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Phosphoinositide 3-Kinase Signaling Mediates β -Catenin Activation in Intestinal Epithelial Stem and Progenitor Cells in Colitis

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Abstract

Background & Aims—Mechanisms responsible for crypt architectural distortion in chronic ulcerative colitis (CUC) are not well understood. Data indicate that Akt signaling cooperates with Wnt to activate β -catenin in intestinal stem and progenitor cells through phosphorylation at Ser552 (P- β -catenin⁵⁵²). We investigated whether phosphoinositide 3-kinase (PI3K) is required for Akt-mediated activation of β -catenin during intestinal inflammation.

Methods—The class IA subunit of PI3K was conditionally deleted from intestinal epithelial cells in mice. Acute inflammation was induced in these mice (*I-pik3r1KO*) and their intestines were analyzed by biochemical and histological methods. The effects of chemically blocking PI3K in colitic *IL-10*^{-/-} mice were examined. Biopsy samples from patients were examined.

Results—Compared to wild type mice, *I-pik3r1KO* mice had reduced T-cell-mediated Akt and β -catenin signaling in intestinal stem and progenitor cells and limited crypt epithelial proliferation. Biochemical analyses indicated that PI3K–Akt signaling increased nuclear total β -catenin and P- β -catenin⁵⁵² levels and reduced phosphorylation of N-terminal β -catenin, which is associated with degradation. PI3K inhibition in *IL-10*^{-/-} mice impaired colitis-induced epithelial Akt and β -catenin activation, reduced progenitor cell expansion, and prevented dysplasia. Human samples had increased numbers of progenitor cells with P- β -catenin⁵⁵² throughout expanded crypts and increased mRNA expression of β -catenin target genes in CUC, colitis-associated cancer, tubular adenomas, and sporadic colorectal cancer, compared with control samples.

Conclusions—PI3K–Akt signaling cooperates with Wnt to increase β -catenin signaling during inflammation. PI3K-induced and Akt-mediated β -catenin signaling are required for progenitor cell activation during the progression from CUC to CAC; these factors might be used as biomarkers of dysplastic transformation in the colon.

Keywords

PI3K; pik3r1; intestinal stem cells; intestinal progenitor cells; ulcerative colitis; inflammatory bowel disease; colon cancer; β -catenin; T-cell activation

INTRODUCTION

In inflammatory bowel disease (IBD), such as chronic ulcerative colitis (CUC) and Crohn's disease (CD), intestinal epithelial cells (IEC) respond to chronic inflammation with crypt cell hyperplasia,¹ apoptosis, and crypt architectural distortion.² The appearance of crypt branching in IBD^{3, 4} suggests that during inflammation, multipotent progenitor cells are activated to form new crypt structures. Intestinal stem cells (ISC) reside in crypt bases where Wnt ligand binding prevents N-terminal phosphorylation and proteosomal degradation of β -catenin.^{5–8} When degradation is impaired by Wnt signaling, β -catenin translocates to the nucleus where it binds T-cell factor/lymphoid enhancing factor (TCF-LEF) and initiates transcription of Wnt target genes such as *c-myc* and *cyclin-D1*.^{6, 9, 10} Nuclear accumulation of β -catenin is a hallmark of activated canonical Wnt/ β -catenin signaling.¹¹ Upon division, ISCs give rise to rapidly cycling progenitor cells, also called transit-amplifying cells.^{12, 13} Bjerknes and Cheng identified crypt-base columnar (CBC) ISCs and multipotent “Mix” progenitors derived from ISCs.¹³ Mix in turn generated progenitors committed to different epithelial lineages. In IBD, increased crypt fissioning or branching and columnar cell hyperplasia suggest that

inflammation promotes activation of ISCs and populations of proliferative progenitor cells within the transit-amplifying zone.

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling has been proposed to induce proliferative signals in IECs.^{14–16} Recent studies suggest PI3K/Akt signaling is upregulated in crypt IECs in CUC and active CD.17 PI3K-mediated generation of phosphatidylinositol-3,4,5-triphosphate (PIP₃) recruits Akt for activation of proliferation and survival signaling.¹⁶ He et al reported that PTEN deficiency increased PI3K/Akt activation resulting in excessive proliferation and crypt fissioning within small bowel (SB) polyps.¹⁵ To investigate the interface between activated PI3K/Akt and β -catenin stabilization, researchers developed a phospho-specific antibody that identifies Akt phosphorylated β -catenin at Ser552 (P- β -catenin⁵⁵²). Data suggested C-terminal phosphorylation at Ser552 was associated with nuclear accumulation and transcriptional activation.^{15, 18} Staining of tissue from SB polyps found P- β -catenin⁵⁵² within nuclei of dividing (activated) ISC and progenitor cells.¹⁵ These results were consistent with the notion that PI3K/Akt activation cooperated with Wnt to activate β -catenin signaling in ISC and progenitor cells.

