CHARACTERIZING THE ROLE OF LRIG1 IN THE DEVELOPMENT OF THE COLONIC EPITHELIUM

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RACHEL ELIZABETH HOPTON

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DISSERTATION APPROVAL PAGE

Student: Rachel Elizabeth Hopton

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This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Adam Miller	Chairperson
Annie Zemper	Advisor
Karen Guillemin	Core Member
Chris Doe	Core Member
Kryn Stankunas	Core Member
Robert Guldberg	Institutional Representative

and

Krista Chronister	Vice Provost for Graduate Studies

Original approval signatures are on file with the University of Oregon Division of Graduate Studies.

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DISSERTATION ABSTRACT

Rachel Elizabeth Hopton

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The digestive system is essential for many higher-order organisms to absorb nutrients and secrete waste. For mammals, the digestive tract develops as a continuous tube along the rostral-caudal axis and is split into two regions, the small intestine and the large intestine. These two sections form two discrete organs, which differ in structure and function. At the cellular level, each section has its own specific composition of epithelial cell types, which enables the small and large intestine to serve distinct roles in digestion. The large intestine is also known as the colon, and its primary functions are to serve as a barrier to the microbe-rich external environment, to absorb water, and to secrete waste. The colonic epithelium is lined with test tube-shaped invaginations called crypts of Lieberkühn, comprised of stem, progenitor, and differentiated cells which facilitate these absorptive and secretory functions of the adult colon.

In the mouse, growth and specification of the intestine occurs *in utero* and completes after birth. The small intestine develops embryonically whereas the colon develops after the mouse is born (postnatally). While a great deal is known about the growth and specification of the small intestine, less is known about the molecular cues that define the structural and cellular parameters for colon crypt formation.

The primary goal of my thesis is to define the cellular, morphological, and

molecular features of colon crypt development during the first three weeks after birth. In this thesis, I characterized the organization of colonic epithelial cells, areas of proliferation, and emergence and expression of a stem and progenitor marker, Lrig1. I show cells expressing Lrig1 are present when the mouse is born and these cells behave as stem cells throughout colon development. To interrogate the requirements for normal mouse colon development, I use an inducible knockout mouse to eliminate *Lrig1*, and demonstrate this leads to an increase in epithelial cell proliferation. Further, I show loss of *Lrig1* does not impact differentiation in the developing colon up to two weeks after birth. My thesis contributes to the understanding of normal mouse colon development and highlights specific molecular requirements for normal colon crypt development.

This dissertation includes previously published co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Rachel Elizabeth Hopton

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene
University of California, Davis

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2023, University of Oregon
Bachelor of Science, Cell Biology, 2016, University of California, Davis

AREAS OF SPECIAL INTEREST:

Molecular Biology
Cell Biology
Developmental Biology

PROFESSIONAL EXPERIENCE:

Teaching Assistant, Department of Biology, University of Oregon, Eugene
2018-2023

Laboratory Manager, Department of Molecular & Cellular Biology, University of California, Davis, 2016-2018

GRANTS, AWARDS, AND HONORS:

Raymond-Stevens Fellowship, University of Oregon. 2023

Raymond-Stevens Fellowship, University of Oregon. 2022

Women in Graduate Science Parenting Award, University of Oregon, 2022

General Scholarship, University of Oregon, 2022

American Gastroenterological Association's Student Abstract Award, 2022

Paul and Helen Weiser Memorial Scholarship, University of Oregon, 2022

National Institute of Child Health and Human Development Institutional Training Grant in Developmental Biology (T32HD007348), University of Oregon, 2019-2021

Stanley David and Lucille Borgen Adamson Memorial Scholarship, University of Oregon, 2018

John Muir Volunteer Leadership Award, John Muir Health, 2016

PUBLICATIONS:

Hopton RE, Jahahn NJ, Zemper AE "Lrig1 Drives Cryptogenesis and Restrains Proliferation during Colon Development." (*In revision, AJP-Gastrointestinal and Liver Physiology*)

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This dissertation is dedicated to my loving husband, Bob, for his endless support and encouragement throughout this adventure. I would also like to dedicate this dissertation to my daughter, Edith. You can do anything you set your mind to.

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CHAPTER I

INTRODUCTION

Function of the Intestine

The digestive system is responsible for nutrient uptake from food through a continuous tube called the gastrointestinal tract and is essential for life in all mammals. In all evolved mammalian species, there are five regions of the gastrointestinal tract: mouth, esophagus, stomach, small intestine, and large intestine. Each of these regions function as independent organs that aid in the uptake of nutrients, vitamins, and minerals (Ogobuiro et al., 2022). The initial processing of food is accomplished by mechanical and enzymatic digestion via the mouth, esophagus and stomach. After this, the small intestine is tasked with the absorption of nutrients and water. In humans, a single meal takes five hours to be shuttled though the small intestine (Fish & Burns, 2022) and by the time the digested material has reached the end of the small intestine, 95% of carbohydrates and proteins and 90% of the water have been absorbed (Fish & Burns, 2022). This absorption largely relies on enzymes and transporters produced by cells on the surface of the small intestine, which enables carbohydrates and proteins to be broken down (Montoro-Huguet et al., 2021). The indigestible waste is then sent into the colon for the absorption of the remaining water. The colon also acts as a barrier between the organism and their commensal microbes located in the luminal space, thereby protecting the animal from the external environment. The colon also plays a pivotal role in absorbing vitamins that are produced by the bacteria that colonizes the gut (Azzouz & Sharma, 2022). The small intestine and colon play different roles in the process of digestion; much of the functional differences are based in gene expression differences between the cells of each organ,

permitting this diversity in function (Said, 2018). Interestingly, the colon is the predominant site of adult digestive diseases including ulcerative colitis and cancer (Azzouz & Sharma, 2022), and diseases of the small intestine are rarer (Chen & Vaccaro, 2018). Despite this difference in disease occurrence, historically, the largest research efforts have focused on investigating the development and molecular regulation of the small intestine (Bry et al., 1994; Dehmer et al., 2011; O. Kwon et al., 2020; Sumigray et al., 2018). The purpose of my thesis was to conduct a careful investigation of colon development to aid in our understanding of how this vital organ comes to be and performs its various functions.

The Mouse as a Model for Colon Crypt Development

The mouse (*Mus musculus*) is a common mammalian model organism for discovering the fundamental molecular mechanisms underlying gastrointestinal diseases. Approximately 70 percent of their protein-coding genes in mice are conserved with humans, making this an excellent model for translational research (Yue et al., 2014). The gastrointestinal tract of humans and mice are homologous in their physiology and their stereotyped anatomical structures. It is important to match developmental stages when modeling human development with mice. In particular, the mouse intestine is largely comparable to the human *in utero* fetal intestine at P10. At P14 and P28 the mouse intestine is markedly similar to the human intestine at 22-23 weeks and at full-term, respectively (Stanford et al., 2020). These parallels make the mouse a textbook-model for investigating intestinal biology and translating those findings into pre-clinical research. Until recently, there has been a lack of data that directly compares intestinal

developmental stages between mice and humans (Stanford et al., 2020). Much of the research conducted on the molecular regulation of the digestive system is focused on the small intestine. Often, many molecular aspects of colonic homeostasis are "assumed" based on conclusions drawn from small intestinal research papers (Chin et al., 2017; O. Kwon et al., 2020). This is particularly true of studies focusing on the development of the intestine. Expanding our knowledge of murine colon development can aid in the understanding of human colon development.

<u>Development of the Murine Intestine</u>

Mice develop over 18.5 days *in utero* and intestinal formation is initiated during this embryogenesis. Advancements in molecular biology techniques have enabled the understanding of developmental processes including cellular movement, gene expression, and signaling mechanisms governing mouse organogenesis (de Santa Barbara et al., 2003; O. Kwon et al., 2020; Noah et al., 2011; Sumigray et al., 2018; Tan et al., 2013). From these data we have learned the gut is composed of all three germ layers and the intestine originates from the endodermal germ layer. The endoderm has distinct gene expression patterns that drive anterior-posterior patterning, which is critical for organ specification. During anterior-posterior patterning, the gut is regionalized into the foregut, midgut, and hind gut during gastrulation at embryonic day 5 (E5) (Sherwood et al., 2009). The small intestine originates from the midgut, whereas the large intestine originates from the hindgut (de Santa Barbara et al., 2003). The endodermal cells from both of these regions form a continuous epithelial sheet which undergoes extensive

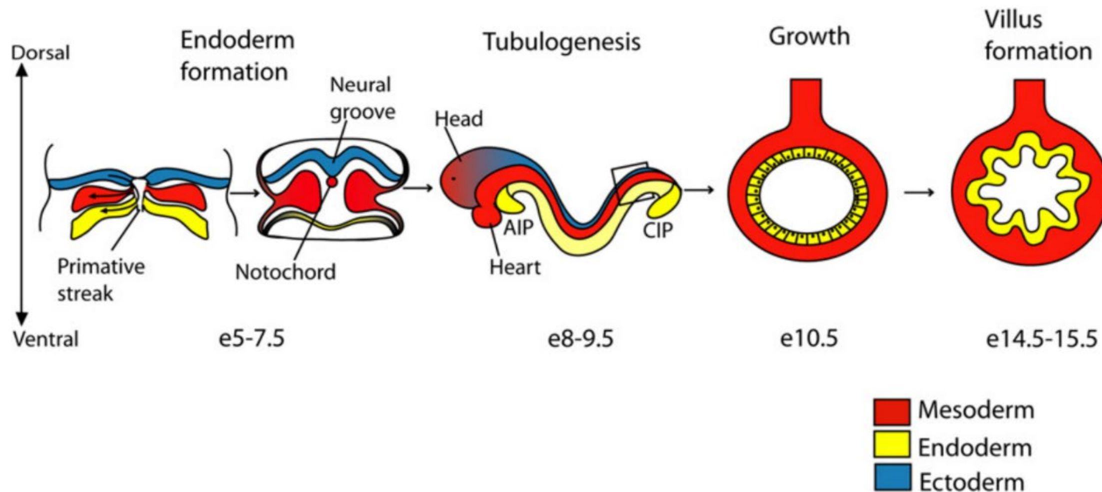

Figure 1. Early Development of the Intestine.
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folding to give rise to a primitive gut tube by E7.5 (Figure 1). This tube further elongates, forms pockets, and is composed of a compact and proliferative pseudostratified cellular layer called the epithelium by E10.5 (G. S. Kwon et al., 2008; Lewis & Tam, 2006). Incredibly, these processes occur within the amniotic space, not within the developing organism. At E14, finger-like projections called villi emerge in the small intestine causing the epithelium to adopt a columnar-like shape. An intervillus region separates the individual villi, is comprised of proliferating epithelial cells which migrate downward to form structures called crypts (Sumigray et al., 2018). The intestine undergoes extensive looping and coiling and returns to the body at E16.5. For the remaining two days of embryonic development, the small intestinal tube continues to lengthen and widen, producing numerous villus-crypt units (Guiu & Jensen, 2015). Additional invaginations of the small intestine continue throughout postnatal development and extensive studies have characterized these morphological changes in-depth as well as the molecular mechanisms governing these changes (Chin et al., 2017;

Radtke et al., 2006; S. Wang et al., 2019). In contrast to the small intestine, the colon develops after the mouse is born and is strictly comprised of colonic crypts (Guiu & Jensen, 2015). It is assumed that the colon develops in the same manner, morphologically and molecularly, as the small intestine. However, very few studies have investigated the morphogenic movements of the colon, let alone the molecular mechanisms involved.

