# Hypoxic macrophages impair autophagy in epithelial cells through Wnt1: relevance in IBD

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**A defective induction of epithelial autophagy may have a role in the pathogenesis of inflammatory bowel diseases. This process is regulated mainly by extracellular factors such as nutrients and growth factors and is highly induced by diverse situations of stress. We hypothesized that epithelial autophagy is regulated by the immune response that in turn is modulated by local hypoxia and inflammatory signals present in the inflamed mucosa. Our results reveal that HIF-1α and Wnt1 were co-localized with CD68 in cells of the mucosa of IBD patients. We have observed increased protein levels of β-catenin, phosphorylated mTOR, and p62 and decreased expression of LC3II in colonic epithelial crypts from damaged mucosa in which β-catenin positively correlated with phosphorylated mTOR and negatively correlated with autophagic protein markers. In cultured macrophages, HIF-1 mediated the increase in Wnt1 expression induced by hypoxia, which enhanced protein levels of β-catenin, activated mTOR, and decreased autophagy in epithelial cells in co-culture. Our results demonstrate a HIF-1-dependent induction of Wnt1 in hypoxic macrophages that undermines autophagy in epithelial cells and suggest a role for Wnt signaling and mTOR pathways in the impaired epithelial autophagy observed in the mucosa of IBD patients.**

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract characterized by impairment of the intestinal epithelium function as a regulator of the host immune response to microbiota.[1,2] In colonic epithelial cells, autophagy has a homeostatic role by engulfing intracellular organelles and endogenous pathogens via double-membrane vesicles that fuse with lysosomes, leading to the degradation of their contents.[3–5] This process is mediated by intracellular microbial sensing via NOD2.[6,7] In recent years polymorphisms in gene loci containing autophagy-related proteins such as ATG16L1, IRGM, and NOD2 have been associated with an increased risk of IBD, which implicates a defective induction of autophagy in the pathogenesis of IBD.[3,6,8–10] Autophagy is regulated mainly by extracellular factors such as nutrients, hormones, and growth factors and is induced by situations of stress including starvation, hypoxia, and endoplasmic reticulum stress.[11,12] We hypothesized that immunological mechanisms modulate autophagy in the mucosa of IBD patients.

Macrophages constitute one of the central components of the inflamed mucosa, where local hypoxia and inflammatory mediators modulate their gene expression through the activity of hypoxia-inducible factors (HIFs).[13,14] These infiltrated macrophages could be the source of signaling molecules such as soluble growth factors or Wnt glycoproteins,[15,16] which by acting on epithelial cells may have an important role in the maintenance of intestinal homeostasis and tissue regeneration.[17–19]

The Wnt glycoprotein family comprises several ligands that mediate close-range signaling.[20,21] Two branches of Wnt signaling have been described: canonical and non-canonical pathways. Binding of canonical Wnt ligands to frizzled receptors results in the inhibition of glycogen synthase kinase 3 (GSK3), which is associated with the accumulation of β-catenin in the cytoplasm and translocation to the nucleus to activate gene expression.[22] Interestingly, stimulation of frizzled receptors has recently been related to activation of mTOR, a central negative regulator of autophagy.[12,23–26] Although most evidence suggests that the canonical Wnt signaling pathway is involved, some studies have shown that Wnt1 activates mTOR through an action that is mediated by both canonical and non-canonical-independent pathways.[27]

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The aim of the present study was to analyze the expression of autophagic protein markers in epithelial cells from the mucosa of IBD patients. Considering the hypoxic microenvironment of inflamed mucosa and the strategic position of macrophages in maintaining communication with epithelial cells, we set out to determine whether HIFs modulate the expression of Wnt1 in macrophages and to explore the role of this ligand on epithelial autophagy and the relevance of this pathway in IBD.

## RESULTS

### An impaired autophagy is observed in epithelial cells of the damaged mucosa of IBD patients

Analysis of the expression of autophagic protein markers in the mucosa of IBD patients revealed an impaired autophagy in the damaged mucosa compared with the non-damaged (**Figure 1b**). Western blot studies showed lower levels of LC3II and increased levels of p62 and phosphorylated mTOR (p-mTOR) in damaged vs. non-damaged mucosa. These differences were also observed in the mucosa of DSS-treated mice compared with the mucosa of control animals (**Figure 1a**), suggesting that activation of mTOR and impaired autophagy are associated with human and murine-injured colon.

The epithelial expression of autophagic protein markers was analyzed in isolated crypts from the mucosa of IBD patients (**Figure 1c**). Western blot protein analysis revealed increased levels of p62 and phosphorylated mTOR and decreased levels of LC3II in isolated crypts of damaged mucosa vs. non-damaged mucosa of the same patients (**Figure 1c**). The evaluation of the

