HIF1α deficiency reduces inflammation in a mouse model of proximal colon cancer

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ABSTRACT

Hypoxia-inducible factor 1α (HIF1α) is a transcription factor that regulates the adaptation of cells to hypoxic microenvironments, for example inside solid tumours. Stabilisation of HIF1α can also occur in normoxic conditions in inflamed tissue or as a result of inactivating mutations in negative regulators of HIF1α. Aberrant overexpression of HIF1α in many different cancers has led to intensive efforts to develop HIF1α-targeted therapies. However, the role of HIF1α is still poorly understood in chronic inflammation that predisposes the colon to carcinogenesis. We have previously reported that the transcription of HIF1α is upregulated and that the protein is stabilised in inflammatory lesions that are caused by the non-steroidal anti-inflammatory drug (NSAID) sulindac in the mouse proximal colon. Here, we exploited this side effect of long-term sulindac administration to analyse the role of HIF1α in colon inflammation using mice with a Villin-Cre-induced deletion of Hif1α exon 2 in the intestinal epithelium (Hif1αIEC). We also analysed the effect of sulindac sulfide on the aryl hydrocarbon receptor (AHR) pathway in vitro in colon cancer cells. Most sulindac-treated mice developed visible lesions, resembling the appearance of flat adenomas in the human colon, surrounded by macroscopically normal mucosa. Hif1αIEC mice still developed lesions but they were smaller than in the Hif1α-floxed siblings (Hif1αIEC). Microscopically, Hif1αIEC mice had significantly less severe colon inflammation than Hif1αIEC mice. Molecular analysis showed reduced MIF expression and increased E-cadherin mRNA expression in the colon of sulindac-treated Hif1αIEC mice. However, immunohistochemistry analysis revealed a defect of E-cadherin protein expression in sulindac-treated Hif1αIEC mice. Sulindac sulfide treatment in vitro upregulated Hif1α, c-JUN and IL8 expression through the AHR pathway. Taken together, HIF1α expression augments inflammation in the proximal colon of sulindac-treated mice, and AHR activation by sulindac might lead to the reduction of E-cadherin protein levels through the mitogen-activated protein kinase (MAPK) pathway.

KEY WORDS: HIF1α, MIF, AHR, E-cadherin, Sulindac, Colon inflammation

INTRODUCTION

The gastrointestinal tract adapts to rapid and drastic changes in tissue oxygen availability (Colgan and Taylor, 2010). Hypoxia-inducible factor (HIF) plays a key role in promoting cell survival under hypoxic conditions. HIF also has a central protective function in maintaining the gut epithelial barrier (Colgan and Taylor, 2010). HIF is rapidly degraded upon oxygen availability and is stabilised in hypoxic conditions. However, HIF is also an inflammatory mediator that can be stabilised under normoxia, as well as other conditions such as the upregulation of pro-inflammatory IL-1β (Jung et al., 2003).

HIF is a heterodimeric transcription factor and comprises a constitutively expressed HIF1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) subunit and one of several inducible HIFα subunits, the best characterised of which are HIF1α and the closely related HIF2α (Semenza, 2003). The role of HIF in colon inflammation is still poorly understood. HIF1α expression in the colon mucosa is protective in the trimetobenzene sulfonic acid (TNBS) and oxazalone models of murine colitis. In these models, the protective function of HIF1α is mediated through upregulation of barrier protective genes and increased resistance to injury in the colon mucosa (Karhausen et al., 2004). HIF1α that is expressed by T cells is also protective in the dextran sodium sulfate (DSS) model of colitis (Higashiyama et al., 2012). By contrast, colon-specific constitutive expression of HIF (through Vhl disruption) augments colitis and increases cytokine expression in the DSS model (Shah et al., 2008).

Mouse models have been used to identify many factors that can initiate or contribute to chronic inflammation in the colon, such as disruption of intercellular signalling, and cross-talk between epithelial and inflammatory cells or injury to the mucosal barrier (Mladenova and Kohonen-Corish, 2012). We have previously shown that HIF1α transcription is upregulated and the protein level is stabilised in inflammatory lesions of the mouse colon, which are caused by the non-steroidal anti-inflammatory drug (NSAID) sulindac (Mladenova et al., 2011). Sulindac is a drug used to treat arthritis that can also be prescribed as a cancer chemoprevention agent, but its use is limited by gastrointestinal side effects. In the mouse, long-term administration of oral sulindac prevents carcinogen-induced cancer in the distal colon but also causes small foci of mucosal surface damage to the proximal colon. These can progress to visible lesions that exhibit acute and chronic inflammation (Mladenova et al., 2011). Microscopic foci of mucosal damage are evident after 1 week of sulindac diet. After 20 weeks of exposure to sulindac, the macroscopic lesions resemble flat adenomas in the human colon. The presence of early microscopic surface erosions suggests that damage to the mucosal barrier plays a role in this model. In knockout mice with defective tumour suppressor genes Msh2, p53 (Mladenova et al., 2011), Mlh1 or Apc (Itano et al., 2009), the inflammatory damage can further progress to cancer. However, the number and size of the visible
TRANSLATIONAL IMPACT

