LOSS OF RAB11A AND RAB11B RESULTS IN DYSREGULATION OF PROLIFERATION AND DIFFERENTIATION DYNAMICS WITHIN INTESTINAL EPITHELIAL CELLS

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ABSTRACT OF THESIS

RAB11A AND RAB11B ARE REQUIRED TO MAINTAIN PROLIFERATION AND DIFFERENTIATION IN INTESTINAL EPITHELIAL CELLS

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The trafficking of molecules to specific targets within a cell is essential for the proper maintenance of a cell. Specifically, the recycling endocytic pathway is essential for the movement of membrane associated proteins to distinct regions of the cell and cell membrane and is required for maintenance of cell polarity in epithelial tissues, including those found in the gastrointestinal tract. Small GTPase Rab proteins are commonly known as master regulators of the endocytic pathway because they primarily aid in the compartmentalization and targeting of trafficking vesicles. In this work we are specifically interested in the small GTPase Rab11 in polarized intestinal epithelial tissue. Rab11 has 3 isoforms, Rab11a, Rab11b and Rab11c with Rab11a and Rab11b having 89% amino acid similarity, indicating that the two isoforms could serve some redundant functions. Rab11 localization apically in polarized epithelial cells and is known to function in indirect recycling of vesicles through association with motor proteins, including Myosin Vb and effector proteins such as Rab11-FIPs. The loss of Rab11a has been shown to have detrimental effects on the cellular functions; with global deletion of Rab11a being embryonically lethal, and inducible intestine specific knockout resulting in
inhibited vesicular recycling and increased colonic tumorigenicity. However, the impact of Rab11b loss has not been well defined. We show here that inducible intestinal specific knockout of Rab11a or global deletion of Rab11b do not result in an immediate change in intestinal architecture, but upon the double knockout of Rab11a and Rab11b there is a dramatic loss of intestinal villus architecture that results in lethality 3 days post knockout. Moreover, double knockout of Rab11a and Rab11b results in differentiated cells reverting to a stem like state and hyperproliferation, likely through a rescue mechanism to restore homeostasis. Lastly, we show that loss of Rab11a and Rab11b results in the formation of monopolar mitotic spindles, likely as a result of an association between Rab11 and motor protein Kif11 being lost in DKO tissues.
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# TABLE OF CONTENTS

Abstract ........................................................................................................................................... ii

Figures ........................................................................................................................................... vi

Abbreviations ................................................................................................................................. vii-viii

Literature Review ............................................................................................................................. 1

Materials and Methods .................................................................................................................. 18

Results ............................................................................................................................................ 26

Discussion ....................................................................................................................................... 49

Conclusion ....................................................................................................................................... 58

Bibliography ..................................................................................................................................... 59

Supplemental Figures ....................................................................................................................... 64

Appendix .......................................................................................................................................... 68
FIGURES

Figure 1 .........................................................................................3
Figure 2 .........................................................................................7
Figure 3 .........................................................................................15
Figure 4 .........................................................................................27
Figure 5 .........................................................................................29-30
Figure 6 .........................................................................................31-32
Figure 7 .........................................................................................34
Figure 8 .........................................................................................35-36
Figure 9 .........................................................................................38
Figure 10 .......................................................................................39
Figure 11 .......................................................................................40
Figure 12 .......................................................................................42
Figure 13 .......................................................................................44
Figure 14 .......................................................................................46
Figure 15 .......................................................................................47
Figure 16 .......................................................................................48
Abbreviations

AKO: Rab11a knockout

BKO: Rab11b knockout

CBC: Crypt Base Columnar

CFTR: Cystic fibrosis transmembrane conductance regulator

CldU: 5-Chloro-Deoxyuridine

DAPI: 4’-6-diamidino-2-phenylindole

DKO: Rab11a/b knockout

EE: Early endosome

ER: Endoplasmic reticulum

FIP: Rab11 Family Interacting Protein

IBD: Inflammatory Bowel Disease

IdU: Iodo-Deoxyuridine

IEC: Intestinal Epithelial Cell

IF: Immunofluorescence

IHC: Immunohistochemistry

ISC: Intestinal stem cell

LE: Late endosome

MVID: Microvillus Inclusion Disease

pHH3: Phosphorylated Histone H3
Rab11BP: Rab11 Binding Protein

RE: Recycling endosome

Rip11: Rab11 Interacting Protein

SNARE: Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

STLC: S-Trityl-L-Cysteine

TA: Transit amplifying

TAM: Tamoxifen

TfR: Transferring Receptor

TGN: Trans golgi network
Literature Review

Vesicular traffic

The transport of intracellular vesicles is essential for the proper functioning of eukaryotic cells. Transport vesicles play a large role in the movement of molecules and proteins into, out of, and throughout the cell to ensure they reach their intended target. Intended targets could be to another intracellular location, the extracellular space, or another cell entirely. On the whole, transport falls into two main categories, exocytosis, in which materials are being transported out of the cell, and endocytosis, in which materials are being transported into the cell. However, these processes are often linked to one another through the trafficking networks within the cell (Tokarev et al 2000).

Vesicular transport allows cells to communicate with other cells within their environment and this communication can be controlled through changes in receptors and ligands on the cell surface, as well as integrins and cell adhesions at cell-cell junctions (Elkin 2016).

The general pathway of cellular traffic follows the cargo from the extracellular space or plasma membrane to endosomes, from which they are sent to either the trans-golgi network (TGN) for sorting or they mature to lysosomes for degradation (Cooper 2000, Schneeberger et al 2018). Vesicles sent to the TGN are either sent back to the plasma membrane through the recycling pathway or they are sent to the endoplasmic reticulum (ER) for further processing, dependent on cargo function. Importantly, not all endocytic vesicles or vesicle cargo are targeted to the same intracellular location, and there are a number of different paths these cargoes can take once within the cell. Within the exocytic pathway, synthesized proteins are trafficked from the ER to the Golgi or TGN where sorting occurs, as with endocytosed cargo, and the synthesized proteins meant for
secretion are trafficked to the plasma membrane for exocytosis (Apodaca et al 2012, Tokarev et al 2000). The recycling endosome pathway is considered to be the link between the endocytic and exocytic pathways (van Ijzendoorn 2006). Notably, recycling to the plasma membrane can occur only through the recycling endosome via an indirect mechanism or can occur through the sorting endosome via a direct mechanism (Ullrich et al 1996).

Within the endocytic pathway, cargo from the plasma membrane is taken into vesicles termed endosomes (Elkin et al 2009, Galvez et al 2012, Gu et al 2001, Grant and Donaldson 2009, Stamatovic et al 2017, Tokarev et al 2000). There are three known types of endosomes; the early endosome (EE), the recycling endosome (RE) and the late endosome (LE) (Galvez et al 2012). All internalized cargoes first enter the early endosome, where they are sorted and either sent on to the TGN for sorting and packaging (Gu et al 2001), to the lysosome for degradation or back to the plasma membrane via recycling endosomes (Elkin 2016). Generally, EE’s receive endocytosed cargo at the plasma membrane and mature into either LE’s or RE’s. While LE’s receive from the EE and traffic cargo to the TGN or mature into lysosomes and RE’s receive from EE’s or the TGN and traffic cargo back to the plasma membrane. An endosomes status as early, late or recycling is determined largely by the pH of the endosome; with recycling, early and late having decreasing pH values respectively, and the lysosome having the lowest pH (Stamatovic et al 2017). In addition to changes in pH, maturation of endosomes can include alterations in phosphatidylinositol lipids as well as activation of a variety of Rab GTPases which allow for the targeted movement of vesicles (Elkin 2016). The basic endosomal pathway is demonstrated as described in Figure 1.
In this work recycling endosome is of specific interest. The recycling endosome is responsible for the cycling of receptors and ligands to and from a cell's surface membrane, and thus is essential for the regulation of many signaling pathways (Grant and Donaldson 2009). Inhibition of the recycling endosomal pathway can result in a signaling pathway to be constitutively active or be disrupted. Due to its role in such a variety of pathways, the recycling endosome can play a role in a large number of processes within the cell, including cell-cell adhesions, cell migration, polarity, division and uptake of nutrients.

![Figure 1: Typical endosomal trafficking pathway](image)

Indicating the movement of endocytosed vesicles from different compartments within the cell; including the recycling endosomal pathway involving cargoes traveling from the early endosome or the TGN to the recycling endosome and to the surface of the membrane.
Vesicular Traffic Regulators - Small GTPases

The cellular trafficking networks aforementioned require regulators. Within the recycling network these regulators are primarily low molecular weight proteins, termed GTPases, including Rab and Arf family proteins (Grant and Donaldson 2009). Both the Rab and Arf families belong to the Ras superfamily, which also includes the Ras, Rho and Ran protein families (Subramani and Alahri 2010). GTPase proteins, often being termed molecular switches change between GTP-bound and GDP-bound states. The enzymatic conversion of GDP to GTP results in a conformational change of the protein and thus, alters the activity status of the protein. It is through these GTP-GDP cycles that trafficking occurs, with (in most cases) GDP-bound GTPase proteins being inactive within the cytoplasm and GTP-bound GTPases being active and having the ability to bind to effector proteins on vesicles or the cellular membrane and allow for targeting of the vesicle to the membrane for fusion (Prekeris et al 2003). These switches are done by utilizing Guanine nucleotide exchange factors (GEFs), which regulate the GTP-bound active state, and GTPase activating proteins (GAPs), which regulate the GDP-bound inactive state (Bhuin and Roy 2015, Grant and Donaldson 2009). Although in some cases, the GTP-GDP switch for Rabs can also be intrinsic, rather than requiring GAP’s for catalysis (Stenmark et al 2009).

