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Loss of prolyl hydroxylase-1 protects in colitis through reduced epithelial cell apoptosis and enhanced barrier function

Short Title: PHD1 loss is protective in colitis.

Murtaza M. Tambuwala^{1,*}, Eoin P. Cummins^{1,*}, Colin R. Lenihan^{1,*}, Judith Kiss³, Markus Stauch³, Carsten C. Scholz¹, Peter Fraisl², Felix Lasitschka⁶, Martin Mollenhauer³, Sean P. Saunders⁵, Patrick H. Maxwell⁴, Peter Carmeliet², Padraic G. Fallon⁵, Martin Schneider^{3#}, & Cormac T. Taylor^{1#}.

¹The Conway Institute, University College Dublin, Ireland, ²Vesalius Research Center, VIB, Katholieke University of Leuven, Belgium, ³Department of General, Visceral and Transplantation Surgery, University of Heidelberg, Germany, ⁴Rayne Institute, University College London, United Kingdom & ⁵Institute of Molecular Medicine, Trinity College Dublin, Ireland, ⁶Institute of pathology, University Hospital, Heidelberg, Germany.

*These authors contributed equally to this study.

#These authors contributed equally to this study.

The Authors declare that there is no conflict of interest.

Address correspondence: Cormac T. Taylor, Ph.D.
UCD Conway Institute,
University College Dublin,
Belfield,
Dublin 4,
Ireland.
Telephone: (353)-1-716-6732
e-mail: cormac.taylor@ucd.ie

Authorship contributions:

(a) Study concept and design, (b) acquisition of data, (c) analysis and interpretation of data, (d) drafting of the manuscript, (e) critical revision of the manuscript for important intellectual content, (f) statistical analysis, (g) obtained funding, (h) technical or material support, (i) study supervision.

Murtaza M. Tambuwala (a,b,c,d)

Eoin P. Cummins (a,b,c,d)

Colin R. Lenihan (a,b,c,d,f)

Judith Kiss (b,c)

Markus Stauch (b,c)

Carsten Scholz (b,c,f)

Peter Fraisl (a,b,c,e)

Felix Lasitschka (b,c)

Martin Mollenhauer (b,c)

Sean P. Saunders (b,c)

Patrick H. Maxwell (e,h)

Peter Carmeliet (a,c,e,h)

Padraic G. Fallon (b,c,d,e,f,h)

Martin Schneider (a,c,d,e,f)

Cormac T. Taylor (a,c,d,e,f,g,i)

Abstract

Background and Aims: HIF prolyl hydroxylase inhibitors are protective in mouse models of inflammatory bowel disease (IBD). Here, we investigated the therapeutic target(s) and mechanism(s) involved. Methods: The effect of genetic deletion of individual HIF-prolyl hydroxylase enzymes (PHD) on the development of dextran sulphate sodium (DSS)-induced colitis was examined in mice. Results: PHD1^{-/-}, but not PHD2^{+/-} or PHD3^{-/-}, mice were less susceptible to the development of colitis than wild type controls as determined by weight loss, disease activity, colon histology, neutrophil infiltration and cytokine expression. Reduced susceptibility of PHD1^{-/-} mice to colitis was associated with increased density of colonic epithelial cells relative to wild type controls which was due to decreased levels of apoptosis resulting in enhanced epithelial barrier function. Furthermore, using cultured epithelial cells it was confirmed that hydroxylase inhibition reversed DSS-induced apoptosis and barrier dysfunction. Finally, PHD1 levels were increased with disease severity in intestinal tissue from IBD patients and in colonic tissues from DSS treated mice. Conclusions: These results imply a role for PHD1 as a positive regulator of intestinal epithelial cell apoptosis in the inflamed colon. Genetic loss of PHD1 is protective against colitis through decreased epithelial cell apoptosis and consequent enhancement of intestinal epithelial barrier function. Thus, targeted PHD1 inhibition may represent a new therapeutic approach in IBD.

Keywords: Colitis: Hydroxylase: Apoptosis: Hypoxia

Introduction

Prolyl hydroxylases (PHDs) are members of a large family of dioxygenases, a subset of which play a key role in intracellular oxygen sensing and signalling responses during hypoxia, primarily via the regulation of the stability of the hypoxia inducible factor (HIF; 1-4). Three PHD enzymes which confer oxygen sensitivity to the HIF pathway have been described (PHD1-3; 15-16). While these isoforms share similar biochemical characteristics, they have distinct tissue-specific expression profiles and as well as regulating HIF, can also impact upon pro-survival signalling pathways including nuclear factor kappaB (NF κ B) (1,17-18,25, 34).

PHDs mediate their effects on HIF through (oxygen-dependent) hydroxylation of key proline residues on the oxygen-dependent degradation (ODD) domain of HIF α subunits. When hydroxylated at these sites, HIF α becomes a target for ubiquitylation by the von Hippel Lindau (VHL) E3 ubiquitin ligase leading to HIF α ubiquitination and degradation. The specific targets for PHDs in the NF- κ B pathway remain to be determined although IKK α and IKK β contain surface expressed consensus sites in their regulatory domains (36). PHDs play a central role in the adaptive response to hypoxia through activation of these pathways with the subsequent expression of genes promoting cell survival, erythropoiesis, angiogenesis and metabolism. As such, PHDs may be an effective target for therapeutic manipulation (1,5). It has recently become clear that hydroxylase activity can impact upon the development of inflammation and that hydroxylase

inhibitors may represent a novel therapeutic approach in chronic inflammatory disease (5,6).

Inflammatory bowel disease is a genetically heterogenous, chronic inflammatory condition with severe pathology and limited therapeutic options (10-11,27). While the underlying causes of IBD remain largely undefined, the fundamental defect involves a loss of intestinal epithelial barrier function. We and others have shown that pan-hydroxylase inhibitors such as dimethyl-oxalylglycine (DMOG) have a protective effect in murine models of colitis and thus represent a potential new therapeutic approach (13-14). However, these studies did not identify which hydroxylase isoforms or effector pathways are involved.

Mice lacking individual PHD enzymes have distinct phenotypes. PHD1^{-/-} mice demonstrate a reprogrammed basal metabolic profile which decreases muscle performance in healthy tissue but provides acute protection in muscle and liver ischemia (7,26). PHD2^{-/-} mice are embryonic lethal with a massive vascular defect during embryogenesis (32) while heterozygous (+/-) mice demonstrate enhanced tumour angiogenesis but decreased metastatic events through what has been termed “endothelial normalisation” (8-9). PHD3^{-/-} mice have reduced neuronal apoptosis, abnormal sympathoadrenal development and reduced blood pressure (12). The diverse phenotypes in mice with different PHD gene deletions strongly suggests that there are distinct isoform-specific functions for the various PHD enzymes *in vivo*.