Clinical issue
Aberrant overexpression of hypoxia-inducible factor 1α (HIF1α) in many different cancers has led to intensive efforts to develop HIF1α-targeted therapies. However, HIF1α is also important in protecting the colon mucosal barrier, and the role of HIF1α is poorly understood in chronic inflammation that predisposes the colon to carcinogenesis. Previous studies have shown that administration of the chemoprevention agent sulindac in mice prevents carcinogen-induced cancer in the distal colon but also causes early foci of mucosal damage to the proximal colon, which can progress to visible lesions with acute and chronic inflammation. The presence of surface erosion suggests that damage to the colon mucosal barrier plays a role in sulindac-induced inflammation. Transcription of HIF1α is upregulated, and the protein is stabilized in sulindac-induced inflammatory lesions. Here, the authors have exploited the side effect of long-term sulindac administration in the proximal colon to analyse the role of HIF1α in colon inflammation using mice that lack HIF1α in the intestinal epithelium (Hif1αΔEC).

Results
Sulindac-treated Hif1αΔEC mice developed colonic lesions but these were smaller than those detected in the genotype control mice (Hif1αF/F) treated with the same drug. Microscopically, Hif1αΔEC mice had significantly less severe colon inflammation than Hif1αF/F mice. Loss of HIF1α reduced the expression of the macrophage migration inhibitor factor (MIF) in sulindac-treated mice, which is compatible with the observed reduction in the severity of inflammation. HIF is a negative regulator of the cell-adhesion protein E-cadherin (reduced expression of which is associated with invasiveness in human carcinomas) and as expected, loss of HIF1α increased E-cadherin mRNA expression in the colon. E-cadherin protein expression was also increased in Hif1αΔEC mice, but treatment with sulindac abolished such an increase.

Implications and future directions
These results indicate that HIF1α expression augments inflammation in the proximal colon of sulindac-treated mice. By contrast, HIF1α expression might be protective against sulindac-induced reduction in E-cadherin protein expression. These findings provide in vivo evidence of the dual role of HIF1α in the colon. As sulindac is a known activator of the aryl hydrocarbon receptor signalling pathway that regulates detoxification of many environmental contaminants and drugs, further studies of this model might be informative in understanding the diverse tissue-specific effects of this important drug detoxification pathway.

lesions, as well as the severity of inflammation is comparable between the knockout mice and their wild-type siblings, despite the difference in neoplasia frequency (Mladenova et al., 2011).

We have exploited this exaggerated inflammatory response to sulindac in the mouse proximal colon in order to analyse the role of HIF1α in colon inflammation. Here, we generated mice with specific knockout of HIF1α in the colon and small intestine epithelium (Hif1αΔEC) using the Cre recombinase transgene under the control of the Villin promoter (Madison et al., 2002). We also further analysed the effects of treatment with sulindac sulfide on the proximal colon and small intestine (Mladenova et al., 2011). Sulindac-induced lesions are localised to a specific region of the proximal colon (labelled thereafter as P2), and show high levels of inflammation and high expression of HIF1α. Therefore, we generated mice with a colon-specific deletion of HIF1α (Hif1αΔEC) using the Villin-Cre mouse line (Madison et al., 2002). The efficiency of Cre-mediated recombination of the HIF1α-lox allele was 91% in the proximal colon mucosa of Hif1αΔEC mice.

We first determined whether Hif1α deficiency in the colon affected the severity of sulindac-induced mucosal inflammation. After 20 weeks of sulindac treatment, the colons were harvested and analysed at the macroscopic level. Visible inflammatory lesions were carefully measured with a fitted eyepiece grid, and their size and location in the colon recorded. Hif1αΔEC mice still developed visible lesions in the P2 region of the proximal colon (Mladenova et al., 2011, but the individual lesions were significantly smaller in Hif1αΔEC mice compared with those in Hif1α-lox floxed mice (Hif1αΔF) mice (P=0.037) (Fig. 1A). The Hif1αΔEC mice also developed fewer large lesions (>10 mm²) (3.6% vs 17%, P=not significant). Three out of 26 Hif1αΔF mice (12%) had macroscopic colon inflammation outside the lesions, involving large areas of the colon, with the affected area in these three cases estimated to cover 10, 15 and 5% of the whole colon, respectively. No Hif1αΔEC mice showed macroscopic damage outside of the lesions.

