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The Role of βFTZ-F1 and MMP2 in Regulating Hormone-Mediated Autophagy and Insulin Signaling in the *Drosophila* Fat Body

by

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This paper was prepared
under the direction of
Professor Craig Woodard
for eight credits.

[for my family]

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ABSTRACT

All animals transition through several different stages during their development. Three major processes are involved in regulating the progression from one developmental stage to the next: cell growth, nutrient utilization and cell death. In *Drosophila melanogaster*, distinct developmental changes occur during feeding and non-feeding periods. The larval stage is characterized by extensive feeding, while metamorphosis involves a long period of starvation (Aguila et al., 2007).

Insulin signaling is a highly conserved pathway that has long been known to play a role in regulating growth, nutrient storage and metabolism. Nutrient storage during the larval stage occurs primarily in the larval fat body, which functions as a hybrid of the mammalian adipose tissue and liver, and allows for the rapid growth of the animal (Britton et al., 2002).

As the animal enters metamorphosis, insulin signaling has to be shut off to allow for the breakdown of stored nutrients via autophagy- a conserved catabolic pathway that targets cytoplasmic constituents for degradation. Even though it is known that autophagy and insulin signaling are antagonistic processes, the molecular mechanisms that regulate these two mutually exclusive processes have not been fully characterized (Scott et al., 2004).

Fly metamorphosis is regulated in large part by the steroid hormone 20-hydroxyecdysone (20E). While most larval organs are destroyed by massive cell death during metamorphosis, the larval fat body escapes this fate and is instead remodeled from a sheet of attached polygonal cells into individual cells that disperse throughout the developing body, providing nutrients. Specifically, a proteinase called matrix metalloproteinase-2 (MMP2) is responsible for the proteolytic degradation of the extracellular matrix and dissociation of the fat body cells. Previous studies have suggested that MMP2 is a potential downstream target of the βFTZ-F1 mediated, 20E signaling cascade. βFTZ-F1 is a nuclear receptor that is required to confer competence upon tissues to respond to 20E (Bond et al., 2011).

In this study, I examined the role of MMP2 and βFTZ-F1 in regulating hormone-induced autophagy and insulin signaling. I hypothesize that MMP2, in addition to functioning in fat body remodeling, also downregulates insulin signaling during metamorphosis, thus triggering nutrient release by autophagy. Moreover, I hypothesize that βFTZ-F1 promotes autophagy in the fat body during metamorphosis. To test these hypotheses, I performed mosaic analyses with reduced *βftz-f1* and *MMP2* expression and looked for the presence of autophagosomal structures. I have demonstrated that βFTZ-F1 is required for autophagy during metamorphosis, while MMP2 does not seem to play a significant role in the regulation of autophagy during this stage.

Elucidating what factors link insulin signaling, hormone signaling and autophagy is important not only for understanding animal development, but it is also critical for understanding numerous diseases characterized by inappropriate regulation of these cellular processes.

INTRODUCTION

CHAPTER 1: AN OVERVIEW

Like most animals, holometabolous insects transition through several different stages during their development. While some animals go through minimal morphological changes (i.e. babies look like miniature adults), other animals can face more drastic changes throughout their life cycle. One of the most extreme examples of developmental change occurs during insect metamorphosis. The fruit fly *Drosophila melanogaster* is capable of resculpturing its whole body on a massive scale: it starts in its juvenile phase as a larva and then develops into an adult fly. This process requires tremendous reorganization and elimination of cells and tissues from one stage to the next. Progression through the different developmental stages involves the accurate coordination of cell differentiation, cell growth, and cell death. Maintaining a strict balance between these cellular processes is critical for regulating animal homeostasis and ensuring survival (Aguila et al., 2007).

Most cellular processes require nutrients. Under favorable conditions, excess nutrients are stored as fat and glycogen. As nutrients become limited, energy homeostasis is disrupted and stored nutrients must be utilized in order to promote animal survival. Failure to adjust in response to stress and changes in nutrient availability can result in numerous diseases, including cancer, diabetes, and high blood pressure (Britton et al., 2002; Scott et al., 2004).

