Understanding the Impact of High Sugar Diet on Intestinal Stem Cell Homeostasis in *Drosophila melanogaster*

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A dissertation submitted for the partial fulfillment of Master degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

May 2021

Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled " **Understanding the Impact of High Sugar Diet on Intestinal Stem Cell Homeostasis in *Drosophila melanogaster*"** submitted by **Koustav Ray (Reg. No. MP18014)** for the partial fulfilment of Master degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rhitoban Roy Choudhury **Dr. Lolitika Mandal** **Dr. Sudip Mandal**

(Supervisor)

Dated: May 9, 2021

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Dr. Sudip Mandal at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institution. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Koustav Ray

(Candidate)

Dated: 09-05-2021

In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sudip Mandal

(Supervisor)

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Abstract

Last few decades have experienced an alarming increase in our consumption of sugar rich diets. Altered food habit has been linked to many metabolic disorders that include obesity and type II diabetes. Gut epithelial cells are the first cells that are exposed to dietary intervention. Any kind of damage to these cells needs to be replenished by new set of cells. The intestinal stem cells (ISCs) housed within the gut epithelium are capable of self-renewal and can differentiate into other cell types of the epithelium. However, our understanding of the mechanism by which altered diet condition like high sugar diet disrupts ISC homeostasis is very limited. In this study we employed *Drosophila melanogaster* posterior midgut as the model system to analyse the impact of high sugar diet (HSD) on ISC homeostasis. Our results revealed that high sugar diet disrupts ISC homeostasis. This includes depletion of the intestinal stem cell (ISCs) and the enteroendocrine (EE) cells and the enhancement of the absorptive enterocytes (EC) and no change in enteroblast cells (EBs). We also observed that there is no change in Notch signaling. We further investigated other signaling pathways and found altered expression of the major ligands Upd2 and Upd3 of JAK-STAT pathway which is mainly involved in ISC proliferation. In conjunction, we also found that the proliferation of the ISCs get compromised. Furthermore, we found that JNK pathway is upregulated in the posterior midgut of HSD fed flies. Together, our results provide a glimpse of the changes in the dynamics of ISC state and fate in the gut epithelium of flies fed on HSD.

CHAPTER I

INTRODUCTION

Gastrointestinal tract:

The gastrointestinal tract is the central organ, situated within the body cavity has become an emerging research field. The gut was contemplated as an obscure organ previously, involved in majorly digestion and absorption but recent past studies has changed this perception that rather gut plays a vital role in modulating several physiological processes which include immune regulation, regulation of various neurotransmitters, secretion of hormones, energy balance, insulin secretion, etc. Any kind of functional dysregulation of intestinal epithelium can give rise to complex diseases such as intestinal cancers, inflammatory bowel disease, obesity etc. Hence, studying gut physiology has become imperative in order to delineate its role in various pathophysiological conditions. Currently, research works related to intestinal stem cell biology, role of gut in modulating immunity through its interaction with microbiota present within it, and understanding metabolic landscape of gastrointestinal tract has become the forefront of research [1], [2].

Last decades research in immune response generated by intestine of *Drosophila melanogaster* has made a remarkable impact on studying and dissecting out intricate mechanism and complex physiology governed by human intestine using the *Drosophila melanogaster* as a model system. In addition to this, *Drosophila melanogaster's* intestine and mammalian intestine share striking similarity in terms of structural, functional as well as signaling cascades which are involved in maintaining intestinal stem cell homeostasis. Thus, *Drosophila* is a promising model organism to investigate human enteric system [3].

Diet and Diseases:

Last few decades have experienced an alarming increase in the prevalence of obesity and associated risks such as heart disease, type II diabetes, hypertension and cancer. Consumption of high caloric diet, specifically the ones which are rich in fat and sugar are the major reason for the increment in obesity-associated diseases. In humans, obesity can be defined as accrual of fat. Excess fat storage can lead to several other metabolic syndromes for instance cardiovascular disease, hyperglycemia, insulin resistance etc. Therefore, understanding the mechanistic basis of genetic and environment induced fat storage and insulin resistance is absolutely imperative in order to circumvent the prevalence of obesity [4].

Drosophila melanogaster a model system:

Drosophila melanogaster, commonly known as the fruit fly or vineger fly, has come up as a wonderful model system for biomedical research. Over hundred years, *Drosophila* is being used to answer some basic questions of fundamental biological processes ranging from human disease modelling to aging, behaviour, cellular morphogenesis. *Drosophila melanogaster* not only provides excellent genetic tools but also it comes up with very low cost and rapid generation time makes it indispensable when compared to other biological model system such as mouse model.

The father of *Drosophila* research is Thomas Hunt Morgan who used fly to substantiate the chromosomal theory of inheritance [5], [6] .However, the first person who introduced *Drosophila melanogaster* as a model organism in work benches is William Ernest Castle [6]. Morgan and his pupils defined various the principles of genetics using the *Drosophila*. The generation of balancer chromosome which are a specific set of chromosome which do not allow the recombination events through DNA inversions, led the researcher to address and solve more intricate problems [5].

In the year of 2000, sequencing and mapping of the entire genome of *Drosophila melanogaster* has been worked out which suggested that a 60 percent of homology exists between flies and humans. Furthermore, 77 percent of genes which are responsible for causing human diseases have homolog in *Drosophila melanogaster* [7], [8].

Presently, MiMIC transposon is being used in *Drosophila* genome to create null mutations, tracking gene expression, protein tagging [9]. These in concert with GAL4-UAS, FLP-FRT, CRISPR-Cas9 toolkits allow the researchers to examine the human disease related genes in *Drosophila* [10]. Numerous number of mutants of various genes of *Drosophila* has been generated and they are available at various stock centres across the globe. Considering all the aspects which are established, make the *Drosophila melanogaster* powerful and amenable model system to demonstrate plethora of biological processes.

Drosophila life cycle:

Drosophila melanogaster is a short lived, holometabolous insect and therefore it is amenable to culture huge number of individuals at a time. *Drosophila melanogaster*'s life cycle is comprised of four stages of development which includes egg, larvae, pupae, and adult (**Figure 1**). The rearing temperature of *Drosophila melanogaster* controls its growth and development.

In the laboratory, it is generally reared at 25^0C. 18^0C is mainly used for maintaining the stock flies. At 25^0C, the generation time which is from fertilized egg to eclosed adult is roughly 10 days. The maximum range of *Drosophila melanogaster*'s life span is 60 to 80 days and although it depends on the culture conditions. *Drosophila* females lay embryos roughly around 100 per day and takes 24 hours to finish the embryogenesis. *Drosophila* larvae proceeds through three 3 stages namely first instar, second instar, third instar. First instar larvae feeds on the surface of the food and it passes through two molts. Second instar larvae delves deeper into the food and when it becomes matured, the stage is called third instar, it leaves the food and starts wandering and searching for a place to prepare for the pupation in the wall of cultured bottle. During the pupal stage, complete body metamorphosis that is larvae to adult transformation takes place. During this process, degradation of larval tissues and development of adult organs form an undifferentiated cells happens. Finally, 9 to 10 days later of egg fertilization adult fly emerges (**Figure 1**) [11].

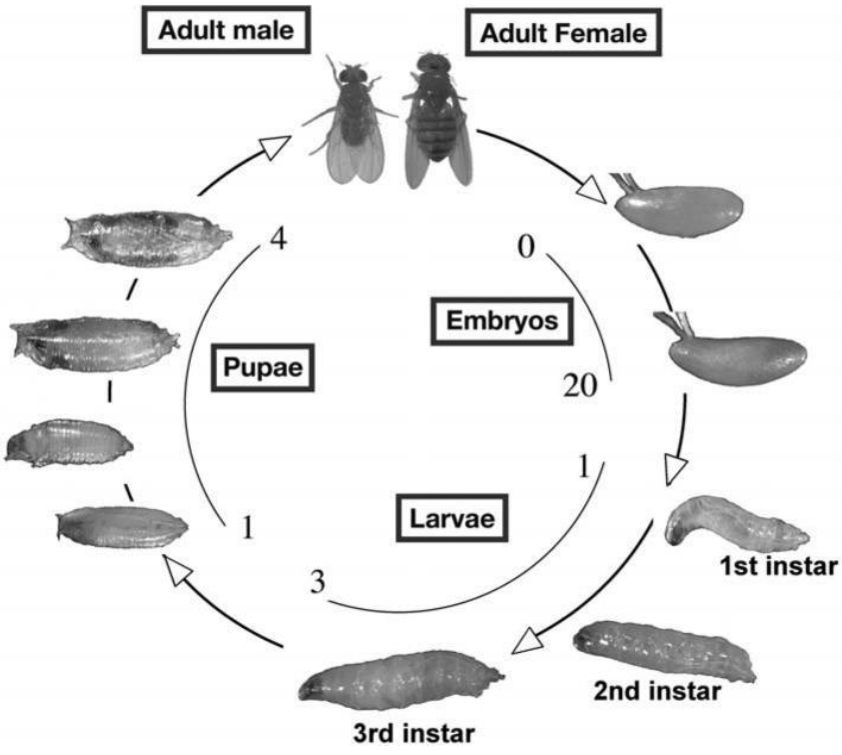

Figure 1: Life Cycle of *Drosophila melanogaster*
Schematic diagram of life cycle of *Drosophila melanogaster* male and female fly [11].

GAL4-UAS system:

The GAL4/UAS system is widely used powerful technique used by fly biologists in order to drive the expression of specific gene of interest. The GAL4 is an 881 amino acid protein which is derived from the yeast *Saccharomyces cerevisiae*, functions as an transcriptional activator. The upstream activation sequence (UAS) is an enhancer. Gal4 protein is specific to the UAS element. Thus spatial as well as temporal regulation of UAS/GAL4 system is achieved (**Figure 2**). Andrea Brand and Norbert Perrimon first discovered this system in 1993 [12].

In activating the specific gene expression, bipartite approach is taken in the UAS/GAL4 system where UAS with a gene of interest (responder) is kept in one fly line and another driver line is used where GAL4 sequence is attached with a tissue specific promoter. When this two driver line and responder line is crossed, then GAL4 will bind to the UAS element and will drive the expression of gene of interest at the specific tissue. This bipartite system allows the researchers to study the effect of various genes through their overexpression or knock-down condition in multiple organs using the tissue-specific promoters available [12], [13].

