Studying the Effects of High Sugar Diet Associated Metabolic Changes on the Gut Homeostasis of *Drosophila melanogaster*

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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

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Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled "**Studying the Effects of High Sugar Diet Associated Metabolic Changes on the Gut Homeostasis of *Drosophila melanogaster*"** submitted by **Greeshma P.Bose (Reg. No. MS14155)** for the partial fulfilment of B.S.-M.S. dual 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.Rajesh Ramachandran **Dr. Lolitika Mandal** **Dr.Sudip Mandal**

(Supervisor)

Dated: November 22, 2019

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.

Greeshma P.Bose

(Candidate)

Dated: 22-11-2019

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

Alterations in diet have profound impact on the health of an individual. Considering the fact that diet is the major modifiable determinant of chronic diseases, through this study, we are investigating the effects of high sugar diet associated metabolism on the gut homeostasis of *Drosophila melanogatster* . The experimental model adopted for this study is based on recent reports wherein the fruit fly has been used to develop a diet – induced disease model. The experimental flies were reared on media containing 1 M sugar while the control flies were fed with 0.15M sugar containing media. Our results demonstrate a significant perturbation in the gut homeostasis. This includes depletion of the intestinal stem cell (ISCs) population and the enteroendocrine (EE) cells and the enhancement of the absorptive enterocytes (EC) .We also observed perturbation of the expression of JAK -STAT signaling pathway ligand *Upd3* which constitutes the major mitogenic signal for the intestinal stem cell proliferation. The high sugar diet fed flies also exhibited higher glucose uptake and altered mitochondrial dynamics. The fatty acid oxidation (FAO) capabilities of these flies were also compromised suggestive of significant alteration in the metabolic landscape.

CHAPTER I

INTRODUCTION

Gastrointestinal tract, one of the largest organs in the body cavity, was previously considered as a passive organ majorly responsible for digestion and absorption of nutrients. But this view has changed drastically since the gut has emerged as the major modulator of various biological processes including insulin secretion, energy balance, food intake, immunity, physiology and behaviour. As a result gastrointestinal biology has come to the forefront of various research fields such as stem cell biology ,metabolism immunity ,development and regeneration [1] [2].

The gastrointestinal tract of *Drosophila melanogaster* remained comparatively uninvestigated until the identification of the adult somatic intestinal stem cells over a decade ago[1].Since then, studies in the insect intestine have presented a number of insights on stem cell homeostasis, regeneration, cell type diversity etc [3].Furthermore, the striking similarity between *Drosophila* and mammalian intestine in terms of structural, functional, cellular and signaling aspects made it an amenable model to investigate.

Diet and Diseases

Alterations in diet have profound impact on the health of an individual. At present, there is growing evidence in support of the view that nutrition is the major modifiable determinant of chronic diseases. Some of the specific dietary components escalate the possible occurrence of these diseases. Current lifestyle and dietary changes have led to the increased incidence of chronic diseases such as obesity, diabetes mellitus, cardiovascular disease (CVD), hypertension and stroke, and some types of cancer[4].

All around the globe sugar is one of the main ingredients in carbohydrate rich diet[5], [6].Over the past few decades the way people eat has changed considerably. One such change in eating habit is the shift to refined carbohydrates – refined grains and added sugars. In the 1985 to 2005 period extensive added sugar intake occurred, especially in consumption of sugar-sweetened beverages and processed foods[7]. There is a robust epidemiological association between sugar sweetened beverages consumption, overweight, obesity[8], hypertension, and type 2 diabetes[9]. High sugar diet is also shown to be associated with steatohepatitis[10],coronary heart diseases[11],certain cancers[12][13][14],kidney disease, tooth demineralisation[15], dementia[16],increased risk factor for gout[17] etc.

Drosophila melanogaster as a model system

Drosophila melanogaster, commonly known as the fruit-fly or vinegar fly, belongs to the order Diptera, family Drosophilidae. The fruit fly *Drosophila melanogaster* is a resourceful model organism that has been used in biomedical research for over a century to study a broad range of phenomena including genetics and inheritance, embryonic development, learning, behaviour, aging and neurodegenerative diseases.

William Ernest Castle (1867-1962) was the first person to introduce *Drosophila* to workbenches, as a model organism. However the "father" of *Drosophila* research is undeniably Thomas Hunt Morgan [18]. After the re-emergence of Mendelian inheritance, in 1910, the discoveries of Thomas Hunt Morgan elucidated several principles of heredity, using *Drosophila melanogaster* as a model system which consisted of gene mapping sex-linked inheritance , multiple alleles, and epistasis [19]

Drosophila melanogaster is an easy inexpensive model to culture which has a short life cycle, small size, small genome and high fecundity. These features make it an amenable model for the generation of large numbers of individuals for the many generations required for genetic analysis. The size of the *Drosophila* genome is around $1/20^{th}$ of the size of a typical mammalian genome, which facilitates molecular genetic analysis[20]

The entire *Drosophila* genome has been sequenced and mapped out in the year 2000.The results suggested a 60 percent homology between human and *Drosophila* genome. Further it was elucidated that 77 Percentage of genes responsible for human diseases have homolog in *Drosophila*[21][22].The availability of various tools for genetic manipulations (Gal4UAS,FLP-FRT,CRISPR-Cas9 mutagenesis, phiC31 integrase-mediated target insertion , etc) and the ease of generating heritable changes makes it the most amenable modal organism to do the genetic studies. The use of phenotypically distinguishable molecular markers for different chromosomes has made the genetic studies less complicated. Over the years, enormous number of mutants has been generated which are available for use in various stock centres across the world. All these aspects established *Drosophila melanogaster* as a powerful model organism for studying various biological phenomenons.

Life Cycle of *Drosophila melanogaster*

Drosophila melanogaster is a holometabolous insect. The life cycle of *Drosophila* consists of four stages of development namely, egg, larvae, pupae, and adult **(Fig.1).**Growth and development of *Drosophila* is dependent on the temperature at which it is reared. At 25°C the life cycle from fertilization to eclosion of fly takes about ten days. Depending on the culture conditions, the maximum life span ranges between 60 to 80 days. *Drosophila* has internal fertilization. Females lay approximately 50-100 embryos per day and the embryogenesis lasts for the next 24 hours. Then the larval stage begins. There are three distinct phases in the larval stage, namely, first instar, second instar, and third instar. Soon after hatching, the first instar larva begins to feed on the surface of the medium and passes through two molting stages. Second instar larvae burrows deep into the medium, and once the third instar larva is mature, it leaves the culture medium and crawls up the dry walls of the culture flask. Once they are on the walls they curb their movements and take on a sedentary mode to prepare for the pupariation event which lasts for 24–48 h [23][24]. The larvae secrete glue proteins necessary for the formation cuticle required for pupal casing which is maintained during metamorphosis. The entire remodelling of the body takes place during the pupal stage which is the most dynamic stage of the fly development. At this stage there is a significant amount of programmed cell-death happening along with the histolysis of the larval organs[25]. 9 to 10 days after egg fertilization, the adult fly emerges from the pupal case[23].

Gal4-UAS System

One of the most powerful techniques for addressing gene function in vivo is to target gene expression in a temporal and spatial fashion.Gal4-UAS system is an elegant genetic tool used extensively by fly geneticists for targeted gene expression. This system of spatiotemporal regulation of gene expression was first discovered by Andrea Brand and Norbert Perrimon in 1993 [26].

GAL4 is an 881 amino acid protein found in the yeast *Saccharomyces cerevisiae* as a regulator of genes. GAL4 binds to an Upstream Activating Sequences (UAS) element comparable to an enhancer element of multicellular eukaryotes. The binding of GAL4 to UAS is essential for the transcriptional activation of these GAL4-regulated genes.

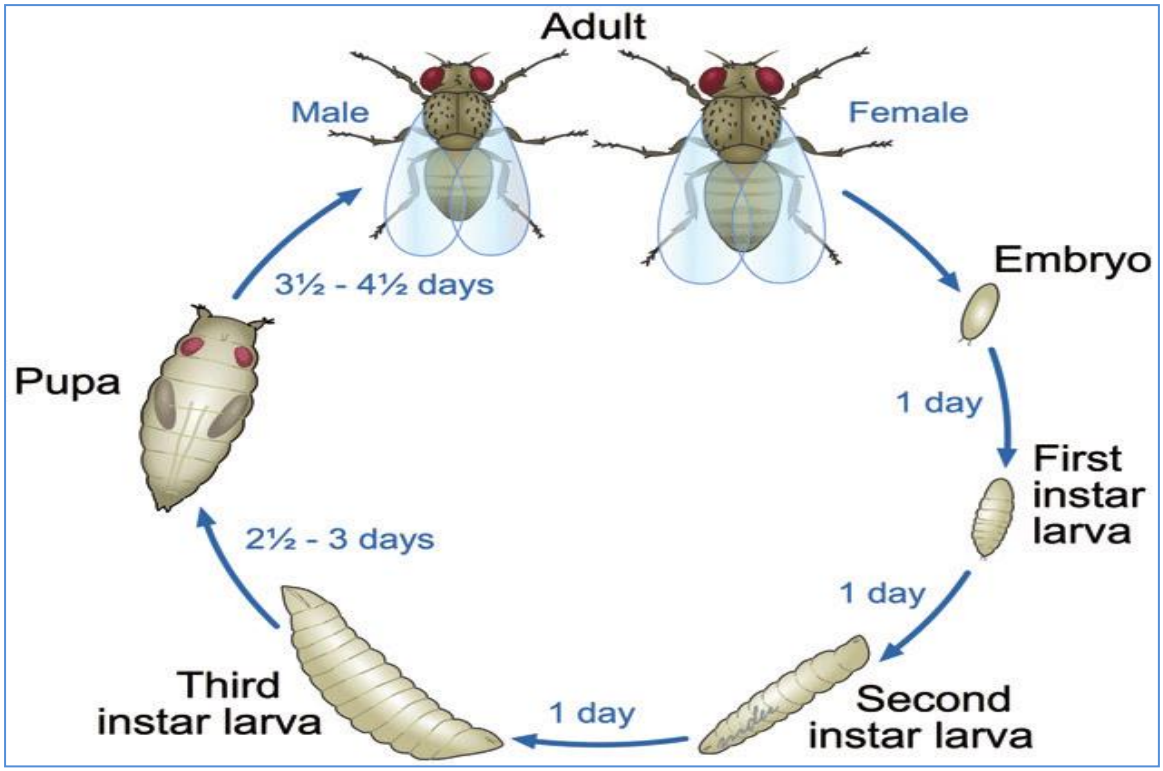


Figure 1 : Life Cycle of *Drosophila melanogaster*

Schematic representation of male and female fly and the life cycle of *Drosophila melanogaster* [24]

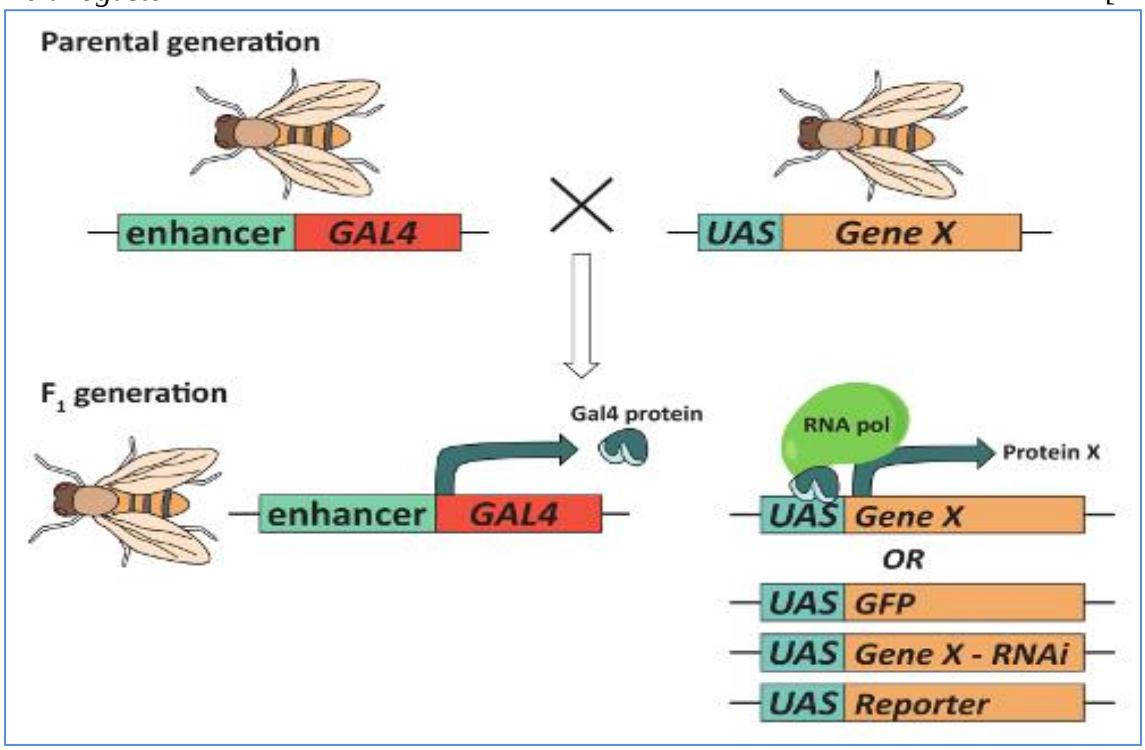


Figure 2: The Gal4-UAS system

Schematic representation of the Gal4-UAS system which is used for targeted gene expression in *Drosophila melanogaster*[27].

