

The NSAID sulindac is chemopreventive in the mouse distal colon but carcinogenic in the proximal colon

Short title: Carcinogenic effect of sulindac in the mouse proximal colon

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Abbreviations: AOM, azoxymethane; IEC, intestinal epithelial cells; MMR, mismatch repair; NSAIDs, non-steroidal anti-inflammatory drugs; WT, wild type

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ABSTRACT

Background and aims: The non-steroidal anti-inflammatory drug sulindac is an effective chemopreventive agent in sporadic colorectal cancer but its potential benefit in mismatch repair deficient cancers remains to be defined. We wanted to determine whether genetic defects that are relevant for colorectal cancer, such as *Msh2* or *p53* deficiency, would influence the efficiency of sulindac chemoprevention or increase the side effects.

Methods: *Msh2* or *p53* deficient and wild type mice received feed containing 160-320 ppm sulindac for up to 25 weeks with or without a concurrent treatment with the carcinogen azoxymethane. Colon tissue was analysed by histopathology and molecular biology methods.

Results: We show that sulindac prevented azoxymethane-induced distal colon tumours in all mice. In the proximal colon, however, sulindac induced new inflammatory lesions on the mucosal folds, which further developed into adenocarcinoma in up to 18-25% of the *p53* or *Msh2* deficient mice but rarely in wild type mice. This region in the proximal colon was characterised by a distinct profile of pro- and anti-inflammatory factors, which were modulated by the sulindac diet, including upregulation of *Hypoxia Inducible Factor 1 α* and *Macrophage Inflammatory Protein 2*.

Conclusions: These data show that the sulindac diet promotes carcinogenesis in the mouse proximal colon possibly through chronic inflammation. Sulindac has both beneficial and harmful effects *in vivo*, which are associated with different microenvironments within the colon of experimental mice. Deficiency for the *Msh2* or *p53* tumour suppressor genes increases the harmful side effects of long-term sulindac treatment in the mouse colon.

SUMMARY

What is already known about this subject

- NSAID sulindac prevents tumours in many animal models of colorectal cancer and has also shown some promise in clinical trials.
- Long-term use of some NSAIDs is associated with significant gastrointestinal side effects.
- There are conflicting reports on the efficacy of sulindac in ApcMin mice, raising questions as to whether certain germline or somatic gene defects reduce the efficiency of chemoprevention or increase the side effects.

What are the new findings

- We show that sulindac prevented carcinogenesis in the distal colon of wild type and *p53* or mismatch repair deficient mice.
- However sulindac induced new inflammatory lesions in the mouse proximal colon, which progressed to malignancy more frequently in *p53* or mismatch repair deficient mice.
- The premalignant changes started from mild surface damage in the colon epithelium, which progressed to chronic inflammation if the sulindac diet was maintained.
- This region in the proximal colon was characterised by a distinct profile of pro- and anti-inflammatory factors, which were differentially modulated by the sulindac diet, including upregulation of *MIP-2* and *Hif1 α* .
- Sulindac has both beneficial and harmful effects *in vivo*, which are associated with different microenvironments within the colon.

How might it impact on clinical practice in the foreseeable future

- It is unlikely that sulindac use in humans leads to as serious side effects as in the genetically modified *Msh2* or *p53* deficient mice.

INTRODUCTION

The great potential of non-steroidal anti-inflammatory drugs (NSAIDs) in cancer chemoprevention has been recognised for decades.[1] For example, in a recent clinical trial of aspirin in the Lynch syndrome, there was a long term reduction in the incidence of colorectal carcinoma after the trial had been concluded.[2,3] Lynch syndrome patients are carriers of DNA mismatch repair (MMR) gene mutations and have a high risk of developing recurrent tumours. The data on sulindac in the Lynch syndrome are limited, but short-term administration of sulindac increased epithelial cell proliferation in the proximal colon of MMR mutation carriers, raising concerns about its potential chemopreventive effect.[4] The NSAIDs celecoxib and rofecoxib reduced the risk of sporadic colorectal adenomas in clinical trials, but were associated with serious side effects, including cardiovascular problems or gastrointestinal ulcers, bleeding and obstructions.[5,6] Subsequently, rofecoxib was withdrawn from clinical use in arthritis but other NSAIDs are among the most commonly used medicines.

It is now widely recognised that apart from gastroduodenal toxicity, NSAIDs also have significant side effects in the colon, such as non-specific, eosinophilic or ischemic colitis, ulcers, strictures and exacerbation of colonic diverticular disease.[7] This implies that NSAIDs can have diametrically opposed effects on the colon, chemoprevention of tumours on the one hand and induction or exacerbation of inflammation on the other. Chronic inflammation can lead to carcinogenesis, as is well known in chronic colitis and the dextran sodium sulfate mouse model of this disease.[8,9]

The mechanism of NSAID chemoprevention and the cause of the side effects *in vivo* remain unclear. NSAIDs are highly effective in preventing tumour development in some animal models, such as the azoxymethane (AOM) model of colorectal cancer in rats and in $p53^{\Delta/+}$ or $p53^{\Delta/\Delta}$ mice.[10,11] Dietary sulindac also caused a significant decrease in the

number of polyps in *ApcMin* mice.[12] However, a subsequent study reported a decrease of tumour development in the small intestine of *ApcMin* mice but an increase of incidence, multiplicity and volume of tumours in the colon, especially in the caecum, with a sulindac diet.[13] In the *Apc^{Δ/+}Msh2^{Δ/Δ}* mice no difference was observed in the numbers of polyps or aberrant crypt foci (ACF) in the colon between mice receiving control diet, sulindac or a specific COX-2 inhibitor, MF-tricyclic.[14]

Therefore, we wanted to determine whether genetic defects that are found in hereditary or sporadic colorectal cancer, such as the MMR or *p53* deficiency, would influence the efficiency of sulindac chemoprevention *in vivo*, or increase the side effects of this treatment. We chose to use sulindac as it has been commonly used in mice and rats, and to compare its effect in wild type, *Msh2^{Δ/Δ}* and *p53^{Δ/Δ}* mice. Deficiency of the *MSH2* gene causes a MMR defect, which is found in Lynch syndrome cancers and a subset of sporadic cancers, and *p53* is a common defect in sporadic cancers. As neither the *p53^{Δ/Δ}* mice nor the *Msh2^{Δ/Δ}* mice develop spontaneous colorectal tumours, we used the carcinogen AOM to induce tumours in the distal colon. Although many features of AOM-induced tumours in wild type mice or rats are consistent with sporadic colorectal cancer in humans,[15] a striking discrepancy is the absence of both the *p53* and *Msh2* gene defects. Here, we have addressed this issue by using genetically modified strains, which allow separate assessment of each defect in the AOM model.

MATERIALS AND METHODS

Mouse lines

HIF1α^{F/F} were crossed with *VILCre* mice (B6.SJL-Tg(vil-cre)997Gum/J; Jackson Laboratories, Bar Harbor, Maine)[16,17] and heterozygous *HIF1α^{Δ/+}* knockout mice to produce the test genotype *VILCre^{cre/+}HIF1α^{Δ/F}*, which are deficient for *HIF1α* in the

intestinal epithelial cells (Δ IEC), and the control genotype *VILcre*^{+/+}*HIF1 α* ^{Δ /F}, which expresses HIF1 α from the floxed (F) allele. Recombination efficiency in the colon was determined as previously described.[18] *Msh2* ^{Δ / Δ} , *p53* ^{Δ / Δ} , *p53* ^{Δ /+}, and the corresponding wild type (WT) siblings were obtained by crossing heterozygotes.[19,20] All mice were on the C57Bl/6J background.

Administration of AOM and sulindac

Mice (8 weeks old) were given three weekly injections (15 mg/kg) of AOM (Sigma-Aldrich, St Louis, MO) or vehicle (saline) only and feed containing either 160 (half) or 320 ppm (full) sulindac (Sigma-Aldrich; Specialty Feeds, Glen Forrest, Western Australia) for 22 weeks (started 2 weeks prior to AOM) or control feed.[10,11] Additional groups of mice received the sulindac diet without AOM treatment for 1-25 weeks. Colons were opened longitudinally and all distal tumours and proximal lesions, which were visible under a dissecting microscope, were counted and measured. The total surface area of tumours or lesions per mouse was determined as previously described.[21] A subset of colons were stained with 0.2% methylene blue for 4 min for the measurement of tumour/lesion dimensions.

Histopathology analysis

All visible lesions and tumours from each colon were biopsied for histopathology analysis together with biopsies of macroscopically normal tissue. The entire length of the biopsies was examined equivalent to 7-14 high power fields. The severity of inflammation was assessed over the entire area that was inflamed. All specimens, including untreated controls, were analysed by an anatomical pathologist (JED). Assessment was conducted

using blinded slides, and findings were peer-reviewed by a second pathologist for concordance.

The features assessed included: acute and/or chronic inflammation, lymphoid aggregates, hyperplastic and /or degenerative changes of the surface epithelium, architectural distortion, fibrosis, and neoplasia, classified as epithelial dysplasia or adenocarcinoma. Dysplasia was graded as negative, indefinite for low-grade dysplasia, low-grade and high-grade dysplasia according to the Riddell classification.[22] Only biopsies that show convincing neoplastic glands within a desmoplastic stroma extending beyond the muscularis mucosae were regarded as invasive adenocarcinoma. Some proximal colon biopsies with severe inflammation showed bland appearing displaced glands extending through the muscularis mucosae, which on occasion even showed some mucin distension associated with these glands. These were regarded as pseudoinvasion or lesions of colitis cystica profunda.