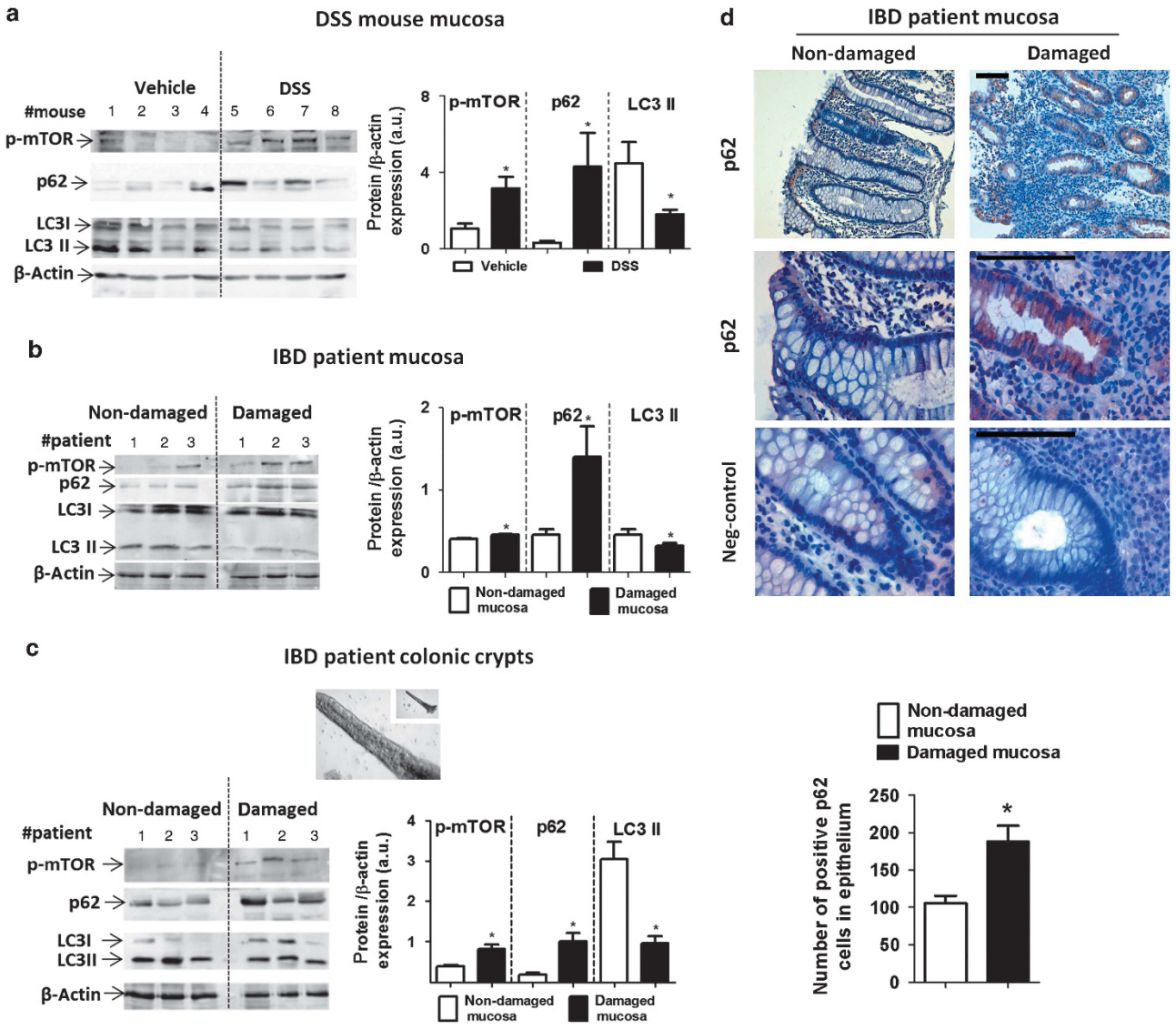


**Figure 1** Autophagic protein markers are reduced in damaged colonic mucosa. Representative western blots and graphs showing p-mTOR, p62, and LC3 expression in panel **a** the mucosa of vehicle- or DSS-treated mice ($n = 10$), (**b**) the mucosa of IBD patients ($n = 14$), and (**c**) the colonic crypts from IBD patients ($n = 14$); representative photographs showing isolated intestinal crypts of human mucosa, magnification $\times 20$ and $\times 40$. (**d**) Representative images showing p62 immunostaining in the damaged and non-damaged mucosa of IBD patients (scale bar 100 μm). Graph shows a quantitative analysis ($n = 14$) of the number of p62-positive cells in a total area of 0.135 $mm^2$. Bars in the graphs represent mean ± s.e.m. Significant difference from the respective non-damaged mucosa is shown by $^*P < 0.05$.

expression of these proteins in crypts obtained from the mucosa of patients with Crohn's disease (CD) and ulcerative colitis (UC) revealed non-significant differences (**Supplementary Figure S1a** online).

Moreover, immunostaining for p62 showed accumulation of this protein in epithelial cells of the damaged mucosa (**Figure 1d**). In this way, by highlighting differences in the expression of autophagic protein markers between damaged and non-damaged areas, our results suggest that epithelial autophagy is modulated by mechanisms associated with damage.

### HIF-1α stabilization and Wnt1 expression are upregulated in the damaged mucosa of IBD patients

We and others have previously reported HIF-1α stabilization in the mucosa of IBD patients.[28,29] In the present study, we perform a comparative study between damaged and non-damaged mucosa of the same patients. Quantitative analysis revealed that protein levels of HIF-1α were higher in the damaged mucosa of human and murine IBD samples than in the respective non-damaged mucosa (**Figure 2a**). In contrast, protein levels of HIF-2α did not differ significantly between injured and non-injured areas (**Figure 2a**). A similar pattern of the expression of these transcription factors was observed in the mucosa of patients with UC and CD (**Supplementary Figure S1a**). Immunohistochemical experiments revealed the presence of HIF-1α and HIF-2α in epithelial cells and cells of the lamina propria. Quantitative analysis showed a higher number of HIF-1α-positive cells in cells of the lamina propria of damaged mucosa vs. non-damaged mucosa, whereas the number of HIF-2-positive cells was similar in the injured and non-injured tissue (**Supplementary Figure S1b**).

Wnt ligands have been reported to regulate multiple aspects of intestinal pathophysiology. Our results show increased protein levels of Wnt1 in the damaged mucosa of both DSS-treated mice and IBD patients with respect to those observed in the respective non-damaged mucosa (**Figure 2a**). Furthermore, a significant increase in the mRNA expression of *Wnt1* was observed in the damaged vs. non-damaged human mucosa (**Figure 2b**). Our results demonstrate that both HIF-1α and Wnt1 are upregulated in the injured mucosa of IBD patients.

### HIF-1α stabilization and Wnt1 expression are detected in macrophages of the mucosa of IBD patients

Immunofluorescence experiments revealed that CD68-positive cells were co-localized with HIF-1α and Wnt1 in the mucosa

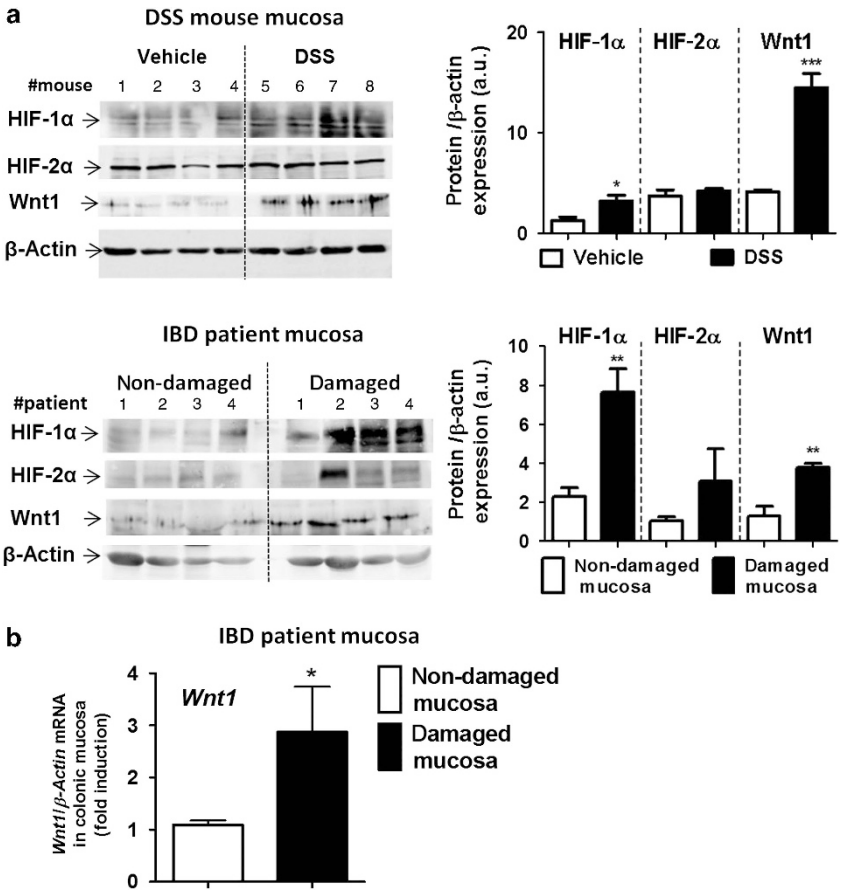


**Figure 2** HIF-1α protein levels and Wnt1 expression are increased in damaged colonic mucosa. (**a**) Representative western blots and graphs showing HIF-1α, HIF-2α, and Wnt1 protein levels in the mucosa of vehicle- or DSS-treated mice ($n=10$) and IBD patients ($n=14$). (**b**) Graph shows *Wnt1* mRNA expression in the damaged and non-damaged mucosa of IBD patients ($n=14$). In all cases, bars in the graphs represent mean ± s.e.m., and significant difference from the respective non-damaged mucosa is shown by $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

(**Figure 3a** and **b**), which shows that these proteins are expressed in gut macrophages. To confirm this, we isolated macrophages from the mucosa and evaluated the expression of Wnt1. Our results demonstrate that the percentage of macrophages that were positive for Wnt1 was significantly higher in macrophages from damaged mucosa than in non-damaged mucosa as well as the mRNA expression of *Wnt1* (**Figure 3c** and **d**).

