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A Dynamic WNT/β-CATENIN Signaling Environment Leads to WNT-Independent and WNT-Dependent Proliferation of Embryonic Intestinal Progenitor Cells

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SUMMARY

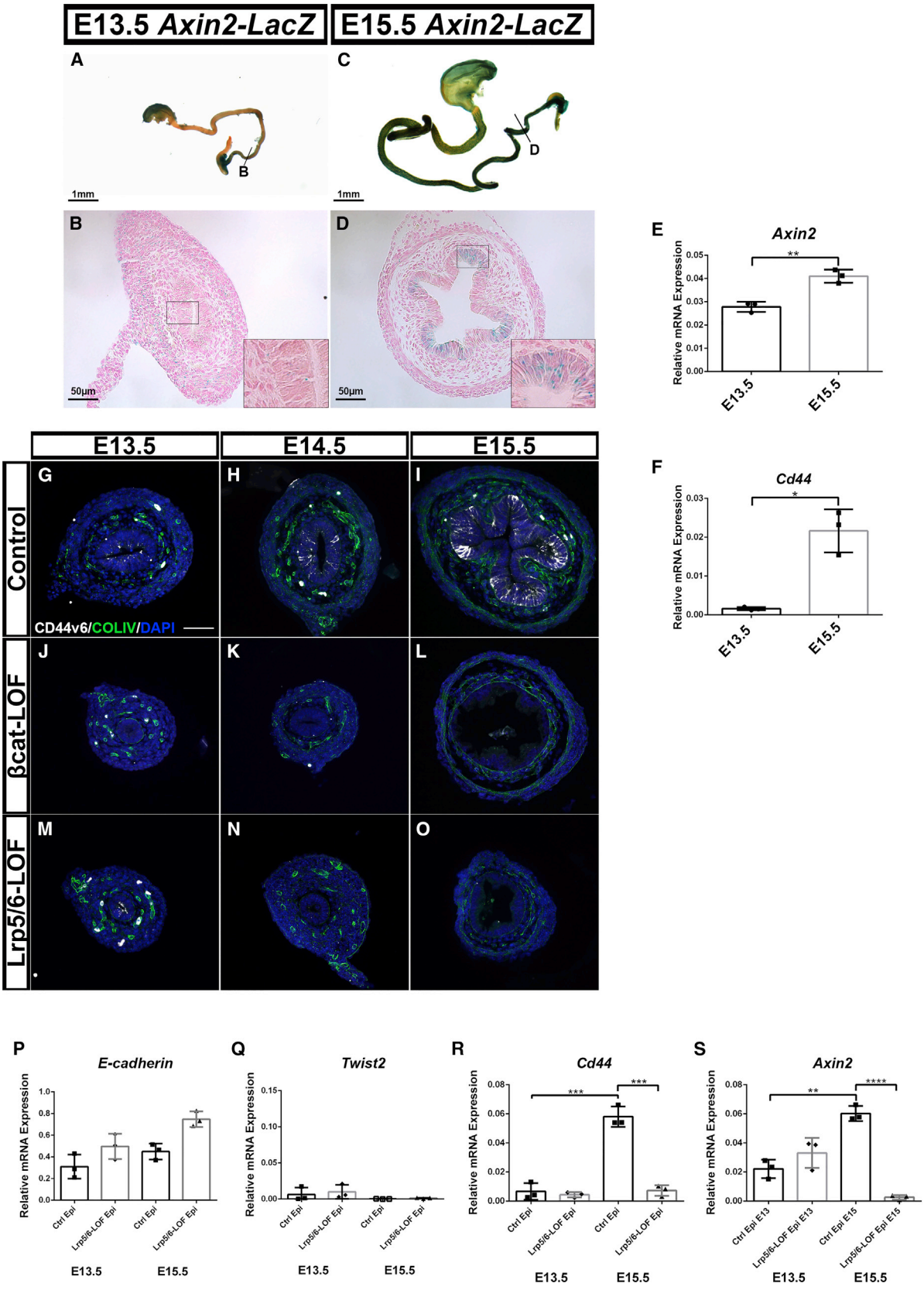
Much of our understanding about how intestinal stem and progenitor cells are regulated comes from studying the late fetal stages of development and the adult intestine. In this light, little is known about intestine development prior to the formation of stereotypical villus structures with columnar epithelium, a stage when the epithelium is pseudostratified and appears to be a relatively uniform population of progenitor cells with high proliferative capacity. Here, we investigated a role for WNT/β-CATENIN signaling during the pseudostratified stages of development (E13.5, E14.5) and following villus formation (E15.5) in mice. In contrast to the well-described role for WNT/β-CATENIN signaling as a regulator of stem/progenitor cells in the late fetal and adult gut, conditional epithelial deletion of *β-catenin* or the Frizzled co-receptors *Lrp5* and *Lrp6* had no effect on epithelial progenitor cell proliferation in the pseudostratified epithelium. Mutant embryos displayed obvious developmental defects, including loss of proliferation and disruptions in villus formation starting only at E15.5. Mechanistically, our data suggest that WNT signaling-mediated proliferation at the time of villus formation is driven by mesenchymal, but not epithelial, WNT ligand secretion.

INTRODUCTION

To keep up with daily demands, the intestine is highly proliferative and has a high rate of cellular turnover. Self-renewing intestinal stem cells (ISCs) located in the crypt at the base of the intestinal epithelium constantly give rise to new progeny. Maintenance of the adult stem cell population requires β-CATENIN-dependent WNT signaling ("canonical" WNT signaling, herein referred to as WNT/β-CATENIN signaling). Inhibition or loss of WNT/β-CATENIN signaling in the epithelium results in loss of stem cells in the crypt (Chiacchiera et al., 2016; Das et al., 2015; Farin et al., 2012; Pinto et al., 2003; Valenta et al., 2016), while activating mutations leading to constitutive WNT activation are causative in colorectal cancer (Barker et al., 2009; Fearon and Spence, 2012; Fearon and Wicha, 2014; Korinek et al., 1997; Morin et al., 1997). Unlike the plethora of information about regulation of the adult ISC, it is much less clear whether and when WNT/β-CATENIN signaling plays a role in the embryonic intestine, and in particular we understand very little about intestine development prior to the formation of villi. For example, studies in mice null for the *β-catenin* transcriptional binding partner *Tcf7l2* (*Tcf4*) or mice in which the FRIZZLED co-receptors *Lrp5* and *Lrp6* have been conditionally deleted both demonstrate a loss of intestinal proliferation and collapse of the intervillus progenitor domain late in fetal development (embryonic day 17.5 [E17.5]) (Korinek et al., 1998; Zhong et al., 2012). However, WNT/β-CATENIN signaling has not been directly interrogated prior to villus morphogenesis, a time when the epithelium is a relatively flat, simple pseudostratified epithelium that proliferates uniformly, and lacks stereotypical intestinal villi and differentiated cell types seen following villus morphogenesis (Grosse et al., 2011; Shyer et al., 2013, 2015; Walton et al., 2012, 2016).