Drosophila development is regulated by three distinct but intertwined processes: insulin signaling, steroid 20-hydroxyecdysone signaling, and autophagy. In response to environmental signals, such as low nutrient levels, animals have evolved strategies to adjust their metabolic and cellular activities. For example, during periods of starvation, animals can survive by breaking down internal stores of energy and reallocating resources. This is achieved through a catabolic process known as autophagy – autophagy promotes cell survival during periods of starvation by sequestering cytoplasmic components into lysosomes for degradation. Conversely, during favorable conditions, energy-demanding functions, such as protein synthesis and growth, are promoted to match the quality of nutrients (Tracy and Baehrecke, 2013).

Even though numerous studies have provided insights into the relationship between cell growth and cell death, the questions of *how* and *why* these signaling processes are integrated still remain unanswered. Studies in model organisms such as *Drosophila* will increase our understanding of the developmental roles and mechanisms of autophagy, insulin signaling and steroid hormones.

CHAPTER 2: FAT BODY REMODELING IN *DROSOPHILA MELANOGASTER*

Drosophila melanogaster, commonly known as the fruit fly, has been extensively studied for over a century as a model organism for genetic studies in eukaryotes. It has been more than 100 years since Thomas Hunt Morgan first

reported the identification of the *white* gene in *Drosophila melanogaster* (Morgan, 1910). After that, genetic approaches dominated the first 50 years of research in *Drosophila*, as scientists tried to unravel the basic principles of inheritance. Soon, investigators realized that the use of *Drosophila* was not limited to genetic research and the study of heredity. The continuous development of new research tools that happened during the past four decades has led to numerous milestone discoveries and will no doubt contribute to the future of biomedical research in many different ways. Today, fruit flies continue to be the choice model system for many scientists (Stephenson and Metcalfe, 2013).

Several features of the fruit fly make it an ideal model organism for genetic investigations. First, flies have a very short generation time. The life cycle of the fruit fly lasts about 12 days at room temperature, so large-scale crosses can be set up and followed through several generations in a short period of time. Because fruit flies are quite small, it is easier to handle large fly quantities. Also, the low, manageable number of chromosomes in the fly's genome allows for easy genetic manipulations (Jennings et al., 2011). Importantly, over 60% of known human disease causing genes have a fly ortholog, thus making the fruit fly an excellent genetic model for numerous human diseases, including many neurodegenerative disorders (Rubin et al., 2000).

2.1. Metamorphosis and fat body remodeling

The life cycle of *Drosophila melanogaster* consists of five distinct phases: embryogenesis, three larval stages, a prepupal stage, a pupal stage, and the adult

stage (Figure 1). During the last three days of larval development, *D. melanogaster* larvae undergo a dramatic increase in body mass, as nutrient reserves accumulate in the larval fat body. The larval fat body serves as an energy reservoir to support the animal during the non-feeding period of metamorphosis. At the end of the last larval stage, the animal stops feeding and wanders for 12-24 hours in search for an adequate site to pupariate. Importantly, after eclosion, the adult remains inactive for approximately 8 hours, until the wings inflate and the cuticle tans. This suggests that larvae must accumulate enough nutrients not only to fuel developmental reorganization of the pupa during metamorphosis but also to ensure that the animal survives during early adulthood (Aguila et al., 2013).

During metamorphosis, the larva undergoes dramatic morphological changes and becomes an adult. During this process, most of the obsolete larval organs undergo massive cell death, while tissues required for further development are retained and modified to meet the needs of the adult fly. The larval fat body escapes cell death and is instead remodeled from a sheet of attached, polygonal cells into individual spherical cells that freely disperse into other parts of the body and provide nutrients (Bond et al., 2011). The remodeling of the larval fat body during early metamorphosis is divided into three stages: retraction, disaggregation, and detachment. During the retraction phase, which begins 4 to 6 hours after puparium formation (APF), the fat body begins to retract from the anterior region of the prepupa. In the disaggregation phase, individual fat cells gradually dissociate from each other. Upon completion of prepupal development,

the detachment phase takes place: anterior fat body cells become spherical and some of them translocate into the head capsule (Nelliot et al., 2006).