In this work, we are particularly interested in Rab proteins. Rab proteins are known to be important in a variety of mechanisms within the cell, specifically in the modulation of vesicle transport, including processes such as vesicular targeting, movement, and the budding/fusion of trafficked vessels (Yu et al 2014). Rab proteins are often termed master regulators of membrane transport (Prekeris 2003). Rab proteins were
first identified in yeast (Parker et al 2018, Ben-Rached and Langley 2013, Bhuin and Roy 2014, Barr and Lambricht 2010), and have since been found in all eukaryotic cells (Parker et al 2018), indicating that they are essential for the life of a eukaryotic cell. There are 11 known Rab proteins within most yeast species while there are over 60 Rab proteins known within the human genome (Barr and Lambricht 2010). Many Rab proteins that are known to be involved in the maintenance of cellular polarity, including Rab4, Rab5, Rab8, Rab11 and Rab14, have been implicated in the last common eukaryotic ancestor (Parker et al 2018) further indicating the importance of Rab proteins, as well as polarity, in the maintenance of cells and tissues. Genetic analysis of Rab proteins indicates that there were likely gene duplications of many Rabs, as a result several isoforms contain high (75-95%) amino acid similarities and many overlapping functions (Stenmark and Olkkonen 2001). In their active (GTP-bound) state, Rab proteins are generally in association with membrane compartments within the cytosol and act alongside effector proteins in order to complete functions necessary for the compartments trafficking (Bhuin and Roy 2014). Rab proteins generally aid in the targeting and docking of vesicles through their associations with SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes and with motor proteins such as Myosin, Dynein and Kinesin family proteins and tether proteins such as Sec15 and Sec3 (Cooper 2000, Parker et al 2018, Shneeberher et al 2018, Welz et al 2014). As shown in figure 2, these processes involve a motor protein binding to vesicles, usually through association with a Rab protein complex, and mediates movement along microtubules or actin filaments to the plasma membrane. At the plasma membrane the endosomes are docked through an association between the vesicle bound v-SNARE and the membrane bound t-
SNARE (Schneeberher et al 2018). Conversion of GTP-Rab to GDP-Rab through GAPs disassociates Rab from the membrane fused vesicle and back into the cytosol. Rab proteins are vastly studied due to their connection with cellular trafficking and have been shown to have connections with genetic diseases, neurodegenerative diseases such as Alzheimer’s, infections and immune disorders, and a number of cancers (Li 2011, Guadagno and Progida 2019). Although Rab proteins are more commonly known as master regulators of the cell, more recent research connecting them to a number of diseases shows that they may be involved in a number of other functions including cell signaling, migration and division (Guadagno and Progida 2019). In some cases, a single Rab protein has been shown to be involved in several diseases through different mechanisms, indicating the vast functions they may have in a cell. For example, Rab11 is involved in neurodegenerative diseases by regulating neurotransmitter traffic as well as being involved in signaling mechanisms that result in the production of amyloid-β (Udayar et al 2013, Guadagno and Progida 2019). Additionally, Rab11 is thought to play a role in cancer progression through signaling mechanisms, including one that promotes hypoxia-driven metastasis (Yoon et al 2005, Guadagno and Progida 2019).
Small GTPase Rab11 in Vesicular Traffic

The specific interest of this research is Rab11, a small GTPase of the Rab family that has three isoforms, Rab11a, Rab11b and Rab25 (also identified as Rab11c). Rab11 is found in most tissue types; Rab11a is found in highest amounts in intestine and kidney epithelial tissue, Rab11b is found in highest concentrations within the brain, although it is also found in epithelial cells, including those of the intestine (Kumar and Lukman 2018) and Rab25 is found in most epithelial cells. All three Rab11 isoforms are known to play roles in vesicular traffic, being involved in both endocytosis and exocytosis of vesicles through mediation of the recycling endosome (Casanova et al 1999, Landry et al 2009, Wang et al 2017, Yu et al 2014). However, Rab11a and Rab11b amino acid sequences are 89% similar to one another, while Rab25 has been shown to have only 61% similarity.
to Rab11a and 66% similarity to Rab11b (Kumar and Lukman 2018). Thus indicating that Rab11a and Rab11b could have some redundancy in their function and/or mechanisms, although this has yet to be determined experimentally. Conversely Rab25, although it is also involved in vesicular trafficking, serves functions apart from those of Rab11a and Rab11b (Wang et al 2017). As a result, this research focuses on Rab11a and Rab11b isoforms and does not evaluate Rab25.

Rab11 is associated with the recycling endosome as well as pericentriolar recycling compartments, internalized transferrin, the TGN and post-golgi vesicles (Ullrich et al 1996, Wilcke et al 2000). Rab11a is most often found to have apical localization within the recycling endosome in epithelial cells (Hales et al 2001, Sobajima et al 2015) and plays a role in the localization of apical proteins in intestinal epithelial cells (Sobajima et al 2015). The trafficking of recycling endosomes is mediated primarily by Rab11 through regulation of the movement of membranes and their associated signaling molecules to the plasma membrane or trans-golgi network (Landry et al 2009) and Rab11 regulates the indirect recycling endosome pathway via cargo proteins such as Myosin Vb (Bouchet et al 2018).

**Small GTPase Rab11 effector proteins - Myosin Vb, Dyneins and Kinesins**

Rab proteins often require effector proteins to allow for proper functioning. Rab11 interacts with a number of proteins, including Myosin Vb, Rab11-FIPs (Rab11 family interacting proteins) and some Dynein and Kinesin proteins (Bouchet et al 2018). Myosin Vb is a motor protein with a homodimer structure containing an N terminal motor domain, a central light chain and a c-terminal domain onto which Rab11 binds
(Knowles et al 2014). Myosin Vb has been found to interact with Rab11a, Rab11b and Rab25, often by associating with traffic vesicles that contain one of the Rab11 isoforms (Hales et al 2001, Hales et al 2002). The binding of Myosin Vb to Rab11-bound recycling vesicles allows for the Rab11 dependent movement of such recycling vesicles along microtubule and actin networks within the cell (Bouchet et al 2018, Feng et al 2017, Hales et al 2001, Hales et al 2002, Schneeberher et al 2018, Stenmark et al 2009). Such movements aid in the establishment of cell polarity in intestinal epithelial cells (Schneeberher et al 2018) and thus, the interaction between Rab11 and Myosin Vb, although not the focus of this research, is important to note when discussing changes in intestinal Rab11 and intestinal epithelial cells.

In addition to Myosin Vb, Rab11 has been shown to interact with Rab11 binding protein (Rab11BP) in its GTP-bound state for the regulation of transferrin receptor (TfR) as well as Rab11 interacting protein (Rip11), forming a complex that is typically located within the apical recycling endosome (Hales et al 2001). Rab11 has also been demonstrated to interact with a number of Rab11-FIPs (Rab11 Family Interacting Proteins), which function as effector proteins for Rab11 (Hales et al 2001). There are 5 Rab11-FIPs known to bind to Rab11 isoforms that are generally grouped into two classes (Bouchet et al 2018, Horgan and McCaffrey 2009). Class I Rab11-FIPs (FIP1, FIP2 and FIP5) are associated with mediation of endosomal trafficking whereas class II (FIP3 and FIP4) are associated with endosome mediation during mitosis (Hogan and McCaffrey 2009). Structurally, Rab11-FIPs contain several domains, one of which regulates the interaction between the FIP and Rab11 in a GTP dependent manner (Bouchet et al 2018). Additionally, Rab11-FIPs have been shown to bind to a number of motor proteins, most
notably Myosin Vb as well as Dynein and Kinesin family proteins. Rab11-FIPs interact with Rab11 and act as effectors for motor protein binding, allowing the linking of Myosin Vb, Dyneins or Kinesins to Rab11 containing recycling vesicles (Setnmark et al 2009). Examples of such interactions include that seen between Rab11, Myosin Vb and Rab11-FIP2 (Hales et al 2002), as well as an association between Kif13A, Rab11 and Rab11-FIPs that results in tubule formation within recycling endosomes (Delevoye et al 2014).

In addition, Schonteich and colleagues (2008) demonstrate an association between Rip11 (Rab11-FIP5) and Kinesin II that plays a role in traffic of endocytosed vesicles, specifically for receptor recycling. Ultimately, Rab11 and Rab11-FIP binding results in a protein complex that aids in vesicle targeting and recruitment within the endocytic pathway (Prekeris et al 2003).

**Consequences of altered Rab11**

The is a substantial number of research projects that have shown that the altering of Rab11 *in vivo* can lead to dramatic changes within the system. Most notably, global knockout of Rab11a in mice was found to be embryonically lethal due a disruption in blastocyst implantation (Yu et al 2014). Research conducted by Ren and colleagues (1998) has demonstrated that a dominant negative Rab11 will result in an inhibition of transferrin recycling that is not present in the wild type or Rab11-constitutively active mice. Additionally, Rab11a knockout mice were shown to have a reduced efficiency of TfR (Transferrin Receptor) recycling, although the recycling was not completely inhibited (Yu et al 2014). More recent work has shown increased proliferation and tumorigenesis of Rab11a intestinal specific knockout (D’Agostino et al 2019).
contrast, the specific functions of Rab11b or the result of constitutively active or lost Rab11b in an in vivo model has not been well defined.

