This paper was prepared

under the direction of

Professor Craig Woodard

for eight credits.

“As you set out for Ithaca
hope that your journey is a long one,
full of adventure, full of discovery.
Laistrygonians and Cyclops,
angry Poseidon-don't be afraid of them:
you'll never find things like that on your way
as long as you keep your thoughts raised high,
as long as a rare sensasion
touches your spirit and your body.
Laistrygonians and Cyclops,
wild Poseidon-you won't encounter them
unless you bring them along inside your soul,
unless your soul sets them up in front of you.

Hope that your journey is a long one.
May there be many summer mornings when,
with what pleasure, what joy,
you come into harbors you're seeing for the first time;
may you stop at Phoenician trading stations
to buy fine things,
mother of pearl and coral, amber and ebony,
sensual perfume of every kind-
as many sensual perfumes as you can;
and may you visit many Egyptian cities
to learn and learn again from those who know.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so that you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaca to make you rich.
Ithaca gave you the marvelous journey.
Without her you would have not set out.
She has nothing left to give you now.

And if you find her poor, Ithaca won't have fooled you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithacas mean.”
(Ithaca, C.P. Cavafy)

~ To my family, friends and every other person in my life who made my journey possible. It is because of their unconditional love and generosity that I’ve been able to keep walking towards my destination. ~

The Genetic Control of Fat Body Development and Function in *Drosophila melanogaster*

By

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Abstract

All living organisms struggle to maintain homeostasis. Organisms have evolved various mechanisms to be able to cope with any changes in temperature, pH or nutrient availability. Mammals, for example put a great amount of effort into maintaining glucose homeostasis. Briefly, they achieve regulation of glucose levels in the blood using primarily two hormones that are produced in the pancreas: insulin and glucagon (Bhagavan 2002). Mammals are not only able to maintain glucose levels within the normal range during long periods of fasting, but they also achieve the same outcome when excessive amount of nutrients are introduced through consumption (Bhagavan 2002). When animals fail to achieve homeostasis multiple complications arise. For example, mammals that are not able to maintain glucose at normal levels in the blood stream, due to lack of insulin, suffer from a disease called diabetes.

In a similar manner, holometabolous insects like *Drosophila melanogaster* try to maintain nutrient homeostasis. The name "holometabolous" describes the metamorphosis that these animals undergo. During metamorphosis, dramatic changes take place such as tissue remodeling and apoptosis. However, in order for these changes to happen, it is critical for the animal to be able to manage its energy resources effectively. The fat body, the main organ responsible for energy storage, responds to environmental changes as it tries to maintain homeostasis, which is probably the reason why it is not destroyed during metamorphosis. For the animal to be ready to initiate this process, great amounts of energy need to be stored in the fat body. This is an important step, as during metamorphosis the animal basically starves. Since the intake of energy is impossible, stored energy must be utilized to fuel the various developmental changes. Therefore, given that fat body plays a pivotal role in nutrient circulation and metamorphosis, experiments are needed to further elucidate its involvement in both metamorphosis and nutrient homeostasis.

With the first set of experiments I sought to understand if there were any similarities between fat-body remodeling and cancer metastasis. In both processes, cells dissociate and migrate to a different tissue. Thus, I sought to identify if there were any functional similarities between βFTZ-F1, a competence factor that is responsible for the initiation of fat-body remodeling, and its mammalian ortholog, SF-1.

With the second set of experiments I sought to understand how insulin signaling is regulated in *Drosophila* larvae. More specifically, I identified MMP2, a matrix-metalloproteinase to be a possible regulator of the process. Having a better understanding of insulin signaling pathway in *Drosophila* offers us the possibility to use *Drosophila* as model organism to study human diseases such as diabetes and high blood pressure.

Introduction

A. Fat-Body Remodeling

Summary

Cancer metastasis is characterized by the migration of tumor cells from the tissue that was initially affected to a new tissue. In addition to that it also describes the formation of new tumor colonies at the new site. Surprisingly, the *Drosophila* fat-body cells resemble a similar type of behavior during a process known as fat-body remodeling.

Drosophila melanogaster, also known as fruit fly, is a well-studied system used for research in genetics and developmental biology. Fruit flies are used as a model for research due to the following characteristics: 1. they are small and easily cultured, 2. they have a short generation time, which allows experiments to precede fast with minor delays, 3. male and female flies are easily distinguishable. As the *Drosophila* life cycle proceeds, an interesting process called metamorphosis takes place. Metamorphosis is the transition from a larva to an adult fly. As metamorphosis proceeds, changes in gene expression occur, leading to changes in the fly's body such as organ/tissue formation, tissue destructions and tissue remodeling. ßFTZ- F1, a competence factor, plays a pivotal role in all these dramatic tissue changes during metamorphosis. To name a few of these changes, in the fat body βFTZ-F1 is involved in tissue remodeling (Bond *et* al 2011) whereas in midgut and

salivary glands βFTZ-F1 is involved in programmed cell death (Broadus *et al* 1999; Lee et al., 2002).

The aim of this study is to understand the mechanisms associated with cell detachment and cell migration during fat-body remodeling and thus possibly gain a better understanding of the mechanisms involved in cancer metastasis. We take advantage of the fact that the mammalian nuclear receptor SF-1was has structural similarities with the *Drosophila* βFTZ-F1 nuclear receptor (Lala *et al* 1992). Thus we sought to perform further experiments with both βFTZ-F1 and SF-1 in an attempt to identify functional similarities associated with the fat-body remodeling between these two orthologs. Any functional similarities might provide us with a better understanding of fat-body remodeling and cancer metastasis mechanisms.

The *Drosophila* Life Cycle

The *Drosophila melanogaster* life cycle consists of 5 distinct developmental stages: the embryonic, larval, prepupal, pupal and adult stages (figure 1). The life cycle begins as soon as the egg gets fertilized. After the embryo develops for one day in a process called embryogenesis, the larva hatches (larval stage) and for the next 8 days it undergoes many developmental changes. There are 3 distinct larval stages, called the 1st, 2nd and 3rd instars. The transition from one instar to the next involves a molting process during which the larval mouth hooks, spiracles and other essential structures are torn apart and reformed (Demerec 1950).

Initially the 1st instar larva has very small mouth hooks, but after the first molt, the resulting 2nd instar develops bigger mouth hooks, forms anterior and posterior spiracles and grows bigger in size. At this particular stage the anterior spiracles are club-shaped (figure 2) and the posterior spiracles have a light orange ring at their extremities. Four days later, as the larva feeds, the second molt occurs, resulting in the formation of a 3rd instar larva with shell-shaped anterior spiracles and dark orange rings on the posterior spiracles.

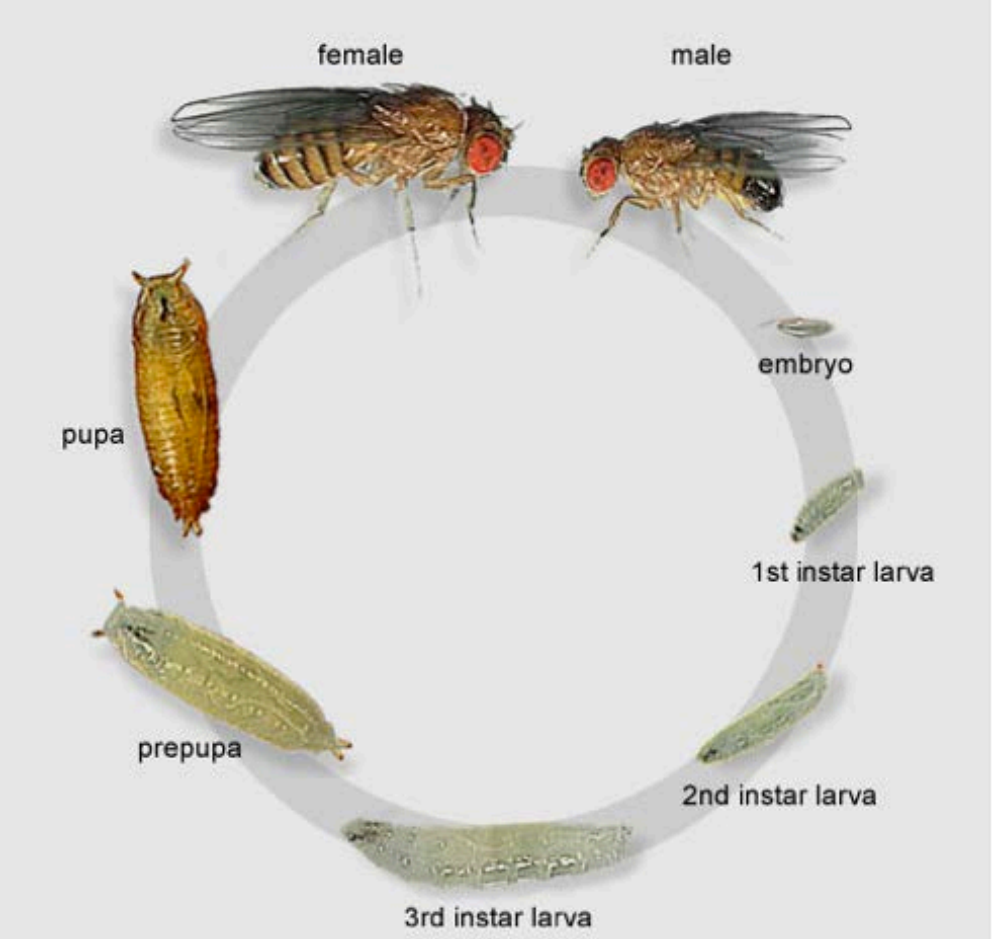

Figure 1. The *Drosophila Melanogaster* Life Cycle. The stages of fruit-fly development from the egg to the adult fly. At the end of the larval stages, the larva forms a puparium and transforms into a prepupa. The transition from the larval to the pupal stage marks the beginning of metamorphosis. **(**Figure from Weigmann *et al*. 2003).

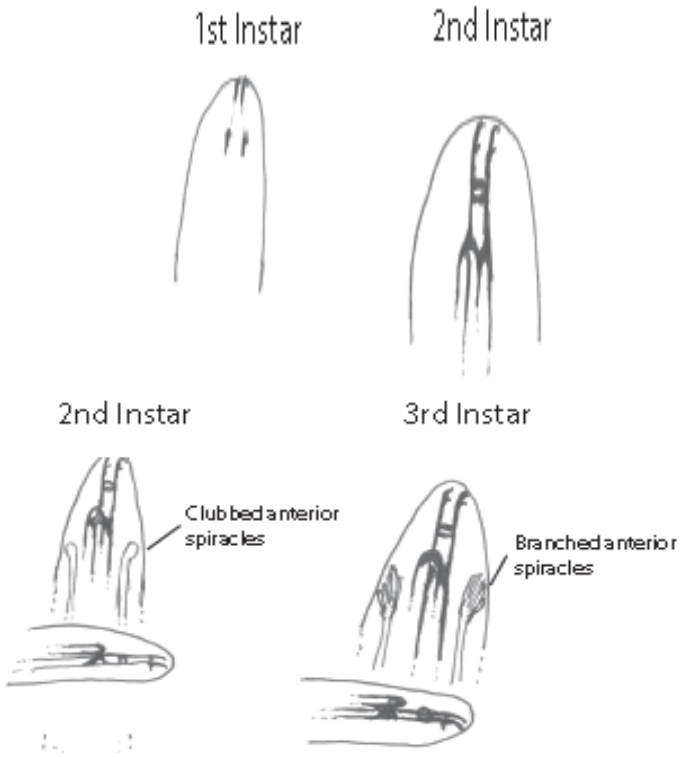

Figure 2. Morphological Differences between Instars as they Result from Molting. 1st and 2nd instar mouth hook size comparison. 2nd and 3rd instar anterior and posterior spiracles comparison (Michigan State Un. Article, https://www.msu.edu/~shingle9/Documents/Instars.pdf).

The 3rd larval instar is divided into two main stages: the feeding stage and the wandering stage (Chung *et al.*, 2009). As the 3rd instar larva feeds, it reaches a critical weight and crawls out from the food, searching for a place to enter the prepupal stage and enclose itself into a puparium. The transition from the wandering stage to the prepupal stage, followed by the puparium formation, marks the beginning of metamorphosis (Bond *et al.*, 2011; Brainbridge and Bownes, 1981). Within an hour after puparium formation, the larval cuticle hardens and changes color – from white to orange-brown (Brainbridge and Bownes 1981). The moment puparium formation occurs is used as a reference point to define the stages of development during metamorphosis. The term 0 h APF (After Puparium Formation) is used to describe the beginning of metamorphosis. During this process the pre-pupa/ pupa undergoes many changes, such as tissue remodeling and tissue destruction by programmed cell death (Brainbridge and Bownes 1981). To compensate for the lost tissue the animal forms new tissue and thus prepares itself for the adult life that will soon begin. For example, 12 hours APF head eversion occurs followed by fat-body cell migration at the head capsule. At the end of metamorphosis, the adult fly ecloses ready to start its reproductive life (Bond *et al.* 2011).

Hormonal Regulation of Post-Embryonic Development

At the end of embryogenesis, there are three major hormones that drive fruit fly development: ecdysone, Juvenile hormone (JH) and prothoracicotropic hormone (PTTH). As post embryonic development begins, PTTH regulates the secretion of ecdysone from the ring gland. Ecdysone is responsible for the regulation of two separate processes: molting and metamorphosis. For molting to occur, JH interferes with ecdysone action (Riddiford and Truman 1993). Ecdysone is inactive until it gets converted into 20-hydroxyecdysone (20E) (Riddiford and Truman 1993). In general, 20E triggers the transcription of genes that are essential for the aforementioned processes (i.e. molting and metamorphosis) by interacting with a nuclear receptor (ecdysone receptor) (Beckstead *et al.* 2005). Ecdysone receptor is a heterodimer composed of the proteins, EcR and USP (Ultraspiricle).

During *Drosophila* development many 20E pulses occur, however there are two distinct 20E pulses that occur during late 3rd instar to the prepupal-pupal transition. The "late larval" 20E pulse occurs the end of the 3rd instar stage, and initiates metamorphosis. At this time, 20E interacts with the ecdysone receptor (see figure 3), to induce the transcription of genes such as *BR-C* (*Broad-Complex*), *E74A* and *E75A* ("early" genes), which encode transcription factors that drive the transcription of "late" genes (see figure 3). Additionally, these early genes repress their own transcription. Next, at approximately 6 hours APF, the competence factor gene *βftz-f1* is transcribed and causes the induction of additional genes (late genes) that are responsible for remodeling (*MMP2*) or apoptosis of various tissues (*E93*). Finally, the

"prepupal" 20E pulse takes place at about 10 hours APF and it induces the transition from the prepupal to pupal stage activating for a second time the early genes (Yao *et al.*, 1993; Woodard *et al.*, 1994; Thummel, 2001). At this time, in both midgut and salivary glands, 20E together with the competence factor βFTZ-F1 turn on the cell death gene *E93* (Lee *et al.* 2000) (See figure 3). As its name suggests *E93* is essential for programmed cell death.