### HIF-1 mediates the hypoxic upregulation of *Wnt1* in macrophages

Next, we set out to determine whether HIFs in cultured macrophages modulated the expression of Wnt1. First, we observed HIF-1α and HIF-2α stabilization in macrophages under hypoxia (**Figure 4a**). Interestingly, hypoxia increased the protein (**Figure 4a**) and mRNA expression (**Figure 4b**) of *Wnt1* with respect to normoxic cells. This was a consistent pattern, as it was observed in U937- and THP-1 macrophages as well as in monocyte-derived macrophages (MDMs) (**Figure 4b**, **Supplementary Figure S2a**). The role of HIF-1 and HIF-2 in *Wnt1* expression was determined using an miRNA approach to selectively knockdown these transcription factors in U937-derived macrophages (**Supplementary Figure S2b**). As shown in **Figure 4c**, hypoxic upregulation of *Wnt1* mRNA expression was significantly reduced in cells transfected with *miHIF1α,* but not in those transfected with *miHIF2α,* suggesting that HIF-1, and not HIF-2, is involved in the induction of this ligand by hypoxia. In line with this observation, the increase in the amount of Wnt1 protein induced by hypoxia was significantly diminished in *miHIF1α* cells (**Figure 4d**). No significant changes in the expression of *Wnt1* were observed in normoxic macrophages treated with *miHIF1α* or *miHIF2α* compared with mock-treated cells, which suggests that neither HIF-1 nor HIF-2 mediates the expression of this ligand in macrophages under normoxia (**Figure 4c**, **Supplementary Figure S2b**). A role for HIF-1 in the expression of Wnt1 in hypoxia was also observed in THP-1 macrophages (**Supplementary Figure S2c**).

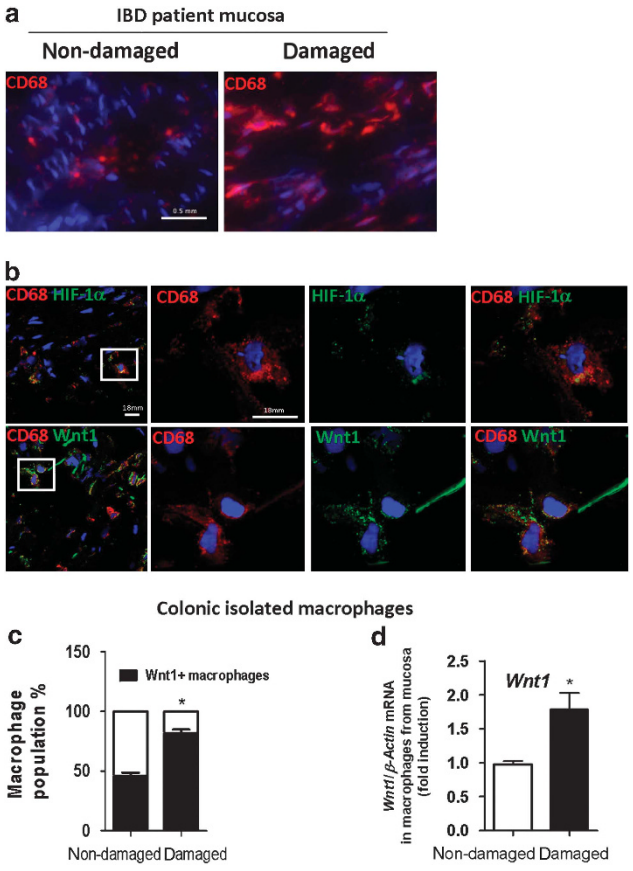


**Figure 3** HIF-1α stabilization and Wnt1 expression in macrophages of the mucosa of IBD patients. (**a**) Representative images of three experiments showing CD68 immunostaining in the damaged and non-damaged mucosa of IBD patients (Fluorescence microscopy). (**b**) CD68 co-localizes with HIF-1α and Wnt1 in the lamina propria of the mucosa (Confocal microscopy). (**c**, **d**) Macrophages were isolated from damaged and non-damaged mucosa of IBD patients ($n=3$), and graphs show the percentage of these cells that were positive for Wnt1 (**c**) and the *Wnt1* mRNA expression in these cells (**d**). Bars in the graphs represent mean ± s.e.m., and significant difference from the respective non-damaged mucosa is shown by $^*P<0.05$.

### Hypoxic macrophages-derived Wnt1 activates Wnt signaling pathways in epithelial cells

Macrophages in the mucosa are strategically located to interact with epithelial cells. The role of Wnt1 from hypoxic macrophages in the activation of the Wnt signaling pathway in colonic epithelial cells was analyzed in a co-culture setup in which cells were treated with *miWnt1* (**Supplementary Figure S2d**). A significant increase in protein levels of total and nuclear β-catenin and the mRNA expression of *Lgr5,* a target gene of Wnt, was detected in Caco-2 cells co-cultured with hypoxic macrophages vs. normoxic macrophages (**Figure 5a**). The increase induced by hypoxic macrophages was prevented when these cells underwent transient transfection with *miHIF1α* or *miWnt1* (**Figure 5a**). Our results suggest that *Wnt1* released from hypoxic macrophages has a role in the activation of the canonical Wnt signaling pathway in epithelial cells. This was confirmed when Wnt1 was administered exogenously to epithelial cells. As shown in **Figure 5b**, Wnt1 significantly increased β-catenin stabilization in Caco-2 cells with respect to the vehicle.

### Hypoxic macrophages-derived Wnt 1 activates mTOR and reduces autophagy in epithelial cells

The role of hypoxic macrophages in the expression of the autophagic protein markers p62 and LC3II and in the activation of mTOR in epithelial cells was evaluated in the aforementioned co-culture system. As can be seen in **Figure 5c**, the amount of LC3II was lower in Caco-2 cells that had been co-cultured with hypoxic macrophages than in those co-cultured with normoxic macrophages, while the amount of p62 and phosphorylated mTOR was higher. These results demonstrate that hypoxic macrophages activate mTOR and reduce autophagy in epithelial cells.

Interestingly, the amount of LC3II, p62, and phosphorylated mTOR in epithelial cells co-cultured with hypoxic macrophages treated with *miHIF-1* or *miWnt1* (**Figure 5c**) did not

