Hypoxia-Inducible Factor-2α Activation Promotes Colorectal Cancer Progression by Dysregulating Iron Homeostasis

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Abstract

Hypoxia-inducible factor (HIF), a key modulator of the transcriptional response to hypoxia, is increased in colon cancer. However, the role of HIF in colon carcinogenesis in vivo remains unclear. In this study, we found that intestinal epithelium-specific disruption of the von Hippel–Lindau tumor suppressor protein (VHL) resulted in constitutive HIF signaling, and increased HIF expression augmented colon tumorigenesis in the Apcmin/+ intestinal tumor model. Intestine-specific disruption of Vhl increased colon tumor multiplicity and progression from adenomas to carcinomas. These effects were ameliorated in mice with double disruption of Vhl and HIF-2α. Activation of HIF signaling resulted in increased cell survival in normal colon tissue; however, tumor apoptosis was not affected. Interestingly, a robust activation of cyclin D1 was observed in tumors of Apcmin/+ mice in which HIF-2α was activated in the intestine. Consistent with this result, bromodeoxyuridine incorporation indicated that cellular proliferation was increased in colon tumors following HIF activation. Further analysis showed that dysregulation of the intestinal iron absorption transporter divalent metal transporter-1 (DMT-1) was a critical event in HIF-2α–mediated colon carcinogenesis. These data provide a mechanistic basis for the widely reported link between iron accumulation and colon cancer risk. Together, our findings show that a chronic increase in HIF-2α in the colon initiates protumorigenic signaling, which may have important implications in developing preventive and therapeutic strategies for colon cancer. Cancer Res; 72(9): 2285–93. ©2012 AACR.

Introduction

Hypoxic microenvironment is a hallmark for solid tumors (1). In response to hypoxia, tumor cells activate genes that are critical in angiogenesis, cell survival, cell proliferation, and glucose metabolism (2). Hypoxia-induced signal transduction is transcriptionally mediated by hypoxia-inducible factor (HIF), a member of the Per-ARNT-Sim family of basic helix-loop-helix transcription factors that bind hypoxia response elements at target gene loci under hypoxic conditions (3–5). Functional HIF is a heterodimer that comprises a constitutive subunit, aryl hydrocarbon nuclear translocator (Arnt, also known as HIF-1β), and a hypoxia-inducible α-subunit (3, 4). Stabilization of the α-subunit is regulated by a family of oxygen and iron-dependent prolyl hydroxylase (PHD) enzymes. PHD enzymes hydroxylate the α-subunit enabling the binding of the von Hippel–Lindau tumor suppressor protein (VHL) coupled to the E3 ubiquitin ligase complex, which leads to proteasomal degradation of HIF-α subunits (6, 7).

Two highly homologous and transcriptionally active subunits have been identified, HIF-1α and HIF-2α (8, 9). Both are expressed in many of the same cells and regulate overlapping and distinct sets of genes critical in the adaptation to hypoxic environments, including cancer development (10). In cancer-derived cell lines and in renal carcinomas, HIF-1α and HIF-2α have opposing roles in cell proliferation. HIF-1α decreases cell proliferation, whereas HIF-2α induces proliferation via an increase in c-Myc activity (11–16). HIF-1α and HIF-2α are overexpressed in a variety of tumor tissues including colon cancer (17, 18). However, the role of HIF-α in colon carcinogenesis is not completely clear. This study shows that Apcmin/+ mice with an intestine-specific activation of HIF signaling via disruption of Vhl using a villin-cre recombinase developed mainly colorectal tumors, with carcinomas seen in 8 of 10 (80%) mice followed for 6 months. Disruption of both Vhl and Hif2α in intestinal epithelial cells prevented colon tumors indicating an HIF-2α–dependent mechanism. Through global gene expression analysis in the Apcmin/+ mice and human colorectal tumor samples, an HIF-2α–dependent dysregulation of colon iron homeostasis was observed. Increase in local iron levels have been implicated in the progression of colon carcinogenesis (19–25), and these data show the increase in tumor iron exacerbated cell proliferation and was critical in colon tumor formation following HIF-2α activation. Together, these data reveal a novel role for...
HIF-2α in initiating a coordinated process that is critical in the progression of colon cancer.