In order to develop our understanding of the mechanisms of protection of pan-hydroxylase inhibitors in colitis, we investigated disease development in PHD1,

PHD2 and PHD3 deficient mice exposed to DSS in their drinking water to induce colitis. We found that PHD1^{-/-}, but not PHD2^{+/-} or PHD3^{-/-} mice, were selectively protected against the development of colitis. This protection was associated with enhanced intestinal epithelial barrier function which was due to diminished epithelial cell apoptosis. These data implicate a positive role for PHD1 in the regulation of intestinal epithelial cell apoptosis during intestinal inflammation and strengthen the case for the development of PHD1-specific inhibitors targeted to intestinal epithelial cells as a new therapeutic approach in the treatment of IBD.

Materials and Methods

Animals.

PHD1^{-/-}, PHD2^{-/+} and PHD3^{-/-} mice against a mixed background strain of Swiss/129 which were used for these experiments have been previously described (7,9,12). **None of the PHD knockout mice demonstrated differential weight gain when compared to wild types.**

DSS colitis model.

The DSS model of colitis was used as described previously (13). Briefly, acute disease was induced by the treatment of mice with 5% DSS in their drinking water for up to 6 days. **The Disease Activity Index (DAI) was determined according to the parameters outlined in Table S1 (30,35).** Upon termination of the experiment, mice were sacrificed by cervical dislocation and colonic tissues were dissected for further analysis. All procedures described were approved by the relevant institutional Animal Research Ethics Committee.

Assessment of tissue inflammation.

Small (approximately 1cm) sections of excised colonic tissue were fixed in 10% paraformaldehyde (pH7.4, PBS buffered) and embedded in paraffin. 4µm sections were cut and stained with hematoxylin and eosin. **Histological assessment of colonic mucosae was carried out in a blinded fashion as described in Table S2 (35).** To assay for markers of inflammation, colonic

tissues were homogenised and levels of TNF α , IL-1 β , IL-6 and myeloperoxidase were measured as described previously (13).

Western blotting.

Whole colon tissues were thawed, homogenised, sonicated in standard western blotting lysis buffer and vigorously vortexed before centrifugation at 12,000rpm for 5 minutes. Supernatant was removed and normalised to 1 μ g/ μ l and separated by SDS-PAGE as described previously (13). Following transfer to nitrocellulose membranes, PHD1 was detected by western blot using an anti-PHD1 primary antibody (Novus Biologicals, Littleton, USA).

In vivo permeability assay.

Mice were exposed to 6 days of DSS treatment prior to oral administration of 0.6 mg/g body weight FITC labeled dextran (4kD) by standard oral gavage. 3.5 hours later, mice were sacrificed and blood was removed by cardiac puncture. Plasma was separated and FITC levels in the plasma were determined by fluorometry. The distribution of FITC-dextran in sectioned colonic tissue was determined by fluorescence microscopy.

In vitro permeability assay.

CaCo-2 epithelial cells (passage 19-26) were seeded on uncoated 6.5mm semi-permeable (3.0mm pore) Transwell^(R) polycarbonate membranes (Costar, Cambridge MA). Monolayers were maintained for 4-5 weeks prior to

experimentation in order to ensure an efficient barrier and development of Trans-epithelial electrical resistance (TEER). Cells were co-treated with DMOG 1mM (or DMSO vehicle) and 4% DSS (w/v) (in DMEM supplemented with 10% FCS and penicillin/streptomycin) in the apical and basolateral compartments of the Transwell^(R) membrane. TEER values were obtained using a REMS Autosampler (World Precision Instruments, Sarasota) at the indicated time points. Values are indicative of triplicate wells per treatment for 4 independent passage numbers.

Immunohistochemistry,

4µm sections were cut from the paraffin blocks, deparaffinised with xylene and rehydrated in a graded series of alcohols. For immunohistochemical staining, endogenous peroxidase activity was quenched using 3% hydroxyl peroxide solution for 10 minutes. Antigen retrieval was performed by immersing the slides in proteinase K solution (20µg/ml in 10mM Tris-HCl, pH 7.5) for 12 minutes. Sections were blocked with 5% goat serum for one hour at room temperature and then incubated overnight with primary antibody at 4°C in a humidified chamber. The primary antibodies used were rabbit anti-KI-67 (1:100 dilution, Abcam), rabbit anti-cleaved caspase-3 (Asp175) (1:200 dilution; Cell Signalling) and rabbit-anti cytokeratin 8 (1:100 dilution, Abcam). The slides were then treated with biotinylated goat anti-rabbit antibody (1:200 dilution Vector Labs). ABC immunodetection (VECTASTAIN Elite ABC Kit; Vector Laboratories) was used to detect cleaved caspase 3 and KI-67. Cytokeratin 8 was detected with avidin-conjugated Texas red (1:50 dilution, Merck Biosciences) followed by

nuclear counterstain with Hoechst. Phosphate buffered saline/1% Tween solution was used to wash sections between steps. The sections were developed using diaminobenzidine (DAB) as a chromagen and counterstained with haematoxylin. Colonic sections from 5-7 animals were stained for KI-67, cytokeratin 8 or cleaved caspase 3. In the case of KI-67 and cleaved caspase 3, for each section, three 200X or 400X fields respectively were randomly selected and the number of positive cells per 100 enterocytes was quantified. All scoring was carried in a blinded fashion.

TUNEL Stain.

Cellular apoptosis was identified using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (Fluorescein In Situ Cell Death Detection Kit, Roche Diagnostic). Following de-waxing, rehydration and proteinase K antigen retrieval colon sections were incubated with the TUNEL reaction mix for 1 hour at 37⁰C in a humidified chamber. Sections were counterstained with Hoechst dye and analyzed using fluorescent microscopy. Colonic sections from 5 animals per group were TUNEL stained. For each section, three 400X fields were randomly selected and the number of positive cells per 100 enterocytes was quantified. All scoring was carried in a blinded fashion.

In vitro apoptosis assay

Caco-2 cells were treated with DMOG (1mM, 24 hours) or transfected with 50nM PHD1 siRNA or control non-target siRNA (Dharmacon). Transfections were carried out using Lipofectamine 2000 (Invitrogen). Following treatment, cells were treated with dextran sulphate sodium (1%, 24 hours). The cells were harvested, resuspended in ice cold phosphate buffered saline and incubated with YO-PRO-1, Hoechst 33342 and propidium iodide (Vybrant[®] Apoptosis Assay Kit #7, Invitrogen) according to manufacturers protocol. Flow cytometric analyses were carried out using the CyAn ADP analyser (Beckman Coulter) and Summit 4.3 software. Cells were defined as living, early apoptotic or late apoptotic/necrotic according to their relative dye uptake.