The detachment of remodeled cells is brought about, in part, by proteases that degrade the extracellular matrix (ECM), which is responsible for maintaining tissue integrity (Bond et al., 2011). A specialized class of Zn^{2+}- and Ca^{2+}-dependent endopeptidases, the matrix metalloproteinases (MMPs), is responsible for the proteolytic degradation of the ECM and is often involved in tissue remodeling. The *Drosophila* genome contains only two MMPs: *MMP1,* and *MMP2*. MMP1 is a secreted protein, while MMP2 is a membrane bound protein with a GPI anchor site. Even though both proteins have the canonical MMP structure, they are not orthologs of any of the 24 mammalian MMPs. It is likely that the two fly MMPs diverged when insect and mammals shared a common ancestor. The expression of both MMPs coincides with the detachment phase of tissue remodeling in the larval fat body and each MMP is required for distinct aspects of tissue remodeling and programmed cell death during metamorphosis. MMP1 is required for larval tracheal remodeling, while MMP2 is involved in the programmed cell death of the larval midgut (Page-McCaw et al., 2003; Bond et al., 2011). Both MMPs seem to be necessary for head eversion and dendrite remodeling (Page-McCaw, 2008).

The catalytic activity of MMPs is inhibited by TIMP (tissue inhibitor of metalloproteinases), which suppresses MMP activity by occupying the active site of the protease. Previous studies have shown that mutants for *MMP2* or animals over-expressing *TIMP* fail to detach and translocate the fat body cells to the head

capsule during the prepupal-pupal transition (Bond et al., 2011). Based on these results, Bond et al. (2011) proposed a model for the tissue autonomous action of MMP2. They postulated that MMP2 might contribute to fat body remodeling by degrading the extracellular matrix between the larval fat cells, thus allowing for cell mobility. This would ultimately result in the breakup and dispersal of fat body cells and the release of nutrients during metamorphosis.

The process of fat body remodeling closely resembles some human disease process. Much like the process of fat body remodeling, proteolytic degradation of the ECM is a critical step in tumor cell invasion and intravasation (Lu et al., 2012). Thus, a deeper understanding of the processes involved in tissue remodeling in *D. melanogaster* can help enhance our understanding of the mechanisms regulating numerous biomedically relevant processes, such as cancer metastasis, air destruction in asthmatics, and wound healing.