Materials and Methods

Animals and diets

The floxed or compound floxed mice hemizygous for villin were mated to each other to generate Vhlfl/F, VhlΔIE, Vhlfl/F; Hif-2αfl/F, Vhlfl/ΔIE/Hif-2αΔIE mice and were described previously (26–28). Apeminc/+ mice were purchased from The Jackson Laboratory. To investigate the role of HIF-2α in colorectal cancer, VhlΔIE and Vhlfl/ΔIE/Hif-2αΔIE mice were crossed with Apeminc/+ mice to generate Vhlfl/F/Apcminc−/−, VhlΔIE/Apcminc−/−, Vhlfl/ΔIE/Hif-2αΔIE/Apcminc−/−, and Vhlfl/F/Hif-2αΔIE/Apcminc−/− mice. All mice were 129S6/SvEv background and maintained in standard cages in a light- and temperature-controlled room and were allowed standard chow and water ad libitum. For low iron study, the 6-week-old Vhlfl/F/Apcminc−/− and Vhlfl/F/Hif-2αΔIE/Apcminc−/− mice were given iron-replete AIN93G diet containing 350 ppm of iron or iron-deficient AIN93G diet (>5 ppm of iron) for 8 weeks (Dyets). All animal studies were carried out in accordance with Institute of Laboratory Animal Resources Guidelines and approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan (approval number: 10299).

Histology, immunohistochemistry, and immunofluorescence

For bromodeoxyuridine (BrdUrd) staining, animals were sacrificed following a 2-hour treatment of 100 mg/kg of BrdUrd (Sigma). Paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene and rehydrated in ethanol gradient. Immunohistochemical analysis was conducted with antibodies for BrdUrd (B290a; eBioscience Inc.) followed by detection with Alexa Fluor 488 goat anti-mouse immunoglobulin G (Molecular Probes Inc.). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Roche Diagnostics) was conducted according to the supplier’s instructions. Briefly, deparaffinized sections were labeled with Tdt and biotinylated dUTP and then were examined under a fluorescence microscope (29). Tissue iron detection was carried out in paraffin-embedded sections stained with Perls Prussian blue and enhanced with 3,3’-diaminobenzidine and H2O2 (30). Histologic analysis was done on hematoxylin and eosin (H&E)-stained paraffin sections and microscopically analyzed by a gastrointestinal pathologist.

Quantitative real-time reverse transcriptase PCR

RNA was isolated from fresh or frozen tissue with Isol-RNA lysis reagent (3 Prime). After quantification with NanoDrop 2000 (NanoDrop products), RNA with a purity of approximately 2.0 (260/280 ratio) was reverse transcribed using M-MLV Reverse Transcriptase (Fisher Scientific). cDNA was quantified by SYBR green dye and run on a 7900HT Fast Real Time RT-PCR system (Life Technologies; primers listed in Supplementary Table S1). Ct values were normalized to β-actin and expressed as fold difference from controls.

Western blot analysis

Tissues were homogenized and lysed in radioimmunoprecipitation assay buffer (RIPA: 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP-40, 0.1% SDS) for whole-cell extracts. Proteins were separated and transferred to nitrocellulose membranes using standard methods. Membranes were incubated with antibodies against cleaved keratin 18 (K18; kindly provided by Prof. Bishr Omary, University of Michigan, Ann Arbor, MI), total caspase 3, cleaved caspase 3 (Cell Signaling Technology Inc.), HIF-2α (Novus Biologicals), divalent metal transporter-1 (DMT-1; Alpha Diagnostic International) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Inc.). Blots were detected by the Enhanced Chemiluminescence Detection kit (Thermo Scientific).

