

β -Catenin activity negatively regulates bacteria-induced inflammation

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Wild-type (WT) *Salmonella typhimurium* causes acute intestinal inflammation by activating the nuclear factor kappa B (NF- κ B) pathway. Interestingly, WT *Salmonella* infection also causes degradation of β -catenin, a regulator of cellular proliferation. Regulation of β -catenin and the inhibitor of NF- κ B, I κ B α , is strikingly similar, involving phosphorylation at identical sites, ubiquitination by the same E3 ligase, and subsequent proteasomal degradation. However, how β -catenin directly regulates the NF- κ B pathway during bacteria-induced inflammation *in vivo* is unknown. Using streptomycin-pretreated mice challenged with *Salmonella*, we demonstrated that WT *Salmonella* stimulated β -catenin degradation and decreased the physical association between NF- κ B and β -catenin. Accordingly, WT *Salmonella* infection decreased the expression of *c-myc*, a β -catenin-regulated target gene, and increased the levels of IL-6 and TNF- α , the NF- κ B-regulated target genes. Bacterial infection directly stimulated phosphorylation of β -catenin, both *in vivo* and *in vitro*. Closer examination revealed that glycogen synthase kinase 3 β (GSK-3 β) kinase activity was increased in response to WT *Salmonella*, whereas non-virulent *Salmonella* had no effect. siRNA of GSK-3 β was able to stabilize I κ B α in response to WT *Salmonella*. Pretreatment for 24 h with LiCl, an inhibitor of GSK-3 β , reduced WT *Salmonella* induced IL-8 secretion. Additionally, cells expressing constitutively active β -catenin showed I κ B α stabilization and inhibition of NF- κ B activity not only after WT *Salmonella* infection but also after commensal bacteria (*Escherichia coli* F18) and TNF- α treatment. This study suggests a new role for β -catenin as a negative regulator of inflammation.

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Bacteria pathogenicity requires overcoming or altering many very effective host defense mechanisms,¹ including the activation of nuclear factor kappa B (NF- κ B).^{2–4} Bacterial invasion of intestinal epithelial cells (IECs) stimulates NF- κ B and, interestingly, degradation of β -catenin,⁵ a potent transcriptional factor responsible for cellular proliferation and differentiation. It is notable that regulation of β -catenin and the inhibitor of NF- κ B, I κ B α , are strikingly similar, involving phosphorylation of the same N-terminal serine sequence sites, ubiquitination by the same E3 ligase complex, and subsequent proteasomal degradation.^{6,7} To date, no publications have reported on the physiological significance of β -catenin's potential inter-relationship with the NF- κ B inflammatory pathway after bacterial infection *in vivo*.

In this study, therefore, we investigated the role of β -catenin in modulating the proinflammatory response mediated by NF- κ B subsequent to *Salmonella* infection *in vivo*. We examined the possibility that β -catenin functions as a nega-

tive regulator of NF- κ B, much in the same way as I κ B α , through physical interaction with NF- κ B. Additionally, we established the role of glycogen synthase kinase 3 β (GSK-3 β), as the negative regulator of β -catenin's stability and subsequent reactivity with NF- κ B in both *in vitro* and *in vivo* *Salmonella*-induced gastroenteritis models. The results of this study suggest a novel role for β -catenin as a negative regulator of NF- κ B activity *in vivo*. Stabilizing of β -catenin has a significant anti-inflammatory effect by reducing NF- κ B-mediated proinflammatory activity, thus controlling intestinal inflammation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains included wild-type (WT) *Salmonella typhimurium* ATCC 14028s, *S. typhimurium* mutant PhoP^c and *Escherichia coli* F18. Bacterial growth conditions were as follows: non-agitated microaerophilic bacterial cultures were

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prepared by inoculation of 10 ml of Luria-Bertani broth with 0.01 ml of a stationary phase culture, followed by overnight incubation (~ 18 h) at 37°C , as described previously.⁵

Cell Culture

HCT116 *CTNNB1*^{WT/ $\Delta 45$} and its β -catenin knockout derivative lines, HCT116 *CTNNB1*^{-/ $\Delta 45$} and *CTNNB1*^{WT/-}, were cultured in McCoy's 5A medium supplemented with 10% (vol/vol) fetal bovine serum (FBS). MDCK II cells were grown on permeable supports (0.4 μm pore size) in DMEM supplemented with 10% FBS. The rat small intestinal IEC-18 cell line was grown in DMEM (high glucose, 4.5 g/l) containing 5% (vol/vol) FBS, 0.1 U/ml insulin, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 50 U/ml penicillin. The NF- κB p65 knockout mouse embryonic fibroblast (MEF p65^{-/-}) and WT MEF p65^{+/+} were cultured in DMEM supplemented with 10% FBS.

Streptomycin Pre-Treated Mouse Model

Animal experiments were performed at the University of Chicago by using specific pathogen-free female C57BL/6 mice (Taconic) that were 6–7 weeks old. Water and food were withdrawn 4 h before oral gavage with 7.5 mg/mouse of streptomycin (75 μl of sterile solution or 75 μl of sterile water (control)). Afterwards, animals were supplied with water and food *ad libitum*. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 h before the mice were infected with 1×10^7 CFU of *S. typhimurium* (50- μl suspension in HBSS) or treated with sterile HBSS (control) by oral gavage as described previously.⁸ At 6 and 18 h after infection, mice were killed, and tissue samples from the intestinal tracts were removed for analysis.

Mouse Colonic Epithelial Cells

Mouse colonic epithelial cells were collected by scraping from mouse colon including proximal and distal regions. Cells were sonicated in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, protease inhibitor cocktail) and the protein concentration was determined (BioRad). β -Actin was used as the loading control for all Western blots. Villin, an accepted marker of epithelial cells,⁹ was used as the control for epithelial cell protein content in all Western blots.

Immunoprecipitation

Immunoprecipitation samples were prepared as described previously.¹⁰ Blots were probed with anti- β -catenin antibody (Transduction Laboratories, USA), stripped and reprobed with anti-p50, anti-p65, anti-I $\kappa\text{B}\alpha$ (Santa Cruz Biotechnology, CA, USA) and anti- α -catenin (Zymed, San Diego, CA, USA) antibodies and visualized by enhanced chemiluminescence (ECL). Chemiluminescent signals were collected and scanned from ECL Hyperfilm (Amersham Pharmacia Biotech) with a Scanjet 7400c backlit flatbed scanner (Hewlett-Packard Co., Palo Alto, CA, USA). For figures, the contrast of

images was adjusted, arranged, and labeled in Adobe Photoshop and Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA, USA). Bands were quantified using NIH Image software. The digital images are representative of the original data.

Immunoblotting

Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium ortho-vanadate, protease inhibitor cocktail) and the protein concentration was measured. Cultured cells were rinsed twice in ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and sonicated. Equal amounts of proteins or equal volumes of total cultured cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with primary antibodies: anti- β -catenin, anti-p50, anti-p65, anti-I $\kappa\text{B}\alpha$, anti-c-myc (Santa Cruz Biotechnology, CA, USA), anti- α -catenin (Zymed, Carlsbad, CA, USA), p-GSK3 β (Y216, BD), p-GSK3 β (Ser9), p- β -catenin (ser33/37/Thr41 and Thr41/Ser45, Cell Signal, MA, USA) or β -actin (Sigma) antibodies and visualized by ECL.

