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The Role of Krüpple-like Factor 5 in Normal Intestinal Homeostasis

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ABSTRACT

The mammalian intestine is a rapidly self-renewing organ with epithelial cell turn over occurring every 4-6 days. Specific differentiated epithelial cell types on the luminal surface aid in digestion, hormone production, waste removal and nutrient absorption to maintain host survival. Due to constant interaction with the external environment, these cells detach from the surface and die at an extensive rate. Crypts of Lieberkühn are invaginations found along the length of the small and large intestine that contain rapidly proliferating progenitors and stem cells that continuously divide and give rise to all cell types. Regulation of this activity requires an intricate network of signaling and gene regulation to maintain homeostasis.

Krüpple-like factor 5 is a zinc-finger transcription factor that is found to be highly expressed in the proliferative crypts. It has been shown to mediate the onset and progression of diseases in the intestine such as colorectal cancer and inflammation; however its function in normal intestinal homeostasis is not well defined. In order to discern its role in this tissue, we first utilized inducible knockout mouse models to delete KLF5 from the adult intestine. We found it to be required for normal proliferation and, further, discovered that loss of KLF5 correlated with decreased expression of active stem cell transcripts, Lgr5, Ascl2, and Olfm4. When we analyzed long-term effects of KLF5 deletions, we saw restoration of protein expression in the crypt within 14 days; however this had no impact on the loss of stem cell expression and did not restore progenitor proliferative to wildtype levels. We found a transient decrease in the enteroendocrine cells, but no other differentiated cell types were affected. These studies indicated that KLF5 is required for stem cell renewal and proliferation.

To continue the analysis of KLF5’s role in the intestine, we specifically overexpressed KLF5 in the intestine discern its effects on proliferation, cytodifferentiation, and stem cell
dynamics. Further, given its role as an intestinal oncogene, we wanted to determine if this would have any neoplastic effects. After 2.5 days of overexpression villus blunting and crypt hyperplasia were associated with decreased villus cytodifferentiation and increased proliferative progenitor cell numbers in mice overexpressing KLF5 in the intestinal epithelium. While no effect was seen on stem cells, we were only able to examine these mice 2.5 days after induction as ectopic KLF5 expression in the intestine proved to be fatal by day 4. Results from these experiments indicated that this transcription factor could maintain and extend the transient amplifying zone and prevent intestinal cell differentiation.

To elucidate a mechanism for KLF5’s effects on proliferation and stem cell maintenance we performed RNA-sequencing on isolated crypts obtained from both wildtype and knockout intestines. Differentially expressed genes were then broadly classified into gene ontologies. Results from this analysis indicated that loss of KLF5 could impact MAPK signaling. Utilizing jejunal tissue from both our knockout and transgenic mice, we discovered that KLF5 expression levels in the intestine correlated with the expression of activated ERK1/2 and MEK. We further investigated this finding by performing rescue experiments in vivo. We found that constitutively active KRAS could rescue proliferation in the absence of KLF5, but had no effect stem cell marker expression. These results were confirmed in vitro when we saw that activation of KRAS could rescue the loss of crypt formation seen in enteroids lacking KLF5. However, the mean survival of enteroids from both groups was significantly decreased when compared to mean survival of wildtype enteroids. This suggests that KLF5’s regulation MEK-ERK signaling is only relevant in the progenitor cell types indicating that KLF5 may differentially regulate stem and progenitor cell populations in the intestinal crypt.
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"If a stem cell dies in the crypt, and no one is around to see it…”

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CHAPTER 1

Intestinal Anatomy & Krüpple-like Factor 5

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GENERAL INTESTINAL ANATOMY

The intestine is the most rapidly renewing organ in the human body. Epithelial turnover occurs every 4-6 days with the aid of rapidly dividing intestinal stem cells. This swift renewal is often accompanied by gene mutations and alterations in signaling pathways that regulate cellular proliferation and differentiation. Consequences of this can have detrimental effects on gastrointestinal health such as inflammation, severe bowel disorders, and cancer often resulting in chronic pain or even death. Therefore it is necessary to unravel the intricate molecular mechanisms required for proper bowel function in order to understand abnormalities. At the helm of proliferative regulation in the intestine is Krüppel-like Factor 5. This oncogenic transcription factor has been found to mediate crucial signaling pathways and cellular processes necessary for epithelial homeostasis. Much research has been performed to elucidate its role in epithelial biology; however, we are the first to use conditional knock-out and inducible overexpression models in the adult mouse to understand its effects on the intestine. Further we performed RNA sequencing on isolated crypts from wildtype and knock-out mice and attempted to unravel its functional role in this context.

Overview of the composition and function of the epithelial barrier

The gastrointestinal epithelium is composed of a stratified columnar monolayer of cells that serves as a protective barrier from the external environment. Adherens, tight junctions, and desmosomes comprise the intercellular protein network that binds single epithelial cells to regulate water, electrolyte, and ion flux into and out of the body (Ballard et al., 1995; Solanas and Batlle, 2011). In the small intestine, finger-like protrusions into the lumen increase the functional surface area of the epithelium. These villi are comprised of differentiated cells that
aid in nutrient digestion and absorption as well as hormone and mucus secretion. Interspersed between them are crypts of Lieberkuhn, pocket-like glands regions made up of proliferative progenitor cells continuously divide and give rise to cell types. At the base of the crypt, intestinal stem cells (ISC) are intercalated between differentiated, anti-microbial secreting Paneth cells that protect from harmful enteric bacteria. The large intestine is also punctuated with crypts; however they lack the same anti-microbial cells found in the large intestine. The luminal, flat epithelial surface does contain similar cell types.

**Rapid renewal depends on the stem cell population**

*Active intestinal stem cells – crypt based columnar cells*

In order to maintain epithelial integrity, continuous proliferation of stem cells is required to produce all cell types. Arguably the most important, and most widely accepted, marker of these actively cycling crypt based columnar cells is LGR5 (Leucine-rich repeat containing G-protein-coupled Receptor 5), a component of the WNT receptor complex. RNF43 and ZNRF3 E3 ligases are two downstream targets of canonical WNT signaling that regulate this pathway through ubiquitination of FRIZZLED receptors. Wheniganded by R-spondin, LGR5 tethers these ligases to the membrane to prevent receptor degradation thus amplifying WNT signaling (de Lau et al., 2014). Cre/LoxP lineage tracing of LGR5-expressing cells was used to visualize their localization between Paneth cells and illustrate that they had the ability to give rise to all cell types (Barker et al., 2007). Because mRNA expression of Lgr5 is low, transgenic reporter mice sometimes do not provide accurate representation of CBCs. Therefore, other markers have been identified to further substantiate studies pertaining to the relevance and mechanisms of stem cell biology. A Notch regulated glycoprotein, Olfactomedin 4 (OLFM4), was found to be
coexpressed with LGR5 in the murine and human small intestine (van der Flier et al., 2009a). Additionally, the basic helix-loop-helix transcription factor, Achaete scute-like 2 (Ascl2), was discovered and confirmed to regulate the expression of LGR5 as conditional ablation of this gene caused a disappearance of the receptor and, ultimately, the CBCs (van der Flier et al., 2009b). Since then, PW1/Peg3 (a gene with an un-known function), Smoc2 (a BMP signaling inhibitor), Igfbp4 (an Igf pathway inhibitor), and Pcdh8 (protocadherin 8) have been identified as putative markers of the active stem cells (Bessona et al., 2011; Merlos-Suárez et al., 2011; Muñoz et al., 2012). Proteins such as Musashi-1, an enhancer of Notch signaling, Prom1/CD133, and the receptor EphB2 have also been localized to CBCs and intestinal tumors, but their expression extends further up the crypt into the proliferative transient amplifying zone thus rendering them ambiguous stem cell markers (Merlos-Suárez et al., 2011; Potten et al., 2003; Snippert et al., 2009; Zhu et al., 2008).

**Quiescent intestinal stem cells – +4 cells**

It has been proposed that tissues in need of a continuous cellular supply retain both active and quiescent stem cell pools to provide a defense mechanism against injury and mutation while simultaneously allowing for expansion and rapid turnover necessary to maintain organ homeostasis. In the intestine, the CBC is not a label retaining cell and is sensitive to both mutation and injury (Escobar et al., 2011; Schepers et al., 2011). In contrast, the quiescent stem cell (+4 ISC or LRC) may provide a “back-up” to render the intestine less vulnerable to damage as they are a means of replacement for the active stem cell pool (Figure 1).

Studies on +4 position stem cell markers fueled the notion that there exists a stem cell hierarchy, with a quiescent or reserve stem cell population giving rise to a more active stem cell.
Unlike the CBCs previously mentioned, these cells have been shown to be resistant to inactivation or perturbations of the cell cycle suggesting a more slowly dividing cell (Lee et al., 2009; Yan et al., 2011). BMI1, a component of the polycomb transcription repressor complex, was found to be expressed in +4 cells and capable of producing all cell lineages within the intestine and forming adenomas in the context of aberrant canonical WNT signaling (Sangiorgi and Capecchi, 2008). It was further found that ablation of these cells with diphtheria toxin lead to the loss of crypts (Sangiorgi and Capecchi, 2008). Simultaneous lineage tracing experiments utilizing LGR5-GFP and BMI1-YPF indicated that BMI1+ cells appeared to be functionally distinct from the active LGR5+ CBC pool. Only LGR5+ cells were affected by manipulation of WNT signaling, a pathway known to affect crypt maintenance and architecture, as either increased or decreased signaling concomitantly resulted in increased or decreased LGR5+ expansion. BMI1+ cells remained unaffected by these perturbations (Yan et al., 2011). In fact, this population could sustain epithelial homeostasis in the absence of LGR5 marked cells (Tian et al., 2011). There has been some investigation of other +4 stem cell markers. HOPX, an atypical homeodomain-containing protein, was found to mark the +4 position along the entire length of the small intestine, and co-localize with BMI1 expression (Takeda et al., 2011). mTERT, or mouse telomerase reverse transcriptase, was found to mark a distinct population of slowly cycling, radiation resistant stem cells throughout the small intestine and colon that can give rise to LGR5 positive cells (Montgomery et al., 2010). A few other putative +4 ISC markers are ambiguous in their specificity: DCLK1, which was first identified to be enriched in ISCs at the crypt base (Giannakis et al., 2006), but has been shown to mark tuft cells (Gerbe et al., 2009). LRIG1, an ERBB inhibitor, was reported to be a specific marker of ISCs (Powell et al., 2012), but also to have a broad pattern of expression through-out the proliferating cells in the
crypt (Wong et al., 2012). Despite the overlap in expression patterns, LGR1-expressing cells exhibited some of the same attributes such as quiescence, injury induced proliferation, and production of LGR5-positive ISCs. PHLDA1, a protein thought to have an anti-proliferative effect, was found to be expressed in the CBCs of all crypts throughout the intestine; however, some crypts contained expression in the +4 stem cells (Sakthianandeswaren et al., 2011).

**Transcriptional networks & signaling pathways regulate intestinal cytodifferentiation**

*Absorptive versus secretory cell fate*

The proliferative ISCs give rise to the numerous transient amplifying cells (TAC), highly proliferative progenitor cells with limited renewal capacity. Under the influence of an assortment of molecular signals, these cells withdraw from the cell cycle and differentiate to become either absorptive enterocytes (colonocytes in the colon) or one of several secretory cell types that collectively constitute the functional intestinal epithelium (Figure 2). Notch signaling is critical for determining the types of cells produced by differentiating ISCs, directing cells toward either the absorptive or secretory fate by controlling the expression of HES1. ATOH1 is a transcription factor required for all secretory cell lineages. In some cells, HES1 represses the activity of this protein and drives them towards an absorptive lineage (Noah and Shroyer, 2013). In addition to Notch regulation, this cell fate can be influenced by other pathways such as WNT/β-catenin. ATOH1 was found to be directly regulated and targeted for proteasomal degradation by GSK3β, a component of the canonical WNT pathway that targets β-catenin for degradation. Inhibition of GSK3β led to stabilization of ATOH1 with consequent upregulation of the goblet cell mucin, MUC2 (Tsuchiya et al., 2007). Conversely, mice with ectopic expression of Dickkopf1 (DKK1), a secreted WNT inhibitor, lacked ATOH1, suggesting that
canonical WNT signaling may be necessary for Atoh1 gene expression (Pinto, 2003). WNT/β-catenin has also been shown to regulate HES1 expression (Peignon et al., 2011). Additional transcription factors such as NEUROG3, GFI1, MTGR1, and SPDEF are thought to function in ATOH1-specified secretory progenitors to determine which of several possible fates are adopted (Li et al., 2011; Noah and Shroyer, 2013; Noah et al., 2011; Vanuytsel et al., 2013b).

Absorptive Enterocytes

The majority of intestinal cells are absorptive enterocytes/colonocytes (EC) which are required for food digestion and nutrient absorption necessary to maintain the overall health of an organism. Following Notch-directed HES1 repression of ATOH1 to commit to the absorptive lineage, further molecular regulation is required for maturation to occur. Depletion of PTK6 was shown to decrease expression of the enterocyte marker I-FABP suggesting a role in EC differentiation (Haegebarth et al., 2006). Additionally, GATA4 is required to maintain the proximal identity of the small intestine including its function in cholesterol and fat absorption (Battle et al., 2008; Bosse et al., 2006). Loss of GATA6 interfered with Notch signaling and altered expression of some absorptive genes involved in lipid metabolism (Beuling et al., 2012). Members of the hepatocyte nuclear family also seem to be involved in absorptive cell fate. Deletion of both HNF1α and β resulted in reduced water absorption and decreased mRNA expression of Dpp4, a brush border enzyme found in ECs. HNF1α was further found to directly regulate CDX2, a gene involved in development and commitment of the intestinal epithelium (D’Angelo et al., 2010). Loss of HNF4α in mice was shown to increase secretory cell numbers (Cattin et al., 2009) and found to be a putative regulator of EC-specific genes such as alkaline phosphatase (Olsen et al., 2005; Stegmann et al., 2006). Despite being the most abundant
cellular component of the intestinal epithelium, much remains to be learned about their molecular regulation.

**M Cells**

M cells, or Microfold cells, are specialized epithelial cells normally found in the follicle-associated epithelium overlying the Peyer’s patches, but can also be found occasionally on the villi intermingled with differentiated intestinal cells. Their primary function is endocytosis and delivery of antigens from the gut lumen to immune cells (Kucharzik et al., 2000). It is not clear how these M cells are differentiated, but it likely involves Notch signaling. Mice with a DLL3 ligand mutation produced more of an intermediate type M cell (characterized by their shape) rather than the fully mature M cells. Furthermore, these studies showed that contact with B-cells is responsible for maintaining the mature M cell phenotype (Mach, 2005). SpiB also regulates their differentiation downstream of RANKL as loss of either of these proteins results in decreased M cell populations (de Lau et al., 2012; Kanaya et al., 2012).

**Enteroendocrine Cells**

The enteroendocrine (EE) cells are hormone-producing cells within the intestine. With approximately 16 different types, these cells are responsible for regulating satiation, immunity, and inflammation (Schonhoff et al., 2004). Following ATOH1-mediated commitment of differentiating ISCs to the secretory lineage, EE differentiation is specified by Neurogenin3 (Neurog3), a transcriptional regulator that is localized to proliferating secretory progenitor cells (Bjerknes and Cheng, 2006; Jenny et al., 2002). Downstream of NEURGO3 is BETA2/NEUROD (Jenny et al., 2002), a transcription factor required for development of
hormones. This, in turn, may regulate PAX6 expression (Larsson et al., 1998; Marsich et al., 2003). Other genes important for maturation of EE cells include Foxa1/a2, Insm1, Nkx2.2, and Arx (Desai et al., 2008; Du et al., 2012; Gierl, 2006; Ye and Kaestner, 2009). Both Pdx1 and Pax4 are regional-specific genes that control EE differentiation within the small intestine (Larsson et al., 1998; Yamada et al., 2001).

**Goblet Cells**

Goblet cells (GC) produce mucous and additional proteins required for physical protection of the epithelium and lubrication of the luminal surface to facilitate fecal expulsion from the gut. Differentiation of these cells from ATOH1-specified secretory progenitors relies on expression of GFI1, a negative regulator of NEUROG3 (Bjerknes and Cheng, 2010; Shroyer et al., 2005). Downstream of ATOH1 and GFI1, SPDEF, an Ets-transcription factor, is also required. Transgenic expression of this gene results in increased GC number, while depletion *in vitro* was shown to have the opposite effect (Noah et al., 2010). Notch signaling may also play a role in GC differentiation through HES5. Overexpression of NICD in post mitotic cells increased both HES5 and GC number specifically above the crypt region (Zecchini et al., 2005). Further, increased HES5 expression was found in mice lacking LKB1 kinase activity, which also increased levels of the well-known GC marker MUC2 (Shorning et al., 2009). Other GC associated genes include FOXA1/A2 that bind to the goblet cell specific Muc2 promoter (Ye and Kaestner, 2009); and KLF4, which when mutated caused a GC deficiency in the embryonic colon and decreased acidic mucin production in the adult small intestine (Katz et al., 2002). Additionally, OASIS was identified downstream of SPEDF to be required for goblet cell maturation in the large intestine (Asada et al., 2012).
Paneth Cells

Unlike the other secretory cells, Paneth cells (PC) reside in the crypt bottom of the small intestine only and have a longer turnover time of about 30 days. Their granulocytic products include lysozyme and defensins which provide antimicrobial defense for the intestine (Clevers and Bevins, 2013). These cells also provide a source of WNT3a, WNT6, WNT9, EGF, TGFα, and DLL4 required for stem cell maintenance (Durand et al., 2012; Sato et al., 2010). Additionally, mTOR expression enables them to be nutritional sensors that can augment stem cell renewal dependent upon calorie intake (Yilmaz et al., 2012). Similar to GCs, PC secretory progenitor fate arises through expression of ATOH1, GFI1, and SPDEF (Gregorieff et al., 2009; Shroyer et al., 2005). Subsequently, WNT signaling through FRIZZLED5 is then required for maturation of the PC lineage. While conditional loss of APC induced proliferation and decreased villus cell differentiation, it also resulted in accumulation of β-catenin and TCF4 leading to the increased expression of PC markers, lysozyme and cryptdin. It was then shown that TCF4 mediates the transcriptional control of several defensin genes (Andreu, 2005; van Es et al., 2005b). Furthermore, SOX9, transcriptional target of WNT signaling, was shown to be a requirement for PC differentiation (Bastide et al., 2007; Mori–Akiyama et al., 2007).

Tuft Cells

Tuft cells, also known as brush cells or caveolated cells, represent a lesser known group of secretory cells. Thought to play a role in the taste receptor pathway and opioid secretion, their only known function is to regulate immunological responses in response to allergy and infection (von Moltke et al., 2015). While there is limited knowledge on the molecular regulation of these
cells, they are known to express DCAMKL1 (Gerbe et al., 2009). ATOH1 was originally thought to be required for their differentiation, while NGN3, SOX9, GFI1, and SPDEF proved to be dispensable in this process (Gerbe et al., 2011). However later studies found that depletion of ATOH1 led to an increase in this lineage (Bjerknes et al., 2012). Further studies are needed to elucidate the mechanism of tuft cell differentiation.

