Intestinal stem cell regulation by the hexosamine biosynthesis pathway in *D. melanogaster*

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Masters's thesis

The intestinal stem cells (ISC) of the *D. melanogaster* midgut, which is the functional analogue to mammalian small intestine, are highly responsive to changes in nutrition. ISC employ the hexosamine biosynthesis pathway (HBP) to monitor nutritional status. HBP activity is an essential facilitator for insulin signaling-induced ISC proliferation. The midgut's compartmentalized structure allows the study of many regulatory pathways. The regions of the midgut are characterized by distinct gene expression patterns, different histology, and physiological functions. The homeostatic regulation of intestine in fluctuating dietary conditions is poorly understood.

In this study the interaction of the HBP and nutrition in the ISC population of the midgut were studied via confocal microscopy and the Longitudinal Analysis of the Midgut (LAM). The HBP was activated in different dietary conditions in ISC by feeding the flies with Glucosamine or by expressing the rate limiting enzyme Gfat2 in the ISC and their progeny. The increased clonal cell numbers suggest higher cellular turnover leading to higher stem cell proliferation rate in comparison fed versus starved dietary conditions. LAM gives a region-specific elevation of the clonal numbers of the cell. When experimenting which nutrients mediate the proliferative capacity of stem cells, we found that removing essential amino acids have similar effect on R4 region compared to the starved versus fed condition. Our results give new insights to the nutritional response and the region-specific activation for further research in connection with the HBP activation.

Pro gradu -tutkielma

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Nisäkkäiden ohutsuolta vastaavan banaanikärpäsen (*Drosophila melanogaster*) keskisuolen kantasolut ovat hyvin herkkiä reagoimaan ravinnon muutoksiin. Heksosamiinin biosynteesireitti (HBP) toimii avainasemassa suolen kantasolujen jakaantumisen säätelyssä muovaamalla suolen kokoa erilaisiin ravinto-olosuhteisiin sopeutumalla. HBP:n aktiivisuus säätelee insuliinin signaloinnin indusoimaa kantasolujen lisääntymistä. Keskisuolen eri alueet vaihtelevat geeniekspression, histologian ja fysiologisten ominaisuuksien suhteen. Suolen jaoteltu rakenne mahdollistaa säätelyreittien tutkimisen. Ravinto-olosuhteiden vaikutusta suolen homeostaasin säätelyssä on tutkittu vähän.

Tässä tutkimuksessa heksosamiinisynteesireitin ja ravinnon vuorovaikutusta tutkittiin konfokaalimikroskopian sekä "Longitudinal Analysis of Midgut"-menetelmän (LAM) avulla suolen kantasoluissa. Heksosaminiisynteesireitti aktivoitiin kantasoluissa joko syöttämällä glukosamiinia tai yliekspressoimalla reitin entsyymiä glutamiini-fruktoosi-6-fosfataasi-aminotransferaasia (Gfat2). Havaitsimme solumäärien lisääntymistä kloonien sisällä verrattaessa dieettiolosuhteita keskenään. LAM:in avulla saadaan mitattua spesifisti suolen eri alueiden välistä eroa kloonien koossa. Dieettiolosuhde, jossa poistettiin välttämättömät aminohapot ravinnosta, sai aikaan samankaltaisia tuloksia suolen R4 alueella kuin starvaation ja täyden ravinto-olosuhteen keskinäinen vertailu. Saadut tulokset antavat uutta tietoa ravinto-olosuhteiden vaikutusksesta sekä HBP:aktivaatiosta suolen eri alueilla.

Content

1. Introduction

1.1 Background

The regulation of intestinal homeostasis is the result of maintenance of specific mutualistic and symbiotic relationships, elimination of microbial pathogens and tight control of tissue regeneration and inflammatory responses (Apidianakis and Rahme, 2011; Garrett et al., 2010). Chronic or acute dysfunction of these processes may lead to several disorders including metabolic imbalance, gastrointestinal infections, inflammatory bowel disease and colorectal cancer (Garrett et al., 2010). The fruit fly *Drosophila melanogaster* is well suited for studying conserved and specific aspects of the human intestinal diseases. Many of the pathways are highly conserved and exploring the function in the fly model may provide new insights to intestinal pathology (Apidianakis and Rahme, 2011).

The fruit fly's gastrointestinal tract is a dynamic organ, which plays a significant role in survival, and reproduction. This organ possesses similar features as that of the mammalian digestive system both in structure and function. It is divided into three different domains (foregut, midgut, hindgut) of discrete developmental origins (Buchon et al., 2013). The midgut has the ability to grow in size when nutrient is abundant (Miguel-Aliaga et al., 2018). On the contrary, in nutrient depleted conditions the division rate of the mitotic intestinal stem cells (ISC) is slowed down (Choi et al., 2011).

This adaptability is regulated by various nutrient sensing and signaling pathways (Choi et al., 2011; O'Brien et al., 2011; Mattila et al., 2018). Unfolding the true mechanism between stem cell activation and nutrition is crucial for understanding epithelial renewal and regulation of homeostasis (Apidianakis and Rahme, 2011).

The *Drosophila* midgut has become an important model in studying how nutrition affects cellular features. The midgut's compartmentalized structure allows the study of many regulatory pathways and mechanisms in a controlled manner (Buchon et al., 2013; Marianes and Spradling, 2013). The regions of the midgut are characterized by distinct gene expression patterns, different histology, and physiological functions. Together these regions form a functional unit where all nutrients are systematically broken down and

absorbed as food moves from the anterior to posterior parts of the midgut (Lemaitre and Miguel-Aliaga, 2013; Miguel-Aliaga et al., 2018).

The intestine is a significant energy consuming tissue (Cruzat et al. 2018) and thus its volume and cellular architecture is tightly regulated. There are frequent fluctuations in nutrient uptake in the intestine, and cellular construction and volume adjustments are adaptive mechanisms. In times of starvation, the epithelial lining siphons fewer calories than required by the body. In response, the overall intestines' mass shrinks, and it also develops fewer enterocytes. However, refeeding triggers the intestine to regain their original state (Mattila et al., 2018). Organ renewal maintains constant cell numbers by coordinating the proliferation of stem cells with the loss of differentiated cells (O'Brien et al., 2011). Tissue homeostasis depends on cell turnover replacing damaged and aged cells through asymmetric stem cell divisions. However the cellular and molecular mechanisms of adaptive growth remains poorly understood. (O'Brien et al., 2011) .

The intestinal epithelial cells are in close contact with the external environment to facilitate nutrient digestion (Losick et al., 2011; Diefenbach et al., 2020). High cell turnover occurs in the intestine and therefore the gut epithelia is under constant renewal through ISC division and differentiation. ISCs and their progenitors are maintained with the supportive microenvironment called the niche (Crosnier et al., 2006). The niche derives signals to control ISC and their progenitor maintenance, differentiation, survival, proliferation and prevention of excessive stem cell production. This environment and the systemic signals originating outside the niche help the ISCs to maintain tissue homeostasis (Moore, 2006; Shim et al., 2013).

The HBP pathway is a key player regulating ISC response to nutrition and midgut adaptation. HBP incorporates intracellular glucose, acetyl-CoA, glutamine and UTP into the synthesis of uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc), a substrate for macromolecule glycosylation (Mattila et al., 2018). HBP and nutrient sensing has a major role in regulating metabolic processes and cellular homeostasis (Akella et al., 2019). In addition, HBP is well conserved across phyla. In this regard, the fruit fly is a good model organism in studies that entail the ISCs and the HBP and how cellular functions are affected by nutrition.

1.2 *Drosophila melanogaster*

Drosophila melanogaster (fruit fly) belongs to the order Diptera, family Drosophilidae. Flies and vertebrates have conserved genetic, developmental, and biochemical blueprints concerning maintaining homeostasis and gut epithelial stability (Southall et al., 2008). The research on human disease have used the fruit fly to study multiple pathological aspects and the fruit fly is undeniably one of the best known model organism (Narbonne, 2018). The fruit fly has been widely used as a model organism in genetic studies for a long time.

There are several concrete reasons why molecular scientists prefer using the fruit fly as a model organism. First, this insect's gene sequence is similar to a significant proportion of human gene sequences. It is approximated that 75 % of genes causing human diseases exist in the fruit fly's genome (Wu and Luo, 2006). The fruit fly's reproductive cycle is about 11 days making this model organism suitable for laboratory experiments. The maintenance of flies in laboratory is also relatively inexpensive, compared to other model organisms. Lastly, their genes are easy to manipulate (Baenas and Wagner, 2019).

1.2.1 The physiology and anatomy of *D. melanogaster* midgut

The adult intestine consists of an epithelial monolayer consisting of four cell types: the intestinal stem cell, the enteroblasts, an immature postmitotic cell type which will differentiate into the absorptive enterocyte, and the secretory enteroendocrine cell (Miguel-Aliaga et al., 2018). This monolayer epithelium is surrounded by a layer of mesodermally derived viscelar muscle with one circular and one longitudinal oriented actin-myosin fibers. Inside the midgut lumen is a chitinous layer: the perithropic matrix which separates the epithelium from the ingested food, and serves as a border against the gut bacteria (Hegedus et al., 2009; Jiang and Edgar, 2011). However, along the midgut's anterior-posterior axis, there are variations both at the gross anatomy and cellular levels. Regional compartmentalization and anatomical specializations both enable subsequent ingestion, storage, digestion, absorption and excretion (Karasov et al., 2011; Miguel-Aliaga et al., 2018).

In the anterior part is the ectodermally derived foregut which is subdivided into esophagus, crop and cardia. Posterior to the cardia is the major digestive and absorptive

part: the endodermally derived midgut. The Malphigian tubules function as tubular excretory organs and discharge at the junction between the midgut and ectodermally derived hindgut where water/ion exchange may occur. The single layered epithelium is surrounded by striated muscles. Circular muscles are located throughout the tract and an outer layer of longitudinal muscles surrounds the midgut. The physiology of the intestine is regulated by hormones and autonomic innervation. Furthermore, the gut is influenced by the tracheal system (Miguel-Aliaga et al., 2018). Multiple physiological processes occur in the midgut, and the cell turnover rate is exceptionally high. There is a need for constant regulation and proliferation of cells, which is checked by the ISCs and a supportive cellular environment, the niche.

Despite the remarkable similarities between the fruit fly midgut and those of vertebrates, considerable disparities exist. The fruit fly has a midgut with an epithelium containing progenitor cells (Capo et al., 2019). Consequently, the fruit fly lacks a crypt-villi like structure. Mammalian intestine epithelial tissue comprises of the extracellular fibrous basement membrane. The vertebrate intestinal column is also distinct in the villi's shape, which assumes a crypt-villi structure. This shape replenishes the new cells that arise from the invaginations, called the Crypts of Lieberkühn. Animal epithelial cells are short-lived and thus regenerated continuously (Apidianakis and Rahme, 2011).

However, Apidianakis & Rahme (2011) put forth that the mammalian intestine contains the Paneth, Goblet, Stromal, and Dendritic cells, which are lacking in the fruit fly. The fruit fly's midgut has chitin and glycoprotein toughened peritrophic matrix that functions like the mammalian goblet cells; their primary function is to shield the membrane from pathogen attack. Lastly, there is no transit amplification of the cell population in the fruit fly (Buchon et al., 2013).

