

NUTRITIONAL STATUS DICTATES THE RATE AND MODE OF INTESTINAL
EPITHELIAL STEM CELL PROLIFERATION THROUGH AN AMPK DEPENDENT
MECHANISM

BY

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THESIS

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Abstract

Obesity is the leading cause of preventable chronic disease, such as diabetes, heart disease and cancer. The most significant factor driving obesity and the development of these secondary disorders is diet. There is significant evidence that the amount of food one eats induces adaptational growth and functional changes of several tissues, including bone, skin, adipose, liver and intestinal tissue. Understanding how the amount of food induces these changes may reveal mechanisms by which we can manipulate to reverse the negative impact of obesity on tissue size and function. One tissue significantly impacted by obesity is the intestinal epithelium. It is known that the amount of food, and not the type of diet, drives changes in tissue size by increasing the number of epithelial cells, villi height and crypt depth. Because increases in tissue size are under the control of stem cells located within the tissue, I investigated if and how the amount of food may drive increases in the rate and mode (asymmetric vs. symmetric) of stem proliferation. *In vivo*, 48 male C57/B6 mice were used. Mice were fed varying amounts of chow diet and were separated into 4 groups; 1) *ab libitum* (adlib) fed 2) fifty-percent (50%) of their average consumption 3) fasted or 4) fasted-refed. Mice were sacrificed and the small intestine was excised and immunohistochemically processed to determine the rate of stem cell proliferation and the mode of division. The results revealed that adlib fed animals showed a higher % of symmetrically dividing cells compared with the 50% and fasted animals. An increase in symmetric division results in an increase in the pool of stem cells and expansion of the tissue size. In order to investigate the mechanism driving nutrient-driven changes in the mode of stem cell division, *in vitro* analysis of intestinal epithelial organoids (primary isolated intestinal epithelial crypts allowed to grow into fully functional tissue) were used. *In vivo* analysis was mimicked by varying the amount of glucose (no, low or high) and measuring the mode of division. In addition, the effect of activating or inhibiting a metabolic pathway known

to be involved in obesity and growth, LKB1-AMPK, on the mode of division was tested. Consistent with our *in vivo* results, high glucose conditions greatly increased the % of symmetrically dividing cells compared with low glucose conditions. Inhibiting LKB1-AMPK blocks the normal switch to asymmetric division under low nutrient conditions and activation induces a greater asymmetric division even under high glucose conditions. Taken together, these data suggest that nutrient availability determines the mode of stem cell division through an LKB1-AMPK dependent mechanism to drive an increase in tissue and may be mechanism by which abnormal tissue function occurs.

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Chapter 1: Literature Review

1.1 Introduction

Organisms reside in dynamic environments and must continuously adapt to maintain homeostasis. Post-developmental tissue is often considered to maintain a constant size, however, there is significant evidence that suggests considerable physiological changes can occur throughout an organism's lifetime. This includes reversible changes in organ size. These changes are often dependent on environmental signals, most importantly, nutrient availability. A primary example of this phenomenon is the plasticity of the small intestine in response to nutrient availability.

1.2 Anatomy and physiology of the small intestine

The small intestine is a part of the gastrointestinal tract between the stomach and the large intestine where most of the digestion and absorption of food takes place. The small intestine is divided into three segments – duodenum, jejunum and ileum. The duodenum is directly connected to the stomach by the pyloric sphincter and surrounds the head of the pancreas. Following a meal, the duodenum receives chyme from the stomach, bile from the gallbladder and digestive enzymes from the pancreas. Bile received from the gallbladder emulsifies fats, allowing for greater surface area by which digestive enzymes can access lipids. Proteins, nucleic acids and carbohydrates are also broken down into their monomer units. Furthermore, Brunner's glands secrete a mucus-rich bicarbonate solution that neutralizes the chyme, protecting the duodenum from the acidic conditions of the stomach contents). Digested products then pass into the jejunum where the majority of nutrient absorption occurs. Simple sugars, water-soluble vitamins, amino acids and short and medium chain fatty acids are absorbed into the blood. Long chain fatty acids are passed into the lymph capillaries. Following the jejunum, the ileum is responsible for absorption of macronutrients that have previously not been

absorbed, fat-soluble vitamins (such as A, D, K and E), cholesterol, sodium and vitamin B12, and bile salts.

The small intestine has four basic layers, the mucosa, submucosa, muscularis externa and serosa. The outermost layer, the serosa, produces serous fluid which lubricates and protects the organ. The second outmost layer, the muscularis externa, is composed of an inner circular and outer longitudinal bands of smooth muscle that are responsible for driving peristalsis. Thirdly, the submucosa contains a rich network of blood and lymph vessels, nerve endings and Peyer's patches. Finally, the mucosa layer is the innermost layer of the alimentary canal and comes in direct contact with the luminal contents. Structurally, the mucosal layer is composed of three sub-layers; the muscularis mucosae, a band of smooth muscle, the lamina propria, a thin layer of loose connective tissue which is richly supplied with capillaries a single lacteal, and the epithelium, which is composed of specialized cells that perform a variety of functions.

The epithelial layer of the mucosa is essential in digestion, absorption and antimicrobial activity. Structurally, the mucosa layer is arranged in two constructs, the crypts and the villi. The villi are composed of the two innermost layers of the mucosa and are fingerlike projections into the lumen that increases the surface area of the mucosa, allowing for maximum absorption of nutrients as digested products pass through the intestine. Between the villi are tubular glands that invaginate into the mucosa, called the crypts of Lieberkühn. The epithelial lining is composed of several different cell types such as, but not limited to, enterocytes which are responsible for nutrient, water and ion uptake and secretion of immunoglobulin complex IgA into the intestinal lumen. Secondly, goblet cells are located along the epithelial lining and produce mucin, a large glycoprotein that is the primary component of mucus. The mucus layer has multiple functions including lubrication and protection. Finally, enteroendocrine cells

secrete gastrointestinal hormones in response to various stimuli. There is significant evidence that the innermost lining of the mucosal layer, the epithelium is most responsive to altered nutrient availability.

1.3 Nutrient availability induces intestinal adaptation

In response to nutrient fluctuations the small intestine displays numerous physiological and morphological adaptations. For example, in response to increased feeding, the small intestine shows hypertrophy, upregulation of intestinal enzymes, and increased absorption (Dailey, 2014). This phenomenon is shown in several different animal models. During lactation in rats, food consumption is nearly twice as high when compared to pregnant rats that are of similar body weight and age and the small intestine is longer, heavier and has a larger diameter (Fell et. al, 1966). The average weight of the small intestine is 77-100% greater between days 12-20 of lactation than virgin control rats (Fell et. al, 1964). Hypertrophy of the villi is characterized by an increase in the height and thickness of the villi, as well as a deepening of the crypt compartment (Fell et. al, 1966). Furthermore, hyperplasia of the epithelial cells and hypertrophy of the nuclei of these cells occurred in lactating animals (Fell et. al, 1966). Similarly, nutrient induced changes are observed in the small intestine of hibernating animals, such as ground squirrels. The feeding pattern of hibernating animals is characterized by varying nutritional availability with respect to season, with periods of rapid food consumption and weight gain prior to hibernation, followed a periods of anorexia and weight loss during hibernation. During their hibernation phase in winter (November-February), animals had no access to food or water and subsequently displayed a decrease in mucosal wet weight (epithelial weight) compared with animals during other seasons (Carey, 1990). Furthermore, the epithelial layer of hibernating animals showed reduced villi height and villus density (Carey, 1990). These morphological

changes in both hibernating and lactating animals can be explained by an increase or decrease in adlib food consumption.

The role of nutrients, excluding other factors associated with lactation or hibernation (body weight, temperature, light exposure, etc), in driving intestinal adaptation is supported by data from patients that have undergone small bowel bypass surgery. During small bowel bypass surgery the size of the stomach is greatly reduced and a Y-shaped section of the small intestine is attached to the stomach to allow food to bypass the lower stomach, the duodenum and the first portion of the jejunum. Following surgery there is a marked reduction in size of the duodenum and jejunum and increase in size of the ileum (Dudrick, 1976). Since there are no nutrients in the duodenum and jejunum and an abundance in the ileum, this suggests that luminal contents dictates growth of the small intestine. These data and the above examples, lactating and hibernating animals, display a wide variety of feeding behaviors but all suggest that high nutrient availability increases intestinal epithelial size and morphology.

1.4 Nutrient-induced changes in cell proliferation

In addition to gross morphological changes, the intestinal epithelium also undergoes cellular changes in response to nutrient availability. Evidence suggests that luminal nutrients increases the rate of cell proliferation and number of mitotic events. For example, mice that overeat a standard rodent chow due to a genetic mutation of the leptin receptor, *db/db* mice, show an increase crypt epithelial proliferation and increases in cell migration up the villi when compared with wild-type control animals (Zhang et. al, 2013). *Drosophila* that are repeatedly fed and fasted show dramatic increases and decreases, respectively, in midgut size. Animals that have been fed for four days show a 300% increase in the midgut cell number when compared with fasted animals (Bilder et. al, 2011). Taking into consideration that daughter cells do not

divide, these cellular changes indicate that a progenitor cell population is responsible for the changes seen in the intestinal epithelial layer during nutrient induced adaptation.

1.5 Nutrient-induced stem cell proliferation

Multiple animal species show a similar phenomenon of increasing the epithelial size under high nutrient availability (*ie.* an increase in food intake or luminal contents of the alimentary canal) even though the cells responsible for this effect are different in the type and location among species. For example, *c. elegans* rely on an increase in the rate of mitosis of the differentiated cells within the tissue. Other animals rely on an increase in the rate and/or a change in the mode of division of the somatic stem cells in order to expand the tissue size (Rothman, 2011). Planarians rely on migrating stem cells (Aziz, 2011), Hydra use interstitial cells located within their body column to produce committed precursors that migrate to the intestine (David, 2007) and *Drosophila* rely on stem cells that reside adjacent to the gut epithelium and are responsible for the increase in cell number as described above (Ohlstein, 2006). Recently there has been substantial research to identify adult stem cell populations in mammals in order to effectively translate these the concepts discovered in simpler organisms.