Due to specific and well-characterized genetic tools such as *Villin-Cre* mice, which allow for epithelium-specific transgene expression or *Cre*-mediated genetic excision of conditional alleles in the intestine, many studies have focused on late development (Madison et al., 2002; El Marjou et al., 2004). *Villin-Cre* lines efficiently mediate recombination after villus morphogenesis begins, around E14.5, and efficient deletion of conditional alleles is often achieved at mid-gestational stages (Bondow et al., 2012; Walker et al., 2014). Therefore, the goal of the current work was to interrogate a functional role for WNT/β-CATENIN prior to villus morphogenesis.

Our results demonstrate that disruption of WNT/β-CATENIN signaling, using *Shh-Cre* (Harfe et al., 2004) to achieve early epithelium-specific conditional deletion



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of *Ctnn1b* (β-catenin) (Brault et al., 2001) or the *Frizzled* co-receptors *Lrp5* and *Lrp6* (Lrp5/6) (Zhong et al., 2012), had little effect on the pseudostratified epithelium, indicating that WNT/β-CATENIN signaling was dispensable for proliferation at this time. Significant defects in proliferation and villus formation were only evident at later times, after villus morphogenesis had begun (E15.5). Furthermore, our results show that conditional deletion of *Wntless*, which is required for proper WNT ligand trafficking and secretion from the cell, from the mesenchymal, but not epithelial compartment, leads to a loss of epithelial proliferation at the time of villus formation. Collectively, our data demonstrate that WNT/β-CATENIN signaling is dispensable for regulating epithelial progenitor cell proliferation in the embryonic gut during the pseudostratified stage of development, whereas active signaling is absolutely required for proliferation and proper villus formation at the time when villus morphogenesis begins.

RESULTS

WNT/β-CATENIN Signaling Activity Increases over Developmental Time

To identify the timing and location of active Wnt signaling in the developing intestine, we first utilized an *Axin2-LacZ* reporter mouse (Lustig et al., 2002). *Axin2-LacZ* reporter activity was very low at E13.5 (Figures 1A and 1B). Activity was more apparent in the E14.5 epithelium (Figures S1A–S1F) while at E15.5, *Axin2-LacZ* reporter activity was also apparent, and was restricted to the intervillus domains (Figures 1C, 1D, and S1G–S1L). Interestingly, as the *Axin2-LacZ* reporter activity increased across developmental time, we observed that the distal small intestine appeared to report WNT/β-CATENIN signaling first (Figures S1A–S1F), and we therefore focused our analysis on this region of the gut. To support our observations made in *Axin2-LacZ* reporter mice, we analyzed mRNA expression in whole-thickness ileum for two downstream targets of WNT/β-CATENIN signaling, *Axin2* and *Cd44*. We found that both *Axin2* and *Cd44* mRNA was significantly upregulated in E15.5 ileum compared with E13.5 ileum (Figures 1E and 1F). In addition, CD44v6 antibody staining indicated increased protein expression as developmental time progressed (Figures 1G–1I, S2A, and S2B).

β-catenin or *Lrp5/6* Loss-of-Function Embryos Have Perturbed Villus Formation

To elucidate a role for WNT/β-CATENIN signaling in the intestinal epithelium at early developmental times, we disrupted WNT/β-CATENIN signaling using two different genetic models: epithelium-specific *Shh-Cre*-driven conditional deletion of *Ctnn1b* (β-catenin) or of *Frizzled* co-receptors *Lrp5* and *Lrp6*. To observe the efficiency of deletion, we stained for β-CATENIN by immunofluorescence and did not detect epithelial β-CATENIN in E13.5 mice with *β-catenin* loss of function (βcat-LOF) (Figure S3A). In addition, while CD44v6 was low in controls at E13.5, βcat-LOF intestines did not have detectable CD44v6 protein at E13.5 (Figures 1J and S2C). It should be noted that while CD44v6 staining is weak in the control epithelium at E13.5, the loss of CD44v6 staining in βcat-LOF at E13.5 suggests that weak protein expression in controls is likely reflective of low levels of WNT/β-CATENIN signaling present in the epithelium (compare Figure 1G with Figure 1J and Figure S2A with Figure S2C). Importantly, loss of WNT/β-CATENIN signaling did not affect intestinal fate, since the βcat-LOF intestines maintained CDX2 protein expression (Figure S3C).

Figure 1. WNT/β-CATENIN Signaling Is Active in Temporally and Spatially Distinct Domains in the Small Intestine

(A and C) Whole-mount X-Gal staining of E13.5 (A) and E15.5 (C) stomach and intestines from *Axin2-LacZ* reporter mice. Black lines indicate plane of section. Scale bar, 1 mm.

(B) X-Gal staining in E13.5 intestinal sections shows low activity in the epithelium (marked in A).

(D) At E15.5, *Axin2-LacZ* reporter activity became restricted to the intervillus domains in the epithelium.

(E and F) qPCR analysis of whole-thickness ileal segments show upregulation of *Axin2* (E) and *Cd44* (F) from E13.5 and E15.5 (n = 3 E13.5 embryos pooled from two litters and n = 3 E15.5 embryos pooled from three litters for one independent experiment).

(G–I) Immunofluorescence staining of E13.5 (G), E14.5 (H), and E15.5 (I) control intestines show increasing CD44v6 staining (white) co-stained with collagen IV (green).

(J–O) WNT/β-CATENIN-deficient ileum shows reduced CD44v6 protein expression where CD44v6 is lost in βcat-LOF as early as E13.5 (J) and Lrp5/6-LOF by E14.5 (N). Scale bar for (G) to (O), 50 μm.

(P and Q) Epithelial isolations from control and Lrp5/6-LOF intestines are enriched for *E-cadherin* (P) and deficient in *Twist2* (Q).

(R and S) Lrp5/6-LOF epithelia are dramatically reduced for *Cd44* (R) and *Axin2* (S) mRNA transcript at E15.5, indicating efficient deletions by *Shh-Cre*.

Both E13.5 genotypes have n = 3 embryos pooled from two litters and both E15.5 genotypes have n = 3 embryos pooled from three litters for one independent experiment. Error bars represent SD. Statistical significance by t test: *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.0001–0.001, ****p = 0.00001–0.0001.

