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Salmonella Transforms Follicle-Associated Epithelial Cells into M Cells to Promote Intestinal Invasion

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SUMMARY

Salmonella Typhimurium specifically targets antigen-sampling microfold (M) cells to translocate across the gut epithelium. Although M cells represent a small proportion of the specialized follicular-associated epithelium (FAE) overlying mucosa-associated lymphoid tissues, their density increases during Salmonella infection, but the underlying molecular mechanism remains unclear. Using in vitro and in vivo infection models, we demonstrate that the *S.* Typhimurium type III effector protein SopB induces an epithelial-mesenchymal transition (EMT) of FAE enterocytes into M cells. This cellular transdifferentiation is a result of SopB-dependent activation of Wnt/β-catenin signaling leading to induction of both receptor activator of NF-κB ligand (RANKL) and its receptor RANK. The autocrine activation of RelB-expressing FAE enterocytes by RANKL/RANK induces the EMT-regulating transcription factor Slug that marks epithelial transdifferentiation into M cells. Thus, via the activity of a single secreted effector, *S.* Typhimurium transforms primed epithelial cells into M cells to promote host colonization and invasion.

INTRODUCTION

Salmonella are important bacterial pathogens that have co-evolved with their hosts to modulate cellular functions to successfully survive and replicate intracellularly. In the case of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), following adherence to epithelial cells via fimbrial adhesins, the bacterium uses the type III secretion system (TTSS)-dependent translocation of effector proteins to actively engage with regulators of the cellular cytoskeleton to induce its uptake (Galan and Zhou, 2000). Although *S.* Typhimurium has been demonstrated to invade different cell types to aid colonization and persistence in the host (Haraga et al., 2008), it can also traverse the epithelial barrier by preferentially entering M cells (Jones et al., 1994).

M cells constitute a small subset of highly specialized follicle-associated epithelium (FAE) enterocytes overlying lymphoid follicles in the gut, and are characterized by an irregular brush border, a reduced glycocalyx and lysosomal apparatus, and a capacity to efficiently transcytose a wide variety of macromolecules and micro-organisms from the gut lumen to the underlying immune inductive Peyer's patches (PPs) (Kraehenbuhl and Neutra, 2000). In addition to Salmonella, many other pathogenic bacteria, viruses, and prions take advantage of these unique features of M cells to gain safe passage across the intestinal barrier (Donaldson et al., 2012; Sansonetti and Phalipon, 1999). Despite the important role of M cells in mucosal immunity, little is known about their lineage and development. Recently, receptor activator of NF-κB ligand (RANKL) was shown to be necessary and sufficient for initiation of M cell development, but the specific underlying mechanism(s) was uncertain (Knoop et al., 2009). Debate continues about whether M cells arise from lymphoid follicle-associated crypts (FACs) or ordinary crypts (OCs) and whether M cells represent a distinct lineage or derive from FAE enterocytes that have the plasticity to transition into M cells following exposure to appropriate stimuli.

Certain microbes appear to exploit the innate plasticity of cells to trigger their transformation into a cell phenotype that suits their habitat, as has been demonstrated for *Mycobacterium leprae* in Schwann cells (Rambukkana et al., 2002). Intestinal epithelial cells can dramatically alter their morphology to become motile, fibroblast-like mesenchymal cells in a process referred as epithelial-mesenchymal transition (EMT). This process and the reverse, mesenchymal-epithelial transition, occur repeatedly during normal embryonic development as well as during pathological changes like tissue fibrosis or tumor metastasis (Thiery et al., 2009) or chronic inflammation following certain bacterial infections (Ferreira et al., 2008). EMT is most commonly associated with loss of epithelial junction protein E-cadherin and an increase in intermediate filament protein vimentin, both of which are under control of the transcription factor Slug (Snail homolog 2; *SNAI2*) (Bolós et al., 2003). Slug gene expression is regulated

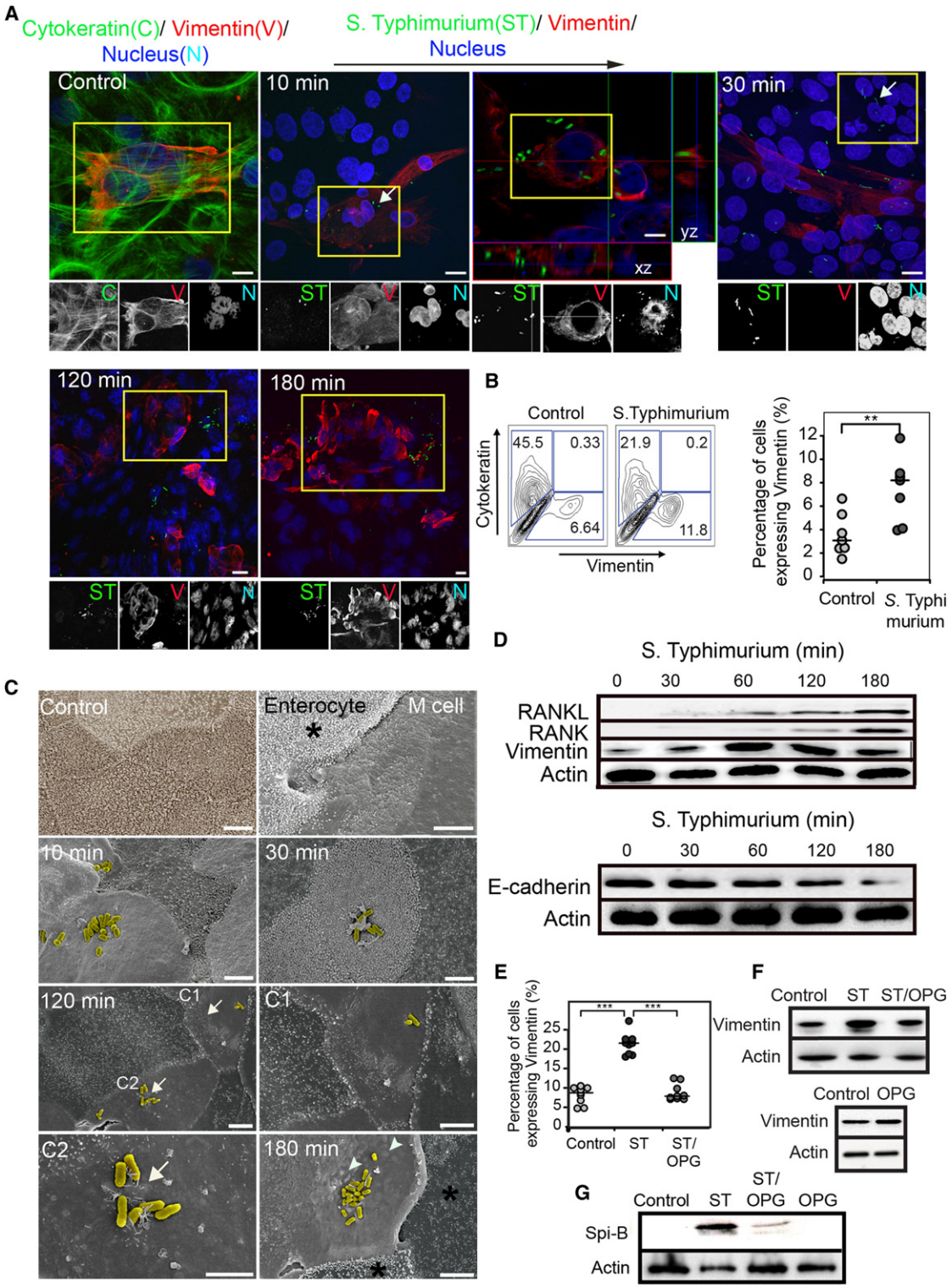

Figure 1. *S.* Typhimurium Transforms Intestinal Epithelial Cells into M-Like Cells