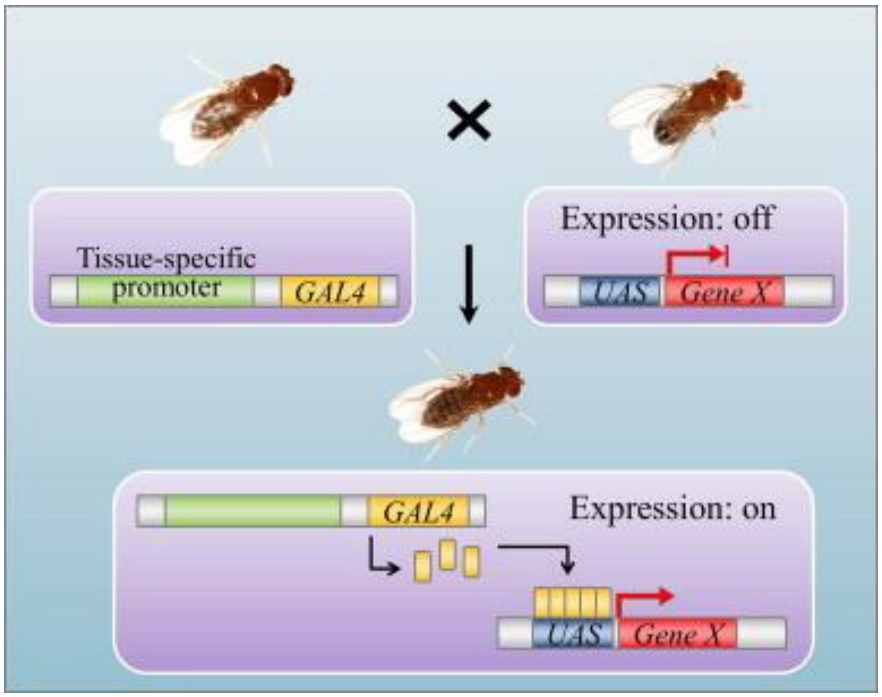

Figure 2: The GAL4-UAS system

Schematic diagram of the UAS-GAL4 system used in *Drosophila melanogaster* for targeted gene expression [14].

Drosophila melanogaster as a model to study different aspects of the gastrointestinal system

Similarities between *Drosophila* gastrointestinal tract and mammalian intestinal tract:

The *Drosophila* digestive tract is comprised of a single epithelial layer which is surrounded by trachea, visceral muscles and the nerves. *Drosophila* adult gut is subdivided into three regions which are foregut, midgut, and hindgut (**Figure 3A**). The inner lining of *Drosophila* midgut is analogous to the small intestine of mammals which is a pseudostratified epithelium further subdivided into six major anatomical regions (R0 to R5). R4 and R5 region constitutes the posterior midgut and this region is very active in metabolism and generating immune response [1], [2]. Unlike anatomical crypt-like niche structure in the mammalian intestine, the posterior midgut of adult *Drosophila* is composed of simple columnar epithelium and it contains intestinal stem cells (ISCs), undifferentiated daughters of ISC cells also termed as enteroblasts (EBs), large sized polyploid cells of having absorptive in nature known as enterocytes (ECs), and ECs are intermingled with enteroendocrine cells (EEs) which are diploid, secretive in nature and its abundance is less in midgut (**Figure 3B**) [15], [16], [17], [18]. Similarly, in mammalian intestine also there are intestinal stem cells which resides at the bottom of the crypts and intermingled between paneth cells. However, instead of EB cells, mammals has the transit amplifying (TA) cells. Mammalian intestine also contains large absorptive cells (enterocytes or ECs) and small enteroendocrine cells (**Figure 4**) [18], [19].

Although there is physiological divergence between vertebrates and insects, high degree conservation has been evidenced between human and *Drosophila melanogaster* intestinal epithelium with respect to anatomy, tissue functioning and signaling pathways which are involved in maintaining the tissue homeostasis, development, regeneration and disease.

Intestinal Stem cells:

Constant turn-over of epithelial cells are absolutely essential to maintain epithelial homeostasis. Failure to maintain the epithelial tissue homeostasis can engender disruption in tissue functioning and loss of control over cell proliferation which can lead to cancer [20]. Likewise, intestinal epithelium goes under constant replenishment of lost cells with new set of cells and thus the protective and absorptive functions of the gut are retained. Intestinal stem cells (ISCs) which are multipotent in nature, are fuelling intestine epithelia renewal process. Similar to other stem cells such as mouse hematopoietic stem cell, intestinal stem cells also possess the capability to self-renewal and also have the ability to generate all differentiated cell

types. Intestinal stem cells can divide symmetrically or asymmetrically. While ISC divides symmetrically it gives rise to two ISCs and when it divides asymmetrically it generates one ISC and another progenitor cell type which can further differentiate into other cell types [19].

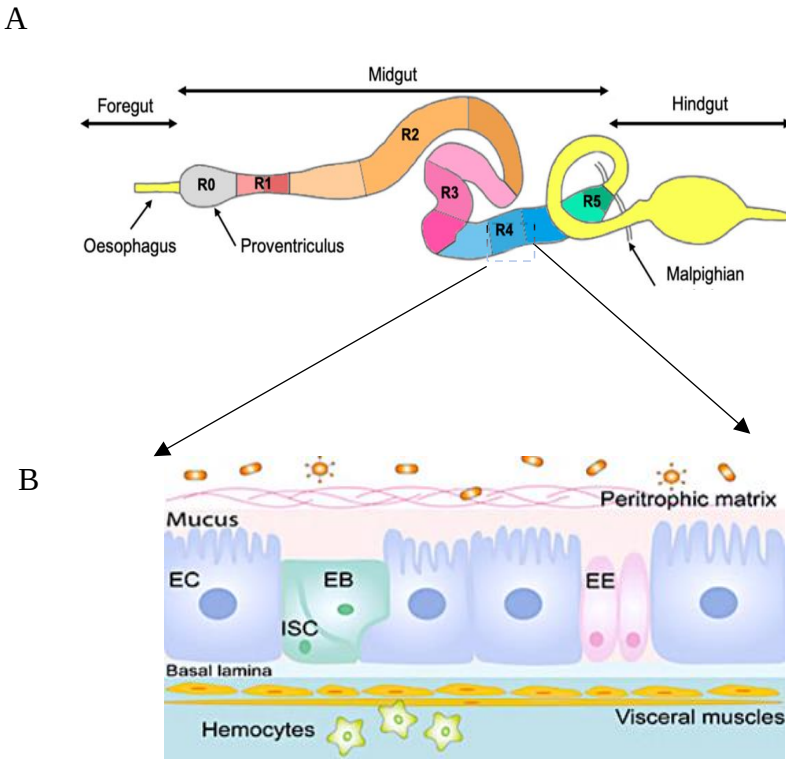

Figure 3: *Drosophila melanogaster* Adult Gut anatomy and cell types present at posterior midgut region.

A) Adult *Drosophila* midgut consists of six major anatomically distinct regions (R0-R5) [3].
B) The fly posterior midgut is composed of absorptive EC cells and secretory EE cells which are generated from the differentiation of ISCs which are situated over the basal lamina. Enteroblasts are transient immature progenitor cells. Peritrophic matrix and thin mucus layer protect the epithelium and they are sheathed in basal lamina and visceral muscle cells [24].

Mammalian Intestinal Stem Cells:

In mammals, monostratified intestinal inner lining undergoes numerous number of invaginations which are called as crypts. Adult mouse's intestine contains innumerous crypts and each crypts produce approx. 300 cells per day [18]. This phenomenal yield is achieved by the population of intestinal stem cells which are present at the base of these crypts. Mammalian ISCs migrate along the axis of crypts while dividing without differentiating into other cell types. Undifferentiated cycling progenitor cells also known as transit amplifying [TA] cells

span the crypt length and at the upper part of the crypt it differentiates into other cell types which are goblet cells, enteroendocrine cells, enterocytes and paneth cells. Paneth cells secretory cells, localized at the bottom of the crypts and intermingled with ISCs [18] (**Figure 4**). Mammalian ISCs can be identified by the expression of Lgr5 [21] which is a wnt target gen codes for an orphan G protein coupled receptor whose function is still not elucidated. Another mammalian ISC marker is recognized in recent studies which is Bmi1, belongs to polycomb family of chromatin remodelers. ISCs which possess Bmi1 have showed long-term regeneration potential and they are localized above the Paneth cells. The presence of both Bmi1+ and Lgr5+ ISCs at distinct localization [21], [22] and their features still need to be elucidated in order to compare these populations [18], [23].

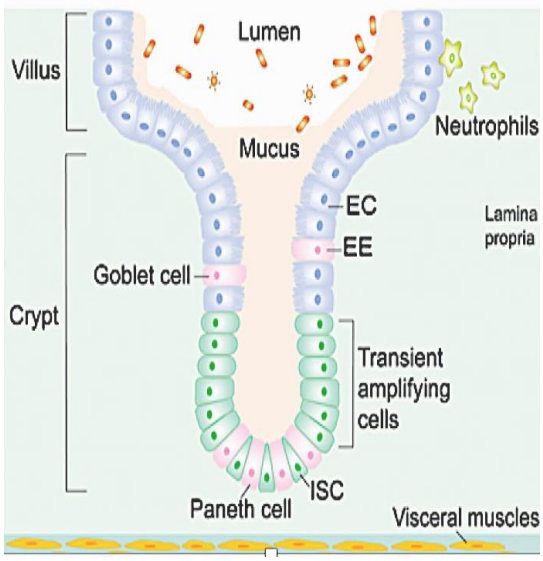

Figure 4: Mammalian intestinal section

Schematic diagram of mammalian intestinal epithelium consist of progenitor and Paneth cells residing at the base of crypts and absorptive EC cells and secretory EE and Goblet cells [24].

***Drosophila* Intestinal Stem cells and other cell types:**

Drosophila intestinal stem cells are those cells which maintains the midgut tissue homeostasis. If any kind of damage of injury to the midguts cells occur, that is replenished by ISCs housed within the epithelium. Drosphila ISCs can divide symmetrically or asymmetrically depending various signaling pathways which dictates the ISC division. Singaling pathways such as JAK-STAT, JNK and Notch are mainly involved in dictating the ISC fate. When ISC divides symmetrically it gives rise to two ISC cell, when it divides asymmetrically it gives rise one ISC and one daughter cell which is undifferentiated known as enteroblasts (EBs). The marker protein which is being used to mark *Drosophila* ISCs and EB cells is escargot (esg), belongs

to SNAIL family transcription factor. The sole marker for *Drosophila* ISCs is Delta. Su(H) is being used to mark the EB cells. *Drosophila* eneterocytes ECs have morphological as well as functional difference within different regions in the midgut. However, most of EC cells express *Myosin31DF* and this is used to mark the EC cells. *Drosophila* EE cells expresss *Prospero* and we used prospero antibody to mark the EE cell types in *Drosophila* posterior midgut (**Figure 5)** [17], [23], [25], [26].

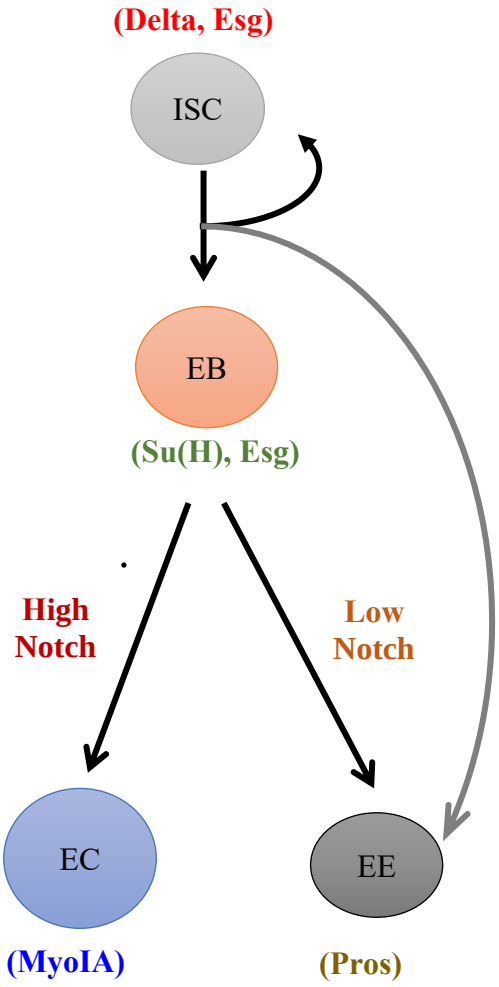

Figure 5: Different cell types present in *Drosophila melanogaster* posterior midgut and markers used to mark those cell types.