Immunohistochemistry

Tissues were fixed in 10% neutral buffered formaldehyde for 2 h, transferred into 70% ethanol, and processed the next day. Immunohistochemistry for β -catenin was performed on paraffin-embedded sections (1 μm) of mouse colons. Paraffin sections were baked in an oven at 56°C for 20 min. The slides were deparaffinized and rehydrated in xylene, followed by graded ethanol washes at room temperature. Antigen retrieval was achieved by boiling of the slides in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0). Slides were then incubated in hydrogen peroxide (3% H_2O_2 and 1% FBS in PBS) for 20 min at room temperature to block endogenous peroxidase activity, followed by incubation for 20 min in 5% FBS/PBS to reduce nonspecific background. The samples were incubated with purified mouse anti- β -catenin antibody (BD Transduction Laboratories, BD Biosciences, San Diego, CA, USA; 1:50) in 1% FBS/PBS overnight at 4°C . Samples were then incubated with HRP-conjugated second antibody (Envision +TM System/HRP Kit, Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature. Antibody staining was visualized with DAB (Envision +TM System/HRP Kit, Dako Cytomation), counterstained with hematoxylin, and coverslipped.

Immunofluorescence

Colonic tissues from the proximal and distal portion of the colon were freshly isolated and embedded in paraffin wax after fixation with 10% neutral buffered formalin. After preparation of the slides as described above, slides were incubated in 3% H_2O_2 for 20 min at room temperature to

block endogenous peroxidase activity, followed by incubation for 20 min in 5% BSA with 0.1% saponin in PBS to reduce nonspecific background. The samples were incubated with rabbit polyclonal NF- κ Bp65 (Santa Cruz Biotechnology, 1:50) and mouse anti- β -catenin antibodies (BD Transduction Laboratories, BD Biosciences, San Diego, CA, USA; 1:50) for 90 min at room temperature. Samples were then incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Invitrogen Detection Technologies, Eugene, OR, USA; 1:200), goat anti-mouse Alexa Fluor 594 (Molecular Probes, CA, USA; 1:200), and DAPI (Molecular Probes 1:10 000) for 1 h at room temperature. Tissues were mounted with SlowFade (SlowFade[®] AntiFade Kit, Molecular Probes) followed by a coverslip, and the edges were sealed to prevent drying. Specimens were examined with a Leica SP2 A OBS Laser Scanning confocal microscope.

siRNA of GSK-3 β : Knock-Down Assay

HuSH 19-21 mer shRNA constructs of GSK-3 β are commercially available from OriGene (Rockville, MD, USA). The 21-nucleotide oligomer against GSK-3 β mRNA is AGGACAAGAGATTAAAGAATC. HCT116 cells were seeded in six-well plates and transfected with 5 μ g plasmids; the empty vector was used as negative control. After transfection for 48 h, cells were colonized with WT *Salmonella* for 30 min. Cell lysates were collected for Western blot to detect GSK-3 β and I κ B α protein levels. After transfection for 48 h, cells were colonized with WT *Salmonella* for 30 min and incubated in fresh DMEM for 4 h. Total RNA was extracted for real-time PCR to evaluate IL-8 mRNA levels.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from epithelial cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) according to the manufacturer's directions. The RT cDNA reaction products were subjected to quantitative real-time PCR using the MyiQ single-color real-time PCR detection system (BioRad) and iQ SYBR green supermix (BioRad) according to the manufacturer's directions. IL-8 cDNA was amplified using primers to the human IL-8 gene that are complementary to regions in exon 1 (5'-TGCATAAAGACATACTCCAAACCT) and overlapping the splice site between exons 3 and 4 (5'-AATTCTCAGCCCTCTTCAAAA). All expression levels were normalized to the GAPDH levels of the same sample, using forward (5'-CTTACCACCATGGAGAAGGC) and reverse (5'-GGCATGGACTGTGGTCATGAG) primers for GAPDH. Percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control cells. All real-time PCR reactions were performed in triplicate. All PCR primers were designed using Lasergene software (DNASStar Inc., Madison, WI, USA).

Salmonella-Induced Mouse IL-6 and TNF- α Secretion

Mouse blood samples were collected by cardiac puncture and placed in tubes containing EDTA (10 mg/ml). Mouse IL-6 and TNF- α were measured using the TiterZyme Enzyme Immunometric Assay kits (Assay Designs Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions.

Salmonella-Induced Human IL-8 Secretion

HT29-C19A or T84 cells plated on inserts were incubated with LiCl (30 nM) in DMEM for 24 h, followed by *Salmonella*-containing HBSS (1.6×10^{10} bacteria/ml) apically for 30 min, washing $3 \times$ in HBSS, and incubation at 37°C for 4 h. Cell supernatants from the basolateral side were removed and assayed for IL-8 by ELISA in 96-well plates (Linbro/Titertek; ICN Biochemicals, Costa Mesa, CA, USA) as described previously.¹⁰

Statistical Analysis

Data are expressed as mean \pm s.d. Differences were analyzed by Student's *t*-test. *P*-values ≤ 0.05 were considered significance.

RESULTS

Direct Physical Interaction between NF- κ B/ β -Catenin in the Mouse Colon

To investigate physical interaction between NF- κ B and β -catenin (NF- κ B/ β -catenin) in mucosal inflammation *in vivo*, we utilized an animal model of bacterial infection after pretreatment with streptomycin. Streptomycin pretreatment reduces the commensal bacteria in the mouse gut and renders the mouse susceptible to *Salmonella*-induced gastroenteritis.⁸ PhoP^c is a PhoP-PhoQ constitutive mutation of the WT *Salmonella* strain 14028s¹¹ that increases the expression of PhoP-activated genes and represses the synthesis of approximately 20 proteins encoded by the PhoP-repressed genes. Neish *et al*⁴ have demonstrated that PhoP^c is able to prevent I κ B α degradation and the subsequent activation of NF- κ B. Mice were infected with either WT or PhoP^c *Salmonella*.

Mice colonized with WT *Salmonella* for 6 h showed a relative decrease in I κ B and β -catenin in colonic epithelial cells (Figure 1a, WT). The intensity of the Western blot bands showed that total β -catenin of colon epithelia in WT *Salmonella*-infected mice was 70% of that in controls. In contrast, PhoP^c treatment stabilized I κ B and increased the amount of total β -catenin (Figure 1a, PhoP^c). These differences were specific to the epithelial cells, because all values were normalized to villin.

Immunoprecipitation with β -catenin pulled down both NF- κ Bp65 and p50 subunits (Figure 1b). The β -catenin/NF- κ B complex was dramatically less in the WT *Salmonella*-infected mouse (Figure 1b, WT); only about 27% of that in the control mice. On the contrary, there was more β -catenin and NF- κ B binding in the PhoP^c-infected mouse, about 121% of control (Figure 1b, PhoP^c).

