Flightless I exacerbation of inflammatory responses contributes to increased colonic damage in a mouse model of dextran sulphate sodium-induced ulcerative colitis

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Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by cytokine driven inflammation that disrupts the mucosa and impedes intestinal structure and functions. Flightless I (Flii) is an immuno-modulatory protein is a member of the gelsolin family of actin-remodelling proteins that regulates cellular and inflammatory processes critical in tissue repair. Here we investigated its involvement in UC and show that Flii is significantly elevated in colonic tissues of patients with inflammatory bowel disease. Using an acute murine model of colitis, we characterised the contribution of Flii to UC using mice with low (Flii⁻/⁻), normal (Flii¹⁺/⁻) and high Flii (FliiTg/Tg). High levels of Flii resulted in significantly elevated disease severity index scores, increased rectal bleeding and degree of colon shortening whereas, low Flii expression decreased disease severity, reduced tissue inflammation and improved clinical indicators of UC. Mice with high levels of Flii had significantly increased histological disease severity and elevated mucosal damage with significantly increased inflammatory cell infiltrate and significantly higher levels of TNF-α, IFN-γ, IL-5 and IL-13 pro-inflammatory cytokines. Additionally, Flii overexpression resulted in decreased β-catenin levels, inhibited Wnt/β-catenin signalling and impaired regeneration of colonic crypts. These studies suggest that high levels of Flii, as is observed in patients with UC, may adversely affect mucosal healing via mechanisms involving Th1 and Th2 mediated tissue inflammation and Wnt/β-catenin signalling pathway.

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with incidences of 7.6 to 13.9 cases per 100,000 people in Westernised industrialized nations. It is defined as a lifelong condition with periods of remission, which manifests in bloody diarrhoea, mucus and abdominal pain¹. It peaks in young adults and to lesser extent in the elderly. Pathogenesis of UC is unknown, although genetic susceptibility, environmental factors, microorganisms, immune dysregulation and chemical mediators have all been suggested as possible contributing factors². Symptoms may relapse and remit, but mucosal inflammation continues with spontaneous remission being uncommon. Treatment includes corticosteroids, aminosalicylates, immunomodulators and biologics such as anti-tumour necrosis factor-α (TNF-α) antibody, and surgical resection³. Randomised controlled trials have

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demonstrated that infliximab and adalimumab, TNF-α antibody therapies, are effective for patients with moderate to severe colitis significantly improving mucosal healing and rates of disease remission hence decreasing the need for colectomy. However, access and cost of this therapy is still a limiting factor for many UC patients, highlighting the need for novel targeted therapies. Additionally, only about two-thirds of subjects with UC respond well to treatment and in severe disease with pancolitis there is a cumulative risk of colon cancer that increases with time due to chronic inflammation.

Mucosal healing is currently accepted as a critical endpoint in the management of UC and regulation of colonic inflammation underpins mucosal healing. Many of the current UC treatments achieve clinical remission without complete mucosal healing which has been demonstrated to correlate with reduced risk of relapse and hospitalisation. Immuno-modulatory protein Flightless I (Flii) impairs skin barrier development, function and recovery post skin blistering and wounding. It negatively affects cellular processes including cellular adhesion, migration and proliferation as well as tight junction formation and macrophage and fibroblast cell secretion of TNF-α in the context of wound healing. Reduced levels of Flii expression, both genetically and using Flii neutralising antibodies, improves skin repair and regeneration in both small and large animal models of healing. Studies have identified Flii as a repressor of estrogen receptor signalling and apoptosis suggesting roles in promotion of both skin cancer and breast cancer progression. In contrast, Flii positively influences tissue regeneration in the context of Wnt signalling pathways during hair follicle regeneration and claw and digit tip regeneration, illustrating the diverse roles of this cytoskeletal protein. Importantly, Flii modulates TLR-4 mediated inflammatory responses, augments Th1/Th2 cell responses as well as autoantibody production and regulates inflammation in a number of inflammatory skin conditions including psoriasis, atopic dermatitis and epidermolysis bullosa acquisita. Its function has not been investigated in the intestine.

Using human UC samples, we sought to examine the levels of Flii in human disease. Additionally, using the dextran sulphate sodium (DSS)-induced colitis model in mice, a reproducible and well documented model of large intestinal damage, in conjunction with mice genetically manipulated to have either high or low levels of Flii, we investigated the role of Flii in UC and mucosal damage. Lastly, we aimed to characterise the effects of altered Flii levels on tissue inflammation in this model of DSS-induced colitis. Our hypothesis was that Flii would alter tissue inflammation and promote colitis development.

