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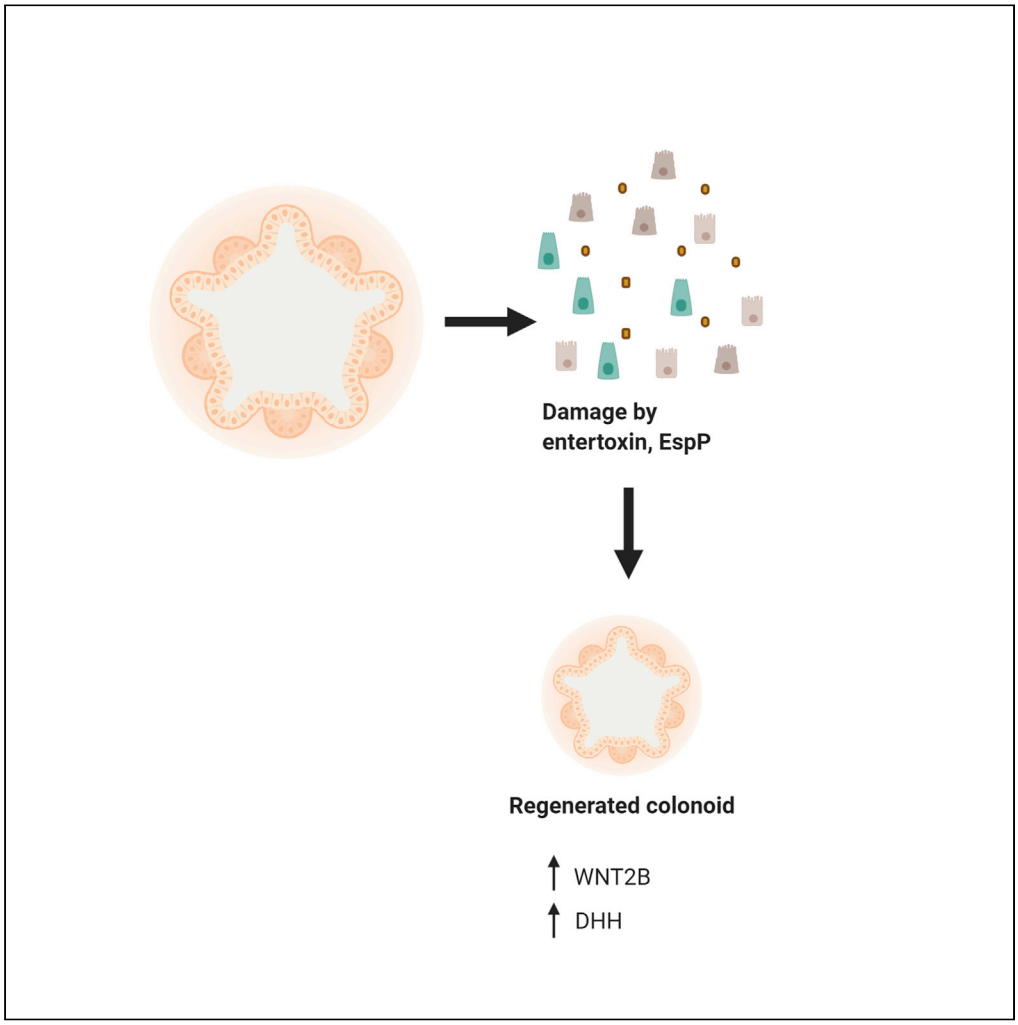

Julie G. In, Jianyi Yin, Roger Atanga, ..., Sarah E. Blutt, Mary K. Estes, Olga Kovbasnjuk

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HIGHLIGHTS

Proteomics on injured colonoids revealed essential pathways in colonic regeneration

Epithelial-produced WNT2B and DHH were found to be drivers of colonoid regeneration

Wnt inhibition prevented regeneration, but was rescued by exogenous WNT2B

Upregulation of DHH was independent of SHH or IHH expression

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Epithelial WNT2B and Desert Hedgehog Are Necessary for Human Colonoid Regeneration after Bacterial Cytotoxin Injury

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SUMMARY

Intestinal regeneration and crypt hyperplasia after radiation or pathogen injury relies on Wnt signaling to stimulate stem cell proliferation. Mesenchymal Wnts are essential for homeostasis and regeneration in mice, but the role of epithelial Wnts remains largely uncharacterized. Using the enterohemorrhagic *E. coli*-secreted cytotoxin EspP to induce injury to human colonoids, we evaluated a simplified, epithelial regeneration model that lacks mesenchymal Wnts. Here, we demonstrate that epithelial-produced WNT2B is upregulated following injury and essential for regeneration. Hedgehog signaling, specifically activation via the ligand Desert Hedgehog (DHH), but not Indian or Sonic Hedgehog, is another driver of regeneration and modulates WNT2B expression. These findings highlight the importance of epithelial WNT2B and DHH in regulating human colonic regeneration after injury.

INTRODUCTION

The adult intestine has the amazing capacity to regenerate following stress, inflammation, or injury (Beumer and Clevers, 2016); however, the mechanisms that regulate regeneration are not well understood. Much of our knowledge in intestinal stem cell renewal and regeneration stems from studies in *Drosophila* (Jiang et al., 2016) and mice (Farin et al., 2016; Metcalfe et al., 2014; Ritsma et al., 2014). Particularly relevant are *Drosophila* studies that revealed the importance of Wnt and Hedgehog signaling in the development, maintenance, and regeneration of the midgut. However, the interplay of these two signaling pathways is not limited to intestinal maintenance. Hedgehog and Wnt signaling are essential pathways in development, homeostasis, and regeneration of many organs. Hedgehog signaling is essential in skin wound healing (Le et al., 2008); cardiac (Wang et al., 2016a), gastric (Konstantinou et al., 2016), lung (Sriperumbudur et al., 2016), hematopoietic (Trowbridge et al., 2006), and liver regeneration (Langiewicz et al., 2016; Wang et al., 2016b); as well as epidermal stem cell homeostasis (Adolphe, 2004). In addition, intestinal regeneration in *Drosophila* is stimulated by active Hedgehog signaling (Tian et al., 2015). *Sonic hedgehog (SHH)* is the most widely expressed mammalian Hedgehog ligand (Varjosalo and Taipale, 2008), but *Indian hedgehog (IHH)* has been shown to be highly expressed in human colon (Van den Brink, 2007; van den Brink et al., 2004). The presence and role, if any, of *Desert hedgehog (DHH)* has not been characterized in the colon, although *DHH* has been linked to the maintenance and regeneration of the corneal epithelium (Kucerova et al., 2012).

Thus far, few studies have detailed the importance of epithelial Wnts in homeostasis or response to injury in the intestine (O'Connell et al., 2018; Suh et al., 2017; Zou et al., 2018), with most studies focused on the role of mesenchymal Wnts (Gregorieff et al., 2005; Greicius et al., 2018; Koch, 2017; Shoshkes-Carmel et al., 2018; Valenta et al., 2016). The majority of data from mouse intestinal injury models suggest that mesenchymal Wnts are necessary for epithelial regeneration, but the role epithelial Wnts may be playing in these processes have not been evaluated.

Human colonoid cultures are a tractable, epithelial-only model that can indefinitely proliferate due to the presence of adult intestinal stem cells (Sato et al., 2011), making them an excellent model to study the role of epithelia-produced factors in intestinal crypt injury and hyperplasia. Foodborne bacterial pathogens, such as enterohemorrhagic *E. coli* (EHEC) or *Citrobacter rodentium*, a mouse-adapted bacterium that

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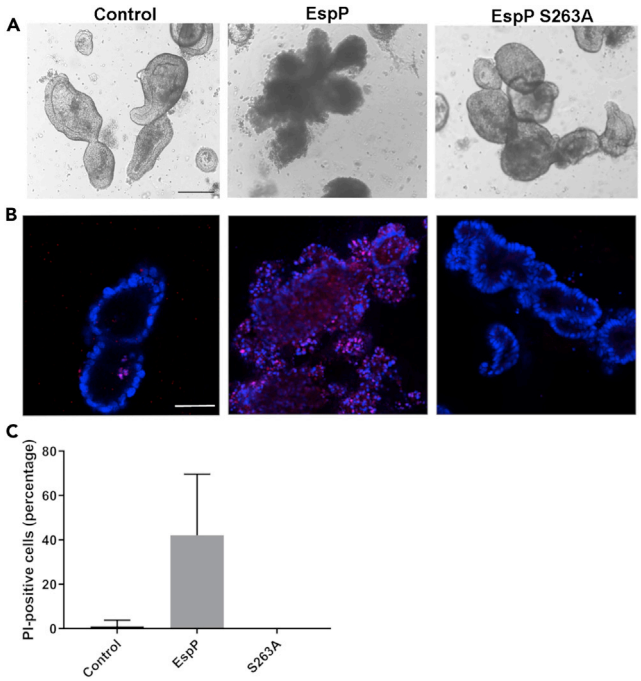

Figure 1. EspP Requires Serine Protease Function to Cause Cytotoxicity of Human Colonoids

(A) Representative images of colonoids after overnight treatment: control (left), EspP-treated (middle), and EspP S263A-treated (right). EspP requires serine protease activity to have a cytotoxic effect on the colonoids; scale bar, 200 μm. N = 3. (B and C) EspP-treated colonoids have more propidium iodide-positive cells compared with control and EspP S263A-treated colonoids; scale bar, 50 μm. Propidium iodide, red; nuclei, blue. N = 3. Data are presented as mean ± SEM.

affects the intestine similarly to EHEC, can cause severe damage to the intestinal epithelia, resulting in hyperproliferation and crypt hyperplasia post-infection (Khan et al., 2006; Vallance et al., 2003; Xicohtencatl-Cortes et al., 2007). We have previously characterized the EHEC-secreted serine protease cytotoxin, EspP, as an important virulence factor in EHEC infection and colonic epithelial damage (In et al., 2013). Cytotoxins in the family of serine protease autotransporters of *Enterobacteriaceae* (SPATEs) are secreted by most pathogenic *E. coli* and have well-characterized functions that aid in bacterial adherence and colonization of epithelial cells (Dautin, 2010). Two SPATEs, Pet and EspC, secreted by enteroaggregative *E. coli* and enteropathogenic *E. coli*, respectively, cause cytotoxicity to intestinal explants (Henderson et al., 1999; Mellies et al., 2001). However, whether or not EspP has cytotoxic properties on intestinal cells has been controversial (Weiss and Brockmeyer, 2012).

In this study, we used EspP to induce epithelial injury and model the intestinal stem cell response, which includes the initiation of regeneration using stem cell-derived human colonoids. Using both molecular and proteomics-based approaches, we found that epithelial-produced WNT2B and DHH-activated Hedgehog signaling interact and are necessary for human colonoid regeneration after EspP-induced damage.

RESULTS

EspP, a Bacterial Autotransporter, has a Serine Protease-Dependent Cytotoxic Effect on Human Colonoids

To determine if EspP induces cytotoxicity in a serine protease-dependent manner in human colonoids, we added recombinant EspP or its serine protease-deficient mutant, EspP 263A (Khan et al., 2011), to normal human undifferentiated colonoids. After an overnight treatment with EspP, all human colonoid lines used in this study (Table S1) exhibited cell shedding, loss of colonoid structure, and positive propidium iodide staining, indicators of cell death (Figure 1). In contrast, overnight treatment with the protease-deficient mutant, EspP S263A, had no visible detrimental effect on the colonoids. Therefore, EspP has a cytotoxic effect on human colonoids, and this activity is serine protease-dependent.