This is known as bipartite approach where in the two components of the system, the driver, and the responder are maintained as separate parental lines. A transgenic line, often known as the driver line, carries a Gal4 sequence under the regulation of a specific promoter sequence **(Fig.2).** In another transgenic line, a target DNA sequence is placed downstream of UAS sequence, called the responder fly line. The absence of GAL4 in the responder fly line sustains them in a silent transcriptional state. Responder fly line is mated with the driver fly line in order to activate transcription of the target DNA. The F1 progeny express the responder in a transcriptional pattern that mirrors the GAL4 pattern of the respective driver. The spatiotemporal control of Gal4 expression is obtained by the choice of tissue-specific or a cell-type specific promoter sequence. One of the advantages of the GAL4-UAS system is that it allows expression of lethal genes for a short duration in specific tissues and eliminates its effects in early developmental stages[28][29].

Drosophila as a model for studying alimentary canal

Similarities between mammalian and Drosophila digestive tract

Drosophila digestive tract consists of a simple epithelium which is surrounded by visceral muscles, tracheae and nerves. The gut is further subdivided into foregut, midgut, and hindgut **(Fig.3)**. The foregut and hindgut epithelium are of ectodermal in origin whereas the midgut epithelium is endodermal in origin similar to mammalian counterpart[2]. There are six distinct anatomical regions (R0 to R5), with specific metabolic and digestive functions, within the midgut [30].

Despite the physiological divergence between vertebrates and insects, there is high degree of conservation between mammalian and *Drosophila* digestive tract with respect to the anatomy, physiological function, tissue and signaling pathways that control intestinal development, regeneration and disease [31][32].Therefore there is appreciable conservation in intestinal pathophysiology as well.

Structural and functional similarity

In mammals, ingested food passes through the esophagus to the stomach where it is temporarily stored. Then it moves to the small intestine for nutrient absorption and then proceeds to the large intestine for additional water, nutrient and electrolyte absorption. Eventually, it arrives at the rectum and anus for excretion. Likewise, in *Drosophila*

ingested food passes through the foregut and is briefly stored in the crop. Then it moves to the midgut where nutrient absorption takes place. Similar to mammals, water and electrolytes are exchanged in the hindgut and then arrives at the rectum and anus for excretion[32][33] **(Fig.4)**.

Cellular and Signalling level

The *Drosophila* midgut is made of a single layer of epithelial cells. Both in flies and mammals, on the basal side of the epithelial monolayer is on an extracellular collagenous matrix known as the basement membrane [34]. Both fly and mammalian guts harbour adult intestinal stem cells (ISCs)[35][36][37][38]. ISCs are multipotent and they are capable of giving rise to all the differentiated cell types of the intestinal epithelium. In *Drosophila* ISCs divide to give rise to a progenitor cell type known as the enteroblast (EB) daughter cell and to self-renew.The enteroblast (EB) cell will differentiate into either an absorptive enterocyte (EC) or a hormone-producing enteroendocrine (EE) cell depending on the amount of notch signalling received [37]. Similarly, the mammalian intestinal epithelium is composed of absorptive cells (ECs) and secretory cells (EE and Goblet cells)[39] (**Fig.5)**.

Considerable homology exists between the mammalian and fly signaling pathways that regulate ISC proliferation as well [32]. *Drosophila* wingless (Wg), secreted from muscle cells located next to the basal epithelium, is required for maintenance of ISCs [40]. Similarly its mammalian counterpart, Wnt, is highly active at the bottom of the crypts and responsible for maintaining ISCs and TA cells in an undifferentiated state[41].In addition to this the Wnt/Wg signaling and STAT pathways that are essential for ISC division, further similarities between the mammalian and *Drosophila* midgut ISCs have been noted. Midgut ISCs divide when the InR pathway is activated and also in the presence of PVF growth factors. The analogous INSR and PDGF pathways, respectively, control mouse ISC homeostasis[32].

On the whole, the intestinal epithelium anatomy, physiology and regeneration time in flies strongly resemble to that of mammals. At the molecular level, various signalling pathways that are involved in the homeostasis, regeneration and immune response are

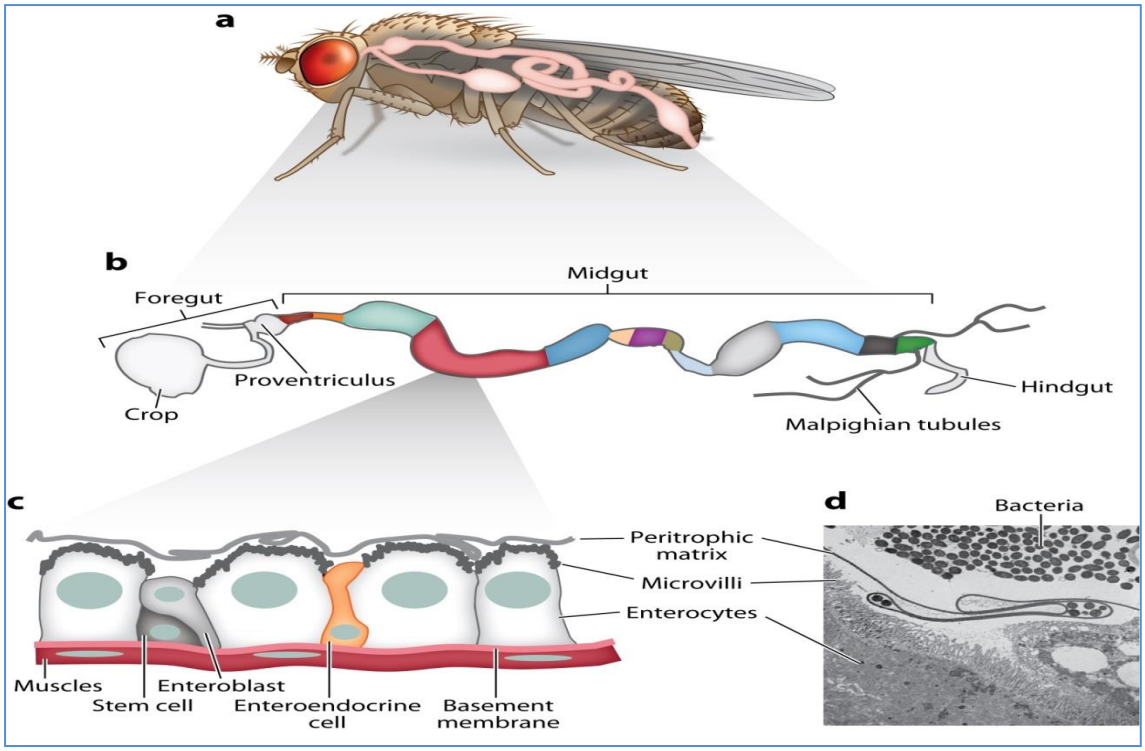


Figure 3 : *Drosophila* digestive tract - Schematic representation of the *Drosophila* digestive tract [2].

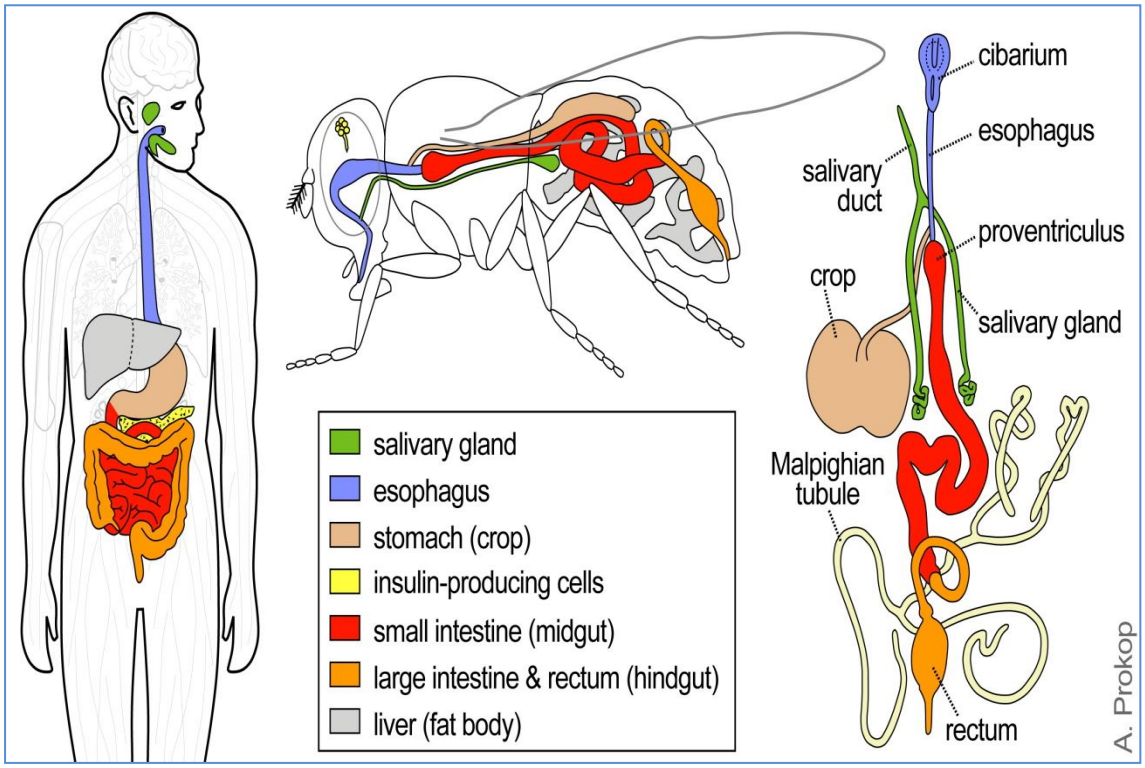


Figure 4: Structural and functional similarities between mammalian and *Drosophila* alimentary canals.

also similar. This reinforces the usage of *Drosophila* as a model for investigating basic mechanisms of mammalian intestinal epithelial development, homeostasis, regeneration and disease. This also suggests that flies can be utilized to investigate diverse features of human disease biology including inflammatory bowel disease (IBD), cancer and epithelial regeneration[32].