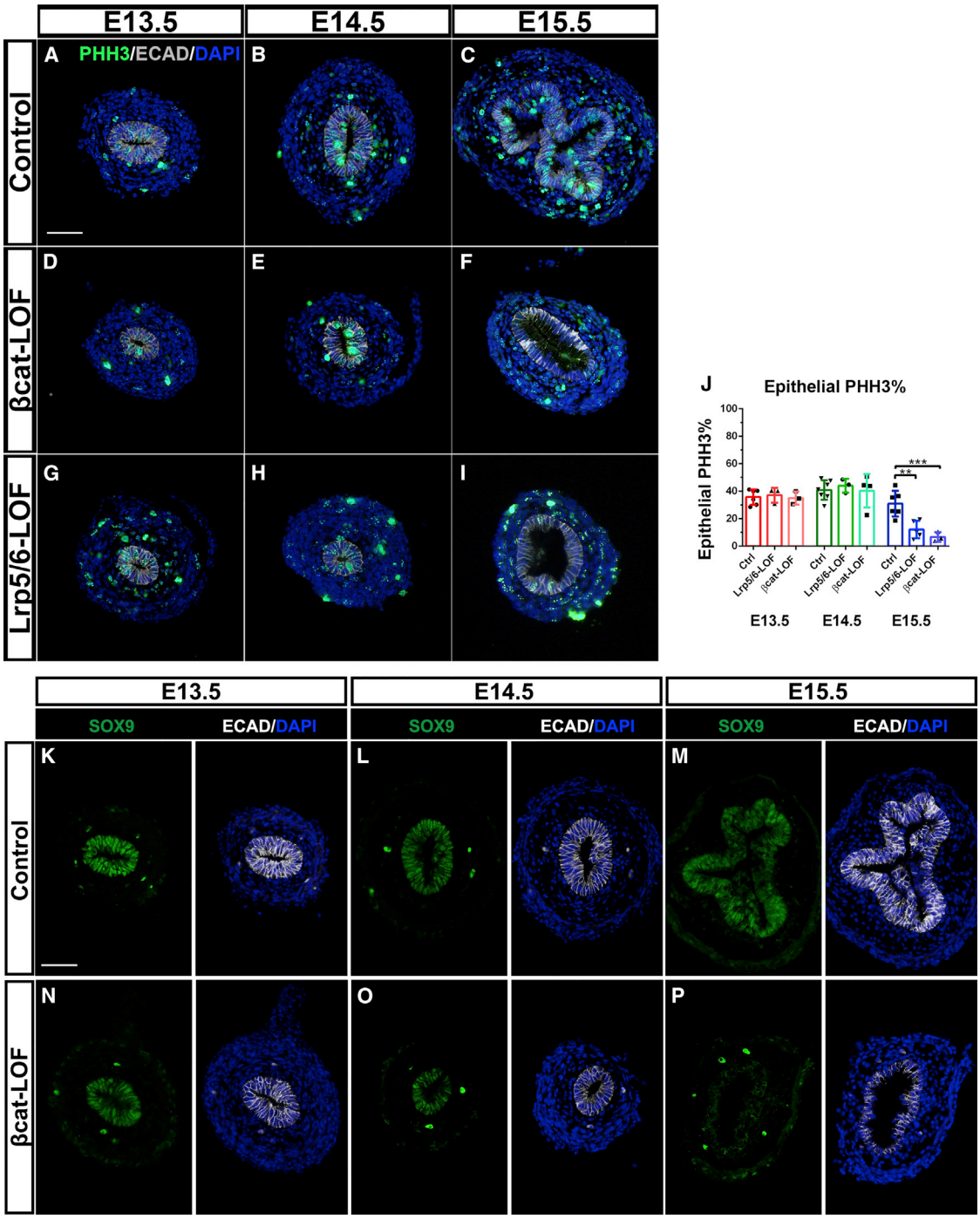

Figure 2. WNT/β-CATENIN Signaling-Deficient Mice Have Epithelial Proliferation Defects and Decreased SOX9 Expression Only at E15.5 and Not at Earlier Time Points

(A–I) Immunofluorescence staining for phospho-histone H3 (PHH3, green) and E-CADHERIN (white) demonstrates that epithelial proliferation was occurring in the distal small intestine of all genotypes at E13.5 and E14.5 (control, A and B; βcat-LOF, D and E; Lrp5/6-LOF, G and H). At E15.5, villus morphogenesis and epithelial proliferation were perturbed in both βcat-LOF (F) and Lrp5/6 LOF (I) compared with control (C). Scale bar, 50 μm.

(J) Quantification of the percentage of $PHH3^+$ epithelial cells ($PHH3^+ECAD^+$/total $ECAD^+DAPI^+$) shows a significant reduction in proliferation only at E15.5. For all genotypes, n = 3–6 embryos pooled from two to five litters for five independent experiments. Statistical significance by t test: **p = 0.001–0.01, ***p = 0.0001–0.001.

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To observe deletion efficiency in *Shh*-Cre-mediated *Lrp5* and *Lrp6* loss-of-function (Lrp5/6-LOF) embryos, we mechanically separated the epithelium and mesenchyme of control and Lrp5/6-LOF embryos and analyzed them using qRT-PCR. We saw a significant reduction of both *Lrp5* and *Lrp6* mRNA transcript in the epithelial fractions of E15.5 Lrp5/6-LOF, but not at E13.5 (Figure S3B). To confirm deletion, we analyzed expression of *Cd44* and *Axin2* mRNA expression in isolated epithelium of Lrp5/6-LOF embryos (Figures 1R and 1S), and CD44v6 protein in tissue sections (Figures 1M–1O, S2E, and S2F). These results showed a loss of CD44v6 protein staining by E14.5 (Figures 1N, 1O, and S2F) and a significant reduction of *Cd44* and *Axin2* at E15.5 (Figures 1R and 1S), suggesting that WNT/β-CATENIN signaling was not efficiently perturbed until E14.5 in this model.

WNT/β-CATENIN Signaling Is Dispensable for Epithelial Proliferation in the Distal Small Intestine during the Pseudostratified Stage of Development

We examined proliferation at E13.5, E14.5, and E15.5 in the distal portion of control, βcat-LOF, and Lrp5/6-LOF intestines (Figure 2). We performed immunofluorescence staining for phospho-histone H3 (PHH3), a marker that detects cells in M phase, along with E-CADHERIN to visualize epithelial-specific proliferation and the formation of nascent villi (Figures 2A–2I). At E13.5 and E14.5, we observed no difference in proliferation in the epithelium of control or mutant intestines. PHH3 staining was easily visualized in all genotypes examined (Figures 2A, 2B, 2D, 2E, 2G, and 2H), and there were no quantitative differences in epithelial proliferation at these stages (Figure 2J). On the other hand, E15.5 epithelial PHH3 staining was reduced in βcat-LOF and Lrp5/6-LOF intestines compared with controls (Figures 2C, 2F, and 2I). Quantitation of the percentage of epithelial cells that are $PHH3^+$ ($ECAD^+PHH3^+$/total $ECAD^+DAPI^+$) showed that the E15.5 epithelium in βcat-LOF and Lrp5/6-LOF intestines had a significant reduction in proliferation (Figure 2J). In addition to proliferation defects, we also observed that mutant intestines failed to begin villus morphogenesis by E15.5 and instead, the epithelium remained flat (Figures 2C, 2F, and 2I). Taken together, our results indicate that the intestinal epithelium does not require WNT/β-CATENIN signaling for proliferation at E13.5 and E14.5 but requires WNT/β-CATENIN signaling for proliferation after initiation of villus morphogenesis by E15.5.

Deletion of *E-cadherin* Does Not Phenocopy βcat-LOF

Given that βcat-LOF and Lrp5/6-LOF embryos showed similar phenotypes, it is likely that the defects observed are due to perturbations in WNT/β-CATENIN signaling. However, given the important role that β-CATENIN plays in the adherens junctions, we wanted to rule out the possibility that cell-cell adhesion defects are leading to the observed phenotypes (Kintner, 1992; Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990). To do this, we conditionally deleted *Cdh1* (*Shh-cre*;*Cdh1-flox/flox*;Ecad-LOF), which encodes E-CADHERIN. In contrast to βcat-LOF intestines, which fail to form nascent villi, we found that Ecad-LOF mutants underwent villus morphogenesis prematurely and had obvious villus formation by E14.5 (Figures S3E and S3F). Consistent with this, Ecad-LOF animals had abundant platelet-derived growth factor receptor α (PDGFRA)-positive mesenchymal clusters under nascent villi whereas controls had much less obvious cluster formation (Karlsson et al., 2000; Walton et al., 2012) (Figures S3I and S3J). These data suggest that loss of WNT/β-CATENIN signaling leads to a phenotype very different from that of Ecad-LOF, and adds supporting evidence that defects in the βcat-LOF phenotype are not due to cell adhesion defects.