An intricate web of signal transduction pathways mediates intestinal proliferative activity

Cellular behavior in the intestine is driven by interaction of various signaling pathways (Figure 3). Wnt signal transduction is perhaps the most important signaling pathway involved in intestinal proliferation and epithelial renewal. In situ hybridization analysis confirmed the expression of Wnt signaling components, WNT-3, WNT-6, WNT-9b, FZ4, FZ6, and FZ7 in the crypt (Gregorieff et al., 2005). Confirmation that canonical Wnt signaling drives intestinal proliferation was found when loss of β-catenin in the adult mouse intestine resulted in premature differentiation of ISCs, followed by loss of crypt compartments, and subsequent depletion of epithelial cells (Fevr et al., 2007). Incidentally, ectopic activation lead to aberrant morphological anatomy indicated by hyperplastic crypts and villus blunting concomitant with increased proliferation, reduced villus cytodifferentiation, increased Paneth cell differentiation, and altered cell migration (Andreu, 2005; Sansom et al., 2004). Downstream effector TCF4 is required to mediate the proproliferative effects of canonical WNT signaling in the intestine while TCF1 and TCF3 are dispensable (van Es et al., 2012).

Notch signaling is well studied in the intestine as it affects not only secretory cell differentiation, but also proliferative activity of stem and progenitor cells. Expression of its
Effector proteins, NOTCH1, NOTCH2, HES1, HES5, HES6, HES7, DLL1, DLL4, JAGGED1, and JAGGED2, are prominent throughout the intestinal crypt epithelium (Sander and Powell, 2004; Schröder and Gossler, 2002) and have been found to influence cellular behavior in this region. Inhibition of Notch signaling in mice by DBZ (a γ-secretase inhibitor), loss of NOTCH1/2 receptors, or loss of downstream effector RBP-J results in abrogation of the proliferative compartment and increased cytodifferentiation toward secretory cell fate (Riccio et al., 2008; van Es et al., 2005a; VanDussen et al., 2011). There was also a significant reduction of Olfm4 mRNA suggesting CBC regulation. Luciferase assays confirmed that HES1 positively regulated promoter activity of the stem cell marker (VanDussen et al., 2011). These effects likely occur though Delta interaction with the Notch receptor as loss of DLL1 and DLL4 resulted in a similar phenotype (Pellegrinet et al., 2011). Downstream HES1 is required repress cyclin dependent kinase inhibitors p27Kip1 and p57Kip2, to maintain proliferative activity of the progenitor population (Riccio et al., 2008). Consistently, ubiquitous expression of NICD suppressed differentiation and expanded progenitor cells (Fre et al., 2005).

The role of receptor tyrosine kinases (RTK) have been extensively explored in the context of intestinal cancer, but its role in intestinal maintenance is less well known. Several lines of evidence point to a role for mitogen-activated protein kinase (MAPK) signaling in the maintenance of proliferative capacity. Inhibition of MAPK signaling in both colorectal cancer cells and nontransformed intestinal epithelial cells (IEC), correlated with cell cycle arrest and increased differentiation (Aliaga et al., 1999). Epidermal growth factor receptor (EGFR) is an ERBB family transmembrane RTK that is expressed highly proliferative cells of the crypts (Suzuki et al., 2010) while its corresponding ligand, EGF, is secreted from the stroma, differentiated IECs, and the Brunner’s glands of the duodenum (Konturek et al., 1989). In mice,
inhibition of EGFR kinase (rendering the receptor inactive) resulted in decreased proliferation followed by increased cell death and loss of differentiated epithelial cells suggesting the inability for cellular repopulation of the epithelium (Suzuki et al., 2010). A requirement for EGF signaling specifically in stem cell proliferation has been found in *Drosophila* intestines (Biteau and Jasper, 2011) however, this pathway may be limited to the transit amplifying population of the normal mammalian intestine. ERBB2 and ERBB3 are other RTKs that is activated in response EGF and EGF-like ligand bonding. The stem cell marker LRIG1 was shown to interact with all ERBB receptors to maintain proper ISC proliferation. Experimentally, deletion of LRIG1 resulted in stem cell expansion as well as increased expression of activated MAPK signaling (Wong et al., 2012). These data indicate that LRIG1 negatively regulates this pathway and prevents unwarranted ISC expansion. Fibroblast growth factor (FGF) signaling is another RTK pathway that may have a direct effect on intestinal proliferation. Knock out of the FGF receptor, FGFR3, increased proliferation in intestinal crypts; complementary in vitro experiments indicated this may be due to increased pERK expression and loss of pAKT (Arnaud-Dabernat et al., 2008). These data suggest that the function of this pathway in normal ISC homeostasis may be to repress proliferation.

Phosphatidylinositol 3-kinase (PI3K) signaling was first shown to induce IEC differentiation through modulation of adherens junctions by p38 signaling in colon cancer cell lines (Laprise et al., 2001). However, its effects dramatically differ in vivo. Administration of PI3K inhibitors to mice, reduced expression of PCNA in the transient amplifying zone. In vitro analysis in rat intestinal epithelial cells indicated that both EGF and TNFα induced phosphorylation of AKT and increased cyclin D1 (CCND1) expression (Sheng et al., 2003). In agreement, depletion of PTEN, a negative regulator of PI3K signaling, increased proliferative
activity in the intestine ultimately resulting in polyposis. This phenotype correlated with increased p-AKT and p-GSK3β as well as other G1/S phase associated genes. Additionally, AKT phosphorylation of β-catenin was found to mediate crypt fission (He et al., 2007). While these data indicate a strong interaction with canonical WNT signaling, hormonal-induced proliferation was mediated through PI3K and EGFR activation of ERK1/2 (Waseem et al., 2014). Therefore PI3K driven proliferation seems to occur largely through interaction with multiple signaling pathways.

Other signaling transduction in the intestine indirectly affects epithelial proliferation. Bone morphogenetic proteins (BMP) are members of the TGF-β superfamily and are well known for their roles in regulation of cell proliferation, differentiation, and tissue pattern formation in several systems. While BMP1/2/7 are expressed in the intestinal epithelium, mesenchymal BMP4 is the most prominent effector of stem cell proliferation (Vanuytsel et al., 2013a). Under physiological conditions, BMP4 ligand is restricted to the mesenchyme of the intervillus region and surrounding area at the base of the crypt (Haramis et al., 2004; He et al., 2004). Defects of BMP signaling in vivo can induce proliferation and cause diseases such as juvenile polyposis syndrome. In mice, transgenic epithelial expression of Noggin, a Bmp antagonist, drove ectopic crypt formation within the villi, induced polyp formation, and increased stromal and epithelial proliferation (Batts et al., 2006; Haramis et al., 2004). Mechanistically, BMP signaling was found to exert its effect as a suppressor of WNT signaling through enhanced activation of PTEN (He et al., 2004). These data indicate that BMP signaling from the mesenchyme maintains a balance of intestinal turnover through negative regulation of stem-cell proliferation via PI3K-AKT inhibition.
Indian hedgehog (IHH) ligand is expressed by mature, differentiated epithelial cells and, via a mesenchymal intermediate, limits proliferation in the crypts (van den Brink et al., 2004; van Dop et al., 2010). Deletion of IHH in the adult intestine resulted in increased nuclear β-catenin and downstream targets of WNT signaling, including ISC markers Lgr5 and Olfm4. Consequently, increased proliferation, crypt fission, crypt depth, and villus length were also found. BMP4 signaling was reduced as evidenced by loss of phosphorylated SMAD1/5/8, possibly owing their absence to the reduced number of mesenchymal cells found in the villus core (Kosinski et al., 2010; van den Brink et al., 2004; van Dop et al., 2010). Conversely conditional activation of IHH signaling in the adult mouse colon led to decreased proliferation, reduced nuclear TCF/β-catenin accumulation within the ISC, crypt hypoplasia, and premature cell differentiation. Additionally, there was a significant accrual of colonic myofibroblasts as well as BMP4 expression (van Dop et al., 2009). Together, these results suggest that, under physiological conditions, IHH functions as part of a negative feedback mechanism to limit production of progenitor cells by ISCs.

**The importance of studying intestinal homeostasis**

Alterations in normal epithelial homeostasis is often accompanied by acute, chronic, or fatal intestinal illness. According to the Center for Disease control, colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States. Deregulation of WNT, NOTCH, PI3K, and MAPK signaling are often associated with the pathology of this disease (Anderson and Wong, 2010; Fang and Richardson, 2005; Krausova and Korinek, 2014; Qiao and Wong, 2009). Furthermore, these pathways have been shown to drive the onset of inflammation and related inflammatory bowel disorders as well be involved their subsequent progression into CRC.
(Khan et al., 2013; Obata et al., 2012; Serafino et al., 2014; Xing et al., 2015). Mechanistically, irregular signaling often involves transcription factors known to regulate homeostatic conditions in the intestine, such as CDX2, HES1 and SOX9. In unhealthy tissue they are often misexpressed, mutated, or absent which contributes to onset and progress of illness (Candy et al., 2013; Matheu et al., 2012; Saad, 2011). KLF5 is one such transcription factor that has been shown to mediate intestinal pathobiology.

**KRÜPPLE-LIKE FACTOR 5**

Originally as cloned BTEB2, KLF5 is part of a large family of Krüpple-like transcription factors and contains three functional domains: the nuclear export signal (NES), the transactivation domain (TAD), and the DNA-binding domain (DBD) (Diakiw et al., 2013; Sogawa et al., 1993) (Figure 4). Like other members of the Krüpple family, the DBD of KLF5 is defined by three C-terminal zinc-finger motifs that bind to GC-rich sequences of DNA to regulate transcription (Bieker, 2001; Shi et al., 1999; Swamynathan, 2010). Additional regulatory functions are governed by protein-protein interactions. KLF5 binds to basal transcription factors; TFIIB, TFIIEβ, TFIIFβ, and TATA-binding protein (TBP) (Kojima et al., 1996) as well as CREB binding protein (CBP/p300) to enhance its transactivation properties (Zhang and Teng, 2003). Though the TAD is required for KLF5-mediated transcriptional activation, it is not required for binding to the basal transcription complex (Kojima et al., 1996).

Genetically, *Klf5* consisting of four exons and three cis-activation domains that bind Sp1, CBFa, and NF-1 (Mori et al., 2003) and is regulated by a variety of mechanisms. Sp1 was confirmed to activate *KLF5* in human epithelial cells (Chen et al., 2004); however, whether or not CBFa and NF-1 play regulatory roles in *Klf5* transcription has yet to be determined. Early
response gene-1 (EGR-1) was later found to bind to the KLF5 GC-rich promoter region in smooth muscle cells downstream of MAPK signaling (Kawai-Kowase et al., 1999; Nandan et al., 2004).

A number of post translational modifications can modify the function of KLF5. For example, in vascular smooth muscle cells (VSMC) phosphorylation of KLF5 by p38, MAPK, and PKC signaling enhances proliferation and transactivation activity (Fujiu, 2005; Liu et al., 2010; Zhang and Teng, 2003; Zheng et al., 2009). However, in breast cancer cell lines GSK3β phosphorylation is required for FBW7-mediated degradation (Zhao et al., 2010). Acetylation of KLF5 by CBP/p300 or deacetylation by HDAC2 alters its promoter binding properties and confers both a positive and negative transcriptional regulatory activity dependent up protein interactions and co-factor recruitment (Guo et al., 2009a; Guo et al., 2009b; Miyamoto et al., 2003; Zheng et al., 2011). Sumoylation is also associated with varied KLF5 function. This modification inhibits its nuclear export activity and facilitates anchorage independent growth of human colon cancer cells (Du et al., 2008). However, sumoylation of KLF5 can exert a repressive effect on genetic activation. In skeletal muscle sumo-KLF5 interacts with PPARδ and co-repressive genes NCoR and SMRT to modulate lipid metabolism by repressing genes involved in fatty-acid oxidation (Oishi et al., 2008). Finally, ubiquitination of KLF5 marks this protein for proteasomal degradation and is dependent upon E3 ligase WWP1 and FBW7 (Chen et al., 2005; Liu et al., 2010; Luan and Wang, 2014). However, degradation can occur independently of this modification (Chen et al., 2007). These modifications are important molecular switches that diversify the function of KLF5.
**KLF5 expression is ubiquitous in the blastocysts and becomes localized in various tissues**

The requirement for KLF5 begins in the embryo where expression is ubiquitous in the blastocyst prior to implantation. Mouse studies have shown that embryonic loss of KLF5 prevents implantation as a result of defective trophoectoderm formation (Ema et al., 2008; Lin et al., 2010). Further, embryonic stem cells (ESC), arising from the inner cell mass of the blastocyst, require KLF5 to maintain normal proliferation and pluripotency, by directly regulating Oct3/4 and Nanog expression (Ema et al., 2008; Parisi et al., 2008; Parisi et al., 2010). KLF also regulates lineage commitment in ESCs through activation of Mix1, a negative regulator of mesodermal genes (Aksoy et al., 2014).

During development KLF5 expression becomes localized to various regions including the stomach, intestine, lung, tounge, trachea, aorta, bladder, uterus, skin and cornea (Ohnishi et al., 2000; Sur, 2002; Watanabe et al., 1999). During this time, alterations in its expression can perturb morphogenesis. Depletion in the bladder urothelium prevented differentiation by e14.5 (Bell et al., 2011). In the lung, KLF5 deletion rendered mice moribund as a result of respiratory failure likely due to lack of cellular differentiation and decreased surfactant protein (Wan et al., 2008). Loss of KLF5 in the developing eye affected glandular maturation, inhibited conjunctival Goblet cell differentiation, disrupted barrier function, and increased corneal proliferation (Kenchegowda et al., 2011). Transgenic expression of KLF5 deregulated tissue development as well. In the skin, hyperproliferation resulted with associated loss of barrier function, stem cell depletion, and craniofacial defects (Sur, 2006). Such drastic effects were not found when KLF5 was overexpressed in the esophagus; however, progenitor basal cell proliferation increased two-
fold (Goldstein et al., 2007). Altogether these data highlight the necessity for KLF5 in normal embryonic and tissue development.

**KLF5 regulates cellular behavior through gene regulation and protein interaction**

*Proliferation*

KLF5 was first shown to have a positive effect on cell proliferation in NIH3T3 embryonic fibroblast cells where transient expression promoted anchorage independent growth (Sun et al., 2001). In both non-transformed IECs and H-Ras transformed NIH3T3 cells, loss of KLF5 decreased proliferation, colony formation, and positively regulated *Ccnd1* promoter activity (Bateman et al., 2004; Nandan et al., 2004). Furthermore, KLF5 was found to increase the promoter activities of *Ccnb1* and *Cdc2* (Nandan et al., 2005) thus illustrating the ability for this transcription factor to regulate cell cycle proteins promoting both G₁/S and G₂/M phase transitions. In the cardiovascular system, KLF5 expression is found early in the development of smooth muscle and later in vascular remodeling. In primary cultured VSMC’s loss of KLF5 resulted in decreased proliferation and CCND1 expression (Liu et al., 2009b; Suzuki et al., 2009). Transfection of COS-7 cell lines confirmed that MAPK-driven phosphorylation of KLF5 facilitated an interaction with c-Jun and increased transcriptional activity of the *Ccnd1* promoter under angiotensin II stimulation (Liu et al., 2009b). Further, KLF5 was shown to regulate the activity of cyclin E (*Ccne*) through recruitment from hHLIM to its promoter (Shi et al., 2012).

KLF5 can also exert a negative effect on proliferation primarily through posttranslational modifications. This is best illustrated by examining the impact of TGFβ signaling on epithelial cell lines. This signaling pathway recruits CBP/p300 to acetylate the lysine 396 residue of KLF5 and facilitate its interaction with SMAD4 to regulate promoter activity (Guo et al., 2009b).
Initially, this interaction was found to positively regulate the promoter of cyclin dependent kinase inhibitor p15\textsuperscript{INK4B} (Guo et al., 2009a; Guo et al., 2009b). This phenomenon was further examined in the context of MYC expression. As expected, induction of KLF5 was correlated with increased proliferation and a concomitant increase of MYC expression; however in the presence of TGFβ, KLF5 acetylation resulted in SMAD4 interaction and was necessary to repress Myc transcription (Guo et al., 2009b). Recently, these effects were recapitulated \textit{in vivo} as the effects of TGFβ signaling was shown to attenuate prostate cancer cell proliferation dependent upon KLF5 acetylation (Li et al., 2014c).

A more complicated example can be seen in the proliferative regulation of VSMCs. KLF5 positively regulates the promoter activity of mitogen PDGF-A after CBP/p300-mediated acetylation. Inhibition of acetylation by the oncogenic regulator SET reduced cellular growth and transcriptional activity (Miyamoto et al., 2003). Further studies found that phosphorylation of KLF5 by p38 signaling facilitates interaction with the retinoic acid receptor (RXR/RAR) on the PDGF-A promoter and stimulates proliferation (Fujiu, 2005; Zhang et al., 2009). RAR stimulation by the synthetic agonist, Am80 resulted in KLF5 dephosphorylation and subsequently dissociation with RAR to prevent activation and VSMC proliferation (Fujiu, 2005; Zhang et al., 2009). Retinoid signaling also modifies proliferative activity through deacetylation. Under basal conditions HDAC2 forms an interaction with acetylated KLF5 and RAR to form a transcriptional repression complex on the cyclin dependent kinase inhibitor, p\textit{21\textsuperscript{Cip1}}, promoter facilitating cell cycle progression. Upon retinoid stimulation, phosphorylated HDAC2 deacetylates KLF5 and dissociates from the p\textit{21\textsuperscript{Cip1}} repression complex (Zheng et al., 2011). Data from these studies indicate that KLF5 plays a dynamic role in the intricate balance of cellular activities.
Apoptosis

While KLF5 is notably thought of as a mediator of proliferation and cell cycle turnover, it also affects cell survival as a regulator of genes involved in apoptosis. It was found to induce the expression of BAX in esophageal squamous cancer cells after exposure to UV radiation (Yang et al., 2014). A direct regulatory function of the Bax promoter was later confirmed by chromatin immunoprecipitation and luciferase assays. (Tarapore et al., 2013). That same study found that KLF5 also increased the promoter activities of genes involved in the JNK signaling pathway, such as Mkk4 and Ask1, upstream of BAX expression (Tarapore et al., 2013). Similarly, in prostate cancer cells, it was found to regulate Mkk4 activity in TNFα-induced apoptosis. KLF5 loss was associated with decreased cell death as evidenced by decreased expression of cleaved-caspase 3 (CC3) and cleaved PARP (Shi et al., 2016).

KLF5 also confers apoptotic resistance through protein interaction and regulation of survival genes. Acetylated KLF5 interacts with PARP-1 in the cardiovascular system and inhibits apoptosis (Suzuki et al., 2007). In HCT116 cancer cells, DNA damage-induced cell death was dependent upon KLF5 regulation of PIM1 expression. Transient depletion of the transcription factor resulted in loss of the survival kinase and associated BAD phosphorylation culminating in increased apoptosis (Zhao et al., 2007). Finally, KLF5 can bind to the promoter and activate expression of SURVIVIN, a protein that inhibits caspase activation (Dong et al., 2013; Zhu et al., 2006). Regulation of this gene is potentiated by KLF5 interaction with p53 as this association prevents suppression of Survivin in acute lymphoblastic leukemia (Dong, 2006).
Differentiation and Migration

To a lesser extent than proliferation and survival, KLF5 plays role in cellular differentiation and migration. In KLF5 heterozygote mice, white adipose tissue formation was delayed compared to control littermates. *In vitro* analysis confirmed that, in conjunction with C/EBPβ/δ, KLF5 regulates the promoter activity of *Pparγ2* to regulate adipocyte generation (Oishi et al., 2005). Reduction of KLF5 by *miR*-448 was accompanied by decreased adipocyte gene expression and triglyceride accumulation (Kinoshita et al., 2010). KLF5 affects migration by altering the expression of kinases involved in activation of cell motility proteins. In keratinocytes, loss of this transcription factor results in decreased expression of integrin-linked kinase (ILK) and ILK-induced activation CDC42 and MLC$_2$ (Yang et al., 2008). Most recently it was found to regulate FYN, a protein-tyrosine kinase necessary for lamellipodia formation and cell migration in bladder cancer cells (Du et al., 2008). Data combined from all of these studies on cell behavior clearly point out a dynamic transcription factor capable modulating a plethora of activities.