(A) Confocal microscopy of *S.* Typhimurium-infected FAC-epithelial cells depicting expression of the bovine M-cell-specific marker vimentin (red) at different time points after infection (min). *S.* Typhimurium typically targeted vimentin-expressing cells within 10 min of infection (arrow). Internalized bacteria are shown in xz and yz view from orthogonal z section of fluorescent images. At 30 min after infection, *S.* Typhimurium was seen to interact with non-vimentin-expressing enterocytes (arrow). At 120 and 180 min postinfection, the number of vimentin-expressing cells increased. Boxed regions are shown as single-channel images underneath. Control represents uninfected cells stained with anti-vimentin (red) and anti-cytokeratin (green). Cell nuclei are counterstained with DAPI (blue). Scale bar, 10 μm.

by Wnt/β-catenin signaling that drives EMT during embryonic development and tumor metastasis (Vallin et al., 2001). β-catenin is typically degraded through the ubiquitin proteosome pathway that involves a complex of proteins including adenomatous polyposis coli (APC), Axin, and the glycogen synthase kinase 3β (GSK3β). Dissociation of this complex by inhibitory GSK3β phosphorylation increases the cytosolic pool of β-catenin. The free β-catenin then translocates to the cell nucleus and mediates transcriptional regulation by forming a complex with members of the T cell factor (TCF) family of transcription factors to induce EMT-specific target genes including *Slug* and *Vimentin* (Nelson and Nusse, 2004).

Given the evidence that *S*. Typhimurium preferentially targets and promotes an increase in numbers of antigen-sampling M cells, we investigated the molecular mechanisms underlying this increase. We show here that *S.* Typhimurium activates Wnt/β-catenin signaling and events similar to EMT that transform a subset of FAC-derived epithelial cells to a cell type that phenotypically and functionally resembles M cells. We demonstrate that the *S.* Typhimurium TTSS effector protein SopB is necessary and sufficient to induce cellular transformation by activating Wnt/β-catenin signaling-mediated RANKL expression. We propose that *S.* Typhimurium facilitates mucosal penetration and host colonization by transforming these primed epithelial cells into M cells.

RESULTS

S. Typhimurium Transforms Intestinal Epithelial Cells into Antigen-Sampling M Cells in a RANKL-Dependent Manner

To investigate Salmonella-mediated M cell transformation, primary epithelial cells cultured from FAC (see Figure S1 online) isolated from regions of bovine terminal rectum rich in lymphoid follicles (Mahajan et al., 2005) were challenged with *S*. Typhimurium, and examined for changes associated with M cell development and differentiation. Prior to infection, 4%–6% of the epithelial cells in these cultures were positive for the M cell marker vimentin (Figures 1A and 1B) (Tahoun et al., 2011). During early stages of infection (at 10 min), Salmonella typically invaded cells that were positive for vimentin (Figure 1A) and expressed sparse, short, or no microvilli or microfolds typical of M cells (Figure 1C). However, at 30 min postinfection, bacteria were also seen to interact with non-vimentin-expressing enterocytes (Figure 1A) with well-developed microvilli (Figure 1C). Over the course of the infection (180 min), the number of vimentin-positive cells (Figures 1A and 1B and Figure S2A), the number of bacteria adherent to these cells (Figure S2B), and the level of vimentin expression both at the protein (Figure 1D) and transcript levels (Figure S1J) increased, and the cells to which Salmonella bacteria adhered almost completely lost their microvilli (arrow) (Figure 1C). Moreover, the expression of E-cadherin, a typical epithelial marker, was reduced over the infection time course (Figure 1D and Figure S1J). Salmonella infection induced the expression of RANKL, which has been shown in mice to be essential for M cell development and differentiation (Knoop et al., 2009), as well as of its receptor RANK (Figure 1D and Figure S1J). The *S.* Typhimurium-mediated increase in number of vimentin-positive cells (Figures 1E), the enhanced expression of vimentin, its transcriptional regulator Slug, and Spi-B, the master regulator of M cell maturation and differentiation (de Lau et al., 2012; Kanaya et al., 2012), were reduced in the presence of osteoprotegerin (OPG) (Figures 1F and 1G and Figure S1K), a competitive decoy receptor for RANKL (Standal et al., 2002) (Figure 1E). Thus, Salmonella infection leads to an increase in number of cells positive for the M cell marker vimentin in this culture model, which is associated with an enhanced expression of the RANKL growth factor and its cellular receptor RANK. No apoptotic changes were observed over the course of *S.* Typhimurium infection (data not shown).

RANKL-Induced Transformation of Intestinal Epithelial Cells into M Cells Is Restricted to FAC-Epithelial Cells

Ultrastructural studies on proximal and lymphoid-dense terminal bovine rectal tissue revealed morphologically distinct crypt populations at these two sites (Figure 2A). Unlike OC, which had apical round orifice in the proximal rectum, most FAC in the terminal rectum exhibited longitudinal or oval orifice morphologically similar to "dome-associated crypts" of mouse PPs previously identified as the source for FAE enterocytes and M cell progenitors (Gebert et al., 1999). We next determined whether epithelial cells cultured from crypts from these distinct regions respond differently to RANKL stimulation. Unstimulated cells derived from both FAC and OC were comparable in expression

(B) Representative flow cytometry contour plots demonstrate a significant increase in the number of vimentin-positive cells after 3 hr of infection. Numbers in rectangles indicate percentage cells in the gate. Dot plot of flow cytometry results from seven independent experiments, with the medians represented by horizontal lines. **p = 0.002.

(C) Scanning electron micrographs of *S*. Typhimurium infected FAC-epithelial cells. (Top panel) Images show a representative control (uninfected) epithelial monolayer with distinct apical microvilli (left); a typical M cell with diffused or membranous microvilli adjacent to an enterocyte (asterisk) is shown on the right. At 10 min, *S*. Typhimurium were seen interacting preferentially with M cells marked by diffused microvilli. At 30 min of infection, bacteria were also seen to interact with enterocytes expressing distinct microvilli. At 120 min of infection, epithelial cells showed partial to complete loss of apical microvilli (arrows), depicted in magnified inset images (C1) and (C2). At 180 min, a typical infected cell with internalized bacteria seen as silhouettes (arrow heads), showed microvilli lost and surrounded by uninfected epithelial cells expressing distinct microvilli (asterisk). To highlight morphogenic changes, bacteria and cells were given artificial colors (*S*. Typhimurium, yellow; uninfected cells, beige). Scale bar, 2.5 μm.

(D) Western blot analysis of cell lysates for indicated proteins in time course infection studies with *S*. Typhimurium. Representative blot from three independent experiments is shown.

(E) Microscopic quantification of vimentin-positive cells following *S*. Typhimurium infection in the presence of OPG (5 μg/ml), the decoy receptor for RANKL. Results from three independent experiments with three replicates each are presented as dot plots with the medians. ***p < 0.001.

(F and G) Western blot analysis of cell lysates for vimentin and Spi-B following *S*. Typhimurium (ST) infection in the presence of OPG (5 μg/ml), the decoy receptor for RANKL. Representative blot from three independent experiments is shown.

See also Figure S1.