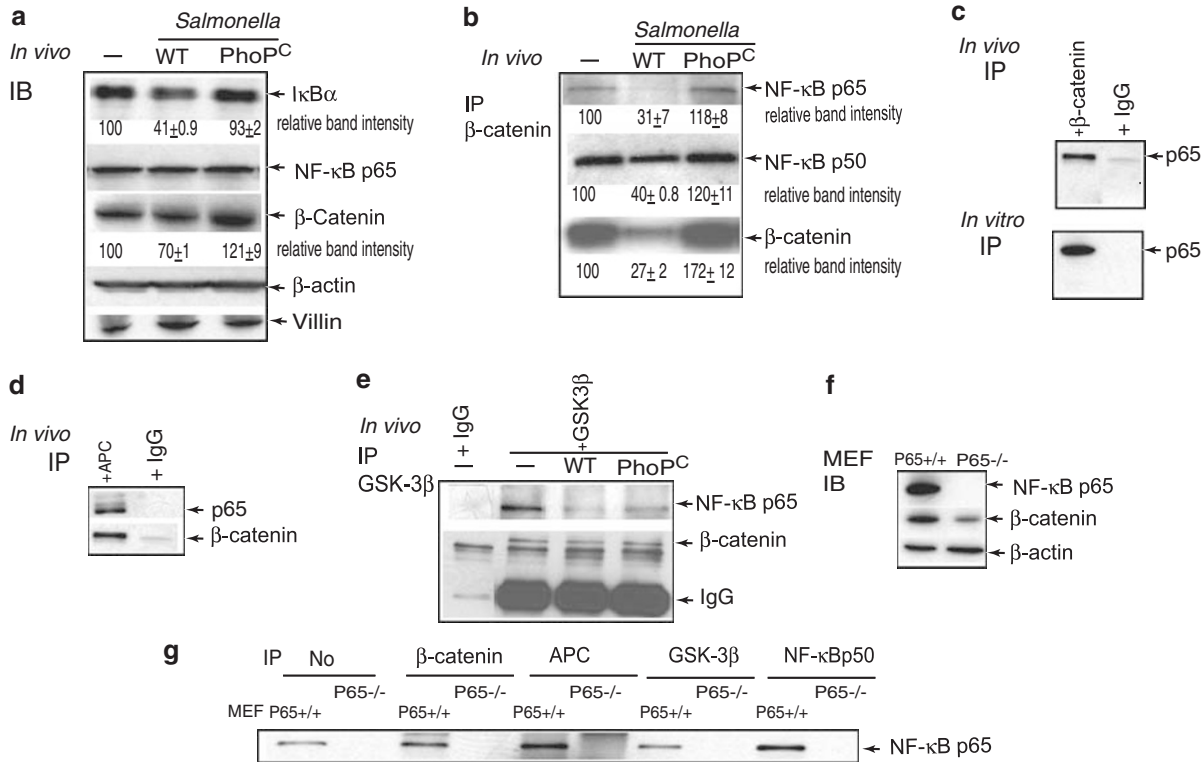


Figure 1 Bacterial infection alters the β-catenin and NF-κB pathways in mouse IECs. Mice were colonized with WT *Salmonella* or PhoPc for 6 h. (a) Large IECs were collected, and cell lysates were analyzed by Western blot for IκBα, NF-κBp65, β-catenin, villin, and β-actin. (b) Large IECs were immunoprecipitated with antibodies against β-catenin, and sequentially Western blotted with antibodies against β-catenin, NF-κBp65, and p50. (c) Large IECs (*in vivo*) and MEF cells (*in vitro*) were immunoprecipitated with antibodies against β-catenin and sequentially Western blotted with antibodies against NF-κBp65. Nonspecific IgG was used as negative control for IP. (d) Large IECs were immunoprecipitated with antibodies against APC and sequentially Western blotted with antibodies against β-catenin and NF-κBp65. Nonspecific IgG was used as negative control for IP. (e) Mice were colonized with WT *Salmonella* or PhoPc for 6 h. Large IECs were immunoprecipitated with antibodies against GSK-3β and sequentially Western blotted with antibodies against β-catenin and NF-κBp65. (f) p65^{-/-} and p65^{+/+} MEF cells were collected, and cell lysates were analyzed by Western blot (WB) for NF-κBp65, β-catenin, and β-actin. (g) MEF cells were immunoprecipitated with antibodies against β-catenin, APC, GSK-3β, or p50, and sequentially Western blotted with antibodies against NF-κBp65. In all instances, the numbers shown represent the relative band intensity compared to control with no treatment (-). Relative band intensities were determined using NIH Image software, and were normalized to control (-) for each sample, which was arbitrarily set to 100. Images shown are representative of 2–3 trials.

In cells not exposed to the stimulation, β-catenin levels are kept low through interactions with GSK-3β, adenomatous polyposis coli (APC), and Axin in the cytoplasm.^{12–14} As positive control, immunoprecipitation with APC was able to pull down β-catenin (Figure 1d). Immunoprecipitation with IgG was used as a negative control and failed to pull down p65, as shown in Figure 1c and d.

We further assessed whether the β-catenin/NF-κB complex was independent of the APC/GSK3β/β-catenin complex. Immunoprecipitation with APC and GSK-3β was able to pull down β-catenin and p65 in mouse epithelial cells (Figure 1d and e). Bacterial infection decreased the p65 pulled down by GSK-3β, but did not change the amount of GSK-3β/β-catenin complex (Figure 1e).

Additionally, we tested the β-catenin/NF-κB complex in NF-κBp65 MEF p65^{-/-}. As shown in Figure 1e, p65 was absent in the p65^{-/-} MEF. The level of β-catenin protein was slightly reduced in p65^{-/-} cells (Figure 1f). Co-im-

munoprecipitation assays showed that anti-β-catenin antibody was able to pull down p65 protein in p65^{+/+} MEFs, but not in p65^{-/-} cells (Figure 1g, β-catenin). Immunoprecipitation of APC or GSK-3β pulled down p65 in WT MEF p65^{+/+}, but not in the p65^{-/-} MEF (Figure 1g, APC and GSK-3β). NF-κBp50 and p65 are two subunits of NF-κB. As positive control, the NF-κBp50 subunit was associated with p65 in p65^{+/+}. The p65/p50 association was abolished in p65^{-/-} (Figure 1g). These data confirm the existence of β-catenin–p65 interaction in mouse fibroblasts. They also suggest that there was binding between NF-κB, GSK-3β, and APC.

Taken together, the findings illustrated in Figure 1 indicate a physical interaction between β-catenin/NF-κB in normal colonic epithelial cells. WT *Salmonella* infection decreased the amount of total β-catenin protein and therefore altered the physical interaction between β-catenin/NF-κB in colonic epithelial cells. Additionally, we found no apparent inter-

action of NF- κ Bp50 with α -catenin, a family member of β -catenin (unpublished data), or with a nonspecific IgG, indicating that the physical interaction between β -catenin and NF- κ B determined *in vivo* is specific.

Localization of β -Catenin and NF- κ B during Inflammation

The intestinal epithelium lining the gastrointestinal tract has a well-defined architecture. In the normal intestine, there are

β -catenin repression, cell-cycle arrest, and terminal differentiation along the crypt to the villus axis. The crypt-villus junction is also under the control of β -catenin signaling. In order to investigate the subcellular distribution of β -catenin and NF- κ Bp65 in the intact colon, tissue sections from mice with or without bacterial colonization were generated and stained with anti- β -catenin and anti-NF- κ Bp65 antibodies. In the normal colon, without any treatment, NF- κ B was localized in the cytoplasm, and most of the β -catenin pro-