**KLF5 mediation of signal transduction alters its effects on cell behavior**

As previously mentioned signal transduction pathways play a crucial role in modulating cellular shape, function, and behavior. Transcription factors can mediate induction and response to signaling through genetic regulation and interaction with effector proteins. Some of the ways in which KLF5 plays a role in signaling have been discussed. It can mediate the effects of JNK-induced and TNFα-induced apoptosis by regulating expression of effector proteins in this pathway (Shi et al., 2016; Tarapore et al., 2013). Additionally, it can mediate proliferative effects of cells through retinoid signaling due to its interaction with the retinoic acid receptor.
(Fujiu, 2005; Zhang et al., 2009; Zheng et al., 2011) and TGFβ signaling through interactions with SMAD4 proteins (Guo et al., 2009b; Li et al., 2014c).

KLF5 mediates other signaling pathways to modulate cellular function. KLF5 induction can occur downstream of MAPK signaling by EGR-1; however, like many other biological feedforward loops, it too can regulate this signaling pathway. Its loss in primary esophageal cells correlated with decreased proliferation, EGFR expression, and phosphorylation of MEK1/2 and ERK1/2. It was found to bind to the 5’ region of the Egfr promoter and have a positive effect on transactivation of the receptor. Reexpression of EGFR in the absence of KLF5 resulted in increased proliferation (Yang et al., 2007). KLF5 also regulates ERK1/2 activity in breast epithelial cells through FGF-BP (Liu et al., 2009a). In angiogenesis, KLF5 can alter PI3K/AKT signaling. Loss of KLF5 in HuVECs inhibited AKT activation resulting in accumulation of HNF1α (Ci et al., 2015). Additionally, in ESCs, concomitant loss of AKT1 activator, TCL1 was found after KLF5 deletion. This subsequently prevented its phosphorylation and had a negative effect on proliferation (Ema et al., 2008). These data demonstrate the importance of KLF5 in signal transduction pathways that influence the cellular activity.

**KLF5 functions in pathophysiology**

Due to the intricate nature of KLF5 involvement in a variety of cell behaviors alterations in its expression patterns or posttranslational modification can affect many pathological conditions. Vascular expression of KLF5 decreases after development; however, reemergence of this transcription factor is found after injury. In rabbit aortic cells, KLF5 was found to regulate the expression of SMemb/NMHC-B, a smooth muscle myosin heavy light chain isoform associated with vascular disease (Watanabe et al., 1999). KLF5 also participates in vascular remodeling.
through activation of PDGF-A indicating a role in the onset of cardiac hypertrophy and fibrosis after acute stress (Shindo et al., 2002).

KLF5 is also a potent mediator of the inflammatory response. It directly regulated the expression of the p65 subunit of NF-κB in human bronchial epithelial cells after lipopolysaccharide (LPS) treatment. This was accompanied by increased expression of inflammatory cytokines TNFα, IL-1β, and IL-6. Tissue from LPS-induced lung inflammation confirmed expression of these elements in vivo (Chen et al., 2014). KLF5 mediates the inflammatory effects of renal tubulointerstitial disease. Decreased expression of TNFα, IL-1β, and macrophage marker, EMR1 was found in unilateral ureteral obstruction mouse models with heterozygote expression of KLF5. Through various experiments, it was discovered that this transcription factor pushed macrophage differentiation toward M2 type which lead to reduced inflammation, but increased fibrosis (Fujiu et al., 2011).

Lastly, KLF5 has been associated with various cancers in epithelial tissues such as the lung, prostate and breast. Due to its context-dependent functionality, it can either be labeled a tumor suppressor or an oncogene. In some lung cancers, KLF5 expression is frequently upregulated along with several cell cycle proteins. In primary lung cancer cells, KLF5 mediated proliferation through direct regulation of Sox4 (Li et al., 2014a). Further, it was found to induce HIF-1α in hypoxic non-small cell lung cancer cells. RNA interference of KLF5 in these cells decreased CCNB1 and SURVIVIN expression with concomitant decrease in cell viability and colony formation indicating that KLF5 confers apoptotic resistance and regulates proliferative activity in these conditions (Li et al., 2014b). KLF5 promotes cell proliferation of bladder cancer by activation of AKT and ERK (Chen et al., 2006). In breast cancer tissue KLF5 expression was correlated with lower patient survival (Tong, 2006). It some contexts it activates cell migration...
and proliferation through a variety of methods such as regulation of TNFAIP2 downstream of TNFα, interaction with TEAD4 downstream of YAP/TAZ signaling to inhibit p27^Kip^, or promotion PGES1 to regulate p21, p27, and SURVIVIN (Jia et al., 2015; Wang et al., 2015; Xia et al., 2013). However, KLF5 expression was also found to be downregulated in human breast cancer and its activity may, in fact, play a role in tumor suppression in this context (Chen et al., 2002). Research has found that the correlation between KLF5 expression and prognosis is dependent upon several other factors including the presence of the estrogen receptor (ER). The proproliferative effect of estrogen stimulated-ER-positive cancer cells was diminished with induction of KLF5 (Guo et al., 2010). Incidentally, a tumor suppressor function has also been identified in prostate and esophageal cancer (Ci et al., 2015; Tarapore et al., 2013). Given the diverse role of KLF5 as a regulator of disease, it’s important to discern its function in homeostatic conditions to understand the onset and progression of illness.

**KLF5 IN THE INTESTINE**

KLF5 expression is found in the mouse embryonic endoderm by e10 and persists through intestinal development eventually becoming confined to proliferative intervillus regions (Bell et al., 2013; Moore-Scott et al., 2007). Conditional loss in the embryonic intestinal epithelium resulted in defects in villus morphogenesis and decreased cytodifferentiation. Expression of CCND1 was absent, but, surprisingly, proliferative activity continued. Luciferase assays suggested that KLF5 blocked the activity of FOXA1-mediated repression of ELF3, another transcription factor involved in maturation of the intestinal epithelium (Bell et al., 2013). Prior to this study, an attempt to determine the role of KLF5 in adult homeostasis was made by deleting it at the time of villus morphogenesis. Although 66% of the mice did not survive, the
remainder were analyzed for defects as adults. Eight weeks after birth, disorganized cellular
differentiation and migration defects were found accompanied by loss of barrier function and
reduced expression of CDX1/2 and WNT-associated genes. No loss was found in proliferative
capacity attributed to the increased expression of SOX9 (McConnell et al., 2011b). Data from
these later experiments implicate that KLF5 regulates homeostasis through regulation of
canonical WNT signaling; however, given the developmental defects that arise in its absence,
other compensatory mechanisms could be contributing to this phenotype. Therefore the role of
KLF5 in normal adult intestine still remains to be elucidated.

The mechanism by which KLF5 mediates intestinal illness has been demonstrated
primarily in vitro and through heterozygote mouse models (due to the lethality of homozygous
mutants). Originally, KLF5 expression was found to be reduced in APC^{min}-induced adenomas
from mice and patients presenting with familial adenomatous polyposis. Further analysis found,
that while KLF5 induced proliferation and colony formation in non-transformed cells, the
inverse was true in Immorto-Min Colon Epithelial cells (IMCE). Additionally, KLF5 expression
had the same effect on RAS-transformed cells (Bateman et al., 2004). These studies
communicated that loss of KLF5 modulated intestinal tumor progression. Later, another group
found its expression was indeed increased in tumors from APC^{min} mice. In fact, KLF5
heterozygosity ameliorated the pathological effects by reducing the number and size of
adenomas. It was concluded that KLF5 facilitated nuclear localization of β-catenin and
cooperatively drove promoter activity of CCND1 (McConnell et al., 2009). Under LPA
induction, it is not required for nuclear accumulation of β-catenin in colon cancer cells, but rather
functions as a co-factor in the β-catenin/TCF complex (Guo et al., 2015).
KLF5’s positive regulatory activity on CCND1 was again demonstrated in HRAS transformed fibroblasts, and was further shown to mediate G2/M transition by increasing promoter activity of Ccnb1 and Cdc2 (Nandan et al., 2005; Nandan et al., 2004). These results, in addition to finding increased KLF5 expression in KRAS-mutated human CRC, necessitated further investigation into its regulatory role. Data from these studies showed that KRAS-MEK-ERK signaling on transformed intestinal epithelial cells could induce KLF5 expression and mediate the effects of proliferative activity (Nandan et al., 2008). In vivo, KLF5 heterozygosity reduced tumor size and formation in tissue expressing APCMIN/KRASV12 mutations. Loss of one allele reduced phosphorylation of downstream KRAS effectors MEK1 and ERK1/2 (Nandan et al., 2010). These data suggest a role for KLF5 in intestinal tumor formation as both downstream mediator and upstream regulator of MEK-ERK signaling.

KLF5 can also regulate the inflammatory response. In bacterial-induced hyperplasia, heterozygous mice were again used to show that this transcription factor has a proproliferative effect on IECs (McConnell et al., 2008). Bacterial endotoxin, LPS, stimulation of IECs resulted in the induction of KLF5 which directly regulated the p50 and p65 subunits of NF-κB and increased TNFα and IL-6 cytokines (Chanchevelap, 2006). Dextran sodium sulfate treatment of Caco-2 cells was accompanied by increased KLF5 expression dependent up on MEK-ERK signaling. Examination of KLF5+/− mice indicated that they incurred greater sensitivity to DSS induced colitis due to decreased proliferative and migratory activity. Interestingly, this colitis sensitivity was also associated with a reduction in EGFR. Complementary in vitro studies showed a positive correlation between KLF5 and EGFR expression (McConnell et al., 2011a) indicating a possible feedback loop present in this disease. In a set of converse experiments, transgenic expression of KLF5 protected mice from DSS injury through increased JAK/STAT
signaling (Tetreault et al., 2012). These studies highlight the diverse mechanisms by which KLF5 can mediate disease in the adult intestine.

**RATIONALE**

The goal for this dissertation was to elucidate the role of KLF5 in intestinal homeostasis. Given its impact as a mediator of cancer and inflammation in these settings, it is important to identify the normal function of this transcription factor to gain further insight on how deregulation might occur. At the onset of this work, the only studies that examined its regulation of adult tissue maintenance resulted from developmental manipulation. Data obtained from this particular approach may not accurately depict the role in normal adult homeostasis as these could be lingering effects from developmental defects or compensatory mechanisms. In adult mice we inducibly deleted KLF5 from the intestine and found surprisingly different results than previously published (Chapter 2). Further, as KLF5 is upregulated in many intestinal diseases, transgenic models were used to determine the effects it may have on driving pathobiology (Chapter 3). Again, our results did not concur with the finding of previous work which suggested that no significant effects resulted from overexpression. Additionally, we report comparative findings for RNA-sequencing in control versus KLF5 knock-out mice from isolated intestinal crypts where expression is prominent (Chapter 3). Finally, we provide data indicating that KLF5 may regulate a signaling pathway common to intestinal disease and transformation (Chapter 3).

Significantly, this project affects intestinal biology in, at least, three ways. First, it provides a novel mouse model that can be utilized in the study of colorectal cancer and inflammation. Due the unfortunate lethality that results from its depletion in embryonic
intestinal development, the mechanism by which KLF5 affects the onset of disease progression is limited to in vitro experiments and heterozygotic mice which may not fully represent the actual effect of KLF5 absence. Second, the temporal depletion of this transcription factor contradicts previously published data and more accurately represents its role in maintaining normal epithelial function. Lastly, we begin to highlight a novel regulatory role for KLF5 in MEK-ERK signaling, a pathway commonly deregulated in intestinal disease, in normal homeostasis in vivo.
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FIGURE LEGENDS

Figure 1 - The intestinal epithelial crypt-villus axis.

The intestinal epithelium is composed of two domains: the crypts of Lieberkuhn, and the villus epithelium. Crypts contain all proliferating cells. Quiescent stem cells, here shown as a Bmi1 positive cell, are thought to be located at position +4 in the crypt. Active stem cells, or crypt based columnar cells, here shown as Lgr5+ cells, are interspersed between Paneth cells at the crypt base. Transit amplifying progenitor cells are rapidly dividing progenitors that reside in the upper crypt. Immunofluorescent images adopted from Yan et al, 2001.

Figure 2 - Intestinal stem cells and their progeny.

Stem cells divide to become an absorptive or secretory progenitor based on Atoh1 expression. Notch-directed Hes1 repression of Atoh1 results in an absorptive progenitor that will divide to become either an absorptive enterocyte or M cell. Expression of Ptk6, Gata4/6, Hnf1α/β, and Hnf4α have been shown to affect absorptive enterocyte differentiation. Both SpiB and RANKL are required for M cell differentiation. Atoh1 expression in stem cell progeny produces a secretory progenitor. Gfi1 repression of Neurog3 leads to a Goblet/Paneth cell progenitor that later expresses the Spdef transcription factor. Klf4, Foxa1, and Hes5 have been shown to be necessary requirements for goblet cell differentiation, while Tcf4 and Sox9 are involved in Paneth cell differentiation. Little information is known about tuft cell origin and differentiation, but Atoh1 seems to be a requirement.
Figure 3 - Integration of signaling pathways effects on Intestinal stem cell regulation.

A gradient of Wnt signaling is highest at the bottom of crypts, providing important signals for ISC maintenance and differentiation. Notch signaling, which is dependent on direct cell-cell contact, has a critical function controlling the fate of ISC. BMP signaling is found in both the epithelium and the stroma of the intestine, and plays various roles in mediating the balance between proliferative and differentiating states. Not shown is PI3K signaling, but BMP signaling may suppress WNT signaling through activation of PTEN, as AKT suppresses GSK3β. Hedgehog signaling plays a role in stem cell maintenance indirectly as it affects the surrounding mesenchymal niche signals needed for ISC homeostasis. EGF/FGF-mediated receptor tyrosine kinase signaling promotes crypt proliferation and epithelial wound healing. YAP signaling, not shown, is thought to interact with both Wnt and Notch pathways.

Figure 4 – The functional domains and posttranslational modifications of Krüpple-like factor 5

KLF5 consisting of three functional domains: the nuclear export signal (NES), the transactivation domain (TAD), and the DNA-binding domain (DBD) consisting of three conserved C2-H2 zinc finger. Multiple post translational modifications can be made to alter its function. Image adopted from Diakiw et al, 2013.
Figure 1
Figure 2

Absorptive Enterocyte

M Cell
Ptk6
Gata4/6
Hnf1α/β
Hnf4α

SpiB
RANKL

Progenitor Cell

Goblet Cell
Klf4
Foxa1
Hes5

Paneth Cell
Tcf4
Sox9

Enteroendocrine Cell
NeuroD/Beta2

Progenitor Cell
Spdef

Progenitor Cell

Progenitor Cell

Progenitor Cell

Progenitor Cell

Neurog3
Gfi1

Tuft Cell

Hes1
Atoh1

Stem Cell
Figure 3
Chapter 2

Krüpple-like Factor 5 is required for proper maintenance of adult intestinal crypt cellular proliferation.

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ABSTRACT

Background: Krüpple-like factor 5 (KLF5) is a transcription factor that is highly expressed in the proliferative compartment of the intestinal crypt. There, it is thought to regulate epithelial turnover and homeostasis.

Aim: In this study, we sought to determine the role for Klf5 in the maintenance of cellular proliferation, cytodifferentiation and morphology of the crypt-villus axis.

Methods: Tamoxifen-induced recombination directed by the epithelial-specific Villin promoter (in Villin-CreERT2 transgenic mice) was used to delete Klf5 (in Klf5loxP/loxP mice) from the adult mouse intestine, and analyzed by immunostaining and RT-qPCR. Control mice were tamoxifen-treated Klf5loxP/loxP mice lacking Villin-CreERT2.

Results: Three days after tamoxifen-induced recombination, the mitosis marker phospho-histone H3 was significantly reduced within the Klf5-mutant crypt epithelium, coincident with increased expression of the apoptosis marker cleaved-caspase 3 within the crypt where cell death rarely occurs normally. We also observed a reduction in Chromagranin A expressing enteroendocrine cells, though no significant change was seen in other secretory or absorptive cell types. To examine the long term repercussions of Klf5 loss, we sacrificed mice 5, 14, and 28 days post recombination and found reemerging expression of KLF5. Furthermore, we observed restoration of cellular proliferation, though not to levels seen wildtype intestinal crypts. Reduction of apoptosis to levels comparable to the wildtype intestinal crypt was also observed at later time points. Analysis of cell cycle machinery indicated no significant perturbation upon deletion of Klf5; however a reduction of stem cell markers Ascl2, Lgr5, and Olfm4 was observed at all time points following Klf5 deletion.
Conclusions: These results indicate that Klf5 is necessary to maintain adult intestinal crypt proliferation and proper cellular differentiation. Rapid replacement of Klf5-mutant crypts with wildtype cells and reduction of stem cell markers suggests further that Klf5 is required for self-renewal of intestinal stem cells.
INTRODUCTION

The intestinal epithelium is a rapidly renewing surface that possesses the ability to regulate nutrient absorption, balance digestive hormone production, and protect the host from noxious lumenal contents (Buddington and Diamond, 1989; Deplancke and Gaskins, 2001; Shimizu, 1999; Strader and Woods, 2005). The dynamic function of this organ relies on specific coordination between proliferation and differentiation to maintain proper homeostatic regeneration. In the adult intestine, the crypts of Lieberkühn are the niche for epithelial stem cells and contain all proliferative stem and progenitor cells. Differentiating cells exit the cell cycle and migrate out of the crypts and onto the surface epithelium of the intestine, where they perform their physiological roles (e.g., nutrient absorption by enterocytes; mucous secretion by goblet cells) and are eventually shed into the lumen (Cheng and Leblond, 1974; Noah et al., 2011). Homeostatic maintenance of these stem and progenitor cell populations is essential to support the rapid turnover of the intestinal epithelium.

Krüpple-like factors are a family of DNA-binding zinc finger transcription factors that regulate a wide variety cellular and context dependent processes such as proliferation, differentiation, and migration (Bieker, 2001a; Kaczynski et al., 2003; McConnell and Yang, 2010). Among these, Krüpple-like factor 5 (KLF5) is a pro-proliferative transcription factor found to be essential in embryogenesis and organogenesis (Dong and Chen, 2009; Ghaleb et al., 2005; Lin et al., 2010b; Parisi and Russo, 2011; Sun et al., 2001). KLF5 is highly expressed in the proliferative compartments of epithelial surfaces such as the skin and gastrointestinal tract (Goldstein et al., 2007a; Sur, 2002; Yang et al., 2007). Cultured fibroblast and intestinal epithelial cell assays were among the first to show that KLF5 is a positive regulator of cellular proliferation. Furthermore, Klf5 mRNA was found in actively proliferating cells, while transient
overexpression of this transcription factor significantly enhanced proliferation, cell growth, and anchorage independent growth (Bateman et al., 2004b; Chanchevalap et al., 2004; Nandan et al., 2004; Sun et al., 2001).