Gut homeostasis of *Drosophila melanogaster*

Most adult tissues and organs are sustained by resident stem cells. The identification of the adult somatic intestinal stem cells in the *Drosophila* gut happened over a decade ago. Intestinal stem cells (ISCs) are present in all regions of the midgut which are capable of regenerating all the cell types of that particular region [42]Both extrinsic niche signals and intrinsic factors control the self-renewal, proliferation, and lineage differentiation of the intestinal stem cells (ISCs)[43]. ISCs are characterized by the expression of *Drosophila* snail family gene Escargot (Esg) and Notch ligand Delta (Dl).ISC divisions are predominantly asymmetric (~70%).Asymmetric division occurs apical basally and the basal daughter cell remains as renewed ISC and the apical daughter cell attains the progenitor fate (EB).Symmetric ISC divisions occur at a lower frequency(~30%) , which will either produce two ISCs or two EBs [36][43][44]. Unlike the transient amplifying progenitors in the mammalian intestine, Drosophila EBs do not proliferate; rather, they differentiate into two ISC lineages: the absorptive enterocyte (EC) and a secretory cell type called enteroendocrine cell (EE) depending upon the amount of notch signaling received [37][45]. The progenitor cell committed to the EC fate undergoes multiple rounds of endoreplication and significantly increase its size to form the major part of the gut epithelium[43]

Tissue homeostasis, within the gut epithelium, demands tight balance between the production and removal of various cell types. Therefore, fast renewing tissues like intestinal epithelium, which undergoes complete replacement within every few weeks, require continuous homeostatic turnover during which the damaged cells are replaced with new healthy cells [31]. Thus the activity of ISCs across the digestive tract is dynamically regulated to accommodate the tissue turnover demands. Multiple signaling pathways that regulate the process of ISC proliferation, differentiation, and maintenance have been identified. Signaling pathways that influence ISC proliferation and

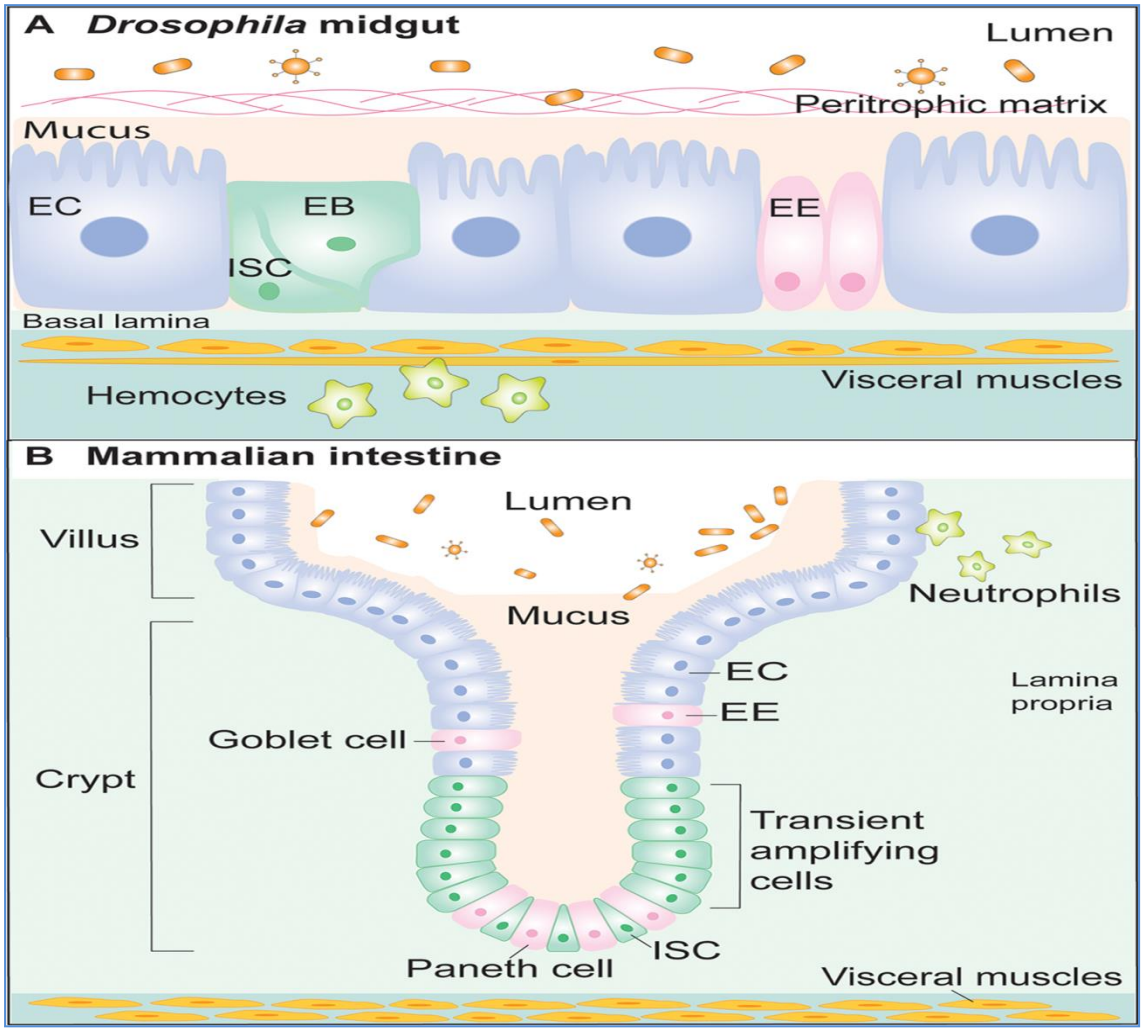


Figure 5: Parallels between the gut epithelia of *Drosophila* and mammals [39].

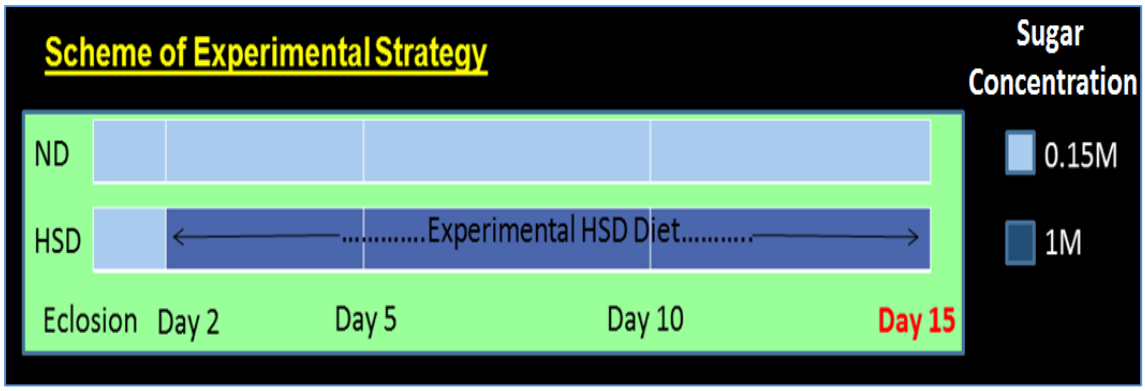


Figure 6 : Schematic representation of experimental strategy

differentiation in Drosophila include Notch [45], Jak/Stat[46][47], Egfr[48],Insulin[49], Jun-N-terminal Kinase (JNK)[50], Wg [40], Bmp/Dpp[51], Hippo[52], Juvenile Hormone (JH) [53], Ret signaling[54] etc.

The multitude of different signals regulating the activity of ISCs is justified by the need to integrate local, paracrine, systemic, and environmental stimuli to evoke appropriate homeostatic responses [42].

Metabolic homeostasis

Over the past few years *Drosophila* is increasingly being utilized to study the fundamental aspects of metabolism. Multicellular complexity of higher order animals demands sensing of their nutritional status and concerted response in order to coordinate growth and energy homeostasis. *Drosophila* and vertebrates share most of its metabolic functions. Similar to vertebrates, fly maintains appropriate amount of circulating sugars and stores excess energy in the form of glycogen and lipid. The stored energy is later mobilized during increased energy demand. The key pathways of intermediary metabolism and regulators of homeostasis are conserved through evolution [55]. Recent studies have led to the understanding that these conserved molecular pathways also posses the potential to give rise to similar phenotypes[56]. This, along with the conservation of metabolic pathways and the availability of large number of genetic tools makes *Drosophila* an attractive model to study metabolism.

Regulation of carbohydrate metabolism in *Drosophila melanogaster*

Carbohydrate metabolism is crucial for maintaing cellular energy balance and for the biosynthesis of cellular building blocks**.** Elaborate regulatory systems coordinate carbohydrate metabolism within various cell types to match with the specific metabolic needs. Variations in the nutrient uptake demands constant homeostatic control of carbohydrate metabolism. Thus the cells constantly detect carbohydrate derived metabolites and accordingly alter the activity of the regulatory pathways. Carbohydrate homeostasis is majorly mediated by intra cellular sugar sensing by the heterodimer of Mondo and Max-like protein transcription factors[57].

Experimental Model

Drosophila as a model system to study the consequences of High Sugar Diet

Recently there is an increased interest towards studying the modulation of epithelial homeostasis by more direct and/or specific nutritional inputs. Studies have found that nutrition affects the activity of progenitors as well as differentiated cell types in the intestinal epithelium[42].

The High Sugar Diet experimental model adopted for the experiments was developed on the basis of recent reports about the diet induced *Drosophila* models [58].There are several recent studies which utilizes the HSD model to explore the important changes in metabolic homeostasis and its possible effects on growth, development and function of an organism.HSD is known to induce obesity, insulin resistance, cardiomyopathy among others[57][58][60].

Studies done in our lab have reported that this experimental model resembles T2DM model in Humans from 13 Day onwards (threshold) of HSD feeding. It displays T2DM characteristics such as increased Glucose level in hemolymph (fluid equivalent to blood), increased Trehalose (sugar consisting of 2 molecules of Glucose, the principal source of energy in Fruitfly), and insulin resistance with unaffected insulin production. Taken together, the HSD diabetic model in Drosophila meticulously captures the prominent features of type-II diabetes.

The earlier studies done in our lab on the effect of high sugar diet on the gastrointestinal tract of Drosophila have shown that there is a significant morphological variation (gradual reduction in the size of gut upon feeding HSD) in the gut of high sugar fed flies. Smurf Assay (*Drosophila* feeding assay to test the feeding, aging and permeability or leakage - functional defects) suggested that there are no feeding defects till Day 15.High sugar diet also showed enhanced membrane integrity and increased cell death in the midgut. Parallel studies conducted to check the impact of HSD on the gut commensal bacteria showed a significant reduction in the amount of gut commensal bacteria in HSD fed flies. As a result the related immune pathway also got affected (reduction in the expression levels of anti microbial peptides in the midgut).

Upon studying the signaling pathways involved in the homeostasis of the gut, it was found that high sugar diet upregulates Notch signaling and downregulates JAK-STAT signaling activity in the midgut.

Thus this experimental model itself serves as a powerful tool to understand the effect of HSD in the gut homeostasis and its correlation with the T2D associated conditions in humans.

Objectives

Over the past decade, it has been demonstrated that the major energy-sensing, metabolic, and endocrine signaling networks of vertebrates are also conserved in flies. Consequently, various studies done on *Drosophila* model are starting to unravel how perturbed energy balance affects the homeostasis[61].Various forms of stress, infection, and damage have been investigated as cues that trigger stem cell proliferation and differentiation. However, little is known about how alterations in cellular metabolism might affect the pathways regulating the cell proliferation and differentiation [62]**.**

Previous studies, done in our lab, have uncovered that the JAK-STAT and Notch signaling pathways, which are critical in maintaining gut homeostasis, are affected in high sugar diet fed flies. Therefore this project aims to analyze the specific metabolic changes that happen in the gut epithelia of *Drosophila* adults upon feeding on high sugar diet.

The specific objectives are:

- The state of different cell types present in gut epithelia of flies fed on high sugar diet
- Study the metabolic changes in the gut upon feeding on high sugar diet

Through the analyses done on this diet induced model, we eventually aim to bridge the gap between metabolic changes and homeostasis of the intestinal stem cells in a diet-induced disease model.

CHAPTER II

MATERIALS AND METHODS

II.I Rearing of flies and maintenance

The flies were reared on standard food made from cornmeal, agar, yeast, and fungicides. The flies were maintained at 25ºC in culture bottles if not mentioned otherwise.