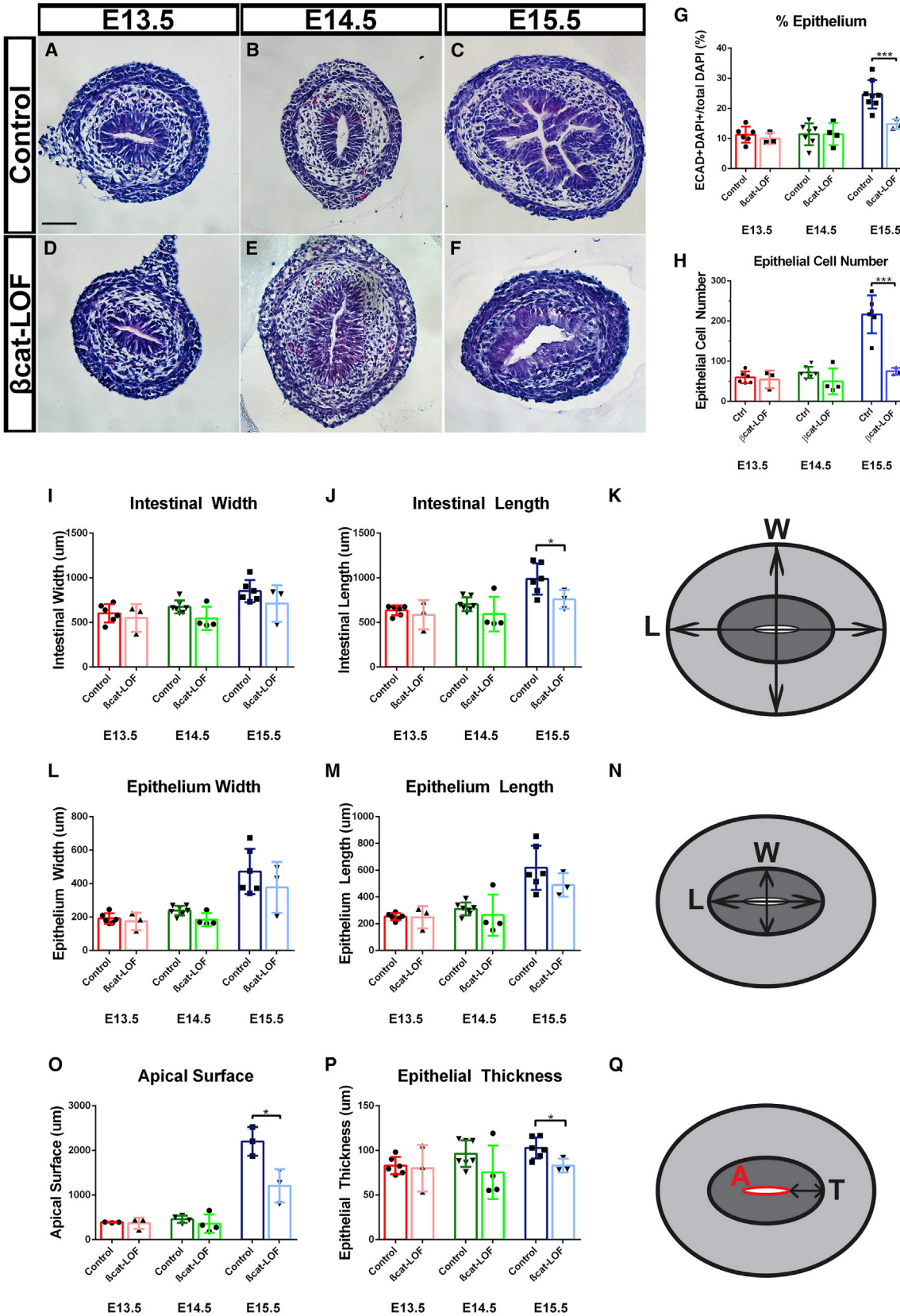
Loss of WNT/β-CATENIN Signaling Does Not Perturb SOX9 Expression in the Intestine at Pseudostratified Stages

Prior to villus morphogenesis, SOX9 is expressed throughout the intestinal epithelium while after villus morphogenesis, expression is restricted to the proliferating intervillus domain and is dependent on WNT signaling (Bastide et al., 2007; Blache et al., 2004) Interestingly, we found that SOX9 expression in the epithelium of βcat-LOF embryos at E13.5 and E14.5 is similar to that in controls (Figures 2K, 2L, 2N, and 2O), and that SOX9 protein expression is lost within βcat-LOF epithelium only at E15.5 (Figures 2M and 2P). These data suggest that *Sox9* is not a sensitive WNT target gene during the pseudostratified stages of intestine development, and corroborate data suggesting that the intestinal epithelium is regulated by different mechanisms before and after villus morphogenesis.

Loss of WNT/β-CATENIN Signaling Severely Disrupts Villus Morphogenesis

Both genetic models used to disrupt WNT/β-CATENIN signaling (βcat-LOF and Lrp5/6-LOF) led to a similar phenotype by E15.5 (Figures 1 and 2). Similarly, both

(K–P) Immunofluorescence staining for SOX9 (green) and E-CADHERIN (white) shows robust nuclear staining in the epithelium of control and βcat-LOF at E13.5 and E14.5 (K, L, N, and O). At E15.5, SOX9 staining in controls is less robust at the tips of nascent villi (M) and is lost in βcat-LOF epithelia (P). Scale bar, 50 μm.
Error bars represent SD.



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βcat-LOF and Lrp5/6-LOF embryos had grossly smaller intestines compared with controls at E15.5 (Figure S3D). Based on these similarities, and the fact that *β-catenin* deletion was more efficient than *Lrp5/6* deletion (Figures S3A and S3B), we focused the remainder of our analysis on βcat-LOF mice. Morphological analysis of βcat-LOF intestines via H&E staining shows that the control and mutant intestines appeared similar at E13.5 and E14.5, whereas abnormal villus morphogenesis in mutants resulted in a loss of nascent villi at E15.5 (Figures 3A–3F). To assess the mutant phenotype in greater detail, we performed several morphometric analyses. The percentage of epithelial cells present relative to all cells (epithelium plus mesenchyme) in a cross-section (represented as [(E-CADHERIN$^+$/DAPI$^+$)/(total DAPI$^+$ cells per section)]), showed that there was no significant difference at E13.5 or E14.5 between mutants and controls. However, a reduction in the percentage of epithelium was observed at E15.5 (Figure 3G). Similarly, counting the absolute number of epithelial cells (E-CADHERIN$^+$ DAPI$^+$) per section showed no difference between controls and mutants until E15.5 (Figure 3H). To further assess any changes in morphology associated with βcat-LOF, we performed a series of measurements (diagrammed in Figures 3K, 3N, and 3Q) including the total cross-sectional length/width (Figures 3I and 3J), cross-sectional length/width of the epithelium (Figures 3L and 3M), and apical surface area and epithelial thickness (Figures 3O and 3P). In several measurements, we did not observe statistical differences at any time point between βcat-LOF and controls (Figures 3I, 3L, and 3M). However, for data shown in Figures 3I–3N, measurements neglected to account for the size of the lumen, which can vary. Therefore, we measured the apical surface (Figure 3Q, "A") as well as epithelial thickness (Figure 3Q, "T"), which both showed a significant decrease in βcat-LOF intestines at E15.5, but not at earlier times (Figures 3O and 3P). These morphometric data are consistent with our findings that loss of WNT/β-CATENIN signaling does not affect intestinal morphology or proliferation during the pseudostratified stage of development.