To better understand the mechanisms by which PI3K/Akt regulates epithelial β -catenin signaling at physiologic and molecular levels, we generated mice with IEC-specific deletion of *pik3r1* (*I-pik3r1KO*), the gene for class IA PI3K (p85 α , p55 α and p50 α subunits). *I-pik3r1KO* mice have reduced T cell-induced β -catenin activation and proliferation in IECs. Inhibition of PI3K signaling in IL-10 knockout (*IL-10*^{-/-}) mouse colitis abrogated β -catenin signaling, crypt proliferation and dysplasia. Furthermore, we observed increased levels of P- β -catenin⁵⁵² (i.e. Akt-phosphorylated nuclear β -catenin) in human IBD, UC-associated dysplasia (UCD) and colitis-associated cancer (CAC). These results are supported by data indicating Wnt/ β -catenin signaling is increased in IBD.^{19, 20}

MATERIALS AND METHODS

Animals

C57BL/6J (B6) and *IL-10*^{-/-} (B6) mice were used (Jackson Laboratory, Bar Harbor, ME). AhCre mice were bred with *pik3r1*^{lox/lox} mice (gift from Lewis Cantley, Boston, MA). Mice were maintained under specific pathogen-free conditions and experiments were approved by the institutional ACUC.

Cre-Recombinase was induced by intraperitoneal (IP) injection of 80mg/kg of β -naphthoflavone (Sigma, St Louis, MO) dissolved in corn oil (8 mg/mL, Sigma) over six days. 0.2mg anti-CD3 monoclonal antibody was administered IP (145-2C11) or control hamster mAb (UC8-IB9) at time zero. Mice were IP-injected with 1mg of BrdU (Sigma) 2 hours prior to sacrifice. To synchronize colitis, 4–6 week old *IL-10*^{-/-} mice were fed pellet-chow containing piroxicam (Sigma) for two weeks; 65mg/250g piroxicam for one week, then 85 mg/250gm the next week (Harlan Teklad custom diet). LY294002 (Sigma) were IP-injected at 50mg/kg dissolved in 20% DMSO (Sigma) every-other day. Untreated mice received DMSO. For long-term retention of BrdU (BrdU-LTR) irradiated (8Gy) mice were IP-injected with BrdU 3 times a day for 2 days as described.²¹

Human colonic specimens

Biopsies were obtained from patients 18 years or older undergoing diagnostic or surveillance colonoscopy, or surgical resections. No patients were pregnant, had a history of intestinal surgery, bleeding diathesis, or coagulopathy. No control or CUC patients were on colitis treatment. Untreated-active CUC patients underwent colonoscopy for increased symptoms

(mean UCDAI score of 9.8+/-2.34). All patient materials were approved by Northwestern University Office for the Protection of Human Subjects.

Histology and Immunohistochemistry

Antigen retrieval of formalin-fixed paraffin sections was performed using Target retrieval solution (Dako, Carpinteria, CA) in a microwave (P-Akt) or a decloaking chamber (all other antibodies; Biocare medical, Concord CA). Sections were incubated with anti-BrdU (Accurate Chemical, Westbury, NY), anti-P-Akt^{T308} (Cell Signaling Technology, Davers, MA), anti-P- β -catenin⁵⁵² (from LL), Ki67 clone TEC-3 (Dako), DCAMKL-1 (Abgent, San Diego, CA), Musashi (Cell signaling), anti-cMyc clone N262 (Santa Cruz, Santa Cruz, CA) and anti-cyclin D1 clone SP4 (Thermo, Fremont, CA) followed by anti-rabbit or mouse-HRP labeled polymer (Dako) or anti-rat VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). LacZ staining was performed as described.¹⁵ Slides were developed using 3,3'-diaminobenzidine tetrahydrochloride or Bajoran Purple Chromogen for double-staining (Biocare Medical LLC, Concord, CA), and counterstained with hematoxylin. H&E staining was performed to determine colitis scores (0–12 based on severity)²² and dysplasia. For counting cells at specific crypt positions, sections of at least 50 well-oriented crypts were analyzed from 3 mice as described.^{23, 24}

Epithelial Cell Isolation and Leukocyte depletion

IECs were isolated as described.^{25, 26} Tissue was washed (0.15M NaCl, 1mM DTT, 40 μ g/mL PMSF), incubated at room temperature with EDTA-buffer [109mM NaCl, 2.4mM KCl, 1.5mM KH₂PO₄, 4.3mM Na₂HPO₄, 2mM EDTA, 10mM glucose, 5mM glutamine, 0.5mM DTT and 40 μ g/ml PMSF (pH7.4)] six times for eight minutes, and finally washed with PBS. Crypts were in washes 4–6. Colon tissue was treated for 45 minutes with dispase (Invitrogen, Carlsbad, CA). CD45-positive cells were depleted using Dynabeads sheep anti-rat-IgG (Invitrogen) and anti-CD45 (BD Pharmingen, San Diego, CA) as described.²⁶ CD45+ cells were <1% of the final isolated cells.