Ulceration was defined as loss of the colonic mucosa associated with an acute inflammatory reaction extending at least through the muscularis mucosae. An erosion was defined as superficial ulceration that involved only the surface epithelium and superficial underlying lamina propria. Mild acute inflammation was arbitrarily defined as the presence of 5-30 neutrophils per high power field in the mucosa. Mild chronic inflammation was arbitrarily defined as 30-200 chronic inflammatory cells in the superficial and deep lamina propria /high power field without significant distortion of the crypt architecture. Severe chronic inflammation involved >500 chronic inflammatory cells/high power field causing expansion of the lamina propria and extending at least into the submucosa. Hyperplastic change was defined as an increase in the number of epithelial cells in the lining mucosa often associated with a “frilly” appearance of the surface epithelium similar to hyperplastic polyps in the human colon. Number of apoptotic cells/crypt column was assessed from H&E stained colon sections as previously described,[13] based on morphological features, including cell

shrinkage, nuclear condensation and presence of apoptotic bodies. Minimum of 20 crypts (40 crypt columns) were counted.

mRNA and protein analysis

The mucosal surface of the proximal lesions and the uninvolved tissue from proximal and distal colon was lightly scraped and snap frozen in liquid nitrogen for RNA extraction. Q-PCR reactions were performed using SYBRgreen, TaqMan assays (Applied Biosystems, Foster City, CA) or UPL assays (Roche Applied Science) on ABI Prism 7900-HT Real Time PCR system (Applied Biosystems). For protein analysis snap frozen mouse tissues were homogenised in standard RIPA buffer and the resulting lysates analysed with western blotting (primary antibodies for cleaved caspase 3, 1:1000, #9661, BclxL, 1:1000, #2764, Bcl-2, 1:1000, #2870s, Cell Signaling Technology, Beverly, MA, Bax, 1:1000, sc-493, p21, 1:500, sc-397-G, Santa Cruz Biotechnology, Santa Cruz, CA, and β -actin, 1:10000, clone AC15, Sigma-Aldrich).

Cell line analysis

HCT15 cells (CCL-225™, ATCC, Manassas, VA, USA) were propagated in RPMI 1640 media supplemented with FBS (10% (v/v), ThermoTrace, Noble Park, VIC, Australia). Cells were incubated overnight in reduced serum conditions (0.2% FBS) and were stimulated with TNF α (Peprotech Inc., Rocky Hill, NJ) or sulindac sulfide (Sigma-Aldrich), dissolved in the vehicle control DMSO (Sigma-Aldrich).

Immunohistochemistry

Colon tissue was fixed in 10% formalin and embedded in paraffin following standard procedures. Sections were incubated with the following antibodies: HIF1 α (1:100,

60 min, sc-8711, Santa Cruz Biotechnology, Santa Cruz, CA); Ki67 (1:200, 60 min, clone SP6, NeoMarkers, Fremont, CA). Positive and negative control for Hif1 α was generated by incubating freshly isolated pieces of colon tissue in special media as previous described in hypoxic (1% oxygen) or normoxic conditions for 4 h.[23] Hif1 α 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).

For analysis of hypoxia, mice were injected with 60 mg/kg pimonidazole HCl (Natural Pharmacia International, Burlington, MA), a well validated hypoxia marker,[24] and sacrificed after 3 h. Hypoxic areas of the colon were detected by incubating tissue sections with monoclonal antibody raised against pimonidazole (1:10, 40 min, Natural Pharmacia International). Antibody binding was visualized with the Animal Research Kit (ARKTM, DAKO). Positive and negative controls were generated by incubating colon tissue with 50 μ g/ml pimonidazole HCl for 2 h in hypoxic or normoxic conditions.

Measurement of drug concentration in colon mucosa

Colon mucosa was lightly scraped and snap frozen, then weighed and homogenised in liquid nitrogen. Sulindac and its metabolites, sulindac sulfone and sulindac sulfide, were extracted as previously described[25] and analysed with a Thermo Scientific Quantum Access triple quadrupole mass spectrometer coupled to a Thermo Scientific Accela UHPLC system (Thermo Fisher Scientific, Waltham, MA). Quantitation was performed using external calibration curves, corrected using the internal standard, piroxicam, 1ng/ μ l.

Statistical analysis

Gene/protein expression and sulindac metabolites in tissue were compared using t-tests, and the overall frequencies of neoplasia with Fisher's Exact Test. Severity of inflammation,

numbers of apoptotic cells/crypt column, number/size of tumours or lesions and Bax/BclxL, Bax/Bcl2 ratios were compared using the Wilcoxon Mann Whitney test (StatXact 8, Cytel Software Corporation, Cambridge, MA).

RESULTS

Sulindac prevents carcinogenesis in the distal colon regardless of mouse genotype.

Carcinogenesis was induced in the distal colon by administration of the carcinogen AOM (fig 1A-C), and both the *Msh2*^{Δ/Δ} and *p53*^{Δ/Δ} mice developed significantly more and larger tumours than their wild type (WT) littermates (p=0.035 and p=0.015, respectively). All mice receiving a diet containing sulindac developed fewer and smaller tumours and this effect was dose-dependent (fig 1B,C). Histopathology analysis showed that the sulindac treatment also reduced the frequency of neoplasia (fig 1D, supplementary table 1).

Sulindac triggers carcinogenesis in the proximal colon.

Examination of methylene blue stained colons revealed new lesions developing as a result of the sulindac diet in the mucosal folds of the proximal colon (fig 1E), in contrast with the AOM-induced tumours, which were located within 2 cm from the anus. Very few proximal lesions were detected when mice received AOM treatment alone, i.e. without sulindac diet, and both doses of sulindac treatment led to a highly significant increase in both the number and size of lesions in all genotypes including the WT (fig 1F,G). The macroscopic appearance ranged from obvious thickening of the mucosal folds to clearly defined flat lesions on the mucosal folds. Histopathology analysis revealed that the lesions ranged from acute and chronic inflammation through to dysplasia and invasive carcinoma (fig 1D, fig 2A-E). Adenocarcinoma was found in up to 25% of *Msh2* and 18% of the *p53* deficient mice, but at the most in only one WT mouse in the sulindac-treated groups

(supplementary table 1). Subsets of *Msh2* or *p53* deficient mice that did not receive the carcinogen AOM treatment also developed neoplasia in the proximal colon either with the full sulindac or the half sulindac diet (fig 1H, supplementary table 1). There was no statistical difference in the frequency of proximal adenocarcinoma between the groups that received AOM+sulindac or sulindac alone. This indicates that the effect of sulindac was not due to an interaction with AOM. There was very good agreement between the two pathologists for diagnosing adenocarcinoma ($\kappa = 0.969$).

We then carried out a systematic pathology comparison between the lesions and tumours in the proximal and distal colon and the surrounding uninvolved mucosa (supplementary tables 2-3). Hyperplastic changes were more common in the proximal lesions (up to 100%) than in the distal tumours (up to 25%). Mucinous adenocarcinomas and tissue ulceration were only found in the proximal colon. Tissue damage (erosion and ulceration) was more frequent in the lesions than surrounding uninvolved tissue in all genotype groups ($p < 0.0001$) but was also found in the surrounding mucosa.

Sulindac metabolites accumulate in the colon mucosa.

Much of the water absorption from the bolus occurs at the mucosal folds in the proximal colon where the faecal material is still in semi-liquid form, while the faeces are solid in the distal colon. To test whether sulindac contained in the semi-liquid bolus causes a higher accumulation of drug in the proximal colon, we measured the concentrations of sulindac and its two metabolites, sulfide and sulfone in the mucosal lining. Sulindac concentration was low (fig 2F), whereas its derivatives accumulated at higher concentrations than the pro-drug. Sulfone and sulfide concentrations were marginally higher in the proximal colon than in the distal colon, but this was not statistically significant.

Premalignant proximal lesions develop in a defined region of the proximal colon.

We treated additional groups of WT littermates from the *p53* line with the full sulindac diet to analyse the development of the proximal premalignant lesions. Small lesions were visible under the dissecting microscope after 1 week but the lesions were significantly larger from 10-20 weeks, and the number of lesions per mouse increased at 20 and 25 weeks (supplementary fig 1). Histopathology analysis revealed infiltration of inflammatory cells as early as one week after sulindac treatment. Depth of inflammation in the lesions progressed from mucosal (1 week sulindac treatment) to transmural (25 weeks), as well as crypt damage from surface crypt and epithelium damage (1 week) to entire crypt and epithelium lost (25 weeks, supplementary fig 1). Most lesions were found in a defined 1 cm region of the mucosal folds (fig 2G). This region was labelled as P2 in subsequent analyses.

Sulindac-induced inflammation is associated with a slight increase of epithelial cell apoptosis

Sulindac-associated carcinogenesis is associated with a decrease of apoptosis in the caecum of *ApcMin* mice.[13] We next determined if we could observe changes in the rate of apoptosis after sulindac treatment. There was a 8.3-fold increase in the number of apoptotic cells/crypt column in the lesions ($p=0.05$) and a 4-fold increase ($p=0.08$) in the surrounding tissue compared to WT mice receiving control food. This increase was not seen in the middle and distal colon in sulindac treated mice (supplementary table 4). Western blot analysis for the apoptotic marker cleaved caspase 3 also demonstrated a slight increase of apoptosis in the P2 region of sulindac treated mice (fig 3A). We next assessed pro- and anti-apoptotic markers Bax, Bcl-xL and Bcl-2 by western blot and qPCR analysis and determined the ratio of Bax/Bcl-xL and Bax/Bcl2, which can indicate changes in the sensitivity of cells to apoptosis.

