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A Cytosolic Multiprotein Complex Containing p85 α Is Required for β -Catenin Activation in Colitis and Colitis-associated Cancer*

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Wnt/ β -catenin signaling is required for crypt structure maintenance. We previously observed nuclear accumulation of Ser-552 phosphorylated β -catenin (p β -Cat^{Ser-552}) in intestinal epithelial cells (IEC) during colitis and colitis-associated cancer. Data here delineate a novel multiprotein cytosolic complex (MCC) involved in β -catenin signaling in the intestine. The MCC contains p85 α , the class IA subunit of PI3K, along with β -catenin, 14-3-3 ζ , Akt, and p110 α . MCC levels in IEC increase in colitis and colitis-associated cancer patients. IEC-specific p85 α -deficient (p85^{ΔIEC}) mice develop more severe dextran sodium sulfate colitis due to delayed ulcer healing and reduced epithelial β -catenin activation. In colonic IEC, p85 α deficiency did not alter PI3K signaling. *In vitro* shRNA depletion of individual complex members disrupts the MCC and reduces β -catenin signaling. Despite worse colitis, p85^{ΔIEC} mice have reduced tumor burden after azoxymethane/dextran sodium sulfate treatment. Together the data indicate that the β -catenin MCC is needed for mucosal repair and carcinogenesis. This novel MCC may be an attractive therapeutic target in preventing cancer in colitis patients.

The generation and maintenance of intestinal crypts require Wnt signaling (1, 2). Canonical Wnt-mediated β -catenin activation induces target gene expression that regulates stem cell self-renewal and progenitor cell generation (3). This pathway is particularly important in the intestine where mutations in genes involved in β -catenin degradation occur in over 90% of sporadic colorectal cancers (CRC)² (4). Our data and that of

others suggest that β -catenin is also activated in colitis (5–7). Interestingly, mutations in β -catenin degradation complex genes occur late in the progression to colitis-induced cancer. Thus, mechanisms that regulate Wnt/ β -catenin signaling may be operative in normal intestinal homeostasis, mucosal inflammation (e.g. colitis), and progression to colorectal cancer.

Cytosolic β -catenin levels are controlled by APC (adenomatous polyposis coli), GSK3 β (glycogen synthase kinase 3 β), Axin2 (axis inhibition protein 2 or conductin), and CK1 (casein kinase 1) proteins that phosphorylate and target β -catenin for ubiquitination and proteasomal degradation (8–10). Active Wnt signaling recruits members of the degradation complex (Axin2, Dishevelled) to the membrane, thereby reducing degradation (11). This results in accumulation of β -catenin and increased nuclear translocation (12). Once in the nucleus, β -catenin induces target gene transcription by displacing Groucho and binding the TCF/LEF transcription complex (13, 14). What is less clear, however, is how β -catenin physically moves to the nucleus. Hood and colleagues (15) showed that β -catenin binds the chaperone protein 14-3-3 ζ in the cytosol, where it likely participates in nuclear translocation. Others have shown that β -catenin binds the PI3K regulatory subunit, p85 α , but whether this event affects nuclear translocation is unclear (16, 17). The current study examines the hypothesis that 14-3-3 ζ and p85 α form a cytosolic complex with β -catenin that regulates its movement to the nucleus and TCF/LEF-dependent transcriptional activation.

In previous studies, Akt was shown to directly phosphorylate β -catenin at serine 552 (p β -Cat^{Ser-552}) within the armadillo repeat domain (6, 18, 19). We examined the role of PI3K signaling by deleting the class IA PI3K subunit p85 α in epithelial cells in the small intestine (5). These studies suggested that Akt cooperates with Wnt to enhance β -catenin signaling in small intestine epithelial cells responding to mucosal inflammation. In the current study, we explore the effect of p85 α deletion in colonic intestinal epithelial cell (IEC) responses to mucosal inflammation. We report that in the colon, p85 α is not required for Akt activation. Rather, p85 α enhances β -catenin signaling by forming a novel multiprotein cytosolic complex (MCC) that

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² The abbreviations used are: CRC, sporadic colorectal cancer(s); CAC, colitis-associated cancer; UC, ulcerative colitis; AOM, azoxymethane; DSS, dextran sodium sulfate; IEC, intestinal epithelial cells; IP, immunoprecipitation; WB, western blotting; LEF, lymphoid enhancer-binding factor; MCC, multiprotein cytosolic complex; p β -Cat^{Ser-552}, Ser-552 phosphorylated β -catenin; p85^{ΔIEC}, Villin-Cre p85 α -deficient mice; TCF4, T cell factor 4; TCF/luc, TCF/LEF luciferase; APC, adenomatous polyposis coli; GSK3 β , glycogen synthase kinase 3 β ; CK1, casein kinase 1; β -TrCP, β -transducin repeat-contain-

ing protein; HBSS, Hanks' balanced salt solution; FOB, fecal occult blood; p, phosphorylated.

delivers β -catenin to the nucleus. This complex, composed of p85 α , p110 α , Akt, 14-3-3 ζ , and β -catenin, is increased during colitis and colorectal cancer. Stability of the MCC requires p85 α , as p85 α deletion disrupts the complex, reduces nuclear β -catenin signaling, and impairs mucosal healing during colitis. Together these findings not only uncover a novel mechanism for regulating β -catenin signaling but also provide the first clear link between β -catenin activation and mucosal repair during colitis.

Experimental Procedures

Human Biopsy Samples—Human colonic biopsy specimens were obtained from patients undergoing diagnostic or surveillance colonoscopy for known or suspected ulcerative colitis (UC) and collected from the Good Samaritan Hospital (Lexington, KY). For patients with UC, the Mayo Clinic UC scores for collected biopsies were not less than 8. For comparison and *ex vivo* stimulation, biopsy specimens were obtained from healthy patients undergoing routine colon cancer surveillance. Colitis-associated cancer (CAC) specimens were obtained from patients undergoing surgery. Collection of all patient materials for this study was approved by Institutional Review Board protocol (IRB #13-0559-F3R).

Animals—C57BL/6 (WT) and Villin-Cre mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Villin-Cre mice were bred with pik3r1^{lox/lox} mice (gift from Lewis Cantley, Boston, MA). C57BL/6 (WT) and Villin-Cre pik3r1^{lox/lox} (p85 ^{Δ IEC}) mice were maintained under specific pathogen-free conditions at the University of Kentucky animal facility. All experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee. Loss of p85 α protein in colon epithelial cells of p85 ^{Δ IEC} mice was confirmed by Western blotting (WB) (see Fig. 1B). To induce colitis, mice were given 2% (w/v) dextran sodium sulfate (DSS) in their drinking water for 7 days, followed by regular water. Mice were given a single intraperitoneal injection of 1 mg of BrdU 2 h prior to euthanasia. Mice were euthanized by CO₂ asphyxiation 8 or 14 days after the end of DSS treatment.

To measure disease activity, mice undergoing treatment were scored every other day using a standard disease activity index based on diarrhea, fecal blood (measured by the Beckman Coulter SENSE Hemocult Test), and percentage of weight loss as described previously (20). Each criterion was assigned a score from 0 (no diarrhea, fecal blood, or weight loss) to 4 (severe diarrhea, visible fecal blood, and up to 20% weight loss).

To induce colon cancer, four WT and five p85 ^{Δ IEC} mice were given a single intraperitoneal injection of 12.5 mg/kg of azoxymethane (AOM). After 1 week, mice were started on three cycles of DSS (2.5% DSS in the drinking water for 1 week followed by 2 weeks of water).