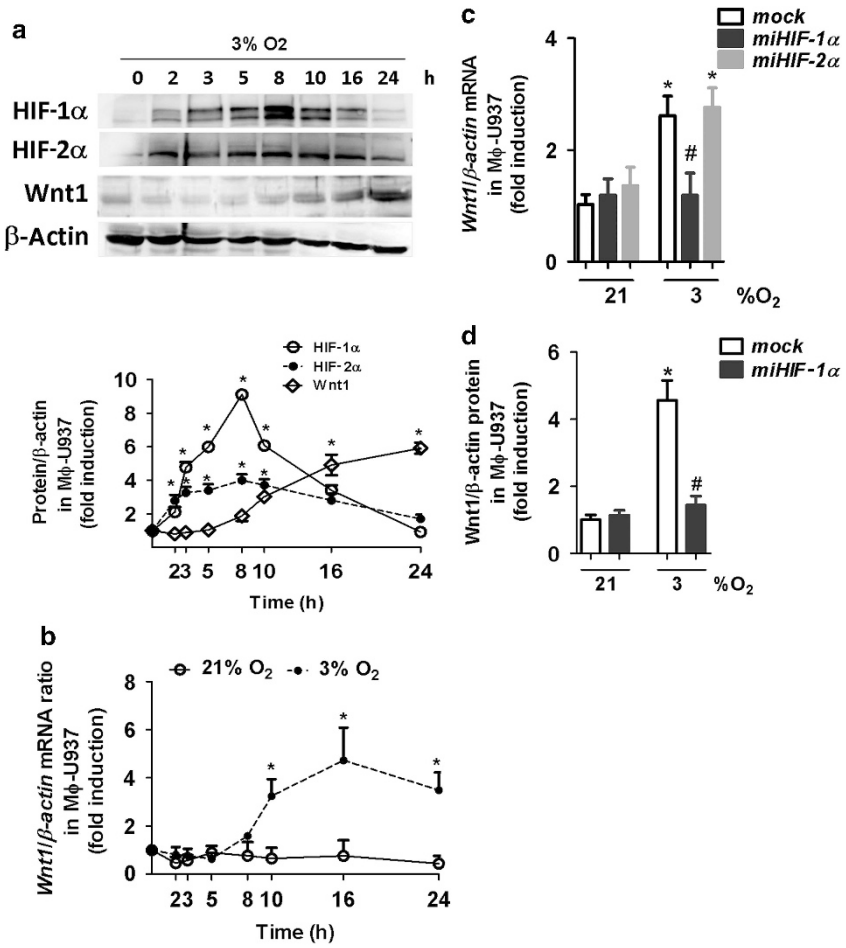


**Figure 4** HIF-1 mediates the hypoxic upregulation of Wnt1 in macrophages. (**a**) Western blots showing HIF-1α and HIF-2α stabilization and Wnt1 expression induced by hypoxia in U937 macrophages. Graph shows a time-course analysis of protein expression in these cells. (**b**) Graph shows a time-course analysis of the effects of hypoxia on *Wnt1* mRNA expression in U937 macrophages. In all cases, points in the graphs represent mean ± s.e.m. ($n > 3$). *$P < 0.05$. vs. time 0 h (**a**) and vs. macrophages in normoxia at the same time point (**b**). (**c**) Graph shows the mRNA expression of *Wnt1* in mock-transfected U937 cells and cells treated with *miHIF-1α* or *miHIF-2α* under normoxic or hypoxic conditions. (**d**) Graph shows Wnt1 protein expression in macrophages in normoxia or hypoxia in mock-transfected U937 cells and cells treated with *miHIF-1α*. In all cases, bars in graphs represent mean ± s.e.m. ($n > 3$). *$P < 0.05$. vs. the same group in normoxia and $^{\#}P < 0.05$. vs. mock-transfected cells in hypoxia.

differ significantly from those observed in normoxic macrophages, showing that the absence of HIF-1 or Wnt1 in hypoxic macrophages prevented the inhibition of epithelial autophagy induced by these cells. Under these co-culture conditions, the exogenous administration of Wnt1 reproduced the effects induced by hypoxic macrophages, which was evident in the lower amount of LC3II, the enhanced expression of p62, and the activation of mTOR (**Figure 5c**). Finally, the role of Wnt1 in epithelial autophagy was further confirmed when Wnt1 was administered exogenously to epithelial cells, which led to activation of mTOR, p62 accumulation, and reduced protein levels of LC3II (**Figure 5b**).

### Wnt signaling correlates with impaired autophagy in epithelial cells from the damaged mucosa of IBD patients

A comparative western blot analysis revealed increased β-catenin protein levels in crypts of damaged vs. non-damaged mucosa (**Figure 6a**), which provided evidence of the activation of the Wnt signaling pathway in the epithelial cells of injured mucosa. Immunohistochemical experiments revealed the presence of nuclear β-catenin immunostaining mainly located in epithelial cells of the damaged mucosa (**Figure 6b**). Interestingly, a detailed analysis of crypts isolated from damaged mucosa revealed a positive and significant correlation between β-catenin protein levels and phosphorylated mTOR (**Figure 6c**). In addition, protein levels of β-catenin positively correlated with those of p62 and negatively correlated with protein expression of LC3II in crypts of damaged mucosa (**Figure 6c**). These results point to a role for the Wnt signaling pathway in the impaired epithelial autophagy detected in the damaged mucosa of IBD patients.

## DISCUSSION

The present study demonstrates impaired autophagy in a murine model of colitis and in the intestinal mucosa of IBD patients. In both cases, differences in the expression of two

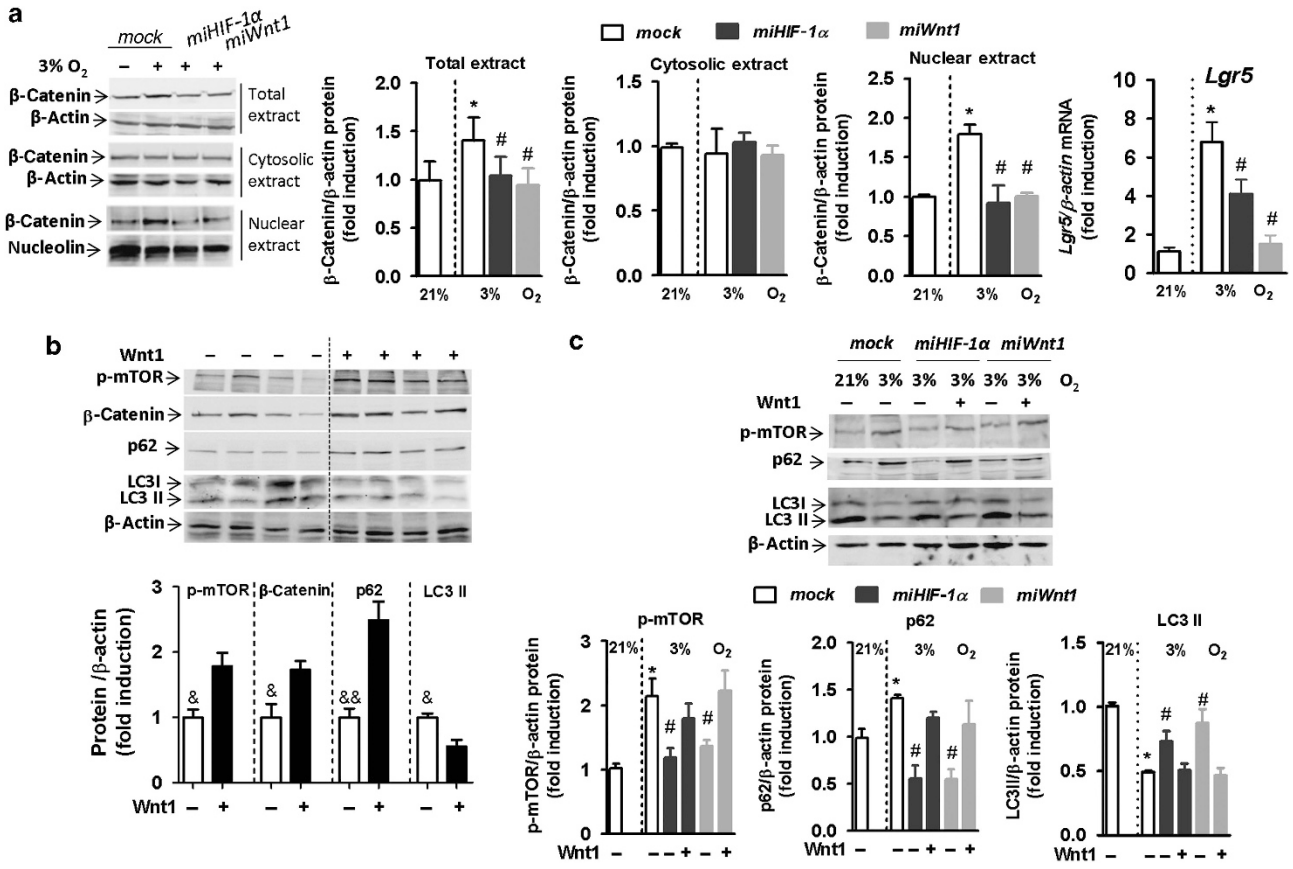


**Figure 5** Wnt1 from macrophages increases the canonical Wnt pathway and decreases autophagy in epithelial cells. (**a**) Caco-2 cells were co-cultured for 24 h with mock, *miHIF-1α* or *miWnt1* transfected U937-derived macrophages under normoxic or hypoxic conditions (16 h). Western blots showing protein expression of β-catenin in Caco-2 cells from total, cytosolic and nuclear extracts. Graphs show quantification of β-catenin protein by densitometry and *Lgr5* mRNA expression in Caco-2 cells. (**b**) Caco-2 cells were treated with Wnt1 (20 ng ml$^{-1}$) or vehicle for 24 h. Western blots and graphs showing protein expression of p-mTOR, β-catenin, p62, and LC3 ($n=3$). (**c**) Caco-2 cells were co-cultured as described in panel **a** and in some cases were treated with Wnt1 (20 ng ml$^{-1}$). Representative western blots and graphs showing protein expression of p-mTOR, p62, and LC3 ($n=3$). In all cases, bars in the graphs represent mean ± s.e.m. $^{*}P<0.05$. vs. mock-transfected U937 cells in normoxia, $^{\#}P<0.05$. vs. mock-transfected U937 cells in hypoxia and $^{\&}P<0.05$ and $^{\&\&}P<0.01$ vs. the respective Wnt1-treated cells.