These ratios remained similar in the mice treated with sulindac compared to control mice, with minor increases in the proximal colon (supplementary fig 2). Thus we did not observe a decrease but a slight increase of apoptosis, which may be explained by increased inflammation as previously reported in ulcerative and experimental colitis.[26,27] We also examined p21, which mediates the chemopreventive effect of sulindac in *Apc1638*^{+/-} mice.[28] p21 expression was not significantly changed in the P2 region, but there was a small but significant increase in the distal colon (fig 3B).

Sulindac modulates expression of pro-inflammatory genes in the colon.

As colon neoplasia was clearly associated with the inflammatory lesions in sulindac fed mice, we next examined a panel of pro- and anti-inflammatory factors, which have been previously implicated in mouse models of colon inflammation, such as HIF1 α and NF κ B target genes. Constitutive expression of *HIF1 α* augments inflammation in experimental colitis[29] and the NF κ B pathway links inflammation and cancer in the AOM/DSS model of colitis.[9] We detected strong upregulation of *HIF1 α* by sulindac in the lesions and in the uninvolved mucosa at the P2 region (fig 4A). Of the NF κ B target genes,[9,29] the most striking effect was seen for *MIP-2*, *IL1 β* and *COX-2*, which were very strongly upregulated by the sulindac diet in the lesions (fig 4A). *A20* an anti-inflammatory and anti-apoptotic factor,[30] was slightly downregulated in the P2 region, but the expression of other NF κ B target genes *IL6*, *TNF α* and *ICAM* was variable and they were not strongly upregulated by the sulindac diet (fig 4A). We also examined *PECAM*, which mediates transendothelial leukocyte migration in experimental colitis,[31] and found that it was slightly upregulated in the lesions. *iNOS* was upregulated by the sulindac diet only in the distal colon.

As we observed strong *MIP-2* upregulation in the mucosal lining throughout the colon in sulindac-treated mice, we next tested whether sulindac has the same effect *in vitro*

independent of inflammatory cells. MIP-2 is the mouse homolog of IL8, a chemokine that is overexpressed in many solid cancers and causes recruitment of infiltrating neutrophils to the tumour microenvironment.[32] We treated the COX-2 deficient human colon cancer cell line HCT15 with sulindac sulfide. This treatment resulted in nearly 40-fold upregulation of *IL8* but only 2-3 fold upregulation of *TNF α* and *ICAM* and had no effect on *IL6* expression (fig 4B). *TNF α* treatment of HCT15 cells was used as a positive control to demonstrate strong upregulation of *IL8*, *ICAM* and *TNF α* upon cytokine stimulation.[33] Therefore, we conclude that the sulindac diet can differentially modulate the expression of pro-inflammatory genes in the proximal and distal colon mucosa and that the sulindac-induced lesions have the highest expression of pro-inflammatory factors.

Sulindac diet induces HIF1 α overexpression in the site susceptible to lesions.

HIF1 α is a transcription factor that regulates many aspects of cancer biology but can also function as a barrier protective factor in the colon.[18,34] HIF1 α protein is rapidly degraded unless it is stabilised by pro-inflammatory cytokines or hypoxic conditions. Therefore, we next investigated if HIF1 α protein was expressed in the lesions apart from its transcriptional upregulation. There was an increase of nuclear HIF1 α protein expression in the lesions compared with the surrounding mucosa and the distal colon (fig 5A). The pattern of HIF1 α overexpression was confined to the surface epithelium of sulindac-induced lesions. However, this overexpression was not accompanied by upregulation of the Hif1 α responsive genes *CD73* and *ITF*, which are important mediators of epithelial barrier protection. For *CD73*, there was a gradient of increasing expression from proximal to the distal colon, where *CD73* was expressed more than 100-fold higher than in the P1 region (fig 5B). *ITF* also showed higher expression in the distal colon compared to the proximal colon.

Mucosal damage was profound in the lesions and resulted in crypt elongation and increased cell proliferation as assessed by immunohistochemistry for the proliferation marker Ki67 compared to normal mucosa (fig 5C,E). Ki67 positive cells were found at the epithelium surface in the lesions, while in normal conditions they were confined to the proliferative zone of the crypt base. Many of the cells that were positive for HIF1 α also stained positive for Ki67 (fig 5D arrows). We conclude that *HIF1 α* is upregulated by the sulindac diet in the P2 region and that the HIF1 α protein is stabilised in epithelial cells that have proliferative potential.

Next we investigated if HIF1 α protein expression at the site of the lesions was associated with hypoxia, as it has been suggested that NSAID-induced tissue ulceration observed in some patients is caused by reduced blood supply to the site of damage.[35] We used pimonidazole, a previously validated hypoxia marker and detected positive staining in the region of the mucosal folds, but also in the rectum and intermittently in other regions (supplementary fig 3). Similar pattern of hypoxia staining was seen in both sulindac fed and control mice. Since any variation in tissue collection or fixation can affect the staining intensity between different mice, it cannot be determined if sulindac increased hypoxia. However, it can be concluded that the site of the lesions was prone to hypoxia, but this was not the only region affected.

Hif1 α expression is pro-inflammatory in the proximal colon lesions.

To determine whether HIF1 α has a pro-inflammatory rather than protective function in the proximal colon, we analysed mice deficient for *HIF1 α* in the intestinal epithelial cells (IEC), *VILcre^{cre/+} HIF1 α ^{Δ /F}* (*HIF1 α ^{Δ /IEC}*) and the control genotype *VILcre^{+/+} HIF1 α ^{Δ /F}* (*HIF1 α ^{Δ /F}*), which has retained *HIF1 α* expression in the colon epithelium from the floxed allele. Recombination efficiency was high for both the proximal (97%) and the distal (95%)

colon epithelium and there was a 20-fold decrease in *HIF1 α* mRNA expression in the mucosa of *HIF1 $\alpha^{\Delta/\Delta IEC}$* mice. The two groups developed similar numbers of macroscopic lesions with the sulindac diet, but there was a small but non-significant decrease in the total surface area of the lesions in *HIF1 $\alpha^{\Delta/\Delta IEC}$* mice. The frequency of adenocarcinoma was 15.4% in *HIF1 $\alpha^{\Delta/F}$* and 5.3% in *HIF1 $\alpha^{\Delta/\Delta IEC}$* mice. There was significantly less inflammation in the *HIF1 $\alpha^{\Delta/\Delta IEC}$* group (fig 6, supplementary fig 4) than in the sibling controls *HIF1 $\alpha^{\Delta/F}$* , indicating that lack of *HIF1 α* expression in the colon alleviates the inflammatory response caused by sulindac. This was seen in both the non-involved tissue (p=0.0006) and in the lesions (p=0.0039). This indicates that HIF1 α expressed by epithelial cells may play a role in modulating the inflammatory response in this mouse model.

DISCUSSION

This study was designed to determine if sulindac chemoprevention was affected by genetic defects that are important in colorectal cancer, such as *p53* deficiency or the MMR defect. We have shown that sulindac was as effective in preventing AOM-induced distal colon tumours on the background of *Msh2* or *p53* deficiency as in the WT mice. However, the sulindac treatment also caused side effects in the proximal colon, which were more pronounced in mice with *Msh2* or *p53* deficiency. These results are in agreement with a recent report of intestinal and proximal colon carcinogenesis in response to sulindac, in mice heterozygous for a mutant *Apc* or the MMR gene *Mlh1*. [36]

It was striking that the sulindac-induced lesions were confined to a specific region (P2) of the proximal colon. Small lesions with inflammatory cell infiltration became visible after just one week of sulindac diet. These early lesions showed surface crypt and epithelium damage suggesting initial mucosal irritation by sulindac. This region may be more susceptible to mechanical surface irritation because most water absorption from the bolus is

completed at the mucosal folds. Although the lesions progressed to neoplasia more frequently in mice with *Msh2* or *p53* deficiency, the macroscopic lesions developed to a similar extent in all mice, including the WT. Therefore, the initial insult to the mucosa was similar in all mice, suggesting that these early lesions were premalignant regardless of the genetic background. Our model does not single out a specific gene that is responsible for the early tissue inflammation in the P2 region, but has identified a combination of factors that characterised its unique response to the sulindac diet.

A number of genes were differentially expressed between different parts of the colon or further regulated by the sulindac diet in the P2 region, including upregulation of *HIF1 α* , *PECAM* and the NF κ B target genes *MIP-2*, *IL1 β* and *COX-2*. *MIP-2* was strongly upregulated throughout the colon in sulindac-treated animals, but was most prominent in the lesions of the P2 region. *MIP-2* is the mouse homolog of chemokine *IL8*, which is emerging as a pro-cancer master regulator of several important pathways.[32] Here we have shown for the first time that sulindac also upregulates *IL8* in human colorectal cancer cells *in vitro*. *IL8* is overexpressed in many solid cancers, where it causes recruitment of infiltrating neutrophils to the tumour microenvironment, and promotes proliferation of human colon cancer cells *in vitro*. [32,37] *MIP-2* also increases neutrophil and lymphocyte recruitment in the mouse intestine and increases inflammation in the DSS model of colitis.[38,39] The active role of epithelial cells as modulators of the inflammatory process is now well accepted. In addition, we observed upregulation of *PECAM* in the lesions. *PECAM* is a cell adhesion factor involved in leukocyte migration, and promotes migration of blood monocytes to tissue, particularly at sites of inflammation, where they mature to tissue macrophages.[31,40] It is unclear if *PECAM* is a tumour promoter but it is expressed in colorectal cancer cells.[41] Thus upregulation of both *MIP-2* and *PECAM* is associated with the presence of infiltrating inflammatory cells in the lesions of the P2 region.