Colon Crypt Formation

In the mouse, growth and specification of the colon occurs after the mouse is born ("postnatally") and is made up of crypts with proliferative cells residing at the base and more differentiated cells at the epithelial surface (Ménard et al., 1994). The distal colon starts out as a flat epithelial sheet and develops from small invaginations on the surface by the second postnatal day (P2).These form the bona fide precursors to colonic crypts by P6 (Ménard et al., 1994). By P14, the colon forms adolescent crypts which are

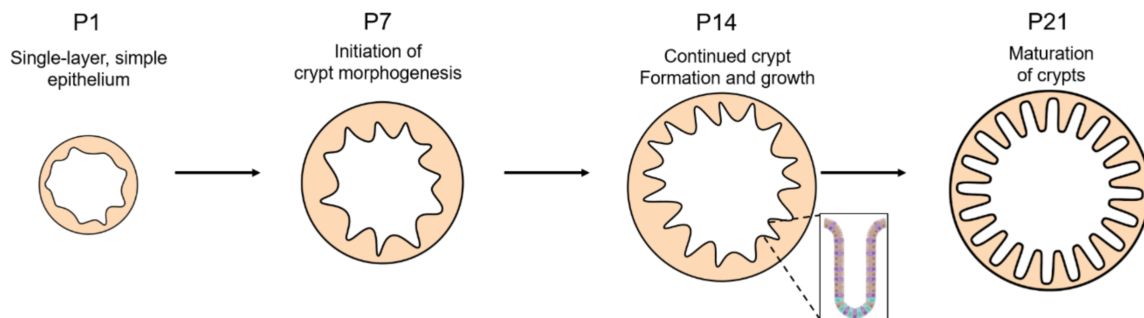

Figure 2. Schematic of Postnatal Colon Crypt Development.

indistinguishable from adult crypts by P21 (Cheng & Bjerknes, 1985; Hopton et al., 2023) (Figure 2). There are only a handful of studies that describe the developing colon in anatomical detail. (Ménard et al., 1994; Yanai et al., 2017). In Chapter II, I illustrate

the morphology and molecular features of colonic crypts, as they develop into their adult-like structures.

Epithelial Architecture of the Adult Colon

The past 60 years of gastrointestinal research have defined the cellular composition and function of adult (12 weeks of age, (Flurkey et al., 2007)) colonic crypts (Barker, 2014; Barker et al., 2007; O. Kwon et al., 2020; Ménard et al., 1994; Snippert et al., 2010; Sumigray et al., 2018; Winton et al., 1988). One of the most notable discoveries from this research is the identification of a long-lived stem cell population in adult crypts (Barker et al., 2007; Bjerknes & Cheng, 1981). This a heterogeneous population of stem cells, located at the base of U-shared crypts, supports the continuous production the single layer of columnar epithelial cells (Barker et al., 2007; Bjerknes & Cheng, 1981; Potten et al., 2009) (Figure 3). The stem cells are positioned adjacent to the basement membrane and divide to create an identical copy of themselves as well as transit-amplifying (TA) cells, which are capable to producing all differentiated cells within the colonic epithelium. TA cells further divide to produce daughter cells migrate toward the lumen of the crypt in a conveyor belt-like fashion. These cells become more

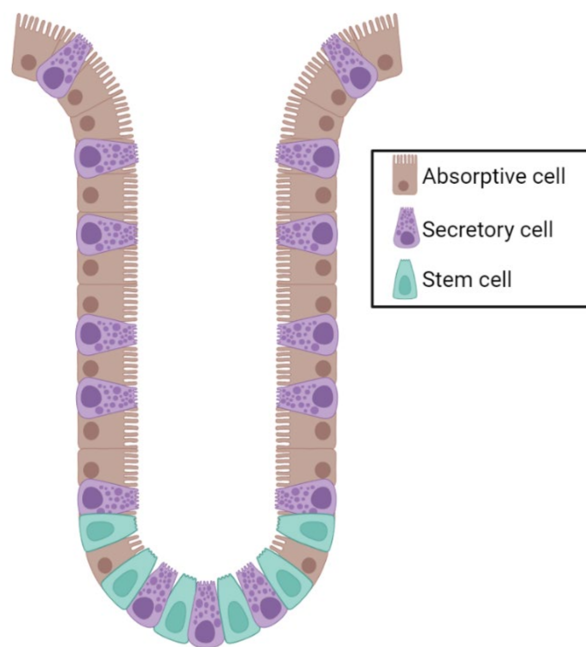

Figure 3. Diagram of a stereotypical adult colonic crypt.

differentiated and commit to either an absorptive or secretory colonic epithelial cell fate (Barker, 2014). When differentiated cells reach the top of the crypt or "cuff", they are subjected to programmed cell death and slough off into the lumen in a process called anoikis (Hall et al., 1994). This process is necessary to maintain homeostasis in the colonic crypt (Barker, 2014). In 2010, Snippert and colleagues demonstrated all of the cells which comprise the crypt are derived from the same stem cell, and this defines them "clonal" (Snippert et al., 2010). Data which support this theory include numerous genetic lineage tracing experiments which permit tracking stem cells over a period of time (Barker et al., 2007; Powell et al., 2012; Winton et al., 1988). These studies also show adult colonic crypts are clonal units, arising from a single stem cell population at the base of each crypt. This concept of clonality is important for the maintenance of the gastrointestinal epithelium, as stem cells have specific molecular mechanisms to guard and support a healthy genome (Potten et al., 2002). Further, genetic changes to colon stem cells are maintained in the entire daughter cell population (Winton and Ponder 1990, Griffiths et al., 1988); disease studies show that genetic mutations in stem cell populations result in widespread aberrant crypts throughout the human colon (Graham et al., 2011). As it is currently unknown when clonality occurs during the process of assembling adult crypts, in Chapter II I examine clonality during colon crypt development.

Lrig1: A Tumor Suppressor and a Stem and Progenitor Cell Marker

One critical molecular feature of adult colonic stem cells is the expression of a protein called Leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) (Powell

et al., 2012; Wong et al., 2012). Lrig1 is a transmembrane protein expressed on the basal lateral surface of stem and progenitor cells in the adult colon (Powell et al., 2012). Lrig1 functions in many tissues to repress growth via the binding to specific receptor tyrosine kinases (Y. Wang et al., 2013). In the gut, Lrig1 facilitates degradation of Egfr, an important growth-promoting receptor tyrosine kinase (Powell et al., 2012; Wong et al., 2012). Egfr signaling is mediated through ligand binding which stimulates the homo- or heterodimerization of Egfr, with ErbB2 or ErbB3, and results in the activation of downstream signaling cascades such as MAPK, AKT, and JAK/STAT (Citri & Yarden, 2006). Egfr has been shown to regulate intestinal epithelial cell proliferation and differentiation. When Egfr binds one of its many ligands, the rate of intestinal epithelial cell differentiation increases, and notably, as does the rate of crypt morphogenesis (Duh et al., 2000). Proper activation and repression of Egfr is critical; as observed in the fruit fly, *Drosophila melanogaster*, ectopic activation of EGFR results in excessive stem cell proliferation leading to gut hyperplasia (Xu et al., 2011).

Consistent with this role of growth repression, *Lrig1* null animals have exogenous proliferation within the colon and sustained expression of activated Egfr, throughout the gut (Powell et al., 2012; Wong et al., 2012). In one model of Lrig1 loss, the mice die by postnatal day 10, due to gut overgrowth harboring enlarged crypts and distended intestines (Wong et al., 2012). In another model of Lrig1 loss, the mice survive into adolescence and adulthood, yet harbor large tumors with sustained activation of Egfr (Powell et al., 2012). The function of Lrig1 has been established in the adult colon, however, the function of Lrig1 in colon development has not been investigated.

Lrig1-expressing (Lrig1+, hereafter) cells have the capability to self-renew and differentiate to create daughter cells in both the small intestine and colon. Powell and colleagues demonstrated Lrig1+ stem cells can be lineage traced from P1 and the lineage labeling was detected eight weeks later, demonstrating that Lrig1+ stem cells exist early on in colon development (Powell et al., 2012). In addition, Lrig1+ cells can act as "cancer stem cells" giving rise to aggressive tumors in mice, if additional genetic alleles are modified (Powell et al., 2014). Ultimately we know Lrig1+ cells are important for the maintenance of the adult gastrointestinal tract, yet it is still how early in development Lrig1+ cells emerge, and if these cells behave as stem cells during the earlier stages of development. In Chapter II, I investigate the emergence and establishment of Lrig1+ cells using lineage labeling techniques. I also sought to determine if Lrig1 restrains proliferation, as observed during adult colonic homeostasis.

In summary, the primary goal of this dissertation is to convey advances I have made in the field of colonic epithelial developmental biology. In Chapter II, I present my morphological and molecular analysis of developing colonic crypts during the first three weeks after birth. I then use this data to lineage trace Lrig1+ cells during colon development. Additionally, I leverage a unique, inducible knockout mouse that allows me interrogate the role of *Lrig1* at various timepoints in colon development.

CHAPTER II

THE ROLE OF LRIG1 IN THE DEVELOPMENT OF THE COLONIC EPITHELIUM

*This chapter contains previously published co-authored material

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Introduction

Over the last 50 years our understanding of the origin, specification and patterning of model mammalian organs and organ systems has exponentially increased (Bardot & Hadjantonakis, 2020; O. Kwon et al., 2020; Montgomery et al., 1999). For digestive organs, these data have contributed to our comprehension of human development (Fair et al., 2018; O. Kwon et al., 2020; Spence et al., 2011; Stanford et al., 2020), informed our knowledge of disease etiology (Dedhia et al., 2016; Fair et al., 2018; O. Kwon et al., 2020; Sprangers et al., 2021), and in some cases, have also facilitated the development of therapeutic interventions (Clevers, 2016; Mochel et al., 2017; Yin et al., 2019). These data sets describe the molecular requirements for growth and development of the small intestine; however, mammalian colon development is less well understood.

A handful of studies from the last 30-40 years (Cheng & Bjerknes, 1985; Chin et al., 2017; Dehmer et al., 2011; Herbst & Sunshine, 1969; Ménard et al., 1994; Montgomery et al., 1999; Powell et al., 2012; Tan et al., 2013; Winton et al., 1988; Wong et al., 2012) provide the groundwork for our current understanding of colonic development, yet the molecular mechanisms that govern colon development from birth to adulthood remain largely unknown.