II.II Experimental setup

Freshly eclosed flies of the preferred genotype were collected and reared on normal diet for two days. After two days, the experimental flies were transferred to a high sugar diet (1M sugar) (2:1 Females to Males ratio) and control flies were transferred to normal diet(0.15 M sugar) (2:1 Females to Males ratio). Approximately 40 healthy female flies and 20 male flies were transferred to each bottle. The number of flies reared on each diet are kept equal. The experimental and control fly bottles were flipped every 3 days to maintain a healthy rearing environment. Female flies were dissected at 3 different time points namely, Day 5, Day 10 and Day 15 after eclosion.**(Fig.6)**

II.III Fly stocks and genotypes

The fly stocks used for this thesis were procured from various stock centres, 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(Chr.2). This stock was obtained from Bloomington Stock Center (67072)

4. w[*];P{w[+mC]=sqh-EYFP-Mito}3

   This transgenic fly line has sqh regulatory sequences drive expression of a fluorescent marker for the mitochondrion. The marker sequence is composed of the mitochondrial targeting sequence fused to yellow fluorescent protein.(7194)

5. 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

Entire guts were dissected from the *Drosophila* adult female flies in 1X PBS followed by fixation in 5% paraformaldehyde for 60 minutes on shaker. After removing the fixative, three washes with 0.3% PBT for 15 minutes each were given for permeabilization (60 rpm shaker). Gut samples incubated in blocking solution, 10% NGS (Normal Goat Serum) for 1 hour (60 rpm shaker). Once blocking is done, the samples were incubated with primary antibody for 18-20 hours at 4ºC in Nunc multiwell plates (All primary antibodies, source, and its dilution are mentioned below). Next day, samples were moved back into cavity blocks. Then samples were washed three times for 15 minutes each using 0.3%PBT at room temperature (60 rpm shaker). Before adding, secondary antibody guts were incubated in 10% NGS for 30 minutes (Secondary antibody, source, and its dilution mentioned below). Samples were incubated in secondary antibody for 16-18 hours at 4ºC followed by three 0.3% PBT washes for 15minutes each. Then samples were incubated with DAPI and Phalloidin (1:500 dilution) for 1 hour. After DAPI and Phalloidin treatment, samples were washed in 1X PBS for two times of 5 minutes each and mounted (Bridge mounting) in VECTASHIELD mounting medium.

II.V Antibodies used

Primary antibodies used

For immunostaining the following primary antibodies were used:

Sl	Antibody	Raised in	Source	Dilution used
1	Anti-GFP	Mouse	DSHB, Iowa	1 : 100

2	Anti-Prospero	Mouse	DSHB, Iowa	1 : 100

Secondary antibody used

For immunostaining the following secondary antibodies were used:

Antibody	Source	Details	Dilution
CyTM3-Conjugated AffiniPure Goat Anti-Mouse IgG(H+L)	Jacksons Immuno Search laboratories Code-711-165-152	Conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm)	1:500

Stains used

- **DAPI**

DAPI (4',6-diamidino-2-phenylindole dihydrochloride). This is a blue fluorescent dye that binds to A-T rich region in double-stranded DNA. It is used to stain nuclei in live (less efficient) as well as in fixed tissues (more efficient). Its absorption maxima/ emission maxima are 351nm/461nm. The working dilution is 1µg/ml.

- **Phalloidin**

It binds and stabilizes F-actin (filamentous actin) and prevents depolymerisation of actin fibres.

Red : Excitation ／Emission (nm): 540／565

Green : Excitation ／ Emission (nm): 495／518

II.VI Imaging

Images of the mounted samples were taken by using confocal microscope (Zeiss LSM 780 and Leica SP8) and processed using Fiji or Image J 9(NIH)software.

II.VI 2- NBDG Glucose uptake assay

The *Drosophila* guts were dissected out in 1X PBS. The 1X PBS was replaced with 0.5 mM 2- NBDG solution (in PBS) and the samples were incubated for 45 minutes at RT.(Note: Here onwards, washing was done in pre-cooled (4°C) PBS solution) .The tissue samples were washed in 1XPBS for 10 minutes on a shaker at RT. Further, the samples were fixed in 5 percentage paraformaldehyde for 20 minutes. After fixation, the samples were washed in 1XPBS twice, each for 5 minutes. In order to stain the nucleus, Dapi staining (1:50) was performed for 15 minutes at RT on a shaker. Finally, the samples were washed with 1XPBS for 5 minutes at RT on a shaker. The samples were mounted in VECTASHIELD medium. Then the samples were immediately imaged under confocal microscope.

II.VIII RNA Isolation

Drosophila adult midguts were dissected out (~20) in 1XPBS. After centrifugation removed the 1X PBS and 200µl TRIzol was added, in eppendorf tubes kept on ice. Samples were then kept at -80°C overnight. Next day, the samples were thawed on ice. Using autoclaved pestles, the samples were homogenized. 800 µl of TRIzol was added to the samples and the following mixture was kept at RT for 30 min along with intermittent pippeting. Then the samples were centrifuged at 10000 rpm for 10 mins (4°C).The supernatants were collected to which 200 µl chloroform was added . The samples were shaken vigorously for 15 seconds at room temperature. The samples were then incubated at room temperature for the next 3 minutes. The resulting mixture was then centrifuged at 4°C for 15 minutes at 10000 rpm. Three layers were separated out, uppermost aqueous layer containing RNA, middle layer containing proteins and lowermost organic layer containing DNA. The upper layer was carefully taken out in to a separate RNase-free tube so that middle layer remained unaffected. Equal volume of isopropanol was added to each sample and incubated overnight at -80°C. On the next day, sample was incubated for 10 minutes at room temperature to precipitate out the RNA and then centrifuge at 10,000 rpm for 15minutes at 4 °C to pellet out the RNA. The supernatant was discarded without disturbing the pellet. Later the pellet was washed using 500 µl 70% chilled ethanol and the samples were centrifuged at 4°C, 13000rpm for 10 mins. The supernatant was

discarded and the pellet was air dried .Finally; the pellet was dissolved in 20µl autoclaved miliQ water. The amount of RNA was quantified using a NanoDrop spectrophotometer (Thermofisher).

II.IX Gel electrophoresis

Preparation of 1% Agarose Gel

To prepare 1% agarose gel **, 1** g of agarose was weighed and added to the conical flask. Next; 100 ml of 1X Tris-acetate-EDTA buffer(1X TAE) was added to the flask. The following mixture was microwaved for 1-3 minutes until the agarose was completely dissolved (swirled the flask every 30 seconds).Then the gel was cooled down for 5 minutes (50 °C) .Then 5 µl of ethidum bromide (10µg/ml) was added to the gel and gently mixed. The gel was then transferred to a casting tray with the well comb in place. The gel was then left to solidify at room temperature for 15-20 minutes.

Loading samples and running an agarose gel

The solidified agarose gel was transferred to the electrophoresis unit. The gel box was then filled with 1X Tris-acetate-EDTA buffer until it completely submerged the gel. Then the 6x loading dye was added to each of the loading sample. Molecular ladder and 15 µl of each sample were added to the wells. The gel was run at 80 V, approximately for an hour, until the dye has reached 70% down the gel. The gel was then visualized using a Gel doc system.

II.X cDNA synthesis

cDNA synthesis was performed using the cDNA synthesis kit (Verso) using the protocol suggested.

Components	**1X**
RNA + water	10µl
cDNA mix	7µl
RNA primer mix	2µl
Verso Enzyme Mix	1µl

Reaction Time:

	Temperature	Time	Number of cycles
cDNA synthesis	42°C	30 min	1
Inactivation	95°C	2 min	1
Forever	4°C	∞	-

Polymerase Chain Reaction

Components	1X
10X PCR Buffer	2.5 µl
5mM dNTPs	0.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Template DNA	1 µl
Taq DNA polymerase	0.1 µl
Water	19.9 µl

Reaction Time:

	Temperature	Time	Number of cycles
Denature template	94°C	0:30 min	35
Anneal template	57°C	0:30 min	
Extension	72°C	1 min	
	72°C	5 min	1
Forever	4°C	∞	-

II.XI Real-Time Polymerase Chain Reaction

Components	1X
Sybr Green	10µl
Forward Primer	1µl
Reverse Primer	1µl
Template DNA	1µl
Water	7µl

Reaction Time:

Temperature	Time	Number of cycles
94°C	0:10 min	40
55°C	0:45 min	
95°C	0:30 min	1
65°C	0:05 min	1
4°C	∞	-

II.XII Buffers and Reagents

10X Phosphate Buffer Solution (PBS) : For a volume of 500ml, 40g NaCl, 1g KCl, 7.2g Na_2HPO_4 and 1.2g K_2HPO_4 were weighed. 490 ml dH_2O was added. pH was adjusted to 7.2. The volume was scaled to 500ml.

10% Paraformaldehyde (PFA): For a volume of 2ml, 0.2g of PFA weighed and 2ml volume was made up with 1XPBS. The eppendorf tubes were then sealed using parafilm and incubated at 65°C in a waterbath until the PFA was completely dissolved (The tubes were provided with intermittent shaking to avoid clumping).

0.3% Phosphate Buffer TritonX (**PBT**): For a volume of 40ml, 120µl of 100% TritonX was added to 40ml 1X PBS. The solution was then kept on a shaker to completely dissolve the TritonX

50 X Tris-Acetate-EDTA_: For a volume of 100 ml, 24.2 grams of Tris-base (MW = 121.14 g/mol) added to 70 ml of deionised water. 5.71 ml of 100 % glacial acid (or acetic acid) and 10 ml of 0.5 M EDTA (pH 8.0) were also added to this solution and the volume was scaled to 100ml.The pH of this solution was adjusted to 8.3.

CHAPTER III

RESULTS

III.I High sugar diet reduces the transcript level of *Upd3* ligand in the midgut.

Previous in vivo studies done in the lab, using the reporter *Upd3-Gal4,UAS-GFP* flies, have provided evidences that the *Upd3* expression is significantly lower in the midgut of high sugar fed flies. This reduction in the *Upd3* expression can potentially be due to the reduction at the transcript level or at the protein stability level. To check the *Upd3* transcript levels mRNA was specifically isolated from the midgut. The *Upd3* transcript levels were analysed by performing RT-PCR followed by gel electrophoresis. Reaction for each sample was also setup without reverse transcriptase to ensure that the RNA samples are devoid of any contamination. The gel image showed a visible change in the *Upd3* transcript level on Day 10 and Day 15 **(Fig 7)**.However the difference between the control and HSD samples did not appear prominent on Day 5.Inorder to quantitatively analyse the data we examined the Upd3 transcript levels using quantitative real time PCR. The results suggested approximately 20%, 50 % and 60% percent reduction in the *Upd3* transcript level on Day 5, Day 10 and Day 15 respectively **(Graph 1)**.

III.II High sugar diet results in the depletion of intestinal stem cell (ISC) population.

As a tissue with high turnover demands, the intestinal epithelial homeostasis is tightly regulated by the activity of intestinal stem cell population. The major mitogenic signal for the ISCs is the JAK-STAT signaling.. Previous studies from our laboratory have evidenced that feeding flies on high sugar diet resulted in the down regulated expression of Upd3, one of the ligands of the JAK-STAT signaling.. This led to the speculation that the turnover of the ISCs may be affected upon feeding high sugar diet. In adult *Drosophila,* the snail homolog *esg* is expressed in the intestinal stem cells and undifferentiated progenitors termed as the enteroblast(EB).Therefore, *esg-Gal4 UAS-GFP* was used to mark the ISCs . The amount of ISCs present in the control and the experimental fly guts were evaluated to figure out the effects of high sugar feeding on the gut homeostasis. The number of ISCs were normalised by accounting for the total number of cells present (by counting the number of nuclei in that area).

Our study suggested that there is a significant reduction in the intestinal stem cell population upon feeding high sugar diet on all the three days analysed **(Fig 8.B-B'').** Upon evaluating the kinetics of the ISC population with age, we found that the ISCs show a positive kinetics in the control scenario i.e. there is a significant enhancement of ISCs by Day 15.However, this kinetics seems to be perturbed in the experimental flies. In the high sugar fed flies ISC pool is significantly depleted from day 5 itself and is steadily maintained at the depleted level (**Graph 2.A-D**). Thus, by Day 15 there is a stark difference in ISC number between the control and HSD fed flies. These results were consistent with our prior finding that the JAK-STAT signaling, which is important for the ISC proliferation, is down-regulated in the experimental fly gut.