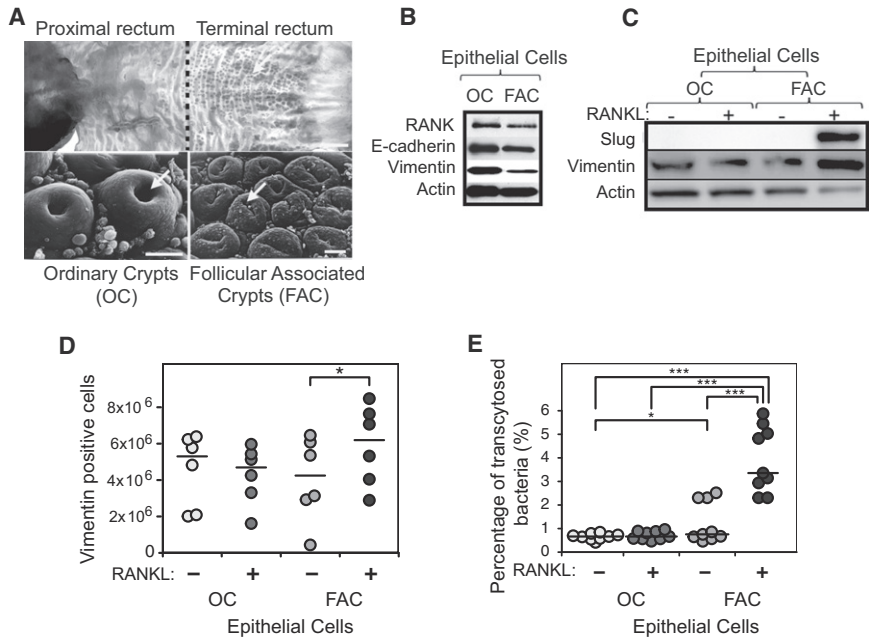

Figure 2. Effect of Purified RANKL on Intestinal Epithelial Cells

(A) In situ localization of lymphoid follicles and epithelial crypts. Rectal tissue fixed in acetic acid (70%, v/v) demonstrates lymphoid follicle-dense (gray nodules) and -deficient areas at the terminal and proximal bovine rectum, respectively. Ultrastructural details of intestinal crypts, ordinary crypts (OC) with apical round orifice (arrow), are structurally distinct from follicle-associated crypts (FAC) with longitudinal or oval orifice (arrow). Scale bar, 2.5 μm.

(B) Western blot analysis of OC- and FAC-epithelial cell lysates for indicated proteins. Data are representative of three independent experiments.

(C) Western blot analysis of OC- and FAC-epithelial cell lysates following treatment with RANKL (100 ng/ml for 5 days) for proteins as indicated. Data are representative of three independent experiments.

(D and E) RANKL priming specifically of FAC-epithelial cells enhanced number of vimentin-positive cells (*p = 0.042) (D) and *S.* Typhimurium transcytosis (***p < 0.001, *p = 0.032) (E). Data from six independent flow cytometry (D) and three transcytosis (E) experiments are presented as dot plots. See also Figure S4.

for RANK, vimentin, and epithelial-specific marker E-cadherin (Figure 2B). In contrast to RANKL-treated cells derived from OC, FAC-epithelial cells treated with RANKL showed increased levels of vimentin as well as an increase in number of cells positive for the M cell marker vimentin (Figures 2C and 2D). We next analyzed RANKL-treated cells for expression of Slug, the transcriptional regulator central to RANKL-induced EMT (Odero-Marah et al., 2008). A RANKL-dependent increase in Slug expression was detected in epithelial cells derived from FAC and not in those from the OC (Figure 2C). Enhanced expression of Slug and vimentin in RANKL-treated FAC-epithelial cells was also confirmed by immunofluorescence (IF) (Figure S3A). Similarly, an increase in *SNAI2* (which encodes Slug), *VIM* (which encodes vimentin), and *TNFRSF11A* (which encodes RANK) transcript expression was observed (Figure S3B). These results indicate that RANKL-induced EMT is the mechanistic basis underpinning epithelial cell transformation into M cells as identified during increased levels of *S.* Typhimurium translocation across RANKL-treated FAC-epithelial cells (Figure 2E).

RANKL-Induced Cellular Transformation Requires NF-κB and Wnt/β-Catenin Signaling Pathways

As both the NF-κB and the Wnt/β-catenin signaling pathways are known to induce the EMT transcription factor Slug (Min et al., 2008; Zhou et al., 2004), we examined their role in RANKL-mediated cell transformation. Epithelial cells derived from FAC were treated with RANKL in the presence of pharmacological inhibitors specific to each of these signaling pathways, and examined for the RANKL-induced expression of vimentin and β-catenin (Figure 3A and Figure S3C). In the presence of NF-κB inhibitor SN50 as well as the β-catenin inhibitor FH535, vimentin expression was suppressed (Figure 3A). In contrast, in the presence of the proteosomal inhibitor MG132, expression of vimentin and β-catenin was increased (Figure 3A and Figure S3C). To examine whether RANKL regulates *SNAI2* and *VIM* promoter activity via the NF-κB and Wnt/β-catenin signaling pathways, luciferase reporter assays were performed in the presence of SN50 and FH535. Both inhibitors suppressed *SNAI2* and *VIM* promoter activity in RANKL-treated epithelial cells, while GSK3β inhibitors (SB415286 and LiCl) enhanced their activity (Figure 3B). Together, these results indicate a role for both the NF-κB and Wnt/β-catenin signaling pathways in RANKL-mediated EMT (Vuoriluoto et al., 2011). In line with these findings, RANKL-mediated enhanced translocation of *S*. Typhimurium was significantly reduced in the presence of OPG and SN50, the RANKL and NF-κB inhibitors, respectively (Figure 3C), suggesting that NF-κB signaling plays a role in enhancing *S.* Typhimurium translocation across epithelial cells. In addition, RANKL treatment also enhanced bacterial uptake by vimentin-positive cells (Figure S4), suggesting a role of RANKL in functional activity of M cells.

These results suggest a role for RANKL in inducing the epithelial transformation of a subset of cells derived specifically from FAC and not from the OC. We reasoned that this cellular restriction was due to differential expression of epithelial factors critical to RANKL signaling. We next compared the expression of RelB, the noncanonical NF-κB transcription factor essential for RANKL-mediated cellular differentiation (Vaira et al., 2008) between cells from each site, as its expression has been shown to be restricted to certain cell types (Yilmaz et al., 2003). RelB expression was restricted to FAC-epithelial cells only (Figure 3D). To further analyze the role of RelB in inducing RANKL-mediated cellular transformation, RelB translation was silenced by RNA interference in FAC-epithelial cells. Epithelial cells treated with RelB siRNAs were not responsive to RANKL, demonstrated by a failure to upregulate Slug expression (Figure 3E) and *S.* Typhimurium translocation across the cultured cells (Figure 3F), suggesting that RelB expression restricts RANKL responsiveness to FAC-derived epithelial cells.

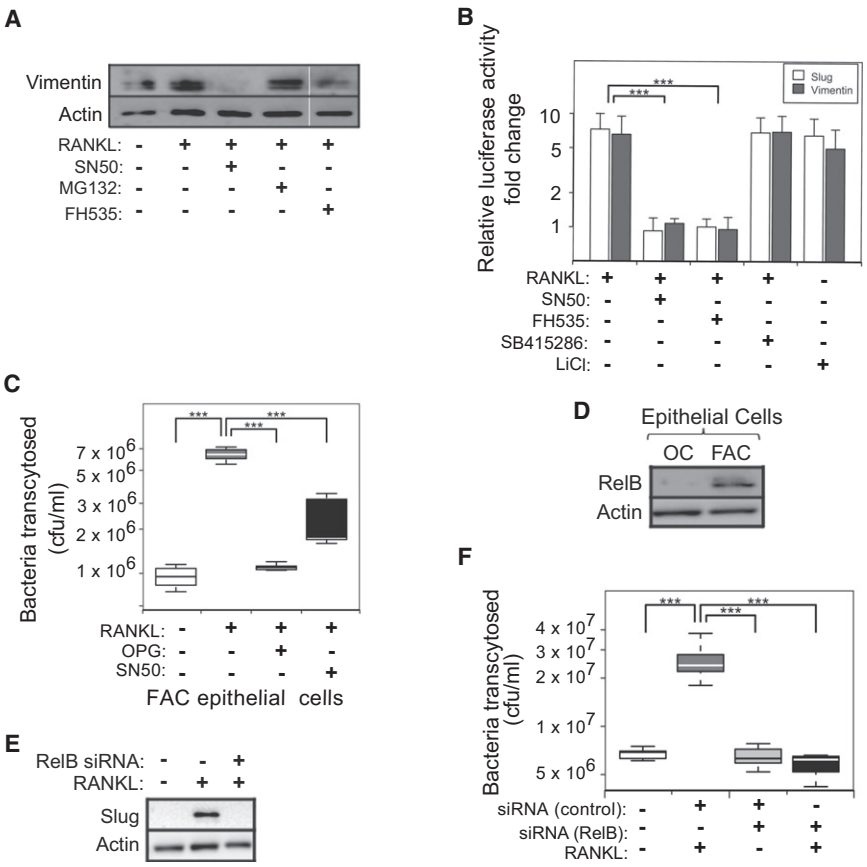

Figure 3. RANKL Activates NF-kB and Wnt/β-Catenin Signaling

(A) Western blot analysis of FAC-epithelial lysates for vimentin following treatment with RANKL (100 ng/ml for 24 hr) in the presence of inhibitors as indicated. Data are representative of three independent experiments.