In the mouse, the majority of the growth and specification of the colon occurs postnatally. In the immediate days and weeks following birth, the colon elongates, and expand to form functional crypt units (Cheng & Bjerknes, 1985; Chin et al., 2017; Dehmer et al., 2011; Fernandez Vallone et al., 2020; Herbst & Sunshine, 1969; Ménard et al., 1994; Powell et al., 2012; Sumigray et al., 2018; Tan et al., 2013; Winton et al., 1988; Wong et al., 2012; Yui et al., 2018). During this timeframe, three functionally distinct regions of the colon are also developing. The region adjacent the cecum is commonly referred to as the "proximal" colon and harbors distinct mucosal folds not present in the "middle" and the "distal" colon, located posterior to the proximal region. The distal colon grows from a flat nascent mucosal layer, forming small invaginations on the surface by the second postnatal day (Ménard et al., 1994). These invaginations form the *bona fide* precursors to adult colonic crypts by P7 (Cheng & Bjerknes, 1985); however, the molecular characteristics of these nascent crypts have not been defined.

In the small intestine, cell proliferation and specification is directed by a heterogeneous population of intestinal stem cells (Barker et al., 2007; Cheng & Bjerknes,

1985; Potten et al., 2009). These stem cells reside at the base of the colonic crypts and divide to create a progenitor cell pool known as transit-amplifying (TA) cells. TA cells further divide, move up the crypt in a conveyor belt-like fashion, and become more differentiated, committing to either an absorptive or secretory colonic epithelial cell fate (Barker et al., 2014). Over recent years, numerous molecular markers of these stem, TA and differentiated cells have been discovered (Barker et al., 2007; Middelhoff et al., 2017; Powell et al., 2012; Rindi et al., 2004). Despite these advances, very little has been done to characterize these markers during colon development (Fernandez Vallone et al., 2020; Yanai et al., 2017) and even less is known about what functional role, if any, these proteins play in crypt development.

The recent expansion in our understanding of adult colon stem cell behavior and the biology of colonic crypts has largely been facilitated by genetically labeling the cells in the stem cell compartment utilizing several lineage-tracing approaches (Barker et al., 2007; Montgomery et al., 2011; Powell et al., 2012; Roth et al., 2012; Sangiorgi & Capecchi, 2008; Takeda et al., 2011). These experimental tactics have provided the basis for our understanding that adult colonic crypts are clonal units, which arise from a single stem cell population at the base of each crypt. Due to their clonal nature, any genetic or epigenetic changes in colon stem cells can persist and are retained in the daughter cell population (Griffiths et al., 1988; Snippert et al., 2010; Winton et al., 1988). Studies examining human genetic and epigenetic variations in crypts demonstrate that aberrant crypts can arise from mutant stem cell populations, and have a widespread, deleterious impact along the length of the adult colon (Graham et al., 2011; Snippert et al., 2014).

Clonality is a defining tenet of colon crypts (Barker et al., 2007; Snippert et al., 2010), yet it is unknown when clonality is established during crypt development.

One important molecular feature of adult intestinal and colonic crypts is the expression of a protein called Leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) (Powell et al., 2012; Wong et al., 2012). Lrig1 is a transmembrane protein which decorates the surface of stem and progenitor cells in the adult colon and functions as a tumor suppressor (Powell et al., 2012). Lrig1 is required for proper intestinal development; complete deletion of *Lrig1* can result in premature postnatal death of mice, with digestive tract abnormalities (Wong et al., 2012). In addition, after acute injury to the epithelium, Lrig1 expression is rapidly expanded in the colon as a part of the regenerative process and lineage tracing from Lrig1+ cells demonstrates the cells proliferate and divide to replenish colonic crypts (Choi et al., 2018; Powell et al., 2012; Yui et al., 2018). Lrig1 is important for mouse development, as Lrig1-expressing (Lrig1+) cells aid in regulating a balance of proliferation and differentiation in the developing brain and skin (Gomez et al., 2013; Jeong et al., 2020; Nam & Capecchi, 2020). Lrig1 is also present in the gastrointestinal tract at birth (Powell et al., 2012), but the role of *Lrig1* and Lrig1-expressing cells have not been defined.

The goal of our study was to identify key cellular, molecular, and morphological features of colon development using a loss-of-function approach. We first defined the morphological and molecular characteristics of wildtype, distal colonic crypt development from birth to three-weeks of age. Next, we examined the role of Lrig1+

cells. To achieve this, we lineage traced Lrig1+ cells using a multicolor reporter during colon development. Further, to test the requirement for *Lrig1* in colon development, we utilized CreERT2/LoxP (Powell et al., 2012) (EUCOMM) mice to inducibly eliminate *Lrig1* five days after birth and examined the impact in the first three weeks of life. Our data indicate Lrig1+ cells are present at P1 and give rise to clonal crypts three weeks later. We show *Lrig1* is not required for crypt cell differentiation but is required to suppress proliferation during colonic crypt development two weeks after birth.

Methods

Mice

Mice were housed in a specific pathogen-free environment under strictly controlled light cycle conditions, fed a standard rodent lab chow and provided water *ad libitum*. All procedures were approved and performed in accordance with the University of Oregon Institutional Animal Care and Use Committee. *C57BL/6* and *Lrig1*$^{tm1.1(cre/ERT2)Rjc}$*/J* (*Lrig1-CreERT2*) mice (Powell et al., 2012) were obtained from Jackson Laboratory (Bar Harbor, ME). Lrig1$^{\mathrm{tm1a(EUCOMM)Wtsi}}$ (*Lrig1-flox/flox*) mice (EUCOMM) were generously provided by Robert J. Coffey (Vanderbilt University Medical Center). *Gt(ROSA)26Sor*$^{tm1(CAG\text{-}Brainbow2.1)Cle}$*/J* mice (*R26R-Confetti*) (Snippert et al., 2010) were generously provided by Kryn Stankunas (University of Oregon). *Lrig1-CreERT2/+* mice were crossed to *R26R-Confetti* mice or *Lrig1-flox/flox* mice. Mice were sacrificed between P1 and 6 weeks of age. For multicolor lineage labeling**,** *Lrig1-CreERT2/+* mice were crossed to *R26R-Confetti* mice; resulting *Lrig1-CreERT2/+;R26R-Confetti* mice were injected via intraperitoneal (IP) injection once with 30mg/kg tamoxifen (Sigma-

Aldrich, T5648, suspended in corn oil) at P1 and the colon was harvested at P5, P17, and P22. For Cre-mediated deletion of Lrig1**,** *Lrig1-CreERT2/+* mice were crossed to *Lrig1-flox/flox* mice and the resulting *Lrig1-CreERT2/ flox* mice and *Lrig1-WT/ flox* (littermate controls) were injected once with 30mg/kg tamoxifen at P5 and the colon was harvested at P7, P14, P21 and at 6 weeks (P42).

Tissue preparation for staining

Colons were dissected from the mice and flushed with ice-cold Phosphate Buffered Saline (PBS, Grow Cells, MRGF-6395) and ice-cold 4% Paraformaldehyde (PFA; Sigma, 158127)/PBS then pinned as a tube or placed into 6-well tissue culture dishes (Falcon, 353046). Colon was then fixed in 4% PFA/PBS for 20 minutes (P1-P7) or 60 minutes (P13-P42) at room temperature with light oscillation. For frozen sections, fixed colons were washed 3×5 minutes in PBS and incubated in 30% sucrose overnight at 4°C, then blocked in Optimal Cutting Temperature Compound (OCT, Tissue-Tek® Sakura, 4583) as tubes, separating the proximal, middle, and distal portions of the colon. Frozen blocks were placed at -80°C for storage. For paraffin sections, fixed colons were washed 3×5 minutes in PBS, incubated in 70% ethanol overnight at room temperature followed by serial ethanol dehydrations and subsequent xylene clearing washes prior to paraffin embedding. Paraffin blocks were stored at room temperature.

Immunofluorescence and Histology

For both frozen and paraffin preparations, 10μm transverse sections of colon were placed on Superfrost™ Plus (Fisher Scientific, 12-550-15) or Millennia Command™ slides

(StatLab, MCOMM). For frozen sections, slides were thawed 15 minutes at room temperature, washed 3×3 minutes in PBS, blocked in blocking buffer composed of 1% Bovine Serum Albumin (BSA), 1M $CaCl_2$, and 0.03% Triton X-100 (Sigma, T8787) for 1 hour before primary antibody was added. All primary antibodies were suspended in blocking buffer. All stains were performed using an overnight incubation at 4°C, except for Ki-67 staining in Figure 5D, D', D''', which was incubated overnight at room temperature. The following primary antibodies were used for frozen sections: anti-Lrig1 (R&D, AF3688 1:250), anti-Non-phospho (Active) β-Catenin (Ser45) (D2U8Y) XP (Cell Signaling Technology, 19807 1:1000), anti-Ki-67 (Invitrogen, 14-5698-82, 1:100), anti-Chromogranin A (LK2H10) (AbD Serotec, MCA845 1:200), anti-DCAMKL1 (Abcam, ab31704, 1:1000), anti-Alexa Fluor™ 488 Phalloidin 488 (Invitrogen, A12379, 1:2000). On the following day, slides were washed 3×3 minutes in PBS then stained with secondary antibody (Jackson ImmunoResearch, 1:500) diluted in the blocking buffer for 1 hour at room temperature. For double staining of Ki-67 and Lrig1, the Ki-67 antibody stain was first accomplished, followed by a one-hour incubation with Lrig1 antibody at room temperature. The secondary antibody staining protocol described above. For phalloidin staining, slides were washed and blocked as described above and the conjugated antibody was incubated for 1 hour at room temperature. Prior to coverslipping, slides were washed 3×3 minutes in PBS, counterstained with DAPI, and mounted with N-propyl gallate/glycerol mounting medium. For Muc2 (anti-Muc2 Cloud-Clone, PAA705Mu01, 1:100), paraffin-embedded tissue sections underwent conventional deparaffinization in xylenes and rehydration via ethanol washes and water. Antigen retrieval was performed using a 1x Citrate Buffer (Sigma, C9999) in a pressure

cooker for 15 minutes on high, followed by quick release and slides placed on ice. Slides were blocked in blocking buffer described above with the addition of 10% donkey serum for 1 hour before addition of the primary antibody. Primary antibody was diluted in PBS and slides were incubated overnight at 4°. On the following day, slides were washed 3×3 minutes in PBS then stained with secondary antibody (Jackson ImmunoResearch, 1:500) diluted in PBS for 1 hour at room temperature. To assess colon morphology, paraffin-embedded tissue sections were stained with Hematoxylin and Eosin (VWR, 95057) using standard pathology protocols.

Image acquisition and statistical analysis

Slides were imaged via confocal microscopy using a Zeiss LSM-880 (Zeiss, Oberkochen, Germany) system for morphometric analysis, or a Nikon Eclipse/Ds-Ri2 (Nikon, Tokyo, Japan) for quantification analysis. Images were false colored using Fiji. All image analysis and quantification were performed using Fiji (Schindelin et al., 2012) in a double-blind fashion and statistical comparisons were analyzed using GraphPad Prism software. Quantification of all images (total "n" and counting metrics) are designated in each figure legend.

<u>Results</u>

Morphological changes in the distal colon during development.