We then analysed the mucosal surface microscopically by scoring the severity of inflammation, depth of inflammation and crypt damage, which were combined to produce a total inflammation score. Hif1αΔEC mice had significantly less severe colon inflammation in the mucosa between lesions in the P2 region compared with mice from the control genotype (Fig. 1B,C), including the score for crypt damage and the total inflammation score (P<0.05). Based on the histological score, the most severely inflamed lesion for each mouse was chosen for further analysis, and there was a trend for lower total inflammation scores in the Hif1αΔEC mice compared with Hif1αΔF mice (P=0.057; Fig. 1D). As in our previous experiment, a subset of sulindac-induced lesions in the colon progressed to adenocarcinoma. The inflammatory microenvironment is rich in mutagenic reactive oxygen species and pro-inflammatory factors that can promote cancer initiation and growth, and lead to tissue remodelling (Mladenova and Kohonen-Corish, 2012). Surprisingly, the frequency of colon adenocarcinoma was similar in Hif1αΔEC (18%; two out of 11) and Hif1αΔF mice (16%; four out of 25) although there was less mucosal inflammation in the Hif1αΔEC mice. The distribution of total inflammation scores in neoplastic and non-neoplastic lesions is shown in Fig. 1E. The photomicrographs in Fig. 2 and supplementary material Figs S1 and S2 show some of the typical pathological changes observed in the proximal colon of sulindac-treated mice, ranging from mild inflammation with no neoplasia to moderate and severe inflammation with dysplasia and adenocarcinoma. A subset of sulindac-fed mice also developed small foci of acute or chronic hepatitis. Sulindac is a rare but known cause of hepatitis in humans (Wood et al., 1985).

Hif1α-knockout defect causes downregulation of macrophage migration inhibitor factor, MIF, but increases Hif2α mRNA levels in sulindac-treated colon mucosa

We observed a 19-fold reduction of Hif1α mRNA expression in Hif1αΔEC mice compared with that of the control genotype (Fig. 3A). The generation of Hif1αΔEC involves deletion of exon 2, which contains sequences encoding the basic helix-loophelix (bHLH) domain that is essential for DNA binding and dimerisation of HIF1α and HIF1β (Jiang et al., 1996), and that results in

RESULTS
Colon-specific deficiency of Hif1α is protective against sulindac-diet-induced mucosal inflammation

We have previously described that administration of a long-term sulindac diet causes similar levels of tissue damage to the colon in tumour suppressor gene knockout mice compared with their wild-type siblings (Mladenova et al., 2011). Sulindac-induced lesions are

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downregulation of HIF1α-target genes (Iyer et al., 1998; Ryan et al., 1998). As we have previously shown in wild-type mice, the sulindac diet increased HIF1α expression in Hif1αΔIEC mice, and this effect was most pronounced in the inflammatory colon lesions (Fig. 3A). HIF1α expression remained low in the colon of the Hif1αΔIEC mice that had been treated with sulindac.

We next examined the HIF1α-target genes phosphoglycerate kinase-1 (Pgk1), glucose transporter (Glut1), vascular endothelial growth factor (Vegfa) and intestinal trefoil factor (ITF) (Fig. 3B-E) (Iyer et al., 1998; Karhausen et al., 2004; Ryan et al., 1998). None of the examined genes were significantly downregulated in the Hif1αΔIEC when compared with their levels in Hif1αF/F mice. Glut1 and Vegfa genes are under the transcriptional control of both HIF1α and HIF2α (Raval et al., 2005). The HIF2α subunit of hypoxia-inducible factor can also drive hypoxia-dependent responses, and HIF1α and HIF2α have been shown to regulate common target genes (Raval et al., 2005). We next assessed the level of Hif2α by using quantitative PCR (q-PCR). The sulindac diet increased Hif2α expression levels in the colon mucosa of Hif1αΔIEC but not of Hif1αF/F mice or in the lesions (Fig. 3F). Therefore, the lack of downregulation of HIF1α-target genes in the colon of Hif1αΔIEC mice might be due to the compensatory role of HIF2α and/or other transcription factors in regulating these genes.

