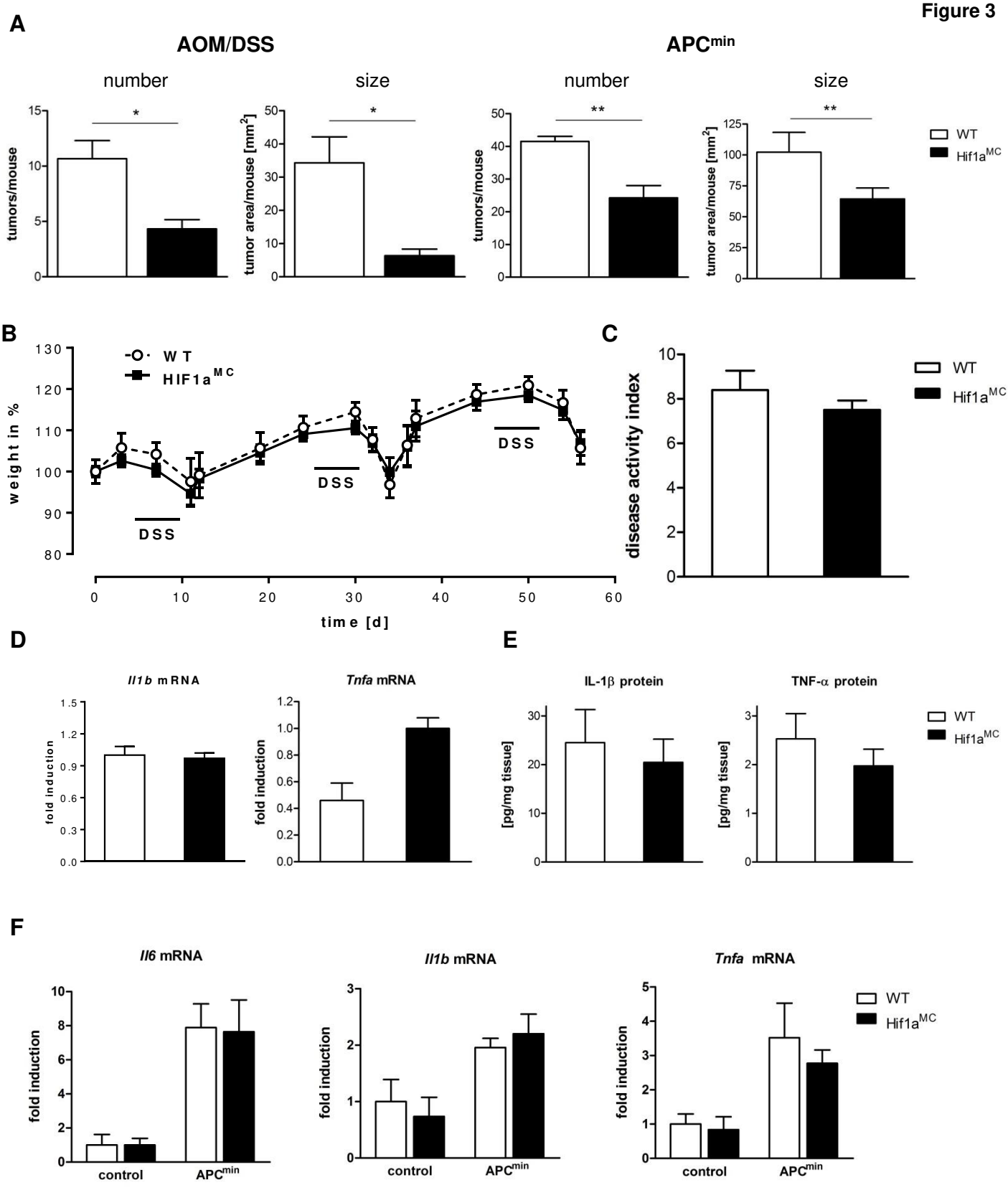
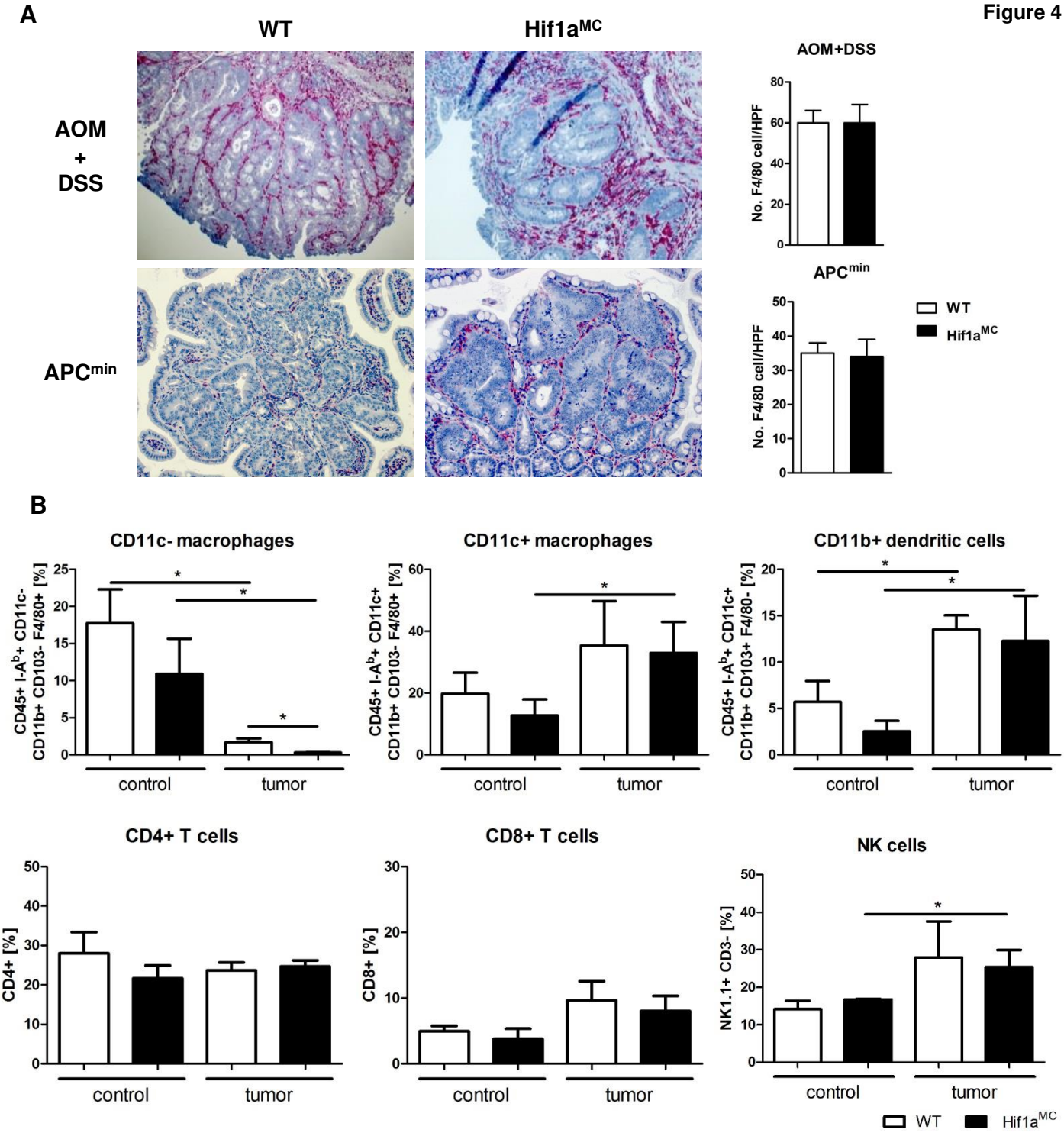
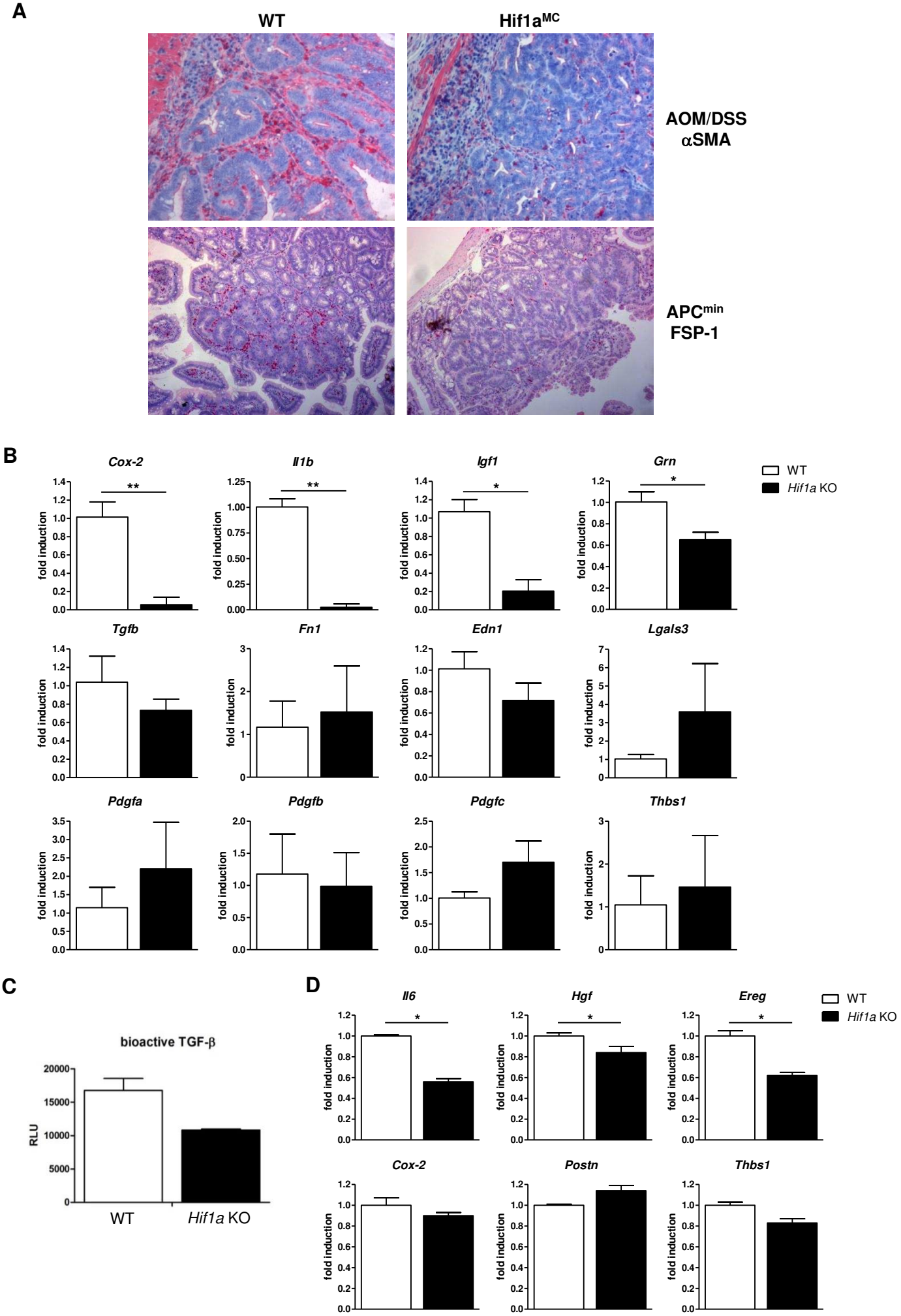
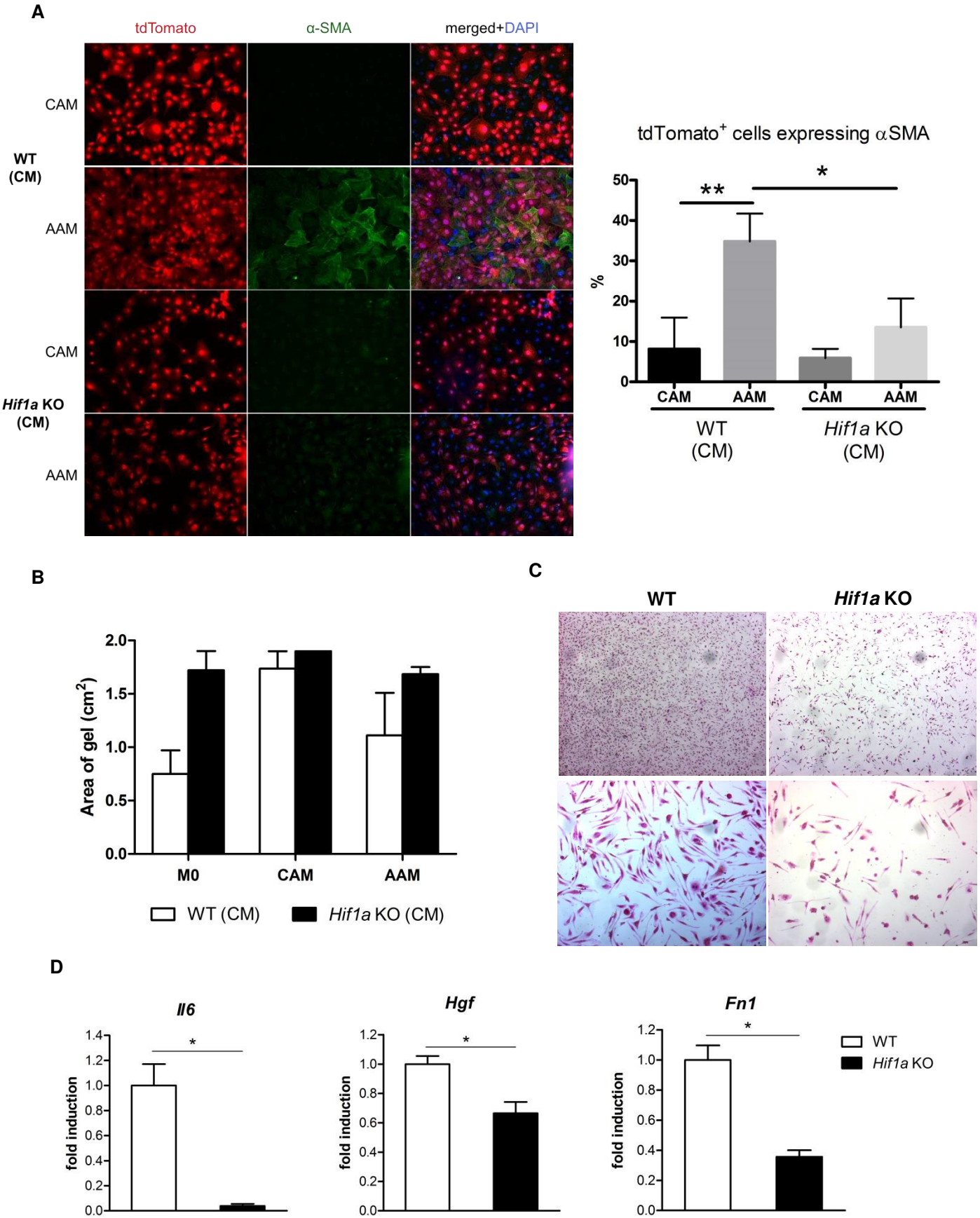
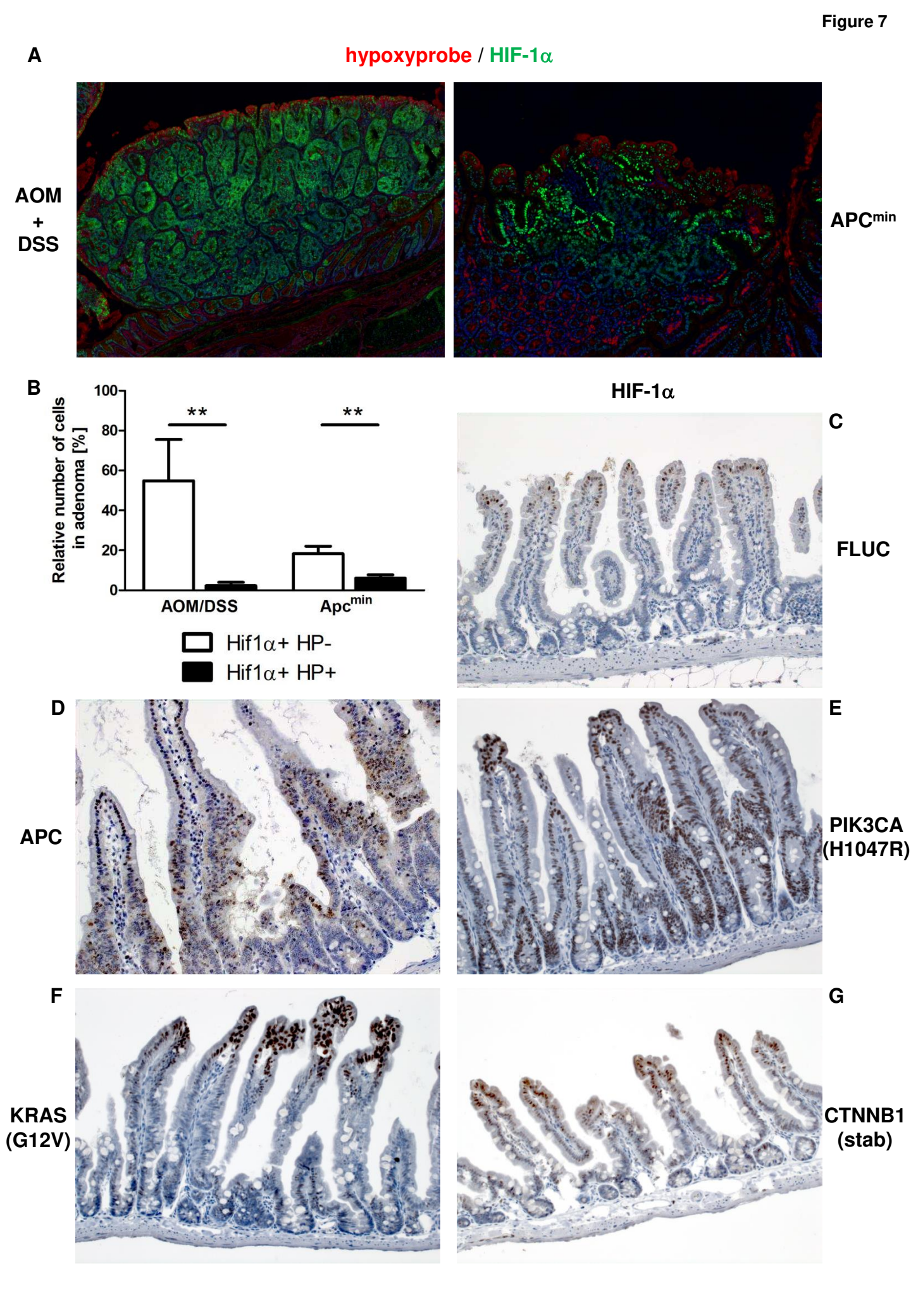

A
AOM/DSS
APCmin
number
size
number
size
tumors/mouse
tumor area/mouse [mm2]
tumors/mouse
tumor area/mouse [mm2]
*
*
**
**
WT
Hif1aMC
B
weight in %
WT
HIF1aMC
DSS
DSS
DSS
time [d]
C
disease activity index