Statistical analysis.

Statistical comparisons were made by analysis of variance (ANOVA) or Students t-test. Values are expressed as mean \pm standard error of mean (sem) for *n* individual experiments.

Results

PHD1 deficient mice are protected against DSS-induced colitis.

Previous studies demonstrated that treatment with pan-hydroxylase inhibitors is protective in DSS-induced colitis in mice (13-14). These studies utilized broad spectrum hydroxylase inhibitors such as DMOG as pharmacologic isoform-specific inhibitors are currently unavailable. In order to determine which PHD enzyme(s) is/are responsible for the protective effects of pan-hydroxylase inhibition, we investigated the development of DSS-induced colitis in PHD1^{-/-}, PHD2^{+/-} and PHD3^{-/-} mice. We found only PHD1^{-/-} mice were significantly protected from DSS-induced colitis as determined by weight loss (Figure 1A) and disease activity index measurements (Figure 1B). **PHD1^{-/-} mice also had significantly attenuated histological signs of inflammation when compared to wild type controls (Figure 1C and Figure 1D).** The reduced severity of DSS-induced colitis in PHD1^{-/-} mice was confirmed by the significantly lower levels of myeloperoxidase enzymatic activity, a marker of neutrophil infiltration, in the colons of PHD1^{-/-} mice relative to wild-type animals (**Figure 1E**). Furthermore, the levels of DSS-induced inflammatory cytokines including interleukin (IL)-1 β , TNF α and IL-6 were significantly lower in PHD 1^{-/-} mice when compared to wild type controls (**Figure 1E**). In contrast, PHD2^{+/-} and PHD3^{-/-} mice did not demonstrate attenuated DDS-induced inflammation in all parameters investigated (data not shown). Taken together, these data indicate that PHD1^{-/-} mice are selectively protected against the development of colitis.

Increased enterocyte density in PHD1 deficient mice in colitis.

We next carried out high magnification (400X) microscopic analysis of mucosal structure in wild type and PHD1^{-/-} mice exposed to DSS. Increased intestinal epithelial cell density was observed in PHD1^{-/-} mice treated with DSS when compared to wild type controls (Figure 2A). **The insets in Figure 2A represent high magnification areas of intestinal epithelial cells demonstrating increased epithelial density in colonic tissues derived from DSS-treated PHD1^{-/-} mice when compared to wild type mice. The enhanced epithelium** was confirmed by epithelial-specific cytokeratin staining in colonic tissues (Figure 2B) which demonstrates decreased ulceration in DSS-treated PHD1^{-/-} mice when compared to wild type controls. Collectively, these data led us to hypothesize that the protective effects of PHD1 deficiency were due to enhanced epithelial barrier function as a result of increased intestinal epithelial cell density.

Enhanced epithelial barrier function in PHD1^{-/-} mice.

To further address the effects of loss of PHD1 on the intestinal epithelial barrier, in vivo intestinal barrier function was measured in wild type and PHD1^{-/-} mice exposed to DSS. Mice were given an oral dose of FITC-dextran on the final day of **DSS exposure** and 4 hours later, FITC levels in the plasma were determined as a measure of intestinal permeability. While DSS induced a characteristic increase in intestinal permeability as reflected by increased appearance of FITC in plasma, this was markedly diminished in PHD1^{-/-} mice (Figure 3A).

Furthermore, fluorescent microscopic detection of FITC in colonic tissues from these animals revealed retention of FITC at the epithelial barrier in PHD1^{-/-} mice whereas in wild type mice, FITC had transcended the epithelial barrier (Figure 3B).

To test whether hydroxylase inhibition can directly impact on epithelial barrier function, we next exposed cultured intestinal epithelial cells (CaCo-2), grown on permeable support inserts to increasing periods of DSS and measured barrier disruption by measuring transepithelial electrical resistance (TEER). Cells treated with the hydroxylase inhibitor DMOG demonstrated reduced barrier dysfunction when compared to cells treated with vehicle and DSS alone indicating that hydroxylase inhibition in isolated enterocytes confers resistance to DSS-induced barrier dysfunction ([supplementary figure S1](#)). Taken together, these data indicate that loss of PHD1 leads to enhanced epithelial barrier function in colitis.

Decreased enterocyte apoptosis in PHD1^{-/-} mice exposed to DSS.

We next investigated whether the increase in enterocyte density (Figure 2) and enhanced barrier function (Figure 3) observed in PHD1^{-/-} mice was due to decreased epithelial cell death or increased proliferation. Immunohistochemical KI67 staining (a marker of proliferating cells) was not different between wild type and PHD1^{-/-} mice either with or without DSS treatment indicating no difference in proliferation between groups (Figure 4A).

We next investigated intestinal epithelial cell apoptosis using both TUNEL staining and detection of cleaved caspase 3. Basal rates of apoptosis detected using both techniques were similar in PHD1^{-/-} and wild type mice. However, PHD1^{-/-} mice exhibited a significantly lower degree of enterocyte apoptosis when exposed to DSS than wild type mice (Figure 4B, 4C). Collectively, these data indicate that PHD1^{-/-} mice are resistant to DSS-induced colitis due to barrier protective effects associated with decreased enterocyte apoptosis.

To investigate whether PHD-1 inhibition could directly affect epithelial apoptosis, cultured enterocytes were exposed to DSS in the presence and absence of the pan hydroxylase inhibitor DMOG or siRNA targeted to PHD1 and the induction of early and late apoptosis was measured. DMOG (Figure 5A) or PHD1 siRNA (Figure 5B) significantly inhibited DSS-induced apoptosis in isolated enterocytes indicating a direct anti-apoptotic effect of pharmacologic hydroxylase inhibition upon epithelial cells.

PHD1 expression is correlated with disease severity in IBD patients.