Research has shown the loss of Myosin Vb in intestinal tissues leads to a loss of microvillus structures as well as changes in the intestinal junctions and trafficking (Knowles et al 2014). Additionally, the loss of the interaction between Myosin Vb and Rab11 was shown to cause microvillus inclusions. Microvillus inclusion disease (MVID), which is identified with a loss of microvilli and apical membrane proteins (Parker et al 2018), as well as a presence of microvillus inclusion bodies within intestinal enterocytes (Feng et al 2017), is closely linked to Myosin Vb. MVID generally develops quickly after birth (within days) and is characterized by persistent diarrhea and malnutrition (Schneeberger et al 2015). MVID has been linked to a genetic mutation in Myosin Vb or in Syntaxin-3, both of which have been shown to play roles in trafficking, specifically in polarized cells, through interactions with Rab8 and Rab11 (Parker et al 2018, Schneeberger et al 2015), and it has been established that loss of function or deletion of Myosin Vb results in MVID (Vogel et al 2015). Further studies evaluating the effects of Rab11a loss shows the mis localization of the apical proteins within the microvilli of the intestines as well as atrophy of the microvillus and, in some cases, microvillus inclusions (Feng et al 2017 and Sobajima et al 2015).

While our focus is within intestinal epithelial tissue, Rab11 loss has also been evaluated in a number of other tissues. Research conducted by Reynier and colleagues (2016) found that loss of Rab11a within human epidermal cells in vitro resulted in a barrier defect as well as a defect in sorting and trafficking. Additionally, research conducted by Xu and colleagues (2011) investigating the impact of Rab11 loss within
follicle stem cells found disruption of stem cell differentiation, which was attributed to loss of cell polarity. Research conducted by Butterworth and colleagues (2012) demonstrated the role of both Rab11a and Rab11b in the trafficking of sodium channels in several epithelial tissues, with the inhibition of Rab11b and to a lesser extent, Rab11a, resulting in a decrease in the sodium channels present in kidney epithelial tissue. Additionally, Silvas and colleagues (2009) demonstrated that loss of Rab11b resulted in a dramatic decrease in cystic fibrosis transmembrane conductance regulator (CFTR) expression at the membranes of polarized epithelial cells. Although these studies are in a range of models, ultimately the consequences of the loss of Rab11 generally involves changes in recycling traffic that impacts polarity, as well as possible changes in proliferation.

**Intestinal Architecture**

This work focuses on the function of Rab11 in the small intestine. The small intestine is made up of projection like structures termed villi, which increase the surface area of the intestinal lumen in order to increase absorption of nutrients during digestion. The small intestine has a crypt-villus architecture and is made up of crypt regions, transit amplifying zones and the villus, with each region housing specialized cells that aid in its function. The crypt regions contain Paneth cells, which function as the secretory cells of the crypts, and stem cells, which function to replace the epithelial cells lost daily in order to maintain a healthy villus. The stem cells within the crypt can be identified utilizing a range of parameters, but in general there are two distinct subsets of stem cells, the actively proliferating crypt-base columnar cells (CBCs) and the label-retaining, or quiescent, stem cells at the +4-6 region on the crypt. The stem cells within the crypt all
express Lgr5 in homeostatic condition, although those that are actively proliferating (CBCs) have a higher expression of Lgr5 in comparison to the quiescent stem cells (Gonzalez et al 2019). There are a number of markers that can be used to differentiate the cells within the crypt, including the widely used intestinal stem cell marker Olfm4, which marks both the active and quiescent stem cells (van der Flier et al 2009). Additionally, Bmi-1 and HopX mark the quiescent stem cells found predominantly at the +4 region of the crypt, Sox9 marks both quiescent and active stem cells, mTert marks a distinct subset of stem cells that are radiation resistant, and Lrig marks highly proliferative stem cells within the crypt (Clevers 2013). All cells within the intestinal crypt and villi are daughter cells of the proliferative stem cells found within the crypt, and as the cells proliferate the daughter cells migrate up towards the +4 position, then to the transit amplifying region and finally to the villus where cells are shed into the lumen through anoikis. The exception to this is the secretory Paneth cells that are found at the base of the crypts. As with all other cell types, Paneth cells arise from the intestinal stem cells. However, while all other cell types differentiate as they move up towards the villus, Paneth cells differentiate and migrate down towards the bottom of the crypt, where the Wnt gradient is highest and allows for Paneth cell maturation (Gasslet 2017 and Stappenbeck 2009). Generally, the loss of contact with a mature Paneth cell at or around the +5 position is when daughter cells are considered to be at the common origin of differentiation, at which point they begin to commit to a specific differentiated state. The transit amplifying zone is located above the crypts and is composed of proliferative and differentiating cells that will become the specialized cells within the villus structure once mature (Clevers 2013). The villus is composed of a single layer of primarily epithelial cells but also
includes goblet cells and enteroendocrine cells, both having a secretory function, as well as tuft cells which aid in the immune response. The basic intestinal structure and various cell types are demonstrated in Figure 3. When the homeostatic levels of different cell types change, either with loss of a specific cell type or a dramatic increase in a specific cell type, it is generally indicative of a response to intestinal insult or injury.

**Intestinal signaling pathways**

The differentiation, migration and maintenance of stem cells in the adult occurs through three major signaling pathways; Wnt, BMP and Notch (Umar 2010). Each pathway operates with a gradient in a specific subset of cell in the intestine and aids in the maintenance of intestinal homeostasis. The canonical Wnt signaling in the adult intestine is highest in cells at the bottom of the crypts, decreases as cells move upwards through the TA zone and is absent in the differentiated cells of the villus (Spit et al 2018). Wnt signaling acts through membrane proteins Frizzled (Fz) or low density lipoprotein receptor-related protein (LRP) to induce transcription of genes thought to promote proliferation and a stem-like state (Umar 2010, Sancho et al 2003). Conversely, BMP (Bone morphogenic protein) signaling in the adult intestine is lowest in differentiated cells closest to the TA zone and increases as cell move towards the tip of the villus (Spit et al 2018). BMP signaling acts through both type I and type II BMP receptors and act by phosphorylating smad proteins that leads to the transcription of genes that drives differentiation of intestinal cells as well as acting as an antagonist of crypt formation and intestinal stem cell renewal (Scoville et al 2008, Zhang and Linheng 2005, Spit et al 2018). Notch signaling is found primarily in the intestinal crypts, with stem cells
expressing the highest amount of Notch and secretory Paneth and Goblet cells having lower expression. Additionally, Hedgehog signaling (Indian hedgehog and Sonic hedgehog) pathways are important in the development of the villi and crypt structures from the endodermal tube in neonatal mice (Madison et al 2004). Disruption of these signaling pathways could result in dramatic consequences for intestinal homeostasis including loss of differentiated or stem cell populations and changes in the migration of differentiating cells up the villus.

**Figure 3:** Typical intestinal architecture; Demonstrating the crypt-villus axis, with active stem cells and Paneth cells residing at the base of the crypt and quiescent stem cells at the +4 position as well as absorptive intestinal epithelial cells with dispersed secretory goblet, enteroendocrine and tuft cells. The crypt, transit amplifying zone, villus, BMP and Wnt signaling, as well as proliferative cells, differentiated cells and apoptotic cells.
Cell Polarity in Intestinal Epithelial Cells

An essential characteristic of intestinal epithelial cells is polarity. Polarity of intestinal epithelial cells (IECs) is essential in the maintenance of barrier formation within the gut, as well as the uptake and transport of nutrients (Schneeberger et al 2018). Cellular polarity is defined as asymmetric localization of cellular compartments that leads to the establishment of an apical and basolateral membrane (Parker et al 2018 and Schneeberger et al 2018). Loss of polarity in IECs has been shown to lead to malnutrition, cancers and IBD (inflammatory bowel disease) (Apodaca 2012, Parker et al 2018, Schneeberger et al 2018). Within intestinal epithelial cells, the apical membrane faces the lumen, and thus encounters the environment within the small intestine, including the nutrients and microorganisms within it (Schneeberger et al 2018), whereas the basolateral membrane faces the basement membrane towards the lamina propria within the villus. Extracellular cell-cell contact and apical interaction with the extracellular matrix, as well as proper intracellular assembly of membrane polarity complexes, are required for maintenance of cell polarity (Parker et al 2018). In order for IECs to achieve polarity, proper localization of vesicle compartments within the cell is required and is achieved through directed traffic of membrane and protein lipids. Several Rab proteins have been found to be involved in the maintenance of cell polarity, specifically, Rab11 has been shown to colocalize the apical recycling endosome (Sobajima et al 2015), and loss of Rab11a results in the loss of cell polarity within the small intestine (Xu et al 2011).