(B) Effects of RANKL treatment on Slug (encoded by *SNAI*) (white bars) and vimentin (encoded by *VIM*) (gray bars) promoter activity. Caco-2 cells transiently transfected with Slug and vimentin promoter constructs were treated with RANKL (100 ng/ml for 24 hr) in the presence of inhibitors as indicated. Luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega). Data are represented as mean relative luciferase activity (normalized to RLuc activity) ±SD. ***$p < 0.001$ for specific post hoc pairwise comparisons.

(C) *S.* Typhimurium transcytosis assays in RANKL (100 ng/ml for 24 hr)-treated FAC-epithelial cells in the presence of selective inhibitors as indicated. Results from three independent experiments are presented as box plots with interquartile range represented by the box, median by the horizontal line, and range by the whiskers. ***$p < 0.001$ for specific post hoc pairwise comparisons.

(D) Western blot analysis for RelB expression in OC- and FAC-epithelial cells. Data are representative of two independent experiments.

(E and F) RelB expression is essential for RANKL signaling. FAC-epithelial cells transiently transfected with siRNA against RelB were treated with RANKL (100 ng/ml); whole-cell extracts were examined for Slug expression by western blotting (E) or used for *S.* Typhimurium transcytosis assays (F). Results from three independent experiments are presented as box plots (as for Figure 3C). ***$p < 0.001$ for specific post hoc pairwise comparisons.
See also Figure S3.

S. Typhimurium Activates Wnt/β-Catenin Signaling by Suppressing GSK3β

We next examined if the *S.* Typhimurium-mediated increased levels of RANKL, vimentin, and Slug were a consequence of GSK3β-dependent Wnt/β-catenin signaling (Gilles et al., 2003; Shin et al., 2005; Vallin et al., 2001). Immunoblot and IF analysis showed that following *S.* Typhimurium infection, β-catenin was redistributed from the adherens junctions to the cytoplasm and nucleus (Figures 4A and 4B). The increased intranuclear localization of β-catenin and Slug (Figures 4C and 4D) suggested a downstream effect of *S.* Typhimurium-mediated GSK3β suppression. GSK3β is a known target for PI3K/Akt kinases (Doble and Woodgett, 2003). In addition to its role in phosphorylation of cytosolic β-catenin and other zinc-finger transcription factors like Snail, which marks these for ubiquitination and subsequent proteosomal degradation (Doble and Woodgett, 2003; Zhou et al., 2004), GSK3β also represses NF-κB-dependent expression of target genes associated with EMT (Bachelder et al., 2005). To confirm the role of GSK3β in *S.* Typhimurium-induced upregulation of β-catenin and thus activation of Wnt/β-catenin pathway, cells were infected in the presence of the following pharmacological inhibitors specific to GSK3β activation and its target substrates: LY294002 (20 μM), an inhibitor of PI3K pathway; SN50 (50 μM), an inhibitor of NF-κB pathway; Akti-1/2 (20 μM), an allosteric inhibitor of Akt1 and Akt2 isozymes; and MG132 (20 μM), a proteosome inhibitor and SB415286 (25 μM), a GSK3β inhibitor. At the concentrations used, none of these inhibitors showed any effect on viability of *S.* Typhimurium or epithelial cells (data not shown). As seen in Figure 4E, treatment with SN50, LY294002, or Akti-1/2 suppressed β-catenin, RANKL, Slug, and vimentin expression, while SB415286 and MG132 elevated their levels in *S.* Typhimurium-infected epithelial cells. Pretreatment of cells with LY294002 and Akti-1/2 completely inhibited *S.* Typhimurium-induced GSK3β phophorylation (Ser-9)/inactivation (Figure 4F). Together, these results suggest that *S.* Typhimurium first activates PI3K and consecutively Akt, which leads to inhibitory phosphorylation (Ser-9) of GSK3β. Inactivation of GSK3β leads to an increase in cytosolic β-catenin levels and induces the Wnt/β-catenin signaling pathway.

SopB Effector Protein Is Critical to *S.* Typhimurium Activation of the Wnt/β-Catenin Signaling Pathway

To establish if Salmonella-specific secreted effector proteins are responsible for EMT, cells were exposed to viable or heat-killed (ST^{HK}) wild-type (WT) *S.* Typhimurium or isogenic derivative strain that lacks a functional Salmonella pathogenicity island-1 (SPI-1)-encoded TTSS. In contrast to WT *S.* Typhimurium, the heat-killed and SPI-1 mutant Salmonella induce much lower levels of expression of RANKL, Slug, and vimentin (Figure 5A),