We have previously shown that pro-inflammatory genes such as IL-1β, MIP-2 and Cox-2 are upregulated by the sulindac diet in the mouse proximal colon (Mladenova et al., 2011, 2013), and impairment in their induction could explain the reduction in the inflammatory response. However, the level of induction of these cytokines was not significantly different between Hif1αΔIEC and Hif1αF/F mice (data not shown). We next examined whether the lower levels of inflammation observed in the colon of Hif1αΔIEC mice were associated with changes in macrophage infiltration or expression of the macrophage migration inhibitory factor (MIF). HIF1α is essential for the function, migration, motility and invasiveness of myeloid cells (Cramer et al., 2003; Scortegagna et al., 2008), and MIF is a direct target of HIF1. MIF is a crucial mediator of HIF-induced pro-inflammatory responses in a mouse model of experimentally induced colitis (Baugh et al., 2006; Cramer...
Apc membrane expression of E-cadherin is a hallmark of the epithelial-cell adhesion protein E-cadherin (Esteban et al., 2006). Reduced encoded by the (Grivennikov et al., 2012). Polymorphisms in E-cadherin, which is tumour-elicited inflammation and contributes to tumour growth protein expression in the colon mucosa loss of HIF1 αΔ mice is repressed through EMT transcription factors, such as Snail, Slug and Twist (Batle et al., 2000; Bolos et al., 2003; Cano et al., 2000; Hajra et al., 2002; Yang et al., 2008). However, no significant differences were observed in the expression of Snail, Slug or Twist between Hif1αΔF and Hif1αΔIEC mice (Fig. 4B-E).

E-cadherin is detected in both the cytoplasm and the membrane of colon epithelial cells. Membrane-bound E-cadherin is crucial in maintaining the integrity of the intercellular junctions. We next determined whether sulindac changed E-cadherin protein expression in the mucosa of the proximal colon, which is most affected by sulindac-induced epithelial damage (Fig. 5). Consistent with the results of mRNA analyses, E-cadherin protein levels were higher in Hif1αΔIEC mice compared with those in Hif1αΔF mice, but this was only seen in the mice that received the control feed. Remarkably, with sulindac exposure, there was a clear decrease in E-cadherin protein level in Hif1αΔIEC mice despite an increase in E-cadherin mRNA level (Fig. 4A). This was due to reduced expression of both cytoplasmic and membrane-bound E-cadherin. Thus, although the Hif1αΔIEC mice showed less inflammatory damage in the colon following treatment with sulindac, these mice displayed a defect in E-cadherin protein expression. This suggests that E-cadherin protein expression in the proximal colon is reduced by sulindac and that this is more pronounced in the absence of HIF1α.

Sulindac metabolites are known to cause degradation of β-catenin in colon cancer cells (Rice et al., 2003). Therefore, we next analysed the expression of the RNA of β-catenin-target genes. There was no difference between Myc, Axin2 or Lgr5 expression between Hif1αΔIEC and Hif1αΔF mice (Fig. 4F-H). However, there was reduced expression of Axin2 and Lgr5 in the inflammatory lesions that had been induced by sulindac, regardless of genotype.

**Treatment of colon cancer cells with sulindac sulfide in vitro causes upregulation of inflammation and cancer-promoting genes through AHR.** We have previously shown that the sulfide metabolite of sulindac can induce NF-xB and AP-1 (c-Jun and JunD) signalling in colon cancer cells, which lead to upregulation of the chemokine IL8...
This resembles the activation of the aryl hydrocarbon receptor (AHR) signalling pathway that regulates detoxification of many environmental contaminants and pharmacological drugs but that can also lead to activation of inflammatory cytokines (Fardel, 2013; Safe et al., 2013). Sulindac is a known ligand and activator of AHR (Ciolino et al., 2006). Therefore, we next tested whether sulindac can activate AHR-associated pathways in our in vitro model. HCT15 cells were treated with 50 µM sulindac sulfide for 1-24 h. Q-PCR analysis of gene expression showed that sulindac sulfide upregulated CYP1A1, the prototype phase I response target of AHR, as well as c-JUN, IL8 and Hif1α. The upregulation of all four genes was abolished with AHR knockdown (Fig. 6). This suggests that sulindac can activate AHR, leading to activation of c-Jun signalling, which is a known EMT-promoting transcription factor. AHR activation through dioxin, the well-known AHR ligand, has been previously shown to lead to loss of cell-cell adhesion in MCF7 breast epithelial cells (Diry et al., 2006). In our model, sulindac-sulfide-induced phosphorylation of c-Jun increased until 24 h, whereas there was a gradual decrease in E-cadherin, β-catenin and p120-catenin levels (Fig. 7).