Pro-inflammatory factors *IL1 β* and *COX-2* were also upregulated in the lesions. It is now well accepted that *IL1 β* is a tumour promoter[42] and that *COX-2*, which is overexpressed in colorectal cancer, plays a key role in intestinal polyp formation.[43] It was unexpected that there was a slight increase of apoptosis in the lesions, as apoptosis is a chemopreventive mechanism linked to sulindac. Also, sulindac-induced tumorigenesis was previously shown to be associated with decreased apoptosis in *ApcMin* mice [13]. As we did not compare the effect of sulindac on apoptosis in all genetic backgrounds, the significance of this is unclear. However, these results are consistent with the findings in ulcerative colitis, as well as in DSS-induced colitis, where increased epithelial cell proliferation is associated with increased apoptosis.[26,27] We also observed slight downregulation of *A20* in the lesions, which is consistent with its role as a major anti-apoptotic and anti-inflammatory protein in a mouse model of experimental colitis.[30] Interestingly, we did not observe a change in p21 levels with sulindac in the proximal colon, whereas there was significant upregulation of p21 in the distal colon. The chemopreventive effect of sulindac in *APC1638^{+/-}* mice is abolished with targeted inactivation of p21.[28] Therefore, our results are consistent with the role of p21-mediated chemoprevention by sulindac in the distal colon.

A striking aspect of the proximal lesions in our study was upregulation of *HIF1 α* by the sulindac diet in the P2 region and protein overexpression of *HIF1 α* in proliferating epithelial cells expressing Ki67. *HIF1 α* is a transcription factor that activates many genes involved in cancer biology, including angiogenesis, cell proliferation/survival, glucose metabolism and invasion.[34] Increased *HIF1 α* signalling in the intestinal epithelial cells leads to a hyperinflammatory reaction in the mouse colon and its overexpression is a feature of serrated colon adenocarcinomas in humans.[29,44] *HIF1 α* is degraded in normoxic conditions unless stabilised by pro-inflammatory cytokines, such as *IL1 β* through the *NF κ B* pathway. The *HIF1 α* pathway has been described as a link between inflammation and

cancer[45,46] but the *HIF1 α ^{Δ IEC}* mice used here were not informative regarding the role of HIF1 α in cancer. The reduction of colon inflammation in these mice suggests that HIF1 α has a pro-inflammatory function in the sulindac model of proximal carcinogenesis. This was consistent with our observation that the HIF1 α responsive genes *ITF* and *CD73* that have been implicated in barrier protection,[47,48] were unaffected by the sulindac diet.

NF κ B activated genes, in particular *IL6*, are important in chronic colitis associated cancer.[9] Here we could not show strong upregulation of *IL6*, *TNF α* or *ICAM* by sulindac, but other NF κ B targets *MIP-2*, *IL1*, and *COX-2* were upregulated in the proximal lesions. Therefore, it appears that colon carcinogenesis in sulindac-induced lesions is associated with a different profile of NF κ B target gene expression compared to the AOM/DSS model of colitis.[9] However, the role of NF κ B activation in the colon carcinogenesis in this model can only be conclusively determined by using mice that have a specific deletion of IKK β in the colon.

This study has implications for proximal colon carcinogenesis in general. It is well known that there are significant differences in the molecular, pathological and clinical characteristics of tumours found in the proximal compared with the distal colon. For example, high microsatellite instability, gene promoter methylation and mucinous tumours are more common in the proximal colon and are associated with the serrated neoplasia pathway.[49,50] The tumours induced by sulindac share some features with proximal cancers, such as hyperplastic changes and a mucinous phenotype. Furthermore, serrated carcinomas have a different gene expression profile when compared with conventional carcinomas, including upregulation of HIF1 α . [44] Although it is unknown whether there is a comparable region in the human colon which is susceptible to sulindac-induced tumours, the sulindac model of cancer may be suitable for the further study of early proximal carcinogenesis.

In conclusion, this is the first report of a simultaneous carcinogenic and chemopreventive effect of sulindac in the mouse colon. The localised effect of sulindac in the proximal colon is associated with the development of inflammatory lesions, which progress into malignancy more rapidly in the absence of *Msh2* or *p53*. Furthermore, we provide evidence that dietary sulindac can modulate gene expression in the colon epithelium and thus may affect the epithelial-inflammatory cell crosstalk and regulation of the inflammatory process. Further investigation is necessary to determine if long-term use of sulindac has procarcinogenic effects in humans.

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FIGURE LEGENDS

Figure 1. Sulindac feed reduces the number and size of tumours in the distal colon but induces new lesions in the proximal colon.

(A) Location of tumours in the distal colon induced by AOM.

(B) Reduction in the total surface area of tumours in $p53^{\Delta/\Delta}$, $p53^{\Delta/+}$, $Msh2^{\Delta/\Delta}$ and corresponding WT siblings with increasing sulindac concentration (* $p \leq 0.05$, compared to AOM-only treated mice).

(C) Mean number of distal colon tumours per mouse showing a decrease in the number of AOM induced tumours with the sulindac treatment (* $p \leq 0.05$, compared to AOM-only treated mice).

(D) Frequency of colon neoplasia in $p53^{\Delta/\Delta}$, $p53^{\Delta/+}$, $Msh2^{\Delta/\Delta}$ and corresponding WT siblings. Sulindac diet decreased the frequency of distal colon neoplasia in AOM treated mice, while increasing the frequency of neoplasia in the proximal colon.

(E) Location of sulindac-induced lesions in the mouse proximal colon.

(F) Measurement of the total surface area of the proximal lesions induced by the sulindac diet in $p53^{\Delta/\Delta}$, $p53^{\Delta/+}$, $Msh2^{\Delta/\Delta}$ and their WT siblings (* $p \leq 0.05$, compared to AOM-only treated mice).

(G) Mean number of proximal lesions per mouse showing increase in the number of lesions with the sulindac treatment (* $p \leq 0.05$, compared to AOM-only treated mice).

(H) Frequency of colon neoplasia in $p53^{\Delta/\Delta}$, $p53^{\Delta/+}$, $Msh2^{\Delta/\Delta}$ and corresponding WT siblings, receiving the sulindac diet without the AOM treatment. Individual frequencies of neoplastic changes, low- and high-grade dysplasia and adenocarcinoma are shown in supplementary table 1.

Figure 2. Proximal colon lesions induced by the sulindac diet progress from acute and chronic inflammation to adenocarcinoma.

(A,B) H&E stained sections of macroscopically normal appearing proximal colon from sulindac-treated mice: low power photomicrographs showing mild hyperplasia of the epithelium and surface erosion (A&B, scale bars 100 μm); at higher power the area of erosion is characterised by a mixed inflammatory cell infiltrate and degenerative change of the surface epithelium without significant fibrosis (B insert, scale bar 20 μm).

(C-E) H&E stained sections from ulcerated areas of the proximal colon from sulindac-treated mice: low power photomicrograph showing mucosal ulceration with early fibrosis (C, scale bar 200 μm ; insert, scale bar 50 μm); well differentiated adenocarcinoma of the colon developing in an area of inflammation (D, scale bar 200 μm and insert, scale bar 50 μm); well differentiated mucinous adenocarcinoma of the colon developing in an area of inflammation (E, scale bar 200 μm and insert, scale bar 50 μm).

(F) Quantification of sulindac and its sulfide and sulfone metabolites in the epithelium of the proximal and distal colon in seven sulindac fed mice (25 weeks). Error bars indicate SEM (* $p \leq 0.05$).

(G) Schematic representation of the mouse colon. Most lesions are found in a well-defined 1 cm section of the proximal colon, 2-3 cm from the caecum (P2). The sum of the lesion surface area per mouse is shown for the proximal and distal colon regions over 25 weeks of sulindac treatment. Error bars indicate SEM.

Figure 3. Sulindac feed causes a slight increase of apoptosis in the mouse proximal colon.

(A) Quantification of apoptosis (western blot analysis for cleaved caspase 3) in the two proximal colon regions (P1 and P2), middle (M) and distal (D) colon. Rate of apoptosis was higher in mice treated with sulindac compared to mice on control feed, particularly in the uninvolved region susceptible to sulindac induced lesions (P2; data for lesions not shown). WT mice received 320 ppm sulindac feed or control diet for 10 weeks. Relative activated caspase 3 expression in sulindac-treated animals is normalised to β -actin and is presented as a fold change to the corresponding colon region of control mice (3 mice per group). Error bars indicate SEM (* $p \leq 0.05$).

(B) Western blot analysis for p21 expression in the colon mucosa of WT mice, treated with the control or 320 ppm sulindac diet for 10 weeks (7-8 per group). Relative p21 expression in sulindac-treated animals is normalised to β -actin and is presented as a fold change to the corresponding colon region of control mice. Error bars indicate SEM (* $p \leq 0.05$).

Figure 4. Sulindac feed modulates the expression of pro-inflammatory genes in specific regions of the mouse colon.