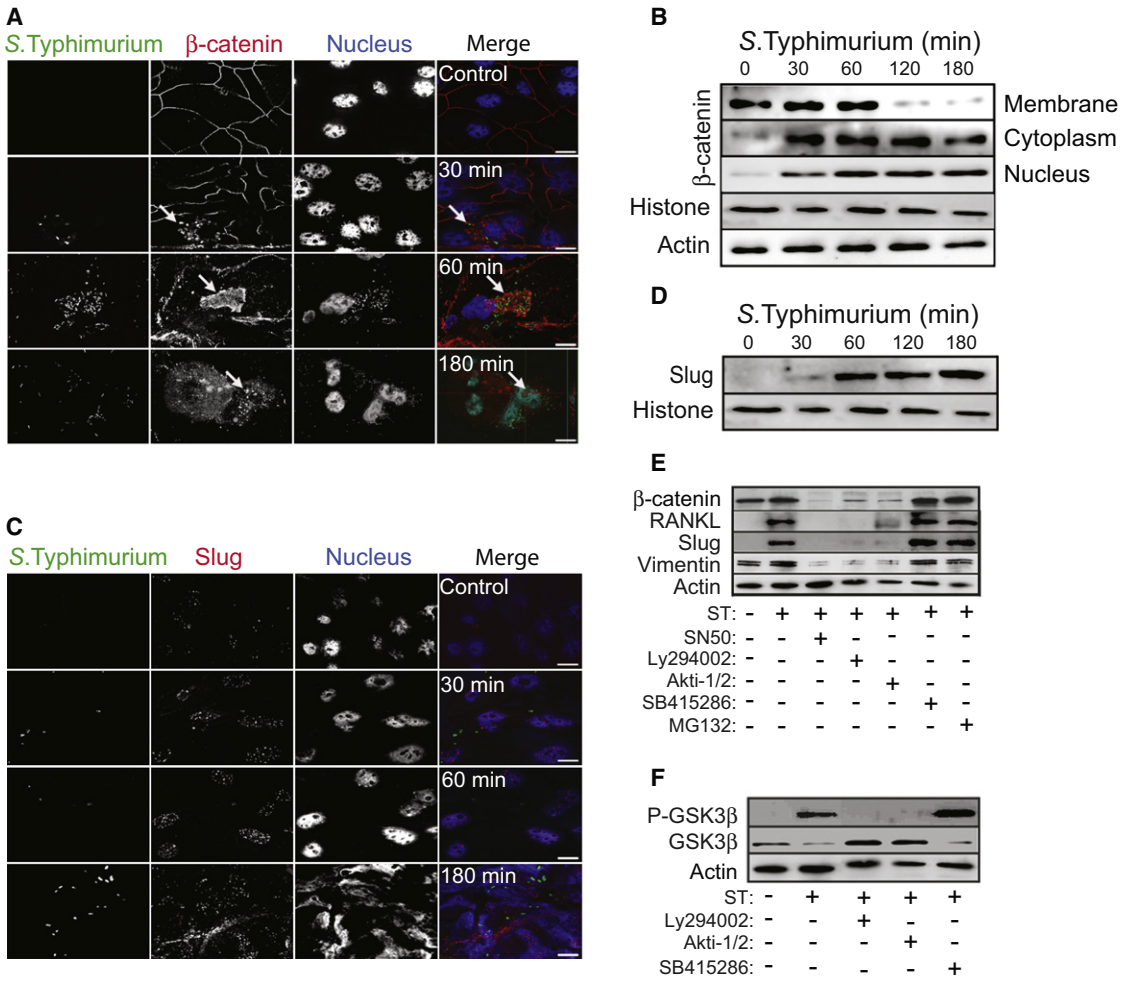

Figure 4. *S.* Typhimurium Activates Wnt/β-Catenin Signaling

FAC-epithelial cells were infected with *S.* Typhimurium strain (SL1344) in time course infection experiments. Infected epithelial cells were processed for localization of β-catenin and Slug proteins by fluorescent microscopy and western blotting at the indicated time points (min). β-actin and total histone H3 were used as equal loading controls for cytoplasmic and nuclear extracts, respectively. Control shows uninfected cells.

(A) Immunofluorescent images demonstrate β-catenin localization at the membranes in uninfected cells, and during the course of infection it was translocated to the cytoplasm and nucleus. Scale bar, 10 μm.

(B) Western blot analysis of subcellular fractions for proteins as indicated.

(C) Immunofluorescent images demonstrate increased Slug levels during the course of infection. Scale bar, 10 μm.

(D) Western blot analysis of Slug in nuclear fractions.

(E and F) FAC-epithelial cells were infected with *S.* Typhimurium strain (SL1344) (ST) for 3 hr in the presence of inhibitors as indicated. The cell lysates were processed to analyze protein expression. Immunoblots were stripped and reprobed with a panel of antibodies as indicated.

(A–F) Data are representative of three independent experiments. See also Figure S2.

suggesting that SPI-1-encoded effector proteins are involved in the transformation process. We next focused on the SPI-1-encoded effector proteins SopB, SopE, and SopE2 that specifically activate Rho family GTPases, and SipA that directly interacts with actin (McGhie et al., 2001), using mutant bacterial strains lacking expression of these proteins. In comparison to WT *S.* Typhimurium and the other isogenic mutants, the *S.* Typhimurium Δ*sopB* strain exhibited a reduced adherence phenotype (data not shown) as well as suppressed induction of RANKL, Slug, and vimentin expression at both protein (Figures 5B) and transcript levels (Figure S5A). Unlike WT, the Δ*sopB* mutant *S.* Typhimurium strain showed no effect on number of vimentin-positive cells in culture (Figure S5C). *S.* Typhimurium suppressed E-cadherin (*CDH1*) transcription in a Sop-B-dependent manner (Figure S5B). Immunoblot analysis of protein lysates revealed an induction of GSK3β phosphorylation (Ser-9) by the WT and other mutants except for the Δ*sopB* strain, which correlated with phosphorylated forms of β-catenin and Akt kinase for each of these respective strains (Figure 5C). Next, we determined whether SopB phosphatase activity was critical for the activation of Wnt/β-catenin signaling. Intestinal epithelial cells were infected with a *sopB*-deficient strain complemented with either catalytically active (*pSopB*) or inactive SopB mutant (*pSopBCys*^{462S}) plasmids. The cell lysates were analyzed for

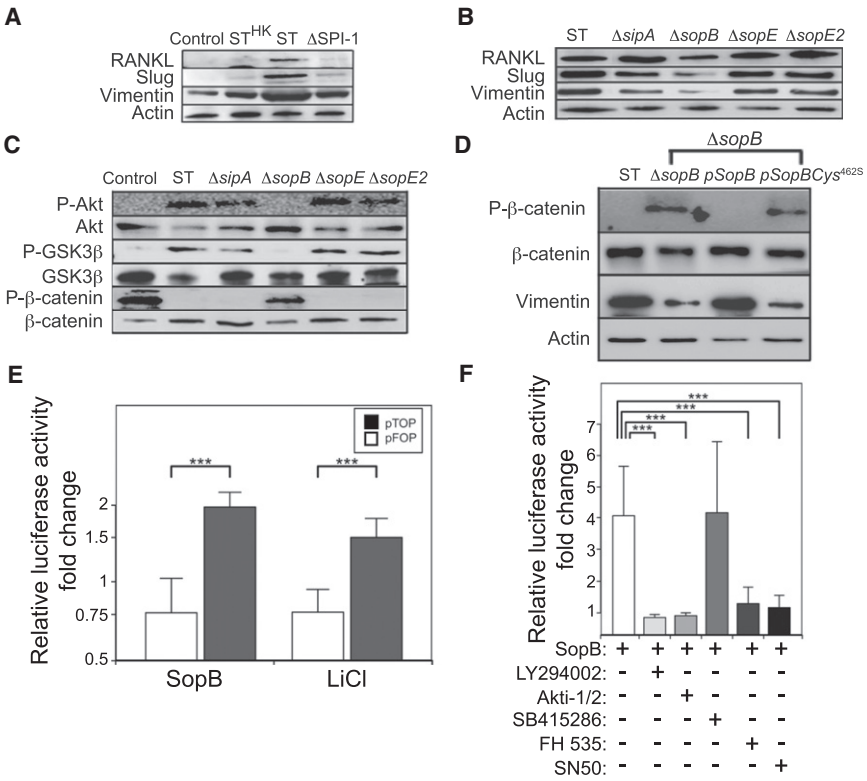

Figure 5. SopB Effector Protein Is Critical to *S.* Typhimurium Activation of the Wnt/β-Catenin Signaling Pathway

(A–C) FAC-epithelial cells were infected with heat-killed (ST^{HK}), WT (ST), ΔSPI-1 (A), or mutant derivatives (Δ*sipA*, Δ*sopB*, Δ*sopE*, and Δ*sopE2*) (B and C) of *S.* Typhimurium strain (SL1344) for 3 hr. Cell lysates were processed to analyze protein expression. Immunoblots were stripped and re-probed with a panel of antibodies as indicated. (A–C) Data are representative of two independent experiments.

(D) FAC-epithelial cells were infected with WT (ST), Δ*sopB* isogenic derivative, Δ*sopB* strain complemented with either catalytically active (*pSopB*) or phosphatase-inactive SopB (*pSopBCys*^{462s}) plasmids. The cell lysates were processed to analyze protein expression as indicated. (A–D) Data are representative of at least two independent experiments.

(E) SopB induces LEF/TCF transactivation. Caco-2 cells transiently transfected with either pTOPflash (gray bars) or pFOPflash (white bars) were transfected with a plasmid encoding SopB or treated with LiCl as a positive control. Luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega). Data are represented as mean relative luciferase activity (normalized to RLuc activity) ±SD. ***$p < 0.001$ for specific post hoc pairwise comparisons.

(F) SopB activates RANKL (encoded by *TNFSF11*) promoter via NF-κB and Wnt/β-catenin signaling. Caco-2 cells transiently transfected with a pGL3 plasmid encoding RANKL-promoter constructs were cotransfected with plasmid encoding SopB in the presence of selective inhibitors as indicated. Luciferase activities were measured by using the Dual Luciferase Reporter Assay System (Promega). Data are represented as mean relative luciferase activity (normalized to RLuc activity) ±SD. ***$p < 0.001$ for specific post hoc pairwise comparisons.

See also Figure S5.

total vimentin and β-catenin phosphorylation, as indicators of Wnt/β-catenin signaling. While the isogenic *sopB* deletion strain (Δ*sopB*) showed enhanced phospho-β-catenin, this was also the case for complementation with the catalytically inactive mutant, SopBCys^{462S} (Figure 5D). However, complementation of the Δ*sopB* strain with a catalytically active SopB (*pSopB*) restored the WT phenotype. This indicates that the conserved cysteine residue Cys-462, which is essential for the inositol phosphatase activity of SopB, is also necessary for activating the Wnt/β-catenin signaling pathway.