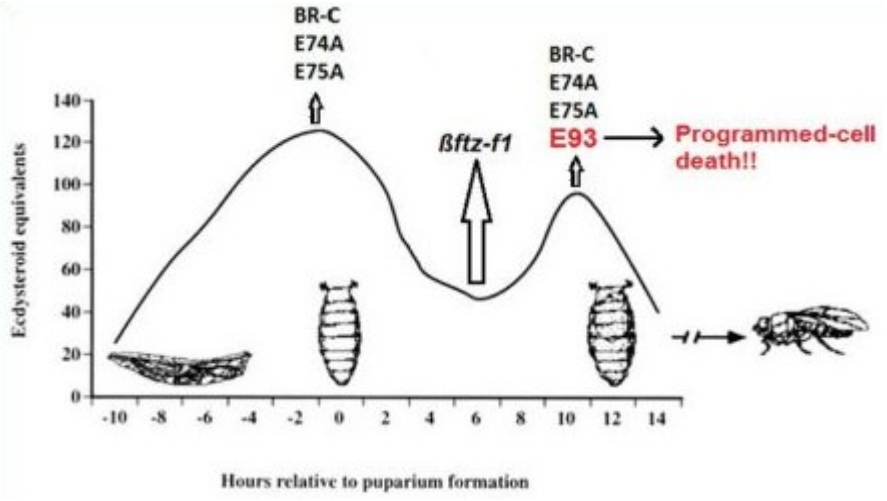

Figure 3. Changes in Ecdysone Hormonal Signaling During Metamorphosis. The "late larval" and "prepupal" ecdysone pulses induce the transcription of genes involved in metamorphosis. The gene encoding the competence factor βFTZ-F1 is expressed in between the two pulses and enables 20E to induce the transcription of genes that determine the fate of the various tissues during metamorphosis (Almonacid 2012).

The *Drosophila* Nuclear Receptor βFTZ-F1

The *Drosophila ftz-f1* gene was discovered by a scientific team that performed studies of the gene *fushi-tarazu* (*ftz*) (Ueda *et al.* 1990). *ftz* is a pair-rule gene that is transcribed during the embryonic stage to induce embryonic segmentation. More specifically, *ftz* expression is regulated by two elements: the zebra element and the upstream element (Hiromi *et al.* 1985). In an attempt to further clarify the interactions between these regulatory elements and *ftz* Ueda and his colleagues (1990) performed a series of experiments that eventually led them to the discovery of the *ftz-f1* gene. They were able to demonstrate that FTZ-F1 (FTZ-Factor 1) is a DNA-binding protein that interacts with the zebra element and therefore is involved in *ftz* regulation.

The *ftz-f1* gene encodes two different FTZ-F1 protein isoforms: αFTZ-F1 (130-kDa) and βFTZ-F1 (97-kDa) (Lavorgna *et al.* 1993). Due to alternative splicing there are two transcripts of *βftz-f1* found at different stages of development (see figure 4) (Pick *et al.* 2006). *αftz-f1* is expressed in early and late embryonic stages (Pick *et al.*, 2006). In contrast, *β-FTZ-F1* is expressed at the end of embryonic development (Woodard *et al.* 1994; Yamada et al, 2000). Both the αFTZ-F1 and βFTZ-F1 proteins bind to signaling molecules (i.e. ßFTZ-F1 binds to 20E) (Pick *et al.*, 2006). As previously stated this binding can induce the transcription of genes involved in programmed cell death (i.e. E93) (Pick *et al.* 2006) or remodeling (i.e. *MMP*2) (Bond *et al.* 2011).

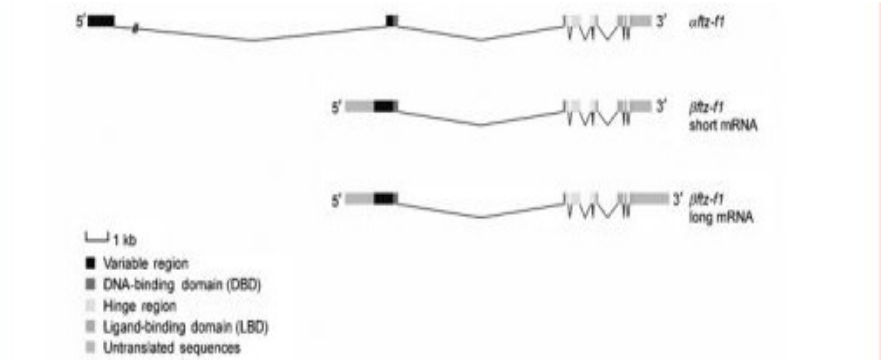

Figure 4. Three *ftz-f1* mRNAs Differ in Size and Structure. *α-ftz-f1* mRNA is the longest and has an extra DNA-binding domain. Both *β-FTZ-F1* isoforms are significantly shorter than *α-ftz-f1*. At the protein level, they differ among themselves at their 3' end. Finally, their transcription occurs at different times during the development (figure from Pick *et al.*, 2006).

As the “late larval” 20E pulse declines, the initiation of fat-body remodeling occurs at approximately 3hours APF. There are three stages of fat-body remodeling: retraction (0-6 hours APF), disaggregation (6-12 hours APF) and detachment (12-18 hours APF). Briefly, during these stages, fat-body cells change shape from a polygonal shape to a spherical shape, lose the attachments between each other, and some of them migrate from the main core body into the head capsule (Figure 5) (Bond *et al.* 2011; Hoshizaki, 2005; Rizki 1978). As previously mentioned, at the end of the retraction stage (6 hours APF) a competence factor called ßFTZ-F1 is expressed and together with 20E modulates the dissociation of fat-body cells (Bond *et al.* 2011).

Studies have shown that when *βftz-f1* is prematurely expressed in the fat body, remodeling occurs at late 3rd instar wandering stage. 20E is necessary for premature remodeling to occur and therefore, if *βftz-f1* premature expression doesn’t coincide with 20E expression then late third instars show no signs of premature remodeling. These findings suggest ßFTZ-F1’s essential role in fat-body remodeling regulation (Bond *et al.* 2011). Alternatively, when the action of *βftz-f1* is blocked by induction of B lymphocyte-induced maturation protein-1 (*dBlimp-1*) most larvae die before reaching the prepupal-pupal stage which demonstrates that *βftz-f1* is also essential for the transition from larval to prepupal-pupal stage (Bond *et al.* 2011). At times, few larvae are able survive through metamorphosis despite the expression of *dBlimp-1*. Even so, these larvae do not proceed into the stages of fat-body cell detachment or fat-body cell migration (Bond *et al.* 2011).

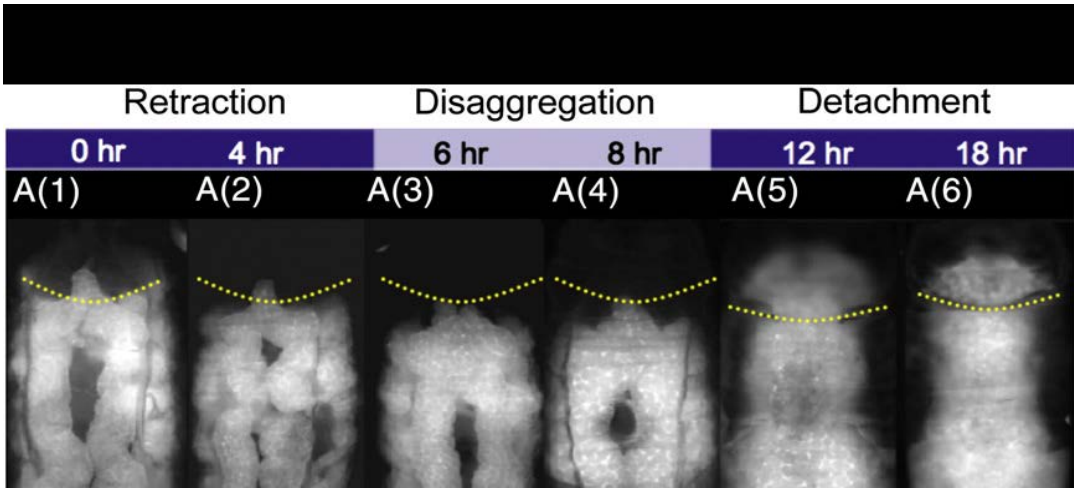

Figure 5. Fat-Body Remodeling Stages. Images of fat body tissue at different times during pupation. It is apparent here that fat-body cells change shape (4-6 hours APF), get detached from each other (12 hours APF) and migrate into the fruit fly head capsule (18 hours APF) (figure from Bond *et al.* 2011).

Drosophila Matrix-metalloproteinase 2 (_MMP_2) and Tissue Inhibitor of Matrix-metalloproteinases (TIMP)

_MMP_2 belongs to the matrix metalloproteinase (MMP) family. MMPs are enzymes responsible for the cleavage of extracellular membrane proteins such as collagen, laminin and fibronectin. When selective cleavage of these ECM proteins occurs, cells lose the previously established attachments. In _Drosophila_, there are two MMPs: MMP1 and MMP2 (Page-McCaw, 2008). Matrix-metalloproteinase 1 (MMP1) is responsible for tracheal development and head eversion during pupation. Matrix-metalloproteinase 2 (_MMP_2) is responsible for fat-body cell detachment (Bond _et al._ 2011).

_MMP_2 expression in the fat body is regulated by the βFTZ-F1 nuclear factor. At the end of the retraction stage during fat-body remodeling, _β-FTZ-F1_ is expressed (Bond _et al._ 2011). In response to _MMP_2 induction, fat-body cells enter the detachment stage. It has been established that _β-FTZ-F1_ together with the "prepupal pulse" of 20E induce _MMP_2 expression during disaggregation stage (see figure 6). This was demonstrated with a set of experiments conducted by Bond and colleagues (2011), who observed that ectopic expression of _ßftz-f1_ results in premature _MMP_2 expression (Bond _et al._ 2011).

MMP function can be inhibited by tissue inhibitors of matrix-metalloproteinases (TIMPs) (Wei _et al._ 2003). _Drosophila_ has only one _TIMP_ gene. The TIMP protein binds to the active site of MMPs, inhibiting their action (Bond _et al._ 2011; Wei _et al._ 2003). _TIMP_ over-expression results in lethality during larval stage as the fat-body cells are unable to migrate to the

head capsule (Bond *et al.* 2011) and complete the fat-body remodeling process.

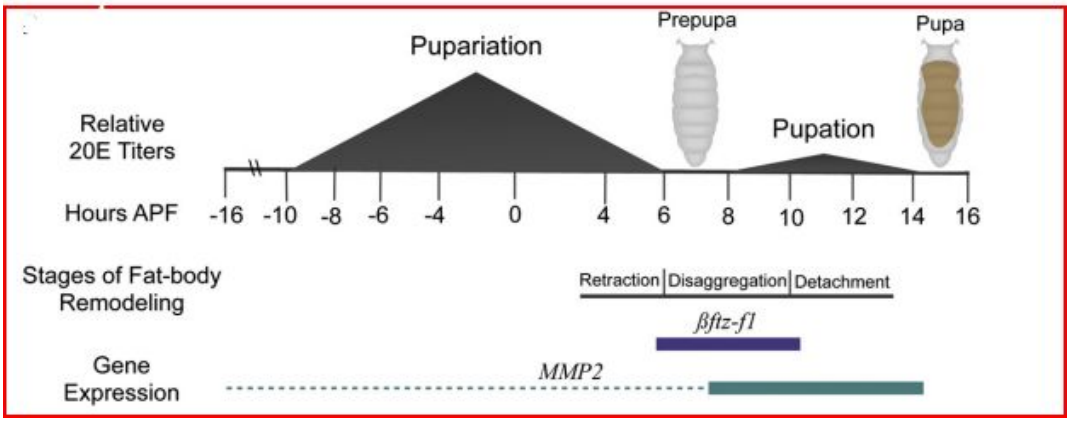

Figure 6. Gene Involvement in Different Fat-Body Remodeling Stages. At 6 hours APF a low 20E titter causes the transcription of *ßftz-f1* (disaggregation stage) which then induces the expression of *MMP*2 at approximately 10 APF. MMP2 proceeds with cleaving ECM proteins that establish the connections among fat-body cells. Soon fat-body cells start to freely float inside the larval body (Bond *et al.* 2011).

The confirmation of MMP2's critical role in fat-body remodeling can be applied in the field of medicine to better understand the nature of various maladies. Since MMP2 liberates the fat-body cells from the attachments they establish among themselves, MMPs might also been involved in cell-detachment of other tissues such as those of cancerous tumors. In other words, there is a possibility that mammalian MMP orthologs play a role in metastatic cancer. It has already been demonstrated that in mice downregulation of MMP9 in the lungs results in the formation of fewer cancerous colonies (Hua and Muschel 1996). Alternatively, scientists working with human subjects that suffered from breast cancer managed to isolate a variety of cancerous cells that were able to metastasize in lungs. In these studies it was shown that, among other proteins, overexpression of MMP1 was proven to mediate cancerous breast cell metastasis in lungs (Minn *et al.* 2005). So far in insects, MMPs have only been found to participate in wound healing and fat-body remodeling (Page-McCaw *et al*. 2008).

SF-1 Member of the Ftz-F1 Family

Steroidogenic factor 1 (*SF-1*), also known as adrenal 4-binding protein (Ad4BP), was initially discovered as a DNA-binding protein that controls the expression of cytochrome P450 steroid hydroxylases (Parker *et al.* 2002; Rice et al 1991). In previous studies, SF-1 has been identified as the mammalian homolog of *Drosophila* αFTZ-F1 and ßFTZ-F1.

SF-1 is expressed in various mammal tissues, such as in the adrenal glands, the gonads, the primordia of the endocrine hypothalamus and the anterior pituitary gland (Ikeda *et al.* 1994). In studies conducted in mice using knockout techniques, SF-1 has been shown to be essential for adrenal gland and gonad development (Ikeda *et al. 1994*). Other studies demonstrate that overexpression of *SF-1* might result in gonad tumourigenesis (Gardiner *et al.* 2012). Finally, studies have found a strong correlation between adenocortical cancer and *SF-1* overexpression in various mammals (Lalli 2010).

Hypothesis and Future Goals

In an attempt to identify similarities between fat-body remodeling and cancer metastasis, I used transgenic flies and ectopically express the mammalian *ftz-f1* homolog, *SF-1*, in fat-body cells. It has been previously shown that ectopic *βftz-f1* expression induces premature expression of *MMP2* and consequently premature fat-body remodeling (Bond *et al*., 2011). Upon MMP involvement in tumor metastasis, experiments need to elucidate how TIMPs might be used to block MMPs action. Performing such studies could give us a better understanding of the causes and restrictions under which cancer metastasis occurs (Baker *et al.* 2002).

Based on the information provided so far, I initially hypothesized that *β-FTZ-F1* and *SF-1*could have functional similarities. I tried to test my hypothesis expressing ectopically *SF-1* in 3rd instar larval fat body seeking to observe premature fat-body remodeling.

B. Larval Fat body and Insulin Signaling

Summary

Insulin signaling is a well known pathway that has been carefully conserved throughout evolution. It is found in various organisms such as insects (i.e. *Drosophila*) and mammals (i.e. mice and humans) that use it in an attempt to regulate their growth and metabolic processes. Diseases such as diabetes and high blood pressure appear when insulin signaling fails to effectively regulate growth and metabolism.