1.6 Mammalian intestinal epithelial stem cells

Mammals have two functionally distinct stem cell populations within the tissue itself that may differentially contribute to nutrient-induced growth of the epithelium (Clevers, 2012). At least two pools of stem cells have been classified in mammals that differ in their position, expression of specific markers and cell cycle characteristics. One is an actively cycling population that is located in the crypt base and termed the crypt base columnar cells (CBC cells; (CBC cells; Clevers, 2012, Yan 2012). These cells are marked by high expression of LGR5 and other markers (Yan, 2012; May, 2008; Van Landeghem, 2012; Clevers, 2012). The other population is located outside of the crypt base predominately in the +4 position (+4 cells), are

quiescent or slow cycling and are marked by low expression of LGR5 and other unique cell markers (Yan, 2012; May, 2008; Van Landeghem, 2012; Clevers, 2012) . It has been postulated that the CBC cells solely contribute to the normal regeneration of the epithelial tissue, whereas the +4 cells remain largely quiescent during homeostasis (Yan, 2012).

Several studies have shown differential responses of BMI1 and LGR5 to common proliferation and survival signals. For example, there is a significant differential response of BMI1 vs. LGR5 to canonical Wnt signaling. Yan and colleagues utilized two mouse models, LGR5-eGFP-IRES-CreERT2 and Bmi1-CreER; Rosa26-YFP and soluble adenoviral secreted factors to achieve Wnt gain and loss of function through the use of common Wnt activators and inhibitors. Under the addition of a Wnt activator, the number of LGR5-GFP cells significantly increased (Yan, 2012). In contrast, the addition of a Wnt activator did not significantly alter the amount or mitotic index of the Bmi1-YFP population (Yan, 2012). Similarly, addition of a Wnt inhibitor greatly decreased LGR5 expression without altering Bmi1 populations (Yan, 2012) . This concludes that LGR5 expressing stem cells are sensitive to gain and loss of Wnt signaling independently of Bmi1 expression, suggesting that there are distinct functional and regulatory responses between the two stem cell populations. Considering there is significant evidence that links nutrient intake and Wnt signaling, it is possible that these two stem cell populations respond differently under varying nutrient conditions.

In addition to canonical Wnt signals, LGR5 and Bmi1 stem cell populations respond differently to insulin-like growth factor-1 (IGF-1). There is a link between the amount of nutrients and increases in IGF-1 levels that could differentially affect the two stem cell populations to induce growth of the tissue. IGF-1 is a potent activator of key proliferation cascades such as the mitogen activated protein kinase network (MAPK) and protein kinase B

(AKT). DIO mice display both increased plasma IGF-1, as well as localized IGF-1 (Van Landeghem, 2015). Increased localized IGF-1 was shown through qPCR analysis of isolated epithelial cells of DIO mice. In DIO mice that showed increased localized IGF-1, there was a differential increase in the two stem cell populations, with increases in the number of LGR5 stem cells per crypt, but not the Bmi1 stem cells (Van Landeghem, 2015). This is consistent with data showing increased IGF-1 receptor (IGF-1R) expression on LGR5 stem cells (Mah, 2011) only. These data are validated using additional methods. When the LGR5 stem cell population was isolated from mice using fluorescent-based flow cytometry, IGF-1 application expands the LGR5 population (Mah, 2011). These data suggest that increased IGF-1, as seen in over nutrition, selectively activates LGR5 stem cells, without affecting the BMI1 population. Similarly, IGF1 stimulation also results in differential mRNA signatures and gene expression profiles in LGR5 vs. Bmi1 stem cells, which are associated with different functional pathways such as stemness, survival and cell cycle arrest (Mah, 2011). Together, this data suggests that IGF1 differentially induces proliferation of LGR5 and Bmi1 stem cells and that this may be a mechanism by which nutrients may differentially affect these populations.

1.7 Nutrient-induced changes in the rate of stem cell proliferation

There is significant evidence that nutritional status dictates the rate of stem cell proliferation in the small intestinal epithelium through intracellular signaling such as the Wnt/ β -catenin pathway. Wnt ligands are excreted by epithelial cells and pericryptal stromal cells. The Wnt pathway is initiated by the binding of Wnt glycoproteins to the N-terminal of the Frizzled receptor. To facilitate Wnt signaling, two co-receptors, LDL receptor-related proteins 5 and 6, are also involved in activation. Upon activation of the receptor an intracellular signal is transduced to Dishevelled, a highly conserved cytoplasmic phosphoprotein. Phosphorylation and subsequent activation of Dishevelled inhibits activation of GSK3, a key molecule in the β -catenin

destruction complex. The phosphorylation of GSK3 allows an accumulation of β -catenin in the nucleus and subsequent activation of TCF target genes such as EGFR and Cyclin D1 which are directly involved in stem cell proliferation and crypt architecture. DIO mice show increased phosphorylation of GSK3 (pGSK), and expression of β -catenin and cyclin D (a cell cycle regulator) (Mao, 2013). Glucose also directly stimulates epithelial cell proliferation directly through β -catenin signaling. When epithelial cells in culture are exposed to high glucose, expression of β -catenin and cyclin D and pGSK3 increased (Mao, 2013). In mice that overconsume standard rodent chow, *db/db* mice, an increase in phosphorylation of GSK3 and correlates with increases in the expression of cyclin D, suggesting that over-nutrition stimulates cell proliferation through phosphorylation of β -catenin /GSK3 (Mao, 2013). Collectively, these data suggest that high nutrient availability increases epithelial cell proliferation by preventing β -catenin activity through pGSK3.

1.8 The mode, in addition to the rate, of stem cell division can affect tissue size

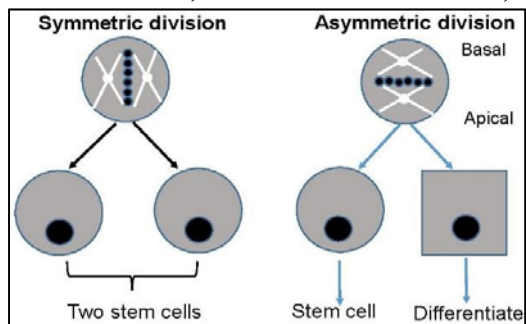


Figure 1.1: Modes of stem cell division. Asymmetric division is determined by the mitotic spindle orientation parallel to the basal-apical boarder, whereas symmetric division is the result of perpendicular mitotic spindle orientation.

In addition to nutrients driving an increase in cell number and expansion of the tissue size through increases in the rate of stem cell proliferation, epithelial stem cells can change the mode of cell division to expand tissue size. Adult stem cells use

asymmetric division to generate a differentiated cell and a stem cell. Alternatively, they divide symmetrically to generate two equal cells that self-

renew or differentiate (figure 1.1). Currently, the *Drosophila* midgut is the only animal model where the mode of stem cell division has been investigated in response to food intake. In fed *Drosophila*, a greater symmetric (68%) versus asymmetric mode of division is found (O'Brien,

2011). In contrast, fasted *Drosophila* show greater asymmetric (69%) compared with symmetric. However, once fasted animals are refed, greater symmetric again is used to increase the pool of stem cells and grow the tissue (O'Brien, 2011). However, whether a switch in the mode of division is responsible for this increase in stem cells in other species has not been characterized. Therefore, I hypothesize that nutrient driven expansion of the mammalian small intestine is also driven by a change in the mode of stem cell division from asymmetric to symmetric.

1.9 A mechanism of nutrient-induced switch in the mode of division

A switch in the mode of division of intestinal epithelial stem cells requires that the amount of luminal nutrients can dictate the spindle orientation at division. Asymmetric division requires alignment of the mitotic spindle in an orientation parallel to the apical-basal axis and polarized localization of cell fate determinants to the apical or basal poles of the cell. This allows for two molecularly distinct cells to be produced and for the two cells to develop in different environments (one towards the apical border and the other towards the basal border) (Quyn, 2010). In order for this to occur, intracellular signaling must be linked to the spindle orientation. This could be accomplished through the activity of adenylate kinases. Adenylate kinases buffer declining ATP production by converting two ADP's to one ATP and one AMP (Hardie, 2007). The resulting accumulation of AMP activates the AMP-activated protein kinase (AMPK) network. The activation of the AMPK pathway is dependent on the tumor suppressor protein LKB1, a serine/threonine kinase, and leads to phosphorylation of several downstream effectors known to improve energy status within cells (Hardie, 2005; Wei, 2012). In non-mammalian species, LKB1, or the homologous Par-4, is known more widely as a PAR protein involved in directing the partitioning of molecules during asymmetric division (Benkemoun, 2014; Bonaccorsi, 2007). For example, mutation of the 6 par genes in *c. elegans* results in

disruption of several aspects of anteroposterior axis polarity in the zygote. This includes alterations in P-granule localization, pseudocleavage and asymmetric placement of the first cleavage spindle (Benkemoun, 2014). Furthermore, the daughter cells that result from Par mutants often have misoriented spindle orientation, divide synchronously and display altered distribution of anterior and/or posterior specific factors (Benkemoun, 2014). Although all par gene mutants affect anterior posterior polarity, the mutant phenotypes between par proteins are unique. The Par 4 mutant results in unequal first cleavage, synchronous division of resulting daughter cells and a complete lack of intestinal differentiation in all embryos. Furthermore, it has been shown that Par4 plays a role in positioning of the mitotic spindle during asymmetric division (Bonaccorsi, 2007).

AMPK, a downstream target of LKB1, has also been implicated in spindle control. During mitosis, pAMPK is localized to the spindle poles, and pAMPK expression increases during division (Thaiparambil, 2012). When AMPK signaling is silenced, cells display mitotic delay and mitotic arrest. The mitotic spindle of pAMPK depleted cells also showed misoriented spindles with spindle poles in different z planes, appeared poorly organized and showed significantly shorter pole-to-pole length (Thaiparambil, 2012). Therefore, the LKB1/AMPK is a likely candidate for linking metabolism with changes in polarity during division in IESCs.

1.10 Significance

There is a connection between obesity and decreased quality of life

Obesity has a substantial impact on physical and psychosocial quality of life. It is well established that obesity is associated with gastroesophageal reflux disease (GERD) (Barak, 2002). More recently, obesity has also been correlated with other functional gastrointestinal diseases such as irritable bowel syndrome (IBS) and dyspepsia (Talley, 2004). There is a positive linear association between body mass index (BMI) and functional gastrointestinal

symptoms such as frequent vomiting, upper abdominal pain, bloating and diarrhea (Talley, 2004), suggesting significant quality of life discrepancies in the obese population. However, the mechanisms explaining gastrointestinal symptoms in obese patients has not been delineated. Furthermore, high BMI has also been linked to higher rates of colon and reproductive cancers (Garfinkel 1985; Dunn, 1967; MacMahon, 1974; Blitzer, 1976; Wynder, 1977; La Vecchia, 1982) and high mortality rates due to cardiovascular disease (Faber, 1949; Amad, 1965; Keys, 1972; Gordon, 1976) and diabetic complications (West, 1971; Hartz, 1983; Pi-Sunyer, 1999). Along with detrimental physical effects, obesity is correlated with several psychosocial complications. For example, lifetime prevalence of mood and anxiety disorders, such as major depression and generalized anxiety, are all higher among people with a BMI of 30 or more (obese), when compared to those with a BMI under 30 (Sullivan, 1993; Stunkard, 1992).