To further confirm the role of SopB in Wnt/β-catenin signaling regulation and RANKL expression, intestinal epithelial cells were cotransfected with a luciferase reporter plasmid containing WT (TOPflash) or mutated (FOPflash) LEF/TCF binding sites and a SopB expression plasmid. As shown in Figure 5E, SopB directly upregulated luciferase activity within 48 hr posttransfection, confirming a role of SopB in regulation of LEF/TCF activity. Recent studies on the regulation of RANKL (encoded by *TNFSF11*) expression have identified several transactivators that cooperate to activate its transcription, including NF-κB and β-catenin/TCF signaling (Shin et al., 2005). To further confirm the role of SopB in the regulation of RANKL expression, RANKL-reporter activity was measured in the presence of pharmacological inhibitors (Figure 5F). In the presence of inhibitors for PI3K (LY294002), Akt1 and Akt2 (Akti-1/2), β-catenin/TCF (FH535), and NF-κB (SN50), the RANKL luciferase activity was significantly reduced. However, luciferase activity was significantly increased by SB415286, a GSK3β inhibitor. In summary, these data indicate that SopB-mediated PI3 kinase, NF-κB, and β-catenin signaling regulate RANKL expression at the transcriptional level.

S. Typhimurium SopB Causes an Increase in the Number of M Cells In Vivo

To confirm that SopB effector protein is responsible for *S.* Typhimurium-mediated induction of M cells in vivo, murine ligated gut loops were infected with WT or the *sopB* mutant (Δ*sopB*) *S.* Typhimurium strains. Glycoprotein 2 (GP2) is a specific surface marker for M cells in the mouse (Nakato et al., 2009). Ultrastructural analysis of WT *S.* Typhimurium-infected PPs revealed morphogenic transition of epithelial cells expressing dense microvilli in to M-cell-like cells with characteristic apical "microfold or membranous" structures (Figures 6A–6F). To determine the changes in M cell numbers, PPs from infected mice were immunostained as whole-tissue mounts for GP2, and positive cells were quantified microscopically. The number of GP2-expressing cells was significantly increased after infection with the WT, but not after infection with the Δ*sopB* *S.* Typhimurium strain (Figure 6G). Furthermore, the immunohistological staining for Slug revealed a statistically significant increase in Slug expression

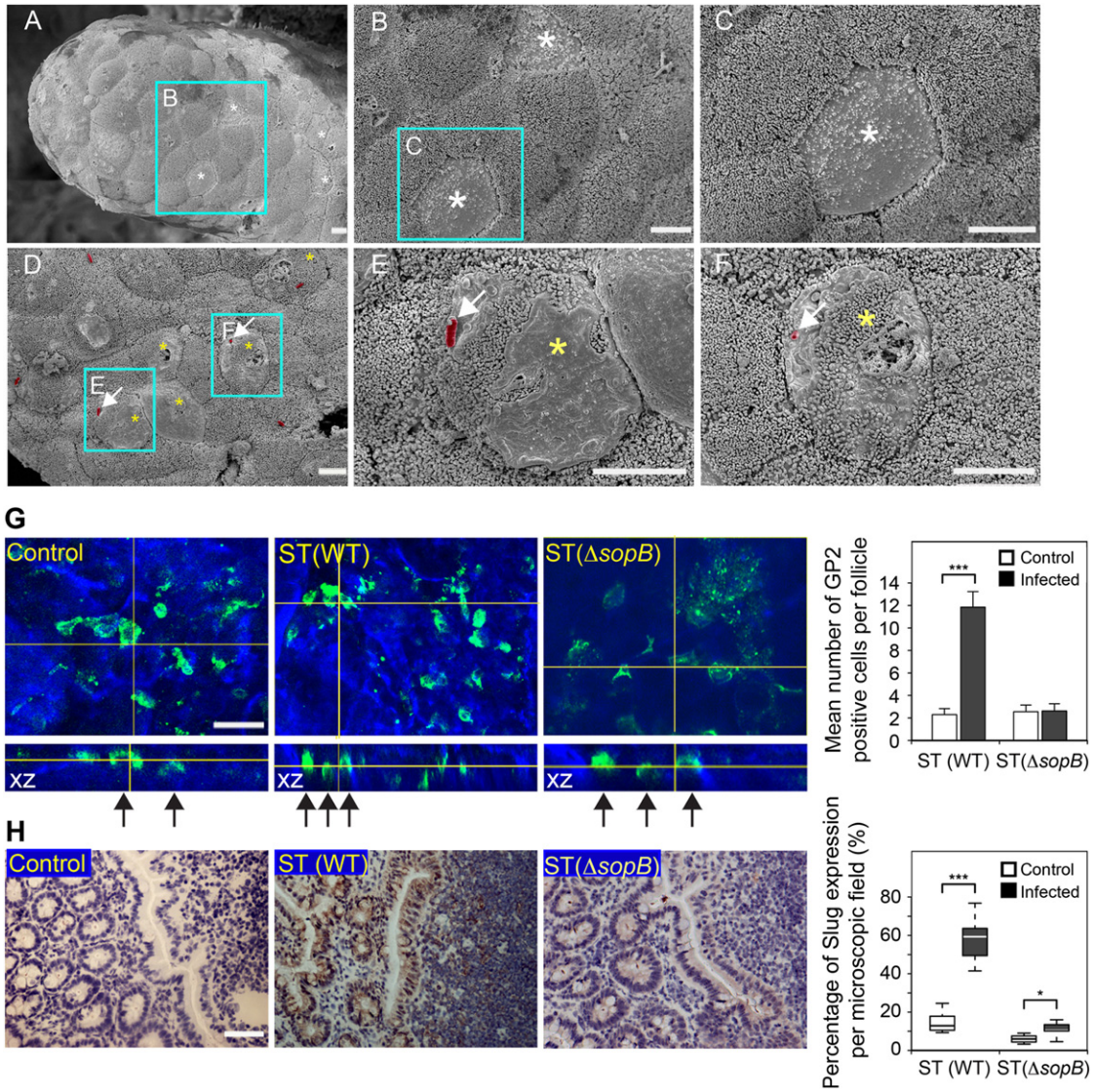

Figure 6. *S.* Typhimurium Transforms Epithelial Cells and Effector Protein SopB Is Critical to Increase in M Cell Number and Slug Expression In Vivo

Murine ligated ileal loops containing Peyer's patches (PP) were infected with the WT *S.* Typhimurium (ST) and Δ*sopB* strains (10^5 CFU) and control PBS (100 μl) for 90 min. PP were fixed as whole-tissue mounts and immune stained for murine M cell marker GP2 (green) and F-actin (blue) as well as processed for immunohistochemistry (IHC) and scanning electron microscopy (SEM) as detailed in the Experimental Procedures.

(A–C) Representative SEM image of an intestinal crypt from an uninfected mouse PP demonstrating M cells (white asterisk) expressing short or no microvilli, surrounded by enterocytes expressing long and dense microvilli. Insets show magnified images of boxed regions. Scale bar, 5 μm.

(D–F) Representative SEM images of WT *S.* Typhimurium-infected mouse PP. Infected epithelial cells show morphogenic changes in cellular architecture (yellow asterisk) characterized by loss of microvilli and acquisition of apical "microfold or membranous" structures characteristic of M cells. Bacteria were given artificial color (red). Scale bar, 5 μm.

(G) Representative fluorescent micrographs demonstrate GP2 (green)-positive cells stained for actin (blue) in FAE of PPs. A representative xz side view of orthogonal confocal image depicts typical an intraepithelial pocket (arrow) indicative of M cell. Bar plot shows total number of GP2-positive cells counted in the follicles from five mice in the control (white bars) and infected (gray bars) group. Data are represented as mean ±SD. ***$p < 0.001$. Scale bar, 20 μm.