In this study, the consequence of the loss of both Rab11a and Rab11b within intestinal epithelial cells is evaluated. Previous work shows the function of Rab11 and the role it plays in the recycling endosomal pathway. Additionally, the importance of the
recycling endosome in the maintenance of a cell and in intestinal epithelial polarity has been previously established. Thus, it is of interest to evaluate the specific role Rab11 has on intestinal homeostasis, and if these roles are shared by the two isoforms A and B. Utilizing previous research it can be hypothesized that the loss of both Rab11a and Rab11b would result in dramatic changes in the intestinal architecture and cell type populations, and could result in disruptions in intestinal polarity and homeostasis. To investigate the hypothesis, changes in crypt-villus intestinal architecture, as well as intestinal stem cell, secretory cell, brush border and proliferative dynamics is evaluated. Additionally, the disruption of bipolar spindle formation upon loss of Rab11 as a result of possible Rab11 association with mitotic spindle associated motor protein Kif11 is evaluated. We find there to be a dramatic loss of intestinal architecture within the small intestine upon the loss of both Rab11a and Rab11b that results in lethality on day 3 post tamoxifen injection, a phenotype that is not observed within single knockout mice. Additionally, we observe changes in stem cell, proliferation and differentiation dynamics as a result of the loss of both Rab11a and Rab11b, likely linked to the lost association between Rab11 and motor protein Kif11 being lost in Rab11a/b double knockout mice.
Materials and Methods

Mice

Rab11a\textsuperscript{FL/FL};VillinCreER mice were obtained as described by Yu et al 2014 and Rab11b\textsuperscript{−/−} was obtained utilizing CRISPR/Cas9 as described by D’Agostino et al 2019. Rab11a/Rab11b double knockout mice were obtained by crossing Rab11a\textsuperscript{FL/FL};VillinCreER mice with Rab11b\textsuperscript{−/−} mice to generate a Rab11a inducible knockout in a Rab11b null background. Double knockout, Rab11a knockout and Wild type mice were injected intraperitoneally with (Z)-4-Hydroxytamoxifen (4-OHT) (20mg/ml) dissolved in corn oil and sacrificed 1, 2, or 3 days post 4-OHT (Tamoxifen) injection, Rab11a knockout and wild type mice were sacrificed 2 days post tamoxifen injection. Intestinal duodenum, jejunum and ileum tissues were collected. Tissues for lysates were stored at -80°C until use and tissues were imaging were cut diagonally and swiss rolled before being fixed in formalin and further imbedded in paraffin for sectioning.

Preparation of lysates

Intestinal tissue samples from DKO and WT mice were placed in lysis buffer (containing 50mM Tris-HCl, pH 7.5, 150mM NaCl, 10mM EDTA, 0.02% NaN\textsubscript{3}, 50mM NaF, 1 mM NaN\textsubscript{3}VO\textsubscript{4}, 0.5% PMSF, 0.5mM DTT and 1 tablet each of Protease and Phosphatase inhibitors) and sonicated until homogenous. Samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant was collected. Lysate protein concentration was determined via Bradford assay. Lysate samples were prepared for western blot analysis at a concentration of 1 ug/\mu L and were prepared with 1X LDS and
10% β-mercaptoethanol before being heat denatured at 95°C for 10 minutes. Prepared samples were kept at -80°C until use.

**Immunoblotting**

Prepared lysates were loaded into a 10% polyacrylamide gel at a concentration of 1 ug/μL and run at around 100V at room temperature within running buffer containing Tris-HCl, Glycine and SDS for about 2 hours. Proteins were transferred to Nitrocellulose membrane via a western blotting apparatus run at 300mA for 1.5 hours in chilled transfer buffer containing Tris-HCl and Glycine at a pH of 7.4. Nitrocellulose membrane with transferred proteins was blocked in 5% milk in TBS with 0.1% Tween-20 for 1 hour at room temperature. Blocked membrane was incubated at 4°C in 5% BSA in TBS-Tween with desired primary antibody overnight, antibodies and concentrations are listed in the appendix. Following primary antibody incubation, membranes were washed with TBS-Tween (3x times quickly followed by 3 times for 10 minute periods). Membranes were incubated with 5% Milk in TBS-Tween with HRP-conjugated secondary antibody for 1 hour. Membranes were again washed with TBS-Tween before being treated with ECL solution in the dark for 3 minutes and placed in a cassette for development. Membranes were developed in a dark room utilizing medical X-ray film with varying, antibody specific, exposure times.

**Genotyping – PCR**

Toe tissues samples from mice pups of unknown genotype were collected and treated with NID buffer with Proteinase K at 55°C overnight. Samples were then denatured at 95°C for 15 minutes and placed on ice until use. Two μL of sample was added to 18μL of PCR master mix containing the following; 11.7μL of Millipore water,
4μL of 5X go taq Flexi Buffer, 1.2 μL of MgCl₂, 0.5 μL of 10 mM dNTPs, 0.5 μL of primer mix and 0.1μL of Taq polymerase. Samples with master mix were placed in a thermocycler programed for the gene of interest. Following the completion of the thermocycler program, samples were loaded onto a 1.5% agarose DNA gel and run at 200V for 1.5 hours. The primers and thermocycler programs for genes of interest are listed in the appendix.

**Histology – Hematoxylin and Eosin**

Formalin fixed paraffin embedded mouse intestinal sections were heated at 55°C for 15 minutes to soften wax. Tissue slides were then deparafinized in Xylene and rehydrated in decreasing concentrations of ethanol; 100% Xylene 2 times for 5 minute incubations, 100% ethanol for 1, 2 minute incubation, 95% ethanol for 2, 1 minute incubations, 75% ethanol for 1, 1 minute incubation, 70% ethanol for 1, 1 minute incubation and 50% ethanol for 1, 1 minute incubation. Tissue slides were washed in running deionized water for 5 minutes. Tissue slides were then incubated in Hematoxylin QS (Vector labs H-3404) for 3-5 minutes and washed in running deionized water for 5 minutes. Tissue slides were then incubated in Eosin for 2 minutes and dehydrated in increasing concentrations of ethanol; 50%, 70%, 75%, 95% (2 times), 100% (2 times) and Xylene (2 times) for 20 dips each. Dehydrated tissue slides were mounted using Cytoseal 60 (Thermo Scientific 8310-4) and cover slipped.

For alcian blue staining, tissue slides were heated and deparafinized in xylene and ethanol as described above. Tissue slides were washed in running deionized water for 5 minutes and incubated in 3% alcian blue 8GX (Sigma, A5268, pH 2.5) for 30 minutes and then washed in running deionized water for 5 minutes. Tissue slides were
counterstained with Nuclear red (Vector H-3403), dried and mounted as described above.

**Immunofluorescence**

Formalin fixed paraffin embedded mouse intestinal sections were heated at 55°C for 15 minutes to soften wax. Tissue slides were then deparaffinized in Xylene and rehydrated in decreasing concentrations of ethanol; 100% Xylene 2 times for 10-minute incubations, 100% ethanol 2 times for 5 minute incubations and 70% ethanol 2 times for 5 minute incubations. Tissue slides were washed in running deionized water for 5 minutes and PBS for 5 minutes. Slides were then boiled in monohydrate citric acid buffer (pH 6) for 15 minutes. Once cooled to 40°C, slides were again washed in deionized water for 5 minutes followed by PBS wash for 5 minutes. Slides were incubated for 1 hour with blocking solution (0.1% Triton-X100, 2% BSA, 2% donkey serum in PBS). Following blocking, primary antibody diluted in blocking buffer was added to the slides and incubated at 4°C overnight. Antibodies used and concentrations are listed in the appendix. Following overnight incubation of primary antibody, slides were washed in PBS for 10 minutes 3 times. Secondary antibodies diluted in blocking buffer were added to slides and incubated for 1 hour. Following incubation with secondary antibody, slides are washed in PBS for 10 minutes 2 times and incubated with DAPI for 15 minutes. Slides are then washed in PBS for 10 minutes, dried and mounted using Prolong Gold Antifade (Invitrogen P36930), cover slipped and dried in the dark for at least 24 hours. All images were taken on a Zeiss LSM 510.

When staining for Sox9, an additional Tyramide amplification step was completed. After rehydration and washing, slides were quenched for endogenous
peroxidase activity in methanol containing 3% H.O. at room temperature prior to wash steps. Antigen retrieval was done in DAKO target retrieval solution (Agilent S1699) and boiled in a rice cooker for 15 minutes. For TSA, secondary antibodies used were poly-HRP-conjugated IgG. Following incubation for 1-hour slides were washed in PBS for 10 minutes 3 times. Slides were then incubated in TSA working solution containing 1% H.O. and AF488 conjugated TSA at a concentration of 1:200 in 1X amplification buffer (provided with kit) for 15 minutes. Slides were then washed with PBS for 10 minutes 3 times and other desired antibodies, such as E-cadherin, were added for overnight incubation. Following second primary incubation slides were washed, incubated with secondaries and mounted as described above.

When staining for Alkaline Phosphatase substrate an additional step for Vector Red staining was completed. After rehydration and washing, slides were incubated in 125mM Tris-HCl, pH 8.4 containing 0.5% Vector red substrate (2 drops of reagent 1, 2 drops of reagent 2 and 2 drops of reagent 3 in 5 mL of deionized water) (Vector SK-5100), for 30 minutes. Slides were then washed in running deionized water for 5 minutes and were continued to antigen retrieval and subsequent staining steps as described above.

When staining cells, cells where fixed in 4% paraformaldehyde and permeabilized in PBS containing 0.3% Triton X-100. Cells were then blocked in PBS containing 5% donkey serum and with primary antibody in blocking buffer 4°C overnight, dilutions in appendix. Following primary antibody incubation cells were washed with PBS and incubated with fluorescent tagged secondary antibody and DAPI in blocking buffer at room temp for 1 hour. Cells were again washed with PBS and mounted using Prolong
Gold Antifade (Invitrogen P36930), cover slipped and dried in the dark for at least 24 hours. All images were taken on a Zeiss LSM 510.