Disrupted Villus Morphogenesis Is Not due to Epithelial Cell Death

To determine whether the perturbed villus formation observed in mutants was due to apoptosis, we conducted cleaved-caspase 3 (CC3) staining on E13.5, E14.5, and E15.5 tissues in control, βcat-LOF, and Lrp5/6-LOF distal small intestines (Lrp5/6-LOF data not shown). Across all time points, no CC3 staining was detected (Figure S4A), indicating that the loss of villus formation is not due to apoptosis. Importantly, positive CC3 staining was detected at the villus tips in the proximal small intestine, a site where apoptosis is normally occurring (Hall et al., 1994) (Figure S4B).

Loss of *β-catenin* in the Epithelium Does Not Affect Smooth Muscle Differentiation

Previous reports have shown that restrictive force from the surrounding smooth muscle is important for villus formation and acts to produce compressive stress on the highly proliferative epithelium and mesenchyme (Shyer et al., 2013). To determine whether the disruption in villus formation observed in βcat-LOF intestines is due to defects in smooth muscle development, we analyzed α-smooth muscle actin via immunofluorescence in E15.5 βcat-LOF and control intestines. We observed no differences between mutants and controls (Figure S4C), suggesting that the inability of the epithelium to properly form villi is not due to perturbations in the smooth muscle layer and is more likely caused by the lack of epithelial proliferation.

Epithelium-Specific Loss of WNT/β-CATENIN Signaling Results in Reduced Aggregation of PDGFRA-Positive Mesenchymal Clusters

Just prior to the emergence of epithelial villus structures, aggregation of the underlying mesenchyme into "clusters" is evident, starting around E14.0 (Shyer et al., 2013, 2015; Walton et al., 2016, 2012). PDGFRA is expressed in mesenchymal clusters that underlie villi, and PDGF signaling is functionally important for normal villus formation (Karlsson et al., 2000). We examined PDGFRA expression in

Figure 3. βcat-LOF Intestines Do Not Display Morphological Defects before E15.5

(A–F) H&E staining of βcat-LOF intestines at E13.5 and E14.5 (D and E) are indistinguishable from controls (A and B). E15.5 βcat-LOF (F) do not have prominent villus structures as in controls (C). Scale bar, 50 μm.

(G–N) Quantification of E-CADHERIN and DAPI double-positive cells (immunostaining not shown) divided by the total number of DAPI-positive cells per section (G) or as absolute cell number (H), reveals significant decrease in βcat-LOF intestines only at E15.5. Morphological analysis of total intestinal width/length (I and J) and epithelium width/length (L and M) was measured according to the schematic diagrams (K and N).

(O–Q) No significant differences were observed at E13.5 or E14.5. However, tracing the apical surface area (O; demonstrated in Q, red), revealed a significant reduction in βcat-LOF at E15.5, reflective of the loss of villus structures. Epithelial thickness (P), measured from the apical to basal surface (Q), was also reduced at E15.5. A, apical surface; T, epithelial thickness.

For all genotypes, n = 3–6 embryos pooled from two to five litters for five independent experiments. Error bars represent SD. Statistical significance by t test: *p = 0.01–0.05, ***p = 0.0001–0.001.

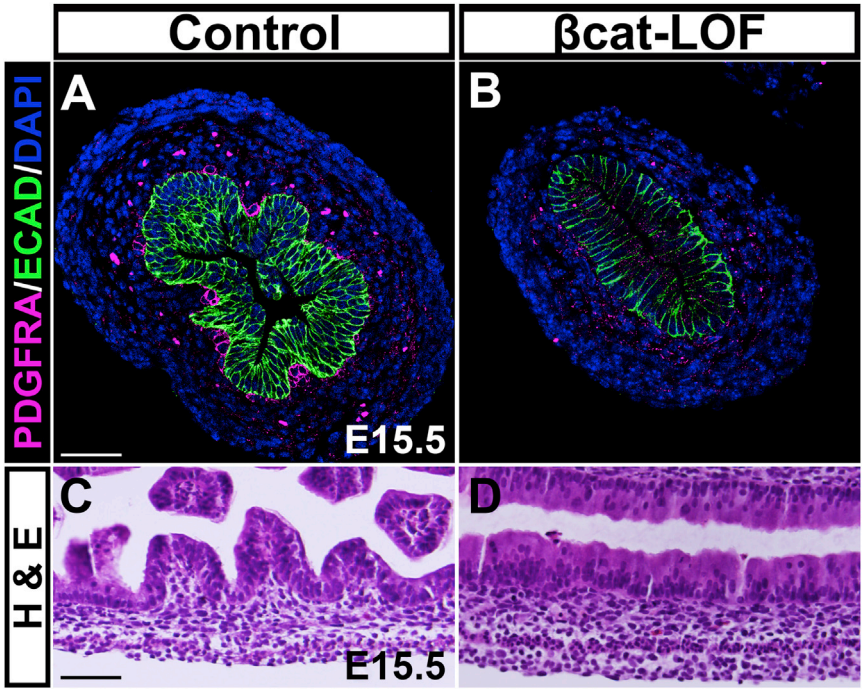

Figure 4. Loss of WNT/β-CATENIN Signaling Results in Perturbed Formation of PDGFRA$^+$ Mesenchymal Clusters

(A) Immunofluorescence staining of E15.5 control distal small intestine shows clusters of PDGFRA$^+$ (magenta) mesenchymal tissue beneath nascent villi.

(B) In bcat-LOF intestines, PDGFRA was still expressed in the mesenchyme, but did not condense into clusters adjacent to the epithelium.

(C and D) Longitudinal sections of E15.5 control intestine stained with H&E display numerous villi (C) while the βcat-LOF epithelium is flat (D).

Scale bars, 50 μm.

control and mutant intestines at E15.5 (Figures 4A and 4B). As expected in controls, the distal small intestine had several nascent villi forming at E15.5, which were present as a buckling of the E-CADHERIN-positive epithelium. In addition, nascent villi were associated with clustered PDGFRA-positive cells of mesenchyme directly adjacent to the buckling epithelium. In contrast, E15.5 βcat-LOF lacked aggregated PDGFRA$^+$ clusters (Figure 4B). It should be noted that PDGFRA staining was still observed in mesenchymal tissue, but that no evidence of cell clusters was present. H&E staining on longitudinal sections showed the flat epithelium in the βcat-LOF intestines, where control tissue showed regularly patterned nascent villi (Figures 4C and 4D). These results suggested that a loss of epithelial WNT/β-CATENIN signaling during villus formation either directly or indirectly affected normal cluster formation.