(A) qPCR analysis of *Hif1 α* , *MIP-2*, *IL1 β* , *Cox-2*, *TNF α* , *IL6*, *ICAM1*, *iNOS*, *A20* and *PECAM* in the colon mucosa of control and sulindac treated WT mice (n=4). mRNA expression was normalised to the housekeeping gene *rpl19*. Graphs represent fold change except for IL6, where no expression was detected in the control mice. Error bars indicate SEM. Only statistically significant comparisons are shown (* $p \leq 0.05$).

(B) qPCR analysis of *ICAM1*, *TNF α* , *IL6* and *IL8* expression in HCT15 cells, unstimulated or stimulated with 50 μ M sulindac sulfide or 20 ng/ml TNF α for 4 hours. Results are mean of

three independent experiments. mRNA expression was normalised to the housekeeping gene *GAPDH*. Error bars indicate SEM (* $p \leq 0.05$).

Figure 5. Sulindac fed mice show stabilisation of HIF1 α protein in the inflammatory lesions (P2) developing in the mouse colon, where HIF1 α is expressed in Ki-67 positive epithelial cells.

(A) Immunohistochemistry analysis of Hif1 α expression. Nuclear HIF-1 α expression was increased in the surface epithelium of sulindac-induced lesions. Quantification of Hif1 α IHC staining (H score) from sulindac treated WT mice.

(B) qPCR analysis for *ITF* and *CD73* mRNA expression normalised to *Rpl 19* in the colon mucosa of control and sulindac treated WT mice. Error bars are SEM. Only statistically significant comparisons are shown (* $p \leq 0.05$).

(C) Immunohistochemistry analysis of the proliferation marker Ki67. Ki67 expression in normal colon (upper panel) is confined to the proliferative zone of the crypt base, but is found throughout sulindac-induced lesions (lower panel). Scale bars 50 μm .

(D) Co-expression of Hif1 α and Ki67 in serial sections of sulindac-induced lesions (red arrows). Scale bars 50 μm .

(E) Quantification of the proliferative index, Ki67 in sulindac treated WT mice showing higher proliferation in lesions than the surrounding uninvolved mucosa. Bars represent mean percentage of crypt Ki67 positive cells. Error bars indicate SEM.

Figure 6. Loss of *Hif1 α* in the colon epithelium reduces sulindac-induced inflammation.

(A) Total surface area of proximal lesions per mouse averaged per genotype in *HIF1 α ^{Δ IEC}* and *HIF1 α ^{Δ f}* mice receiving the sulindac diet. Error bars indicate SEM (p=NS).

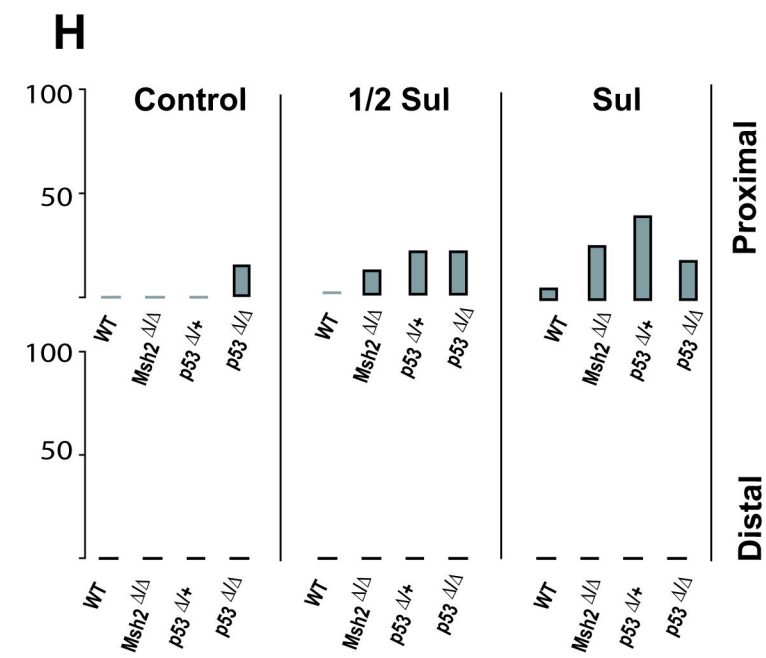
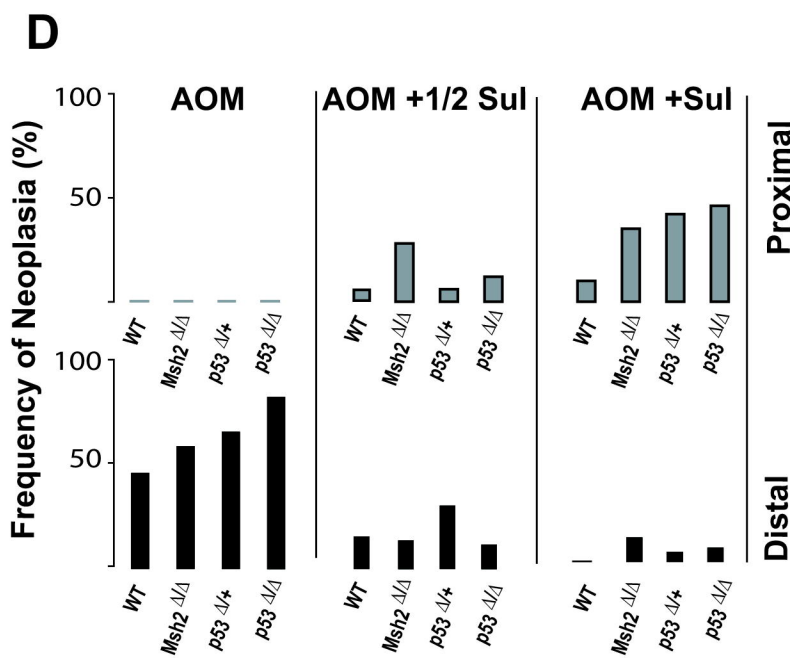
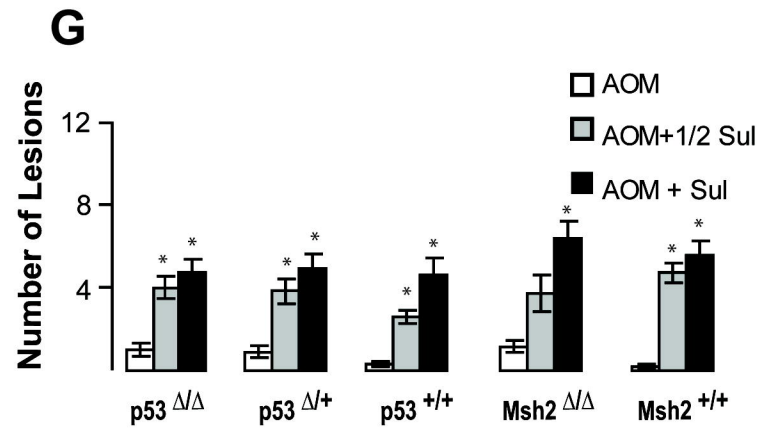
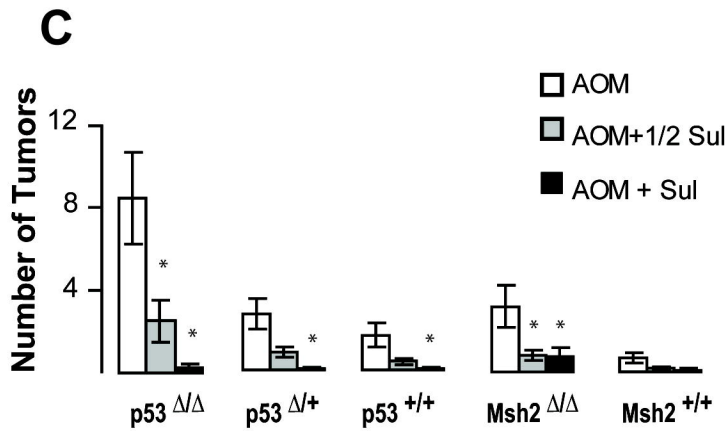
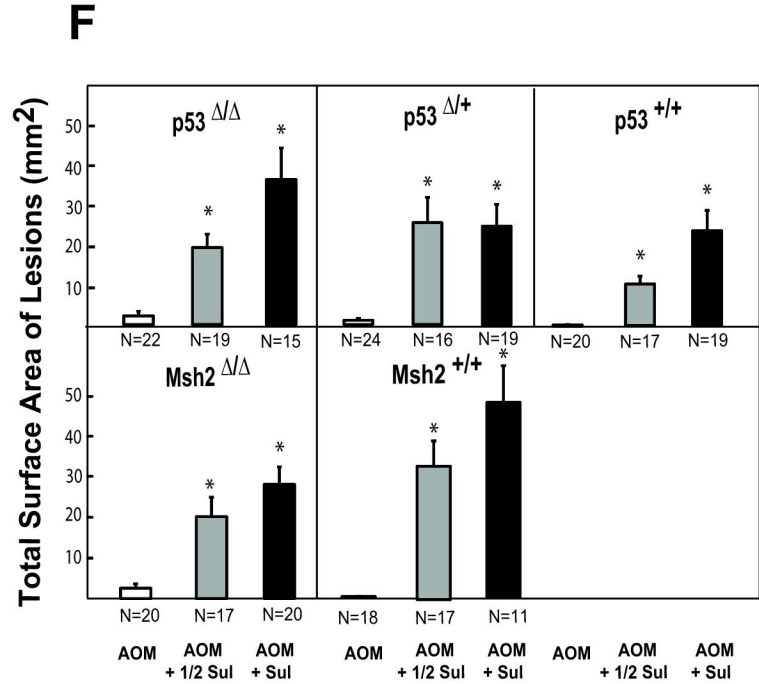
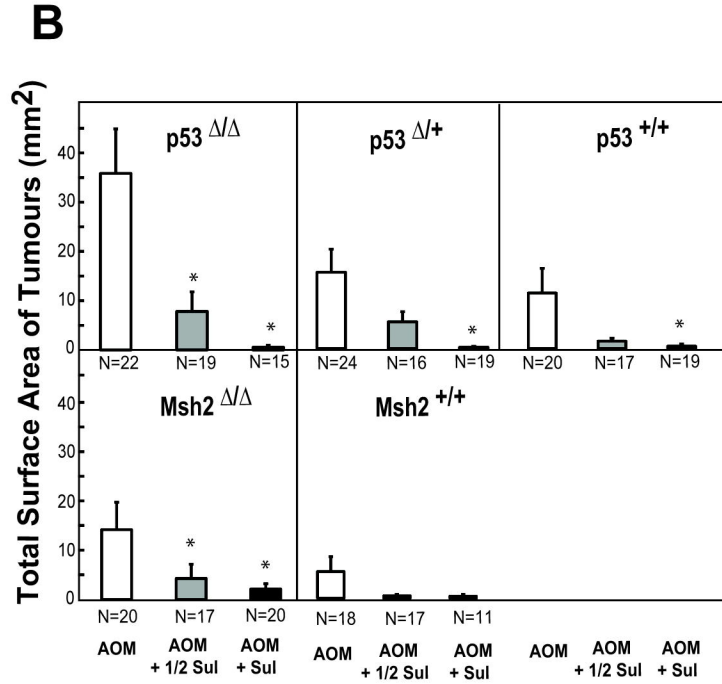
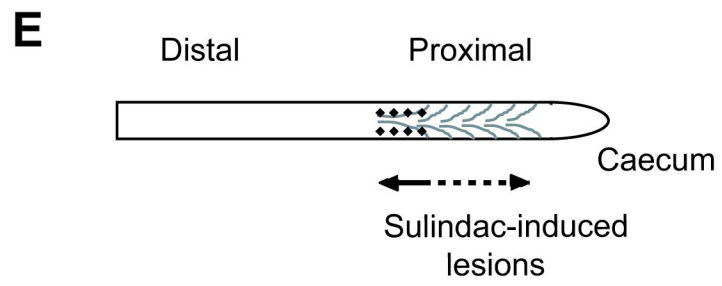
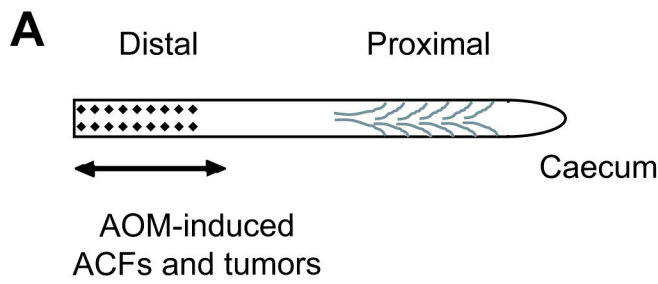
(B) Histopathology assessment of inflammation in proximal lesions and uninvolved colon biopsies. Inflammation score is significantly reduced in *HIF1 α ^{Δ IEC}* mice compared with *HIF1 α ^f* mice (p=0.0039, lesions; p=0.0006, uninvolved tissue). Bars indicate percentage of specimens in each inflammation category; 0= no inflammation, 1= mild, 2=moderate and 3=severe inflammation. The inflammation scores for specific regions of the mouse colon are shown in supplementary figure 4.

