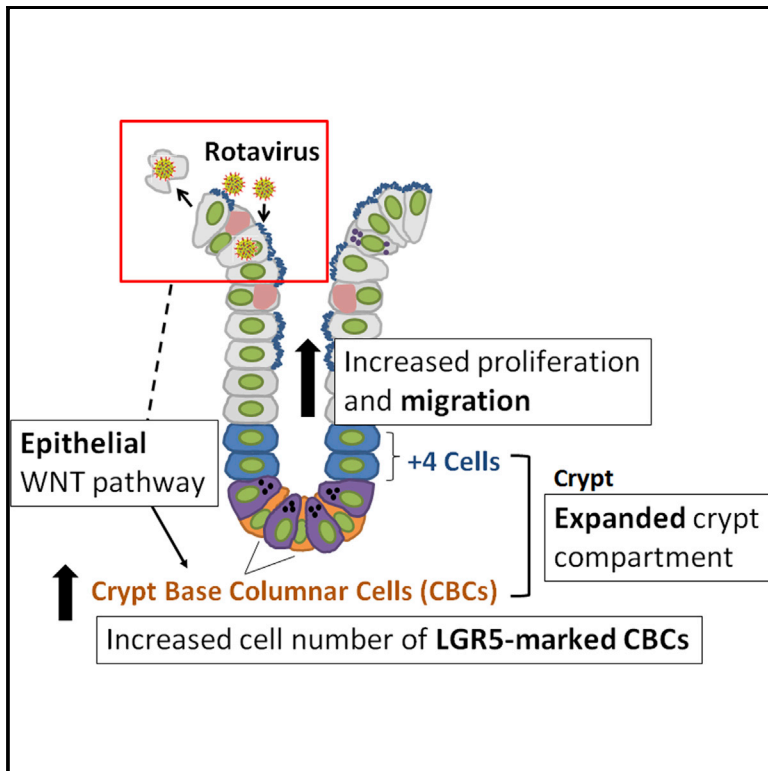Article

Epithelial WNT Ligands Are Essential Drivers of Intestinal Stem Cell Activation

Graphical Abstract

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In Brief

Using rotavirus infection as an intestinal epithelial injury model, Zou et al. demonstrate that crypt-based columnar cells are the predominant cell type responding to epithelial villus injury, and WNT ligands secreted from the intestinal epithelium are essential for this regenerative process.

Highlights

- Rotavirus infection is an excellent model for investigating intestinal villus injury

- Villus damage stimulates the active crypt-based columnar intestinal stem cells

- Reserve intestinal stem cells play a minimal role in repair of villus injury

- Regeneration following villus injury depends on epithelial-secreted WNT

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Epithelial WNT Ligands Are Essential Drivers of Intestinal Stem Cell Activation

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SUMMARY

Intestinal stem cells (ISCs) maintain and repair the intestinal epithelium. While regeneration after ISC-targeted damage is increasingly understood, injury-repair mechanisms that direct regeneration following injuries to differentiated cells remain uncharacterized. The enteric pathogen, rotavirus, infects and damages differentiated cells while sparing all ISC populations, thus allowing the unique examination of the response of intact ISC compartments during injury-repair. Upon rotavirus infection in mice, ISC compartments robustly expand and proliferating cells rapidly migrate. Infection results specifically in stimulation of the active crypt-based columnar ISCs, but not alternative reserve ISC populations, as is observed after ISC-targeted damage. Conditional ablation of epithelial WNT secretion diminishes crypt expansion and ISC activation, demonstrating a previously unknown function of epithelial-secreted WNT during injury-repair. These findings indicate a hierarchical preference of crypt-based columnar cells (CBCs) over other potential ISC populations during epithelial restitution and the importance of epithelial-derived signals in regulating ISC behavior.

INTRODUCTION

The small intestinal epithelium is one of the fastest renewing tissues in the human body, regenerating every 4–5 days (van der Flier and Clevers, 2009; Mezoff and Shroyer, 2015). This regenerative capacity is critical for maintaining the epithelium, protecting against constant insults from the luminal environment. Intestinal stem cells (ISCs) in the crypts maintain and repair the epithelial surface by giving rise to differentiated cells on the villi. Differentiation of ISCs in the crypts produces daughter cells that migrate up in a "conveyer belt" fashion to the villi, where they mature into both absorptive and secretory cells that play a major

role in nutrient absorption and other intestinal functions (Potten, 1997; van der Flier and Clevers, 2009; Mezoff and Shroyer, 2015; Henning and von Furstenberg, 2016; Beumer and Clevers, 2016). One exception to this migratory pathway is the mature Paneth cells, which remain in the crypts instead of migrating upward, interact closely with the ISCs, and secrete stem cell maintenance factors, including WNT (Henning and von Furstenberg, 2016; Mezoff and Shroyer, 2015; Beumer and Clevers, 2016).

The crypts are thought to contain two types of ISCs (Henning and von Furstenberg, 2016; Mezoff and Shroyer, 2015; Beumer and Clevers, 2016). The best studied is the crypt-based columnar cells (CBCs) located at the base of the crypt. CBCs express the cell-surface marker leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), among others, and continually proliferate under homeostasis (Cheng and Leblond, 1974a, 1974b; Barker et al., 2007). There is also evidence for an alternative reserve ISC population, which can be referred to as +4 cells or quiescent ISCs, as well as early absorptive and secretory progenitors that reside above the base of the crypt (Potten and Hendry, 1975; Potten, 1977; van der Flier and Clevers, 2009; Mezoff and Shroyer, 2015). Traditionally, tissue renewal after injury in the intestine has been studied using γ-irradiation, chemotherapy treatments, or genetic ablation, in which proliferating crypt-based columnar ISCs are ablated (Beumer and Clevers, 2016). CBC loss can activate reserve ISCs or the dedifferentiation of committed progenitors to repopulate the CBC pool and provide epithelial restoration (May et al., 2008; Potten et al., 2009; Takeda et al., 2011; Tian et al., 2011; Hua et al., 2012; Powell et al., 2012; van Es et al., 2012; Van Landeghem et al., 2012; Yan et al., 2012; Yu, 2013; Metcalfe et al., 2014; Poulin et al., 2014; Roche et al., 2015; Tetteh et al., 2016; Buczacki et al., 2013). These studies highlight the dynamic nature of the ISC niche that can readily regenerate following functional stem cell loss. However, while ISCs are well characterized under homeostatic conditions or situations where they are damaged directly, very little is known about the differential activation of these populations under intestinal epithelial dysbiosis in which ISCs remain undamaged.