1.2.2 Regionalization of the *Drosophila* midgut

The Drosophila midgut is divided into distinct regions (Capo et al., 2019). The gross subdivision includes the anterior midgut, the middle midgut and the posterior midgut but it has been molecularly and morphologically subdivided into further regions (Buchon et al., 2013; Marianes and Spradling, 2013; Murakami et al., 1994). The anatomical regionalization includes 5 major regions (Figure 1). which are further subdivided into 8 histological and 14 genetic subregions (Buchon and Osman, 2015). Each subregion has a sharp boundary with its neighbors suggesting that it carries out distinctive purpose. The

regionalization is an essential feature of the digestive tract because it regulates digestion by enabling sequential features ranging from the uptake and processing of food to nutrient absorption and waste elimination (Karasov et al., 2011; Buchon et al., 2013).

Each midgut region is characterized by specific cellular and histological features, stem cell proliferation rates, physicochemical properties and gene expression profiles (Murakami et al., 1994; Marianes and Spradling, 2013; Miguel-Aliaga et al., 2018). These variable cell compositions are considered functional units. The regionalization is not limited to the epithelium but it is also observable in the visceral muscles, trachea and neurons that surround the midgut (Cognigni et al., 2011; Marianes and Spradling, 2013; Miguel-Aliaga et al., 2018).
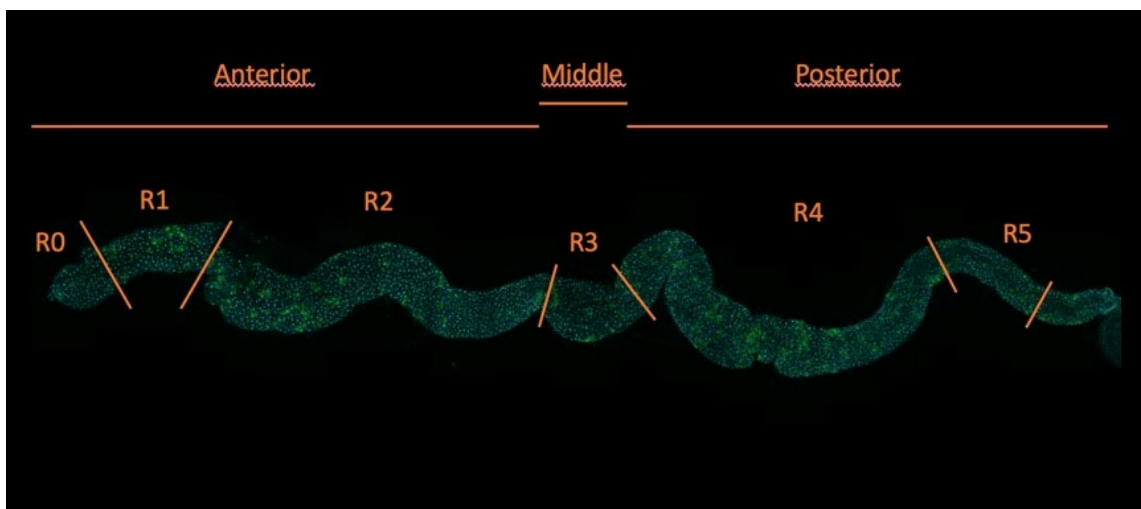

Figure 1. The regions of the adult midgut. *The anterior, middle and posterior regions of the midgut and the primary region divisions R0-R5 described by Buchon et al. (2013)*

While notable differences in cellular function and composition exist in the different regions of the intestinal epithelium, all regions of the midgut contain ISCs able to regenerate all cell types of their particular region (Buchon and Osman, 2015). Midgut ISCs are interspersed among their differentiated progeny and they are heterogenous in both their gene expression and cellular behavior which may contribute to maintaining regional differences (Marianes and Spradling, 2013). Coherent with this, mosaic analysis has shown that ISCs in certain region tend to sustain their region's progeny and rarely contribute to the production of differentiated cells in adjacent regions (Miguel-Aliaga et al., 2018). Although there are lot of evidence of the regional behavior there is still lacking

knowledge describing and integrating the relationship between the structure and function of different gut regions (Buchon et al., 2013).

1.3 Stem cells of the intestine

The architecture of the midgut epithelium contains multipotent intestinal stem cells and their differentiated daughter cells (Figure 2). The midgut turns over at a rate of about once per 1-3 week depending on the exploited lineage tracing system, and the experimental conditions (Jiang and Edgar, 2011). There are two progenitor cell types in the midgut: stem cells and their immature daughter cells, the enteroblasts (EB). Stem cells are the only mitotic cells whereas EBs terminally differentiate without further divisions (O'Brien et al., 2011; Ohlstein and Spradling, 2006). Both progenitors are diploid cells which express the SNAIL family transcription factor *escargot* as a genetic signature (Capo et al., 2019; O'Brien et al., 2011). EBs also express the Notch activity reporter *Suppressor of hairless (Su(H))*. EBs are localized apically to their mother stem cell. Ten percent of the EBs differentiate into enteroendocrine cells whereas 90 % differentiate into enterocyte cells (Ohlstein and Spradling, 2007, 2006).
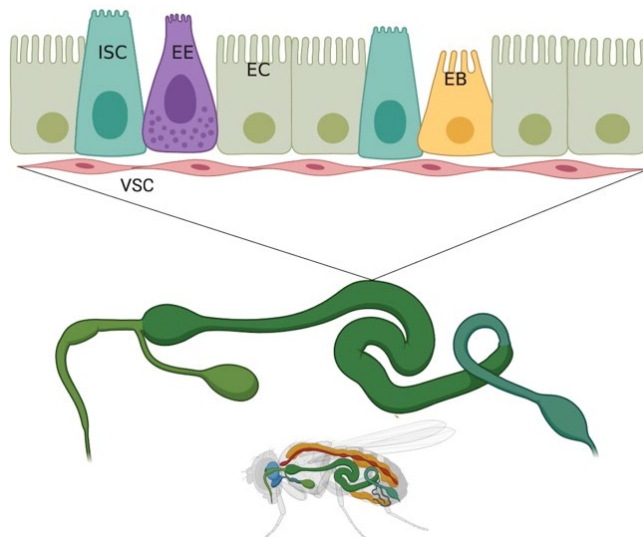
Figure 2. The Drosophila midgut and the architecture of the epithelium. *On the left side is the foregut including the crop. The midgut is in the middle and on the posterior side is the hindgut with malphigian tubules which are the Drosophila analogue to kidneys. Intestinal stem cells (ISC), enteroendocrine cells (EE), enterocytes (EC) and enteroblasts (EB) populate the midgut, which is surrounded by visceral muscle cells (VSC).*

Enteroendocrine cells (EE) express the marker gene*, Prospero.* The EEs primary function is to secrete hormones regulating gut function and mobility in response to abiotic and

biotic stimuli coming from the external environment (Capo et al., 2019). The variety of hormones are produced region-dependently (Ohlstein and Spradling, 2006). EEs release peptides and hormonal signal that potentially control inter-organ communication such as the gut-brain axis to transmit the organismal nutritional status and to modulate metabolism and behavior in response to nutrient availability (Reiher et al., 2011; Capo et al., 2019).

The enterocytes differ especially in function and morphology within different regions of the gut. Most of them express the gene *Myosin31DF* (Capo et al., 2019). The ECs primary role is the absorption and transportation of nutrients and secretion of digestive enzymes (O'Brian et al., 2011). According to Zielke and colleagues (2013) enterocyte cells stop proliferating but remain in a final differentiated state that may regulate tissue size or adapt to environmental conditions or stress or injury. Another important feature of the enterocytes is the ability to increase their size via endoreplication. The cells are programmed to exit the mitotic cycle in G2 phase and undergo multiple S phases excluding mitosis and cytokinesis. These cells become into nonproliferating polyploid cells which can increase the expression and copy number of cell-type relevant genes and increase cell and tissue size (Zielke et al., 2013).

1.4 Hexosamine synthesis pathway

Mattila et al (2018) revealed a novel mechanism of ISC regulation connecting the ISC extrinsic growth signals to intrinsic nutrient metabolism. The key player regulating ISC response to midgut adaptation and nutrition sensing is the hexosamine biosynthesis pathway (HBP). The pathway activity is functioning through a Warburg effect-like regulatory switch in central metabolism into ISC division rate. Warburg effect regulates the balance between glycolysis and oxidative phosphorylation supporting the proliferation of ISC and adaptation to nutrient content. HBP activity also defines the responsiveness of insulin (InR)-mediated signaling in the ISC (Mattila et al., 2018).

Glucose, glutamine, amino acids and fatty acids act as substrates for the HBP pathway. Cell growth is firstly supported by growth factor-driven glutamine and glucose intake. The HBP's end product is uridine diphosphate N-acetyl glucosamine (UDP-GlcNAc) that, along with other charged nucleotide sugars, functions as the basis for biosynthesis of glycoproteins and other glycoconjugates (Figure 3). The pathway and its end product

are also important players in cell signaling that favor tumor promotion. The nutrient driven post-translational modifications are highly altered in cancer and cancer-associated protein functions (Akella et al., 2019).
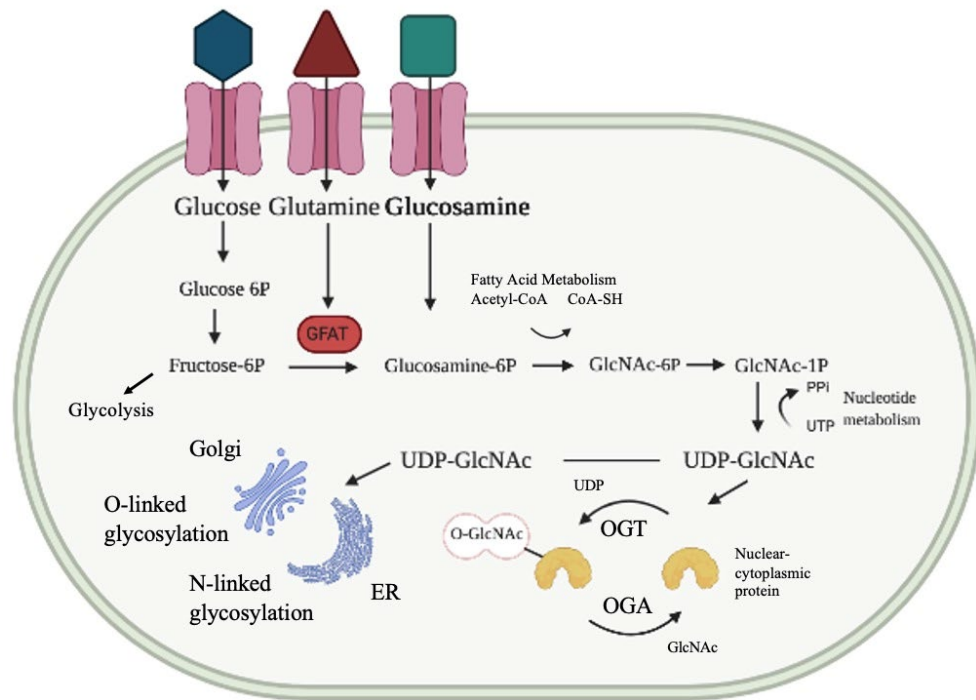

Figure 3. The hexosamine biosynthetic pathway. *After entering the cell glucose undergoes two-step conversion to fructose-6P after which around 95% of it proceeds to glycolysis and 3-5% of it is converted to glucosamine-6P by the GFAT enzyme utilizing glutamine that enters the cell. GFAT catalyzes the first and rate-limiting step of the pathway. It is the key regulator of the HBP. After that glucosamine-6P is converted into GlcNAc-6P also utilizing acetyl-CoA that is made from fatty acid metabolism. This can also be made by glucosamine entering the cell. This is then converted to GlcNAc-1P and further to UDP-GlcNAc by utilizing UTP from the nucleotide metabolism pathway. The end product UDP-GlcNAc is then used for O-linked and N-linked glycosylation in the Golgi in the ER and for O-GlcNAc modification of cytoplasmic and nuclear proteins by OGT. More detailed description of the pathway is described by Akella et al. (2019).*

UDP-GlcNAc is regulated in large part by the metabolism of glucose. The synthesis of UDP-GlcNAc is regulated by glutamine-fructose-6-phosphate amidotransferase (GFAT), which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine as the amine donor. As the first and rate-limiting enzyme in the HBP, GFAT is of crucial importance since it governs the availability of the end product UDP-GlcNAc (Ma and Hart, 2013; Akella et al., 2019).