There is a connection between obesity and dysfunction of the intestinal epithelium

The negative physiological impact of obesity is seen in alterations of the form and function of many organs, including growth of specific tissues and metabolic disturbances that contribute to the persistence of hyperphagia and weight gain and the incidence of associated diseases. The intestine is significantly affected by obesity, with the epithelial tissue showing increased size (i.e. number of cells, villi height, and crypt depth), increased absorption, decreased satiety hormone release and decreased immune barrier function (Dailey, 2014). It is also becoming evident that these epithelial changes contribute to the increasing incidence of intestinal discomfort, irritable bowel syndrome, dyspepsia and cancer discussed above. Thus, identifying the mechanisms by which obesity alters the morphology and function of the intestinal epithelium will allow for the development of therapeutic strategies to attenuate or reverse the negative effects.

Obesity produces long-lasting effects on the stem and daughter cells of the epithelium

The cells of the intestinal epithelium are continually renewed every 3-5 days. New cells are produced by active proliferation of stem cells localized near the base of the crypts, progression of these cells up the crypt–villus axis, cessation of proliferation and subsequent differentiation into one of the many cell types. In the process of differentiation, these cells acquire structural features of mature cells and all but the Paneth cells continue to move up the villus towards the tip (Heath, 1996). As the mature cells reach the villus tip, they undergo apoptosis and are extruded into the lumen as new differentiated cells take their place (Heath, 1996). The constant renewal of spent epithelial cells allows for tissue integrity to be maintained, but it is not clear how constancy in the function of daughter cells is preserved across generations of new cells. For example, the enteroendocrine cells of an obese individual produce less satiety hormones than those of their lean counterparts (Hellstrom, 2013). Is it the case that the enteroendocrine cells are responding to their local environment in order to show different physiological responses to nutrients or could these cells have been programmed to be “obese” type cells earlier in the proliferation or differentiation process. The latter can be argued by looking at growth potentials of isolated stem cells. When stem cells are cultured from obese and lean mice, the cells isolated from obese individuals have a reduced growth potential to develop into an organoid in vitro and that this is maintained for multiple weeks (Mah, 2014). There is also significant data that supports long lasting effects of obesity on cellular function. For example, mammary cells (De Angel, 2014), bone marrow stem cells and adipose stem cells retain obese properties even after weight loss in humans and non-human animals (Wu, 2013). In addition, maternal diet and obesity can alter epigenetic gene regulation in the offspring during development in utero, which impacts the function of cells long after birth (Tamashiro, 2010). Taking these data into consideration, it is highly possible that obesity produces persistent

changes in the IESCs that could alter the proliferation, differentiation and function of the daughter cells, perpetuating the physiological characteristics of obesity.

Identifying the mechanisms by which obesity alters the morphology and function of the intestinal epithelium will allow for the development of therapeutic strategies to attenuate or reverse the negative effects. Understanding the cellular characteristics and protein kinase cascades of intestinal epithelial stem cells that contribute to obesity will provide valuable information on how we can utilize pharmaceuticals to target obesity at the cellular and molecular level.

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Chapter 2: Food intake influences the rate and mode of stem cell division both *in vivo* and *in vitro*

2.1 Introduction

Despite increased recognition and resolution, the obesity epidemic continues to plague the United States and the world. Currently, obesity is the leading cause of preventable chronic disease, such as diabetes, heart disease and cancer, resulting in job absenteeism and decreased work production that costs the United States 4.3 billion dollars each year (Amad, 1965; Blitzer, 1976; Gordon, 1976; Barak, 2002). Several factors work in conjunction to drive obesity such as environmental, genetic, behavioral and cultural. In part, these factors can be reduced to diet, either what you eat, or how much you eat. There is significant evidence that the amount you eat drives adaptational growth of several tissues, including bone, skin, adipose, liver and the small intestinal epithelium. For this study, we have chosen to investigate the effects of nutrient intake on the small intestinal epithelium.

The amount of luminal nutrients in the intestine is shown to be a driving factor behind the intestinal epithelial growth under a variety of conditions. Numerous animal models show that whenever there is an increase in luminal nutrients, there is epithelial growth indicated by an increased epithelial cell number, increased rate of cell turnover, and increased mitotic index (Mah, 2014; Crean, 1971; Toloza, 1991; Kaushik, 2005). In contrast, a reduction in the luminal nutrients causes a reduction in tissue size under conditions of fasting, hibernation and surgical gastric bypass (Gleeson, 1972; Robinson, 1980; Carey, 1991). Collectively, these studies indicate that the amount of nutrients in the intestinal lumen is the driving factor behind epithelial tissue growth and shrinkage.

Intestinal epithelial growth is caused by a change in total epithelial cell number, which is dictated by proliferation patterns of the epithelial stem cells located in the crypt base of the

tissue. Stem cells can increase their rate of division and/or change the mode in which they divide in order to ultimately increase the number of daughter cells within the tissue. It is found that both occur in response to an increase in luminal nutrients in *Drosophila*. Fasted *Drosophila* show greater asymmetric division when tissue size is held constant, but once refed, greater symmetric self-renewal is used to increase the pool of stem cells and growth the tissue (O'Brien, 2011). Similarly, increased cell proliferation and growth of the intestinal stem cell pool in response to nutrient intake has been observed in mammalian models such as diet induced obesity mouse models (Mah, 2014). However, these studies did not include analysis of the mode of division. ***Thus, we utilized both in vivo and in vitro systems to investigate whether nutrient driven expansion of the mammalian small intestine, is a result of a change in the mode and/or a change in the rate of division.***

2.2 Materials and Methods

***In Vivo* Methods**

Animals

Male C57BL/6 mice (Jackson Laboratories, n=24 at 2.5 months of age). Mice were caged individually in single shoe box cages lined with brown Kraft paper so that food spillage could be quantified. During this time mice had *ad libitum* (adlib) access to standard rodent chow diet (Tecklad 22/5, Tecklad Diets, Madison, WI) and tap water unless otherwise stated. They were maintained on a 12:12 light:dark cycle, with lights on at 1000 h and lights off at 2200 h. Mice were weighed daily at 0900 h, 1 h before the lights came on, under red light (lux). Room temperature was maintained at 22 ° ±1 with 60% relative humidity. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Animal Husbandry

Following one week acclimation to the housing conditions, food intake was measured each day for 4 d and an average intake for each animal was calculated. The average food intake and body weight were used to split the animals equally into 4 groups; 1) adlib, 2) food restricted (50%) , 3) fasted and 4) 12 hour fast/6 hour refeed (refed). The refed group was included to model growth of the small intestine after feeding, since a DIO group was not included. Using a refed group in lieu of DIO ensures that observed effects are not caused by confounding factors such as body weight or secondary obesity complications. The average food intake across the 4 days was used to calculate 50% average daily intake for the food restricted group. At 1600 h, food was taken away from the fasted and fast/refed group while the Ad lib was given fresh food in excess of what the mice would normally eat. The food restricted group was also given fresh food but only 50% of their average daily consumption. At 1000 h, the animals were sacrificed by decapitation under isoflurane anesthesia (Henry Schlein Animal Health, Dublin, OH). An abdominal incision was made and the intestine was exposed. Three, 2 cm long intestinal segments from each region of the intestine (duodenum, jejunum and ileum) were excised and flushed with cold 1x phosphate buffered saline (PBS) (Lonza, Walkersville, MD) and collected for separate processing for immunohistochemistry, Western blotting and RT-PCR.

Tissue processing for immunohistochemistry

A 2cm long segment from each intestinal region was fixed with 15 μ M Taxol (S1150, Selleckchem, Boston, MA) diluted in 4% EM grade paraformaldehyde (PFA; Ted Pella, Redding, CA). Taxol was diluted from 10 mM to a working concentration of 15 μ M in 1X PBS. Fixed intestine was placed into 3 mL 15 μ M Taxol/PFA (Selleckchem). Following a 6 h fixation in Taxol/PFA at room temperature tissue was immersed in 30% sucrose diluted in 1X PBS, overnight at 4°C. Tissue was then embedded using Tissue-Tek O.C.T medium (25608-930,

VWR, Radnor PA). Sections were cut on a cryostat at 8 μ m thickness and then mounted on charged slides. Slides were left to dry overnight and then stored in slide boxes at room temperature before immunohistochemical processing.

Immunohistochemistry for mitotic spindle orientation

Slides were briefly washed with PBS and permeabilized in 0.5% Triton X-100 (Triton-X, Sigma-Aldrich) diluted in 1X PBS (Lonza, Walkersville, MD) for 5 minutes at room temperature. An ImmEdge hydrophobic marker (Vector Laboratories, Burlingame, CA) was used to mark the slides along the perimeter, allowing a hydrophobic barrier for on-slide solution application. All solutions applied directly to slides used a volume of 400 μ L per slide. Blocking solution (0.3% Triton-X, Sigma-Aldrich, St. Louis, MO and 1% Bovine Serum Albumin, diluted in 1x PBS) was applied directly to slides, and allowed to incubate for 20 min at room temperature. After blocking, slides were incubated overnight at 4°C in rabbit monoclonal anti-alpha tubulin (DM1A) Alexa Fluor 488 conjugated antibody (Ab195887, Abcam, San Francisco, CA) at a 1:100 concentration diluted in the blocking solution previously described. After rinsing in 1x PBS, slides were incubated in phalloidin AlexaFluor⁵⁶⁸ (A12380, ThermoFisher Scientific, Waltham MA) at 165nM and 1 μ g/ml DAPI (D1306, ThermoFisher Scientific, Waltham MA) diluted in 1x PBS for 15 minutes. Slides were coverslipped with Permount mounting media (Fisher Scientific, Fair Lawn, NJ). Slides were stored short-term at room temperature and long term at 4 degrees in light-proof boxes. Images of the sections were captured by digital camera attached to a Zeiss LSM700 Confocal Imager (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Calculation of spindle orientation

Several cell inclusion criteria were set prior to analysis. This included that cells had be clearly adjacent to, and not overlapping with, other cells, and they must be localized below the +4 position to exclude transit amplifying cells as described previously (Clevers, Potten, 2009).

Furthermore, the crypt had to have a clear basal border, apical border and intercrypt lumen. Exclusion criteria included tissue sections without clearly defined crypts, or other tissue defects caused by tissue processing. Cells without two defined spindle poles, or with spindle poles in various image planes were also excluded.