The maintenance and regeneration of the intestinal epithelium is regulated, at least in part, by canonical WNT/β-catenin signaling (Clevers et al., 2014; Clevers and Nusse, 2012; Kühl
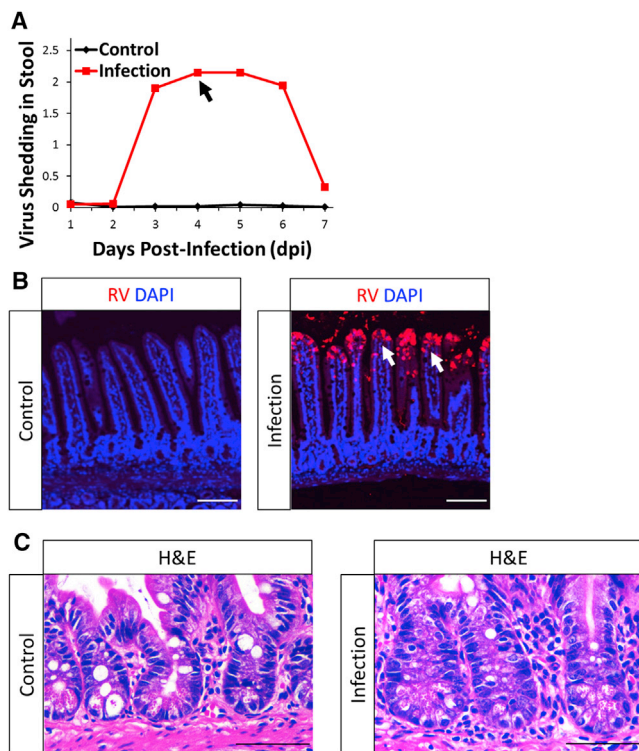
Figure 1. RV Infection Is Limited to the Tip of the Villi, Preserving an Intact Crypt Compartment

(A) Representative stool ELISA monitoring RV infections. Points represent means of respective groups (n = 2). Peak viral shedding was observed at 4 days post-infection (dpi; black arrow).

(B) Representative confocal images of control- and RV-infected mouse epithelium. RV-infected differentiated cells were detected using a laboratory-generated, polyclonal anti-RV antibody. Infected villi are noted by white arrows.

(C) Representative H&E images showing intact crypts in both control- and RV-infected mouse epithelium.

See also Figures S1 and S2.

and Kühl, 2013; Nusse and Varmus, 2012; Shroyer et al., 2015; Yan et al., 2017b). Extracellular WNT ligands bind to membrane Frizzled (FZD) receptors to trigger intracellular translocation of the transcriptional co-activator β-catenin, which can then drive the expression of well-established WNT pathway target genes (Nusse and Varmus, 2012). Studies in mice have indicated that cells in the intestinal epithelium and mesenchyme are two independent sources of WNT secretion (Farin et al., 2012; Gregorieff et al., 2005; Valenta et al., 2016). Paneth cells in the intestinal epithelium secrete WNT3, WNT6, and WNT9B; and myofibroblasts in the mesenchyme express WNT2, WNT4, and WNT5A (Gregorieff et al., 2005; Farin et al., 2012; Aoki et al., 2016; Stzepourginski et al., 2017; Valenta et al., 2016). Importantly, the epithelium and the mesenchyme are thought to be redundant sources of WNT secretion, with epithelial secreted WNT ligands being nonessential (Farin et al., 2012; Kabiri et al., 2014; San Roman et al., 2014; Valenta et al., 2016). When WNT3 or genes essential for WNT secretion (e.g., *Porcupine* and *Wntless*) were ablated in the intestinal epithelium, no apparent phenotype

was observed (Farin et al., 2012). Knockouts (KOs) of genes specific to Paneth cells (e.g., *Atoh1* and *Gfi1*), the primary cellular source of epithelial WNT, also do not affect WNT pathway activation (Durand et al., 2012; Kim et al., 2012; Shroyer et al., 2005, 2007). These studies led to the prevailing notion that the epithelium is a redundant source of WNT and is not essential for CBC maintenance. By contrast, *in vitro*, mouse intestinal enteroids that contain only epithelial cells can be propagated without supplementing WNT ligands (Sato et al., 2009, 2011), suggesting that epithelial-secreted WNT is sufficient for stem cell proliferation. These discordant results warrant additional analyses on the role of epithelial-secreted WNT under nonhomeostatic situations.

We use rotavirus (RV) infection as an infection/injury model to test ISC responses to villus damage and whether mesenchymal or epithelial WNT plays a role in ISC activation. RV, a well-characterized, noninflammatory small intestinal viral pathogen, infects terminally differentiated mature cells, but not ISCs, in the small intestinal epithelium, resulting in watery diarrhea with vomiting, fever, and abdominal pain (Greenberg and Estes, 2009). RV infection alters the cytoskeleton and impairs host secretory pathways, leading to mislocalization of brush border enzymes and malabsorption, disruption of cell-cell junctions, increased permeability, cytotoxic effects, and activation of cell death (Ramig, 2004; Beau et al., 2007; Jourdan et al., 1998; Burns et al., 1995). Infection in adult mice is accompanied by a notable lack of villus blunting or epithelial ulceration, suggesting the induction of epithelial repair mechanisms that compensate and presumably replace the infected, lost cells (Ward et al., 1990; Burns et al., 1995; O'Neal et al., 1997; Blutt et al., 2012). Importantly, both the active and reserve ISC populations remain uninfected and undamaged during RV infection, providing an elegant model system to examine the ISC response within an intact stem cell compartment after injury. Our studies show that epithelial-derived signals regulate the hierarchical stem cell response when the niche remains intact and provide evidence for a functional role for epithelial WNT signaling.

RESULTS

RV Infection Does Not Infect the Crypt Compartment and Induces Crypt Expansion and Crypt-Villus Migration

Oral inoculation of adult mice with murine RV results in viral infection that is detected by viral shedding in stool (Figure 1A) (Ward et al., 1990; Burns et al., 1995; O'Neal et al., 1997; Blutt et al., 2012). Infected cells detected by anti-RV antibody were limited to the tips of the villi from 2 to 4 days post-infection (dpi) (Figures 1B and S1A). Maximum levels of viral shedding were observed 4 dpi (Figure 1A); therefore, all subsequent studies were performed at this time point. Importantly, the stem cell compartment is never infected (Figure 1B and S1A), and all cell types, including CBCs, reserve ISCs, and Paneth cells within the crypts, remained intact in RV-infected animals (Figures 1C, 3D, 3H, and S2).

To determine whether infected cells at the tip of the villus are damaged by infection, we examined cell morphology via H&E staining (Figure S1B), which showed increased cell shedding, nuclear mislocalization, and distorted and enlarged nuclei with

hypodense hematoxylin staining. Immunohistochemical staining of junctional protein E-cadherin showed its mislocalization from the cell membrane into the cytoplasm in addition to necrotic shedding cells (based on fragmented nuclei) (Figure S1C). To characterize the integrity of the brush border after infection, we evaluated villin, sodium-hydrogen antiporter 3 (NHE3), and ezrin, which are all normally located on the villus brush border (Figures S1D–S1F). On the infected villi, irregular and intensified villin staining was present on the apical cell surface with protein mislocalization into the cytoplasm (Figure S1D). NHE3 and ezrin protein staining was lost in parts of RV-infected villi (Figures S1E and S1F). To decipher the mechanism of cell death following infection, we probed for the anoikis marker phosphorylated myosin light chain (P-MLC) (Bullen et al., 2006) and the apoptosis marker cleaved caspase-3 (Figures S1G and S1H). We found that the number of P-MLC-labeled cells increased following infection but found few cleaved-caspase-3-labeled cells, suggesting that the majority of RV- infected cells undergo anoikis related cell death (Figures S1G and S1H).