Histological Analysis—For histological analysis, tissues were fixed in 4% neutral buffered formalin overnight, processed through paraffin, sectioned at 5 μ m, and stained with H&E. Colitis scores were calculated based on a graded scale of inflammation (0–3), extent (0–3), regeneration (0–4), crypt damage (0–4), and percentage of involvement (1–4) as described previously (21). Combined colitis scores are the sum of the scores

TABLE 1
Antibodies used in the study

Specificity	Protocol	Vendor, catalog number
14-3-3 ζ	WB, IP	Santa Cruz Biotechnology, sc-1019
Akt1	WB, IP	Santa Cruz Biotechnology, sc-5298
APC	WB, IP	Santa Cruz Biotechnology, sc-896
BrdU	IHC ^a	ABD Serotec, MCA2060GA
CK1 α	IP	Novus, NBP1-18880
c-Myc	WB	Cell Signaling, 9402
Cyclin D1	WB	Santa Cruz Biotechnology, sc-450
E-cadherin	WB, IP	Cell Signaling, 3195
Fibrillarlin	WB	Santa Cruz Biotechnology, sc-374022
GSK3 β	WB, IP	Cell Signaling, 9315
HistoneH3	WB	Millipore, 06-755
Lamin B1	WB	Zymed, 33-2000
p110 α	WB, IP	BD Biosciences, 611389
p85 α	WB, IP	Abcam, ab135253
pAkt Ser-473	WB	Cell Signaling, 4060
pGSK3 β Ser-9	WB, IP	Cell Signaling, 9336
PTEN	WB, IP	Cell Signaling, 9559
p β -catenin Ser-552	WB, IP	Cell signaling, 9566
TCF4	WB, IP	Santa Cruz Biotechnology, sc-166699
Tubulin	WB	Sigma, F1804
β -Actin	WB	Sigma, A3854
β -Catenin	WB, IP	Abcam, ab19450
β -Catenin	WB, IP	BD Biosciences, 610154
β -TrCP	IP	Cell signaling, 4394

^a IHC, immunohistochemistry.

for inflammation, extent, and crypt damage/regeneration. To determine the percentage of ulceration, slides were scanned using an Aperio ScanScope XTTM slide scanner and measurements were made using ImageScope version 11. Survival statistics were calculated using a Kaplan-Meier survival curve (SigmaPlot, Systat Software, San Jose, CA).

Immunohistochemistry—Antigen retrieval of paraffin sections was performed using Target Retrieval Solution (Dako, Carpinteria, CA), pH 6.0, in a decloaking chamber. Sections were incubated with anti-BrdU antibody followed by the rat VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). Sections were developed using 3,3'-diaminobenzidine tetrahydrochloride chromogen (Dako). For analysis, cells in at least 30 well oriented colonic crypts per mouse were counted.

Statistical Analysis—All experiments were repeated three times with at least four mice in each group. Comparisons among multiple groups were assessed by analysis of variance. $p < 0.05$ was considered statistically significant.

Antibodies and Inhibitor Used—Antibodies used in this study are listed in Table 1. The inhibitor MK2206 was obtained from LC Laboratories (Woburn, MA) and used in concentration 1 μ M.

Murine Intestinal Epithelial Cell Isolation—Lengthwise sections of murine colon were incubated in 4 °C Ca²⁺- and Mg²⁺-free HBSS (CMF-HBSS) containing 10 mM DTT and 50 nM calyculin A (Calbiochem) for 30 min. Tubes were shaken, and then tissue was transferred to fresh tubes containing CMF-HBSS with 50 nM calyculin A and 10 mM EDTA and incubated at 4 °C for 1 h. Epithelial cells were then dislodged by vigorous shaking. Large pieces of tissue were discarded. Epithelial cells were harvested by centrifugation at 16 \times g for 5 min. Cells were snap-frozen in liquid nitrogen and stored at -80 °C until use. Flow cytometry confirmed that CD45⁺ cells were <1% of the remaining isolated epithelial cells.

Human Biopsy Epithelial Cell Isolation—Human colon epithelia samples were delivered from the operating room in ice-cold PBS. Samples were washed once with ice-cold PBS and

Nuclear β -Catenin Activation in Colitis

incubated at 4 °C with rotation in PBS with 10 mM DDT and 50 nM calyculin A for 30 min, 4 °C. Then samples were centrifuged at 300 rpm for 5 min. Cells were snap-frozen in liquid nitrogen and stored at –80 °C until use. WB of both murine and human epithelial cell lysates obtained by these protocols was negative for anti-CD45 staining.

Ex Vivo TNF Stimulation—Human colon biopsies from healthy patients were treated as above, supernatant was discarded, and fresh HBSS with or without 10 ng/ml TNF was added. Samples were incubated at 4 °C with slow rotation for 2 h and centrifuged at 300 rpm for 5 min, and supernatant was removed. Samples were frozen and stored at –80 °C until use.

Cell Culture—NCM460 cells (normal derived colon mucosa cells (22)) were received by a cell licensing agreement with INCELL Corp. (San Antonio, TX) and were routinely propagated under standard conditions in M3:10A medium with the addition of the conditioned medium (33%) from previously cultured NCM460 cells (22). Cells were treated overnight with 1 ng/ml TNF or TNF plus MK2206, harvested the next morning, and fractionated. For experiments with Wnt3a stimulation, we used a Wnt3a-expressing NCM460 cell line (22).

Caco2 cells (ATCC, HTB-37) were cultured in DMEM supplemented with 10% fetal bovine serum under standard conditions. The cells were harvested in log phase and fractionated.

RNA Interference, Lentiviral Constructs, and Transductions—For knockdown experiments, we used at least two different shRNA constructs directed to different parts of the corresponding gene. For each construct, a stable cell line was generated, and expression levels of the protein of interest were verified. Immunoprecipitation experiments, as well as TCF/LEF luciferase (TCF/luc) and wound healing assays, were made with each cell line and showed similar results. The most compelling data are presented in the study.

The pGIP lentiviral plasmids encoding shRNA against p85 α , 14-3-3 ζ , Akt, and shRNA control were provided by the RNAi/Throughput Core, Northwestern University, Evanston, IL. The pGIP plasmids for shRNA to p110 α was purchased from GE Healthcare Dharmacon. The reporter construct containing TCF/luc was generated in the DNA/RNA Delivery Core, Skin Disease Research Center (SDRC) at Northwestern University (Chicago, IL) by inserting six copies of the TCF/LEF response element in the lentiviral pGF1 vector (System Biosciences, Mountain View, CA). The Wnt3a-expressing construct was generated in the facility mentioned above and inserted in the pGF1 vector.

Vesicular stomatitis virus G pseudotyped lentivirus stocks were made in the DNA/RNA Delivery Core, SDRC (Chicago, IL). NCM460 cells were infected in the presence of 1 μ g/ml Polybrene (Sigma), and stable cell lines were generated. Cells were maintained under selection pressure with 5 μ g/ml puromycin. Expression levels of p85 α , 14-3-3 ζ , and Akt in NCM460 cells infected with shRNA were assessed by WB (see Fig. 6A). Luciferase activity of cells co-infected with TCF/luc was detected with Luciferase reagent (Promega, Madison, WI).

Caco2 Cell Transfection—Caco2 cells were plated in 10-cm dishes at 60% confluence 1 day before transfection. Cells were transfected with pGIP lentiviral plasmids encoded shRNA against p85 α , 14-3-3 ζ , Akt, and p110 α by using the jetPRIME

transfection reagent (Polyplus-Transfection, New York, NY). 48 h after transfection, cells were harvested and used for analysis. Expression levels of p85 α , 14-3-3 ζ , p110 α , and Akt transfected with shRNA were assessed by WB (see Fig. 6B). Stable Caco2 cell lines expressing shRNA against p85 α , 14-3-3 ζ , Akt, and p110 α were generated and maintained under selection pressure with 6 μ g/ml puromycin. Caco2 shRNA-expressing cell lines were co-transfected with the reporter construct containing TCF/luc to evaluate β -catenin transcription activity.

Caco2 Cell Proliferation and Wound Assays—Proliferation assays were performed by using the CyQUANT[®] cell proliferation assay kit (Invitrogen) according to the manufacturer's instructions. For the wound assay, Caco2 cells transfected with shRNA against p85 α , and control shRNA were plated in 6-well plates near confluence. Using a sterile 200- μ l pipette tip, three separate wounds through the cells were scratched on each dish. Pictures were taken immediately and 48 h after. Wound sizes were measured by using Adobe Photoshop tools. The experiment was repeated three times with 10 scratches for each cell line.

Subcellular Protein Fractionation—The subcellular protein fractionation (murine and human epithelial cells) protocol was modified from described procedures (23). All buffers used contained Protease ArrestTM protease inhibitor mixture (G-Biosciences, St. Louis, MO), as well as phosphatase inhibitor mixture I and II (Sigma) at 1:100. Murine and human epithelial cells were homogenized in buffer I (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.01% digitonin), and lysates were passed through a 26-gauge needle and then centrifuged at 4 °C for 10 min at maximum speed. The supernatants were collected and used as the cytosolic fraction. Pellets were resuspended in buffer II (50 mM Tris-HCl, pH 7.4, 2% Triton X-100, 100 mM NaCl), and incubated on ice for 30 min, and then centrifuged as above. The supernatants were used as the membrane/organelle fraction. Pellets were dissolved in buffer III (50 mM Tris-HCl, pH 7.4, 0.25% *n*-dodecyl- β -D-maltoside, 100 mM NaCl) and with two units of Benzonase (Sigma) per 100 μ l of lysate, and then incubated for 30 min at room temperature. Following centrifugation, the supernatants were used as nuclear fractions.