UDP-GlcNAc is required for O-GlcNAcylation which is a single sugar conjugation catalyzed by O-GlcNAc transferase (OGT) in the cytoplasm, mitochondria, and nucleus. As important is the N- and O-linked glycosylation of proteins occurring in the Golgi apparatus and endoplasmic reticulum (ER). (Butkinaree et al., 2010; Akella et al., 2019) O-GlcNAc modifies a wide variety of proteins, including signaling molecules, transcription factors and metabolic enzymes (Love and Hanover, 2005; Bond and Hanover, 2015.)

The HBP applies up to 2-5 % of glucose that enters a non-cancer cell and along with glutamine, acetylcoenzyme A (Ac-CoA) and uridine-5' triphosphate (UTP) are used to produce the amino sugar UDP-GlcNAc (Marshall et al., 1991). According to (Akella et al., 2019) the HBP has emerged as a major regulator and contributor of cancer phenotypes and pathways (Figure 4). The elevated HBP and O-GlcNAcylation has been reported in nearly all examined cancers. HBP regulates the characteristic features of cancer including growth, angiogenesis, metabolism and metastasis (Ferrer et al., 2016).
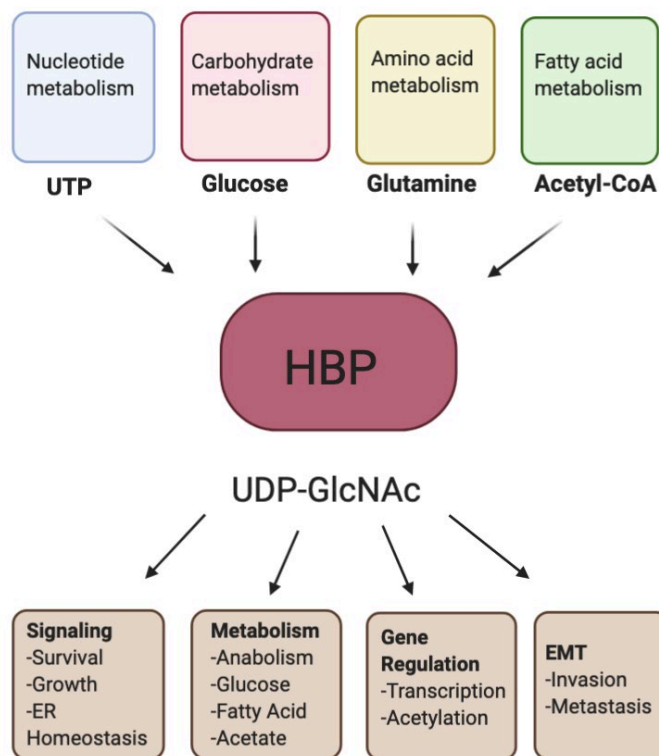
Figure 4. The HBP is highly related to the nutrient state of a cell. It is heavily dependent on dietary molecules like glucose and glutamine as well as other metabolic pathways such as fatty acid and nucleotide metabolism. The end product UDP-GlcNAc plays a key role in many downstream glycosylation processes that in turn control proteins and processes involved in metabolism gene regulation, signaling and EMT. The HBP also plays a as a key role in many cancer processes (Akella et al., 2019).

1.4.1 Glucosamine

N-Acetylglucosamine (GlcNAc) is a monosaccharide derivative of glucose and has an important role in many biological systems. GlcNAc is the monomeric unit of the polymer chitin, the second most abundant carbohydrate after cellulose. Chitin is widely distributed in many organisms. GlcNAc is also constituent of heterogenous polysaccharides such as hyalorunic acid and murein. In addition, glycoproteins that contain GlcNAc are found in the mucous membranes of the digestive tract (Chen et al., 2018). Glucosamine is utilized for biosynthesis of glycosaminoglycans and glycoproteins (Runkel and Cupp, 1999).

Dietary GlucNAc is taken up by cells via glucose transporters and incorporated into the HBP flux (Wellen and Thompson, 2010). Mattila et al. (2018) found that feeding flies with the intermediate of HBP promoted ISC proliferation in midgut clones. They utilized MARCM clones (explained in materials and methods) within the R4c region as a model for midgut adaptation. Their main result was that dietary GlcNAc can maintain midgut clone size during calorie restriction independent of food intake.

1.4.2 Nutrient sensing and signaling

The midgut is a highly dynamic organ which response to physiological cues and environmental condition. The midgut is highly sensitive to nutrition. When food is abundant the ISCs are signaled to increase proliferation rate and as a result the midgut can grow. The cells also have considerably larger volumes of cytoplasm in well fed animals compared to animals in starvation (O'Brien et al., 2011). Under condition of low food supply, the midgut shrinks in size through reduced proliferation, EC apoptosis and natural turnover to preserve resources (Choi et al., 2011; O'Brien et al., 2011).

Nutritional status is known to greatly influence regulation of growth factors which in turn can change expression patterns and ISC activity (Choi et al., 2011; Buchon et al., 2013). Midguts retain their anatomical shape however they shrink during fasting and enlarge during feeding both in development and feeding/fasting cycles. Midguts conserve this growth response which is reversible and repeatable with dietary change (O'Brien et al., 2011).

One of the key factors regulating these changes is the insulin/IGF signaling pathway. The modulation of symmetric and asymmetric ISC division is regulated by insulin-like peptide 3 (ILP3) signaling produced by the visceral muscle during rotations of feeding and starvation. Midgut dilp3 fluctuates dynamically during feeding and fasting in tandem with organ size (O'Brien et al., 2011). Insulin signaling promotes proliferation during development, homeostasis and adaptive growth.

However the signaling by the insulin pathway is not clear and entails modulation by other mechanism and it's activation can lead to either prompted or suppressed proliferation (Choi et al., 2011). The activation of HBP pathway has been shown to regulate ISC proliferation through InR signaling. (Mattila et al., 2018)

1.5 Aims of the study

Although many studies have expanded the information of the intestine, an inclusive, multiscale analysis integrating the relationship between the function and structure of different gut regions is still lacking (Buchon et al., 2013). One possible way to study nutrient-dependent cellular regulation is by altering the diet of the model organism and then quantifying any appearing effects. In this study the effects of various nutritional manipulations were studied by exploiting a chemically defined holidic medium. (Piper et al., 2014) The intermediate of HBP, N-acetyl-D-glucosamine (GlcNAc) can maintain midgut clone size during calorie restriction independent of food intake (Mattila et al., 2018). The dietary GlucNAc is taken up by cells via glucose transporters and incorporated into the HBP flux (Wellen and Thompson, 2010).

In this study we investigated the interaction of the Hexosamine biosynthesis pathway (HBP) and nutrition in the intestinal stem cell population by utilizing Linear Analysis of Midgut (LAM) which allows quantitative regionally defined phenotyping of the whole *Drosophila* midgut (Viitanen et al., 2021). HBP has shown to increase ISC proliferation in animals kept in calorie restricted conditions (Mattila et al 2018). By using a chemically defined diet together with means to activate the HBP in the ISCs, it is possible to investigate in more detail the mechanism of the HBP's proliferative signal. The Hexosamine biosynthesis pathway was activated in stem cells by two means: (1) by feeding the flies with Glucosamine, or (2) by expressing the rate limiting enzyme Gfat2

specifically in the ISCs and in their progeny. In addition, the response of the ISCs in the different regions of the fly midgut was investigated.

Hence, the specific objectives were:

i. To investigate the interaction between the HBP and specific nutrients, i.e., essential and non-essential amino acids and lipids, in the ISCs.
ii. To investigate if different regions of the midgut react differently to the proliferative signal emanated by the HBP.

2. Materials and methods

2.1 Diet preparation

Holidic medium (Appendix A) is a chemically defined diet available for *Drosophila melanogaster* (Piper et al., 2014). This synthetically prepared diet supports the whole lifespan and reproduction. Piper et al. (2014) assert that the holidic media offers steadier experimental setups and results. Holidic media was used in the experiments in order to manipulate the nutritional conditions of the flies. Some components of the diet were either removed or reduced (Table 1). Full recipe for the starvation media is described in Appendix B.

Table 1. Experimental dietary conditions

Dietary group +/- Glucosamine
Full holidic media
Starvation media
Calorie restricted diet (25% holidic)
Holidic without Essential amino acids (EAA)
Holidic without Non-essential amino acids (NEAA)
Holidic without Lipids

2.2 GAL4 system

Brand and Perrimon (1993) elaborated the GAL4 system which use the transcriptional machinery required for galactose metabolism in yeast. The GAL4 protein binds to its upstream activating sequence (UAS) and activates gene expression (Figure 5). The system allows the selective activation of any cloned gene in large variety of cell and tissue-specific patterns.

In *Drosophila* the gene of interest is under the control of UAS, and GAL4 expression is controlled by a tissue specific enhancer. The system ensures that the target gene is silent until GAL4 is introduced in a genetic cross. When GAL4 driver line is crossed to a line with UAS-target gene the progeny will indicate the gene of interest only in cells which the GAL4 is present (Brand and Perrimon, 1993; Southall et al., 2008).
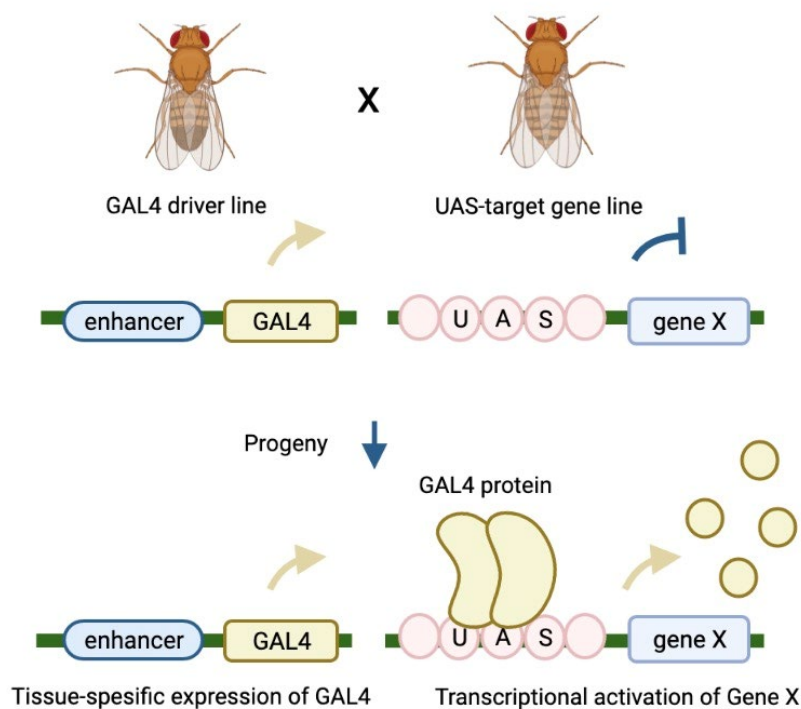

Figure 5. Targeted ectopic gene expression utilizing GAL4. The GAL4 gene, encoding a transcriptional activator from yeast, is exhibited into the genome under the control of a specific endogenous promoter as part of an enhancer vector. Drosophila lines expressing the GAL4 protein in specific tissues and cells are crossbred to lines carrying a target gene of interest. The target gene is expressed in the progeny only in those tissues and cells where GAL4 is present (Brand and Perrimon, 1993; Southall et al., 2008.)