autophagic protein markers, LC3II and p62, were observed between the damaged and non-damaged mucosa. Furthermore, the activation of mTOR, a central negative regulator of autophagy, was detected in the damaged area, which led us to suspect that colonic inflammation and injury may be associated with impaired autophagy. At the intestinal level, autophagy proteins are required for a variety of cellular functions, including antigen presentation by dendritic cells, cytokine secretion by macrophages, and antimicrobial peptide secretion by paneth cells.[3] The impaired autophagy detected in isolated crypts of damaged human mucosa indicates that inflammation and injury specifically affect epithelial autophagy in IBD.

Hypoxia and inflammatory cytokines are present in the mucosa of IBD patients[14,29] and may modulate the activity of HIFs. Our results show that levels of HIF-1α, but not of HIF-2α, are higher in damaged vs. the non-damaged mucosa of both IBD patients and DSS-treated mice. The upregulation of HIF-1 in damaged mucosa is in line with previous studies relating HIF-2 with mild hypoxia and HIF-1 with intense hypoxia,[30] a condition that is likely associated with mucosal inflammation and injury.[14,29] Previous studies have highlighted the presence of HIF-1α in epithelial cells of the mucosa of IBD patients,[29] where it promotes epithelial barrier function.[31] Our results demonstrate that HIF-1, in addition to epithelial cells, is also present in macrophages that infiltrate the mucosa of these patients, and we have analyzed whether epithelial function is modulated by the transcriptional activity mediated by HIF-1 in these cells.

Wnt glycoproteins are small signaling molecules that have a crucial role in the regulation of epithelial proliferation and differentiation.[14,20,21] Our immunohistochemical experiments reveal that macrophages in the lamina propria express Wnt1, and the studies we have performed in macrophages isolated from mucosa indicate that the proportion of cells expressing this ligand is higher in damaged mucosa than in normal tissue. This suggests that the enhanced expression of Wnt1 observed in damaged mucosa is due to infiltrated macrophages, which is

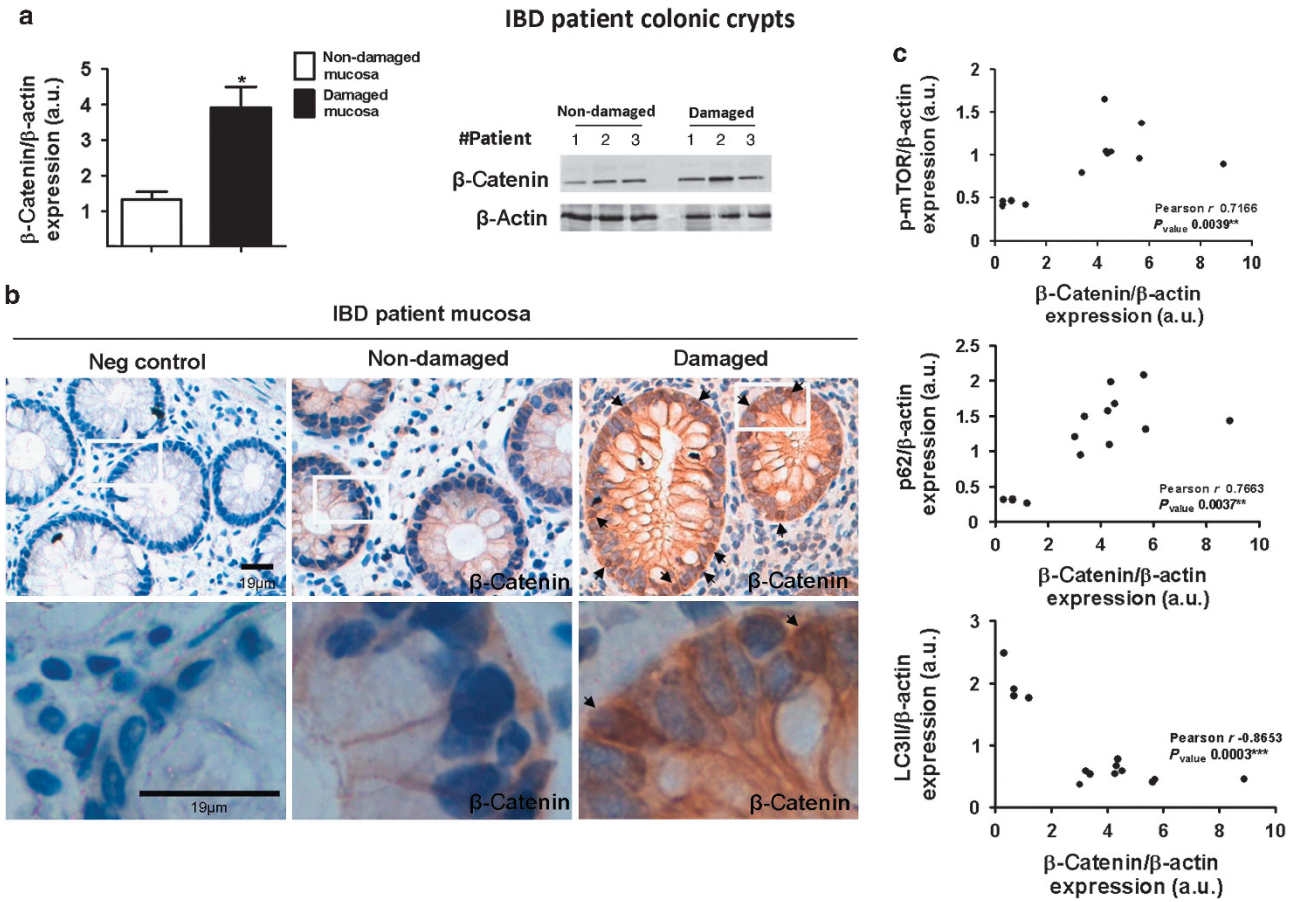


**Figure 6** Increased Wnt signaling correlated with decreased autophagy in epithelial cells of damaged mucosa. (**a**) Representative western blots and graph show β-catenin protein levels in crypts of the mucosa of IBD patients. Bars in the graph represent mean ± s.e.m. ($n = 14$); $^*P < 0.05$. vs. non-damaged mucosa. (**b**) Representative microphotographs of 14 patients showing β-catenin immunostaining in the mucosa of IBD patients. Arrows show nuclear staining. (**c**) A positive and significant correlation was observed between protein levels of β-catenin and the expression of p62 or p-mTOR in crypts of damaged human mucosa, whereas a negative and significant correlation was detected between protein levels of β-catenin and LC3II in the same crypts ($n = 14$).

consistent with the low levels of *Wnt1* mRNA expression reported in the colonic epithelial cells of IBD patients.[16,32]

Immunofluorescence analysis revealed the co-localization of HIF-1 and Wnt1 in macrophages of the mucosa and the presence of HRE sequences in the promotor region of the *Wnt1* gene led us to determine whether HIFs were involved in the expression of this ligand. We observed that hypoxia, which induced HIF-1α and HIF-2α stabilization, enhanced the expression of *Wnt1* in macrophages derived from two different cell lines and human monocyte-derived macrophages in a time-dependent manner. Interestingly, specific silencing studies revealed that HIF-1, but not HIF-2, mediated Wnt1 induction, which points to *Wnt1* being a target gene of HIF-1.