NCM460 and Caco2 cells were fractionated according to the Pierce manufacturer's protocol (subcellular protein fractionation kit, Thermo Scientific). Protein concentration was measured by BCA assay (Thermo Scientific). The purity of the fractions was confirmed by WB with anti- α -tubulin, anti-E-cadherin, anti-laminB1, anti-histoneH3, and anti-fibrillarin antibodies (Table 1).

Immunoprecipitation and Western Blotting—500 μ g of cytosolic protein and 2 μ g of antibody were used for each immunoprecipitation (IP) reaction. The mixture was incubated overnight at 4 °C. 20 μ l of protein A/G plus agarose (Santa Cruz Biotechnology, Dallas, TX) were added to the mixture, and incubation was continued for another 30 min at 4 °C with gentle rotation. Agarose beads were washed four times with ice-cold radioimmunoprecipitation assay buffer (20% in HBBS) and resuspended in LDS NuPAGE sample buffer (Invitrogen) with 10% 2-mercaptoethanol. The samples were boiled and resolved with SDS-PAGE, followed by WB detection. For T-cell factor 4 (TCF4) IP, 200 μ g of nuclear fraction and 2 μ g of TCF4 primary

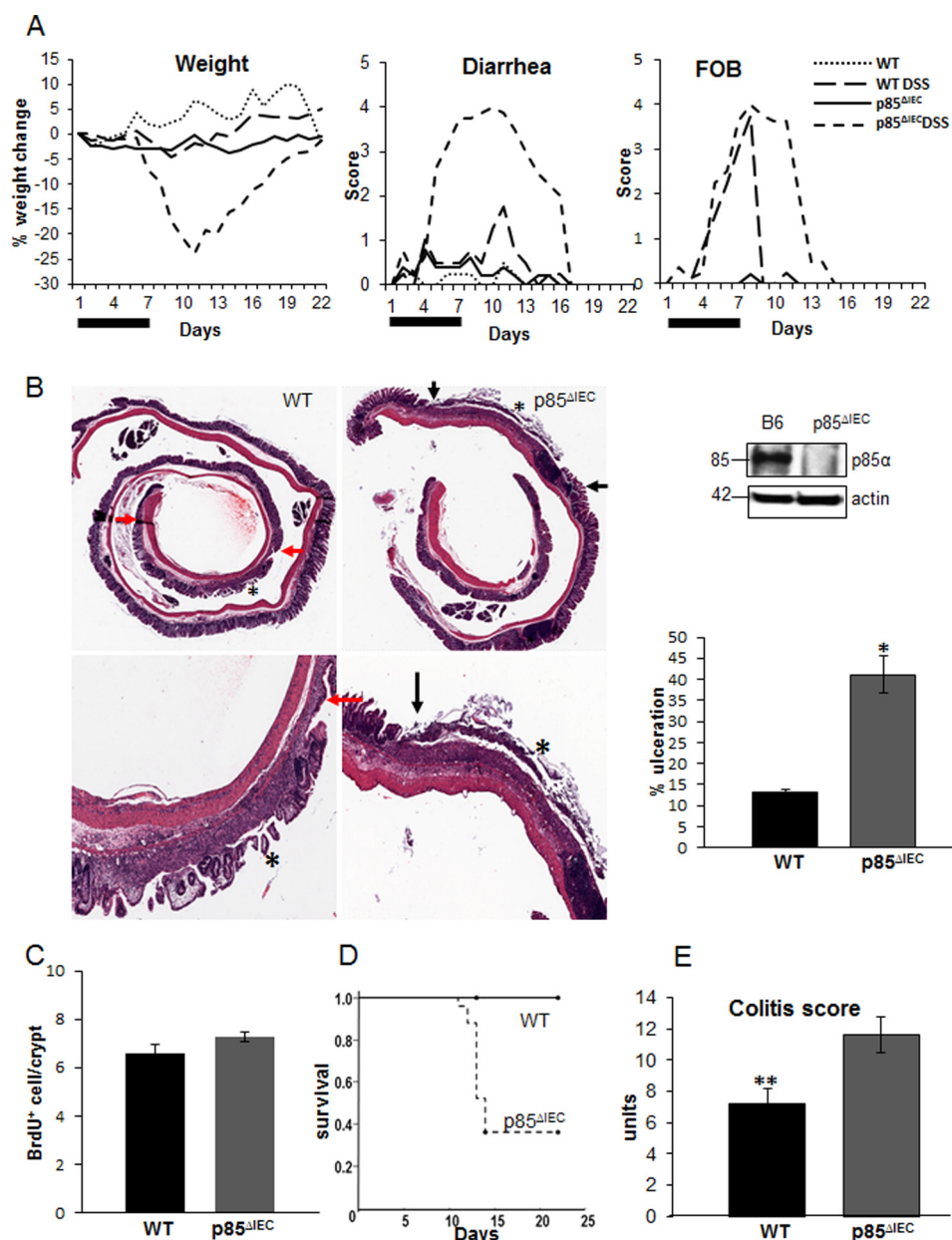


FIGURE 1. p85 α promotes wound healing in DSS colitis. *A*, disease parameters of the percentage of weight change, diarrhea, and FOB scores are shown for WT, WT DSS, p85^{ΔIEC}, and p85^{ΔIEC} DSS mice as indicated. DSS was given 2% w/v in drinking water *ad libitum* for days 0–7 (solid bars) followed by water. *B*, H&E-stained sections at low and high magnification from DSS mice are shown with the percentage of ulcerated mucosa (mean \pm S.E.). Areas of ulceration are demarcated by asterisks, and margins are demarcated by arrows. *n* = 4 (WT), *n* = 5 (p85^{ΔIEC}). The right upper panel represents WB analysis of p85 α expression in intestinal epithelium cells of WT and p85^{ΔIEC} mice. *C*, BrdU incorporation in WT and p85^{ΔIEC} mice (*, *p* < 0.9). *n* = 4 (WT), *n* = 5 (p85^{ΔIEC}). *D*, survival curves are shown for WT and p85^{ΔIEC} mice *, *p* < 0.005. *E*, composite colitis scores are compared between strains (see "Experimental Procedures"). **, *p* < 0.05. For *A*, *D*, and *E*, *n* = 16 (WT), *n* = 25 (p85^{ΔIEC}).

antibody coupled to agarose beads (Thermo co-immunoprecipitation kit, Thermo Scientific) were incubated overnight. The beads were washed, and proteins were eluted, acetone-precipitated, and resolved using SDS-PAGE followed by WB. Proteins were transferred on Immobilon FL (Millipore, Billerica, MS) by semi-dry transfer (Bio-Rad), and membranes were blocked in Pierce Protein-Free T20 blocking buffer (Thermo Scientific) for 1 h and incubated overnight at 4 °C in 1:1000 primary antibody solution. Membranes were extensively washed, incubated in 0.02 μ g/ml secondary antibody for 1 h, washed again, and developed using West Pico, Dura, or Femto reagent (Thermo Scientific).

Results

p85 α Deletion Delays Wound Healing in DSS Colitis—To study the role of p85 α in colitis, p85^{ΔIEC} mice were examined after induction of DSS colitis. Data show that after 2% DSS, p85^{ΔIEC} mice exhibit more severe diarrhea, weight loss, and intestinal bleeding as compared with WT controls (Fig. 1A). Prolonged intestinal bleeding from 9 to 15 days in p85^{ΔIEC} mice suggested that repair of mucosal ulceration was delayed. Examination of mice at day 15 revealed that ulcers in p85^{ΔIEC} mice were 68% longer than in WT mice (Fig. 1B). These effects occurred without altering IEC BrdU incorporation (Fig. 1C).