Signaling pathways involved in maintenance of ISC homeostasis:

Delta/Notch Signaling pathway:

Notch signaling, an evolutionary conserved pathway which is involved in various essential developmental events both in mammals and *Drosophila*. This signaling pathway gets activated when notch ligands Delta (Dl) and serrate (Ser) binds to their receptor Notch which is displayed by another neighbouring cell This leads to cleavage of the Notch intracellular domain (NICD) that enters into the nucleus and along with other co-factors for instance Suppressor of Hairless (Su(H)), it regulates gene expression [27], [28]. In *Drosophila* intestine, Notch signaling dictates the decision of whether the dividing ISC will undergo self-renewal or differentiate into other cell type. Thus, Notch controls the identity of ISC progeny in the adult *Drosophila* gut. High level notch activation prevents EB cells to become ISCs rather promotes differentiation towards enterocytes and low notch signaling in EBs promotes differentiation into EEs [29]. The notch ligand Delta is solely expressed by the ISCs whereas notch receptor is exhibited by both ISCs and EBs. Knockdown of Notch signaling induces ISC loss and leads to enteroendocrine hyperplasia in both intestine of *Drosophila* and mice. Furthermore, ectopic expression of notch signaling leads to EC differentiation [30]. A study from Ohlstein group demonstrated that bidirectional notch signaling is necessary to maintain the *Drosophila* ISC multipotency [31]. EE cells induce notch signaling in the future ISC daughter cells and thus prevents ISC to EE fate. This study has been performed in both pupal ISCs and adult ISCs. These studies underline the important role played by Delta/Notch signaling in regulating ISC division modes and fates during the intestinal epithelium homeostasis [26], [28], [32], [33].

JNK signaling pathway:

In order to maintain the tissue homeostasis, many animal tissues employ regenerative as well as cytoprotective strategies. As *Drosophila* gut cells are the first cells which are going to experience any kind of dietary intervention so to maintain the *Drosophila* intestine homeostasis, proliferation of ISC is required to replace the damaged cells in the epithelium [33]. Bacterial infection, DNA damage, or any kind stress can lead to activation of JNK in the *Drosophila* midgut. Studies from Jasper's group has showed that JNK signaling is essential to provide the protection to the gut cells from oxidative stress during the regeneration process and also induces the ISC proliferation [30], [33]. Another study demonstrate that JNK signaling in the enterocytes activates the production of cytokines (Upd1, Upd2, Upd3) in ECs and they activate the JAK/STAT signaling by binding to the Dome receptor present at the ISCs. JAK-

STAT signaling promotes the rapid division of ISCs [34]. Recent past studies also suggest that the crosstalk between cytoprotective JNK signaling and Notch signaling which controls cell proliferation and differentiation need to have critical balance to ensure the gut homeostasis is maintained [30].

JAK-STAT and EGFR Signaling pathway:

In the *Drosophila* adult midgut, during regeneration EGFR and JAK-STAT signaling pathways are required to activate the ISC division [33], [35]. If any of the pathway is activated ectopically, it leads to hyper-proliferation of ISCs and results into hyperplasia. These pathways get activated by the ligands produced by visceral muscle cells (VMs), EC cells and progenitor cells. A myriad of signaling pathways including Hippo, Hedgehog, BMP, Wingless and JNK pathways regulate the production of these ligands. Some studies suggested that JAK-STAT pathway is required during differentiation of EB to mature midgut cells during midgut homeostasis [33], [34], [35]. When JAK-STAT is downregulated, it has been found that EBs become accumulated in the midgut [36]. However, how JAK-STAT is regulating this lineage differentiation process is still not known [36].

Other signaling pathways:

Drosophila Wnt/Wingless pathways has been reported to be involved in specifying the ISC fate. Studies from clonal analysis has shown that loss of Wg signaling results in gradual loss of ISCs and ectopic activation of Wg pathway expands the ISCs [33]. Another major signaling pathway which is involved in mainating midgut tissue homeostasis is Hh signaling. It has been showed that Hh signaling controls the ISC proliferation in the midgut. Studies in finding role of BMP signaling in *Drosophila* midgut homeostasis suggest that BMP signaling plays multiple functions including ISC self-renewal and also promotes ISC proliferation [28], [33]. The Hippo pathway, a newly emerged pathway has also been shown to have role in controlling intestinal stem cell activity. It has been reported that Hpo signaling restricts the ISC proliferation [28]. Insulin/Insulin-like (IIS) signaling has also role in regulating induction of ISC divisions and promotes the symmetric division of ISC. In summary, various signaling pathways are responsible for regulation and maintenance of ISC activity and midgut homeostasis [28], [33].

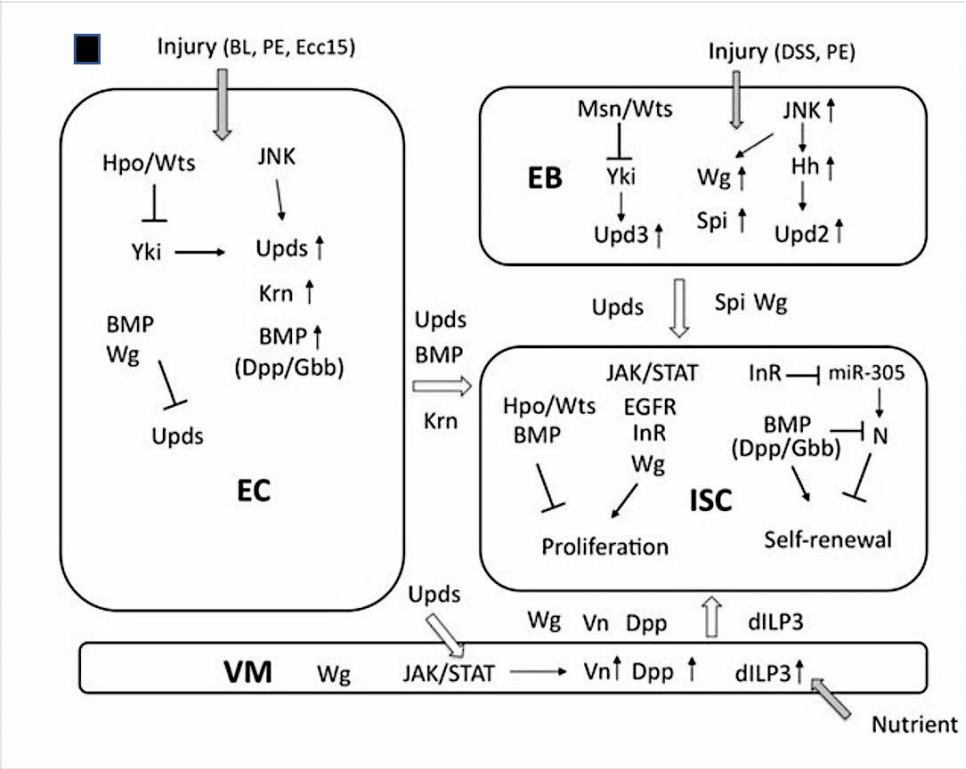

Figure 6: Signaling network that maintains the state and fate of *Drosophila melanogaster* ISCs.

Schematic diagram of different signaling pathways involved in maintaining stem cell homeostasis. JNK, JAK-STAT, EGFR are major mitogenic signals that regulate ISC activity [33].

Experimental model

Drosophila as a model to study the effects of High Sugar Diet

Drosophila has emerged as a wonderful model system for studying diet dependent changes in different tissues [37], [38]. Specifically, it has been demonstrated that high amount of sugar uptake in flies can lead to increased glucose level in the hemolymph, insulin resistivity and obesity like conditions, that are hallmarks of the type II diabetes [39], [37], [38]. In that direction we have been successful in generation diet-induced type II diabetic flies in our lab. Previous studies in the lab demonstrated that rearing of flies on HSD results in flies that exhibit typical attributes of type II diabetes, such as increased glucose in the hemolymph, increased trehalose levels, and insulin resistivity with no effect in the production of insulin. Furthermore it has been evidenced that the gut of the flies reared on HSD demonstrate a gradual reduction in both length and breadth, the gut epithelial cells demonstrate reduction in cell size and increased cell death.

For my studies, I employed the same system to explore the effects of HSD in the midgut stem cell homeostasis. The regimen of feeding followed for the study is as shown in **Figure 7**.

Experimental Strategy:

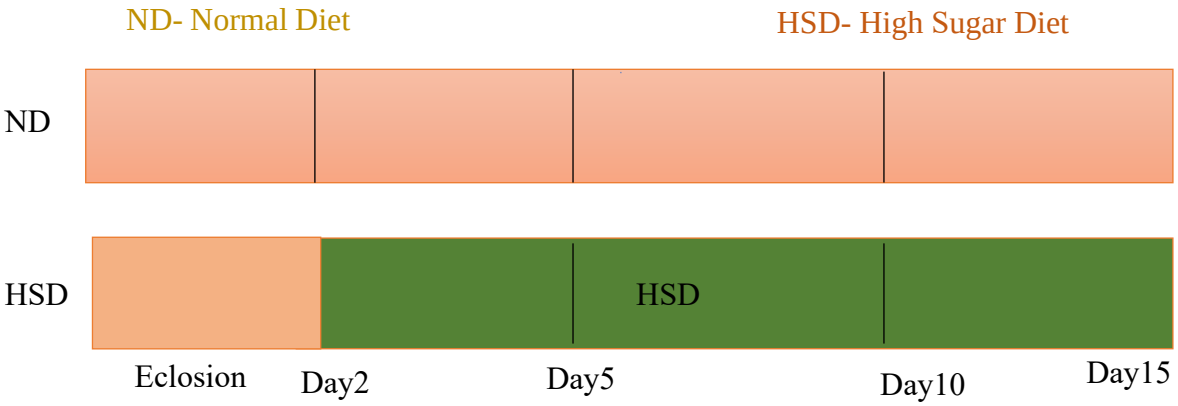

Figure 7: Schematic diagram of experimental strategy.

Objectives

In the recent past studies have uncovered that changes in dietary components can alter the ISC homoeostasis either by triggering the ISC proliferation and differentiation [40] or by depleting the ISC proliferation [41]. However, how high sugar diet affects the ISC homeostasis is still not known.

Previous studies performed in our lab demonstrated that high sugar diet alter the gut morphology and increase the cell death of cells. But the mechanism is not clear.

Therefore, this project aims to analyse the changes happening in gut epithelial cells upon high sugar diet. The specific objectives of this study are:

1. Study the impact of high sugar diet in altering the ISC homeostasis, if any.
2. Understand the mechanistic basis of the changes happening in ISC homeostasis due to high sugar diet.

CHAPTER II

MATERIALS AND METHODS

II.I Rearing of flies and maintenance

For rearing flies standard food was made from agar, maize, sugar, methyl paraben, propionic acid. Flies were maintained at 25^0C in culture bottles.