In *Drosophila,* the fat body is the main organ for energy storage. When the larva feeds, insulin signaling is turned on and nutrients are stored in the form of lipids in the fat-body cells (Britton *et al.* 2002). There are various pathways that assist this process: the insulin/insulin-like growth factor signaling pathway (IIS), the phosphatidylinositol-3 signaling pathway (Pl3) and the target of rapamycin signaling pathway (TOR) (as reviewed on Teleman 2010). In *Drosophila* there are seven different ILPs (Garofalo 2002).

In contrast, when insulin signaling is turned off nutrient release occurs. Throughout the *Drosophila* life cycle, there is only one instance in which despite nutrient availability, insulin signaling is shut down. This has been observed during the 3rd instar larval stage when the larvae crawl out from the food and stop feeding. However, due to the complexity of insulin signaling, scientists have not been able to determine what regulates this process. In order for insulin signaling to be turned off an inhibitor must act to prevent the

binding of ILPs to the insulin receptor (Inr). It has been hypothesized by the Woodard laboratory that *Matrix metalloproteinase 2* might be the regulator responsible for this inhibition (Bond et al). Starvation experiments using *Drosophila* larvae and *MMP2* expression detection techniques could either confirm or refute its direct/indirect involvement.

By understanding how insulin signaling is regulated in *Drosophila,* we hope to gain a better understanding of human diseases such as diabetes or high blood pressure. In other words, we can use fruit flies as a model system to study human diseases so that great progress in the treatment, cure and prevention of these diseases can occur.

The *Drosophila* Larval Fat Body: Nutrient Storage

The larval fat body is a single cell layer organ spread along the larval body cavity (Rizki, 1978). The fat body and the prothoracic gland are the main organs of the *Drosophila* endocrine system. Interestingly, the insect's endocrine system has a number of similarities with the mammalian one (Hoshizaki 2005). *In Drosophila,* the larval fat body stores great amounts of energy –in the form of lipids and glycogen– and it is responsible for their proper distribution throughout the organism (Arrese and Soulages 2010; Demereck 1994). In fat body tissue energy is stored in lipid droplets (Brown 2001).

The significance of the larval fat body is made apparent during metamorphosis; while most larval tissues experience programmed cell death, the fat body is conserved. As the animal enters metamorphosis it stops feeding and undergoes a long period of starvation. For the animal to survive, nutrients previously stored in the fat body are utilized to satisfy the system's energy demands. In particular, 3rd instar larvae significantly increase their fat body mass before entering the prepupal stage (Bate, 1993; Grönke *et al.* 2003; Arrese and Soulages 2010). During prepupal/pupal stage, when fat-body remodeling occurs, all these stored nutriens get dispersed in the newly formed tissues in order to satisfy their energy demands.

Experiments have showed that over-expression of the lipid storage droplet protein 2 (lsd2) in larvae resulted in fat body mass increace (Grönke *et al.* 2003). Interestingly as metamorphosis phenocopies starvation, a dramatic decrease in lsd2 levels is detected together with an increase in lsd1. Finally, it

has been found that lsd1 is involved in fat body lipolysis and glycogenolysis (Arrese and Soulages 2010).

Insulin/ Insulin-Like Growth Factor Signaling Pathway

Lipid and glycogen deposition and lysis are controlled in mammals and insects via a metabolic pathway called insulin signaling. This pathway is responsible for changes in growth and size (Leevers *et al.* 1996) (see figure 7). Mammalian insulin has many structural and functional similarities with that identified in *Drosophila* (dILP) (Yamaguchi et al 1995). The functional similarities of these proteins are such that it is known mammalian insulin can bind to the *Drosophila* Insulin Receptor (dInR) (Taguchi and White 2008). *Drosophila* insulin-like protein as its name suggests, has an affinity for dInR (*Drosophila* Insulin-like receptor) (Teleman 2010). Animals lacking dInR do not survive through larval stage (Chen *et al.* 1996; Britton et al).

There are seven different dILP forms (dILP1-7) that are very similar structurally (Wu and Brown 2006) but their function differs, and their expression takes place in various tissues (Teleman 2010). It has been verified that all dILPs bind to dInR. Studies have demonstrated that overexpression of any ILP in *Drosophila* larvae results in body enlargement (Ikeya *et al.* 2002; Teleman 2010). There is a reciprocal relationship between dILPs and nuntrient availability. dILPs are not recruited only when nutrients are present to assist nutrient storage, but they are also recruited to dictate feeding behaviour. Recent findings suggest the insulin-like peptide binding at the InR requires the formation of a trimeric complex between insulin-like peptide, dALS and Imp-

L2. dALS and Imp-L2 are found to be insulin-like growth factor-binding proteins (IGF-BPs) (Arquier *et al.*2008).

The Phosphatidylinositol-3 Signaling Pathaway

The Pl3K pathway is responsible for growth regulation, as it responds appropriately to fluctuations in nutrient availability (Britton *et al.* 2002). phosphatidylinositol-3 (Pl3K) signaling pathway is activated when insulin-like proteins phosphorylate dInR, a process which is mediated by the insulin receptor substrate, Chico (Britton et al 2002; Giannakou and Partridge 2007). Even though Chico responds immediately upon ligand bindind to InR, InR is still able to transduce the signal upon its absence (Garofalo, 2002). This is apparent in studies conducted with *chico* null mutants, which do not die, but display several growth defects (Garofalo2002).

Pl3K together with its regulatory subunit Dp60 (Weinkove *et al.* 1997) induces the modification of Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_{2]}$ to PtdIns(1,4,5)P_3] (Giannakou and Partridge 2007; Maehama et al 2004). Deactivation of PtdIns(1,4,5)P_3 is conducted by the tumour suppressor gene *dPTEN* which ,in fact, dephosphorylates both PtdIns(4,5)P_2 and PtdIns(1,4,5)P_3 (Maehama *et al.* 2004). dPTEN mutants do not survive the embryonic stage (Maehama *et al.* 2004).

There are three *dPTEN* isoforms identified and although all of them are able to dephosphorylate PtdIns(4,5)P_2 it has been suggested that dPTEN3 is responsible for PtdIns(1,4,5)P_3 phosphorylation (Maehama *et al.* 2004). At low levels of *dPTEN* expression, PtdIns(1,4,5)P_3 are free to recruit dPDK1 and

dAkt that are responsible for the inhibition of a *Drosophila* transcription factor called dFOXO. If dFOXO is over-expressed, cell growth is inhibited; a response analogous to the cell's response at times when nutrients are not available (Kramer et al 2003).

The Target of Rapamycin (TOR) Signaling Pathway

PlK3 signaling also regulates the Target of rapamycin (TOR) pathway by phosphorylating S6K (Giannakou and Partridge 2007). When insulin signaling is inhibited, TOR signaling is inhibited as well. S6K is active after it gets phosphorylated by TOR and dPDK1 at different sites (see figure 7) (Teleman 2010). TOR is found to be involved not only in cell growth regulation but also in carbohydrolyisis, lipolysis and autophagy (i.e. in fat body) (Teleman 2010; Scott *et al.* 2004). This signaling pathway senses the nutrient availability and the nutrient needs of the cell and dictates the actions that must be performed (Teleman 2010). TOR overexpression leads to cell growth and cell proliferation (Zhang *et al.* 2000) whereas TOR loss of function has the opposite result (Hennig *et al.* 2006).

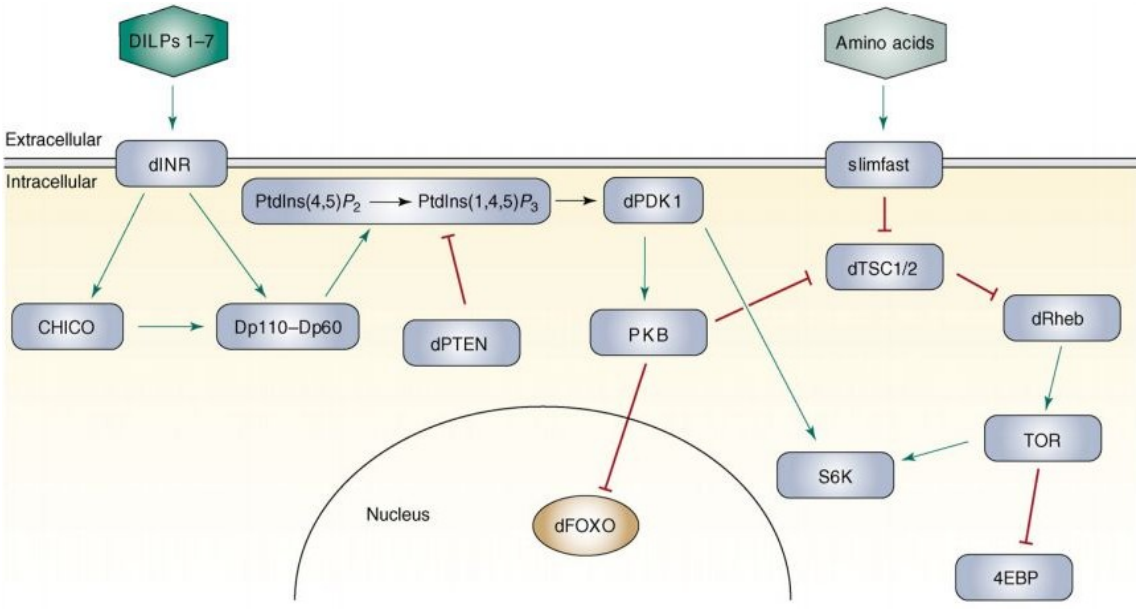

Figure 7. *Drosophila* Insulin-like Signaling: Activation of PlK3 Singaling and TOR Signaling Pathways. Upon ligand binding to dInR PlK3 singaling pathway is turned on. The activated Chico, dPDK1 and PKB act downstream of dInR in order to suppress the transcription factor dFOXO and activate TOR pathway. dFOXO gets phosphorylated and can no longer act inside the cell nucleus. Downstream of TOR, a protein essential for cell growth regulation gets activated. (Figure from Giannakou and Partridge 2007)

Insulin Singaling Regulation by 20-Hydroxyecdysone (20E)

It has been hypothesized that 20-hydroxyecdysone (20E), in addition to playing a pivotal role in larval development and metamorphosis, is also involved in insuling signaling regulation. It has been suggested that 20E together with Insulin signaling regulate growth during larval development (Colombani *et al.* 2005). The hypothesis is as follows: 20E acts as an upstream repressor of the insulin signaling pathway (Rusten et al, 2004; Colombani *et al.* 2005) thus indirectly blocking the TOR signaling pathway (Bond *et al.* 2010).

It is already known that during metamorphosis, 20E directs the transition from the larval to the prepupal stage. Briefly, as 3rd instars reach their so-called "critical weight" they stop feeding, crawl out from the food and soon after that they enter the prepupal stage in order to initiate metamorphosis. Since 20E binds to its receptor to promote the transcription of various target genes, it is possible that 4 hours before puparium formation a high titter of 20E blocks insulin signaling forcing the animal to start metabolizing the stored energy.

Experiments done by Colombani *et al.* (2005) have shown that inhibiting 20E signaling in larval fat body results in size reduction in pupae. Controversially, another group of scientists didn't observe any changes in size, however they reported cases where inhibition of 20E signaling results in death in pupal stage (Cherbas *et al.* 2003). Some years after these studies were published, Nicole Bond (2010) in her dissertation showed evidence that

confirm 20E involvement in pupal development and reject 20E involvement in pupal size changes.

Moreover, 20E singaling is also responsible for *Matrix metalloproteinase 2* (*MMP2*) expression. In particular, *MMP2* expression is regulated by the EcR in fat-body cells (Bond *et al.* 2011). Experiments performed with larvae that had a dominant negative form of EcR showed a significant decrease in *MMP2* expression when compared to wild type larvae (Bond *et al.* 2011). However, both wild type larvae and EcR-DN accumulate nutrients on the same rate (Bond *et al.* 2010). Nutrient rate accumulation is not analogous to insulin signaling regulation. Based on this observation, Bond *et al.* (2010) hypothesized that 20E indirectly effects insulin signaling by directly effecting *MMP2* expression during larval development.

So far it is known that MMP2 plays a pivotal role in fat-body remodeling as its expression causes fat-body cell dissociation to occur (Bond *et al.* 2011). As fat-body cells freely float at the larval cavity they get dispersed in various places where they release stored nutrients to satisfy the animal's energy demands during this prolonged period of starvation. During this time, 20E is also present. Since *MMP2* is expressed in a time in the development at which the animal phonocopies starvation, it is hypothesized that it is not only involved in fat-body cell dissociation but also in nutrient release and consequently in insulin signaling downregulation.

Mammalian Insulin-Like Growth Factor Binding Proteins and Matrix-Metalloproteinases

In mammals, insulin-like growth factor-binding proteins (IGF-BPs) are known to be involved in the insulin signaling pathway by interaction with insulin-like growth factors (IGFs). This interaction results in IGF transportation, IGF prolonged half life and IGF protection from degrading factors (Jones *et al.* 1995; Hwa *et al.* 1995; Honegger *et al.* 2008). For example, the mammalian IGF-BP-7 is thought to bind with insulin and consequently down-regulate insulin signaling (Yamanaka *et al.* 1997).

In *Drosophila*, two proteins that have similar function with that of mammalian IGF-BPs have been discovered recently (dALS and Imp-L2), (Arguier *et al.* 2008; Honneger *et al.* 2008). Both dALS and Imp-L2 are involved in insulin signaling in *Drosophila*. Imp-L2 has been identified as the mammalian ortholog of IGFBP-rP1 as it shares many functional and structural similarities with IGFBP-rP1 (Arquier *et al.* 2008). Some studies suggest that Imp-L2 suppresses insulin signaling as it binds to dilp-2. It is thought that, this Imp-L2-dilp-2 interaction unables dilp-2 to phosphorylate InR (Honegger *et al.* 2008). Interestingly, during starvation periods when nutrient release is essential to ensure animal survival, Imp-L2 expression increases causing glycogen and lipid release (Honegger *et al.* 2008). Finally, over-expression of dilp-2, a condition know generally as hyperinsulinemia, seems to require Imp-L2 to be controlled (Honegger *et al.* 2008).

The mammalian homolog of insulin-like growth factor-binding protein acid-labile subunit (IGF-BP-ALS) is known as dALS (Arquier *et al.* 2008).

The mammalian form, ALS, binds with IGF-1 and together with other IGF-BPs form various complexes that depend upon interaction of at least three molecules (Boisclair *et al.* 2001). Respectively, dALS binds to both dILPs and Imp-L2 forming a trimeric complex (Arquier *et al.* 2008). If binding between dILP and Imp-L2 does not occur, dALS is unable to bind dILP (Arquier *et al.* 2008). Interestingly, when this complex is formed its involvement in growth regulation becomes apparent (Arquier *et al.* 2008). dALS regulates growth and metabolism through this trimeric complex as it responds to fluctuations in nutrient availability. Upon starvation, dALS prolongs the half-life of the dILPs that are to be found in limiting numbers. Such interactions provide *Drosophila* with some type of resistance to starvation (Arquier *et al.* 2008). Alternatively, when there is an excess of nutrients, dALS acts as an inhibitor of animal growth (Arquier *et al.* 2008).