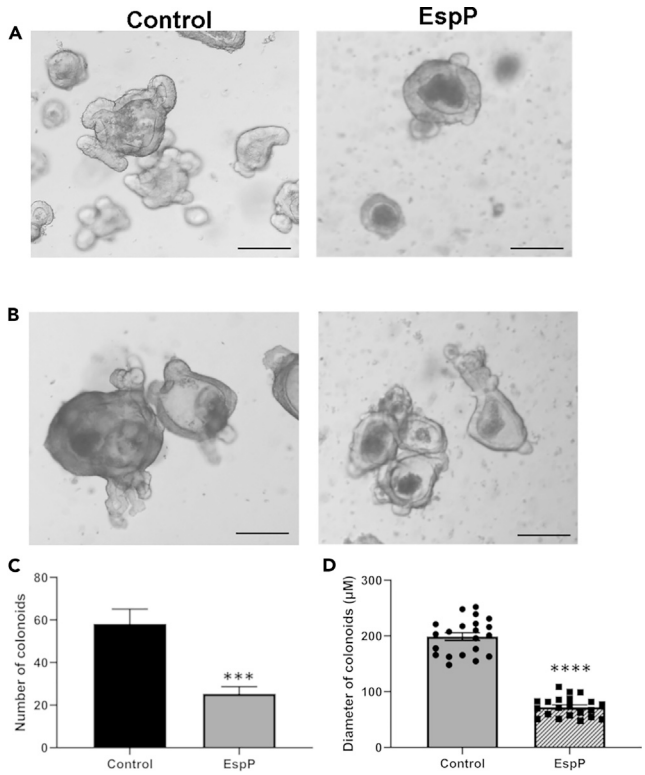

Figure 2. Colonoids can Model Crypt Regeneration after EspP Washout

(A and B) Representative images of colonoids after washout and replating: (A) 24 h post-washout and (B) 48 h post-washout; scale bar, 200 μm. N ≥ 3.

(C) Average numbers of colonoids in each condition 24 h post-washout. ***p = 0.0001; N ≥ 3.

(D) Average diameter of colonoids in each condition 24 h post-washout. ****p < 0.0001; N ≥ 3. Data for (C) and (D) are presented as mean ± SEM.

We hypothesized that EspP-induced injury would model the EHEC-induced denuded colonic epithelia and crypt hyperplasia, the latter mimicked by colonoid regeneration after EspP-induced injury. To test this hypothesis, control and EspP-injured colonoids were harvested after overnight EspP treatment and replated to monitor for colonoid regeneration. The formation of colonoids in the EspP-treated cultures was observed at 24 and 48 h post-replating (Figure 2A). At 24 h, the colonoids were generally smaller in size compared with control and primarily spheroids. The starting colonoid density for both control and EspP-injured colonoids ranged from approximately 50 to 100 colonoids for each condition. After EspP injury and subsequent replating, the average number of control colonoids was 58.2 ± 6.9 and the average number of EspP-injured colonoids was 25.4 ± 3.3 (Figure 2C). The average diameter of the control colonoids at 24 h post-replating was 199.03 ± 6.9 μM, whereas the EspP-injured colonoid diameter averaged 72.5 ± 3.9 μM (Figure 2D). In contrast, at 48 h, the regenerating colonoids more resembled the control culture, with colonoids beginning to form multi-lobular structures (Figure 2B). Therefore, human colonoids can regenerate after injury by the bacterial cytotoxin EspP.

Proteomics Analysis Shows that WNT2B and Desert Hedgehog Are Upregulated during Regeneration

To begin to identify key regeneration-associated pathways, we employed a proteomics approach. Control, EspP-, and EspP S263A-treated colonoids from the same patient-derived biopsy were harvested and lysed and the proteins were identified and quantified with tandem mass spectrometry and iTRAQ. Over 5,000 proteins in the EspP-injured culture were found up- or downregulated compared with the control culture, with very little overlap of differentially expressed proteins between the EspP- and EspP S263A-treated cultures (Figure S1A). The majority of proteins identified in the proteomics assay were cytosolic or nuclear (Figure S1B). A key group of proteins that was upregulated in the EspP-injured cultures were those associated

Accession	Description (*Homo sapiens*)	# PSMs	% Coverage	E-value	Ratio (EspP/Control)
630044901	Protein Wnt2b isoform 3	2	4.35	3.8E-001	3.101
27883842	Polycomb complex protein BMI-1	2	2.15	1.2E-001	2.057
4502805	chromogranin-A isoform 1 preproprotein	7	39.61	7.8E-002	2.009
31542745	protein wntless homolog isoform 1 precursor	2	6.10	1.9E-001	1.997
24431935	reticulon-4 isoform A	12	12.08	5.1E-005	1.995
4506055	cAMP-dependent protein kinase catalytic subunit alpha isoform 1	9	36.47	2.2E-002	1.807
20544145	casein kinase I isoform delta isoform 2	3	16.63	8.2E-005	1.734
225903437	glycogen synthase kinase-3 beta isoform 2	39	20.95	5.6E-008	1.678
395394053	disheveled-associated activator of morphogenesis 1 isoform 2	3	7.49	1.7E-002	1.585
34485714	ras-related protein Rab-23	6	12.24	1.7E-003	1.477
33636738	cAMP-dependent protein kinase catalytic subunit beta isoform 1	6	28.14	1.6E-003	1.464
25121993	RNA-binding protein Musashi homolog 2 isoform b	6	27.49	1.4E-002	1.391
14916475	protein Wnt-3a precursor	21	27.56	2.3×10^{-13}	1.269
578808446	PREDICTED: slit homolog 2 protein isoform X5	12	13.65	2.4E-004	1.259
4885523	noggin precursor	12	22.84	1.5E-005	1.247
578808417	PREDICTED: prominin-1 isoform X5	3	3.68	3.9E-005	1.194
339276103	R-spondin-1 isoform 3 precursor	12	31.50	2.8E-006	1.167

Table 1. EspP-Injured Colonoids Upregulated Proteins in the Wnt and Hedgehog Pathways

with Wnt, Hedgehog, and stem cell regulation. An abbreviated list of these proteins is shown in Table 1. WNT2B isoform 3, WNT3A, Wntless, and numerous downstream targets of GLI1 (active hedgehog signaling) were upregulated in the EspP-treated culture. Many of the proteins listed in Table 1 either were not changed or were downregulated in the EspP S263A-treated (no cytotoxicity) culture suggesting that EspP specifically induced activation of Wnt and Hedgehog signaling as part of the colonic damage and regenerative response.

We performed qRT-PCR to validate the key pathway molecules identified in the proteomics screen. The mRNA expression of select stem cell, Wnt, and Hedgehog genes was compared between EspP-injured regenerating (at the 24 h time point) and control colonoids. Although the injured colonoids regenerate to re-form their 3D structure after EspP washout, the putative intestinal stem cell markers *LGR4* and *LGR5* were not upregulated. *LGR4* was significantly downregulated, whereas *LGR5* was unchanged (Figure 3). *BMI1* was significantly upregulated in the proteomics result, but mRNA expression showed no statistical difference between control and injured colonoids. The proteomics screen identified WNT2B isoform 3 as significantly upregulated in the EspP-injured regenerating colonoids. The EspP-injured regenerating colonoids had a slight downregulation of *WNT2B2* (previously known as *WNT13A*), an upward trend of *WNT2B1* (*WNT13B*), and a significant upregulation of *WNT2B3* (*WNT13C*) (Figure 3). WNT2B3's upregulation following EspP-induced injury confirmed the proteomics assay, but was still unexpected as it is not thought to be a classical epithelial-produced Wnt.

As numerous downstream targets of Hedgehog signaling were upregulated in the regenerating colonoids, we evaluated whether the canonical Hedgehog effectors *GLI1* and *GLI2* were changed in the regenerating colonoids. Both genes have been found upregulated in colon cancer cell lines (Mazumdar et al., 2011; Zhang et al., 2017) and implicated in cancer cell proliferation. *GLI2* transcripts were not detected in either the control or regenerating colonoids. However, *GLI1* was significantly upregulated in the regenerating colonoids (Figure 3). Only the hedgehog ligand *DHH* was significantly upregulated in the regenerating colonoids (Figure 3). Both hedgehog ligands *SHH* and *IHH* were significantly downregulated. Overall, the

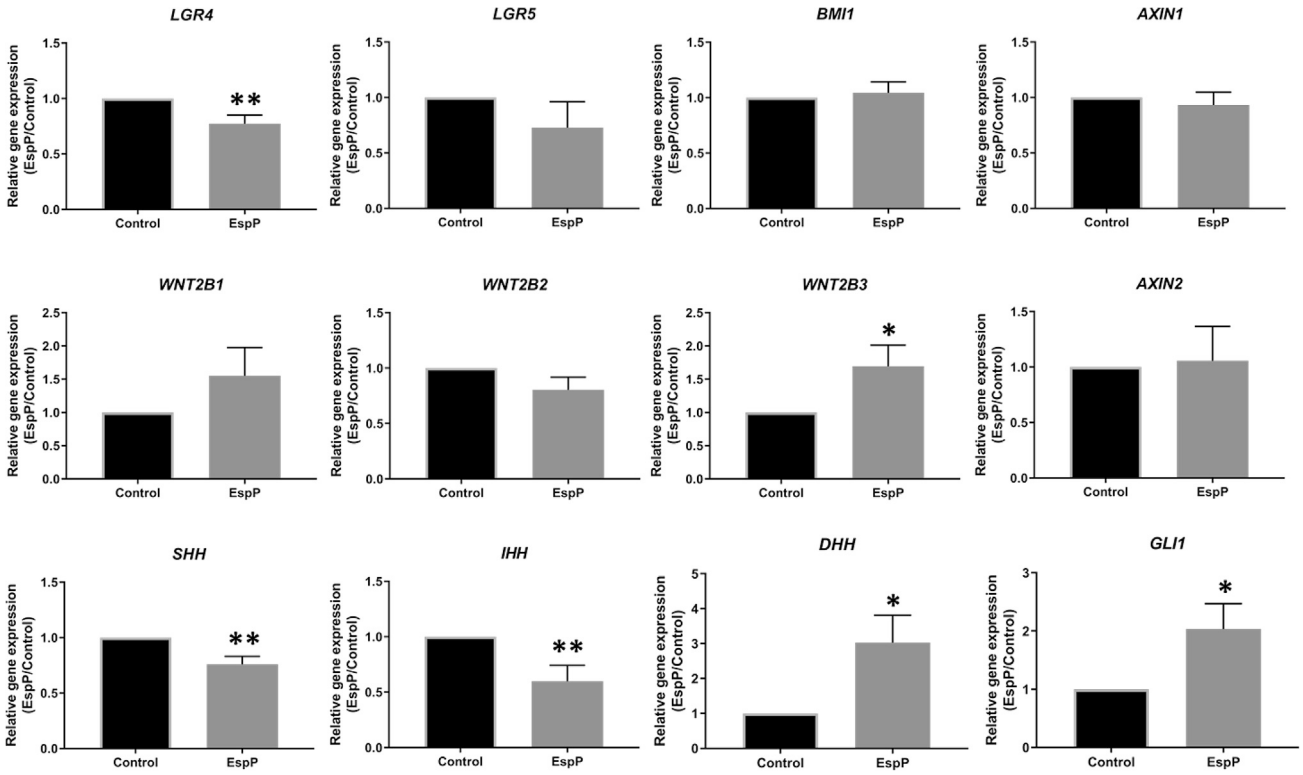

Figure 3. EspP-Treated Colonoids Upregulate WNT2B and DHH during Regeneration

Gene expression of regenerating colonoids was analyzed by qRT-PCR. Relative gene expression is shown as a ratio of EspP-treated compared with control colonoids and normalized to 18S. *$p \leq 0.05$; **$p \leq 0.01$. $N \geq 11$. Data are presented as mean ± SEM.

EspP-injured regenerating colonoids lead to upregulation of hedgehog signaling, specifically via the hedgehog ligand, DHH.