WT
Hif1aMC
D
Il1b mRNA
Tnfa mRNA
fold induction
fold induction
E
IL-1β protein
TNF-α protein
[pg/mg tissue]
[pg/mg tissue]
WT
Hif1aMC
F
Il6 mRNA
Il1b mRNA
Tnfa mRNA
fold induction
fold induction
fold induction
control
APCmin
control
APCmin
control
APCmin
WT
Hif1aMC

Figure 4


A
WT
Hif1a^MC
AOM + DSS
APC^min
AOM+DSS
No. F4/80 cell/HPF
APC^min
No. F4/80 cell/HPF
WT
Hif1a^MC
B
CD11c- macrophages
CD45+ I-A^b+ CD11c- CD11b+ CD103- F4/80+ [%]
control
tumor
CD11c+ macrophages
CD45+ I-A^b+ CD11c+ CD11b+ CD103- F4/80+ [%]
control
tumor
CD11b+ dendritic cells
CD45+ I-A^b+ CD11c+ CD11b+ CD103+ F4/80- [%]
control
tumor
CD4+ T cells
CD4+ [%]
control
tumor
CD8+ T cells
CD8+ [%]
control
tumor
NK cells
NK1.1+ CD3- [%]
control
tumor
WT
Hif1a^MC
C
WT (CM)
Hif1a KO (CM)
Apc^min spheroids
Spheroid number per well