DISCUSSION

The role of HIF in promoting carcinogenesis has been extensively studied, but its role in intestinal inflammation is poorly understood (Bracken et al., 2003; Semenza, 2003). Amongst the factors contributing to inflammatory bowel disorders are dysregulation and inappropriate activation of inflammatory signalling, and defects in the mucosal barrier. HIF1 signalling has been reported to play a role in both the immune response and in the maintenance of the integrity of the mucosal epithelial barrier (Mladenova and Kohonen-Corish, 2012). Epithelial cells are now seen as important players in the gut immune response because they have the ability to secrete a range of...
pro-inflammatory factors and other mediators that modulate the function of the immune cells (Mladenova and Kohonen-Corish, 2012). Constitutive expression of HIF1α in basal epidermal keratinocytes in transgenic mice primes an increase in inflammatory infiltrate (Scortegagna et al., 2008). Similarly, HIF overexpression in colon epithelial cells exacerbates DSS-induced colitis, and results in an increased inflammatory infiltrate and colon oedema, even without treatment with DSS (Shah et al., 2008). Consistent with these data, in our previous study, we showed that treatment with sulindac induced significantly less colon inflammation in Hif1αΔIEC mice (Mladenova et al., 2011). Those mice had a Villin-Cre driven deletion of Hif1α in the...
intestine and a heterozygous Hif1α-null mutation in the whole body – i.e. including the inflammatory cells infiltrating the colon mucosa.

Here, we deleted HIF1α specifically in mouse intestinal epithelium and achieved a good efficiency of recombination (91%). Hif1α mRNA levels were significantly reduced in Hif1αΔIEC mice compared with those in mice that expressed HIF1α from the floxed allele (Hif1αF/F). However, Hif2α mRNA levels were not affected in Hif1αΔIEC mice, and Hif2α levels were significantly increased in the colons of Hif1αΔIEC mice that were fed with the sulindac diet when compared with those of mice on the control diet. HIF1α and HIF2α regulate overlapping or distinct sets of target genes in different tissues (Raval et al., 2005). Therefore, it is possible that HIF2α expression compensates to some extent for the lack of HIF1α expression in the colon mucosa. In support of this hypothesis, we did not observe downregulation of the HIF-target genes Glut1, Pgk1, Vegfa, ITF, Snail or Twist.

As we have previously reported, treatment of mice with sulindac triggers colon inflammation and the formation of visible lesions in the proximal colon, most pronounced in a region that we designated as P2 (Mladenova et al., 2011). Histopathology analysis showed that deficiency of HIF1α in the colon epithelium resulted in a decrease in the inflammatory response in both the macroscopically normal (uninvolved) proximal P2 mucosa and in the lesions. The reduction of the inflammatory response was accompanied by a significant decrease in MIF expression in Hif1αΔIEC mice. MIF is a direct target of HIF1α (Baugh et al., 2006) and is described as a link between inflammation and cancer, contributing to a microenvironment favouring cancer progression (Conroy et al., 2010). MIF has been found to play a role in a mouse model of experimentally induced colitis, and MIF plasma concentrations are increased in individuals with Crohn’s disease (de Jong et al., 2001). Similarly, transgenic mice that overexpress MIF are more susceptible to DSS-induced colitis (Ohkawara et al., 2005). Conversely, antibodies against MIF ameliorate DSS-induced colitis in mice (Ohkawara et al., 2002). Shah and colleagues have reported that HIF expression exacerbates colitis through a MIF-dependent mechanism (Shah et al., 2008). Therefore, we speculate that reduced MIF expression contributes to the reduction of colon inflammation in Hif1αΔIEC mice.

HIF is one of the known factors promoting EMT in tumour cells and is a negative regulator of E-cadherin expression, potentially through several mechanisms (Esteban et al., 2006; Lamouille et al., 2014). Membrane-bound E-cadherin plays an important role in maintaining the homeostasis of the gut epithelial lining to prevent invasion of pathogenic bacteria (Schneider et al., 2010). Reduced membrane E-cadherin is also observed in the development of invasive carcinomas. As expected, gut-specific ablation of Hif1α resulted in an increase in E-cadherin mRNA expression in the mice that received either the sulindac or control feed. Also, E-cadherin protein expression was increased in Hif1αΔIEC mice, but only in the mice receiving the control feed. In sulindac-treated Hif1αΔIEC mice, this increase was abolished.