To assess WNT2B expression in the colonoids post-EspP injury, we performed immunostaining. WNT2B expression was concentrated in specific, rare epithelial cells in normal human colon crypt (Figure 4A) and in undifferentiated colonoids (Figure 4B). Not every colonic crypt or colonoid had WNT2B-positive cells, suggesting a mosaic expression pattern. However, colonoids regenerating 24 h after EspP-induced injury contained more WNT2B+ cells and diffuse WNT2B staining throughout the colonoid (Figure 4C and 4C′). To confirm the immunostaining results, we performed RNAscope staining for *WNT2B* and *DHH*. RNAscope showed that *WNT2B* is localized to the deep crypt in human colonic tissue (Figure 4D) and is present in a mosaic pattern, with varied amounts of *WNT2B* present from crypt to crypt. A higher longitudinal cut of the colonic tissue showed that *WNT2B* mRNA is also present in the mesenchymal cells of the lamina propria (Figure S2), as previously reported (Shoshkes-Carmel et al., 2018). Control colonoids had an indiscriminate expression pattern for both *WNT2B* and *DHH* with an average of 2.2 ± 0.2 *WNT2B* dots and 1.96 ± 0.1 *DHH* dots per nuclei (Figures 4E, 4G, and 4H; control bars). The regenerating colonoids increased the number of *WNT2B* and *DHH* dots per nuclei (3.5 ± 0.4 and 4.3 ± 0.5, respectively) (Figures 4F, 4G, and 4H; EspP bars) suggesting an increase in transcription of both genes following EspP injury and washout.

Epithelial Wnt Is Indispensable for Colonoid Regeneration

To determine whether epithelial produced Wnts are important for colonoid regeneration, control and EspP-injured colonoids were monitored in the absence (Figure 5A) or presence (Figure 5B) of IWP-2 (2.5 μM), a Porcupine inhibitor that inhibits palmitoylation of all Wnts and results in inhibition of processing and secretion of Wnts (Farin et al., 2012). The colonoid media containing 50% v/v Wnt3A-conditioned media was maintained in all experimental conditions. As previously shown, the EspP-injured colonoids were able to regenerate and re-form 3D colonoids after EspP is removed (Figure 5A). In the continued presence

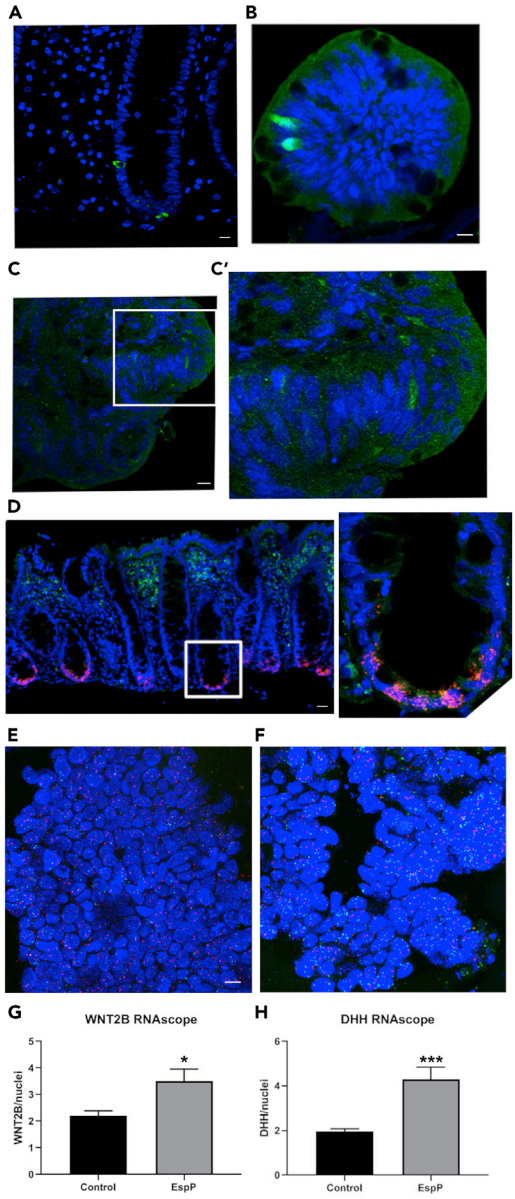

Figure 4. WNT2B Marks a Specific Cell in the Colonic Crypt

(A and B) Representative immunofluorescence staining shows WNT2B concentrated in a specific cell in (A) human colonic tissue and (B) human colonoids; scale bar, 10 μm. WNT2B, green; nuclei, blue.

(C) EspP-treated colonoids regenerating 24 h post-EspP washout. Representative immunofluorescence staining shows WNT2B is more diffuse with more WNT2B+ cells, seen in the zoomed inset (C′); scale bar, 10 μm. WNT2B, green; nuclei, blue.

(D) Representative RNAscope staining of WNT2B in human colonic tissue shows localization to the crypt and a mosaic pattern; scale bar, 10 μm. WNT2B, red; autofluorescence, green; nuclei, blue.

(E and F) Representative RNAscope staining of WNT2B and DHH in control (E) and regenerating (F) colonoids 24 h post-EspP washout; scale bar, 10 μm. WNT2B, red; DHH, green; nuclei, blue.

(G and H) The RNAscope dots per nuclei were quantified for both conditions. *p = 0.012; ***p = 0.0004. N = 3. Data are presented as mean ± SEM.

See also Figure S2.

of IWP-2 (pre-treatment before EspP addition, during EspP treatment, and during the 24-h regeneration period), EspP-injured colonoids were unable to re-form 3D colonoids. Interestingly, the control culture showed no morphologic difference in the presence of IWP-2 for 72 h (Figure 5B) and no significant changes

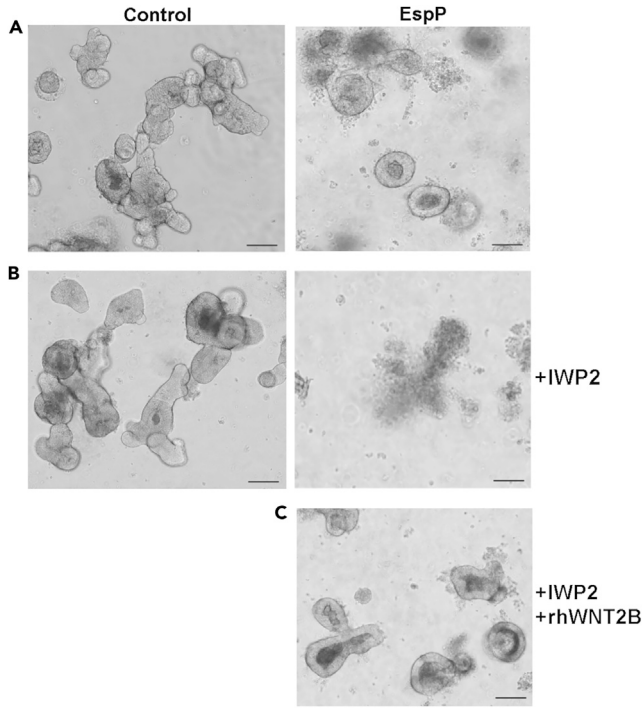

Figure 5. WNT2B Compensates for Inhibition of Epithelial wnts in Regenerating Colonoids

(A–C) Control (left) and EspP-treated (right) colonoids after washout, at 24-h regeneration. (A) Representative images of colonoids at 24-h regeneration. (B) Representative images of colonoids in the continued presence of IWP2 at 24-h regeneration. (C) Representative image of colonoids in the continued presence of IWP2 and recombinant human WNT2B (rhWNT2B) at 24-h regeneration; scale bar, 200 μm. N ≥ 4.

See also Figures S3 and S4.

in gene expression levels of *WNT2B1, 2, 3, LGR5, OLFM4,* or *BMI1* compared with untreated control (Figure S3). This suggests that Wnt3A-conditioned media is sufficient to maintain homeostatic growth and proliferation of colonoids, but is not sufficient for regeneration following EspP-induced injury. Inhibition of epithelial Wnt secretion (by IWP-2) prevents human colonoid regeneration, indicating that epithelial Wnt(s) are necessary for regeneration.

Inhibition of GSK3β stimulates Wnt/β-catenin (canonical) signaling and drives proliferation (Wu and Pan, 2010). EspP-treated regenerating colonoids show a slight upward, but not significant, trend in β-catenin expression (Figure S4). Addition of CHIR99021, a small molecule GSK3β inhibitor, did not lead to enhanced growth of colonoids and also did not significantly increase mRNA expression of *β-catenin* in the EspP-treated colonoids compared with the regenerating colonoids without CHIR99021. Although expression levels of both *WNT2B3* and *DHH* increase in the regenerating colonoids, addition of CHIR99021 led to a sharp decrease in mRNA expression of both genes. The putative stem cell marker, *LGR5*, does not change in the EspP-treated regenerating colonoids, but in the presence of CHIR99021, its expression increased (Figure S4). Thus, although GSK3β inhibition may drive proliferation with increased *LGR5* expression, it does not lead to an increase in *DHH* or *WNT2B3* expression in the EspP-treated regenerating colonoids.

The proteomics screen identified upregulation of WNT2B3 in the EspP-injured colonoids. We evaluated if WNT2B alone could stimulate regeneration. Recombinant human WNT2B (rhWNT2B) was added to colonoids at the same time as IWP-2 and kept in the cultures during the course of the experiment. Although IWP-2 inhibited colonoid regeneration, rhWNT2B was sufficient to rescue and promote regeneration after EspP-induced injury (Figure 5C). Addition of rhWNT2B alone to colonoids for 2–3 days, the length of the injury/regeneration experiment, had no effect on morphology or growth over that time period. To determine the direct effect of epithelial WNT2B on colonoid regeneration post EspP injury, we used a lentiviral

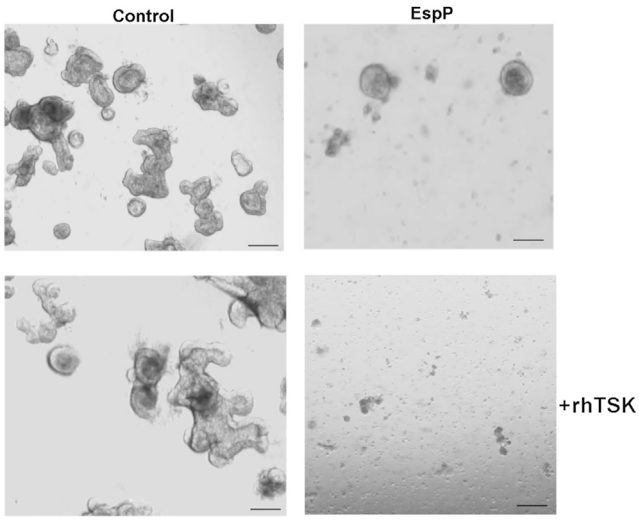

Figure 6. TSK Inhibits Colonoid Regeneration Post EspP-Treatment

Representative images of control (left) and EspP-treated (right) colonoids after washout, at 24-h regeneration. Control and EspP-treated colonoids were in the continued presence of recombinant human Tsukushi (rhTSK) (bottom panel); scale bar, 200 μm. N = 3.

See also Figure S5

short hairpin RNA (shRNA) approach to knockdown *WNT2B* in the colonoids. As a technical control, colonoids were transduced with a lentiviral scrambled shRNA. Puromycyin selection was started 2 days post-transduction. The scrambled colonoids thrived (Figure S5, top panel), but the colonoids with *WNT2B* shRNA sharply declined after selection and were unable to propagate (Figure S5, bottom panel). This result is consistent with the published study by O'Connell et al. (2018), in which the enteroids and colonoids derived from WNT2B-deficient individuals were not stable and unable to form robust cultures. They found that addition of recombinant murine WNT2B allowed for the formation of short-term enteroid cultures from these patients.

Studies in chick retinal explants found that Wnt2b overexpression leads to increased cell proliferation and the growth of large, folded retinal tissue (Ohta et al., 2011). However, co-overexpression of Wnt2b with the small, leucine-rich proteoglycan Tsukushi (Tsk) led to inhibition of the Wnt2b-dependent hyperproliferation. TSK functions as a competitive inhibitor against WNT2B by binding to the same receptor, Frizzled4 (Ohta et al., 2011). As we could not create a viable *WNT2B* KD line, we examined whether TSK could inhibit WNT2B function in colonoids. Colonoids were treated with recombinant human TSK (rhTSK). Similar to the presence of IWP-2, control colonoids showed no morphologic difference in the presence of rhTSK (Figure 6). However, the EspP-injured colonoids were unable to regenerate in the presence of rhTSK. Taken together, these data indicate that epithelial WNT2B is necessary for human colonoid regeneration after EspP-induced injury and is not functionally redundant with Wnt3A.