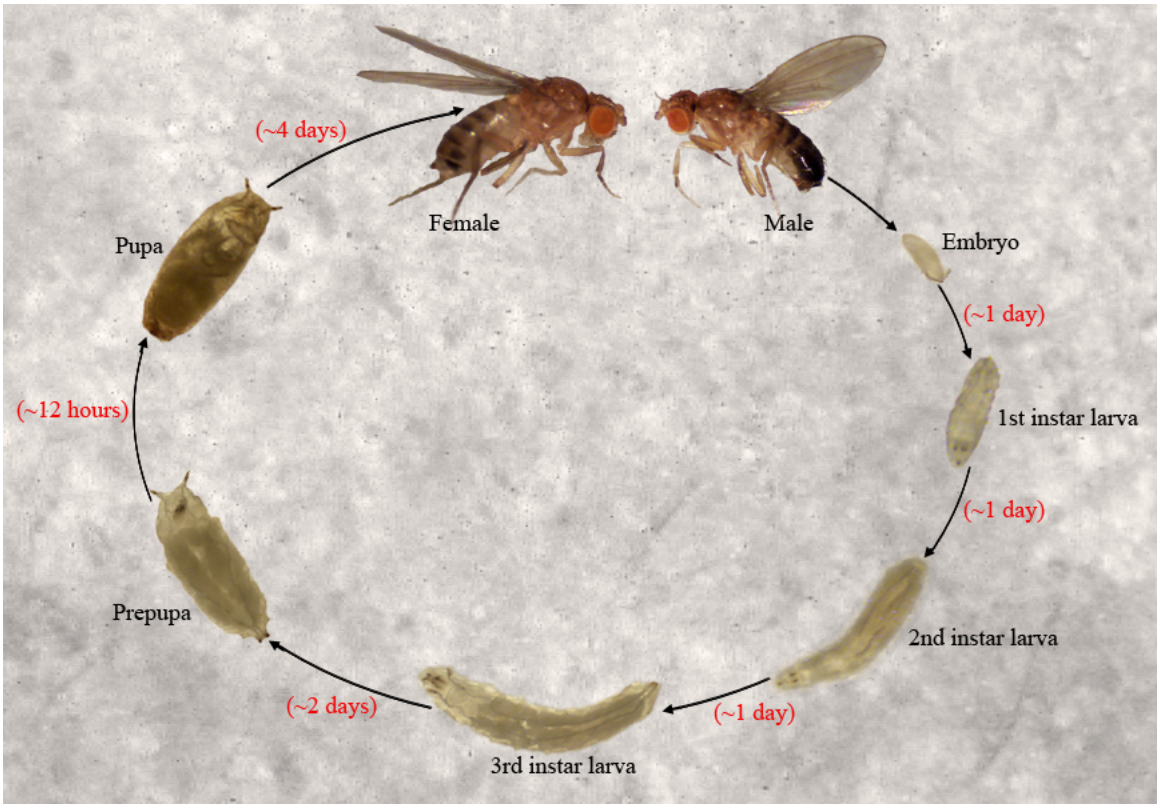


Figure 1. The life cycle of *Drosophila melanogaster*. The life cycle of *Drosophila melanogaster* takes about 12-14 days. A fruit fly begins its lives as an embryo. After one day, the embryo hatches and enters the larval stage, which is divided into first instar, second instar and third instar. During the three larval stages, the organism is constantly feeding and growing. About 24 hours before pupariation, the larva stops feeding and crawls out of the food source. At this time, the animal enters the wandering state, looking for a substrate where it can attach. Once it attaches to the substrate, the larva becomes a white, motionless prepupa. The transition into the prepupal stage marks the onset of metamorphosis, which lasts until eclosion (Aguila et al., 2013).

CHAPTER 3: ECDYSONE SIGNALING DURING METAMORPHOSIS

Steroid hormones (including testosterone, estrogens, progesterone, the corticosteroids and ecdysone) are synthesized from cholesterol and produced by endocrine glands. Because of their lipophilic character, steroid hormones can easily pass through cellular and nuclear membranes by diffusion. Once inside the

cell, they bind to intracellular receptors, which are regulated by hormonally responsive target cells and function as signal transducers and transcription factors by modulating gene expression without the need for an intervening cascade of protein kinase signal transducers. After binding to the ligand, steroid receptors often dimerize and can either augment or suppress gene transcription. Some receptors can also function as repressors in the absence of a ligand, thus maintaining gene silencing (King-Jones and Thummel, 2005).

Although numerous studies have provided some insights into the molecular mechanisms by which steroid hormones regulate the transcription of target genes, much remains to be known. Steroid hormones regulate various biological processes, including metabolism, reproduction, embryonic development, and cell death. Steroids also regulate major transitions in the life cycle in a stage-specific way (Xiaochun et al., 2012; Falkenstein et al., 2000). Consistent with these crucial regulatory roles, mutations in the steroid hormone signaling pathway can lead to numerous diseases, such as cancer, diabetes and heart disease (King-Jones and Thummel, 2005).

3.1. 20-hydroxyecdysone

In *Drosophila*, pulses of the steroid hormone 20-hydroxyecdysone (20E), also commonly known as ecdysone, are responsible for driving the metamorphosis of larval tissues into adult structures. Each pulse of ecdysone is unique in amplitude and duration – two parameters that are derived from the rate of hormone synthesis, how efficiently the hormone in converted into its active

form, and how quickly it is degraded (Moeller et al., 2013). Ecdysone belongs to the family of ecdysteroids, which play a role in cell proliferation, differentiation and apoptosis during insect development. In particular, ecdysone is responsible for upregulating the expression of genes required to control apoptosis and cell differentiation during metamorphosis. These processes are essential for tissue remodeling and the removal of larval structures that have become obsolete (Schwedes and Carney, 2012). As all steroid hormones, ecdysone is characterized by the steroid nucleus, composed of three six-membered rings, attached to one five-membered ring (Figure 2) (Browning et al., 2007).

During larval stages, ecdysteroids are produced primarily in the prothoracic gland (PG), a component of the ring gland, which also includes the corpora allata and the corpora cardiaca (Gilbert et al., 2002). The ring gland is the major endocrine organ of *Drosophila* and releases two types of ecdysteroids: α-ecdysone and 20-deosymakisterone. These hormones are believed to be largely inactive (Baker et al., 2003). Once released into the hemolymph, α-ecdysone is converted to its active form, 20-hydroxyecdysone, by P450 monooxygenase and reaches the target tissues (Gilbert et al., 2002). Some of the ecdysteroid-target tissues include the epidermis, fat body, salivary glands, wing imaginal discs and the midgut (Kamimura, 1997).