Statistical analysis

All experiments were repeated three times with at least 8–110 mice (3 mice in siRNA study) in each group. Comparison was assessed by Student's t test or Mann-Whitney U test. $p < 0.05$ was considered statistically significant.

RESULTS

IEC PI3K/Akt signaling is abolished in *I-pik3r1 KO* mice

P- β -catenin⁵⁵² levels are increased in proliferative regions and sites of crypt fissioning within SB polyps.¹⁵ We found that P- β -catenin⁵⁵² is enhanced in patients with CUC and reduced by mesalamine treatment.²⁷ To establish a conditional genetic model to investigate whether class I_A PI3K is required in IECs for β -catenin activation, mice carrying *loxP*-flanked exon 7 of *pik3r1* (*pik3r1^{lox/lox}*)²⁸ were crossed with mice carrying Cre recombinase regulated by the promoter for the cytochrome P4501A1 (*Cyp1a1*) gene (referred to here as *AhCre*).²⁹ *Cyp1a1* is upregulated on exposure to the lipophilic xenobiotic β -naphthoflavone (BNF). Previous data showed that within the SB, Cre recombinase activity specifically localized to epithelial cells. However, outside the intestine, recombination was reported in the liver and pancreas with BNF induction. This conditional knockout avoids perinatal lethality observed in germline knockout mice.³⁰ Cre-mediated deletion of exon 7 of *pik3r1* ablates expression of p85 α , p55 α and p50 α proteins in IECs (hereafter referred to as *I-pik3r1 KO* mice). Nearly 100% SB recombination is achieved in *AhCre* mice after Cre recombinase induction as scored using *AhCre/Rosa26R* reporter mice (Supplemental Figure 1 A–D). Since IP injection of BNF

resulted in incomplete recombination in the colon (Supplemental Figure 1E) only SB tissue was examined.

Western blot analysis of purified epithelial extracts from *I-pik3r1KO* mice revealed the complete loss of p85 α and greater than 90% reduction in p85 and p110 α in BNF-induced *I-pik3r1KO* mice relative to control (*pik3r1^{lox/lox}*) mice (Figure 1). Reduction of p110 α catalytic subunit levels are consistent with findings that p85 α is necessary for p110 α stability.²⁸ Next, the role of PI3K signaling in epithelial responses to T cell activation was evaluated in control and *I-pik3r1KO* mice. To simulate epithelial responses in IBD, we utilized an *in vivo* model of T cell activation induced by intraperitoneal injection of anti-CD3 monoclonal antibody (mAb).^{31–33} Flow cytometry of lamina propria T cells revealed that IEC deletion of *pik3r1* did not affect T cell activation (Supplemental Figure 2A–D). In addition T cell induced intestinal fluid accumulation and cytokine production were largely unaffected by IEC *pik3r1* deletion (Supplemental Figure 2E,G). In control mice, T cell activation increased epithelial p85 α levels whereas total p85 and p110 α levels were slightly increased. In contrast, levels of p85 α , p110 α and total p85 were essentially ablated in *I-pik3r1KO* mice, with or without T cell activation (Figure 1). Thus, IEC *pik3r1* deletion attenuated p85 α , total p85 and p110 α protein levels in control and anti-CD3-treated mice.

***I-pik3r1KO* mice display reduced IEC activation of PI3K and β -catenin after T cell activation**

To determine if PI3K is involved in the molecular mechanism of inflammation-induced epithelial β -catenin signaling, PI3K and β -catenin signaling in IECs were examined after T cell activation in control and *I-pik3r1KO* mice. Data in Figure 2A show that T cell stimulation increased Akt activation as detected by phosphorylation at Ser473 of Akt in nuclear and cytoplasmic fractions of control mice. Reduced nuclear forkhead transcriptional factor Foxo1 (FOXO1), with elevated cytoplasmic levels of FOXO1, further supports the model that Akt is indeed activated by T cell stimulation. Complete inhibition of PI3K signaling in *I-pik3r1KO* mice was demonstrated by the ablation of Akt activation in anti-CD3-treated mice and of FOXO1 translocation from the nucleus. As data suggest *I-pik3r1 KO* mice exhibit attenuated PI3K signaling, we next evaluated T cell induced β -catenin signaling in IECs.