We have previously shown that sulindac sulfide can induce pro-inflammatory NF-kB and AP-1 signalling, and concurrent apoptosis in the same experimental conditions in colon cancer cells (Mladenova et al., 2011, 2013). Here, we show that the upregulation of EMT-promoting factors c-Jun and IL8 through
sulindac is likely to be the result of activation of AHR, the major pathway that is activated by environmental carcinogens and pharmacological drugs. Sulindac is a known ligand and agonist of AHR (Ciolofo et al., 2006; Safe et al., 2013), and the prototype AHR-target gene CYP1A1 is also upregulated by sulindac sulfide. In MCF7 breast epithelial cells, AHR activation through dioxin leads to activation of Jun NH2-terminal kinase (JNK), a reduction in E-cadherin protein levels and the loosening of cell-cell contacts (Diry et al., 2006). Sulindac sulfide is also known to cause phosphorylation of JNK (Singh et al., 2011), which we have confirmed in HCT15 colon cancer cells (data not shown). Thus, sulindac sulfide might reduce E-cadherin protein levels through activation of JNK targets, such as c-Jun, through the AHR pathway.

Sulindac is known to cause degradation of β-catenin, and this is thought to contribute to its chemopreventive effects (Han et al., 2008; Rice et al., 2003). Here, we show that the reduction in β-catenin protein level in sulindac-sulfide-treated cells is associated with the reduction of E-cadherin and p120-catenin, two proteins controlling cell-cell adhesion. Also, β-catenin-target genes Lgr5 and Axin 2 were downregulated in sulindac-induced inflammatory lesions. It remains unexplained as to why the reduction in E-cadherin appears to be more pronounced in the absence of HIF1α signalling in colon epithelial cells. It is possible that this is due to some functional interaction between the AHR and HIF1α pathways (Nie et al., 2001), which share the same binding partner HIF1β (ARNT). Dioxin can also activate AP-1 transcription factors and reduce E-cadherin protein levels independently of HIF1β through non-canonical AHR pathways (Dietrich and Kaina, 2010). Here, we show that both AHR and HIF1α are transcriptionally activated through sulindac sulfide and that this upregulation is abolished by AHR knockdown.

In summary, this study shows that HIF has both pro-inflammatory and protective roles in the proximal colon of sulindac-treated mice. We found that loss of Hif1α expression in the Hif1αIEC mice protects the colon mucosa against sulindac-induced tissue damage and inflammation but that sulindac treatment also causes a defect of E-cadherin protein expression in the proximal colon of these mice. Thus, this study further clarifies the molecular mechanism of sulindac-induced tissue toxicity in the mouse proximal colon.

**MATERIALS AND METHODS**

**Generation of Hif1αIEC mice and administration of sulindac**

Hif1αIEC mice (Ryan et al., 2000) were first crossed with Villin-Cre (Vill-Cre) mice [B6.SJLt(vil-cre)997Gum/J (Madison et al., 2002); Jackson Laboratories, Bar Harbor, Maine]. Heterozygous Vill-Cre-Hif1αF/F mice were then backcrossed with Hif1αIEC to obtain Vill-Cre-Hif1αIEC/F/F, wild-type (WT-Hif1αIEC/F/F) and the corresponding Hif1αIEC/F/F heterozygotes. The Mendelian ratios of the four genotypes were 0.22 (Vill-Cre-Hif1αIEC/F/F), 0.32 (WT-Hif1αIEC/F/F), 0.20 (Vill-Cre-Hif1αIEC/F/F) and 0.26 (WT-Hif1αIEC/F/F) of 269 mice born. Vill-Cre-Hif1αIEC/F/F mice are deficient for Hif1α in the intestinal epithelial cells (IEC), and the sibling controls WT-Hif1αIEC/F/F retain HIF1α expression from two floxed alleles. The intestinal epithelium HIF1α-deficient mice were designated Hif1αIEC and the sibling control mice Hif1αIEC/F/F. Mice were bred at specific-pathogen-free conditions. Hif1αIEC mice appeared normal and healthy with similar weight gain compared to Hif1αIEC/F/F mice. Cre-mediated recombination for the HIF1α conditional mutant was determined by using q-PCR analysis of colon mucosal DNA as previously described (Karhausen et al., 2004; Mladenova et al., 2011). The strain background of the Villin-Cre mice was C57BL/6J, and the parent Hif1αIEC mice were at least 91% C57BL/6J and the rest (ppm) sulindac in the diet before colon collection, as previously described (Mladenova et al., 2011). Mice that were given control food were age-matched with mice on the sulindac diet. Both males and females were used. After examination under a dissecting microscope, individual biopsies from standardised areas of the mouse colon were collected. From each colon, six standard biopsies of flat mucosa were collected, and every visible lesion was dissected. The ‘Australian code for the care and use of animals for scientific
purposes’ was followed in all experimentation and the project was approved by the Garvan Institute and St Vincent’s Hospital Animal Ethics Committee.