To understand colon development, we first characterized the morphology of colonic crypts after birth (P1) through adolescence (P21). To accomplish this, we performed Hematoxylin and Eosin (H and E) staining of formalin-fixed, paraffin-

embedded tissue sections from wildtype (WT) mice (Figure 4). At P1, the colonic epithelial cells formed a continuous columnar sheet, with occasional folded structures,

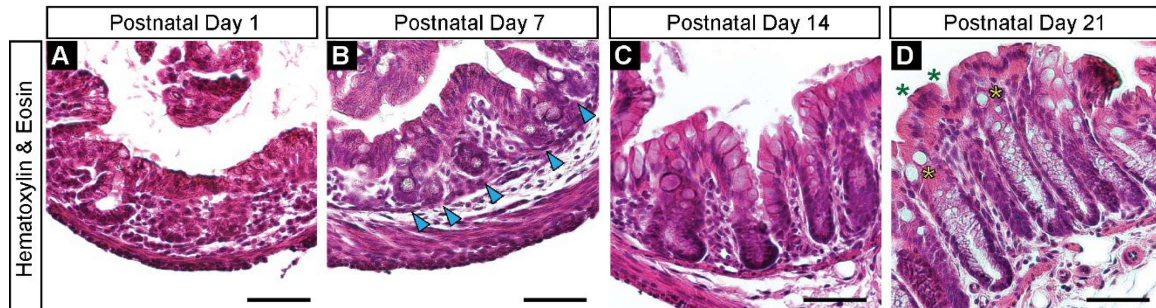


Figure 4. Morphological changes in the distal colon during development. Characterization of the morphology of colonic crypts after birth (P1) through adolescence (P21). Representative colon transverse sections of wildtype mice stained with Hematoxylin and Eosin (A-D). A) At P1, the colonic epithelial cells form a continuous epithelium, populated by columnar cells that reside adjacent to the lamina propria and muscle layers. B) Widespread crypt initiation was detected by the presence of frequent folds within the epithelium at P7 (blue arrowheads). C) At P14, epithelial cell layer was comprised of distinct crypt structures. D) At P21, crypt-like structures are readily apparent, containing secretory and absorptive cells (yellow and green asterisks, respectively). N=5 C57BL/6 mice surveyed per timepoint. Representative images for each timepoint are shown. Scale bars=50μm.

residing above the non-epithelial cells of the developing lamina propria and muscle tissue (Figure 4A). One week after birth (P7), frequent folds within the epithelium indicated widespread crypt formation (blue arrowheads, Figure 4B). The mucosal and submucosal layers were larger than at P1, the columnar shape of the epithelial cells was more evident, and we detected cells which appear to have similar morphology to adult secretory cells within the epithelial cell layer. Two weeks after birth (P14), the colonic epithelium harbored numerous, deep crypt-like structures with cells organized linearly into a clearly defined crypt-base to crypt-cuff axis. Consistent with the growth from P7 to P17, the mucosa and submucosa were larger (Figure 4C). Lastly, three weeks after birth (P21), the epithelial layer consisted exclusively of crypt-like structures, which contained

numerous secretory (yellow asterisks) and absorptive cells (green asterisks). These crypts, as well as the underlying lamina propria and muscle layers, were morphologically indistinguishable from those of adult distal colonic crypts (Figure 4D). In sum, we show the colon undergoes numerous morphological changes as it grows from birth to three weeks of age.

Molecular characteristics of the developing colon.

In order to better understand the morphological changes we detected from P1-P21, we sought to molecularly characterize the epithelial cells during the same developmental timeframe. To accomplish this, we performed immunostaining of the junctional marker β-catenin, the proliferative marker Kiel-67 (Ki-67), and the stem and progenitor cell marker Leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1). β-catenin is localized to adherens junctions and basal-lateral borders of cells (Valenta et al., 2012). At P1, we observed β-catenin (yellow) localized to epithelial cells lining the mucosal layer and notably, in the small invaginations we had detected by H and E (Figure 5A, A'). At this timepoint, β-catenin was also expressed by non-epithelial cells within the submucosa and muscle tissue (Figure 5A'). At P7, β-catenin was detected in all cells that populate the deep invaginations of developing colonic crypts and was also present within the muscle layer (Figure 5B, B'). The same pattern was detected through P14 and P21 (Figure 5C-D, C'-D'). To visualize proliferation in the developing colon, we assessed protein expression of Ki-67 which is expressed by cells in all active phases of the cell cycle (Bruno & Darzynkiewicz, 1992). At P1, Ki-67 expression (magenta) was detected throughout the mucosa and submucosa (Figure 5A, A'') but by P7 and P14, Ki-67

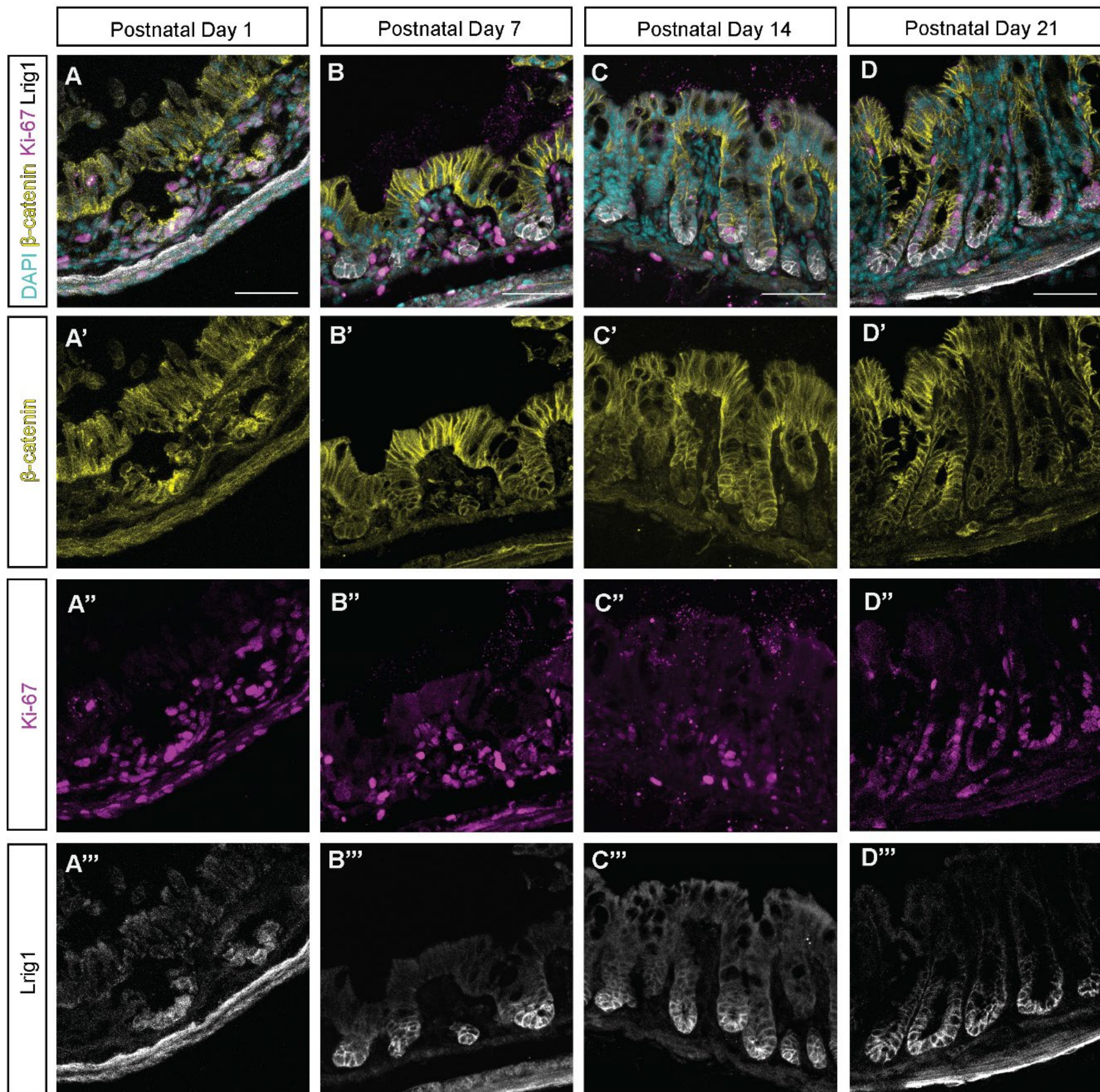


Figure 5. Molecular characteristics of the developing colon.

Protein expression of the junctional marker β-catenin, the proliferative marker Ki-67, and the stem and progenitor cell marker Leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1) from P1-P21in distal colonic crypts (A-D). A'-D') β-catenin (yellow) detected at cell junctions in the mucosal and submucosal layers. A''-D'') Punctate expression of Ki-67 (magenta) detected in the mucosal and submucosal layers. A'''-D''') Lrig1 (white) was detected in cells in both early and late crypt development, as well as in the submucosa. DAPI, (cyan) is marking all nuclei displayed in the merged images (A-D). Images shown are maximum intensity projections of confocal z-stacks taken from 7.5μm to 12μm. N=5 *C57BL/6* mice surveyed per timepoint. Representative images for each timepoint are shown. Scale bars=50μm.

expression was restricted to a subset of cells within both compartments (Figure 5B, B'', C, C''). At P21, Ki-67 was largely constrained to the epithelial cells in colonic crypts (Figure 5D, D''). We next examined Lrig1 expression which is detected on stem and progenitor cells within the adult colon (Powell et al., 2012; Wong et al., 2012). At P1, we observed Lrig1 protein expression (white) restricted to a subset of epithelial cells located within the small invaginations in the mucosa (Figure 5A, A'''). Lrig1 was also detected in the submucosa, consistent with expression of Lrig1 in the Interstitial Cells of Cajal in the adult colon (Kondo et al., 2015; Poulin et al., 2014) (Figure 5A, A'''). As these invaginations enlarge through P7 and P14, Lrig1 expression was confined to cells located near the base of the epithelial folds (Figure 5B, B''', C, C'''). Three weeks after birth, Lrig1 remained restricted to epithelial cells at the base of individual crypts, in a similar expression pattern as is seen in the adult colon (Powell et al., 2012; Wong et al., 2012) (Figure 5D, D'''). Together, these data describe epithelial cell location, areas of proliferation and expression of Lrig1 in the colon from birth to three weeks of age.

Lrig1+ cells act as stem cells during colon development.