Reduced β -catenin degradation and enhanced nuclear localization of stabilized β -catenin are key events in stem cell activation in a variety of systems including the intestine.^{34, 35} Western Blot analysis for β -catenin activation in IECs showed that T cell activation enhanced IEC β -catenin stabilization as nuclear total (T) β -catenin levels rose and cytoplasmic levels dropped with anti-CD3 mAb treatment (Figure 2A). Cytoplasmic P- β -catenin^{33/37,41} levels also declined suggesting β -catenin degradation lessened. Results showed that T cell activation induced Akt-mediated activation of β -catenin as levels of P- β -catenin⁵⁵² in nuclear fractions rose. Analysis of β -catenin target gene induction was assessed by real-time PCR (cMyc and CD133, supplemental Figure 2F), immunoblotting (cyclin D1, Figure 2A) and immunohistochemistry (cMyc and cyclin D1, Figure 2B). Data showed both cyclin D1 and cMyc levels rose within crypt IECs indicative of T cell activation-induced β -catenin signaling.

To test whether PI3K/Akt signaling was required for T cell-induced β -catenin activation, IEC responses were examined in *I-pik3r1KO* mice. Reduced PI3K/Akt signaling in *I-pik3r1KO* IECs attenuated nuclear T- β -catenin localization (Figure 2A). Increased cytoplasmic P- β -catenin^{33/37,41} levels, along with the appearance of lower molecular weight bands of T- β -catenin, suggest that loss of *pik3r1* enhanced cytoplasmic degradation of β -catenin. Immunohistochemistry showed that induction of nuclear cyclin D1 and cMyc were attenuated in *I-pik3r1KO* mice (Figure 2B). Furthermore, nuclear P- β -catenin⁵⁵² levels were attenuated in *I-pik3r1KO* mice. Taken together, *pik3r1* expression is required for IEC β -catenin activation in this model.

***pik3r1* is required for immune-mediated β -catenin activation in proliferating ISC/PC populations**

To examine the role of IEC *pik3r1* expression on T cell-induced Akt and β -catenin activation in IECs, SB sections from control and anti-CD3-treated mice were stained for P-Akt and P- β -catenin⁵⁵². Both of these markers were localized within crypt epithelial nuclei. In the resting state, cells positive for P-Akt or P- β -catenin⁵⁵² localized to the +4 position, but they were also found sporadically at lower levels throughout transit-amplifying zones (Figure 3A,B). By comparison, the markers of active Akt and β -catenin signaling were dramatically increased following T cell stimulation. Notably, both markers localized to cells with enlarged or dividing nuclei (insets). Similarly, BrdU-positive proliferative cells also localized to mid-crypt regions (Figure 3C). However, in *I-pik3r1KO* mice epithelial deletion of *pik3r1* nearly eliminated the activation of Akt and β -catenin and reduced BrdU incorporation in lower and mid-crypt areas. These data were confirmed quantitatively as there were less IECs with P-Akt, P- β -catenin⁵⁵², and BrdU incorporation in *I-pik3r1KO* compared to control mice (Figure 3D).

To determine the spatial relationship of sites of IEC proliferation and β -catenin activation, biomarker-positive cells were scored by position (Figure 4A,C). Three hours after T cell activation, P- β -catenin⁵⁵² staining increased at the +4 position and within the transit-amplifying zone (positions 6–10). By comparison, IEC BrdU incorporation peaked 18 hours after T cell activation, within the transit-amplifying zone. *pik3r1* deletion severely attenuated T cell-induced β -catenin activation as numbers of P- β -catenin⁵⁵² positive cells decreased by 80% in *I-pik3r1KO* mice. Strikingly, *pik3r1* deletion reduced BrdU incorporation in lower crypts to levels below controls. TUNEL staining indicated that IEC apoptosis precipitously rose in activated *I-pik3r1 KO* mice 18 hours after anti-CD3 treatment (Figure 4D,E). Thus, PI3K serves dual functions in coordinating IEC responses to immune-activation. PI3K-induced β -catenin signaling commits transit-amplifying cells to enter the cell cycle; and PI3K generated anti-apoptotic signals are required for complete proliferative responses.

To examine the potential that PI3K regulates β -catenin activation in ISCs, the frequency of P- β -catenin⁵⁵² positive CBCs was determined. CBCs are defined as the cells at the crypt base between Paneth cells.³⁶ More recently, these cells were shown to exhibit pluripotent stem cell function.²⁴ We observed P- β -catenin⁵⁵² positive CBCs (Figure 4F). In WT mice, T cell activation increased numbers of P- β -catenin⁵⁵² positive CBCs 3-fold (Figure 4F). In *I-pik3r1 KO* mice, base-line numbers of P- β -catenin⁵⁵² positive CBCs were similar to WT while there was no change following T cell activation. Therefore PI3K regulates activation of P- β -catenin in putative stem cells located in the crypt base.