In mammals, 24 MMPs have been identified (Page-McCaw et al. 2007). They are involved in many different processes such as extracellular matrix and bone cartilage remodeling (Krane and Inada 2008), angiogenesis, cancer metastasis, cell proliferation, and wound healing (Rodríguez *et al.* 2009). Various MMP isoforms have been shown to serve as IGF-BP-cleaving proteins. In general this characteristic facilitates the release of insulin growth factor that regulates growth. Studies have shown that MMP7 cleaves all IGF-BPs in mammalian tumors resulting in insulin growth factor bioavailability. In other words, MMP-7 it is found to be responsible for enhancing cancer cell growth (Nakamura *et al.* 2005).

In *Drosophila* it is not yet known exactly how insulin signaling is regulated. If mammalian IGF'BPs and MMPs are not only structurally similar but also functionally, then the *Drosophila* MMP1 or MMP2 might play a pivotal role in insulin signaling regulation. As previously stated, MMP2 is involved in fat-body cell dissociation during metamorphosis. When fat-body cells get dispersed nutrient release occurs which suggests that insulin signaling downregulation occurs. This line of thinking made us to hypothesize that MMP2 is also acting as an insulin signaling inhibitor during metamorphosis.

Based on this hypothesis Nicole Bond (2010) proposed a model which describes that during larval development the *Drosophila* IGF-BPs (dALS and Imp-L2) get cleaved by MMP2 (see figure 8). As a result, insulin signaling is shut off and nutrients are released to satisfy the energy needs of the animals. This model is partially supported by the findings of Arquiel *et al* (2008) and Honegger (2008). As mentioned above, their findings suggest a direct involvement of dALS and Imp-L2 in nutrient accumulation as they respond appropriately in environmental changes. dALS and Imp-L2 help IGF-BPs' bind at the InR promoting nutrient accumulation. But during starvation while the animal needs to utilize the stored energy MMP2s cleave dALS and Imp-L2, thus blocking IGF-.BPs' binding at the InR.

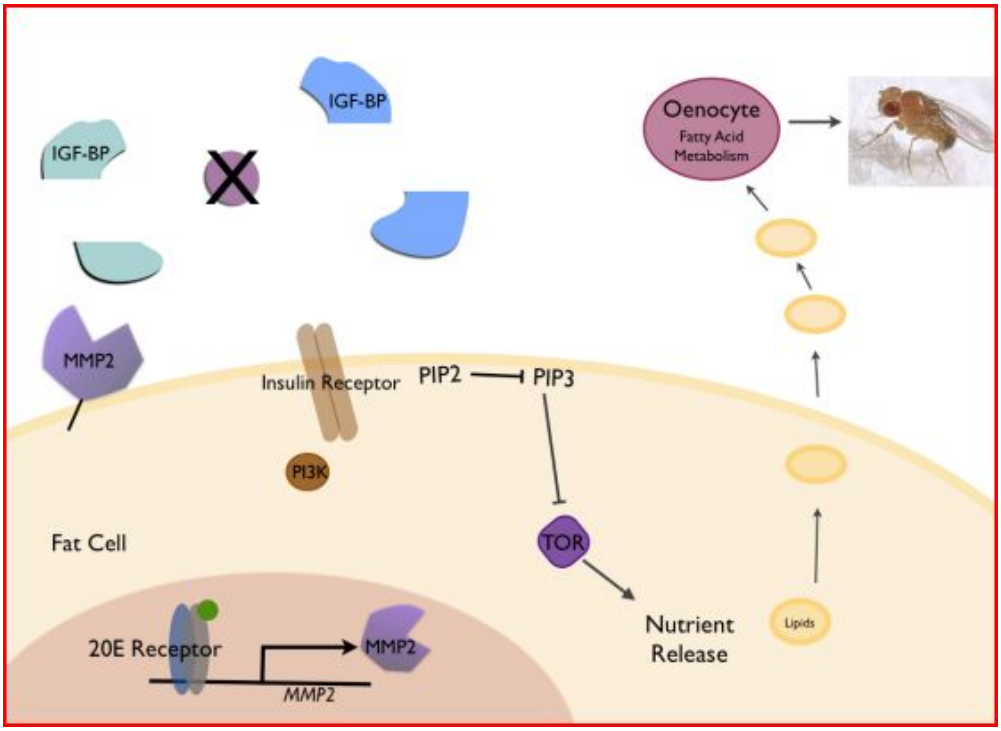

Figure 8. Proposed Model for Insulin Signaling Regulation During Larval Development. Upon binding by the 20E-receptor complex, the *MMP2* gene is expressed. MMP2 protease moves from the nucleus to the ECM and breaks apart the IGF-BPs-IGF trimeric complex by cleaving the IGF-BPs. As a result insulin remains unprotected, its bioavailability decreases and it is unable to bind at the InR. Insulin signaling is turned off as the ligand is unable to bind at the InR. Inhibition of insulin signaling pathway leads to TOR signaling pathway inhibition which results in nutrient release (Bond, 2010).

Hypothesis

By understanding how insulin signaling regulation occurs in *Drosophila*, we hope to gain a better understanding of human diseases such as diabetes and high blood pressure (Teleman 2010). *Drosophila melanogaster* can be used in as a model to try and develop ways to treat, cure or prevent such diseases from occurring.

In an attempt to understand MMP2's role in insulin signaling, I performed experiments to demonstrate, indirectly, its involvement in insulin signaling during larval development. My hypothesis was that MMP2 in addition to playing a pivotal role in fat-body remodeling (Bond *et al.* 2011), is also involved in regulation of insulin signaling during the larval development. This hypothesis was first described at the model proposed by Nicole Bond (2010). Bond's findings suggest that *MMP2* is present at the extracellular matrix of the fat-body cells cleaving proteins that establish the cell to cell attachments during fat-body remodeling (Bond et al. 2011). Since, it is through the process of remodeling that the larva effectively distributes its energy fuels at the appropriate tissues, Bond (2010) hypothesized that *MMP2* might also act as an inhibitor of insulin signaling by cleaving dALS, dILP or other unverified IGF-BPs that are highly involved in the regulation of insulin pathway.

For this project, I used starvation as a way to turn off insulin signaling. By comparing fed and starved animals at three different larval developmental stages, I sought to identify the metabolic phenotypes involved in insulin signaling. First I tried to replicate the results presented from Britton *et al.*

(2002) which demonstrated that insulin signaling is turned off upon starvation. To test this, I took advantage of the GPH flies that express an insulin signaling marker fused with a fluorescent protein. Next, I hypothesized that *MMP2* gene expression in fat body should increase in response to starvation. I tested my hypothesis by quantifying the levels of *MMP2* expression for both starved and fed animals.

Materials and Methods

Fruit Fly Care

All fly stocks were kept at 25^{o}C in bottles containing the appropriate standard fly food. The food was rich in corn syrup, cornmeal, yeast, agar, malt, propionic acid (antifungal agent) and Tegosept (antifungal agent).

***Drosophila* Stocks and Crosses**

For the experiments conducted with transgenic flies the following genotypes were kept in stocks: *UAS-βftz-f1*-LA276, *y w; Lsp2-Gal4, UAS-GFP* (referred to here as *Lsp2::syb), UAS-SF-1* and w^{1118}. After several stocks were established, virgin flies were isolated from *UAS-βftz-f1*-LA276, *Lsp2::syb*. Finally, virgin females were crossed with males of the opposite genotype in an attempt to generate *UAS-βftz-f1-LA276 /Lsp2::syb* flies.

For the starvation experiments GPH^{8163} flies were used. In these flies the gene encoding the plekstrin homology domain is fused with a gene encoding the Green Fluorescent Protein (GFP) (Britton et al. 2002). When insulin signaling is on, the plekstrin homology domain is recruited to the membranes of the lipids in the fat-body cells because it has a high affinity for an intermediate insulin signaling product called PIP3. If insulin signaling is off we expect to see very weak fluorescence at the plasma membrane and signal localization in the fat-body cell nuclei. In contrast, when insulin signaling is on, we expect to see strong fluorescence signal localized at the plasma

membrane. Control w^{1118} flies were also used to perform a different set of starvation experiments.

UAS/Gal4 System

Gal4 is a transcription factor identified for the first time in *Saccharomyces cerevisiae*. This transcription factor has an affinity for the Upstream Activation Sequence (UAS) element (Duffy 2004). In *Lsp2::syb* flies *Gal4* expression is driven by the *Lsp2* gene. *Lsp2* is expressed in the larval fat body during the 3rd instar larval stage, which indicated that *Gal4* is driven during a specific time interval, at a specific tissue (Deustch *et al.* 1989). In *Lsp2::syb* flies, *syb*, a construct containing the GFP gene under the control of the UAS promoter is on the same chromosome as the Lsp2-Gal4 driver. Thus, the fat body in *Lsp2::syb* flies emits a fluorescent signal that can be detected using the microscope.

The UAS element acts upstream of a gene of interest to regulate the gene expression (in our case, *βftz-f1*gene expression in the UAS-*βftz-f1*-LA276 flies or *SF-1* gene expression in UAS-*SF-1* flies). For the expression of the gene to take place *Gal4* needs to bind at UAS which suggests that when *Gal4* is absent the expression of the gene of interest is blocked. In other words, flies with UAS-*βftz-f1*-LA276 genotype are unable to express the *βftz-f1*gene. However, when flies with UAS-*βftz-f1*-LA276 and *Lsp2::syb* genotypes are crossed, their progeny prematurely express the *βftz-f1* gene (as well as the GFP gene) specifically in the larval fat body during the 3rd instar larval stage (See figure 9). Similarly, when flies with UAS-*SF-1* and *Lsp2::syb* genotypes

are crossed, their progeny prematurely expresses the *SF-1* gene (as well as the GFP gene) specifically in the larval fat body during the 3rd instar larval stage (see figure 9).

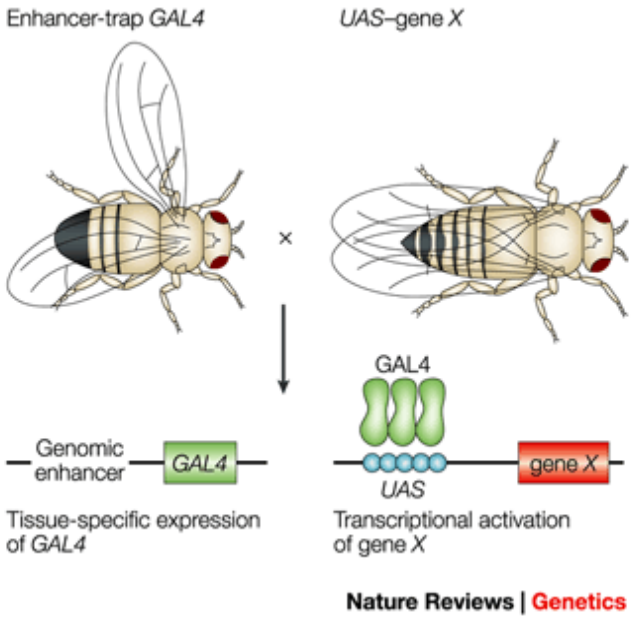

Figure 9. UAS/Gal4 System. In *drosophila* the UAS/Gal4 system is used to achieve the expression of a gene *x* in a specific tissue at a certain type of the development. The type of the "driver" determines the time and place of the gene *x* expression (Duffy, 2002). When Gal4 and UAS are separated in different stocks the gene *x* is inactive. Only upon crossing of these two genotype the gene *x* is expressed (Johnston 2002).

Staging 3rd Instar Larvae

In order to distinguish third instars at the feeding and wandering stage food containing 0.05% bromophenol blue was prepared (Andres and Thummel 1994). Larvae at the feeding stage have dark blue guts whereas the ones at wandering have guts with almost no blue dye.

Starvation Experiments

10-15 animals were put into 15% sucrose so that they get separated from food. Animals were washed with distilled water and then left for 3-4 hours in 20% sucrose in PBS prior to dissection (Scott et al. 2004, Reis et al. 2010). Dissection was time restricted. Animals were dissected after 3-4 hours of starvation.

Fat Body Dissection and RNA Isolation

Fat body tissue was collected from 2^{nd} instars, 3^{rd} instars (feeding phase) and 3^{rd} instars (wandering phase) in separate tubes. Each tube contained fat body tissue collected from approximately 4-5 larvae. Control animals were picked up for dissection from the bottles and starved animals were placed for 3 to 4 hours in 20% sucrose in PBS. The fat body tissue was collected in tubes containing 30µl Robb's PBS solution. 300µl of TRIzol reagent was added in each tube. For the completion of RNA isolation a protocol was used as described by Ryan Baugh. Finally the concentration of RNA in each tube was quantified and samples were stored at -20^{o}C.

DNA-Free/DNAse Treatment: Removal of Contaminating Genomic DNA

To ensure the purity of the RNA samples genomic DNA was removed using the Ambion DNA-free Kit. In each tube, it was added 1µl of 10X DNase buffer together with 1µl of rDNase-1. The samples were incubated at 37^{o}C for 30 minutes. The reaction was terminated with the addition of 1µl DNase inactivation reagent followed by the incubation of the samples at room temperature for 2 minutes.

First Strand cDNA Synthesis

The First strand synthesis kit from *Ambion* was used in order to convert RNA to a single strand DNA. The protocol as modified by Professor Craig Woodard was followed throughout the process. The RNA samples were treated with two different master mixes at different temperatures (Tables 1 and 2).

Table 1. Master Mix 1 added at 65 ^{o}C.

Component	Amount for 1 Reaction
RNA	**1µl**
10mM dNTP mix	**1µl**
Primer (0.5µg/µl oligo (dt))	**1µl**
DEPC-treated water	**7µl**

Table 2. Master Mix 2 added at 42 ^{o}C.

Component	Amount for 1 Reaction
10X RT Buffer	**2µl**
25mM MgCl2	**4µl**
0.1M DTT	**2µl**
RnaseOUT (40 U/µl)	**1µl**

The DNA synthesis was initiated by adding 1µl of SuperScript^{TM} II Reverse Transcriptase to each sample. The reaction was terminated at 70 ^{o}C. Finally, 1µl of RNase H was added and the samples were incubated at 37 ^{o}C for 20mins. cDNA samples were stored at -20 ^{o}C for later use.

RNA/DNA Quantification

The concentration of RNA/DNA samples was estimated using a nanodrop spectrophotometer (ND-1000 spectrophotometer). The ND-1000 V3.1.0 software was used to view the concentrations.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

In the experiments with the transgenic flies Polymerase Chain Reaction (PCR) was performed in order to detect the presence of *βftz-f1* and *SF-1* transcripts. Forward and reverse primers for both *βftz-f1* and *SF-1* genes were designed by previous students (Ayer 2008; Singh *et al* 2012) (see table 3).

Table 3. *βftz-f1*and *SF-1*Primer Sequences.

Primer	Sequence
***βftz-f1* (forward)**	5'-TTCGGACCCATGTACAAACGGGAT-3'
***βftz-f1* (reverse)**	5'-AGGAGGAGGAACCAATTCCAACGA-3'
***SF-1* (forward)**	5'-GCCAGGAGTTCGTCTGTCTC-3'
***SF-1* (reverse)**	5'-ACCTCCACCAGGCACAATAG-3'

To perform the RT PCR a *Techgene* thermocycler was used. The Stages of PCR reaction --specific to *βftz-f1*and *SF-1*-- together with the temperature, duration and number of repeats of each cycle is shown in Table 4.