DHH-Activated Hedgehog Signaling Modulates WNT2B

The regenerating colonoids also had a significant upregulation of *DHH* and *GLI1* (Figure 3) suggesting an active role for hedgehog signaling following EspP-induced injury. To determine whether there was a link between Hedgehog signaling and WNT2B in regeneration, we treated colonoids with either Smoothened agonist (SAG) or recombinant human DHH (rhDHH) before EspP exposure. SAG binds to Smoothened and induces activation of the Hedgehog pathway (Chen et al., 2002). Its function is thought to be Hedgehog ligand-independent. DHH, as a Hedgehog ligand, also activates the Hedgehog pathway. Colonoids present 24 h after regeneration were collected and analyzed for gene expression of stem cell markers, WNT, and Hedghog pathway molecules. mRNA expression in the presence of the agonists was compared with control (no agonists). The intestinal stem cell markers *LGR4* and *LGR5* were further downregulated in the presence of SAG compared with control. However, both genes were upregulated in the presence of rhDHH compared with control (Figure 7). *BMI1* remained largely unchanged with SAG treatment, but was significantly upregulated in the EspP-injured, rhDHH-treated colonoids, similar

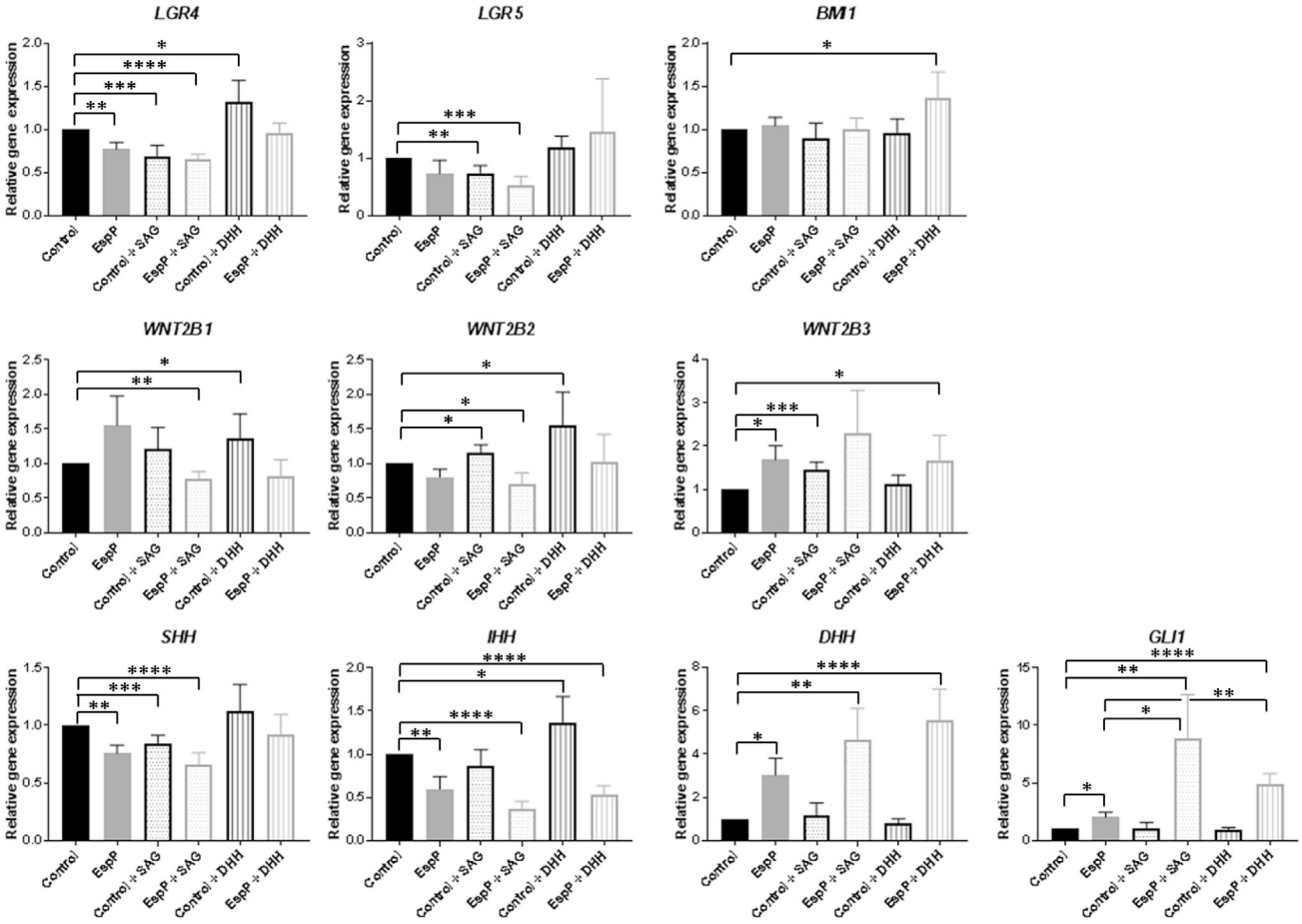

Figure 7. Hedgehog Agonists Upregulate WNT2B3 and DHH during Regeneration

Control and EspP-treated colonoids were treated with Smoothened agonist (SAG) or recombinant human Desert Hedgehog (DHH). Gene expression of regenerating colonoids was analyzed by qRT-PCR. Relative gene expression is shown as a ratio of treated (EspP and/or SAG or DHH) compared with control colonoids and normalized to 18S. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. N ≥ 3. Data are presented as mean ± SEM.

to the upregulation of *LGR4* and *LGR5*. This suggests that addition of recombinant DHH or SAG does not lead to the same mRNA changes. DHH-activated signaling has a direct effect on the intestinal stem cell markers.

SAG treatment significantly downregulated *WNT2B1* and *WNT2B2* in EspP-injured colonoids. In contrast, *WNT2B3* expression continued to trend upward. rhDHH treatment had no effect on *WNT2B1* and *WNT2B2* expression in EspP-injured colonoids. However, *WNT2B3* was significantly upregulated (Figure 7). This suggests that DHH positively modulates WNT2B3 expression during colonoid regeneration. Similar to the stem cell markers that we evaluated, SAG and rhDHH treatment leads to different expression patterns of the three *WNT2B* isoforms.

SAG treatment either significantly downregulated or had no effect on the expression of *SHH* and *IHH* in both control and EspP-injured colonoids, but significantly upregulated *DHH* and *GLI1* expression in EspP-injured colonoids. EspP-injured colonoids showed significant upregulation of *GLI1* and *DHH* in the presence of rhDHH, compared with control (Figure 7). These data show that both SAG (hedgehog activation) and rhDHH can modulate *WNT2B* expression, but only *WNT2B3* is upregulated in EspP-injured colonoids with these hedgehog agonists. DHH acts in a specific manner to activate hedgehog signaling following injury to the colonoids. Although SAG and rhDHH treatment similarly upregulated *DHH* and *GLI1* in EspP-injured colonoids, they had different effects on the expression levels of the other genes interrogated. Overall, these results show that human colonoids are dependent on WNT2B to regenerate

after bacterial cytotoxin-induced injury. DHH is upregulated during this process and possibly interacts with WNT2B in a yet unknown mechanism.

DISCUSSION

Intestinal regeneration is dependent on Wnt signaling to stimulate stem cell proliferation. Most studies have focused on the identity of the intestinal stem cells that drive proliferation and crypt hyperplasia in mouse models under both normal and post-injury conditions, particularly post-radiation (Hua et al., 2012, 2017; Kuruvilla et al., 2016; Metcalfe et al., 2014; Zhou et al., 2013). The regenerative pathways and key players in these pathways are not well understood. In this study, we focused on characterizing the molecules that drive a regenerative response following exposure to a virulence factor in a bacterial diarrheal disease: EspP, an EHEC-secreted bacterial cytotoxin that causes epithelial damage. Colonic regeneration is dependent on epithelial signals, namely, WNT2B and DHH. These two molecules activate Wnt and Hedgehog signaling interaction during colonic regeneration.

Using the human colonoid model, which contains no mesenchyme, we employed a proteomics screen to characterize the pathways that are active following EspP-induced injury. WNT2B and numerous proteins downstream of active Hedgehog signaling were upregulated, suggesting Wnt and Hedgehog signaling are important in colonoid regeneration. Both pathways have been implicated in organ development and maintenance (Clevers, 2006; Petrova and Joyner, 2014), with Hedgehog signaling described as important in regeneration of most organs (Adolphe, 2004; Konstantinou et al., 2016; Langiewicz et al., 2016; Le et al., 2008; Sriperumbudur et al., 2016; Trowbridge et al., 2006; Wang et al., 2016a, 2016b). Although recent studies have focused on the crosstalk between Wnt and Hedgehog signaling in cancer progression (Jiang et al., 2014; Regan et al., 2017; Song et al., 2015), these two pathways also have been implicated in regeneration of bladder epithelia, bone, and adrenal glands (Day and Yang, 2008; Finco et al., 2018; Shin et al., 2011).

Our results indicate that epithelia-produced WNT2B and DHH are important regulators of human colonoid regeneration, with DHH modulating *WNT2B3* expression following EspP-induced injury. Activation of this particular Hedgehog pathway is not redundant between the three mammalian Hedgehog ligands. Sonic and Indian Hedgehog transcripts were either downregulated or unchanged during regeneration. Most of our understanding of Hedgehog signaling focuses on Sonic Hedgehog, likely because it is the most widely expressed mammalian Hedgehog ligand (Varjosalo and Taipale, 2008). The implications of downregulated *SHH* in colonoid regeneration are not clear; however, previous studies have shown that *IHH* downregulation initiates intestinal wound healing and abrogates adenoma development (Büller et al., 2015; van Dop et al., 2010). Until now, DHH function has not been well understood. It is primarily described as an essential factor in gonad (O'Hara et al., 2011; Rothacker et al., 2018; Yao et al., 2002) or peripheral nerve development (Bajestan et al., 2006; Parmantier et al., 1999). However, one study demonstrated an essential role for DHH in corneal homeostasis and regeneration (Kucerova et al., 2012). Our results highlight a novel role for DHH-activated Hedgehog signaling in human colonic regeneration.

In human colonoids and colonic tissue, WNT2B is localized to a rare cell that is not present in every colonoid or crypt. The identity of this cell in human colonoids is currently unknown but under further investigation. Regeneration following cytotoxin-induced injury results in diffuse WNT2B staining with a higher number of WNT2B+ cells, similar to a study that showed upregulation of Wnt2b in mouse intestinal crypts post-irradiation (Suh et al., 2017). This correlates with the upregulation of *WNT2B3* mRNA in the regenerating colonoids. Although WNT2B has been characterized as having two isoforms in cancer cells (Katoh, 2001), three WNT2B isoforms have been identified in multiple mammalian cells and have been shown to function disparately from each other (Bunaciu et al., 2008). As our proteomics screen identified the WNT2B isoform 3, we used the primers described by Bunaciu et al. to distinguish between the WNT2B isoforms. The three isoforms were regulated differently during regeneration and in the presence of Hedgehog agonists, SAG and rhDHH.

Mesenchymal Wnts are clearly essential for regeneration (Gregorieff et al., 2005; Greicius et al., 2018; Koch, 2017; Shoshkes-Carmel et al., 2018; Valenta et al., 2016), but only a few studies have highlighted the importance of epithelial Wnts in intestinal development and injury response (O'Connell et al., 2018; Suh et al., 2017; Zou et al., 2018). Importantly, a recent study found that patients with a homozygous nonsense *WNT2B* mutation experience neonatal-onset chronic diarrhea, with inflammation seen in the stomach,

duodenum, and colon (O'Connell et al., 2018). The *WNT2B* mutation was associated with loss of crypt architecture and decrease in stem cell numbers in these patients. In addition, enteroids from the *WNT2B*-deficient patients could not form stable cultures, although addition of recombinant murine Wnt2b stabilized the cultures for a short period. These results place WNT2B in an essential role for intestinal development and also emphasizes the significant differences between the mouse and human intestinal epithelium. Knockout or knockdown of *Wnt2b* in the whole mouse or mouse organoids, respectively, results in no detrimental phenotype. However, human intestinal epithelial WNT2B is indispensable in intestinal development and regeneration following injury. Taken together, our studies indicate that data gained on mouse models of intestinal development, homeostasis, and injury may not directly translate to human intestinal physiology and pathophysiology.