To determine the mode of division the angle between the spindle poles and apical border was calculated using the ImageJ angle tool. An angle less than thirty degrees was considered a symmetric division and an angle over 30° indicated an asymmetric division. Results from the duodenum, jejunum and ileum were pooled, thus presented data is of the whole organ. Mode of division was calculated as the ratio of symmetric to asymmetric division for each animal. The ratios from each animal was averaged with the other animals in that feeding group, and data is expressed as mean \pm SEM.

Immunohistochemistry for Ki67

Slides were prepared as described above and heated for 30 min at 67° prior to Immunohistochemical processing. Slides were rinsed in 1X phosphate buffer and antigen retrieval was performed by immersing slides in sodium citrate buffer (Sodium Citrate Hydrochloride, Fisher Scientific and Tween 20, Sigma- Aldrich, Germany, pH 6.0) for 20 min in a water bath at 95°C. Slides were allowed to cool for 30 min at room temperature, and then rinsed in PB. Blocking solution (0.3% Triton-X, Sigma-Aldrich, St. Louis, MO and 3% Normal Donkey Serum, Jackson Immuno Research, West Grove, PA) diluted in 1X PB was applied to slides, and allowed to incubate for 20 min at room temperature. After blocking, slides were incubated overnight at four degrees in sheep monoclonal anti-Ki67 antibody (Santa Cruz Biotech, Dallas TX) at a 1:100 concentration, diluted in the blocking buffer described above. After rinsing in 1X PB, slides were incubated in a donkey anti-sheep biotinylated secondary antibody (Ab6899, Abcam, San Francisco, CA) at a 1:500 concentration diluted in 1X PB for 2 h

at room temperature. Slides were rinsed in PBS, and then incubated in an avidin–biotin complex (Vectastain Elite reagents, Vector Labs, Burlingame, CA) for 1 h at room temperature. After rinsing in PB, a diaminobenzidine kit (SK-4100, Vector Laboratories, Burlingame, CA) was used for visualization. After rinsing in 1X PB, slides were left to dry overnight, then dehydrated in a series of graded ethanols, and cleared in xylenes. Slides were cover slipped with Permount mounting media (Fisher Scientific, Fair Lawn, NJ) and stored at room temperature.

Quantification of proliferating intestinal stem cells

Intestinal tissue sections were visualized and quantified using the NanoZoomer Digital Pathology System (Hamamatsu, Hamamatsu City, Japan) and NDP View 2 software using a 40x objective. Nine crypts per slide were quantified based on intact crypt morphology with distinct apical and basal borders. Several cell inclusion criteria were also set prior to analysis, such as cells had to be clearly stained darker than surrounding (paneth) cells, and they must be localized below the +4 position to exclude transit amplifying cells, as previously described (Clevers, Potten, 2009). Quantification of proliferation was performed in two different ways; 1) number of positive Ki67 cells and 2) ratio of positive Ki67 to total number of cells in the proliferative zone. Furthermore, these data were analyzed both as whole organ (duodenal, jejunal and ileum data combined) as well as by individual segments to detect differences from the proximal to distal axis.

Statistical Analysis

Values are expressed as the mean \pm SEM. All data was analyzed using a one way ANOVA with feeding conditions as the independent measure. Tukey-Kramer post hoc analysis was utilized and differences among groups were considered statistically significant if $P < 0.05$.

In Vitro Methods

Isolation of Small Intestinal Crypts and Small Intestinal Crypt Culture

One male C57BL/6 mice (Jackson Laboratories) at 2.5 months of age were sacrificed under isoflurane anesthesia and the intestine was exposed. Approximately 2 cm of each intestinal segment was excised, opened longitudinally and flushed with ice-cold PBS. Villi were scraped off using a coverslip and the tissue was washed with ice-cold PBS in a 50 mL conical tube. In a sterile cell culture hood, intestinal fragment were cut into 1mm x 1 mm squares and washed by gentle trituration in 30 mL of ice-cold 1X PBS. Supernatant was discarded and the procedure was repeated 5 to 8 times. Fragments were incubated in 2 mM Ethylenediaminetetraacetic acid (Sigma Aldrich, Germany) diluted in 1X PBS at 4°C for 30 min with gentle rocking. The supernatant was removed and fragments were washed with 20 mL of ice cold 1X PBS. This was considered fraction 1. Fraction 1 was discarded and fragments were resuspended in 10 mL of 1X PBS. After gentle trituration fragments were allowed to settle and the supernatant (fraction 2) was removed and put in a 50 mL conical tube. This was repeated two more times, each time adding the supernatant to the tube containing fraction 2. These are considered fractions 3 and 4. Crypt fractions were passed through a 70 µm cell strainer and spun down at 300 x g for 5 min. The pellet was resuspended in 10 mL of ice cold advanced DMEM/F12 (11320082, Gibco, Elmhurst, IL) supplemented with 2 mM GlutaMax (35050061, Gibco, Elmhurst, IL), 10 mM HEPES (15630080, Gibco, Elmhurst IL), and 100 U/mL penicillin/100 ug/mL streptomycin (15140148, Gibco, Elmhurst, IL). Crypt fractions were spun at 200 x g for 2 min to separate single cells and crypts were counted using tryphan blue exclusion and an inverted light microscope.

Crypts were spun down and resuspended in Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (356231, Corning, Corning, NY) at 1000 crypts/50 µl. Fifty

microliters of resuspended MatrigelTM was added to the center of a pre-warmed Millicell ® EZ slide (1.7 cm² growing area per well) (EMD Millipore, Kankakee, IL). The plate was incubated in a CO₂ incubator (5% CO₂, 37°C) for 10 min to allow the MatrigelTM to solidify and 500 µl of complete culture media (Basal culture medium with N2 supplement (1×), B27 supplement (1×), and 1 mM N-acetylcysteine, 50 ng/mL EGF, 100 ng/mL Noggin, 1 mg/mL R-spondin.). Primary crypt cultures were kept at 5% CO₂, 37°C, media was replaced every 4 days and crypts were passaged every 7-14 days as needed.

In vitro experimental design

Following primary culture, crypts were allowed to grow for 4 d. At 0800h, 5 d post plating, crypts were changed to glucose free media (Basal culture medium with N2 supplement (1×), B27 supplement (1X), and 1 mM N-acetylcysteine, 50 ng/mL EGF, 100 ng/mL Noggin, 1 mg/mL R-spondin.) and incubated at 37° at 5% CO₂ for 4 h. Organoid cultures were then supplied with glucose at one of three concentrations, 0mM, 5mM or 20mM. Organoid cultures were then fixed, processed and visualized as described for the *in vivo* experiment above.

Statistical Analysis

Values are expressed as the mean ± SEM. All data was analyzed using a one way ANOVA with glucose concentration as the independent measure and symmetric division as the dependent measure. Tukey-Kramer post hoc analysis was utilized and differences among groups were considered statistically significant if $P < 0.05$.

2.3 Results

Body weight and food intake

There was no significant difference in body weight or food intake between groups at the start of the experiment (figure 2.4.a). Following the 18 h in each feeding condition, fasted mice displayed significantly reduced body weight compared with the other groups (figure 1A; $p < 0.05$).

The refed group was given adlib access to chow diet, following a 12 hour fast. Food intake in the refed mice during the 6 h feeding period following the 12 h fast was not significantly reduced when compared to the previous daily food intake (figure 2.4.b).

Number of proliferating cells by Ki67

When the duodenum, jejunum and ileal values were collapsed and analyzed as a whole organ, there was no significant difference in positive Ki67 cell number (figure 2.5a) or ratio of positive cell number to total number of cells in the stem cell zone (figure 2.5.b) across feeding groups. There were also no significant differences in the number of proliferating cells or the ratio of proliferating cells to total cells between feeding groups when each intestinal segment was analyzed separately: duodenum (figure 2.6.a, 2.7.a), jejunum (figure 2.6.b, 2.7.b), or ileum (figure 2.6.c, 2.7.c).

Mode of division *in vivo*

When the ratio of asymmetric to symmetrically dividing cells of all of the intestinal sections were combined, there was a significant increase in asymmetric division between adlib and the fasted group and the adlib and the refed group (figure 2.8).

Mode of division *in vitro*

There was a significant increase the ratio of symmetric to asymmetric divisions in the 20mM glucose group compared with the 0mM glucose group (figure 2.9).

2.4 Tables and Figures

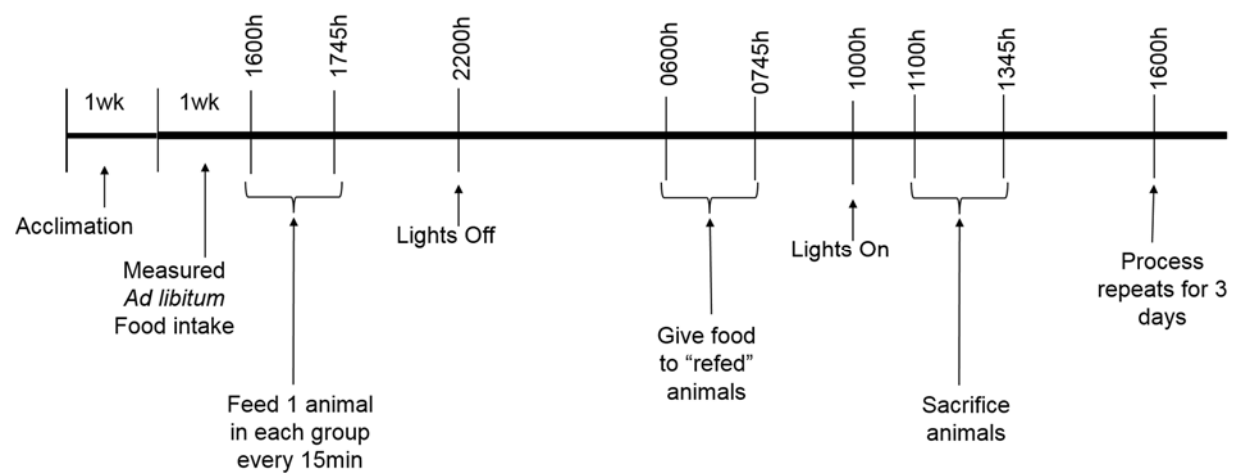


Figure 2.1: Pictorial representation of *in vivo* timeline

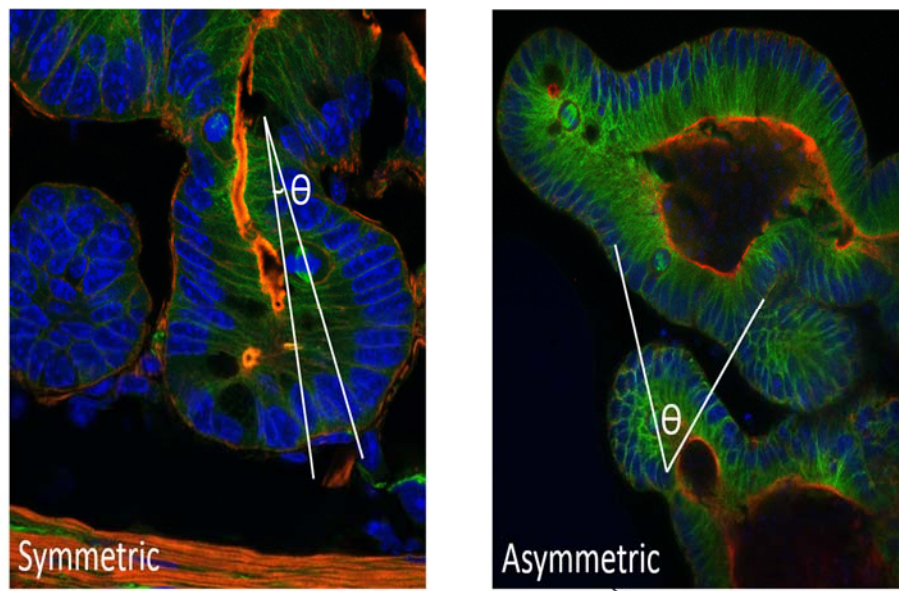


Figure 2.2: Fluorescent photomicrograph of symmetric (left) and asymmetric (right) stem cell divisions.