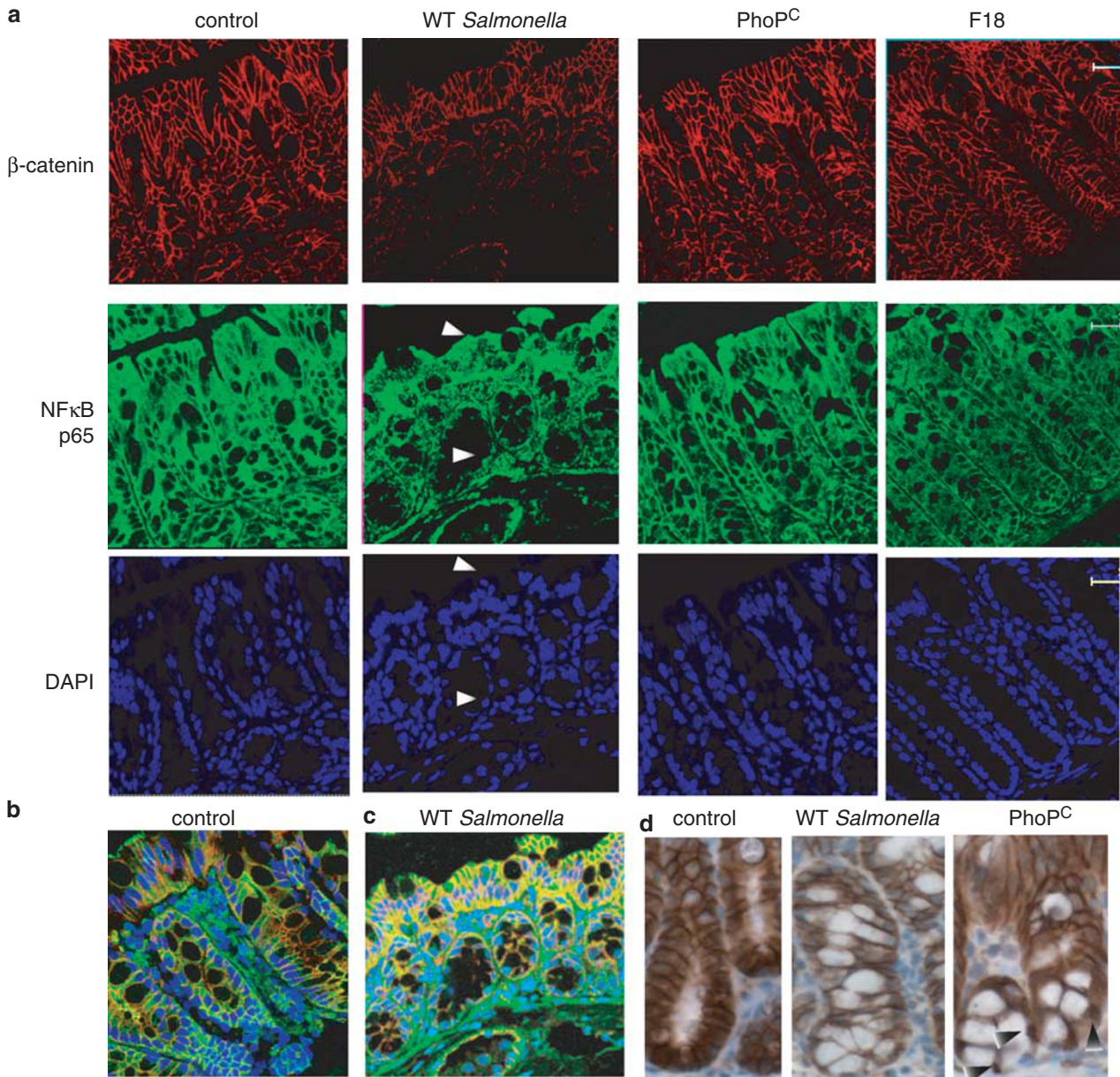


Figure 2 Location of β -catenin and NF- κ B in mouse colon by immunofluorescence. (a) Immunostaining of β -catenin and NF- κ Bp65 in mouse colon 24 h after infection with WT *Salmonella*, PhoP^C, or *E. coli* F18. Tissues were fixed, permeabilized, and stained with anti- β -catenin and anti-p65 antibodies, followed by A488 anti-goat secondary antibodies, A594 anti-mouse FITC-conjugated secondary antibodies, and DAPI. Arrowheads indicate cell nuclei. Images shown are from a single experiment and are representative of three separate repeats. (b) Overlap staining of DAPI, β -catenin and NF- κ B in normal mouse colon. (c) Overlap staining of DAPI, β -catenin, and NF- κ B in WT-infected mouse colon. (d) β -Catenin in control, WT *Salmonella*-, and PhoP^C-infected mouse colon. Data are representative of at least two independent experiments with similar results.

teins were located around the cell membrane and in the cytoplasm (Figure 2a, control). There was obvious colocalization of β-catenin and NF-κB (Figure 2b), as evidenced by the orange or yellow color, which resulted from a combination of red (β-catenin) and green (NF-κB). In contrast, WT bacterial infection for 18 h induced dramatic changes in the localization and distribution of β-catenin. Unlike finding in the control tissue, β-catenin was distributed to the tops of crypts, with very weak β-catenin staining in the lower parts of the crypts (Figure 2a, WT). We noted that NF-κBp65 was translocated to the nucleus after WT *Salmonella* infection, as indicated by white arrowheads (Figure 2a, WT, NF-κBp65 green, DAPI blue). As shown in the overlap image of Figure 2c, WT *Salmonella* induced a combination of blue (DAPI) and green (NF-κB). There also was less colocalization of β-catenin and NF-κB, especially in the lower crypts (Figure 2c), as evidenced by the reduction in yellow color, which results from a combination of red (β-catenin) and green (NF-κB). In tissue sections from mice receiving PhoP^C or commensal bacteria *E. coli* F18 treatment, β-catenin was distributed diffusely throughout the membranes (Figure 2a, PhoP^C and F18), with nuclear staining visible in the lower parts of the crypts (Figure 2d, PhoP^C), as indicated by black arrowheads. There was no p65 nuclear staining under this condition.

The immunohistochemistry results shown in Figure 2d support the fluorescence staining in Figure 2a, b, and c. In the normal colon, most of the β-catenin proteins were located around the cell membrane and in the cytoplasm (Figure 2d, control). The β-catenin staining in WT *Salmonella*-treated colons was very light compared to that of the controls and PhoP^C-treated mice in the lower crypts and cell membrane. In PhoP^C-treated mice, the nuclear staining of β-catenin was visible in the lower part of the crypts (Figure 2d, PhoP^C), as indicated by black arrowheads.

Bacterial Infection Changes the Expression of Target Genes in β-Catenin Pathways

The effect of bacterial colonization on the expression of the β-catenin transcriptionally regulated target gene, *c-myc*,¹¹ was investigated in a streptomycin-pretreated mouse model. After infection with either the WT or the avirulent PhoP^C, colonic *c-myc* levels were determined from IEC lysates. As shown in Figure 3a, *c-myc* expression decreased to about 40% of the normal control after infection with WT *Salmonella* for 18 h, but did not decrease when infected with PhoP^C. *C-myc* has an essential function in cellular growth and proliferation.¹⁵ In addition, *cyclin D1*, a target gene of the β-catenin pathway, and PCNA, a marker of proliferation, were also decreased by WT *Salmonella* infection (data not shown).

Bacterial Infection Changes the Secretion of IL-6 and TNF-α, the NF-κB Target Genes

Colonization of cells with WT *Salmonella* resulted in the dissociation of NF-κB from IκBα allowing NF-κB to translocate to the nucleus, where it binds to its recognition sites in the promoter region of a wide variety of genes, including IL-8 (humans) or IL-6 (mouse). IL-6 secretion in mouse plasma (Figure 3b) was observed after WT *Salmonella* colonization. *In vivo* colonization with avirulent PhoP^C and commensal *E. coli* F18 induced IL-6 secretion (98.3 ng/ml in PhoP^C and 71.4 ng/ml in F18), which was less than 354.5 ng/ml after WT infection (Figure 3b). Additionally, our real-time PCR data indicated that WT *Salmonella* colonization induced a 12-fold increase of IL-6 at the mRNA level, whereas colonization with avirulent PhoP^C and commensal *E. coli* F18 only induced only a onefold increase of IL-6 mRNA (data not shown). The other cytokine levels, such as that of TNF-α (Figure 3c), were also significantly greater in WT *Salmonella*-infected mice (287 ng/ml) than that in PhoP^C- (51 ng/ml) or *E. coli* F18- (58 ng/ml) infected mice.