In summary, using the bacterial cytotoxin EspP to model damage, we showed that human colonoids can be used to study the role of epithelia in regeneration. Epithelial WNT2B and Desert Hedgehog likely interact and play a significant role during regeneration following EspP-induced injury. Importantly, the hedgehog ligands, Desert, Indian, and Sonic, are not redundant in human colonic regeneration. Understanding the mechanisms that specifically drive WNT2B3 and DHH in colonic development and regeneration may provide the basis for useful therapeutics in controlled regeneration in patients with colonic diseases.

Limitations of the Study

Our study uses a model that consists of only epithelia. It would be strengthened by showing the effects of WNT2B and/or DHH on intestinal regeneration in a fuller intestinal model, such as those derived from iPS cells, which contain mesenchyme and immune components. In addition, direct inhibitors or colonoids stably expressing knockdowns of either gene would strengthen the results presented here.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Julie In (jgin@salud.unm.edu).

Materials Availability

All unique reagents generated in this study are available from the Lead Contact without restriction.

Data and Code Availability

The proteome dataset was deposited into figshare under the https://doi.org/10.6084/m9.figshare.12964619.

These data were previously published as a preprint on bioRxiv https://doi.org/10.1101/434639.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101618.

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Conflict of Interest statement: All authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

J.G.I., M. Donowitz, N.C.Z., and O.K. conceived of and designed the experiments. J.G.I., J.Y., R.A., M. Doucet, R.N.C., and L.D. performed the experiments. J.G.I., J.Y., M. Donowitz, N.C.Z., S.E.B., M.K.E., and O.K.

analyzed the data. J.G.I. wrote the manuscript with critical edits from J.Y., M. Donowitz, N.C.Z., S.E.B., M.K.E., and O.K. All authors reviewed the final version.

DECLARATION OF INTERESTS

All authors have declared no conflict of interest.

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REFERENCES

Adolphe, C. (2004). An in vivo comparative study of sonic, desert and Indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis. Development *131*, 5009–5019.

Bajestan, S.N., Umehara, F., Shirahama, Y., Itoh, K., Sharghi-Namini, S., Jessen, K.R., Mirsky, R., and Osame, M. (2006). Desert hedgehog-patched 2 expression in peripheral nerves during Wallerian degeneration and regeneration. J. Neurobiol. *66*, 243–255.

Beumer, J., and Clevers, H. (2016). Regulation and plasticity of intestinal stem cells during homeostasis and regeneration. Development *143*, 3639–3649.

Büller, N.V.J.A., Rosekrans, S.L., Metcalfe, C., Heijmans, J., van Dop, W.A., Fessler, E., Jansen, M., Ahn, C., Vermeulen, J.L.M., Westendorp, B.F., et al. (2015). Stromal Indian hedgehog signaling is required for intestinal adenoma formation in mice. Gastroenterology *148*, 170–180.e176.

Bunaciu, R.P., Tang, T., and Mao, C.D. (2008). Differential expression of Wnt13 isoforms during leukemic cell differentiation. Oncol. Rep. 195–201.

Chen, J.K., Taipale, J., Cooper, M.K., and Beachy, P.A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Development *16*, 2743–2748.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469–480.

Dautin, N. (2010). Serine protease autotransporters of Enterobacteriaceae (SPATEs): biogenesis and function. Toxins *2*, 1179–1206.

Day, T.F., and Yang, Y. (2008). Wnt and hedgehog signaling pathways in bone development. J. Bone Joint Surg Am *90*, 19–24.

Farin, H.F., Jordens, I., Mosa, M.H., Basak, O., Korving, J., Tauriello, D.V.F., de Punder, K., Angers, S., Peters, P.J., Maurice, M.M., et al. (2016). Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. Nature *530*, 340–343.

Farin, H.F., Van Es, J.H., and Clevers, H. (2012). Redundant sources of wnt regulate intestinal stem cells and promote formation of paneth cells. Gastroenterology *143*, 1518–1529.e1517.

Finco, I., Lerario, A.M., and Hammer, G.D. (2018). Sonic hedgehog and WNT signaling promote adrenal gland regeneration in male mice. Endocrinology *159*, 579–596.

Gregorieff, A., Pinto, D., Begthel, H., Destree, O., Kielman, M., and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology *129*, 626–638.

Greicius, G., Kabiri, Z., Sigmundsson, K., Liang, C., Bunte, R., Singh, M.K., and Virshup, D.M. (2018). PDGFRalpha(+) pericryptal stromal cells are the critical source of Wnts and RSPO3 for murine intestinal stem cells in vivo. Proc. Natl. Acad. Sci. U S A *115*, E3173–E3181.

Henderson, I.R., Hicks, S., Navarro-Garcia, F., Elias, W.P., Philips, A.D., and Nataro, J.P. (1999). Involvement of the enteroaggregative *Escherichia coli* plasmid-encoded toxin in causing human intestinal damage. Infect. Immun. *67*, 5338–5344.

Hua, G., Thin, T.H., Feldman, R., Haimovitz–Friedman, A., Clevers, H., Fuks, Z., and Kolesnick, R. (2012). Crypt base columnar stem cells in small intestines of mice are radioresistant. Gastroenterology *143*, 1266–1276.

Hua, G., Wang, C., Pan, Y., Zeng, Z., Lee, S.G., Martin, M.L., Haimovitz-Friedman, A., Fuks, Z., Paty, P.B., and Kolesnick, R. (2017). Distinct levels of radioresistance in Lgr5+Colonic epithelial stem cells versus Lgr5+Small intestinal stem cells. Cancer Res. *77*, 2124–2133.

In, J., Lukyanenko, V., Foulke-Abel, J., Hubbard, A.L., Delannoy, M., Hansen, A.-M., Kaper, J.B., Boisen, N., Nataro, J.P., Zhu, C., et al. (2013). Serine protease EspP from enterohemorrhagic Escherichia coli is sufficient to induce shiga toxin macropinocytosis in intestinal epithelium. PLoS One *8*, e69196.

Jiang, H., Li, F., He, C., Wang, X., Li, Q., and Gao, H. (2014). Expression of Gli1 and Wnt2B correlates with progression and clinical outcome of pancreatic cancer. Int. J. Clin. Exp. Pathol. *7*, 4531–4538.

Jiang, H., Tian, A., and Jiang, J. (2016). Intestinal stem cell response to injury: lessons from Drosophila. Cell Mol. Life Sci. *73*, 3337–3349.

Katoh, M. (2001). Differential regulation of WNT2 and WNT2B expression in human cancer. Int. J. Mol. Med. *8*, 657–660.

Khan, M.A., Ma, C., Knodler, L.A., Valdez, Y., Rosenberger, C.M., Deng, W., Finlay, B.B., and Vallance, B.A. (2006). Toll-like receptor 4 contributes to colitis development but not to host defense during Citrobacter rodentium infection in mice. Infect Immun. *74*, 2522–2536.

Khan, S., Mian, H.S., Sandercock, L.E., Chirgadze, N.Y., and Pai, E.F. (2011). Crystal structure of the passenger domain of the Escherichia coli autotransporter EspP. J. Mol. Biol. *413*, 985–1000.

Koch, S. (2017). Extrinsic control of Wnt signaling in the intestine. Differentiation *97*, 1–8.

Konstantinou, D., Bertaux-Skeirik, N., and Zavros, Y. (2016). Hedgehog signaling in the stomach. Curr. Opin. Pharmacol. *31*, 76–82.

Kucerova, R., Dora, N., Mort, R.L., Wallace, K., Leiper, L.J., Lowes, C., Neves, C., Walczysko, P., Bruce, F., Fowler, P.A., et al. (2012). Interaction between hedgehog signalling and PAX6 dosage mediates maintenance and regeneration of the corneal epithelium. Mol. Vis. *18*, 139–150.

Kuruvilla, Jes G., Kim, C.-K., Ghaleb, Amr M., Bialkowska, Agnieszka B., Kuo, Calvin J., and Yang, Vincent W. (2016). Krüppel-like factor 4 modulates development of BMI1+ intestinal stem cell-derived lineage following γ-radiation-induced gut injury in mice. Stem Cell Rep. *6*, 815–824.

Langiewicz, M., Schlegel, A., Saponara, E., Linecker, M., Borger, P., Graf, R., Humar, B., and Clavien, P.A. (2016). Hedgehog pathway mediates early acceleration of liver regeneration induced by a novel two-staged hepatectomy in mice. J. Hepatol. *66*, 560–570.

Le, H., Kleinerman, R., Lerman, O.Z., Brown, D., Galiano, R., Gurtner, G.C., Warren, S.M., Levine, J.P., and Saadeh, P.B. (2008). Hedgehog signaling is essential for normal wound healing. Wound Repair Regen. *16*, 768–773.

Mazumdar, T., DeVecchio, J., Shi, T., Jones, J., Agyeman, A., and Houghton, J.A. (2011). Hedgehog signaling drives cellular survival in human colon carcinoma cells. Cancer Res. *71*, 1092–1102.

Mellies, J.L., Navarro-Garcia, F., Okeke, I., Frederickson, J., Nataro, J.P., and Kaper, J.B. (2001). espC pathogenicity island of enteropathogenic Escherichia coli encodes an enterotoxin. Infect Immun. *69*, 315–324.

Metcalfe, C., Kljavin, Noelyn M., Ybarra, R., de Sauvage, and Frederic, J. (2014). Lgr5+ stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell *14*, 149–159.

O'Hara, W.A., Azar, W.J., Behringer, R.R., Renfree, M.B., and Pask, A.J. (2011). *Desert hedgehog* is a mammal-specific gene expressing during testicular and ovarian development in a marsupial. BMC Developmental Biol. *11*, 72.

O'Connell, A.E., Zhou, F., Shah, M.S., Murphy, Q., Rickner, H., Kelsen, J., Boyle, J., Doyle, J.J., Gangwani, B., Thiagarajah, J.R., et al. (2018). Neonatal-onset chronic diarrhea caused by homozygous nonsense WNT2B mutations. Am. J. Hum. Genet. *103*, 131–137.

Ohta, K., Ito, A., Kuriyama, S., Lupo, G., Kosaka, M., Ohnuma, S.-i., Nakagawa, S., and Tanaka, H. (2011). Tsukushi functions as a Wnt signaling inhibitor by competing with Wnt2b for binding to transmembrane protein Frizzled4. Proc. Natl. Acad. Sci. U S A *108*, 14962–14967.

Parmantier, E., Lynn, B., Lawson, D., Turmaine, M., Namini, S.S., Chakrabarti, L., McMahon, A.P., Jessen, K.R., and Mirsky, R. (1999). Schwann cell-derived Desert hedgehog controls the development of peripheral nerve sheaths. Neuron *23*, 713–724.

Petrova, R., and Joyner, A.L. (2014). Roles for Hedgehog signaling in adult organ homeostasis and repair. Development *141*, 3445–3457.

Regan, J.L., Schumacher, D., Staudte, S., Steffen, A., Haybaeck, J., Keilholz, U., Schweiger, C., Golob-Schwarzl, N., Mumberg, D., Henderson, D., et al. (2017). Non-canonical hedgehog signaling is a positive regulator of the WNT pathway and is required for the survival of colon cancer stem cells. Cell Rep. *21*, 2813–2828.

Ritsma, L., Ellenbroek, S.I.J., Zomer, A., Snippert, H.J., de Sauvage, F.J., Simons, B.D., Clevers, H., and van Rheenen, J. (2014). Intestinal crypt homeostasis revealed at single-stem-cell level by in vivo live imaging. Nature *507*, 362–365.

Rothacker, K.M., Ayers, K.L., Tang, D., Joshi, K., van den Bergen, J.A., Robevska, G., Samnakay, N., Nagarajan, L., Francis, K., Sinclair, A.H., et al. (2018). A novel, homozygous mutation in desert hedgehog (DHH) in a 46, XY patient with dysgenetic testes presenting with primary amenorrhoea: a case report. Int. J. Pediatr. Endocrinol. *2018*, 2.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G., Van Es, J.H., Van den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D., et al. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology *141*, 1762–1772.