In yeast the GAL4 transcription activation is prevented by GAL80 which binds to the transactivation domain of GAL4 (Lue et al., 1987). In *Drosophila* the GAL80 can be

expressed ubiquitously under the control of the tubulin 1α promoter. This feature represses GAL4 activity in all tissues and enabled Lee and Luo (1999) to develop the technique to generate marked mutant clones (Southall et al., 2008).

2.3 Mosaic analysis with a repressible cell marker = MARCM

Mosaic analysis allows the study of mutant cells surrounded by wild type tissues. The advantage of this technique is that it allows the studies of mutant cells and their characteristics that would otherwise cause lethality (Lee and Luo, 1999; Southall et al., 2008). Wu & Luo (2016) describe the technique of using MARCM system for generating homozygous mutant cells from heterozygous precursors via mitotic recombination (Figure 6).
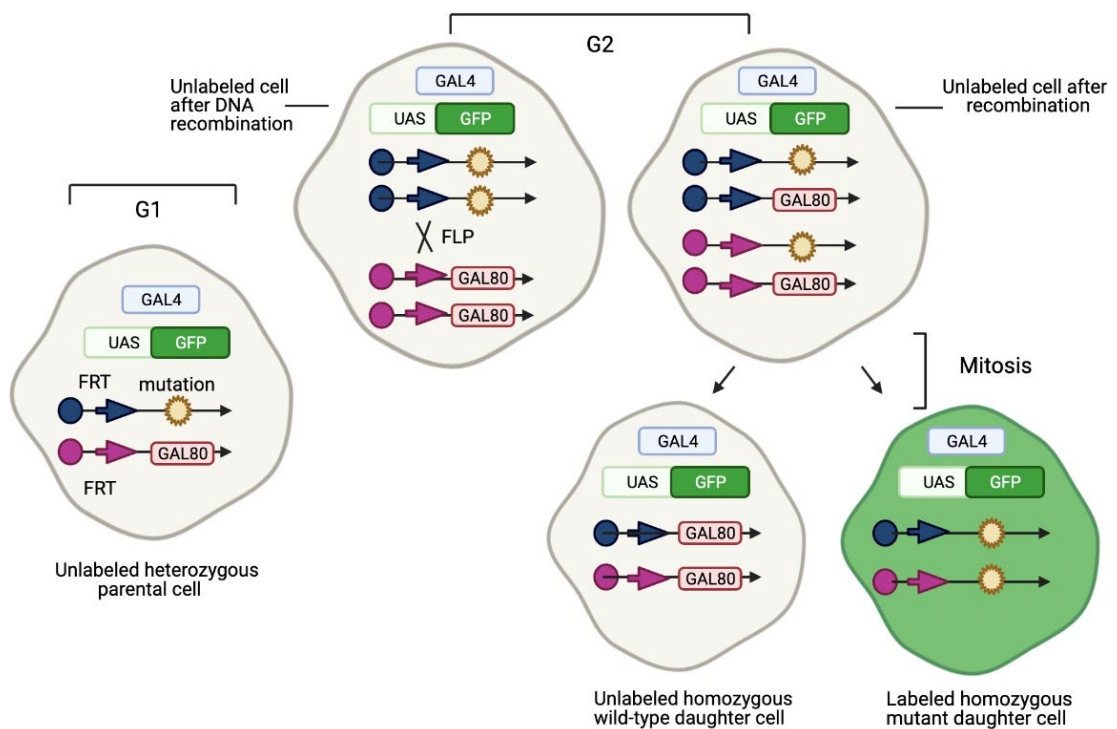

Figure 6. *MARCM entails 1) two FRT sites located at the same position on homologous chromosomes, 2) FLP recombinase located anywhere in the genome 3) GAL80 located distal to one of the FRT sites, 4) GAL4 located anywhere in the genome except distal to the FRT site on the FRT,GAL80 recombinant chromosome arm, 5) UAS-marker located anywhere in the genome except distal to the FRT site on the FRT,GAL80 recombinant chromosome arm, and optionally 6) a mutation distal to FRT, in trans to but not on the FRT, GAL80 recombinant chromosome arm. Site-specific mitotic recombination at FRT sites (arrowheads) gives rise to two daughter cells, each of which is homozygous for the chromosome arm distal to the FRT sites. Ubiquitous expression of GAL80 represses GAL4-contingent expression of UAS-marker GFP gene. Loss of GAL80 expression in homozygous mutant cells yields in specific expression of GFP. Adapted from Wu and Luo (2016).*

The system employs FRT sequence and Flipase enzyme facilitated somatic recombination to produce cells which are homozygous for a given mutant allele and marked by GFP (Lee and Luo, 2001). The MARCM- and UAS-Gfat2 transgene fly stocks used in this study were already generated. MARCM flies were crossed with either animals with the FRT site bearing chromosome (control), or with animals with the FRT site, and UAS-Gfat2 bearing chromosomes.

2.3.1 TM6B balancer chromosome

TM6B balancer found in the third chromosome carries the Tb^1 dominant mutation which results in a tubby phenotype presenting squat larvae and pupae. (Lattao et al., 2011) Balancer chromosomes prevent genetic recombination between homologous chromosomes and carry dominant markers which can be used for visual identification to select for heterozygotes. They are often lethal or negatively affect the reproductive fitness when carried homozygous. The progeny carrying *TM6B* was discarded and only the wild type pupae were collected.

2.4 Feeding and fly care

For the control group, 20 MARCM-ready virgins were crossed with 6-8 males containing FRT alone on a standard growth media. For the second group, 20 MARCM-ready virgins were crossed with 6-8 males carrying FRT and UAS-transgene (Table 4). To enhance egg laying dry yeast was provided for the flies. The MARCM crosses were maintained in a 25 °C incubator (Figure 7).

Table 4. Drosophila strains

w-;;Frt82B/TM6B	Bloomington	Control FRT line
UAS-mCD8::GFP,hsFLP;tub-GAL4/CyO;FRT82B,tub-GAL80	Osamu Shimmi	MARCM line
w-;UAS-Gfat2/Cyo;Frt82B/TM6B	Jaakko Mattila	Gfat2 overexpression

MARCM-ready flies crossed with FRT line, and UAS-transgene flies

MARCM82B   Frt82B / UAS-Gfat2, Frt82B

X   25°C 8-9 days

Collect semi-late/late
pupae into starvation
vials

25°C 3-4 days

Transfer to experimental diets
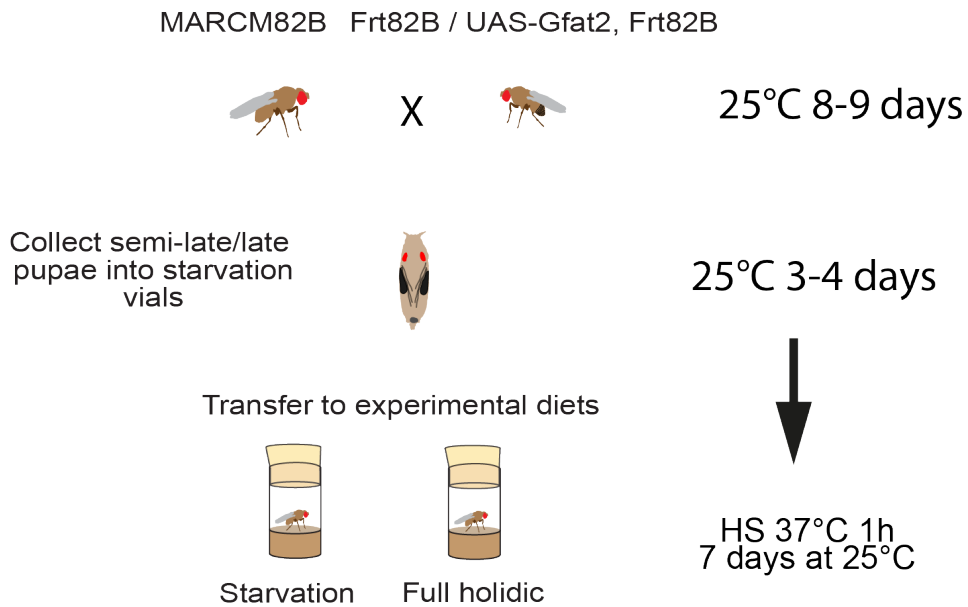
Starvation   Full holidic

HS 37°C 1h
7 days at 25°C

Figure 7. Experimental setup. Three strains of D. melanogaster were crossbred and kept at 25 °C. Resulting pupae were collected to starvation media and grown for 3-4 days. After this the flies were heat shocked to activate lineage labeling. Non-virgin flies were then collected to the experimental diets and grown for 7 days at 25 °C

After 8 to 9 days the flies were discarded, and pupae were collected selectively into vials containing starvation media. The purpose for this was to establish a baseline nutritional status for studying the effects of the manipulated dietary conditions. All the pupae with the balancer chromosome presenting the tubby phenotype were discarded. After eclosion, flies were given heat shock and transferred to the different dietary conditions (Table 5).

To induce clones flies were transferred into empty vials and heat-shocked at 37 °C for one hour in a water bath. After the heat-shock the flies were left to recover for one hour before transferring them on the indicated dietary conditions. The developing progeny was returned to 25°C until 7 days for the clones to be examined.

Table 5. The experimental setups.

Experimental setup	Genotype	Control group	Compared with
1) Comparison between dietary groups	MARCM82B x Frt82B	holidic	starvation 25% holidic -EAA -NEAA -lipids
2) The effects of feeding glucosamine (GlcNAc)	MARCM82B x Frt82B	All dietary groups - GlcNAc	All dietary groups + GlcNAc
3) Gfat2 overexpression	MARCM82B x Frt82B/UasGfat2	Genotype MARCM82B x Frt82B	The two genotypes compared with each other within dietary groups

2.5 Dissection and mounting

After 7 days of rearing at +25°C the flies were dissected. Flies were dissected in phosphate buffered saline (PBS) after anaesthetized with carbon dioxide. Dissection was operated on a nine well glass plate under a stereo microscope with precision forceps. The fly was dipped into PBS where the abdomen was detached carefully from the thorax. After this the gut was pulled out cautiously while the thorax was held by the forceps. The head was removed while the gut was captured very gently. Finally, the thorax was removed by holding it with the left hand from the harder chitinous layer and pulling the rest away with the right hand. The dissection was finished by removing the Malpighian tubules and any debris and placing the gut into a clean well filled with PBS.

After dissection the guts were washed and fixed. This was performed by placing the guts to an Eppendorf tube containing 8 % paraformaldehyde (PFA) in PBS for one hour. Next the midguts were rinsed twice with PBS solution containing 0.1 % triton (PBT). After the second wash the guts were left in PBT for 30 minutes. PBT was removed completely before adding the mounting media. The midguts were mounted in 20 µl of VECTASHIELD® Mounting Medium containing 4',6-diamidino-2-phenylindole

(DAPI). The guts were incubated at least overnight before microscopy. The prepared microscope slides were stored at 4°C.

2.6 Microscopy

Aurox Clarity spinning disk confocal microscope with 20x objective was used for whole-midgut imaging (Table 6). Only one side of the epithelial layer of the flattened tube (midgut) was imaged. Studying only one side of the wall has been considered adequate approximation of the whole. The midgut has not been shown to contain variation along its circumference.

DAPI signal was included in the Vectashield Mounting medium. DAPI fluoresces when bound to DNA of the nuclei of the cells by binding to A/T- rich regions. DAPI excites at about 360 nm and emits at about 460 nm when bound to DNA by producing a blue fluorescence. GFP signal was produced by lineage labeled cells. It emits light at a maximum of 509 nm.