Results
Flightless I is significantly increased in human UC colonic tissue. Histological analysis of human samples revealed classic morphological characteristics associated with UC including increased inflammatory infiltrate in lamina propria, crypt distortion and shortening when compared to healthy control (Fig. 1A). Inflammatory infiltrate included neutrophils with infiltration of crypts and formation of crypt abscesses (not shown). Crypts were shortened and separated from muscularis mucosae with occasional branching (not shown) indicating chronicity of poorly controlled disease. No Flii staining was observed in colonocytes lining the lumen or colonic crypts. However strong Flii staining was observed in the inflammatory infiltrate present in the lamina propria (Fig. 1A). Cell counts of Flii positive cells in the human colonic tissue revealed significantly elevated...
levels of Flii in UC patients compared to healthy controls (Fig. 1B). Isotype control staining revealed negligible fluorescence (data not shown).

**Overexpression of Flightless I increases clinical disease severity following DSS consumption.** While only 37.5% of wild-type mice exhibited evidence of rectal bleeding, 100% of Flii overexpressing mice (FliiTg/Tg) showed signs of rectal bleeding and all had significantly higher average disease activity index on day 7 compared to either Flii−/− mice or wild-type counterparts (Fig. 2A). In contrast, mice with low Flii (Flii+/−) exhibited no rectal bleeding and significantly decreased average disease activity index from day 3 of the experiment (Fig. 2A). Examining the visceral and gastrointestinal organ weights showed no significant differences between the three genotypes (data not shown). However, when we compared the degree of colon shortening in colitis-induced mice by analysing the change in colon lengths in colitis-induced vs water control mice results showed that colitis-induced Flii−/− mice had reduced percentage of colon shortening compared to wild-type counterparts while FliiTg/Tg mice had significant increase in percentage of colon shortening compared to both Flii−/− and wild-type counterparts suggestive of greater disease severity in response to higher levels of Flii (Fig. 2B).

**Increased histological disease severity is observed in colitis-induced FliiTg/Tg mice.** Distal colonic tissue from Flii−/−, wild-type and FliiTg/Tg colitis-induced animals was examined and a clear increase in colitis severity was observed in mice with elevated levels of Flii including elevated polymorphonuclear infiltration (Fig. 3A). Overall there was a statistically significant increase in histological disease severity between FliiTg/Tg colitis-induced animals compared to Flii−/− counterparts (Fig. 3A). Additionally, colitis-induced FliiTg/Tg mice showed significantly delayed healing of damaged mucosal tissue as demonstrated by significantly reduced distal colon crypt depth compared to both Flii−/− and wild-type mice counterparts (Fig. 3B). Evidence of increased disease histological severity in colitis-induced FliiTg/Tg mice was also observed following analysis of crypt area index revealing a significantly decreased crypt area index in FliiTg/Tg mice compared to Flii−/− and wild-type mice counterparts (Fig. 4). Conversely, mice with low levels of Flii showed a significant increase in crypt area index compared to both normal and FliiTg/Tg mice suggestive of decreased colitis severity.

**Decreased Flii levels lead to a reduced inflammation in DSS-induced colitis.** Flii is a known regulator of cellular proliferation and inflammation[6,10]. To ascertain the effect of differential Flii on mucosal healing of colitis-induced mice, enterocyte proliferation and total tissue inflammation were assessed. No effect of Flii altering levels were observed on enterocyte proliferation as demonstrated by analysis of the numbers of PCNA positive cells in the crypts of colitis-induced Flii−/−, wild-type and FliiTg/Tg mice (Supplementary Fig. 1A). However, assessment of total tissue inflammation by MPO analysis revealed significantly decreased levels of tissue inflammation in colitis-induced Flii−/− mice compared to both wild-type and FliiTg/Tg counterparts (Supplementary Fig. 1B). Additionally, distal colons of colitis-induced Flii−/− mice showed significantly lower levels of TNF-α compared to wild-type and FliiTg/Tg counterparts with staining observed in only apical enterocytes (Fig. 5A,B). Increased levels of Flii also resulted in exacerbation of both Th1 and Th2 immune responses in colitis-induced mice with significantly greater levels of TNF-α, IFN-γ, IL-5 and IL-13 (Fig. 5C) being observed. Similarly, mice with low Flii exhibited a reduced inflammatory response in their distal colons with significantly decreased levels of TNF-α, IL-17A and IL-5 (Fig. 5C).