Shin, K., Lee, J., Guo, N., Kim, J., Lim, A., Qu, L., Mysorekar, I.U., and Beachy, P.A. (2011). Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. Nature *472*, 110–114.

Shoshkes-Carmel, M., Wang, Y.J., Wangensteen, K.J., Toth, B., Kondo, A., Massassa, E.E., Itzkovitz, S., and Kaestner, K.H. (2018). Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. Nature *557*, 242–246.

Song, L., Li, Z.-Y., Liu, W.-P., and Zhao, M.-R. (2015). Crosstalk between Wnt/β-catenin and Hedgehog/Gli signaling pathways in colon cancer and implications for therapy. Cancer Biol. Ther. *16*, 1–7.

Sriperumbudur, A., Breitzig, M., Lockey, R., and Kolliputi, N. (2016). Hedgehog: the key to maintaining adult lung repair and regeneration. J. Cell Commun. Signal. *11*, 95–96.

Suh, H.N., Kim, M.J., Jung, Y.-S., Lien, E.M., Jun, S., and Park, J.-I. (2017). Quiescence exit of tert + stem cells by Wnt/β-catenin is indispensable for intestinal regeneration. Cell Rep. *21*, 2571–2584.

Tian, A., Shi, Q., Jiang, A., Li, S., Wang, B., and Jiang, J. (2015). Injury-stimulated Hedgehog signaling promotes regenerative proliferation of Drosophila intestinal stem cells. J. Cell Biol. *208*, 807–819.

Trowbridge, J.J., Scott, M.P., and Bhatia, M. (2006). Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. Proc. Natl. Acad. Sci. *103*, 14134–14139.

Valenta, T., Degirmenci, B., Moor, Andreas E., Herr, P., Zimmerli, D., Moor, Matthias B., Hausmann, G., Cantù, C., Aguet, M., and Basler, K. (2016). Wnt ligands secreted by subepithelial mesenchymal cells are essential for the survival of intestinal stem cells and gut homeostasis. Cell Rep. *15*, 911–918.

Vallance, B.A., Deng, W., Jacobson, K., and Finlay, B.B. (2003). Host susceptibility to the attaching and effacing bacterial pathogen Citrobacter rodentium. Infect. Immun. *71*, 3443–3453.

Van den Brink, G.R. (2007). Hedgehog signaling in development and homeostasis of the gastrointestinal tract. Physiol. Rev. *87*, 1343–1375.

van den Brink, G.R., Bleuming, S.A., Hardwick, J.C.H., Schepman, B.L., Offerhaus, G.J., Keller, J.J., Nielsen, C., Gaffield, W., van Deventer, S.J.H., Roberts, D.J., et al. (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat. Genet. *36*, 277–282.

van Dop, W.A., Heijmans, J., Büller, N.V.J.A., Snoek, S.A., Rosekrans, S.L., Wassenberg, E.A., van den Bergh Weerman, M.A., Lanske, B., Clarke, A.R., Winton, D.J., et al. (2010). Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine. Gastroenterology *139*, 1665–1676.e1610.

Varjosalo, M., and Taipale, J. (2008). Hedgehog: functions and mechanisms. Genes Development *22*, 2454–2472.

Wang, Y., Lu, P., Zhao, D., and Sheng, J. (2016a). Targeting the Hedgehog Signaling Pathway for Cardiac Repair and Regeneration (Herz).

Wang, Z., Li, W., Li, C., Yang, Y., Li, W., Zhang, L., Sun, S., Li, J., and Cai, Y. (2016b). Small hepatocyte-like progenitor cells may be a Hedgehog signaling pathway-controlled subgroup of liver stem cells. Exp. Ther. Med. *12*, 2423–2430.

Weiss, A., and Brockmeyer, J. (2012). Prevalence, biogenesis, and functionality of the serine protease autotransporter EspP. Toxins *5*, 25–48.

Wu, D., and Pan, W. (2010). GSK3: a multifaceted kinase in Wnt signaling. Trends Biochem. Sci. *35*, 161–168.

Xicohtencatl-Cortes, J., Monteiro-Neto, V., Ledesma, M.A., Jordan, D.M., Francetic, O., Kaper, J.B., Puente, J.L., and Girón, J.A. (2007). Intestinal adherence associated with type IV pili of enterohemorrhagic Escherichia coli O157:H7. J. Clin. Invest. *117*, 3519–3529.

Yao, H.H.-C., Whoriskey, W., and Capel, B. (2002). Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Development *16*, 1433–1440.

Zhang, L., Song, R., Gu, D., Zhang, X., Yu, B., Liu, B., and Xie, J. (2017). The role of GLI1 for 5-Fu resistance in colorectal cancer. Cell Biosci *7*, 17.

Zhou, W.-J., Geng, Z.H., Spence, J.R., and Geng, J.-G. (2013). Induction of intestinal stem cells by R-spondin 1 and Slit2 augments chemoradioprotection. Nature *501*, 107–111.

Zou, W.Y., Blutt, S.E., Zeng, X.-L., Chen, M.-S., Lo, Y.-H., Castillo-Azofeifa, D., Klein, O.D., Shroyer, N.F., Donowitz, M., and Estes, M.K. (2018). Epithelial WNT ligands are essential drivers of intestinal stem cell activation. Cell Rep. *22*, 1003–1015.

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Supplemental Information

Epithelial WNT2B and Desert Hedgehog Are Necessary for Human Colonoid Regeneration after Bacterial Cytotoxin Injury

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Figure S1

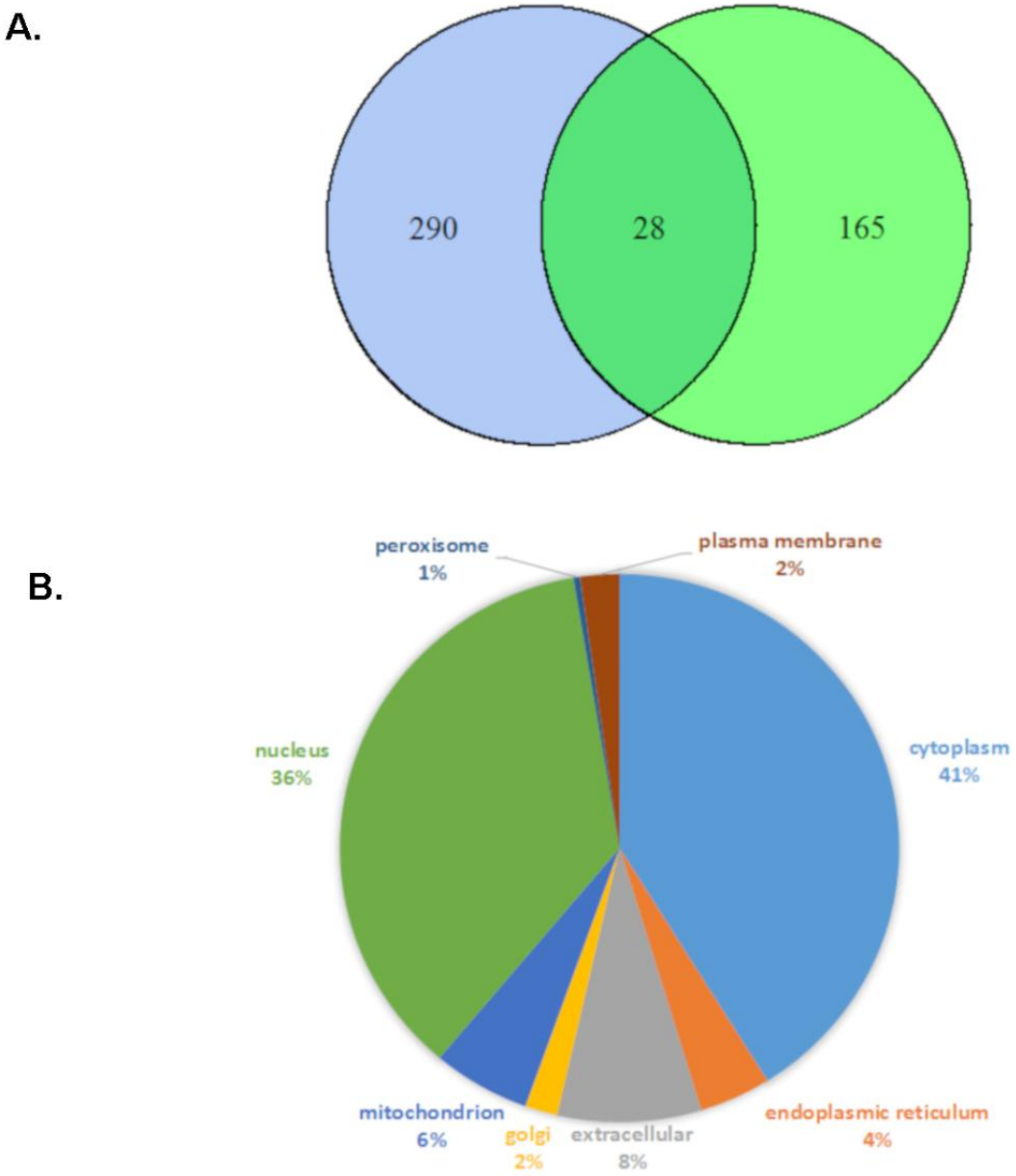

Figure S1. Proteomics analysis of differentially expressed proteins after EspP or EspP 263A treatment compared to control, Related to Table 1

(A) The Venn diagram depicts the number of differentially expressed proteins in the EspP S263A-treated (blue circle) and the EspP-treated (green circle) colonoids compared to control. Note the minimal overlap between the two treatments.

(B) The distribution of subcellular localization of differentially expressed proteins in the EspP-treated compared to control colonoids.

Figure S2

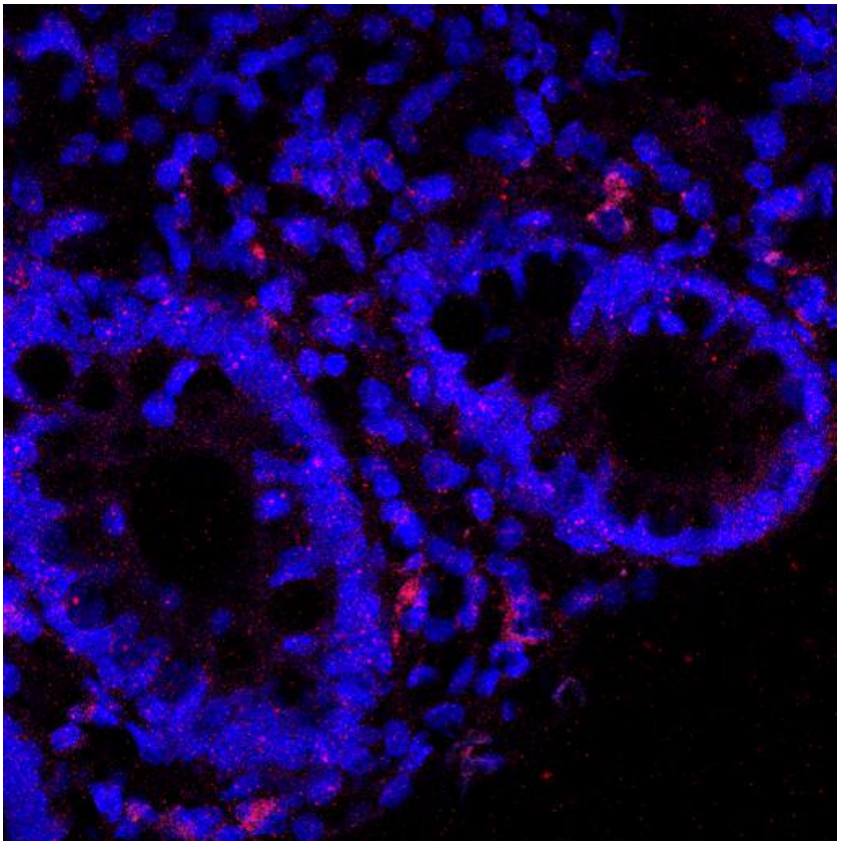

Figure S2. WNT2B is present in specific cells in the lamina propria, Related to Figure 4

RNAscope staining of WNT2B in human colonic tissue shows WNT2B in the lamina propria. Note that this is a higher longitudinal cut of the tissue and not in the deep crypt. WNT2B, red; nuclei blue.