II.II Experimental setup

Flies of particular genotypes were used in order to set up the experiment. Flies which were freshly eclosed were collected and then reared on the normal diet for two days at 25^0C in incubator. Thereafter, the flies are divided into two separate batches. In 2:1 Females to males ratio some set of flies were reared on normal sugar diet containing sugar concentration of 0.15M. While some other set of flies were transferred to a high sugar diet containing 1M sugar concentration. Approximately 40 healthy female flies and 20 male flies were kept in each bottle for both normal and high sugar diet. In order to maintain a healthy culture environment in every 3 days flies were flipped to fresh normal diet and high sugar diet containing bottles. We have performed our analysis mainly on Day 15 after eclosion.

II.III Fly stocks and genotypes

The fly stocks used for this thesis were obtained from various stock centres(B, while some were gifted fly lines.

1. ***Oregon ^{R}***

Wild type laboratory stock of *Drosophila*

2. ***w[*];P{w[+Mw.hs]=GawB}Myo31DF[NP00001]/Cyo;P{y[+t7.7]w[+mC]=UAS-Cas9.P2}attP2/TM6B, Tb[1]***

This transgenic fly line expresses GAL4 in the Myo31DF expression pattern. This stock was obtained from Bloomington Stock Center (67088).

3. ***w[*];P{w[+mW.hs]=GawB}NP5130P{w[+*]=UAS-GFP.U}2;P{w[+mC]=UAS-3xFLAG.dCas9.VPR}attP2.P{w[+mC]=tubP-GAL80[ts]}2***

This transgenic fly line expresses GAL4 in the esg expression pattern and GFP under UAS control in chromosome number 2. This stock was procured from Bloomington Stock Center (67072).

4. w[1118]; P{w[+m*]=NRE-EGFP.S}5A

This transgenic line expresses EGFP under the control of a Notch Response Element (NRE) on the second chromosome. This stock was obtained from Bloomington Stock Center (30727).

5. w[1118]; 12XSu(H) LacZ; +/+

This transgenic line contains 10 tandem Su(H) consensus binding sites are fused upstream of a HLHmy promoter fragment (which contains two Su(H) binding sites) which drives lacZ expression. The transgene insertion is viable to choromosome 2. This stock fly line was a generous gift from A. Tsakonas, Boston, USA.

6. w[*]; P{w[+mC]=UAS-2xEGFP}AH2

This is a transgenic line that contains a UAS-driven green fluorescent protein. The transgene insertion is homozygous viable on chromosome 2. This stock was obtained from Bloomington Stock Center (6874).

II.IV. Immunohistochemistry:

II.IV.I. Delta Antibody Staining:

Posterior midguts from adult *Drosophila* female flies (*Oregon* ^{R}) were dissected in 1X PBS and fixed in a 1:1 (v/v) mixture of n-heptane and 5% formaldehyde for 15 mins at room temperature. After replacing the mixture, an equal volume of 100% methanol was being used to dip the tissues and kept at room temperature for 5 mins. 100% methanol was slowly removed and replaced with 75% methanol in 0.1% PBT and incubated in room temperature for 5 mins. After that 75% methanol in 0.1% PBT was replaced by 50% methanol in 0.1% PBT and incubated at room temperature for 5 mins. After slowing removing 50% methanol in 0.1% PBT, 25% methanol in 0.1% PBT was added to the samples and kept at room temperature for 5 mins. After the fixation, samples were washed three 3 times in 0.1% PBT for 12 mins each.

Following the washing, samples were incubated in 5% NGS in 0.1% PBT as the blocking solution at room temperature for 60 mins in 60 rpm shaker. Once blocking is finished, gut samples were incubated in Delta antibody solution (1:100) made in 5% NGS for 18-20 hours at 4^0C in Nunc plates. Samples were transferred to cavity blocks containing 0.1% PBT and a quick was given to the samples followed by three times washing in 0.1% PBT for 12 mins each were done. Samples were blocked in 5% NGS solution for 60 mins. After that, blocking solution is replaced by secondary antibody (Cy3 conjugated anti-mouse IgG) made in 5% NGS solution of 1:1000 dilution and incubated for 2 hours at room temperature followed by two 0.1% PBT washes for 17 mins and then one 1X PBS wash for 20 mins were given to the samples. The samples were incubated in Hoechst solution (1:1000) for 20 mins. After Hoechst treatment, gut samples were washed in 1X PBS for three times 15 mins each. Sample were mounted (bridge mounting) in VECTASHIELD (Vector laboratories #H1000) mounting medium.

II.IV.II. β–Gal Antibody Staining:

Posterior midguts from adult *Drosophila* female flies were dissected in 1X PBS and fixed in 5% paraformaldehyde for 1 hour at room temperature in 60 rpm shaker. After fixation gut sample were washed in 0.1% PBT three times for 12 mins each. Samples were incubated in 5% NGS in 0.1% PBT solution for 60 mins at room temperature in 60 rpm shaker. Then gut samples are transferred into the Nunc plates containing β-gal antibody of dilution 1:50 in 5% NGS solution for 18-20 hours at 4^0C. Then gut samples were moved to a cavity block containing 0.1% PBT solution and 0.1% PBT and a quick was given to the samples followed by three times washing in 0.1% PBT for 12 mins each were done. Samples were blocked in 5% NGS solution for 60 mins. After that, blocking solution is replaced by secondary antibody (Cy3 conjugated anti-mouse IgG) made in 5% NGS solution of 1:1000 dilution and incubated for 2 hours at room temperature followed by two 0.1% PBT washes for 17 mins and then one 1X PBS wash for 20 mins were given to the samples. The samples were dipped in Hoechst solution (1:1000) for 20 mins. After Hoechst treatment, gut samples were washed in 1X PBS for three times 15 mins each. Sample were mounted (bridge mounting) in VECTASHIELD (Vector laboratories #H1000) mounting medium.

II.IV.III. Prospero Antibody Staining:

Posterior midguts from adult *Drosophila* female flies were dissected in 1X PBS and fixed in 5% paraformaldehyde for 1 hour at room temperature in 60 rpm shaker. After fixation gut sample were washed in 0.1% PBT three times for 12 mins each. Samples were incubated in 5% NGS in 0.1% PBT solution for 60 mins at room temperature in 60 rpm shaker. Then gut samples are transferred into the Nunc plates containing Prospero antibody of dilution 1:50 in 5% BSA solution for 18-20 hours at 4^0C. Then gut samples were transferred to a cavity block containing 0.1% PBT solution and immediately the solution was removed and the samples were washed with 0.1% PBT for 12 mins each were done. Samples were blocked in 5% NGS solution for 60 mins. After that, blocking solution is replaced by secondary antibody (Cy3 conjugated anti-mouse IgG) made in 5% NGS solution of 1:500 dilution and incubated for 2 hours at room temperature followed by two 0.1% PBT washes for 17 mins and then one 1X PBS wash for 20 mins were given to the samples. The samples were incubated in Hoechst solution (1:1000) for 20 mins. After Hoechst treatment, gut samples were washed in 1X PBS for three times 15 mins each. Sample were mounted (bridge mounting) in VECTASHIELD (Vector laboratories #H1000) mounting medium.

II.V Antibodies used

Primary antibodies used:-

Primary antibodies which are used as followed-

Serial No	Antibody	Raised in	Source	Dilution
1	Delta	Mouse	DSHB, Iowa	1:100
2	b-gal	Mouse	DSHB, Iowa	1:50
3	Prospero	Mouse	DSHB, Iowa	1:50

Secondary antibody used:-

For immunostaining, the secondary antibody which is mainly used:

Antibody	Source	Details	Dilution
CyTM3- Conjugated AffiniPure Goat Anti-Mouse IgG(H+L)	Jacksons Immuno Search Laboratories Code-711-165-162	Conjugated with cyanine CyTM3 dye (Absorption maxima/ emission maxima is 550 nm/ 570 nm)	1:1000

Hoechst Staining:

Hoechst(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a blue fluorescent dye and it preferentially binds to A-T regions of DNA. It is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. The working dilution is 1 μg/ml.

II.VI. Edu Staining:

Drosophila adult posterior midguts were dissected out in 1x PBS of PH 7.2 within 10 min at room temperature. 1X PBS was replaced with EdU+ solution (1:1000) made in 1x PBS and kept at room temperature in a shaker (60 rpm) for 45 min. Then, EdU+ solution was replaced by 5% Paraformaldehyde and kept at room temperature in a shaker (60 rpm) for 1 hour. The gut samples were then washed with 0.1% PBT for four times for 10 min each. After washing, staining solution was added in nunc plates containing gut samples were dipped into it and kept it at room temperature for 30 min. Guts were transferred to cavity block containing 0.1% PBT and then washed three times for 10 min each. The samples were incubated in Hoechst solution (1:1000) for 20 min and then three 1x PBS washes were given to the samples for 20 min each. Sample were mounted (bridge mounting) in VECTASHIELD (Vector laboratories #H1000) mounting medium.

II.VII. Imaging

Mounted sample were imaged in confocal microscope (Zeiss LSM 780 and Leica SP8) and processed using Fiji (NIH) software and Imaris software.

II.VIII. RNA Isolation:

Drosophila adult posterior midguts were dissected out (nearly 70) in 1X PBS and kept in a Eppendorf tube. Followed by a short centrifugation 1X PBS was replaced by 200 μl of TRIzol. Samples were stored at -80°C overnight. Next day, samples were thawed on ice. The samples were homogenized by using autoclaved pestels. After that, 800 μl of TRIzol was added to the samples and kept at room temperature for 30 mins along with intermittent pipetting. Samples were centrifuged at 10000 rpm for 10 mins at 4^0C. The supernatants were transferred to another Eppendorf tube and 200 μl of chloroform was added. The samples were shaken vigorously for some minutes and incubated at room temperature for 30 mins. Then the samples were

centrifuged at 15000 rpm for 15 mins at 4^0C. The uppermost layer containing RNA was taken very carefully in a RNAase-free tube. After that equal volume of isopropanol was added to the samples and mixed very well and followed by glycogen (1:500) was added. The samples were stored at -20^0C for 2 hours. After that samples were centrifuged at 10000 rpm for 15 mins at 4^0C to pellet out the RNA and the supernatants were discarded carefully without disturbing the pellet. Then the pellet was washed by chilled 70% molecular grade ethanol and incubated the samples for 2 mins and then the samples were centrifuged at 13000 rpm for 10 mins at 4^0C. After discarding the supernatant, the pellet was air dried and finally it was dissolved in 20µl autocloaved molecular grade water. The amount of RNA was quantified using Nanodrop spectrophotometer (Thermofisher).

II.IX. cDNA synthesis :

Using the cDNA synthesis kit (Verso) cDNA was synthesized from the RNA isolated from posterior midgut.

Components	**Volume (1X)**
RNA+Water	10 µl
cDNA mix (RT enhancer, dNTPs, 5X cDNA Synthesis buffer in ratio of 1:2:4 respectively)	7 µl
RNA primer mix (Anchored oligo DTs and Random hexamers in ratio of 1:1)	2 µl
Verso Enzyme mix	1 µl

Reaction time:

	Temperature	Time	Number of cycles
cDNA Synthesis	42^0C	45 min	1
Inactivation	95^0C	2 min	1
Forever	4^0C	∞	-

II.X. Real-Time Polymerase Chain Reaction:

Components	1X
Syber Green	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Template DNA	1 µl
Water	5 µl

Reaction Time:

Temperature	Time	Number of cylces
94^0C	0:10 min	40
58^0C	0:45 min	
95^0C	0:30 min	1
65^0C	0.05 min	1
4^0C	∞	-

II.XI. Buffers and Reagents:

10X Phosphate Buffer Solution (PBS) :

40g NaCl, 1g KCl, 7.2g Na_2HPO_4 and1.2g K_2HPO_4 were weighed in a weighing machine for a volume of 500ml 10X PBS solution. Autoclaved dH_2O was added to scale upto 500 ml and then pH was set to 7.2.