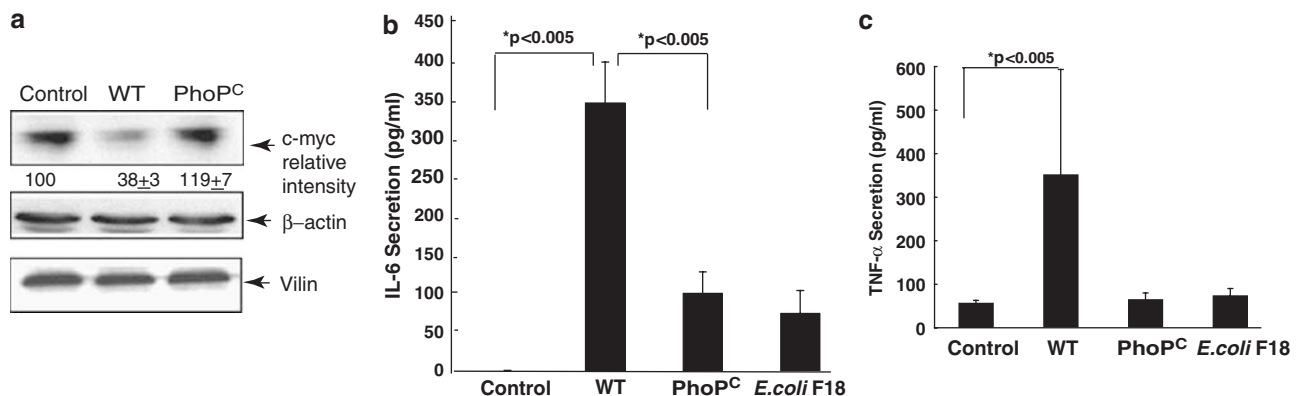


Figure 3 Target gene expression in bacterial-infected mice. (a) Mice were colonized with WT *Salmonella* or PhoP^C for 18 h. Mouse colons were collected, and epithelial cells were scraped, lysed, and analyzed for *c-myc*, villin, and β-actin levels by immunoblot. Images shown are representative of three trials. $P < 0.05$, control compared with WT *Salmonella*. (b) IL-6 levels in mouse plasma samples 18 h after WT *Salmonella*, PhoP^C, or *E. coli* F18 infection. (c) TNF-α levels in mouse plasma samples 18 h after WT *Salmonella*, PhoP^C, or *E. coli* F18 infection. Data shown in (b) are mean ± s.d. for $n = 3$ experiments. Significance was at $P \leq 0.05$.

Histological examination of tissues for *in vivo* WT *Salmonella*-treated mice supports the cytokine profile, demonstrating tissue inflammation in response to WT *Salmonella*. *In vivo* colonization with avirulent PhoP^c induced less inflammation (data not shown).

Bacteria Modulate β -Catenin Phosphorylation

To determine where bacteria are acting to modulate β -catenin activity, we assessed phospho- β -catenin (p- β -catenin) levels in mouse colons after infection with WT and PhoP^c *Salmonella*. β -Catenin has been shown to be primed at S45 by CKI (casein kinase I), allowing efficient subsequent serial phosphorylations by GSK-3 β on residues T41, S37, and S33.¹⁶ The phosphorylation of β -catenin on S33 and S37 is essential for β -catenin’s recognition by the ubiquitin ligase β -TrCP, which targets it for subsequent degradation.¹⁷

As demonstrated in Figure 3a, WT *Salmonella* infection increased p- β -catenin, whereas PhoP^c and F18 decreased p- β -catenin in mouse colonic epithelial cells. The increased phosphorylated I κ B (p-I κ B α) is an indicator of the increased NF- κ B activation. We further tested the p-I κ B α in mouse colonic epithelial cells. WT *Salmonella* infection increased p-I κ B α , whereas PhoP^c and F18 decreased p-I κ B α .

A similar trend for p- β -catenin was also found in the cultured epithelial cell models—rat small IEC, IEC-18, Madin-Darby canine kidney (MDCK) epithelial cells, and the human epithelial cell line HCT116 (Figure 4d). Phosphorylation of β -catenin, expressed as p- β -catenin ser33/37/Thr41, increased in rat small IECs (IEC-18 cells) with WT bacterial colonization (Figure 4b). MDCK cells demonstrated a similar response to WT *Salmonella in vitro* (Figure 4c), indicating that this response was not specific to rodent cells.

Bacterial Modulation of GSK-3 β Phosphorylation

GSK-3 β , a critical negative regulator of β -catenin, also phosphorylates I κ B, the inhibitor of NF- κ B activity. Because of the potential interaction in the NF- κ B and β -catenin pathways as a result of shared GSK-3 β activity, we evaluated the activity of GSK-3 β during bacterial infection.

GSK-3 β is a constitutively active serine-threonine kinase. Site-dependent phosphorylation of GSK-3 β can suppress or enhance its kinase activity.¹⁸ Specifically phosphorylation of GSK-3 β at Ser9 leads to inactivation of its kinase activity.¹⁹ On the contrary, GSK-3 β phosphorylation within the catalytic domain at Tyr216 stimulates GSK-3 β kinase activity, resulting in phosphorylation and degradation of β -catenin.¹⁹ Phosphorylated GSK-3 β levels at Tyr216 and Ser9 are two opposite valid indicators of GSK-3 β activity.¹⁸ Increase of p-Tyr216 and decrease of p-Ser9 indicate the elevated GSK-3 β activity, resulting in β -catenin degradation (\uparrow p-Tyr216 = \uparrow GSK-3 β activity = \downarrow total β -catenin; \downarrow p-Ser9 = \uparrow GSK-3 β activity = \downarrow total β -catenin). In the streptomycin mouse model, WT *Salmonella* infection increased phosphorylated GSK-3 β (pY216) in colonic epithelia by 50% within 6 h, whereas PhoP^c decreased phosphorylated GSK-3 β

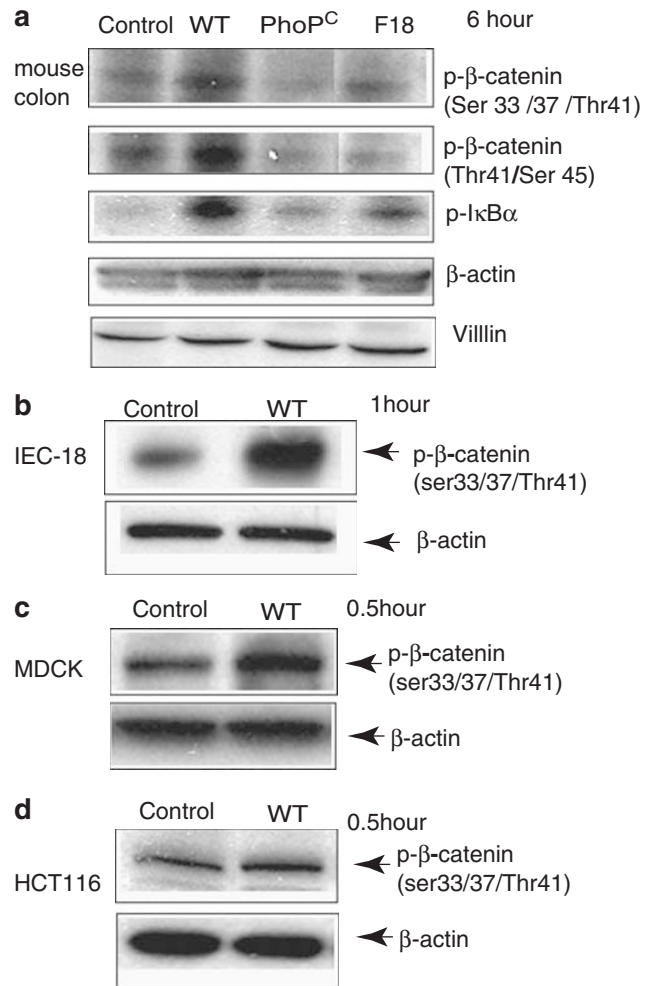


Figure 4 P- β -catenin induced by bacteria *in vivo* and *in vitro*. (a) IECs were harvested from control, WT *Salmonella* PhoP^c, and *E. coli* F18-treated mice 6 h after treatment. Cells were lysed and analyzed for p- β -catenin, p-I κ B α , villin, and β -actin. (b) P- β -catenin (ser 33/37/Thr41) in IEC-18 cells 1 h after treatment with WT *Salmonella*. The images shown are representative of three trials with comparable results. (c) P- β -catenin (ser33/37/Thr41) in MDCK cells treated with WT for 0.5 h. (d) P- β -catenin (ser33/37/Thr41) in HCT116 cells treated with WT for 0.5 h. The images shown are representative of three trials with comparable results.

by 31%, compared to the control without any bacterial infection. In contrast, WT *Salmonella* infection decreased phosphorylated GSK-3 β (Ser9) in colonic epithelia by 48% within 6 h, whereas PhoP^c increased phosphorylated GSK-3 β (Ser9) by 21% compared to the control without any bacterial infection (Figure 4a). Bacterial infection did not change the total amount of GSK-3 β (Figure 5a) or GSK-3 α , another member of the GSK-3 family (data not shown). Briefly, our data suggest that bacterial colonization alters GSK-3 β activity by stimulating its phosphorylation, which, in turn, influences the β -catenin-mediated pathways by phosphorylating β -catenin in colonic epithelial cells.