Primary colon organoid and other cell culture

For organoid whole-colon cultures, colon cells were opened longitudinally and were rinsed gently and quickly placed in culture medium, RPMI-1640 plus 10% heat inactivated FBS and 1% antibiotic/antimycotic (1 unit/mL of penicillin, 1 μg/mL of streptomycin, and 2.5 ng/mL of amphotericin B; Life Technologies). The colonos were cut into 5 to 7 mm pieces and incubated in the culture medium described above for 2 hours at 37°C, 5% CO2/21% O2. After incubation, tissues were homogenized and lysed in RIPA buffer to conduct Western blot analysis as described above. HCT116 cells were obtained from American Type Culture Collection and maintained at 37°C in 5% CO2 and 21% O2. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1% antibiotic/antimycotic. Early passed (P10) stable HIF-2α and parental HCT116 cells were generated by transfection of a normoxially stable HIF-2α or pRES puro 3 empty vector with Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions and selected by 2 μg/mL puromycin. The cells were maintained in growth media described above containing 1 μg/mL of puromycin.

PolyHema apoptosis assay

Cell adhesion to proper extracellular matrix is essential for epithelial cell survival. Failure of attachment leads to a form of apoptosis termed anoikis (31). Poly(hydroxy ethyl methacrylate) (PolyHema) is a polymer which can form a surface that prevents cell adherence when coated on cell culture plate (32). Twenty-four–well plates were coated with 250 μL per well PolyHema (20 mg/mL) to achieve nonadherent conditions. HIF-2α overexpressing or parental HCT116 cells were plated at a concentration of 1 × 105 cells/mL in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic into PolyHema coated plates or noncoated control plates. After 24- or 48-hour culture, 125 μL 5 mg/mL MTT (Sigma) reagent was added to each well and incubated for 30 minutes. Dimethyl sulfoxide was added and absorbance was measured at 570 nm.
cDNA microarray analysis

RNA was extracted from colon mucosal scrapings from 5-week-old Vhl^{F/F}, Vhl^{DIE}, Vhl^{F/F}/Apc^{min/+}, and Vhl^{DIE}/Apc^{min/+} mice. Isolated RNA with RNA integrity number values more than 6.5 examined by Agilent 2100 bioanalyzer (Agilent Technologies, Inc.) was amplified, reverse transcribed, labeled, and hybridized to mouse 430 2.0 Affymetrix GeneChips (Affymetrix), and data were analyzed as previously described (28). The full data set is available on the Gene Expression Omnibus database accession number GSE36091.

Statistics

Results are expressed as mean ± SD. P values were calculated by independent t test, one-way ANOVA, Dunnett t test, and two-way ANOVA. P < 0.05 was considered significant.

Results

Activation of HIF-2α potentiates colon carcinogenesis following adenomatous polyposis coli mutation

Mice with an intestine-specific disruption of Vhl (Vhl^{str}) activate HIF signaling, whereas no induction of HIF signaling is observed in littermate controls (Vhl^{F/F}; refs. 28, 33). To assess the role of HIF in intestinal tumorigenesis, these mice were crossed to the Apc^{min/+} mice (Supplementary Fig. S1A). Apc^{min/+} mice have a nonsense mutation at codon 850 of the murine Apc gene leading to a truncated protein (34). Adenomatous polyposis coli (APC) is a tumor suppressor protein highly relevant in colon cancer; more than 80% of patients with sporadic colon cancer have a somatic mutation of the Apc gene (35), and mutations are observed in early and late colitis–associated neoplasms (36–38). In patients with
Interestingly, Vhl and genesis is independent of HIF-2
month-old Vhl plays a critical role in the development of colon cancer (Fig. Vhl they were still signi
numbers were observed in the colon of 3-month-old were signi
(tumor incidence: 11 of 15 mice; tumor multiplicity: 2.80). However, a signi
multiplicity of 32.24, whereas 4 of 17 Vhl IE mice showed early signs of carcinoma formation (Fig. 2B and D). In 6-month-old Vhl IE/Apc min/+ mice, 8 out of 10 mice had colon tumors that displayed complex glands with cribriform architecture and desmoplastic stroma, and thus were classified as carcinomas (Fig. 2C and D). To assess whether the increase in colon cancer progression was HIF-2α dependent, tumor analysis was conducted on 6-month-old Vhl IE/Hif-2α min/Apc min/+ mice (Fig. 2D). Although adenomas were observed, no tumors progressed to carcinomas. These data show that activation of HIF-2α is critical for the progression of colon tumors.