5% Paraformaldehyde:

For a volume of 4ml, 0.2g of PFA was weighed and 4 ml of 1X PBS was added to it. After sealing the eppendorf tube with parafilm, it was incubated at 65^0C in a waterbath and shked intermittently, until it is fully dissolved.

0.1% PBT:

For a volume of 40ml, 40µl of 100% TritonX was added to 40ml 1X PBS (pH 7.2). To dissolve the solution, it was kept on shaker at 60 rpm.

Edu Components:

For a volume of 50 μl, staining solution was made from-

Components	**Volume**
1x Click it buffer	44 μl
Copper protectant	1μl
Alexa fluor picoyl azide	0.12 μl
Reaction buffer additive	0.5 μl

II.XII. Genetic Constructs:

Primers used:

rp49:

Fw: CTAAGCTGTCGCACAAATGGC

Rw: TTCTGCATGAGCAGGACCTC

Upd1:

Fw: TTCGACTGGCGCTTTCCACGTC

Rw: CGCAGCTCCACCTTGAATGGCA

Upd2:

Fw: CCACAAGTGCGGTGAAGCTA

Rw: TGCTGATCCTTGCGGAACTT

Upd3:

Fw: AGCACCTACAGAAGCGTTCC

Rw: TGCAGGATCCTTTGGCGTTT

CHAPTER III

RESULTS

III.I High Sugar diet disrupts Intestinal Stem Cell Homeostasis:

III.IA: Feeding flies with High Sugar diet depletes ISC population.

To analyse the status of the ISCs in the gut epithelium of the flies reared on HSD, immunostaining with an antobody against Delta was performed. Shortly after asymmetric ISC division, the cell that retains the high amount of Delta stays as ISC and the other cell that looses Delta expression become the EB cell. We found a drastic depletion in the number of Delta positive ISCs in the midgut of the adult flies reared on HSD for 15 days (**Figure 8A and 8B**). Quantitative analyses revealed a significant drop in the average number of ISCs in the midgut of these HSD fed flies (**Figure 8C**). We also observed that the levels of expression of Delta (based on fluorescence intensity analysis) in the ISCs of the HSD fed flies were much less as compared to that in the ISCs of the control flies. This could be one of the reasons for losing ISCs.

The other marker that we used to study the ISCs is Escaragot (esg). *Drosophila* adult flies express *esg*, a snail homolog, in both intestinal stem cells (ISCs) and undifferentiated progenitor cells (EBs). We drove the reporter *UAS-2xEGFP*, with *esg-Gal4* driver line to mark both the ISCs and EBs. We found that in day 15, HSD fed flies demonstrated a drop in the number of *esg* positive cells (**Figure 9A and 9B**). Quantitative analysis revealed a modest drop in the number of *esg* positive cells in the gut of HSD flies as compared to those reared on ND (**Figure 9C**).

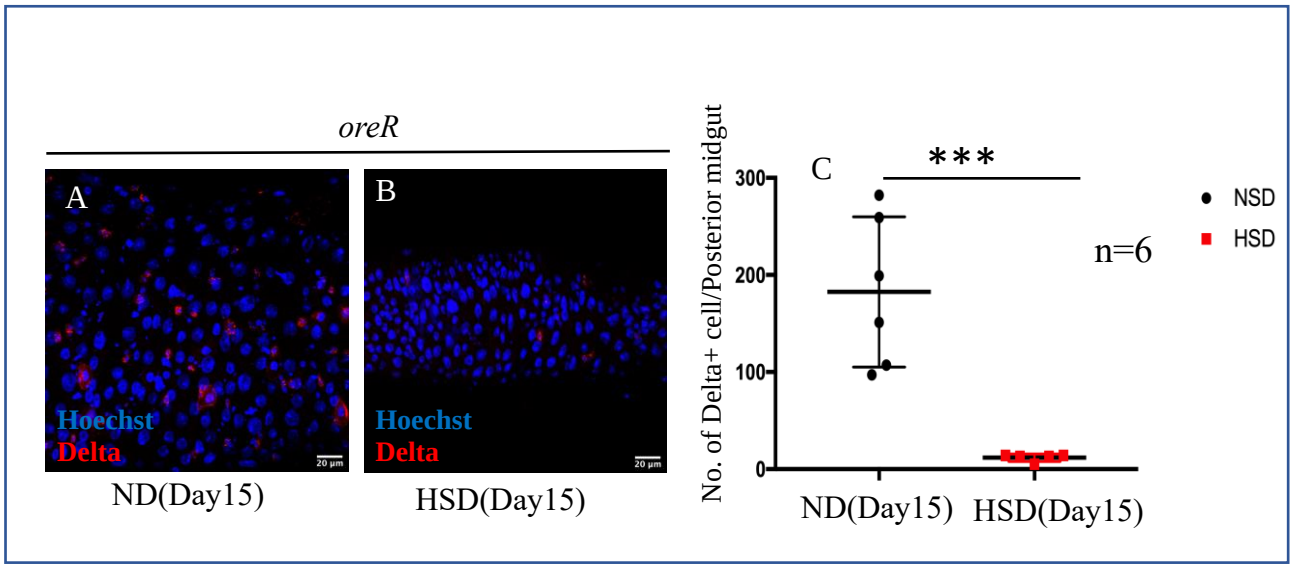

Figure 8: Depletion in Intestinal Stem Cells (Delta+ve).

Scale bar - 20μm. Expression pattern of Delta antibody in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis (**C**) of number of ISC cells in the posterior midgut. Compared to ND posterior midgut, HSD posterior midgut shows a significant decrease in ISC number.

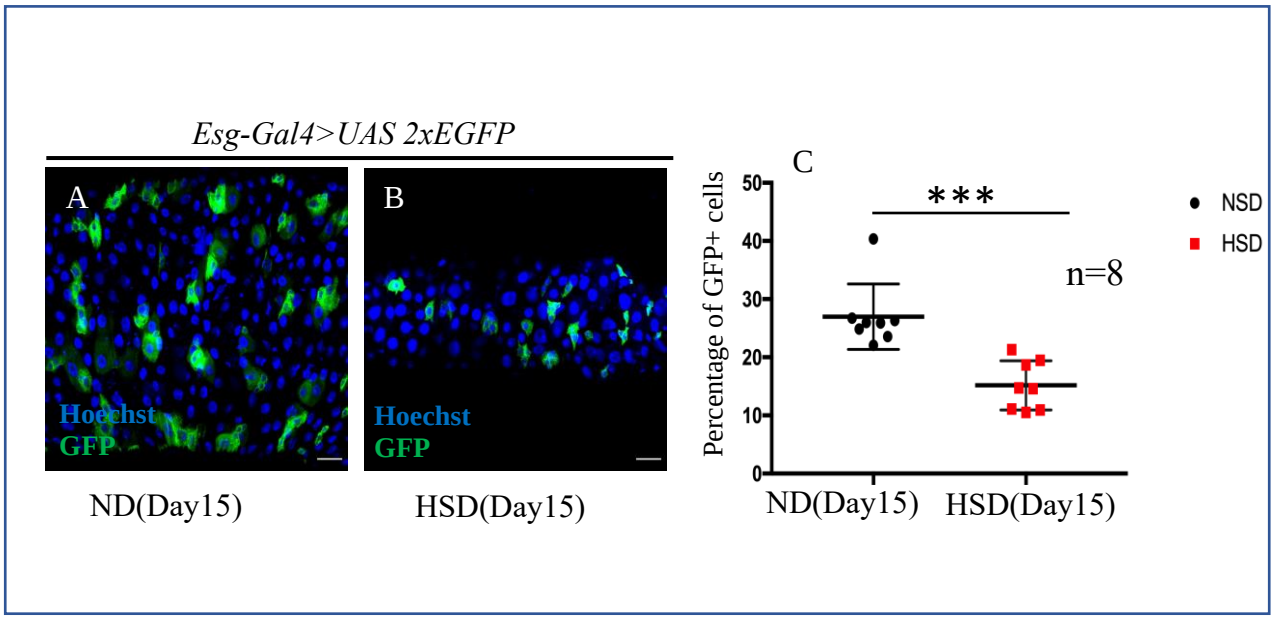

Figure 9: Drop in intestinal stem cell (ISC) and Enteroblast (EB) cell population.

Scale bar - 20μm. Expression patter of *esg-Gal4>UAS-2xEGFP* in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis (**C**) of percentage of esg positive cells in posterior midgut. Compared to ND posterior midgut, HSD posterior midgut shows a modest drop in *esg* (ISC and EB) number.

III.IB: High Sugar Diet Does not affect the EB population

Next, we employed the Su(H)LacZ flies to analyse the expression of Su(H), a Notch pathway target gene, that specifically marks the enteroblasts (EBs) [25], [17]. Immunostaining of the gut with antibody against β-galactosidase demonstrated that there was no significant change in the EB cell number in the posterior midgut of the HSD-fed flies when compared to those reared on ND (**Figure 10A and 10B**). Upon quantification we observed that the number of EBs in the midgut of HSD-fed flies were comparable to that observed in the midgut of ND-fed flies (**Figure 10C**).

From these results, we argue that since the number of EB positive cells remained unchanged, the drop observed for esg positive cells (marking both ISCs and EBs) was not as prominent as that observed for Delta positive cells (that specifically marks the ISCs).

III.II: High Sugar Diet Does not affect Notch signaling:

Regulation of the specification and differentiation of midgut progenitors is mediated by the Notch signaling. In adult *Drosophila* midgut epithelium, Notch signaling is instrumental in determining the fate of ISC progeny into either EC or EE cells. While high Notch activity leads to generation of EC cells, low Notch induces the differentiation of EE cells [18], [29]. Therefore, we examined how Notch signaling is getting affected upon feeding the flies on HSD. We investigated this by employing Notch response element EGFP (NRE-EGFP) fly line and when we quantified the intensity of GFP positive cells, no significant change in intensity was detected (**Figure 11**) which suggests that there is no significant change in Notch signaling.