**Immunohistochemistry**

Formalin fixed paraffin embedded mouse intestinal sections were heated at 55°C for 15 minutes to soften wax. Tissue slides were then deparaffinized in Xylene and rehydrated with decreasing concentrations of ethanol; 100% for 2 10-minute incubations, 90%, 80% and 70% for 5 minute incubations. Tissue slides were washed in deionized water and PBS for 5 minutes each and then quenched for endogenous peroxidase activity in 3% hydrogen peroxide in methanol for 5 minutes. Antigen retrieval was performed in boiling monohydrate citric acid buffer (pH 6) for 15 minutes using a microwave and cooled to 40°C. Slides were washed under running DI water for 5 minutes, then washed in PBS 5 minutes and blocked for non-specific antibody binding in PBS with 2% BSA, 10% Triton-X100 and 2% donkey serum for 1 hour. Primary antibody in blocking buffer was added and incubated at 4°C overnight, concentrations are listed in the appendix. After overnight incubation, tissue slides were washed in PBS three times for 10 minutes. Biotin conjugated secondary antibody was added in blocking solution and incubated for 1 hour at room temperature and subsequently washed 3 times for 10 minutes each wash. Following secondary antibodies, tissue slides were incubated in an Avidin Biotin Complex (ABC) solution containing 2 drops of reagent A and 2 drops of reagent B (Vector Labs ABC standard kit, SK-4100) in 5ml of PBS (ABC solution was prepared 30 minutes prior to use) for 1 hour. Tissue slides were then washed again in PBS for 10 minutes 3 times. Following washing, tissue slides were incubated in DAB solution containing 2 drops of buffer, pH 7.4, 4 drops of DAB substrate and 2 drops of hydrogen
peroxide. DAB reaction time was antigen specific, ranging in time from 30 seconds to 5 minutes. Following DAB incubation slides were placed in deionized water and washed for 5 minutes. Tissue slides were incubated in Hematoxylin QS (Vector labs H3404) for 3 minutes and again washed in deionized water for 5 minutes. Tissue slides were then dehydrated in the reverse order they were rehydrated in. Following Xylene slides were mounted with Cytoseal 60 (Thermo Scientific 8310-4) and cover slipped.

**CldU/IdU experiments**

Wild type mice (Rab11a\textsuperscript{fl/fl}) and double knockout mice (Rab11a\textsuperscript{fl/fl}-Rab11b\textsuperscript{-/-};VillinCreER) were treated intraperitoneally 200μl of (Z)-4-Hydroxytamoxifen (4-OHT) (20mg/ml) dissolved in corn oil at hour 0 and were treated with CldU (5-Chloro-2’-deoxyuridine) intraperitoneally at 24 hours and IdU (Iodo-Deoxyuridine) at 48 hours. Mice were sacrificed at 51 hours post tamoxifen injection and the small intestine was collected and divided into Jejunum, Duodenum and Ileum segments. Collected tissues were fixed with formalin and embedded in paraffin for sectioning.

**Co-Immunoprecipitation**

HEK293T cells were transiently transfected with 3xFlag-Rab11a and 3xFlag-Rab11b plasmid. Transfected cells were lysed in lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and Roche proteinase inhibitor) and 2 mg of protein lysate was added to 40 μl of anti-Flag affinity beads (A2220, Sigma), followed by overnight incubation at 4°C. Lysate and bead solution was centrifuged at 8200g for 1 minute and washed 3 times (washing buffer 50mM Tris-HCl pH 7.4 and 150mM NaCl). Following wash proteins were eluted and analyzed by immunoblotting, as described above.
**Kif11 Inhibition**

HEK293T cells were grown to 50% confluency in DMEM media containing 20% FBS in a four well chamber slide. Cells were then treated with either 0.5% DMSO as a control, 50µM Monastrol, 2.5µM STLC or 100nM Ispinesib, all within DMSO, for 24 hours and then fixed, permeabilized and stained as described above. For Co-immunoprecipitation HEK293T cells were treated with Monastrol, STLC, or Ispinesib 24 hours post transfection and were lysed 24 hours post Kif11 inhibitor treatment.

**Quantification and Statistical Analysis**

Quantification of mean grey scale values of IHC images was done utilizing the IHC tool in ImageJ. Images were converted to grayscale and inverted, and the mean grey scale of each region of interest (ROI) was measured. Grey scale distribution of pixels within Olfm4 images was derived from these values, and high (≥ 165 intensity) vs low (164-60 intensity) values were derived from the distribution histogram, shown in the appendix. Quantification of mean grey intensity of Lysozyme and Sox9 IF images was done by converting IF images to grayscale and utilizing ROIs to measure mean grey intensity. Distribution of pixel intensity for Sox9 was utilized to determine high (intensity of 101-256) or low (intensity of 1-100) expression. Quantification of Alcian blue, Cleaved Caspase, pHH3 and CldU/IdU positive cells was done by counting the number of positive cells per each continuous crypt-villus axis or crypt within images. Quantification of Kif11 inhibition in Rab11 Co-immunoprecipitation was standardized to Flag and Kif11 CT band size and completed by measuring band intensity in ImageJ. All statistical analysis was done on GraphPad Prism software with unpaired T-tests.
Results

Rab11 double knockout results in compromised intestinal structure

For analysis of the role of Rab11a and Rab11b within mouse intestinal tissue, inducible deletion of Rab11a within intestinal epithelial cells (IEC) using Rab11a<sup>fl/fl;</sup> VillinCreER (AKO) mice, Rab11b knockout mouse (Rab11b<sup>−/−</sup>) (BKO) and Rab11a/Rab11b double knockout (DKO) inducible with tamoxifen mice were obtained. Analysis of Rab11a and Rab11b intestinal tissue concentration through immunoblotting show Rab11b absent in BKO and DKO mice, and Rab11a is decreased 24 hours and 48 hours post tamoxifen injection and absent 72 hours post injection in AKO and DKO mice (Figure 4A). Intestinal phenotypic changes were then assessed in both the single and double knockout mice. Analysis of mouse intestinal structure utilizing Hematoxylin and Eosin (H&E) staining show that the single loss of Rab11a or Rab11b does not result in any dramatic change in intestinal structure, with the typical crypt-villus structure found in wild type mice maintained in both the Rab11a and the Rab11b single knockout animals (Figure 4B). These findings are consistent with those previously reported (D’Agostino et al 2019, Yu et al 2014). Conversely, when both Rab11a and Rab11b are removed the architecture of the intestine changes dramatically (Figure 4B), resulting in lethality on the third day post tamoxifen injection (Unpublished data). The crypt-villus structure is maintained in DKO mice in the first day following tamoxifen injection, however, by the second day the villus structure becomes blunted and the crypts elongated. The intestinal structure on the third day is severely compromised, with the villi being almost completely absent throughout the tissue.
Figure 4: Loss of Rab11 results in disrupted intestinal architecture.

A: Western blots for Rab11a and Rab11b using intestinal lysates from wild type (WT, lane 1), Rab11a<sup>FL/FL</sup>; VillinCreER 48 hours post tamoxifen treatment (AKO Lane 2), Rab11b<sup>−/−</sup> (BKO lane 3), and Rab11a<sup>FL/FL</sup>; VillinCreER; Rab11b<sup>−/−</sup> 24 hours (DKO day 1, lane 4), 48 hours (DKO day 2, lane 5) and 72 hours (DKO day 3, lane 6) post tamoxifen treatment. B: Histological sections of AKO, BKO and DKO 1, 2 and 3 days following tamoxifen injection.
Dysregulation of intestinal stem cells in Rab11 double knockout

In order to begin assessing possible mechanisms leading to the observed phenotype and due to the intestinal stem cell’s role in repopulating the villus, changes in the stem cell niche were assessed. Immunohistochemistry (IHC) for the intestinal stem cell marker Olfm4 indicates the dysregulation of stem cells upon the loss of both Rab11a and Rab11b (Figure 5A). The wild type, AKO, BKO and DKO day 1 stem cells have robust brown staining and show the typical triangular shaped stem cells at the bottom of crypts. Upon the loss of both Rab11a and Rab11b, the robust concentrated staining is lost. In DKO day 2 and day 3 tissue, although Olfm4 is present, the staining is less intense and more diffuse within the cells of the crypt. This is further demonstrated when observing the distribution of Olfm4 staining intensity, with a decrease in the number of pixels at high intensity in the double knockout tissue (Figure 5B, Supplemental Figure 1). Observing the average percentage of pixels at high intensity vs low intensity shows a decrease in high and an increase in low intensity Olfm4 staining within the crypts of DKO day 2 and day 3 tissues, as well as Rab11b although to a lesser extent, when compared to the wild type (Figure 5C). No significant difference in the intensity of staining was observed in the AKO or DKO day 1 tissues when compared to the wild type (Figure 5C). Additionally, we observed changes in Sox9, a transcription factor that has been previously shown to be present in intestinal stem cells, with quiescent stem cells having higher levels than actively proliferating stem cells (Prévostel and Blache 2017). Within all genotypes Sox9 staining can be found localized in the nucleus at the base of the crypt within ISCs as well as within cells in the transit amplifying zone, with an observed decrease in Sox9 staining in all knockout tissues (Figure 6A). Distribution of
pixel intensity of Sox9 staining shows a decrease in the number of high intensity pixels in double knockout tissue (Figure 6B, Supplemental Figure 2). Quantitative analysis of the percent staining at low intensity and high intensity Sox9 staining indicates a decrease in high expressing cells in all knockout tissues (Figure 6C)
Figure 5: Changes in Olfm4 expression in double knockout tissue
A: Immunohistochemistry for Olfm4 shows robust levels of Olfm4 in the WT, AKO, BKO and DKO day 1 tissues and diffuse levels of Olfm4 in Rab11 DKO day 2 and day 3 tissues. Black arrows indicate intestinal stem cells of robust (high) staining with triangular shape.
B: Histogram analysis of the pixel intensity of Olfm4 staining shows there to be a significant decrease in intense staining (pixels with an intensity of ≥165) and increase in low intensity staining (pixels with an intensity between 164 and 60) in DKO day 2 tissues when compared to the wild type.
C. Percent pixels in the high and low intensity range show a decrease in high intensity and increase in low intensity pixels in BKO, DKO Day 2 and DKO Day 3 tissue and no significant change in the AKO or DKO day 1 intensity values is observed. ***p≤0.001, t-test.
A