Deregulated cellular proliferation can influence life-threatening intestinal pathophysiology such as inflammation or cancer. Studies investigating the role of Klf5 in DSS-induced colitis determined it was requisite for the proliferative and migratory responses required for epithelial repair (McConnell et al., 2011a). Furthermore, it was found to increase the proliferative capacity of the colon after bacterial infection (McConnell et al., 2008a). Interestingly, the KLF5 gene is amplified in colorectal cancers and frequently overexpressed in KRAS mutated colorectal cancers, suggesting a contributory role in tumorigenesis (Cancer Genome Atlas, 2012; Nandan et al., 2008a). In vitro analysis showed that KLF5 mediates the hyperproliferative phenotype found in HRAS and KRAS transformed cells through induction of mitogen-activated protein kinase signaling and cell cycle related genes (Nandan et al., 2005; Nandan et al., 2004; Nandan et al., 2008a). In vivo studies further concluded that Klf5 mediated tumorigenesis in intestines harboring KRAS mutations, loss of the tumor suppressor adenomatous polyposis coli (APC), or both KRAS and APC mutation: heterozygosity for Klf5 attenuated both number and size of intestinal adenomas in both Apc\textsuperscript{min}/KRAS and Apc\textsuperscript{min} induced tumors (McConnell et al., 2009; Nandan et al., 2010). These data implicate KLF5 as a key mediator of intestinal health; however, its primary role in regulation of normal epithelial homeostasis remains unclear.

A recent study examined the role of Klf5 in the intestine by generating a mouse model in which conditional deletion of Klf5 was directed by the epithelial-specific Villin promoter. It was concluded that loss of Klf5 in the adult results in the loss of crypt architecture, loss of barrier
function, impaired differentiation, migration, and proliferation (McConnell et al., 2011b).

However, this study also reported a 66% mortality rate shortly after birth in mice lacking \(\text{Klf5}\) in the intestinal epithelium. Subsequently it was determined that \(\text{Klf5}\) is integral part of villus formation and cellular differentiation in the embryonic small intestine at the time of villus formation (Bell et al., 2013). Based on this result, it was unclear whether the reported adult phenotype of \(\text{Klf5}\) mutant mice was due to dysfunction of \(\text{Klf5}\) in the adult intestine or a result of maldeveloped tissue thus prompting the need for a novel inducible knockout of this gene.

Therefore, we set out to distinguish these possibilities by determining the effect of \(\text{Klf5}\) loss in the adult intestinal epithelium utilizing an inducible \(\text{Klf5}\) deletion mouse model. In this present study, we found that absence of \(\text{Klf5}\) acutely inhibited proliferation and concomitantly increased cell death in the crypts. Long term loss of \(\text{Klf5}\) was not sustainable, and strong selection for expansion of the remaining undeleted epithelium allowed repopulation of the crypt with \textit{wildtype} epithelium along with abatement of cell death. However, loss of stem cell markers, \(\text{Lgr5}\), \(\text{Ascl2}\), and \(\text{Olf4}\) as well as decreased proliferation remained through 28 days after recombination.
METHODS

Animals

Vil\textsuperscript{CreERT2} mice (el Marjou et al., 2004) were mated with \textit{Klf5}\textsuperscript{loxP/loxP} mice (Wan et al., 2008) to produce an F2 generation of \textit{Klf5}\textsuperscript{loxP/loxP}; Vil\textsuperscript{CreERT2} experimental mice and \textit{Klf5}\textsuperscript{loxP/loxP}; \textit{Vil}\textsuperscript{WT} controls. Eight week old mice were given an intraperitoneal injection of 80mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in sesame oil, for two consecutive days. Animals were sacrificed 3, 5, 14, and 28 days after first injection. DNA was extracted from tail clippings and utilized for PCR to determine genotype.

Tissue Staining

All intestinal specimens were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and cut into 5µm sections. For immunofluorescence, antigen retrieval was performed in 10mM sodium citrate solution (pH 6.0). Tissue was blocked in 4% normal donkey serum and then stained with antibodies raised against KLF5 (a gift from Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital, OH; 1:500), Ki67 (Leica Biosystems, New Castle, UK; 1:1000), Chromagranin A (CHGA) (Immuostar, Hudson, WI; 1:5000), Mucin2 (MUC2) (Santa Cruz, Dallas, TX; 1:1000), Lysozyme (LYZ) (Zymed Laboratories, San Francisco, CA; 1:5000), or phospho-Histone H3 (PHH3) (Cell Signaling, Boston, MA; 1:1000). The sections were then mounted with Vectashield containing DAPI stain. For immunohistochemistry, antigen retrieval was performed in 10mM Tris, 1mM EDTA, 0.05% Tween solution (pH 9.0). Tissues were blocked with 0.3% H\textsubscript{2}O\textsubscript{2} in Methanol, then Avidin/Biotin(Vector Laboratories, Burlingame, CA), and finally in 4% normal goat serum. Staining was performed for cleaved-caspase 3 (CC3) (Cell Signaling, Boston, MA; 1:100) using the Vector ABC kit (Vector Laboratories,
Burlingame, CA). Development was completed utilizing liquid DAB (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (Leica Biosystems, New Castle, UK). Intestinal tissue was evaluated from at least three animals per time point.

**RNA Isolation and qRT-PCR**

Jejunal segments of approximately 1 cm were excised from the small intestine and immediately placed in TRIzol reagent (Life Technologies, Carlsbad, CA). RNA was isolated as described in the manufacturer’s instructions and subsequently purified with the RNeasy kit (QIAGEN, Valencia, CA) with DNase treatment (QIAGEN, Valencia, CA). Five micrograms of RNA were used to synthesize cDNA using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. Taqman assays were carried out utilizing ABSolute Blue QPCR Low ROX Mix (Thermo Scientific, Waltham, MA) with commercially available probe and primer sets were used for Klf5 (Mm00456521_m1), Ccnd1 (Catalog # Mm00432359_m1), Ccnb1 (Catalog # Mm03053893_gH), and Cdk1 (Catalog # Mm00772472_m1) (Life Technologies, Carlsbad, CA) with 3 to 6 animals per group. Relative RNA expression was found by absolute quantification with standard curves created from pooled cDNA, normalized to Actb (Mm00607939_s1), and reported expression relative to the control group. SYBR green assays were carried out utilizing Brilliant III Ultra Fast SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA) with ChgA (F: 5’-CCCAGACTGCAGCATCCAGTT-3’, R: 5’-AGTCCGACTGACATCTCTTTC-3’), Muc2 (F: 5’-ATGCCACCTCCTCAAAGAC-3’, R: 5’-GTAAGTTTCGTTGGAACAGTGAA-3’), Fabp1 (F: 5’-ATGAACCTTCTCCGGCAAGTACC-3’, R: 5’-CTGACACCCCTTGTGTGC-3’), Gata4 (F: 5’-CCCTACCCCAGCCTACATGG-3’, R: 5’-ACATATCGAGATGGGGTGTCT-3’).
3’), Lgr5 (F – ACATTCCCAAGGGAGCGTTC, R – ATGTGGTGGCATCTAGGCG), Ascl2 (F – AAGCACACCTTGACTGTACG, R-AAGCACACCTTGACTGTACG), and Olfm4 (F – CAGCCACTTTCCAATTTCACTG, R – GCTGGACATACTCCTTCACCTTA) primers. Relative RNA expression was found by absolute quantification with standard curves created from pooled cDNA, normalized to *Gapdh* (F: 5’-AGGTCGGTGTGAACGGATTTG-3’, R: 5’-TGTAGACCATGTAGTTGAGGTCA-3’), and reported as expression relative to the control group.

**Cell Quantification**

Proliferation was calculated as an average of PHH3 positive cells per total crypt cell numbers for at least 20 crypts per region per mouse. Apoptosis was calculated as an average number of CC3 positive cells per crypt. The final numbers were averaged per mouse group (*wildtype* or *Klf5*-mutant at 3, 5, 14, or 28 days following tamoxifen treatment) to give the average proliferation or apoptosis rate for each segment of the intestine. These rates were then compared for each segment of the intestine, with significance assessed by Student’s t-test. Data is reported as mean ± SD.
RESULTS

KLF5 expression overlaps with proliferative cells in the small and large intestine

In the intestine, expression of KLF5 is predominantly localized in the proliferative crypt cells (Conckright et al., 1999) extending up to the lower 1/3 of the villi in the small intestine (Conckright et al., 1999; McConnell et al., 2011b; Nandan et al., 2010). This was confirmed by examining the co-expression of KLF5 with the proliferative marker Ki67. Along the epithelium, KLF5 expression is highest in the top two-thirds of the crypt with expression fading towards the bottom third. The expression of Ki67 is confined to the proliferative population of the crypts in both the large and small bowel. Immunoflourescent staining showed extensive overlap between Ki67 and KLF5 indicating that proliferative cells express this transcription factor (Figure 1A-C).

To determine the role of KLF5 in the adult intestine, we utilized conditional Klf5 knockout mice that contain loxP sites flanking exons 2 and 3, as previously described (Wan et al., 2008). Immunofluorescence was used to determine the efficiency of recombination in the adult mouse. Three days after the first intraperitoneal injection, histological staining showed efficient depletion of KLF5 throughout the proximal small intestine and a large decrease in KLF5 positive cells in the ileum and colon (Figure 1D-E). As Klf5 loss was previously reported to be detrimental to barrier function and overall intestinal maintenance (McConnell et al., 2011b), we wanted to examine the long-term effects of Klf5 absence. Interestingly, we found that Klf5 loss was not maintained for 28 days for any experimental mice (Figure 1B). Intermediate time points were examined to determine when KLF5 reemerged. KLF5 remained low 5 days after tamoxifen treatment, whereas extensive reemergence of KLF5 could be seen by 14 days after recombination (Figure 1A, 1G-H). The results were confirmed by RT-qPCR on isolated
jejunal segments which showed almost complete loss of Klf5 mRNA at 3 and 5 days after recombination and restoration of Klf5 expression at days 14 and 28 (Figure 1I).

Loss of KLF5 affects cellular differentiation.

It was previously reported that loss of Klf5 in the adult intestine has a significant effect on villus formation and inhibits cytodifferentiation (McConnell et al., 2011b; Shindo et al., 2002). To examine these effects, we utilized immunofluorescence analysis to visualize cellular differentiation within the intestine 3 and 5 days after tamoxifen treatment coincident with Klf5 depletion. Furthermore, it is at these time points that turnover of the villus epithelium is nearly complete. The overall crypt-villus architecture of the small and large intestines was preserved in Klf5-mutant mice (Figure 2). Immunofluorescence indicated a decrease in the number of enteroendocrine cells (CHGA-positive cells) within the small intestine of the 3 and 5 day animals (Figure 2A-C). RNA analysis indicated a significant reduction in ChgA was maintained for 14 days, but returned to baseline levels by 28 days (Figure 2M). Normal numbers and placement of goblet cells (MUC2-positive) (Figure D-F) and enterocytes (DPP4-positive cells) (Figure G-I) were seen within the small and large intestines of Klf5-mutant mice (Figure 2D-F). RNA expression analysis confirmed normal expression of goblet cell marker, Muc2, and enterocyte markers, Gata4 and Fabp1, 3 days post tamoxifen treatment (Figure M).

Paneth cell lifespans (approximately 60 days) are longer than cells migrating up the villi and thus deletion of Klf5 for 3-5 days is not expected to perturb the census of these long-lived cells. However, it was previously reported that Klf5 loss interfered with proper cell migration, specifically in Paneth cell placement (McConnell et al., 2011b). Therefore we examined their localization in our knockout mice. Paneth cells (LYZ-positive) remained localized along the
bottom of the small intestinal crypts of Klf5-mutant mice (Figure 2J-L). Together these data suggest that loss of Klf5 results in enteroendocrine cell loss within the small intestine, but does not significantly affect the other cell types during the time periods examined.

**Loss of Klf5 reduces proliferation.**

KLF5 is a well-known pro-proliferative transcription factor, and its loss is associated with decreased proliferation in other epithelial cell types (Ghaleb et al., 2005; Sun et al., 2001). As the expression of KLF5 overlaps with the proliferative transient amplifying population of cells, we hypothesized that loss of Klf5 would have an effect on proliferation. Tissue analyzed three days after recombination showed a significant decrease in proliferating crypt cells as measured by the mitotic marker, PHH3. After 3 days, the number of cells in mitosis dropped significantly in all regions of the intestine. This drop was nearly three-fold in the duodenum (9.13±0.97% to 3.22±0.79%), jejunum (8.86±0.83% to 3.28±0.63%), and colon (8.82±0.67% to 3.68±0.12%). While magnitude of effect was slightly less, probably due to a less robust loss of Klf5, the loss in proliferation was also significant in the ileum (7.05±0.35% to 3.22±0.34%) (Figure 3A-B, 3F). Reduced proliferation was similar in the intestines after loss of Klf5 for 5 days with proliferation rates also reduced nearly three-fold in (3.75±1.26% in the duodenum, 2.78±0.65% in the jejunum, 2.64±0.29% in the ileum, and 3.73±0.38% in the colon) (Figure 3C, 3F).

Because KLF5 expression returned between 14 and 28 days after tamoxifen-induced recombination, we hypothesized that proliferation would increase accordingly. While there were increased numbers of PHH3+ mitotic figures found after 14 days (4.92±0.45% in the duodenum, 4.04±0.26% in the jejunum, 3.62±0.94% in the ileum, and 4.72±0.86% in the colon) (Figure 3D,
3F), the rate of proliferation, as measured by this marker, was significantly improved only 28 days after Klf5 deletion. The number of PHH3 positive cells rose in all regions of the epithelium: to 5.34±0.25% in the duodenum, 5.82±0.66% in the jejunum, 5.26±0.28 in the ileum, and 5.31±0.28% in the colon (Figure 3E-F). Although a restoration in the level of proliferation was seen 28 days after deletion of KLF5, this level of proliferation remained less than wildtype (Figure 3F). Altogether, these data indicate that disruption of Klf5 has an immediate and perdurant effect on normal intestinal proliferation.

**Loss of KLF5 increases apoptosis in the crypt.**

In the uninjured intestine, cell death within the epithelium is rare and typically observed only at the villus tips. After cells migrate up the villi, they detach from the basement membrane and are shed into the luminal cavity, where they undergo detachment-induced apoptosis, called anoikis (Grossman, 2002; Rosen et al., 2002). Due to the decrease in proliferation and the lack of change in tissue morphology, we sought to determine the fate of cells within the crypt. Interestingly, the loss of Klf5 was correlated with an acute increase in apoptosis in the crypts as visualized by CC3. On average, the number of apoptotic cells was about 1.5 cells per crypt three days after ablation of Klf5, with the number of dying cells per crypt ranging from 1-5 (Figure 4A-B, 4F). Apoptosis was still apparent in the crypt 5 days after loss of Klf5, but did was not significantly increased as seen in 3 days (Figure 4A, 4C, and 4F). Intestinal tissues examined 14 and 28 days after deletion of Klf5 had negligible amounts of crypt cell death comparable to the wildtype controls (Figure 4A, 4D-F). This may be explained by the reemergence of the KLF5 and restoration of normal homeostatic function. Collectively, these data indicate that acute loss of KLF5 expression correlates with increased apoptosis.
**Loss of KLF5 does not globally affect cell cycle machinery in vivo, but does affect stem cell marker expression.**

As KLF5 has been shown to play a role in the regulation of cell cycle machinery such as CyclinD1 (Ccnd1), CyclinB1 (Ccnb1), and Cdc2 (Cdk1) (Dong and Chen, 2009; Liu et al., 2010b; Nandan et al., 2005; Nandan et al., 2004), we examined the RNA expression levels of these proteins from jejunal segments where deletion of Klf5 was most robust (Figure 1). In contrast to the previous reports, no significant change was found in the expression of any of these genes (Figure 5A).

KLF5 has also been shown to affect canonical Wnt signaling, a positive regulator of intestinal cellular proliferation, as reduction of Klf5 reduced nuclear localization of β-catenin in the context of Apc<sup>min</sup> mice (McConnell et al., 2009). Therefore we examined RNA expression of well-known downstream targets of Wnt signaling in the intestine, Ccnd1, Axin2, Lgr5 and Ascl2. No significant decrease was seen in Ccnd1 or Axin2 levels; however loss of stem cell markers Lgr5 and Ascl2 were found to be significantly decreased (Figure 5B-C). Loss of Lgr5 expression dropped to 55.2±15.0% of wildtype levels after 3 days, and continued to drop to 26.8±4.6% of wildtype levels 14 days after Klf5 loss. Twenty-eight days after recombination, Lgr5 expression remained below half of wildtype expression at 41.9±13.8%. Ascl2 exhibited a more dramatic effect after recombination as expression dropped to 35.8±2.7% in 3 days, and continued to lower to 8.0±1.9% in 14 days. After 28 days, mRNA levels were still low at 16.0±5.1% of wildtype. This prompted us to examine another well-known stem cell marker Olfm4, which is thought to depend on Notch activity rather that beta-catenin transactivation (VanDussen et al., 2011) (Figure 5C). Expression of Olfm4 plummeted to 17.5±4.4% of wildtype levels after 3 days, and
continued to fall slightly to 15.8±4.8% after 14 days. As seen with the other two stem cell markers, there was some restoration of expression after 28 days (25%±5%), coincident with restored KLF5 protein expression, but this still remained significantly less than *wildtype* expression. Overall these data indicate that loss of *Klf5* does not globally disrupt cell cycle machinery nor canonical beta-catenin transactivation, but rather points to a role for this transcription factor in the maintenance of stem cells.
DISCUSSION

The goal of this study was to determine the effects of $Klf5$ loss in the adult intestine. Previously loss of $Klf5$ was reported to have adverse effects on barrier function, cytodifferentiation, and migration. However, that prior study used the Villin-Cre driver to delete $Klf5$ in the intestinal epithelium, resulting in perinatal death in two-thirds of the mutant offspring (McConnell et al., 2011b). It was later discovered that this transcription factor plays an important role in intestinal morphogenesis wherein deletion of $Klf5$ from the embryonic intestine disrupted villus emergence and cellular differentiation (Bell et al., 2013). As these phenotypes emerged at the onset of villus outgrowth, we hypothesized that the effects of acute loss of $Klf5$ in the adult would be distinct from those phenotypes reported in the previous study using the continuously active Villin-Cre transgenic line, which we suggest may have developmental origins. To test this hypothesis, we used the tamoxifen-inducible Villin-CreERT2 driver to delete $Klf5$ in the adult intestine as a means to test its function in this context.