(H) Representative IHC sections show Slug expression in FAE of PPs. Slug expression across control and infected tissue sections was calculated using Image Analysis software package as detailed in the Experimental Procedures. Labeling index for the Slug expression is presented as box plots (as for Figure 3C). *$p = 0.023$ and ***$p < 0.001$ for specific pairwise comparisons. Scale bar, 200 μm.

in tissue sections of PPs from mice infected with WT, but not in those infected with the Δ*sopB S.* Typhimurium strain (Figure 6H), confirming that SopB is critical to *S.* Typhimurium-mediated induction of the EMT regulator Slug and M cells in vivo.

DISCUSSION

Here we show that *S.* Typhimurium is able to transform follicle-associated intestinal epithelial cells into M cells in order to

enhance bacterial translocation across the intestinal mucosa. Specifically, the TTSS effector protein SopB plays a central role in inducing RANKL- and Wnt/β-catenin-regulated signaling that converges to trigger differentiation of a topographically restricted subset of enterocytes into M cells. This study provides explanation to a long-standing question in the field and reveals a host-pathogen interaction involving pathogen-driven transformation of host cells to promote invasion.

Previous work has reported an increase in M cell number and/or enhanced transcytosis activity within the FAE following microbial challenge. Specifically, *S.* Typhimurium was shown to cause an increase in the number of M cells in mouse FAE at 12 hr of infection (Savidge, 1996). Similarly, other pathogens have been reported to induce rapid (<1 hr) formation of vimentin-positive M cells in rabbit PPs (Borghesi et al., 1999; Meynell et al., 1999). Using in vitro and in vivo infection models of Salmonellosis, we observed a Salmonella-mediated increase in M cell numbers. To determine the molecular basis underlying such observations, we tested the hypothesis that Salmonella provides signals to epithelial cells that secrete a set of cytokine(s) which could either induce de novo formation of M cells or transdifferentiate epithelial cells into M cells. Critically, *S.* Typhimurium infection of epithelial cells resulted in the release of RANKL, a cytokine crucial to M cell development and maturation (Knoop et al., 2009).

Using a panel of *S.* Typhimurium mutant strains, we demonstrated that SopB is an essential effector protein that mediates the RANKL-dependent differentiation of M cells in culture. Of the range of different effector proteins that *S.* Typhimurium injects into host cells, the phosphoinositide phosphatase SopB is one of the most versatile proteins and controls a number of cellular pathways during different stages of infection (Hernandez et al., 2004; Knodler et al., 2005; Patel et al., 2009). Remarkably, all of these activities are strictly dependent on the phosphatase activity of SopB. In the present study, we show that SopB-dependent activation of the Akt kinase (Steele-Mortimer et al., 2000) leads to inhibitory phosphorylation (Ser-9) of GSK3β, which results in an increase in cytosolic β-catenin and downstream Wnt/β-catenin-mediated EMT (Figure 7). This includes the induction of transcriptional regulator Slug that represses expression of the tight junction protein E-cadherin (Bolós et al., 2003). E-cadherin binds and restricts β-catenin translocation from epithelial junctions to the nucleus, accounting for the β-catenin/LEF-1 transactivation (Orsulic et al., 1999). The Slug-mediated decreased E-cadherin transcription could release β-catenin from adherens junctions and might consequently lead to an increased concentration in the cytosolic β-catenin pool. E-cadherin is important for epithelial integrity, and suppression of its expression is identified with early changes typical of EMT. Taken together, these findings indicate that SopB-mediated inhibition of the central regulator GSK3β triggers a signaling cascade converging at activation of transcription effectors like β-catenin and Slug, as well as downregulation of the epithelial-junction protein E-cadherin. In concert with the SopB-mediated increase in RANKL, the Wnt/β-catenin signaling governs Salmonella-mediated transformation of epithelial cells into M cells.

A key finding was that the RANKL-mediated increased M cell activity was restricted to epithelial cells cultured specifically from crypts isolated from lymphoid-dense terminal rectum. Although it is accepted that intestinal enterocytes or FAE and M cells have a common precursor, it has been postulated that FAE enterocytes and M cells may originate in the specialized FAC as a cell lineage distinct from stem cells via an independent differentiation program (Gebert et al., 1999; Lelouard et al., 2001) or M cells may transdifferentiate from enterocytes when exposed to specific stimuli including the localized cytokine milieu (Man et al., 2008; Knoop et al., 2009; Debard et al., 2001) in specialized niches such as lymphoid-rich PPs. Our data suggest that the cultured FAC either typically contain predetermined, progenitor M cells or generate epithelial cells akin to FAE (Gebert et al., 1999; Lelouard et al., 2001). Since M cell numbers increased as soon as 90 min following infection, de novo synthesis of M cells from lineage-specific stem cells in FAC is unlikely. Therefore, we propose that a proportion of the epithelial cells cultured from FAC rapidly differentiate into M cells following stimulation with RANKL.

Although enterocytes within FAE resemble intestinal enterocytes morphologically, recent analysis of gene expression profiling data reveals that these cells show a distinct pattern of gene expression (Hase et al., 2005), including expression of unusual extracellular matrix and extracellular matrix-interacting proteins (Sierro et al., 2000), and surface ligands like CD137 that are typical of cells of myeloid origin (Hsieh et al., 2010). FAE secrete distinct chemokines such as CCL20/Mip3 (Lügering et al., 2005) and CXCL16 (Hase et al., 2006). FAE and FAC are constitutively positive for expression of RelB, which suggests a role for nonclassical NF-κB signaling in FAE development (Yilmaz et al., 2003). Collectively, these findings confirm FAE as a specialized compartment in the gut, which can respond to signals in a specific manner. In addition, the increased number of M cells following microbial challenge reflects the highly dynamic state of FAE. Recombinant RANKL induced formation of M cells from FAC-epithelial cells in a RelB-dependent manner. These results indicate that following microbial infection or appropriate stimuli, such as exposure to specific cytokines like RANKL, it is only the RelB-expressing epithelial cells, typically associated with FAC or FAE, that are sensitized to transform into M cells. *S.* Typhimurium-mediated M-cell expansion may be critical to its long-term colonization of the intestinal tract rather than a factor for acute disease in the reservoir host species. And the absence of SopB in *S. typhi* may undermine its relative short colonization of the intestinal tract.

In summary, we have described here a remarkable mechanism by which a single bacterial virulence factor can transform epithelial cells into specialized antigen-sampling M cells. The current work provides evidence for a unified signaling mechanism driven by convergence of GSK3β-regulated signaling molecules that confer the enhanced acquisition of M cell phenotype during Salmonellosis.

EXPERIMENTAL PROCEDURES

A detailed description of materials and methods used in this manuscript can be found in the Supplemental Experimental Procedures in the Supplemental Information.