**HIF-2α regulates cell survival in the colon**

Analysis of apoptosis in colon tumors between Vhl F/F/Apc min/+ and Vhl IE/Apc min/+ was conducted with TUNEL assay. No significant difference was observed in tumors from Vhl F/F/Apc min/+ and Vhl IE/Apc min/+ mice (Supplementary Fig. S3).

![Image](https://cancerres.aacrjournals.org/)

**Figure 3.** HIF-2α increases cell survival in the colon. A, Western blot for cleaved caspase 3 (cCasp3), cleaved K18, and GAPDH in colon organoid cultures from Vhl F/F, Vhl IE, Vhl F/F/Apc min/+ , and Vhl IE/Hif-2α min/+ mice incubated for 0 or 2 hours at 37°C. B, Western blot analysis for HIF-2α and GAPDH from HCT116 stably transfected with empty vector (EV) or HIF-2α plasmid. C, cell survival rate of HCT116 cells stably transfected with EV or HIF-2α plasmid in an anokia-induced apoptosis assay. Survival rates were compared with uncoated control plates. Each bar represents the mean value ± SD (n = 3), *P < 0.05; and **P < 0.01 versus EV. D, Western blot analysis for cCasp3, total caspase 3 (casp3), and GAPDH from HCT116 cells stably transfected with EV or HIF-2α plasmid 24 hours following induction of anoikis.

**Activation of HIF-2α increases tumor progression in the Apc min/+ mice**

All tumors assessed in the small intestine or colon from 3- and 6-month-old Vhl F/F/Apc min/+ mice showed well-organized glandular structures and were classified as adenomas (Fig. 2A). However, 2 out of 15 tumors from 3-month-old Vhl IE/Apc min/+ mice showed early signs of carcinoma formation (Fig. 2B and D). In 6-month-old Vhl IE/Apc min/+ mice, 8 out of 10 mice had colon tumors that displayed complex glands with cribriform architecture and desmoplastic stroma, and thus were classified as carcinomas (Fig. 2C and D). To assess whether the increase in colon cancer progression was HIF-2α dependent, tumor analysis was conducted on 6-month-old Vhl IE/Hif-2α min/Apc min/+ mice (Fig. 2D). Although adenomas were observed, no tumors progressed to carcinomas. These data show that activation of HIF-2α is critical for the progression of colon tumors.