Having identified PHD1 as a potential target for pharmacologic intervention in IBD, we next examined the expression of PHD1 in intestinal tissue taken from patients undergoing restorative colectomy for surgical treatment of IBD. Intestinal tissues from patients diagnosed with IBD were independently scored by two clinical pathologists and categorized for histopathological grade of inflammation according to the Truelove & Richards scale (33) (grade 0 – no inflammation; grade 1 - chronic inactive inflammation (increased content of

mononuclear cells within the mucosal lamina propria); grade 2 - mild active inflammation (plus few circumscribed foci with neutrophilic intraepithelial infiltrates/cryptitis or single crypt abscesses); grade 3 - moderate active inflammation (plus several foci with neutrophilic intraepithelial infiltrates/cryptitis or crypt abscesses). Semi-quantitative analysis of immunoblots revealed significantly higher expression of PHD1 protein in colon samples displaying active mucosal inflammation (Figure 6A,B). Indeed, PHD1 was relatively over-expressed in 100% of tissue samples displaying high inflammatory activity (Truelove and Richards score 2-3). By contrast, relative over-expression of PHD1 was only observed in 33% of samples with inactive inflammation (Truelove and Richards score 0-1). This difference was significant when applying the Fisher's Exact Test ($P=0.015$, $n=8$). Similarly, colonic tissue from mice demonstrated increased PHD1 expression following DSS exposure (supplementary figure S2). In summary, while PHD deficiency or inhibition is associated with decreased disease activity, increased PHD expression is associated with disease severity.

Discussion

A common feature of all IBD regardless of the underlying cause is the loss of intestinal epithelial barrier function due to excessive epithelial cell death, an event that allows the non-specific movement of luminal antigenic material into the submucosa (31). The resulting inflammatory response drives further barrier dysfunction and the symptoms associated with the disease thus establishing a positive feed forward loop between developing inflammation and barrier dysfunction. The intestinal epithelial barrier relies heavily upon the controlled turnover of intestinal epithelial cells, which originate from stem cells in the intestinal crypts and migrate to the top of the crypt or villus before undergoing apoptosis and being released into the intestinal lumen. The regulation of the intestinal epithelial cell turnover is a complex balance between epithelial proliferation and cell death in which stem cells play a key role (20). In the physiologic state, intestinal epithelial cells have a relatively short lifespan and their programmed cell death through apoptosis is a carefully controlled process which is likely critical for the maintenance of normal barrier function (19). The regulatory factors which are responsible for the initiation of physiologic intestinal epithelial cell apoptosis remain to be fully elucidated, however increased rates of apoptosis with resultant dysfunction of the intestinal epithelial barrier is central to the pathology of inflammatory bowel disease (21). Thus, therapies directed towards delaying or suppressing intestinal epithelial cell apoptosis may allow

time for mucosal healing to occur in IBD and may thus represent a therapeutic approach.

We, and others, have demonstrated that pan hydroxylase inhibition is protective against the development of colitis in multiple models (13,14). In the current study, we find that PHD1^{-/-} mice selectively demonstrate a significant decrease in DSS-induced apoptosis, an event that we hypothesise to be linked to enhanced barrier function and subsequent protection against the development of colitis. This is supported by studies investigating the link between hydroxylase activity and apoptosis in other model systems, which have demonstrated an anti-apoptotic effect of pharmacologic hydroxylase inhibition (22-24).

Hydroxylase inhibition impacts upon multiple transcriptional pathways including HIF and NF- κ B, both of which have been demonstrated to be protective in epithelial cells during IBD (28). HIF activates the expression of a range of factors including intestinal trefoil factor, CD73 and the adenosine A2B receptor that have been demonstrated to be barrier protective (28). Furthermore, NF- κ B activation results in increased expression of anti-apoptotic factors (29). We have proposed that the combined effects of barrier protective and antiapoptotic gene expression in epithelial cells which occurs via these pathways underpins the effectiveness of pan-hydroxylase inhibitors in models of IBD (28). In the current study, we have identified that PHD1 is the key target hydroxylase in mediating the protective effects of hydroxylase inhibitors in colitis and thus represents a likely therapeutic target for IBD. **Importantly, because of the lethality of a homozygous PHD2 knockout mouse, we cannot rule out a possible role for a protective effect of**

complete PHD2 inhibition during colitis. Future studies will determine whether the protection afforded by hydroxylase inhibition is via activation of HIF, NF- κ B or other transcriptional regulators. Increased PHD1 correlates with disease severity in IBD patients reflecting a positive relationship between the expression levels of PHD1 and disease severity. In a previous study, Robinson et al. demonstrated that PHD isoform mRNA expression levels in the intestinal mucosa were in the order PHD2=PHD3>PHD1. While the mRNA expression levels of PHD1 are lowest in non-inflamed tissue, we found that PHD1 levels were increased in inflamed tissues from IBD patients raising the intriguing possibility that increased PHD1 expression / activity may be contributing to disease progression through increasing epithelial cell apoptosis and reducing barrier function. In summary, we propose that, as a positive regulator of intestinal epithelial cell apoptosis in colitis, PHD1 represents a new therapeutic target in the treatment of inflammatory bowel disease.

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Figure Legends

Figure 1: *PHD1*^{-/-} mice are selectively protected against DSS-induced colitis.

(A) Weight change measurements over 6 days in wild type, *PHD1*^{-/-}, *PHD2*^{+/-} or *PHD3*^{-/-} mice exposed to 5% DSS. (B) Disease activity indices over 6 days in wild type, *PHD1*^{-/-}, *PHD2*^{+/-} or *PHD3*^{-/-} mice exposed to DSS. (C) Representative 100X histological images and (D) histological scores of colonic tissue taken from control and DSS-treated wild type (WT) or *PHD1*^{-/-} mice (scale bar=100 μ m). (E) Myeloperoxidase, TNF α , IL-1 β and IL-6 levels were determined in wild type and *PHD1*^{-/-} mouse colonic tissue 6 days after initiation of administration of DSS. Each control and experimental group contained a minimum of n=6 individual mice; *P<0.05; **P<0.01; nsd=not significantly different.

Figure 2: *Increased epithelial density in homozygous PHD1 knockout mice exposed to DSS.*

(A) High magnification (400X) histological images of colonic tissue from wild type and *PHD1*^{-/-} mice with and without DSS treatment reveals increased intestinal epithelial density in tissues from *PHD1*^{-/-} animals (scale bar=25 μ m). Insets demonstrate examples of areas of epithelium in high magnification (1600X). L=Lumen, U= Region of ulceration). (B) 200X Immunohistochemical analysis of cytokeratin (staining red) and nuclei (staining blue) in colonic tissue from wild type and *PHD1*^{-/-} mice with DSS treatment

reveals increased mucosal ulceration and decreased epithelial density in wild type mice when compared with PHD1^{-/-} mice (scale bar=50µm). Each control and experimental group contained a minimum of n=6 individual mice (L=Lumen; U=area of ulceration).