**Figure 2.** Increased clinical disease severity is observed in Flii overexpressing mice following DSS consumption. (A) All Flii overexpressing mice showed evidence of rectal bleeding and a significantly increased disease activity index on day 7 of the experiment compared to wild-type mice. Flii deficient mice show no evidence of rectal bleeding and significantly decreased disease activity index from day 3 of the experiment. (B) Colon lengths of colitis-induced Flii−/−, wild-type and FliiTg/Tg mice were compared to colon lengths in water control counterpart mice, and percentage change in colon shortening analysed. Colitis-induced Flii−/− mice have significantly reduced percentage of colon shortening while FliiTg/Tg have significantly increased percentage of colon shortening compared to wild-type counterparts, suggestive of increased UC damage in mice with high Flii levels. n = 8/genotype. Mean +/- SEM. *p < 0.05.
Flii over-expression inhibits Wnt/β-catenin signalling and impairs regeneration of colonic crypts in DSS-induced colitis.

Flii has previously been shown to modulate Wnt/β-catenin signalling and regulate tissue regeneration16,17,26. To determine the effect of differential Flii gene expression on regeneration of distal colonic crypts in colitis-induced mice, Wnt/β-catenin signalling was assessed. Flii overexpression was found to inhibit Wnt/β-catenin signalling, with significantly decreased levels of Lgr6 receptor and intracellular β-catenin levels while Flii deficiency resulted in a significantly decreased number of Axin-2 positive cells (Fig. 6A–E). These findings were further confirmed using PCR and Western Blotting (Fig. 6E–G). These findings suggest that Flii effects on Wnt/β-catenin signalling pathway may underpin the impaired regeneration of colonic crypts observed in Flii over-expressing mice (Fig. 6H).

Figure 3. Increased disease histological severity is observed in Flii over-expressing mice. (A) Flii overexpressing mice showed significantly higher histological disease severity compared with Flii deficient mice, with significantly damaged mucosal tissue and high level of pro-inflammatory cell infiltrate. (B) Flii overexpression resulted in significantly delayed healing of blistered mucosal tissue with significantly reduced distal colon crypt depth compared to both wild-type controls and Flii deficient mice counterparts. Magnification ×4 and ×20. Scale Bar = 200µm. n = 8/genotype. Mean ±/− SEM. *p < 0.05.

Figure 4. High Flii levels promote mucosal damage in DSS model of UC. (A) Flii overexpressing mice show significantly decreased crypt area index indicative of increased disease severity compared to wild-type controls, while Flii deficient mice have significantly increased crypt area index indicative of decreased mucosal blistering and gut damage in the DSS model of UC. n = 8/genotype. Magnification ×4 and ×20. Scale Bar = 200µm. Mean ±/− SEM. *p < 0.05.
Discussion

UC is a chronic inflammatory disease that can lead to severe consequences including colectomy and significantly increased risk of colorectal cancer. Retrospective and prospective studies with UC patients have highlighted the importance of mucosal healing as the critical endpoint in disease management. This study has shown that human UC lesions have significantly elevated levels of Flil, a cytoskeletal protein previously shown to impair healing responses and to be upregulated in response to tissue inflammation in a number of different inflammatory skin disease conditions including human psoriasis, dermatitis and inflammation mediated epidermolysis bullosa acquisita. In the current study, Flil was prominent in the inflammatory infiltrate of human lamina propria surrounding the distal colon crypts suggesting its potential involvement in the inflammatory pathway of human colitis. Studies have previously demonstrated Flil expression in organs susceptible to inflammation and fibrosis including liver, lung and kidney. Although Flil levels in normal gut are low, it has previously been shown to be upregulated in response to injury and inflammation hence its involvement in inflammatory mediated conditions like human UC is not surprising.

This study therefore set out to determine the extent of Flil involvement in UC and mucosal healing using an acute model of DSS-induced colitis which closely resembles clinical and histopathological features of human UC. While the mechanism of DSS-induced damage in the colon remain unclear, damage is most prominent in the distal colon and is believed to be caused by alterations in colonic microflora, direct cytotoxic effects on the epithelium and increased macrophage and neutrophil activity resulting in free radical production. Clear differences in disease severity were observed in response to altered Flil levels including higher degree of colon shortening, decreased crypt depth and increased inflammation in animals with high Flil. In contrast, reducing Flil expression resulted in significantly reduced levels of colon shortening, no evidence of rectal bleeding, significantly decreased disease severity and significantly higher crypt area index compared to mice with normal levels of Flil. Together, these results suggest that high levels of Flil in the gut of patients with UC, may exert a negative influence on clinical disease progression and recurrence.