Table 6. Sample sizes for the analysis.

Genotype	Channel	N Holidic	N Starved	N EAA	N NEAA
MARCM82B x Frt82B	DAPI	8	3	6	3
MARCM82B x Frt82B/UasGfat2	DAPI	5	4		
MARCM82B x Frt82B + GlucNAc	DAPI	10			
MARCM82B x Frt82B	GFP	8	3	6	3
MARCM82B x Frt82B/UasGfat2	GFP	5	4		
MARCM82B x Frt82B + GlucNAc	GFP	10			

2.7 Image analysis

The multistacks contain plenty of image data outside the midgut which had to be filtered out leaving only the region of interest (ROI). The microscope slides still contained an abundance of debris like shed cells or larger cell aggregations. The filtering was performed with Image J software (Schneider et al., 2012) with hand-drawn surface borders around every single midgut. After selecting ROI image tiff images were

converted into Imaris (Bitplane Inc, 2018) files by Imaris file converter. Imaris was used for segmenting DAPI nuclei, through the spot detection algorithm. Once identified, the DAPI spots were filtered for GFP fluorescence to identify GFP expressing cells of the MARCM clones. The object data, i.e., nuclear co-ordinates, was then transferred into LAM. See next section.

2.7.1 Linear analysis of the midgut (LAM)

Linear analysis of the midgut (LAM) functions as an extension to image processing and feature detection after Imaris analyses. Although imaging of the whole midguts is feasible by using fast tile scan imaging, several biological features makes full midgut analysis difficult to downstream analysis. For example, the midguts have coiled structure and the length of the midgut is variable. Therefore, each midgut has a unique morphology, which need subjective and time consuming manual work in order to align, identify and compare the intestinal regions (Viitanen et al., 2021).

Viitanen et al. (2021) describe a widely applicable phenotyping method (LAM) to achieve spatially defined quantitative data on midgut cells. In their studies they use LAM to quantitively analyse regional distributions of ISCs, EBs and EE cells. They developed means to transform the three-dimensional midgut images into one dimension by creating an algorithm enabling midline vector creation along the A/P axis. The coupling of cellular identities into a certain position on the linear vector enables binning of cell-specific data along the midgut. LAM's qualities achieve robust quantitative phenotyping of midguts with subregional resolution (Viitanen et al., 2021). LAM was used in this thesis to create regionally defined plots of cellular parameters.

The total statistics feature of LAM creates comparisons of sample group data regarding total cell numbers on each channel and averages of additional data in each bin of sample groups. The statistical testing between sample groups in LAM is calculated to all bins of a channel sequentially with Mann-Whitney-Wilcoxon U-test with LAM. The cell counts at a specific longitudinal location from all the samples belonging to the control group are compared to the cell counts at the comparable location in the other sample groups (Viitanen, 2019).

3. Results

3.1. Regional quantification of the Drosophila midgut

The midgut regions were identified based on their morphological features. To this end, nuclei were first separated based on their size (Figure 8). Intestinal stem cells are diploid cells whereas enterocytes are non-proliferating polyploid cells which have both increased their expression and the copy number of genes along with increased cell size. The size of the enterocytes are known to vary depending on the midgut region (Buchon et al. 2013). Hence by plotting the enterocyte nuclei area along the midgut anterior-posterior axis it was possible to identify distinct border regions constituting to the R1+R2, R3, R4 and R5 region borders (Buchon et al. 2013). The border between R1 and R2 was not possible to identify by this method and was therefore left undetected (Figure 9).
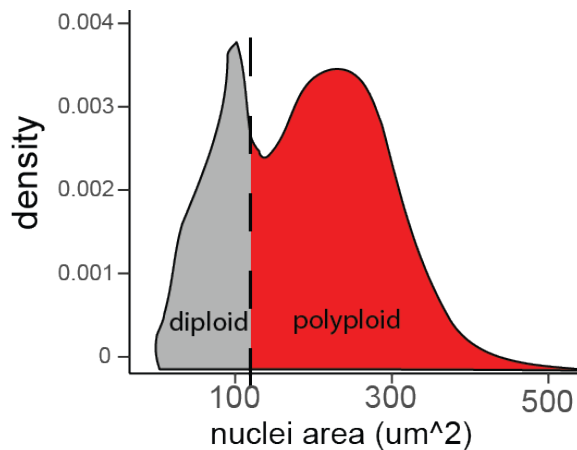

Figure 8. Separation of diploid and polyploid cells.
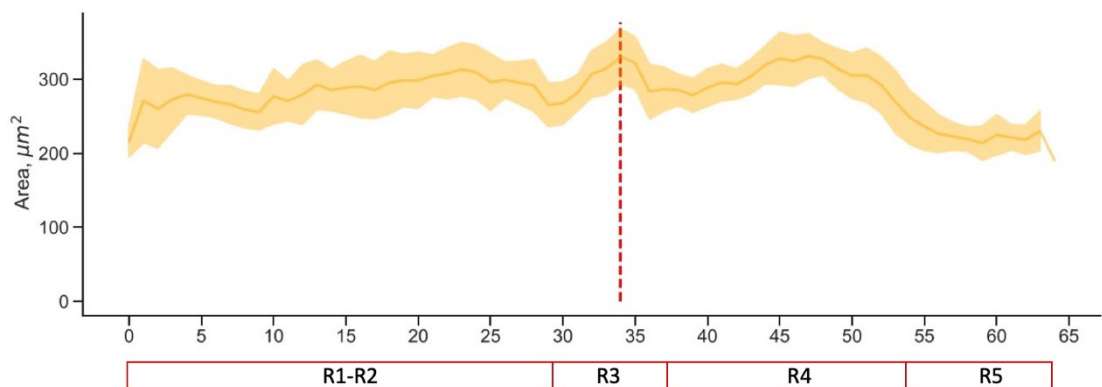*Distribution of the midgut nuclei area.*

Figure 9. Regional distribution of polyploid cell area along the midgut anterior-posterior axis.
Vertical dashed red line indicates the midpoint of R3, which was manually marked to the images at the time of image processing.

Figure 10. Regional distribution of polyploid cell count along the midgut anterior-posterior axis. *Vertical dashed red line indicates the midpoint of R3, which was manually marked to the images at the time of image processing.*
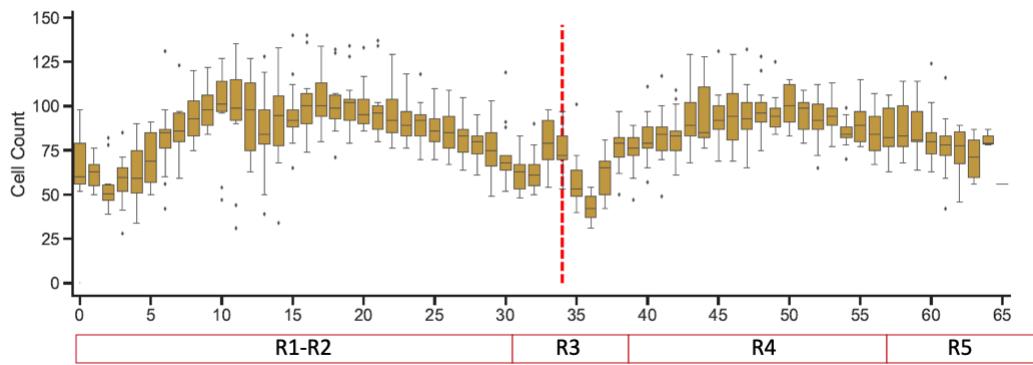
In addition to the nuclei area, polyploid cell counts also vary in a region dependent manner along the midgut anterior-posterior axis (Figure 10). Furthermore, polyploid cell distribution along the anterior-posterior axis was also investigated by mapping the average minimum distance between two nearest nuclei (Figure 11). The parameters described above (Figures 8-11) are tools that can be used for detection of regional properties of the *Drosophila* midgut and, hence, identify region borders in high accuracy. These boundaries were then used to refine the analysis of the regional variation of HBP's effect to ISC proliferation.
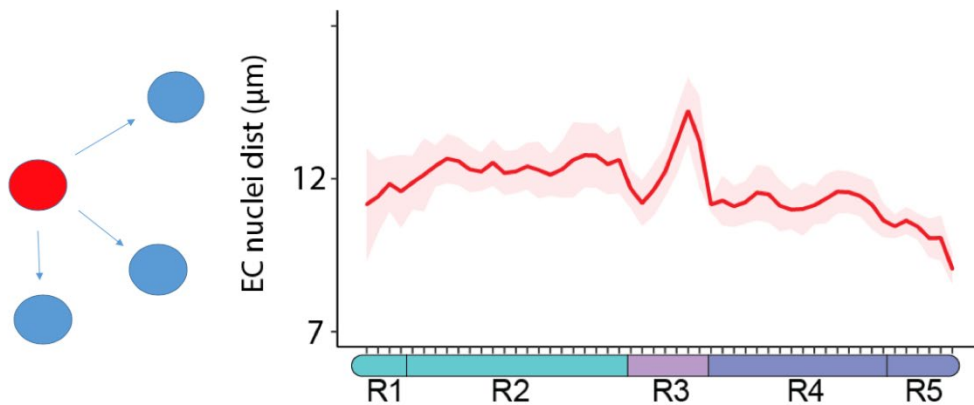

Figure 11. Regional distribution of polyploid cell distances along the midgut anterior-posterior axis. *Polyploid cell distribution along the anterior-posterior is mapped by the average minimum distance between two nearest nuclei.*

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3.2 Quantification of stem cell proliferation in starved versus fed midguts

Feeding has been shown to increase cell number of the fly midgut (O'Brien et al 2011). Cell growth along the midgut within clones obtained from DAPI/GFP channel show a faster cell turnover in the holidic treatment compared to starved condition. However, when only the clonal cell number was compared, the number of cells was found to be higher in the fed condition (Figure 12A). Hence the total cell number was quantified in starved versus fed midguts (Figure 12B). The total cell numbers describe the equilibrium between cell division and cell death. Total cell numbers were not significantly different between the diets. These results suggest that in these dietary conditions the net total cell number is not changed. However, increased clonal cell number suggests higher cellular turnover leading to higher stem cell proliferation rate.
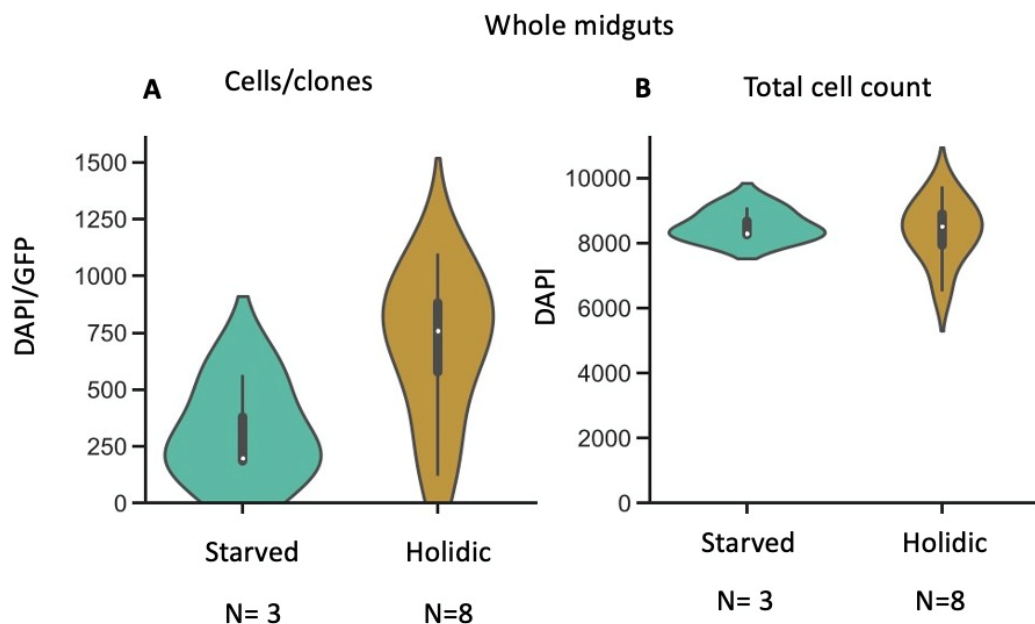