Figure S3

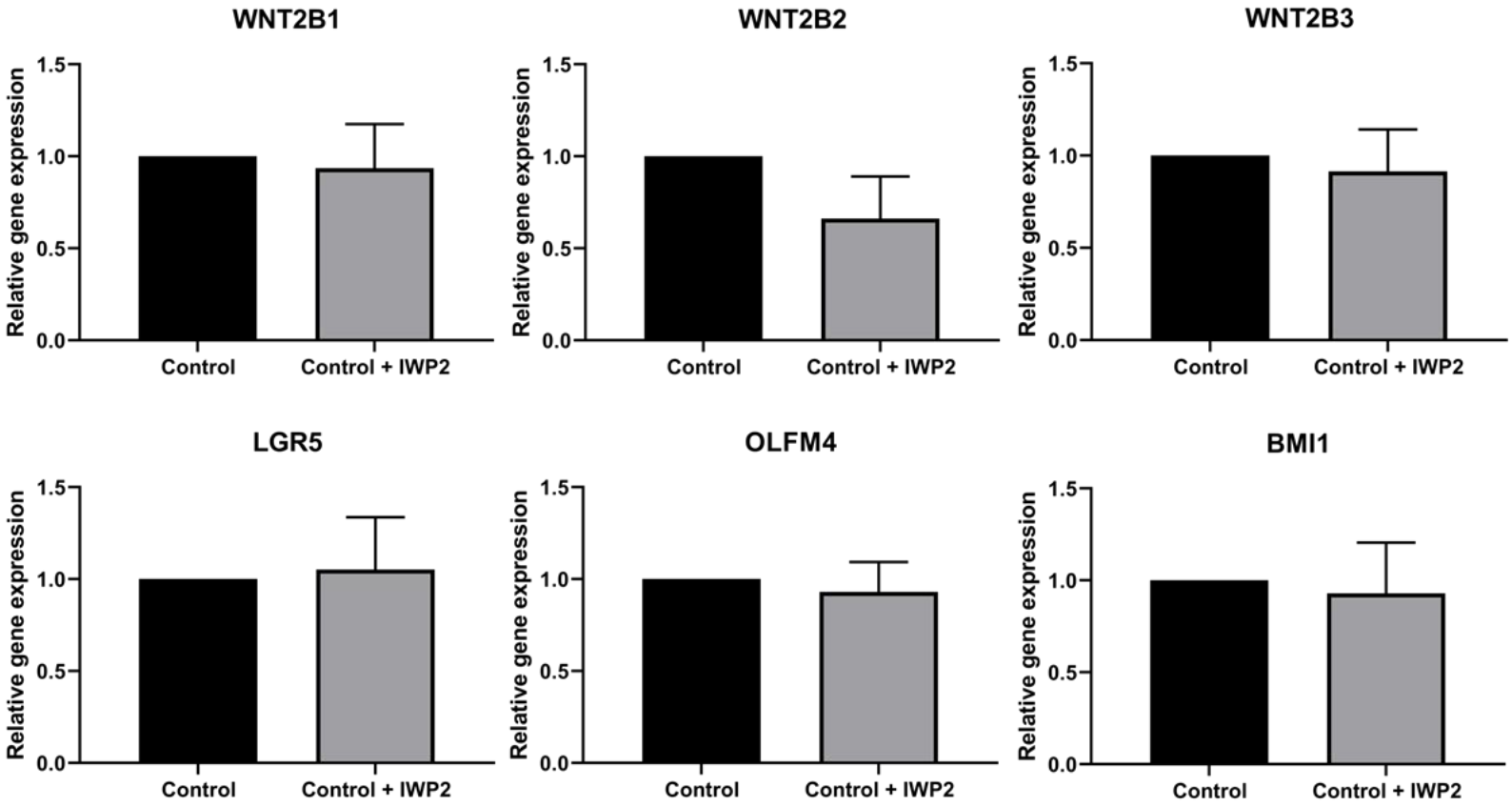

Figure S3. IWP2 treatment does not change gene expression of select genes in normal colonoids, Related to Figure 5

Control colonoids in the presence (grey bars) or absence (black bars) of IWP2 for approximately 72h, or the total length of an injury-regeneration experiment. Gene expression of selected stem cell-related genes and WNT2B was analyzed by qRT-PCR. Relative gene expression is shown as a ratio of IWP2 treated compared to control (untreated) colonoids, and normalized to 18S. N≥3. Data are presented as mean ± SEM.

Figure S4

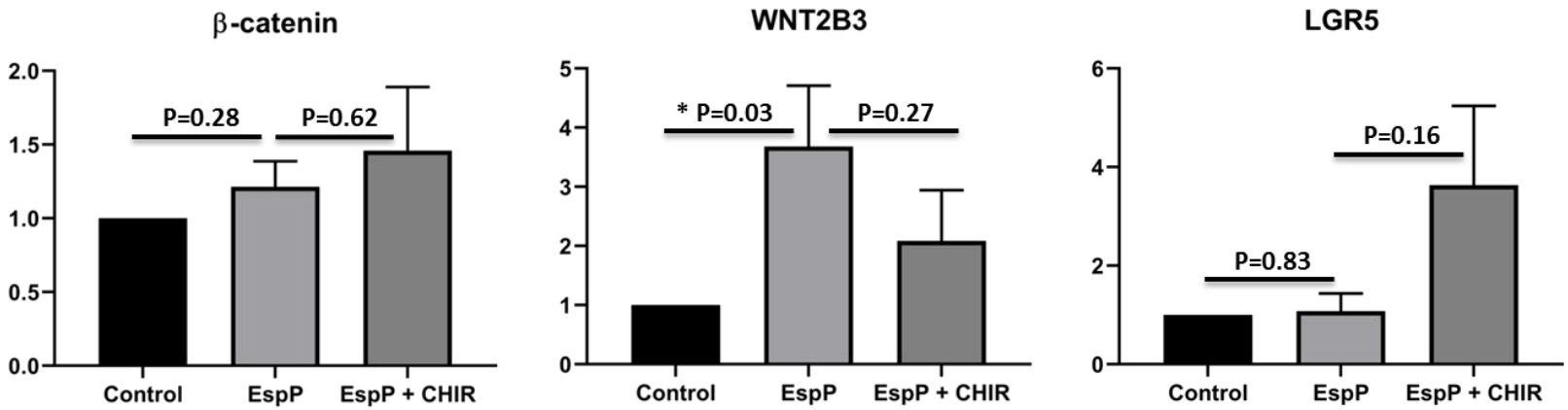

Figure S4. GSK3β inhibition does not increase WNT2B or DHH expression during regeneration, Related to Figure 5

Gene expression of colonoids in the presence or absence of the GSK3β inhibitor, CHIR99021 (10μM), for approximately 72h, or the total length of an injury-regeneration experiment, was analyzed by qRT-PCR. Relative gene expression is shown as a ratio of CHIR/EspP or EspP-treated compared to control colonoids, and normalized to 18S. $N \geq 3$ * $p \leq 0.05$.

Data are presented as mean ± SEM.

Figure S5

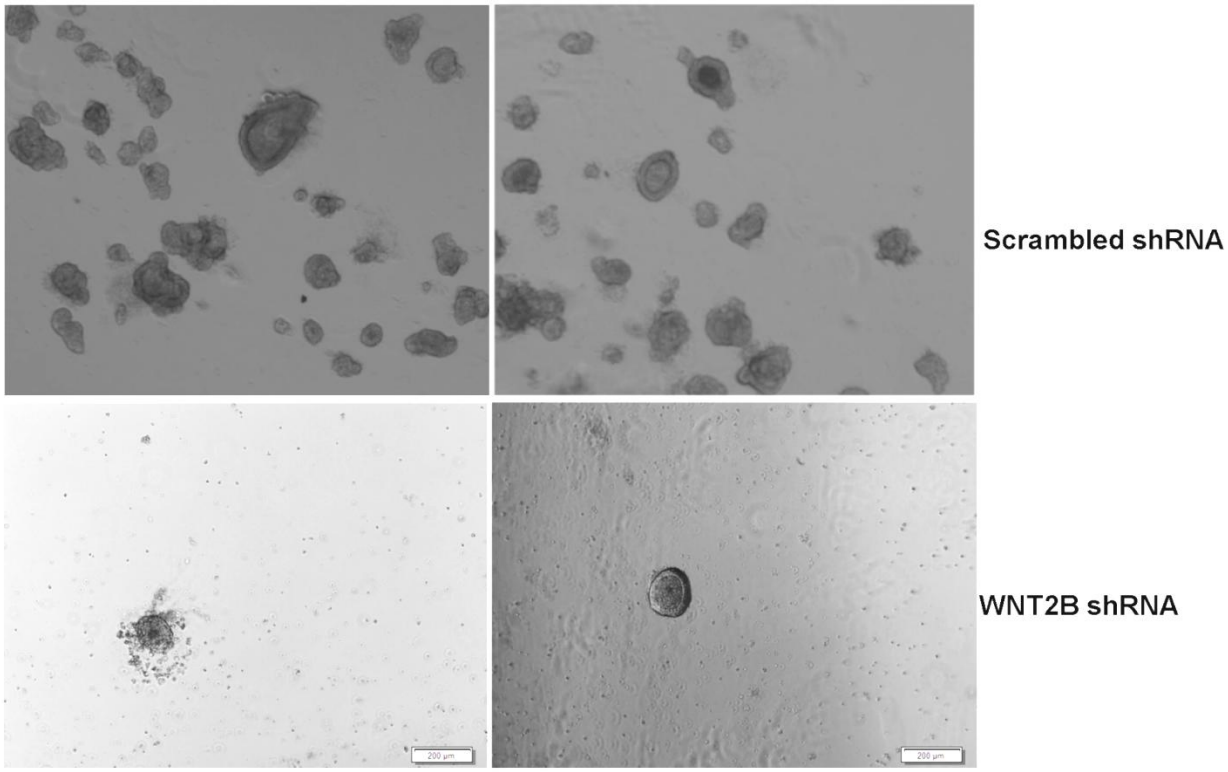

Figure S5. Knockdown of WNT2B results in non-viable colonoids, Related to Figure 6
Scrambled shRNA (top panel) and WNT2B shRNA (bottom panel) was introduced into colonoids via lentivirus transduction. Images were taken 7-17 days post-transduction, showing healthy scrambled colonoids but lackluster WNT2B KD colonoids.