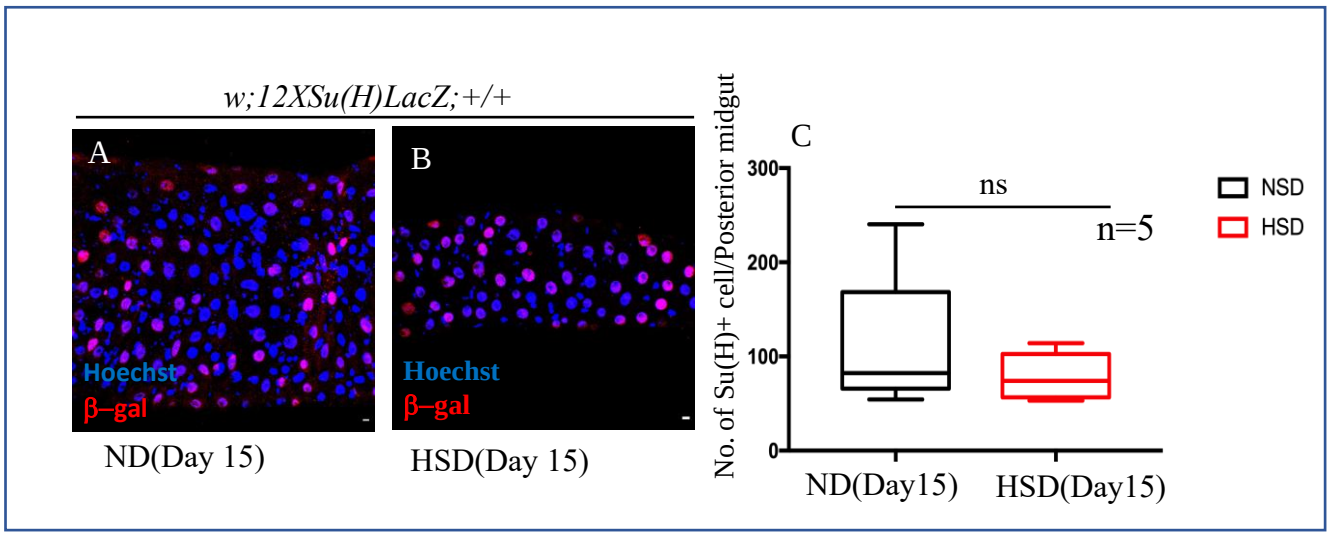

Figure 10: No significant change in number of EB cells (Su(H)+ve) in posterior midgut.

Scale bar - 20μm. Expression pattern of β-galactosidase antibody in ND and HSD posterior midgut on Day 15 (**A**, **B**) and Quantitative analysis (**C**) of number of EB cells in whole posterior midgut. Compared to ND posterior midgut, HSD posterior midgut shows no significant change in EB number when compared to ND posterior midgut.

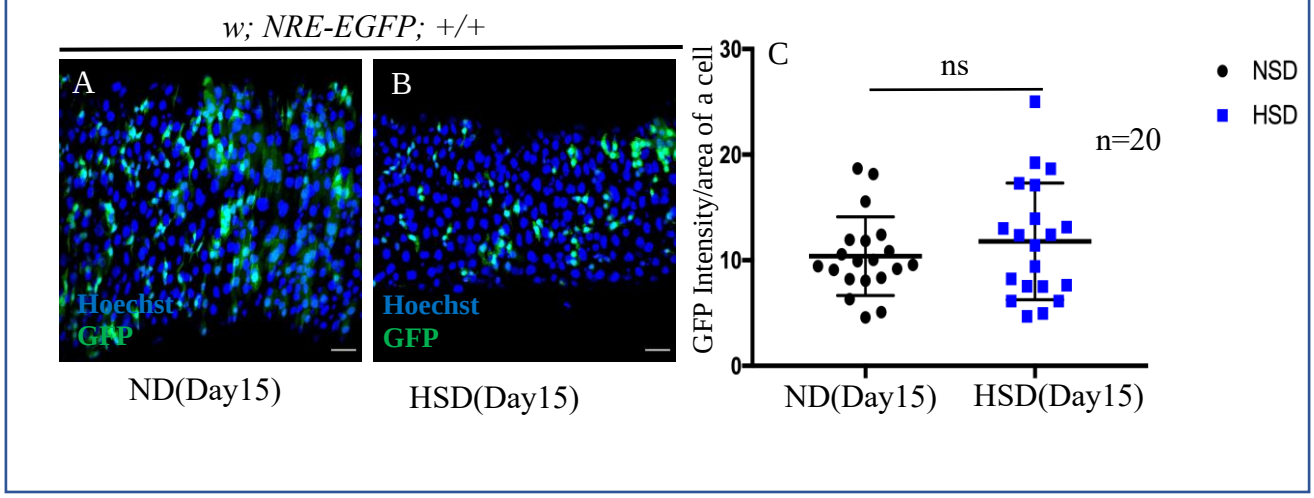

Figure 11: No significant change in intensity of NRE-EGFP.

Scale bar - 20μm. Expression pattern of NRE-EGFP in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis **(C**) of intensity of GFP positive cells per unit area. No significant change in GFP intensity of HSD posterior midgut cells suggest that there is no change in Notch signaling.

III.IC. High Sugar diet increases absorptive enterocytes population of the posterior midgut

Results, till now have demonstrated that a drastic depletion in the number of ISCs, while the number of EBs remain unaltered in the posterior midgut of HSD-fed flies. No significant alternation in the intensity of NRE-EGFP expression was also observed. As a next step, we looked at the status of the differentiated cell types, the ECs and EEs. The ECs constitute the most abundant cell type of the midgut epithelium. They are mainly involved in absorption of nutrients and also has a role in aiding the digestion of secreting digestive enzymes [1], [23], [26]. *MyoIA-Gal4/UAS-2xEGFP* marks the EC cell types. By using this fly line, we observed a significant increase in EC population in the midgut of HSD-fed flies when compared to that of ND-fed flies (**Figure 12A and 12B**). Quantitative analyses of the data revealed a significant increase in EC population. The quantitative analysis was performed by counting the GFP+ cells and the count was normalized by the total nuclei (Hoechst staining) present in that area (**Figure 12C**).

III.ID. High Sugar Diet decreases the enteroendocrine population in the posterior midgut

Enteroendocrine cell population are the less abundant population in the posterior midgut during the normal midgut homeostasis. Enteroendocrine cells are mainly involved in secretion of hormones. There are different types of EE cells which secretes different subset of hormones. However, overall population of EC cells can be identified by looking at the expression of the homeodomain protein Prospero (Pros) [1], [26]. So in order to determine whether the EE population and its number in the posterior midgut is getting affected in HSD fed flies, we immunostained the guts with an antibody against Prospero to mark the EEs. We observed a significant depletion in the number of EE cells (Pros+) in the posterior midgut (**Figure 13A and 13B**). The data was further quantified by calculating total number of Prospero positive cells in the whole posterior midgut. As shown in the **Figure 13C** almost fifty percent reduction in the number of EE cells was observed.

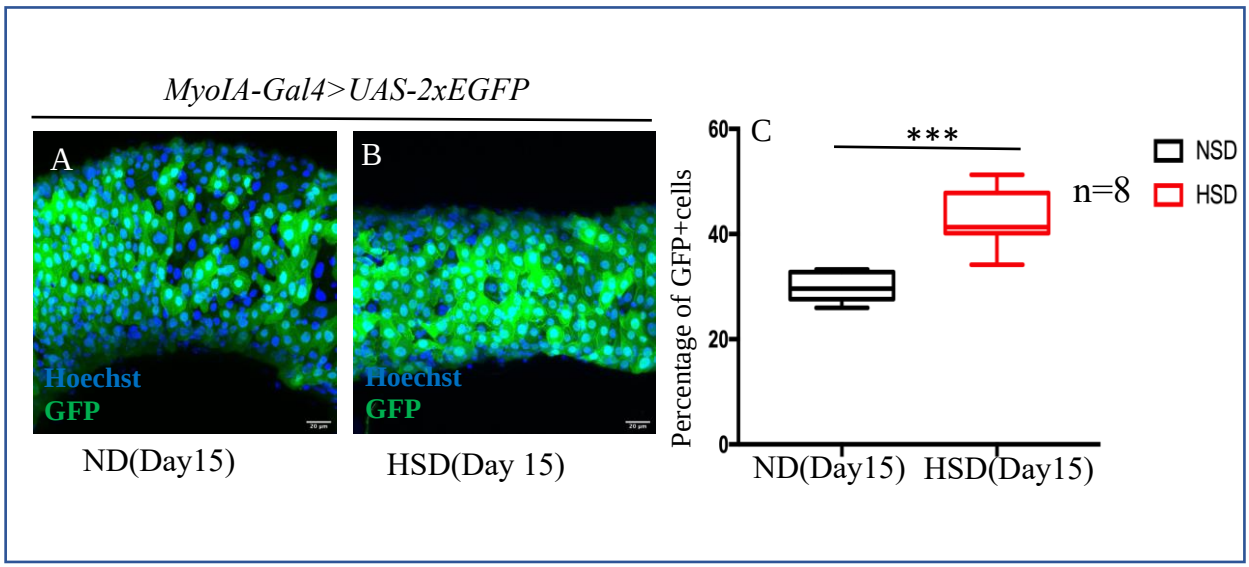

Figure 12: Enhancement of EC cells (MyoIA+ve).

Scale bar - 20μm. Expression pattern of *MyoIA-Gal4>UAS-2xEGFP* in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis of percentage of EC cells in posterior midgut. HSD posterior midgut shows a significant increase in EC cell number compared to ND posterior midgut (**C**).

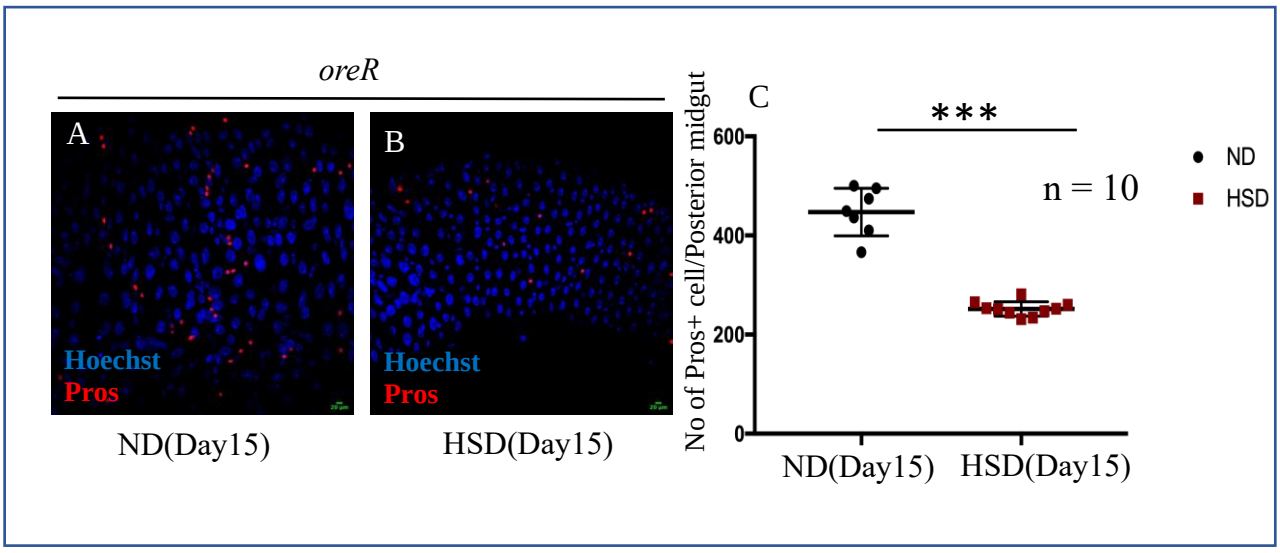

Figure 13: Depletion in number of EE cells (Pros+ve) in posterior midgut.

Scale bar - 20μm. Expression pattern of Prospero antibody marking the EE cells in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis (**C**) of number of EE cells in whole posterior midgut. Compared to ND posterior midgut, HSD posterior midgut shows a significant drop in EE number when compared to ND posterior midgut.