Mesenchymal WNT Ligand Secretion Is Required for Normal Epithelial Proliferation

Collectively, our data suggest that WNT/β-CATENIN signaling activity is low in the pseudostratified stages of intestine development, and that deletion of *β-catenin* or *Lrp5/6* has no discernible effect on proliferation at this time, but that active signaling is required for epithelial proliferation once villi are present. We wanted to elucidate the mechanism regulating the change in WNT/β-CATENIN signaling activity that occurs during the time of villus morphogenesis. One possibility is that expression of WNT ligands are increased as intestine development progresses. To determine whether WNT ligand expression increases over developmental time, we analyzed whole-thickness ileum from control intestines at E13.5 and E15.5 and looked for changes in mRNA for all 19 *Wnt* ligands (MacDonald et al., 2009) (Figures 5A and S5). Of the 19 *Wnt* ligands examined, only four ligands showed significant changes between E13.5 and E15.5. These included *Wnt5a* and *Wnt11*, which are involved in non-canonical WNT signaling, both of which were higher at E13.5 than E15.5. In contrast, we found that *Wnt3* and *Wnt7b* were upregulated (Figures 5A and S5A). To further characterize where *Wnt3* and *Wnt7b* are expressed, we mechanically separated E13.5 and E15.5 ileum into epithelial and mesenchymal fractions, as demonstrated by qRT-PCR for *E-cadherin* and *Vimentin*, respectively (Figure 5B). *Wnt3* was higher at E15.5 in both compartments while *Wnt7b* mRNA transcript was higher in the mesenchymal fraction (Figure 5B). To determine whether WNT ligands were functionally important at different times during development, we conditionally deleted *Wntless* in the epithelium or mesenchyme, which has been shown to block all WNT ligand secretion (Belenkaya et al., 2008; Franch-Marro et al., 2008a, 2008b). *Wntless-floxed* mice were crossed with *Twist2-Cre* for mesenchyme-specific deletion (Šošić et al., 2003) (MesWntless-LOF) and *Shh-Cre* for epithelium-specific deletion (EpWntless-LOF). MesWntless-LOF animals are embryonic lethal around E13.5, due to other organ defects (Cornett et al., 2013; Lange et al., 2014). Therefore we analyzed E13.5 embryos, and also explanted E13.5 intestinal tissue for ex vivo

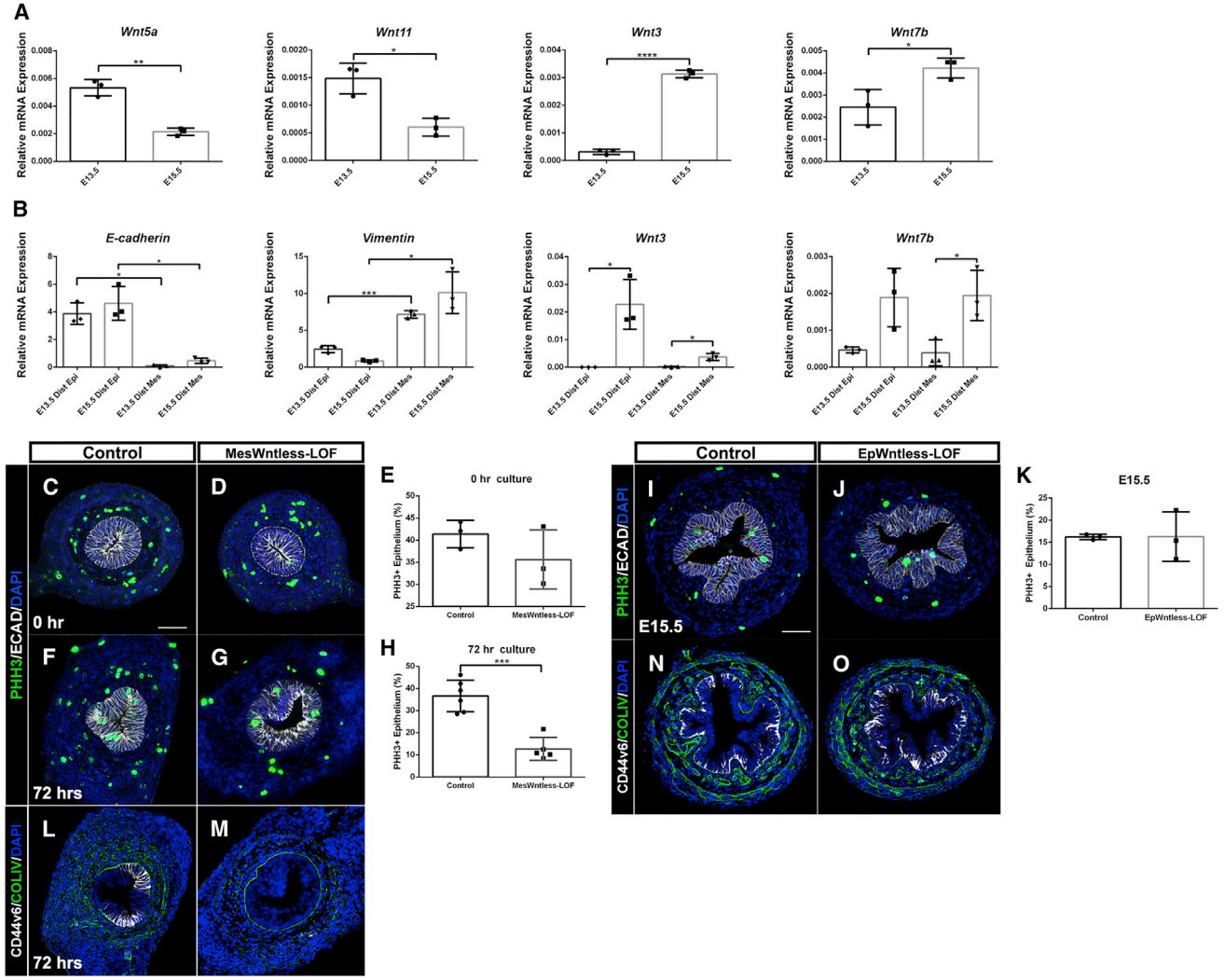

Figure 5. Mesenchymal WNT Ligand Secretion Regulates Epithelial Proliferation

(A) qPCR on whole-thickness control ilea from E13.5 (n = 3 embryos pooled from two litters) and E15.5 (n = 3 embryos from one litter) showed downregulation of *Wnt5a* and *Wnt11* transcript and upregulation of *Wnt3* and *Wnt7b* transcript.

(B) E13.5 and E15.5 epithelial isolations and mesenchymal isolations (each from n = 3 embryos for one independent experiment) are enriched for *E-cadherin* and *Vimentin*, respectively. *Wnt3* is significantly upregulated at E15.5 in both epithelial and mesenchymal compartments, while *Wnt7b* is only significantly upregulated in the mesenchyme and insignificantly increased in the epithelium.