Once preserved in 70% ethanol, mouse colons were opened and flattened longitudinally, and were examined using a Leica stereomicroscope (MZ8, Leica Microsystems GmbH, Wetzlar, Germany) with a fitted grid eyepiece. Visible lesions were carefully measured with a fitted eyepiece grid in two dimensions, and the size was calculated as the surface area (mm²). The exact location of lesions along the colon length in reference to the caecum was also recorded. Caecum, proximal P1, P2, middle and distal colon segments were collected precisely from standardised colon regions; caecum (the tip of caecum), P1 (1 cm from the caecum-colon junction), P2 (the end of the V-shaped mucosal folds), middle colon (4 cm from the caecum-colon junction) and distal colon (1 cm from the anus). All visible lesions were also biopsied.

For a subset of mice, the mucosal surface of the proximal colonic tissue was lightly scraped or whole colon biopsies were collected. Tissue was snap-frozen in liquid nitrogen for RNA extraction.

Histopathology analysis
Histopathology assessment was conducted by an anatomical pathologist (J.E.D.) from de-identified slides, as previously described (Mladenova et al., 2011). The features assessed included: acute and/or chronic inflammation, lymphoid aggregates, hyperplastic and/or degenerative changes of the surface epithelium, crypt architectural distortion, fibrosis and neoplasia – classified as epithelial dysplasia or adenocarcinoma. Inflammation was assessed using a scoring system modified from the literature (Cummins et al., 2008; Vowinkel et al., 2004). Three independent parameters were measured: severity of inflammation, depth of injury/inflammation and crypt damage, and scored as shown below. The total histological score was calculated through summing of the three independent scores with a maximum score of 12. For severity of inflammation, representative tissue images are shown in supplementary material Fig. S1 (0=no inflammation; 1=slight – presence of mucosal inflammatory cell infiltrate without significant distortion of the crypt architecture; 2=erosion – superficial ulceration that involved only the surface epithelium and lamina propria; 3=ulceration – defined as loss of the colonic mucosa associated with an acute inflammatory reaction extending at least through the muscularis mucosae). The depth of inflammation was scored as 0=none; 1=mucosal; 2=mucosal and submucosal; 3=transmural. For crypt damage, representative tissue images are shown in supplementary material Fig. S2 (0=none; 1=only surface epithelium damaged; 2=surface crypt and epithelium damaged; 3=basal 1/3 crypt damaged; 4=basal 2/3 crypt damaged; 5=entire crypt lost and surface epithelium damaged; 6=entire crypt and epithelium lost; grades 3 and 4 are not a feature of this model).

Immunohistochemistry analysis
Sections from tissue paraffin blocks were cut on a Leica microtome. Antigen retrieval was performed in a pressure cooker for 25 s using target retrieval solution at pH 6 (S1699, DAKO, Carpinteria, CA). A Dako autostainer was used for immunohistochemistry. The protocol included an initial step of 3% hydrogen peroxide block (K4011, DAKO) for 5 min in order to quench endogenous peroxidase activity, followed by an avidin-biotin block (Biotin Blocking system, X0590, DAKO) and a serum-free protein block for 30 min. Endogenous biotin was blocked with Biotin Blocking solution at pH 6 (S1699, DAKO, Carpinteria, CA). A Dako autostainer was used for immunohistochemistry. The protocol included an initial step of 3% hydrogen peroxide block (K4011, DAKO) for 5 min in order to quench endogenous peroxidase activity, followed by an avidin-biotin block (Biotin Blocking system, X0590, DAKO) and a serum-free protein block for 30 min (X0909, DAKO). A primary mouse antibody against E-cadherin (1:500; BD Transduction Laboratories™, NJ) was biotinylated with Dako ARK™ Hematoxylin counterstaining with Mayer’s hematoxylin. Stained tissue sections were counterstaining with Mayer’s hematoxylin. Stained tissue sections were coverslipped using Ultramount No. 4 (Fronine Laboratory Supplies, New South Wales, Australia).

The HIF1α expression intensity (H-score) was calculated by summing the products of the percentage of positively stained surface epithelial cells (0-100) and the staining intensity (1, 2 or 3). The slides were scored in a blind analysis by two researchers (D.M. and F.B.).