The results obtained in our co-culture system suggest that the increased synthesis of Wnt1 induced by HIF-1α in hypoxic macrophages acts in a paracrine way to modulate the epithelial compartment, since we detected the activation of Wnt signaling pathways in co-cultured epithelial cells. This is consistent with previous reports showing that epithelial cells respond to rather than produce Wnt ligands.[32] Of interest, we observed the activation of Wnt signaling in epithelial cells, which is associated with a decreased expression of autophagic protein markers. This effect was prevented after knocking down Wnt1 in macrophages and was reproduced by the exogenous administration of Wnt1. As a whole, these results suggest that Wnt1 mediates the impaired epithelial autophagy induced by hypoxic macrophages, which is in accordance with the role of this ligand in the epithelial activation of mTOR. Wnt ligands have been shown to activate the mTOR pathway through β-catenin-dependent[23,26] and canonical- and non-canonical-independent pathways.[27] Although we have not analyzed the precise molecular pathway involved, our results showing that hypoxic macrophages increase the protein levels of nuclear β-catenin in parallel to those of phosphorylated mTOR suggest that Wnt1 activates mTOR through the inhibition of GSK3. These results together with previously published evidence of autophagy negatively modulating Wnt signaling[33] suggest a regulatory feed-back mechanism between Wnt signaling and autophagy.

Finally, we set out to analyze whether Wnt signaling modulates autophagy in epithelial cells of the mucosa of IBD patients. Activation of the canonical Wnt pathway has previously been reported as a response to injury.[34,35] Our results endorse this by showing that canonical Wnt signaling is activated specifically in colonic epithelial crypts from damaged mucosa of IBD patients, as demonstrated by the increased protein levels of β-catenin. Interestingly, levels of β-catenin in these crypts correlated negatively with LC3II and positively with p62, suggesting that the activation of Wnt signaling is involved in impaired epithelial autophagy. In addition, our results show a positive and significant correlation between β-catenin and the activation of mTOR in the same crypts. The crucial role had by mTOR pathways in the negative regulation of autophagy[12] and our *in vitro* results showing a role for Wnt1 in the activation of mTOR strongly suggest that Wnt signaling impairs autophagy in epithelial cells of the damaged mucosa of IBD patients through activation of mTOR pathways.

In summary, our results demonstrate a HIF-1-dependent induction of Wnt1 in hypoxic macrophages that impairs autophagy in epithelial cells. In the damaged mucosa of IBD patients, macrophage-derived Wnt1 may be involved in the epithelial activation of Wnt signaling and mTOR pathways, which mediate the impaired epithelial autophagy. A better understanding of the pathophysiological role had by autophagy in colonic epithelial cells will help to clarify the relevance of the HIF-1-dependent induction of Wnt1 in macrophages on epithelial function.

## METHODS

**DSS-induced colitis in mice**. Male 6- to 8-week-old C57BL/6 mice received vehicle ($n = 10$) or Dextran Sulfate Sodium ($n = 10$) (DSS, 40 kDa, Sigma-Aldrich, St Louis, MO) via their drinking water in a 3% (w/v) solution (7 days). Body weight and clinical signs of disease were recorded from day 1. On day 7, mice were killed, and colon tissue samples were collected for further analysis. All the animals were housed under appropriate conditions and treated according to institutional guidelines.

**Intestinal mucosal samples**. Colonic surgical resections from both damaged and non-damaged mucosa were obtained from IBD patients (**Table 1**). The study was approved by the Institutional Review Board of The Hospital of Manises (Valencia). Written informed consent was obtained from all patients.

**Isolation of colonic crypts**. Human intestinal crypts were isolated from both damaged and non-damaged mucosa of surgical resections obtained from IBD patients ($n = 14$). The intestinal epithelial cell isolation procedure was performed using a non-enzymatic dissociation technique based on short-term EDTA treatment, as described previously.[36] The resulting crypt suspension was analyzed by light microscopy (**Figure 1c**).

**Isolation of macrophages from human intestine**. Macrophages were isolated from both damaged and non-damaged mucosa of surgical resections obtained from IBD patients ($n = 3$) by a previously described technique[37] with modifications. The mucosa was transported in cold (4 °C) 0.9% NaCl and washed with Hanks' balanced salt solution without calcium and magnesium (cmHBSS). Epithelial cells were removed by shaking the mucosa (5–10 mm strips of mucosa) with 1 mM DTT and 5 mM EDTA for 20 min at 4 °C, followed by washing with cmHBSS. This was repeated twice, and, after the last step, washing was performed with Hanks' balanced salt solution with calcium and magnesium (HBSS). The mucosa was then cut into 2 mm pieces and digested with collagenase (from Clostridium histolyticum; Lonza, Basel, Switzerland) at a concentration of 100 mg per 100 ml in culture medium (10% inactivated bovine fetal serum (FBS, Lonza, Basel, Switzerland)) in RPMI (Sigma-Aldrich) by shaking for 3 h at 37 °C. After digestion, the cells were filtered through a 100 μ nylon mesh and washed thoroughly with HBSS. Cells were cultured for 24 h with RPMI medium with 10% inactivated FBS with 100 U ml$^{-1}$ penicillin (Lonza), and 100 μg ml$^{-1}$ streptomycin (Lonza) in the presence of phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich). Adhered cells were washed thoroughly with HBSS and stained with a specific monoclonal antibody CD68 (1:100, Biolegend, Madrid, Spain); Tex-Red-labeled anti-mouse was used as the secondary antibody. Hoechst 33342 (Sigma-Aldrich) was used for nuclear staining. The fluorescent signal (18 images per well) was quantified using the static cytometer software (Olympus, Barcelona, Spain) 'Scan' version 2.03.2, and results showed that 82 ± 2% of the cells were macrophages.

**Table 1 Patient characteristics**

| | Ulcerative colitis | Crohn's disease |
|---|---|---|
| Number of patients | 5 | 9 |
| *Age* | | |
| 17–40 years | 3 | 1 |
| >40 years | 2 | 8 |
| *Gender* | | |
| Male | 4 | 4 |
| Female | 1 | 5 |
| *Concomitant medication* | | |
| Azathioprine | | 3 |
| Anti-TNF | 4 | 9 |
| Mesalazine | 2 | |

**Cell culture**. Caco-2 cells (American Type Culture Collection, VA) were cultured in MEM medium (Sigma-Aldrich) supplemented with 20% inactivated FBS with 100 U ml$^{-1}$ penicillin 100 μg ml$^{-1}$ streptomycin, 2 mM L-glutamine (Lonza), 100 mM sodium pyruvate (Lonza), and 1% of non-essential amino acids (Lonza).

Human monocytes (U937 and THP-1, European Collection of Cell Culture, Salisbury, UK) were cultured in RPMI medium with 10% inactivated FBS with 100 U ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. Monocytes (U937 or THP-1) were differentiated into macrophages by culturing them in the presence of PMA for 48 h.[28]

Hypoxia (3% $O_2$) was established by incubating macrophages in a $CO_2/O_2$ incubator (model INVIVO2 400, RUSKINN Technology Ltd, Pencoed, UK) with a blend of 5% $CO_2$ and the appropriate percentage of $O_2$ and $N_2$ up to a total of 100%. Normoxic controls were obtained by incubating the cells at 21% $O_2$.