To determine whether viral infection at the tip of the villi produced any response within the crypt region, we first examined proliferation of the crypts following RV infection. Expression of the proliferative marker PCNA indicated that crypts in RV-infected animals remained intact and that the PCNA$^+$ compartment was expanded (Figure 2A). Quantification of this expansion showed a 15-μm (or nearly one-third) increase in the height of PCNA$^+$ cells, with increased PCNA$^+$ cells per crypt following RV-infection (Figure 2B). The proliferative markers *Ki67* and *Pcna* were also transcriptionally upregulated in isolated crypts following infection (Figure 2C). Together, these experiments demonstrate an expanded and more proliferative PCNA$^+$ zone following RV infection.

Renewal of the intestinal epithelium depends on an upward migration of proliferating and differentiating cells from the crypt to the villus compartment. Previous studies have indicated that RV infection can affect cell migration in neonatal mice (Boshuizen et al., 2003; Preidis et al., 2012). Having documented that RV infection results in increased proliferation within the crypt region, we examined whether this expansion might impact upward migration of the differentiated progeny (Figure 2D). 2 hr after injecting the thymidine analog EdU, EdU$^+$ cells were restricted to the proliferating crypt zone in both control- and RV-infected animals (Figure 2D). 24 hr after EdU labeling, EdU$^+$ cells were observed to migrate out of the crypts and onto the villi (Figure 2D). Cell migration was assessed by measuring the absolute distance from the crypt-villus junction (just outside of the trans-amplifying zone) to the EdU$^+$ cell that had migrated the furthest. We determined the migration distance to be 22 μm, or 87%, faster in the RV-infected group than in the control group in 24 hr (Figures 2D and 2E, left). 48 hr after EdU injection, positive cells were already present at the tip of the villi in RV-infected mice, replacing RV-infected, damaged cells that had been shed lumenally, whereas EdU$^+$ cells were still migrating up the villi in control mice. (Figure 2D). Despite this more rapid cell migration up the villi, the total villus height was not different between RV- and control-infected animals, indicating that faster-migrating cells in RV-infected mice were replacing shed cells rather than extending the villus length (Figure 2E, right). These experiments demonstrate that RV infection stimulates a faster migration of proliferating cells from the crypt to the villi.

Active CBCs, but Not Alternative Reserve ISCs, Respond to RV Infection

In addition to the active CBC population, reserve ISCs have been postulated to exist within the small intestinal crypt (Potten, 1977; Potten and Hendry, 1975; Cheng and Leblond, 1974a; Henning and von Furstenberg, 2016; Mezoff and Shroyer, 2015; Beumer and Clevers, 2016). To determine whether a specific cell type responds to the injury from RV-induced damage, we evaluated the expression of putative ISC markers. Markers associated with CBCs alone (*Lgr5*, *Olfm4*, and *Ascl2*) (Barker et al., 2007; van der Flier et al., 2009a, 2009b) and with both CBCs and reserve ISCs (*Sox9* and *Lrig1*) (Van Landeghem et al., 2012; Powell et al., 2012) were significantly upregulated in crypts isolated from RV-infected mice, whereas markers associated with reserve ISC populations (*Bmi1*, *Hopx*, and *Dclk1*) (Yan et al., 2012; Takeda et al., 2011; Giannakis et al., 2006) showed minimal upregulation following infection (Figure 3A). These data suggest that the predominant response following RV infection occurs in the CBC population.

Given that some CBC markers would also be direct targets of the WNT and other signaling pathways, we tested whether transcriptional upregulation of CBC markers during RV infection indicates an increase in the CBC population. We utilized *Lgr5^{GFPCreERT}* mice (Barker et al., 2007) in which LGR5-expressing cells are directly tagged with GFP (Figure 3B). A significant increase in the percentage of crypt cells expressing LGR5^{GFP} and LGR5$^{GFP-high}$ was seen following infection by flow cytometry (Figures 3C and S3). Immunofluorescence staining indicated that LGR5-labeled CBCs were increased in number in crypts from RV-infected animals compared to control animals (Figure 3D). While the number of crypts per micrometer of intestine is unchanged (Figure S4C), an increase in LGR5-labeled crypts per micrometer of intestine was also observed when the entire small intestine was examined from infected mice (Figures S4A and S4B). It is possible that the increase in LGR5$^+$ crypts might be an artifact of the mosaic nature of the *Lgr5^{GFPCreERT}* mice; however, assessment of OLFM4 (van der Flier et al., 2009a), another marker of CBCs, showed increased expression in crypts when comparing RV-infected and control animals (Figure 3E). Together, these data indicate an expansion of crypt-based columnar ISCs in response to RV infection.

To assess whether expansion of reserve ISCs also occurred in small intestinal crypts in response to RV infection, we first examined the reserve ISC marker BMI1. In *Bmi1^{CreERT};R26^{mTmG}* mice (Yan et al., 2012), BMI1-labeled and reserve ISCs can be tagged with membrane-GFP following a dose of tamoxifen injection 1 day before harvest (Figure 3F). The BMI1$^+$ cell population remained very stable and comparable in control and RV-infected animals (Figures 3G and 3H). While *Bmi1* transcription was significantly upregulated with a small change (1.6-fold), a similar percentage of BMI1 cells in control- and RV-infected animals was observed by flow cytometry and immunofluorescence (Figures 3G and 3H). Lineage tracing also showed no change in BMI1 lineage following RV infection (Figure S5). Together, our data suggest that alternative reserve ISC populations do not
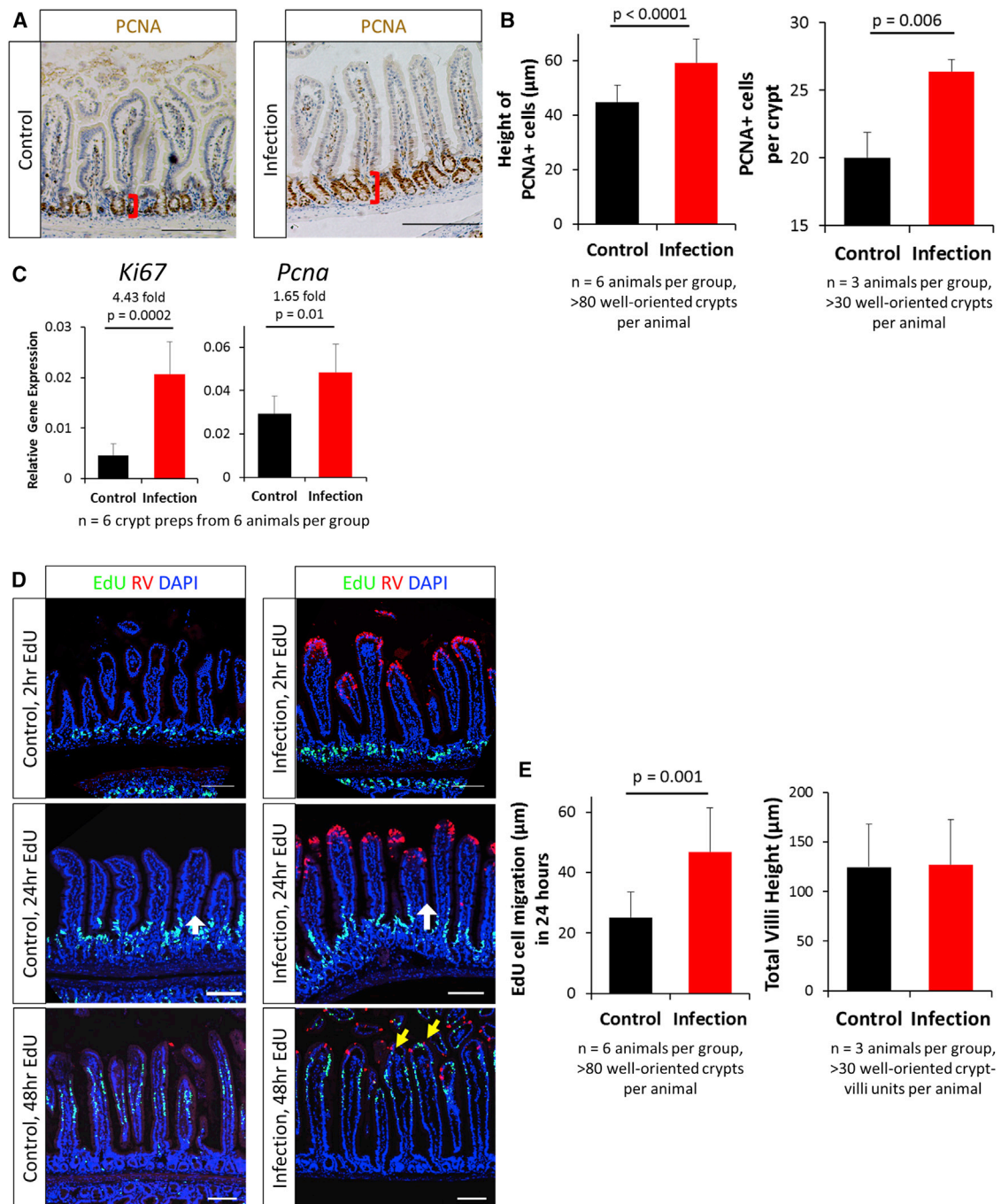
Figure 2. RV Infection Led to Increased Epithelial Proliferation and Increased Migration