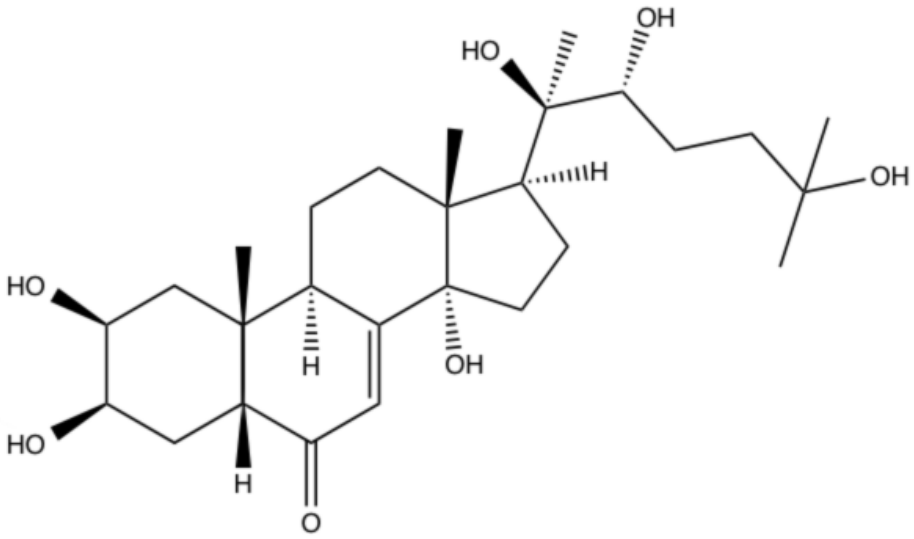


Figure 2. Chemical structure of 20-hydroxyecdysone. 20-hydroxyecdysone is an ecdysteroid that is primarily produced in the prothoracic gland and is transported via the hemolymph fluid. Like all steroid hormones, 20E consists of three six-membered rings and one five-membered ring.

The production and release of ecdysone is regulated, in part, by the insect neuropeptide prothoracicotropic hormone (PTTH). PTTH is a small, diffusible peptide that is produced by a pair of lateral neurosecretory neurons in the brain and travels along axons to the corpus allatum, where it is released into the circulation (Agui et al., 1979). Release of PTTH from PTTH-producing neurons is under the control of various environmental factors (e.g. photoperiod and temperature) and physiological cues (e.g. nutrition). Once released, PTTH binds to an unknown receptor on the prothoracic gland and triggers ecdysteroidogenesis (Yamanaka et al., 2013). The pathways within the PG that mediate the response to PTTH are not known. However, previous studies have demonstrated that the Ras/Raf pathway in the PG regulates the timing of metamorphosis (Caldwell et al., 2005) and that PTTH expression increases the levels of Erk (Rybczynski et

al., 2001). Taken together, these results raise the possibility that PTTH activates ecdysone synthesis via the Ras/Raf/Erk pathway (Figure 3). In line with the predicted role of PTTH in stimulating ecdysone synthesis, larvae lacking PTTH producing neurons show reduced ecdysone titers. However, ecdysone levels are never completely ablated, thus suggesting that other factors might be involved in modulating the onset of ecdysteroidogenesis. In fact, loss of PTTH does not result in metamorphosis arrest but rather in delayed larval development and eclosion (McBrayer et al., 2007).