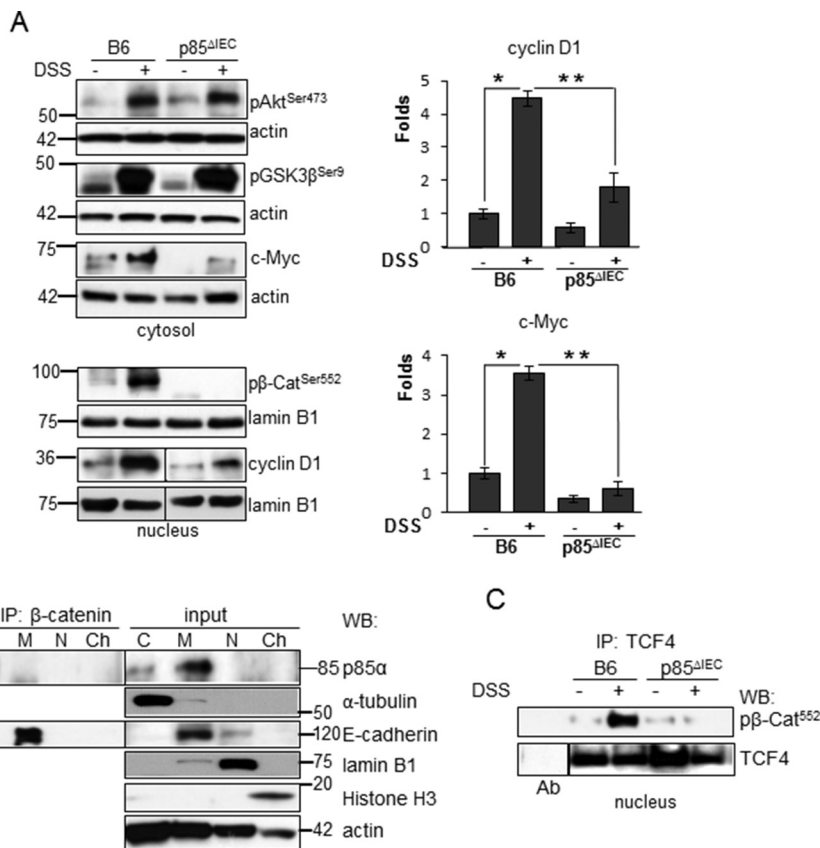


FIGURE 2. p85 α is required for β -catenin activation independent of PI3K signaling. *A*, WBs are shown for cytosolic and nuclear fractions of colonic IEC probed for targets of PI3K (pAkt^{Ser-473}) and Akt (GSK3 β ^{Ser-9}) activation as well as p β -Cat^{Ser-552} and the β -catenin target proteins cyclin D1 and c-Myc. The bar graphs represent cyclin D1 (*, $p < 0.001$, ** $p < 0.01$) and c-Myc (*, $p < 0.0015$, ** $p < 0.002$) expression. *B*, p85 α binds β -catenin in the cytosol of colonic epithelial cells. Protein fractions (C, cytosol; M, membrane; N, nuclear soluble; Ch, chromatin-bound) from NCM460 cells stimulated with TNF were immunoprecipitated with anti- β -catenin antibody (BD Biosciences, Table 1) and probed for p85 α and E-cadherin. WB of the fraction's input is shown on the right with markers of the fraction's purity. *C*, IEC nuclear fractions were immunoprecipitated by TCF4 and probed for p β -Cat^{Ser-552}. Each experiment was run at least three times with independent samples.

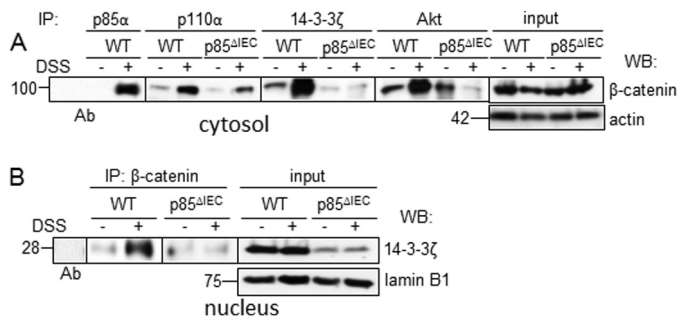


FIGURE 3. Cytosolic p85 α forms an MCC with β -catenin, 14-3-3 ζ , Akt, and p110 α . *A*, cytosolic fractions from WT and p85 Δ IEC control (-) and DSS-treated (+) mice were immunoprecipitated for proteins as shown and then probed for β -catenin (BD Biosciences, see Table 1). Data show p85 α , p110 α , 14-3-3 ζ , and Akt bound to cytosolic β -catenin increases in DSS colitis. p85 α knock-out in p85 Δ IEC mice destroys co-precipitation between β -catenin and p110 α , 14-3-3 ζ , and Akt. *B*, p85 α knock-out limits 14-3-3 ζ transfer into nucleus and its precipitation with β -catenin. Nuclear fractions from WT and p85 Δ IEC control (-) and DSS-treated (+) mice were immunoprecipitated with anti- β -catenin antibody (Abcam, see Table 1) and probed for 14-3-3 ζ . Each experiment was run at least three times with independent samples.

Greater ulceration correlated with reduced survival and significantly more mucosal inflammation (Fig. 1, *D* and *E*). Interestingly, fecal occult blood (FOB) results in Fig. 1*A* suggest that resolution of colonic bleeding was delayed only 4 days in p85 Δ IEC as compared with WT mice on DSS. The differences in

mucosal bleeding was, however, likely greater than these results would indicate. In fact, p85 Δ IEC mice with persistent FOB positivity were more likely to die by day 15. The “removal” of mice that failed to survive actually gives the impression that FOB resolved. Thus, the absence of euthanized mice skewed the appearance of the data in Fig. 1*A*. In fact, the only p85 Δ IEC mice that resolved FOB (albeit delayed) were also the only mice that survived. Together these data suggest that epithelial p85 α is required for optimal mucosal healing in colitis.

p85 α Participates in Colitis-induced β -Catenin Signaling—
To explore the mechanism of how p85 α affects mucosal healing, IEC were sorted for the intestinal stem cell and progenitor cell marker, CD44v6 (24), and examined by WB. As p85 α participates in PI3K signaling, we first examined whether colonic epithelial cells from p85 Δ IEC mice exhibited alterations in phosphorylation of Akt at serine 473 (pAkt^{Ser-473}) or its target GSK3 β at serine 9 (pGSK3 β ^{Ser-9}). WB for pAkt^{Ser-473} and pGSK3 β ^{Ser-9} indicated that the increased colonic IEC PI3K activation seen in colitis was unaffected by p85 α deletion. Thus, as reported in other cell types (25–27), colonic epithelial p85 α expression was dispensable for PI3K signaling (Fig. 2*A*).

Data from studies in Ref. 28 reported that p85 α binds to β -catenin *in vitro* in epithelial cell lines in both cytosolic and whole cell lysates. To examine the possibility that p85 α bound

β -catenin in colonic IEC, cell fractions from NCM460 cells were immunoprecipitated with anti- β -catenin antibody and then probed for p85 α . Results revealed relatively high levels of p85 α precipitated by β -catenin in cytosolic fractions but not membrane-, nuclear, or chromatin-bound compartments. As a

control for this experiment, we probed a membrane with anti-E-cadherin antibody. As expected, we detected β -catenin/E-cadherin binding in the membrane fraction (Fig. 2B).

Next, we examined whether epithelial p85 α deficiency altered β -catenin signaling *in vivo* using p85 Δ IEC mice. Studies of β -catenin activation in WT mice showed that colitis increased nuclear levels of activated p β -Cat^{Ser-552} as well as protein levels of its targets, cyclin D1 (83%) and c-Myc (60%). In contrast, findings in p85 Δ IEC mice revealed that p85 α deletion attenuated the increase in nuclear p β -Cat^{Ser-552} seen in colitic WT mice. A parallel effect was seen for the β -catenin targets cyclin D1 and c-Myc where epithelial p85 α deletion impaired colitis-induced increases in these proteins (Fig. 2A).