(A) Representative immunohistochemistry images of PCNA+ proliferating compartments. Red brackets represent the height of the PCNA+ cells measured in (B).

(B) Quantification of height of PCNA+ cells (left) and PCNA+ cells per crypt (right).

(C) qRT-PCR results showing upregulation of the proliferative markers *Ki67* and *Pcna* in isolated epithelial crypts.

(D) Representative confocal images of cell migration in control- and RV-infected animals with 2-, 24-, and 48-hr EdU labeling. EdU+ cells were restricted to the crypts after 2-hr EdU labeling. The length of 24-hr EdU migration was measured from the crypt-villi junction to the farthest EdU-labeled cells (white arrows). After 48 hr of labeling, EdU+ cells were observed near RV-infected cells (yellow arrows).

(E) Quantification of cell migration 24 hr after EdU labeling (left) and total villi height (right). EdU cell migration distance is an absolute measure of distance from the crypt-villus junction (just outside of the transient amplifying [TA] zone) to the cell that had migrated the furthest.

Bars represent means ± SD. All statistical analyses were performed using Student's t test. Scale bars, 100 μm. See also Figures S3–S6.
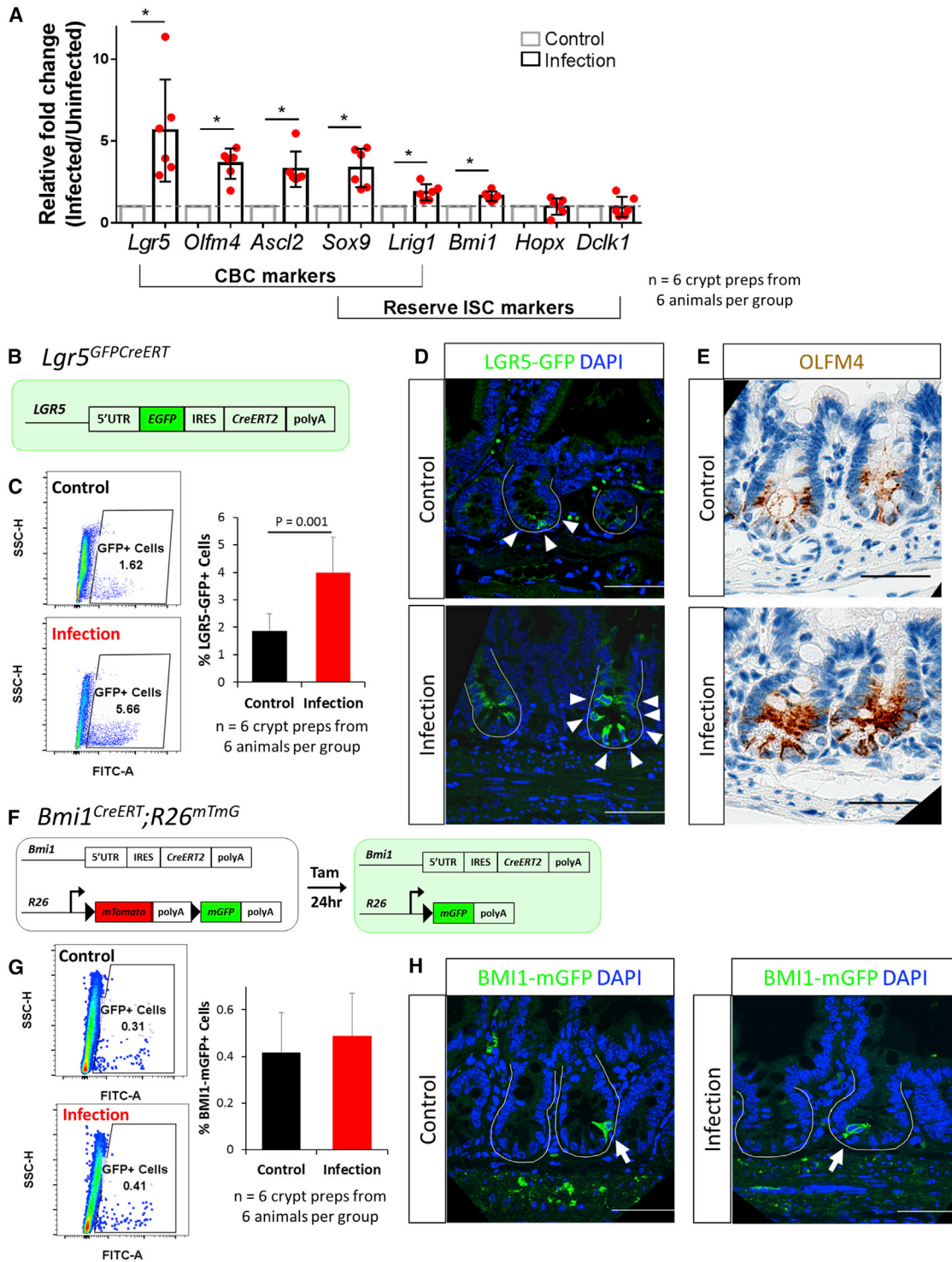
Figure 3. CBCs Are Induced following RV Infection

(A) qRT-PCR results showed upregulation of all CBC markers and some reserve ISC markers.