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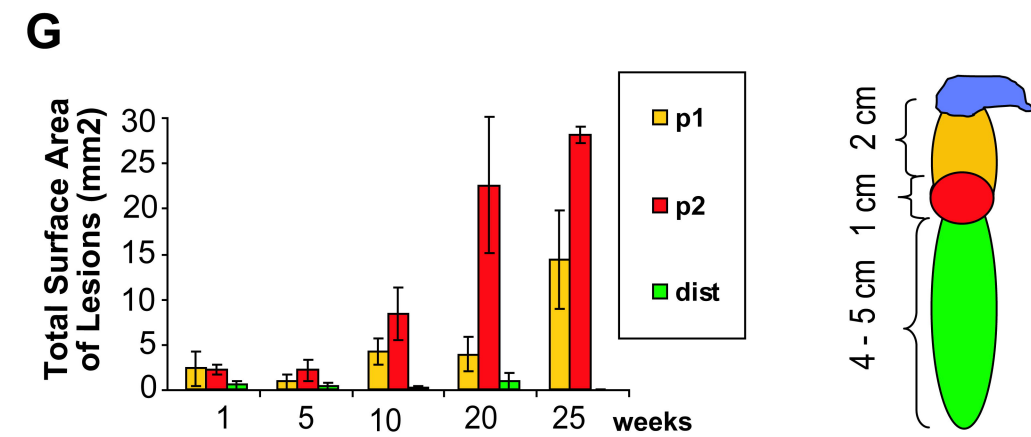
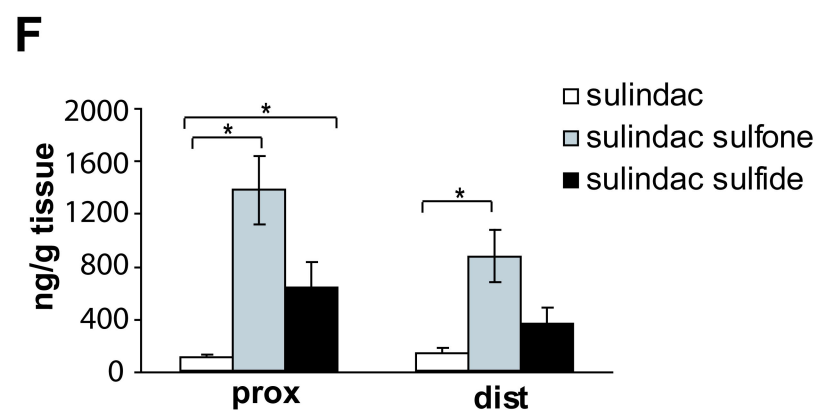
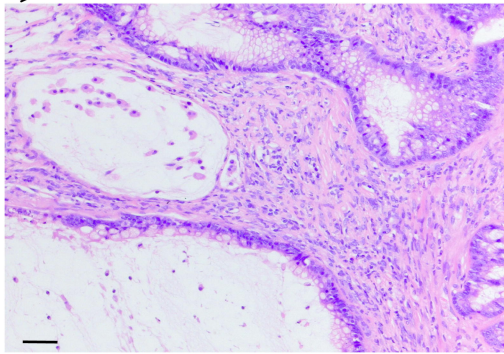
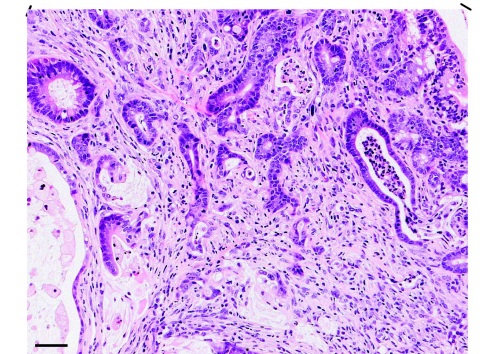
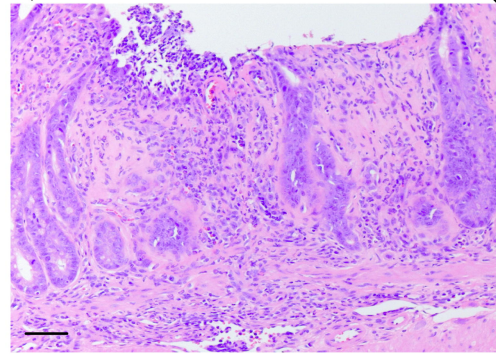
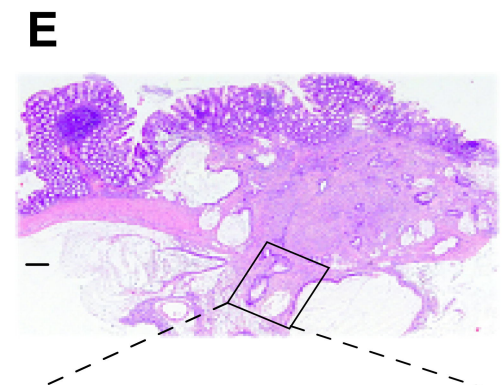
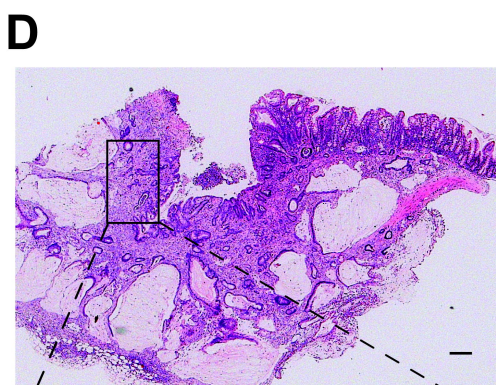
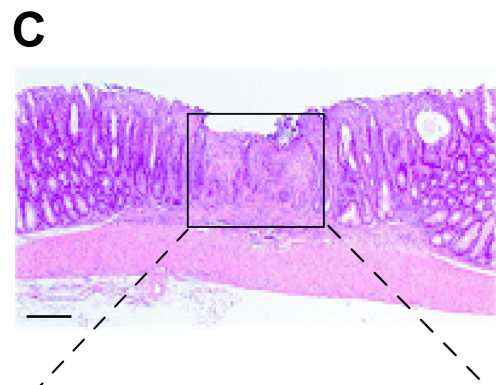
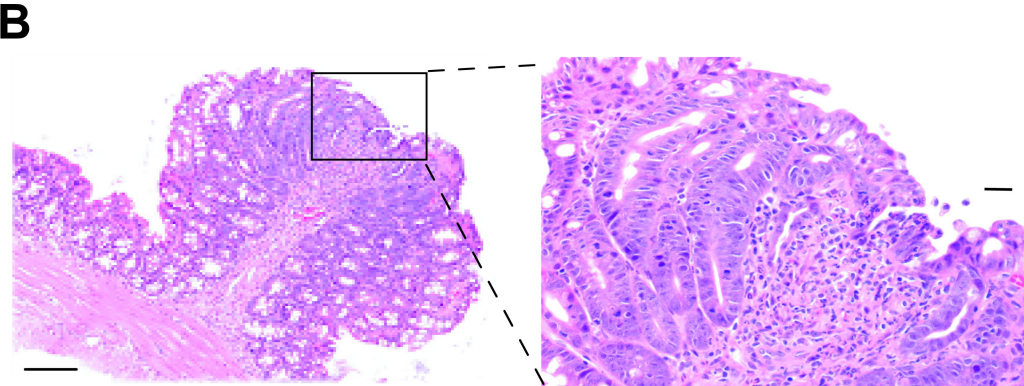
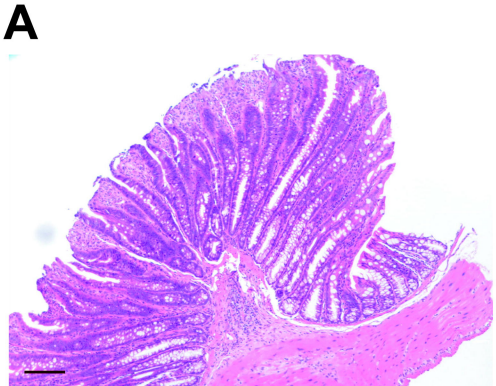