Western blot analysis
HCT15 cells (CCL-225; American Type Culture Collection) were treated with 50 μM sulindac sulfide (Sigma-Aldrich) for 1-24 h and analysed by western blotting following our previously established protocols (Mladenova 2011). The features assessed included: acute and/or chronic inflammation, lymphoid aggregates, hyperplastic and/or degenerative changes of the surface epithelium, crypt architectural distortion, fibrosis and neoplasia – classified as epithelial dysplasia or adenocarcinoma. Inflammation was assessed using a scoring system modified from the literature (Cummins et al., 2008; Vowinkel et al., 2004). Three independent parameters were measured: severity of inflammation, depth of injury/inflammation and crypt damage, and scored as shown below. The total histological score was calculated through summing of the three independent scores with a maximum score of 12. For severity of inflammation, representative tissue images are shown in supplementary material Fig. S1 (0=no inflammation; 1=slight – presence of mucosal inflammatory cell infiltrate without significant distortion of the crypt architecture; 2=erosion – superficial ulceration that involved only the surface epithelium and lamina propria; 3=ulceration – defined as loss of the colonic mucosa associated with an acute inflammatory reaction extending at least through the muscularis mucosae). The depth of inflammation was scored as 0=none; 1=mucosal; 2=mucosal and submucosal; 3=transmural. For crypt damage, representative tissue images are shown in supplementary material Fig. S2 (0=none; 1=only surface epithelium damaged; 2=surface crypt and epithelium damaged; 3=basal 1/3 crypt damaged; 4=basal 2/3 crypt damaged; 5=entire crypt lost and surface epithelium damaged; 6=entire crypt and epithelium lost; grades 3 and 4 are not a feature of this model).

Immunohistochemistry analysis
Sections from tissue paraffin blocks were cut on a Leica microtome. Antigen retrieval was performed in a pressure cooker for 25 s using target retrieval solution at pH 6 (S1699, DAKO, Carpinteria, CA). A Dako autostainer was used for immunohistochemistry. The protocol included an initial step of 3% hydrogen peroxide block (K4011, DAKO) for 5 min in order to quench endogenous peroxidase activity, followed by an avidin-biotin block (Biotin Blocking system, X0590, DAKO) and a serum-free protein block for 30 min (X0909, DAKO). A primary mouse antibody against E-cadherin (1:500; BD Transduction Laboratories™, NJ) was biotinylated with Dako ARK™ Hematoxylin counterstaining with Mayer’s hematoxylin. Stained tissue sections were counterstaining with Mayer’s hematoxylin. Stained tissue sections were coverslipped using Ultramount No. 4 (Fronine Laboratory Supplies, New South Wales, Australia).

The HIF1α expression intensity (H-score) was calculated by summing the products of the percentage of positively stained surface epithelial cells (0-100) and the staining intensity (1, 2 or 3). The slides were scored in a blind analysis by two researchers (D.M. and F.B.).

Western blot analysis
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et al., 2011, 2013). Only adherent cells were analysed for protein expression. The membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4°C (β-actin, 1:10,000, clone AC15, Sigma-Aldrich), phosphorylated c-Jun (Ser73) no. 9164, c-Jun no. 9165 (Cell Signaling Technology), E-cadherin (1:2000, no. 610181, BD Biosciences), β-catenin (1:2000, no. 610153, BD Biosciences), p120-catenin (1:1000, clone H-90, no. sc-13957, Santa Cruz Biotechnology). ImageJ densitometry software (National Institutes of Health) was used for quantitative densitometry analysis.

AHR knockdown
Knockdown of AHR was performed using three unique 27mer AHR small interfering (si)RNA duplexes as follows: A– 5′-GGAAUGUACAGAGAACAUAAGCT–3′; B– 5′-GGACUAGAAGUAAACUACC–3′; C– 5′-UGCUGCGCAAAAGAUAUUUGGT–3′. Non-targeting siRNA (SR30004; OriGene) was used as control. Transfection was performed in triplicate with 10 nM siRNA using X-tremeGENE HP DNA Transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. Cells were treated with 50 µM sulindac sulfide following 24-h transfection and then harvested for mRNA quantification at the indicated time points.

Q-PCR analysis
RNA was extracted using QIAeasy mini (Qiagen GmbH, Germany). Q-PCR reactions were performed using SYBRgreen (Applied Biosystems) as ligands: implications for toxicity and xenobiotic detoxification. The transcription factor snail represses E-cadherin in breast cancer. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell. Mol. Life Sci. 60, 1376-1393.

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