To examine p β -Cat^{Ser-552} binding to transcriptional cofactor, TCF4, nuclear extracts were immunoprecipitated with anti-TCF4 antibody and probed for p β -Cat^{Ser-552}. WB of IPs showed that colitis increases nuclear β -catenin/TCF4 binding, whereas epithelial p85 α deletion markedly reduces nuclear β -catenin/TCF4 binding in colitis (Fig. 2C). These findings support the notion that p85 α participates in β -catenin signaling.

p85 α Forms an MCC That Contains β -Catenin—Given observations that p85 α binds β -catenin in the cytosol and that p85 deficiency reduces β -catenin signaling, we considered the possibility that p85 α participates in the formation of an MCC that contains β -catenin. Cytosolic fractions from IEC in normal and colitic WT and p85 Δ IEC mice were isolated and immunopre-

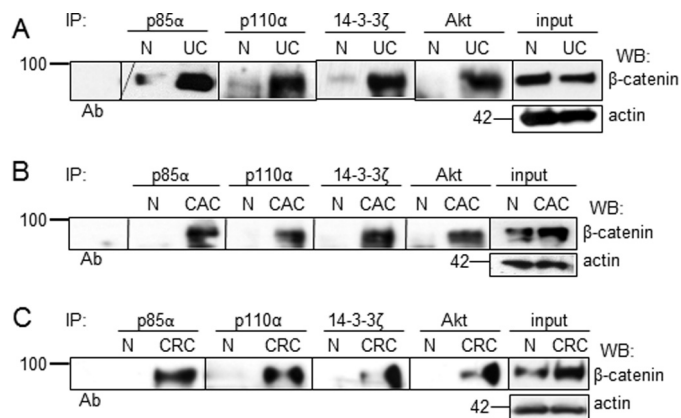


FIGURE 4. UC and colon cancer increase β -catenin-containing MCC. Cytosolic protein lysates from IEC isolated from biopsies from normal (N), UC, CAC, and CRC patients were immunoprecipitated for proteins as shown and then probed for β -catenin (BD Biosciences, see Table 1). Input levels are on the right. A–C, immunoprecipitations for UC (A), CAC (B), and CRC (C). Results indicate that both colitis and colon cancer increase levels of MCC as compared with normal tissue. Each immunoprecipitation was done at least three times with different biopsy samples.

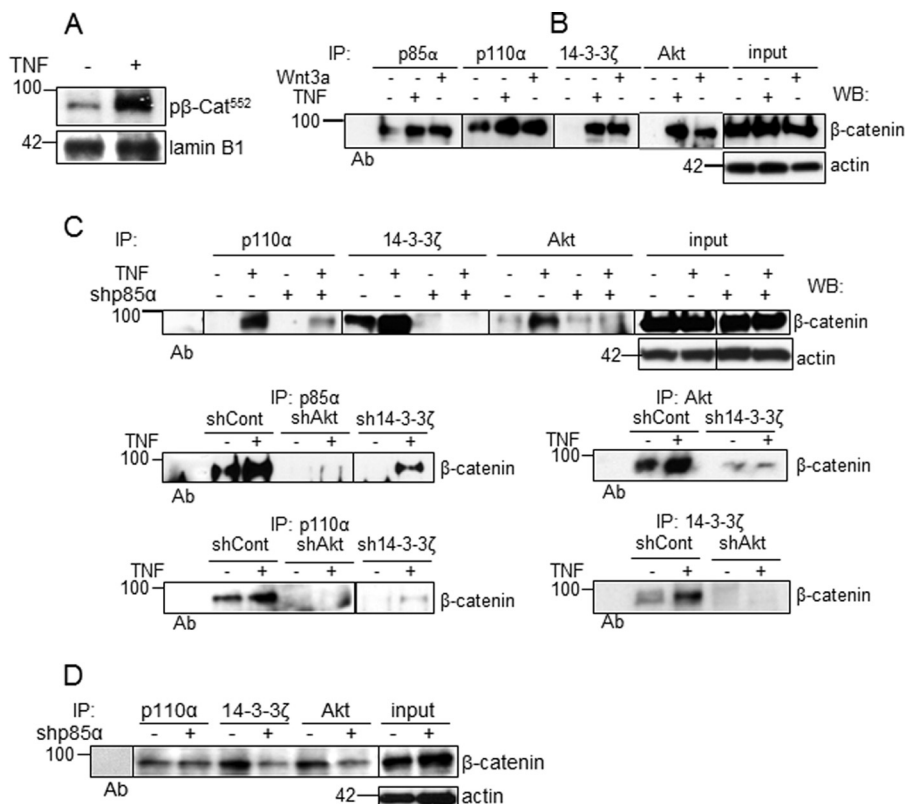


FIGURE 5. Cytokine-induced β -catenin signaling requires formation of the MCC. A, TNF increases nuclear p β -Cat^{Ser-552} in human colon epithelial cell *ex vivo*. Human biopsy samples were treated as described under “Experimental Procedures.” WBs were run to assess expression of proteins. Lamin B1 was used as a loading control. B, cytosolic fractions of NCM460 cells infected by Wnt3a-expressing construct and NCM460 cells treated with TNF were immunoprecipitated with p85 α , p110 α , 14-3-3 ζ , and Akt and probed for β -catenin (BD Biosciences, see Table 1). C, NCM460 cells infected with shRNA control (*shCont*) or shp85 α , shAkt, or sh14-3-3 ζ were incubated with TNF, and cytosolic fractions were immunoprecipitated for proteins as shown and then probed for β -catenin (BD Biosciences, see Table 1). D, cytosolic lysates from Caco2 cells treated with shRNA control or p85 α were immunoprecipitated for p110 α , 14-3-3 ζ , and Akt and probed for β -catenin. Each IP/WB was done at least three times.

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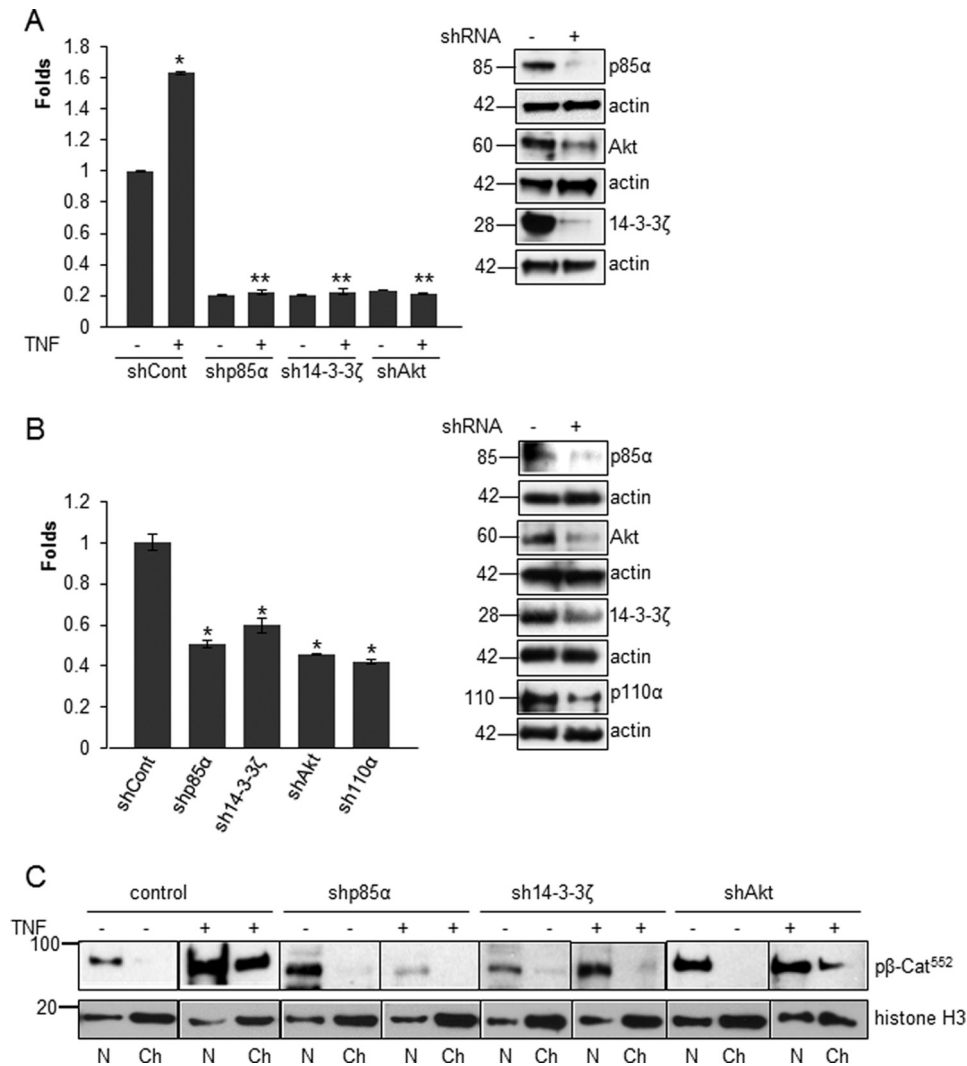