Supplemental Table 1

Number	Patient pathology	Colonic segment	Age	Gender
1	Normal, routine screening	sigmoid	53	M
2	Normal, routine screening	distal	66	M
3	Normal, routine screening	transverse	50	M
4	Normal, routine screening	proximal	56	F
5	Normal, routine screening	proximal	58	M
6	Normal, routine screening	proximal	50	M
7	Normal, routine screening	proximal	67	F

Table S1. Description of colonoid donors used, Related to Methods: Tissue collection and colonoid generation

Supplemental Table 2

Reagent	Supplier	Catalog number	Concentration
IWP-2	MilliporeSigma	10536	2.5 µM
Propidium Iodide	MilliporeSigma	P4864	3 µM
Recombinant human Desert Hedgehog (rhDHH)	R&D systems	4777-DH	2 µg/ml
Recombinant human Tsukushi (rhTSK)	R&D systems	3940-TS	2 µg/ml
Recombinant human WNT2B (rhWNT2B)	MyBioSource	MBS1352751	1 µg/ml
Smoothened Agonist (SAG)	MilliporeSigma	566661	500 nM
Hs-DHH	ACDBio	520141	--
Hs-WNT2B	ACDBio	453361-C2	--

Table S2. Detailed experimental regeants, Related to Methods: EspP treatment and colonoid regeneration

Supplemental Table 3

Gene Name	Forward Primer	Reverse Primer
AXIN1	GACCTGGGGTATGAGCCTGA	GGCTTATCCCATCTTGGTCATC
AXIN2	GCGCTGACGGATGATTCCAT	TGGGTTCTCGGGAAATGAGGT
BMI1	CCATTGAATTCTTTGACCAGAA	CTGCTGGGCATCGTAAGTATC
CTNNB1	TCTGAGGACAAGCCACAAGATTACA	TGGGCACCAATATCAAGTCCAA
DHH	CGAGCGTTGTAAGGAGCGG	CCCTCAGTCACTCGTAGGC
GLI1	AACGCTATACAGATCCTAGCTCG	GTGCCGTTTGGTCACATGG
GLI2	CCCCTACCGATTGACATGCG	GAAAGCCGGATCAAGGAGATG
IHH	TGCATTGCTCCGTCAAGTC	CCACTCTCCAGGCGTACCT
LGR4	GATAACAGCCTCCAGGACCA	TTCAAGAGTGCTTGTGACATTTG
LGR5	ACCAGACTATGCCTTTGGAAAC	TTCCCAGGGAGTGGATTCTAT
SHH	GCTTCGACTGGGTGTACTACG	GCCACCGAGTTCTCTGCT
WNT2B-1	GATCCTTGAGGACGGCAGTA	GCATGATGTCTGGGTAACGC
WNT2B-2	CGTAGACACGTCCTGGTGGTA	GCATGATGTCTGGGTAACGC
WNT2B-3	CTAAAAGTACATTGGGGCAC	GCATGATGTCTGGGTAACGC
18S	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC

Table S3. Primer sequences used in this study, Related to Methods: RNA isolation and gene expression analysis

Transparent Methods

Tissue collection and colonoid generation. Human colonoid studies were reviewed and approved by the Johns Hopkins University School of Medicine Institutional Review Board (IRB# NA_00038329). Colonic biopsies were obtained from healthy individuals undergoing screening colonoscopies who had given informed written consent. The colonoids used in this study are detailed in Supplemental Table 1. Colonic crypt isolation and colonoid generation were prepared as previously reported (In et al., 2016; Jung et al., 2011). Briefly, biopsy tissue was minced, washed several times in freshly prepared cold chelating solution (CCS; 5.6mM Na2HPO4, 8mM KH2PO4, 96.2mM NaCl, 1.6mM KCl, 43.4mM sucrose, 54.9mM D-sorbitol, and 0.5mM DL-dithiothreitol) and incubated 1 hour at 4°C in 10 mM EDTA in CCS on an orbital shaker. Isolated crypts were resuspended in Matrigel (Corning, Tewksbury, MA) and 30 ul droplets were plated in a 24-well plate (Corning). After polymerization at 37°C, 500 ul of expansion media (EM) was added for 2 days (Advanced Dulbecco's modified Eagle medium/Ham's F-12 (ThermoFisher, Waltham, MA), 100 U/mL penicillin/streptomycin (Quality Biological, Gaithersburg, MD), 10 mM HEPES (ThermoFisher), and 1X GlutaMAX (ThermoFisher), with 50% v/v WNT3A conditioned medium (ATCC CRL-2647), 15% v/v R-spondin1 conditioned medium (cell line kindly provided by Calvin Kuo, Stanford University), 10% v/v Noggin conditioned medium (cell line kindly provided by Gijs van den Brink, Tytgat Institute for Liver and Intestinal Research), 1X B27 supplement (ThermoFisher), 1mM N-acetylcysteine (MilliporeSigma), 50 ng/mL human epidermal growth factor (ThermoFisher), 10 nM [Leu-15] gastrin (AnaSpec, Fremont, CA), 500 nM A83-01 (Tocris, Bristol, United Kingdom), 10 μM SB202190 (MilliporeSigma), 100 mg/mL primocin (InvivoGen, San Diego, CA), 10 μM CHIR99021 (Tocris), and 10 μM Y-27632 (Tocris)). After 2 days, the EM (without CHIR99021 and Y-27632) was replaced every other day. Colonoids were passaged every 7 days

by harvesting in Cultrex Organoid Harvesting Solution (Trevigen, Gaithersburg, MD) at 4°C with shaking for 30'. Colonoids were fragmented by trituration with a P200 pipet 30-50 times, collected and diluted in Advanced DMEM/F12, centrifuged at 300 xg for 10' at 4°C. The pellet was resuspended in Matrigel and plated as described for crypt isolation. All colonoid cultures were maintained at 37°C and 5% CO_2. Unless noted, colonoid lines have been passaged >20 times.

Recombinant EspP generation and collection. AD202 cells transformed with the plasmid encoding wild-type EspP (pRLS5) and serine protease-deficient mutant EspP S263A was kindly provided by H. Bernstein, NIH (Szabady et al., 2004). The cells were grown at 37°C in Luria-Bertani (LB) broth (ThermoFisher) overnight. They were then pelleted, washed, and grown at 37°C in fresh LB broth for approximately 15'. IPTG (100 μM) was added to induce *espP* or *espP S263A* expression. The culture was grown until reaching an OD_{550} 2.0. Bacterial cells were removed by centrifugation (9000 rpm, 30', 4°C, Sorvall RC6, SLA-3000 rotor). EspP and EspP S263A was collected from the cell-free supernatant by ammonium sulfate precipitation (60%, o/n, 4°C), followed by centrifugation (9000 rpm, 30', 4°C, Sorvall RC6, SLA-3000 rotor). The pellet was resuspended in PBS, syringe filtered (0.2 μm), then diluted with 15% glycerol to allow for freezing. Each batch of recombinant EspP and EspP S263A was separated on SDS-PAGE and stained with Coomassie Blue to check purity. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Serine protease activity was determined by pepsin A-cleavage assay (Brockmeyer et al., 2007).

EspP treatment and colonoid regeneration. All experiments described in this study used undifferentiated colonoids (grown in high WNT3A conditions). Colonoids were plated in Matrigel in 24 well plates and separated into experimental conditions (control, EspP treatment, EspP plus inhibitors or agonists). The control colonoids were also treated with vehicle of the tested inhibitor or agonist. EspP and EspP S263A treatment was 50 μg of the recombinant protein. Since the mechanics of passaging colonoids includes fragmenting their 3D structure and therefore causing injury, we attempted to minimize this by not triturating the colonoids, but instead, harvesting them without fragmentation and replating into new Matrigel. Colonoids were pre-treated with inhibitors or agonists at least 8h prior to overnight EspP treatment. After overnight treatment, colonoids were harvested in Cultrex Organoid Harvesting Solution, washed twice in Advanced DMEM/F12, and pelleted at 300 xg for 10' at 4°C, and replated in Matrigel for 24h regeneration. After replating, colonoids were kept in the presence of any inhibitors or agonists using during the experiment. After the 24h regeneration period, colonoids were imaged or processed for further studies. All experimental reagents used are detailed in Supplemental Table 2.

Brightfield imaging. Colonoids plated in Matrigel in 24 well plates were imaged during the course of experiments on a Zeiss Axio Observer A1 inverted microscope (Zeiss, Oberkochen, Germany) with images captured on CellSense imaging software (Olympus, Tokyo, Japan). Images were viewed and processed using OlyVia (Olympus).

Immunofluorescence, RNAscope and confocal imaging. Fixed tissues were frozen in OCT and sectioned (10 μm thick). Colonoids were harvested from Matrigel using Cultrex Organoid

Harvesting Solution. They were pelleted (300 xg, 10', 4°C), and fixed for 40 min in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Both fixed tissue and colonoids were permeabilized and blocked simultaneously for 1h using 10% Fetal Bovine Serum (Atlanta Biologicals, Flowery Branch, GA), 0.1% saponin (MilliporeSigma) solution prepared in PBS. After three PBS washes, 100 µl of primary antibody against WNT2B (HPA060696, MilliporeSigma) prepared at 1:100 dilution in PBS was added to the cells and incubated overnight at 4°C. Afterwards, cells were washed 3 times with PBS, and 100 µl of AlexaFluor secondary antibodies, AlexaFluor-647 phalloidin, and Hoechst 33342 (1 mg/ml, all ThermoFisher), diluted 1:100 in PBS, were added for 1h at room temperature. After three PBS washes, 50 µl of FluorSave Reagent (Calbiochem) was added to the cells and they were mounted between a glass slide and a number 1 coverslip.

Fixed colonoids were prepared for whole mount staining with RNAscope Multiplex Fluorescent V2 using probes Hs-WNT2B and Hs-DHH. Sample preparation and labeling were performed according to the manufacturer's protocol (ACDBio, Newark, CA). Opal fluorophores (Perkin Elmer, Waltham, MA) and DAPI were used for visualization. 50 µl of FluorSave Reagent was added to the colonoids and they were mounted between a glass slide and a number 1 coverslip.

Confocal imaging was carried out in the Imaging Core of the Hopkins NIH/NIDDK Basic and Translational Research Digestive Disease Core Center using a LSM510 META laser scanning confocal microscope running ZEN 2012 imaging software (Zeiss) and in the University of New Mexico Cancer Center Fluorescence Microscopy Shared Resource using a LSM 800 AiryScan confocal microscope (Zeiss).

Protein extraction and proteomic analysis. Colonoids were harvested in Cultrex Organoid Harvesting Solution and centrifuged at 300 xg for 10' at 4°C. The cells were washed with ice cold PBS 5 times. Cells were lysed in 250 µl of lysis buffer (60 mM HEPES pH 7.4, 150 mM KCl, 5 mM Na_3EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 2% SDS (all MilliporeSigma)) supplemented with 1:100 of protease inhibitor cocktail (P8340, MilliporeSigma). Cells incubated with lysis buffer were sonicated on ice 3 times for 10 sec using 30% energy input. The lysed cells were centrifuged for 10 min at 5000 rpm at 4°C (MC2 Centrifuge, Sarstedt Desaga) to remove any unbroken cells. Protein concentration was determined by Bradford assay (Bio-Rad). Proteomic analysis was carried out by the Mass Spectrometry and Proteomics Facility, Johns Hopkins University School of Medicine. Raw data was sent to and analyzed by Creative Proteomics (Shirley, NY). Figure S1A and B were generated by Creative Proteomics.

RNA isolation and gene expression analysis. Colonoids were harvested from Matrigel using Cultrex Organoid Harvesting Solution and centrifuged at 300 xg for 10' at 4°C. Supernatant was removed and pellet was stored at -80C until RNA extraction. RNA isolation was carried out using PureLink RNA Mini Kit (ThermoFisher) according to the manufacturer's protocol. RNA concentration was determined using a DU 800 spectrophotometer (Beckman Coulter, Brea, CA). 500 ng to 2 ug of RNA was retro-transcribed into cDNA using SuperScript VILO Master Mix (ThermoFisher). DNA Real-time qPCR were run using PowerUp SYBR green Master Mix and QuantStudio 12K Flex Real-Time PCR instrument (all Applied Biosystems, Foster City, CA). Each sample was analyzed in triplicate. The primer oligonucleotide sequences are listed in Supplemental Table 3 (Xiaowei Wang, Athanasia Spandidos, Huajun Wang and Brian Seed:

PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update) AND (Bunaciu RP et al. 2008). The relative fold changes in mRNA levels between EspP-injured and control colonoids were determined using the $2^{-\Delta\Delta CT}$ method with normalization to *18S* ribosomal RNA.

shRNA lentiviral transduction. Colonoids were harvested from Matrigel and pelleted. The colonoids were digested with TrypLE (ThermoFisher Scientific) at 37°C for 90s. The cells were then washed with Advanced DMEM and pelleted. shRNA lentiviral transduction was performed as previously described (Van Lidth de Jeude et al., 2015). Selection antibiotic (puromycin, 1 ug/ml) was added 48h after transduction.

Statistics. Data are represented as mean ± SEM. Statistical significances were calculated using Student's *t*-test. Significance was represented as at least $p < 0.05$. All experiments were performed on a minimum of 3 different colonoid lines derived from separate normal human subjects, with a total of 7 colonoid lines used throughout these studies (Supplementary Table 1). N refers to number of independent replicates performed. All analyses were performed on GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA).