Elucidating a novel WNT/β-CATENIN signaling-independent environment that precedes villus morphogenesis in the embryonic intestine

by

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“Courage is being afraid to do something but doing it anyway.”
-Dr. Yvonne Wong, DDS-- my childhood dentist before my tooth extraction

This is dedicated to everyone who has ever been afraid to aim high.
Have courage.

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ABSTRACT

The intestine is a vital organ responsible for several functions, including excretion of waste, acting as a major site of host immunity, and most importantly, absorption of nutrients. In order to fulfill the body's daily demands for energy and nutrients, the intestine evolved to expand the absorptive surface area by undergoing a morphogenetic process that generates finger-like units called villi. These villi house specialized cell types critical for both absorbing nutrients from food and for protecting the host from commensal and pathogenic microbes. Villus morphogenesis occurs in the developing embryo and is a complex process that requires the successful coordination of many events, during which the epithelium, initially a tube with a flat apical surface, remodels into a complex structure with stereotypical villus units. During this process, the epithelium and mesenchyme undergo rapid cell division to support tissue expansion. Concomitantly, cell signaling crosstalk between the epithelium and mesenchyme drives the formation and patterning of regularly distributed mesenchymal cell clusters, which aggregate adjacent to the pseudostratified epithelium and demarcate nascent villi.

Currently, our understanding of the molecular mechanisms regulating these processes is incomplete. This thesis work focuses on one part of villus morphogenesis, specifically the propagation of epithelial progenitors before and during the remodeling of the flat epithelia into protruding villus structures. It is unclear what signaling pathway

drives epithelial proliferation before villus formation. One candidate pathway is canonical WNT/β-CATENIN signaling, as WNT/β-CATENIN signaling is essential for maintenance of the adult intestinal stem cell population and loss of WNT/β-CATENIN signal transduction results in the collapse of villus and crypt structures.

In this work, I elucidated the role of WNT/β-CATENIN signaling before and during villus morphogenesis. First, I characterized WNT/β-CATENIN signal transduction using a reporter mouse model and found that before villus formation, reporter activity was very low in the pseudostratified epithelium, but after the emergence of nascent villi, reporter activity was robust and restricted to the intervillus domains. Next I conducted loss-of-function studies with two genetic mouse models that perturb WNT/β-CATENIN signal transduction in the epithelium and observed defects in epithelial proliferation and villus formation at E15.5, while mutant animals displayed no defects at earlier time points (prior to E14.5). Additionally, I found that secretion of mesenchymal WNT ligands, possibly WNT3 and WNT7b, were required for normal epithelial proliferation. Together, these data indicate that there are two phases of growth during villus morphogenesis: One before villus morphogenesis, in which WNT/β-CATENIN signal transduction is low and dispensable for epithelial proliferation, and another after villus emergence, characterized by robust and patterned epithelial WNT/β-CATENIN signal transduction (requiring mesenchymal WNT ligands) that is critical for epithelial proliferation. In sum,

this dissertation provides novel insight into the role of WNT/β-CATENIN signaling to drive proliferation of epithelial progenitors during villus morphogenesis.

CHAPTER 1

INTRODUCTION*

Intestine overview

The mature intestine is a highly complex organ with several essential functions. The small intestine interacts with food after it has been broken down in the stomach. Carbohydrates, proteins, and other nutrients are absorbed by intestinal enterocytes into a highly integrated vascular network.

In addition to absorbing nutrients, the intestine has important roles in host immunity. Within the intestine, luminal contents come into contact with an epithelial layer, which must serve as a barrier to the outside environment and protect the body against indigenous (commensal) microbes and pathogens. Critical to this barrier are epithelial tight junctions which selectively limit the passage of luminal contents in between epithelial cells (Turner, 2009). In addition, the epithelium secretes mucus,

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which lines the intestinal tract and serves as a dense barrier that can trap microbes to inhibit infection (Turner, 2009), and can also provide a rich source of nutrients for commensal bacteria (Ley et al., 2006; Dethlefsen et al., 2007). Specialized cells of the intestinal epithelium also play an important role in host immunity by secreting antibacterial and antifungal peptides (Salzman et al., 2003; Chu et al., 2012; Clevers and Bevins, 2013). Moreover, colonization by commensal bacteria at birth stimulates immune system development and is necessary for proper immune function (Round and Mazmanian, 2009).

To adequately fulfill the cellular demands of these complex functions, the small intestinal epithelium is organized into villi, which are finger-like structures that protrude into the lumen. The intestine has a high rate of epithelial cell turnover, driven by proliferative epithelial stem cells housed at the base of the villi in domains called crypts (Figure 1.1). Stem cell-driven proliferation fully regenerates the intestinal lining every 5-7 days (Creamer et al., 1961; Cheng and Leblond, 1974a; 1974b; Potten et al., 1974; Barker et al., 2007; Sato et al., 2009). As these stem cells divide, they differentiate and move along the villus structures like a conveyor belt. Once they reach the villus tips, cells undergo programmed cell death and slough off into the lumen.

The highly archetyped crypt-villus structures of the adult intestine emerge over developmental time through the coordination of several complex processes that govern tissue patterning, cell fate, and morphogenesis. Early in embryonic development, the intestinal epithelium is a relatively flat, tube-shaped structure which undergoes a

process called villus morphogenesis through which the relatively flat tube-shaped intestine gives rise to villus structures. Villus structures project into the lumen, expanding the apical surface area of the absorptive epithelium. Morphogenesis of these projections is evolutionarily conserved, and therefore a positive adaptation of fitness, found in many vertebrates including the chicken and mouse, but also in zebrafish, seahorses, snakes, and amphibians (McAvoy and Dixon, 1978; Shyer et al., 2013). This morphogenesis is responsible for a massive expansion of intestinal surface area; it is estimated that the absorptive surface area of the adult human intestine is 30 m^2, with villus structures amplifying the surface area by 6.5 fold (Helander and Fändriks, 2014). Abnormal loss of absorptive surface area hinders nutritional uptake and can lead to malabsorption or intestinal failure (Goulet et al., 2004).

Here, I will discuss the molecular, biochemical, and biophysical events that guide normal intestine development, with a focus on mammalian development including human intestinal development where possible. Specifically, I will cover developmental events starting after gut tube formation and through early postnatal life.

Models of the developing intestine

Historically, many studies of vertebrate intestinal development have been carried out in the chick and mouse. Chick embryos are easy to acquire, develop rapidly, are low cost, and can be easily manipulated experimentally. However, tools for genetic manipulation in a tissue specific manner are more limited in the chick. Additionally, there are significant differences between avian and mammalian gut development that may

limit cross-species comparisons (Walton et al., 2016a). The embryonic mouse develops in a similar time frame to the avian embryo (19-21 days), and as an advantage, has an extensive set of tools for tissue specific genetic manipulation. Drawbacks include larger housing costs, long breeding times to obtain genetic crosses, relatively small litter sizes, and internal development which hinders experimental access to the developing tissue. Most importantly, it is not entirely clear which aspects from these models may be directly applicable to human intestine development, since our understanding of human intestine development is severely limited at this time.

However, access to human fetal tissue and in vitro tissue culture techniques using human pluripotent stem cells (hPSCs) have begun to shed some light into human intestine development. Indeed, recent access to high-resolution 3D-reconstructions of early stage human embryos in addition to histological sections will likely improve our understanding of the early stages of human fetal gut development (de Bakker et al., 2016). However, most studies of human fetal tissue are limited to descriptive analyses. hPSCs, which include both embryonic and induced pluripotent stem cells, provide a highly tractable solution to the limitations inherent to fetal tissue. hPSCs can be differentiated into complex 3-dimensional (3D) intestinal tissue using soluble growth factors and/or small molecules in a step-wise process known as directed differentiation (Spence and Wells, 2007; Finkbeiner and Spence, 2013; Wells and Spence, 2014). Directed differentiation aims to recapitulate key developmental stages in vitro. In the case of intestinal tissue, hPSCs undergo a gastrulation-like process that gives rise to a mixed endoderm/mesoderm population, followed by posterior patterning events,

intestinal specification and gut-tube morphogenesis which gives rise to small self-assembling 3D structures that can be expanded into ‘organoids’ (McCracken et al., 2011; Spence et al., 2011; Forbester et al., 2016; Turner et al., 2016). Intestinal organoids have been reviewed extensively elsewhere (Wells and Spence, 2014; Finkbeiner et al., 2015; Sato and Clevers, 2015; Aurora and Spence, 2016; Dedhia et al., 2016).

Recent studies have shown that intestinal organoids derived from hPSCs are most similar to fetal intestine but do not form villi in culture (Spence et al., 2011; Watson et al., 2014; Finkbeiner et al., 2015; Aurora and Spence, 2016; Hill and Spence, 2016). Intestinal organoids transplanted into the mouse kidney capsule engraft, form villus and crypt structures, and undergo enhanced cellular, molecular and structural maturation, resulting in more adult-like tissue (Watson et al., 2014; Finkbeiner et al., 2015). In addition to hPSC-derived organoids, *in vitro* culture of primary human fetal intestinal epithelium (fetal organoids) is also shedding light on the cellular dynamics of the human fetal intestine (Fordham et al., 2013). Collectively, hPSC-derived organoids and fetal organoids provide a powerful new platform for investigating human development, since both systems are experimentally tractable, allowing for long-term growth, and genetic and pharmacologic manipulation.

Intestinal specification and gut tube patterning.

In the case of human gastrulation, like the chick, the endoderm, mesoderm and ectoderm lineages are specified and are present as a flat, layered disc-shaped structure

(reviewed elsewhere: (Zorn and Wells, 2009; Spence et al., 2011; Le Guen et al., 2015; Deglincerti et al., 2016; Shahbazi et al., 2016). As development progresses, the body of the embryo rotates from a flat to a fetal position where the ectoderm is present on the outside of the embryo and the endoderm, wrapped by mesoderm, is present on the inside of the embryo (Lewis and Tam, 2006). Conceptually, the endoderm can be visualized as a flat sheet of paper that is folded into a tube that must be sealed in the middle as the two sides come together. In the mouse, gut tube closure is complete by embryonic day (E) 9.0 (reviewed in Lewis and Tam, 2006; Spence et al., 2011), but mutant mice lacking GATA4, SOX17, and FURIN/SPC1 fail to rotate properly and have open gut tubes (Molkentin et al., 1994; Kuo et al., 1997; Roebroek et al., 1998; Constam and Robertson, 2000; Kanai-Azuma et al., 2002).

During embryo rotation and coinciding with complex morphological events that shape the tissue, the nascent gut tube is patterned into different domains along the anterior-posterior axis. Secreted morphogens help to establish region-specific gene regulatory networks, segmenting the gut tube into domains with distinct molecular characteristics that will ultimately give rise to different organs (Wells and Melton, 2000; McLin et al., 2007; Rankin et al., 2011). This process is reviewed in detail elsewhere (Wells and Melton, 1999; Lewis and Tam, 2006; Spence and Wells, 2007; Sherwood et al., 2009; Zorn and Wells, 2009; Sherwood et al., 2011; Spence et al., 2011; Tanaka et al., 2011; Arkell et al., 2013; Wells and Spence, 2014). For example, the foregut and hindgut domains of the endoderm are separated by expression of SOX2 and CDX2, respectively (Que et al., 2007; Gao et al., 2009; Sherwood et al., 2009; 2011). The

anterior region of the gut tube, which gives rise to the esophagus and stomach in addition to the lungs, liver, and pancreas, initially expresses SOX2, which sets up a sharp boundary at the pylorus (Li et al., 2009; Sherwood et al., 2009). Adjacent to this SOX2 boundary is the posterior region of the gut tube, which will give rise to the small and large intestine, marked by CDX1, 2, and 4 expression (Dufort et al., 1998; Martinez Barbera et al., 2000; Chawengsaksophak et al., 2004; Kinkel et al., 2008; Gao et al., 2009; Sherwood et al., 2009; Zorn and Wells, 2009; Grainger et al., 2010).

Interestingly, while CDX (CDX1, 2, 4) proteins have been shown to play redundant roles in intestinal patterning during development (van den Akker et al., 2002; Savory et al., 2009; Verzi et al., 2011), *Cdx2* is considered to be a master regulator of intestinal identity; conditional deletion of *Cdx2* in the epithelium resulted in complete loss of the intestinal gene expression program as well as loss of intestinal structure (Gao et al., 2009; Grainger et al., 2010). In these mouse mutants, the gut tube formed normally; however, mutant tissue adopted an esophagus-like fate suggesting *Cdx2* is absolutely required for intestinal commitment. Conditional deletion of *Cdx2* later in development, around E13.5, resulted in transformation of the intestine into stomach-like tissue (Grainger et al., 2010). Collectively, these studies suggest that *Cdx2* is critical for not only for specification but also for maintenance of intestinal identity during development. Interestingly, in the adult, loss of *Cdx2* does not lead to homeotic transformations, but instead impairs enterocyte differentiation, suggesting that *Cdx2* affects intestinal identity only in the developing embryo (Verzi et al., 2010; 2011).

The WNT/β-CATENIN signaling pathway and its role in gut tube patterning

WNT signaling is critical for mid- and hindgut development, and plays a central role in inducing *Cdx2* gene expression and specifying the intestinal endoderm both *in vivo* and *in vitro* (Gregorieff et al., 2004; McLin et al., 2007; Sherwood et al., 2011; Spence et al., 2011; Ren et al., 2015). WNT ligands are secreted molecules that bind to cognate receptors and transduce downstream signaling. In vertebrates, WNT ligands are lipid modified with the addition of a palmitate group by the palmitoyltransferase PORCUPINE (Najdi et al., 2012). These lipid groups are recognized by the transmembrane protein WNTLESS and shuttled to the plasma membrane for secretion. Deletion of *Wntless* or *Porcupine* prevents secretion of WNT ligands and abrogates WNT signaling (Willert et al., 2003; Galli et al., 2007; Komekado et al., 2007; Kurayoshi et al., 2007). Secreted WNT ligands interact with neighboring cells and bind to FRIZZLED and lipoprotein receptor-related proteins (LRP) receptors (Figure 1.2). Unpalmitoylated WNT proteins cannot bind to FRIZZLED receptors (Komekado et al., 2007; Kurayoshi et al., 2007). It is thought that the hydrophobic palmitylated lipid side chains (Willert et al., 2003) create a high affinity to cell membranes, and therefore operate as short-range signals, rather than long-range morphogens; although how exactly specific WNT ligands travel and bind to their targets is not well understood.

In canonical WNT signaling, also known as WNT/β-CATENIN-dependent signaling, WNT ligands bind to FRIZZLED receptors and LRP co-receptors, allowing them to form a receptor complex and undergo conformational change that allows their phosphorylation by protein kinases (Tamai et al., 2004; Davidson et al., 2005; Zeng et

al., 2005). When the cytoplasmic tail of LRP5/6 receptors are phosphorylated, AXIN is sequestered and glycogen synthase kinase 3 (GSK3) activity is inhibited. Without WNT ligand binding, GSK3 forms a destruction complex with AXIN and anaphase-promoting complex (APC) (Clevers, 2006) where GSK3 phosphorylates co-regulator β-CATENIN, marking it for ubiquitination by E3 ubiquitin ligases and proteasomal degradation (Figure 1.2). Simultaneously, TCF/LEF transcription factors are bound to the corepressor GROUCHO, which keeps transcription off (Arce et al., 2006). However, with WNT ligand binding to FRIZZLED and LRP5/6 receptors, phosphorylated LRP5/6 sequesters AXIN, preventing the formation of the destruction complex and subsequent GSK3 phosphorylation of β-CATENIN (Tamai et al., 2004; Zeng et al., 2005). This allows β-CATENIN to accumulate in the cytosol. Once cytosolic β-CATENIN accumulates to a certain threshold, β-CATENIN translocates into the nucleus, replaces GROUCHO to bind to TCF/LEF transcription factors, and activates transcription of downstream target genes. Some well-characterized target genes include *Axin2*, *Lgr5*, *Cd44*, *Myc*, and *Ccnd1* (Clevers and van de Wetering, 1997; He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999; Blache et al., 2004; Li et al., 2009; Shyer et al., 2015). Additional modulators of canonical WNT signaling include Dickkopf (DKK) and secreted Frizzled-related proteins (SFRP) inhibitors, which disrupt ligand-receptor binding (reviewed in Kawano and Kypta, 2003) (Figure 1.3). Agonists of canonical WNT signaling include R-spondin (RSPO) proteins, which stabilize FRIZZLED receptors by antagonizing their degradation by E3 ubiquitin ligases RNF43/ZNRF3 (Hao et al., 2012; Koo et al., 2012) (Figure 1.3).

Several studies have shown that WNT/β-CATENIN signaling transduction is present at higher levels on the posterior end of the developing embryo and several WNT ligands are highly expressed in this region (Christian et al., 1991; Krauss et al., 1992; Moon et al., 1993; Kelly et al., 1995; McLin et al., 2007; Hong et al., 2008; Hikasa and Sokol, 2013). In addition, as the embryo elongates, WNT/β-CATENIN signaling transduction remains active in the posterior end of the embryo after WNT/β-CATENIN signaling transduction turns off in the anterior. This sets up both temporal and spatial gradients within the embryo, where the developing midgut endoderm is exposed to WNT signaling at lower levels and for shorter periods of time whereas hindgut endoderm is exposed to WNT signaling at higher levels for a longer period of time. There is emerging evidence that these early signaling gradients in the embryo endoderm may help to establish intestinal regional identity, setting up the different domains of the intestine: the duodenum, jejunum, ileum and colon. This notion is supported by studies using mouse embryonic explants. Stimulating endoderm with high levels of WNT signaling transduction led to induction of posterior small intestine and colonic gene expression in the endoderm (Sherwood et al., 2011). Interestingly, stimulating mouse embryonic stem cell (mESC) derived endoderm with high levels of WNT signaling induced a CDX2-positive small intestinal fate, but was unable to induce a colonic fate, suggesting that other factors may cooperate with WNT signaling to drive colonic specification (Sherwood et al., 2011). In line with these studies in mouse embryos and mESCs, recent studies using hPSC-derived intestinal organoids demonstrated that the duration of Wnt/β-catenin stimulation in hPSC-derived endoderm cultures was associated with intestinal patterning, with shorter durations specifying

proximal small intestine-like organoids (duodenum) and longer durations specifying distal small intestine-like organoids (ileum). Interestingly, and similar to mESC-derived endoderm, colonic gene expression was not induced in these studies, suggesting that colonic specification may require additional signals (Tsai et al., 2016b).

Non-canonical WNT signaling as a regulator of gut tube elongation

WNT ligands can also signal independently of β-CATENIN through non-canonical pathways (Kestler and Kühl, 2008). These pathways are less understood than the β-CATENIN dependent pathway, and manifest in at least three distinct mechanisms (for detailed reviews, please see (Veeman et al., 2003; Fanto and McNeill, 2004; Kohn and Moon, 2005; Semenov et al., 2007; van Amerongen et al., 2008). Briefly, one of these is the WNT/calcium pathway where certain WNT ligands bind to FRIZZLED receptors and activate calcium/calmodulin-dependent kinase II (CAMKII) and protein kinase C (PKC) (Kühl et al., 2000). Alternately, certain FRIZZLED receptors can interact with GTP-binding proteins to activate phospholipase C (PLC) and phosphodiesterase (PDE). Lastly, the planar cell polarity pathway (PCP) occurs when FRIZZLED activates Jun-N-terminal kinase (JNK) (Qian et al., 2007). The PCP pathway has been implicated in gut tube elongation during development through WNT5a (Qian et al., 2007; Cervantes et al., 2009) (further discussed below).

After gut tube patterning, by E9.5 in the mouse the CDX2+ epithelium becomes a simple pseudostratified epithelium (Grosse et al., 2011). From E9.5 to E14.5 in the mouse, the epithelium and mesenchyme rapidly proliferate, resulting in elongation of the

gut tube and increased intestinal length, circumference, and luminal area (Lepourcelet et al., 2005; Cervantes et al., 2009). It is known that the increase in intestinal length/girth during early development is mediated in part by non-canonical WNT signaling through the PCP pathway (Qian et al., 2007; Cervantes et al., 2009). Misregulation that either decreases or increases WNT5a signaling leads to defects in gut lengthening. Studies on *Wnt5a* null mice demonstrated significantly shorter gut tubes with bifurcation of the duodenum and perturbed midgut elongation as well as a truncation at the cecum (Cervantes et al., 2009). Defects were apparent by E10.5, at the onset of midgut elongation, and corresponded to reduced epithelial proliferation. Similarly, mice lacking *Sfrp*, an inhibitor of WNT5a, display shortened guts with ectopic clumps of epithelia that protrude into the lumen at E13.5. Epithelial clumps displayed aberrant localization of aPKC, β1-INTEGRIN, and E-CADHERIN, indicating defects in apicobasal polarity (Matsuyama et al., 2009). Notably, improper cell intercalation in frogs also results in gut lengthening defects (Dush and Nascone-Yoder, 2013).

HH signaling and its role in the early intestine

In addition to WNT signaling, Hedgehog (HH) signaling is an important regulator of intestinal development as it is required for differentiation of smooth muscle and villus formation. Mechanistically, in the absence of HH ligands, Patched (PTCH) receptors, 12-transmembrane pass proteins, inhibits the 7-transmembrane pass protein Smoothened (SMO) and renders it inactive (Figure 1.4). When HH ligands Sonic Hedgehog (SHH), Indian Hedgehog (IHH), or Desert Hedgehog (DHH) (Marigo et al., 1995) bind to PTCH receptors, PTCH releases inhibition of SMO (Alcedo et al., 1996;

van den Heuvel and Ingham, 1996; Taipale et al., 2002). Active SMO activates glioma-associated oncogene (GLI) transcription factors, GLI1, GLI2, and GLI3, whose behavior varies in different contexts. In most contexts, GLI1 is both the main activator and target gene of the pathway, while GLI2 and GLI3 can act as activators or repressors in different systems. During development of the gut, GLI2 and GLI3 appear to be the main activators of the pathway, as mice null for *Gli2* display esophageal defects while mice null for *Gli3* display stomach defects (Motoyama et al., 1998; Mo et al., 2001; Kim et al., 2005). *Gli1* null mice display normal embryonic development (Park et al., 2000; Bai et al., 2002). In the developing intestine, GLI2, and not GLI3, appears to be the major effector of HH signaling. Loss of *Gli3* is dispensable for normal growth while activation of GLI2 fully rescues *Smo*-null intestinal defects (Huang et al., 2013). Several transcriptional targets and faithful readouts of the pathway include *Ptch1*, *Ptch2*, and *Gli1*. Other mediators of the HH pathway include co-receptors CDO, BOC, and GAS1 that also bind HH ligands and can interact with PTCH to positively regulate signaling (Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006).

The HH signaling pathway is required for normal intestine lengthening during early intestinal development. Conditional epithelial-specific deletion of *Ihh* by E10.5 resulted in loss of mesenchymal proliferation and dramatically shortened intestines. E12.5 IHH-deficient intestines were 10% the length of their control counterparts (Mao et al., 2010). Also taking place during this early developmental time is formation of the smooth muscle layers, which surround the gut tube to provide structure and later aid in peristalsis (reviewed in McHugh, 1996). Smooth muscle differentiation starts around

E11 in the mouse and proceeds in a proximal to distal wave along the length of the intestine. At E12, a layer of mesenchymal cells become circularly arranged and forms a distinct layer of alpha smooth muscle actin (αSMA) expressing circular muscle (Sbarbati, 1982; Geske et al., 2008; Walton et al., 2016b). Through the remainder of development, three distinct layers of smooth muscle are patterned—the circular smooth muscle and longitudinal smooth muscle of the muscularis propria, and the longitudinal smooth muscle of the muscularis mucosae (McHugh, 1996). Smooth muscle differentiation is dependent on HH signaling from the epithelium. HH ligands, SHH and IHH, are expressed in the epithelium and signal to PTCH1 and GLI1 expressing mesenchymal cells at early developmental stages (Ramalho-Santos et al., 2000; Kolterud et al., 2009). Classical experiments conducted in chick and mouse demonstrate that overexpression of SHH expands gut mesoderm and induces smooth muscle differentiation (Roberts et al., 1995; Apelqvist et al., 1997; Roberts et al., 1998). Mice deficient in SHH or IHH display a 20-30% reduction in thickness of the circular smooth muscle layer at E18.5 (Ramalho-Santos et al., 2000). Epithelial-specific conditional deletion of *Ihh* by *Shh-Cre* results in the complete loss of smooth muscle actin (SMA) expressing cells at E12.5 (Mao et al., 2010). Gain of function studies expressing constitutively active SMO in early gut mesenchyme or overexpression of IHH in the epithelium demonstrated that ectopic activation of HH signaling resulted in an expansion of SMA expressing cells, indicating that HH drives expansion of smooth muscle cell progenitors (Mao et al., 2010; Zacharias et al., 2011).

Early intestine development

Once the smooth muscle has formed, muscular contractions controlling peristalsis are coordinated by the enteric nervous system (Hatch and Mukouyama, 2015; Hao et al., 2016). The gut tube becomes innervated upon migration of vagal neural crest cells starting at E9. These neural crest cells proliferate and migrate caudally throughout the myenteric region and later populate the submucosa. Around E14, neural crest progenitors give rise to sensory and motor neurons, which project nerve fibers into the gut, allowing colonization of Schwann cell precursors. Neurons and glial cell differentiation occurs and continues postnatally (McHugh, 1996; Uesaka et al., 2016). The detailed molecular mechanisms surrounding the enteric system in the gut exceeds the scope of this review and are reviewed elsewhere (Furness et al., 1990; McHugh, 1996; Kuo and Erickson, 2010; Uesaka et al., 2016), but of note the RET/GDNF signaling pathway is perhaps the best characterized. Absence of RET/GDNF signaling abrogates the migration and differentiation of enteric neural crest cells and leads to enteric nervous system disorders like Hirschsprung's disease (Manié et al., 2001; Burns, 2005). Also of note, tissue engineered systems using hPSC-derived intestinal organoids and/or neural crest progenitors are now being implemented to better study human mutations that lead to innervation defects, causing improper gut function and dysmotility at birth (Fattahi et al., 2016; Workman et al., 2016).

Simultaneous with ENS development, the early intestine becomes vascularized. PECAM+ endothelial cells are present in the gut by E9.5 (Hatch and Mukouyama, 2015). By E11 in the mouse, the serosal mesothelium begins to form on the surface of

the gut and covers the peritoneal cavity (Hatch and Mukouyama, 2015), and at E12.5, mesothelial cells undergo EMT and enter the submesothelial space of the gut and over the next few days, they differentiate into vascular smooth muscle of the newly forming vascular network of intestinal arteries and veins (Wilm et al., 2005).

Rapid proliferation of the epithelium also takes place in the early intestine, and is required for intestinal lengthening and expansion of the surface area. Prior to villus morphogenesis at E14.5, the pseudostratified intestinal epithelium is uniformly proliferative, but upon the emergence of villus architecture, epithelial proliferation becomes restricted to the intervillus domains (Korinek et al., 1998). The signaling mechanisms that drive epithelial proliferation during the pseudostratified stages remain unknown. It is well documented that proliferation in the intervillus domains that emerge following villus morphogenesis (starting around E15.5 in mice) and in the crypts of the adult intestine, is highly dependent on WNT/β-CATENIN signaling (Korinek et al., 1998; Pinto et al., 2003; Farin et al., 2012; Das et al., 2015; Chiacchiera et al., 2016; Valenta et al., 2016). Yet, recent studies have suggested that epithelial proliferation in the pseudostratified epithelium prior to villus formation is regulated by mechanisms independent of WNT/β-CATENIN signaling. TOPGAL reporter mice suggested that WNT/β-CATENIN signal transduction was low at E14.5 and dramatically increased over developmental time in the intestinal epithelium (Kim et al., 2007). Supporting the notion that WNT signaling is low in the pseudostratified epithelium, mice null for *Tcf4* (*Tcf7l2*), which is a transcriptional binding partner of β-CATENIN and is required for β-CATENIN-dependent WNT signaling, did not display proliferation defects in the pseudostratified

epithelium, but completely lost epithelial proliferation after villus formation (Korinek et al., 1998). Collectively, these studies suggest that WNT/β-CATENIN signaling is dispensable for proliferation during pseudostratified intestinal development. In this context, it is interesting to note that several separate studies have shown that the WNT/β-CATENIN target gene, and well described adult intestinal stem cell marker, *Lgr5* (Barker et al., 2007), is expressed during this time of low WNT activity (Shyer et al., 2015; Tsai et al., 2016a; Nigmatullina et al., 2017). Recent lineage tracing experiments in *Lgr5-creER* mice have shown that lineage tracing can occur as early as E12.5 (Tsai et al., 2016a). Mechanistically, it appears that the transcription factor ID2, restricts WNT activity during this window of development and *Id2*-deficient intestinal epithelial tissue had more LGR5+ cells starting at E9.5 compared to controls (Nigmatullina et al., 2017). Evidence also suggested that *Id2* deletion increased WNT/β-CATENIN signaling activity in these animals. Collectively, these studies point to an interesting and unexplained paradox. While *Lgr5* is considered a sensitive WNT/β-CATENIN target gene in the adult intestine, it appears that it is already expressed during a time when WNT/β-CATENIN signal transduction is very low in the fetal gut (E12.5-E13.5) (Shyer et al., 2015; Tsai et al., 2016a; Nigmatullina et al., 2017). On the other hand, removing ID2, which presumably leads to an increase in WNT/β-CATENIN signal transduction, increased the number of cells expressing LGR5 (Nigmatullina et al., 2017). Thus, it is unresolved if, how and why LGR5 is present when WNT-signaling is very low but still seems to respond as a target gene when WNT-signaling is activated in *Id2*-null epithelium. It is possible that LGR5 is regulated by multiple mechanisms. In addition, given that *Id2* is a Bone Morphogenetic Protein (BMP) target gene in other systems (Hollnagel et al., 1999;

Miyazono and Miyazawa, 2002), this work would suggest that BMP is highly active in the epithelium during the pseudostratified stage of development; however, this idea has not yet been experimentally tested.

One candidate regulator of proliferation prior to villus development is GATA4. ChIP-Seq of adult mouse intestinal epithelia shows that GATA4 binds to many cell-cycle genes (Kohlnhofer et al., 2016). Additionally, conditional epithelial deletion of *Gata4* disrupts epithelial cell proliferation by E10.5, resulting in delayed villus morphogenesis (Kohlnhofer et al., 2016). It is interesting to speculate that GATA4 may act through retinoic acid signaling to regulate proliferation as it has been shown to be a downstream target of retinoic acid in the intestine and other endoderm derived tissues (Arceci et al., 1993; Ghatpande et al., 2000).

Villus formation

Villus morphogenesis is a process where the flat pseudostratified intestine begins to remodel and give rise to villus structures, which consist of finger-like epithelial protrusions into the intestinal lumen with an underlying mesenchymal core (Spence et al., 2011; Walton et al., 2016a). Villus formation massively expands the intestinal epithelial surface area, allowing for sufficient nutrient absorption to sustain life. As such, villus morphogenesis is a complex process that is driven by a combination of inductive cues and physical forces, which coincide to coordinate this morphological process (Walton et al., 2016a). Individual villi are connected to neighboring villi by proliferative intervillus domains (also called intervillus zones) (Figure 1.5). Within the past 5 years, a

plethora of work in the mouse and chick has shed significant light on the regulation of villus morphogenesis, and has highlighted significant species-specific differences in this process (Grosse et al., 2011; Walton et al., 2012; Shyer et al., 2013; 2015; Walton et al., 2016b). In addition, recent studies of human fetal development revealed that villi begin to form between 51-54 days of gestation correlating to the beginning of villus morphogenesis at E14.5 in the mouse embryo (Karlsson et al., 2000; Walton et al., 2012; de Bakker et al., 2016). In mice, villi emerge in a proximal to distal wave, arising first in the duodenum and spreading to the ileum over a span of 36 hours (Walton et al., 2012), and this trend appears to be consistent in the human fetal intestine (de Bakker et al., 2016). Interestingly, for many years it was thought that the human and mouse intestine initially formed micro-lumens in the flat epithelium, which then went on to fuse, giving rise to villi (Spence et al., 2011); however this was recently shown not to be the case in mice, and 3-dimensional reconstructions demonstrated that the lumen was continuous during villus formation (Grosse et al., 2011). Interestingly, new data from the 'three-dimensional digital atlas and quantitative database of human development' (de Bakker et al., 2016) has shown that micro lumens may be present in the developing human intestine representing an interesting species-specific difference, although it should be noted that more detailed follow up studies will be needed to definitively show any potential differences (Figure 1.6).

While little-to-nothing is known mechanistically about villus formation in humans, it is well appreciated in the mouse that signaling molecules secreted from the rapidly proliferating pseudostratified epithelium act as critical regulators of villus

morphogenesis. HH and Platelet Derived Growth Factor (PDGF) signaling are well established signaling pathways that regulate this process. Epithelial HH and PDGF ligands signal to the underlying mesenchymal cells, which express the pathway receptors PTCH1 and PDGFRA, respectively (Madison et al., 2005; Kolterud et al., 2009; Walton et al., 2012). As HH signaling is activated in the mesenchyme adjacent to the epithelium, it stimulates these cells to exit the cell cycle and aggregate into small dense clusters (Madison et al., 2005; Kolterud et al., 2009; Walton et al., 2012). Cluster formation coincides with the initiation of a nascent villus in the epithelium overlying the cluster. While the cluster itself expresses several signaling molecules, including Bone Morphogenetic Protein (BMP) ligands, it is not clear how the epithelium-cluster unit initiates the formation of a nascent villus (Karlsson et al., 2000; Walton et al., 2016b). Nonetheless, formation of the cluster is an absolute prerequisite for villus formation, since mutations in the HH or PDGF pathways perturb normal mesenchymal cluster formation and disrupt subsequent villus formation, with HH signaling being the most critical to this process as blocking HH signaling can completely block all mesenchymal clustering and villus formation (Madison et al., 2005; Mao et al., 2010; Walton et al., 2012; 2016b). Alternately, increased HH signaling in explant cultures by the addition of a pathway agonist (Smoothened agonist; SAG) increased the size of cluster and villus structures (Walton et al., 2012).

Although mesenchymal clusters form and express BMP ligands, it does not seem that mesenchymal BMP's immediately signal back to the epithelium, since genetic deletion of BMP receptors in the overlaying epithelium does not lead to perturbations in

villus formation (Harfe et al., 2004). Conversely, genetic deletion of BMP receptors in the mesenchyme lead to fused mesenchymal clusters and enlarged villi (Walton et al., 2016b). In addition, mesenchymal clusters also express inhibitors of BMP signaling, including Noggin (NOG) and Twisted Gastrulation 1 (TWSG1), and functional experiments have shown that perturbing BMP signaling affects the spatial distribution of the mesenchymal clusters (Walton et al., 2016b). These functional experiments have led to the hypothesis that BMP signaling establishes the regular spacing and patterning of mesenchymal clusters in an activator-inhibitor reaction-diffusion style mechanism (Walton et al., 2016b).

The reaction-diffusion model that may explain the distribution and patterning of mesenchymal clusters in the intestine as recently suggested by Walton et al., (Walton et al., 2016b) was first proposed by mathematician Alan Turing, who described a model where an activator and inhibitor emanating from the same source interact to establish a self-organized and predictable pattern (Turing, 1952). It is interesting to note that in his manuscript, Turing noted, "…the description of the state consists of two parts, the mechanical and the chemical". He then goes on to state, "One cannot at present hope to make any progress with the understanding of such systems except in very simplified cases. The interdependence of the chemical and mechanical data adds enormously to the difficulty, and attention will therefore be confined, so far as is possible, to cases where these can be separated" (Turing, 1952). Thus Turing acknowledged, but did not address, the mechanical forces that would normally be present in a biological system. In this light, it is interesting to note the work of Oster and colleagues many years later, who

used mathematical modeling of mesenchymal cell behavior to propose that mechanical traction forces exerted by the mesenchyme on the surrounding extracellular matrix could deform the matrix and affect both the direction of mesenchymal movements and the formation of the pattern (Oster et al., 1983; Murray and Oster, 1984b; 1984a). In this work, it was proposed that mesenchymal traction forces would eventually lead to a uniform distribution of cells breaking up into local cell condensations, characterized by "islands of high cell density alternating with regions of low cell density" (Oster et al., 1983). Oster's model also predicted that as tissues grow and mature, developmental waves of mesenchymal condensations could form behind maturing tissue in regular patterns (Oster et al., 1983). For example, it was predicted that an anterior-to-posterior gradient of mesenchymal condensations could form in a developing tissue as a population of cells became developmentally competent to form cell aggregates in an age/time dependent manner (Oster et al., 1983). It is interesting to speculate that such traction forces could cooperate with morphogen signals, and that both may play a role in the formation or propagation of the anterior-posterior wave of mesenchymal clusters that condense during villus morphogenesis, since it is well described that the intestine matures in an anterior-posterior fashion. At this point in time there is no evidence in the developing mouse intestine to show how signaling and biomechanical forces may cooperate during the process of mesenchymal clustering in mice.

On the other hand, it has been predicted that tension/force placed on the epithelium by the underlying mesenchymal clusters may instruct the overlying epithelium to start forming a villus (Freddo et al., 2016). Here, it has been proposed that

nascent clusters deform the epithelium as they form on the basal side of the epithelium, placing compression forces on the epithelium and placing strain on the apical epithelial surface. When coupled with reduced tension in the apical F-ACTIN cortical network due to a mitotic event in the highly proliferative epithelium, sufficient strain from below the epithelium coupled with a local reduction in apical surface tension would lead the epithelium to deform and buckle in between the mesenchymal clusters, effectively forming nascent villi above the clusters (Freddo et al., 2016).

Recent work has also linked tensile forces produced by radial smooth muscle on the development of villus structures in the developing chick gut (Shyer et al., 2013; 2015). This work demonstrated that as the smooth muscle layers differentiate, they place a global compressive force on the intestine. As the highly proliferative epithelium continues to expand in a uniform manner circumferentially, the compressive forces created by the smooth muscle cause the epithelium to buckle. As each subsequent muscle layer differentiates in the developing gut – the circumferential muscle layer, followed by the exterior longitudinal muscle and then the interior longitudinal muscle – new mechanical strains are placed on the intestine, leading to an initial pattern of epithelial ridges, followed by a zig-zag pattern and finally, villus structures (Shyer et al., 2013). It is interesting to note that there are significant differences between chick and mouse intestine development. In the chick, the epithelial folding induced by mechanical constraint is suggested to help concentrate morphogenetic signals in the underlying tissue; epithelial HH ligands are concentrated in the underlying mesenchyme after epithelial buckling to induce changes in the mesenchyme (Shyer et al., 2015). However,

in the mouse HH signals drive formation of mesenchymal clusters and are a prerequisite to villus formation (Walton et al., 2012; Walton et al., 2016b). Collectively, these data suggest that the mechanisms driving villus development and regulating epithelial and mesenchymal differentiation in the two species are dramatically different, but also show the importance of both signaling molecules and biomechanical forces in tissue morphogenesis in both species (Shyer et al., 2013; 2015; Freddo et al., 2016; Walton et al., 2016b).

Development and maturation of the intestine following villus formation

Following villus formation, epithelial proliferation becomes rapidly restricted to the intervillus domains at the base of the villi for the remainder of development (Korinek et al., 1997; Bell et al., 2013). Two major signaling pathways that are important for intestinal proliferation in the late neonatal and adult intestine are the WNT/β-CATENIN and NOTCH signaling pathways (Harada et al., 1999; Pinto et al., 2003; He et al., 2004; Ireland et al., 2004; Kuhnert et al., 2004; van Es et al., 2005; van der Flier and Clevers, 2009; VanDussen et al., 2012; Tsai et al., 2014). For detailed reviews on WNT and NOTCH signaling in the intestinal stem cell, see: (Demitrack and Samuelson, 2016; Mah et al., 2016). Although proliferation in the pre-villus intestinal epithelium in mice can occur in the absence of WNT/β-CATENIN signaling, once villi form at E15.5, proliferation is dependent on WNT/β-CATENIN signaling. Blocking signaling activity using a variety of genetic methods results in a complete loss of epithelial proliferation (Korinek et al., 1997; Garcia et al., 2009; Joo et al., 2010; Zhong et al., 2012). In addition, several WNT/β-CATENIN target genes become restricted to the proliferative

intervillus domain following villus morphogenesis, including *Axin2*, *Cd44*, *Ccnd1*, *Sox9* and *Lgr5*, reinforcing the location of WNT/β-CATENIN signaling activity following villus formation (Korinek et al., 1997; Blache et al., 2004; Li et al., 2009; Zhong et al., 2012; Shyer et al., 2015). And while NOTCH signaling is a critical regulator of intestinal stem cells postnatally and in the adult (VanDussen et al., 2012; Tsai et al., 2014; Carulli et al., 2015; Tian et al., 2015), the role that NOTCH signaling plays at earlier times of development is not clear. NOTCH genetic gain-of-function studies have been conducted in the developing intestinal epithelium, and have shown that developmental misregulation of Notch Intracellular Domain (NICD) expression can lead to increased epithelial proliferation (Fre et al., 2005), or can lead to a block in proliferation (Stanger et al., 2005). These opposing results are likely explained by the different *Cre* drivers used and the developmental timing of NICD expression. Recent studies in the developing mouse intestine, the human fetal intestine, and in hPSC-derived intestinal organoids have shown that the NOTCH target gene, *OLFM4*, is expressed at extremely low levels relative to the adult intestine (Fordham et al., 2013; Finkbeiner et al., 2015). Interestingly, inhibition of NOTCH function in fetal mouse intestine cultures resulted in secretory cell hyperplasia (VanDussen et al., 2012), consistent with NOTCH inhibition studies in the adult (Kazanjian et al., 2010; van Es et al., 2010; Kim and Shivdasani, 2011).

Shortly after villus emergence, around E16.5 in mice, the epithelium on the villi begins to undergo cytodifferentiation into the functional cell types of the small intestine, including secretory cells - mucus producing goblet cells and hormone-producing

enteroendocrine cells- and absorptive enterocytes, which comprise more than 80% of the intestinal epithelial cells in the proximal small intestine and are responsible for absorbing nutrients from the lumen (van der Flier and Clevers, 2009; Noah et al., 2011; Spence et al., 2011; Noah and Shroyer, 2013). A detailed review of the molecular mechanisms controlling differentiation in the intestinal epithelium is outside the scope of this discussion, but readers are encouraged to see the following reviews: (Noah et al., 2011; Vooijs et al., 2011; Noah and Shroyer, 2013; Sancho et al., 2015). In brief, NOTCH signaling is known to play an important role regulating the choice to differentiate into a secretory cell (NOTCH OFF) or into an absorptive enterocyte (NOTCH ON) (VanDussen and Samuelson, 2010; VanDussen et al., 2012; Milano et al., 2004; Wong et al., 2004; van Es et al., 2005; Noah and Shroyer, 2013; Yin et al., 2014). While most of our understanding about cellular differentiation in the intestine has been established through studies in the postnatal intestine, in the developing gut, GATA4 and GATA6 function redundantly to suppress proliferation and regulate cytodifferentiation of goblet cells by modulation of NOTCH signaling (Walker et al., 2014). In addition, the transcription factor, KLF5, is required for initiation of differentiation in the developing gut, as genetic deletion of *Klf5* from the intestinal epithelium lead to the reduction of goblet and enteroendocrine cells as well as the loss of the apical brush border (Bell et al., 2013).

In mice, the crypt of the intestine emerges around postnatal day 14. The mechanisms by which the embryonic intervillus domains give rise to the postnatal/adult crypt are completely unknown. However, crypt emergence coincides with differentiation

of Paneth cells in the intestine (Calvert and Pothier, 1990; Kim et al., 2012). In humans, Paneth cell differentiation occurs around week 20 of fetal gestation (Moxey and Trier, 1978; Mallow et al., 1996). Paneth cells initially emerge at the 5-7th cell position in the crypt and then migrate downwards to the base of the crypt adjacent to LGR5+ stem cells (Bjerknes and Cheng, 1981; Kim et al., 2012). Paneth cells secrete defensin proteins (Ouellette et al., 1992; Bevins and Salzman, 2011; Ouellette, 2011), which are known to have antimicrobial properties to protect against pathogen infection and also play a role as a niche cell, supporting intestinal stem cell maintenance (Salzman et al., 2003; Sato et al., 2011; Chu et al., 2012; Clevers and Bevins, 2013). Paneth cell differentiation is initially controlled through a NOTCH dependent mechanism during secretory progenitor specification and further Paneth cell maturation is regulated by WNT signaling (van Es et al., 2005; Farin et al., 2012). Deletion of *Lgr5* in the embryonic intestine led to increased levels of WNT signal transduction and precocious Paneth cell differentiation (Garcia et al., 2009). Further differentiation of Paneth cells requires the expression of the WNT target SOX9 (Blache et al., 2004; Formeister et al., 2009) and mice with conditional deletion of *Sox9* lack Paneth cells in the crypts (Bastide et al., 2007; Mori-Akiyama et al., 2007).

One of the hallmarks of intestinal epithelial maturation is the acquisition of fully functional epithelial cell types (Fordham et al., 2013; Mustata et al., 2013; Finkbeiner et al., 2015) and reviewed in (Guiu and Jensen, 2015). In the human fetal intestine and in hPSC-derived intestinal organoids, this includes the differentiation of Paneth cells and many enzymes that function in nutrient absorption that are present on the enterocyte

brush boarder (Finkbeiner et al., 2015). In the mouse, epithelial maturation is regulated by the transcriptional repressor BLIMP1 (also known as PRMD1) (Harper et al., 2011; Muncan et al., 2011). BLIMP1 is expressed broadly throughout the intestinal epithelium of the embryonic intestine, with expression becoming restricted shortly after birth, when it is excluded from the proliferative domain (intervillus domain and emerging crypt) over the first 2 weeks postnatally. By the third week of life and through adulthood, BLIMP1 is expressed only in the tip of the villus. *Blimp1* mutant mice show early differentiation of Paneth cells, an increased differentiation of goblet cells, and a major metabolic shift towards the adult phenotype by postnatal day 7 at the expense of suckling-period-specific enzymes (Harper et al., 2011; Muncan et al., 2011). Mechanistically, ChIP-seq experiments showed that BLIMP1 is able to bind to DNA associated with metabolic genes in the epithelium adding evidence to the notion that BLIMP1 repressed gene expression of adult genes during the embryonic period (Mould et al., 2015). Further, it was shown that BLIMP1 was bound to many of the same regions as the transcriptional activator, IRF1. IRF1 binds and can activate transcription of MHC class I pathway genes, and it was postulated that an additional role for BLIMP1 was to repress IRF1-bound genes while the neonatal gut acquires tolerance during microbial colonization over the first few weeks of life (Mould et al., 2015).

Recent work has also suggested that major shifts in the metabolism of the developing intestinal epithelium play a significant role in maturation of the intestinal epithelium (Kumar et al., 2016). This study found that there was an increase in the expression of genes involved in oxidative phosphorylation coincident with villus

development, and that expression of these genes continued to increase throughout embryonic development. It is interesting to note that metabolism in the adult intestine shifts from glycolysis-to-oxidative phosphorylation along the crypt-villus axis (Stringari et al., 2012), indicating that this developmental switch may be critical in preparing the epithelium for postnatal life. The embryonic shift in oxidative phosphorylation genes was controlled by the transcription factor YY1, as genetic deletion of *Yy1* in the epithelium led to reduced gene expression and stunted villus growth (Kumar et al., 2016). Further supporting the connection between oxidative phosphorylation and intestinal growth, pharmacological inhibition of the electron transport chain caused a similar stunting of villus growth (Kumar et al., 2016). Interestingly, oxidative phosphorylation was reduced in human neonates with necrotizing enterocolitis (NEC), indicating that this metabolic shift may be critical for the intestine to mature and adapt to neonatal life (Kumar et al., 2016).

Conclusion and goals of this thesis:

The gastrointestinal tract is a highly complex and multifunctional organ system. Decades of work in multiple disciplines have resulted in a framework for our understanding of the development of the structures and functions that comprise the mature intestine. However, formation of an integrated, comprehensive understanding of the growth, development and maturation of the intestine and its dynamic function throughout life will require the continued use of multidisciplinary approaches that allow for new ways to further our understanding. Classical animal models such as the frog and chick have proven to be powerful experimental tools for understanding the cellular

and genetic basis of gut development, and have allowed us to explore the molecular pathways important during gastrulation, formation of the endoderm, and gut morphogenesis. Mice have also been an excellent model to study mammalian intestinal development and provided insights into similarities and differences between species; most notably are the distinct mechanisms behind the extension of luminal surface area and formation of villus structures.

However, there are still many questions left unanswered. Our understanding of the process of villus formation is incomplete and warrants further investigation into the molecular mechanisms that instruct cellular behavior. The initiation of villus formation begins a complex process where the uniformly pseudostratified epithelium gives rise to alternating villus and intervillus domains. These previously uniform epithelial progenitors adopt distinct villus and intervillus properties that differ in cell shape, proliferative capacity, and gene expression. These changes occur quickly and between a brief window of developmental time (E13.5-E15.5), the uniform epithelium already transforms into distinct villus and intervillus domains. However, the molecular mechanisms that regulate the propagation of epithelial progenitors during this precise window of developmental time, is unknown. The work in this dissertation aims to gain insight into the signaling pathways that regulate epithelial proliferation during villus morphogenesis. We found that WNT/β-CATENIN signaling activity is dynamic before and after initiation of villus morphogenesis. Prior to villus formation, the intestine is independent of WNT/β-CATENIN signaling and WNT/β-CATENIN signal transduction is very low. But after villus formation has begun, WNT/β-CATENIN signal transduction is robust and now

required for epithelial proliferation and villus formation. Lastly, we determined that reception of WNT signals occurs in a paracrine manner as secretion of WNT ligands from the mesenchyme, and not the epithelium, induces WNT/β-CATENIN-mediated epithelial proliferation.

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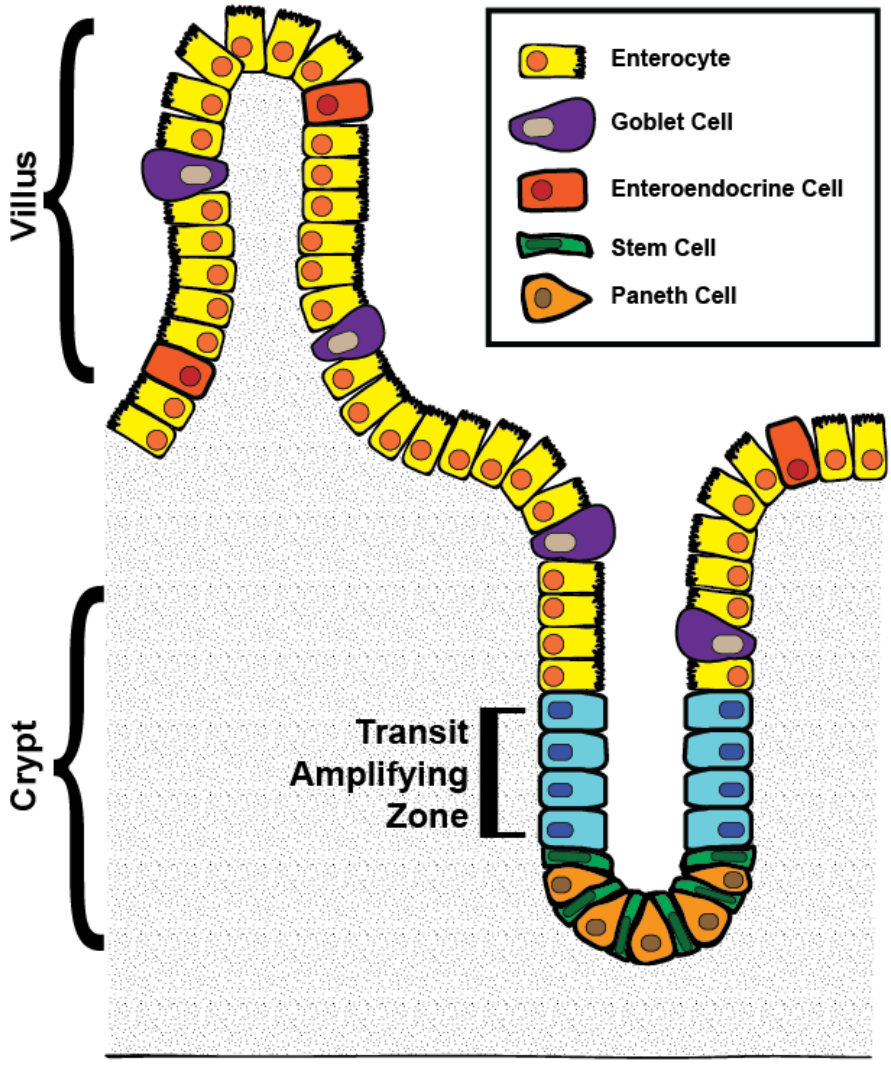

Figure 1.1. The adult epithelial crypt-villus unit[*]
The adult small intestinal epithelium is arranged in crypt-villus units. Intestinal stem cells and Paneth cells are housed in the crypt. A Transit Amplifying zone is a site for rapid proliferation and amplification of undifferentiated progenitor cells as they begin to make cell fate choices. Differentiated cell types continue to move up the villus in a conveyer-belt fashion where they carry out their day-to-day function, until they reach the villus tip where they undergo apoptosis and slough off into the lumen. Villus cell types include enterocytes, goblet cells and enteroendocrine cells, as well as tuft cells and M-cells (not shown).

[*] Figure is from the review article in press:
Alana M. Chin, David R. Hill, Megan Aurora, Jason R. Spence. Morphogenesis and maturation of the embryonic and postnatal intestine. *Seminars in Cell and Developmental Biology.* doi.org/10.1016/j.semcdb.2017.01.011

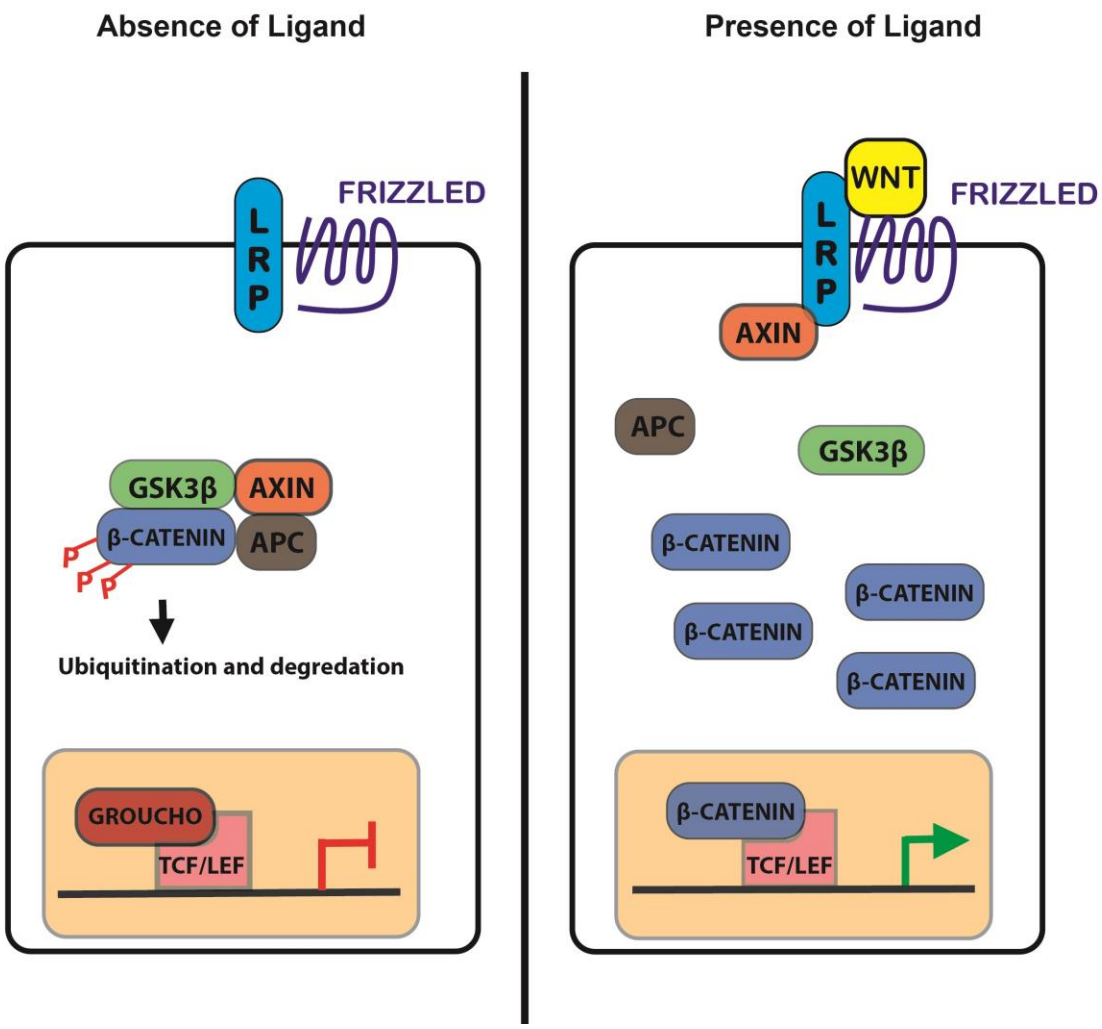

Figure 1.2. The canonical WNT/β-CATENIN signaling pathway
In the absence of WNT ligand binding, GSK3 forms a destruction complex with AXIN and anaphase-promoting complex (APC) where GSK3 phosphorylates co-regulator β-CATENIN, marking it for ubiquitination by E3 ubiquitin ligases and sent for proteasomal degradation. Simultaneously, TCF/LEF transcription factors are bound to the corepressor GROUCHO, which keeps transcription of target genes turned off. When WNT ligands bind to FRIZZLED receptors and lipoprotein receptor-related proteins (LRP) co-receptors, AXIN is sequestered and glycogen synthase kinase 3 (GSK3) activity is inhibited. This allows β-CATENIN to accumulate in the cytosol and translocate into the nucleus. β-CATENIN replaces GROUCHO to bind to TCF/LEF transcription factors and activate transcription of downstream target genes.

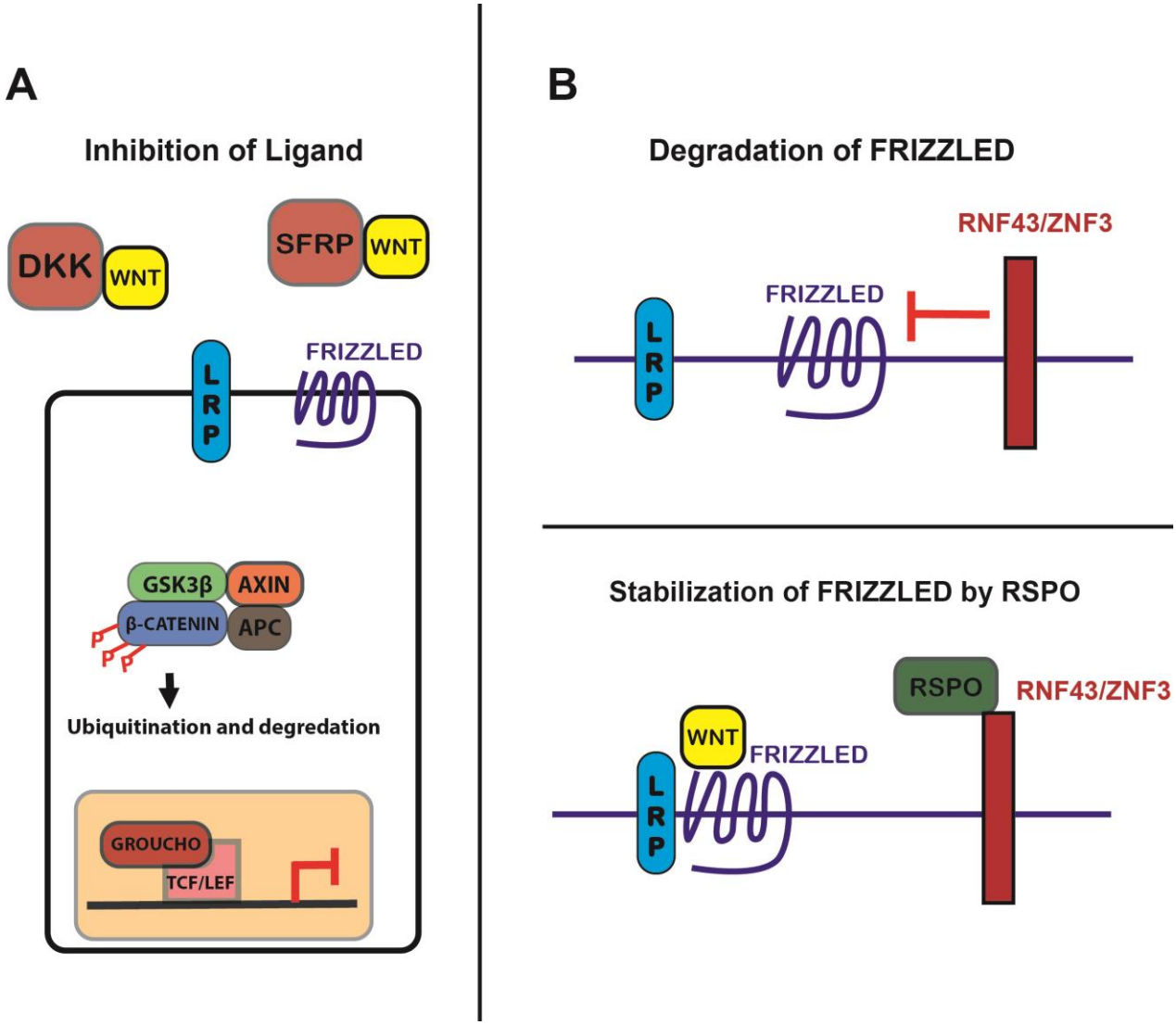

Figure 1.3. Modulation of the WNT/β-CATENIN signaling pathway
(A) DKK and SFRP are inhibitors of WNT/β-CATENIN signaling and interfere with ligand binding. Competitive binding prevents WNT ligands from interacting with LRP and FRIZZLED co-receptors, permitting the degradation of cytosolic β-CATENIN. (B) RSPO proteins are agonists of the WNT/β-CATENIN signaling pathway. Without RSPO activity, E3 ubiquitin ligases RNF43 and ZNRF3 degrade FRIZZLED receptors, preventing LRP/FRIZZLED receptor complex formation. RSPO proteins interact with RNF43/ZNRF3 to prevent their degradation of FRIZZLED receptors.

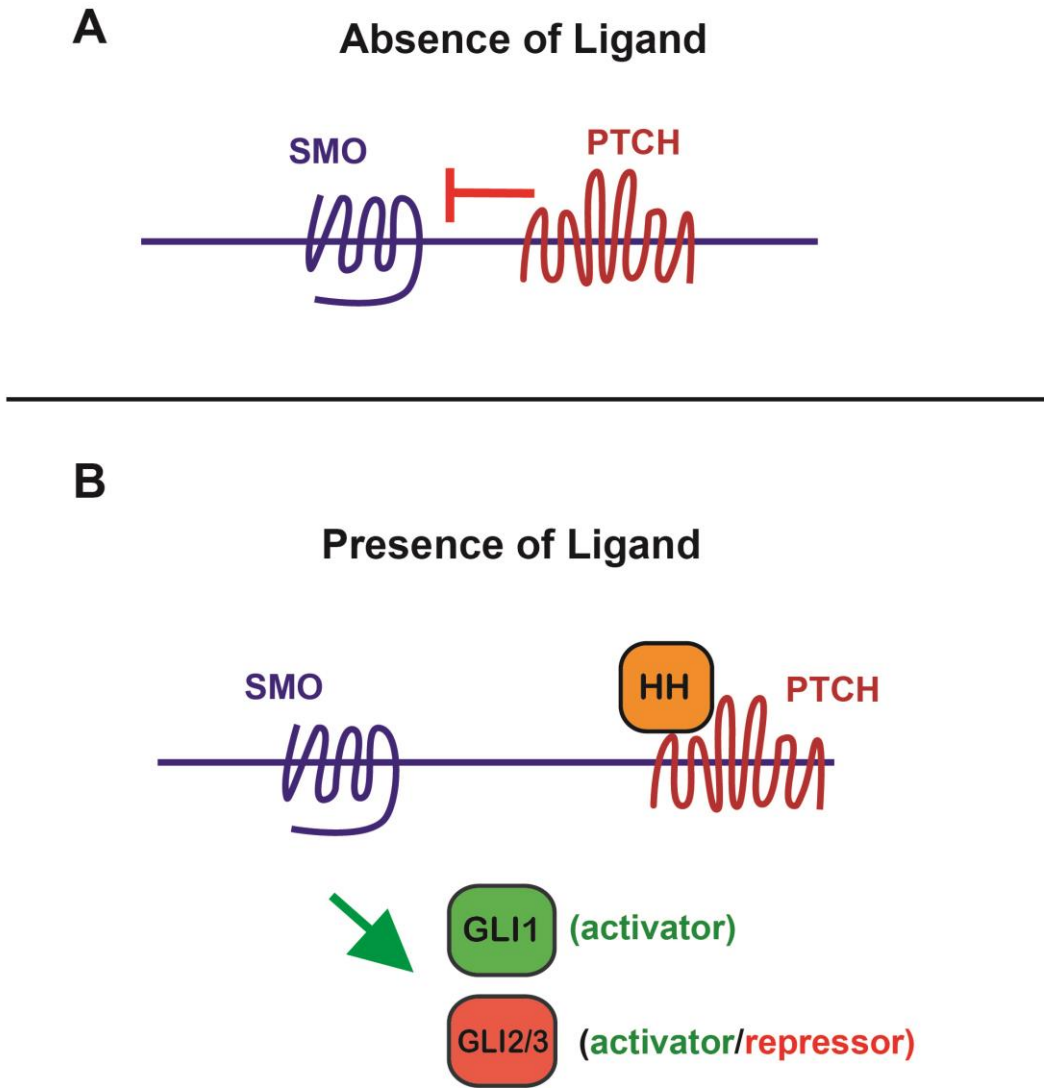

Figure 1.4. The HH signaling pathway
(A) In the absence of HH ligands, PTCH receptors inhibit the 7-transmembrane pass protein SMO and renders it inactive. (B) When hedgehog ligands bind to PTCH receptors, PTCH releases inhibition of SMO. Active SMO activates various GLI transcription factors which can then either behave as transcription activators or repressors in different contexts.

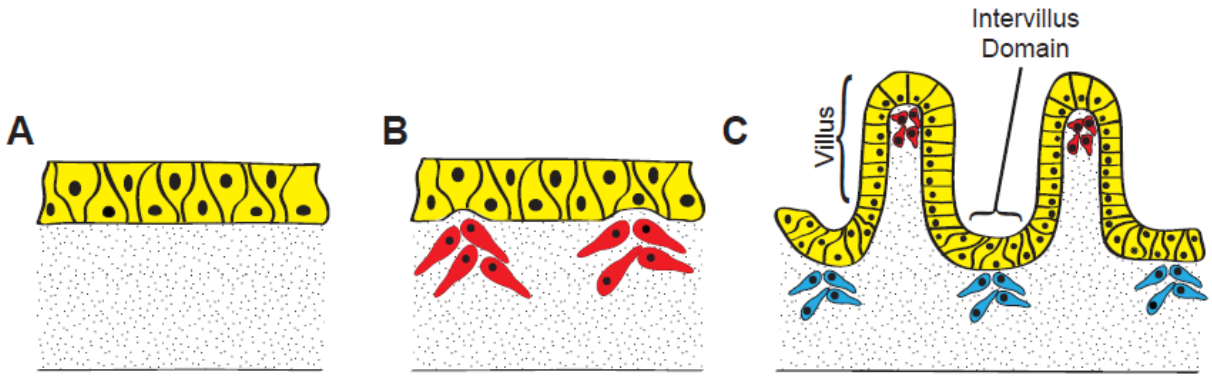

Figure 1.5. Developmental epithelial transitions and mesenchymal cluster formation in the mouse intestine*

(A) The early murine intestinal epithelium (yellow), between E9.5-E13.5, is present as a flat pseudostratified epithelium within the gut tube. (B) Beginning around E14.5, mesenchymal clusters (red) aggregate adjacent to the epithelium where a nascent villus will form. Cluster formation causes a deformation in the epithelium above the cluster. (C) Villi form above the cluster, establishing the highly proliferative intervillus domain between villi. Several rounds of villus morphogenesis will occur, and new clusters will form (blue) adjacent to the intervillus domain following completion of the prior round of cluster-villus formation (red clusters).

* Figure is from the review article in press:
Alana M. Chin, David R. Hill, Megan Aurora, Jason R. Spence. Morphogenesis and maturation of the embryonic and postnatal intestine. *Seminars in Cell and Developmental Biology.* doi.org/10.1016/j.semcdb.2017.01.011

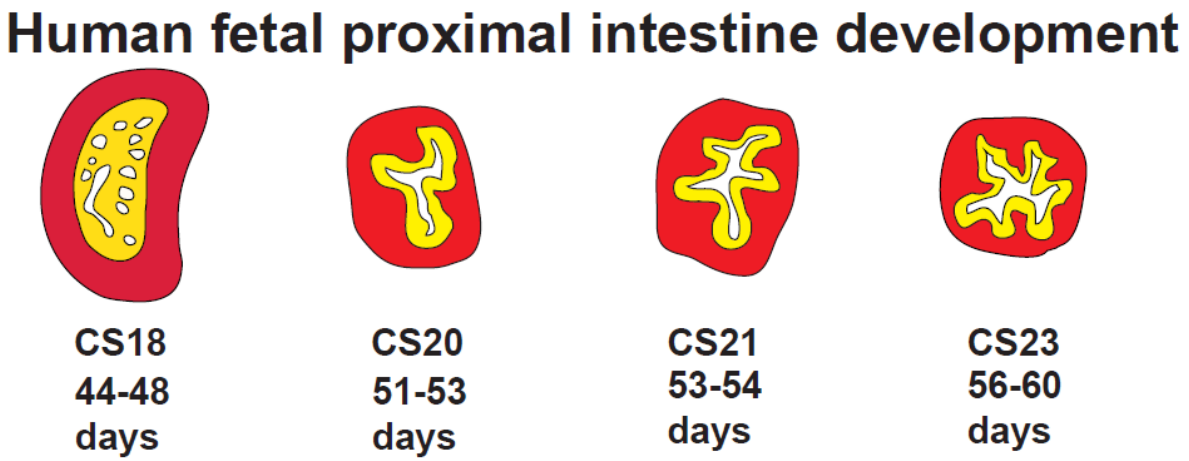

Figure 1.6. Human fetal intestine development[*]
Sections through different Carnegie Stages (CS) of the developing human embryo were obtained (http://www.3dembryoatlas.com and de Bakker et al., 2016) and traces of the proximal small intestine (duodenum) were generated. The intestinal epithelium (yellow) appeared to have multiple lumens prior to villus morphogenesis (CS18), and nascent villi formation was apparent by CS20. Image resolution was not sufficient to determine if the human intestine formed villus clusters in the mesenchyme (red). Villus structures became more pronounced, and greater in number as development progressed (CS21-CS23).

[*] Figure is from the review article in press:
Alana M. Chin, David R. Hill, Megan Aurora, Jason R. Spence. Morphogenesis and maturation of the embryonic and postnatal intestine. *Seminars in Cell and Developmental Biology.* doi.org/10.1016/j.semcdb.2017.01.011

CHAPTER 2

A DYNAMIC WNT/β-CATENIN SIGNALING ENVIRONMENT LEADS TO WNT-INDEPENDENT AND WNT-DEPENDENT PROLIFERATION OF EMBRYONIC INTESTINAL PROGENITOR CELLS*

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

* This chapter represents the following manuscript:

Alana M. Chin, Yu-Hwai Tsai, Stacy R. Finkbeiner, Melinda S. Nagy, Emily M. Walker, Nicole J. Ethan, Bart O. Williams, Michele A. Battle and Jason R. Spence. A dynamic Wnt/β-catenin signaling environment leads to Wnt-independent and Wnt-dependent proliferation of embryonic intestinal progenitor cells. *Stem Cell Reports* (2016) 7, 826-839. Doi:10.1016/j.stemcr.2016.09.004

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 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 2.1-A and 2.1-B). Activity was more apparent in the E14.5 epithelium (Figures 2.2-A–2.2-F) while at E15.5, *Axin2-LacZ* reporter activity was also apparent, and was restricted to the intervillus domains (Figures 2.1-C, 2.1-D, and 2.2-G – 2.2-L) 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 2.2-A- 2.2-F), 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 down- stream 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 2.1-E and 2.1-F). In addition, CD44v6 antibody staining indicated increased protein expression as developmental time progressed (Figures 2.1-G– 2.1-I, 2.3-A, and 2.3-B).

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 2.4-A). In addition, while CD44v6 was low in controls at E13.5, βcat-LOF intestines did not have detectable CD44v6 protein at E13.5 (Figures 2.1-J and 2.3-C). 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 2.1-G with Figure 2.1-J and Figure 2.3-A with Figure 2.3-C). Importantly, loss of WNT/β-CATENIN signaling did not affect intestinal fate, since the βcat-LOF intestines maintained CDX2 protein expression (Figure 2.4-C).

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 2.4-B). To confirm deletion, we analyzed expression of *Cd44* and *Axin2* mRNA expression in isolated epithelium of Lrp5/6-LOF embryos (Figures 2.1-R and 2.1-S), and CD44v6 protein in tissue sections (Figures 2.1-M– 2.1-O, 2.3-E, and 2.3-F). These results showed a loss of CD44v6 protein staining by E14.5 (Figures 2.1-N, 2.1-O, and 2.3-F) and a significant reduction of *Cd44* and *Axin2* at E15.5 (Figures 2.1-R and 2.1-S), 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 2.5). 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 2.5-A–2.5-I). 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 2.5-A, 2.5-B, 2.5-D, 2.5-E, 2.5-G, and 2.5-H), and there were no quantitative differences in epithelial proliferation at these stages (Figure 2.5-J). On the other hand, E15.5 epithelial PHH3

staining was reduced in βcat-LOF and Lrp5/6-LOF intestines compared with controls (Figures 2.5-C, 2.5-F, and 2.5-I). 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 2.5-J). 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 2.5-C, 2.5-F, and 2.5-I). 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 2.4-E and 2.4-F). 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 2.4-I and 2.4-J). 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 2.5-K, 2.5-L, 2.5-N, and 2.5-O), and that SOX9 protein expression is lost within βcat-LOF epithelium only at E15.5 (Figures 2.5-M and 2.5-P). 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 2.1 and 2.5). Similarly, both βcat-LOF and Lrp5/6-LOF embryos had grossly smaller intestines compared with

controls at E15.5 (Figure 2.4-D). Based on these similarities, and the fact that *β-catenin* deletion was more efficient than *Lrp5/6* deletion (Figures 2.4-A and 2.4-B), 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 2.6-A–2.6-F). 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 2.6-G). Similarly, counting the absolute number of epithelial cells (E-CADHERIN+ DAPI+) per section showed no difference between controls and mutants until E15.5 (Figure 2.6-H). To further assess any changes in morphology associated with βcat-LOF, we performed a series of measurements (diagrammed in Figures 2.6-K, 2.6-N, and 2.6-Q) including the total cross-sectional length/width (Figures 3I and 3J), cross- sectional length/width of the epithelium (Figures 2.6-L and 2.6-M), and apical surface area and epithelial thickness (Figures 2.6-O and 2.6-P). In several measurements, we did not observe statistical differences at any time point between βcat-LOF and controls (Figures 2.6-I, 2.6-L, and 2.6-M). However, for data shown in Figures 2.6-I– 2.6-N, measurements neglected to account for the size of the lumen, which can vary. Therefore, we measured the apical surface (Figure 2.6-Q, ‘‘A’’) as well as epithelial thickness (Figure 2.6-Q, ‘‘T’’), which both showed a significant decrease in

βcat-LOF intestines at E15.5, but not at earlier times (Figures 2.6-O and 2.6-P). 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 2.7-A), 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 2.7-B).

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 2.7-C), 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 control and mutant intestines at E15.5 (Figures 2.8-A and 2.8-B). 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 2.8-B). 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 2.8-C and 2.8-D). 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 2.9-A and 2.10). Of the 19 *Wnt* ligand genes 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 2.9-A and 2.10-A). 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 2.9-B). *Wnt3* was higher at E15.5 in both compartments while *Wnt7b* mRNA transcript was higher in the mesenchymal fraction (Figure 2.9-B). 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 (Sosic 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 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 2.9-C–22.9-E). 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 2.1, 2.5, and 2.6). However, following 72 hr of culture, MesWntless-LOF intestines had a significant reduction in the percentage of PHH3+ epithelial cells compared with controls (Figures 2.9-F–2.9-H). 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 2.9-L and 2.9-M). In contrast, EpWntless-LOF did not show any changes in epithelial proliferation (PHH3) or CD44v6 staining at E15.5 (Figures 2.9-I– 2.9-O). 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/B-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-GA*L 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 RSPO 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). More- over, *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.

Methods

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), *β-catenin 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* (Sosic et al., 2003), and *Wntless f/f* (Carpenter et al., 2010). Control mice

used were of the following genotypes: *β-catenin f/f, β-catenin f/+, Shh-cre; β-catenin 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), 13 HEPES (Invitrogen 15630080), 13 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 37C with 5% CO2 with medium changes every 24 hr.

Tissue preparation

For histology, *Shh-Cre; Lrp5 f/f;Lrp6 f/f, Shh-Cre; β-catenin 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 (1x 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 mm 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 (99C) 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 13 R-Buffer A (Electron Microscopy Sciences 62706-10). Sections were blocked in donkey serum (5% serum in 1x PBS + 0.5% Triton X-100) for 1 hr. Antibody information and dilutions are presented in Table 2.1. Primary antibodies were diluted in blocking buffer and incubated on tissue sections overnight at 4C. Slides were washed in 13 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 40x. Higher-magnification images were taken on a Nikon A1 confocal microscope at 60x 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-mm 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 2.2 for primer sequences.

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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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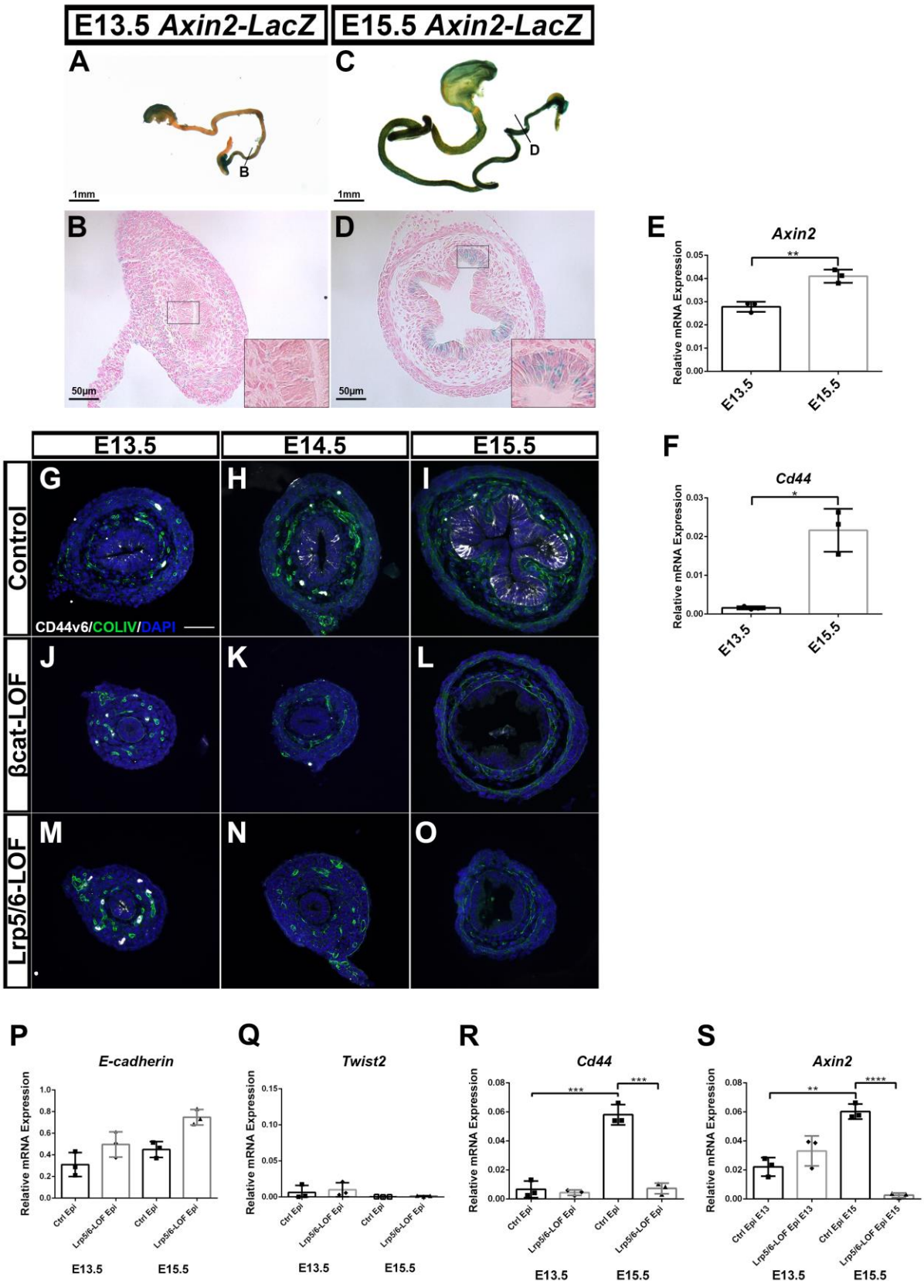
E13.5 Axin2-LacZ
E15.5 Axin2-LacZ
A
B
C
D
1mm
50μm
E
Axin2
Relative mRNA Expression
E13.5
E15.5
F
Cd44
E13.5
E14.5
E15.5
Control
βcat-LOF
Lrp5/6-LOF
G
H
I
J
K
L
M
N
O
CD44v6/COLIV/DAPI
P
E-cadherin
Ctrl Epi
Lrp5/6-LOF Epi
Q
Twist2
R
Cd44
S
Axin2
Ctrl Epi E13
Lrp5/6-LOF Epi E13
Ctrl Epi E15
Lrp5/6-LOF Epi E15

Figure 2.1. WNT/β-CATENIN signaling is active in temporally and spatially distinct domains in the small intestine. (A, C) Whole mount X-Gal staining of E13.5 and E15.5 stomach and intestines from *Axin2-LacZ* reporter mice. Black lines indicate plane of section. Scale bar: 1mm. (B) X-GAL staining in E13.5 intestinal sections shows low activity in the epithelium. (D) At E15.5, *Axin2-LacZ* reporter activity became restricted to the intervillus domains in the epithelium but at lower levels in the duodenum (C). (G-I) Immunofluorescence staining of E13.5, E14.5 and E15.5 control intestines show increasing CD44v6 staining (white) co-stained with Collagen IV (green). (J-O) Wnt/β-catenin deficient ileums show efficient downregulation of CD44v6 target gene expression where CD44v6 is lost in βcat-LOF as early as E13.5 (J) and Lrp5/6-LOF by E14.5 (N). Scale bars: 50μm. (E, F) qPCR analysis of whole thickness ileal segments show upregulation of *Axin2* and *Cd44* from E13.5 and E15.5 (n=3 E13.5 embryos pooled from 2 litters and n=3 E15.5 embryos pooled from 3 litters for one independent experiment). (P-Q) Epithelial isolations from control and Lrp5/6-LOF intestines are enriched for *E-cadherin* and deficient in *Twist2*. (R, S) Lrp5/6-LOF epithelia are dramatically reduced for *Cd44* and *Axin2* mRNA transcript at E15.5, indicating efficient deletions by *Shh-Cre*. Both E13.5 genotypes have n=3 embryos pooled from 2 litters and both E15.5 genotypes have n=3 embryos pooled from 3 litters for one independent experiment. Statistical significance by t-test. *p-value 0.01-0.05, **p-value 0.001-0.01, ***p-value 0.0001-0.001, ****p-value 0.00001-0.0001.

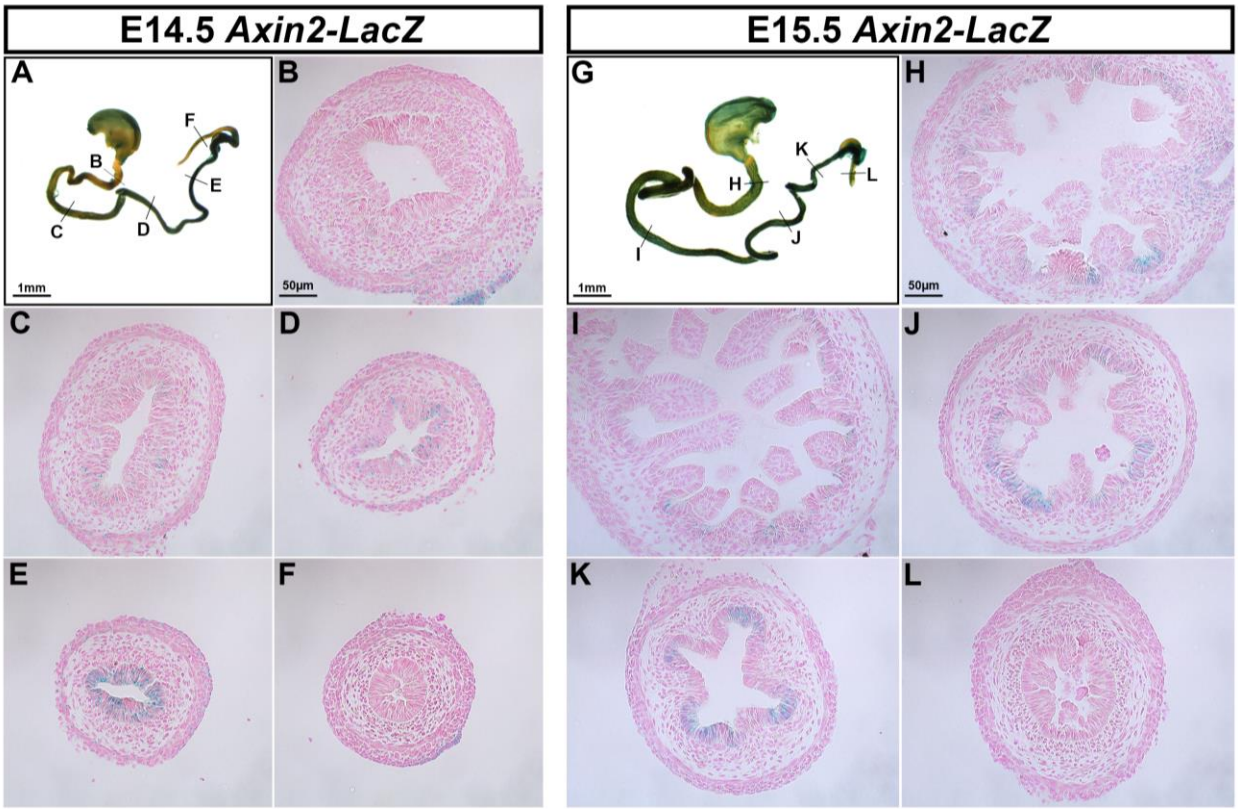

Figure 2.2. *Axin2-LacZ* reporter activity at E14.5 and E15.5. (A) *Axin2-LacZ* reporter mice show a gradient of WNT-signaling activity along the proximal-distal axis of the intestine at E14.5 and E15.5. Whole mount image of E14.5 gut tube stained for LacZ. Black bars indicate location of section in B-F. (B-E) Epithelial *Axin2-LacZ* reporter activity appears to be "patchy" with higher activity at putative proliferative intervillus domains. (F) *Axin2-LacZ* is very low to undetectable in the colon. (G) Whole mount E15.5 gut tube shows low WNT activity in the proximal duodenum and high WNT activity through the distal intestine (jejunum and ileum). Black bars indicate location and plane of section in H-L. (H-L) Sections from proximal to distal small intestine display a gradient of reporter activity in the epithelium and very low to undetectable levels in the colon (L). **Note:** the image shown in (K) is the same image that is shown in Figure 1D. Scale bars: 50µm.

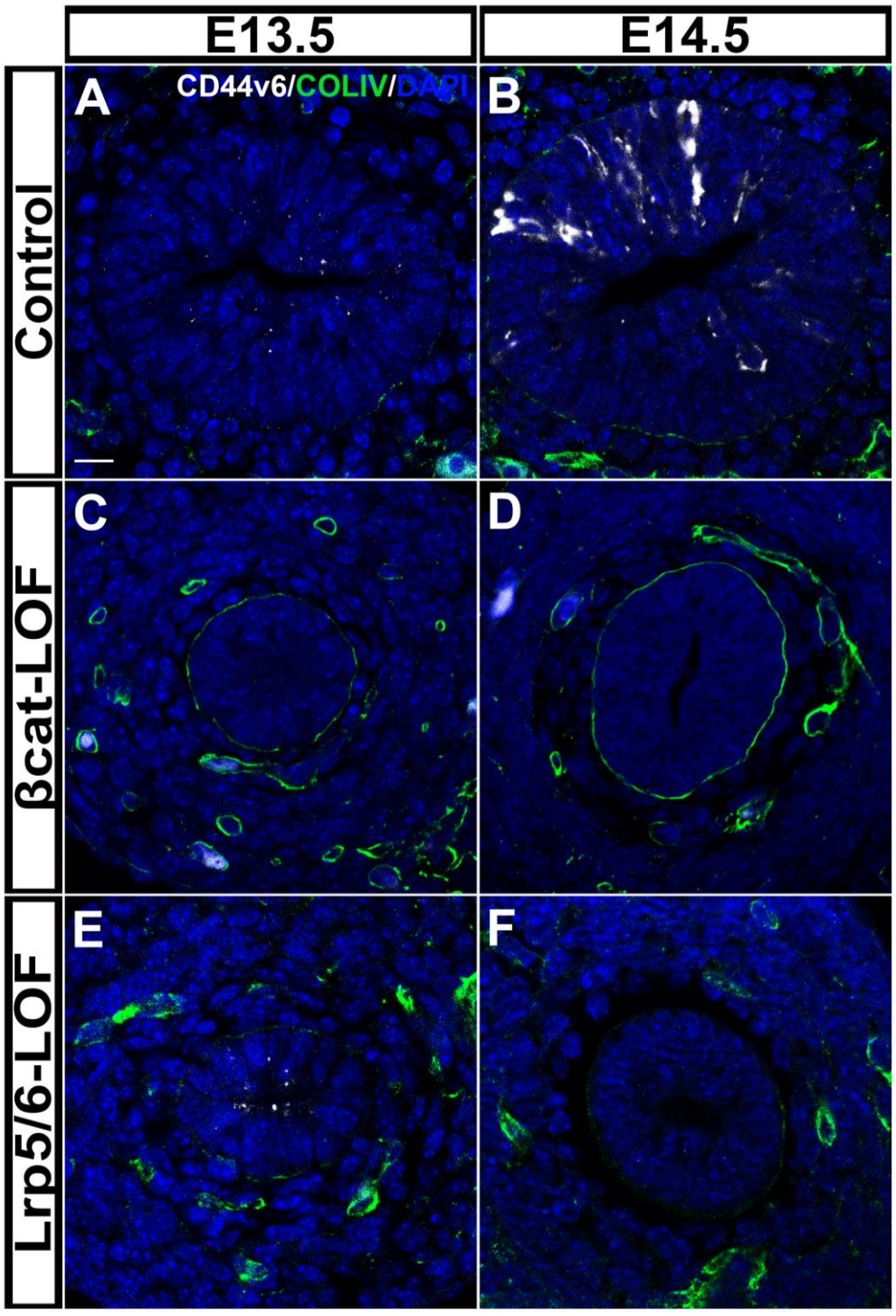

Figure 2.3. High magnification immunofluorescence images of CD44v6 protein in distal small intestines. (A) Co-stained with Collagen IV (green), punctate CD44v6 protein (white) is detected at low levels in E13.5 control intestines. (B) At E14.5, more robust membrane-bound CD44v6 is detected. (C-F) WNT/β-CATENIN signaling deficient intestines show efficient loss of CD44v6 in βcat-LOF as early as E13.5 (C) and Lrp5/6-LOF by E14.5 (F). Scale bar: 10μm.

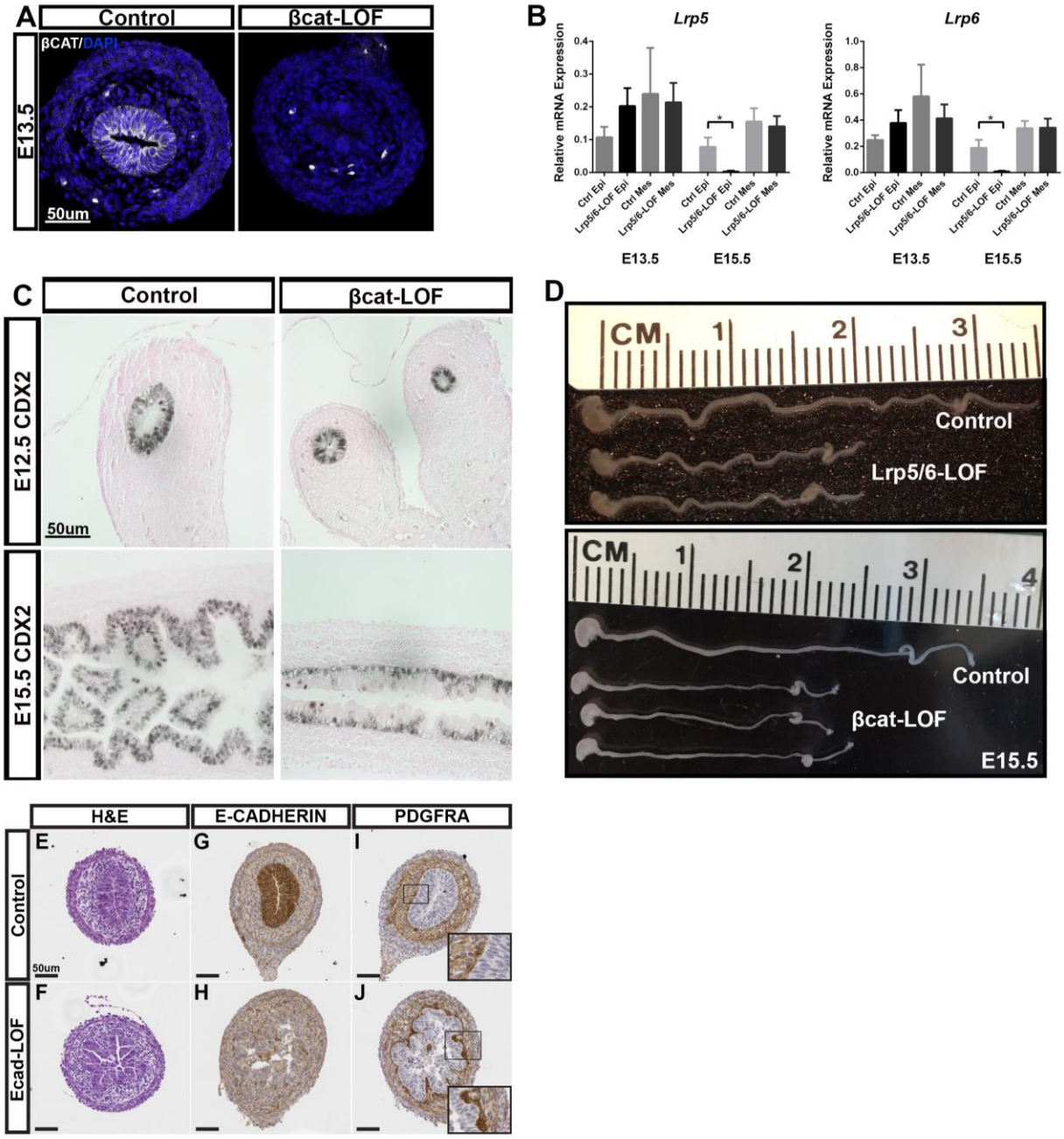

Figure 2.4. β-catenin and Lrp5 and Lrp6 is efficiently deleted by *Shh-Cre*. (A) Immunofluorescence staining for β-CATENIN displays complete deletion in mutants compared with controls at E13.5. β-CATENIN (white), DAPI (blue). (B) qPCR of epithelial and mesenchymal tissue isolations in Lrp5/6-LOF and control at E13.5 (n=3 embryos pooled from 2 litters) and E15.5 (n=3 embryos pooled from 3 litters) reveal efficient decrease in *Lrp5* and *Lrp6* transcript in E15.5 Lrp5/6-LOF epithelia. Asterisks indicate statistical significance of $p<0.05$. (C) Positive staining by DAB immunohistochemistry for intestinal epithelial marker CDX2 in βcat-LOF intestines at E12.5 and E15.5 indicates that the loss of *β-catenin* did not affect intestinal cell identity. (D) Whole mount images demonstrate that E15.5 Lrp5/6-LOF and βcat-LOF intestines

were drastically shorter and thinner than controls. (E-J) Disrupted villus morphogenesis is not due to cell-cell adhesion defects. (E, F) H&E histological staining of E14.5 control and Ecad-LOF jejunum shows that Ecad-LOF epithelium is able to form villi. (G, H) E-CADHERIN immunostaining showed that the protein was efficiently deleted in Ecad-LOF (H). (I, J) Ecad-LOF intestines formed PDFRA+ mesenchymal clusters similar to controls undergoing normal villus morphogenesis. Scale bars: 50μm. Error bars represent SD.

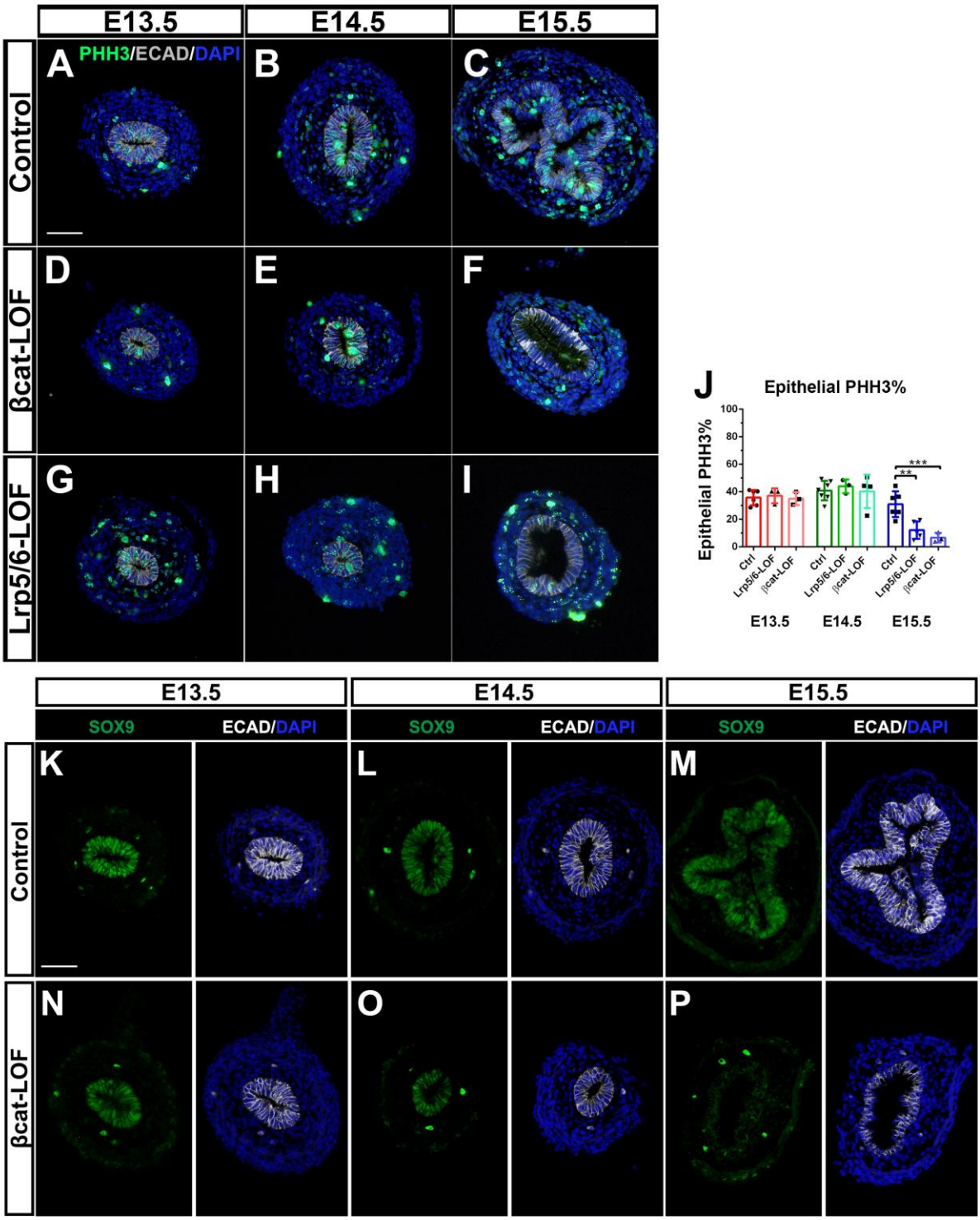

Figure 2.5. WNT/β-CATENIN signaling deficient mice have epithelial proliferation defects and decreased SOX9 expression only at E15.5 and not at earlier time points. Immunofluorescence staining for phospho-histone H3 (PHH3, green) and E-CADHERIN (white) demonstrates that epithelial proliferation was occurring in the distal small intestine of all genotypes at E13.5 and E14.5 (Control (A-B); βcat-LOF (D-E)

Lrp5/6-LOF (G-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). (J) Quantification of the percent of PHH3+ epithelial cells (PHH3+ECAD+ /total ECAD+DAPI+) shows a significant reduction in proliferation only at E15.5. For all genotypes, n=3 to 6 embryos pooled from 2 to 5 litters for 5 independent experiments. Statistical significance by t-test. **p-value 0.001-0.01, ***p-value 0.0001-0.001. (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, 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 bars: 50μm.

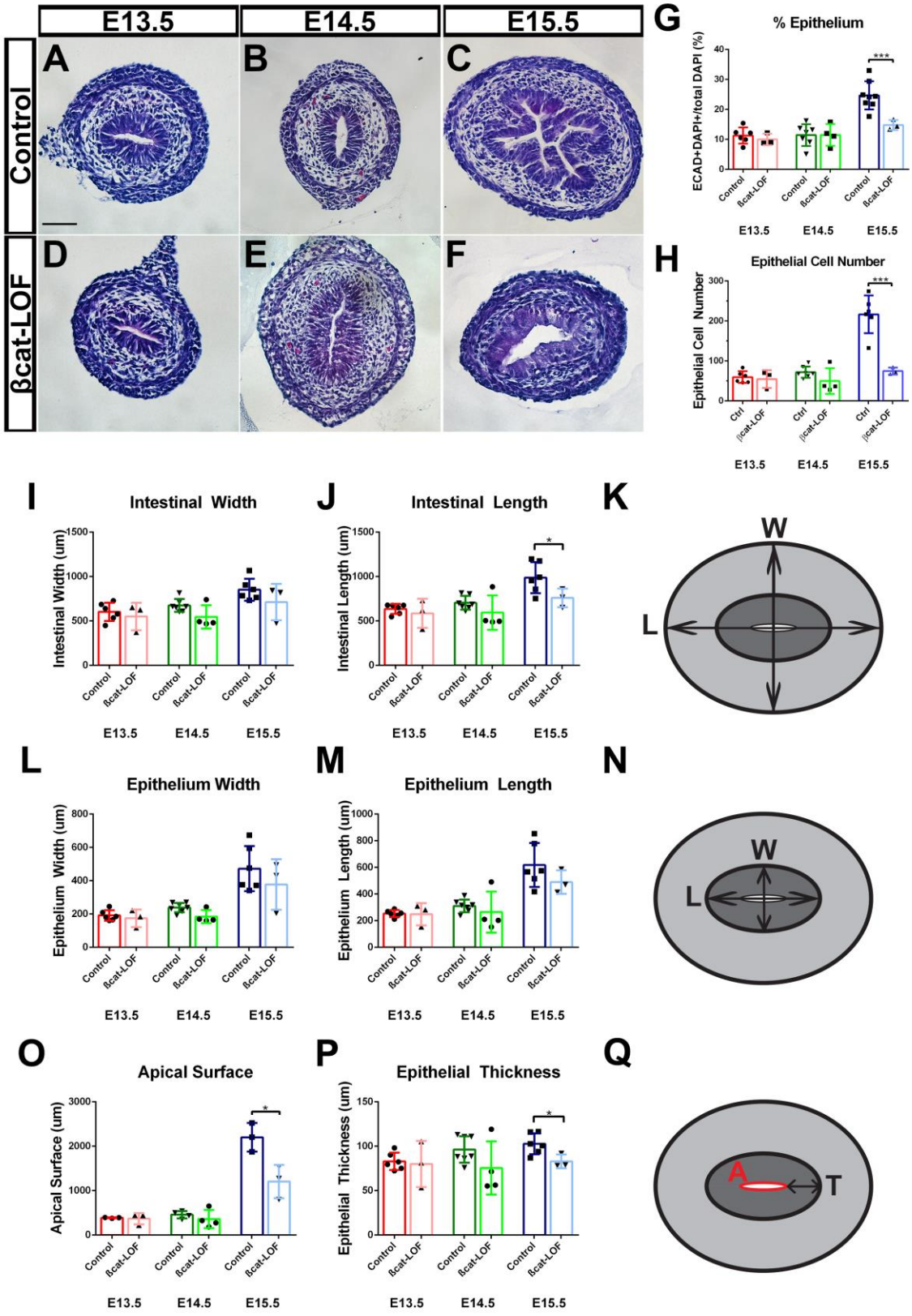
E13.5
E14.5
E15.5
Control
βcat-LOF
A
B
C
D
E
F
G
% Epithelium
ECAD+DAPI+/total DAPI (%)
H
Epithelial Cell Number
Epithelial Cell Number
I
Intestinal Width
Intestinal Width (um)
J
Intestinal Length
Intestinal Length (um)
K
W
L
L
Epithelium Width
Epithelium Width (um)
M
Epithelium Length
Epithelium Length (um)
N
W
L
O
Apical Surface
Apical Surface (um)
P
Epithelial Thickness
Epithelial Thickness (um)
Q
A
T

Figure 2.6. βcat-LOF intestines do not display morphological defects before E15.5. (A-E) H&E staining of βcat-LOF intestines at E13.5 and E14.5 (D, E) are indistinguishable from controls (A, B). (F) E15.5 βcat-LOF do not have prominent villus structures as in controls (C). 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, J), and epithelium width/length (L, M), was measured according to the schematic diagrams (K, N). No significant differences were observed across all time points. However, tracing the apical surface (demonstrated in Q, red), revealed a significant reduction in βcat-LOF at E15.5, reflective of the loss of villus structures. (P) Epithelial thickness, measured from the apical to basal surface (Q), was also reduced at E15.5. For all genotypes, n=3 to 6 embryos pooled from 2 to 5 litters for 5 independent experiments. Statistical significance by t-test. *p-value 0.01-0.05, **p-value 0.001-0.01, ***p-value 0.0001-0.001. Scale bar: 50μm.

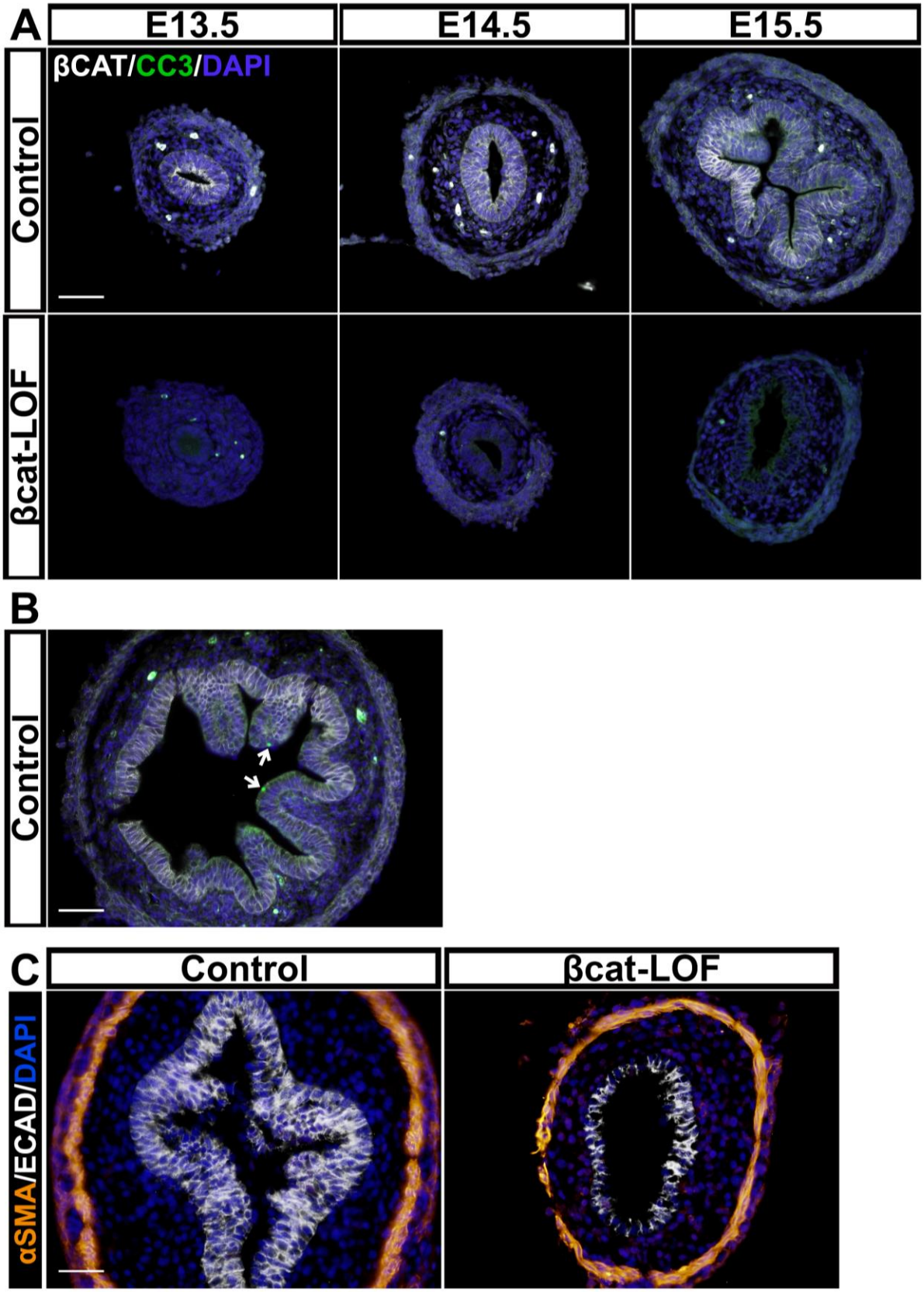
A
E13.5
E14.5
E15.5
βCAT/CC3/DAPI
Control
βcat-LOF
B
Control
C
Control
βcat-LOF
αSMA/ECAD/DAPI

Figure 2.7. Epithelial defects do not influence cell death or smooth muscle differentiation. (A) Immunofluorescence staining for apoptosis marker Cleaved-Caspase3 (CC3, green) shows no CC3+ cells in βcat-LOF distal small intestine. β-CATENIN (white) is completely deleted across all time points. (B) Absence of CC3+ cells in βcat-LOF distal small intestine was not an artifact of immunostaining as CC3+ cells were seen in the proximal duodenum at the tips of villi (arrows). (C) βcat-LOF distal small intestine displayed normal alpha-smooth muscle actin at E15.5 compared with controls. Alpha-smooth muscle actin, αSMA (orange). E-cadherin (white), DAPI (blue). Scale bars: 50μm.

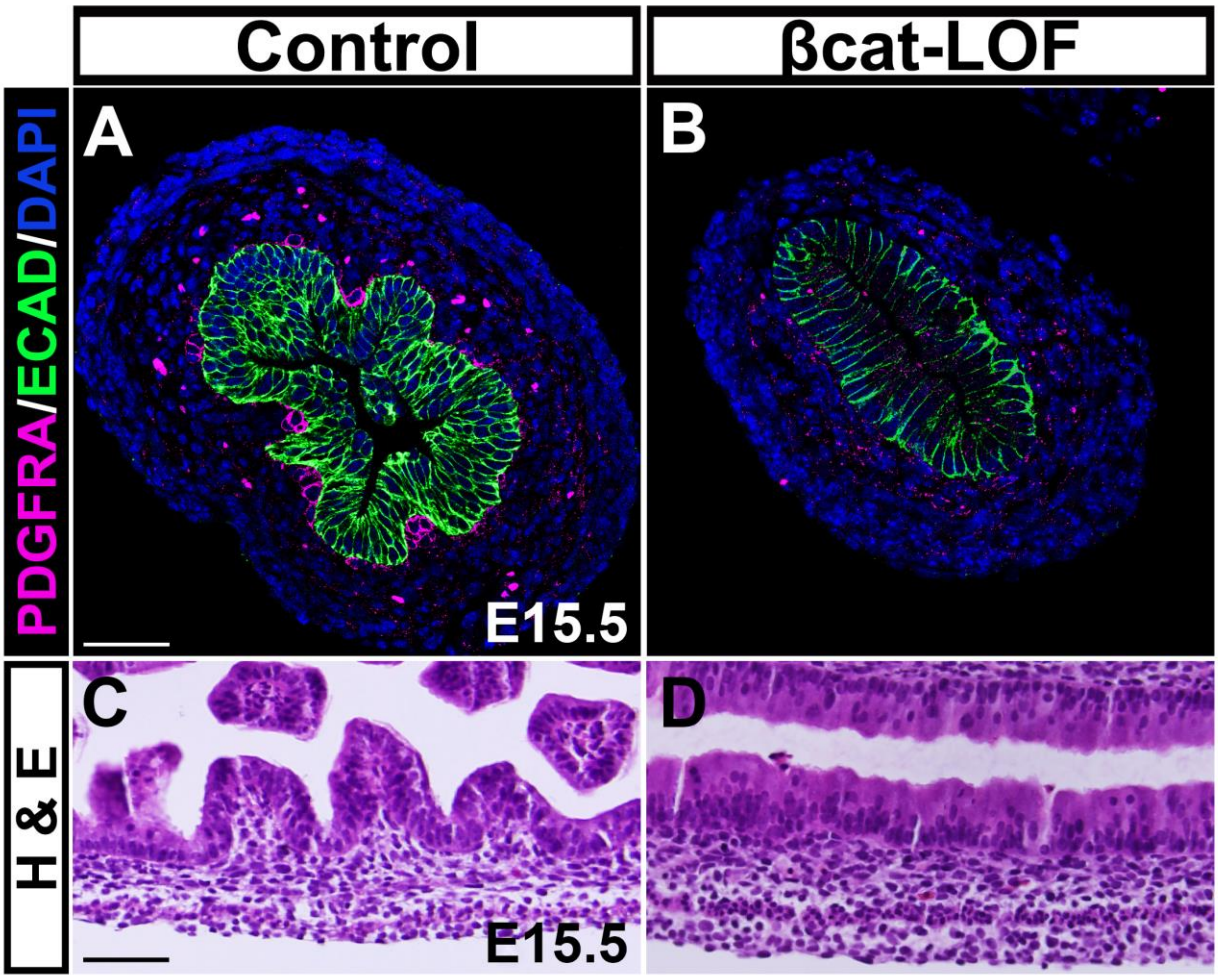

Figure 2.8. Loss of WNT/β-CATENIN signaling results in perturbed formation of PDGFRA+ mesenchymal clusters. (A) Immunofluorescence staining of E15.5 control distal small intestine shows clusters of PDGFRA+ (magenta) mesenchymal tissue beneath nascent villi. (B) PDGFRA was still expressed in the mesenchyme, but did not condense into clusters adjacent to the epithelium. (C) Longitudinal sections of E15.5 control intestine stained with H&E display numerous villi while βcat-LOF epithelial is flat (D). All samples are biological replicates with an n≥3. Scale bars: 50μm.

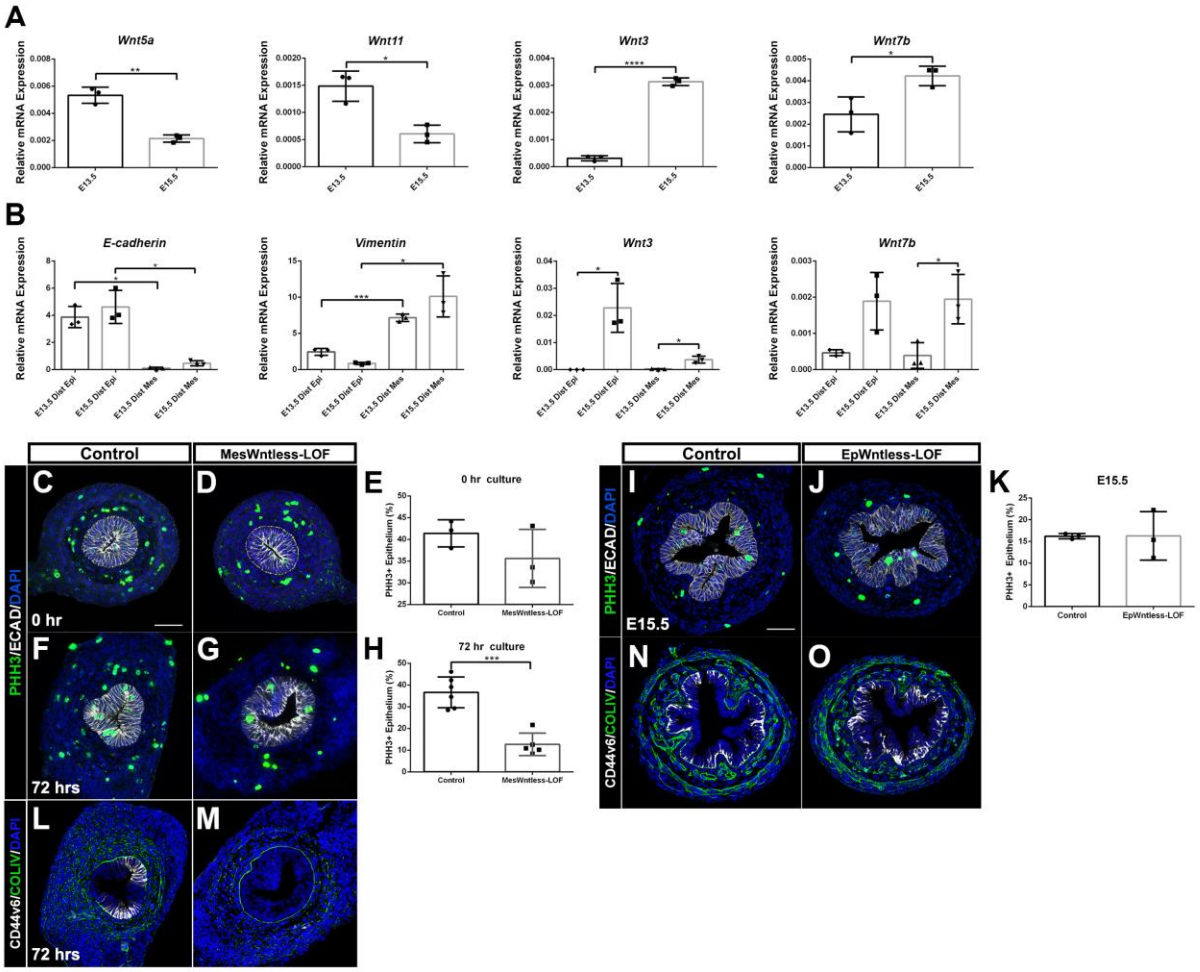

Figure 2.9. Mesenchymal WNT ligand secretion regulates epithelial proliferation. (A) qPCR on whole thickness control ileums from E13.5 (n=3 embryos pooled from 2 litters) and E15.5 (n=3 embryos from 1 litter) showed downregulation of *Wnt5a* and *Wnt11* transcript and upregulation of *Wnt3* and *Wnt7b* transcript. (B) E13.5 and E15.5epithelial 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-H, L-M) MesWntless-LOF E13.5 intestines (n=3 embryos pooled from 2 litters for one independent experiment) cultured ex vivo for 0 hours show no proliferation defects, visualized by PHH3 (green) and E-CADHERIN (white) staining, compared to littermate controls (n=3 embryos) (C, D). At 72 hours in culture, MesWntless-LOF intestines (n=6 embryos pooled from 2 litters) have a significant reduction in epithelial proliferation compared to controls (n=5 embryos pooled from 2 litters for two independent experiments) (F, G). PHH3+ epithelial cells were quantified in E and H. EpWntless-LOF intestines showed no significant differences in PHH3+ epithelial cells at E15.5 compared to controls (I-K). Wnt/β-catenin signaling target CD44v6 (white) is undetected in MesWntless-LOF when cultured for 72 hours (M) while controls exhibit robust membrane-bound epithelial staining (L). (N-O) EpWntless-LOF

E15.5 intestines do not show any differences in CD44v6 staining compared to controls. EpWntless-LOF and controls each have n=3 for one independent experiment. Statistical significance by t-test. *p-value 0.01-0.05, **p-value 0.001-0.01, ***p-value 0.0001-0.001, ****p-value 0.00001-0.0001.