We found that loss of $Klf5$ significantly decreased the number of proliferating cells per crypt along the entire region of the small and large intestines. Simultaneously, we observed an acute increase the number of apoptotic cells in $Klf5$-deficient crypts, and a decreased number of enteroendocrine cells in the small intestine. The diminished number of these cell types may indicate a role for $Klf5$ specific to enteroendocrine cell differentiation. Additionally, we saw no change in intestinal morphology, suggesting that production of new epithelial cells continues in $Klf5$-mutant intestines. In contrast to the minimal changes in intestinal cellular differentiation, we observed decreased proliferation coincident with increased apoptosis in $Klf5$ mutant intestines. We suggest that the lack of cell cycle progression potentiated the onset of cell death early after loss of $Klf5$. Caspase-3 is known to induce apoptosis after G1 phase cell cycle arrest.
As KLF5 is known to regulate expression of CyclinD1 (Dong and Chen, 2009; Liu et al., 2010b), which is required for G1/S phase transition, it is possible that loss of this transcription factor could result in a G1 phase arrest and activation of caspase activity and thus cellular apoptosis. However we did not see a significant change in CyclinD1 mRNA expression. We note that RNA was isolated from whole jejunum, and therefore, these results measure the entire tissue including mesenchyme, and may have reduced the sensitivity of our assay to detect subtle changes in CyclinD1 expression. As an alternative mechanism underlying the observed increased cell death, Klf5 deletion may have caused a loss of a prosurvival gene such as survivin, which has previously been linked to KLF5 in leukemia (Zhu et al., 2006b), and thus may play a similar role in the intestine. Spontaneous crypt cell apoptosis is generally a sign of stress or toxicity in the epithelium (Watson and Pritchard, 2000) therefore we interpret our results to suggest that lack of KLF5 triggered a stress response within the intestinal epithelium.

Interesting, Klf5-deficient cells were rapidly replaced with wildtype cells, such that crypts had restored KLF5 expression within 14 to 28 days of induced deletion. While this is probably the result of the lack of 100% efficient knock out of Klf5, we suggest that, given the increased rate of apoptosis, there could be selection against Klf5-mutant cells that is required to reestablish the proliferative capacity of the crypt to maintain proper epithelial turnover. Our results are consistent with the previous studies of Klf5-deficient intestinal epithelium, in which surviving mice showed re-expression of KLF5 in tissues examined 8 weeks after birth (McConnell et al., 2011b) and, very recently, in inducible loss of Klf5 in the colon (Nandan et al., 2014). Regardless of its origin, the reemergence of this transcription factor and incomplete loss of proliferation could explain the lack of disruption in cellular differentiation and intestinal morphology in Klf5-mutant mice.
The main conclusion of this study is that KLF5’s primary function in the intestine is to maintain epithelial proliferation. Due to extensive turnover of the epithelium, three to five days is sufficient to see potential differentiation and morphological defects caused by acute KLF5 loss; however, loss of proliferation is the prominent phenotype observed in Klf5-mutant mice. Increased apoptosis is likely coupled to this effect early after recombination, but the number of PHH3 positive cells remains significantly lower than wildtype 28 days post tamoxifen treatment suggesting a lasting effect from the mutation.

KLF5 has been linked to both the Wnt and MAPK pathways (McConnell et al., 2009; Nandan et al., 2004; Nandan et al., 2008a; Yang et al., 2007), and has been reported to regulate cell cycle components such as Cyclin D1, Cyclin B1, and Cdc2 (Liu et al., 2010b; Nandan et al., 2005). However, we did not see a significant decrease in the expression of any of these genes. As previously mentioned, analysis of whole jejunal tissue may have diminished the sensitivity of our assay to detect cell-specific changes in gene expression, for example specifically in transient amplifying or stem cells. It is interesting that in the developing intestine, Klf5 deletion causes a profound loss of CyclinD1 expression, however loss of these genes had no effect on proliferation of the embryonic intestine (Bell et al., 2013). Therefore the reduction in proliferation seen here may suggest that KLF5 regulates cell cycle in the adult intestine via mechanisms distinct from identified in tumors and cell lines.

Reemergence of Klf5 coupled with the lasting effects of reduced proliferation indicates that the initial loss of Klf5 may have an effect on another important element of homeostasis. While it is thought that expression is rare or weak in adult stem cells (Dong and Chen, 2009), loss of Klf5 in Lgr5 positive stem cells has resulted in suppression of oncogenic transformation (Nakaya et al., 2014) indicating a prominent role for this transcription factor in this cell type.
Indeed we saw a significant reduction in active intestinal stem cell markers, \textit{Lgr5}, \textit{Ascl2}, and \textit{Olfm4} which can explain a decreased proliferative response in the intestine in the absence of an overt change in cell cycle machinery or canonical Wnt signaling. In contract to what we expected, there seems to be no lack of crypt cell numbers in these intestines which is possibly due to an incomplete loss of stem cells entirely. In any instance, it is clear that given its potential roles in the initiation of tumorigenesis and in stress induced epithelial repair, understanding the molecular mechanisms by which KLF5 controls homeostatic cellular proliferation and stem cell maintenance could provide valuable answers for future therapies of intestinal diseases.


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FIGURE LEGENDS

Figure 1 – KLF5 is expressed in proliferative crypts of the small and large intestine.

(A) Immunofluorescence staining for coexpression of KLF5 (red) and Ki67 (green). DAPI (blue) was utilized as a nuclear stain. Immunofluorescence staining of the crypt with DAPI (B) and without DAPI (C) shows regions of KLF5 and Ki67 expression overlap. Colocalization between these two proteins is seen in yellow. (D-H) Regional histological analysis of tamoxifen-induced recombination in Klf5<sup>loxP/loxP</sup>;Vil<sup>WT</sup> control mice (D) and Klf5<sup>loxP/loxP</sup>;VilCreERT2 experimental mice 3 (E), 5 (F), 14 (G), and 28 days (H). (I) Comparison of Klf5 mRNA expression analysis by RT-qPCR from jejunal segments of the small intestine. Student’s t test was performed to determine P values; expression was normalized to Actb (Mean ± SD, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; n = 6)

Figure 2 – Loss of KLF5 does not affect intestinal morphology or cytodifferentiation.

Histological comparison of Klf5<sup>loxP/loxP</sup>;Vil<sup>WT</sup> (WT) and Klf5<sup>loxP/loxP</sup>;VilCreERT2 (Klf5-mutant) intestinal tissues for cellular differentiation utilizing immunofluorescent staining for CHGA (green; enteroendocrine cells) (A-C), MUC2 (green; goblet cells) (D-F), LYZ (green; Paneth cells) (G-I), and DPP4 (green; enterocytes) (J-L). Nuclear stain is DAPI (blue). (M) Comparison of Chga, Muc2, Gata4, and Fabp1 mRNA expression analysis by RT-qPCR from jejunal segments of the small intestine. Student’s t test was performed to determine P values; expression was normalized to Gapdh (Mean ± SD, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; n = 6)
Figure 3 – Loss of KLF5 attenuates cellular proliferation in the adult intestine.

(A-E) Histological analysis for the comparison of cellular proliferation utilizing immunofluorescent staining for mitotic marker, PHH3 (green) in *wildtype* and *Klf5* loss-of-function mice (3 and 28 days post tamoxifen-induced recombination). Nuclear stain is DAPI (blue). (F) Quantification of average percentage of PHH3 positive cells per crypt. Significance determined between groups of two (*wildtype* vs. loss of *Klf5* for 3 days, *wildtype* vs. loss of *Klf5* for 28 days, and loss of *Klf5* for 3 vs. 28 days). (Mean ± SD, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n = 3)

Figure 4 – Intestinal crypt cell death is increased early after loss of KLF5, but disappears over time.

(A-E) Immunohistochemical analysis of apoptosis is shown using CC3 (brown) counterstained with hematoxylin (blue) in *wildtype* and *Klf5* loss-of-function mice (3, 5, 14, and 28 days post tamoxifen-induced recombination). (F) Quantification of the number of CC3 positive cells per crypt. Significance determined between groups of two (*wildtype* vs. loss of *Klf5* for 3 days, *wildtype* vs. loss of *Klf5* for 28 days, and loss of *Klf5* for 3 vs. 28 days). (Mean ± SD, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n = 3)

Figure 5 - Loss of Klf5 results in loss of active stem cell marker expression in the small intestine.

Results of mRNA of expression for (A) cell cycle proteins (*Ccnd1, Ccnb1*, and *Cdk1*), (B) downstream targets of canonical Wnt signaling (*Lgr5, Ascl2, Axin2*, and *Ccnd1*), and (C) active intestinal stem cell markers (*Lgr5, Ascl2, Axin2, CyclinD1*) by RT-qPCR from jejunal segments of the small intestine. Student’s t test was performed to determine P values; expression was
normalized to *Gapdh* (SYBR) or *Actb* (Taqman) (Mean ± SEM: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; n = 6). Note that some RT-qPCR results for relative RNA expression appear on two graphs for comparison purposes.
Figure 1 - KLF5 is expressed in proliferative crypts of the small and large intestine.
Figure 1 – Continued

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I) Klf5

- Wildtype
- 3-day KO
- 5-day KO
- 14-day KO
- 28-day KO

Relative RNA Expression

- `*` denotes significance at p < 0.05
- `**` denotes significance at p < 0.01
Figure 2 - Loss of KLF5 does not affect intestinal morphology or cytodifferentiation.

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Figure 2 – Continued
Figure 3 - Loss of KLF5 attenuates cellular proliferation in the adult intestine.
Figure 4 - Intestinal crypt cell death is increased early after loss of KLF5, but disappears over time.

A) Wildtype

B) Klf5+/− - 3 day

C) Klf5−/− - 5 day

D) Klf5−/− - 14 day

E) Klf5−/− - 28 day

G) Average Number of CC3 Positive Cells Per Crypt

- Wildtype
- 3 day KO
- 5 day KO
- 14 day KO
- 28 day KO

*** p < 0.001
Figure 5 - Loss of Klf5 results in loss of active stem cell marker expression in the small intestine.
Krüpple-Like Factor 5 regulates proliferation, cytodifferentiation, apoptosis, and MEK/ERK signaling in the intestinal epithelial progenitors.

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A portion of this work will be submitted for publication
ABSTRACT

Krupple-like factor 5 (KLF5) is a transcription factor required for proliferation and stem cell homeostasis in the intestine. KLF5 has been shown to mediate signaling pathways involved in colorectal cancer and other intestinal diseases; however, is still unclear how it regulates normal homeostasis and basal proliferative activity. To better understand how this transcription factor functions to regulate progenitor proliferation and stem cell maintenance, we analyzed the effects of transgenic over-expression of KLF5 on the intestinal epithelium. Doxycycline-inducible expression of KLF5 throughout the intestinal epithelium of transgenic mice (Villin-Cre; Rosa26-rtTA-IRES-EGFP; TRE-KLF5) was fatal by day 4. Histological analysis illustrated aberrant morphological changes such as villus blunting and hyperplastic crypts, accompanied by loss of cytodifferentiation and increased apoptosis. These changes were not associated with loss or increase of stem cell activity, suggesting that the observed phenotype was due to abnormal differentiation of transient amplifying progenitor cells at the crypt-villus axis. Furthermore, we found that increased KLF5 expression correlated with increased activation of MAPK effectors phospho-MEK and phospho-ERK1/2. We next examined the requirement for KLF5 in activation of the MAPK pathway utilizing conditional knock out animals (Villin-CreERT2; Klf5floxflox). After tamoxifen-induced recombination, histological and immunoblot analysis of phospho-ERK1/2 and phospho-MEK indicated that KLF5 was required for activation of MAPK signaling. To test the requirement of MAPK to mediate the functions of KLF5, we performed rescue experiments by simultaneously deleting Klf5 and expressing activated KRAS (Villin-CreERT2; Klf5floxflox; KrasG12D). KRASG12D expression was able to rescue proliferation that was decreased in the absence of KLF5. In vitro enteroid assays showed that KRASG12D could rescue proliferation and crypt formation associated with KLF5 depletion, however, KRASG12D could not rescue overall survival of enteroids. Furthermore, active stem cell markers Ascl2, Lgr5, and
$Olfm4$, were significantly decreased after loss of KLF5 and were not restored by KRAS$^{G12D}$ expression. These results suggest that KLF5 regulates proliferation via MAPK signaling but controls stem cell self-renewal via a separate pathway. To identify potential mechanism underlying these phenotypes, RNA-sequencing was performed on isolated crypts from both conditional knock out and *wildtype* animals. Of those differentially expressed genes, we identified a subset involved in the negative regulation of MAPK signaling indicated a potential mechanism by which KLF5 regulates progenitor maintenance and proliferation.
INTRODUCTION

The mammalian intestine is a rapidly proliferating organ with epithelial renewal occurring every 4-6 days. This speedy turnover begins with active intestinal stem cells (ISCs) that reside at the base of crypts that cover the surface of the small and large intestine. As ISCs divide, proliferative progenitor daughter cells migrate upwards along the crypt walls to make up the transient amplifying zone. Upon reaching the luminal epithelial surface, these transient amplifying (TA) cells exit the cell cycle and differentiate into the functional absorptive enterocytes and secretory cells that aid in nutrient digestion, hormone regulation, and waste expulsion.

Krüpple-like factor 5 (KLF5) belongs to a family of transcription factors containing three C-terminal zinc fingers that bind to GC-rich sites of DNA (Sogawa et al., 1993). It plays a many roles in the regulation of cellular processes such as proliferation, migration, differentiation, and apoptosis in epithelial, adipose, and vascular tissues (Chen et al., 2006; Oishi et al., 2005; Sun et al., 2001; Yang et al., 2008; Zhao et al., 2007). Throughout the intestinal tract, KLF5 expression is confined to proliferative crypt cells. Previously we showed that conditional loss of this transcription factor in adult mice resulted in acute crypt cell death followed by significantly reduced proliferation. This effect was transient as wildtype tissue outcompeted cells without KLF5; however, a persistent decrease in proliferation was seen in long-term experiments even after restoration of KLF5 expression. Upon further investigation, active ISC marker transcripts also remained significantly reduced suggesting that KLF5 is required for stem cell homeostasis (Bell and Shroyer, 2014). In agreement with this, loss of KLF5 in LGR5-positive stem cells diminished their proliferation and survival (Nakaya et al., 2014; Nandan et al., 2015). Other studies in the intestine have found KLF5 to be required for dextran sodium sulfate- and
radiation-induced injury remodeling as well as bacterial induced hyperplasia after infection (Li et al., 2015; McConnell et al., 2008; McConnell et al., 2011a). These data demonstrate an indispensable role for KLF5 in homeostatic maintenance and repair in the intestine.

KLF5 has frequently been associated with various epithelial cancers, and was found to be amplified in a subset of colorectal cancers (TCGA, 2013). Several in vitro assays have shown that transient knockdown of Klf5 abrogates proliferation and anchorage-dependent growth in HRAS and KRAS transformed cell lines due to regulation of cell cycle related genes involved in G1/S and G2/M transition (Bateman et al., 2004; Nandan et al., 2005; Nandan et al., 2004; Nandan et al., 2008). Complementary in vivo studies have further revealed that KLF5 mediates signaling pathways involved in adenoma formation and growth in several mouse models containing alterations in canonical Wnt signaling and KRAS mutations which are commonly affected in colorectal cancers. Specifically, Klf5 hemizygosity attenuated the amount and size of tumors in mice with loss of the tumor suppressor, adenomatous polyposis coli (APC) alone and also when combined with constitutively activated KRAS (McConnell et al., 2009; Nandan et al., 2010). Furthermore, loss of Klf5 in Lgr5-positive stem cells prevented beta-catenin gain-of-function-induced adenoma formation in mice (Nakaya et al., 2014). These data indicate that augmented expression of KLF5 may play an important role in intestinal tumorigenesis.

Transgenic studies of KLF5 overexpression in vivo have been a useful way to analyze its function in epithelial biology. For example, increasing KLF5 in the basal layer of the epidermis was used to discern its role in development and homeostasis, where overexpression of KLF5 disrupted epidermal barrier function by embryonic day 17.5. In adult mice, overexpression for six weeks resulted in a hyperproliferative epidermis leading to hyperkeratosis and hair loss. Erosions on the skin were associated with loss of regenerative potential due to a depleted CD34-
positive population of stem cells (Sur, 2006). In addition to proliferative regulation, this animal model uncovered a role for KLF5 in the epidermal differentiation program. In the esophageal epithelia, increased KLF5 induced hyperproliferation in the basal cell layer, but was not sufficient for induction of esophageal dysplasia or cancer after one year. Further, novel targets of KLF5 were identified as mediators of its function (Goldstein et al., 2007). Due to the importance of KLF5 in normal intestinal homeostasis and its oncogenic potential, we took advantage of this model to reexamine the nature of this transcription factor in to discern its effects on proliferation, cytodifferentiation, and stem cell dynamics. Previous reports of transgenic Klf5 expression in the intestine showed no morphological or proliferative defects in the intestine after one year, but did confer resistance to DSS-induced colitis in adult mice (Tetreault et al., 2012). However, expression was induced at the time of villus morphogenesis, therefore compensatory mechanisms may have emerged to prevent malfunctions in development of the gastrointestinal tract. Here we found surprising differences from the previous study (Tetreault et al., 2012) including hyperproliferation, morphological abnormalities, and loss of cytodifferentiation. KLF5 is sufficient to expand the TA population and prevents villus cytodifferentiation, but interestingly, has no acute effects on stem cells. Further, we implicate RAS/ERK signaling in mediating the proliferative effects of KLF5 in the intestine.
METHODS

Animals

To induce KLF5 expression specifically in the intestine, mice containing constitutively active Cre recombinase driven by the Villin promoter (Madison et al., 2002) were crossed to mice containing Rosa26-rtTA-IRES-EGFP (Belteki, 2005). Offspring were bread to mice containing the TRE-Klf5 allele (Sur, 2006) to produce TRE-KLF5; Vil-Cre; R26rtTA/rtTA experimental mice and Vil-Cre; R26rtTA/rtTA littermate controls. Animals 6-8 weeks old were fed doxycycline chow for up to 4 days and then sacrificed. Conditional deletion of the Klf5 allele in the intestine was achieved by crossing $\text{Klf5}^{\text{loxP/+}}$ (Wan et al., 2008) mice with tamoxifen-inducible Vil-CreER$^{T2}$ (el Marjou et al., 2004) mice to create $\text{Klf5}^{\text{loxP/loxP}}; \text{Vil-CreER}^{T2}$ experimental mice and $\text{Klf5}^{+/+}; \text{Vil-CreER}^{T2}$ controls. To constitutively activate MAPK signaling, these animals were then crossed to mice harboring the Cre-activated $\text{KRAS}^{G12D}$ allele (Johnson et al., 2001). Intraperitoneal injections of 80mg/kg of tamoxifen (Sigma-Aldrich, St. Louis, MO) were given for five consecutive days prior to sacrifice on the following day.

Mouse Enteroid Assay & Whole-Mount Staining

Viable crypts were isolated from freshly-harvested jejunal tissue and grown as previously described (Mahe et al., 2013). Enteroids were kept at 37°C, and media supplemented with growth factors was changed every 4 days. Whole mount staining was also performed exactly as outlined in the referenced protocol. The following primary antibodies were used to visualize protein expression: KLF5 (a gift from Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital, OH; 1:500), Ki67 (Catalog # NCL-Ki67p, Leica Biosystems, New Castle, UK; 1:1000), and DAPI
All secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were utilized at a 1:200 dilution. Images were obtained with confocal microscopy.