III.III High sugar diet enhances the absorptive Enterocyte population of the midgut

Since the ISCs pool is being depleted upon feeding high sugar diet, we further investigated the state of the two differentiated cell types. Enterocyte constitutes the major cell type of the intestinal epithelium which has role in the secretion of digestive enzymes and in the absorption of nutrients. *Myo-Gal4 UAS-2xEGFP* transgene is expressed in the ECs cells .Thus it was used as a marker for the enterocyte population. When the enterocyte population of the control and experimental guts were compared, the results suggested a significant increase in the enterocyte population **(Fig 9.A-F)**. When we quantitatively analysed this data, the experimental flies showed significant enhancement of ECs from day 5 itself (**Graph 3 A-C)**. When the overall kinetics of the ECs with age was analysed, we observed that in control scenario the ECs are steadily maintained, where as in the HSD flies the ECs almost exhibit a positive kinetics **(Graph 3 D)**. This result is in alignment with the earlier data which suggested that the Notch signaling is enhanced in the flies fed on high sugar diet. Enhanced Notch signaling is already known to promote the EC fate [36].

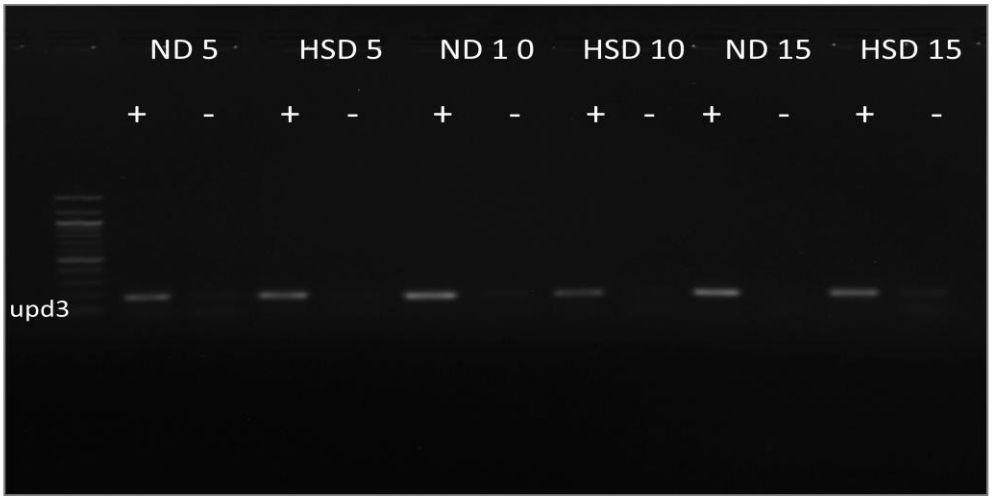
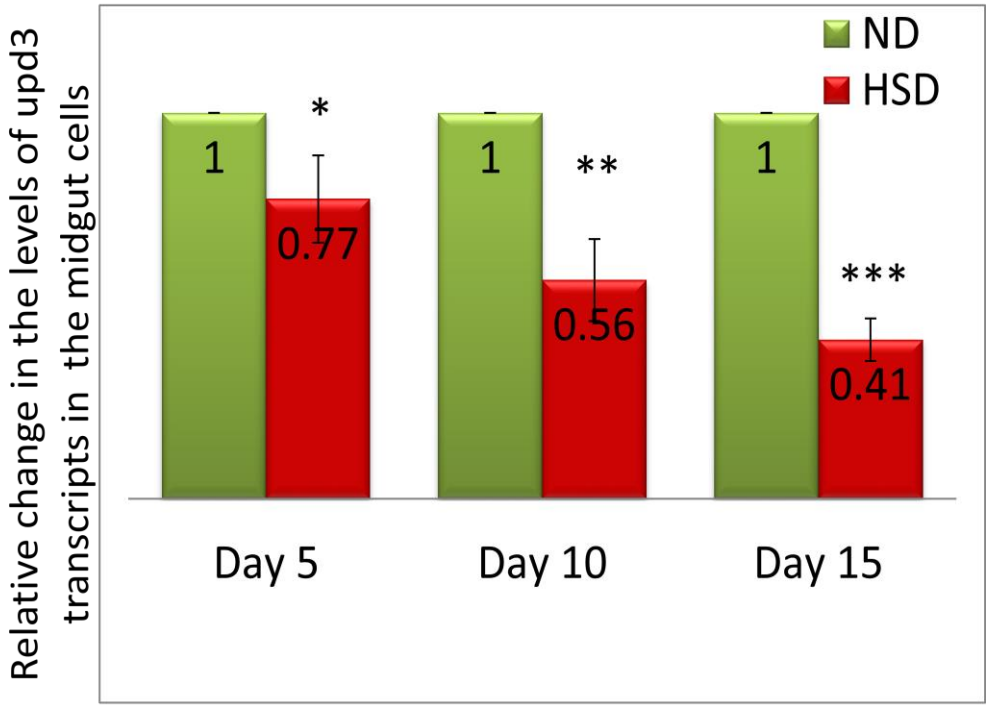


Figure 7: Upd3 transcript levels in the midgut

Agarose gel image showing the levels of *Upd3* transcript in the midgut of control and HSD flies on Day 5, Day 10 and Day 15 respectively. The plus (+) sign signifies the +RT reactions and the minus (-) sign signifies the –RT reactions. **Upd3~135 bp**



Graph 1: Upd3 transcript levels in the midgut

Graphical representation of depression in *Upd3* transcript levels, of the HSD fly gut compared to the control, on Day 5, Day 10 and Day 15.

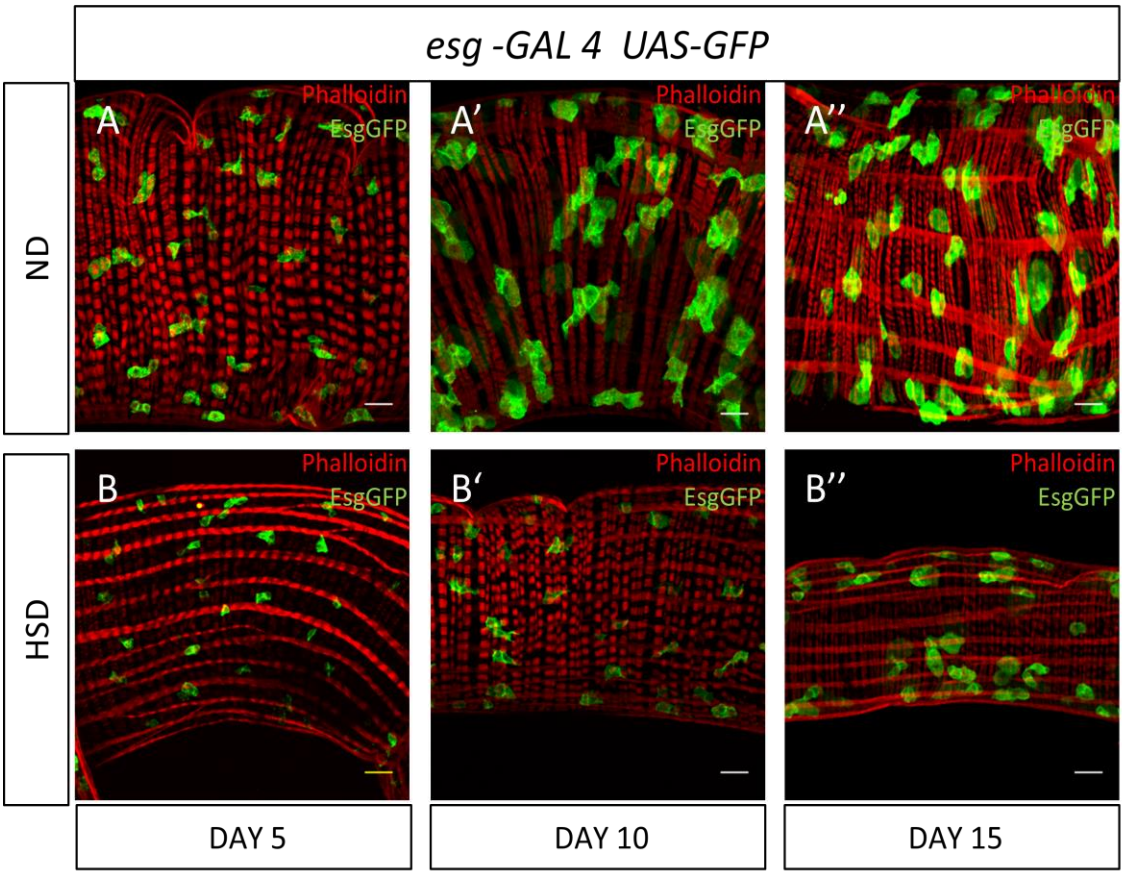
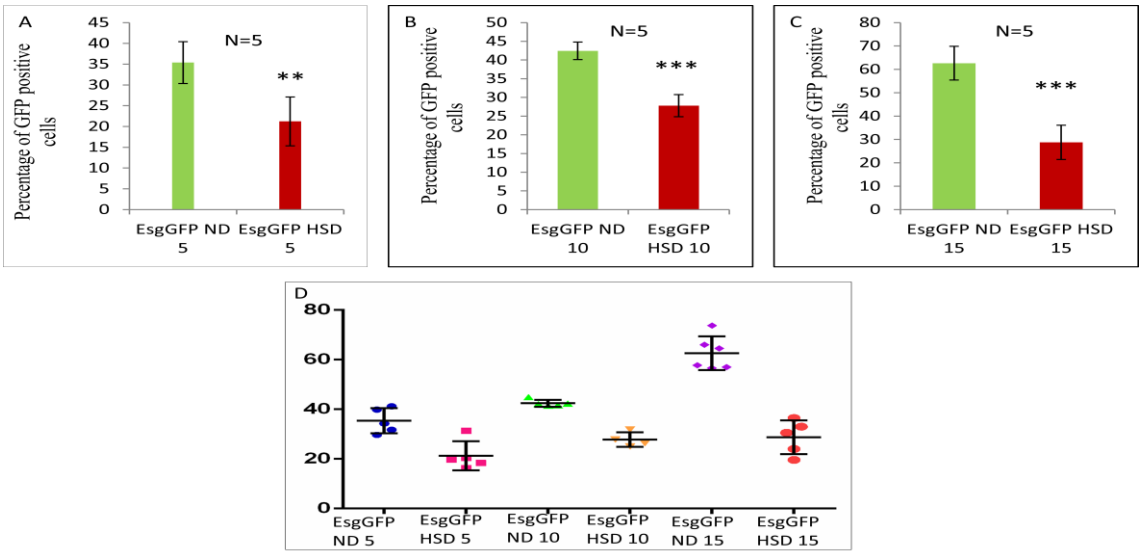


Figure 8 : Depletion in the intestinal stem cell population

Scale bar-20µm. Expression pattern of *esg-Gal4 UAS-GFP* reporter line in ND and HSD posterior midgut on Day 5(A, B), Day10 (A', B'), and Day 15 (A'', B''). HSD midgut shows depleted stem cell pool.



Graph 2 : Percentage of Esg positive cells in the midgut.

Graph showing the **(A-C)** comparison of percentage of ISCs in ND and HSD on Day 5 **(A)**, Day 10 **(B)** and Day 15 **(C)**.**(D)**Graphical representation of overall ISC population (in ND and HSD) kinetics with age.

III.IV High sugar diet depletes the EE population of the midgut

Enteroendocrine cells are the other major cell type present in the midgut epithelium which simultaneously fulfills the functions of both EE and Paneth cells in mammalian system. The overall population of secretory cells in the midgut are called as the EEs. However, different subtypes of EEs producing different subsets hormones are also known to exist[63]. The overall population of EEs can be marked by the expression of the homeodomain protein Prospero (Pros) [35].So we performed immunostaining with Prospero antibody ,on *Myo-Gal4 UAS-GFP* fly line ,in order to mark the EE population of the midgut.

When the EE population of experimental and control flies were compared, from day 5 itself, a significant reduction was observed **(Fig 9.A''-F'')**. Quantitative data suggested that the percentage of EE population has gone down to 5 percent by day 5 itself and it maintained at this depleted level **(Graph 4 A-C)**. Age dependent dynamics of the EE population exhibited a steady kinetics in both control and experimental flies. However, the experimental flies' possessed limited number of EE cells **(Graph 4D)**. This result further validated our prior evidence that the Notch signaling, which is important for EC fate, is upregulated.