Table 4. Thermocycler Profile for RT PCR (specific to *βftz-f1*and *SF-1* primers).

Stage	Temperature (^{0}C)	Time	Cycle Count
Separation	94	30 seconds	35 Cycles
Annealing	63	30 seconds	
Extension	72	30 seconds	
Final extension	72	5 minutes	1 Cycle
Final hold	4	-	-

Similarly, for the starvation experiments, PCR was used to detect *MMP2* transcripts at different larval developmental stages. Gene specific primers were used to perform the technique. Forward and reverse primers for both *MMP2* and *Actin5c* genes had been previously designed. The primer sequences were obtained by Kathryn Gorrski (2006) and are shown in table 5.

Table 5. *Actin5c* and *MMP2* Primer Sequences.

Primer	Sequence
***Actin5c* (forward)**	5'- TCTACGAGGGTTATGCCCTT-3'
***Actin5c* (reverse)**	5'-GCACAGCTTCTCCTTGATGT-3'
***MMP2* (forward)**	5'-AGCAATCCGGAGTCTCCAGTCTTT-3'
***MMP2* (reverse)**	5'-TGGAGCCGATTTCGTGATACAGGT-3'

Once again the *Techgene* thermocycler was used to conduct the experiment. The Stages of PCR reaction --specific to *Actin5c* and *MMP2*-- together with the temperature, duration and number of repeats of each cycle is shown in Table 6.

Table 6. Thermocycler profile for RT PCR (specific to *MMP2* and *Actin5c* primers).

Stage	Temperature (^{0}C)	Time	Cycle Count
Separation	94	30 seconds	35 Cycles
Annealing	55	30 seconds	
Extension	72	30 seconds	
Final extension	72	5 minutes	1 Cycle
Final hold	4	-	-

The reagents used for both PCR reactions described on this section were added in the order that they are presented in table 7. Gel electrophoresis was performed to detect the presence of *Actin5c* and *MMP2* in the PCR products and that of *βftz-f1* and *SF-1* respectively.

Table 7. RT PCR Reagents amounts used for each reaction.

Reagent	Amount added
10X PCR Buffer	**5µl**
50mM MgCl2	**3µl**
10nM dNTPs	**1µl**
10µM forward primer	**2µl**
10µM reverse primer	**2µl**
cDNA	**2µl**
Water	**34.6µl**
Taq Polymarese	**0.4µl**

Quantitative PCR

This technique was used to quantify the levels of expression of *Actin5c* and *MMP2* at different developmental stages. The master mixes used for both genes are shown in Tables 8 and 9. In each well 2.5 µl of cDNA were added together with 22.5 µl the appropriate master mix. The stages of qPCR reaction are shown in Table 10. The qPCR machine was a *7300 Real Time PCR System* and the software used to view the data was *7300System.* The data were analyzed as described in Pfaffl's paper (2001).

Table 8. *Actin5c* Master Mix for qPCR.

Reagent	Amount for 1 reaction
2.5X Real SYBR ROX	**11.25µl**
***Actin5c* (forward) 500nM**	**1.25µl**
***Actin5c* (reverse) 300nM**	**0.75µl**
Nuclease-free water	**9.25µl**

Table 9. *MMP2* Master Mix for qPCR.

Reagent	Amount for 1 reaction
2.5X Real SYBR ROX	**11.25µl**
***MMP2* (forward) 500nM**	**1.25µl**
***MMP2* (reverse) 500nM**	**1.25µl**
Nuclease-free water	**8.75µl**

Table 10. Thermocycler Profile for qPCR.

Stage	Temperature (^{o}C)	Time	Cycle Count
Taq Activation	**95**	**2 minutes**	**1 cycle**
Separation	**95**	**15 seconds**	**40 cycles**
Annealing	**55**	**30 seconds**	
Extension	**72**	**30 seconds**	
Dissociation	**-**	**-**	**1 cycle**

Standard Curves

Each qPCR experiment required the calculations of standard curves for the *Actin5c* and *MMP2* primers. These calculations were used to specify the efficiency of the primers used in each specific amplification experiment. A 12µl cDNA (1:1 dilution) sample taken from w^{1118} 3rd instar wandering animals was used to obtain 4 consecutive two-fold cDNA dilutions (1:2, 1:4, 1:8 and 1:16) for each standard curve. Each time, 6µl nuclease free water was added to the cDNA in order to achieve the desired dilution. Every well on the qPCR plate contained 2.5µl of the appropriate cDNA dilution. At the end of the experiment the C_t values from the samples were plotted against the log of

the cDNA concentrations and a linear standard curved was obtained. The primer efficiency was calculated using the formula presented above. Primer efficiencies were thought to be optimal if they were approaching the value of 2.

$$\text{Primer efficiency} = 10^{(-1/\text{standard curve slope})}$$

Experimental qPCR

The qPCR technique was used to compare the expression of *Actin5c* (reference gene) and *MMP2* (experimental gene) in fed and starved animals. cDNA samples taken from w^{1118} 3rd instars (wandering stage), 3rd instars (feeding stage) and 2nd instars –both fed and starved– were used. *Actin5c* was used as a house-keeping gene to ensure the success of the experiment. House-keeping genes are expressed at any time of the development in any tissue and are good markers to indicate that all the techniques were successfully performed.

For every cDNA sample 4 reactions were set: two w^{1118} (at the appropriate developmental stage) with *Actin5c* primers and two w^{1118} with *MMP2* primers. Additionally, there were two reactions set with the noRT cDNA as a negative control. In one reaction *Actin5c* primers were added whereas in the other *MMP2* primers were added. The aforementioned reactions took place in a 96-well plate (see table 9).

qPCR Calculations

For each duplicated reaction on the plate the average C_t value was calculated. These averages were used to calculate the $\Delta C_{t(MMP2)}$ and $\Delta C_{t(Actin5c)}$ which gives as the difference between reference gene expression (*Actin5c*) and experimental gene expression (*MMP2*).

$$\Delta C_{t(MMP2)} = C_{t(MMP2\ starved\ animals)} - C_{t(MMP2\ fed\ animals)}$$

$$\Delta C_{t(Actin5c)} = C_{t(Actin5c\ \text{starved animals})} - C_{t(Actin5c\ \text{fed animals})}$$

Next, the Pfaffl method (2001) was used to calculate the gene expression ratio between starved and fed animals. The ratio is expressed as the *MMP2* primer efficiency raised to the $\Delta C_{t(MMP2)}$ over the *Actin5c* primer efficiency raised to the $\Delta C_{t(Actin5c)}$.

$$ratio = \frac{E(MMP2)^{\Delta Ct(MMP2)}}{E(Actin5c)^{\Delta Ct(Actin5c)}}$$

Fluorescent Microscopy

To obtain images from the transgenic *Lsp2::syb*, UAS-*βftz-f1*-LA276 and UAS-*SF-1* flies the inverted microscope (Nikon TE2000) was used.For the starvation experiments, fat-body cells were observed once again under the inverted microscope (Nikon TE2000) in order to see theinsulin signaling. *MetaVue* imaging software was used to obtain pictures of fat-body cells.

Statistical Analysis

The Student's t-test (one-tailed) was used to decide whether the results obtained from qPCR were statistically significant. Results were considered statistical significant if the p value was less than 0.05.

Results

A. ***Transgenic Fly Experiments on SF-1***

Presence of βftz-f1 Transcripts

Gel electrophoresis was performed to view the RT-PCR results. RT lanes represented RNA samples that were treated with the enzyme reverse transcriptase. These samples were successfully converted into a cDNA. As I expected *βftz-f1* gene expression in *lsp2::syb/UAS-βftz-f1-LA276* late 3rd instar larvae was confirmed. The *βftz-f1* primer detected the βftz-f1 cDNA construct and amplified it (See arrow on RT lane, figure 10). In contrast, the *βftz-f1* primer was unable to detect any βftz-f1 cDNA constructs in w^{1118} (wild type control) and *lsp2::syb*/*UAS-SF-1* late 3rd instar larvae (wandering stage). noRT lanes represent RNA samples that lacked the enzyme reverse transcriptase and they were used as a negative control to ensure the quality of the results. Lack of this enzyme does not permit cDNA synthesis to occur. As we expected, noRT lanes displayed no bands on the gel. Finally, bands with less than 100bp were identified as dimers of the *βftz-f1* primer.

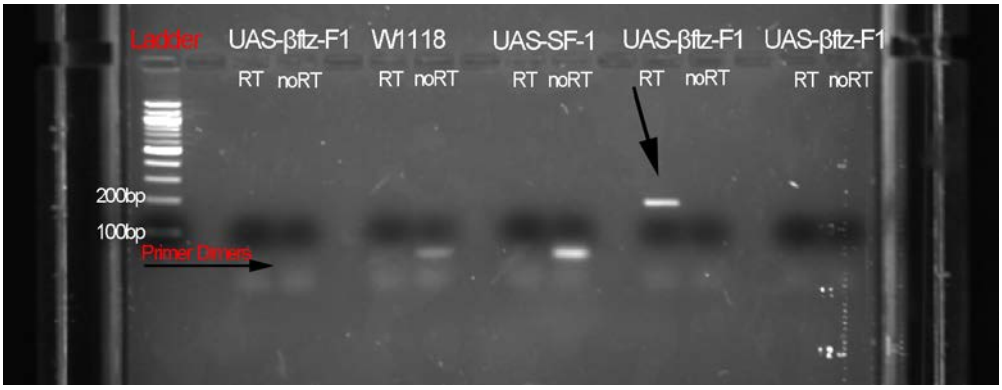

Figure 10. *βftz-f1* Primer-Specific RT-PCR Gel. Gel electrophoresis was used to detect gene expression. The black arrow shows that *βftz-f1* was expressed in late 3rd instar larvae. A 1000bp ladder was used to approximate the length of the detected transcript (far left lane). *βftz-f1* transcript is roughly 195bp long. Primer dimers are marked with the appropriate label on the gel.

Presence of SF-1 Transcripts

An additional RT-PCR experiment, in which *SF-1* primers were used, showed that the *SF-1*gene expression in *lsp2::syb/UAS-SF-1* larvae occurred during late 3rd instar wandering stage. In other words, *SF-1* primers were able to detect and amplify the SF-1 cDNA construct (See black arrow on RT lane, figure11).On the contrary, *SF-1* primers were unable to detect any SF-1 cDNA transcript in w^{1118} and *lsp2::syb/UAS-βftz-f1*late 3rd instar larvae. noRT lanes did not display any bands on the gel. As pointed out previously, bands with less than 100bp were marked as *SF-1* primer dimers.

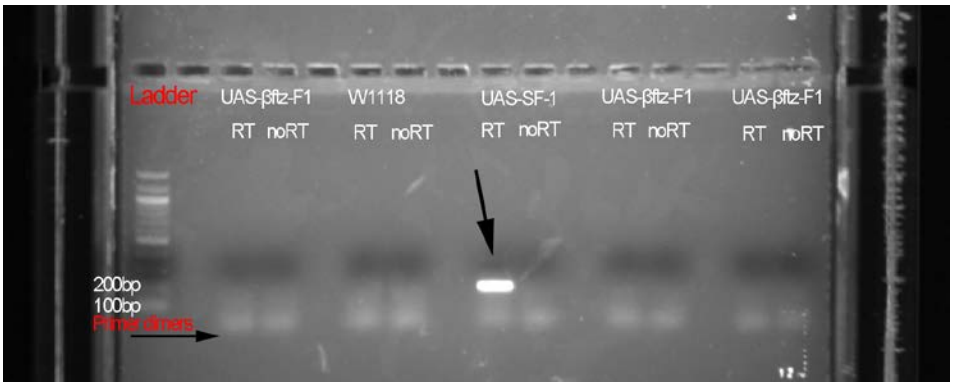

Figure 11. *SF-1* Primer-Specific RT-PCR Gel. Gel electrophoresis was used to detect gene expression. The black arrow shows that *SF-1* was expressed in *lsp2::syb/UAS-SF-1* late 3rd instar larvae. A 1000bp ladder was used to approximate the length of the detected transcript (far left lane). *SF-1* transcript is roughly 210bp long. Primer dimers are marked with the appropriate label on the gel.

Premature Fat-Body Remodeling

Fluorescence microscopy using *lsp2::syb*/*UAS-βftz-f1* 3rd instars (wandering stage) confirmed premature expression of *βftz-f1* as it showed premature fat-body cell remodeling. There were two fat-body remodeling stages observed: disaggregation and detachment. In particular, fat-body cells were amidst the dissociation stage (see figures 12c-e). Unlike *lsp2::syb/UAS-βftz-f1*fat-body cells, *lsp2::syb* and *lsp2::syb/UAS-SF-1* late 3rd instar larval fat-body cells were still intact and they retained their polygonal shape. In other words there were no indications suggesting that premature fat-body remodeling occurred at this stage (See figures 12a and 12b).

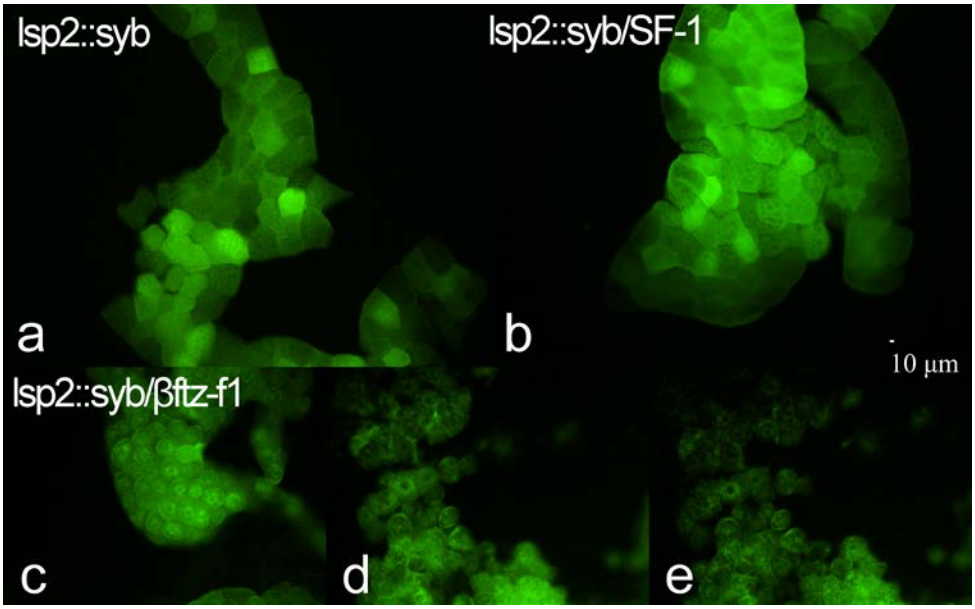

Figure 12. Premature Fat-Body Remodeling in Late 3rd Instar Larvae. Fat-body cells dissected in 5µl PBS solution**. (a)** Fat-body cells isolated from control *lsp2::syb* late 3rd instar larvae. The maintained their polygonal shape as they did not dissociate from each other. **(b)** Fat-body cells isolated from *lsp2::syb/ UAS-SF-1* late 3rd instars. Cells are similar, identical to the fat-body cells from *lsp2::syb* late 3rd instars. (**c-e)** Fat-body cells isolated from *lsp2::syb/UAS-βftz-f1*late 3rd instar larvae. Immediately after dissection, the cells' spherical shape was noted (12c). In some cases fat cells were unable to stay intact and instead they floated around (12d and 12e). Images were obtained using the 10x objective.