**Histological Staining & In situ Hybridization**

Intestinal segments were fixed overnight in 4% paraformaldehyde in PBS overnight at 4°C, paraffin embedded, and cut into 7µm sections. After rehydration of tissue, antigen retrieval was performed in 10mM sodium citrate (pH 6.0) in a Decloaking Chamber (Biocare Medical) at 125°C for 30 seconds. For immunofluorescent staining, tissues were blocked in 4% normal donkey serum in PBS for 1 hour at room temperature. Primary antibodies raised against KLF5 (a gift from Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital, OH; 1:500), Ki67 (Catalog # NCL-Ki67p, Leica Biosystems, New Castle, UK; 1:1000), phospho-histone H3 (PHH3) (Catalog # 3377P, Cell Signaling, Boston, MA; 1:1000), chromagranin A (CHGA) (Catalog # 20085, Immuostar, Hudson, WI; 1:5000), mucin 2 (MUC2) (Catalog # sc-15334, Santa Cruz, Dallas, TX; 1:1000), lysozyme (LYZ) (Catalog # 18-0039, Zymed Laboratories, San Francisco, CA; 1:5000), and dipeptidyl-peptidase 4 (DDP4) (Catalog # AF954, R&D Systems, Minneapolis, MN; 1:1000) were diluted in blocking buffer and incubated on sections overnight at 4°C. Secondary antibodies were supplied by Jackson ImmunoResearch (West Grove, PA) and used at a 1:200 dilution. For immunohistological staining of cleaved-caspase 3 (CC3) (Catalog # 9661S, Cell Signaling, Boston, MA; 1:50), bromodioxyuridine (BrdU) (Catalog # G3G4, DSHB, Iowa City, IA;1:50), and phospho-ERK (pERK) (Catalog # 4376S, Cell Signaling, Boston, MA; 1:100), antigen retrieval was performed 10mM sodium citrate (pH 6.0). Tissues were then blocked with Avidin for 15 minutes, washed in PBS for 5 minutes, and then blocked with Biotin for 15 minutes in dilutions indicated by the manufacturer (Vector Laboratories, Burlingame,
Subsequently, tissues were submerged in a 3% peroxide solution (diluted in methanol) to block for endogenous peroxidase. For CC3 and pERK detection, tissues were stained with the anti-rabbit Vector ABC kit (Vector Laboratories, Burlingame, CA). Prior to the blocking steps in the BrdU staining protocol, tissues were pretreated with 0.2N HCl for 20 minutes and 0.1N sodium borate for 2 minutes. After blocking, BrdU staining was completed with the M.O.M. peroxidase kit (Vector Laboratories, Burlingame, CA). Liquid DAB (Vector Laboratories, Burlingame, CA) was used for the development of the stains. In situ hybridization was performed as outlined (Roche, 2006) using Ascl2 (IMG: 1078130) mouse cDNA cloned into the pT7T3D-Pael vector. Antisense probes were generated by restriction of the plasmid with EcoR1 and transcription with T3 polymerase incorporating the digoxigenin label.

**Immuno Blot Analysis**

Whole jejunal segments were flushed with PBS and snap frozen in liquid nitrogen following excision from the mouse. Tissues were homogenized in RIPA (Final concentrations of the following: 150mM NaCl, 1% NP40, 0.1% SDS, 50mM Tris-HCl (pH 7.5), 25mM NaF, 25mM Na₃PO₄, 0.5mM PMSF, 1:500 dilution commercial protease inhibitor (Catalog #P8340, Sigma), 1:500 dilutions of phosphatase inhibitor cocktails (Catalog #P0044 & #P5726, Sigma) lysis buffer and protein concentration was determined utilizing the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, MA). Equal concentrations of protein were loaded into 12% SDS-Page gels and run at 100V for 2 hours. Protein transfer onto PVDF membranes occurred at 100V for 2.5 hours at 4°C. The membranes were subsequently washed with TBST and blocked in 5% BSA at room temperature for 1 hour. Antibodies for KLF5 (a gift from Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital, OH; 1:1000), CHGA (Catalog # 20085, ImmunoStar, Hudson, WI;
1:1000), trefoil factor 3 (TFF3) (Catalog # sc-28927, Santa Cruz, Dallas, TX; 1:1000), DPP4 (Catalog # AF954, R&D Systems, Minneapolis, MN; 1:1000), CC3 (Catalog # 9661S, Cell Signaling, Boston, MA; 1:100), pERK (Catalog # 4376S, Cell Signaling, Boston, MA; 1:1000), phospho-MEK (pMEK) (Cell Signaling, Boston, MA; 1:1000), total ERK1/2 (Catalog # 9102S, Cell Signaling, Boston, MA; 1:1000), total MEK (, Cell Signaling, Boston, MA; 1:1000), and Actin (DSHB; 1000) were all diluted in 5% BSA and incubated with the membranes overnight at 4°C. Incubation with secondary antibodies (guinea pig HRP & goat HRP & rabbit HRP) diluted 1:1000 in 5% nonfat milk occurred for 1 hour after washing the next day. Luminol detection with Pierce™ ECL Western Blotting Substrate (Thermo Fisher, Waltham, MA) was used to visualize protein bands.

**RNA Isolation & RT-qPCR**

Fresh samples of whole jejunal tissue, jejunal crypts, or enteroids were placed immediately in TRIzol reagent (Life Technologies, Carlsbad, CA) followed by phenol-chloroform extraction. RNA clean-up was performed per manufacturer’s instructions utilizing an RNeasy kit (QIAGEN, Valencia, CA) with DNase treatment (QIAGEN, Valencia, CA) omitting the step for cell lysis in RLT. cDNA was generated from whole jejunal tissue and enteroids utilizing iScript™ Reverse Transcription Supermix (BioRad, Hercules, CA) while subsequent quantitative PCR reaction prepared with Brilliant III Ultra Fast SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA). Quantity of mRNA was calculated from standard curves generated from pooled cDNA normalized to *Gapdh* expression (F: 5’-AGGTCGGTGTGAACGGATTTG-3’, R: 5’-TGTAGACCATGTAGGTGAGGTCA-3’). Primers sets included those for *Lgr5* (F – ACATTCCCAAGGGAGCGTTC, R – ATGTGGTGGCATCTAGGCG), *Ascl2* (F –
AAGCACACCTTGACTGGTACG, R-AAGCACACCTTGACTGGTACG), and Olfm4 (F – CAGCCACTTTCCAATTTCACTG, R – GCTGGACATACTCCCTTCACCTTA). Expression was reported relative to the control group.

RNA-sequencing

A double-stranded DNA library was created using 250ng of total RNA, quantitated by picogreen, preparing the fragments for hybridization onto a flowcell. cDNA was created using the fragmented 3’ poly (A) selected portion of total RNA and random primers. Libraries were created from the cDNA first blunt ending the fragments, attaching an adenosine to the 3’ end, and ligating unique adapters to the ends. The ligated products were then amplified using 15 cycles of PCR. The resulting libraries were quantitated using NanoDrop and fragment size assessed with the Agilent 2100 Bioanalyzer. A qPCR assay was performed determine the concentration of adapter ligated fragments using the Applied Biosystems ViiA 7 instrument and a KAPA Library Quant Kit. All samples were pooled equimolar and re-quantitated by qPCR and reassessed on the Bioanalyzer. Pooled concentration from the qPCR assay, the library was loaded onto a rapid run flow cell at a concentration of 12pM for on-board cluster generation and sequencing on the HiSeq 2500, at a read length of 100bp, paired-end. TopHat was used to align the reads to the mouse genome. Samples were mapped as follows: *wildtype* sample 1 contained 45589099 paired-end reads (95.2% mapped), *wildtype* sample 2 contained 50147474 paired-end reads (95.0% mapped), *wildtype* sample 3 contained 45676445 paired-end reads (94.7% mapped), knockout sample 1 contained 52022786 paired-end reads (93.6% mapped), knockout sample 4 contained 50327272 paired-end reads (94.5% mapped), and knockout sample 3 contained 4176659 paired-end reads (94.9% mapped). The relative abundance of transcripts was
found using Cufflinks to determine FPKM. Differential expression was then found using Cuffdiff. From this output, significant differentially expressed genes (q≤0.05) having at least 2-fold change were used to generate a heatmap. PANTHER was then used to broadly categorize these into specific gene ontologies.

**Enteroid Survival Rate, Cell Quantification, and Statistics**

For each animal, the average number of BrdU positive, pHH3 positive, or total number of cells for 20 crypts was calculated for each region. The Student’s t-test was used to determine the significance between groups. Data is reported as mean ± SEM. To determine the mean survival of enteroids 3 animals were used per group. Crypts were plated in three wells each (a total of 27 wells) and counted on day one, Counting was repeated for 6 consecutive days before being fixed for whole-mount staining. The percentage of surviving enteroids for each day was compared to day 1. For each group, a mean percentage was calculated per well. A two-way ANOVA with Bonferonni correction was used to determine the significance of mean percentage survival over time between groups.
RESULTS

*KLF5 overexpression results in aberrant intestinal morphology and decreased villus cytodifferentiation.*

Previously we found that KLF5 was required for progenitor proliferation and maintenance of active stem cells (Bell and Shroyer, 2014). To continue investigation of KLF5 function in the intestine, we asked if it was sufficient to drive intestinal proliferation and stem cell renewal. Further, considering its role as an oncogenic mediator, we wanted to determine if it could drive neoplastic changes in the epithelium. To address these questions we generated transgenic mice allowing tetracycline/doxycycline-inducible intestinal epithelial expression of KLF5 (*TRE*-KLF5; *Vil*-Cre;R26<sup>rtTA/rtTA</sup>) and littermate *Vil*-Cre;R26<sup>rtTA/rtTA</sup> controls. Doxycycline inducible expression of KLF5 was fatal after 3-4 days. This effect was not due to intestinal neoplasms or destructive inflammatory response, but instead appeared to be due to diarrhea/dehydration. We therefore selected the 2.5 day time point to examine the effects of increased KLF5. At this time, 100 percent of the transgenic mice displayed a fluid-filled bowel (Figure 1A). Histological analysis confirmed ectopic expression of KLF5 throughout the entire gastrointestinal tract (Figure 1B). Villi in these mice were blunted throughout the small intestine while the Ki67-positive proliferative crypt compartments, which normally harbor most KLF5 expressing cells, were expanded throughout all regions of the transgenic tissue (Figure 1C & D, Figure 2C). Thus, pan-epithelial KLF5 overexpression was lethal, potentially due to malabsorptive diarrhea.

Given the profound morphological abnormalities, we then examined the effect of increased KLF5 on cytodifferentiation utilizing immunofluorescence and immunoblot analysis for common intestinal secretory and absorptive cell markers. We found decreased expression of
enteroendocrine marker, chromogranin A (CHGA) (Figure 2A & D), Goblet cell markers, mucin2 (MUC2) and trefoil-factor 3 (TFF3) (Figure 2B & D), and enterocyte marker, dipeptidyl peptidase-4 (DPP4) (Figure 2C & D). It has been previously reported that KLF5 can incite reprogramming events that lead to cellular dedifferentiation and hyperproliferation in cancerous tissue (Liu et al., 2011). To address this as a possible effect in our transgenic intestines we examined the Paneth cell population. Located at the bottom of the crypt intercalated between the actively cycling stem cells, Paneth cell turnover occurs approximately every thirty days (Clevers and Bevins, 2013). Lysozyme staining indicated that these cells remained intact at the bottom of the crypt (Figure 2E). These data indicate that increased KLF5 expression expands the proliferative zone and prevents differentiation rather than reverses it.

**Overexpression of KLF5 increases the number of proliferative progenitor and apoptotic cells.**

KLF5 is a proproliferative transcription factor that has been shown to directly regulate various proteins that mediate G1/S and G2/M cell cycle progression. *In vitro* analyses confirm that KLF5 enhances the promoter activity of cyclin D1 (CCND1), cyclin B1 (CCNB2), and CDC42 (Nandan et al., 2005; Nandan et al., 2004). Combined with data that KLF5 is required for progenitor proliferation and its known role in regulating cell cycle proteins, we wanted to analyze the effects of KLF5 on crypt expansion. To examine this bromodeoxyuridine (BrdU) was incorporated into proliferating cells two hours prior to sacrifice (Figure 3A). As expected, the number of cells per crypt was indeed significantly greater in each region (Figure 3C) confirming the expansion observed by H&E and Ki67 staining (22.25±1.62 to 42.01±1.74 in the duodenum, 19.48±1.06 to 34.63±2.22 in the jejunum, 19.78±0.68 to 35.03±2.08 in the ileum, and 24.35±1.16 to 38.22±2.58 in the colon). Furthermore, the number of BrdU-positive cells was
also increased (8.43±0.32 to 17.05±1.26 in the duodenum, 8.61±0.21 to 15.53±1.46 in the jejunum, 7.95±0.74 to 14.17±1.39 in the ileum, and 6.73±0.12 to 13.73±0.55 in the colon) (Figure 3D). Of interest, RT-qPCR analysis of cell cycle genes showed a significant increase in the relative mRNA expression of Ccnb1, but not in Ccnb2 or Cdc42 (Cdk1) (Figure 3B). These data indicate that KLF5 sufficiently expands and maintains the transient amplifying zone as it effectively increased the progenitor population and number of proliferating cells.

To further analyze the effect of KLF5 on perturbations of cytodifferentiation and villus blunting, we examined cell death in the epithelium. In the normal intestine cell death is mostly found at the tips of the villi where cells detach from the basement membrane and are shed into the lumen, a process referred to anoikis (Bullen et al., 2006). Ectopic expression of KLF5 increased CC3 (Figure 3F-G), specifically at the crypt-villus junction where differentiation would normally occur. The effect could partially explain the loss of cellular differentiation as cells attempting to differentiate preferentially underwent apoptosis, however increased apoptosis did not counterbalance the increase in TA proliferation as crypt expansion was still quite evident in transgenic animals. Combined with loss of differentiation and crypt hyperplasia, this implies that KLF5 function must be regulated to induce villus cytodifferentiation.

**Increasing KLF5 has no effect on stem cell marker expression.**

We, and others, have previously shown that loss of KLF5 reduces active stem cell renewal throughout the intestines (Bell and Shroyer, 2014; Nandan et al., 2015) and loss of its expression in the LGR5-positive active stem cell population prevented beta-catenin driven adenoma formation (Nakaya et al., 2014). Given the expansion of the crypt zone and deregulation of proliferation in our mice, we wanted to examine potential stem cell depletion or
expansion. *In situ* hybridization for the active stem cell marker, *Ascl2*, suggested that the stem cell compartment may be slightly increased (Figure 4A); however, RT-qPCR analysis of *Ascl2* and other common active stem cell markers, *Lgr5* and Notch-regulated *Olfm4*, showed no change in expression (Figure 4B). Altogether these data suggest that crypt hyperplasia is not due to an acute stem cell-related effect, but rather to expansion of TA cells.

**KLF5 is necessary and sufficient to activate MEK/ERK signaling in the intestine.**

Both loss and gain of function mice clearly implicate a role for KLF5 in progenitor proliferation and maintenance; however, the mechanism of these functions has yet to be elucidated. *In vitro* assays in primary esophageal epithelial cells have linked KLF5 with the RAS-RAF-MEK-ERK pathway as loss of KLF5 correlated with decreased phosphorylation of p-MEK and p-ERK1/2 due to direct regulation of *Egfr* (Yang et al., 2007). Most importantly, this regulatory mechanism was found to mediate the effects of colitis in mice (McConnell et al., 2011a). Additional evidence points to MEK-ERK signaling as a regulator of intestinal homeostasis as it is required for progenitor proliferation through EGFR (Suzuki et al., 2010). Further, this pathway was found to regulate differentiation such that constitutive activation of MEK maintained a progenitor state in intestinal cell lines, with MEK inactivation required for proper differentiation (Lemieux et al., 2011). Because we found that overexpression of KLF5 increased both proliferation and prevented cytodifferentiation, and due to previous findings that KLF5 regulates MEK-ERK signaling, we tested the hypothesis that KLF5 regulates MEK/ERK activation *in vivo*. In mice overexpressing KLF5 we found an increase in activated effectors of MAPK signaling. 100% (5/5) showed increased p-ERK1/2 expression throughout the crypt villus epithelium (Figure 5A). We confirmed this finding with immunoblot analysis and also
found concomitant increase in p-MEK (Figure 5B). We then examined the requirement of KLF5 for MAPK signaling by utilizing previously reported conditional knock out mice (Bell and Shroyer, 2014). After 5 consecutive days of tamoxifen injections, $Klf5^{flox/flox};Vil-CreERT^2$ experimental mice and $Klf5^{+/+};Vil-CreERT^2$ controls were sacrificed the following day and resulted in efficient deletion (Figure 5C). Immunohistochemical staining of jejunal sections showed loss of p-Erk1/2 in $Klf5$-deficient mice, but with variable penetrance: 18% (2/11) of the mice showed total loss of p-Erk1/2, 36% (4/11) of the mice showed partial loss of p-Erk1/2, while the remaining 46% (5/11) showed no change (Figure 5D). These results were confirmed by immunoblot (Figure 5E). We extended these findings by examining p-MEK expression, which showed a pattern similar to pERK, with loss of p-MEK in 6/11 $Klf5$-deficient mice (Figure 5E). Altogether, these data indicate that KLF5 expression can regulate MEK-ERK activity in the intestine.

**Constitutively active KRAS rescues proliferation of progenitor cells, but does not rescue stem cell expression, in Klf5-deficient mice.**

Based on our finding that KLF5 regulates MEK/ERK activity in the intestine, we hypothesized that constitutive activation of KRAS, the key regulator of MEK/ERK in the intestine, would rescue the phenotypes previously reported in KLF5 loss-of-function mice. $Klf5^{floxflox};Vil^{CreERT2}$ mice and $Klf5^{+/+};Vil^{CreERT2}$ controls were crossed with mice that inducibly express the constitutively active GTP-bound $KRAS^{G12D}$ mutant allele. Tamoxifen was administered for 5 days to simultaneously delete $Klf5$ and activate KRAS specifically in the intestine. Mice were then sacrificed the next day. Histological analysis confirmed that p-ERK1/2 was decreased in $Klf5^{floxflox};Vil^{CreERT2}$ mice and was restored to supra-physiological levels in
Importantly, activated KRAS was able to restore proliferation in Klf5-deficient intestines, as measured by expression of the mitotic marker phospho-histone H3 (pHH3) (Figure 6B). As expected, PHH3 was significantly decreased in intestines lacking KLF5 (number of PHH3-positive cells was reduced from 1.96±0.21 to 0.973±0.10 in the duodenum; 1.71±0.74 to 1.12±0.05 in the jejunum; 1.64±0.13 to 0.99 in the ileum; 1.91±0.18 to 0.99±0.25 in the colon). However, proliferation was restored to normal levels upon activation of KRAS in Klf5-deficient intestines (number of PHH3-positive cells was increased to 2.15±0.11 in the duodenum; 2.23±0.12 in the jejunum; 1.98±0.34 in the ileum; 2.07±0.21 in the colon) (Figure 6C).