III.V Higher glucose uptake by the midgut cells in the high sugar fed flies

Before analyzing the role of high sugar associated metabolism in altering the gut homeostasis, we found it appropriate to establish that the high sugar flies are indeed high on sugar uptake. For *in vivo* assessment of glucose uptake, we utilized 2-NBDG as a fluorescent indicator of glucose uptake. This assay is based on the direct incubation of the tissue with a fluorescent d-glucose analog 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) followed by confocal imaging.

The 2-NBDG assay revealed that there is a stark difference in the glucose uptake between the control and experimental gut **(Fig10.A-A', B-B')**. Quantification data suggested that there is approximately two fold increase in the glucose uptake by the midgut cells on Day 10.On day 15 , there is a significant fourfold increase in the sugar uptake by the midgut

cells **(Graph 5)**. This is also consistent with the earlier reports which suggested that administration of various glucose containing carbohydrates have shown to cause postprandial glycemia which triggers the release of insulin and subsequent glucose uptake by numerous cell types [64]**.**

III.VI Altered mitochondrial dynamics in the high sugar fed flies

Once the sugar is absorbed, it is converted to pyruvate in the cytoplasm which are then transported into the mitochondrial matrix. Then the substrate-derived metabolites are oxidized, leading to the formation of reducing equivalents, which are subsequently utilized to supply the electron transport system (ETS) located in the inner mitochondrial membrane. Therefore, at the subcellular level, mitochondria integrate multiple metabolic pathways. It is known that Mitochondria can utilize various fuels depending on physiological and nutritional conditions to promote cellular homeostasis[65]**.** Mitochondrial functioning is already known to be important for the *Drosophila* intestinal homeostasis during stress and aging [62]. Therefore, to investigate how enhanced sugar uptake is altering the mitochondrial dynamics we utilized *Mito sqh-EYFP* reporter line in which the Sqh regulatory sequences drive expression of yellow fluorescent marker for the mitochondrion. The marker sequence is composed of mitochondrial targeting sequence fused to EYFP. We also did immunostaining with an antobody against Prospero to specifically mark the enteroentocrine (ee) population. The EC population could be easily distinguished from the ee population by the presence of big polyploidy nuclei. The purpose of this was to analyze if the alteration in mitochondrial dynamics (if any) shows any specificity in terms of cell type.

Our study found that the overall *Mito-YFP* expression is reduced in the high sugar fed flies compared to the control flies on all the three days analysed **(Fig11.A-C)**. The high sugar fed fly guts showed diffused expression of mitochondria where as the control flies showed a prominent punctae like appearance. Image J software was used to quantitatively analyze the fluorescence intensity. When the mean fluorescence intensities were compared, it was evident that the mean *Mito-YFP* intensities have gone significantly down from Day 3 of high sugar feeding (Age 5) and this depletion is maintained throughout. Though it appears that there is no specificity in loss of mitochondria in terms of cell type, as the ee cells are very small in size we need to image at higher magnification to confidently comment on this.

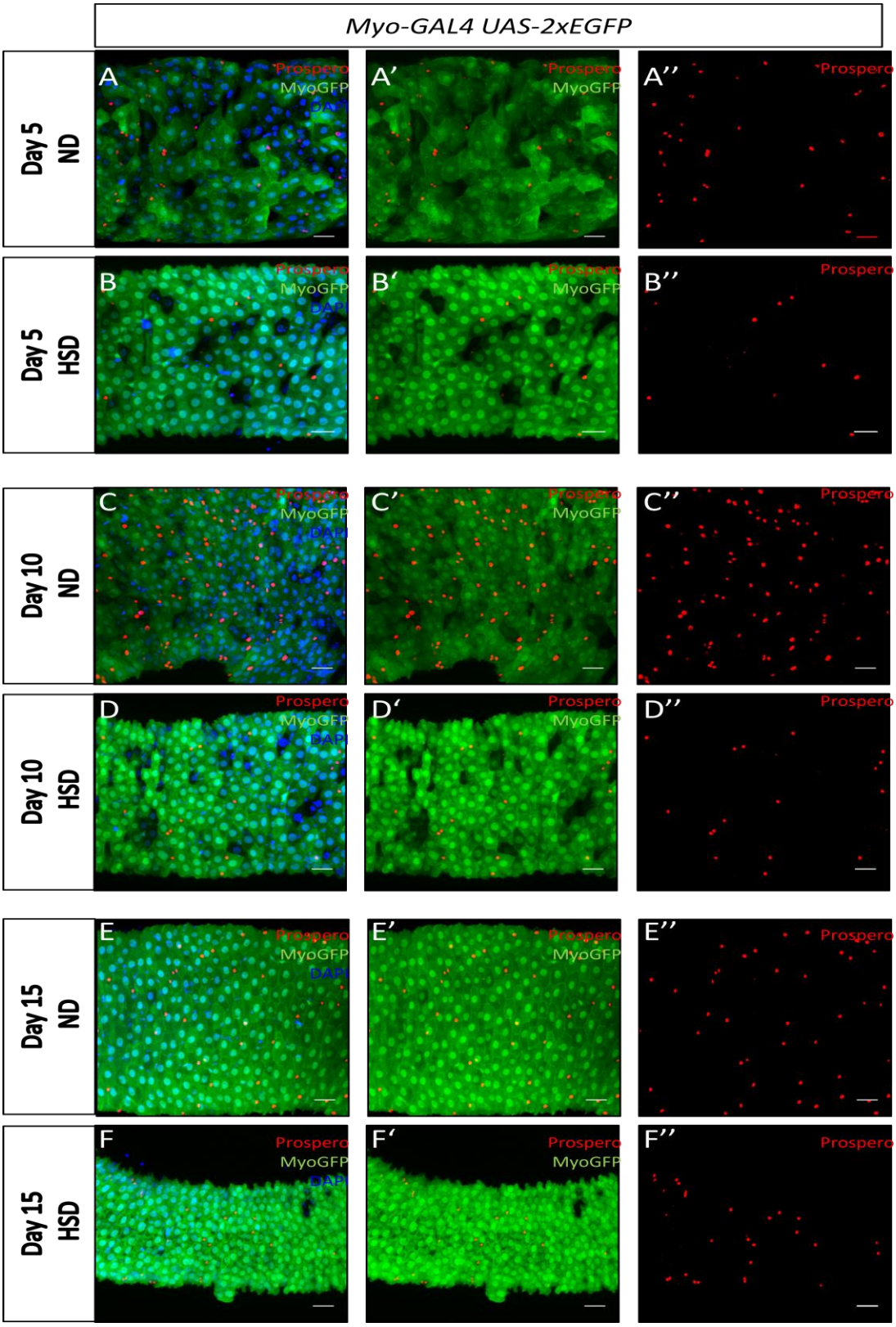
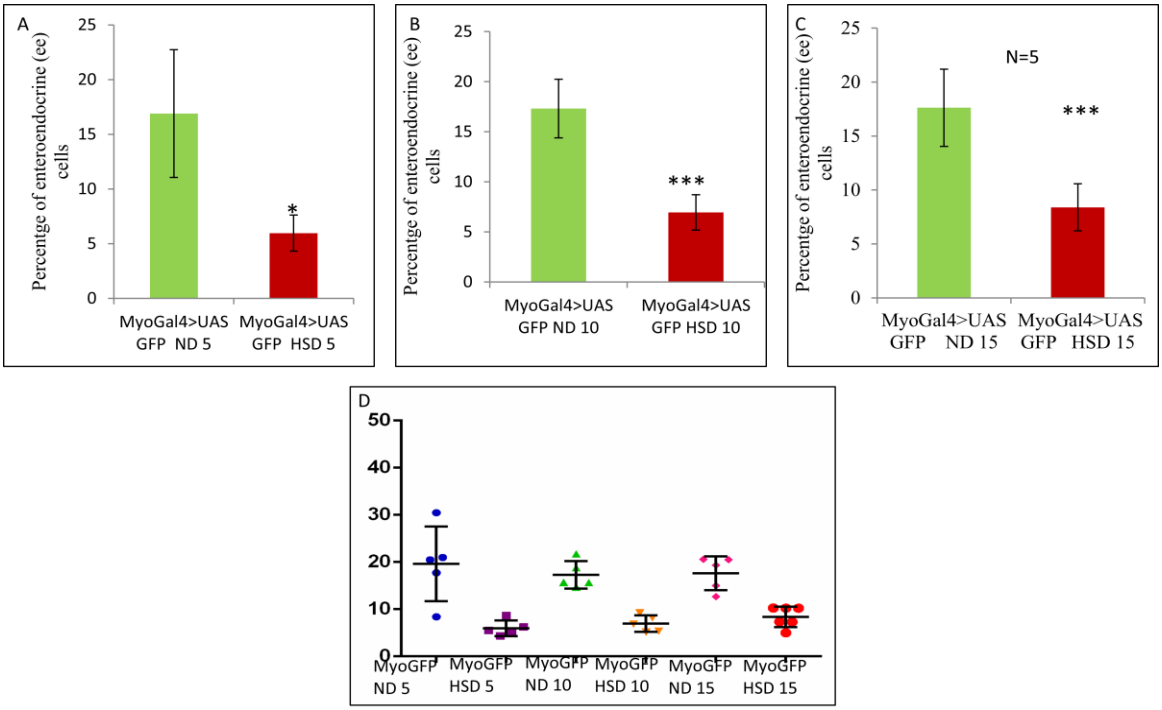


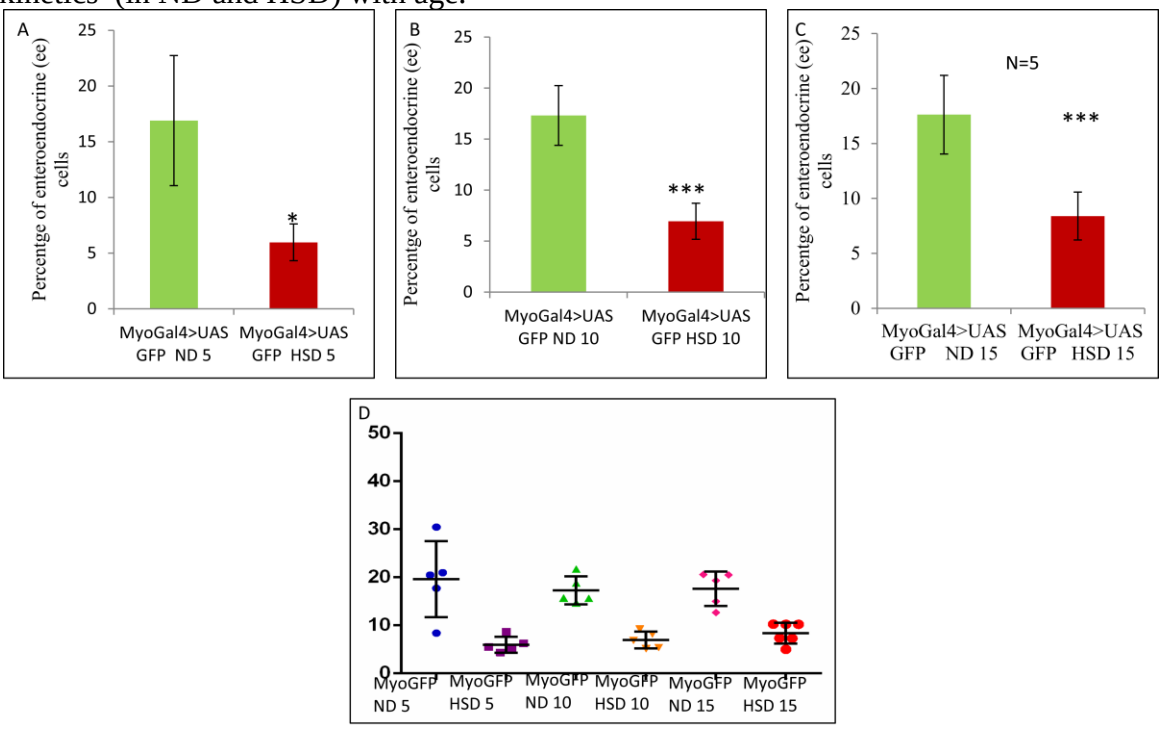
Figure 9 : Enhancement of ECs and depletion of EEs in the HSD fly gut.