(B) Schematic of *Lgr5*[GFPCreERT] mice. Cells expressing LGR5 are green.

(C) Representative flow cytometry analysis on control- and RV-infected *Lgr5*[GFPCreERT] mice. Quantification of GFP+ cells using flow cytometry analysis on crypt-enriched epithelial preparations.

(D) Representative confocal images of *Lgr5*[GFPCreERT] mice following control- and RV-infection. White arrowheads denote GFP+ cells.

(E) Representative light microscopy images of OLFM4 immunohistochemistry staining in control- and RV-infected animals.

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respond to RV infection. These results demonstrate that, when intact, the CBC population remains the primary source of epithelial restitution and does not rely on alternative reserve ISC populations.

Epithelial WNT Secretion Is Essential for ISC Induction following RV Infection

The WNT signaling pathway is known to play a major role in stem cell proliferation and expansion (Fevr et al., 2007; Kuhnert et al., 2004; Pinto et al., 2003; Yan et al., 2017b). To determine whether RV infection induces WNT signaling, we examined the transcriptional profile of WNT ligands in isolated epithelial crypt and mesenchymal compartments of the small intestine (Gregorieff et al., 2005; Farin et al., 2012). Epithelial-expressed *Wnt3* and *Wnt9B* in crypt preparations were significantly upregulated in RV-infected mice compared with control mice (Figure 4A); however, the expression pattern of *Wnt3* in Paneth cells and other crypt cell types did not change when comparing control and RV-infected mice (Figure 4B). *Wnt2B*, *Wnt4*, and *Wnt5A* expression in mesenchymal preparations of infected mice were not affected (Figure 4A). Additionally, when we examined the WNT signaling amplifier R-spondin (R-spo) family members, we saw that *R-spo1* is upregulated in the mesenchymal preparations of infected mice (Figure S6). The expression of several well-established downstream WNT target genes (*Axin2, EphB2*, *Myc*, *Ccnd1*, *Cd44*, and *Tert*) was also upregulated in the crypts isolated from RV-infected animals (Figure 4C). RNA *in situ* hybridization confirmed WNT signaling upregulation by the expansion of *Axin2* expression in the crypts of the infected animals (Figure 4D). Immunofluorescence showed that expression of CD44v6, a pan crypt cell-surface marker that is regulated through the WNT pathway, was expressed on more crypt cells following infection (Figure 4E). Additionally, β-catenin, the intracellular transducer of the WNT signaling pathway, translocated from the cytoplasm to the nucleus in the crypts of RV-infected animals (Figure 4F). Collectively, these experiments show that RV-induced epithelial damage results in the increased transcriptional expression of epithelial WNT molecules, and they provide evidence of WNT signaling within the crypts.

Traditionally, the epithelium and the mesenchyme are thought to be redundant sources of WNT secretion during homeostasis, with the important WNT pathway signals derived from mesenchymal cells (Kabiri et al., 2014; Farin et al., 2012; Valenta et al., 2016; San Roman et al., 2014; de Groot et al., 2013). Whether there are unique functions of epithelial-secreted WNT ligands is currently unknown. The transcriptional upregulation of the epithelial-secreted WNT ligands *Wnt3* and *Wnt9B* (Figure 4A) suggested that epithelial WNTs might play an important role in the activation of LGR5⁺ stem cells within the crypt following villus damage. To assess whether secretion of epithelial WNT was related to crypt expansion following infection, $Villin^{CreERT};WLS^{f/f};R26^{mTmG}$ (WLS KO) mice, in which WNT

secretion in the epithelium is conditionally impaired, were infected with RV (Figures 5A and S7A) (Bänziger et al., 2006; Carpenter et al., 2010). Although RV infection induced upregulation of *Wnt5A* in mesenchymal preparations from WLS KO mice (Figure S7B), WNT pathway target genes were no longer upregulated in small intestinal crypts following infection (Figure 5B) compared to the upregulation seen in wild-type (WT) infected mice (Figure 4C). Further, CBC markers, including *Lgr5* and *Olfm4*, were not induced when epithelial WNT secretion was impaired (Figure 5C). The crypts were not expanded (Figures 5D and 5E), nor was there faster migration induced by infection, suggesting that epithelial WNT secretion plays an important role in these processes (Figures 5F and 5G). Additionally, while no overt phenotype was observed in the RV-infected WLS KO mice and RV shedding remained similar in the WT and WLS conditional KO model (Figure 5H), RV-infected cells in the WLS KO model were no longer localized to the tips of the villi (Figures 5F and S7A) compared to infected WT mice (Figures 1B and S1A). These findings indicate that epithelial-secreted WNT ligands are important for the regenerative crypt response following epithelial damage, and they may help restrict RV infection to the tip of the villi.

DISCUSSION

Understanding activation of stem cells within the niche environment is important for dissecting pathways that regulate epithelial repair. Intestinal regeneration after injury has been studied extensively using several models. For example, radiation-induced damage targets and kills LGR5⁺ cycling CBCs. In this situation, the epithelium regenerates due to the plasticity of the reserve ISCs labeled by BMI1, HOPX, LRIG1, SOX9, and others, which repopulate the LGR5⁺ cycling population within the base of the crypt (Hua et al., 2012; May et al., 2008; Metcalfe et al., 2014; Potten et al., 2009; Powell et al., 2012; Takeda et al., 2011; Yan et al., 2012; Yu, 2013). Chemotherapy treatments, such as doxorubicin, elicit injury patterns similar to radiation treatment, with loss of LGR5⁺ populations within the crypt. Activation and increased proliferation of surface glycoprotein CD24-labeled ISCs following injury can reconstitute the damaged epithelium (Dekaney et al., 2009; Seiler et al., 2015). Genetic mouse models resulting in the direct ablation of LGR5-labeled CBCs have also shown the importance of the reserve ISCs as well as the newly characterized secretory and absorptive progenitors after LGR5⁺ cell loss (Tetteh et al., 2016; van Es et al., 2012; Buczacki et al., 2013). Together, these studies suggest a plastic ISC environment in which many different stem cell types and progenitor cells can fulfill the demand of injury repair during CBC damage and loss. In the present work, we extend these studies by exploring how the crypt stem cell populations respond to villus-specific injury. RV infection has been linked to increased proliferation in the epithelium