III.III High Sugar Diet impacts differentiation of EBs

Our results demonstrated that while there was an enhancement in the number of EC cells, a drastic reduction in the number of EE cells was observed. One possible argument for this kind of result would be a differentiation bias for ECs. To determine that, we checked for the subset of EB cells that are contributing towards the formation of EE cells. Co-immunostaining of the midgut with antibodies against Prospero (marks the EEs) and *esg* (marks the EBs) detected a significant decrease in the pool of *esg* and Pros double positive cells in the gut epithelium of HSD-fed flies (**Figure 14A and 14B**). While in normal diet fed flies, the double positive cells constitute 3.47% of the population, in HSD-fed flies only 1.5% of the cells were double positive for both *esg* and Pros (**Figure 14C**).

In an alternate experiment, we checked for Prospero (marking EEs) in the gut of HSD–fed flies that were otherwise expressing NRE-GFP (marking mostly the EBs). Here also, we observed a significant drop in the number of cells double positive for both NRE and Pros (**Figure 15).** Given that the number of EBs remain unaltered in the mid gut of HSD-fed flies, as compared to that in ND-fed flies, these results clearly demonstrate that the EB population in the posterior midgut of HSD-Fed flies contributes less towards the EE fate.

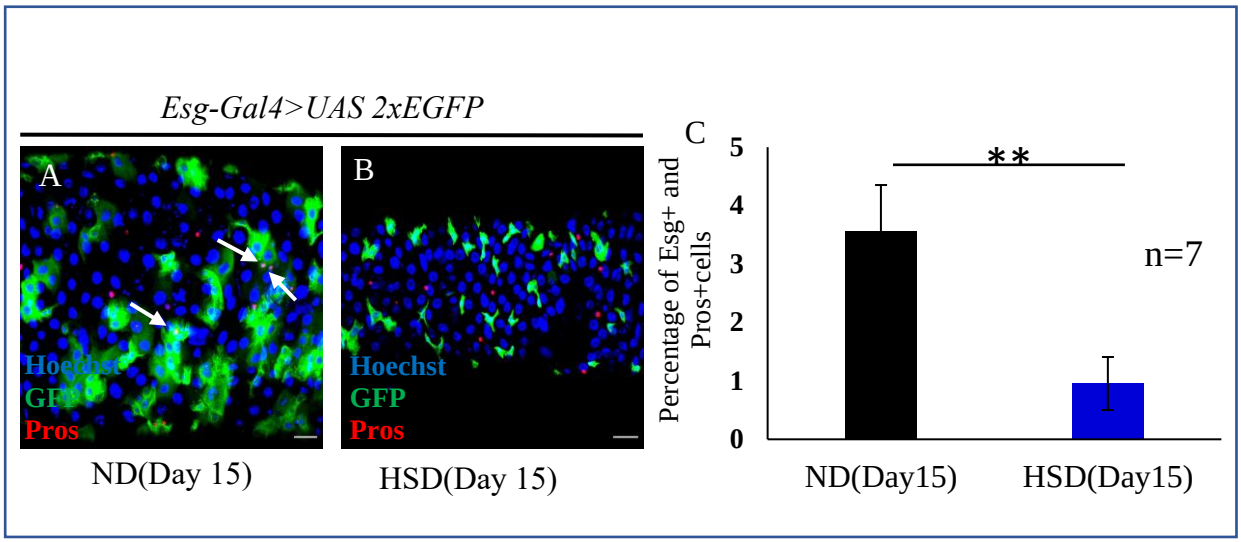

Figure 14: Decrease in Esg+ve and Pros+ve (double positive) cells in total Esg+ve cell population.

Scale bar - 20μm. Escaragot and Prospero positive cell in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis of both positive cell type. HSD posterior midgut shows a significant drop in both positive cell type when compared ND posterior midgut (**C**).

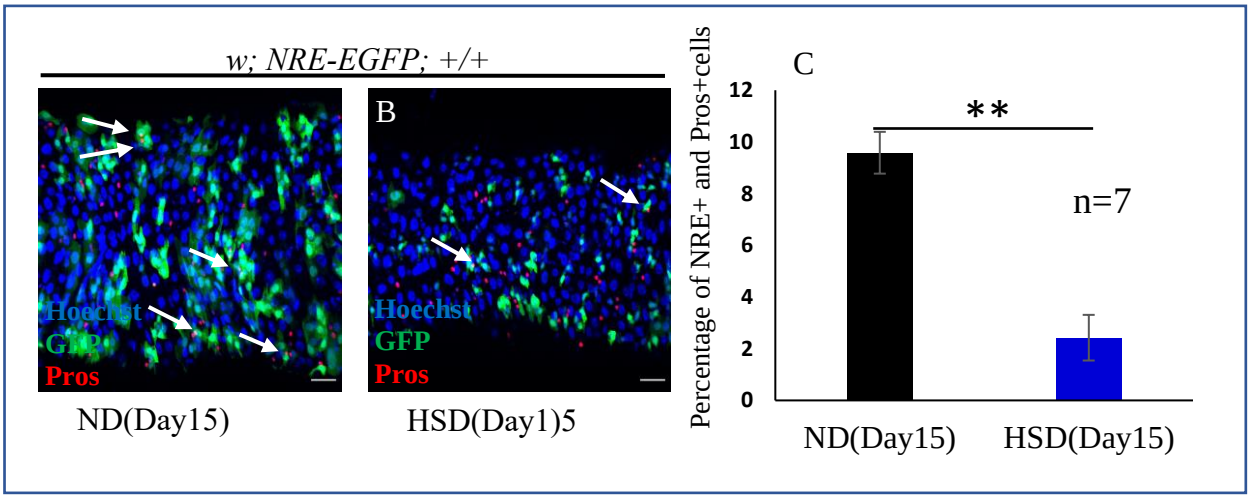

Figure 15: Drop in both NRE+ve and Pros+ve (double positive) cells in total NRE+ve cells.

Scale bar - 20μm. NRE and Prospero positive cell in ND and HSD posterior midgut on Day 15 (**A, B**) and Quantitative analysis of both positive cell type. HSD posterior midgut shows a significant drop in both positive cell type when compared ND posterior midgut (**C**).

III.IV. HSD decreases the proliferation rate of ISC population:

Previous studies performed in the lab had reported drastic shrinkage in the gut length and breadth when flies were fed in HSD. It was also observed that a severe reduction in the cell size of the gut epithelial cells contributed for this apparent shrinkage. The other possible contributing factor might be a drop in the proliferation rate of the cells. To determine whether the proliferation of ISCs gets affected in the gut of HSD fed flies, we used *esg-Gal4/UAS-2xEGFP* fly line and performed the Edu staining. Edu staining would mark the S phase of the cell which is replicating stage of DNA. Compared to flies fed on ND, HSD-fed flies exhibited a significant depletion in the proliferation rate (**Figure 16C and 16C'**). Though the number of tissues analysed was very low, quantitation of the data revealed almost (50 percent) drop in the proliferation rate of the ISCs (**Figure 16D**).

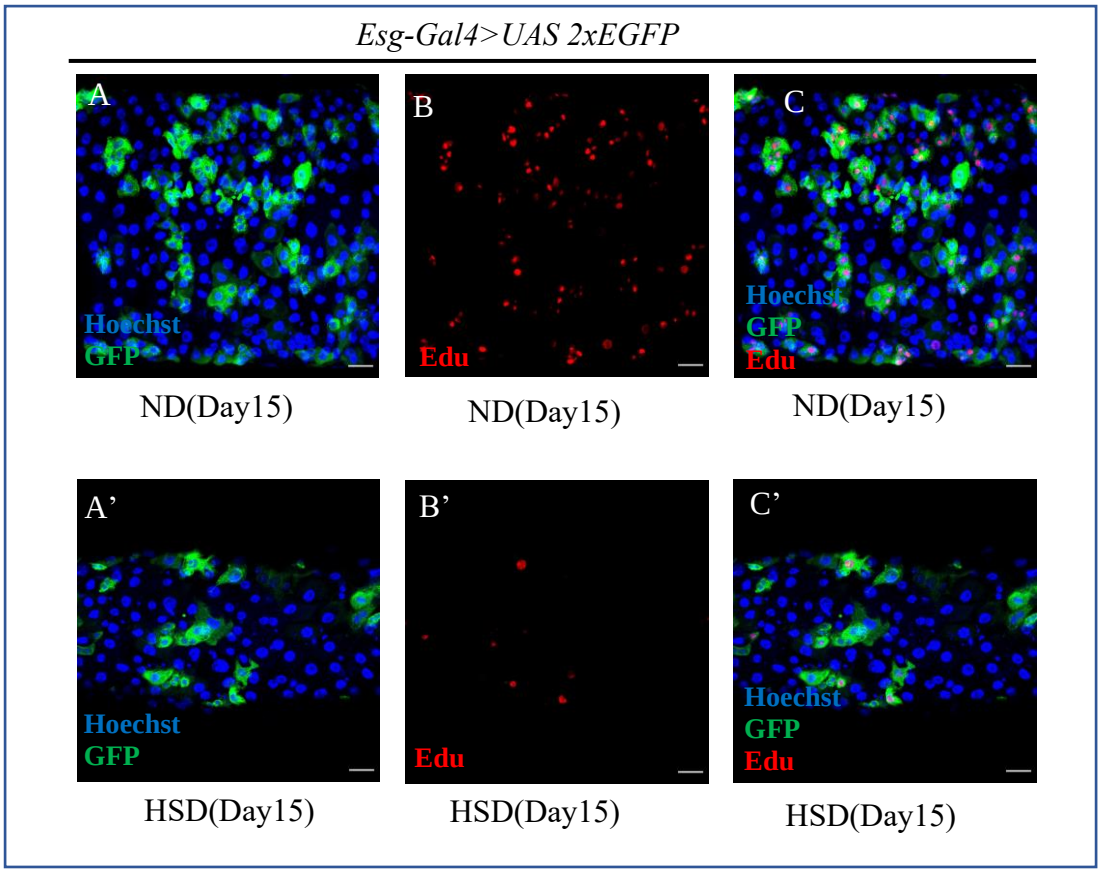

Figure 16: Drop in proliferation rate in ISC and EB cell type.

Scale bar - 20μm. Escaragot expression (marks both ISC and EB cell) in ND and HSD posterior midgut on Day 15 (**A, A'**). Proliferation pattern in ND and HSD posterior midgut on Day 15 (**B, B'**). Proliferation pattern of *esg* positive (ISCs and EBs) in ND and HSD posterior midgut on Day 15 (**C, C'**) HSD posterior midgut exhibits significant drop in proliferation rate compared to ND posterior midgut.

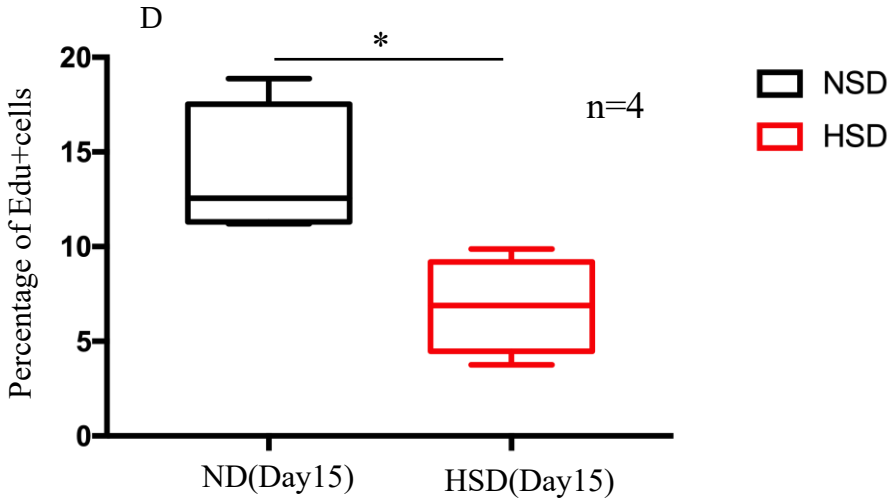

Figure 16D: Percentage of Edu+ cells in posterior midgut.