We also established a GSK-3 β siRNA system to knock down the total expression of GSK-3 β in HCT116 cells. As

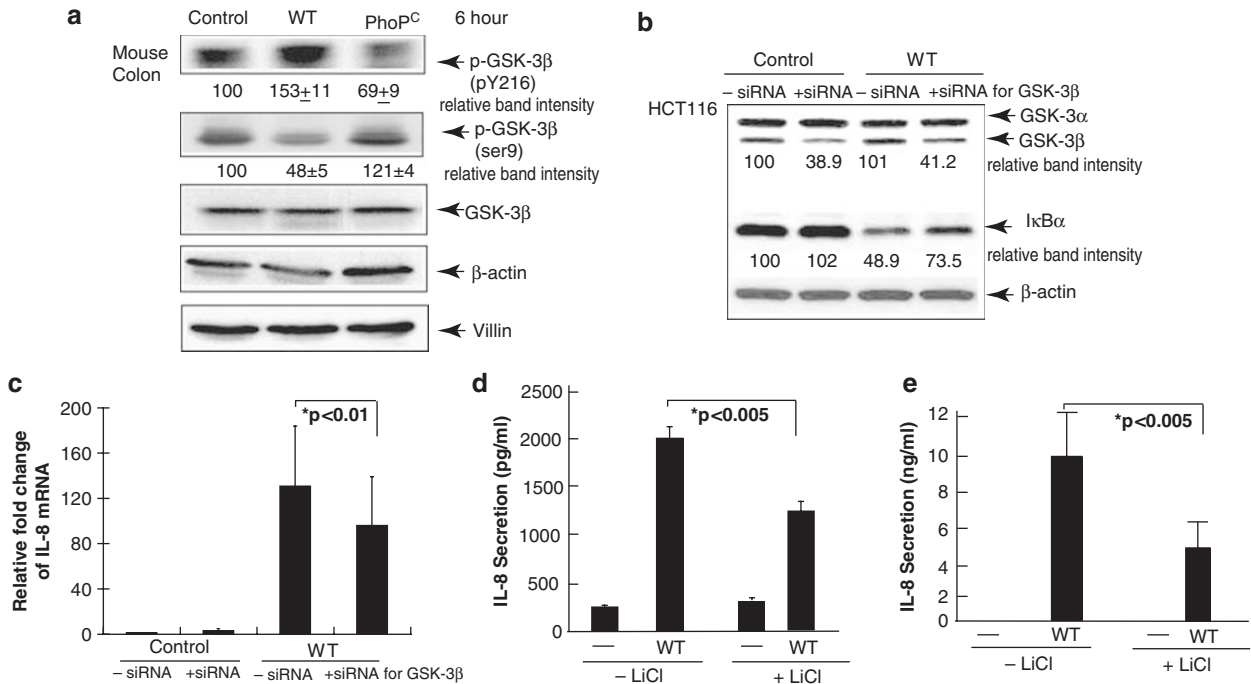


Figure 5 Bacterial infection changed GSK-3 β activity. **(a)** P-GSK-3 β activity in mouse colonic epithelial cells 6 h after WT *Salmonella* or PhoP^C infection. Bands were quantified using NIH Image software. Data presented are mean \pm s.d. from a single experiment assayed in triplicate, and are representative of results obtained in three separate experiments. **(b)** GSK-3 β siRNA reduced the WT *Salmonella*-induced I κ B α degradation. Bands were quantified using NIH Image software. The GSK-3 β siRNA was able to low the total GSK-3 β to about 40% of the control cells. The images shown are representative of two trials with comparable results. **(c)** GSK-3 β siRNA reduced the WT *Salmonella*-induced IL-8 mRNA. **(d)** IL-8 secretion in HT29Cl.19A cell culture supernatants on the basolateral side. **(e)** IL-8 secretion in T84 cell culture supernatants on the basolateral side. Data presented are mean \pm s.d. from a single experiment assayed in triplicate, and are representative of results obtained in three separate experiments.

shown here, the expression of total GSK-3 β and phospho-GSK-3 β was decreased by about 43% (Figure 5b, + siRNA for GSK3 β), whereas the GSK-3 α expression was not altered. The degradation of the total I κ B α was less in the siRNA-transfected cells compared to the non-siRNA-transfected cells in response to the WT *Salmonella* colonization (Figure 5b). Additionally, real-time PCR data showed that the IL-8 mRNA was decreased in the siRNA-transfected cells compared to the non-siRNA-transfected cells in response to the WT *Salmonella* colonization (Figure 5c).

Further to examine the effects of GSK-3 β on *Salmonella*-induced activation of the NF- κ B pathway, we tested the protective function of LiCl, an inhibitor of GSK-3 β , in a polarized human colonic epithelial cell line, HT29Cl.19A. Pretreatment for 24 h with LiCl reduced IL-8 secretion in response to WT *Salmonella* (Figure 5d and e). This response was not cell line specific, because polarized human colonic epithelial T84 cells demonstrated the same response as did the HT29Cl.19A cells to WT *Salmonella* after with LiCl pretreatment (Figure 5d). Less WT *Salmonella*-induced IL-8 secretion was detected in the medium of T84 cells after LiCl pretreatment (Figure 5e). These data are consistent with these in our previous report, which demonstrated that LiCl pretreatment can inhibit WT *Salmonella*-induced GRO/CINC-1 (rat IL-8) secretion in a non-transformed rat small intestine

cell line.¹⁰ Taken together, our siRNA and LiCl pretreatment data suggested that inhibition of GSK-3 β is able to reduce the NF- κ B activity in response to the *Salmonella* colonization.

β -Catenin Activation Negatively Regulates the NF- κ B Pathway during Bacterial Colonization

To address the possible biochemical and physiological effects of β -catenin on the NF- κ B pathway, we used a unique cell model: the human epithelial cell line HCT116 with only the mutant β -catenin allele.²⁰ The mutant β -catenin gene $\Delta 45$ represents a 3-bp deletion (TCT) that removes codon 45.²¹ *CTNNB1* is the β -catenin gene. Parental HCT116 cells (*CTNNB1*^{WT/ $\Delta 45$}) possess the mono-allelic β -catenin mutation $\Delta 45$ and WT *CTNNB1*. Chan *et al*²⁰ disrupted the *CTNNB1* allele in the parental HCT116 cell line and generated two new cell lines with only one allele: HCT116 (*CTNNB1*^{-/ $\Delta 45$}) possesses only mutant *CTNNB1*, and HCT116 (*CTNNB1*^{WT-}) possesses only WT *CTNNB1*. These genetically modified cell lines allowed us to test the possible effects of β -catenin activity on the NF- κ B pathway. In cell line HCT116 (*CTNNB1*^{-/ $\Delta 45$}) expressing constitutively active β -catenin, I κ B α was stabilized; NF- κ B/DNA binding, and NF- κ B transcriptional activity were repressed after WT *Salmonella* colonization. Accordingly, constitutively active β -catenin was found to inhibit the secretion of IL-8.¹⁰