**Apc mutations, the predominant cancer is colon cancer and very rarely (~10-fold less) are small intestinal tumors observed (39). However, Apc min/+ mice develop mostly small intestinal tumors (34). Consistent with previous data, Vhl F/F/Apc min/+ mice developed predominantly small intestinal tumors. In the small intestine, tumors were found in 17 of 17 3-month-old Vhl F/F/Apc min/+ mice, with a tumor multiplicity of 32.24, whereas 4 of 17 Vhl F/F/Apc min/+ mice developed tumors in the colon with a tumor multiplicity of 0.59. Interestingly, Vhl IE mice crossed to the Apc min/+ background had a dramatic shift in the tumor localization to the colon in 3-month-old mice (Fig. 1A–C). Small intestinal tumors were significantly decreased in the 3-month-old Vhl IE/Apc min/+ compared with Vhl F/F/Apc min/+ mice (tumor incidence: 11 of 15 mice; tumor multiplicity: 2.80). However, a significant increase in tumor incidence and numbers were observed in the colon of 3-month-old Vhl IE/Apc min/+ mice (tumor incidence: 15 of 15 mice; tumor multiplicity: 13.87). Consistent with this, Western blot analysis showed an increase of HIF-2α protein expression in both normal colon and tumor tissues from Vhl IE/Apc min/+ mice (Supplementary Fig. S2). To assess whether the changes in tumor number and incidence were dependent on HIF-2α, a double knockout mouse model of Vhl and Hif-2α on an Apc min/+ background (Vhl IE/Hif-2α min/Apc min/+ ) was generated and compared with littermate controls (Vhl F/F/Hif-2α IE/Apc min/+ ; Supplementary Fig. S1B). Interestingly, small intestinal tumors in Vhl IE/Hif-2α IE/Apc min/+ mice were significantly repressed compared with the Vhl F/F/Hif-2α IE/Apc min/+ mice. These data were similar to that observed in the Vhl IE/Apc min/+ mice indicating that VHL-mediated decrease in small intestinal tumorigenesis is independent of HIF-2α (Fig. 1B and C). However, the increase in the colon tumorigenesis in Vhl IE/Apc min/+ mice was completely ablated following compound knockout of Vhl and Hif-2α in the Apc min/+ background, showing that HIF-2α plays a critical role in the development of colon cancer (Fig. 1B and C). These results were further confirmed in the 6-month-old Vhl IE/Apc min/+ and Vhl IE/Hif-2α IE/Apc min/+ mice (Fig. 1D and E). Compared with 3-month-old Vhl F/F/Apc min/+ mice, the tumor multiplicities in 6-month-old Vhl F/F/Apc min/+ mice were increased in the small intestine (tumor multiplicity: 62.50, tumor incidence: 6 of 6 mice) and colon (tumor multiplicity: 1.33, tumor incidence: 3 of 6 mice). However, small intestinal tumors were still significantly decreased in the 6-month-old Vhl IE/Apc min/+ mice (tumor incidence: 10 of 10 mice; tumor multiplicity: 15.10) compared with 6-month-old Vhl F/F/Apc min/+ mice. The tumor incidence and numbers observed in the colon of 6-month-old Vhl IE/Apc min/+ mice (tumor incidence: 10 of 10 mice; tumor multiplicity: 14.50) were not further increased compared with 3-month-old Vhl IE/Apc min/+ mice. However, they were still significantly increased compared with 6-month-old Vhl F/F/Apc min/+ mice. Assessing the double knockout mice (Vhl IE/Hif-2α IE/Apc min/+ ) at 6 months further showed that the decrease in small intestinal tumorigenesis is independent of HIF-2α, whereas the increase in colon carcinogenesis is completely dependent on HIF-2α.
Interestingly, a dramatic increase in cell survival was observed in normal colon epithelial cells of VhlAIE mice compared with VhlF/F mice. Intestinal epithelial cells undergo a spontaneous form of apoptosis termed anoikis as the differentiated cells reach the villus tips and are shed (40). Capturing this by TUNEL or caspase 3 expression revealed no significant decrease in cleaved K18 and cleaved caspase 3 in colons isolated from VhlF/F and VhlAIE mice (Fig. 3A). However, in colons cultured from VhlAIE mice, significant decreases in cleaved K18 and cleaved caspase 3 expressions were observed (Fig. 3A). In contrast, cleaved K18 and cleaved caspase 3 expressions were partially restored in colons cultured from VhlAIE/Hif-2αAIE mice (Fig. 3A). To further confirm these results, a stable HIF-2α over-expressing colon-derived HCT116 cell line was generated. As expected, in parental HCT116 cells, HIF-2α was not detectable, whereas in the stable overexpressing cells, a dramatic increase in HIF-2α expression was observed (Fig. 3B). Cell survival was assessed in an anoikis-induced apoptotic assay. Cells were incubated in PolyHema-coated plates to inhibit attachment, leading to increased apoptosis. Parental cells showed decreased survival rate following 24 and 48 hours of incubation. The survival rate was significantly increased in HCT116 cells overexpressing HIF-2α (Fig. 3C) with a concordant decrease in the activation of caspase 3 following 24 hours of incubation on PolyHema-coated plates (Fig. 3D). Although no significant difference is observed in tumor apoptosis (Supplementary Fig. S3), activation of HIF-2α induces cell survival that may play an important role in colon tumor progression.