**Isolation of mononuclear cells**. Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation at 400 *g* for 40 min. Monocyte-derived macrophages were obtained from monocytes seeded in 12-well tissue culture plates and differentiated into macrophages through culture in X-Vivo 15 medium (Lonza) supplemented with 1% human serum, 100 U ml$^{-1}$ de penicillin, 100 μg ml$^{-1}$ streptomycin, and 20 ng ml$^{-1}$ recombinant human M-CSF (Peprotech, London, UK) at 37 °C in 5% $CO_2$ for 6 days.

**Co-culture with Caco-2 cells**. Caco-2 cells were co-cultured with U937 macrophages using Transwell inserts (Corning Incorporated,

**Table 2 Specific antibodies used for immunohistochemical and western blot analysis**

| | Immunohistochemistry | Western blot |
|---|---|---|
| Antibody | Antigen retrieval | Antibody dilution |
| P62 (Santa Cruz Biotechnology, Heidelberg, Germany) | Sodium citrate buffer pH = 9, 97 °C 20 min. 1:100 | 1:1,000 |
| HIF 1α (Santa Cruz Biotechnology) | Sodium citrate buffer pH = 9, 97 °C 20 min. 1:100 | 1:250 |
| HIF 2α (Sigma-Aldrich) | Sodium citrate buffer pH = 9, 97 °C 20 min. 1:200 | 1:250 |
| Wnt1 (Santa Cruz Biotechnology) | | 1:1,000 |
| β-Catenin (Sigma-Aldrich) | Sodium citrate buffer pH = 6, 97 °C 20 min. 1:200 | 1:1,000 |
| LC3 (Novus Biologicals, CO) | | 1:1,000 |
| p-mTOR (Ser 2448) (Santa Cruz Biotechnology) | | 1:1,000 |
| β-Actin (Sigma-Aldrich) | | 1:10,000 |

MA) with a 0.4-μm porous membrane.[38] U937-derived macrophages were seeded on the inserts and differentiated, after which they were incubated in hypoxia for 16 h. Subsequently, the inserts were placed on top of Caco-2 cells and maintained in co-culture for 24 h. Some Caco-2 cells were treated with Wnt1 (20 ng ml$^{-1}$, 24 h, Sigma-Aldrich).

**Immunohistochemical studies**. Immunostaininig for HIF-1α, HIF-2α, p62, and β-catenin was performed with 5 μm sections of paraffin-embedded tissues (**Table 2**). A horse anti-mouse/rabbit biotinylated antibody (Vector Laboratories, CA, 1:200) was used as a secondary antibody. The VECTASTAIN elite ABC system Kit (Vector Laboratories) was employed for signal development. All tissues were counterstained with hematoxylin, and the specificity of the immunostaining was confirmed by the absence of signal when primary or secondary antibodies were omitted. An area of 0.135 mm$^2$ was selected for quantitative analysis.

For immunofluorescence studies, colonic mucosa from IBD patients was frozen in liquid nitrogen, and sections of 4–8 μm were cut with the cryo-microtome. The slides were blocked with PBS/ 0.5% BSA for 20 min and incubated with diluted primary antibodies (CD68, Wnt1 or HIF-1 (**Table 2**), dilution 1:100) in PBS/ 0.5% BSA for 30 min at 37 °C in a humidified atmosphere. After washing with PBS, fluorescent double-labeling was performed with the secondary antibodies, goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC, HIF-1 and Wnt1) and goat anti-mouse IgG-Tex-Red (1:200, Abcam, Cambridge, UK, CD68); 1 mM Hoechst 33342 was added to stain nuclei (30 min at 37 °C). The specificity of the immunostaining was confirmed by the absence of fluorescence when primary antibodies were omitted. Samples were analyzed with a Olympus FLUOVIEW FV1000 confocal or a Olympus IX81 fluorescent microscopes (Olympus, Hamburg, Germany).

**Static cytometry**. Isolated macrophages from both damaged and non-damaged mucosa of surgical resections obtained from IBD patients were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton-X100, and then stained with antibodies against CD68 and Wnt1 (dilution 1:100). Tex-Red-labeled goat anti-mouse or FITC goat anti-rabbit, respectively, were used as the secondary antibodies (dilution 1:200), and Hoechst 33342 was added to stain nuclei. The fluorescent signal (18 images per well) was quantified using the static cytometer.

**RNA interference and cellular transfection**. U937 or THP-1 cells were transfected with a vector-targeting human HIF-1α (*miHIF-1α,* targeting sequence: 5′-AGCTATTTGCGTGTGAGGAAA-3′, GenBank Accession No. NM_001530), HIF-2α (*miHIF-2α*, targeting sequence: 5′-CTCCAACAAGCTGAAGCTGAA-′3, GenBank Accession No. NM_001430), Wnt1 (*miWnt1*, targeting sequence: 5′-TGACTTGT TAAACAGACTGCGAA-3′, GenBank Accession No. NM_005430) or a non-targeting control vector (mock), as described previously.[38] Lipofectamine-2000 (Invitrogen Life Technologies, Carlsbad, CA) was employed as a transfection reagent according to the manufacturer's instructions. Twenty-four hours post transfection, the cells were incubated for 16 h in normoxic or hypoxic conditions, as described above.

**Protein extraction and western blot analysis**. Equal amounts of protein from macrophages or Caco-2 cells (nuclear, cytosolic or total extracts),[39,28] or from colonic tissues[40] were loaded onto SDS/PAGE gels and analyzed by western blot. Membranes were incubated overnight at 4 °C with different primary antibodies (**Table 2**). Subsequently, membranes were incubated with a peroxidase-conjugated anti-mouse IgG (Thermo Scientific, Rockford, IL, 1:5,000) or anti-rabbit IgG (Thermo Scientific, 1:10,000). Following treatment with supersignal west pico chemiluminescent substrate (Thermo Scientific), protein bands were detected by a LAS-3000 (Fujifilm, Barcelona, Spain). Protein expression was quantified by means of densitometry using the Image Gauge Version 4.0 software (Fujifilm). Data were normalized to β-actin.

**RNA extraction and qPCR analysis**. Total RNA from colonic tissue was isolated using the Tripure Isolation reagent (Roche Diagnostics, Barcelona, Spain), and total RNA from macrophages (isolated macrophages, THP1- or U937-derived macrophages or from MDMs cells) was obtained by using an extraction kit (Illustra RNA spin mini isolation kit, GE Health Care Life Science, Spain). In all cases, cDNA was obtained with the Prime Script RT reagent Kit (Takara, Otsu, Japan). The protocol was followed as described previously.[28] Real-time PCR was performed with the Prime Script Reagent Kit Perfect Real Time (Takara) in a thermo cycler LightCycler (Roche Diagnostics). Specific oligonucleotides for human *Wnt1* (5′-cgcccacccgagtacctcca-3′, 5′-ttcatgccgccccaggcaag-3′) or *Lgr5* (5'-tgctccgacctggggctctc-3', 5'- tcggaggctaagcaactgctgga-3') were designed according to the reported sequences, and human *β-actin* (5′-ggacttcgagcaagagatgg-3′, 5′-agcactgtgttggcgtacag-3′) expression was used as a housekeeping gene. The threshold cycle (CT) was determined, and relative gene expression was expressed as follows: change in expression (fold) = $2^{-\Delta(\Delta CT)}$ where $\Delta CT = CT\ (target) - CT\ (housekeeping)$, and $\Delta(\Delta CT) = \Delta CT\ (treated) - \Delta CT\ (control)$.