To test the global effect of β -catenin activity on inflammatory stimulation, we measured relative I κ B levels as an indicator of NF- κ B activation after treatment with WT *Salmonella*, TNF- α , and commensal *E. coli* F18. In *CTNNB1*^{WT/ Δ 45}, there was I κ B degradation after treatment with WT *Salmonella*, *E. coli* F18, and TNF- α (Figure 6). These

cells responded like normal mouse tissue incubated with WT *Salmonella* (Figure 1). In HCT116 (*CTNNB1*^{WT/-}), which possesses only WT *CTNNB1*, the responses to the WT *Salmonella*, *E. coli* F18, or TNF- α stimulation were comparable to that in the parental HCT116 cells. However, in *CTNNB1*^{-/ Δ 45}, there was less I κ B degradation induced by

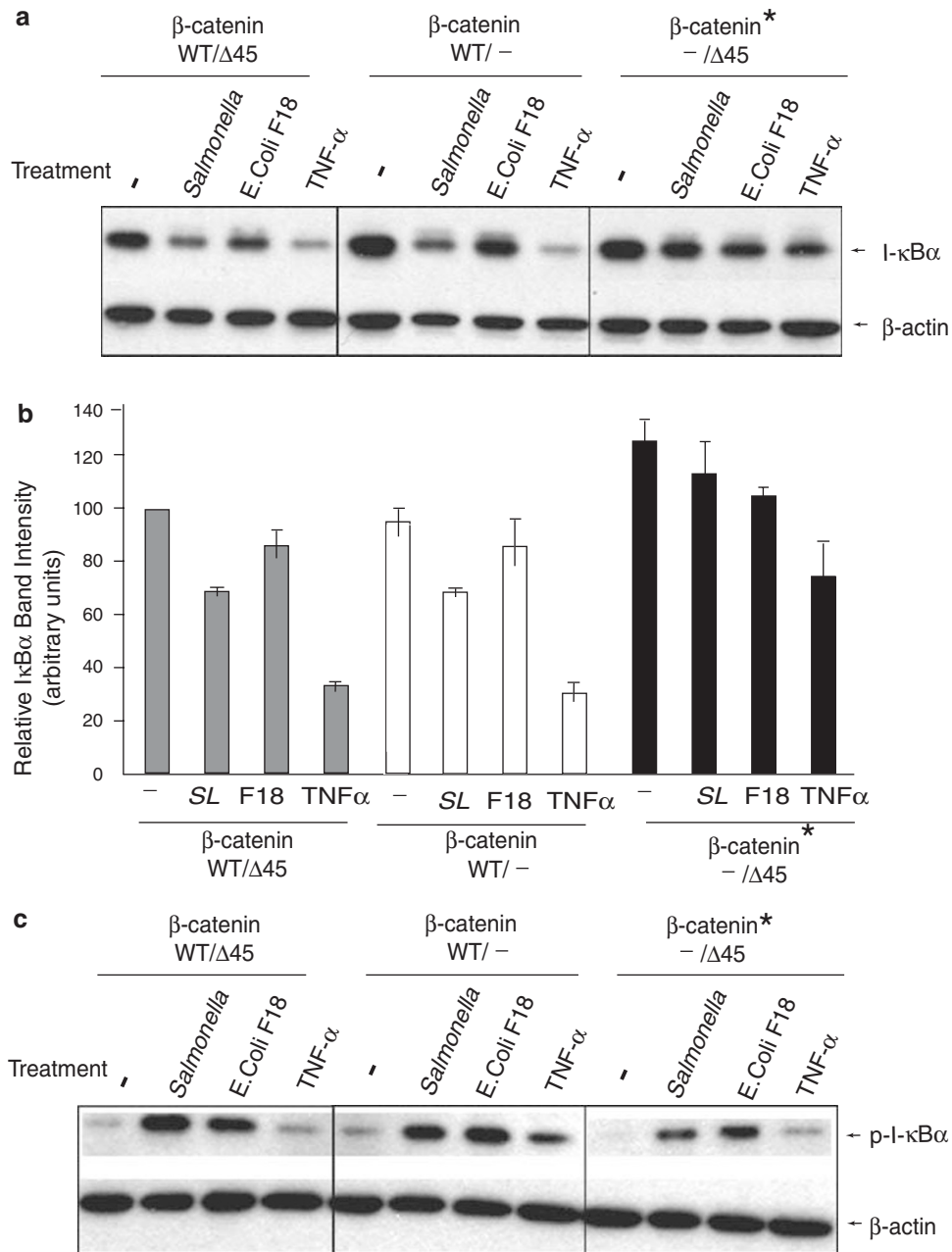


Figure 6 Stabilization of β -catenin inhibits inflammatory responses. (a) I κ B α levels were determined in HCT116 β -catenin WT/ Δ 45, HCT116 β -catenin WT/-, and HCT116 β -catenin -/ Δ 45 (*) after treatment with WT *Salmonella*, *E. coli* F18, and TNF- α for 30 min. Equal amounts of total cell lysate were sequentially immunoblotted with antibodies against I κ B α and β -actin (loading control). Data shown in (a) are representative of four trials. (b) Relative intensity of the Western blot images. Data plotted in (b) are the mean \pm s.d. of $n = 4$. SL: *Salmonella*. (c) p-I κ B α levels were determined in HCT116 β -catenin WT/ Δ 45, HCT116 β -catenin WT/-, and HCT116 β -catenin -/ Δ 45 after treatment with WT *Salmonella*, *E. coli* F18, and TNF- α for 30 min. Equal amounts of total cell lysate were sequentially immunoblotted with antibodies against p-I κ B α and β -actin. These data are from a single experiment that is representative of results obtained in three separate experiments.

Salmonella, TNF- α , and the commensal *E. coli* compared to the HCT116 parental cells (Figure 6a). The relative intensities of the Western blot bands demonstrated that the baseline of I κ B is higher in *CTNNB1*^{-/ Δ 45} cells possessing only the β -catenin mutant *CTNNB1*^{-/ Δ 45} than that in the parental HCT116. Moreover, *CTNNB1*^{-/ Δ 45} cells showed similar protection of I κ B α after *Salmonella*, *E. coli* F18, or TNF- α stimulation (Figure 6b) relative to the parental cells, and HCT116 (*CTNNB1*^{WT}) possessed only WT *CTNNB1*. Interestingly, the baseline of the p-I κ B α was relatively low in *CTNNB1*^{-/ Δ 45} cells. After *Salmonella*, *E. coli* F18, or TNF- α stimulation, the p-I κ B α was still lower compared to the parental cells and HCT116 (*CTNNB1*^{WT}) (Figure 6c). This suggests that the protective effect of constitutively active β -catenin is not strictly limited to *Salmonella*-induced I κ B α degradation.

DISCUSSION

This study demonstrates a novel role for β -catenin in IECs as a constitutive negative regulator of inflammation *in vivo*. By physically binding to NF- κ B and preventing its activation, β -catenin acts in much the same way as I κ B α to inhibit NF- κ B activity. In response to pathogenic bacteria, the physical interaction between β -catenin and NF- κ B is compromised as a result of phosphorylation of β -catenin by GSK-3 β . P- β -catenin is subsequently degraded, liberating NF- κ B from its physical connection and repression by β -catenin. I κ B α , the well-established negative regulator of NF- κ B, is degraded in a similar manner as β -catenin after WT *Salmonella* infection. Following β -catenin and I κ B α degradation, free NF- κ B

translocates to the nucleus, stimulating the production of proinflammatory cytokines such as IL-6 and IL-8. In contrast, colonization by the non-virulent *Salmonella* strain PhoP^c repressed GSK-3 β kinase activity, thus stabilizing total β -catenin and the β -catenin/NF- κ B complex. As a result, IL-6 and TNF- α secretion were suppressed compared to the mice with WT infection.