Figure 2.3: Photomicrograph of Ki67 in the crypt

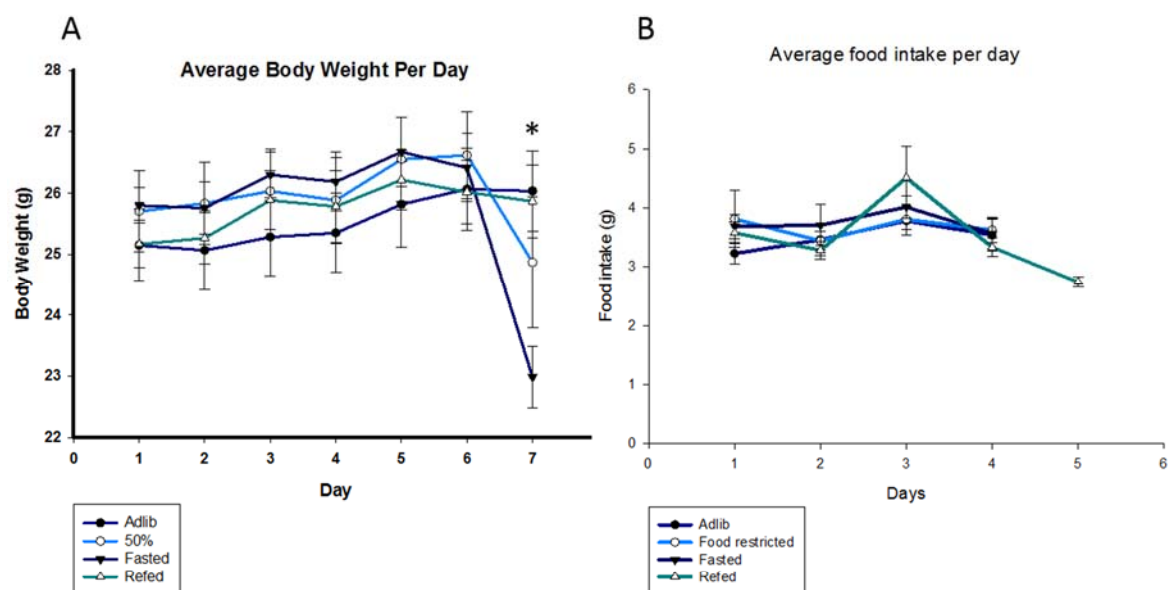


Figure 2.4: Body weight and food intake of C57/B6J mice under altered feeding conditions. Body weight and food intake showed no significant changes between feeding groups during acclimation. A) On the day of sacrifice, fasted mice showed significantly decreased body weight when compared to previous days. B) There was no significant overall reduction in food intake between any group both during acclimation or experimental data points. Data is expressed as mean \pm SEM

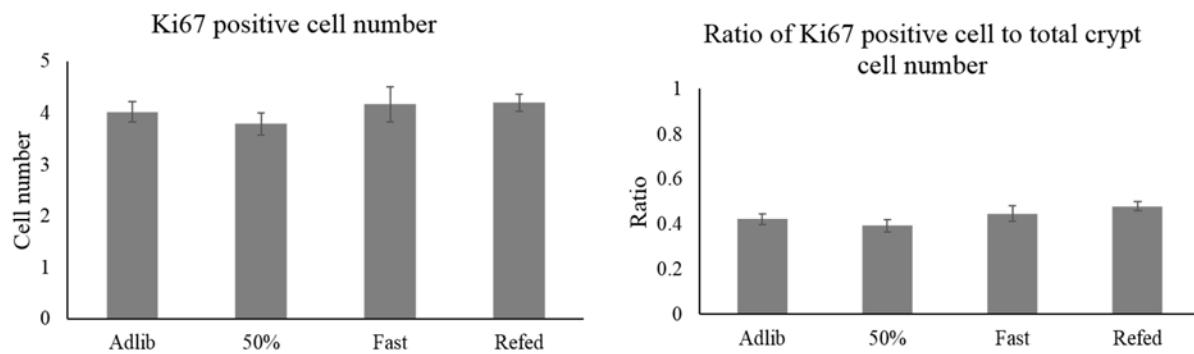


Figure 2.5: Influence of food intake on cell proliferation. A) There is no significant difference in positive Ki67 cell number between any feeding group. B). There is also no significant difference in the ratio of positive Ki67 to total crypt cell number.

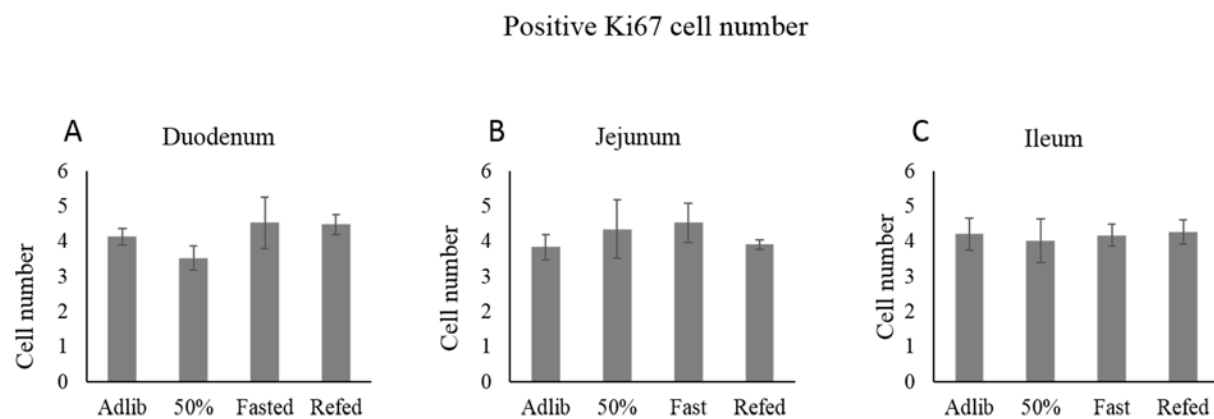


Figure 2.6: Impact of food intake on the number of proliferating intestinal epithelial stem cells from the proximal to distal axis. There is no significant difference in number of Ki67 positive cells in the proliferative zone between feeding groups in the duodenum (A), jejunum (B) or ileum (C). Data is expressed as mean \pm SEM

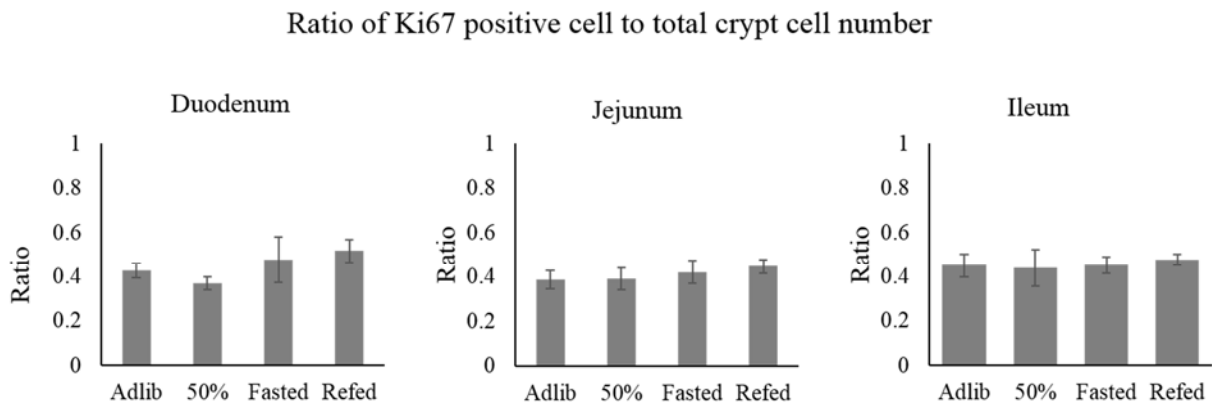


Figure 2.7: Impact of food intake on the number of proliferating intestinal epithelial stem cells from the proximal to distal axis. There is no significant difference in number of Ki67 positive cells in the proliferative zone between feeding groups in the duodenum (A), jejunum (B) or ileum (C). Data is expressed as mean \pm SEM