Figure 2

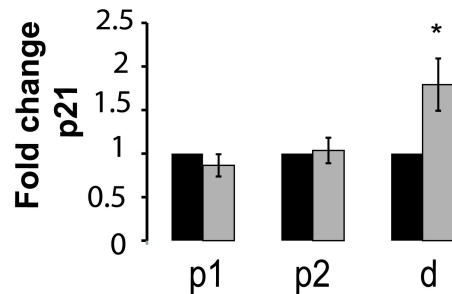
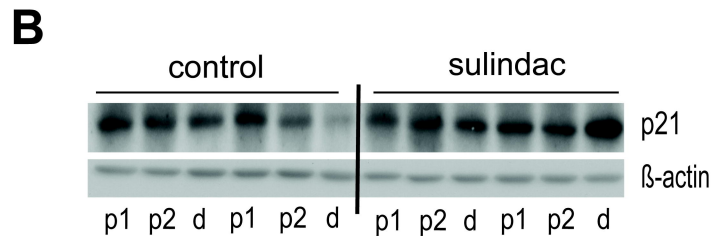
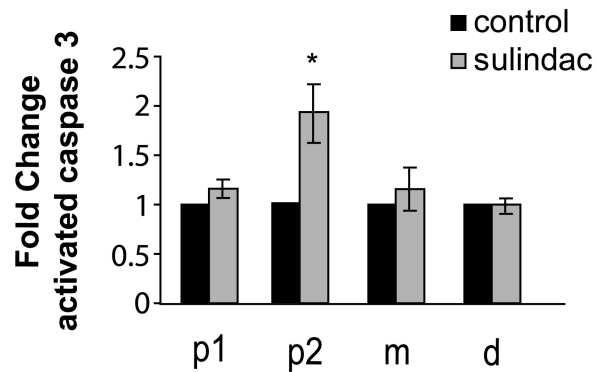
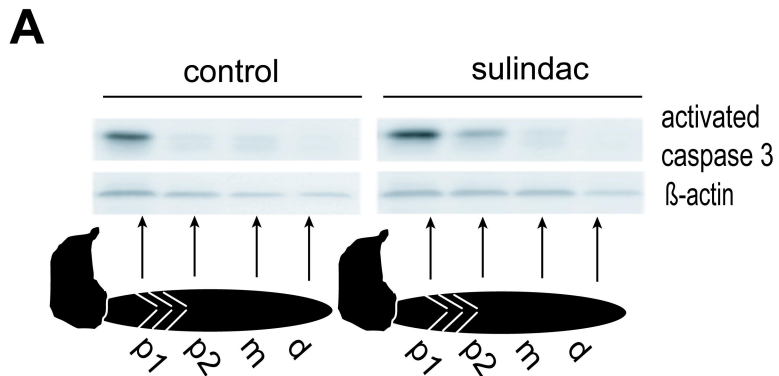
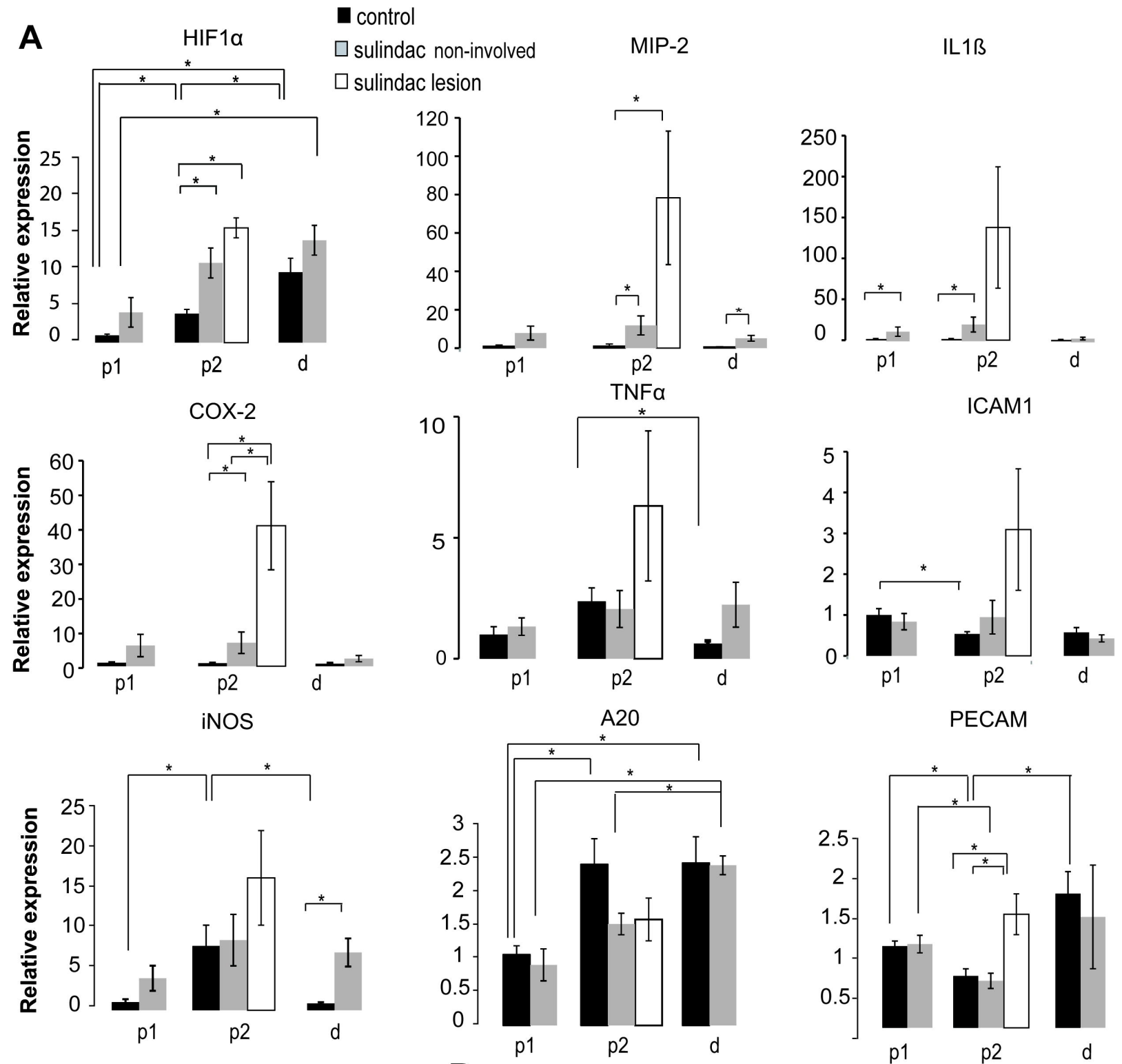
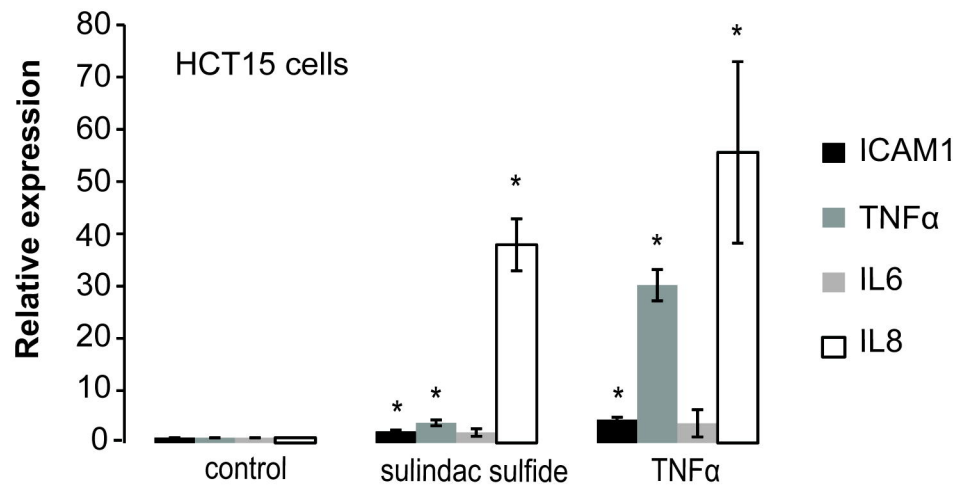
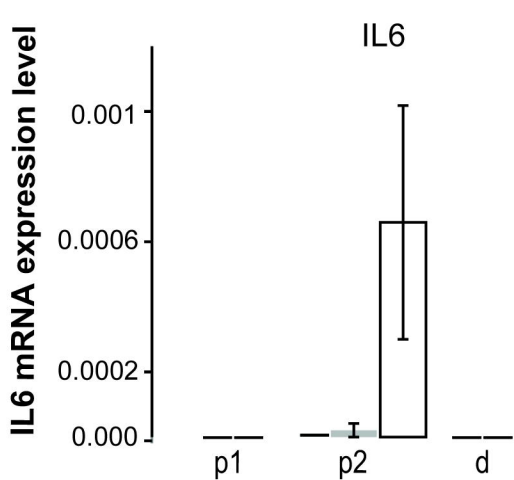