FIGURE 6. Cytokine-induced β -catenin transcriptional activation requires formation of the MCC. *A*, luciferase activities of TCF/LEF reporter (see “Experimental Procedures”) are shown for cells treated with shRNA for p85 α , Akt, 14-3-3 ζ , and control (*shCont*) (mean \pm S.E.). *, $p < 0.0001$ for NCM460 TNF-stimulated cells as compared with control. **, $p < 0.000015$ for shRNA-treated cells as compared with TNF-stimulated control cells as indicated. $n = 4$ for each cell line. The right panel represents WB analysis of p85 α , Akt, and 14-3-3 ζ in control and knock-out cells. *B*, Caco2 cell line expressing TCF/LEF luciferase reporter was transiently co-transfected with shRNA to p85 α , 14-3-3 ζ , Akt, p110 α , and control. 48 h later, luciferase activity was assessed. *, $p < 0.0001$ for shRNA-treated cells as compared with control. The right panel represents WB analysis of p85 α , Akt, 14-3-3 ζ , and p110 α in control and knock-out cells. *C*, nuclear soluble (N) and chromatin-bound (Ch) protein fractions from NCM460 cells treated with shRNA and stimulated with TNF were probed for β -Cat^{Ser-552}. Data show that knockdown of MCC proteins reduces β -catenin localized to chromatin-bound fractions and β -catenin transcriptional activity. Each IP and WB experiment was repeated at least three times.

precipitated with antibodies against p110 α , 14-3-3 ζ , and Akt and probed for β -catenin. In these studies, we found that p85 α deletion reduced levels of p110 α , 14-3-3 ζ , and Akt bound to β -catenin in IEC from colitic tissues (Fig. 3A). These observations were supported by reciprocal IP experiments (p110 α and 14-3-3 ζ IPs probed for Akt, and Akt IP probed for 14-3-3 ζ) (data not shown). These data suggest that p85 α is required for β -catenin binding to p110 α , 14-3-3 ζ , and Akt in the cytosol.

Prior studies proposed that 14-3-3 ζ performs a chaperone function in the nuclear translocation of β -catenin (15). We observed lower levels of 14-3-3 ζ bound to β -catenin in the MCC for p85^{ΔIEC} mice (Fig. 3A). Data in Fig. 3B show that colitis increased 14-3-3 ζ bound to β -catenin in the nucleus of WT mice. However, in p85^{ΔIEC} mice, nuclear levels of 14-3-3 ζ were reduced as well as levels of 14-3-3 ζ bound to β -catenin. These data are consistent with the hypothesis that β -catenin

binding to 14-3-3 ζ in the MCC participates in nuclear translocation of β -catenin.

p85 α Is Required to Form the MCC in Human Colitis and in Colon Cancer—To examine the presence of the MCC in human colitis, subcellular fractions were isolated from biopsy-derived IEC (see “Experimental Procedures”). WB of cytosolic fractions from IEC from normal and colitic human biopsy specimens shows that colitis increased levels of p85 α , p110 α , 14-3-3 ζ , and Akt bound to β -catenin (Fig. 4A). Because we and others have reported that immunohistochemistry for nuclear β -catenin is increased in CAC (5–7), tissues from CAC resection were examined. Results in Fig. 4B show that, as compared with control, MCC formation increased in CAC. Similar findings were detected in CRC samples where enhanced MCC levels were seen in tumors (Fig. 4C). Together these findings support the notion that enhanced

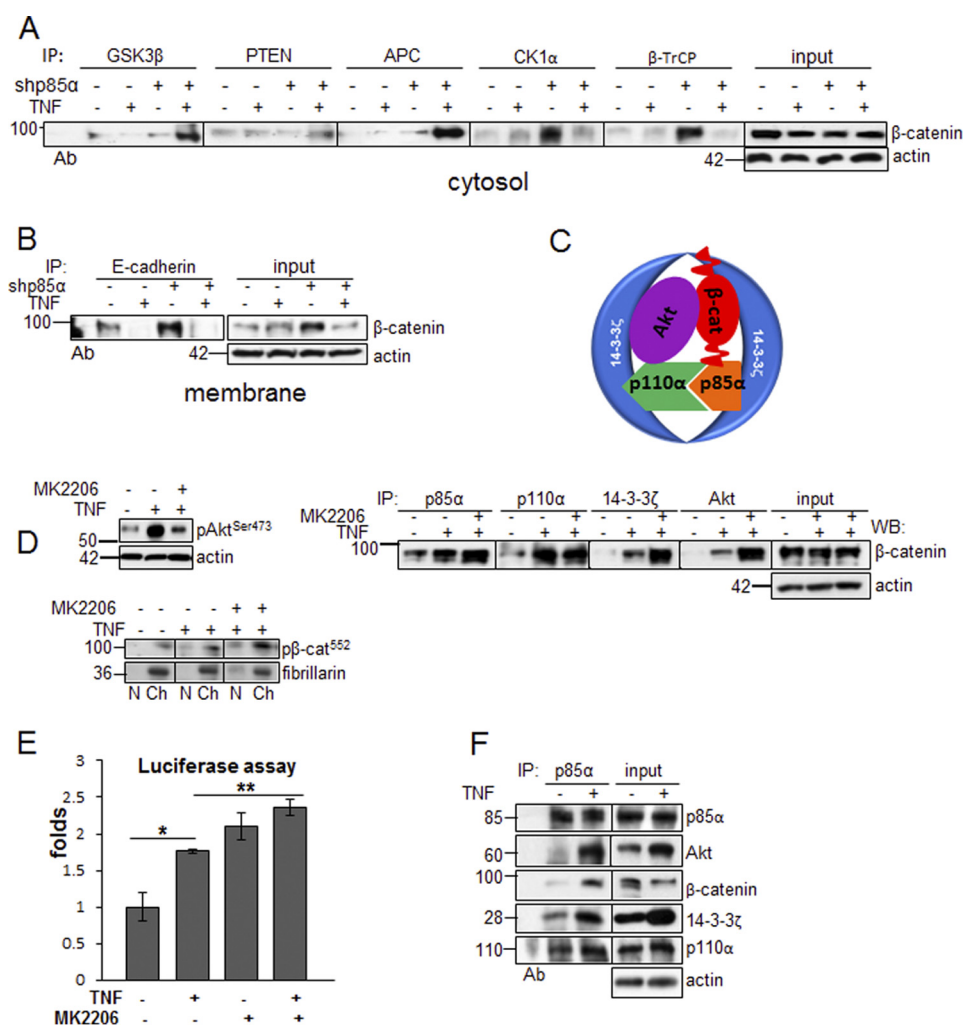


FIGURE 7. Formation of the MCC is independent from β -catenin destruction complex, tight junction complex, and PI3K signaling pathway. *A*, cytosolic lysates from NCM460 cells treated with shRNA (control and shp85 α), incubated with TNF, and immunoprecipitated for GSK3 β , PTEN, APC, CK1 α , and β -TrCP. WB was run for β -catenin. *B*, membrane protein fraction from NCM460 cells treated as above was immunoprecipitated for E-cadherin. WB was run for β -catenin. *C*, proposed model of MCC. A graphic model of MCC is shown with homodimeric 14-3-3 ζ proteins bound to p85 α , p110 α , Akt, and β -catenin. *D*, NCM460 cells were treated with TNF and Akt inhibitor MK2206. The *left panel* represents reduced pAkt^{Ser473} after MK2206 application. The *right panel* shows immunoprecipitation results with antibodies to indicated proteins. *E*, nuclear and chromatin-bound fractions of NCM460 cells treated as above were used for p β -Cat^{Ser552} WB analysis. The bar graph represents TCF/LEF luciferase analysis in NCM460 cells treated as indicated (*, $p < 0.01$, **, $p < 0.025$). *F*, cytosolic lysates from NCM460 cells treated with TNF and control were immunoprecipitated with anti-p85 α antibody and consequently probed for indicated proteins. Each IP and WB experiment was repeated at least three times.

MCC formation associates with increased β -catenin signaling in colitis and colon cancer.