The association of P- β -catenin⁵⁵² with known markers of resting and dividing ISC/progenitor cells was investigated. SB tissue was stained for BrdU incorporation (2hr; LTR), doublecortin and CaM kinase-Like (DCAMKL)-1 (Supplemental Figure 3A,B), and Musashi (Msi; Supplemental Figure 3C,D). Anti-P- β -catenin⁵⁵² co-stained a subset of BrdU-labeled dividing cells localized to the stem cell or transit-amplifying zone (Supplemental Figure 3C) further suggesting the cells are actively proliferating. Cells with nuclear P- β -catenin⁵⁵² were distinct from populations of BrdU-LTR cells (Supplemental Figure 3D). Similarly, Msi or DCAMKL-1 staining indicated P- β -catenin⁵⁵² staining was excluded from these populations (Supplemental Figure 3). Detection of P- β -catenin⁵⁵²/BrdU-labeled cells within CBC and transit-amplifying populations imply immune-mediated β -catenin signaling occurs in proliferating (activated) ISC/PCs.

PI3K signaling is required for IBD-associated carcinogenesis

As acute inflammation induces PI3K-dependent activation of β -catenin, we postulated that active PI3K and β -catenin signaling play key roles in colitis and colitis-induced dysplasia.

IL-10^{-/-} colitis has several features that resemble human IBD. In these mice, colitis is mediated by Th1 responses³⁷ and aggravated by exposure to bacteria³⁸ or non-steroidal anti-inflammatory agents such as piroxicam (Px).³⁹ *IL-10*^{-/-} mice also develop chronic inflammation-induced “flat” colonic dysplasia and cancer.^{40, 41} The induction and severity of colitis was synchronized with Px treatment for two weeks (D0–D14; Figure 5A). On days 14–35, some mice were treated with the selective PI3K inhibitor, LY294002, or with small-interfering RNA specific for *pik3r1* (Supplemental Figure 6).^{42, 43} Gross examination of unstained (Figure 5B) and methylene blue-sprayed colons (Figure 5C) at day 42 revealed numerous ulcerated, dysplastic mass lesions that were verified by histology (Figure 5D) in 70% of untreated colons. PI3K inhibition significantly reduced the prevalence of dysplasia by 55%. Colitis scores were also reduced in the LY treated mice (supplemental figure 5). Therefore, attenuating PI3K signaling reduces the onset of colitis-induced dysplasia (CID) in mice.

Further examination of the effects of PI3K inhibition on epithelial P-Akt and P-β-catenin⁵⁵² levels were analyzed by immunoblotting of lysates from purified IECs and immunohistochemistry. While colitis-induction increased P-Akt and P-β-catenin⁵⁵² levels, PI3K inhibition by LY294002 (Figure 5E, 6A) or siRNA (Supplemental Figure 6B) prevented colitis-induced activation of both proteins. P-Akt, P-β-catenin⁵⁵², cyclin D1, and BrdU incorporation were all increased in lower/mid crypt regions of inflamed colons (Figure 6A). Numbers of P-Akt, P-β-catenin⁵⁵², cyclinD1, and BrdU-stained cells increased further in areas of dysplasia largely due to extension of staining to the upper crypt and surface regions (Figure 6).⁴⁴ Chronic inflammation in *IL-10*^{-/-} mice increased P-β-catenin⁵⁵² positive cells at points of crypt branching (Supplemental Figure 4). PI3K inhibition reduced numbers of P-Akt, P-β-catenin⁵⁵², cyclin D1, and BrdU-stained cells (Figure 6B). Taken together, PI3K/Akt-induced β-catenin signaling mediates epithelial responses to inflammation (e.g. crypt hyperplasia/branching) and contributes to the emergence of dysplasia in chronic colitis.