Figure 12. The number of cells/clones and total cell count in comparison with starved and holidic condition.
*A. Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. Holidic treatment had a faster cell turnover compared to starved condition. **B.** Total cell count from whole midguts. Total cell number describes the equilibrium between cell death and cell division. There was no difference observed between the holidic treatment and starved group.*

3.2.1 Regional Stem cell proliferation in starved versus fed midguts

Since total cell counts in starved versus fed condition did not show any statistically significant difference, the samples were then compared at the level of different regions. LAM (see material and methods) divides the midguts into user defined bins and aligns the average cell numbers in each bin for bin-to-bin comparison between samples. The

total cell number plot (Figure 13) shows that whereas in most part of the gut there is no change, a region at the posterior R4 is showing increased cell numbers in the starved sample. On the other hand, when looking at the clonal cell numbers, a region in anterior R4 is showing elevated number of clonal cells in the fed condition. These results show how significant, region specific, differences can be missed if only measuring total cell numbers.
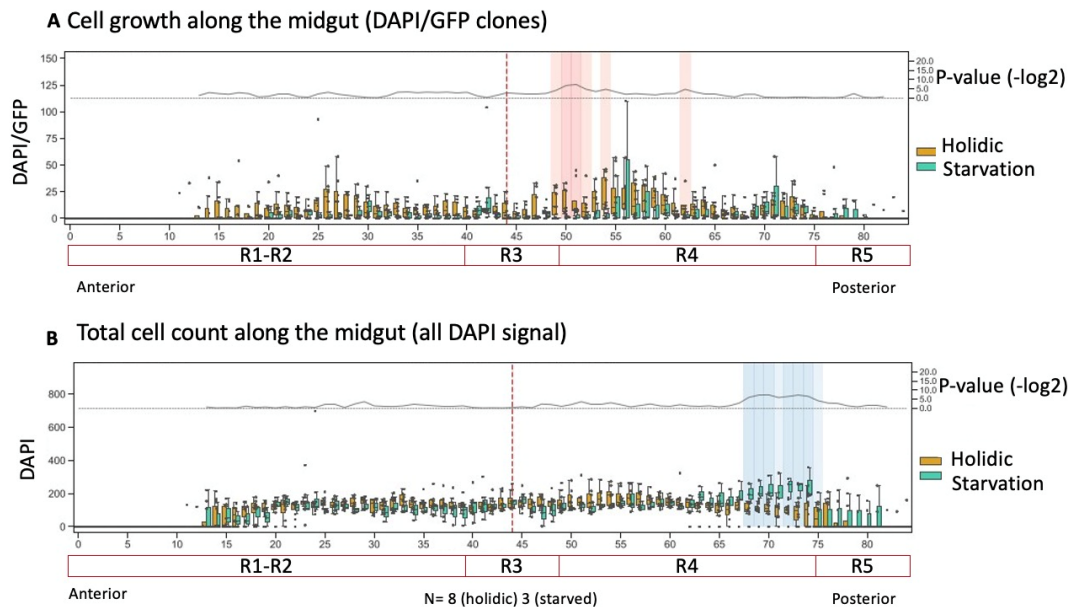

Figure 13. The regional variation of clonal cell numbers and total cell count.
*Regional variation along the whole midguts in comparison with holidic treatment and starved condition. **A.** Proliferation rate expressing the size of clones obtained from DAPI/GFP channels in a regional variation. Improved nutrition by the holidic treatment caused faster cell turnover in R4 region compared to starved condition. **B.** Total cell numbers obtained from DAPI-channel. Total cell count shows the equilibrium between cell death and cell division. There was no difference observed between the two dietary conditions.*

3.3 The role of EAA and NEAA in the midgut nutrient response

The essential amino acids (EAA) were omitted from the holidic diet and the total cell number as well as the clonal cell number were scored. The role of EAA in the midgut nutrient response resulted similar results as the starved versus fed comparison. Total cell numbers were not affected whereas the clonal cell number was decreased in the diet lacking EAA (Figure 14). Cell growth is represented in the clonal growth obtained from DAPI/GFP channel. Cell growth describes the proliferation rate of the ISC. The regional comparison showed that clonal cell numbers were reduced specifically in the anterior R4 region, like the starved versus fed comparison (compare Figures 13A and 15A). The total

cell numbers were not affected in any region (Figure 15B). Total cell numbers describe the equilibrium between cell death and cell division obtained from DAPI channel.
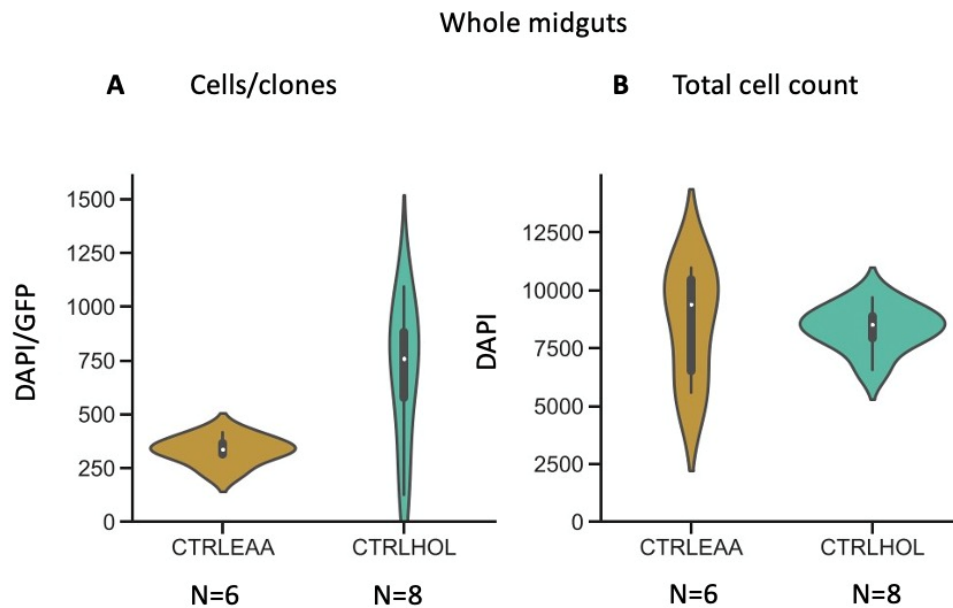

***Figure 14. The number of cells/clones and total cell count in comparison with holidic-essential amino acids (EAA) and holidic condition. A.** Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. Holidic treatment had a faster cell turnover compared to holidic-EAA condition **B.** Total cell count from whole midguts. Total cell number describes the equilibrium between cell death and cell division. There was no significant difference observed between the holidic treatment and starved group.*
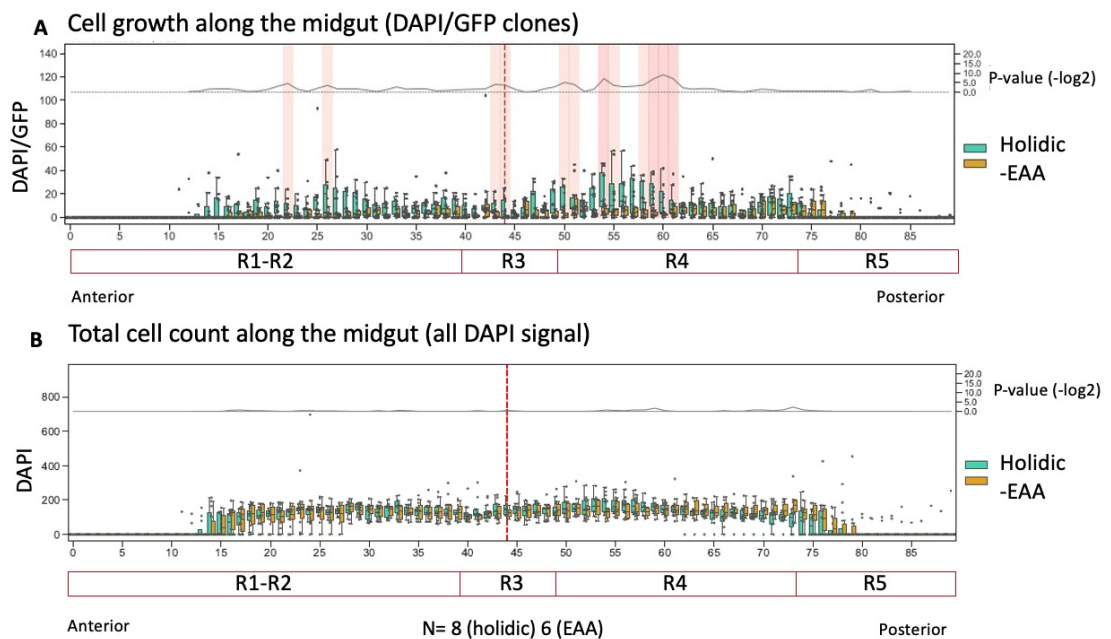

***Figure 15. The regional variation of clonal cell numbers and total cell count.** Regional variation along the whole midguts in comparison with holidic-EAA treatment and holidic condition. with the regional differentiation. **A.** Proliferation rate expressing the size of clones obtained from*

*DAPI/GFP channels in a regional variation. Improved nutrition by the holidic treatment caused faster cell turnover in R4 region compared to -EAA condition. **B.** Total cell numbers obtained from DAPI-channel. Total cell count shows the equilibrium between cell death and cell division. There was no difference observed between the two dietary conditions.*

The role of non-essential amino acids (NEAA) was examined to the total and clonal cell number of the midgut. Removing NEAA from the holidic diet had no effect to the total or clonal cell numbers (Figure 16 & 17). Furthermore, regional comparison did not show any apparent regions with significant differences. Hence, it was concluded that the NEAA were not required in the midgut nutrient response. Cells/clones are showing the proliferation rate of the intestinal stem cells along the midgut within clones obtained from DAPI/GFP channel. There was no significant difference observed between the two groups. Total cell number describes the equilibrium between cell death and cell division. There was no difference observed between the two different dietary condition obtained from DAPI channel from whole midguts.
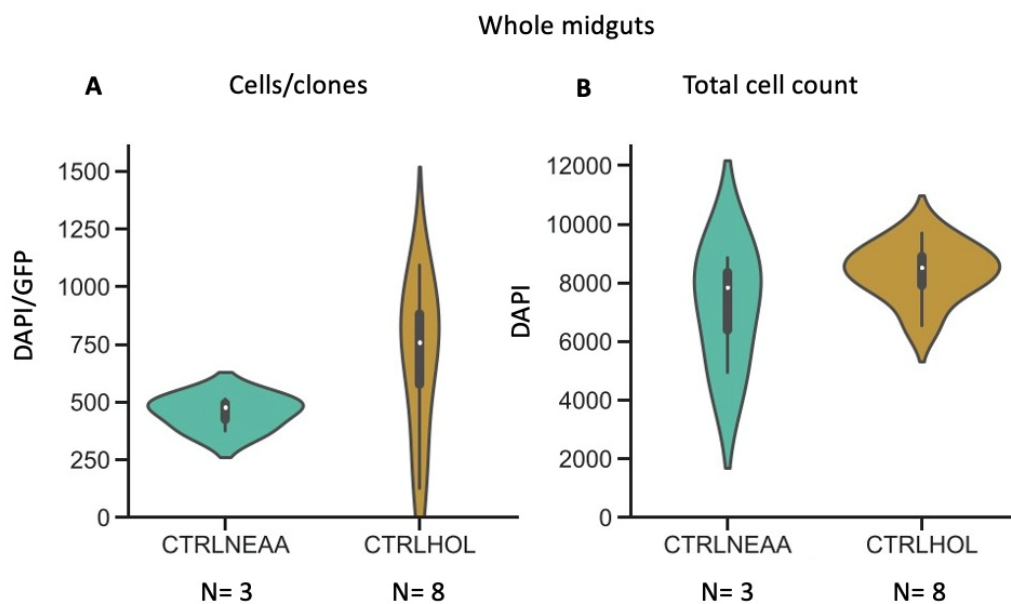