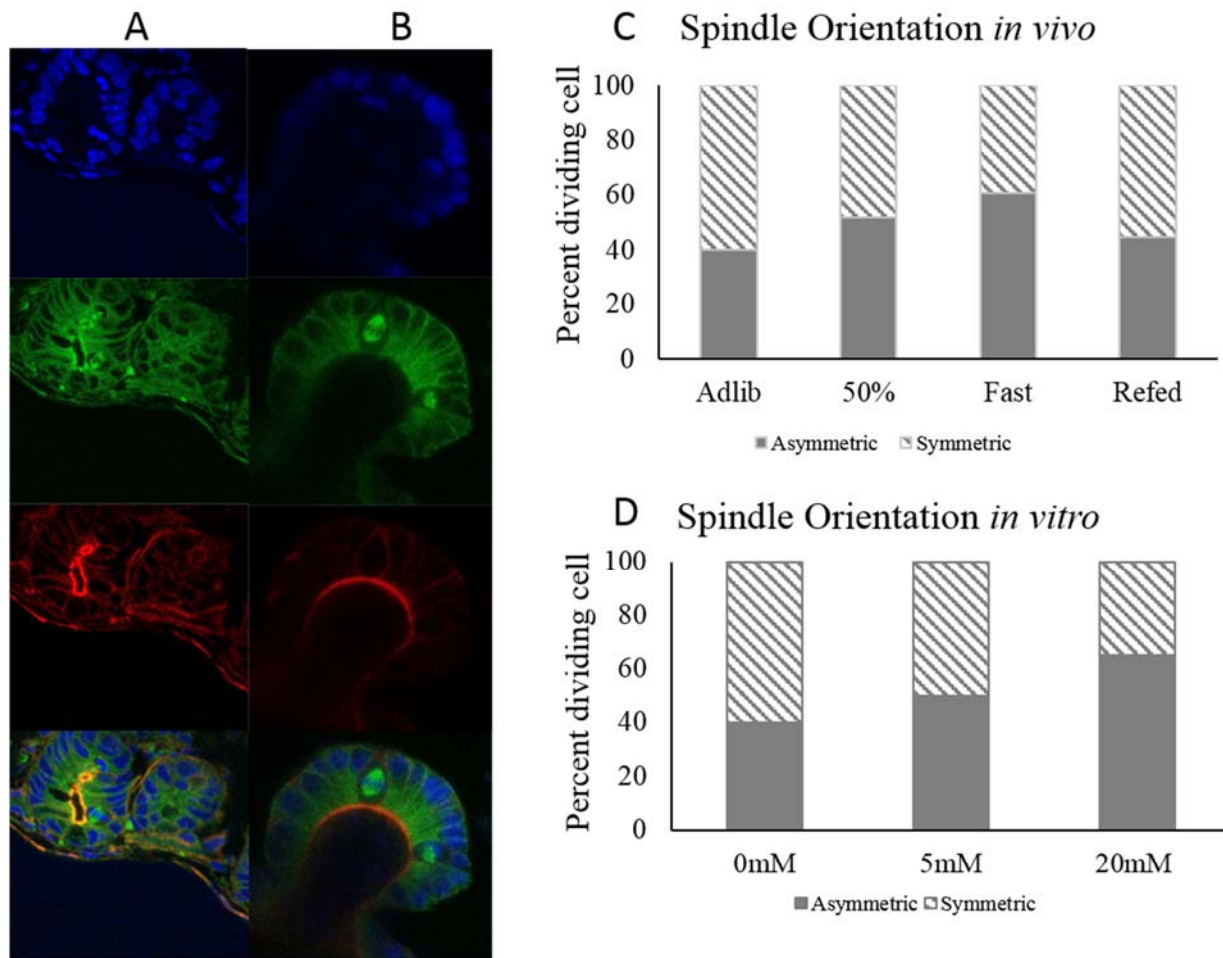


Figure 2.8: Mitotic spindle orientation is influenced by food intake *in vivo*. A) Representative fluorescent image of a symmetrically dividing stem cell. B). Representative fluorescent image of an asymmetrically dividing stem cell. C) There is a significant increase in asymmetric division as food intake decreases *in vivo*. D) There is a significant increase in symmetric to asymmetric divisions in the 20mM glucose group when compared to the 0mM group. Data is expressed as mean \pm SEM.

2.5 Discussion

A major goal of this study was to determine whether nutrient-induced intestinal expansion was driven by a change in the rate and/or a change in the mode of stem cell division, both *in vivo* and *in vitro*. The major findings were: 1) the amount of food intake does not influence the number of proliferating stem cells in the crypt, either when analyzed as a whole tissue, or by individual segments, 2) food intake significantly alters ratio of asymmetric to symmetric divisions *in vivo* and 4) there is a glucose concentration-dependent increase in symmetric division *in vitro*.

There is conflicting evidence in the literature concerning increased cell proliferation under high nutrient conditions. In our experiment, increased food intake did not significantly increase proliferating cell number. These results are consistent with data from David Sabatini's lab at MIT. Sabatini concluded that caloric restriction increased Bromodeoxyuridine (BrdU) positive stem cells in the crypt, while reducing the numbers of BrdU positive cells in the greater transit amplifying (TA) compartment (Yilmaz, 2012). These data indicate that caloric restriction, not increased caloric intake, increases stem cell proliferation. Conversely, several other groups have published data that suggests the opposite. For example, P. Kay Lund has shown that DIO mice show increased stem cell proliferation through S-phase marker, EdU, when compared to healthy body weight controls (Mah, 2014). The discrepancies between our studies and previous literature can possibly be accounted for by our use of Ki67, in lieu of BrdU. Ki67 and BrdU can be visualized during different phases of the cell cycle. Ki67 can be seen during all phases, where BrdU is only incorporated during the S-phase. In future experiments we aim to delineate the discrepancies seen in the literature regarding stem cell proliferation and nutrient availability.

Our results show that food intake significantly influence spindle orientation *in vivo*.

There is a significant increase in asymmetric division, as food intake decreases. These data are consistent with previous findings in *Drosophila* (O'Brien, 2011). Furthermore, we investigated this phenomenon in a 3D matrigel based culture system to determine whether glucose had a direct effect on the mode of stem cell division in vitro. Several studies, including our own, have shown that intestinal organoids are a promising model for studying cellular and molecular functions of the small intestine (Sato, 2009; Sato, 2011). These organoids are composed of differentiated daughter cells that are replenished by epithelial stem cells in the crypt region. Furthermore, differentiated daughter cells undergo routine apoptosis, express glut receptors and produce common intestinal hormones, such as PYY and CCK. Thus, it is not surprising that the mode of division was influenced by media glucose concentration. Under high glucose conditions, organoids exhibited a high rate of symmetric division. Conversely, under low nutrient conditions the majority of stem cells divided asymmetrically.

The task of translating media glucose concentration into physiological relevance is particularly tricky. We believe that luminal contents, including glucose, do not reach the crypt base. This implies that the crypt is not under the control of systemic glucose concentrations, but that of a localized glucose source within the mesenchyme. At this time we are unable to measure localized glucose concentration in the crypt, meaning we cannot fully compare in vitro and in vivo responses. Furthermore, cellular utilization of other macromolecules further complicates translation between in vitro and in vivo phenomena. For example, under fasting or low caloric intake mice can utilize several other substrates, such as amino acids and fatty acids, from the adipose and liver to compensate for low nutrient intake. In culture, organoids have access to low levels of essential amino acids, such as glutamine, but cannot derive energy from stored sources or utilize fatty acids.

Conclusion

Under high nutrient conditions, such as chronic obesity, mammals can change both the rate and mode of ISC division to grow the intestinal epithelium. By identifying the mechanisms by which obesity alters the morphology of the intestinal epithelium we will allow for the development of therapeutic strategies to attenuate or reverse the negative effects. These data suggest a promising therapeutic target for obesity in the control of spindle orientation

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Chapter 3: Changes in the mode of division under varying nutrient conditions are driven by LKB1/AMPK signaling

3.1 Introduction

A switch in the mode of division in stem cells requires that the amount of luminal nutrients can dictate the spindle orientation at division. Asymmetric division requires alignment of the mitotic spindle in an orientation parallel to the apical-basal axis and polarized localization of cell fate determinants to the apical or basal poles of the cell. This allows for two molecularly distinct cells to be produced and for the two cells to develop in different environments (one towards the apical border and the other towards the basal border). In order for this to occur, nutrient metabolism must be linked to the spindle orientation. This could be accomplished through the activity of adenylate kinases. Adenylate kinases buffer declining ATP production by converting two ADP's to one ATP and one AMP (Hardie, 2007; Kahn, 2005). The resulting accumulation of AMP activates the AMP-activated protein kinase (AMPK) network. The activation of the AMPK pathway is dependent on the tumor suppressor protein LKB1, a serine/threonine kinase, and leads to phosphorylation of several downstream effectors known to improve energy status within cells. The LKB1/AMPK pathway is known to be involved in energy regulation in many tissues, including the small intestine (Shakelford, 2008; Wei, 2012). In high nutrient conditions this pathway has been shown to be suppressed. In contrast, low nutrient conditions result in phosphorylation of AMPK and activation of downstream effectors. Furthermore, AMPK has been shown to directly interact with microtubule spindle poles and affect the number and integrity of astral microtubules (Thaiparamibil, 2012). It has not been shown whether LKB1/AMPK signaling plays a direct role in nutrient induced stem cell division. Thus, we investigated whether LKB1/AMPK plays a role in this switch. In order to test this hypothesis, we utilized both an *in vivo* mouse model and *in vitro* murine intestinal epithelial

organoids and measured changes in the mode of division and LKB1/AMPK gene expression and protein signaling in response to varied amounts of nutrients.

3.2 Materials and Methods

***In vitro* Methods**

Crypt isolation was performed as described in 2.3 Methods and Materials. A subset of crypts were immediately processed for gene expression and protein levels. A separate subset were utilized for culture of organoids.

Protein Isolation and Analysis

Isolated crypts were collected and protein extracted using TPer protein extraction reagent (ThermoFisher, Waltham, MA) per manufacturer's instructions.

Western Blot

Intestinal segments from the duodenum, jejunum and ileum were immediately frozen on dry ice and stored at -80. Total protein was extracted using 1X RIPA buffer (89900, ThermoFisher, Waltham, MA). Proteins were separated using a 4-12% Bolt BIS/TRIS gel system (NW04125BOX, ThermoFisher Scientific, Waltham MA) at 200volts for 25 minutes. The gel was transferred onto a nitrocellulose membrane (ThermoFisher, Waltham, MA)) at 100 volts for 1hr and then blocked in 10% milk diluted in 1X Tween/PBS (Catalog number) for 1 hour at room temperature with shaking. The membrane was then incubated in Rabbit monoclonal primary antibodies against AMPK α (1:100), phospho-AMPK α (Thr172) (1:100), GAPDH (1:1000), Cell Signaling Technologies, Beverly, MA) and LKB1 (1:500) (ab15095, Abcam, San Francisco, CA) diluted in blocking buffer described above, overnight at 4 degrees with gentle shaking. Membrane was rinsed in PBS and incubated in goat monoclonal HRP-conjugated anti-rabbit secondary antibody (ab6721, Abcam, San Francisco, CA) at room

temperature for 1 h with gentle shaking. Protein was detected using SuperSignal™ West Pico Chemiluminescent Substrate (34080, ThermoFisher Scientific, Waltham, MA) per manufacturer's instructions and imaged with an ImageQuant LAS 410 Luminescent image analyzer (GE Healthcare, Chicago, IL). Relative protein expression was estimated using band intensity quantified by imageJ software and, normalized to that of GAPDH.