P-β-catenin⁵⁵² increases in UC, IBD-associated dysplasia, and cancer

Previously we found an increase in IEC P-β-catenin⁵⁵² in patients with CUC.²⁷ Thus, we wanted to determine if our results from *IL-10*^{-/-} colitis recapitulate molecular events that occur in human disease and investigate whether Akt-mediated β-catenin activation also increases in UCD and cancer. Sections from patients with active untreated CUC, UCD, and CAC were stained for P-β-catenin⁵⁵² and Ki67. In un-inflamed control human colon specimens, relatively low numbers of P-β-catenin⁵⁵²-stained cells localized to areas of Ki67 staining within crypt bases and lower crypt areas (arrows; Figure 7A). CUC revealed chronic architectural changes, including elongated crypts with areas of expanded proliferation and crypt branching (dotted line). As seen in our animal studies, nuclear P-β-catenin⁵⁵² was increased within areas of proliferation and at sites of crypt branching (block arrow). Similar features were detected in Crohn's colitis (Supplemental Figure 7C). By comparison, tissue from high grade UCD (Figure 8A, Supplemental Figure 7A) demonstrated elongated, pleomorphic, hyperchromatic nuclei within stratified epithelial layers. Compared to CUC, dysplastic tissue contained approximately twice as many P-β-catenin⁵⁵² and Ki67 positive cells (Figure 7B, C). A distinguishing feature of UCD were P-β-catenin⁵⁵² positive cells within surface epithelial populations, as also reported for Ki67 staining.⁴⁵ Tubular adenomas (TA) also showed abnormal P-β-catenin⁵⁵² in surface epithelia of (Supplemental Figure 7C, D). In CAC, P-β-catenin⁵⁵²-stained cells were distributed throughout carcinomatous structures (Figure 7A). P-β-catenin⁵⁵² patterns in sporadic CRC resembled CAC (Supplemental Figure 7C). Real-time PCR analysis of human tissues revealed increased β-catenin target gene (e.g. *Myc*, *Ptgs2*, *Lgr5*) mRNA expression in CUC and UCD (Figure 7D). Serial sections stained for P-β-catenin⁵⁵² and the mitosis marker, Phospho (P)-histone H3 indicated that >40% of P-histone H3 positive cells were P-β-catenin⁵⁵² positive and nearly all P-β-catenin⁵⁵²-positive cells were P-histone H3⁺

(Supplemental Figure 7B). Altogether, nuclear P- β -catenin⁵⁵² staining is therefore a marker for expanded populations of activated progenitor cells increased in intestinal inflammation and areas of dysplasia and cancer.

DISCUSSION

The current study indicated that class IA PI3K-induced Akt activation is required to enhance Wnt-mediated IEC β -catenin signaling in mucosal inflammation. IECs with deficient *pik3r1* failed to respond to immune-mediated signals that induce nuclear accumulation of β -catenin. Chemical inhibition and siRNA of *pik3r1* blocked activation of β -catenin in colitis. Also, human colitis tissue revealed nuclear P- β -catenin⁵⁵² increased with the level of mucosal inflammation and degree of dysplasia. The latter findings provide clinical relevance to the proposed pathway developed in mouse models.

pik3r1-deficient mice were instrumental in elucidating several aspects of PI3K/Akt-mediated Wnt/ β -catenin signaling. In WT mice, T cell activation induced PI3K signaling, reduced cytoplasmic P- β -catenin^{33/37,41} levels, and increased nuclear accumulation of β -catenin. However, in *I-pik3r1KO* mice, loss of PI3K signaling enhanced cytoplasmic P- β -catenin^{33/37,41} and reduced nuclear T- β -catenin and P- β -catenin⁵⁵². Attenuation of nuclear β -catenin and P-Akt in T cell-stimulated *I-pik3r1KO* mice suggests that PI3K/Akt signaling impaired cytoplasmic N-terminal phosphorylation and enhanced nuclear C-terminal phosphorylation of β -catenin. Therefore the data suggest expression of class IA PI3K coordinates immune-mediated activation of Akt and β -catenin in IECs.

Our data indicate context, tissue-type, and the nature of stimulation are all important for Akt/ β -catenin cross-talk. This pathway may not be operative in other systems. For example, Ng *et al* reported that in breast and prostate cancer cell lines, β -catenin transcription was not affected by PI3K signaling.⁴⁶ Their findings differ from data where Akt regulated β -catenin signaling using TOP-GFP-lentivirus-infected mammospheres.⁴⁷ In an *in vivo* system, Marsh *et al* reported that mice with PTEN-deficient IECs had increased PI3K signaling but bore no phenotype, while mice with APC-deficient (*APC*^{+/-}) IECs produced benign adenomas.⁴⁸ Significantly, they showed that mice with combined *PTEN/APC* IEC deficiencies progressed to ulcerating adenocarcinomas. Our data show the reduction in N-terminal phosphorylation (33/37, 45) of β -catenin in the cytosol of stimulated IECs consistent with enhanced Wnt signaling. Thus, in the context of mucosal T cell activation, enhanced PI3K signaling increases β -catenin activation.