B. The Effects of Starvation on Insulin Signaling and *MMP2* Expression in *Drosophila Melanogaster* Larvae

Insulin Signaling Inhibition in Starved Larvae

Fluorescence microscopy revealed disparities in the levels of green fluorescent signal in larvae exposed to different feeding conditions. In particular, differences in fluorescence were apparent in the extracellular matrix fat-body cells of fed and starved larvae. In 2nd instars the fluorescent signal in the fed larvae was very strong whereas in the starved larvae it was weak or in some cases absent (figure 13). In other words, at the periphery of the cells from the fed larvae there was found a high localization of the fluorescent signal and lipid droplets were visible (Figure 13A). In starved larvae the fluorescent signal was localized mostly at the fat-body cells' nuclei and the lipid droplets were not distinguishable (Figure 13B).

Interestingly, some fat-body cell samples taken from 2nd instar starved larvae displayed different signaling characteristics. Specifically, there were areas in the fat body displaying strong fluorescent signal and other areas where the signal was absent (Figure 14B). To ensure the presence of fat-body cells on the specimen, a bright field image was taken. The comparison between the fluorescent and bright field image revealed that in some areas despite the presence of fat-body cells there was no fluorescent signal detected (Figures 14A and 14B).

In a similar manner, fat body tissue dissected from fed 3rd instar larvae (feeding stage) emitted a strong fluorescent signal that was concentrated at the periphery of the fat-body cell lipid droplets (Figure 15A). The fluorescent signal was able to confine the fat-body cells' extracellular matrix (Figure 15A). In contrast, fat body dissected from starved 3rd instars emitted a weak fluorescent signal that was mainly concentrated at the fat-body cells' nuclei (Figure 15B).

In contrast with the aforementioned findings, 3rd instar larvae (wandering stage) were constantly emitting the fluorescent signal independently of the feeding conditions (fed animals vs starved). For both fed and starved animals the fluorescent signal was found to be strong at the periphery of the lipid droplets (Figures 16A and 16B).

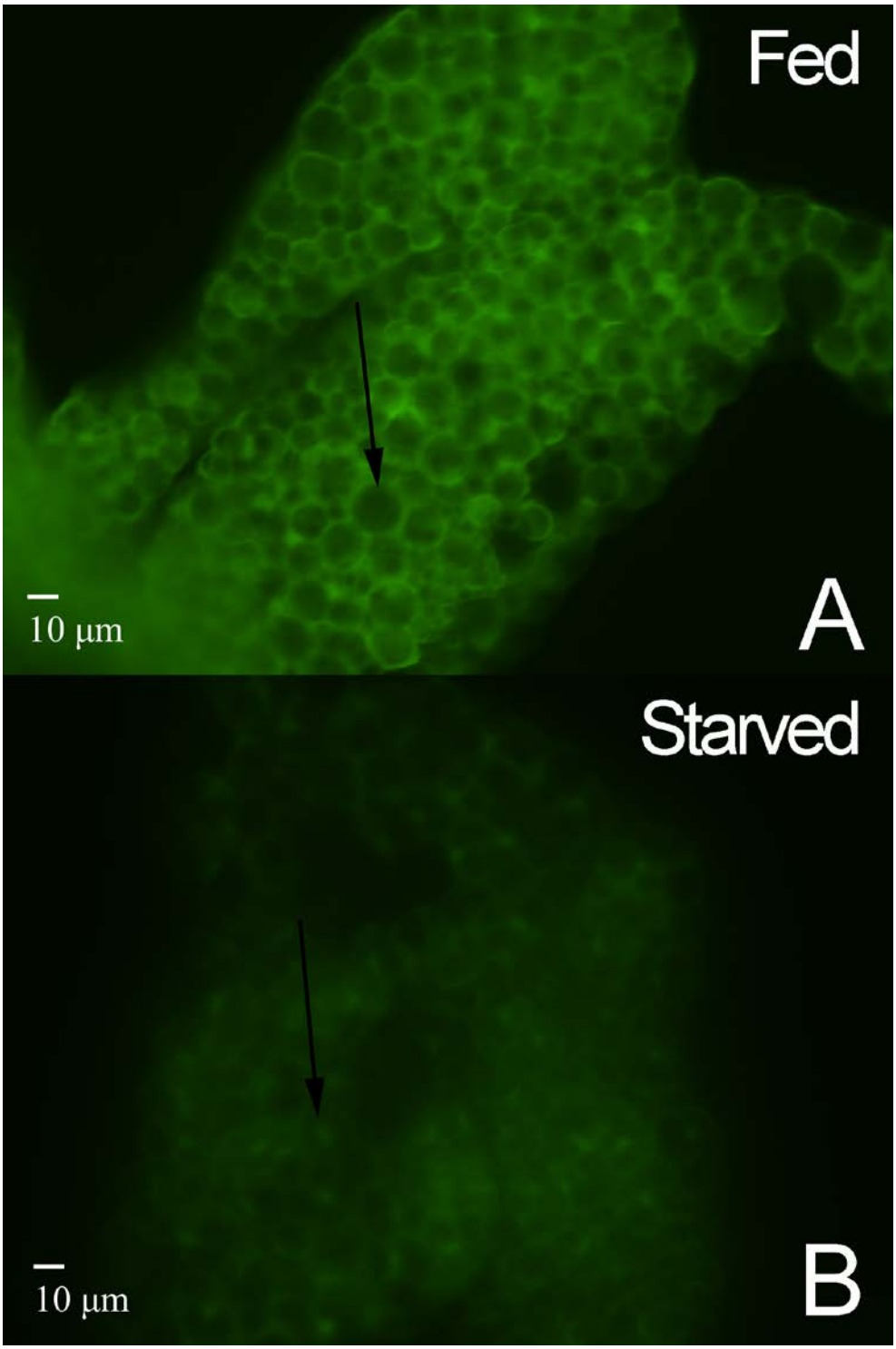

Figure 13. Insulin Signaling Detection in 2nd Instar Larvae Using the GPH System. (A) Fed larvae were displaying strong fluorescent signal. Black arrow indicates a fat body lipid droplet. **(B)** Starved larvae displaying a weak fluorescent signal. Black arrow indicates a fat-body cell nucleus. Pictures were obtained using the 40x objective.

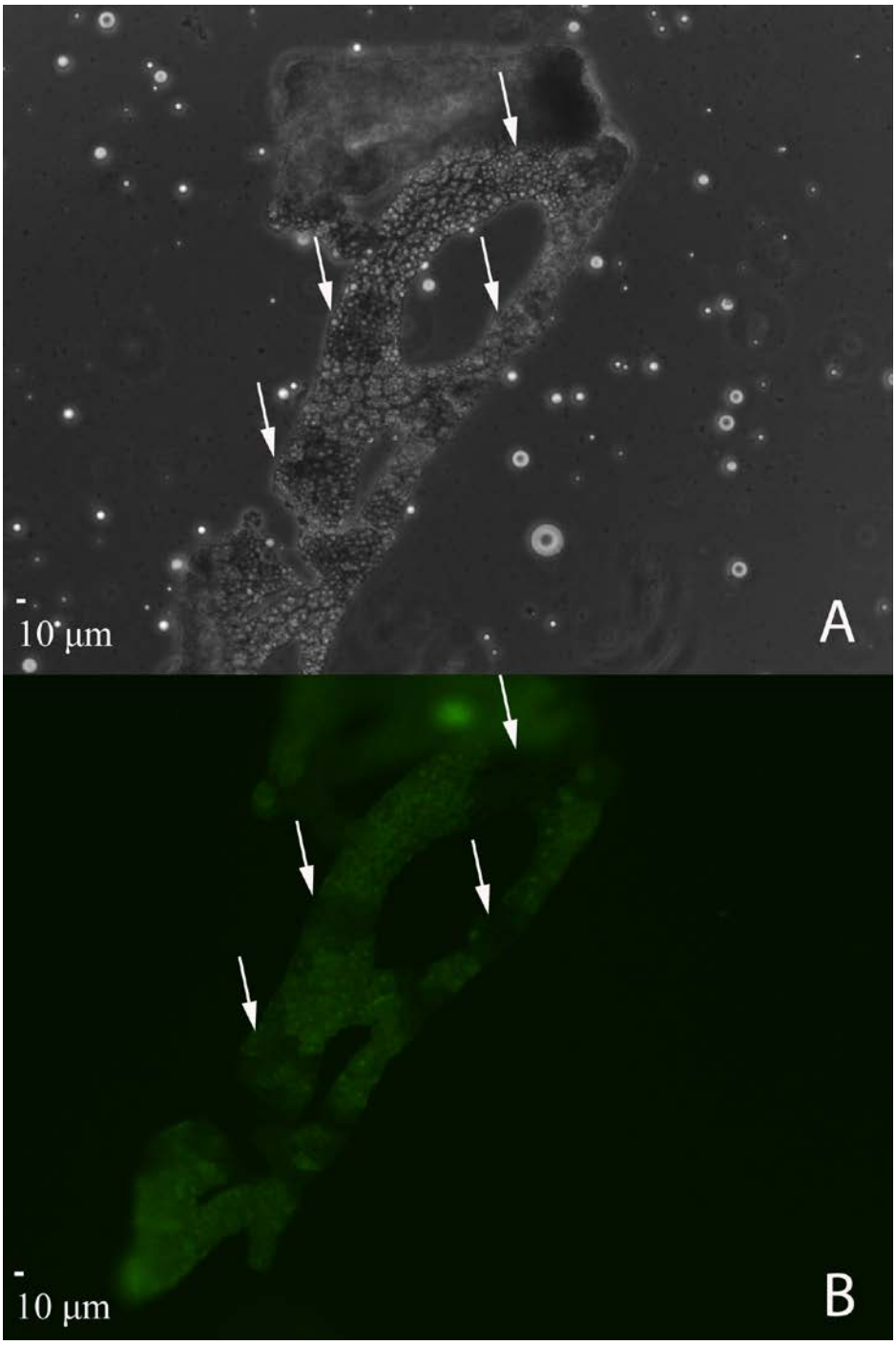

Figure 14. Partial Disruption of Insulin Singaling in 2nd Instar Starved Larvae. **(A)** Bright field microscopy displaying fat-body cell structure on the tissue. White arrows indicating the areas that were compared with the fluorescent microscopy. **(B)** Fluorescent microscopy showing partial disruption of insulin signaling in fat-body cells (see white arrows). Pictures were obtained using the 10x objective.

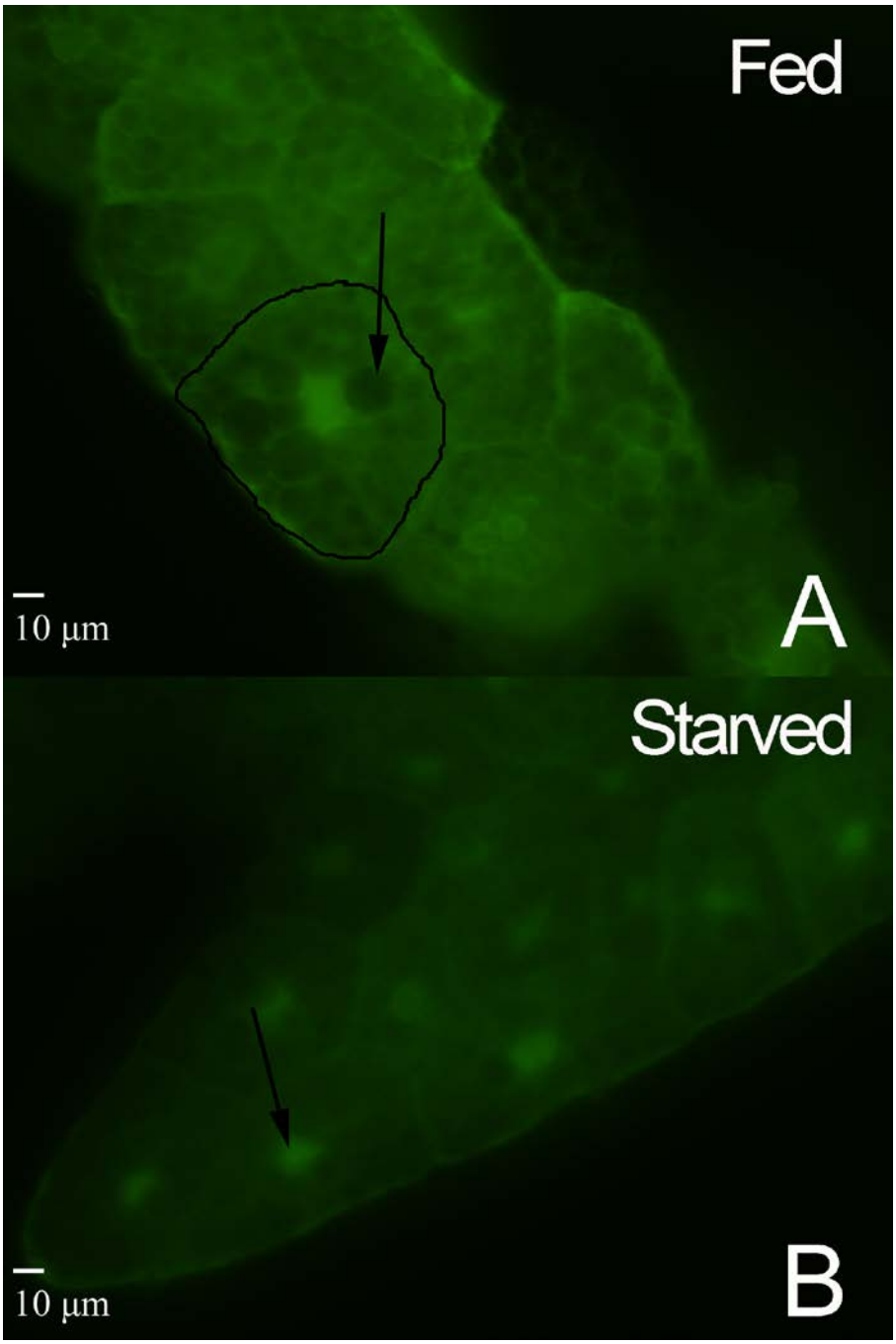

Figure 15. Insulin Signaling Detection in 3rd Instar Larvae (Feeding Stage) Using the GPH System. (A) Fat-body cells isolated from fed larvae displayed strong signal localization on the periphery of the lipid droplets. Black arrow shows a lipid droplet. A fat-body cell's extracellular matrix is traced with black color. **(B)** Fat-body cells isolated from starved larvae displayed overall a weak fluorescent signal. In addition, the signal was localized at the fat-body cells' nuclei. Black arrow shows a cell's nucleus. Images were obtained using the 40x objective.