***Figure 16. The number of cells/clones and total cell count in comparison with holidic-nonessential amino acids (NEAA) and holidic condition** A. Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. There was no significant difference observed between the two groups. B. Total cell count from whole midguts. Total cell number describes the equilibrium between cell death and cell division. There was no difference observed between the two different dietary condition.*
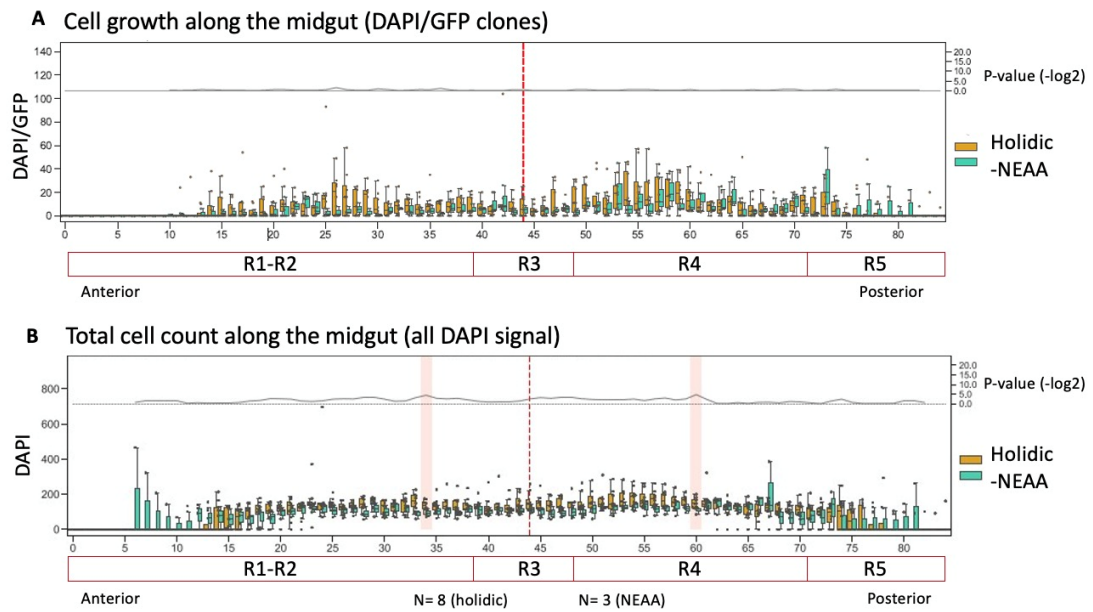
Figure 17. The regional variation of clonal cell numbers and total cell count.
*Regional variation along the whole midguts in comparison with holidic-NEAA treatment and holidic condition. The figure is representing the same condition as above (Figure 5.) with the regional differentiation. **A.** Proliferation rate expressing the size of clones obtained from DAPI/GFP channels in a regional variation. There was no difference observed between the two dietary conditions. **B.** Total cell numbers obtained from DAPI-channel. Total cell count shows the equilibrium between cell death and cell division. There was no difference observed between the two dietary conditions.*

3.4 The effect of Glucosamine in the midgut nutrient response

Dietary glucosamine was shown to increase the midgut clonal cell numbers in dietary restricted flies (Mattila et al. 2018). The effect of glucosamine was tested on the holidic diet. Adding Glucosamine to the holidic diet had no effect to the total or clonal cell number of the midgut (Figure 18). Cells/clones are showing the proliferation rate of the intestinal stem cells. Feeding glucosamine had a faster cell turnover compared to holidic treatment obtained from DAPI/GFP channel. There was a slight increase in the clonal cell numbers at the R4 region (Figure 19).
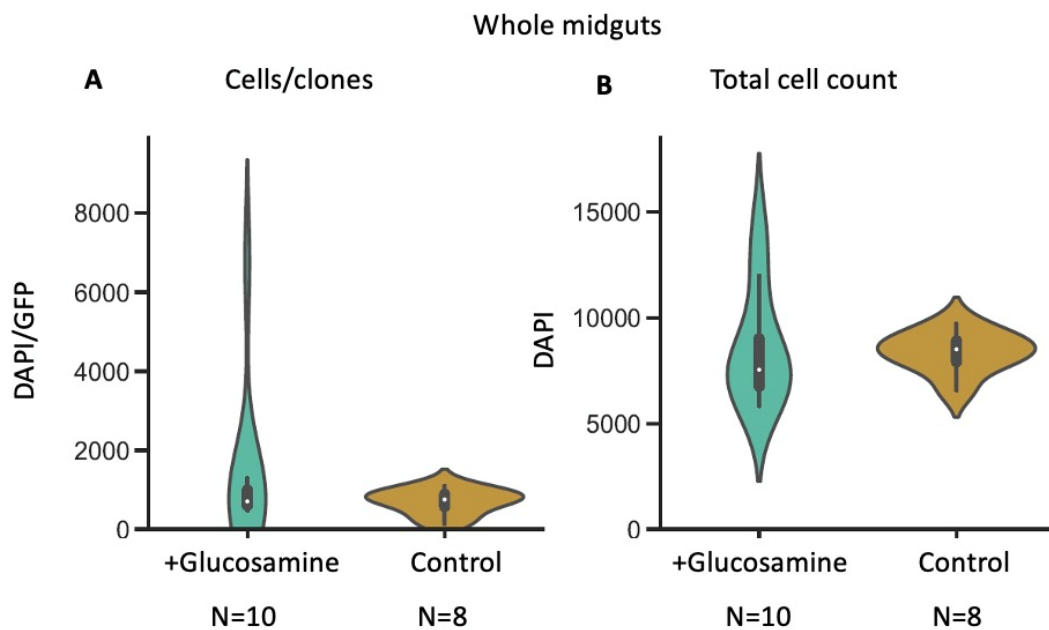
Figure 18. The number of cells/clones and total cell count in comparison with holidic media (control group) + glucosamine treatment. *A. Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. Feeding glucosamine had a faster cell turnover compared to holidic treatment.* ***B.*** *Total cell count from whole midguts. Total cell number describes the equilibrium between cell death and cell division. There was no significant difference observed between the two groups.*

Figure 19. The regional variation of clonal cell numbers and total cell count.
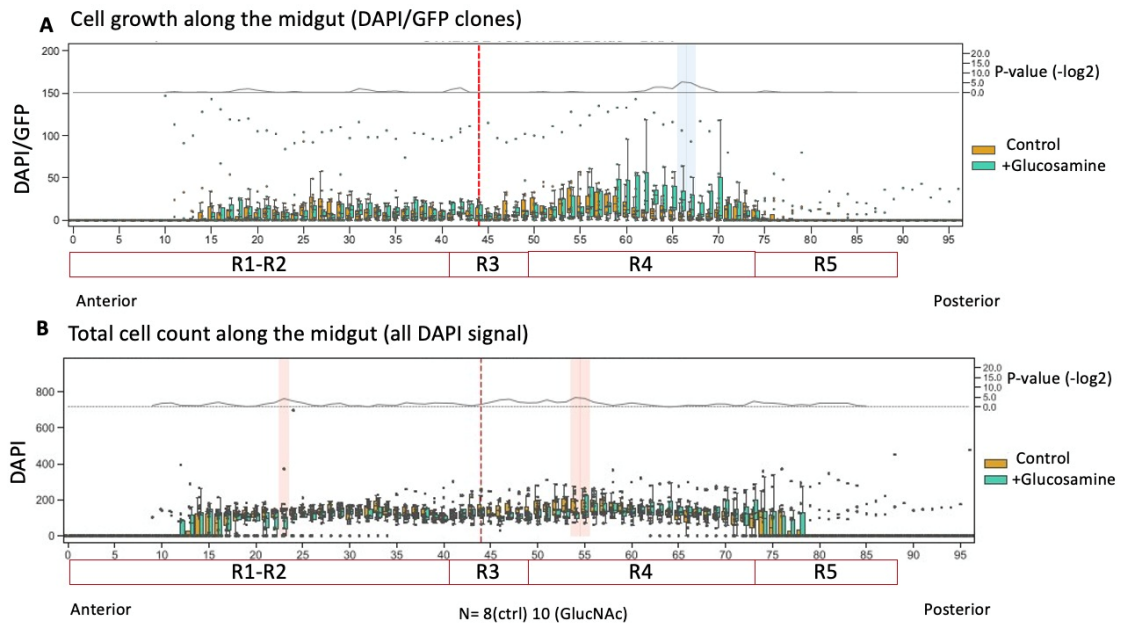*Regional variation along the whole midguts in comparison with holidic treatment +/-
glucosamine. **A.** Proliferation rate expressing the size of clones obtained from DAPI/GFP
channels in a regional variation. Glucosamine treatment had a faster cell turnover compared to
holidic group. **B.** Total cell numbers obtained from DAPI-channel. Total cell count shows the
equilibrium between cell death and cell division.*

Increasing the activity of the HBP genetically by overexpressing the Gfat2 enzyme in
midgut clones increased the number of total clonal cells in flies kept in starvation. This
result was not significant though, likely due to the small sample sizes (Figure 20).
Cells/clones are showing the proliferation rate of the intestinal stem cells. The Gfat2
overexpression had a faster cell turnover compared to the control genotype showing
increase in the number of total clonal cells. Regionally, the increase in clonal cell numbers
was observed throughout the midgut. Again, the result was not statistically significant
(Figure 21). However, overexpressing the Gfat2 enzyme in midgut clones in animals fed
in the holidic diet, had no effect to the total clonal cell numbers (Figure 22). Furthermore,
clonal cell numbers were not changed in any of the regions in this condition (Figure 23).
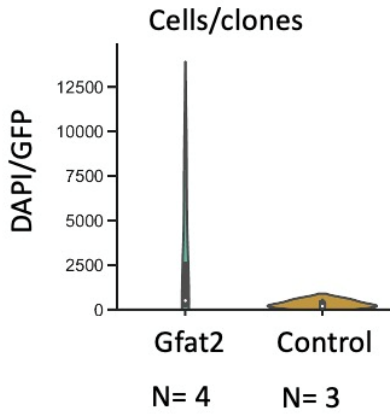
Figure 20. The number of cells/clones in comparison with the genotype overexpressing Gfat2 and control genotype in starved condition. *Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. The Gfat2 overexpression had a faster cell turnover compared to the control genotype.*
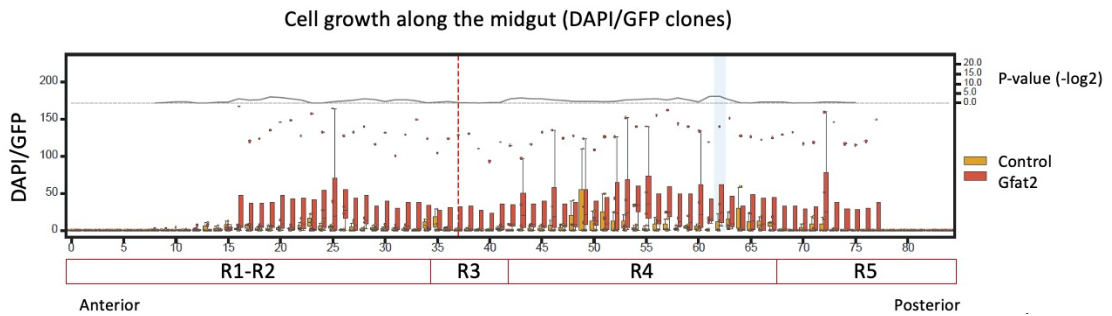