(F) Schematic of $Bmi1^{CreERT};R26^{mTmG}$ mice.
(G) Representative flow cytometry analysis on control- and RV-infected $Bmi1^{CreERT};R26^{mTmG}$ mice. Quantification of BMI1-mGFP⁺ cells using flow cytometry analysis on crypt-enriched epithelial preparations.
(H) Representative confocal images of BMI1-mGFP⁺ mice following control- and RV-infection. White arrows denote BMI1-mGFP⁺ cells.
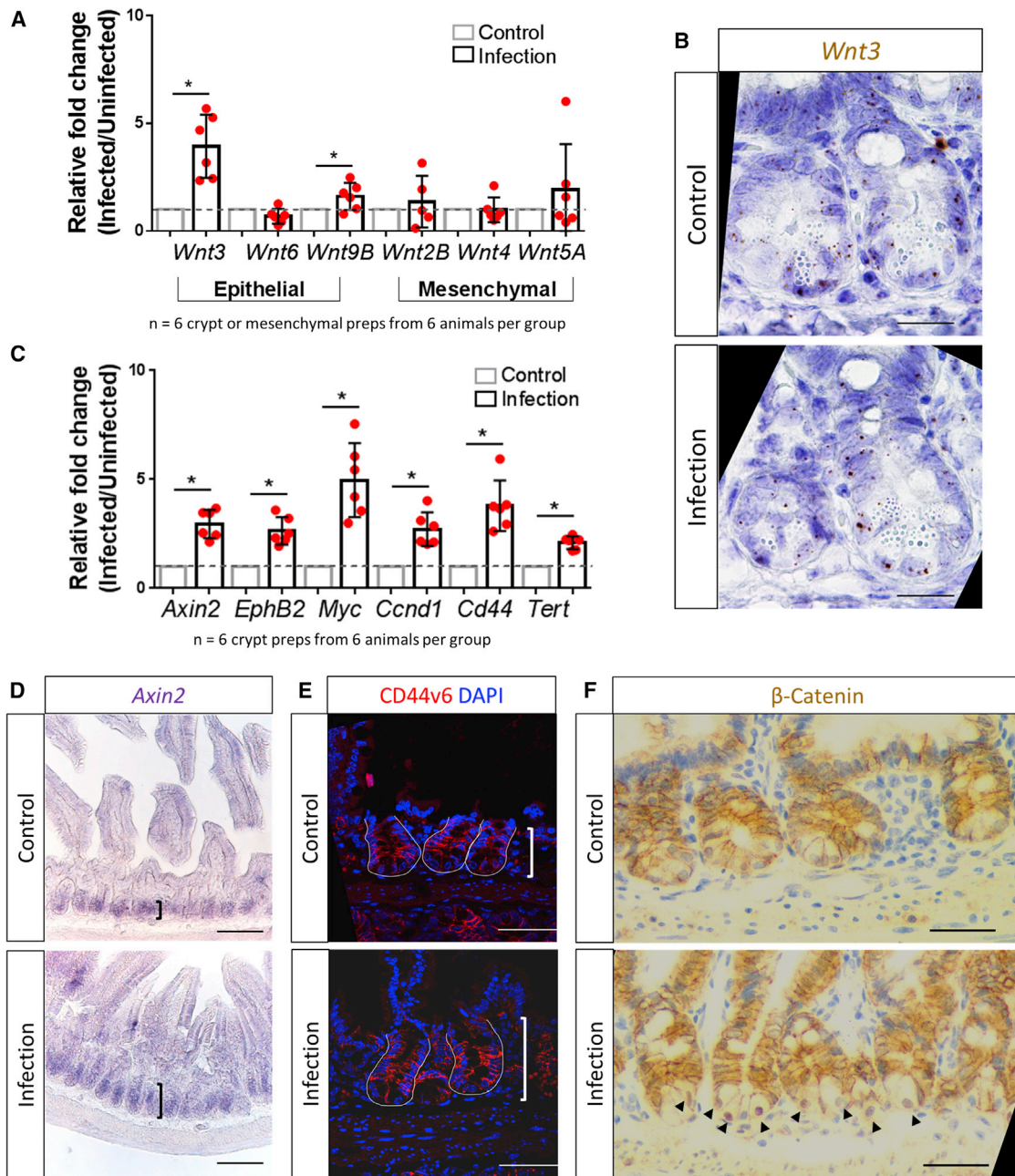Scale bars, 50 μm.

Figure 4. WNT Signaling Pathway Is Stimulated in Crypts following RV Infection

(A) qRT-PCR results showing upregulation of the epithelial-expressed *Wnt3* and *Wnt9B* in isolated epithelial crypt preparations. *Wnt2B*, *Wnt4*, and *Wnt5A* remained stable in mesenchymal preparations.

(B) RNAScope analysis showing *Wnt3* expression in Paneth cells and other crypt cell types in both control and RV infection.

(C) qRT-PCR results showing upregulation of well-established WNT signaling pathway target genes in isolated epithelial crypt preparations after RV infection.

(D) Representative RNA *in situ* hybridization images showed expansion of *Axin2* expression in RV infected animals.

(E) Representative immunofluorescence images showing that the WNT-target gene CD44v6 is expressed on more cells in the RV-infected animal.

(F) Representative immunohistochemistry staining of β-catenin, the intracellular transducer of the WNT signaling pathway. RV infection induces cytoplasm to nuclear translocation of β-catenin in the crypts (black arrowheads).

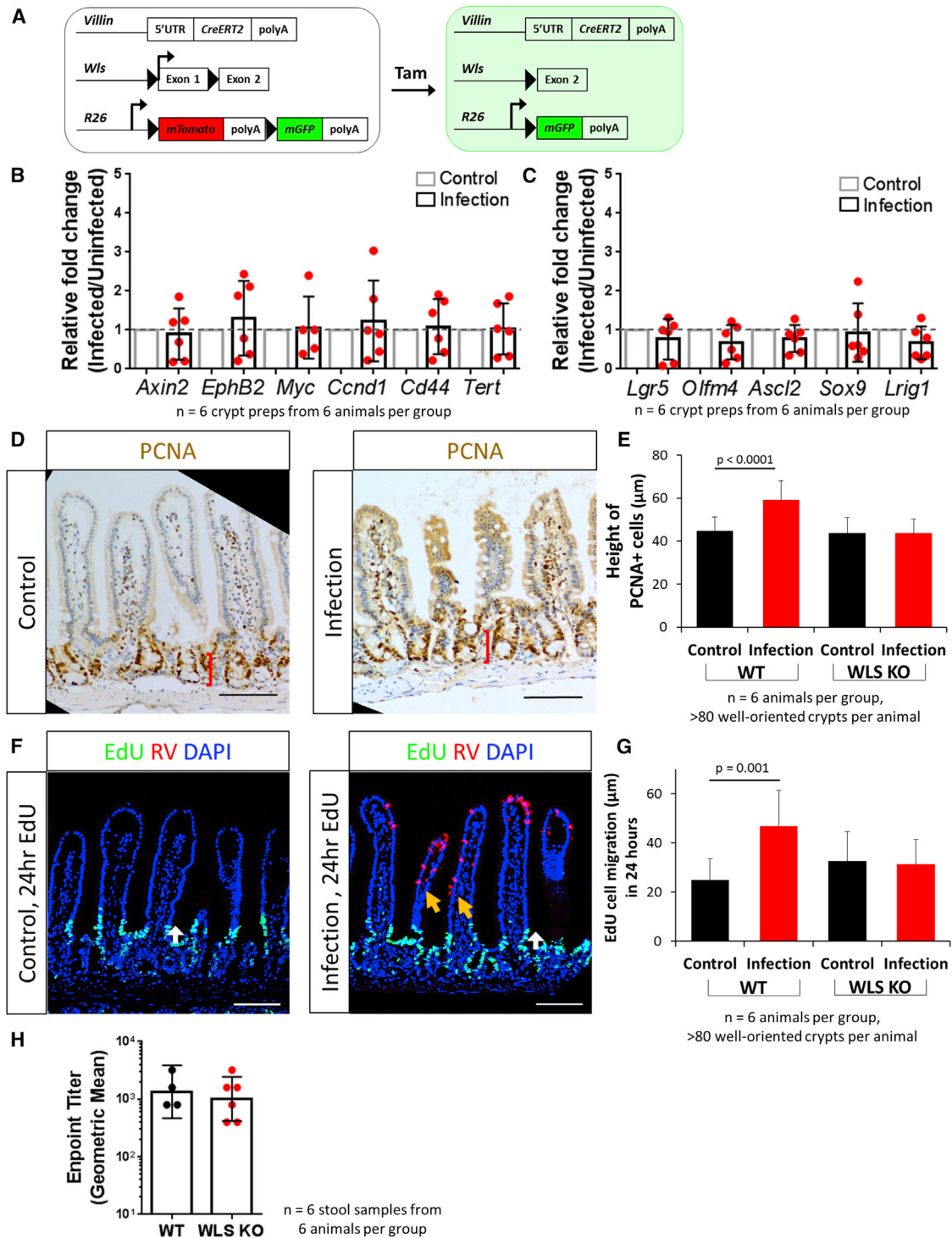Scale bars represent 50 μm in (B), (E), and (F) and 100 μm in (D). See also Figure S6.