Scale bar-20µm. Expression pattern of MyoGFP (marking the ECs) in ND and HSD posterior midgut on Day 5(A, B), Day10 (C, D), and Day 15 (E, F). Depletion of EEs (marked by Prospero) in HSD gut on Day 5(B''), Day 10(D'') and Day 15(F'') compared to the respective controls (A'', C'', E'')



Graph 3: Percentage of ECs in the midgut

Graph showing the **(A-C)** comparison of percentage of ECs in ND and HSD on Day 5 **(A)**, Day 10 **(B)** and Day 15 **(C)**.**(D)**Graphical representation of overall EC population kinetics (in ND and HSD) with age.



Graph 4: Percentage of EEs in the midgut .

Graph showing the (A-C) comparison of percentage of EEs in ND and HSD on Day 5 (A), Day 10 (B) and Day 15 (C). (D)Graphical representation of overall EE population kinetics (in ND and HSD) with age.

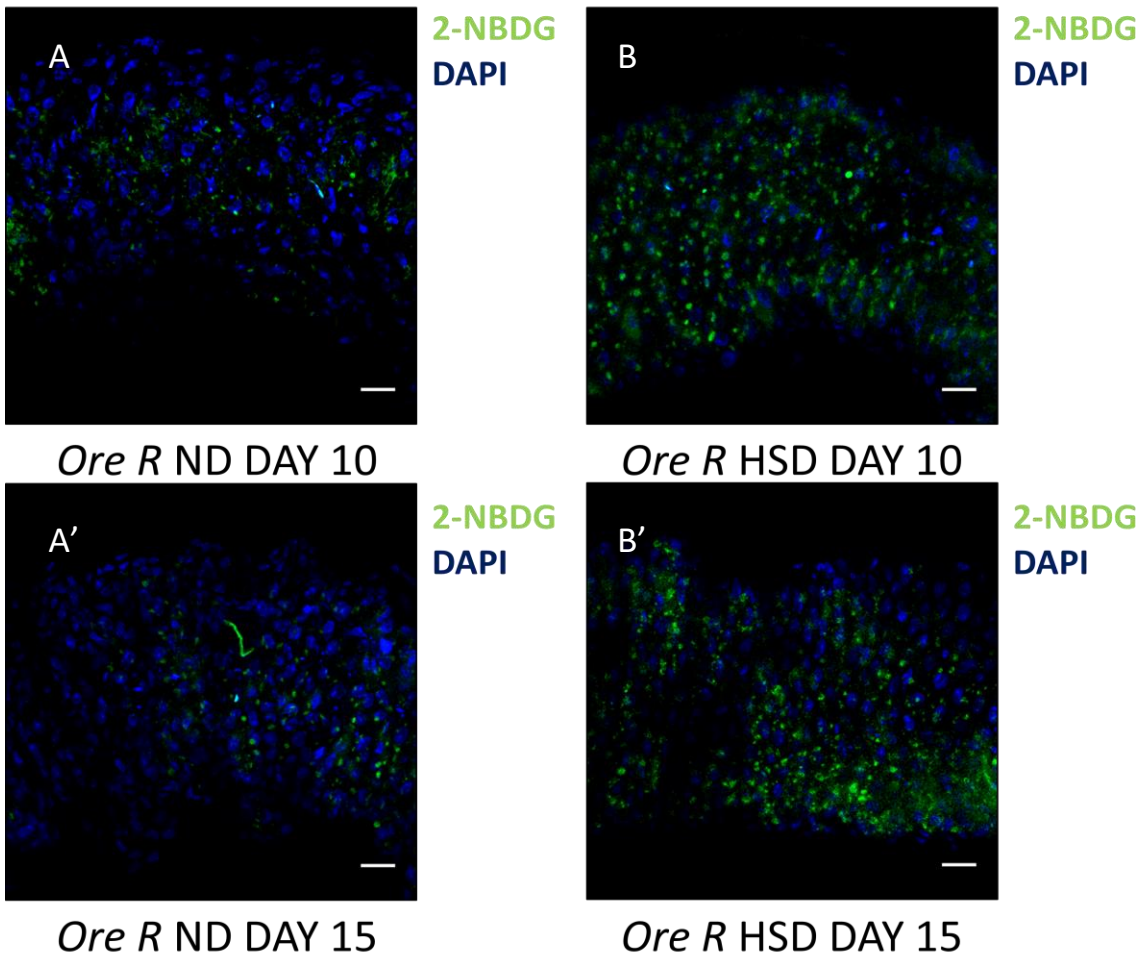
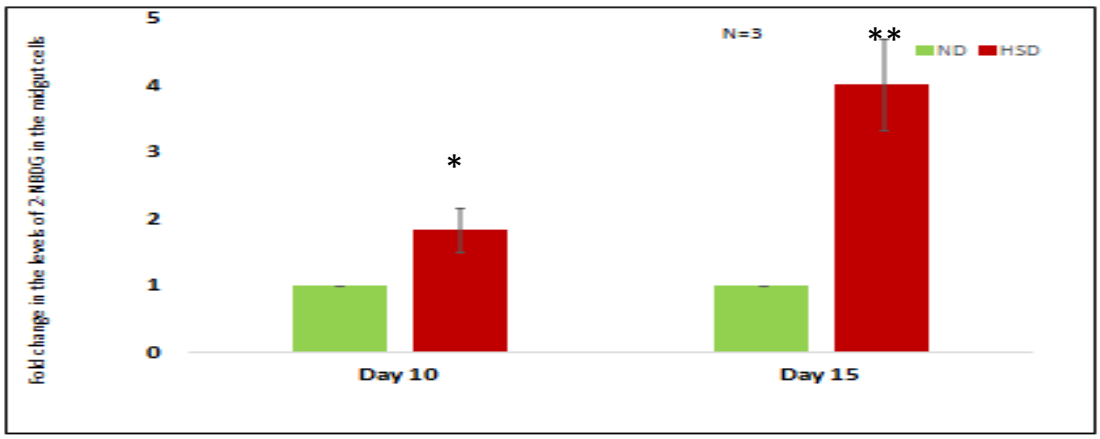


Figure 10 : *In vivo* glucose uptake assay, using fluorescent d-glucose analog 2-NBDG, showing higher glucose uptake by the midgut cells of the high sugar fed flies.

(A-A') 2-NBDG uptake by the ND (A) compared to the HSD (A') midgut cells on Day 10. 2-NBDG uptake by the ND (B) and HSD (B') midgut cells on Day 15.**Scale bar-20µm**



Graph 5: Quantification data of 2-NBDG uptake by the midgut cells.

Graph showing the fold change in the level of 2-NBDG uptake by the gut on day 10 and day 15 compared to the respective controls.

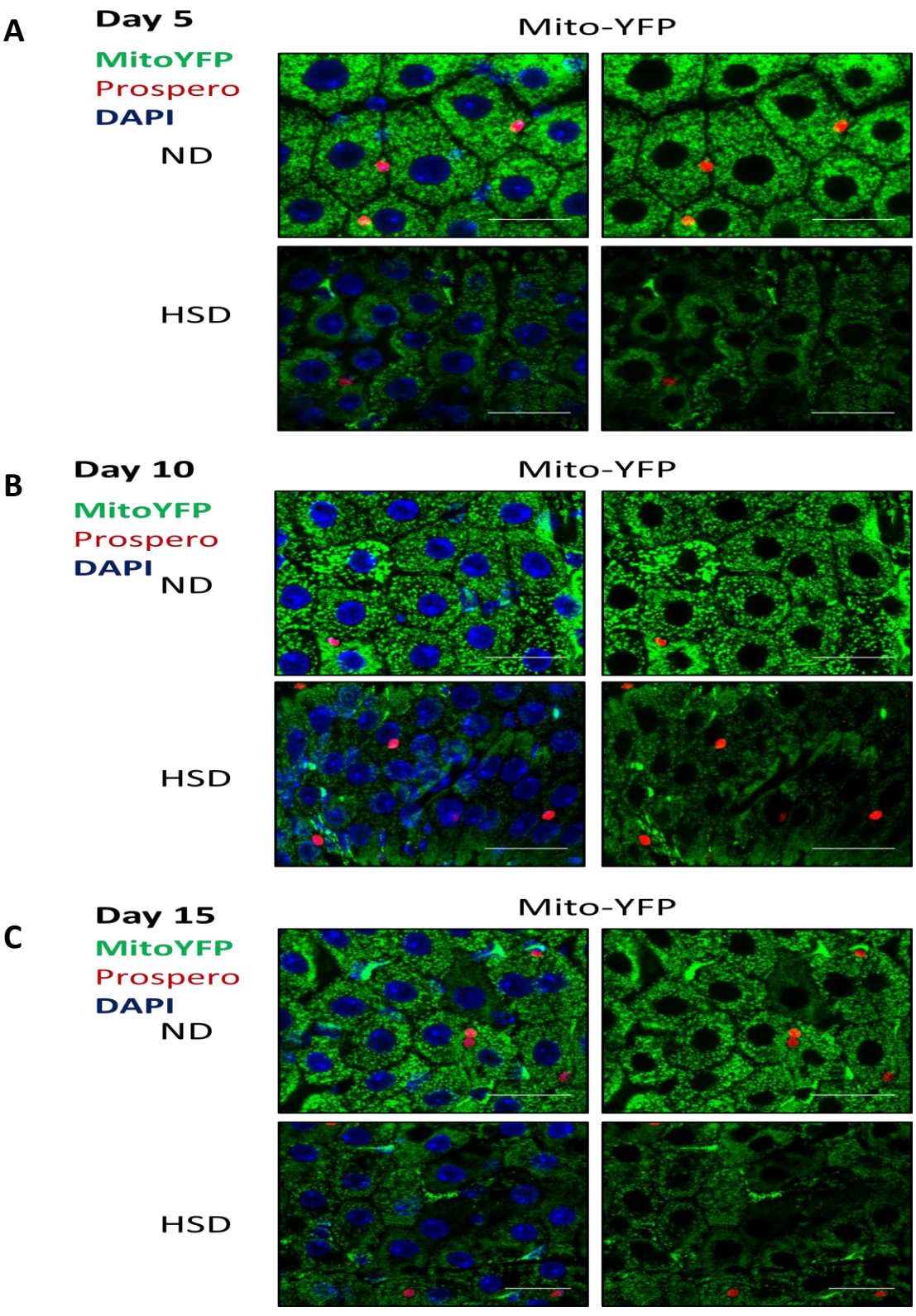
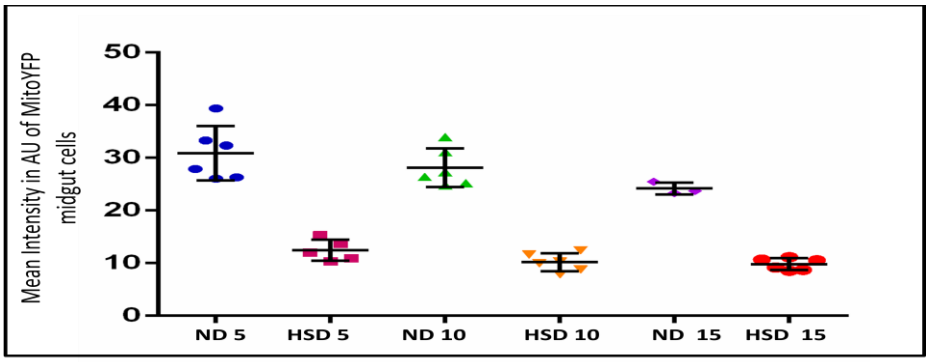


Figure 11 : Mitochondrial distribution in the gut epithelial cells

Mitochondrial distribution in the gut epithelial cells of normal and high sugar fed flies on Day 5 (A) , Day 10 (B) and day 15(C) marked by the expression of Mito-YFP. Prospero staining marks the EEs. Scale bar-20µm

III.VII High sugar diet results in the reduction of Fatty Acid Oxidation (FAO) on Day 10

Apart from sugar/carbohydrate metabolism, Fatty acid oxidation (FAO) is the primary source of energy for many tissues and it exclusively happens in the mitochondria. Since there is a significant reduction in the amount of mitochondria in the HSD fed flies, we analysed the status of FAO. We utilized a protein-trap transgenic fly construct *scu-GFP*. Scully or the hydroxy-acyl-CoA dehydrogenase is an enzyme implicated in FAO. The results indicated that the *scu-GFP* expression is significantly reduced in the HSD fed flies on Day 10 **(Fig12.B)**. This result was further validated by analysing the *scully* transcript levels. Results of qRT-PCR clearly demonstrated a a dip in the *scully* transcript levels. There was approximately 40 percentage reduction in the *scully* transcript levels (**Graph 7**). This data was also consistent with the overall reduction in the amount of mitochondria suggesting that not only the mitochondrial amount but mitochondrial activity also have gone down in the cells of the midgut of HSD fed flies..