Spheroid diameter [µm]
WT (CM)
Hif1a KO (CM)


A
WT
Hif1a^MC
AOM/DSS
αSMA
APC^min
FSP-1
B
Cox-2
Il1b
Igf1
Grn
WT
Hif1a KO
fold induction
Tgfb
Fn1
Edn1
Lgals3
Pdgfa
Pdgfb
Pdgfc
Thbs1
C
bioactive TGF-β
RLU
WT
Hif1a KO
D
Il6
Hgf
Ereg
WT
Hif1a KO
fold induction
Cox-2
Postn
Thbs1


A
tdTomato
α-SMA
merged+DAPI
CAM
WT (CM)
AAM
CAM
Hif1a KO (CM)
AAM
tdTomato+ cells expressing αSMA
%
50
40
30
20
10
0
**
*
CAM
AAM
CAM
AAM
WT (CM)
Hif1a KO (CM)
B
Area of gel (cm2)
2.0
1.5
1.0
0.5
0.0
M0
CAM
AAM
WT (CM)
Hif1a KO (CM)
C
WT
Hif1a KO
D
Il6
Hgf
Fn1
fold induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
Hif1a KO


A
hypoxyprobe / HIF-1α
AOM + DSS
APCmin
B
Relative number of cells in adenoma [%]
100
80
60
40
20
0
**
**
AOM/DSS
Apcmin
Hif1α+ HP-
Hif1α+ HP+
HIF-1α
C
FLUC
D
APC
E
PIK3CA (H1047R)
F
KRAS (G12V)
G
CTNNB1 (stab)