Figure 3: *Enhanced epithelial barrier function in PHD1^{-/-} mice.* (A) Intestinal permeability was measured by the appearance of orally administered FITC-labelled dextran in plasma from wild type and PHD1^{-/-} mice exposed to DSS (bars represent mean \pm SEM; *P<0.5, n=6). (B) Fluorescence microscopy (100X) of the intestinal mucosa from DSS-treated wild type and PHD1^{-/-} mice exposed to orally administered FITC-labelled dextran demonstrates FITC permeation into tissue is enhanced in wild type animals treated with DSS when compared to PHD1^{-/-} animals where FITC-dextran is retained at the epithelium (scale bar=100µm).

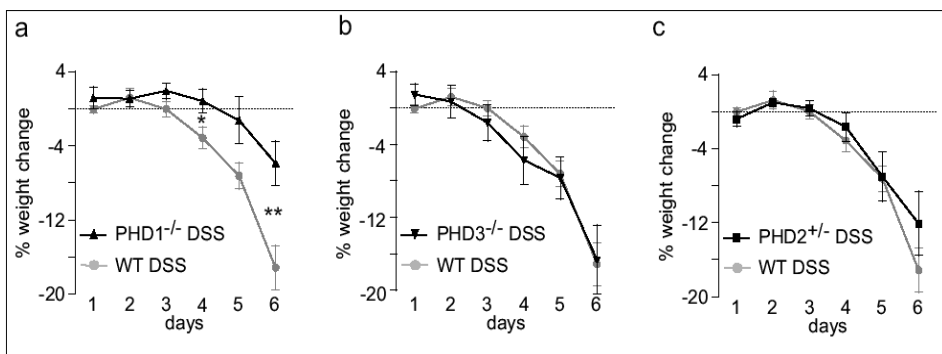
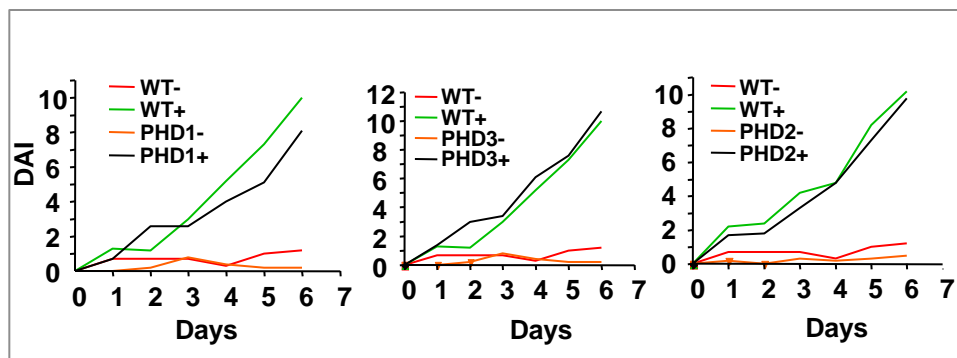
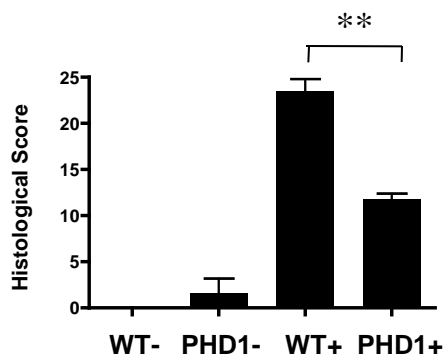
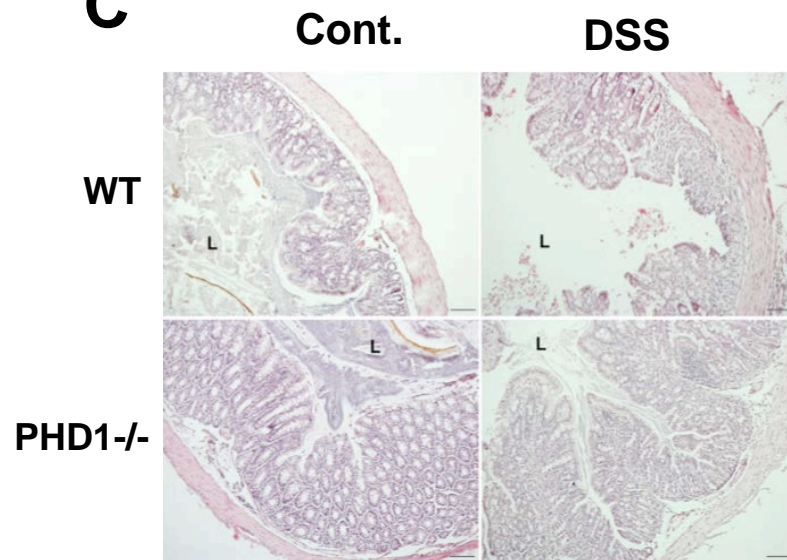
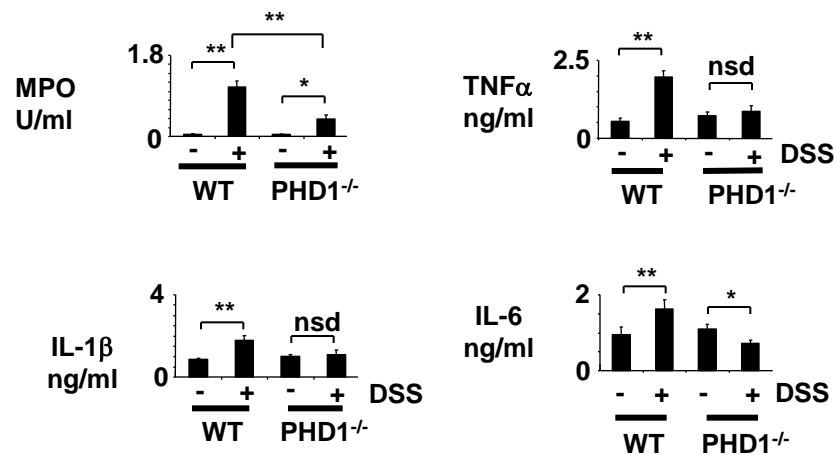
Figure 4: *Decreased enterocyte apoptosis in PHD1^{-/-} mice exposed to DSS.* (A) Representative 100X immunohistochemical images (left panel) and quantification of intestinal epithelial cell proliferation (right panel) in wild type and PHD1^{-/-} mice exposed to DSS as measured by KI-67 staining (scale bar=50 µm). (B) Representative 100X Immunofluorescence images (left panel) and quantification of intestinal epithelial cell apoptosis (right panel) in wild type

and PHD1^{-/-} mice exposed to DSS as measured by TUNEL staining (scale bar=50 μm). (C) Representative 200X immunohistochemical images (left panel) and quantification of intestinal epithelial cell apoptosis (right panel) in wild type and PHD1^{-/-} mice exposed to DSS as measured by cleaved caspase-3 staining (scale bar=25μm). Each control and experimental group contained a minimum of n=6 individual mice; *P<0.05.