Graph 6: Quantitative comparison of mean Mito-YFP intensities

Graph showing the reduced mean Mito-YFP intensities of HSD fed fly guts of day 5, day 10 and day 15 with their respective controls

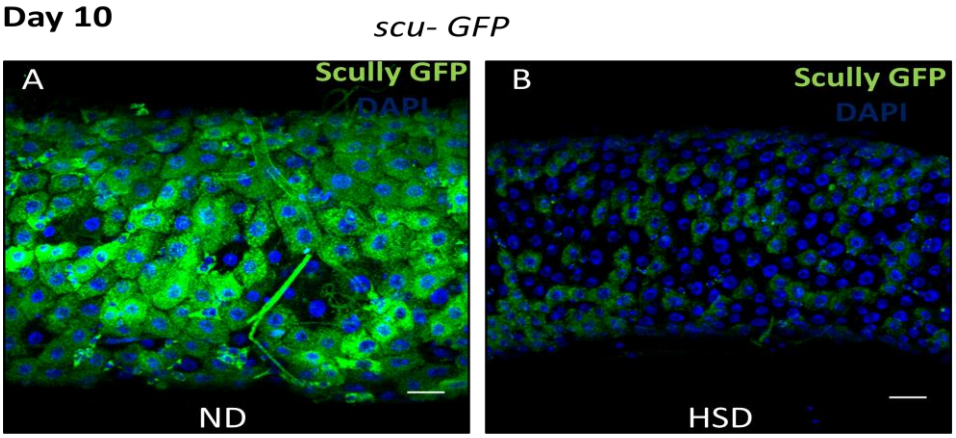
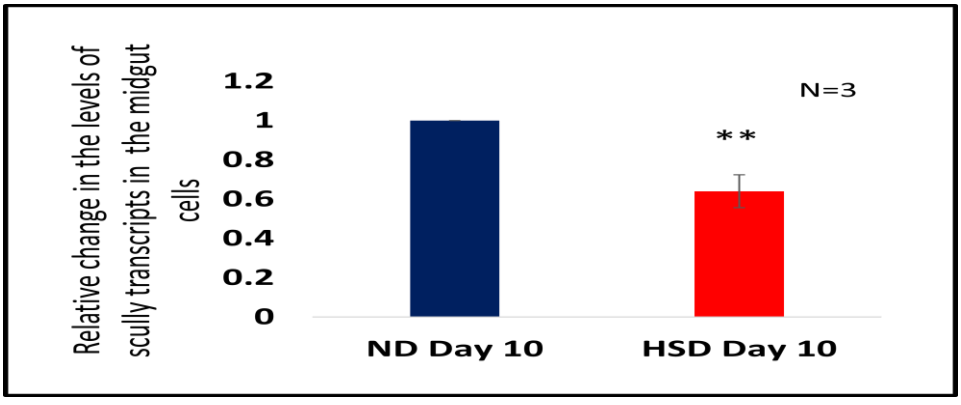


Figure 12 : Reduced fatty Acid Oxidation in HSD fly gut.

Comparison of *scu-GFP* expression between control(A) and HSD (B) fiy gut on Day 10



Graph 7: Scully transcript level

Graphical representation of depression in *scully* gene transcript levels in high sugar diet fed gut (red) compared to control midgut (blue).

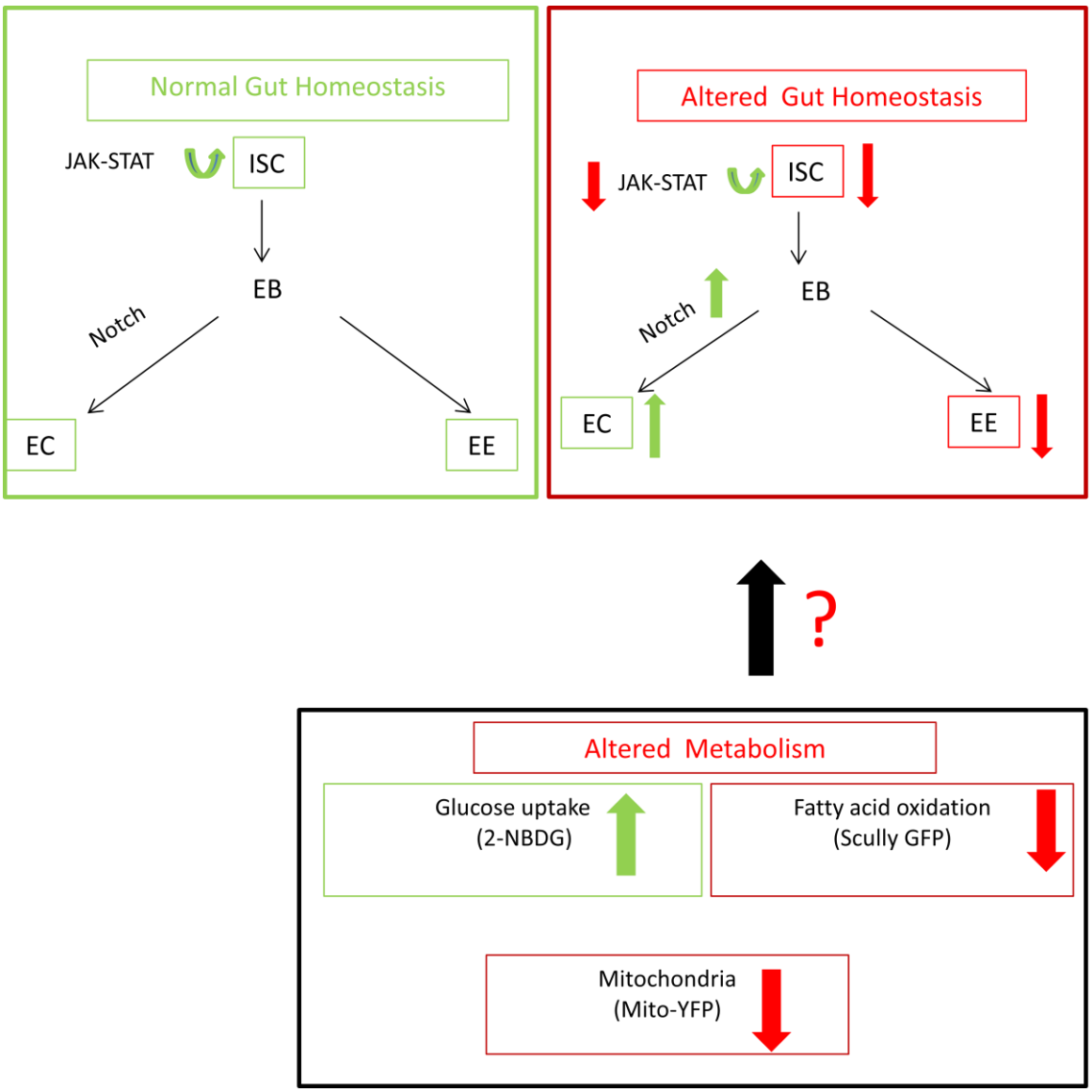


Figure 13 : Proposed model of altered gut homeostasis in high sugar diet fed flies

CHAPTER IV

DISCUSSIONS AND FUTURE DIRECTIONS

Growing incidence of diabetes and obesity are evidence to the impacts of change in life style and diet on human health. The current studies support the fact that diet is a major modifiable determinant in chronic diseases. Gastrointestinal tract, one of the largest organs in the body cavity, was previously considered as a passive organ majorly responsible for digestion and absorption of nutrients. But this view was changed drastically since the gut has emerged as the major modulator of various other biological processes. Gut being the primary organ of interaction with the food is expected to function as a mediator of diet induced metabolic and functional changes. Thus through our study we addressed the issue of how high sugar diet can induce metabolic changes which results in the alteration in the gut homeostasis. Our investigations resulted in the finding that the two major signaling pathways JAK-STAT and Notch are significantly altered in the midgut of high sugar diet fed flies. Since these pathways govern the division and differentiation of ISCs, which plays critical role to maintain the gut homeostasis, we speculated significant alteration in the gut homeostasis. We clearly demonstrate a significant depletion in the amount of ISCs and EEs. We also observed a concomitant enhancement of EC population of the gut. These results were cross validatory as enhanced Notch is known to promote the EC fate and down regulated JAK-STAT compromises the proliferative capacity of the ISCs. This suggested that the enhanced Notch signaling is promoting the EC fate at the cost of EE fate. This enhancement of the absorptive cell type can be interpreted as an adaptive shift to balance the nutrient uptake during nutrient abundance. ECs are known to integrate information about the carbohydrate status of the fat body and the sugar uptake (sensed by Mondo-Bigmax) to adjust the expression of the carbohydrate digestive enzymes. Therefore, though we can see a concomitant increase in EC number, their functionality must be analysed as specific nutrients can probably change the absorptive properties of the enterocytes [42]**.** The Upd3 ligand of JAK-STAT is mainly produced by the ECs [43].Though we have seen an enhancement of ECs in the high sugar scenario the expression of Upd3 has gone down significantly. Thus it further strengthens the argument that the functionality of the ECs needs to be analysed. The age dependent enhancement of the ISCs observed also correlated with the studies which suggested that that the ISCs become hyper proliferative in aging flies[66]. Moreover, there are evidences which suggest that insulin sigaling is important for the proliferation of ISCs[67]. In our diet induced model we have established that the tissues are insulin resistant. Thus it can be

speculated that the reduction in the number of ISCs can be a combinatorial effect of the reduced JAK-STAT signaling and insulin resistance. Flies lacking all EE cells are also known to display abnormal intestinal homeostasis[63].There are links which connects the EE cells and ISC proliferation as well. Flies lacking EE cells are also known to exhibit narrower midguts. EE are known to produce Tachykinin (Tk) which promotes expression of the visceral muscle-derived Ilp3. This peptide is demonstrated to sustain the stem cell proliferation and nutrient-dependent midgut growth [67]**.** The overall reduction in the midgut size is also consistent with the depletion of the EEs. Therefore, our results clearly demonstrated that the gut homeostasis is significantly altered upon feeding high sugar diet.

The elevated glucose uptake by the high sugar fed flies might be suggestive of higher utilization of glucose within the midgut cells.At sub-celluar level mitochondria integrates the various metabolic pathways involved in the metabolism of carbohydrates. Thus investigating the mitochondria associated parameters gave a significant clue towards the alteration in metabolism. The drop in Mito-YFP expression suggested that there are lesser number of mitochondria present in the experimental flies. This diminished pool of mitochondria can potentially be due to the effect of high sugar induced metabolism on the mitochondrial biogenesis or due to the degradation of the mitochondria itself. Nutrient overload could possibly have resulted in metabolic inflexibility which can cause mitochondrial dysfunctions. Moreover, increased Fluorodeoxyglucose (F-FDG) uptake and in vitro glucose consumption has previously been strongly correlated to loss of mitochondrial markers in the lung cancers [68].The enhanced glucose uptake can potentially be metabolized by glycolysis or by the pentose phosphate pathway. The elevated levels of G-6-P can shut down the generation of Acetyl CoA through Fatty acid oxidation[57].This along with the reduction in mitochondrial number can possibly be the reasons for reduced fatty acid oxidation status indicated by the Scully-GFP expression. This can likely lead to higher fat accumulation in the high sugar fed fly guts. This can be investigated by Nile red staining.In order to quantify the various metabolites involved in the central pathways Gas chromatography –mass spectrometry (GC-MS) analytical technique can be utilized. This will provide a global view regarding the high sugar associated metabolic changes.

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