Given that previous studies have shown that KLF5 is required for stem cell maintenance (Bell and Shroyer, 2014; Nandan et al., 2015), we utilized the crypt-derived enteroid assay to assess stem cell viability after KRAS rescue. Jejunal crypts were isolated from Klf5<sup>+/+;</sup>Vil-CreERT<sup>2</sup>, Klf5<sup>flox/flox;</sup>Vil-CreERT<sup>2</sup>, and Klf5<sup>flox/flox;</sup>Vil-CreERT<sup>2;</sup>KRAS<sup>G12D</sup> mice and grown as enteroids in Matrigel for 6 days. While normal intestinal enteroids form proliferative budding crypt-like structures that protrude outward, enteroids from mice lacking KLF5 formed intact spheroid structures but failed to proliferate or form crypt domains (Figure 7A). Interestingly, constitutive activation of KRAS rescued both proliferation and crypt formation in enteroids lacking KLF5 (Figure 7A). Enteroid survival was quantified as a percentage of initial crypts plated (Figure 7B). Analysis of variance revealed that the loss of KLF5 significantly impacted the survival of enteroids (F (2, 6) = 42.82, p < 0.0001). Over 6 days a significant decrease was observed in the percentage of surviving Klf5<sup>flox/flox;</sup>Vil-CreERT<sup>2</sup> enteroids (37.02±2.45%) and, surprisingly, Klf5<sup>flox/flox;</sup>Vil-CreERT<sup>2;</sup>KRAS<sup>G12D</sup> (28.43±1.49%) when compared to wildtype controls (63.55±7.73%) (F (5, 30) = 93.62, p < 0.0001). We did observe KLF5 expression in the
remaining enteroids. Overall, a significant interaction was found between the depletion of KLF5 and survivability over time (F (10, 30) = 8.059) p = 0.0003); however, activation of KRAS had no significant impact on this measure (F (6, 30) = 1.475, p = 0.223). Given that perdurance of enteroids depends on weekly passaging due to buildup of cytotoxic signals from cell shedding into the central luminal space, we presumed that the hyperproliferative effect of KRAS activation may have hastened this cytotoxic effect. Therefore we passaged these enteroids at day 6 and assessed them after 5 days. In both Klf5^{flox/flox};Vil^{CreERT2} and Klf5^{flox/flox};Vil^{CreERT2};KRAS^{G12D} virtually no survival was seen. Thus, while KRAS activation could rescue crypt formation and proliferation in KLF5-deficient enteroids, stem cell activity was not rescued. To determine if this was due to depleted stem cells, we isolated RNA from jejunal tissue found that KRAS activation could not rescue stem cell marker expression in the absence of KLF5 (Figure 7C). These results suggest that KLF5 regulation of MEK-ERK signaling is uniquely important for TA cell proliferation, and suggests that KLF5 controls stem cell self-renewal via a distinct mechanism.

**Loss of Klf5 affects genes related to MAPK signaling.**

To determine how KLF5 could be affecting MAPK pathway, we performed RNA-sequencing on jejunal crypts isolated from Klf5-deficient and wildtype mice, using three biological replicates per genotype. We identified 1,480 genes that showed significant differential expression with at least a 2-fold difference between Klf5-deficient and wildtype (q-value ≤ 0.05) (Figure 5A). Klf5 expression was significantly downregulated 2.7-fold. We used the PANTER classification system for ontological analysis. Not surprisingly, we identified several GO terms related to cell cycle and apoptosis. Interestingly, we found significant enrichment of genes
related to G-coupled protein receptor signaling and negative regulation of MAPK (Figure 5B).
Analysis of differentially expressed genes in the latter indicated significant upregulation of phosphatases and other genes that could possible alter this pathway in the intestine (Figure 5D). Though KLF5 has been shown to directly regulate Egfr, we did not find differential expression of this transcript indicating that KLF5 affects MEK-ERK signaling through an alternative mechanism.
DISCUSSION

*Klf5* has been reported to play a variety of roles in the pathology of intestinal diseases. Not only is it indispensable for normal proliferative function and stem cell homeostasis, it has been shown to mediate the effects of wound healing, bacterial-induced hyperplasia, and tumorigenesis (Bell and Shroyer, 2014; Chanchevalap et al., 2006; McConnell et al., 2008; McConnell et al., 2009; McConnell et al., 2011a; Nandan et al., 2015; Nandan et al., 2010; Nandan et al., 2008). Given this, we were driven to further elucidate the role of KLF5 by increasing its expression to study the regulatory mechanisms of progenitor and stem cell function. Overexpression in the intestinal epithelium was previously reported to have no morphological, proliferative, or differentiation defects; however, expression was induced at the time of villus morphogenesis (Tetreault et al., 2012). Here we have shown that increasing KLF5 in the adult intestine resulted in crypt zone expansion, villus blunting, and loss of cytodifferentiation after 2.5 days. The increase in the number of transient amplifying progenitors suggests that KLF5 expression is tightly correlated with the continued proliferation of this population *in vivo*. We also found an escalation in the number of apoptotic cells. This phenotype may be due to the upregulation of CCND1 by KLF5 as ectopic expression of this cell cycle protein has been reported to promote apoptosis (Duquesne et al., 2008; Han et al., 1999; Katayama et al., 2001). Regardless of the mechanism of cell death, this effect could not compensate for the increased cell proliferation, as indicated by crypt expansion. This suggests that KLF5 primarily regulates proliferation and progenitor cell maintenance.

KLF5 has previously been shown to play a role in active intestinal stem cell maintenance (Bell and Shroyer, 2014; Nakaya et al., 2014; Nandan et al., 2015) and, due to the expansion in the proliferative crypt zone, we hypothesized that stem cells would be affected by ectopic *KLF5*
expression; however, we did not observe any change in stem cell markers in our transgenic animals. Unfortunately, KLF5 transgenic mice became moribund after three days of doxycycline treatment. Based on a ~24-hour cycling time, active stem cells were exposed to increased KLF5 for 1-2 cell divisions, potentially limiting our ability to discern the effect of increased KLF5 expression on ISCs. Utilizing a stem cell specific promoter like Lgr5-CreER to induce KLF5 expression could help discern any stem cell-specific roles for KLF5. Further, enteroid studies could be used to substantiate any findings. Results from these assays could potentially uncover differential roles governing active stem cell dynamics.

To determine potential mechanisms that regulate the maintenance and proliferation of the transient amplifying population, we examined signaling pathways that have been associated with these cell types. An earlier study determined that loss of KLF5 correlated with decreased nuclear β-catenin accumulation in crypt cells and disrupted canonical Wnt signaling (McConnell et al., 2011b), but this phenotype was found in adult mice in which KLF5 was deleted at the time of villus morphogenesis, therefore this result could have been caused by compensatory mechanisms. Our previous data in Klf5-deficient adult intestines showed reduced expression only seen in beta-catenin target genes also known for marking active intestinal stem cells, Lgr5 and Ascl2 (Bell and Shroyer, 2014). As a first approach to reconcile our gain-of-function and previously reported loss-of-function phenotypes, we performed RNA-sequencing on Klf5-deficient crypts. These data showed that downstream targets of Wnt signaling Sox9, Axin1, Axin2, Tcf4, and Lef1 were not differentially expressed. There was, however, a significant change in Ccnd1 and Myc, but this could be explained by the reduction in proliferation rather than disruption of the entire pathway. Regulation of these specific transcripts can also be induced by MEK-ERK signaling. Interestingly, we identified this as a potential KLF5-pathway
in normal intestinal tissue. KLF5 has previously been shown to regulate MEK-ERK signaling in colitis models and esophageal epithelial cells, specifically by direct regulation of \textit{Egfr}. (McConnell et al., 2011a; Yang et al., 2007). While no significant changes in \textit{Egfr} were found in our RNA-seq data, we did not analyze the active receptor, leaving open the possibility of post-transcriptional regulation of EGFR. We did, however, find an increase in genes that are known to specifically downregulate MAPK signaling such as \textit{Dusp1}, \textit{Dusp9}, \textit{Ptprr}, and \textit{Spry4}. Interestingly, these genes are generally regulated by MAPK pathways. Due to the observed downregulation of MEK-ERK signaling in our knockout model, it is possible that other MAPK pathways like p38 could be affected. In the intestine, p38 signaling has been found to regulate cytodifferentiation (Houde et al., 2001). We have shown here that increased KLF5 expression activates MEK and ERK while preventing differentiation. It is therefore possible that loss of KLF5 may incite a differentiation program involving p38 activity that results in phosphatase transcription.

It is interesting that loss of KLF5 does not abolish the expression of pERK1/2 in all instances. One explanation could be due to residual expression of KLF5 as \textit{Villin} recombination is not always 100% effective. Further, if KLF5 is regulating the differentiation program as our work suggests, MEK-ERK signaling may be indirectly downregulated and therefore loss would not necessarily be completely penetrant. It may prove useful to analyze effectors of alternative signaling that can control MEK-ERK activity such as p38. Alternatively, activation of the MEK-ERK pathway could be the result of compensatory mechanisms such as PI3K signaling. Well-documented evidence has demonstrated the interaction between MEK-ERK and PI3K signaling in a variety of settings (Aksamitiene et al., 2010; Grammer and Blenis, 1997; Jiang et al., 2010).
It may therefore be useful to analyze PI3K signaling in this context. Perhaps inactivation of AKT could potentiate the effects of KLF5 depletion.

Interestingly, while constitutive activation of KRAS was sufficient to restore proliferation and crypt formation in enteroids, and proliferative activity in $Klf5$-deficient intestinal tissue, this had no bearing on the survival of stem cells as determined by RT-qPCR and lack of enteroid survival. These findings agree with other research indicating activation of KRAS induces intestinal hyperplasia without affecting the stem cell pool (Feng et al., 2011). The unexpected capacity of KRAS activation to restore crypt formation in the setting of KLF5 loss indicates that crypt formation is possible in the absence of stem cells. Collectively, these results demonstrate that there are separate mechanism by which KLF5 controls stem cell renewal and TA proliferation. Separate RNA-sequencing on these two populations could identify the putative underlying mechanisms.

Ectopic KLF5 expression had a profound effect on MEK-ERK signaling with a hyperplastic phenotype that mirrored KRAS activation reported in previous studies (Feng et al., 2011; Haigis et al., 2008; Trobridge et al., 2009). However, activation of this pathway alone is not fatal, thus other downstream targets of KLF5 mediate this lethal phenotype. In all of our mice, overexpression of KLF5 was associated with a fluid-filled bowel. A simple explanation for this is a lack of absorptive cell differentiation which could result in malabsorptive diarrhea. Alternatively, fluid accumulation could suggest an alteration in barrier function. In support of this, previous data have demonstrated that transgenic expression of KLF5 perturbs barrier function in the epidermis (Sur, 2006; Yang et al., 2008). Changes in cell adhesion can effectively influence proliferation and differentiation and mediate the effects of intestinal disease; therefore it is possible that KLF5 could regulate these changes by affecting proteins or pathways related to
intestinal epithelial cell adhesion. Further analysis of this interaction would help to decipher the mechanisms by which KLF5 regulates intestinal homeostasis and disease.
LIST OF REFERENCES


FIGURE LEGENDS

**Figure 1 – Overexpression of KLF5 results in aberrant intestinal morphology.**

(A) Distended, fluid-filled bowels are seen 2.5 days after doxycycline administration in mouse intestines overexpressing KLF5. (B) Representative immunofluorescent sections depicting the increase of KLF5 (red) and its expansion out of the crypt region where normal expression occurs. (C) H&E immunohistological staining comparing the morphology of the control versus the transgenic intestines. (D) Comparative immunofluorescent stains of the large and small intestine. Illustrated are the morphological changes that occur including an elongated proliferative crypt zone as indicated by Ki67 (green).

**Figure 2 – Overexpression of KLF5 decreases villus cytodifferentiation.**

Immunofluorescent stains indicate chromogranin A (CHGA), an enteroendocrine marker (A), mucin 2 (MUC2), an intestinal goblet cell marker (B), and dipeptidyl peptidase-4 (DPP4), an enterocyte marker, (C) are reduced when KLF5 is overexpressed in the intestine. (D) Western blot analysis of protein from whole jejunal tissue confirms loss of villus cell differentiation markers, ChgA, trefoil factor 3 (Tff3) (a Goblet cell marker), and DPP4 in whole jejunal tissue of *wildtype* versus KLF5-overexpressing intestine. (E) Immunofluorescent staining of lysozyme (LYZ) a Paneth cell marker, indicate no difference is seen in crypt cell differentiation.

**Figure 3 – Overexpression of KLF5 results in crypt expansion with increased proliferative progenitors and cell death.**

(A) Immunohistochemistry depicts BrdU incorporation of proliferating cells within the crypts of doxycycline treated *wildtype* and KLF5 transgenic mice after a 2 hour pulse. (B) Relative mRNA
expression of cell cycle proteins indicates a significant increase in *Ccnd1*, but not *Ccnb1* or *Cdk1*. Student’s t-test was performed to determine p-values; expression was normalized to β-actin. (Mean ± SEM, T-test: * p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, n = 5)

Quantification of the number of cells indicates a significant increase in the mean number of cells per crypt (C) and the mean number of BrdU positive cells per crypt (D) (in 20 crypts per mouse); however, there is no significant increase in the number rate of proliferation in the small intestine (E). Student’s t-test was used to calculate p-values (Mean ± SEM, T-test: * p-value ≤ 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001, n = 9); (F) Cleaved caspase 3 (CC3) staining was used to analyze the amount of cell death within the intestine. (G) Western blotting in whole jejunal tissue segments confirms an increase in CC3.

**Figure 4 – Increased expression of KLF5 has no effect on stem cell placement or expression.**

(A) *In situ hybridization* of active stem cell marker *Ascl2* does not show any comparable difference between wildtype and transgeneic crypts. (B) Relative mRNA expression of other active stem cell markers (*Ascl2*, *Lgr5*, and *Olfm4*) does not show any significant difference. Student’s t-test was performed to determine p-values; expression was normalized to GAPDH. (Mean ± SEM, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n = 11)

**Figure 5 – Loss or gain of KLF5 expression affects the phosphorylation of ERK and MEK proteins.**

(A) Immunohistochemical staining of jejunal segments show an increase in p-ERK compared to wildtype tissue. (B) Western blot analysis of jejunal tissue confirms that phosphorylation of
ERK1/2 and MEK is increased. (C) Immunofluorescent staining indicates that KLF5 (red) is sufficiently deleted in intestinal tissue. (D) Immunohistochemical staining of Klf5flox/flox jejunal segments depicts complete or partial loss of phospho-ERK (p-ERK). (E) Western blot analysis of jejunal tissue indicates incomplete penetrance of the phenotype by complete, partial, or no loss of phosphorylation in phospho-ERK1/2 and phospho-MEK.

**Figure 6 – Increased expression of KRAS after loss of Klf5 rescues proliferation in vivo.**

(A) Immunohistochemistry comparing pERK expression in the jejunum in *wildtype*, Klf5 knock-out, and Klf5KO/KRAS induced intestine. (B) Immunofluorescent histology compares the expression of KLF5 (red) and the proliferation marker phospho-histone H3 (pHH3) (green). (C) The number of PHH3 cells per crypt in each region from wildtype, KO, and KRAS/KO mice shows a significant increase of proliferation when KRAS is expressed in the knockout. (Mean ± SEM, T-test: * P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, n = 9)

**Figure 7 – Activation of KRAS in mouse-derived enteroids recues budding, but not enteroid survival or stem cell expression.**

(A) Whole mount immunofluorescent stained enteroids with proliferative marker Ki67 (green), KLF5 (red), and DAPI (blue) illustrates that KRAS can rescue budding after jejunal crypts from recombined mice were plated in Matrigel for 6 days. (B) The percentage of live enteroids indicates a significant difference in the survival between *wildtype* and those without KLF5 expression. Expression of activated KRAS could not rescue this phenotype. (C) Relative mRNA expression shows a significant decrease in Klf5 mRNA in mice containing recombined Klf5. Active stem cell markers Ascl2 and Olfm4 are significantly decreased as well. Activation
of KRAS does not rescue loss of stem cell markers including \textit{Lgr5}. Student’s t-test was performed to determine p-values; expression was normalized to GAPDH. (Mean ± SEM, T-test: * \(P \leq 0.05\), **\(P \leq 0.01\), ***\(P \leq 0.001\), \(n = 3\))

\textbf{Figure 8 – Loss of Klf5 in crypts affects genes related to MAPK signal transduction and cell cycle.}

\textit{(A)} A heatmap was generated by hierarchical clustering of 1,480 genes that were differentially expressed with at least a 2-fold change (q-value \(\leq 0.05\)) comparing isolated RNA from \textit{wildtype} crypts to those lacking \textit{Klf5}. Normalized values reported are Log2(FPKM) and pseudocolored to represent the intensity of expression. \textit{(B)} The PANTHER classification system was used to group differentially expressed genes into specific significantly enriched ontologies such as cell cycle, apoptosis, and signal transduction. \textit{(C)} Bar plot depicting the fold change (log2 scale) of significantly upregulated or downregulated genes involved in the regulation of MAPK signaling.
Figure 1 – Overexpression of Klf5 results in aberrant intestinal morphology.
Figure 1 – Continued

(D) Duodenum Jejunum Ileum Colon

Ki67/DAPI
Kif5^WT^, ^VII^Cre, R26^rtTA/rtTA
Kif5^Tre^, ^VII^Cre, R26^rtTA/rtTA
Figure 2 – Overexpression of Klf5 decreases villus cytodifferentiation.
Figure 2 – Continued
Figure 3 – Overexpression of KLF5 results in crypt expansion with increased proliferative progenitors and cell death.
Figure 3 – Continued

(F)  

E)  

Mean % of BrdU+ Cells/Crypt

Duodenum    Jejunum    Ileum    Colon

Klf5wt; VillCre
Klf5Tre; VillCre

(G)  

CC3  
Actin

Klf5wt; VillCre
Klf5Tre; VillCre
Figure 4 – Increased expression of Klf5 has no effect on stem cell placement or expression.
Figure 5 - Loss or gain of Klf5 expression affects the phosphorylation of ERK and MEK proteins.
Figure 6 – Increased expression of KRAS after loss of Klf5 rescues proliferation in the *in vivo*. 

(A) pERK 

(B) Klf5/pHH3/DAPI 

(C) 

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<th>Tissue</th>
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Number of pH3+ cell/crypt 

- Klf5+/+; VilCreERT2
- Klf5f/f; VilCreERT2
- Klf5f/f; VilCreERT2; KRAS
Figure 7 – Activation of KRAS in mouse-derived enteroids rescues budding, but not enteroid survival or stem cell expression.
Figure 8 – Loss of Klf5 in the crypts affects genes related to MAPK signal transduction and cell cycle.
CHAPTER 4

General Discussion
SUMMARY OF MAJOR FINDINGS

Given its function as a mediator of disease and inflammation in the intestine (Bateman et al., 2004a; Chanchevalap et al., 2006; Dong and Chen, 2009; McConnell et al., 2008; McConnell et al., 2009; McConnell et al., 2011a; Nandan et al., 2005; Nandan et al., 2010; Nandan et al., 2008b; Tetreault et al., 2012), elucidation of the role of KLF5 in normal homeostasis has been a relevant topic of study in gastrointestinal biology. The overall goal of this research was to determine how this transcription factor mediates progenitor and stem cell dynamics and affects proliferation and cytodifferentiation. Previous studies have undertaken this task; however, we hypothesized that their experimental models may have been incomplete and, thus, inaccurately represented its function in the bowel. The first published work on the role of KLF5 in vivo effectively deleted this transcription factor at the time of villus morphogenesis around e14.5. Two-thirds of the mice did not survive, and the remainder were analyzed for alterations in homeostasis after 8 weeks. Defects in cytodifferentiation were evidenced by an increase in goblet cell numbers and a decrease in enteroendocrine cell numbers. Migration was also impaired as Paneth cells did not remain localized at the bottom of the crypt. Further, proliferation did not cease in these intestines, but Ki67-positive cells were found positioned between non-cycling cells suggesting further that KLF5 regulated crypt-villus patterning. This paper concluded that the effects of KLF5 depletion were caused by decreased nuclear accumulation of β-catenin and perturbations in canonical Wnt signaling. (McConnell et al., 2011b). A few years later, it was discovered that KLF5 plays an important process in villus morphogenesis in the intestine (Bell et al., 2013) which could easily explain the fatality rate of mice in the previous study. We reasoned that the earlier reported data may have been attributed to developmental defects, therefore we decided to reopen investigation into this vital component.
of intestinal function. Our key findings differed immensely as we found a reduction in proliferation, but no defects in cytodifferentiation or migration.