Statistical Analysis

Values are expressed as the mean \pm SEM. All data was analyzed using a one way ANOVA with feeding group as the independent measure and protein concentration (AMPK or pAMPK) as the dependent measure. Tukey-Kramer post hoc analysis was utilized and differences among groups were considered statistically significant if $P < 0.05$.

Metformin/Compound C in vitro

Isolated crypts were allowed to grow for 4 days. At 0800h on the 5th d, crypts were changed to glucose free media (Basal culture medium with N2 supplement (1 \times), B27 supplement (1 \times), and 1 mM N-acetylcysteine, 50 ng/mL EGF, 100 ng/mL Noggin, 1 mg/mL R-spondin.) and incubated at 37° at 5% CO₂ for 4 h. After a 4 h glucose starvation, organoids were supplemented with 0mM or 20mM glucose and either 1mM metformin HCL, 1mM Compound C or a vehicle control. Following a 5 h incubation, organoids were fixed, processed, mitotic spindle orientation visualized and calculated as described in 2.2b

Statistical Analysis

Values are expressed as the mean \pm SEM. All data was analyzed using a one way ANOVA with drug group as the independent measure and ratio of symmetric division as the dependent measure. Tukey-Kramer post hoc analysis was utilized and differences among groups were considered statistically significant if $P < 0.05$.

3.3 Results

Protein levels

There was no significant difference in AMPK levels between groups in any section (figure 3.1.a-c). There was no significant differences in pAMPK expression between groups in the duodenum and the jejunum (Figure 3.2.a-b). In the ileum, there is a significant increase in the levels of pAMPK in the adlib group compared with the fasted group and a significant increase in adlib group compared with the refed group (figure 3.2.c; $p < 0.05$).

Activation or inhibition of LKB1-AMPK signaling

Activation of AMPK through application of metformin results in a significant increase in asymmetric division under high glucose conditions (figure 3.3; $p < 0.05$). Similarly, inhibition of AMPK using compound C significantly increases symmetric division under low glucose conditions (figure 3.4; $p < 0.05$). Furthermore, stem cells inhibited with Compound C displayed abnormal spindle development (figure 3.5.a) and displayed significantly shorter spindles when compared to vehicle controls (figure 3.5.b).

3.4 Tables and Figures

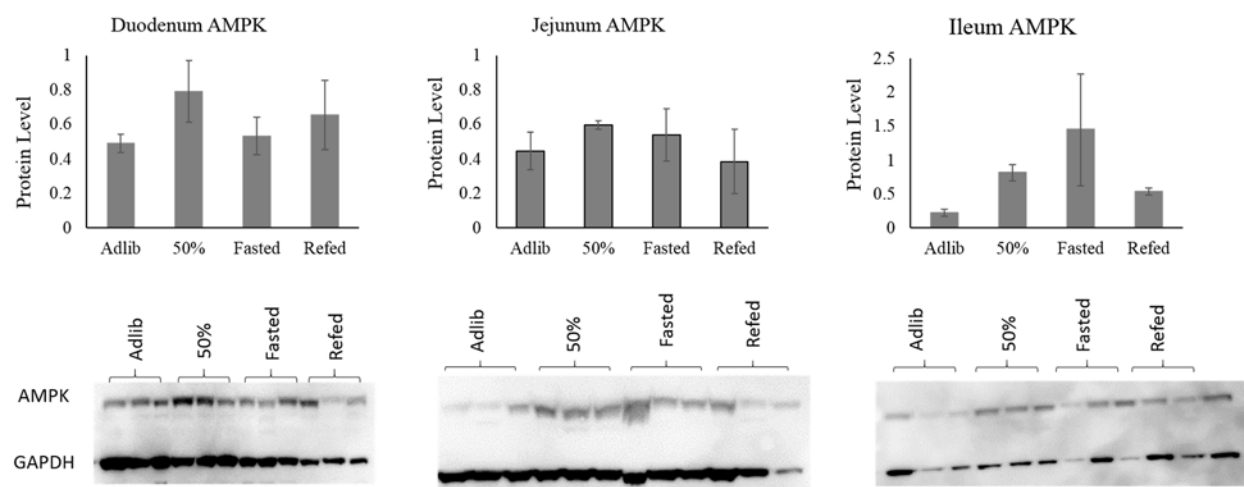


Figure 3.1: Western blot analysis for AMPK. There is no significant difference in AMPK levels in the A) duodenum, B) jejunum or C) ileum between feeding groups. Data expressed as the mean \pm SEM

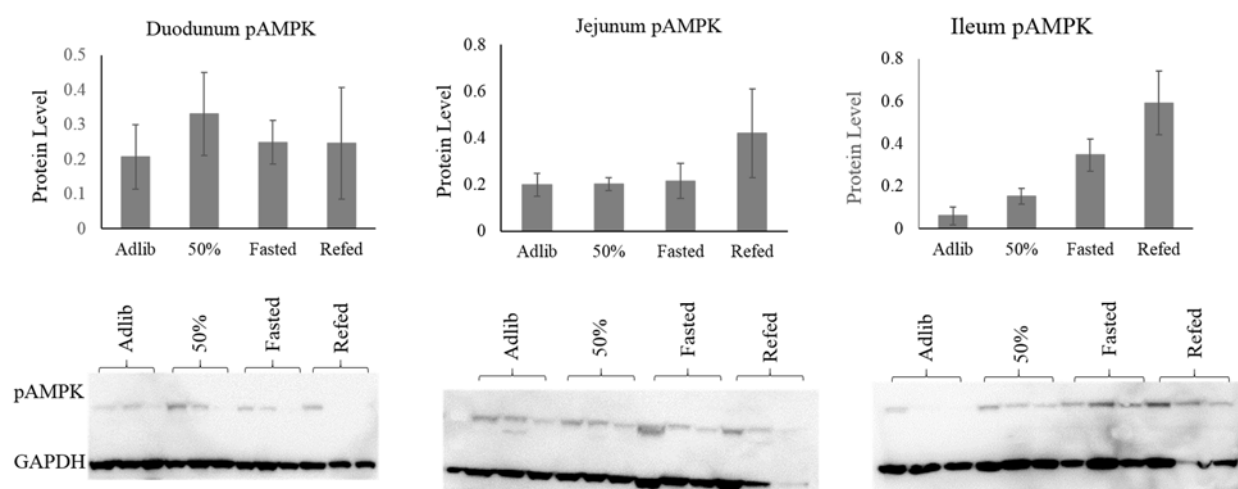


Figure 3.2: Western blot analysis for pampk. There is no significant differences in pAMPK levels between feeding groups in the A) duodenum or B) jejunum. C) There is a significant increase in pAMPK in fasted and refed animals in the ileum. Data expressed as the mean \pm SEM

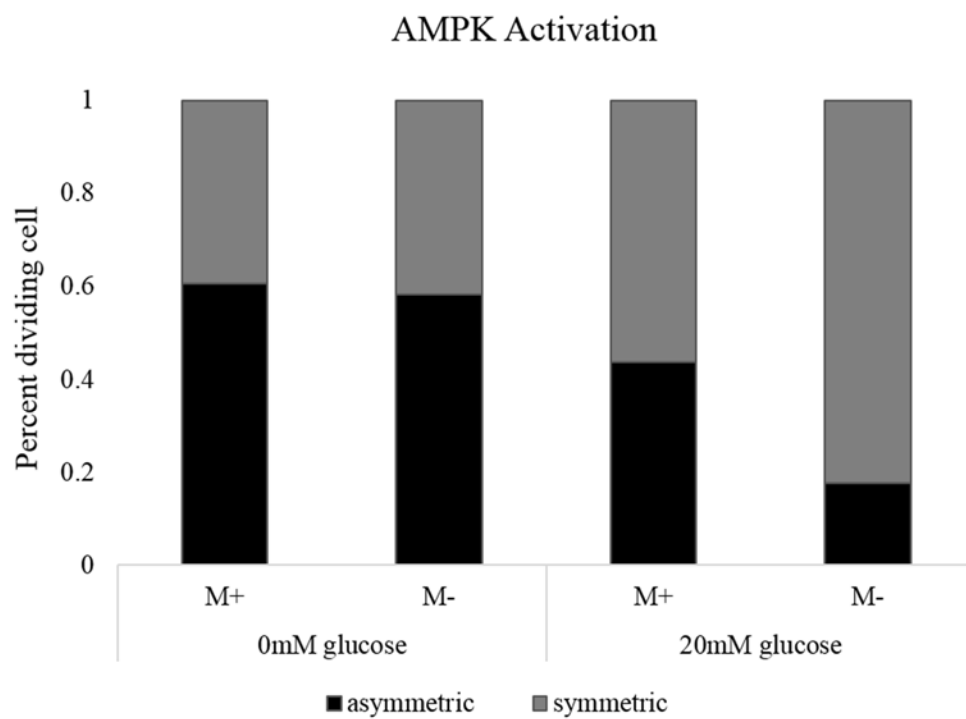


Figure 3.3: Activation of AMPK increases asymmetric division under high glucose conditions.