Quantitative analysis of percentage of Edu+ cells in posterior midgut in ND and HSD on Day 15 (**D**). Graph shows that there is 50 percent drop in Edu+ve cells in HSD-fed flies compared to ND-fed flies.

III.V. HSD does not upregulate the expression of the members of the Unpaired family of proteins in the posterior midgut:

Next we wanted to determine the status of the expressions of Upd1, Upd2 and Upd3 in the midgut of the flies reared on HSD. Upd2 and Upd3 are the major cytokines which are secreted mainly by the EC cells and the progenitor cells. These cytokines are the ligands of JAK-STAT pathway, a major signaling pathway involved in maintaining the midgut homeostasis. JAK-STAT pathway promotes ISC proliferation and differentiation during homeostasis [34], [36]. So we hypothesized that HSD might affect the JAK-STAT pathway for which the ISCs demonstrate low rate of proliferation. In order to check that, we did the real time PCR analysis of Upd1 , Upd2 and Upd3 transcripts in the midgut of the HSD fed flies. As evident from **Figure 17** a modest increase in the level of Upd1 transcripts was observed. Conversely, the transcript levels of the two major cytokines, Upd2 and Upd3, was drastically reduced (**Figure 17**), indicating low activation of JAK-STAT pathway in the gut epithelium of HSD fed flies. This could be one of the possible reasons for which the proliferation of ISCs gets compromised in HSD-fed flies.

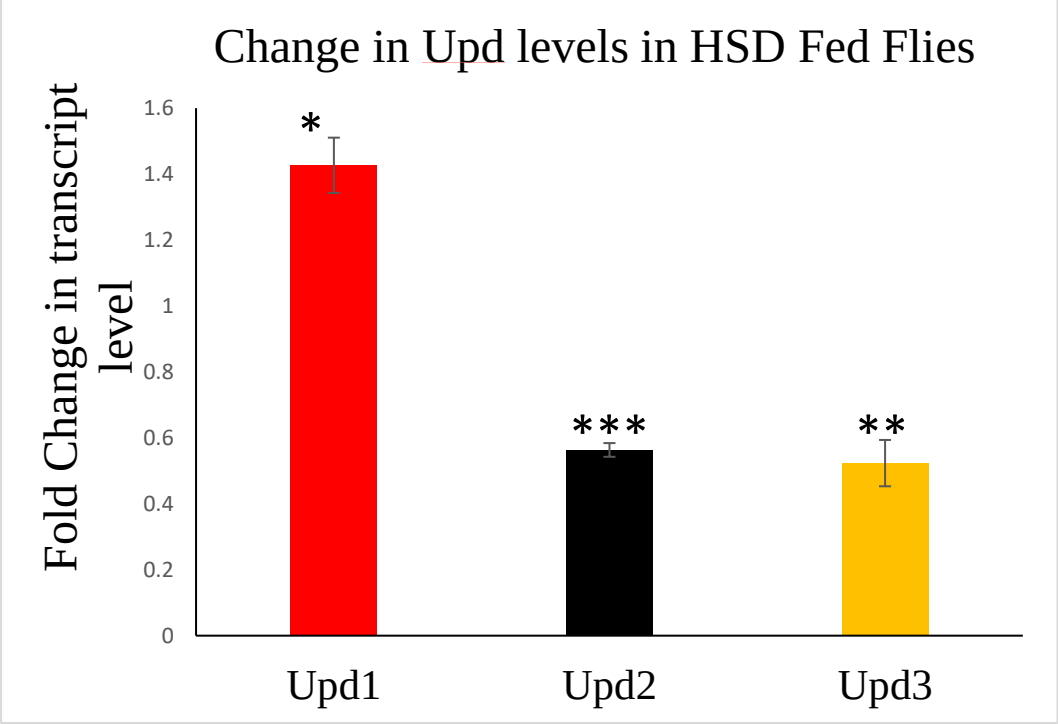

Figure 17: Transcripts levels of Upds.
Quantitative analysis of transcripts levels of Upds shows a modest increase in Upd1 transcript level whereas there is drastic depletion in Upd2 and Upd3 transcript levels, of the HSD-fed flies compared when flies reared on ND-fed flies.

III.VI. HSD upregulates JNK signaling pathway

JNK pathway has a cytoprotective role and also gets activated when there is any kind of stress [42]. As in our case, cells are under constant metabolic stress, so we addressed that whether JNK pathways is getting activated in the gut of the HSD-fed flies. For this purpose we employed the TRE-DsRED transgenic line, that reports JNK activity [40]. We observed that the gut of flies reared on HSD had significantly higher levels of TRE-DsRED expression when compared to those reared on ND (**Figure 18**). From this preliminary result, we conclude that that HSD leads to elevated activation of JNK pathway in the midgut. However, the role of JNK and how it's getting activated in HSD condition need to elucidated.

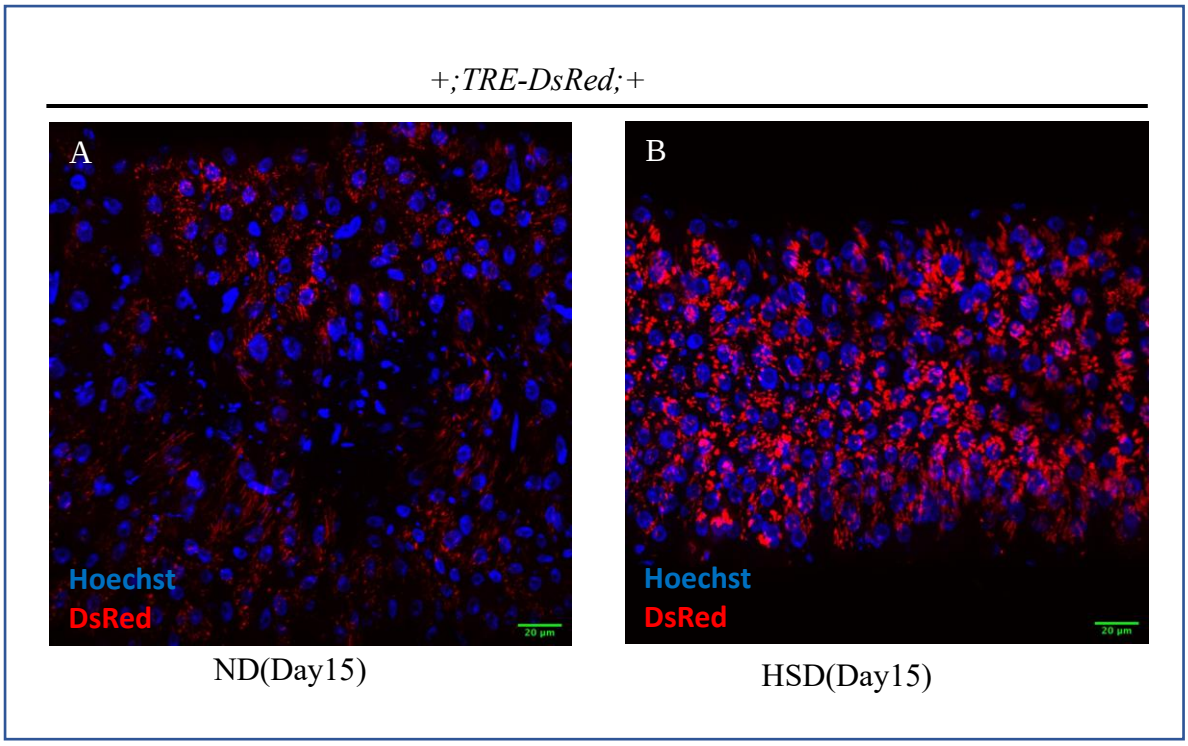

Figure 18: Increase in JNK reporter expression in posterior midgut cells.
Scale bar - 20μm. Expression pattern of JNK reporter element (TRE-DsRed) in ND and HSD posterior midgut on Day 15 (**A, B)**. HSD posterior midgut shows higher level of TRE-DsRed expression.

CHAPTER IV
DISCUSSIONS AND FUTURE DIRECTIONS

In our study, we focused on the effects of High Sugar Diet on the intestinal stem cell homeostasis as intestine is the first organ which are going to experience any kind of change in the food intake. Our investigation resulted in finding that high sugar diet disrupts Intestinal Stem cell (ISC) homeostasis by depleting the number of ISCs as well as that of the enteroendocrine (EE) cells. Although enteroblasts (EB) number remains unchanged, we have found there is increment in enterocyte (EC) production. The low proliferation of ISC population as observed can be the one of the reasons behind the depletion of ISC numbers in posterior midgut in HSD condition. JAK-STAT signaling pathway which in one of the major signaling pathways primarily responsible for proliferation of ISCs, is also altered in HSD condition. Attenuated JAK-STAT signaling pathway might be responsible for the drop in proliferation of the ISCs. Since, EB number is unchanged and EE numbers are significantly dropped in posterior midgut of HSD-fed flies, we got interested to know how much EB population is contributing towards the EE population. Our findings suggest that the contribution of EB population towards the EE fate is very less. Further studies on EB to EC fate may elucidate that the EB population is mainly contributing towards EC production. EC cells are absorptive in nature and ECs can integrate information regarding the sugar uptake and the carbohydrate status of fat body by relaying signaling cascades which in turn adjust the expression of certain carbohydrate digestive enzymes [1], so increment in EC number can be interpreted as the metabolic adaptation shift which is employed by the intestinal epithelium in order to circumvent the burden of huge amount of nutrient uptake. From recent past studies, it is reported that enteroendocrine cells aids a connecting link between the diet and visceral muscle which secretes insulin like-peptides mainly Dilp3 which stimulates the tissue growth as well as promotes ISC proliferation [43]. Depletion in EE number can result into reduction in tissue growth and ISC homeostasis. In our findings, a reduction in midgut size as well as depletion in EE number have been evidenced.

It is well established that high notch signaling leads to EBs differentiation towards the ECs [29]. In our study, we have found that interestingly Notch signaling remain unaltered which is indicating that HSD does not create the differentiation bias by modulating the Notch pathway. HSD might disrupt ISC homeostasis by altering other major signaling pathways which are involved in maintaining state and fate of ISCs. Our investigation on JNK signaling, clearly shows that there is high level expression of JNK reporter element which suggests that HSD induces JNK signaling. Although we don't know the function of the JNK in this context yet. In future studies, the function of JNK will be elucidated whether it is involved in creating the differentiation bias of EBs towards EC fate, need to be checked. In summary, HSD alter the

ISC homeostasis by altering the signaling pathways involved in maintaining the midgut tissue homeostasis.

Last few decades have observed an alarming increase in intaking carbohydrate rich and sugar rich diets. Many studies have implicated the altered food habit with the manifestation of a large number of metabolic disorders that include hypertension, obesity and type II diabetes. Therefore, understanding the mechanistic basis of HSD induced alterations in ISC homeostasis will provide the basic information to design strategies to prevent them.

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