Acute DSS-induced colitis is characterized by an increase in pro-inflammatory cytokines TNF-α and IFN-γ which are the major proinflammatory cytokines that synergistically drive epithelial barrier dysfunction and apoptosis, particularly during colitis, while chronic DSS-induced colitis comprises focal Wnt/β-catenin mediated epithelial regeneration and both Th1 and Th2 pro-inflammatory cytokine profiles. While the atypical cytokine profile of a Th2 reaction is more prevalent in patients with UC, the absence of prototypic IL-4 combined with Arthurs reaction of neutrophil infiltration contributes to disease chronicity. Additionally, in patients, IFN-γ has been causatively involved in UC epithelial homeostasis and intestinal inflammation while IL-17A is associated with increased...
UC disease activity and ability to trigger and amplify multiple inflammatory pathways regulating gut inflammation. Flii has been demonstrated to regulate inflammation through its effects on TLR4 signalling pathway both intracellularly and extracellularly. Its intracellular effect on TLR4 signalling and subsequent NF-κB secretion is mediated via interactions with Myd88 and has been shown to affect inflammation signalling in inflammatory mediated psoriasiform dermatitis. Flii is secreted through a non-classical late endosome/lysosome mediated pathway by both fibroblasts and macrophages, and is present in both acute and chronic human wound fluids. Like its family member gelsolin, plasma Flii functions to scavenge extracellular actin following injury and mediate inflammatory responses. Plasma Flii binding to lipopolysaccharide alters macrophage activation and subsequent macrophage secretion of TNF-α. Additionally, a recent study has shown that Flii alters inflammatory responses in inflammation mediated atopic dermatitis, where high Flii correlates with increased inflammatory responses resulting in a skewed Th2 response. Indeed, this atypical Th2 response with increased IL-5 and IL-13 levels has been observed in chronic UC patients. The observed effects of Flii on Th1/Th2 immune responses are also in agreement with previous reports showing high Flii correlates with increased inflammatory responses resulting in a skewed Th2 response.

In this study, a significantly increased inflammatory cell infiltrate was observed in the distal colon of colitis-induced Flii overexpressing animals compared to controls while colitis-induced mice with low levels of Flii showed significantly decreased MPO activity in the distal colon suggesting Flii may augment UC mediated inflammation and mucosal healing. Furthermore, examining the effect of Flii on cytokines known to drive UC mediated tissue inflammation revealed that reducing Flii expression results in a decrease in tissue inflammation and significantly lower levels of pro-inflammatory cytokines including TNF-α, IL-17A and IL-5; all of which would favour decreased UC disease severity. In contrast, but in agreement with increased UC disease severity observed in FliiTg/+ mice, distal colons of these colitis-induced mice showed an exacerbated immune response with significantly increased expression of Th1 and Th2 cytokines including TNF-α, IL-17A and IL-5. Indeed, this atypical Th2 response with increased IL-5 and IL-13 levels has been observed in chronic UC patients. The observed effects of Flii on Th1/Th2 immune responses are also in agreement with previous reports showing high levels of Flii alter immune responses in inflammation mediated conditions including psoriasiform dermatitis and atopic dermatitis. Together, these findings suggest that Flii plays an important role in inflammatory mediated conditions, like UC, and that its effect on inflammation promotes a Th2 mediated response in UC which would favour more chronic disease state. Additionally, numerous studies to date have postulated that this exacerbated Th2 mediated response in UC patients is an attempt to activate mucosal Wnt/β-catenin signalling known to...
regulate intestinal epithelial stem cell proliferation required for regeneration of colonic crypts\(^{41-43}\). Cooperative interaction between Wnt and R-spondin ligands establishes a molecular precedent for regulation of intestinal stem cells required for colonic tissue regeneration\(^{44}\). Our recent study has described the Flii regulation of Wnt signalling during skin homeostasis and wound healing indicating that Flii negatively regulates epidermal stem cell activation via its effects of Wnt signalling pathway\(^{45}\). How Wnt/\(\beta\)-catenin signalling pathway contributes to wound healing during colitis has yet to be formally established. However, it is well accepted that Wnt signalling pathway is crucial for development and renewal of the intestinal epithelium\(^{46}\). Here we demonstrate that Flii overexpression leads to inhibition of Wnt signalling with decreased expression of \(\beta\)-catenin and leucine-rich repeat-containing G protein-coupled receptor 6 (Lgr6) receptor required for R-spondin amplification of canonical Wnt signalling. This agrees with previous studies showing that Flii can inhibit Wnt signalling by binding to negative regulators of the Wnt signalling pathway through Dishevelled (Dvl) protein interactions\(^{47}\).