**Sox9/DAPI/ECAD**

<table>
<thead>
<tr>
<th>WT</th>
<th>AKO</th>
<th>BKO</th>
</tr>
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<tbody>
<tr>
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<td><img src="image2.jpg" alt="Image" /></td>
<td><img src="image3.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>

**DKO Day 1**

**DKO Day 2**

**DKO Day 3**

B

**Sox9 Intensity Distribution**

![Chart](chart.png)

- **WT**
- **DKO Day 2**
Figure 6: Changes in Sox9 expression in double knockout tissue

A: Immunofluorescence for Sox9 shows a decrease in crypt Sox9 expression in all knockout tissues. B: Histogram analysis of the pixel intensity of Sox9 staining shows there to be a significant decrease in intense staining (pixels with an intensity of ≥100) and increase in low intensity staining (pixels with an intensity <100) in DKO day 2 tissues when compared to the wild type. C. Percent pixels in the high intensity range and percent pixels in the low intensity range show a decrease in high intensity and increase in low intensity pixels in all knockout tissues. ****p≤0.0001, **p≤0.01, *p≤0.05, t-test.
Loss of differentiated intestinal cell types in Rab11 double knockout

We next investigated several differentiated cell type markers, including the brush border associated alkaline phosphatase (AP) and Villin, and secretory Goblet and Paneth cells, in order to assess possible changes in differentiation upon loss of Rab11. AP, known to be present on the borders of villi and have roles in inflammation and pH balance (Fawley and Gourlay 2016), is dramatically reduced in DKO tissues (Figure 7A). Similarly, there is a dramatic reduction in the expression of the brush border associated protein Villin (Figure 7B). Additionally, the number of secretory goblet cells within double knockout mice are reduced significantly by day two post tamoxifen injection and are even further reduced on day three (Figure 8A and 8B). There is no significant change in the AKO, BKO and DKO day 1 tissues when compared to wild type. The presence of lysozyme, indicative of the lysozyme producing Paneth cells within the crypts, is also significantly reduced in all DKO, as well as BKO, tissues (Figure 8C and 8D).
Figure 7: Brush border Disruption in Double knockout tissue. A: Immunofluorescence for Alkaline Phosphatase (Red) shows a dramatic decrease in expression in double knockout tissues days 2 and 3 post tamoxifen injection. B: Immunofluorescence for Villin (Red) shows a dramatic decrease in expression in double knockout tissues days 2 and 3 post tamoxifen injection.
Figure 8: Disruption of Secretory Cells upon Rab11 double knockout

A: Histological staining for Alcian blue, indicating Goblet cells shows a decrease in goblet cells in DKO day 2 and 3 tissues.
B: Quantitative analysis of number of Alcian blue positive cells per crypt shows a significant decrease in AKO, day 2 and DKO day tissues. ****p≤0.0001, **p≤0.01, t test.
C: Immunofluorescence for lysozyme (green) shows decreased expression in DKO day 2 and DKO Day 3 tissues D: quantitative analysis of Lysozyme expression in crypts illustrates decreased expression of lysozyme in Rab11 DKO tissues 2 and 3 days following tamoxifen injection, with no significant change in day 1, AKO or BKO when compared to wildtype. ****p≤0.0001, t test.
**Rab11 DKO results in disrupted differentiation and proliferation dynamics**

Due to the severe loss of villus structure observed, possible changes in the proliferation of cells within the small intestine were investigated. c-Myc, a protein that marks proliferative cells within the transit amplifying and is down-regulated upon differentiation and movement out of the TA zone (Bettess et al. 2005), was investigated. Immunohistochemistry identified a significant increase in c-Myc upon the loss of both Rab11a and Rab11b when compared to wild type and single knockout tissue (Figure 9A and 9B). Immunohistochemistry for proliferative marker Ki67, which accumulates in cells during S, G2 and M phase of the cell cycle (Miller et al 2018), shows an increase in Ki67 staining intensity in the DKO tissues at day 2, but a decrease at both day 1 and day 3 (Figure 10A and 10B). There is also a significant increase in Ki67 staining in the Rab11a single knockout, which is consistent with previous findings indicating Rab11a’s role in intestinal adenoma formation (D’Agostino et al 2019). We further assessed proliferative changes through immunofluorescence of the proliferative marker pHH3, which is present in mitotic cells and highest during metaphase (Hendzel et al 1997) (Figure 11A). Similar to that seen in Ki67 analysis, we found there to be a significant increase in pHH3 expression within AKO and DKO day 2 tissue, and a significant decrease in pHH3 on day 3 of double knockout (Figure 11B). There was no significant difference in BKO and DKO day 1 expression of pHH3 when compared to the wildtype.
Figure 9: Increase in c-Myc expression in Double Knockout

A: Immunohistochemistry for c-Myc shows robust increase in double knockout tissues and little staining in WT and single knockout tissues.

B: Quantitative analysis for c-Myc staining intensity shows a significant increase in levels of c-Myc in Rab11 DKO tissues 1, 2 and 3 days post tamoxifen injection but no significant change in Rab11a or Rab11b single knockout, when compared to wildtype. ****p<0.0001, t test
Figure 10: Increase in Ki67 expression Knockout tissue
A: Immunohistochemistry for Ki67 shows robust increase in AKO and DKO tissues when compared to the WT. B: Quantitative analysis for Ki67 staining intensity shows a significant increase in AKO and DKO day 2 tissues and a significant decrease in BKO, DKO Day 1 and DKO day 3 when compared to WT. ****p≤0.0001, ***p≤0.001, t test.
**Figure 11: Increase in pHH3 expression**

**Knockout tissue**

A: Immunofluorescence for pHH3 (Red) shows an increase in pH3 staining in AKO and DKO Day 2 tissue.

B: Quantitative analysis for pHH3 positive cells indicates a significant increase in AKO and DKO day 2 tissues, a significant decrease in DKO Day 1 and DKO day 3 and no change in BKO when compared to WT. ****p<0.0001, **p<0.01, t test
Pulse-Chase shows Rab11 KDO results in more incorporation of Thymidine analogs

In order to further understand increased proliferative markers on day 2 of double knockout and what may be happening *in vivo* over the course of Rab11a knockout, a pulse-chase experiment was performed in which we assessed the incorporation of thymidine analogs CldU and IdU within the intestinal tissues of wild type and DKO day 2 tissue (Figure 12A). Within the wildtype we observed CldU or IdU positive cells primarily within the crypt or transit amplifying zone, with a limited number of cells being positive for both CldU and IdU and with some CldU positive differentiated cells in the villi (Figure 12B). Similarly, within the DKO day 2 tissue, CldU or IdU positive cells were also observed residing primarily within the proliferative and differentiation zone and some CldU positive cells in the villus structure. However, significantly more IdU positive cells was observed (Figure 12C), as well as significantly more CldU/IdU double positive cells (Figure 12E) in the double knockout tissue. Additionally, significantly more CldU positive cells that had shed off of the villi of DKO day 2 tissue when compared to the wild type was observed (Figure 12F). Conversely, no significant change in the number of CldU positive cells was found in the DKO tissue when compared to the wild type (Figure 12D).
Figure 12: Rab11a/b double knockout results in increased incorporation of Thymidine Analogs

A: Schematic indicating CldU/IdU experimental protocol

B: Immunofluorescence for CldU (green) and IdU (red) shows an increase in the number of IdU+ cells, CldU/IdU+ cells and CldU+ cells shedding from the villus.

C: Quantification of IdU positive cells per crypt-villus axis shows there to be an increase upon the loss of Rab11a and Rab11b. **p≤0.01, t test

D: Quantification of CldU positive cells per crypt-villus axis shows there to be no significant change upon loss of Rab11a and Rab11b

E: Quantification of CldU/IdU double positive cells per crypt-villus axis shows there to be an increase upon loss of Rab11a and Rab11b, ****p<0.0001, t test

F: Quantification of CldU positive cells being shed per crypt-villus axis shows there to be an increase in double knockout day 2 tissue, ****p<0.0001, t test
**Rab11a and Rab11b associate with motor protein Kif11**

Through proteomic analysis of Rab11 knockdown Caco2 cells completed by Gao lab (unpublished data), the motor protein Kif11 was identified as an interacting protein for both Rab11a and Rab11b. Kif11 is a plus-end mediated microtubule motor protein that aids in the formation of bipolar mitotic spindles (Bartoli et al 2011).