The location of Lrig1 expression throughout colonic epithelial development suggested Lrig1 might be marking adolescent stem cells. We hypothesized if Lrig1 expressing (Lrig1+) cells were acting as stem cells, they may divide and give rise to daughter cells that would populate the crypts. To test this hypothesis, we took a multicolor lineage tracing approach by intercrossing *Lrig1-CreERT2/+* (Powell et al., 2012) and R26R-Confetti (Snippert et al., 2010) mice. The resulting *Lrig1-CreERT2/+;R26R-Confetti* mice received one intraperitoneal injection (IP) of 30mg/kg

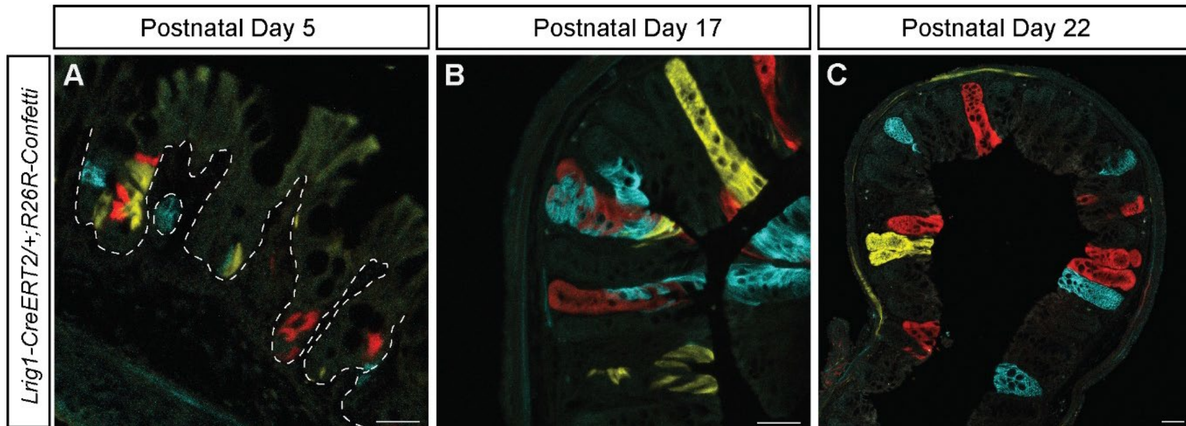


Figure 6. Lrig1+ cells give rise to clonal crypts during colon development.

Colon images from *Lrig1-CreERT2/+;R26R-Confetti* mice injected with tamoxifen at P1 and harvested at P5, P17, and P22. A) At P5, multiple populations of Lrig1 lineage-labeled colored cells within the colonic epithelium were detected. White dashed line outlines the epithelial layer. B) At P17, polyclonal (differently colored) crypts and monoclonal (single color) crypts are detected. C) At P22, monoclonal (single color) crypts predominate. N=5 *Lrig1-CreERT2/+;R26R-Confetti* mice per timepoint. Representative images for each timepoint are shown. For A-B, images shown are maximum intensity projections of 12µm confocal z-stacks. For C, 16 confocal images were tiled by the confocal software with 10% overlap in a rectangular grid. Scale bars=50µm.

tamoxifen at P1 to initiate lineage tracing in Lrig1+ cells. We then sacrificed the mice at discrete timepoints to examine fluorescent reporter expression in the colonic epithelium. At P5 we observed multiple populations of Lrig1 lineage-labeled cells (yellow, cyan and red) within the colonic epithelium (Figure 6A). Nearly two weeks later (P17), colon crypts harbored multiple, differently colored cells (polyclonal labeling) or expressed a single color in all cells (monoclonal labeling, Figure 6B). To determine if polyclonal crypts became monoclonal over time, we allowed a cohort of labeled mice to develop to P22 (Figure 6C). At this timepoint, labeled crypts cross-sections were a single color indicating monoclonality. We also detected lineage tracing within the submucosa of the colon at P22 consistent with Lrig1 expression in the Interstitial Cells of Cajal in the adult colon (Kondo et al., 2015; Powell et al., 2012). Our lineage tracing data show multiple

Lrig1+ cells present at birth can give rise to the epithelial cells in the nascent crypts and these Lrig1+ cells drive clonality within individual crypts over the first three weeks of life.

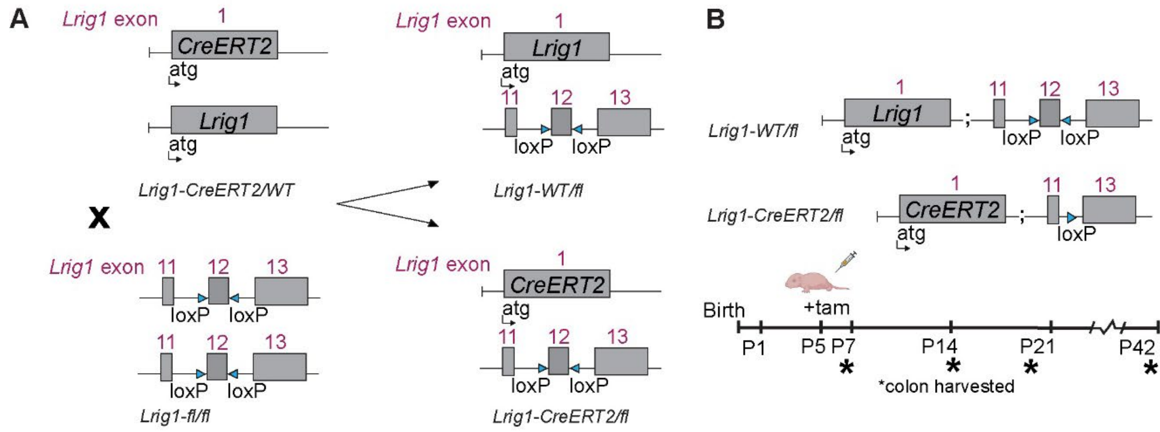
Generating the inducible loss of Lrig1 during development.

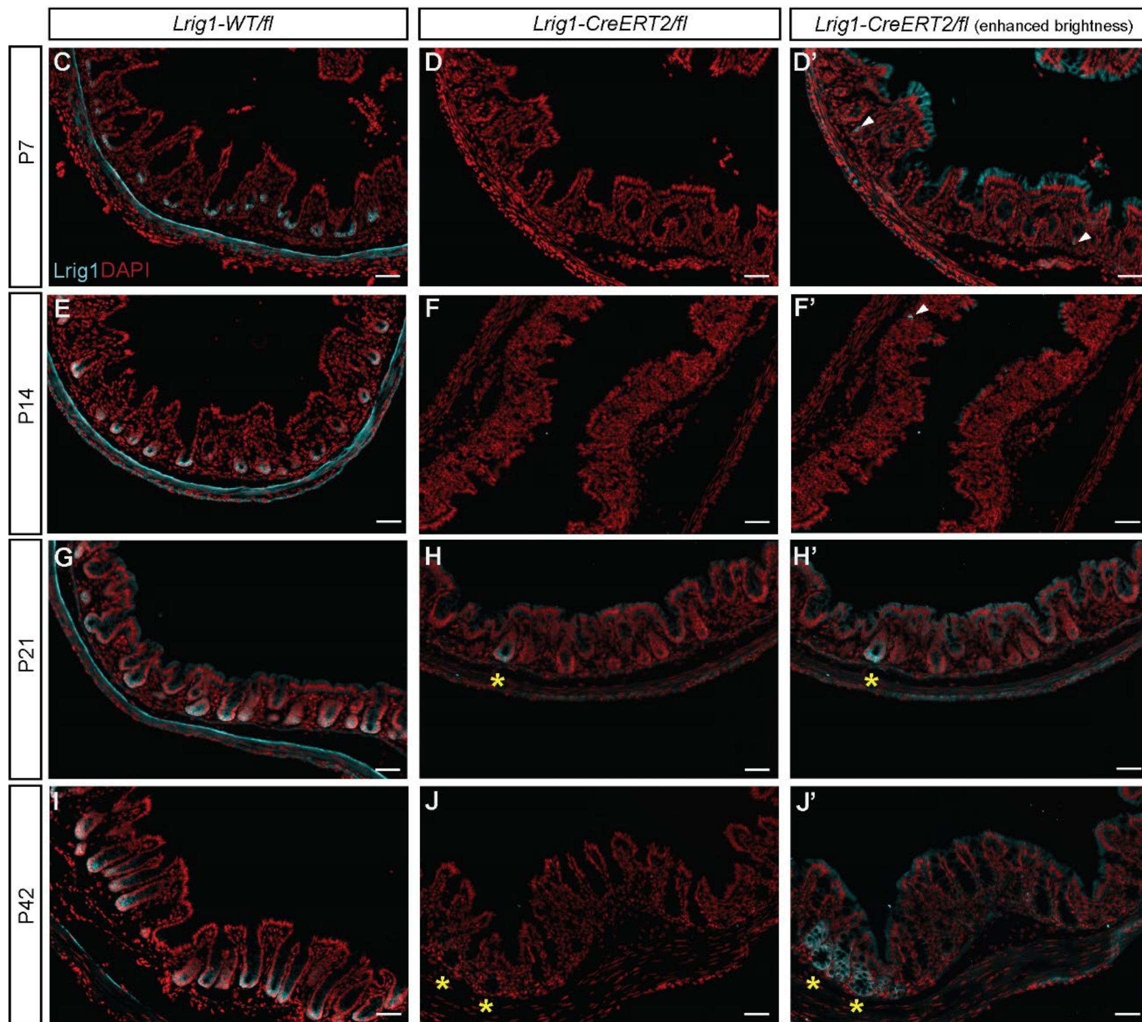
Our data thus far indicate that Lrig1 is expressed in stem cells in the developing colon and we next tested the role of Lrig1 in colon development. In the adult mouse, Lrig1 regulates growth in numerous epithelial tissues (Jensen et al., 2009; Jensen & Watt, 2006; Kondo et al., 2015). During brain development, Lrig1 is also important for regulating cell fate decision making (Nam & Capecchi, 2020). A complete knockout of Lrig1 can result in postnatal death (Wong et al., 2012), therefore we devised an approach to perform inducible, loss-of-function experiments with the goal of defining the role of Lrig1 without deleting it entirely. To accomplish this, we combined *Lrig1-CreERT2/+* mice (Powell et al., 2012) with *Lrig1-flox/flox (Lrig1-fl/fl)* mice (EUCOMM; Wellcome Trust Sanger Institute) (Skarnes et al., 2011) to create *Lrig1-CreERT2/flox* (*Lrig1-CreERT2/fl)* experimental mice and *Lrig1-WT/flox* (*Lrig1-WT/fl*) littermate controls (Figure 7A). All mice were injected with tamoxifen at P5, which induced recombination of the remaining *Lrig1* allele in the experimental mice (Figure 7B). To examine if this recombination resulted in the loss of Lrig1 protein, we examined *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* control colons for Lrig1 protein expression (Jensen et al., 2009; Powell et al., 2012; Y. Wang et al., 2013; Wong et al., 2012) at specific stages of development (Figure 7C-J). Two days after the induction of recombination (P7), Lrig1 protein (cyan) was uniformly distributed at the base of the epithelial invaginations of the

Lrig1-WT/fl littermate control colons (Figure 7C), as we observed in WT tissue (Figure 5B, 2B’’’).

Figure 7. Generating the inducible loss of Lrig1 during development.

Colon images from *Lrig1-CreERT2/fl* mice injected with tamoxifen at P5 and harvested at P7, P14, P21, and P42. A) Schematic for the generation of *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice. B) Experimental schematic for the injection of *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice at P5, and colon tissue harvested at P7, P14, P21, and P42 (asterisks). C-J’) Epifluorescent images displaying protein expression of Lrig1+ cells (cyan) and DNA (DAPI; red) at the timepoints surveyed. D', F’, H’ and J’) Enhanced images by increasing the brightness of D, F, H and J images, to showcase Lrig1+ cells (white arrowheads) and Lrig1+ crypts (yellow asterisks) in *Lrig1-CreERT2/flox* mice. N=6 mice per genotype per timepoint. Representative images are shown. Scale bars=50µm.