Cell Culture

Primary rectal epithelial cells were isolated, cultured, and characterized as previously described (Tahoun et al., 2011). FAC- and OC-epithelial cells were cultured to 70%–80% confluency over 4–6 days. Epithelial cells were infected with *S.* Typhimurium and the isogenic mutants at a multiplicity of

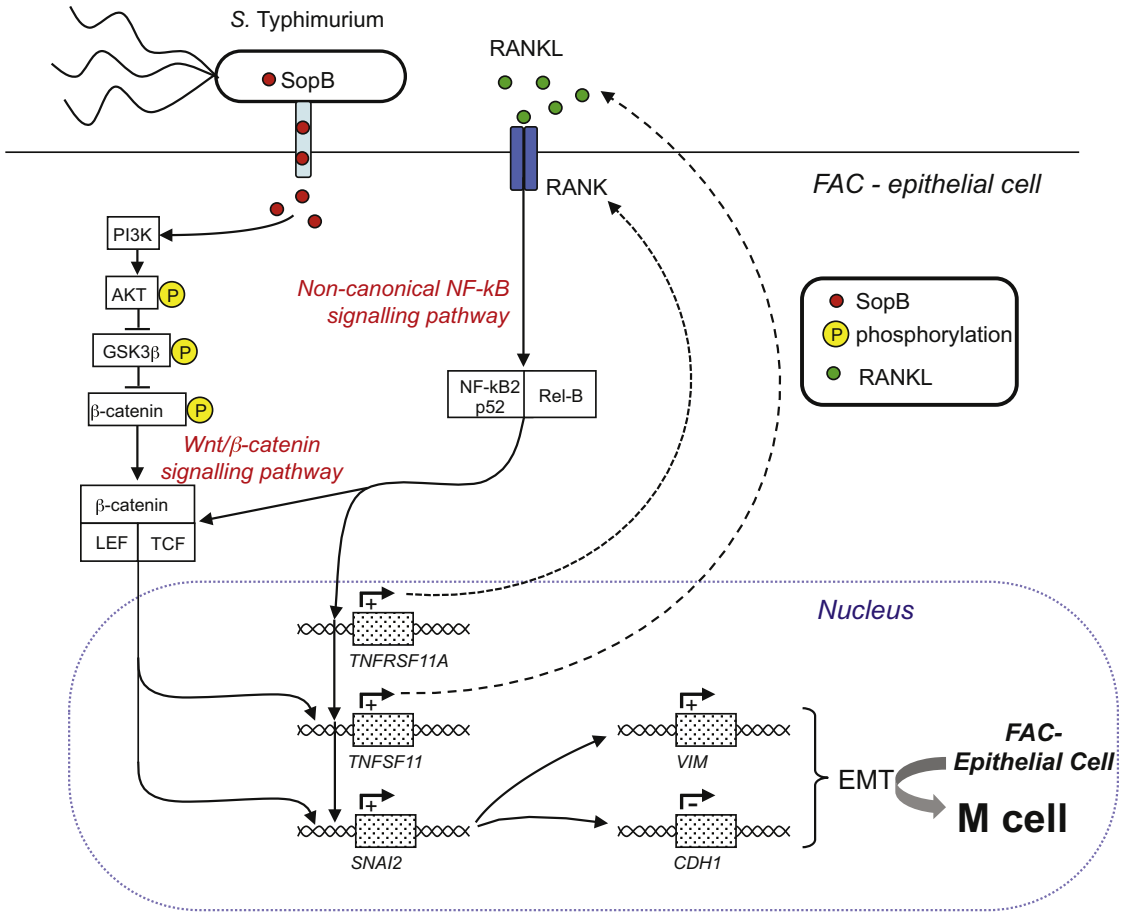

Figure 7. Schematic Diagram Depicts *S.* Typhimurium-Mediated Activation of Wnt/β-Catenin Signaling and Its Role in Induction of Cellular Transdifferentiation

S. Typhimurium type III effector protein SopB in a PI3K-dependent manner activates Akt kinase (phosphorylation) that phosphorylates (Ser-9) and inactivates GSK3β, resulting in increased levels of cytosolic β-catenin. The free β-catenin binds to members of the TCF family of transcription factors (including LEF-1), then translocates to the cell nucleus and regulates transcription of *TNFSF11* (RANKL) and its receptor *TNFRSF11A* (RANK), the epithelial-mesenchymal transition (EMT) transcription regulator *SNAI2* (Slug) and its target genes *VIM* (Vimentin) and *CDH1* (E-cadherin). RANKL functions by binding to its receptor RANK and in a RelB-dependent manner activates β-catenin signaling. The autocrine activation of RelB expressing follicle-associated crypt (FAC)-epithelial cells by RANKL/RANK acts in synergy to induce β-catenin signaling-mediated EMT. FAC-epithelial cell-restricted RelB expression is critical to their RANKL-dependent cellular transdifferentiation into M cell phenotype.

infection (moi) of 20 in time course infection experiments as previously described (Steele-Mortimer et al., 2000). Bacterial transcytosis experiments were done on the FAC-epithelial cells cultured on 0.4 μm pore size polycarbonate filters of 35 μm Transwell chambers (Corning Incorporated, USA) as described (Tahoun et al., 2011). To examine effect of recombinant RANKL on FAC- and OC- epithelial cells, the cells were cultured in the presence or absence of RANKL (100 ng/ml) for 5 days.

Detailed procedures on bacterial infections and pharmacological treatments are provided in the Supplemental Experimental Procedures.

Bacterial Strains and Plasmids

Details are provided in the Supplemental Experimental Procedures.

Transfection and Reporter Gene Assay

Bovine vimentin (−862 to +93), bovine Slug (*SNAI2*) (−946 to +61), and human RANKL (*TNFSF11*) (−1,539 to +107) promoter regions were PCR amplified from genomic DNA isolated from bovine or human tissues (Table S1) and cloned into NheI and XhoI restriction sites of the pGL3-basic luciferase reporter plasmid (Promega). SopB was cloned by PCR amplification from *S.* Typhimurium SL1344 strain (Table S1) into the pDONR-207 entry vector (Gateway, Invitrogen) and subsequently into a Gateway-compatible pCR3-derived eukaryotic expression vector. All clones were sequence verified. Luciferase-based reporter assays were applied with minor modifications as described (Medici et al., 2008). A detailed description on reporter constructs, reporter gene assays, and siRNA transfections has been included in the Supplemental Experimental Procedures.

Flow Cytometry, Western Blotting, Fluorescence, and Scanning Electron Microscopy

FAC- and OC-epithelial cells following infection with *S.* Typhimurium and the isogenic mutants were processed for flow cytometry analysis, immune blotting, and confocal and scanning electron microscopy as described previously (Mahajan et al., 2005; Tahoun et al., 2011). Detailed descriptions about these techniques and the antibodies are listed in the Supplemental Experimental Procedures.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

RNA extractions were performed using the RNeasy Mini kit (QIAGEN). Reverse transcription-polymerase chain reaction (RT-PCR) was conducted using the

Super-ScriptII kit (Invitrogen, Paisley, UK) and corresponding protocol. Samples were amplified using PTC-100 thermal cycler (MJ Research, Waltham, MA). The primer sequences for target genes are provided in Table S1 (see the Supplemental Experimental Procedures).

Animal Experiments

S. Typhimurium infection studies were performed using murine ileal ligated loop assays and processed for anti-GP2 staining of PPs as described previously (Hase et al., 2009). Experiments were approved by the Roslin Institute's Ethical Review committee and were conducted according to regulations of the UK Home Office Animals (Scientific Procedures) Act 1986. Briefly, 7- to 8-week-old C57BL/6J female mice were purchased from Jackson Laboratory and maintained in the specific-pathogen-free barrier facility of the Small Animal Unit of the Roslin Institute. For intestinal loop ligations, mice were anaesthetized and kept on a heated pad during the experiment. Overnight LB grown cultures of *S.* Typhimurium (WT) or isogenic mutant Δ*sopB* with appropriate antibiotic were resuspended in PBS and injected (10^5 cfu) into one of the two ligated intestinal loops in each mouse. The other ligated loop was injected with the PBS and served as a control. After incubation for 60–90 min, the mice were euthanized and PPs excised and processed for whole-mount anti-GP2 staining as previously described (Hase et al., 2009) or cryo-fixed for immunohistochemistry. All experiments were approved under a Project License granted by the Home Office (UK) and conducted in accordance with local guidelines after approval of all experimental procedures by the local ethical committee.

Statistical Analysis

Data were analyzed by applying general linear models with binomial errors (*GLMb*) or standard analyses of variance (*AOV*) or general linear models with poisson errors (*GLMp*). Details are provided in the Supplemental Experimental Procedures. In all analyses, the data were added first into the model as a fixed effect, and standard post-hoc Tukey pairwise comparisons were carried out if overall differences were found. $p < 0.05$ was taken to indicate statistical significance, and all analyses were carried out in R (version 2.15.0 R Foundation for Statistical Computing).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, Supplemental References, and one table and can be found with this article at http://dx.doi.org/10.1016/j.chom.2012.10.009.

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