Induction of PI3K-mediated Wnt/ β -catenin signaling by mechanisms shown here help explain IEC proliferation and crypt architectural distortion seen in CUC. Previous studies showed that Wnt/ β -catenin signaling promotes IEC proliferation.⁴⁹ PI3K inhibition prevented insulin-mediated β -catenin signaling in colorectal cancer cells.⁵⁰ It was also proposed that Akt-phosphorylated FOXO is prevented from binding β -catenin, allowing β -catenin/TCF4 to initiate transcription.⁵¹ Our murine and human colitis data indicate that nuclear P-Akt and P- β -catenin⁵⁵² increase within cells in areas of crypt hyperplasia and branching. Reductions in P-Akt and P- β -catenin⁵⁵² in mice with *pik3r1* deletion or PI3K inhibition support the role of the class IA PI3K p85 α subunit in mediating β -catenin signaling and IEC proliferation. In *IL-10*^{-/-} mice, PI3K inhibition reduced IEC proliferation and cyclin D1 levels. Although colitis scores were reduced by LY treatment, mice in both groups displayed the same severe colitis at the end of Px induction therapy (i.e. d14) regardless of later treatment. Interestingly P- β -catenin⁵⁵² consistently co-stains with P-histone H3, a marker for mitotic cells. These findings are consistent with data that β -catenin functions during mitosis; β -catenin localizes to centrosomes in mitosis where it helps establish the bipolar spindle and plays a role in centrosome separation.^{52, 53} The anti-apoptotic role of PI3K/Akt signaling was supported by

detection of substantial apoptosis in activated *I-pik3r1-KO* mice, albeit after β -catenin activation. Therefore, coordinated Wnt and β -catenin signaling allows the pro-survival effects of PI3K/Akt to combine with the mitogenic impact of Wnt/ β -catenin target gene transcription to promote clonal expansion of crypt IECs (Figure 7E).

P- β -catenin⁵⁵² staining of CID, sporadic dysplasia, and cancer suggest increased PI3K/Akt-mediated β -catenin signaling may be a biomarker of neoplastic transformation in the colon. In murine models of colitis, nuclear P-Akt and P- β -catenin levels increased as colitis progressed to dysplasia. PI3K inhibition reduced P- β -catenin⁵⁵² staining and progression to dysplasia. Thus, PI3K/Akt-mediated β -catenin activation participates in the generation of CID in mice. Data from mouse and human studies show that IECs with mutant APC have enhanced β -catenin nuclear accumulation.^{12, 54} We found increased P- β -catenin⁵⁵² staining in sporadic TA and CRC along with polyps in APC ^{Δ 468} mice²⁷ further suggesting Akt plays a role in activating nuclear β -catenin.

The current studies explore a novel area of crosstalk between PI3K and Wnt/ β -catenin signaling *in vivo* (Figure 7E). These areas are being explored in metabolic disorders such as diabetes, cancer, cardiovascular disease and osteoporosis.^{50, 55, 56} Data here indicate that PI3K is required for increased P- β -catenin⁵⁵² in CBC and TA populations. P- β -catenin⁵⁵²-staining therefore identifies an ISC/PC population highly responsive to immune-cell activation. The increased number of P- β -catenin⁵⁵²-positive cells seen with dysplasia and cancer suggest that this pathway may be activated during neoplastic transformation. Further investigation into the mechanisms responsible for these interactions will advance our knowledge of colitis pathogenesis, colorectal cancer, and other disorders associated with aberrant PI3K-related stem and progenitor cells activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CUC	chronic ulcerative colitis
ISC	intestinal stem cell
CAC	colitis-associated cancer
PI3K	phosphoinositide-3 kinase
P- β -catenin ⁵⁵²	Akt-phosphorylated β -catenin
CRC	colorectal cancer
IEC	intestinal epithelial cells
UCD	UC-associated dysplasia

CID	colitis-induced dysplasia
CBC	crypt base columnar cell

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Figure 1. Loss of p85α protein expression in IECs of *I-pik3r1 KO* mice

Western blot analyses of total p85, p85α and p110α subunits of PI3K in cytosolic fractions from purified crypt epithelial cells at 0 and 3 hours after T cell activation with anti-CD3 mAb in *pik3r1^{lox/lox}* and *I-pik3r1 KO* mice. h, hours.

Figure 2. Epithelial deletion of *pik3r1* attenuates T cell-mediated Akt and β-catenin signaling in SB crypts

(A) Western blot analyses of P-Akt, FoxO1, total (T) β-catenin, P-β-catenin^{33/37, 41}, P-β-catenin⁵⁵², and cyclin D1 in nuclear or cytoplasmic extracts of purified SB crypt epithelial cells from *pik3r1^{lox/lox}* and *I-pik3r1 KO* mice following T cell activation. Fibrillarin and α1-Tubulin are subcellular fraction controls. (B) Cyclin D1 and cMyc in SB crypts detected by immunohistochemistry (arrows). Bar graphs show mean ± standard deviation (±SD) of positive cells/crypt in indicated groups. Scale bar size is 50μm. p-value represents the results of student t-test.