Figure 5: *PHD1 inhibition blocks apoptosis in cultured enterocytes.* (A) CaCo2 intestinal epithelial cells were exposed to 1% DSS (+) or vehicle (-) for 24 hours with and without pre-treatment with DMOG (1mM). Numbers of live and apoptotic epithelial cells was determined by flow cytometric analysis. (B) CaCo2 cells were exposed to siRNA directed against PHD1. Specific siRNA treatment resulted in effective knockdown of PHD1 (upper panel) and resulted in decreased numbers of DSS-induced apoptotic cells (lower panel). Data shown reflects a minimum of n=4 individual experiments; *P<0.05 ; **P<0.01 .

Figure 6: *PHD1 levels correlate with inflammatory score in IBD patients.* (A) Representative immunoblots of colon tissues from ulcerative colitis patients with either inactive (left lanes) of active (right lanes) mucosal inflammation. Equal amounts of protein were loaded. (B) Densitometric analysis of PHD1 protein concentrations in colon mucosa from patients with inactive (Truelove and Richards score 0-1) or active mucosal inflammation (score 2-3), normalized to

the expression of beta-actin. Bars represent mean \pm SEM of relative band intensity values (a.u., arbitrary units); *P<0.05; n=8.

A**B****D****C****E****Figure 1**

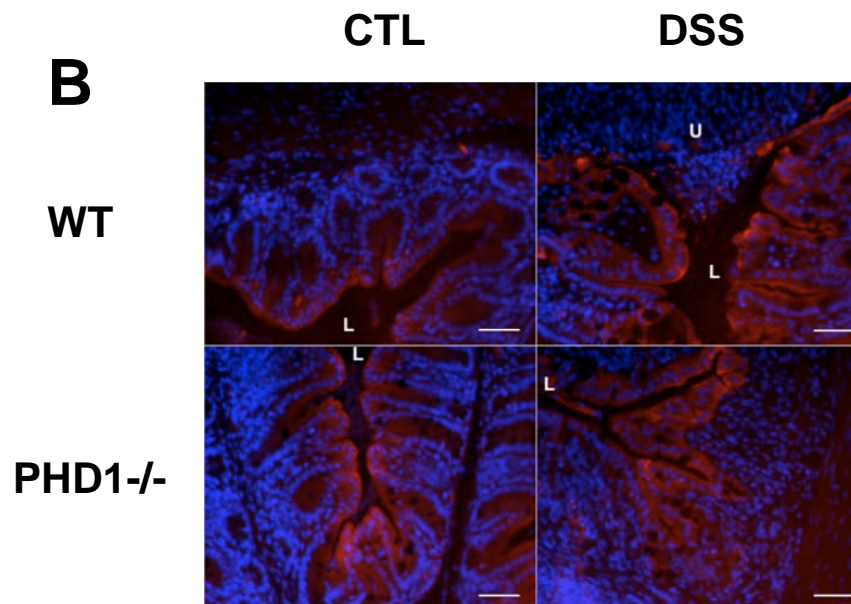
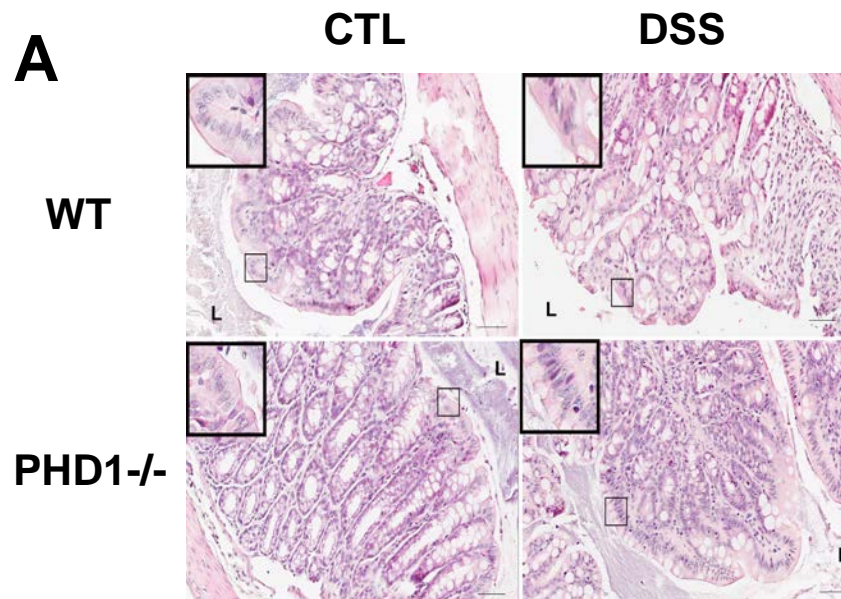
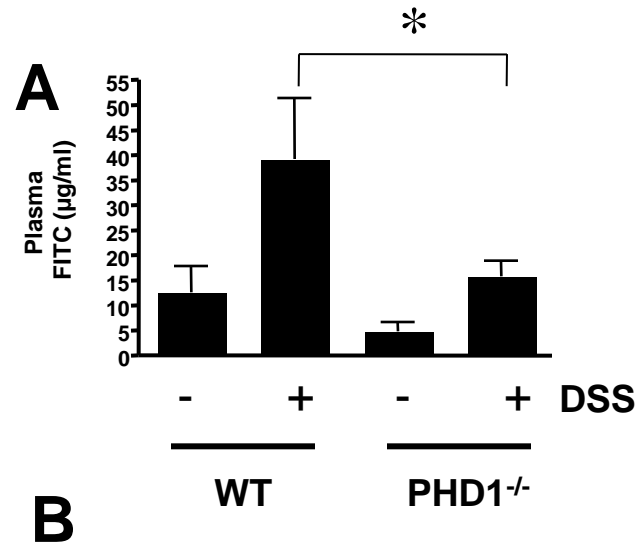