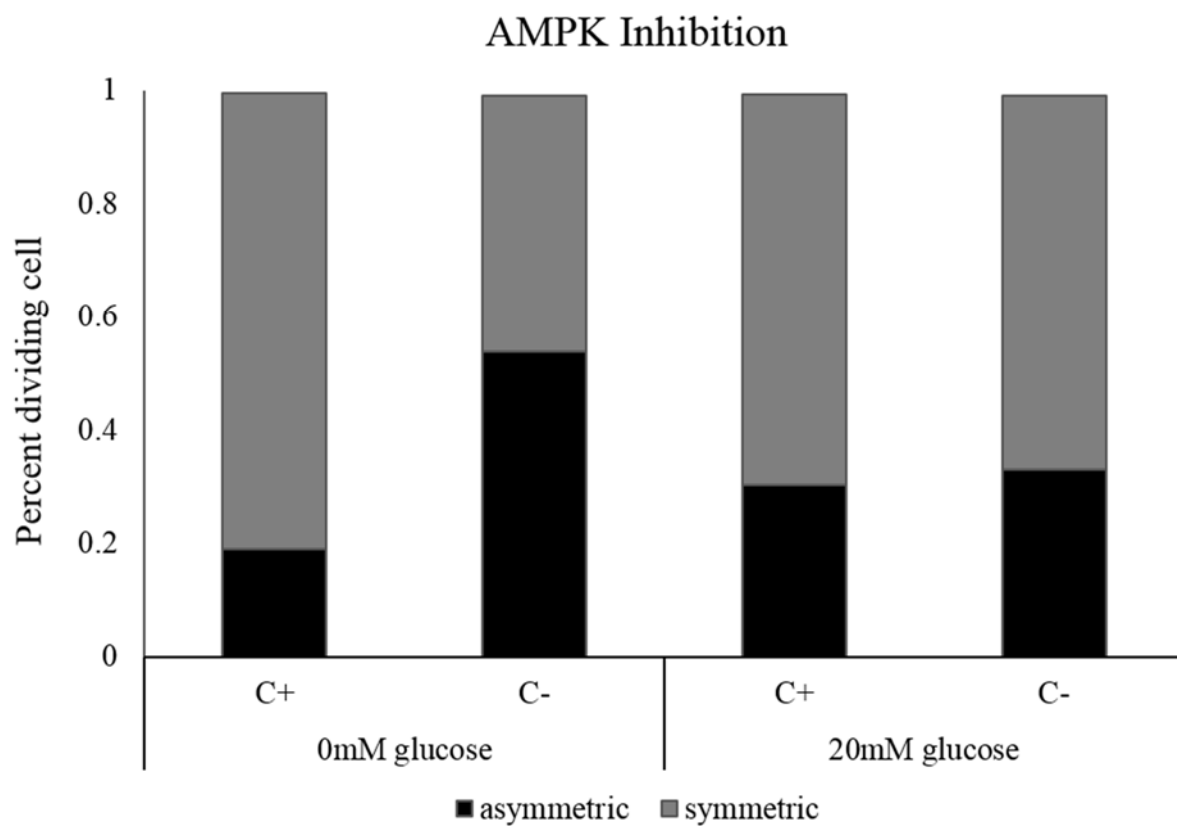


Figure 3.4: Inhibition of AMPK increases symmetric division under low glucose conditions. Data expressed as mean \pm SEM

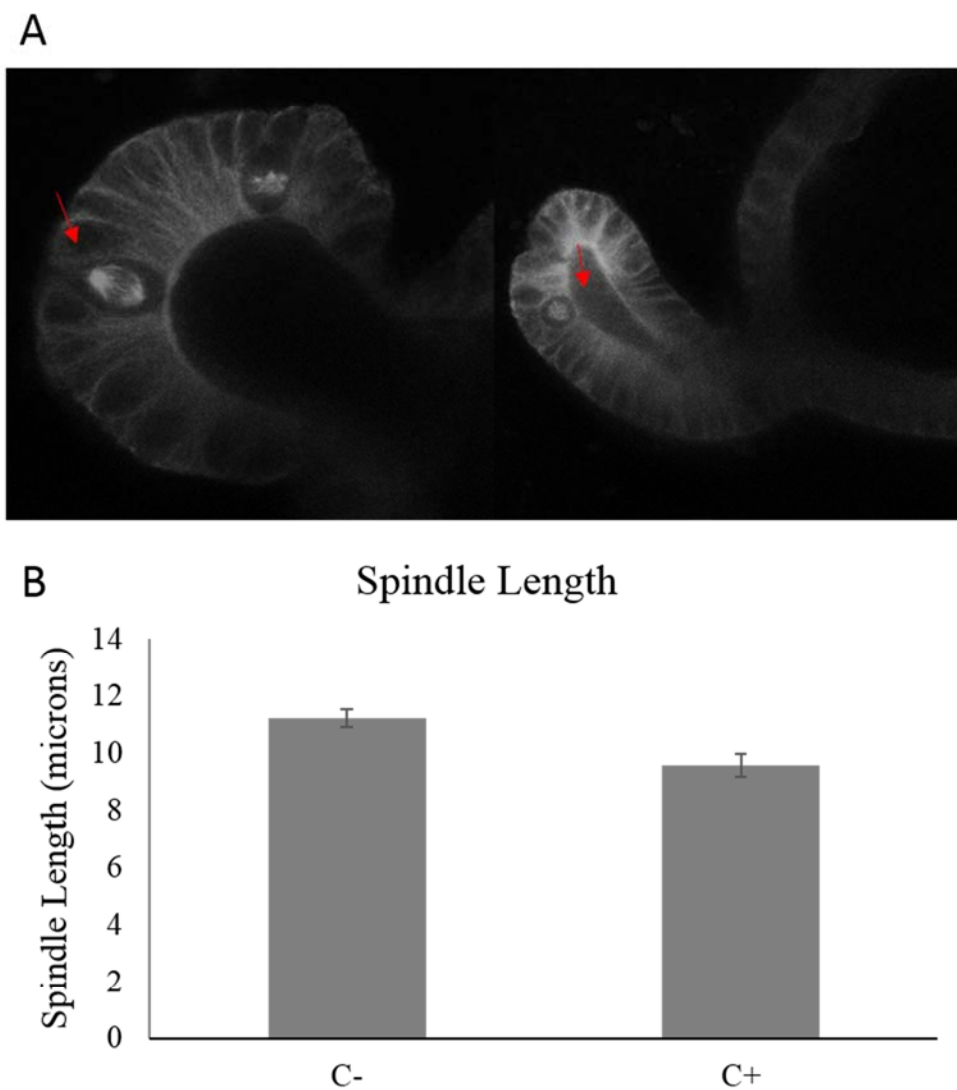


Figure 3.5: Inhibition of AMPK through compound C results in A) disorganized spindle microtubules in the mitotic spindle and B) significantly shorter mitotic spindles pole to pole. Data expressed as mean \pm SEM

3.5 Discussion

A major goal of this study was to determine whether differences in the mode of division is driven by the LKB1/AMPK pathway. The major findings were 1) food intake does not influence LKB1 or AMPK gene expression or protein levels *in vivo* 2) decreased food intake increases pAMPK in the ileum, but does not affect the or the jejunum *in vivo* 3) activation of AMPK drives asymmetric division in high glucose conditions *in vitro* 3) inhibition of AMPK drives symmetric division under low glucose conditions *in vitro* 4) inhibition of AMPK results in disorganized spindle structures and shortened spindle lengths *in vitro*.

The role of the LKB1-AMPK pathway is well known in metabolism and growth (Hardie, 2007; Kahn, 2005). Alterations in this pathway result in many of the cellular changes seen in obesity, and mutations of the LKB1 (aka. STK11) gene leads to Peutz-Jeghers syndrome in humans, characterized by the development of non-cancerous growths in the gastrointestinal tract and an increased risk of developing cancer (Mehenni, 1998). In non-mammalian species, LKB1, or the homologous Par-4, is known more widely as a PAR protein involved in directing the partitioning of molecules during asymmetric division (Benkemoun, 2014; Bonaccorsi, 2007). Therefore, LKB1 is a likely candidate for linking metabolism with changes in polarity during division in IESCs. LKB1 directly phosphorylates AMPK under low nutrient conditions which acts as a central regulator to restore energy balance (Wei, 2012). This is accomplished by attenuating muscle and liver adipogenesis and fatty acid synthesis and upregulating gluconeogenic genes. Interestingly, in this study we did not find that food intake altered the level of AMPK phosphorylation in crypt cells when comparing fed and fasted animals. However, when we manipulate this pathway *in vitro* we see significant changes in the division patterns of ISCs. Organoids under AMPK inhibition show statistically significant increases in

symmetric division in low glucose conditions. Although we have been unable to delineate the mechanism behind these discrepancies we propose several hypotheses.

Primarily, in this study we have isolated protein from the whole crypt. It is possible that the phosphorylation of AMPK and subsequent alteration in spindle orientation is cell specific. There is significant data that supports that LGR5 and BMI1 ISCs respond differently to several cell signaling mechanisms. For example there are significant differential response of BMI1 vs. LGR5 to canonical Wnt and IGF1 signaling (Yan, 2012; van Landeghem, 2015). Thus, it is possible that these two stem cell populations respond differently to food deprivation and AMPK signaling. We would be interested in investigating whether the levels of pAMPK and/or a change in spindle orientation is occurring in only one population of stem cell. This would explain why the pAMPK levels in the whole crypt do not change, but why we see a switch in the mode of division under differing levels of AMPK phosphorylation.

Similarly, there is significant evidence that suggests that BMI1 and LGR5 respond differently under exogenous stress, such as radiation (Metcalf, 2014). Under radiation, LGR5 expression is abolished, whereas, there are no discernable effects to the BMI1 population (Metcalf, 2014). If we consider fasting an assault to the small intestinal epithelium it is very possible that the LGR5 population is reduced, while the BMI1 population is unaffected. This strengthens the argument that this could be a cell selective process, where an increase in pAMPK and subsequent change in mode of division is only occurring in a single population of cell, and not the entire crypt.

Finally, we must take into account the selectivity of protein kinase inhibitors. Compound C has an IC_{50} of 0.1-0.2 μ M (Bain, 2007), however at this concentration, compound C also inhibits several other protein kinases with similar or greater potency. Other inhibited protein

kinases include extracellular-regulated kinase 8 (ERK 8, also known as mitogen-activated protein kinase (MAPK) 15), Map kinase interacting serine-threonine kinase 1 (MNK1), maternal embryonic leucine zipper kinase (MELK), Dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) (Bain, 2007). The functions of these genes range from interacting with microtubule-associated proteins (MAP), protein coding and translation and regulating cell proliferation (Abe, 2002; Chrestensen, 2007; Nakano, 2005; Aranda, 2011). Thus, it is possible that the effect we are seeing under assumed AMPK inhibition is either compounded by, or a result of, inhibition of other kinases. Although the specificity of metformin is not as well characterized we can postulate that it also cross reacts with other protein kinase networks.

Consistent with previous findings (Tirnauer, 2012; Marcus et. al, 2012) organoids treated with compound C, an AMPK inhibitor, displayed disorganized microtubule structures and significantly shorter spindle length. At this time, the mechanism behind this phenotypic change has not been characterized. However, there are several cytoskeletal signaling complexes downstream of AMPK, such as the Clip170/Tau complex which is responsible for binding newly polymerized distal ends of growing microtubules and controlling the speed of microtubule polymerization (Takashima, 2010). Similarly, Marcus et. al (2012) proposed that AMPK indirectly phosphorylates MRLC, which is indicated in actin turnover. When MRLC is phosphorylated by AMPK actin turnover is halted, creating actin bundling and subsequent inhibition of astral microtubule formation by myosin light chain II, a critical step in spindle positioning and division (Marcus et. al, 2012). Our study does not address downstream signaling of AMPK and further studies are needed to test these proposed mechanisms.

Conclusion

The LKB/AMPK pathway plays a significant role in the mode of division under varying nutrient conditions. However, at this time we are unsure how LKB1/AMPK influences the mode of

division and whether this is a cell specific response.

3.6 References Cited

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