The MCC Requires Expression of Each Member to Maintain Composition—To determine the requirement for each protein component to maintain the MCC composition and function, NCM460 cells were examined after knockdown of p85 α , 14-3-3 ζ , and Akt. Cells were treated with both Wnt3a and TNF, as we found that co-culture with TNF increased nuclear β -catenin in human biopsies (Fig. 5A). Data in Fig. 5B indicate that both TNF and Wnt3a increased levels of p110 α , 14-3-3 ζ , and Akt bound to β -catenin. Fig. 5C demonstrates that p85 α knockdown abrogated MCC levels. Similar reductions in MCC levels were observed when 14-3-3 ζ and Akt proteins were knocked down (Fig. 5C). These findings were supported by IP studies in Caco2 cells, in which reduced p85 α attenuated formation of the MCC (Fig. 5D). In both cell lines, the requirement for MCC member expression was tested on β -catenin transcriptional activity. Data show that knockdown of p85 α , Akt, 14-3-3 ζ , and

p110 α reduced luciferase activity of TCF/LEF (Fig. 6, A and B) and decreased p β -Cat^{Ser552} expression in nuclear soluble and chromatin-bound fractions of NCM460 cells (Fig. 6C).

Based on these results, we defined criteria for the identity of MCC members. We proposed that MCC proteins should bind other putative members in IP reactions. Furthermore, we considered it essential that MCC levels were reduced when levels of individual protein members were reduced (e.g. in shRNA knockdown cells). Examples of proteins that can bind β -catenin in other cytosolic complexes include GSK3 β , PTEN (phosphatase and tensin homolog), APC, CK1 α , and β -TrCP (β -transducin repeat-containing protein) (4, 19, 29, 30). IP experiments reveal that shRNA against p85 α fails to diminish levels of β -catenin binding to these proteins in TNF-stimulated NCM460 cells. (Fig. 7A). Similarly, p85 knockdown did not affect levels of β -catenin bound to E-cadherin in the membrane (Fig. 7B). The model that emerges for these facts posits that β -catenin signaling in the cytosol involves formation of an

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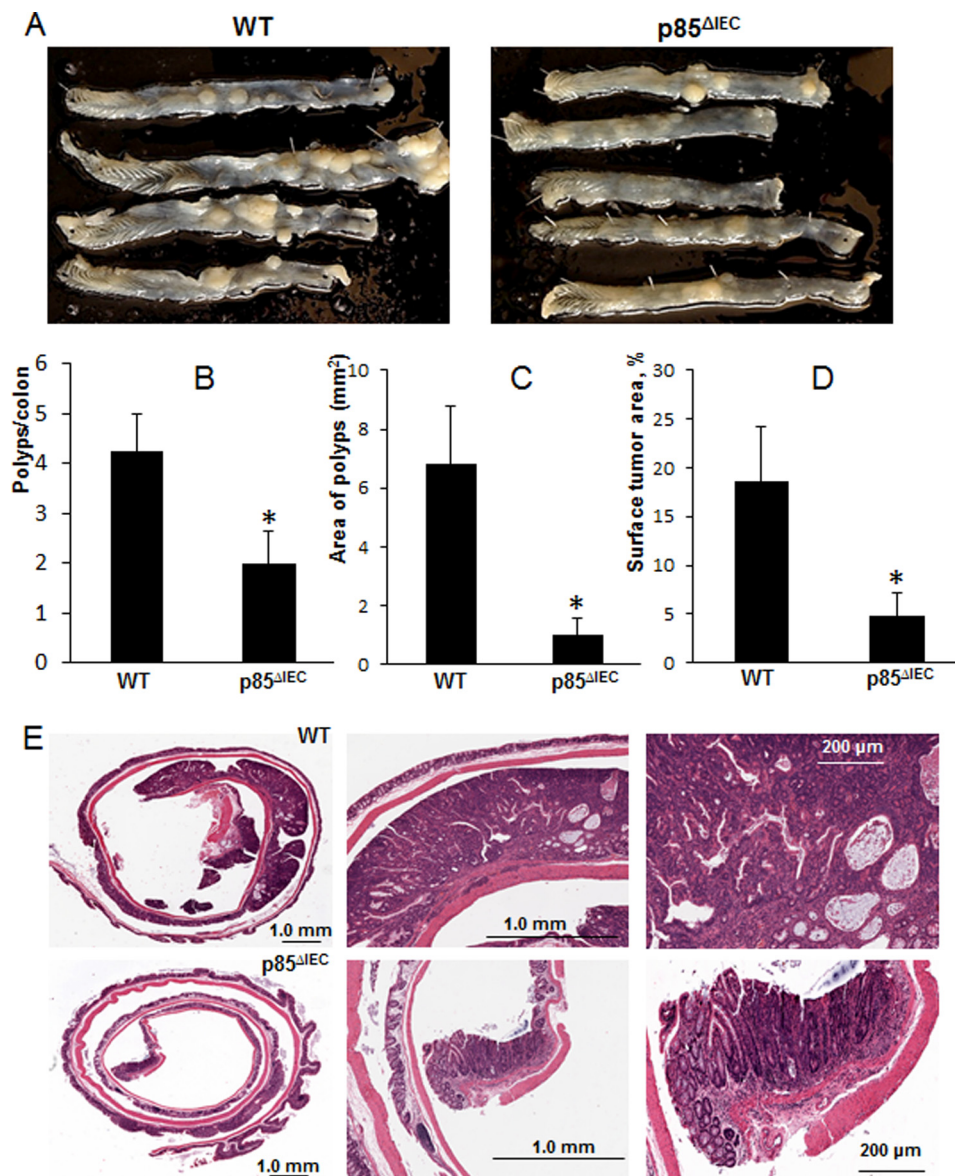


FIGURE 8. Loss of p85 α reduces CAC. WT and p85 Δ IEC mice were given AOM and three cycles of 2.5% DSS (see “Experimental Procedures”). A, representative photographs of colon from day 70. B, number of polyps in the distal colon. (mean \pm S.E.) * p < 0.05. C, polyp area (mm²) (mean \pm S.E.) * p < 0.015. D, the percentage of colon surface covered by polyps (mean \pm S.E.) * p < 0.031. E, photomicrographs of polyps from WT (top) and p85 Δ IEC (bottom) mice. n = 4 (WT), n = 5 (p85 Δ IEC).

MCC that requires p85 α , 14-3-3 ζ , Akt, and p110 α binding. In Fig. 7C, we propose a model where the MCC is composed of two 14-3-3 ζ molecules that bind β -catenin and Akt along with p85 α and p110 α . Whether p85 α binds β -catenin in *trans* (as illustrated) or in *cis* is unknown. We propose it to be in *trans*, given previous studies that show Akt phosphorylating β -catenin at Ser-552 (18). 14-3-3 ζ typically exists as a dimer (31) and has been shown to bring kinases together with their targets (32). Given that we do not detect p85 α or p110 α in the nucleus, we suspect that the complex helps to deliver β -catenin to the nucleus during active signaling.

To examine the role of PI3K/Akt signaling in β -catenin activation, we utilized a newly developed allosteric inhibitor of Akt activation, MK2206. Data presented in Fig. 7D show that MK2206 inhibits TNF-induced pAkt, yet had no effect on β -catenin binding to p85 α , p110 α , 14-3-3 ζ , and Akt. These

findings were further supported by results from nuclear p β -Cat^{Ser-552} accumulation and the TCF/LEF luciferase assay (Fig. 7E) where the Akt inhibitor MK2206 failed to inhibit TNF-induced β -catenin signaling and TCF/LEF transcriptional activation.