(C–M) MesWntless-LOF E13.5 intestines (n = 3 embryos pooled from two litters for one independent experiment) cultured ex vivo for 0 hr show no proliferation defects, visualized by PHH3 (green) and E-CADHERIN (white) staining, compared with littermate controls (n = 3 embryos) (C and D). At 72 hr in culture, MesWntless-LOF intestines (n = 6 embryos pooled from two litters) have a significant reduction in epithelial proliferation compared with controls (n = 5 embryos pooled from two litters for two independent experiments) (F and G). $PHH3^+$ epithelial cells are quantified in (E) and (H). EpWntless-LOF intestines (J) showed no significant differences in $PHH3^+$ epithelial cells at E15.5 compared with controls (I) (quantified in K). WNT/β-CATENIN signaling target CD44v6 (white) is undetected in MesWntless-LOF when cultured for 72 hr (M) while controls exhibit robust epithelial staining (L).

(N and O) EpWntless-LOF E15.5 intestines (O) do not show any differences in CD44v6 staining compared with controls (N). EpWntless-LOF and controls each have n = 3 for one independent experiment.

Error bars represent SD. Statistical significance by t test: *p = 0.01–0.05, **p = 0.001–0.01, ***p = 0.0001–0.001, ****p = 0.00001–0.0001.

culture experiments. At E13.5 (0 hr of culture time), MesWntless-LOF intestines did not display any differences in proliferation compared with controls, as shown by the percentage of $PHH3^+$ epithelial cells (Figures 5C–5E). This is consistent with βcat-LOF and Lrp5/6-LOF data demonstrating that WNT/β-CATENIN signaling is not driving

epithelial proliferation at this developmental time (Figures 1, 2, and 3). However, following 72 hr of culture, MesWntless-LOF intestines had a significant reduction in the percentage of PHH3$^+$ epithelial cells compared with controls (Figures 5F–5H). Consistent with these findings, MesWntless-LOF intestines, but not controls, cultured for 72 hr showed a loss in epithelial CD44v6 protein staining by immunofluorescence, suggesting that WNT/β-CATENIN signaling is reduced in the epithelium (Figures 5L and 5M). In contrast, EpWntless-LOF did not show any changes in epithelial proliferation (PHH3) or CD44v6 staining at E15.5 (Figures 5I–5O). Collectively, our data show that blocking WNT ligand secretion at E13.5 from the mesenchyme or the epithelium does not result in proliferation defects. In contrast, we show that WNT ligands secreted from the mesenchyme at E15.5 are required for WNT/β-CATENIN target gene expression and proliferation in the epithelium.

DISCUSSION

Previous embryonic studies have shown that deletion of the *β-catenin* transcriptional binding partner *Tcf7l2* (*Tcf4*) or the WNT ligand co-receptors *Lrp5* and *Lrp6* resulted in a loss of proliferation and collapse of the intervillus compartment at late stages of fetal development (E17.5–E18.5), indicating that WNT signaling is critical for proliferation at this developmental time (Korinek et al., 1998; Zhong et al., 2012). In contrast, results from transgenic *Wnt* reporter mice (*TOP-GAL*) have suggested that WNT/β-CATENIN activity was absent from the proliferating intervillus domain until postnatal life (Kim et al., 2007). Our results collectively show that WNT/β-CATENIN has biphasic activity, with very low WNT signaling activity during the pseudostratified stages, and with robust WNT signaling activity after the onset of villus morphogenesis. Thus, it is possible that previously published studies have touched on both of these modes of regulation without full appreciation that there are different levels of WNT signaling at different developmental times. In addition, some conclusions in published literature have been drawn from transgenic reporter mice, which may not accurately report signaling activity in certain contexts. For example, while the *TOP-GAL* mouse has been shown to faithfully report WNT/β-CATENIN signaling in the adult intestine (Davies et al., 2008), side-by-side comparisons of *TOP-GAL* and *Axin2-LacZ* reporter activity have indicated that multimerized *Tcf/Lef* reporter mice may not always be faithful (Al Alam et al., 2011; Barolo, 2006).

Here, we presented several lines of evidence that suggest that there are two distinct mechanisms regulating fetal intestinal progenitor cell proliferation. During the pseudostratified stage of development at E13.5 and E14.5, epithelial progenitor cell proliferation occurs normally in the absence of WNT/β-CATENIN signaling, whereas after villus morphogenesis (E15.5), proliferating progenitor cells require WNT/β-CATENIN signaling. Mechanistically, our data point to increased WNT ligand expression in the mesenchyme as a major player in this developmental switch to WNT-dependent proliferation. However, our data do not totally rule out alternative scenarios. For example, it is also possible that ligands that augment WNT signaling, such as R-SPONDIN proteins, also change over developmental time (Kamata et al., 2004; Kim et al., 2008); and yet a second alternative possibility exists whereby an inhibitor of WNT signaling, such as DKK proteins, may be reduced over developmental time (Bafico et al., 2001; Mao et al., 2001; Tamai et al., 2000).

A current unresolved question that still remains is how proliferation is regulated during the pseudostratified stage. Interestingly, we also observed that SOX9 expression, which is a strong WNT/β-CATENIN signaling target gene in the late embryonic and adult intestine (Bastide et al., 2007; Blache et al., 2004), was still present in mutant mice during the pseudostratified stages, and SOX9 expression was not lost until WNT-dependent proliferation began after villus morphogenesis. Interestingly, studies in the embryonic lung have shown that *Sox9* is not regulated by WNT/β-CATENIN; rather, it is likely downstream of FGF signaling (Chang et al., 2013; Rockich et al., 2013). Moreover, *Fgf10* has been demonstrated to play a role in suppressing cytodifferentiation in the developing intestine (Nyeng et al., 2011). Thus, it is interesting to speculate that fibroblast growth factor signaling may play a role regulating progenitor cell proliferation during the pseudostratified stage. In addition, recent work has shown that GATA4 binds to several cell-cycle genes, and that epithelial deletion of *Gata4* at the pseudostratified stage leads to a loss of proliferation, which recovers following villus morphogenesis (Kohlnhofer et al., 2016). Given that *Gata4* is a retinoic acid (RA) signaling target gene in some contexts (Arceci et al., 1993; Ghatpande et al., 2000), it is also possible that an RA-GATA4 signaling axis controls early progenitor proliferation. Future studies aimed at elucidating the mechanisms regulating progenitor cell proliferation during the pseudostratified stages will no doubt prove interesting, as will studies demonstrating how stem/progenitor cells change across developmental time to acquire their adult state.

Our results showing that mesenchymal, but not epithelial WNT ligands are required for epithelial proliferation are consistent with recent studies in the adult intestine showing that epithelial WNT ligands are dispensable for epithelial proliferation, and that the mesenchyme is the

primary source for WNT ligand-driven epithelial proliferation (San Roman et al., 2014; Valenta et al., 2016). Interestingly, our qRT-PCR screen identified two *Wnt* ligands, *Wnt3* and *Wnt7b*, which increase between E13.5 and E15.5. While additional studies are needed to determine whether these ligands are responsible for the transition from a WNT-independent stage of growth to a WNT-dependent stage of growth, it is interesting to note that *Wnt7b* is not expressed in the adult intestine, and *Wnt3* is strongly expressed in the epithelium (Farin et al., 2012). In the adult, evidence suggests that mesenchymal WNT2b may be a critical WNT ligand for epithelial proliferation, although there are likely redundant sources and redundant WNT ligands that support the epithelium in the adult (Farin et al., 2012; Valenta et al., 2016). Therefore, it is also interesting to speculate that the specific WNT ligands responsible for WNT-driven proliferation may be different in the E15.5 intestine when compared with the adult intestine.