In Chapter 2 we examined the loss of KLF5 in the adult intestine by inducing recombination in 6 week old mice. Notably we found a significant reduction in proliferation and increased apoptosis throughout the small and large bowel 3 days after induced KLF5 depletion; however, no defects were seen in cytodifferentiation or migration. We then decided to examine these mice at different time points to discern possible differential effects between acute and long-term loss. Unfortunately, we found that KLF5 expression had reemerged after 2 weeks. These effects were likely attributed to outcompeting wildtype tissue expansion given that Villin-creER-induced recombination in the epithelium does not result in total depletion. Our histology in Chapter 2 does depict a few remaining KLF5-positive cells. This phenomenon suggests that this transcription factor is indispensable for intestinal homeostasis. Further we found that while proliferation had significantly increased following acute loss, this activity was not restored to basal levels after KLF5 levels were restored. This effect could not be explained by crypt cell death as apoptosis was only an acute effect. Interestingly, we discovered a reduction in active stem cell marker (CBC) expression that persisted for up to 28 days. This provides some explanation for the overall decrease in proliferation at this time point. Despite this fact, the intestine displayed no major morphological or physiological defects. As discussed in Chapter 1, the intestine is thought to have a reserve, quiescent stem cell population that can overcome the loss of CBCs (Tian et al., 2011). Retrospectively it would have been ideal to examine the relative mRNA expression of quiescent stem cell markers such as Bmi1 and Hopx during this investigation; however, RNA-sequencing data from Chapter 3 indicates that there was no differential expression in the Bmi1 transcripts, but there was a 2-fold increase in Hopx. This
could explain why we found no significant morphological defects. We also investigated the published role of KLF5 as a mediator of canonical WNT signaling (McConnell et al., 2009; McConnell et al., 2011b), but we did not find any significant differences in downstream targets of this pathway other than those of actively cycling stem cells at the time of investigation. Data from our RNA-seq supports these findings as known downstream targets in the intestine such as Sox9, Axin1, Axin2, Tcf4, or Lef1, were not differentially expressed. There was a significant change in Ccnd1 and Myc, but this could be explained by the reduction in proliferation rather than disruption of the entire pathway. While we would have been excited to learn more about the transactivation function of KLF5 in the intestine through ChIP analysis, the lack of high-quality antibodies rendered this task impossible. Overall our results differ greatly from previously published data; however, we shouldn’t discount what they have shown. Those effects could manifest in absence of KLF5 after a long period of time.

Transgenic expression of proteins is often utilized to analyze their relevant biological functions in specific settings. Given its identified capacity as a proto-oncogene in the intestines of both mice and humans (Bateman et al., 2004b; McConnell et al., 2009; Nandan et al., 2010; Nandan et al., 2008a), we felt that it was particularly important to make use of this approach for continued assessment on KLF5 in Chapter 3. Previous research found that ectopic expression in the intestine presented with no morphological abnormalities or proliferative defects; however, this group also manipulated expression at the time of villus morphogenesis (Tetreault et al., 2012). In our work, we induced ectopic expression of KLF in adult mice which had a fatal result after 4 days. Histological examination revealed dramatic differences in intestinal morphology including crypt hyperplasia and villus blunting in just 2.5 days. Further, we found an increase in proliferative and overall progenitor cell numbers concomitant with loss of villus
cytodifferentiation. This suggests that persistent KLF5 expression is sufficient to maintain the transient amplifying zone.

We further correlated the expression of KLF5 with the activation of MEK and ERK indicating a role for KLF5 in regulating this pathway. Histological analysis of intestinal tissue confirmed a loss of phosphorylated ERK1/2 expression; however, this effect was not 100% penetrant. Only 18% of our animals were devoid of pERK1/2 expression while 36% showed some downregulation. The remainder did not seem to be affected at all (discussed below). We did see increased activation of MEK-ERK in all KLF5-expressing transgenic mice, indicating that this transcription factor can activate this pathway. To test the contribution of dysregulated MEK-ERK activity to the phenotype of Klf5-deficient mice, we performed rescue experiments in vivo with constitutively activated KRAS. We found this to rescue intestinal proliferation. To examine the effect KRAS on stem cell dynamics, we utilized the enteroid system to determine if loss of this pathway could account for the reduction in stem cell marker expression. Isolated crypts are plated in laminin-rich Matrigel and bathed in recombinant growth factors to mimic the intestinal environment. Under these conditions, the stem cells within in each crypt will continuously divide and produce a structure containing multiple crypt buds and all intestinal cell lineages. Passaging of these structures involves mechanical dissociation of crypts and allows for maintenance of enteroid lines for an extended period of time. This assay has become a popular method for analyzing stem cell functionality due to the ease of manipulation and ability for multivariable testing. In short, it can provide large amounts of reproducible data in a short period of time. As expected, budding and overall survival were significantly decreased in enteroids derived from intestinal tissue lacking KLF5. KRAS was able to rescue budding, but had no effect on the survival of Klf5-deficient enteroids. RT-qPCR analysis confirmed that
activation of this pathway had no effect on CBC expression. This agrees with current literature indicating that KRAS activation induces intestinal hyperplasia, but has no bearing on ISC activation (Feng et al., 2011). It is interesting that despite this fact, budding could be rescued. We attributed this result to persistence of residual stem cells as virtually no survival was seen after enteroid passaging. Regardless, KRAS activation affected only the progenitor population suggesting that KLF5 differentially regulates stem cell expression.

Additional insight could be provided from RNA-sequencing data from isolated crypts from these mice. The comparison to data obtained from our knockouts would be useful in isolating genes that are potentially regulated by KLF5. Additionally, the fatal effect of transgenic overexpression of KLF5 prevented us from examining potential long-term effects on stem cell function. Enteroid assays may prove to be useful determining if KLF5 expanded or depleted this population. Regardless of this, these data still provide interesting results and incite further questions regarding its function in homeostasis and disease.

**ADDITIONAL QUESTIONS AND FUTURE DIRECTIONS**

*Does KLF5 differentially control the stem cell survival and progenitor cell proliferation?*

Data generated from our work discerning the role of KLF5 in the normal intestine suggests the possibility of differential regulatory mechanisms governing the self-renewal of ISCs and the proliferative maintenance of the progenitor cells. Our work (Chapter 2), and others, have demonstrated that loss of KLF5 results in decreased CBC expression and loss of LGR-positive cells despite reemergence of KLF5 expression (Nandan et al., 2015) suggesting that acute loss of this transcription factor affects stem cell survival. Further, localized activation of proliferative
WNT signaling in CBCs was shown to induce lethal adenoma formation in wildtype mice. However, when KLF5 was depleted simultaneously in these cells no abnormal phenotype was found suggesting its role as an oncogenic effector in intestinal stem cells (Nakaya et al., 2014). The data indicate that there is a discrete role for KLF5 in this population of cells. KLF5 loss in the progenitor population did not alter cell survival over time. Further, these cells were capable of responding to outside signaling cues as evidence by the induction of KRAS at the time of KLF5 deletion (Chapter 3). Our transgenic studies demonstrated that short-term, ectopic expression was sufficient to increase the number of crypt cells and proliferative progenitors, while having no effect on stem cells at this time. These effects convey the possibility of differential function.

One of the most plausible roles for KLF5 in the CBCs could be as a transactivator of Survivin/BRIC5, an inhibitor of apoptosis. Our sequencing data indicate that there is a significant downregulation in its expression levels in knockout crypts. KLF5 has previously been shown to bind to the Survivin promoter and directly regulate gene expression cancer cells (Dong et al., 2013; Zhu et al., 2006). Very recent studies on the effects of Survivin loss in the intestine show similar stem cell phenotypes when compared with the effects of KLF5 depletion (Martini et al., 2016). Both enteroid survival and expression of mRNA from active stem cell markers are significantly decreased when either of Klf5 or Survivin is knocked out in the intestinal epithelium. Therefore KLF5 may directly regulate Survivin expression in order to prevent loss of CBCs. The hypothesized mechanism for direct regulation of Survivin could also occur in the progenitor cells. (Chapter 2); however, when we compare the overall morphological and systemic phenotypes of intestinal tissue lacking SURVIVIN expression to those lacking KLF5 expression we see profoundly different effects suggesting this is unlikely. SURVIVIN
depletion had a significantly increased crypt cell death, induced inflammation and ultimately had fatal results (Martini et al., 2016). While these pathological changes do not occur in our knockout mice, it does not mean that KLF5 does not directly regulate *Survivin* expression in the stem cell population. The lack of inflammation and fatality in our knockout animals could be attributed to persistence of the protein in proliferative progenitor daughters of stem cells.

If KLF5 can not directly regulate *Survivin* expression, or is insufficient alone, Hippo signaling could be involved. Interaction with membrane receptors activate a signaling cascade that results in the phosphorylation of YAP and TAZ proteins. Activation of these proteins facilitate cell adhesion and polarity due to their integration into the cytoskeletal complex specifically through interaction with 14-3-3 proteins (Wang et al., 2009). In the absence of the phosphorylation, YAP and TAZ accumulate in the nucleus and interact with TEAD transcription factors to regulate gene transcription (Wang et al., 2009). Nuclear YAP/TAZ were found to regulate intestinal stem cell survival. Both disruption of YAP-TEAD interaction and depletion of TEAD4 effectively depleted the number of Lgr5+ and OLFM4+ stem cells and suppressed the growth of enteroids (Imajo et al., 2014) demonstrating a requirement for these transcription factors in stem cell renewal. Interestingly, YAP has been found to induce the expression of *Survivin* (Bai et al., 2012; Dong et al., 2007), and KLF5 has been shown to interact with TEAD4 to regulate promoter activity (Wang et al., 2015) suggesting a possible mechanism where KLF5 and TEAD4 interaction is required to regulate the *Survivin* expression necessary for ISC survival. Directly testing this hypothesis could be achieved in the future by performing examining KLF5 and TEAD4 effects on *Survivin*.

If KLF5 is indeed required for SURVIVIN expression in the CBCs, but not the progenitor cells, what is the possible differential mechanism regulating this population? Given
our results from Chapter 3, KLF5 may maintain a proliferative progenitor fate through activation of MEK-ERK signaling. Evidence in literature indicates that this is a likely possibility. Activation of ERK was found to stimulate progenitor cell proliferation without increasing crypt fission ((Park et al., 1997)). Additionally, this pathway was shown to prevent apoptosis and differentiation in IEC cell cultures (Suzuki 2010). Further, EGF, and RTK ligand, is required for long-term enteroid survival (Date and Sato, 2015; Suzuki et al., 2010). Therefore KLF5 could regulate progenitor cell maintenance and proliferation of MEK-ERK signaling. Although loss of MEK and ERK activation in our mutants is not 100% penetrant three days after recombination of KLF5, it is possible that other effectors are responsible for alternative activation of this pathway such as PI3K (discussed in Chapter 3). If this is the case, it might be useful to examine MEK and ERK activation at an earlier time point than 3 days in an attempt to see an increased effect in penetrance prior to alternative activation. Collectively these data provide potential mechanisms by which KLF5 could be differentially regulation the CBC and progenitor cell populations.

Does KLF5 differentially affect barrier function and progenitor cell maintenance?

KRAS mutations are found in nearly 50% of patients with CRC or small bowel adenocarcinoma (Bos et al., 1987; Sutter et al., 1996)Histological analysis from human CRC with KRAS mutations displayed an upregulation of KLF5 expression (Nandan et al., 2008b). Data collected from mice harboring both Apc\text{min} and KRAS mutations, indicated that hemizygosity of KLF5 could reduce both nuclear accumulation of β-catenin and activation of MEK and ERK (Nandan et al., 2010). These data provide evidence for oncogenic properties of KLF5 in CRC. Given this effect we wanted to examine the role of ectopic expression of KLF5
under basal conditions to further elucidate a role for this transcription factor and determine if there was some effect on tissue transformation. Due to early fatality, we did not witness any neoplastic effects in the intestines. We did, however, find a lack of cytodifferentiation and increased fluid retention in the small bowel. This phenotype was accompanied by increased activation of MEK-ERK signaling in the epithelium which could explain both of these effects. However, because intestinal barrier function is mediated by different effects such as defects in cell-cell junction adhesion and ion flux it is possible that KLF5 could be regulating some other gene or pathway involved in intestinal permeability.

The first point to address is how KLF5 could regulate villus cell differentiation effects. Transgenic overexpression of KLF5 increased the progenitor cell population and prevented villus cell differentiation. This effect was accompanied by increased expression of phosphorylated MEK and ERK. As previously mentioned, ectopic activation of MEK-ERK signaling inhibits cell-cycle arrest and subsequent differentiation of intestinal epithelial cells (Lemieux et al., 2011; Suzuki et al., 2010). Therefore this phenomenon could explain the hyperplastic crypts and loss of cytodifferentiation described in Chapter 3. Pharmacological inhibition of MEK activity in mice with transgenic expression of KLF5 would be a convenient way to determine if that is the case. Incidentally, an upregulation of differentiation markers was found in our RNA sequencing data of Klf5-deficient intestines; however there was no evidence of increased protein expression (Chapter 2). This result suggests that factors involved in cytodifferentiation may be direct targets of KLF5, but require additional transcriptional and post-transcriptional regulation for full protein expression.

Activation MEK-ERK signaling could also explain the deregulation of intestinal barrier function seen in our transgenic mice. Regulation of proteins involved tight junctions, adherens
junctions and desmosomes are known to affect intestinal barrier function (Groschwitz and Hogan, 2009). E-cadherin, a major component of adherens junctions was shown to be downregulated in RAS transformed breast epithelial cells. Suppression of MEK with pharmacological inhibitors could effectively restore E-cadherin cell-cell junctions (Li and Mattingly, 2008). These data provide evidence that ectopic MEK-ERK signaling could affect cell adhesion in other epithelial tissues. A simple way to test if this pathway affects both hyperplasia and barrier function alterations would be to administer MEK inhibitors to our transgenic mice after inducing ectopic expression of KLF5. If barrier function and differentiation are regulated by the same phenomenon, it is expected that MEK inhibition will effectively restore intestinal cell differentiation mice will not suffer the same moribund fate. Then we would have to discern if suppression of MEK-ERK signaling restored cell-cell junctions or if simply restored absorptive function restoration of enterocyte differentiation. Regardless, this would indicate that KLF5 regulates both barrier function and progenitor maintenance through MAPK signaling.

Other alternatives could explain the deregulation in barrier function if MEK-ERK signaling affects only progenitor cell state. Our RNA sequencing data indicates that loss of KLF5 upregulates the transcripts of cytoskeletal-associated proteins known to mediate E-cadherin such as Rhob, insinuating that that ectopic KLF5 could downregulate its expression. Transient depletion of RHOB has been shown to reduce E-cadherin expression in prostate cancer cells (Vega et al., 2015). Further, loss of this protein in colon cancer cells promotes proliferation and invasion (Liu et al., 2011). Although defects in E-cadherin could explain deregulation of barrier function in our transgenic animals, this is unlikely as loss of this protein is usually associated with migratory defects which are not found in our mice. Again, administration of MEK inhibitors would be useful in discerning this information. If MEK inhibition has no effects on
barrier function we would expect that that fatality will occur. It would then be important to
determine possible causes of this effect as simple manipulations could be made to mouse model
in order to prolong its life and continue the study of the effects of KLF5 on stem cell dynamics in vivo. FITC-dextran experiments could be employed to determine if there is an alteration in pericellular permeability. There is also the possibility that transcellular permeability could also be affected causing osmotic swelling into the luminal space due to deregulated ion flux. A number of ion channels exist in the apical and basal membranes of intestinal epithelial cells and create an electrochemical gradient that regulates the secretion and adsorption of water and electrolytes (Field, 2003). An ongoing collaboration with Hogan et al. has accumulated evidence that KLF5 directly regulates the CFTR channel increasing chloride ion secretion. Whatever the mechanism may be, loss of barrier function has been shown to mediate intestinal disease (Fondacaro, 1986; Groschwitz and Hogan, 2009; Martin and Jiang, 2009; Turner, 2009) and it is clear that ectopic expression of KLF5 can disrupt homeostasis and subsequently cause death in mice. Uncovering the link between this transcription factor and barrier function could provide us with vital information regarding intestinal pathobiology.

CONCLUSION & SIGNIFICANCE

The overall objective of this work was to determine the function of KLF5 in normal intestinal homeostasis. We have found that KLF5 is required for normal homeostasis and CBC marker expression. Additionally, it is sufficient to increase proliferative progenitor cells and prevent villus cytodifferentiation. Further, it was shown to affect MEK-ERK signaling in the intestine. Many questions still remain. For example, we still do not know the transactivation function of KLF5 in the intestine. We do not know if this transcription factor regulates stem cell
homeostasis in the same manner as the progenitor cells. We do not know whether KLF5 regulates intestinal barrier function. Data collected from these studies provides the groundwork for new hypothesis and research objectives. KLF5 may directly regulate Survivin expression to maintain CBC survival. KLF5 may maintain a progenitor cell fate through activation of MEK-ERK. Downregulation of KLF5 may be required for villus cytodifferentiation. These details are outlined in Figure 1.

Our work has impacted the field of intestinal biology studies in several ways. We provide RNA sequencing data that may prove to be useful in discerning the regulatory roles of a potential oncogenic transcription factor. We utilize mouse models that possibly supplant the use of heterozygote mice in intestinal disease models to more accurate represent pathological defects. Finally, we provide novel insight about the possible differential regulation of intestinal stem cells and proliferative progenitors.
LIST OF REFERENCES


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FIGURE LEGENDS

Figure 1 – Hypothesized roles for KLF5 in the intestine.

Evidence from our research, and others, indicates that KLF5 may play a differential role in regulating stem cell viability and maintaining a proliferative progenitor state in the transient amplifying population.
Hypothesis: Downregulation of KLF5 decreases MEK-ERK signaling and induces differentiation.
- Increased expands crypt zone
- Overexpression of Klf5 decreases cytodifferentiation
- Increased expression of activated effector proteins (pERK1/2 and pMEK)

Hypothesis: KLF5 activation of MEK-ERK signaling is required to maintain a proliferative progenitor fate in the TA cells.
- Loss of Klf5 significantly decreases proliferation
- Some loss of MEK-ERK activation
- KRAS induction can rescue this effect
- mRNA expression of some differentiation markers were increased

Hypothesis: KLF5 directly regulates Survivin expression to maintain ISC population.
- ISC population is depleted in the absence of KLF5
- Stem cell functionality is lost
- Rescue experiments active proliferative function but cannot restore ISC expression
- BIRC5/Survivin mRNA is significantly decreased