Figure 3



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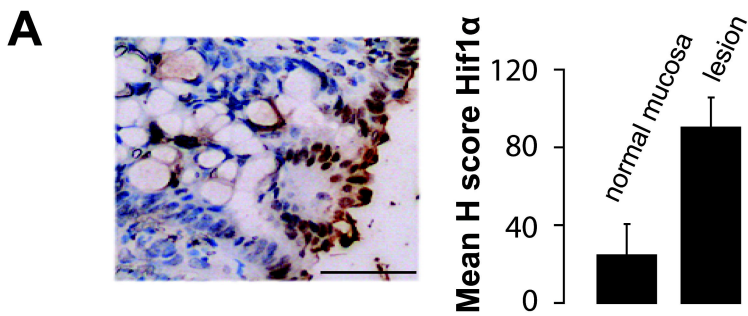


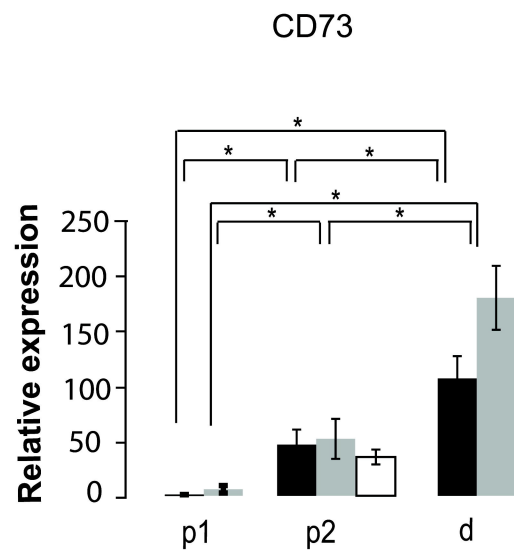
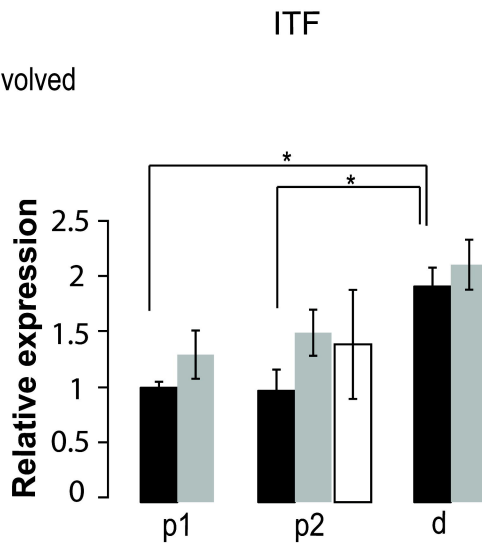
Figure 5

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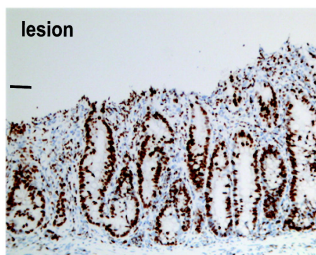
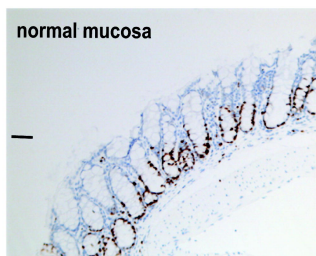
■ control

■ sulindac non-involved

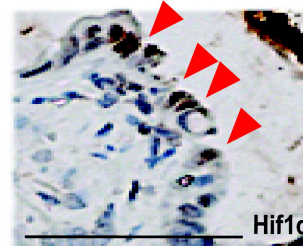
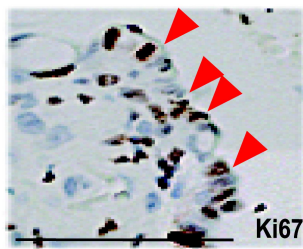
□ sulindac lesion



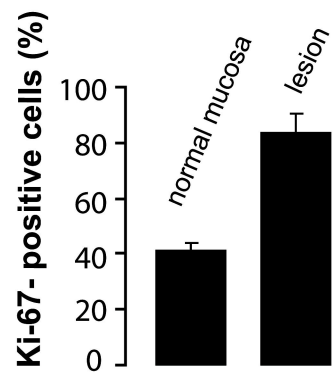
C



D



E



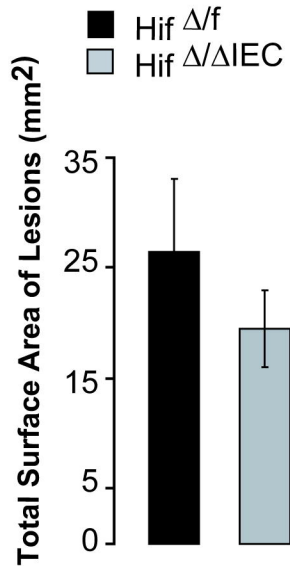
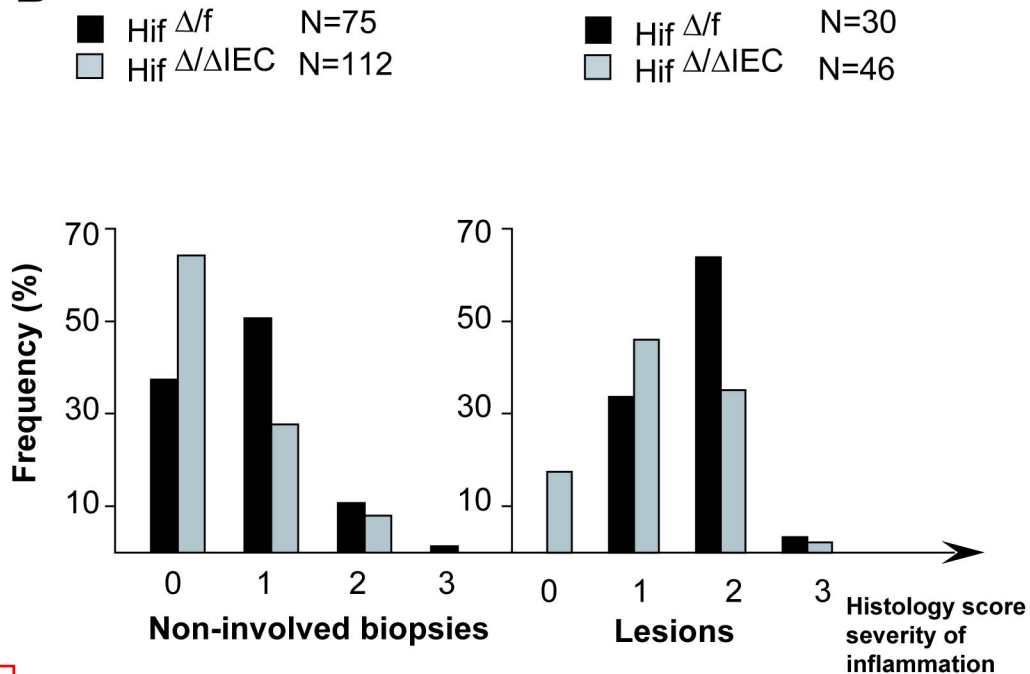
A**B**

Figure 6