A
Lrig1 exon
1
CreERT2
atg
Lrig1
atg
Lrig1-CreERT2/WT
X
Lrig1 exon
11
12
13
loxP
loxP
Lrig1-fl/fl
Lrig1-WT/fl
Lrig1-CreERT2/fl
B
Lrig1-WT/fl
Lrig1-CreERT2/fl
Birth
+tam
P1
P5
P7
P14
P21
P42
*colon harvested


Lrig1-WT/fl
Lrig1-CreERT2/fl
Lrig1-CreERT2/fl (enhanced brightness)
P7
P14
P21
P42
C
D
D'
E
F
F'
G
H
H'
I
J
J'
Lrig1 DAPI

In our *Lrig1-CreERT2/fl* inducible knockout animals, Lrig1 was virtually undetectable (Figure 7D). The same pattern was observed at P14 for both the control (Figure 7E) and knockout colons (Figure 7F). At P21, Lrig1 was expressed at the base of every control crypt (Figure 7G), as we observed in WT colons (Figure 5D, D'''). However, in our P21 experimental *Lrig1-Cre/fl* animals, we detected some individual crypts expressing Lrig1 at the crypt-base (yellow asterisk, Figure 7H). In adult colons (P42), Lrig1 was expressed in every control crypt (Figure 7I), as has been reported previously (Powell et al., 2012; Wong et al., 2012), whereas our knockout animals lacked Lrig1 in most crypts, yet we did observe small groups of Lrig1+ crypts (yellow asterisks, Figure 7J), similar to a mosaic pattern. Given this expression of Lrig1 in discrete cells within our experimental colons, we then revisited the same experimental images from Figure 7D, F, H and J to test if enhancing the brightness of Lrig1 in the image would allow us to detect any Lrig1+ cells at P7 and P14. With these enhanced images, we detected single Lrig1+ cells (white arrowheads, Figure 7D' and F') at the P7 and P14 timepoints. The same groups of Lrig1+ cells present in the P21 and P42 experimental colons were also more readily visible with increased brightness (yellow asterisks, Figure 7H' and J'). In sum, our inducible knockout approach generated efficient knockout of Lrig1 as early as two days post-induction (P7) and this was maintained through P14. At the later timepoints, Lrig1 expression was readily detectable in the *Lrig1-CreERT2/fl* inducible knockout mice, therefore we focused on the P7 and P14 timepoints to evaluate the impact of the loss of Lrig1.

Lrig1 restrains proliferation in developing colonic crypts.

Lrig1 is a known regulator of growth and proliferation (Faraz et al., 2018; Jensen & Watt, 2006; Kondo et al., 2015; Powell et al., 2012; Simion et al., 2014). In addition, *Lrig1* complete knockouts have significant exogenous proliferation within the intestine before their postnatal death (Wong et al., 2012) and expression of Lrig1 is rapidly expanded in the colon as a part of the regenerative process after injury to the epithelium (Yui et al., 2018). Given these data, we hypothesized Lrig1 regulates proliferation during colon development and loss of *Lrig1* in our inducible knockout mice would result in hyperproliferation. In Figure 4, we show widespread crypt formation and cell proliferation occurred during the first week of birth (Figure 4B), so we next examined whether loss of *Lrig1* affected cell proliferation in the *Lrig1-CreERT2/fl* inducible knockout mice compared to our *Lrig1-WT/fl* littermate control animals at P7 (Figure 8). To assess proliferation, we examined the P7 colonic epithelium for the expression of the proliferative marker Ki-67. After analyzing both cohorts of animals, we detected indistinguishable patterns of Ki-67 expression between our injected inducible knockout mice, two days after induction, compared to their littermate controls (Figure 8A-B'). We further quantified this observation by counting the total Ki-67+ cells per 100µm^2 of total

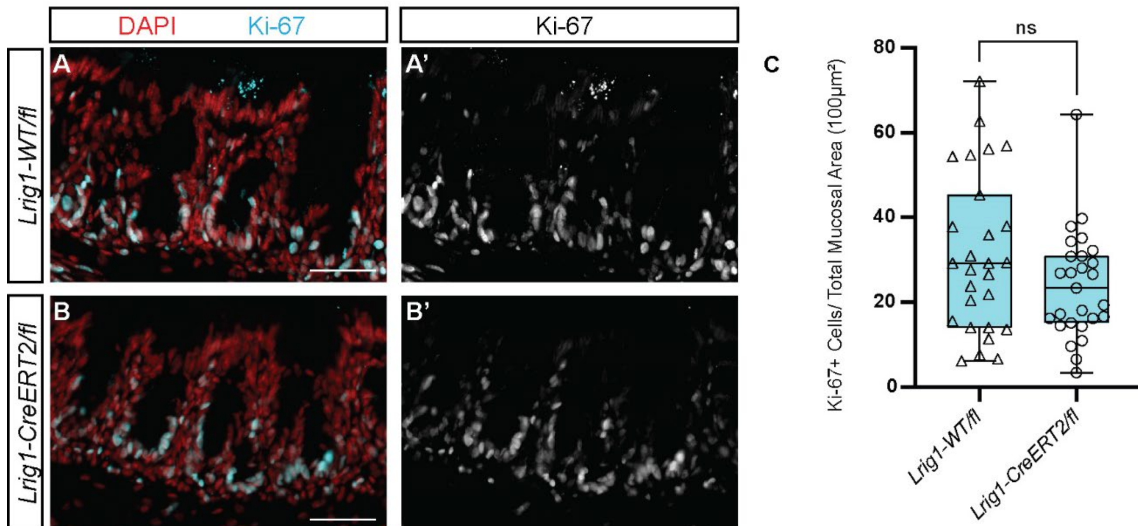


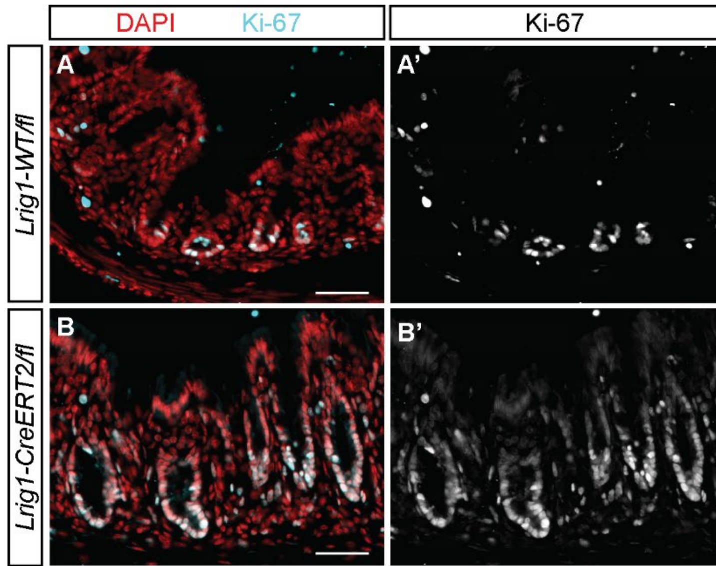
Figure 8. Loss of Lrig1 does not affect proliferation at P7.

Colon images from *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice injected with tamoxifen at P5 to induce the loss of Lrig1, and mid and distal colon tissue was harvested at P17 (A-C). A-B') Proliferation detected by expression of Ki-67 (cyan) expression in *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice. All cells present are indicated by the nuclear marker DAPI (red). C) Quantification of Ki-67+ cells per total mucosal area (100µm^2). n=6 mice per genotype, 110,000µm (+/-15k µm) mucosal area analyzed per mouse. Box-whisker plot represents individual data points with whiskers representing minimum to maximum values. Significance was determined by an unpaired *t*-test and *p* values are indicated. All images are epifluorescence images. Scale bars=50µm.

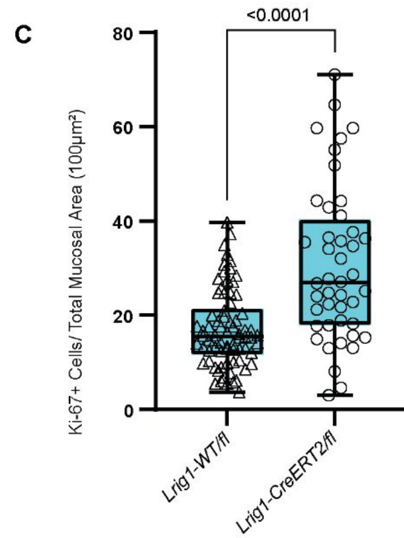
mucosa area (Figure 8C). As colon proliferation is ongoing but moderate from P8 to P16, compared to earlier developmental timepoints (Ménard et al., 1994), we reasoned that examining proliferation at P14 in our knockout *Lrig1-CreERT2/fl* and *Lrig1-WT/fl* littermate control animals, would be an appropriate timepoint to measure any potential increase in proliferation in the loss-of-function animals. In agreement with ^{3}[H] assays (Ménard et al., 1994), Ki-67 protein was detected in a few, discrete cells at the base of colonic crypts in our littermate controls (*Lrig1-WT/fl* mice; Figure 9A-A'). This was consistent with what we observed in WT crypts at this same developmental stage

Figure 9. Lrig1 restrains proliferation in developing colonic crypts.

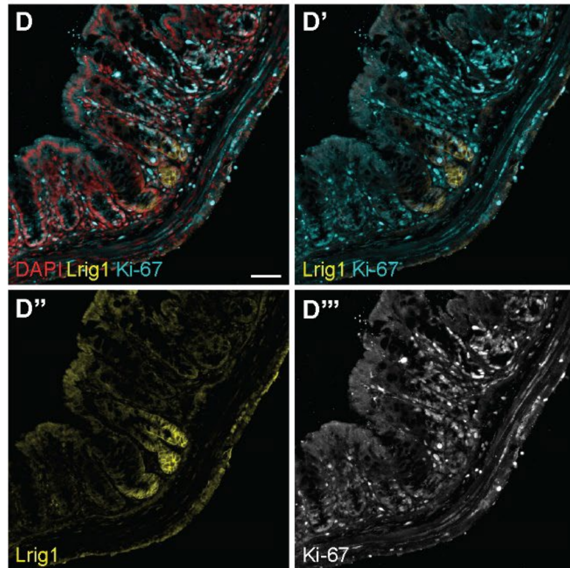
Colon images from *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice injected with tamoxifen at P5 to induce the loss of Lrig1, and tissue was harvested at P14 (A-C) and P21 (D-E). A-B') Proliferation detected by expression of Ki-67 (cyan) expression in *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice. All cells present are indicated by the nuclear marker DAPI (red). C) Quantification of Ki-67+ cells per total mucosal area (100µm^2). N=6 mice per genotype, 110,000µm (+/-15k µm) mucosal area analyzed per mouse. Box-whisker plot represents individual data points with whiskers representing minimum to maximum values. Significance was determined by an unpaired *t*-test and *p* values are indicated. D-E) Analysis of Lrig1 and Ki-67 expression in mosaic crypts D-D''') Protein expression of Lrig1 and Ki-67analyzed in *Lrig1-CreERT2/fl* experimental mice displaying representative adjacent Lrig1-expressing and Lrig1-missing crypts. D) Composite image of DAPI (red), Lrig1 (yellow), and Ki-67 (cyan). D') Overlay of Lrig1 and Ki-67 protein expression. D'') Single channel of Lrig1 protein expression. D''') Single channel of Ki-67 protein expression. E) Quantification of number of Ki-67+cells per crypt in n=6 *Lrig1-CreERT2/fl* mice. Bar graph represents individual data points in Lrig1-expressing and Lrig1-missing crypts with error bars representing the standard error of the mean. Significance was determined by a nested paired *t*-test and *p* values are indicated. For A-E, images are epifluorescence images. Scale bars=50µm.