Figure 5. Epithelial-Secreted WNT Ligands Are Essential for RV-Induced Stem Cell Regeneration

(A) Schematic of $Villin^{CreERT};WLS^{f/f};R26^{mTmG}$ (WLS KO) mouse. Injections of tamoxifen allows for the conditional knockout of the $Wntless$ gene in villin-expressing cells, impairing WNT secretion specifically in the epithelium.

(B) qRT-PCR results showed expression of WNT pathway target genes remain stable in isolated epithelial crypts following RV infection in WLS KO mice.

(C) qRT-PCR results showed expression of putative CBC markers remain stable in isolated epithelial crypts following RV infection in WLS KO mice.

(D) Representative immunohistochemistry staining of PCNA in control- and RV-infected WLS KO animals.

(E) Quantification of height of PCNA+ cell measurement in WT and WLS KO mice. Bars represent means ± SD.

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(Boshuizen et al., 2003; Preidis et al., 2012). Our data demonstrate the LGR5$^+$ CBC population within the crypt environment is the one that expands during RV infection. In contrast to radiation, chemotherapy, and direct ablation, BMI1-marked cells do not respond to villus-specific damage (Figure 3). Because the crypt compartment is highly plastic, it is possible that BMI1-marked cells may have reverted back to the CBC state following RV infection, resulting in LGR5$^+$ cell expansion. However, this is unlikely, as we did not observe BMI1$^+$ cells at the CBC position (Figure 3), nor did we observe increased lineage tracing of BMI1$^+$ daughter cells following RV infection (Figure S5). Recent work suggests that BMI1$^+$ cells bear signatures of enteroendocrine cells, a differentiated, secretory cell type in the small intestine, making BMI1$^+$ cells a possible early progenitor committed to the enteroendocrine lineage (Jadhav et al., 2017; Yan et al., 2017a). Together, these results argue that RV-induced villus damage also does not affect the enteroendocrine progenitor population. These data build a more complex model of regeneration initiated in the stem cell niche in which LGR5$^+$ CBCs are important for homeostatic maintenance of the intestinal epithelium and are preferred to respond to villus epithelial damage by increasing proliferation and migration, while reserve ISCs and other progenitor cell types are only recruited during CBC loss and play an important role in regeneration of the crypt itself. Importantly, these data indicate there is a hierarchical control of the niche and the stem cells that comprise it.

WNT signaling is an important component of the ISC niche. Inhibition of global WNT secretion reduces proliferation and impairs epithelial homeostasis (Fevr et al., 2007; Kuhnert et al., 2004; Pinto et al., 2003; Valenta et al., 2016). While both the epithelium and mesenchyme secrete WNT molecules, epithelial-secreted WNT ligands are thought to be redundant and nonessential (Farin et al., 2012; Kabiri et al., 2014; San Roman et al., 2014; Valenta et al., 2016). When WNT3 and WNT secretion pathway genes (e.g., *Porcupine* and *Wntless*) were ablated in the intestinal epithelium, no apparent phenotype was observed (Farin et al., 2012). KOs of *Atoh1* and other genes specific to the Paneth cell, a known source of epithelial WNT secretion, also give no aberrant loss in WNT signaling activation (Durand et al., 2012; Kim et al., 2012). These findings have led to the hypothesis that mesenchymal WNT secretion is the primary mechanism for regulating the proliferative response in the stem cell niche. This idea is supported by several recent studies that have identified key mesenchymal cell types, marked by transcription factor *Foxl1* and surface antigen CD34, which can potentiate WNT signaling (Aoki et al., 2016; Stzepourginski et al., 2017). By contrast, our results show that epithelial-secreted WNT ligands are essential for the expansion of LGR5$^+$ cells and that proliferation and migration occur following virus-induced villus damage (Figures 3 and 4). Thus, although epithelial WNTs are thought to be nonessential for maintaining homeostasis, they are an impor-

tant component of epithelial repair. It is important to note that our findings do not exclude the involvement of the mesenchyme. The upregulation of the WNT signaling amplifier *R-spo1* in the mesenchyme following infection raises the interesting possibility that epithelial WNT may be one arm of a broad inter-compartmental response to RV infection in which the induction of R-spo family proteins in the mesenchyme requires the priming by epithelial WNT to carry out injury repair after RV infection. This is in line with the recent publication on the nonequivalent yet cooperative role of R-spo and WNT on stem cell self-renewal (Yan et al., 2017b). Further, while RV-infected cells are restricted to the tip of the villi in WT mice, epithelial *Wntless* deletion resulted in an aberrant infection pattern in which infected cells can be observed midlength on the villi (Figure 5F). Since RV infects the terminally differentiated enterocytes that are present at the tips of the villi (Figures 1 and S1A) (Ward et al., 1990; Burns et al., 1995; O'Neal et al., 1997; Blutt et al., 2012), one possibility may be that the differentiation status of the enterocytes has been altered in the absence of epithelial-secreted WNT. Although previous studies did not find any significant differences in the differentiation pattern of epithelial-WNT impaired mice compared to WT mice (Kabiri et al., 2014; San Roman et al., 2014), it is possible that subtle cellular changes that are relevant to RV susceptibility and pathogenesis have occurred that were not detected in the initial comparison studies. Another explanation may be that epithelial WNT ligands, through their proliferative effects, affect the rate of cell migration and epithelial repair (Figures 2 and 5). Therefore, in the absence of epithelial WNT ligands, mature cells simply do not migrate as fast to the tip of the villi; thus, RV-infected cells are found in nontraditional locations. Future studies are needed to determine why RV-infected *Wntless* KO mice exhibit an altered infection pattern.