We show that Flii overexpression leads to decreased \(\beta\)-catenin expression suggesting that Flii may decrease \(\beta\)-catenin stabilisation and increase ubiquitin-mediated and proteasomal \(\beta\)-catenin degradation. This important finding is in agreement with previous studies which suggested that Flii inhibition of Wnt signalling occurs via \(\beta\)-catenin binding and inhibited lymphoid enhancer factor (LEF) and T-cell factor (TCF) transcriptional factor-mediated expression of Wnt target genes\(^{48}\). Tight regulation of Wnt/\(\beta\)-catenin signalling by Flii was also evident in Flii deficient mice which showed decreased Axin-2 expression, supporting our earlier findings which suggested that Flii may impact \(\beta\)-catenin via Axin-2 regulation at the transcriptional level\(^{49}\). Further studies are required to identify the specific molecular patterns governing Flii involvement in Wnt signalling pathway and subsequent effects on activation and proliferation of intestinal stem cells.

In conclusion, we have demonstrated that Flii is upregulated in the distal colon of human UC patients. High levels of Flii correlate with greater inflammation and exacerbated Th1/Th17 immune responses resulting in increased disease severity in mouse models of DSS-induced colitis, while reducing Flii levels promotes decreased gut inflammation and improved mucosal healing. Although the exact mechanisms of Flii function in UC are yet to be elucidated, our results suggest that Flii negatively regulates Wnt/\(\beta\)-catenin signalling required for regeneration of colonic crypts. Together these results suggest that manipulation of Flii levels may lead to potential novel therapeutic interventions by which UC disease severity, tissue inflammation and mucosal healing may be improved.

**Materials and Methods**

**Human studies.** Colonoscopies were performed at The Queen Elizabeth Hospital (TQEH, Adelaide). Ten adult subjects with UC, and 10 normal adult subjects with non-inflammatory conditions, such as irritable bowel syndrome or who attended for colon cancer screening were included in the study. All experimental protocols were approved by the Human Ethics Committee of the TQE in accordance with relevant guidelines and regulations. Approval was given to perform additional biopsy for research and to archive biopsies for future studies and written informed consent was obtained from all participants\(^{48}\). Colonic biopsies in histologic paraffin blocks were retrieved and histological sections (4 \(\mu\)m) stained with haematoxylin and eosin (H&E) and standard immunohistochemistry staining protocols (see below) for Flii (2 mg/ml; anti-Flightless I sc-30046 rabbit IgG; Santa Cruz Biotechnology, CA, USA) and 4′-diamidino-2-phenylindole (DAPI; 0.1 mg/ml; D1306; Live Technologies, VIC, AUS) as previously described\(^{48}\).

**Animal studies.** Female Balb/c mice were maintained according to the Australian Standards for Animal Care under the protocols approved by the Child, Youth and Women’s Health Service Animal Ethics Committee, The University of Adelaide Animal Ethics Committee and University of South Australia Animal Ethics Committee (AEC 962/12/16 and AEC 137a/13). All strains were BALB/c-congenic and were maintained as homozygous colonies or by continuous backcrossing to BALB/c animals. Wild-type controls were obtained from BALB/c inbred litters. The murine alleles of Flii used in this study include: a heterozygous carrier of the murine Flightless I gene via homologous recombination in embryonic stem cells and passage of these cells through the germ line following chimera production\(^{48}\). The generation of Flii\(^{+/−}\) mice and the resulting mutation are described in detail in Campbell et al., (2002) and a diagram of the targeting strategy is illustrated in Supplementary Fig. 2A. The heterozygous mice were identified using three primer PCR sets that amplified products specific to the wild-type or targeted allele as illustrated in Supplementary Fig. 2B. The PCR was performed on DNA extracted from ear biopsies of potential heterozygotes. The animals with one wild-type copy of the Flii gene and one mutant copy of the Flii gene express no more than 50% of the normal Flii gene expression\(^{49}\).