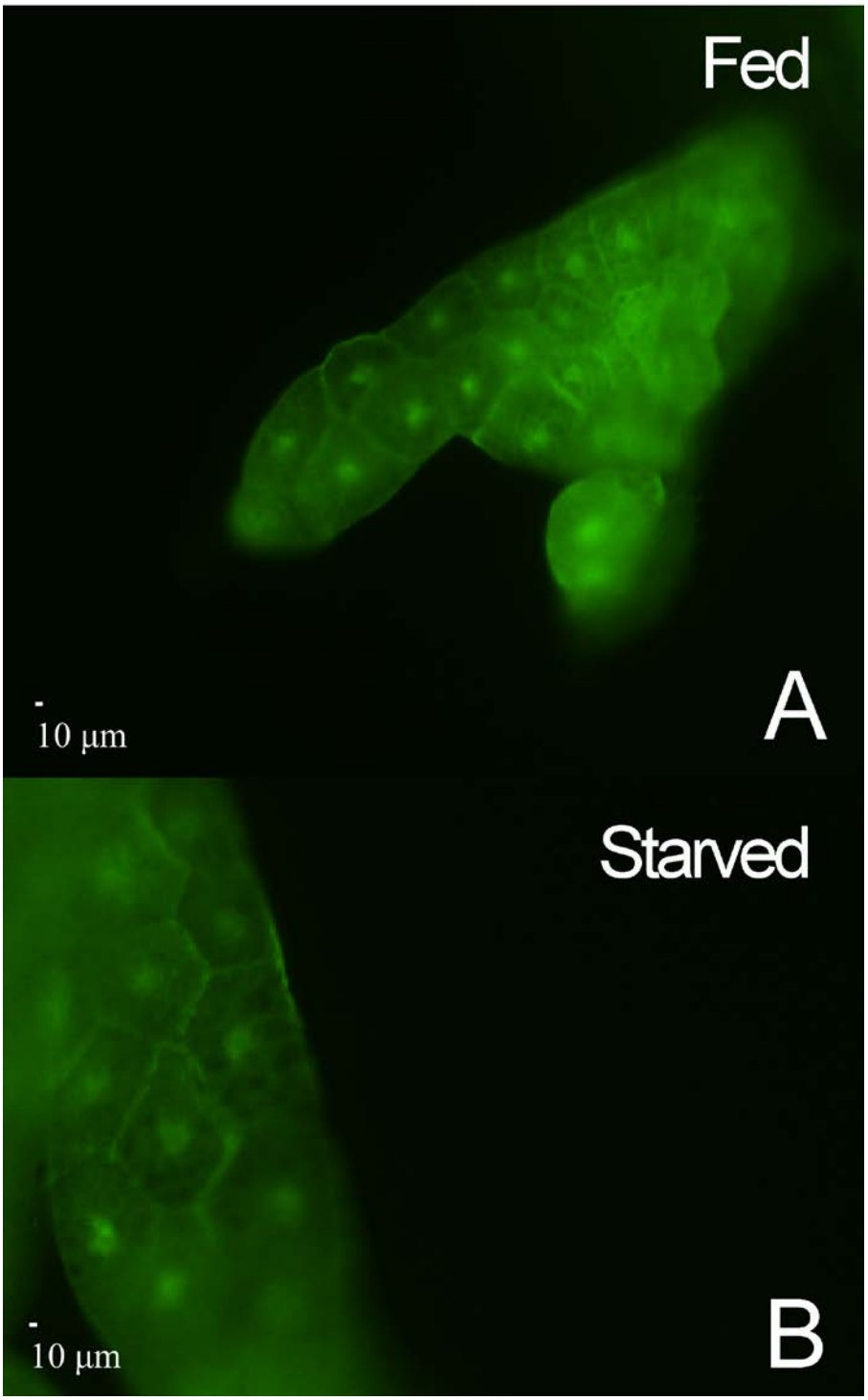

Figure 16. Insulin Signaling Detection in 3rd Instar Larvae (Wandering Stage) Using the GPH System. (A) Fed larvae displaying a strong fluorescence signal at the periphery of the lipid droplets. **(B)** Starved animals displaying a strong fluorescent signal at th e periphery of the lipid droplets as well. Images obtained using the 10x objective.

Presence of MMP2 Transcripts in Fed w^{1118} 3rd Instar Larvae

Gel electrophoresis was performed to view the RT-PCR results. RT lanes represented RNA samples that were treated with the enzyme reverse transcriptase. These samples were successfully converted into a cDNA. Both *Actin5c* and *MMP2* gene expression in fed w^{1118} 3rd instar larvae was confirmed. The primers detected both *Actin5c* and *MMP2* cDNA constructs and amplified them (See black arrows on RT lanes, figure 17). noRT lanes represent RNA samples that lacked the enzyme reverse transcriptase and they were used as a negative control to ensure the quality of the results. Lack of this enzyme does not permit cDNA synthesis to occur. Bands with less than 100bp were identified as dimers that both *Actin5c* and *MMP2* primers formed.

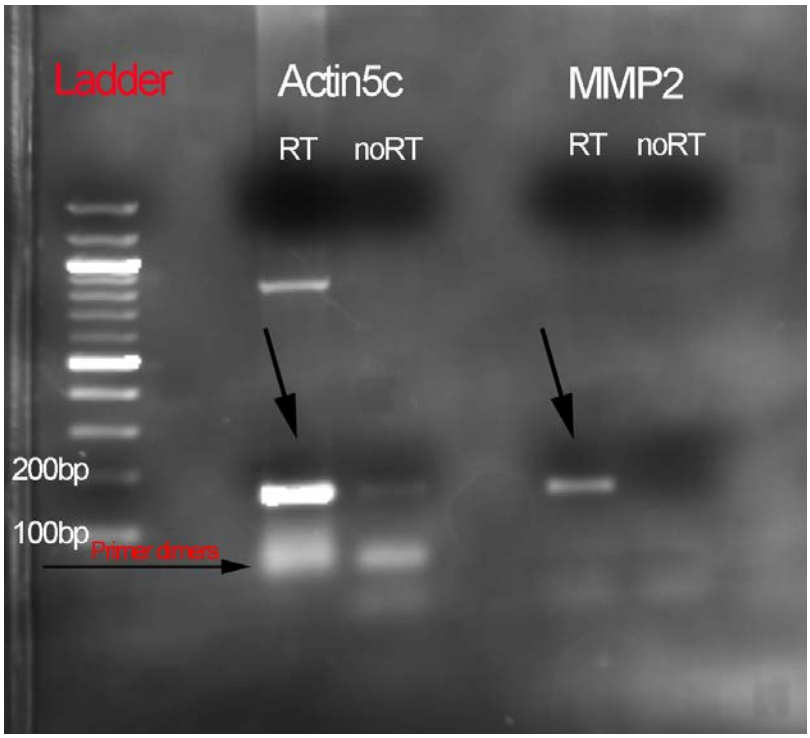

Figure 17. *Actin5c*/*MMP2* Primer-Specific RT-PCR Gel of Fed 3rd Instar Larvae. Gel electrophoresis was used to detect the presence of *Actin5c* and *MMP2* transcripts in 3rd instar larval fat body. The black arrow on the left shows that *Actin5c* was expressed in w^{1118} third instar larvae. Similarly, the black arrow on the right shows that *MMP2* was expressed. A 1000bp ladder was used to approximate the length of the detected transcript (far left lane). *Actin5c* transcript is roughly 180bp long whereas *MMP2* transcript is roughly 190bp. Primer dimers are marked with the appropriate label on the gel.

Presence of MMP2 Transcripts in Starved w^{1118} 3rd Instar Larvae

RT-PCR was performed using cDNA samples constructed from the fat-body cells dissected from starved 3rd instars. Both *Actin5c* and *MMP2* gene expression in these w^{1118} 3rd instar larvae was confirmed. The primers detected both *Actin5c* and *MMP2* cDNA constructs and amplified them (See black arrows on RT lanes, figure 18). noRT lanes represent RNA samples that lacked the enzyme reverse transcriptase and they were used as a negative control to ensure the quality of the results. Lack of this enzyme does not permit cDNA synthesis to occur. noRT lanes displayed no bands on the gel. Bands with less than 100bp were identified as dimers that both *Actin5c* and *MMP2* primers formed.

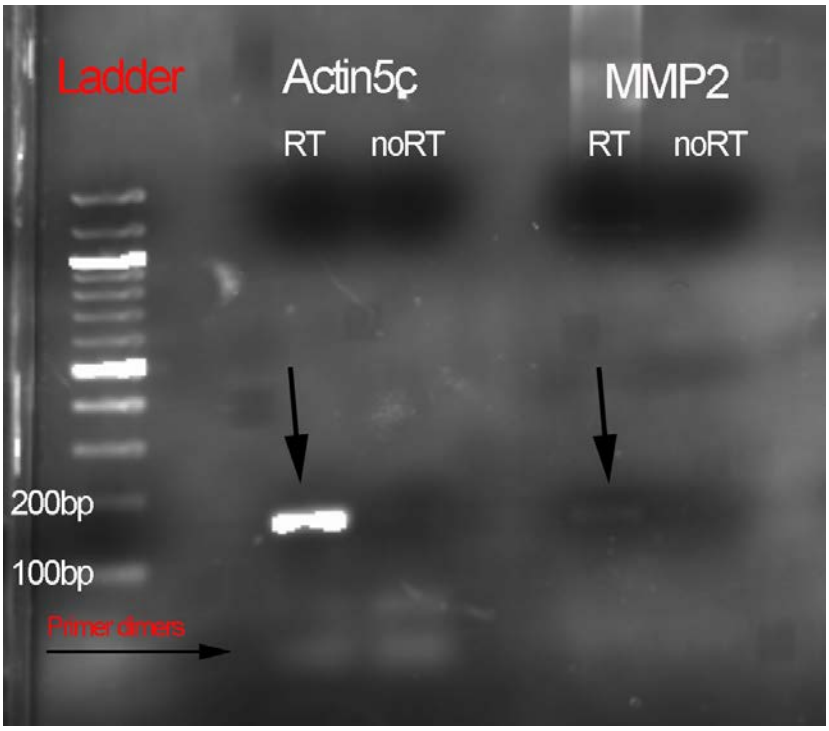

Figure 18. *Actin5c*/*MMP2* Primer-Specific RT-PCR Gel of Starved 3rd Instar Larvae. Gel electrophoresis was used to detect the presence of *Actin5c* and *MMP2* transcripts in 3rd instar larval fat body. The black arrow on the left shows that *Actin5c* was expressed in w^{1118} third instar larvae. Similarly, the black arrow on the right shows that *MMP2* was expressed. A 1000bp ladder was used to approximate the length of the detected transcript (far left lane). *Actin5c* transcript is roughly 180bp long whereas *MMP2* transcript is roughly 190bp. Primer dimers are marked with the appropriate label on the gel.

Experimental Quantitative PCR Analysis of MMP2 Gene Expression in Fed and Starved Larvae

I. Standard Curves and Primer Efficiency

Standard curves were set up for every experimental qPCR that was performed. Every experiment had standard curves for *Acti5c* and *MMP2.* The protocol used to set up standard curves is described in the materials and methods section. At the end of each experiment, standard curves were used to approximate the primer efficiency values. Below there are two graphs showing the standard curves of *Actin5c* and *MMP2* (figure 19 and 20). C_t values were plotted against the log of cDNA concentration corresponding with each cDNA dilution. The slope taken from the standard curve equations was used to calculate primer efficiency (table 1).

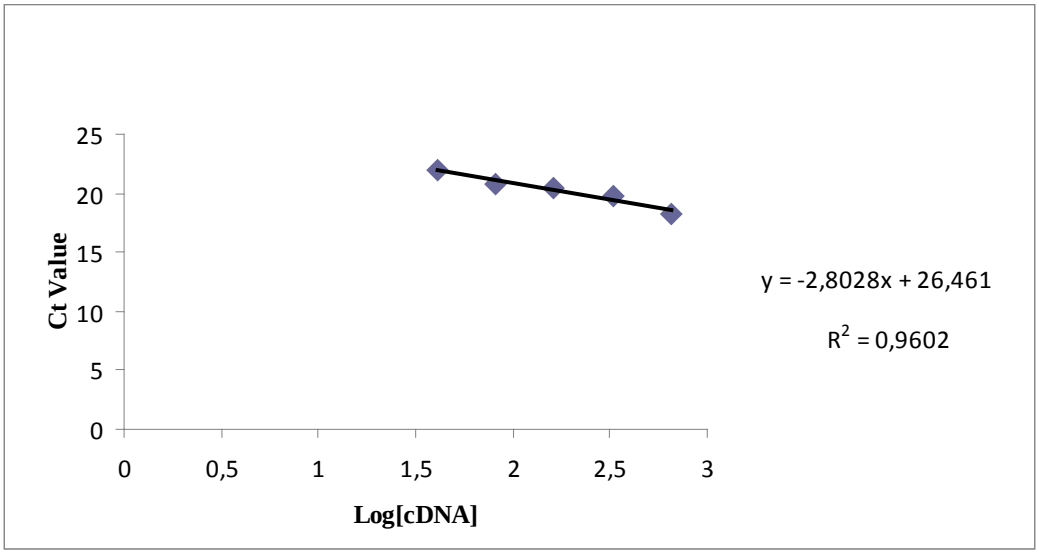

Figure 19. *Actin5c* Standard Curve. An equation was obtained from the trend line on this graph. The slope of the equation was used to calculate *Actin5c* primer efficiency.

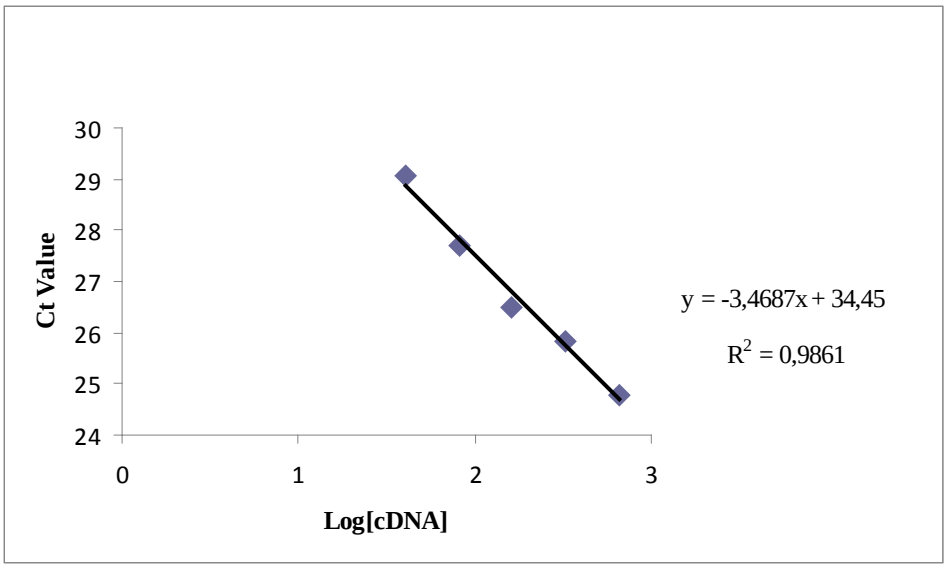

Figure 20. *MMP2* Standard Curve. An equation was obtained from the trend line on this graph. The slope of the equation was used to calculate *MMP2* primer efficiency.

Table 11. Primer efficiency values.