One of the key aspects to determining the mechanisms through which villus damage results in WNT secretion will be identifying which cell type is producing WNT following RV infection. A likely candidate is the Paneth cell, which has already been shown to secrete WNT (Sato et al., 2009, 2011). Our experiments, however, did not show changes in this cell type (Figure S2). Hence, there may exist an additional cell type with WNT-secreting ability that produces WNT only in the context of epithelial damage. Apoptotic cells during hydra head injury have been shown to be an unexpected source of WNT3 and are able to drive regeneration (Chera et al., 2009; Lengfeld et al., 2009). Similar mechanisms may exist in RV infection, where infected, apoptotic cells directly secrete WNT ligands, leading to increased stem cell proliferation within the crypt. Alternatively, other nonapoptotic damage signals produced by infected villus cells may indirectly stimulate WNT secretion from other cell types (e.g., Paneth cells), leading to stem cell activation. Such feedback mechanisms from differentiated cells to stem cells are not well understood in mammalian

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(F) Representative image of EdU-labeled cell migration in control- and RV-infected animals in WLS KO mice. There was no induction of EdU$^+$ cell migration (white arrows). RV-infected cells were no longer restricted to the tip of the villi (orange arrows).

(G) Quantification of cell migration with 24 hr of EdU labeling in WT and WLS KO mice (white arrows in E). Bars represent means ± SD.

(H) Endpoint titer from stool ELISA of WT and WLS KO animals. Bars represent geometric mean ± 95% CI.

Scale bars, 100 μm. See also Figure S7.

systems, but several studies in the *Drosophila* gut have shown the importance of the JAK-STAT pathway (Beebe et al., 2010; Buchon et al., 2009; Jiang et al., 2016; Lin et al., 2010). Stress, injury, and bacterial infection can lead to cytokine production in damaged cells, stimulating the JAK-STAT pathway in WNT-producing cell types, which manifests into ultimate ISC proliferation (Jiang et al., 2016; Xu et al., 2011). Our previous study observed a robust upregulation of the interferon pathways in the intestinal epithelium following RV infection in the human intestinal enteroids (Saxena et al., 2017). Interferon production, with the subsequent activation of the JAK-STAT pathway, may serve as a bridge from infected cells to epithelial WNT production and CBC expansion (Saxena et al., 2017; Nava et al., 2010; Arnold et al., 2013). Future studies will focus on elucidating the cell type and mechanisms that elicit WNT signaling in response to RV infection.

Finally, while our data point to a necessary role in epithelial-secreted WNT ligands, we were not able to study whether epithelial-secreted WNT ligands are sufficient for the proliferative responses seen following RV infection. Future studies using the intestinal enteroid cultures that do not contain the mesenchyme will address the sufficiency of epithelial-signals in stem cell induction following infection. In addition, these cultures may facilitate the identification of additional epithelial sources of WNT ligands that are induced following infection. Alternatively, the mesenchyme may still potentiate regenerative responses in villus damage. Such close communication between epithelial and mesenchymal WNT signaling pathways has been shown in both the *Drosophila* midgut as well as mammalian small intestine (Kux and Pitsouli, 2014; Le Guen et al., 2015; Roulis and Flavell, 2016; Karlsson et al., 2000). Well-established feedback loops from Hedgehog and WNT signal pathways could serve as a potential link between the mesenchyme and the epithelium after RV infection (Büller et al., 2012; Kosinski et al., 2010). Several cell types recently identified to secrete R-spo and other growth factors in the mesenchyme could also serve as important catalysts for the WNT signaling pathway following epithelial injury (Stzepourginski et al., 2017; Aoki et al., 2016).

In summary, this study used RV infection as a model to study the injury-repair response from an intact ISC niche. We showed that LGR5^{+} CBCs, when present, remain the primary source of epithelial restitution, and no other reserve cell types were needed for the response. In addition, epithelial-secreted WNT ligands are nonredundant, essential components of injury repair. These discoveries provide a framework for how cell types communicate in the intestinal tract and lay a foundation for developing regenerative treatments for intestinal injury.

EXPERIMENTAL PROCEDURES

Genetic Mouse Lines and Viral Infection
Lgr5[GFPCreERT], *R26*[mTmG], *Bmi1*[CreERT], and *Villin*[CreERT] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *WLS*[f/f] mice were a generous gift from Dr. Jim Wells (Cincinnati Children's Hospital) (Carpenter et al., 2010). Mice aged 8–16 weeks (both males and females) were randomly assigned to control- or RV-infected groups with at least three mice per group per experiment. RV strain EC$_{WT}$ (P[17], G3) or PBS-control gut homogenate from previously infected mice was administered by oral gavage (O'Neal et al., 1997). All

animals were housed in a physically separated BSL-2 animal facility. A 1×10^5 50% infectious dose (ID50) was used to obtain adequate infection (O'Neal et al., 1997). Fecal samples from each mouse were collected daily. Fecal ELISA was used to monitor RV infection as described previously (O'Neal et al., 1997). Mice were sacrificed 4 days following infection at the peak of viral shedding. To induce lineage tracing through *CreERT*, tamoxifen in 10% EtOH/ corn oil was intraperitoneally injected. To label BMI1^{+} cells and BMI1 lineage, 1 dose of 1 mg tamoxifen was injected in *Bmi1*[CreERT];*R26*[mTmG] mice 1 or 7 days before harvest, respectively. To conditionally knock out *Wntless*, 3 daily doses of 1 mg tamoxifen were injected in *Villin*[CreERT];*WLS*[f/f];*R26*[mTmG] mice 3 days before infection. To determine proliferation in the intestinal epithelium, 1 mg EdU in 10% DSMO/PBS was intraperitoneally injected at 2, 24, or 48 hr before harvest. All protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC).

Crypt Purification and Mesenchymal Isolation
Intestinal crypts were prepared using previously described protocols, with modifications (Mahe et al., 2013; Gracz et al., 2012). Briefly, the entire small intestine was dissected out and flushed with ice-cold $Ca^{2+}/Mg2^+$-free Dulbecco's PBS (DPBS). Intestines were opened lengthwise and cut into 1-cm pieces. Tissues were incubated with chelating buffer (2 mM EDTA in DPBS) on ice for 30 min by gentle shaking. Chelating buffer was then replaced with shaking buffer (1% [43.3 mM] sucrose, 1% [54.9 mM] sorbitol in DPBS) and manually shaken for ~1–2 min to disassociate crypts. Intestinal crypts were filtered through a 70-μm cell strainer (BD Falcon) and then spun down at 150 × *g*, 4°C for 5 min. Remaining tissue after epithelial purification was harvested as mesenchyme.

Statistical Analysis
Data are presented as means ± SD. The n in all experiments refers to biological replicates (different animals) in each group. n for each experiment is listed in each figure. Statistical comparisons between two groups were analyzed using the Student's t test. Significance was taken as p < 0.05. All analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA).

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the flow cytometry data reported in this paper are Flow Repository: FR-FCM-ZYF8 and FR-FCM-ZYF9.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.093.

AUTHOR CONTRIBUTIONS

S.E.B. and M.K.E. conceptualized the study. M.K.E., S.E.B., and N.F.S. supervised all studies performed. W.Y.Z., M.-S.C., X.-L.Z., and D.C.-A. designed and performed experiments. W.Y.Z., Y.-H.L, S.E.B., N.F.S., and O.D.K. critically analyzed experimental data. W.Y.Z. wrote the manuscript. S.E.B., N.F.S., M.K.E., and M.D. provided critical input in manuscript revision. All authors contributed to the writing or editing of the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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