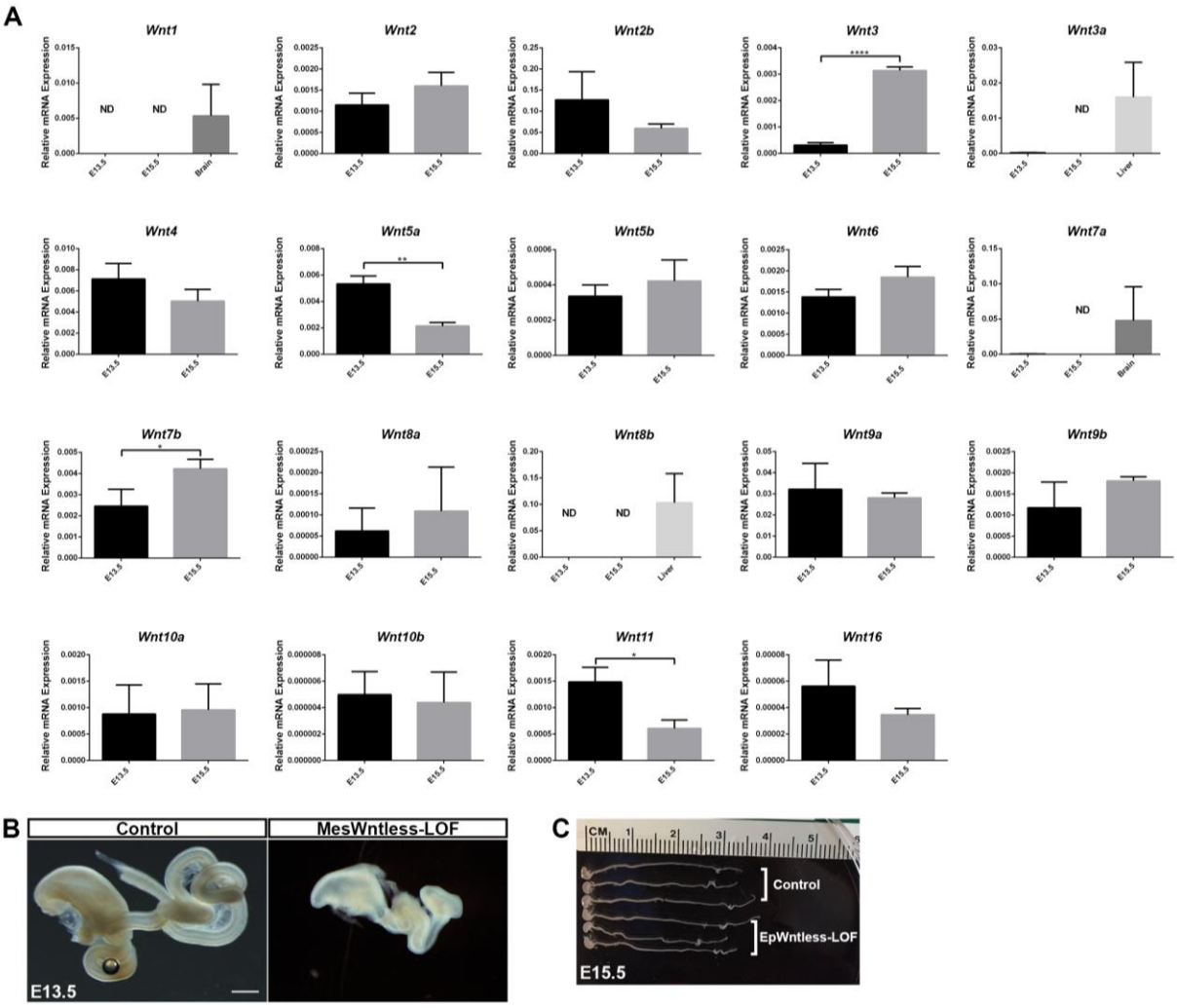

Figure 2.10. Mesenchymal-specific deletion of *Wntless* results in defects in gross morphology. (A) Whole thickness control ileums at E13.5 and E15.5 were analyzed for expression of the 19 *Wnt* ligands. Not detected, ND, reflects qPCR Ct value of 45. Asterisks indicate statistical significance: * = $p<0.05$, ** = $p<0.01$, ****= $p<0.0001$. All samples are biological replicates with n=3 embryos for one independent experiment. (B) Whole mount image of E13.5 MesWntless-LOF whole intestine shows a dramatic shortening of the small intestine and truncation at the ileum. Scale bar: 500μm. (C) Whole mount image of E15.5 EpWntless-LOF whole intestine do not display dramatic phenotypes and appear indistinguishable from controls. Error bars represent SD.

Table 2.1: Primary and Secondary Antibodies

Primary Antibody	**Source**	**Catalog #**	**Dilution**
Anti-Actin, α Smooth Muscle-Cy3	Sigma-Aldrich	C6198	1:300
Chicken anti-GFP	Abcam	Ab13970	1:500
Goat anti-B-catenin	Santa Cruz Biotech	sc-1496	1:250
Goat anti-E-Cadherin	R& D Systems	AF748	1:500
Goat anti-Sox9	R& D Systems	AF3075	1:500
Mouse anti-CDX2	BioGenex	MU392A-UC	1:500
Mouse anti E-Cadherin	BD Transduction Laboratories	610181	1:500, 1:4000
Rabbit anti-Cleaved Caspase3	Cell Signaling Technology	9664	1:500
Rabbit anti-Collagen IV	Millipore	AB756P	1:500
Rabbit anti-Ki67	Thermo Scientific	RM-9106	1:400
Rabbit anti-PDGFRa	Santa Cruz Biotech	sc-338	1:500,1:1000
Rabbit anti-PhosphoHistone H3	Millipore	06-570	1:500
Rabbit anti-Shh	Santa Cruz	sc-9024	1:20
Rat anti-CD44v6	eBioscience	BMS145	1:1000
Secondary Antibody	**Source**	**Catalog #**	**Dilution**
Biotin anti-rat	Jackson Immuno	712-065-150	1:1000
Biotin anti-rabbit	Jackson Immuno	711-065-152	1:1000
Donkey anti-chicken 488	Jackson Immuno	703-546-155	1:500
Donkey anti-goat 488	Jackson Immuno	705-545-147	1:1000
Donkey anti-goat 647	Jackson Immuno	705-605-147	1:1000
Donkey anti-mouse 647	Jackson Immuno	415-605-350	1:1000
Donkey anti-rabbit Cy3	Jackson Immuno	711-165-102	1:1000
Donkey anti-rat Cy3	Jackson Immuno	712-165-153	1:1000
Streptavidin 488	Jackson Immuno	160-540-084	1:1000

Table 2.2: qPCR Primer Sequences

Primer Name	Forward Sequence	Reverse Sequence
Axin2	TGCATCTCTCTCTGGAGCTG	ACTGACCGACGATTCCATGT
CD44	CACATATTGCTTCAATGCCTCAC	CCATCACGGTTGACAATAGTTATC
E-cadherin	GAGGTCTACACCTTCCCGGT	AAAAGAAGGCTGTCCTTGGC
GAPDH	TGTCAGCAATGCATCCTGCA	CCGTTCAGCTCTGGGATGAC
Lrp5	CTGTACTGCAGCTTGGTCCC	ACTCCAGCTTCACTCCGC
Lrp6	TCTGCGTGCTGCTGAGAG	ATCGTTGCATTCTCTTTGCC
Twist2	GCCTGAGATGTGCAGGTG	GTCTCAGCTACGCCTTCTCC
Vimentin	AGAGAGAGGAAGCCGAAAGC	TCCACTTTCCGTTCAAGGTC
Wnt1	AAATGGCAATTCCGAAACC	GAAGATGAACGCTGTTTCTCG
Wnt2	CCAACGAAAAATGACCTCGT	GGGAAGTCAAGTTGCACACA
Wnt2b	CTGCTGCTGCTACTCCTGACT	GGGGATGTTGTCACAGATCA
Wnt3	CTGCTACTCGGCCTCCTG	GAG ATGTGTACTGCTGGCCC
Wnt3a	CACCACCGTCAGCAACAG	TCACTGCGA AAGCTACTCCA
Wnt4	CCTGCGACTCCTCGTCTTC	GTTTCTCGC ACGTCTCCTCT
Wnt5a	ACGCTTCGCTTGAATTCCT	CCGGGCTTAATATTCCAATG
Wnt5b	GGGGAGAGACAGTGTGGAAG	AACATCTTCCAAAGCGGAGC
Wnt6	ACTGCTGCTGCTGCTCTTGT	CCTGCAGATGCTGGTAGGAT
Wnt7a	TACACAATAACGAGGCGGGT	TGTGGTCCAGCACGTCTTAG
Wnt7b	ACGTGTTTCTCTGCTTTGGC	CCAGGCCAGGAATCTTGTT
Wnt8a	GGTGGAATTGTCCTGAGCAT	GGATGGCATGAATGAAGGAT
Wnt8b	CCCGTGTGCGTTCTTCTAGT	AGACCAGGTAAGCCTTTGGA
Wnt9a	GATGCTGGATGGGTCCCT	GGGAGGATAGTCAGGGGTTC
Wnt9b	CGAGGAGATGCGAGAGTGC	GGAAGGGTGTCAGGACCTC
Wnt10a	GAGTGCCAGCATCAGTTCC	GCACTCTCTCGAAAACCTCG
Wnt10b	AACTGCTCGGCACTGGAG	GCATGGAGAAGGAGAAAGCA
Wnt11	CTGCGAGGCTCTGCTCTTT	TCTGATTCAGTGCCAAGGCT
Wnt16	TCTACACAACAACGAAGCGG	TTTTCCAGCAGGTTTTCACA

CHAPTER 3

DISCUSSION

Summary of findings

The research described in this thesis provides novel insight into the molecular mechanisms regulating intestinal development surrounding the formation of villi. In Chapter 2, I find that WNT/β-CATENIN signal transduction by the epithelium is dynamic during intestinal development. This conclusion is supported by evidence from reporter mice and loss-of-function genetic studies. *Axin2-LacZ* mice report very low WNT/β-CATENIN signal transduction in the intestinal epithelium at E13.5, prior to villus formation, which becomes much more robust 48 hours later. Using a *Shh-Cre* mouse to drive conditional epithelial-specific deletion of the β-catenin gene *CDH1* or of co-receptors *Lrp5* and *Lrp6*, we examined the distal intestine for morphological defects before, at the onset of, and after initiation of villus morphogenesis (E13.5, E14.5, and E15.5) and found that the intestine was indistinguishable from controls at E13.5 and E14.5, but displayed dramatic villus defects and loss of epithelial proliferation at E15.5. Together this represents two different stages of intestinal growth: one before villus morphogenesis has begun where WNT/β-CATENIN signal transduction is low and dispensable for epithelial proliferation, and another after villus morphogenesis has

begun where WNT/β-CATENIN signaling activity is high and required for epithelial proliferation. In order to understand how WNT/β-CATENIN ligand expression is behaving over this time, we analyzed the expression of *Wnt* ligand genes at E13.5 and E15.5 and found increased abundance of *Wnt3* and *Wnt7b* transcripts in both the epithelium and mesenchyme. To determine whether WNT/β-CATENIN signal transduction in the epithelium is dependent upon epithelial- or mesenchymal-expressed WNT ligands, we used genetic mouse models to inhibit total WNT ligand secretion in a compartment-specific manner. We analyzed mice with either *Shh-Cre* or *Twist2-Cre* driven *Wntless* loss of function, which consequently prevents WNT ligand secretion from either the epithelium or mesenchyme respectively. These studies showed that loss of mesenchymal WNT secretion resulted in reduced epithelial proliferation and abrogated WNT/β-CATENIN signal transduction, while mice with loss of epithelial WNT secretion were indistinguishable from controls. While more work is required to identify the expression pattern and effects of WNT signaling modulators, these data suggest that mesenchymally expressed WNT ligands are critical for proliferation of the intervillus regions as villi begin to emerge.

Contribution of work

This work advances the field of intestinal biology and describes an interesting difference between the adult and embryonic intestine. While canonical WNT/β-CATENIN signal transduction is required for stem cell maintenance in the adult intestinal epithelium, it appears that regulation of epithelial proliferation by WNT/β-CATENIN signal transduction prior to birth is much more dynamic. As the epithelium

converts from pseudostratified growth to villus emergence, the proliferation of the epithelium changes from WNT/β-CATENIN-independence to dependence. Prior to villus formation, the pseudostratified epithelium is uniformly proliferative; then upon emergence of nascent villi, the villus epithelium above clusters becomes columnar and withdraws from the cell cycle (Grosse et al., 2011). After villus emergence, proliferation becomes restricted to the intervillus domains and is driven by WNT/β-CATENIN signaling (Korinek et al., 1997; Garcia et al., 2009; Joo et al., 2010; Nigmatullina et al., 2017). Before my studies, it was not clear whether WNT/β-CATENIN signaling drives epithelial proliferation prior to villus formation. One study analyzing the loss of *Tcf4* at E14.5 showed no proliferation defects (Korinek et al., 1997), consistent with my findings. But a thorough analysis of the role of WNT/β-CATENIN signaling surrounding the initiation of villus morphogenesis had not been conducted. My work characterizes the role of WNT/β-CATENIN signaling to regulate proliferation of the intestinal epithelium before and after the onset of villus formation in order to enhance our understanding of the mechanisms guiding tissue morphogenesis. Additionally, my work uncovers how WNT/β-CATENIN signaling activity changes over developmental time and how mesenchymal versus epithelial WNT ligands affect epithelial proliferation, providing an excellent example of how mesenchymal-epithelial crosstalk can influence tissue morphogenesis. In light of the findings presented herein, newly published literature by others, and our unpublished data, this thesis raises new questions for future study. I will describe several of these questions and propose future experiments below.

Future Directions

What drives epithelial proliferation before villus formation when the epithelium is pseudostratified?

At E12.5 and E13.5, prior to villus formation, the pseudostratified epithelium is highly proliferative. Our studies show that proliferation at this time is not driven by WNT/β-CATENIN signal transduction, so the mechanism driving proliferation of the pseudostratified epithelia is still unknown. Recent work has shown that GATA4 regulates proliferation of early epithelial progenitors from E10.5-E11.5, but not from E12.5-E16.5 nearer to the emergence of villus structures (Kohlnhofer et al., 2016). Noncanonical WNT signaling may also be regulating epithelial proliferation during pseudostratified growth as *Wnt5a*-null mice show elongation defects due in part to reduced epithelial proliferation, although cell apoptosis had not been investigated (Cervantes et al., 2009). Both of these studies conclude that epithelial proliferation was only moderately reduced, suggesting that other pathways may also be driving proliferation at these times.

In other tissues, proliferation of pseudostratified epithelia is regulated by various signaling pathways. Proliferation of the developing vertebrate neural epithelium has been shown to be regulated by SHH and FGF signaling through the upregulation of CYCLIN and MYC proteins (Kenney et al., 2003; Oliver et al., 2003; Lobjois et al., 2004). However, these are not attractive candidates in the pseudostratified intestinal epithelium because HH ligands signal in a paracrine manner to the mesenchyme (Kolterud et al., 2009; Ramalho-Santos et al., 2000) and *FGF9*-null mice display

elongation defects at E14.5 due to impaired mesenchymal proliferation (Geske et al., 2008). A more attractive candidate may be mitogen-activated protein kinase (MAPK). In the pseudostratified Wolffian duct of the developing kidney, MAPK has been shown to regulate the G1/S transition during mitosis, which is required for sustained proliferation (Ihermann-Hella, et al, 2014). *In vitro* studies demonstrate that high levels of P42/44 MAPK activate epithelial proliferation in human colon cancer cells (Aliaga et al., 1999) and E14.5 mouse intestinal enteroids require EGF for proliferation in culture through activation of ERK1/2-mediated signaling (Suzuki et al., 2010). Interestingly, adult mice with inducible *p38 MAPK*-deletion in colonic epithelium displayed increased proliferation and tumorigenesis, suggesting that MAPK may be suppressing epithelial proliferation in the adult mouse colon (Wakeman et al., 2012).

Additional studies need to be done in order to determine if these pathways or others regulate epithelial proliferation during pseudostratified growth. Experiments using genetic mouse models that manipulate specific signaling pathways or explant intestine cultures treated with small molecule libraries could be used to identify the signaling pathway (or multiple pathways) stimulating epithelial proliferation before villus formation. After identifying the pathway driving epithelial proliferation before villus formation, it would be interesting to determine if it is then inactivated upon villus emergence, or if it plays a redundant role with WNT/β-CATENIN to drive epithelial proliferation after villus emergence. If it acts redundantly with WNT/β-CATENIN, compound loss of function experiments disrupting signal transduction of both the newly identified pathway and WNT/β-CATENIN may reveal more severe proliferation defects after villus emergence.

Or if this pathway is inactivated upon villus emergence, then two distinct regulatory mechanisms control epithelial proliferation before and after villus formation.

Epithelial WNT/β-CATENIN signal transduction between E13.5 and E15.5: How is the transition made?

Together with findings from published literature, the data presented in this thesis describes a dramatic morphological change that takes place over a very short period of time. At E13.5, the intestinal epithelium is entirely pseudostratified, proliferating and producing SHH, with no mesenchymal clusters and low WNT/β-CATENIN signal transduction (Figure 3.1- A). At E15.5, villus structures with PDGFRA+ and GLI1+ mesenchymal clusters are established and villus epithelial cells in direct contact with the clusters withdraw from the cell cycle and become columnar. Between clusters, discrete intervillus domains are composed of proliferating epithelial cells that transduce WNT/β-CATENIN signals and express SHH (Figure 3.1- C). But importantly, the morphogenetic processes that occur between these two times (from E14.0-E14.5) and result in the restriction of WNT/β-CATENIN signal transduction to the intervillus domains are less clear. There are two distinct possibilities. One is that there is a transient intermediate stage at E14.0, prior to mesenchymal cluster formation, where all pseudostratified epithelial cells robustly transduce WNT/β-CATENIN signals and as a result, emit other signals that are necessary for cluster formation. Subsequently, the newly formed clusters signal to the overlying epithelium to silence WNT signal transduction, possibly by secretion of a WNT inhibitor (Figure 3.1- B). This would implicate WNT/β-CATENIN signal transduction as an indirect driver of mesenchymal

cluster formation and also attribute mesenchymal clusters as local inhibitors of WNT/β-CATENIN signal transduction. Alternatively, WNT/β-CATENIN signal transduction may be activated *de novo* in a patterned way, only in intervillus cells between clusters (Figure 3.1- B'). This implies that mesenchymal clusters and defined intervillus domains arise before the selective activation of WNT/β-CATENIN signal transduction in intervillus epithelium.

In support of the first possible mechanism, our data shows increased *Axin2-LacZ* reporter expression in all pseudostratified epithelial cells at E14.5, consistent with a transient intermediate stage of uniform WNT/β-CATENIN signal transduction (Figure 2.2 –E). We also found that epithelial-specific deletion of *β-catenin* or *Lrp5/6* lacked PDGFRA+ mesenchymal clusters (Figure 2.8- A-B) and showed reduced epithelial SHH (Figure 3.2) and mesenchymal *Ptch1* expression (Figure 3.3- A-B) compared to controls. It will be important in the future to carefully examine *Shh* and *Ptch1* expression at E14.5, just before and during the time of cluster formation, in order to determine exactly when this expression is diminished. Additionally, 80% of *β-catenin*-deleted intestines examined displayed rescued mesenchymal cluster formation when cultured *ex vivo* in the presence of Smoothened agonist (SAG) (Figure 3.3- E, F). These data suggest that epithelial WNT/β-CATENIN signal transduction may induce mesenchymal cluster formation by promoting expression of epithelial SHH ligand. Interestingly, *β-catenin*-deleted intestines cultured with SAG retained epithelial proliferation defects (Figure 3.3- I), suggesting that epithelial WNT/β-CATENIN signaling may mediate SHH expression and proliferation independently. However, the possibility that these genetic

deletions of epithelial WNT signal transduction leads to dramatic changes in the epithelial cells themselves, indirectly causing reduced SHH expression, cannot be ruled out at this time.

In support of the second mechanism in which WNT/β-CATENIN signal transduction is activated *de novo* in a patterned manner in intervillus epithelium, our data show that expression of the WNT target CD44 is heterogeneous in the epithelium of E14.5 wildtype mice (Figure 2.1- H). We can speculate that patterned activation of WNT/β-CATENIN signal transduction could be due to upregulation of FRIZZLED receptors, reception of the WNT agonist RSPO, or down-regulation of WNT inhibitors, only in epithelial cells between clusters. Or aside from patterned activation or suppression of WNT ligands or modulators, mechanical signals may activate WNT/β-CATENIN signal transduction. It has been shown that upon aggregation of mesenchymal clusters, epithelial cells overlying mesenchymal clusters undergo cell shape changes to become shorter and wider (Freddo et al., 2016: Walton et al., 2016). This widening puts intraepithelial compressive forces on epithelial cells between clusters, potentially causing mechanotransduction that may activate WNT/β-CATENIN signal transduction (Freddo et al., 2016). But further evidence that mesenchymal cluster formation and establishment of intervillus domains precedes activation of WNT/β-CATENIN signal transduction needs to be provided to support this model.

In order to distinguish between these two models, we need to first understand when WNT/β-CATENIN signal transduction is activated in relation to cluster formation.

Because mesenchymal clusters arise in a wave-like fashion that travels proximally to distally (Walton et al., 2012), we can observe WNT/β-CATENIN signal transduction at the "wave front" of cluster formation, analyzing epithelial and mesenchymal morphology and gene expression in pseudostratified epithelium immediately before and immediately after cluster formation. If we see WNT/β-CATENIN signal transduction in all pseudostratified epithelium before clusters are present, this will be consistent with (but not definitively prove) the first model (Figure 3.1- B), suggesting the existence of an important transient stage in intestinal development that has not been previously recognized or explored. It is also consistent with the notion that WNT/β-CATENIN signaling is involved in induction of cluster formation, perhaps through activation of SHH. If WNT/β-CATENIN signal transduction induces cluster formation through activation of SHH, then we would predict that intestinal explants cultured with beads coated with WNT inhibitors would display reduced SHH and subsequent loss of cluster formation. Furthermore, explant culture of *Lrp5/6*-deleted intestines would show rescued SHH expression and cluster formation upon addition of WNT agonist. Alternately, the second model (Figure 3.1- B') would be supported if we observe cluster formation preceding activation of WNT/β-CATENIN signal transduction and WNT/β-CATENIN only turns on in intervillus epithelium. This would suggest that patterned activation of WNT/β-CATENIN signal transduction is possibly due to upregulation of WNT receptors or activators, downregulation of inhibitors, or mechanotransduction. We would predict that gene expression analysis after laser capture microdissection of intervillus epithelium versus epithelium overlying clusters and mesenchymal clusters versus mesenchyme adjacent to clusters would reveal distinct expression patterns. For example, we would

predict discrete expression of *Frizzled* in the intervillus, *Rspo* in the intervillus or mesenchyme beneath the intervillus, or WNT inhibitors in epithelia above clusters or in mesenchymal clusters. Additional investigation is also needed to elucidate the potential activation of mechanotransduction in the intervillus domains.