Immunofluorescence for Kif11 and Rab11 (non-isoform specific) in HEK293T shows colocalization in mitotic cells (Figure 13A) and co-immunoprecipitation for Rab11a and Rab11b shows Kif11 association (Figure 13B).
Figure 13: Rab11 associates with Motor Protein Kif11. Immunofluorescence for Rab11 (non isoform specific) and Kif11 in HEK239T cells shows colocalization in mitotic cells, indicating association. Immunoblot of immunoprecipitates of Flag in 3xFlag-Rab11a and 3xFlag-Rab11b shows Rab11-Kif11 association.
**Rab11 double knockout results in monopolar spindle formation**

Due to the found association of Kif11 with both Rab11a and Rab11b isoforms, we preformed immunofluorescence for Kif11 in Rab11 DKO tissue to observe changes in Kif11 positive mitotic spindle formation. Rab11 DKO day 2 tissue shows disrupted mitotic spindle formation (Figure 14A). Comparison of the number of normal bipolar mitotic spindles and abnormal spindles reveals a dramatic increase in the percentage of abnormal spindles within the double knockout tissue (Figure 14B). To further analyze the role Kif11-Rab11 association may play in monopolar spindle formation we utilized several inhibitors for Kif11, each of which inhibit GDP release resulting in the inhibition of movement of Kif11 along the microtubule (Cochran et al 2004, DeBonis et al 2004, Lad et al 2008). Kif11 inhibitors Monastrol, STLC and Ispinesib have all been previously shown to cause Monoastral formation in mitotic cells and mitotic arrest in a number of cell lines. Treatment of HEK293T cells with Monastrol, STLC or Ispinesib results in the loss of bipolar spindles in mitotic cells (Figure 15). The number of monopolar and bipolar spindle formation in treated cells indicates that all drugs result in the formation of monopolar spindles, with STLC and Ispinesib resulting in a higher percentage of monopolar spindles than Monastrol (Figure 15B). Importantly, the loss in spindle formation in Kif11 inhibited cells is remarkably similar to that seen in tissues of Rab11 DKO mice. Immnoblot of co-immunoprecipitates of Flag-Rab11a and Flag-Rab11b cell lysates treated with Monastrol, STLC or Ispinesib shows decreased Kif11 precipitation, indicating a decreased association between Rab11 and Kif11 when Kif11 has been inhibited (Figure 16)
Figure 14: Loss of Rab11a/b results in abnormal spindle formation in mitotic cells
A: Immunofluorescence for Kif11 (green) shows disruption of mitotic spindles upon loss of Rab11a and Rab11b intestinal tissue
B: Quantitative analysis of Kif11 mitotic spindles shows there to be a decrease in normal mitotic spindles upon the loss of Rab11a and Rab11b.
Figure 15: Inhibition of Kif11 results in monopolar spindle formation in mitotic cells
A: Immunofluorescence for Kif11 (red) and α-Tubulin (green) shows disrupted spindle formation when HEK239T cells are treated with Kif11 inhibitors. C: Quantitative analysis of spindle formation shows loss of bipolar spindles when Kif11 is inhibited.
Figure 16: Rab11-Kif11 association is reduced in Kif11 inhibited cells.
Co-Immunoprecipitation for Flag-Rab11a and Flag-Rab11b in HEK293T cells treated with DMSO, Monastrol, STLC or Ispinesib A: Immunoblot of treated Flag-Rab11a immunoprecipitates indicates reduced Rab11a-Kif11 association in Kif11 inhibited cells. B: Quantitative analysis of relative amount of Kif11 captured in the Flag immunoprecipitation indicates reduced Rab11a-Kif11 association when Kif11 is inhibited. C: Immunoblot of treated Flag-Rab11b immunoprecipitates indicates reduced Rab11b-Kif11 association in Kif11 inhibited cells. D: Quantitative analysis of relative amount of Kif11 captured in the Flag immunoprecipitation indicates reduced Rab11b-Kif11 association when Kif11 is inhibited.
Discussion

**Rab11a/b Redundancy and Intestinal Tissue changes**

Rab11 is known to play a role in cellular trafficking and be involved in cellular maintenance within the gut (Bhuin et al 2014, Bouchet et al 2018, Bryant et al 2010, Feng et al 2017, Galvez et al 2012, Grand and Donaldson 2009, Grimsey et al 2015, Khandewai et al 2013, Landry et al 2009, Parker et al 2018, Prekeris et al 2003, Roland et al 2011, Schafer et al 2016, Sobajima et al 2015, Stamatovic et al 2017, Vogel et al 2015, Wang et al 2017, Wilcke et al 2000 and Yu et al 2014). It has been previously shown that global deletion of Rab11a is embryonically lethal (Yu et al 2014) and intestinal deletion is associated with colonic tumorigenicity (D’Agostino et al 2019). However, the effect of double knockout of Rab11a and Rab11b has not yet been evaluated and due to their close similarity in amino acid sequence, it is possible for the Rab11 isoforms to play some redundant roles within the intestine. Histological analysis of Rab11a single knockout showed no dramatic change in the intestinal crypt-villus architecture and Rab11b single knockout appeared to also have no immediate impact on the architecture of the small intestine. Conversely, the loss of both Rab11a and Rab11b through a Rab11a<sup>fl/fl</sup>; VillinCreER; Rab11b<sup>−/−</sup> inducible knockout model leads to dramatic loss of intestinal architecture 2 and 3 days post tamoxifen injection, suggesting possible redundant roles for Rab11a and Rab11b. Thus, it is possible that upon the single loss of only Rab11a or Rab11b within intestinal epithelial cells, the remaining Rab11 isoform is capable of compensating for the loss and maintain a normal intestinal structure. However, global deletion of Rab11b is not found to be embryonically lethal, as seen in global Rab11a deletion, indicating that not all functions are shared. Analysis of intestinal concentration
of Rab11a post tamoxifen injection indicates that Rab11a is decreased 24 hours post tamoxifen injection and completely lost at 72 hours (Figure 4A). The observation of an increasingly dramatic loss of structural architecture of the intestinal tissue each day post tamoxifen injection is consistent with the decreasing amount of Rab11a within the tissues each day post tamoxifen injection observed. As a result, based on our observed concentrations of Rab11a, we would expect double knockout tissues to be relatively healthy at only one day post injection, and to become increasingly unhealthy with each consecutive day, as observed in Figure 4B. The loss of villus structure and expansion of crypts within the DKO tissue indicates possible disruption of proliferative or differentiation mechanisms.

**Change in Intestinal Stem Cell Dynamics in Rab11a/b knockout**

Because the intestinal stem cells within the crypt are responsible for the repopulation of the epithelium and we observed a loss of villus structure upon double knockout, analysis of possible changes of the stem cell population was of interest. We observed a loss of CBC Olfm4 positive cells as well as a dramatic reduction in strong Olfm4 expression (Figure 5). However, there remained a presence of Olfm4 in the DKO tissues at a decreased intensity and displayed a more dispersed pattern in the crypt region. These data suggest that there is a loss of CBC stem cells, but daughter cells within the transit amplifying zone are possibly reverting back to a more proliferative state. This is likely a response to the observed villus loss, as an attempt to rescue the shortened villus and restore the intestinal homeostasis. These differences were observed in DKO day 2 and day 3 tissues only, as would be expected based on histology and Rab11a
concentration analysis discussed previously (Figure 4). In addition, there is a significant decrease in the high intensity staining in BKO tissue however, but there remains a presence of triangular CBC cells. Therefore, it is possible that Rab11b plays a larger role in the changes in stem cells observed in double knockout tissue and could indicate an independent function of Rab11b.

Sox9 is a Sry-related transcription factor that has been shown to play a role in the development of several tissues, including the pancreas, lungs, nervous system and hair follicles, among others (Furuyama et al 2010). Beyond embryonic expression, it is expressed in some adult tissues, including the pancreas, hair follicles, testis and notably, the intestine, and is essential to maintaining cells in an undifferentiated state within adult pancreatic cells, although this has not been explicitly shown to be true within adult intestinal cells (Furuyama et al 2010). In the gastrointestinal tract, Sox9 is expressed within intestinal stem cells, as well as in lower amounts in intestinal enteroendocrine cells (Formiester et al 2009, Prévostel and Blache 2017). Recent studies have shown that activation of intestinal stem cells to a proliferative state is likely Sox9 dose dependent, with a lower expression leading to active intestinal stem cells and a higher expression leading to quiescent stem cells, generally in the +4 position (Prévostel and Blache 2017, Roche et al 2015). Furthermore, Prévostel and Blache (2017) show in intestinal injury models in which the crypt-base columnar cells are lost, there is an observed decrease in Sox9 expression within the quiescent stem cell population as a means of repopulating the lost active stem cell niche and restoring intestinal homeostasis. Based on our Olfm4 observations, a hypothesis that there is a loss of active CBC stem cells and an increase in proliferation of cells in the TA zone could be made. If there is an increase in the
proliferative activity of cells in the TA zone, it is possible there is also an increase in the proliferative activity of quiescent stem cells. Therefore, it could be expected that there be a decrease in the expression of Sox9 within DKO tissues. Analysis of Sox9 staining intensity supports this hypothesis, showing there to be a significant decrease in high intensity Sox9 staining in all genotypes, including single Rab11a and Rab11b tissues (Figure 6). Together, these data indicate that loss of Rab11a and Rab11b dramatically alter the dynamics of the stem cells within the intestinal crypt, likely due to an attempt to alter proliferation and differentiation dynamics in order to repopulate the lost villus. Although, based on the lethality of the phenotype, these changes do not appear to be dramatic enough to overcome the loss of both Rab11 isoforms within the gut.

**Epithelial Differentiation Loss in Rab11a/b Knockout**

It is expected that upon dramatic loss of intestinal architecture there will be a dramatic loss of the brush border integrity. Alkaline phosphatase (AP) has been shown to be present at the brush border within the small intestine and plays a role in inflammation, pH balance and regulation of the intestinal microbiome (Fawley and Gourlay 2016). The almost complete loss of AP observed within DKO day 2 and day 3 tissues (Figure 7A), as well as the loss of brush border associated Villin (Figure 7B), is likely due to the loss of differentiated enterocyte cells within the intestinal villus and thus, a loss of the AP and Villin producing intestinal epithelial cells. It could be hypothesized that the loss of brush border integrity results in increased risk for bacterial infection or increased inflammation, however this has yet to be investigated. Potential consequences of the lost brush border seen in Rab11 DKO tissue on both commensal and pathogenic microbiota and how this
may change the systems response to infection has not yet been investigated, and due to
the lethality of Rab11a/b double knockout, it is not possible to observe long term effects
of the loss of the brush border may have on the system. In addition to AP and Villin
producing enterocyte loss, the observed loss of secretory Goblet cells and Lysozyme
producing cells (Figure 8) also suggests a loss of differentiated cell types in DKO tissue.
Interestingly, the decrease in lysozyme in BKO and DKO tissues that is not observed in
AKO tissues indicates a possible independent function of Rab11b. Increased expression
of c-Myc in all DKO tissues (Figure 9) further suggests a decrease in the differentiation
of cells and supports the hypothesis that cells are reverting back to a more stem like state.