In summary, we report a stage of growth during the pseudostratified stage of intestine development whereby progenitor cell proliferation does not require WNT/β-CATENIN signaling. Our data show that WNT target gene expression is low during this stage, and genetically blocking WNT/β-CATENIN signaling has no observable effect. In contrast, following the onset of villus morphogenesis, mesenchymal WNT ligands are required for β-CATENIN-dependent epithelial proliferation. These findings show that stem/progenitor cells are not regulated in the same way across development and into adulthood, and open up exciting opportunities to explore how ISCs acquire their adult identity and how embryonic progenitors differ functionally from their adult counterparts.

EXPERIMENTAL PROCEDURES

Mice

All experiments conducted in this study were approved by the University of Michigan, the Van Andel Research Institute, and the Medical College of Wisconsin's institutional animal use and care committees. All mice used in this study have been previously reported: *Shh-Cre* (Harfe et al., 2004), *βcat f\f* (Brault et al., 2001), *Lrp5/6 f\f* (Zhong et al., 2012), *Axin2-LacZ* (Lustig et al., 2002), *E-cadherin f\f* (Boussadia et al., 2002), *Twist2-Cre* (Šošić et al., 2003), and *Wntless* (Carpenter et al., 2010). Control mice used were of the following genotypes: *βcat f/f*, *βcat f/+*, *Shh-cre;βcat f/+*, *Lrp5 f/f;Lrp6 f/f*, *Lrp5 f/f;Lrp6 f/+*, *Lrp5 f/+;Lrp6 f/f*, *Lrp5 f/+;Lrp6 f/+*, *Shh-cre;Lrp5 f/+;Lrp6 f/f*, *Shh-cre;Lrp5 f/+;Lrp6 f/+.Wntless f/f*, *Wntless f/+*, and *Twist2-cre;Wntless f/+*.

Ex Vivo Culture

Ex vivo cultures were performed as described by Walton et al. (2012). In brief, E13.5 intestines were dissected from the embryo and placed on 6-well transwell plates (Costar 3428) in basal media: Advanced DMEM/F12 (Gibco 12634-010) supplemented with 1% penicillin-streptomycin (v/v) (Invitrogen 15140-122), 1× HEPES (Invitrogen 15630080), 1× B27 (Invitrogen 0080085-SA), and 10% fetal bovine serum (FBS) (Invitrogen). E13.5 control and *Twist2-Cre;Wntless f/f* intestines were cultured for 72 hr in basal medium at 37°C with 5% CO_2 with medium changes every 24 hr.

Tissue Preparation

For histology, *Shh-Cre;Lrp5 f/f;Lrp6 f/f*, *Shh-Cre;βcat f/f*, *Twist2-Cre;Wntless f/f*, *Shh-cre;Wntless f/f*, and control tissues were fixed overnight in 4% paraformaldehyde and dehydrated through a 25:75, 50:50, 75:25, 100% methanol to PBSt (1× PBS with 0.5% Triton X-100) series. Following dehydration the intestines were cut into equal segments, representing the proximal, middle, and distal thirds of the small intestine, and set into Histogel (Thermo Fisher HG-4000-012) to maintain orientation. Tissues were then equilibrated in 100% ethanol and embedded into paraffin. Sections were cut 7 μm thick by a microtome.

Epithelial/Mesenchymal Isolations

For epithelium and mesenchymal isolations, E13.5 and E15.5 intestines were dissected from the embryo in cold PBS. Connective tissue was removed and the distal one-third of the small intestine (ileal segment) was placed into a fresh Petri dish on ice-cold PBS. PBS was removed from the Petri dish and tissues were incubated in Dispase (Corning 40-235) for 30 min on ice. The Dispase was then removed and tissues were incubated in 100% FBS (Invitrogen) for 15 min on ice to stop Dispase activity. An equal volume of Advanced DMEM/F12 (Gibco 12634-010) was added to the Petri dish, and the epithelium and mesenchyme were mechanically separated with tungsten needles.

Immunohistochemistry

Paraffin sections were deparaffinized in Histoclear and rehydrated into PBS. Antigen retrieval for all primary antibodies (except anti-CD44v6 staining), was performed by heating slides to near boiling (99°C) in a rice steamer in sodium citrate buffer for 20 min. Antigen retrieval for anti-CD44v6 was conducted in a 2100 Antigen Retriever (Electron Microscopy Sciences 62700-10) in 1× R-Buffer A (Electron Microscopy Sciences 62706-10). Sections were blocked in donkey serum (5% serum in 1× PBS + 0.5% Triton X-100) for 1 hr. Antibody information and dilutions are presented in Table S1. Primary antibodies were diluted in blocking buffer and incubated on tissue sections overnight at 4°C. Slides were washed in 1× PBS and incubated in secondary antibody in blocking buffer for 2 hr at room temperature, then counterstained with DAPI. Slides were washed and mounted using Prolong Gold antifade reagent. DAB staining was performed as previously described (Spence et al., 2009). Immunohistochemistry for CD44v6 was additionally amplified with Tyramide Signal Amplification kits (Life Technologies T20935 and T20932) according to the manufacturer's protocol. Images were taken on an Olympus IX71 microscope at 40×. Higher-magnification images were taken on a Nikon A1 confocal microscope at 60× plus digital zoom.

LacZ Staining and Histology Analysis

LacZ staining was performed as previously described (Spence et al., 2009). β-Galactosidase activity was detected in fixed whole tissue

using the Histomark X-gal substrate system (Kireguard and Perry Laboratories). For H&E staining, 6-μm paraffin sections were deparaffinized in xylene, rehydrated, and stained.

Morphometric Analysis, Immunofluorescence Quantification, and Statistical Analysis

Morphometric measurements were conducted with ImageJ software using the Cell Counter plugin. Differences between two groups were evaluated using an unpaired two-tailed Student's t test. Homogeneity of variance was validated for these parametric tests using the Bartlett test. A p value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 6. For all genotypes, $n \geq 3$.

RNA Isolation and qRT-PCR Analysis

Embryos were dissected and tissues were frozen with liquid nitrogen for storage. For RNA extraction, tissues were ground with a pestle before RNA was extracted using the Purelink RNA Mini Kit (Life Technologies). RNA quantity and quality was assessed with a Nano Drop 2000 (Thermo Fisher Scientific). Reverse transcription was conducted using the SuperScript VILO kit (Invitrogen) according to the manufacturer's protocol.

qRT-PCR was conducted using Quantitect Sybr Green Mastermix (Qiagen) on a Step One Plus Real-Time PCR system (Life Technologies). Reactions for *Wnt* ligands were run for 45 cycles while all other reactions were run for 40 cycles. Gene expression analysis was determined using a standard curve and was normalized to the housekeeping gene *GAPDH*. See Table S2 for primer sequences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.09.004.

AUTHOR CONTRIBUTIONS

A.M.C. and J.R.S. conceived of the study, designed and conducted experiments, analyzed data, and wrote the manuscript. Y.-H.T., S.R.F., M.S.N., E.M.W., N.J.E., M.A.B., and B.O.W. conducted experiments and provided critical revisions to the manuscript.

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