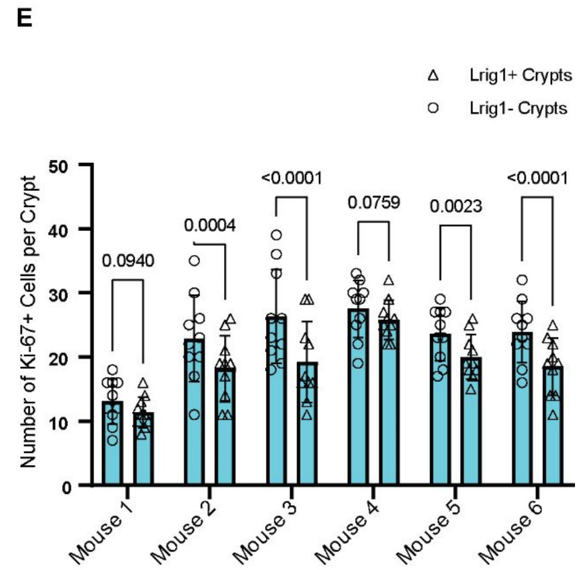
DAPI Ki-67
Ki-67
A
A'
B
B'
Lrig1-WT/fl
Lrig1-CreERT2/fl



C
<0.0001
Ki-67+ Cells/ Total Mucosal Area (100µm²)
80
60
40
20
0
Lrig1-WT/fl
Lrig1-CreERT2/fl



D
D'
D''
D'''
DAPI Lrig1 Ki-67
Lrig1 Ki-67
Lrig1
Ki-67

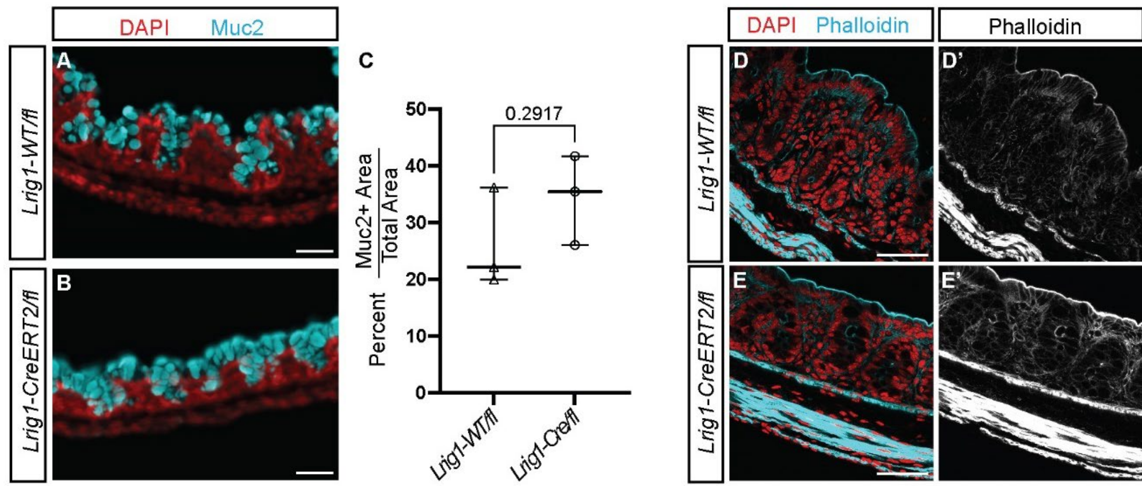


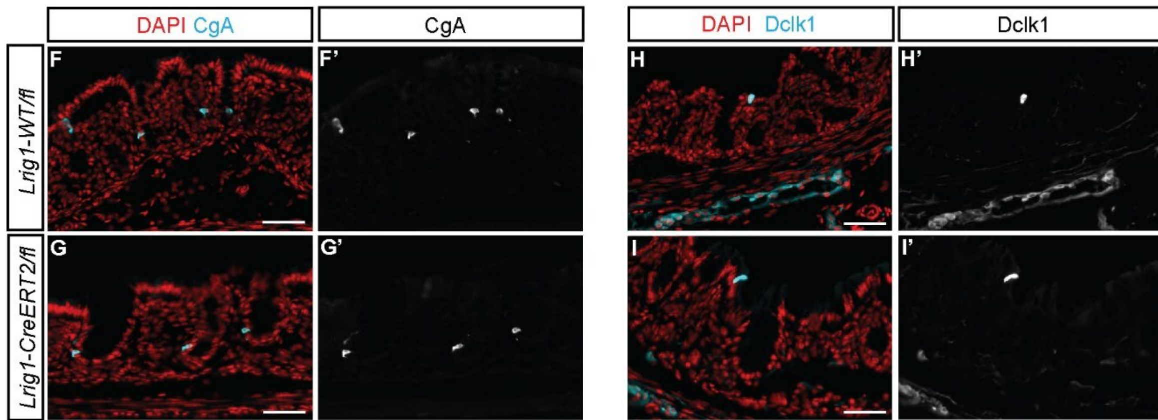
E
Lrig1+ Crypts
Lrig1- Crypts
Number of Ki-67+ Cells per Crypt
50
40
30
20
10
0
0.0940
0.0004
<0.0001
0.0759
0.0023
<0.0001
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6

(Figure 5C, C''). In contrast, we detected expanded Ki-67 expression within the crypts of our *Lrig1-CreERT2/fl* mice (Figure 9B-B'). We quantified this observation across all mice, as in Figure 8, and show proliferation was significantly increased in our P14 knockout mice, compared to the littermate controls (Figure 9C, $p<.0001$, n=6 for each genotype). As experimental *Lrig1-CreERT2/fl* mice displayed a mosaic pattern of Lrig1 expression at P21 (shown in Figure 7H'), we hypothesized crypts retaining Lrig1 would have less proliferation than knockout crypts from the same animal. To test this, we examined Ki-67 protein expression in Lrig1-expressing and Lrig1-missing adjacent crypts (Figure 9D-D'''). In agreement with our hypothesis, we detected a significant

Figure 10. *Lrig1* does not regulate differentiation in P14 developing colonic crypts.

Protein expression of the goblet cell marker Mucin 2 (Muc2), enteroendocrine cell marker Chromogranin A (CgA), tuft cell marker Doublecortin-like kinase 1 (Dclk1), and absorptive cell marker phalloidin in P14 *Lrig1-CreERT2/fl* experimental and *Lrig1-WT/fl* littermate control mice. For all images, the protein of interest is labeled in cyan, and all nuclei are labeled with DAPI in red. A-B) Epifluorescence images of Muc2 protein expression in *Lrig1-CreERT2/fl* and *Lrig1-WT/fl* mice. C) Graph displaying percent positive Muc2 area per total epithelial area for *Lrig1-CreERT2/fl* and *Lrig1-WT/fl* mice. For the whisker plot, the individual data points represent the mean percent positive Muc2 area per total epithelial area for each mouse. N=3 mice and 10 random images were acquired per mouse for each genotype. Significance was determined by an unpaired *t*-test and the *p* value is indicated. D-E') Expression of F-actin detected by the presence of phalloidin (cyan) using confocal microscopy (single slice shown). F-G') Epifluorescence images of Chromogranin A (CgA; cyan) protein expression in *Lrig1-CreERT2/fl* and *Lrig1-WT/fl* mice. H-I') Epifluorescence images of Doublecortin-like kinase 1 expression (Dclk1; cyan) protein expression in *Lrig1-CreERT2/fl* and *Lrig1-WT/fl* mice. All images are representative and acquired from P14 colon. Scale bars=50μm.


DAPI Muc2
A
B
Lrig1-WT/fl
Lrig1-CreERT2/fl
C
0.2917
Percent Muc2+ Area / Total Area
Lrig1-WT/fl
Lrig1-Cre/fl
DAPI Phalloidin
Phalloidin
D
D'
E
E'


DAPI CgA
CgA
F
F'
G
G'
Lrig1-WT/fl
Lrig1-CreERT2/fl
DAPI Dclk1
Dclk1
H
H'
I
I'

decrease in the number of Ki-67+ cells in Lrig1-expressing crypts compared to their Lrig1-missing adjacent counterparts, in many of the mice we examined (Figure 9E).

Loss of *Lrig1* does not impact differentiation at P14.

In the brain and the skin, Lrig1 controls a balance between proliferation and differentiation (Gomez et al., 2013; Jeong et al., 2020; Nam & Capecchi, 2020). As the loss of Lrig1 increased proliferation in the developing colon at P14, we next wanted to examine if the loss of Lrig1 inhibits differentiation of colon epithelial cells at the same developmental timepoint. To examine this, we performed immunofluorescent antibody staining for common differentiation markers of both secretory and absorptive cell types in our *Lrig1*-CreERT2/fl inducible knockout mice and Lrig1-WT/fl littermate controls (Figure 10). We used Mucin2 (Muc2, cyan) (Gum et al., 1999) to detect emerging secretory cells at P14 (Figure 10A-B) and compared the two genotypes. We detected no quantitative difference in Muc2 expression between the genotypes both qualitatively and quantitatively (Figure 10C). To detect absorptive cells, we used the brush border marker phalloidin (cyan) (Stidwill & Burgess, 1986) and we detected no qualitative difference in phalloidin expression between the genotypes (Figure 10D-E'). We next examined the presence of emerging enteroendocrine and Tuft cells by staining with Chromogranin A (CgA, cyan, Figure 10F-G') (Mazzawi et al., 2015; Rindi et al., 2004) and Doublecortin-like kinase 1 (Dclk1, cyan, Figure 10H-I') (Gerbe et al., 2009), respectively. As with Muc2 and phalloidin, there was no obvious difference in the differentiation pattern of these cells in both genotypes (Figure 10F-I'). In sum, these data show loss of *Lrig1* does

not impact colon epithelial cell differentiation during the developmental time window from P5 to P14.

Discussion

Our research is one of the first to define the morphological and molecular characteristics of the developing distal mouse colon three weeks after birth. Our data describes three key molecular characteristics of the developing colon: epithelial cell location, areas of proliferation, and emergence and expression of a stem and progenitor cell marker, Lrig1. Using a novel *Lrig1-CreERT2/+;R26R-Confetti* reporter mouse, we demonstrate that Lrig1+ cells are present at birth and these cells can give rise to colonic crypts. To test the role of Lrig1 in development, we created an Lrig1 inducible knockout mouse (*Lrig1-CreERT2/fl)* and after knocking out Lrig1 at discrete timepoints after birth, our data indicate that inducible loss of Lrig1 leads to hyperproliferation two weeks after birth. In addition, we show this phenotype does not impact differentiation in the developing colon. In sum, our data is the first to show Lrig1 is required to restrain proliferation during colon crypt development, specifically during the first two weeks after birth and unlike the adult colon, hyperproliferation does not lead to a loss of differentiation during colon development.