**HIF-2α regulates cell proliferation in colon cancer**

Because apoptosis could not fully explain the increase in colon tumor formation in the VhlAIE/Apcmin/+ mice, the contribution of cell proliferation to HIF-2α-induced colon cancer was assessed. Cyclin D1 expression was assessed in VhlAIE/Apcmin/+ and VhlF/F/Apcmin/+ mice by quantitative PCR (qPCR) analysis. No significant difference of cyclin D1 expression was observed in the normal colon tissues of VhlF/F/Apcmin/+ and VhlAIE/Apcmin/+ mice. However, cyclin D1 was significantly induced in the colon tumors of VhlAIE/Apcmin/+ mice compared with tumors isolated from VhlF/F/Apcmin/+ mice (Fig. 4A). Consistent with cyclin D1 expression, BrdUrd incorporation revealed no significant difference in the normal colon tissues of VhlF/F/Apcmin/+ and VhlAIE/Apcmin/+ mice (Fig. 4B and C). However, the numbers of BrdUrd-positive proliferating epithelial cells in the colon tumors of VhlAIE/Apcmin/+ mice were significantly higher than those in tumors of VhlF/F/Apcmin/+ mice (Fig. 4B and C). Elevated tumor proliferation was ablated in the colon tumors of VhlAIE/Hif-2αAIE/Apcmin/+ mice (Fig. 4D). These data show that HIF-2α increases tumor cell proliferation, which may be critical for the increase in colon tumor formation and progression.

**DMT-1 and iron uptake represent a cellular target and regulator of HIF-2α–induced colon cancer**

To identify the precise molecular mechanisms that could contribute to the increase in colon carcinogenesis, microarray gene expression analysis was conducted on colon RNA...
isolated from 5-week-old Vhl\textsuperscript{F/F} and Vhl\textsuperscript{AE}, Vhl\textsuperscript{AE}/Apc\textsuperscript{min/+} and Vhl\textsuperscript{F/F}/Apc\textsuperscript{min/+} mice. The data identified 469 genes that were significantly increased/decreased in both Vhl\textsuperscript{AE} and Vhl\textsuperscript{AE}/Apc\textsuperscript{min/+} compared with their littermate controls and 29 genes that were significantly increased/decreased in both Vhl\textsuperscript{F/F}/Apc\textsuperscript{min/+} and Vhl\textsuperscript{F/F}/Apc\textsuperscript{min/+} compared with their littermate Apc wild-type controls (Fig. 5A). The top 10 genes that were regulated in an HIF-2\textalpha-dependent manner were further assessed using ONCOMINE (Supplementary Table S2), a cancer microarray data mining platform (41). Those genes that were increased in the colons of Vhl\textsuperscript{AE}/Apc\textsuperscript{min/+} mice and in human colon cancer samples were identified. This analysis showed a specific increase in DMT-1 expression in colon adenomas and carcinomas isolated from patients compared with normal controls in 3 independent studies (refs. 42–44; Fig. 5B). DMT-1 is the major apical iron transporter in the intestine (45–47). It is essential for iron absorption and its expression in the small intestine is directly regulated by HIF-2\textalpha (26). Colon tumors have been shown to accumulate iron (23). Moreover, mouse models of colon cancer and population-based studies show a direct correlation between an increase in intestinal iron and increased incidence of colon cancer (24, 25). These studies suggest that dysregulation of local iron homeostasis is critical in colon carcinogenesis. Consistent with these results, measuring tissue iron using enhanced Prussian blue staining showed a specific increase in iron accumulation in colon tumors compared with normal tissue (Fig. 5C). To assess whether HIF-2\textalpha was critical for DMT-1 regulation during colon tumorigenesis, DMT-1 expression was measured by qPCR. DMT-1 was significantly induced in the colons of Vhl\textsuperscript{F/F}/Apc\textsuperscript{min/+} mice compared with wild-type littermates (Vhl\textsuperscript{F/F}), which was further potentiated in Vhl\textsuperscript{AE}/Apc\textsuperscript{min/+} mice compared with wild-type littermates.
iron is essential for colon cancer progression and a critical pathway by which HIF-2α signaling contributes to colon tumorigenesis.