Figure 21. The regional variation of clonal cell numbers. *Proliferation rate expressing the size of clones obtained from DAPI/GFP channels in a regional variation. Gfat2 overexpression had a faster cell turnover compared to the control genotype. There was a significant effect obtained in the R4 region.*
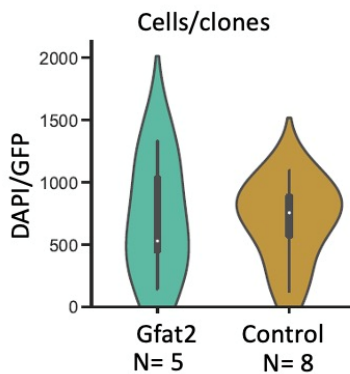
Figure 22. The number of cells/clones in comparison with the genotype overexpressing Gfat2 and control genotype with holidic dietary treatment. *Cell growth along the midgut within clones obtained from DAPI/GFP channel. Cells/clones are showing the proliferation rate of the intestinal stem cells. There was no difference between the two genotypes.*
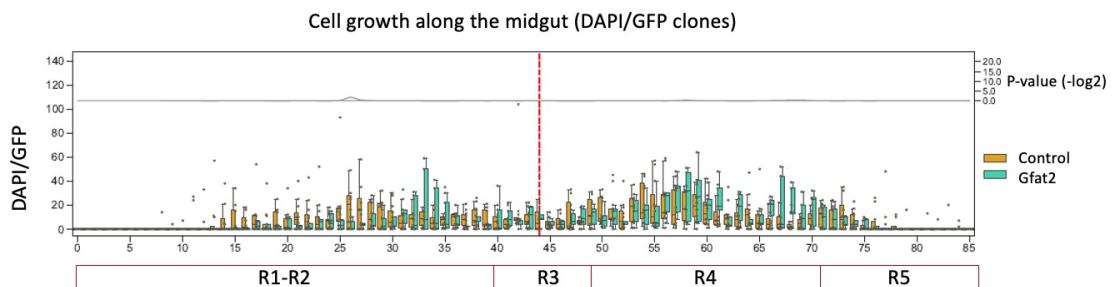
Figure 23. The regional variation of clonal cell numbers *Proliferation rate expressing the size of clones obtained from DAPI/GFP channels in a regional variation. There was no significant difference obtained between the two genotypes with holidic dietary treatment.*

4. Discussion

Our results on fed condition and removed essential amino acids show difference in clonal cell numbers which suggest higher cellular turnover leading to higher stem cell proliferation rate in specific regions along the midgut. In contrast to the fed condition the clonal cell numbers are reduced specifically in the anterior R4 region in starved condition. When experimenting which nutrients mediate the proliferative capacity of stem cells, we find that removing essential amino acids from the diet have similar effect on the R4 region compared to the starved versus fed condition. In addition, the total cell numbers did not change statistically significantly in any of the experimental setups which is representing the equilibrium between cell division and cell death.

The effects of nutrient dependent conditions and nutrient repleted conditions have been previously studied in the *Drosophila* midgut (Choi et al., 2011; O'Brien et al., 2011; Lemaitre and Miguel-Aliaga, 2013). The *Drosophila* midgut is a highly adaptive organ that responds to nutritional changes such as highly altered nutrition or starvation by either activation of metabolic pathways, driving proliferation and overall growth or vice versa (Choi et al., 2011; O'Brien et al., 2011). The key player of this adaptive growth are the intestinal stem cells. The mechanism of specific nutrition induced changes as well as the activation or deactivation of nutrient dependent pathways is still under investigation.

The regionalization of the midgut causes some difficulty for the study of the mechanisms found in the intestine (Buchon et al., 2013; Marianes and Spradling, 2013). Previous research to nutrient induced stem cell activation have not regarded each region separately or have mainly focused on subsections of the midgut (Choi et al., 2011; O'Brien et al., 2011). In this study the effects of various different dietary conditions specifically the manipulation of a chemically defined holidic medium were studied using a region-by-region whole midgut analysis. In addition, the effects of glucosamine supplementation and the effects of overexpression of Gfat2 enzyme on the cell populations of the midgut were examined.

There is a common understanding of renewal programs which uphold homeostatic tissue state. The stem cells divide to replace damaged or lost cells while keeping overall stem cell number constant (Pellettieri and Alvarado, 2007). In addition, tissue homeostasis is flexible in its modulation. In the fly midgut the cell number oscillates in response to the

dietary load, a specific external cue. The adult tissue exploits its renewal program to adapt to environmental change. The ability of midgut cell number to alternate indicates that homeostasis in this organ is metastable (O'Brien et al., 2011).

Adaptive growth can be characterized by a homeostasis breaking increase in progenitor and total cell numbers. However, the relative proportion of stem cells appears to be homeostatically controlled and remain 15-20 % of the total cell population (O'Brien et al., 2011).

Total cell count from whole midguts describes the equilibrium between cell death and cell division. In our conditions the total number of cells were not significantly increased in any of the dietary comparisons. However, the total number of cells within MARCM clones were slightly higher in the fed condition. The increase was further shown to take place in the R4 region. Having more cells in a clone, yet total cell numbers are not changed, indicates higher cellular turnover, i.e., the rate of stem cell proliferation is increased due to higher cell death rate. The best explanation to our opposing results is that in our experiments the measurement changes were obtained along the whole midgut whereas the determinations by O'Brien et al. (2011) were done at a specific region.

In response to nutritional changes the insulin/IGF-signaling pathway is responsible for proliferation during homeostasis, development, and adaptive growth (O'Brien et al., 2011). The fasting and feeding cycles are accompanied by changes in local insulin production and modulating the insulin responsiveness of the ISC have alterations to the adaptation of the midgut to nutrient content (Mattila et al., 2018). The existing literature describes how the insulin source of the visceral muscle niche upregulates *dilp3* with immediate and sensitive responses to ingested nutrition and signals directly to adjacent stem cells. Midgut *dilp3* oscillates during fasting and feeding in together with organ size (O'Brien et al., 2011). In this connection the fly gut is similar to the mouse small intestine, in which insulin-like growth factor I (IGF-I) is expressed in smooth muscle and subepithelial myoblasts (Pucilowska et al., 2000) and grows during adaptation (Winesett et al., 1995).

However the signaling by the insulin pathway is not clear and entails modulation by other mechanism and it's activation can lead to either prompted or suppressed proliferation (Choi et al., 2011). The activation of HBP pathway regulates ISC proliferation through a

Warburg effect-like metabolic switch and HBP interacts with InR signaling. HBP regulates the balance between glycolysis and oxidative phosphorylation. The midgut's ISC employ a cell intrinsic nutrient-sensing dependent on HBP activity to adjust the rate of cell division into the existing dietary content (Mattila et al., 2018).

Previous research suggests that low ISC intrinsic HBP activity, activated InR signaling advances ISC growth, cessation of cell divisions and subsequent ISC loss. In order to stimulate ISC division additional ISC activation in required through HBP mediated metabolic rewiring which highlights the role of ISC nutrient sensing through HBP (Mattila et al., 2018). In this study the effects of HBP activation were examined via excess glucosamine supplementation and Gfat2 enzyme overexpression.

Glucosamine has been shown to increase cellular turnover of calorie restricted midguts (Mattila et al., 2018). In this study we asked if supplementing glucosamine could rescue the effects of removal of EAA from the diet. In this regard we did the experiment with the starved died. Adding GlucNAc to the holidic diet had little or no effect to cellular turnover or total cell number of the midgut. However, there is a slight increase in the cell turnover at the R4 region. Alternatively, increasing the activity of the HBP genetically by overexpressing the Gfat2 had little or no effect to the cellular turnover or total cell number of the midgut in the holidic diet. However, overexpressing Gfat2 in the starvation diet increased the cell turnover process. However, this was not statistically significant due to the small sample size.

The characterization of the regional compartmentalization of the midgut has shed light to the different gene expression patterns, varying histology and distinct physiological functions (Buchon et al., 2013). In this study the regional stem cell proliferation in starved versus fed midguts takes place in the R4 region. In our experiments the R4 region is the hot spot for the nutrient induced cellular turnover. In our experiments there is difference between fed and starved guts in the cell numbers in the R5 region. The artefact is caused by improper alignment of the midguts in the analysis.

The role of EAA in the midgut response is very similar to the starved versus fed experiment. The number of cells in MARCM clones are significantly higher in the fed condition in comparison to the dietary manipulation when EAA are removed. Again, the total number of cells was not increased. The regional stem cell proliferation takes place

particularly in the R4 region as well as in the starved condition. Worth noticing is the disappearance of the artefact which was observed in the cell numbers with the starved guts. In contrast to this there is no difference in the cell turnover after the removal of NEAA from the diet. In conclusion NEAA are not required in the midgut nutrient response.

When the midgut is regionalized, the resolution is more clear and higher in the specific areas. The statistical testing used for the total cell count is a constant non-parametric test. When the midgut is divided into subsections more significant results are obtained. On the other hand, when the number of statistical tests is higher the source for error rate increases. The high variation in our results was probably caused by the small sample groups. The experiments were made several times because there were so many phases in the experiments where an error could be done. Some of the midguts did not remain- intact and had to be discarded from the statistical testing.

For the sake of the global Covid-19 pandemic situation our experiments were disrupted in the laboratory during May 2020. In the end we could not finish our experiments and several experimental setups were left in the incubator. We could not carry out the dissection and imaging for some of the dietary conditions. Hence, the obtained dataset is less than planned, and only data obtained thus far is presented at the thesis. The statistical part of this thesis was conducted via remote connections to the lab computer.

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7. Appendices

Appendix A. The complete holidic medium described by Piper et al. (2014)

Holidic media	
Agar	2g
Isoleucine	0.29g
Leucine	0.41g
Tyrosine	0.21g
sucrose	4.28g
cholesterol stock	3.75ml
buffer	25ml
CaCl2	0.25ml
MgSO4	0.25ml
CuCO4	0.25ml
FeSO4	0.25ml
MnCl2	0.25ml
ZnSO4	0.25ml
total	40.44ml
dH20	to 200ml
boil in microwave	
Wait until cool down	
EAA (essential amino acids)	15.13ml
NEAA (non-essential amino acids)	15.13ml
glutamate stock (Glutamic acid)	4.55ml
cysteine	1.32ml
Glutamine	0.38g
Vitamin stock	5.25ml
Lipid stock	2ml
folic acid	0.25ml
propionic acid	1.5ml
nipagin	3.75ml
total	47.255ml
dH2O	to 250ml

***Appendix B.**. The basic starvation media.*

Starvation media	
Agar	2g
sucrose	4.28g
dH20	to 200ml
boil in microwave	
Wait until cool down	
propionic acid	1.5ml
nipagin	3.75ml
5M NaOH	2.5ml
dH20	to 250ml