Mice homozygous for the transgene were used in this study: had two copies of Flii gene and two copies of human FLII transgene (Flii\(^{+/+}\); FLII\(^{+/+}\)) with significantly elevated levels of Flii protein compared to wild-type\(^{48}\) and are denoted as Flii\(^{+/−}\) throughout the article. Mice carrying additional copies of the Flii gene were generated by introduction of a cosmids construct into the mouse genome using transgenesis. At the time of strain production, the cosmids contained the human Flii gene and the surrounding sequences with the extent of the construct being defined via restriction mapping\(^{48}\). The availability of the mouse genome allowed estimation of the extent of the cosmids. Currently, it is known that the cosmids contains all the neighbouring Smcr7 gene and parts of the Topo and LLGL1 genes (Supplementary Fig. 2C). The transgenic strain was backcrossed to Balb/c for 10 generations before being intercrossed; and homozygous animals were classified via progeny testing following established protocols\(^{49,50}\). The mouse colony was subsequently maintained by intercross of animals homozygous for the transgene. The expression of Human FLII gene was examined using species specific RT-PCR showing FLII expression in all tissues examined (adult brain, heart, lung, muscle, spleen and skin) (Supplementary Fig. 2D)\(^{50}\).
An upregulation of Flii protein levels was confirmed using semi-quantitative Western analysis that showed total (mouse + human) protein levels up to 1.52 fold greater than wild-type levels (Supplementary Fig. 2E) 

Colonic inflammation was induced in mice using Dextran Sulphate Sodium (DSS; colitis grade; MW 36,000–50,000; #02160110; MP Biomedicals, Jomar Life Research, SA, AUS) 

Histology and immunohistochemistry. Paraffin embedded, fixed tissue samples were stained with H&E or subjected to antigen retrieval and immunohistochemistry following manufacturer’s protocols (DAKO Corporation, DK). H&E stained sections were used for standardised measurements of colon length, crypt depth, crypt area index and histological disease severity following established protocols 

Q-PCR. Harvested tissue was snap-frozen in liquid nitrogen and total RNA was isolated from 1 cm of distal colon per sample (n = 6/genotype) using Ultraclean Tissue and Cell RNA Isolation Kit (MoBio Laboratories, CA, USA) according to the manufacturer’s protocol. Total cDNA was reverse-transcribed from equal amount of RNA (200 ng) per sample using iScript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) according to manufacturer’s protocol. The PCR reaction mix consisted of 2 μl RT reaction mix, 5 μl 5 × PCR buffer, RNA and water making a total volume of 20 μl. The reaction was initiated by incubation at 25 °C for 5 min, followed by annealing at 42 °C for 30 min and final incubation at 85 °C for 5 min followed by 10 min at 4 °C. Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA) in triplicate reactions in CFX connect real-time PCR system and analysed by CFX Maestro software (Bio-Rad Laboratories, CA, USA). Each Q-PCR reaction mix consisted of 10 μl supermix, 1 μl of cDNA, primers and water making a total volume of 20 μl. A three-step PCR was carried out with initial denaturation for 30 s at 95 °C, followed by 39 cycles of denaturation for 5 s at 95 °C and annealing for 20 s at 60 °C with a final extension of denaturation for 10 s at 95 °C and annealing for 5 s at 60 °C. CypA and GAPDH were used as reference genes and the inter-reaction calculator method was applied for all plates. For relative comparison, the cycle threshold value (Ct) was analysed using the ΔΔCt method and data reported as Ct normalized to reference genes. Gene expression was expressed as fold change of ΔΔCt.
Primary antibodies including anti-β-catenin sc-7963 rabbit polyclonal IgG (1:400), anti-Axin-2 (ab32197) rabbit polyclonal IgG (1:400) and anti-α-tubulin sc-51670 mouse monoclonal IgG (1:3000) were diluted in buffer and applied to the membrane at 4 °C overnight. Species-specific secondary horseradish peroxidase-conjugated antibodies were diluted in 5% milk-blocking buffer and applied to the membrane at room temperature for 1 hour. Protein bands were detected using Super Signal West Femto (Pierce Biotechnology, Rockford, IL) and visualized with GeneSys analysis software (Syngene, MD).

Statistical analysis. Parametric data were expressed as mean ± standard error of the mean (SEM). Histological crypt depth and MPO activity were analysed using a one-way ANOVA with Tukey’s post hoc tests. Disease activity index were analysed using repeated measures ANOVA with least significance difference to compare the differences both between and within groups. Non-parametric data included histological damage severity scores and were analysed using a Kruskal Wallis test with Mann Whitney U tests, expressed as median range. p < 0.05 was considered statistically significant.

Data Availability
All data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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