To clarify that p85 α interacts directly with MCC proteins in the same complex, we performed additional IP experiments showing that β -catenin, 14-3-3 ζ , Akt, and p110 α are detected in immunoprecipitates of p85 α on the same WB membrane (Fig. 7F). Together with the data in Fig. 5, these findings support the model proposed in Fig. 7C that MCC proteins combine to translocate β -catenin to the nucleus.

p85 α Is Required for Colitis-associated Cancer—Given the importance for cytoplasmic p85 α in nuclear translocation of β -catenin, we tested the AOM/DSS mouse model of colitis-induced cancer. In this model, treatment with AOM enhances

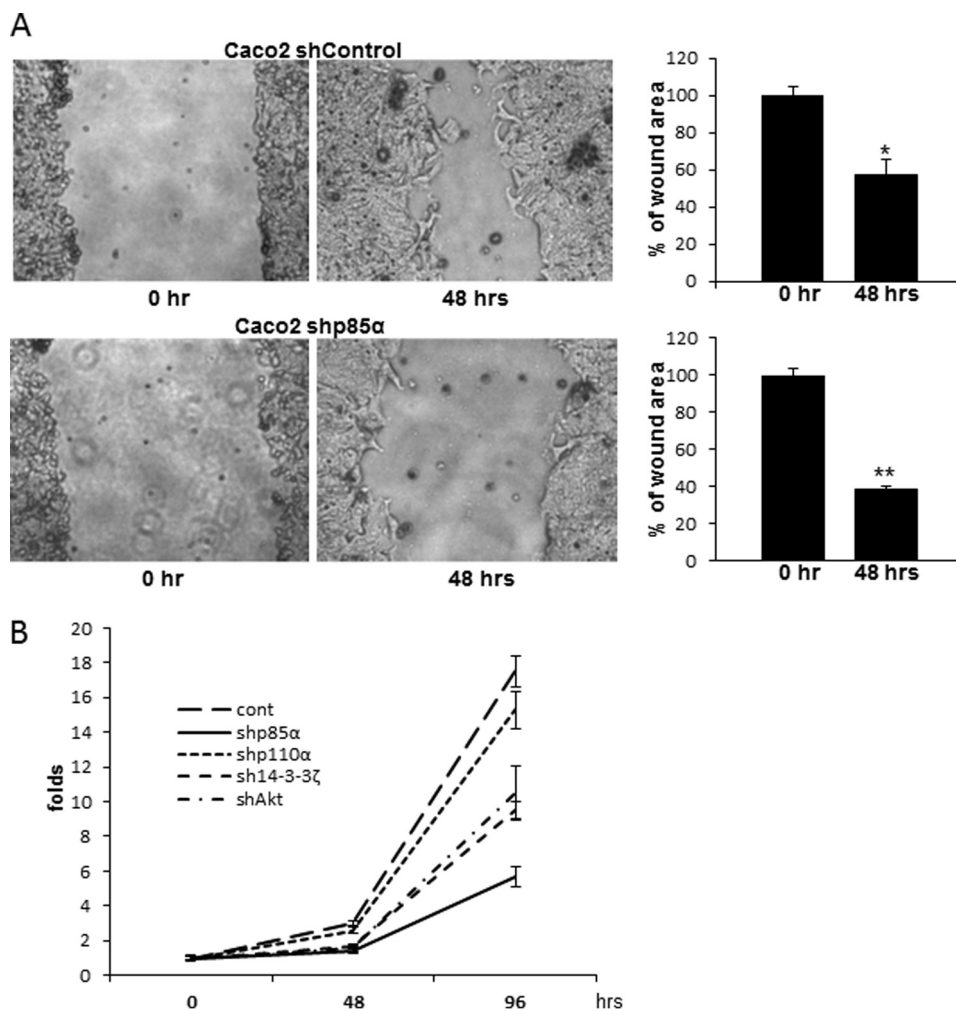


FIGURE 9. Stable knockdown of p85 α suppresses migration and proliferation of Caco2 colon cancer cells. *A*, confluent monolayers of p85 α knockdown or control Caco2 cells were scratched with a micropipette tip and imaged in zero time point and 48 h after on an inverted microscope. Quantification was performed on three separate experiments, 10 images of each cell line. Wound areas were quantified with Photoshop analysis tools. *shControl*, shRNA control. *, $p < 0.00001$, **, $p < 0.00001$. *B*, proliferation of Caco2 cells was measured as described (see "Experimental Procedures") in three independent experiments.

nuclear translocation of β -catenin by inducing exon 3 mutations (33). At the end of the AOM + DSS treatment regimen, colons of p85^{ΔIEC} mice showed a reduced number of polyps (2.0 ± 0.6) as compared with those of WT mice (4.3 ± 1.5) shown in Fig. 8, *A* and *B*. Reductions in polyp area and in the percentage of colon surface covered by polyps are shown in Fig. 8, *C–E*. Thus, reduced levels of p85 α lowered the number and size of polyps formed in AOM/DSS-treated mice.

In vitro studies in Caco2 cells demonstrated that knockdown of p85 α suppressed cell migration by 30% and lowered cell proliferation by 67% (Fig. 9, *A* and *B*). These data are consistent with the notion that formation of the MCC participates in dysplastic transformation of CAC.

Discussion

The data presented here identify a critical role for p85 α in forming an MCC required for enhanced β -catenin signaling in colitis. Understanding the biochemical role of this complex in β -catenin signaling also helped determine the role of intestinal stem cell activity in mucosal wound repair in colitis. Deletion of p85 α reduced nuclear translocation of β -catenin along

with lowering *c-Myc* (a β -catenin target) and TCF/LEF transcriptional activity. *In vivo*, this correlated with delayed wound healing along with more extensive ulceration, mucosal inflammation, and colitis activity. The observation that IEC BrdU incorporation did not change suggests that proliferation of transit-amplifying cells may have compensated for defects in intestinal stem cell activation. Given that Wnt/ β -catenin signaling plays a critical role in stem and progenitor cell activity, we postulate that p85 α deletion reduced mucosal healing as a result of its effect on reducing β -catenin signaling and intestinal stem cell activation. If validated in future studies, these data may lead to an important role for epithelial stem cells in healing epithelial surfaces damaged during colitis.

Our data enable us to propose a novel function for p85 α in Wnt/ β -catenin signaling. In prior studies, deficiency in p85 α has been linked to increased insulin sensitivity without altering PI3K signaling (27, 34). We found that p85 α deletion in small bowel IEC reduced inflammation-induced PI3K and β -catenin signaling, resulting in increased degradation of the catalytic PI3K subunit p110 α (5). Results in small bowel IEC are different from colonic IEC (Fig. 2). Colonic IEC p110 α levels were

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unchanged and PI3K signaling was left intact in p85 α -deficient IEC. Our results also challenge the model that p85 α deficiency leads to increased levels of inhibitory p110 α (Fig. 3A). Rather, the data presented here and elsewhere (16, 35) are consistent with the model that p85 α exists in cytosol bound to β -catenin and 14-3-3 ζ . At this time, we are unsure how other binding partners (p110 α , Akt) participate in β -catenin signaling. It is attractive to speculate that p110 α dimerizes with p85 α and Akt phosphorylates β -catenin in the MCC. More studies are needed to clarify the physical relationship of these proteins and how their functions contribute to β -catenin signaling. This model would assign functional significance to the dimeric complex proposed in Fig. 7.

We propose that results shown here provide an important means toward development of a stem cell-specific therapy for CAC. Based on observations that PI3K signaling is increased in multiple cancers, several companies have developed agents that target PI3K and Akt activities. However implementation of these therapies has been hampered by serious side effects (hypoglycemia, myelosuppression, etc.) due to the prominent role of PI3K/Akt signaling in metabolism and cell proliferation (36). Elevation of apoptosis in p85 α -deficient colon epithelial cells raised the possibility that targeting p85 α may be an attractive therapeutic approach in cancer patients (37). Data here indicate that targeting p85 α would also diminish β -catenin signaling in cancer stem cells. Given this potential benefit, it is important to note that p85 α deletion reduces β -catenin signaling without altering PI3K and Akt activity. These findings suggest that reduced p85 α binding to β -catenin will effectively reduce cancer stem cell activation without producing systemic toxicities. Given that p85 Δ ^{IEC} mice displayed normal growth and tissue histology prior to colitis, we predict that toxicity to normal intestinal stem cells would be minimal.

Author Contributions—T. G. designed the study, performed, and analyzed the experiments shown in Figures 2–7 and 9, interpreted data, and wrote the manuscript. E. M. B. designed, performed, and analyzed the experiments shown in Figures 1 and 8. H. R. contributed to Figure 1. M. T. provided technical assistance for Figures 2 and 4. M. P. M. provided the NCM460 cell line and reviewed the manuscript. T. Gao and L. L. contributed in critical revision of the manuscript for important intellectual content. T. A. B. designed the study, interpreted data, wrote a manuscript and supervised study. All authors reviewed the results and approved the final version of the manuscript.

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A Cytosolic Multiprotein Complex Containing p85 α Is Required for β -Catenin Activation in Colitis and Colitis-associated Cancer

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