Figure 2



B

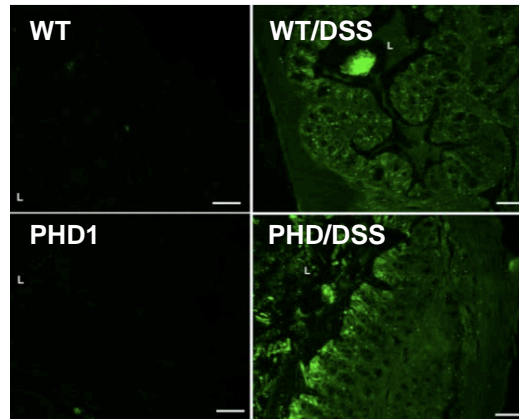


Figure 3

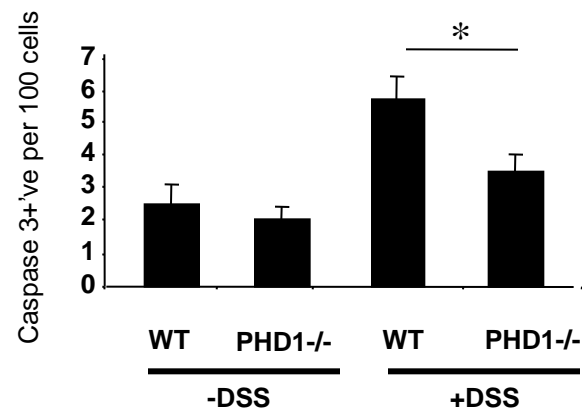
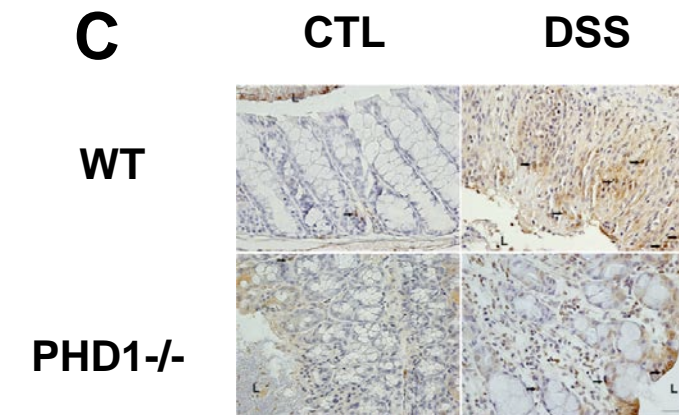
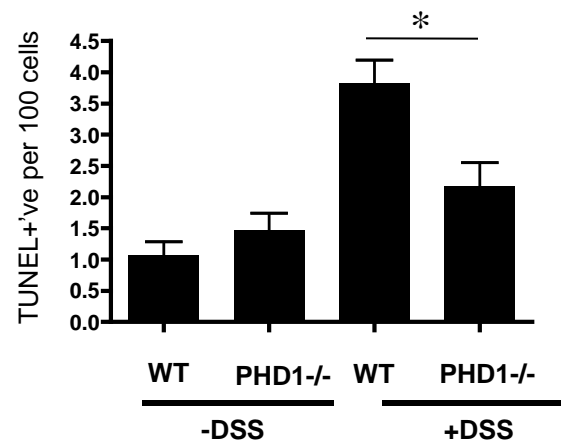
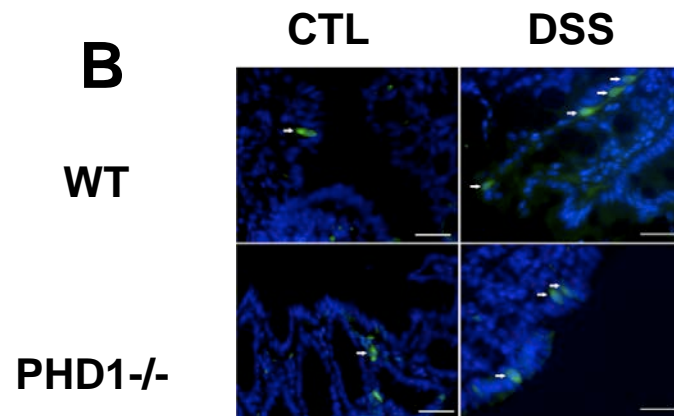
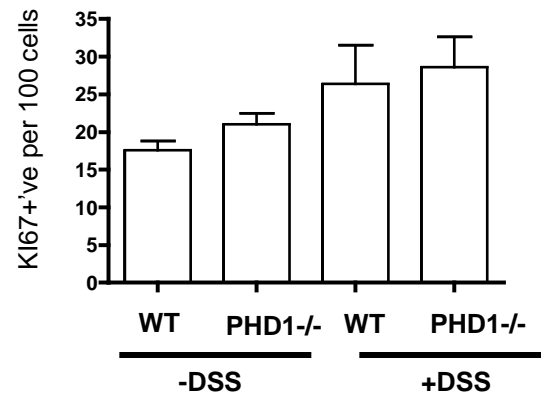
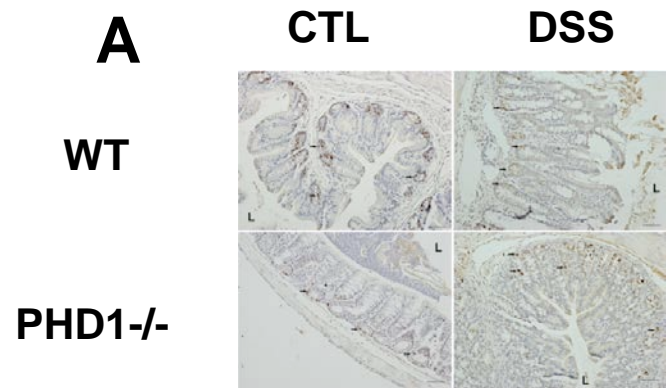
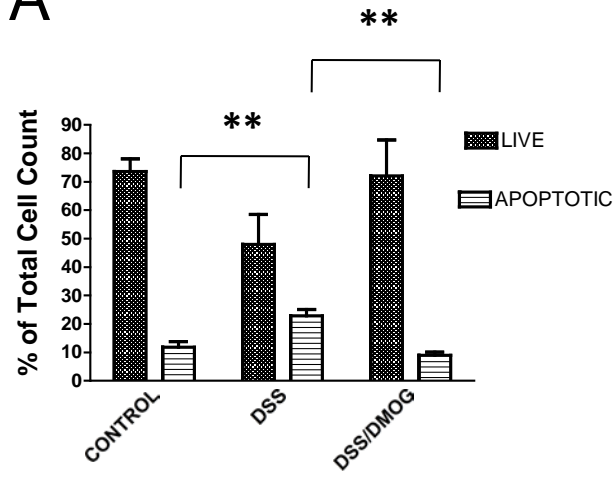
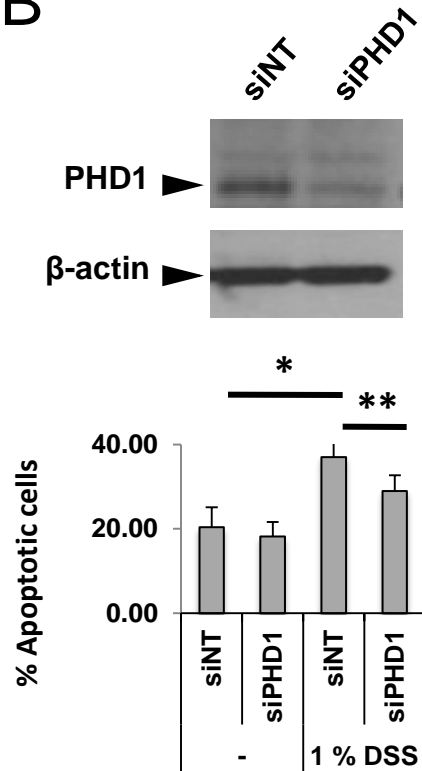
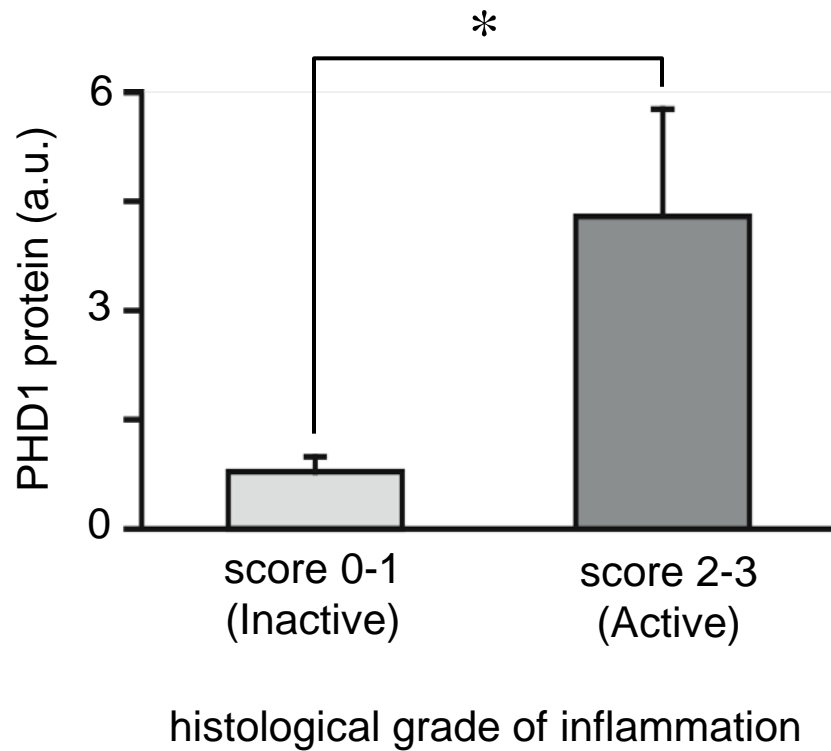
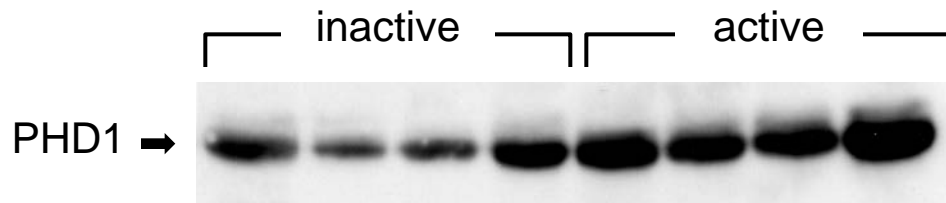
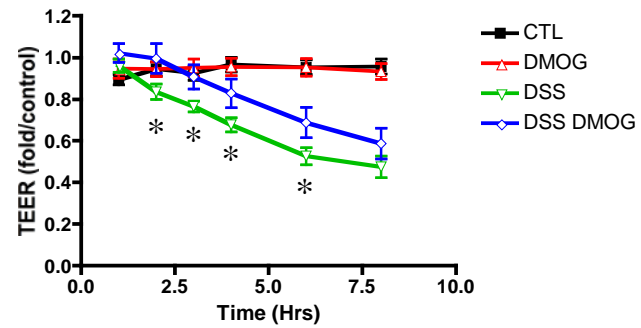