How is WNT ligand expression upregulated at the time of villus emergence?

Another important finding from these studies is that the onset of villus formation occurs concomitantly with upregulated WNT ligand expression. Figure 2.9- A-B reports increased *Wnt3* and *Wnt7b* mRNA abundance in the mesenchyme at E15.5 compared to mesenchyme at E13.5. We also determined that mesenchymal WNT ligands, and not epithelial WNT ligands, are required for normal epithelial proliferation and expression of the WNT target gene *CD44* (Figure 2.9- C-O). This prompts two questions: What is the mesenchymal cell population secreting WNT ligands? What promotes increased expression of *Wnt* ligands?

Subepithelial myofibroblasts in the adult mouse have been proposed to be a vital source of WNT ligands. Previously, researchers had detected expression of *Wnt2b*, *Wnt4*, and *Wnt5a* in subepithelial cells by *in situ* hybridization (Gregorieff and Clevers, 2005). Indeed, these mesenchymal cells appear to secrete ligands important for epithelial proliferation, since isolated human epithelium (enteroids) can be sustained for 60 days in culture when grown with subepithelial myofibroblasts, compared to just 2-3 days without (Lahar et al., 2011). However, *Myh11-Cre* specific deletion of *Porcupine* to abrogate total WNT ligand secretion in adult mouse subepithelial myofibroblasts did not

yield any defects (San Roman et al., 2014). Thus, the particular mesenchymal cell population that provides WNT ligands and drives epithelial proliferation is still unknown.

Another recent study attempting to find the source of WNT ligands identified a non-myofibroblastic CD34+ GP38+ αSMA- population (Stzepourginski et al., 2017). They found that these cells localized in close proximity to LGR5+ stem cells, produce WNT2 and RSPO1 and are sufficient to maintain LGR5+ stem cells in human intestinal organoids (Stzepourginski et al., 2017). However, these cells develop after birth and therefore would not be the critical cells producing WNT ligands in the developing intestine (Stzepourginski et al., 2017). Another study ablating FOXL1+ cells in adult mice by diphtheria toxin administration observed dramatic reduction of epithelial proliferation and loss of WNT/β-CATENIN signal transduction (Aoki et al., 2016). *In situ* hybridization data suggested that these FOXL1+ cells produce *Wnt2b*, *Wnt4*, and *Wnt5a* (Aoki et al., 2016). And because *Foxl1* knockout mice displayed hyperproliferation and increased WNT/β-CATENIN signal transduction (Kaestner et al., 1997; Perreault et al., 2001), there is a clear difference in removing FOXL1 transcription factors and removing FOXL1+ cells. It is possible that these are the key cells that are producing WNT ligands. Further investigation may target FOXL1+ cells as an attractive candidate for the source of WNT ligands in the developing intestine. But first it would need to be elucidated if FOXL1+ cells exist in the embryonic intestine and if emergence of FOXL1+ cells coincides with epithelial WNT/β-CATENIN-dependent signaling. If so, functional experiments ablating FOXL1+ cells in the developing intestine would predict a loss of mesenchymal WNT ligand expression.

If FOXL1+ cells are the source of mesenchymal WNT ligands, we then ask, does FOXL1 play a functional role during intestinal development? Do FOXL1 transcription factors promote the transcription of *Wnt* ligands? FOXL1 is a member of the *forkhead* family of transcription factors. The forkhead box, or *Fox*, family of transcription factors has been shown to play important roles during development and are implicated in various human diseases (reviewed in Carlsson and Mahlapuu, 2002; Benayoun et al., 2011; Golson and Kaestner, 2016). They are expressed in the intestinal mesenchyme (Kaestner et al., 1996; Mahlapuu et al., 2001; Ormestad et al., 2006; Nik et al., 2013) and have been shown to differentially affect epithelial proliferation at different times. While postnatal *Foxl1* null mice demonstrate hyperproliferation of the epithelium, increased nuclear β-catenin, and enhanced tumorigenesis in mice with mutations in APC (Kaestner et al., 1997; Perreault et al., 2001; 2005), in late fetal stages (after E16.5) *Foxl1* null mice develop fewer and blunted villi (Kaestner et al., 1997), suggesting that FOXL1 may have different roles in the intestine before and after birth. Another subfamily of *Fox* factors, the FOXF proteins (FOXF1 and FOXF2) negatively regulate proliferation and WNT signaling in both fetal and adult stages. *Foxf1* and *Foxf2* mutants display hyperproliferation in colon epithelia at E18.5. The authors suggest that FOXF2 may be down regulating proliferation indirectly through upregulation of BMP4 (Ormestad et al., 2006). Loss of *Foxf2* promotes adenoma formation in adult *Apc* mutants (Nik et al., 2013). FOXF2 appears to regulate WNT signaling directly by promoting the expression of the WNT inhibitor SFRP1 (Nik et al., 2013). This evidence suggests functional differences between *Fox* subfamilies, where FOXL transcription

factors promote WNT signaling and epithelial proliferation during development, while FOXF transcription factors inhibit WNT signaling and restrict proliferation at all time points. And both FOXL and FOXF have been shown to be downstream of HH signaling (Ormestad et al., 2004; 2006; Madison et al., 2009). FOXL1 and FOXF1 loci are bound by GLI proteins, suggesting direct regulation (Madison et al., 2009). Together, we can imagine a mechanism in which epithelial HH ligands in the developing intestine activate mesenchymal FOXL transcription factors, promoting their secretion of WNT ligands, which signal back to the epithelium and drive epithelial proliferation. To support this model, it will be important to demonstrate that HH ligands induce FOXL expression in the developing intestine prior to epithelial WNT-dependence and that FOXL transcription factors directly regulate transcription of WNT ligands. This information can expand our understanding of how WNT ligands in the mesenchyme are upregulated before the onset of villus morphogenesis.

In conclusion, the work presented in this dissertation provides insight into the molecular mechanisms that control epithelial proliferation around the time of villus formation. This work describes dynamic WNT/β-CATENIN signaling activity in the developing intestine and elucidates a previously unrecognized transition during which WNT/β-CATENIN signal transduction increases at the onset of villus formation. Further loss of function studies show that during the pseudostratified stage of growth, proliferation in the intestinal epithelium is independent of WNT/β-CATENIN signal transduction. However, at the start of villus formation, the epithelium makes an important transition, requiring WNT/β-CATENIN signals for normal epithelial

proliferation. Yet many questions still remain. If not WNT/β-CATENIN signaling, what is driving proliferation of the pseudostratified epithelium? How does WNT/β-CATENIN signal transduction increase at the onset of villus formation and how is it restricted to the intervillus domains? These questions and others will drive further investigation and promise an interesting study.

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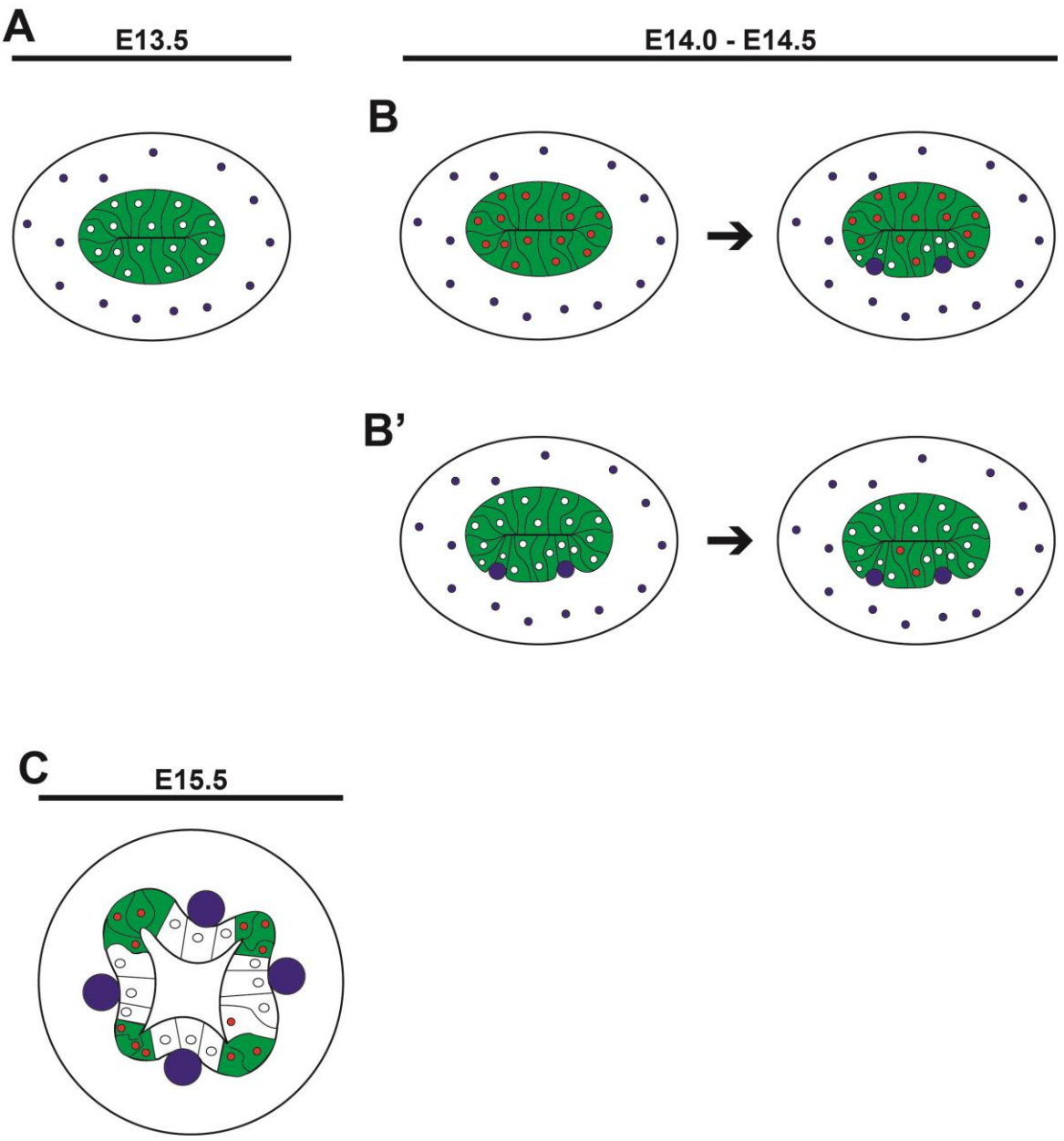

Figure 3.1. Schematic of morphogenetic changes during villus formation. (A) At E13.5, the intestinal epithelium is pseudostratified and expressing SHH (green) WNT/β-CATENIN signaling activity (red) is very low in the epithelium. (B) From E14.0-E14.5, WNT/β-CATENIN signal transduction becomes restricted to the intervillus domains by either one of two possible mechanisms: WNT/β-CATENIN signal transduction is briefly activated in all pseudostratified cells and then turns off in epithelial cells overlaying mesenchymal clusters (blue) (B), or the formation of mesenchymal clusters patterns the intervillus domains and WNT/β-CATENIN signal transduction is activated *de novo* in intervillus cells (B'). (C) At E15.5, villus and intervillus domains are established. Epithelial proliferation, SHH expression, and WNT/β-CATENIN signal transduction becomes restricted to the intervillus domains.

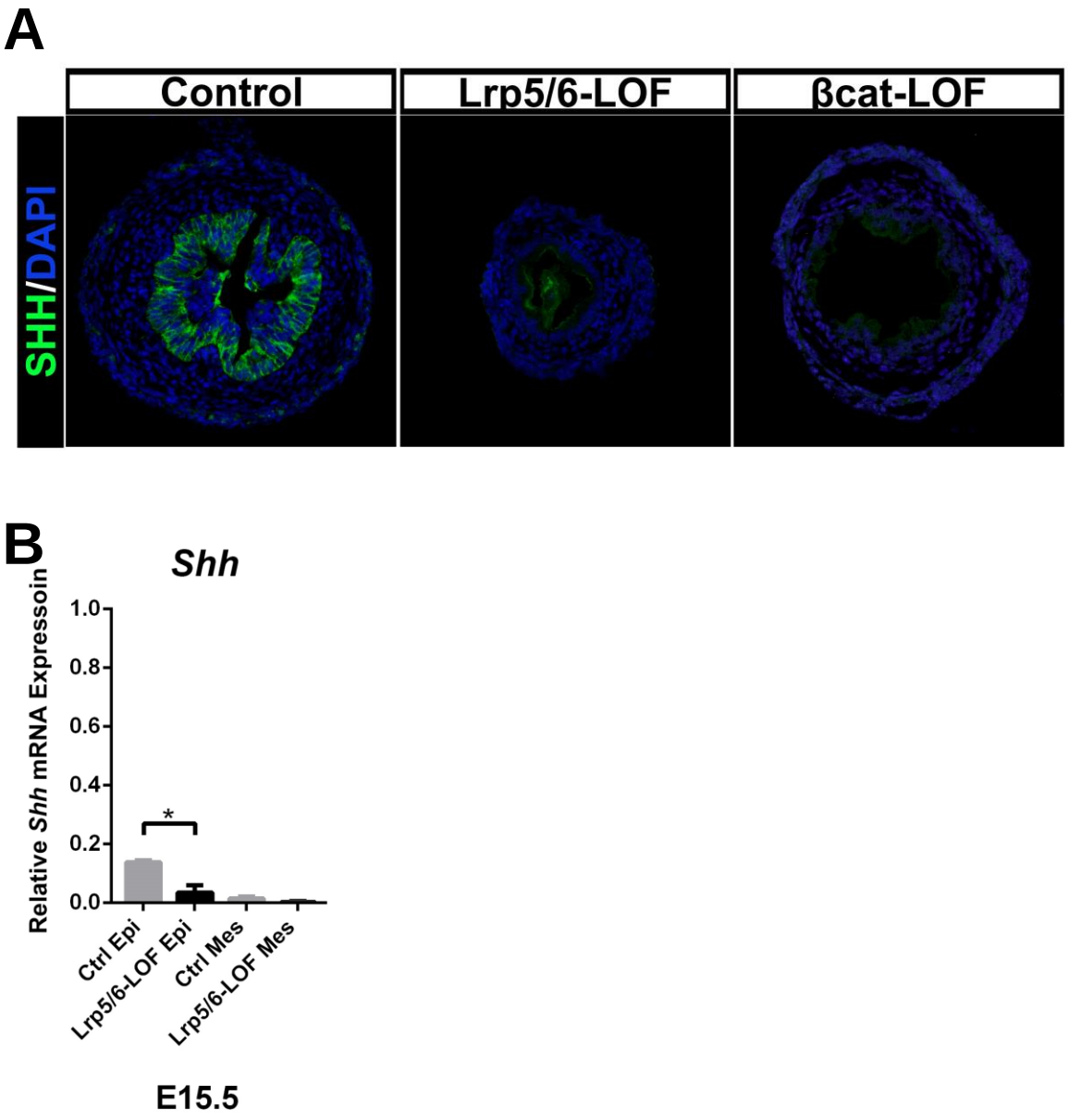

Figure 3.2. *Lrp5/6* and *β-catenin*-deleted intestines display defects in epithelial SHH expression. (A) Immunofluorescence staining against SHH does not detect appreciable levels of SHH protein in Lrp5/6-LOF or βcat-LOF at E15.5. (B) qPCR analysis of epithelial (Epi) versus mesenchymal (Mes) isolations display significant reduction of *Shh* mRNA transcript in Lrp5/6-LOF epithelium compared to controls. Statistical significance by t-test. *p-value ≥ 0.05

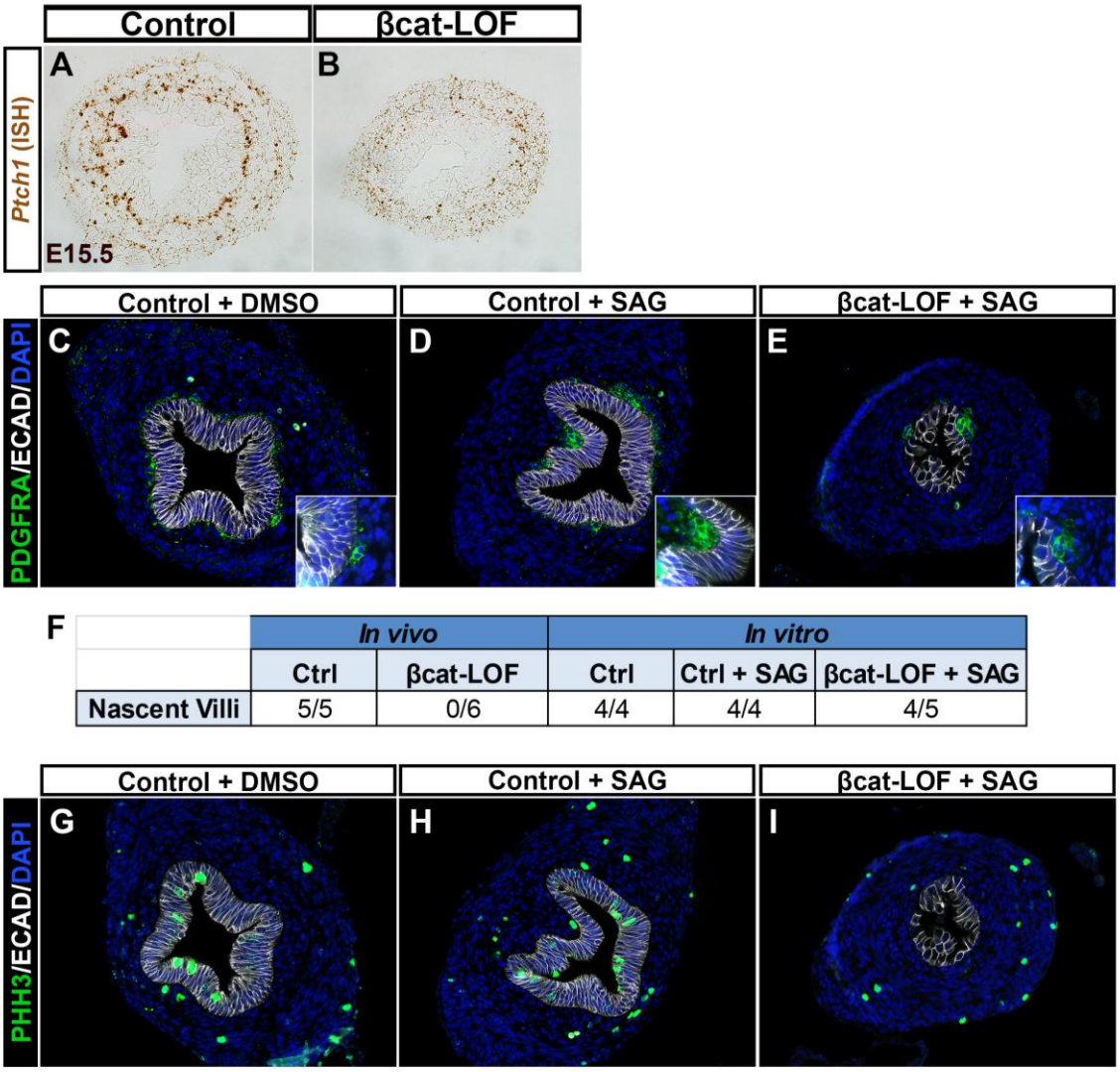

Figure 3.3: *β-catenin*-deleted mice display loss of mesenchymal cluster formation due to defects in HH signaling (A-B). *In situ* hybridization shows that large condensations of mesenchymal cells positive for *Ptch1* mRNA transcript aggregate adjacent to the epithelium in controls (A), but not in βcat-LOF at E15.5 (B). (C-I) To allow for the application of SAG, E14.5 distal small intestines were cultured *ex vivo* for 48 hours and then analyzed by immunofluorescence. Staining for PDGFRA (green) and E-CADHERIN (white) revealed small clusters in Controls + DMSO (C). Controls + SAG displayed larger clusters and larger villi (D). Unlike the *in vivo* βcat-LOF which lacked cluster formation, βcat-LOF with SAG treatment had PDGFRA positive mesenchymal clusters surrounded by buckling epithelium (E). Insets show the cluster and nascent villus at higher magnification. (G-I) Immunostaining for PHH3 (green) and E-CADHERIN (white) demonstrated highly proliferative epithelium in controls with DMSO and SAG (G, H), whereas βcat-LOF + SAG epithelium was not proliferative (I). (F) Table summary of the number of biological samples in either *in vivo* or *in vitro* experiments that display nascent villi as defined as having both PDGFRA+ mesenchymal cluster and adjacent buckling.