Primer	Efficiency = 10^(-1/slope)	Efficiency (%)
Actin5c	**2.27**	**127%**
MMP2	**1.94**	**94%**

II. Experimental qPCR Analysis

Quantitative PCR analysis showed that the levels of expression of *MMP2* are higher in starved animals than they are in fed animals. Figure 21 shows the average log of expression ration between fed and starved larvae at three different developmental stages (calculated from three different qPCR experiments). A positive value of the log of expression ratio indicates up-regulation of *MMP2* when animals were starved. As seen in the graph *MMP2* is upregulated in all three developmental stages in starved animals. The greatest upregulation value was observed in 3rd instars at wandering stage and the lower one was observed in 2nd instars. Statistical analysis showed that the differences observed between starved and fed animals were significant.

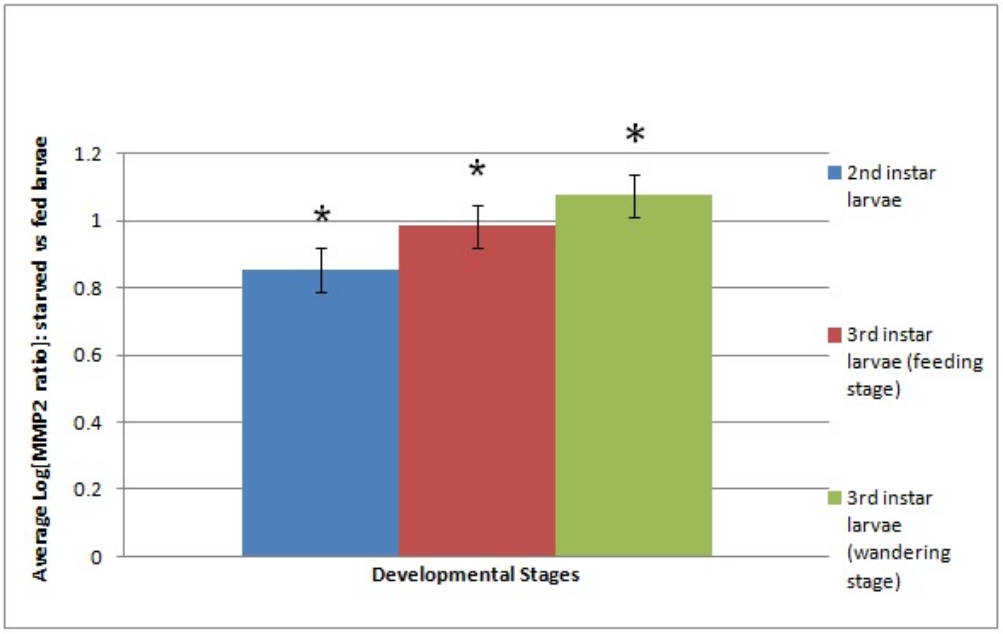

Figure 21. Quantitative PCR Analysis of *MMP2* Expression in *Drosophila melanogaster* Developmental Stages. The average log of expression ratio in three different developmental stages is shown. For all stages the log of expression ratio had a positive value. Standard error was calculated and error bars were placed on the graph (St.dev.= 0.11091469, St.er. = 0.064). *Statistical analysis was performed to ensure that the results were significant ($t_{statistical}$= 15.1596, $t_{critical}$=4.303, df=2, p value < 0.05).

Discussion

A. Premature *SF-1* Expression does not Induce Premature Fat-Body Remodeling

In this study I tried to identify functional similarities between βftz-f1 and its mammalian ortholog SF-1. The structural similarities between these two proteins (Lala et al 1992) suggested that they might also perform the same function. I expected that premature expression of SF-1 in the *Drosophila* fat body will cause premature fat body remodeling.

RT-PCR and gel electrophoresis revealed that the expression of *βftz-f1* and *SF-1* occurred successfully in both *lsp::2syb/UAS-βftz-f1* and *lsp2::syb/UAS-SF-1* transgenic flies respectively. However, fluorescence microscopy demonstrated that only *lsp2::syb*/*UAS-βftz-f1* 3rd instar larvae showed signs of premature fat-body remodeling which means that the results do not coincide with our prediction (figures 10a-c). The fat-body cells of these flies had already become spherical in shape and they showed signs of dissociation. In contrast, *lsp2::syb/UAS-SF-1* and *lsp2::syb* fat body showed no signs of fat-body remodeling (figures 10d-e). These findings suggest that premature expression of *SF-1*did not have the same effect in the fat body as *βftz-f1* expression had. Based on this observation, my initial hypothesis that there are functional similarities between these two orthologs is not supported.

Further experiments are necessary to understand whether SF-1 is involved in fat body remodeling indirectly or alternatively in other processes. Alternatively, It might be wise to consider using a different animal model to

directly study SF-1's involvement in cell remodeling and cell migration. Elucidating the mechanisms of the aforementioned processes will allow us to better comprehend wound healing and cancer metastasis.

B. MMP2: A Potential Insulin Singaling Regulator

Drosophila melanogaster responds to the various environmental changes in an attempt to maintain its internal conditions stable. One mechanism that helps the animal achieve homeostasis is insulin signaling. While, during larval development the animal prepares itself to undergo metamorphosis, it is to our great interest to observe how insulin signaling serves the animal's ultimate purpose. In this study, I sought to identify the key component responsible for nutrient homeostasis through out larval development. More specifically, I performed a series of experiments in order to identify a possible insulin signaling regulator. I hypothesized that matrix-metalloproteinase 2 could be the regulator in question. Being able to confirm MMP2's involvement in insulin signaling regulation we would gain great insight in *Drosophila* larval development and metamorphosis. Understanding in depth the mechanism involved in nutrient homeostasis would also give us the opportunity to use *Drosophila* as a model organism to study maladies such as diabetes or high blood pressure.

Insulin Signaling Regulation in Starved Larvae

Fluorescent microscopy performed using fat-body cells from 2nd and 3rd instars (feeding stage) revealed that insulin signaling is very week upon starvation (figure 12A-B and figure 13A-B). These results were anticipated since other scientists in the past have been able to obtain them (Britton et al. 2002). However this study shows for the first time what happens to 3rd instars (wandering stage) during starvation. Fluorescent microscopy revealed that 3rd

instars (wandering stage) do not respond to starvation (15A-B), meaning that the animals are unable to turn off insulin signaling.

It is apparent that both control and starved wandering 3rd instars ultimately experience starvation. Thus it was anticipated that both groups would show a significantly low fluorescent signal indicating insulin signaling downregulation. To our surprise the findings did not met our expectations. Our control wandering 3rd instars were in an environment where food was easily accessible even after they crawled out from food. Thus, it is possible they kept accumulating nutrients while searching for a place to form a puparium. However, we should have not observed the same with the experimental population. The starved wandering 3rd instars were taken away from any possible food sources which suggests that in this case insulin signaling should have successfully been turned off.

Since, in starved wandering 3rd instars insulin signaling is on, we can suggest a very interesting mechanism that larvae have developed over the years to ensure a successful transition from a 3rd instar (wandering stage) to a pre-pupa/pupa (metamorphosis). As a 3rd instar enters its wandering stage and while it searches for a place to attach itself it wants to avoid losing any previously stored energy that it is essential to the animal's survival during metamorphosis. As a result, wandering 3rd instars do not seem to respond at variations in nutrient availability in an attempt to maintain their energy sources intact.

Finally, although it was confirmed that insulin signaling in 2nd instars responds to changes in nutrient availability in a manner similar (figure 12A-B)

to that described from Britton *et al* (2002), it should be noted that there were cases in which insulin signaling was only partially disrupted in the fat body (13A-B). This observation might help us understand whether fat-body cells respond independently at these changes or as a whole. In fact, the previously presented findings suggest that fat-body cells sense nutrient availability in an independent manner.

Insulin Signaling Downregulation by MMP2 during Starvation

RT-PCR results showed that *MMP2* is expressed in the fat body of both fed and starved animals. Since it was hard to draw any significant conclusions from such findings it was necessary to assess the relative levels of *MMP2* expression in both groups. In all larval developmental stages, qPCR indicated that the level of *MMP2* expression in fat-body cells was greater in starved larvae then in fed larvae. Increased levels of *MMP2* expression in the fat body upon starvation suggest that MMP2 down regulates insulin signaling when larvae experience conditions of great nutritional deprivation in order to allow for nutrient release to occur.

MMP2 does not Act Independently to Regulate Insulin Signaling

As previously discussed wandering 3rd instars regulate insulin signaling differently. Despite this, qPCR demonstrated that upon starvation these animals had higher levels of *MMP2* expression than the control population. These rather controversial findings might suggest that MMP2 does not regulate insulin signaling in an independent manner. In fact this

observation coincides with Nicole Bond's hypothesis (2010) that insulin signaling regulation requires both the formation of the IGF-BPs-IGF trimeric complexes and the expression of *MMP2.* Alteration on the levels of expression of *MMP2, dALS* and *Imp-L2* can up regulate or down regulate insulin signaling accordingly (Arquiel *et al.* 2008 and Honegger 2008).

Future Studies

To further elucidate the molecular basis of insulin signaling in *Drosophila* it is important to perform additional experiments. First, it is necessary to confirm that MMP2 does not regulate insulin signaling in an independent manner. Being able to perform starvation experiments with MMP2 mutant flies will allow us to observe any differences in the fat body cells. If Insulin signaling is not properly regulated then we could except to see differences in cells and/or lipid sizes. In particular if our hypothesis is correct we would expect fat-body cells isolated from MMP2 mutants to store more energy in the form of lipid droplets.MMP2 mutants could also be used to perform feeding behavior assays. If insulin signaling cannot be turn off at the absence of MMP2, we could predict that MMP2 mutant larvae will feed more than wild type larvae.

Alternatively it would be useful to quantify the levels of *MMP2* expression at all larval stages and at various time points of the pupal stage. Any changes in the levels of *MMP2* expression can provide us with useful information about the process of metamorphosis. In particular we could better

understand how insulin signaling and fat-body remodeling are regulated simultaneously by MMP2.

Appendix

Table 12. Abbreviations found in this study.

Abbreviation	Name
20E	20-Hydroxyecdysone
APF	After Puparium Formation
bp	Base Pair
BR-C	Broad Complex
dALS	*Drosophila* Insulin-like Growth Factor-Binding Protein Acid-Labile Subunit
dBlimp-1	B Lymphocyte-Induced Maturation Protein-1
dILP	*Drosophila* Insulin-Like Protein
EcR	Ecdysone Receptor
EcR-DN	Dominant Negative form of Ecdysone Receptor
Ftz	Fushi-Tarazu
GFP	Green Fluorescent Protein
IGF	Insulin-Like Growth Factor
IGF-BP	Insulin-Like Growth Factor-Binding Protein
IIS	Insulin/Insulin-Like Growth Factor Signaling Pathway
Imp-L2	Ecdysone-Inducible Gene L2
InR	Insulin Receptor
JH	Juvenile Hormone

Lsd2	**Lipid Storage Protein-2**
MMP2	**Matrix-metalloproteinase 2**
PBS	**Phosphate-Buffered Saline**
PCR	**Polymarase Chain Reaction**
PI3	**Phosphatidylinositol-3**
PTTH	**Prothoracicotropic Hormone**
qPCR	**Quantitative Polymerase Chain Reaction**
RT	**Reverse Transriptase**
SF-1	**Steroidogenic Factor 1**
TOR	**Target of Rapamycin**
TIMP	**Tissue Inhibitor of Matrix-Metalloproteinases**
UAS	**Upstream Activation Sequence**
USP	**Ultraspiracle**

Table 13. qPCR 96-well Plate Layout Used to Set up the Experiment with *Actin5c* and *MMP2* Primers and the Appropriate cDNA samples.

	1	2	3	4	5	6
A	St. C. und. *Actin5c*	St. C. 1:2 *Actin5c*	St. C. 1:4 *Actin5c*	St. C. 1:8 *Actin5c*	St. C. 1:16 *Actin5c*	Actin5c no cDNA template
B	St. C. und. *MMP2*	St. C. 1:2 *MMP2*	St. C. 1:4 *MMP2*	St. C. 1:8 *MMP2*	St. C. 1:16 *MMP2*	MMP2 no cDNA template
C	w^{1118} ctrl 3rd instar wand. RT *Actin5c* (1)	w^{1118} ctrl 3rd instar wand. RT *Actin5c* (2)	w^{1118} ctrl 3rd instar wand. RT *MMP2* (1)	w^{1118} ctrl 3rd instar wand. RT *MMP2* (2)	w^{1118} ctrl 3rd instar wand. nRT *Actin5c* (1)	w^{1118} ctrl 3rd instar wand. nRT *MMP2* (1)
D	w^{1118}3h starv. 3rd instar wand. RT *Actin5c* (1)	w^{1118}3h starv. 3rd instar wand. RT *Actin5c* (2)	w^{1118}3h starv. 3rd instar wand. RT *MMP2* (1)	w^{1118} 3h starv. 3rd instar wand. RT *MMP2* (2)	w^{1118}3h starv. 3rd instar wand. nRT *Actin5c* (1)	w^{1118} 3h starv. 3rd instar wand. nRT *MMP2* (1)
E	w^{1118} ctrl 3rd instar food RT *Actin5c* (1)	w^{1118} ctrl 3rd instar food RT *Actin5c* (2)	w^{1118} ctrl 3rd instar food RT *MMP2* (1)	w^{1118} ctrl 3rd instar food RT *MMP2* (2)	w^{1118} ctrl 3rd instar food nRT *Actin5c* (1)	w^{1118} ctrl 3rd instar food nRT *MMP2* (1)
F	w^{1118} 3h starv. 3rd instar food RT *Actin5c* (1)	w^{1118} 3h starv. 3rd instar food RT *Actin5c* (2)	w^{1118} 3h starv. 3rd instar food RT *MMP2* (1)	w^{1118} 3h starv. 3rd instar food RT *MMP2* (2)	w^{1118} 3h starv. 3rd instar food nRT *Actin5c* (1)	w^{1118} 3h starv. 3rd instar food nRT *MMP2* (1)
G	w^{1118} ctrl 2nd instar RT *Actin5c* (1)	w^{1118} ctrl 2nd instar RT *Actin5c* (2)	w^{1118}ctrl 2nd instar RT *MMP2* (1)	w^{1118}ctrl 2nd instar RT *MMP2* (2)	w^{1118} ctrl 2nd instar nRT *Actin5c* (1)	w^{1118} ctrl 2nd instar nRT *MMP2* (1)
H	w^{1118} 3h starv. 2nd instar RT *Actin5c* (1)	w^{1118} 3h starv. 2nd instar RT *Actin5c* (2)	w^{1118} 3h starv. 2nd instar RT *MMP2* (1)	w^{1118} 3h starv. 2nd instar RT *MMP2* (2)	w^{1118} 3h starv. 2nd instar nRT *Actin5c* (1)	w^{1118} 3h starv. 2nd instar nRT *MMP2* (1)

Table 14. Loading Scheme for RT-PCR Using βftz-f1 and SF-1 Primers

Lanes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Genotype	1000 bp Ladder		*UAS-βftz-f1*			w^{1118}			*UAS-SF-1*			*UAS-βftz-f1*			*UAS-βftz-f1*	
				NO			NO			NO			NO			NO
			RT	RT		RT	RT		RT	RT		RT	RT		RT	RT

Table 15. Loading Scheme for RT-PCR Using Actin5c and MMP2 Primers.

Lanes	1	2	3	4	5	6	7
Genotype	1000bp Ladder		w^{1118} *(Actin5c)*			w^{1118} *(MMP2)*	
				NO			N
				RT		RT	O
			RT				T

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