Figure 3. PI3K is required for T cell mediated Akt and β -catenin signaling in SB crypts
Immunohistochemistry staining for (A) P-Akt, (B) P- β -catenin⁵⁵² (arrows) performed in ileal sections of control and anti-CD3 treated *pik3r1*^{lox/lox} or *I-pik3r1* KO mice. Insets indicate P-Akt and P- β -catenin⁵⁵² positive epithelial cells with enlarged nuclei within the crypt. (C) BrdU incorporation was examined 18 hrs after anti-CD3 treatment. (D) Bar graphs display mean $\pm$ SD of positively stained cells/crypt for P-Akt, P- β -catenin⁵⁵², and BrdU.

Figure 4. PI3K is required for epithelial cell proliferation induced by T cell activation
 (A) Single crypt picture indicating enumeration of crypt cells. (B) P-β-catenin⁵⁵² and (C) BrdU labeled cells per position of SB crypt determined 3 or 18 hours after T cell activation, respectively. (D) TUNEL staining of control *pik3r1*^{lox/lox} or *pik3r1*^{lox/lox} and *I-pik3r1* KO mice 18hr after anti-CD3 mAb injection. (E) Bar graph quantifying **D** shows mean ±SD of TUNEL-positive cells/crypt. Scale bar size is 50μm. p-value represents results of student t-test. (F) Representative image and quantification of a P-β-catenin⁵⁵² positive CBC (block arrow) with 2 neighboring paneth cells (small arrows) in the SB. 100 well-oriented SB crypts were counted 3hrs after control or anti-CD3 treatment.

Figure 5. PI3K is required for progression of chronic CID

(A) Timeline of colitis induction with Px in *IL10*^{-/-} mice and LY294002 treatment. (B) Gross findings in colons of WT, or vehicle only and LY294002 D42 treated *IL10*^{-/-} mice. (C) Ulcerating masses (red arrows) in methylene blue-stained colonic mucosa. (D) Prevalence of dysplasia in indicated group over time. (E) Western blot data showed cytosolic P-Akt and nuclear P-β-catenin in IECs. Bar size=500μm.

Figure 6. Immunohistochemical evidence that PI3K is required for progression of CID
(A) Immunohistochemistry for P-Akt, P-β-catenin⁵⁵², cyclin D1, or BrdU incorporation in colonic sections of control, D42-colitis, D42-dysplasia, D42-LY294002-treated *IL10*^{-/-} mice.
(B) Bar graph shows mean ±SD of positively stained cells of P-Akt, P-β-catenin⁵⁵², cyclin D1, and BrdU in each group. **p*<0.01, compared to control; ***p*<0.01, compared to D42-colitis, #*p*<0.01, compared to D42-dysplasia. Bar size=50μm. p-value represents results of student t-test.

Figure 7. β-catenin activation and Ki67 levels are increased in CUC, UCD, and CAC patients
(A) Human colonic specimens stained for P-β-catenin⁵⁵² and Ki67. Arrows indicate positively stained cells in representative control (C), active non-treated CUC, UCD, and CAC samples. Block arrow shows P-β-catenin⁵⁵² stained cells at points of crypt branching (dotted line). Scale bar size=50μm. **(B)** Bar graphs show mean ±SD of P-β-catenin⁵⁵²- and **(C)** Ki67-stained cells/100 epithelial cells in each group. The number of patients in each group in **B** is 20 C, 13 CUC, 10 UCD, and 2 CAC, and in **C** is 8 C, 10 CUC, 4 UCD, and 2 CAC. **(D)** Real-time RT-PCR for β-catenin target genes in human colonic tissues. Bar graphs show mean ±SD of mRNA fold-induction of cMyc (*Myc*), COX2 (*PTGS2*), and GPR49 (*LGR5*) in the indicated groups. Data represents specimens from 11–13 C, 19–24 CUC, 4–5 UCD, and 5–7 CAC patients. p-

value represents the results of a Mann-Whitney U test. (E) Wnt signaling inhibits the destruction complex, impairs N-terminal phosphorylation (degradation), and stabilizes β -catenin which accumulates in the nucleus. In the inflammatory setting PI3K/Akt signaling is activated and β -catenin is phosphorylated at Ser552. The location for this phosphorylation event is unknown, but P- β -catenin⁵⁵² is only detected in the nucleus in mice. Nuclear P- β -catenin⁵⁵² binds to the Tcf4 to induce transcription.