**Statistical analysis**. Data were expressed as mean ± s.e.m. and were compared by analysis of variance (one way-ANOVA) with a Newman-Keuls *post hoc* correction for multiple comparisons or a *t*-test when appropriate. A *P*–value $<0.05$ was considered to be statistically significant. Clinical correlations were analyzed in the human samples using Pearson's correlation coefficient.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at http://www.nature.com/mi

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**DISCLOSURE**

The authors declare no conflict of interest.



**REFERENCES**

1. Maloy, K.J. & Powrie, F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* **474**, 298–306 (2011).
2. Khor, B., Gardet, A. & Xavier, R.J. Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474**, 307–317 (2011).
3. Patel, K.K. & Stappenbeck, T.S. Autophagy and intestinal homeostasis. *Annu. Rev. Physiol.* **75**, 241–262 (2013).
4. Sokollik, C., Ang, M. & Jones, N. Autophagy: a primer for the gastroenterologist/hepatologist. *Can. J. Gastroenterol.* **25**, 667–674 (2011).
5. Benjamin, J.L., Sumpter, R. Jr., Levine, B. & Hooper, L.V. Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host. Microbe* **13**, 723–734 (2013).
6. Kaser, A. & Blumberg, R.S. Autophagy, microbial sensing, endoplasmic reticulum stress, and epithelial function in inflammatory bowel disease. *Gastroenterology* **140**, 1738–1747 (2011).
7. Fritz, T., Niederreiter, L., Adolph, T., Blumberg, R.S. & Kaser, A. Crohn's disease: NOD2, autophagy and ER stress converge. *Gut* **60**, 1580–1588 (2011).
8. Cadwell, K., Patel, K.K., Komatsu, M., Virgin, H.W. & Stappenbeck, T.S. A common role for Atg16L1, Atg5 and Atg7 in small intestinal Paneth cells and Crohn disease. *Autophagy* **5**, 250–252 (2009).
9. Baumgart, D.C. & Sandborn, W.J. Crohn's disease. *Lancet* **380**, 1590–1605 (2012).
10. Strober, W. & Watanabe, T. NOD2, an intracellular innate immune sensor involved in host defense and Crohn's disease. *Mucosal. Immunol.* **4**, 484–495 (2011).
11. Choi, A.M., Ryter, S.W. & Levine, B. Autophagy in human health and disease. *N. Engl. J. Med.* **368**, 651–662 (2013).
12. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132**, 27–42 (2008).
13. Wynn, T.A., Chawla, A. & Pollard, J.W. Macrophage biology in development, homeostasis and disease. *Nature* **496**, 445–455 (2013).
14. Glover, L.E. & Colgan, S.P. Hypoxia and metabolic factors that influence inflammatory bowel disease pathogenesis. *Gastroenterology* **140**, 1748–1755 (2011).
15. Lin, S.L. *et al.* Macrophage Wnt7b is critical for kidney repair and regeneration. *Proc. Natl Acad. Sci. USA* **107**, 4194–4199 (2010).
16. Farin, H.F., van Es, J.H. & Clevers, H. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* **143**, 1518–1529 (2012).
17. Pull, S.L., Doherty, J.M., Mills, J.C., Gordon, J.I. & Stappenbeck, T.S. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc. Natl. Acad. Sci. USA* **102**, 99–104 (2005).
18. Mowat, A.M. & Bain, C.C. Mucosal macrophages in intestinal homeostasis and inflammation. *J. Innate. Immun.* **3**, 550–564 (2011).
19. Anderson, E.C. & Wong, M.H. Caught in the Akt: regulation of Wnt signaling in the intestine. *Gastroenterology* **139**, 718–722 (2010).
20. Yeung, T.M., Chia, L.A., Kosinski, C.M. & Kuo, C.J. Regulation of self-renewal and differentiation by the intestinal stem cell niche. *Cell Mol. Life Sci.* **68**, 2513–2523 (2011).
21. Nakamura, T., Tsuchiya, K. & Watanabe, M. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *J. Gastroenterol.* **42**, 705–710 (2007).
22. Clevers, H. & Nusse, R. Wnt/beta-catenin signaling and disease. *Cell* **149**, 1192–1205 (2012).
23. Jin, T., George, F.,I. & Sun, J. Wnt and beyond Wnt: multiple mechanisms control the transcriptional property of beta-catenin. *Cell Signal.* **20**, 1697–1704 (2008).
24. Inoki, K. *et al.* TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* **126**, 955–968 (2006).
25. Ashton, G.H. *et al.* Focal adhesion kinase is required for intestinal regeneration and tumorigenesis downstream of Wnt/c-Myc signaling. *Dev. Cell* **19**, 259–269 (2010).
26. Valvezan, A.J. & Klein, P.S. GSK-3 and Wnt Signaling in Neurogenesis and Bipolar Disorder. *Front Mol. Neurosci.* **5**, 1 (2012).
27. Castilho, R.M., Squarize, C.H., Chodosh, L.A., Williams, B.O. & Gutkind, J.S. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. *Cell Stem Cell* **5**, 279–289 (2009).
28. Ortiz-Masia, D. *et al.* Induction of CD36 and thrombospondin-1 in macrophages by hypoxia-inducible factor 1 and its relevance in the inflammatory Process. *PLoS. ONE* **7**, e48535 (2012).
29. Giatromanolaki, A. *et al.* Hypoxia inducible factor 1alpha and 2alpha overexpression in inflammatory bowel disease. *J. Clin. Pathol.* **56**, 209–213 (2003).
30. Koh, M.Y. & Powis, G. Passing the baton: the HIF switch. *Trends Biochem. Sci.* **37**, 364–372 (2012).
31. Karhausen, J. *et al.* Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J. Clin. Invest.* **114**, 1098–1106 (2004).
32. Hughes, K.R., Sablitzky, F. & Mahida, Y.R. Expression profiling of Wnt family of genes in normal and inflammatory bowel disease primary human intestinal myofibroblasts and normal human colonic crypt epithelial cells. *Inflamm. Bowel. Dis.* **17**, 213–220 (2011).
33. Gao, C. *et al.* Autophagy negatively regulates Wnt signalling by promoting dishevelled degradation. *Nat. Cell Biol.* **12**, 781–790 (2010).
34. Lee, G. *et al.* Phosphoinositide 3-kinase signaling mediates beta-catenin activation in intestinal epithelial stem and progenitor cells in colitis. *Gastroenterology* **139**, 869–881. 881 (2010).
35. Brown, J.B. *et al.* Mesalamine inhibits epithelial beta-catenin activation in chronic ulcerative colitis. *Gastroenterology* **138**, 595–605. 605 (2010).
36. Branka, J.E. *et al.* Early functional effects of Clostridium difficile toxin A on human colonocytes. *Gastroenterology* **112**, 1887–1894 (1997).
37. Mahida, Y.R., Wu, K.C. & Jewell, D.P. Respiratory burst activity of intestinal macrophages in normal and inflammatory bowel disease. *Gut* **30**, 1362–1370 (1989).
38. Ortiz-Masia, D. *et al.* iNOS-derived nitric oxide mediates the increase in TFF2 expression associated with gastric damage: role of HIF-1. *FASEB J.* **24**, 136–145 (2010).
39. Hernandez, C. *et al.* Induction of trefoil factor (TFF)1, TFF2 and TFF3 by hypoxia is mediated by hypoxia inducible factor-1: implications for gastric mucosal healing. *Br. J. Pharmacol.* **156**, 262–272 (2009).
40. Riano, A. *et al.* Nitric oxide induces HIF-1alpha stabilization and expression of intestinal trefoil factor in the damaged rat jejunum and modulates ulcer healing. *J. Gastroenterol.* **46**, 565–576 (2011).