Our findings indicate that stabilization of β -catenin by inhibiting GSK-3 β kinase activity has a significant effect in downregulating the NF- κ B pathway in IECs. A recent report on Toll-like receptor (TLR) agonist-stimulated monocytes demonstrated that GSK-3 β is a pivotal factor in controlling whether a pro- or anti-inflammatory response is elicited after TLR activation.²² Inhibition of GSK-3 β activity switched the response after LPS stimulation from proinflammatory, IL-12 releasing to anti-inflammatory, IL-10 producing. This anti-inflammatory response to TLR stimulation in monocytes demonstrated that GSK-3 β could regulate the inflammatory responses.²²

A recent study in colon cancer and breast cancer cells has shown that inhibition of GSK-3 β with LiCl can alter NF- κ B activity, and that this cross-regulation occurs through β -catenin.²³ Our published¹⁰ and unpublished data showed that LiCl inhibition of GSK-3 β significantly decreased the amount of IL-8 secretion in response to WT *Salmonella* in small and large IECs. siRNA of GSK-3 β was able to reduce I κ B α degradation and IL-8 mRNA expression. Collectively, these data indicate that the regulation of NF- κ B activity by β -catenin stabilization is not restricted to epithelial cells, but is active in other cell lines as well.

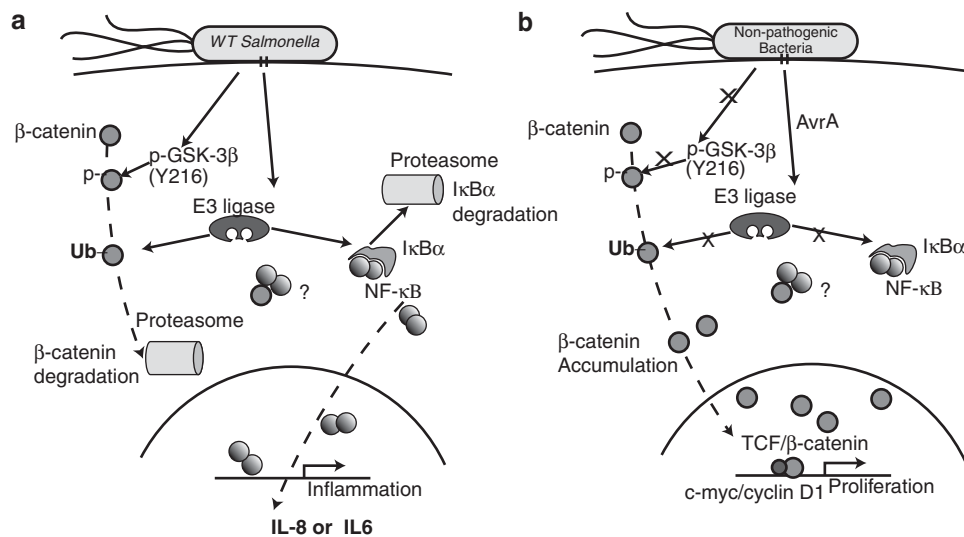


Figure 7 Proposed model for β -catenin inhibition of *Salmonella*-induced proinflammatory cascades. (a) WT *Salmonella* colonization of IECs stimulated phosphorylation of β -catenin and I κ B α through GSK-3 β , and ubiquitination through E3 ligase. β -Catenin and I κ B are subsequently degraded in the proteasome, reducing their concentration within the cell. As a result, there is less NF- κ B/ β -catenin binding, less I κ B, and NF- κ B freely translocates to the nucleus. Once in the nucleus, NF- κ B activates transcription of IL-8 and other proinflammatory genes. (b) When avirulent *Salmonella* colonize epithelial cells, bacteria secrete AvrA, a bacterial effector protein. That may inhibit the phosphorylation and ubiquitination of β -catenin and I κ B. I κ B is stabilized, and β -catenin is not degraded and accumulates in the nucleus. This allows for activation of the β -catenin signaling cascades and inhibition of NF- κ B-dependent proinflammatory gene transcription.

The involvement of GSK-3 β phosphorylation and β -catenin in regulating the inflammatory cascade in response to bacterial challenge is not unique to *Salmonella* infection. The type three secretory system (TTSS)-dependent pathogen *Yersinia* stimulates GSK-3 β phosphorylation during the early stages of macrophage infection.²⁴ It is known that the TTSS Ipa effector proteins play prominent and diverse roles in *Shigella*-induced infection.²⁵ Ipa is able to bind with β -catenin. IpaC-associated β -catenin is tyrosine phosphorylated and destabilized, thus allowing for bacterial invasion.²⁵ A study on *Listeria* showed that β -catenin and α -catenin are recruited to the site of bacterial entry, which is necessary for the dynamic events that induce the cytoskeletal rearrangement and plasma membrane extensions in bacterial uptake.²⁶ Our unpublished data also showed that WT enteropathogenic *E. coli* infection was able to reduce the total β -catenin expression in epithelial cells, whereas the phosphorylation of GSK-3 β (Ser9) was increased. These results suggest that phosphorylation of GSK-3 β and β -catenin stabilization provides important control points in the inflammatory cascade.

Our published data showed that AvrA, a bacterial effector in *Salmonella*, mediates the ubiquitination of β -catenin.⁵ Additionally, we showed that AvrA inhibited the NF- κ B pathways and consequently modulated cellular inflammation.²⁷ We believe that the bacterial effector AvrA regulates the activation of NF- κ B through β -catenin stabilization. Future characterization of the precise function of AvrA *in vivo* will give new clues concerning microbial–host interaction in inflammation.

We propose that the *Salmonella*-induced proinflammatory cascade involves both the β -catenin and NF- κ B pathways (Figure 7). WT *Salmonella* triggers the proinflammatory response, including I κ B α degradation, NF- κ B activation, and IL-8 secretion in epithelial cells. However, we have shown that the same stimulation by WT *Salmonella* also causes β -catenin degradation by increasing the activity of phosphorylated GSK-3 β (Figure 7a). We propose that phosphorylation of GSK-3 β provides a pivotal control site for the stability of β -catenin, which not only controls the cell's fate, apoptosis *vs* proliferation, but also modulates the magnitude of inflammation, through the activity of NF- κ B. Phosphorylation of β -catenin by GSK-3 β destabilizes β -catenin and thus prevents its interference with NF- κ B signaling. In contrast, non-pathogenic bacteria stabilize β -catenin by inhibiting β -catenin phosphorylation through GSK-3 β and ubiquitination of β -catenin.⁵ Stabilization of β -catenin represses NF- κ B-dependent inflammatory pathways and inhibits IL-8 secretion during bacterial colonization (Figure 7b).

In conclusion, we examined the role of GSK-3 β in modulating the β -catenin response to WT *Salmonella*. Our data showed that bacteria modulate β -catenin stabilization by stimulating β -catenin phosphorylation through the kinase activity of GSK-3 β . Pathogenic bacteria increased β -catenin phosphorylation by increasing GSK-3 β activity, thus redu-

cing the total β -catenin expression and β -catenin/NF- κ B binding. On the other hand, non-pathogenic bacteria are capable of stabilizing β -catenin and activating the β -catenin pathway by inhibiting GSK-3 β activity. Consequently, β -catenin levels are maintained and activation of the NF- κ B pathway is prevented. This idea is in agreement with our results showing that stabilization of β -catenin represses NF- κ B pathway activation and inhibits IL-8 or IL-6 secretion during bacterial colonization both *in vitro*²² and *in vivo*. Thus, our findings suggest that activated β -catenin may play an important role in modulating bacteria-induced inflammatory responses.

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