**Epithelial Proliferation Changes in Rab11a/b Knockout**

Due to the dramatic loss of intestinal structure observed within DKO tissue, it
may be expected for there to be an equally dramatic loss of proliferation within the
tissues. However, we observe a significant increase in proliferative markers c-Myc
(Figure 9), Ki67 (Figure 10) and pH3 (Figure 11) within DKO tissues, specifically
when observing day 2 tissues. This is likely due to the loss of both Rab11a and Rab11b
resulting in the activation of rescue mechanisms that result in a hyperproliferative
phenotype. Notably, the dramatic increase in both Ki67 and pH3 markers were found in
only the AKO and DKO day 2 tissues. The increase in proliferation within AKO tissues
supports previous reports of hyperproliferation within Rab11a+/−; VillinCre mice leading
to tumorigenesis (D’Agostino et al 2019). However, this phenotype is not observed in
BKO tissues, further indicating that although there are redundant functions between
Rab11a and Rab11b, not all functions are shared equally between the two isoforms, and
there are likely also a number independent of functions for the two isoforms.

Additionally, although there is an increase of Ki67 and pHH3 expression in DKO tissues at day 2, there is a decrease at day 3 compared to the wild type, and a slight decrease (for Ki67) or no significant change (for pHH3) in day 1 tissues. It is very likely that, due to the tissue at day 1 maintaining a relatively healthy structure and a higher amount of Rab11a remains within the tissue, that the tissues have not yet reached a point where rescue through hyperproliferation is required for homeostasis. Conversely, the tissues on day 3 are so severely damaged that the cells are unable to maintain the hyperproliferative state found at day 2. Thus, the tissue 2 days post tamoxifen injection is at a state of compromise severe enough that several rescue mechanisms are likely activated, but not compromised to the level that the tissue cannot maintain these rescue mechanisms.

Immunofluorescent and immunohistochemistry analysis of endogenous proliferative markers with the double knockout tissue aids in our understanding of potential changes that occur post Rab11a knockout. However, a pulse chase experiment aids in further understanding what occurs in vivo over the course of Rab11a knockout. Analysis of cells that were proliferative at 24 and 48 hours following tamoxifen injection supports the trend observed in Ki67 and pHH3 staining, with no significant change in the number of cells incorporating CldU injected 24 hours post tamoxifen but observing a significant increase in the number of cells incorporating IdU injected 48 hours post tamoxifen (Figure 12). Thus, the increased proliferation does not appear to occur until around the same time as the blunted villus phenotype. Interestingly, in addition to single IdU positive cells, there is a significant increase in the number of cells that incorporated both thymidine analogs within the DKO day 2 tissue. These double positive cells could
suggest that although there is an increase in proliferative markers within the DKO tissue, these cells may not be successfully completing the cell cycle and thus, not successfully proliferating to replace the lost villus. In this case, cells that have both CldU and IdU represent cells that are likely able to enter S phase of the cell cycle, and thus incorporate the thymidine analogs into their genome but are unable to exit S phase and complete mitosis. However, the double positive cells could also suggest that cells are undergoing multiple rounds of cell division without differentiating and thus incorporating both analogs. It cannot be said which of these are occurring from the pulse-chase data alone.

In addition, the observed increase in CldU positive cells shedding from the villus in DKO day 2 tissue suggest that there is a possible increased apoptotic response, despite the additional phenotype of increased proliferation.

Changes in differentiation and proliferation of cells could also be due, in part, to changes in the BMP, Wnt and/or Notch signaling pathways. Disruption of the recycling endosome due to the loss of Rab11 could result in changes in the expression of proteins at the membrane surface, resulting in loss of expression or constitutively active expression. It can be hypothesized, due to the observed loss of differentiated cell type, that there is a dramatic decrease in the BMP signaling in the intestine and a possible increase in Wnt signaling. This could be due to rescue mechanisms meant to increase the proliferative activity of the tissue, or due to disruption of the mediation of receptor expression within Rab11 DKO cells. Future work to evaluate the expression of receptors, including Fz, LRP and BMPR, as well as downstream proteins and mRNA of differentiation and proliferation promoting genes would be necessary to better understand how these
signaling pathways are impacted by loss of Rab11 and if they play a role in the observed phenotype.

**Rab11-Kif11 association is important for mitotic spindle formation**

Finally, analysis of spindle formation with Rab11 DKO tissue indicates a possible role of Rab11 in mitosis. The loss of Rab11 results in the formation of monopolar spindles, resulting in cells being unable to complete mitosis (Figure 14). These data aid in further interpretation of the pulse chase experiment (Figure 12). Together, they suggest that upon the loss of Rab11, proliferative intestinal cells are able to enter S phase of the cell cycle, but are unable to complete mitosis, likely as a result of incomplete spindle formation and therefore continue to incorporate thymidine analogs. Furthermore, comparison of a phenocopy of the observed spindle phenotype in Rab11 DKO tissue with HEK293T cells (Figure 15), as well as decreased Rab11-Kif11 association, when Kif11 is inhibited (Figure 16), suggests that an association between Rab11 and Kif11, verified through immunofluorescence and co-immunoprecipitation (Figure 13), is essential for cell division within proliferative intestinal cells. The exact mechanism by which this occurs has yet to be determined and warrants further analysis.

Future work is required to develop a stronger understanding of the mechanisms involved in the processes observed and the roles of Rab11a and Rab11b in maintaining intestinal homeostasis. This should include in depth analysis of specific functions of both Rab11a and Rab11b in order to better understand the degree to which the two isoforms overlap in function and if this overlap is specific to individual functions or mechanisms. In addition, investigation into the Rab11-Kif11 interaction, as well as other potential
proteins that may be involved in their association, and how it is involved in mitotic spindle formation and mitosis progression, is required for understanding the changes in proliferative and differentiation dynamics observed in Rab11 double knockout mice. Possible change in WNT, BMP and/or Notch signaling in single and double knockout tissues is also of interest. Finally, investigation into potential apoptotic or necrosis mechanisms involved in the dramatic loss of villi observed in double knockout mice as well as analysis of potential impacts Rab11 double knockout may have on the intestinal microbiome or immune response are of interest.
Conclusion

Vesicular trafficking within cells is essential for the maintenance and proper functioning of the cell. Vesicular trafficking is of particular importance within polarized cells, as it is essential for the maintenance of the apical and basolateral membranes through targeted endo and exocytosis. Within polarized intestinal epithelial cells cellular trafficking has been closely studied, and small GTPase proteins termed Rab11 have been indicated as essential mediators of vesicle traffic within the recycling endocytosis network. Here, we show that Rab11a and Rab11b are essential for the maintenance of the intestinal epithelium, and loss of both Rab11a and Rab11b is detrimental to the health of intestinal tissue. However, single loss of Rab11a or Rab11b do not exhibit as dramatic a phenotype, indicating possible redundant roles for the Rab11 isoforms within the intestinal epithelium. Loss of both Rab11a and Rab11b results in blunted villi and elongated crypts within the small intestine as well as hyperproliferation, changes in stem cell activity and reduced differentiation of cells, likely through mechanisms activated to restore intestinal homeostasis. In addition, we demonstrate that loss of Rab11a and Rab11b results in a disruption in mitotic spindle formation, possibly due to the interaction between Rab11 and motor protein Kif11 being lost. Further analysis of the Rab11-Kif11 interactome and specific mechanisms leading to mitotic spindle formation is required. Additionally, further analysis of specific Rab11 functions that are shared by the A and B isoforms, and those that cannot be compensated when only one isoform is lost would aid in our understanding of Rab11’s role in intestinal homeostasis and diseases.
Bibliography


Clevers H. 2013. The Intestinal Crypt, a prototype stem cell compartment. Cell. 154:274-284


Spit M, Koo B, Maurice M.M. 2018. Tale from the crypt: intestinal niche signals in tissue renewal, plasticity and cancer. *Open Biol.* 8:180120


Supplemental Figures

![Olfm4 Intensity distribution](image1)

![Olfm4 Intensity distribution](image2)
**Supplemental Figure 1: Olfm4 intensity distribution.**

Histogram of pixel intensity distribution of Olfm4 staining for BKO, AKO, DKO Day 1, and DKO Day 3.
Sox9 Intensity distribution

Pixel Intensity

Number of Pixels

WT vs AKO

Sox9 Intensity distribution

Pixel Intensity

Number of Pixels

WT vs BKO
Supplemental Figure 2: Sox9 intensity distribution. Histogram of pixel intensity distribution of Sox9 staining. High and Low pixel intensities used in figure 5D indicated.
### Appendix

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PCR Programs

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PCR Primer Sequences

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Reverse: TGTGAGGCCACCCCGCATGGT

Rab11b;

E2F: CATTCTTGACTTACTCAGCTGTCA
E2R: TGCTATCTCTAGGTCTTGACCCTA
E4R: GAGGGTTCCCAGGCACAGGCAAGT

VillinCreER;

Forward: CGCGAACATCTTCAGGTTCT
Reverse: CAAGCCTGGCTCGACGGCC