Figure 4

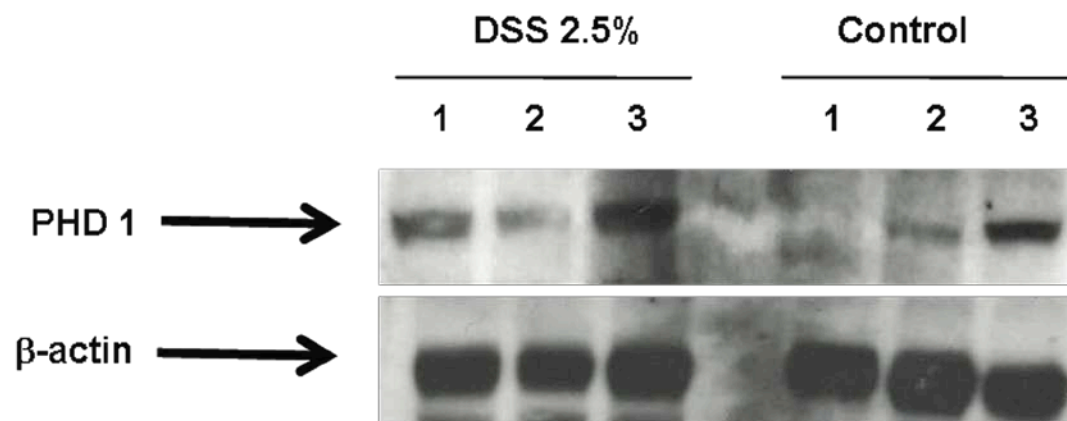
A**B****Figure 5**



Active mucosal inflammation is associated with enhanced *PHD1*



Supplementary Figure S1: Trans-epithelial electrical resistance measurements in CaCo-2 intestinal epithelial cells exposed to DSS with and without treatment with DMOG. Experiments were carried out 3 times in duplicate.



Supplementary Figure S2: (A) Western blot analysis of PHD1 expression in colonic tissues taken from wild type C57/BI 6 mice exposed to 6 days of 2.5% DSS in drinking water. n=3.