Discussion

Hypoxia is an adaptive response in many solid tumors. However, its role in tumor development in vivo is still not clear. This study found that the activation of HIF signaling through HIF-2α enhanced colon cancer incidence and progression in the Apcmin/+ mice. The Apc tumor suppressor is a gatekeeper gene mutated in a majority of patients with familial, sporadic, and colitis-associated colon cancer (35–38). However, Apcmin/+ mice develop mostly small intestinal tumors, and colon tumors are observed with low incidence and multiplicity (34). HIF-2α activation in the intestines of Vhl+/Apcmin/+ mice dramatically increased colon tumor multiplicity and incidence. Moreover, at 6 months of age the majority of colon tumors observed were histologically defined as carcinomas, whereas no tumors identified in the Vhl+/Apcmin/+ mice progressed further than adenomas, showing that HIF-2α may be a critical transcription factor involved in colon cancer progression.

Interestingly, small intestinal tumors observed in the Vhl+/Apc+/min/+ mice were rare and this decrease in tumorigenesis was independent of HIF-2α expression. Despite the profound decrease in tumorigenesis, no changes in small intestinal proliferation were observed between Vhl+/Apc+/min/+ and Vhl+/Apc+/min/+ mice (Supplementary Fig. S5). Moreover, the Vhl+/Apc+/min/+ mice had increased DMT-1 expression in the small intestine, suggesting that decreased small intestinal tumorigenesis by the HIF-2α–independent mechanism is downstream of the iron accumulation effects. HIF-1α was shown to have an antagonistic role in cell growth and VHL has several HIF-independent functions (11, 48–52), and these pathways are currently being assessed in the small intestine. There is some evidence that colon tumors in the Apc+/min/+ do not form because of the large tumor load of the small intestine compromise the mice, therefore the slower forming colon tumors do not develop. However, in the Vhl+/Hif-2α+/Apc+/min/+ mice, the decrease in small intestinal tumors did not lead to a refractory increase in colon tumors. Confirming that the increase in colon tumors in the Vhl+/Apc+/min/+ mice is mediated by HIF-2α activation. The Vhl+/Apc+/min/+ mice, through HIF-2α–dependent and HIF-2α–independent mechanisms, can recapitulate downstream events following Apc mutation that is observed in sporadic, familial, and colitis-associated colon cancer and is an optimal preclinical animal model to study colon cancer.

The mechanisms that decrease small intestinal tumorigenesis are currently not known. However, the increases in colon tumor incidence, multiplicity, and progression in Vhl+/Apc+/min/+ mice was completely ablated following double knockout of Vhl and Hif-2α in the Apc+/min/+ background, showing that HIF-2α plays a critical role in the development of colon cancer following Apc+/min/+ mutation. Activation of HIF-2α in the intestine increased epithelial cell survival. This was measured with colon organoids that may be in
undergoing apoptosis very early following the culture of the colons and using colon cancer–derived cells lines. Therefore, the anokisia detected may not be a physiologic response, and a more detailed analysis of HIF-2α–mediated cell survival is needed. However, this study clearly shows a role of HIF-2α in tumor proliferation. To understand the mechanisms involved in the increase in colon tumorigenesis, the HIF-2α–dependent gene expression profile was analyzed by the ONCOMINE database (41). The HIF-2α target gene DMT-1 was highly induced in colon adenomas and carcinomas compared with normal colon tissue isolated from patients. DMT-1 is a critical intestinal iron transporter, and its overexpression can lead to iron accumulation (45, 46, 53, 54). Iron accumulation is a critical factor in colon carcinogenesis and studies have shown that both body iron stores and dietary iron intake are positively associated with subsequent risk of colon cancer (22). In mouse models of colon cancer, iron-enriched diets increase the development of colon tumors (21). Iron activates free radical formation, which clearly show that the activation of HIF-2α contributes to the increase in cell proliferation, dysregulation of local iron homeostasis, and eventually an increase in the incidence of colon cancer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**References**

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