The first aim of our study was to morphologically characterize the development of distal colonic crypts using modern pathology staining techniques. In addition, despite the numerous distinctions between the colon and the small intestine, most of the research conducted on the developing intestine has historically been focused on the small intestine

(Dehmer et al., 2011; Karlsson et al., 2000; Madison et al., 2005; Rao-Bhatia et al., 2020; Sumigray et al., 2018; Walton et al., 2012) and does very little to address colon development. Therefore, we thought it was pertinent to examine the formation of colonic crypts as colon development proceeds. Our data demonstrate distal colonic crypt formation is widespread by the first postnatal week and hallmarks of epithelial cell differentiation can be observed by the second postnatal week. During this early timeframe, the distal colon is already comprised of cells displaying morphological features of classically differentiated cells While the purview of our study was kept to a relatively small developmental time window, several interesting areas for further research arise from these morphological data. In Figure 1 we show small invaginations, which represent nascent crypts, are present the day after birth. Going forward, it will be important to examine the formation of the colon from the late embryonic days up until birth to map tube development and observe the cellular organization that occurs to generate these nascent crypts. It will also be interesting to examine whether or not this crypt formation in the colon mimics the same process as the small intestine. Our data from P21 mice indicate that the adolescent distal colonic crypts present appear indistinguishable from adult crypts, however testing whether they are truly adult-like in nature, in terms of size, width, and function will be crucial to our understanding of colonic function during development. Finally, a careful examination of crypt bifurcation and expansion of the crypts during this time frame will promote our understanding of how the colon crypts distribute themselves along the rostral to caudal axis.

The extensive morphological changes we show in Figure 1 are accompanied by the molecular marker expression shown in Figure 2. Our molecular characterization illustrates the tight epithelial organization, the highly proliferative epithelial cells present, and the expression of the stem and progenitor marker Lrig1, throughout colon development. Most of the previous studies examining the cellular characteristics of the developing colon were performed before advanced molecular markers were developed and a clear next step is to more fully examine additional molecular features of developing colonic crypts. These analyses could include several different avenues of investigation; one path that might be particularly interesting would be examining the emergence of stem cell support populations during crypt emergence and expansion. Studies like these will help us understand how these cells come to reside next to each other in adult homeostasis and if they have a symbiotic relationship, as in adult colonic tissue. In the developing small intestine, stem cell support cells called Paneth cells emerge during the first week after birth and a dramatic increase in Paneth cell number occurs between two and four postnatal weeks (Bry et al., 1994). Paneth cells produce factors that promote stem cell homeostasis in the small intestine (Sato et al., 2011) and are important for the process of crypt expansion (Langlands et al., 2016). As Paneth cells do not exist in the mouse colon, cells marked with Regenerating Family Member 4 (Reg4) have been defined as deep crypt secretory cells, and often act as Paneth cell equivalents (Bry et al., 1994; Sasaki et al., 2016). It is currently unknown when Reg4+ cells emerge during development and if they are required for developmental stem cell homeostasis, or crypt growth. This will be an important area for future investigation and could be accomplished by combining any number of colon stem cell fluorescent reporters (Barker et al., 2007; Hosen et al., 2007;

Poulin et al., 2014; Rutlin et al., 2020) with the Reg4-dsRed-DTR mouse to examine patterning and crypt formation in the presence or absence of Reg4+ cells. Certainly, the possibilities for expanding our molecular knowledge in colon development are numerous and could also include examining of the emergence of cellular transporters, junctional proteins, and additional differentiation markers. Ultimately, discovering the molecular underpinnings of how colonic crypts develop may be informative for our understanding of colonic disease progression and crypt regeneration in humans.

Our study is the first to use multicolor lineage tracing in Lrig1+ cells throughout colon development to examine the clonality potential of young epithelial stem cells. Lrig1 expressing (Lrig1+) cells are present at P1 in the base of immature crypt-like folds and using our multicolor approach, we demonstrate these Lrig1+ cells continuously produce daughter cells, which give rise to fully labeled, clonal crypts by P22. It is intriguing that multiple populations of Lrig1+ cells establish clonal crypts three weeks after birth, as this is a distinct timeline from clonality studies in adult mice (Snippert et al., 2010). We show Lrig1+ cells can be detected as early as P1 and are localized to the base of nascent and developing crypts throughout colon crypt development, suggesting that Lrig1+ cells may be involved in crypt establishment. In the future it will be important to define where the Lrig1+ cells arise from, both in cellular lineage and in anatomical location. Answering these questions may give us a fuller picture of the origins of these cells, which are critical for appropriate proliferation in developing crypts.

Our inducible, loss-of-function approach for eliminating Lrig1 enabled us to circumvent the birth defects associated with the Lrig1 knockout (Wong et al., 2012) and ultimately allowed us to define the molecular function of Lrig1 during colon development. Loss of Lrig1 resulted in increased proliferation in developing colonic crypts two weeks after birth (P14), yet our data also revealed populations of Lrig1+ "escaper cells" detected at P7 and P14 and Lrig1+ "escaper crypts" were detected and persist one week later (P21). These data illustrate a selection against Lrig1 elimination during colon crypt development and a dominant push for Lrig1 expression as crypts are forming. Examining the molecular regulators of this selection towards homeostasis will be important. Likely, this push is dictated by a multitude of signaling mechanisms which regulate a balance between proliferation and differentiation, as in adult mice (He et al., 2004; Qi et al., 2017; Sasaki et al., 2016; Sato et al., 2011, 2011; Schuijers & Clevers, 2012; van Es et al., 2005; Yan et al., 2017). To this end, we show Lrig1 suppressed proliferation in a narrow developmental window, yet this level of proliferation can vary, even between adjacent crypts. It is currently unknown how Lrig1 is regulating proliferation during colon development, but studies from the small intestine may offer clues. It is well-established that the Notch and Wnt cell signaling cascades regulate epithelial cellular proliferation and differentiation (Carulli et al., 2015; Fre et al., 2005; Gregorieff & Clevers, 2005; Jensen et al., 2010) in the adult small intestine and colon. In addition, an interplay between Wnt, BMP, and Hedgehog signaling cascades are important for crypt formation in the small intestine (Batts et al., 2006; Bettess et al., 2005; Madison et al., 2005). While we know Lrig1 is important for regulating proliferation, we do not yet know where Lrig1 fits in between these signaling cascades.

Further studies examining the cellular mechanisms impacted due to both the loss and overexpression of Lrig1, will be informative to help us build a molecular model for colon crypt development.

Perhaps the most striking observation from our developmental studies is the hyperproliferation we observe in our Lrig1-Cre/fl experimental mice at P14 does not result in a loss of differentiation, as commonly seen in adult mice (Dow et al., 2015; Spit et al., 2018). Our data indicate that both absorptive and secretory cell populations are still present in the highly proliferative epithelium, suggesting these differentiated cells are actively proliferating. As these differentiated cells do not proliferate in adult tissues, our data support a model where molecular signals may govern newly differentiated cells in the developing colon differently, compared to those which regulate the rapidly renewing adult intestinal epithelium.

In sum, our studies clearly define the importance of studying Lrig1 and its role in colon development. We address a critical gap in the intestinal development literature and provide new information about the molecular cues that guide colon development. Using a novel inducible knockout of Lrig1, we show Lrig1 is required for appropriate colon epithelial growth and illustrate the importance of Lrig1 in the establishment of developing colonic crypts.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

Despite extensive studies on the developing gastrointestinal tract, there is a paucity of research examining colon development. In this dissertation, I have presented my morphometric and molecular analysis of the developing mouse colon to add to our knowledge of the process by which the colon reaches adult homeostasis. Specifically, I histologically characterized the morphological changes that occur during postnatal distal colon crypt development and showed protein expression of key epithelial markers during this timeframe. Notably, my experiments demonstrated when a stem and progenitor population can be universally detected in the developing colon, which is important data to expand our knowledge of colon stem cell biology. Finally I utilized an inducible, loss-of-function, mouse to decipher the role of that stem cell population in postnatal colon development. In sum, this thesis has laid a firm foundation for colonic epithelial developmental biologists on which to build further experiments interrogating colonic development.

In Chapter II, I presented experimental data characterizing distal colonic crypt development and observed how elimination of a stem and progenitor marker, Lrig1, effects colonic crypt development. Over the course of these experiments, I made several novel observations. These observations include mapping the formation of distal colonic crypts and defining and quantifying the presence of the epithelial differentiated cells by P14. I addition, my experiments showed Lrig1 expressing cells were present in the developing distal colonic epithelium as early as P1 and Lrig1 was restricted to the base of developing colonic crypts throughout postnatal colon development. Taking this

observation further, I used lineage tracing experiments to illustrate multiple, different populations of Lrig1 lineage-labeled cells present during colon development, which behave as stem cells. These data are the first to show the developmental stage in which clonality occurs during colon development. . The final part of Chapter II explored the role of Lrig1 in colonic crypt development. Through temporally controlled deletion, my experiments demonstrated loss of Lrig1 leads to increased proliferation in developing colonic crypts two weeks after birth, yet this had no impact on cell differentiation within developing crypts. Further studies investigating cell morphology and gene expression patterns will aid in our understanding of how colonic crypts develop.

One aspect of Lrig1 in colon developmental biology that remains unexplored in my dissertation is the mechanistic understanding of how Lrig1 is restraining proliferation in P14 developing colonic crypts. Lrig1 is a known negative regulator of Egfr, however my preliminary data (not shown) suggests Egfr is not elevated when Lrig1 is lost, two weeks after birth. If these observations are true after additional repeated experiments, another mechanism worth exploring is whether Lrig1 is regulating proliferation through Wnt signaling. Wnt signaling controls cell fate decision making and tissue patterning and is arguably one of the most important signaling pathways during embryonic and late-term development (Buechling & Boutros, 2011). Activation of the Wnt pathway drives the expression of genes that promote cell proliferation, cell growth, and survival of genes necessary for growth (Nusse & Clevers, 2017). Additionally, Wnt signaling is a major driver of proliferation in the developing gut (Gregorieff & Clevers, 2005) and is required for proliferation in adult crypts (Nusse & Clevers, 2017). It will be interesting to

investigate a potential relationship between Lrig1 and Wnt signaling during colon development, in future studies

In conclusion, for my doctoral dissertation, I set out to identify how colonic crypts develop. I was able to address the morphology and some molecular features of developing crypts over the first three weeks of life. More notably, I illustrated the presence of Lrig1 stem cells as early as P1, show they drive clonal expansion, and investigated the function of Lrig1 throughout colon crypt development. My thesis contributes key information to our understanding of mammalian colon development and has provides a framework for future studies comparing mouse and human colon epithelial biology during development.

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