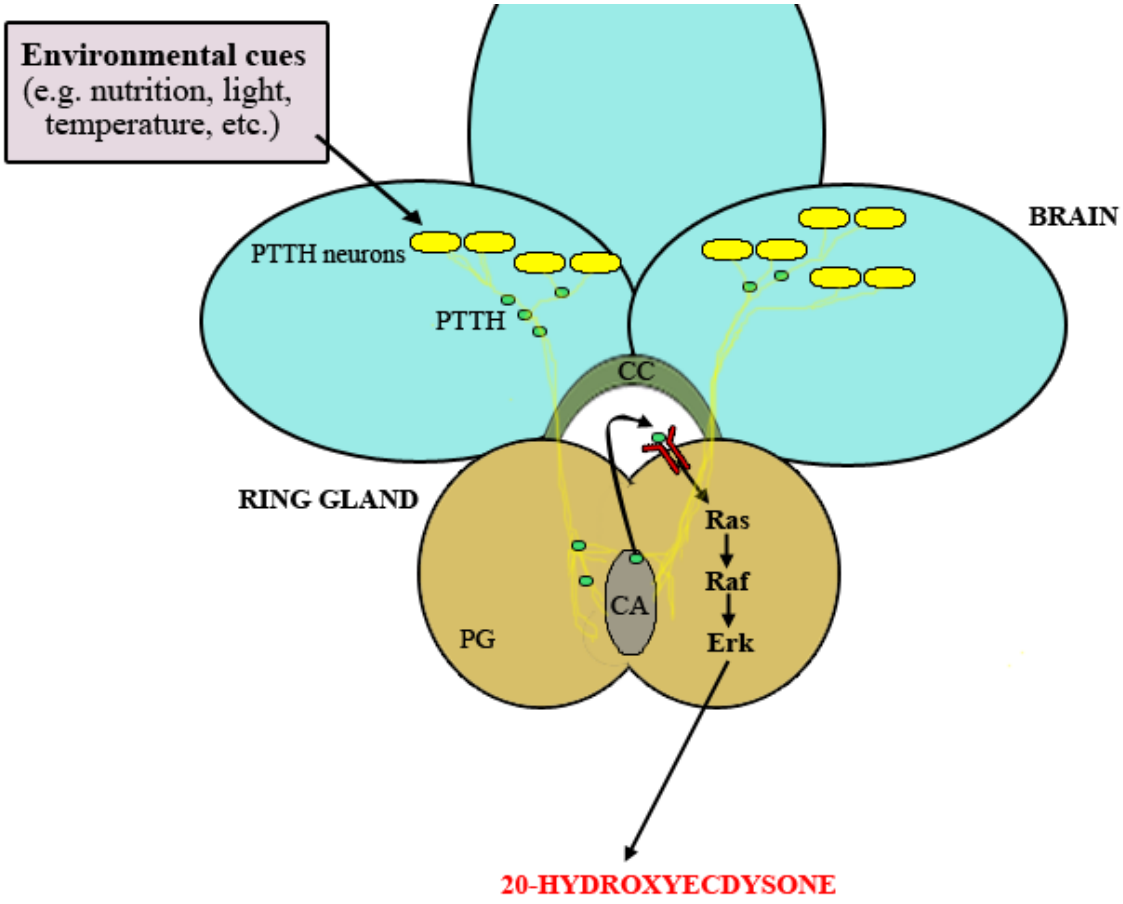


Figure 3. The regulation of 20-hydroxyecdysone synthesis by PTTH. Ecdysone synthesis is triggered when PTTH is released from PTTH-producing neurons, whose axons terminate on the corpus allatum (CA), a component of the ring gland, which also contains the prothoracic gland (PG) the corpus cardicum (CC). Once released from the corpus allatum into the hemolymph, PTTH targets the prothoracic gland via an unknown receptor and stimulates the release of ecdysone. The production and the release of 20-hydroxyecdysone require one (or more) second messenger pathways, including a MAP kinase cascade, as well as the conversation of the prohormone α-ecdysone into functional 20-hydroxyecdysone.

3.2. The Ashburner model

The first evidence that ecdysone plays a role in mediating gene expression came in 1959, when Becker noted puffing of the salivary gland polytene chromosomes when incubated with the ecdysteroidogenic ring gland. Becker

showed that these characteristic puff patterns enlarge and regress during development. Puffs are enlargements of specific loci detected in these oversized chromosomes and indicate the presence of transcriptionally active chromosomal sites. Moreover, it was found that some of the puffs responded rapidly to ecdysone (early puffs) while others only appeared later (late puffs) (Becker, 1959). Within a few minutes after the addition of the hormone to a medium containing 3rd instar *Drosophila* salivary glands, six early puffs are detected: 22B4-5, 23E, 63F, 74EF, 75B and 74C. After 4 hours, the early puffs begin to regress and ultimately disappear. A few hours later, up to 100 new puffs appear at unique chromosomal sites. These newly formed puffs, called late puffs, have been divided into early-late and late-late puffs, depending on their time of appearance (Ashburner, 1972).

Clever (1965) and later Ashburner (1974) tested whether protein synthesis was necessary for the induction of early and late puffs. The results showed that, in the presence of protein inhibitors, early puffs were still induced, while late puffs were no longer detectable. Ashburner also found that cycloheximide treatments prevent the regression of some early puffs. From these results, Ashburner postulated that early puffs are induced by ecdysone, while gene regression and later puffs require the synthesis of proteins encoded by the early genes (Ashburner et al., 1974). Based on these findings, Ashburner and colleagues proposed that the binding of a hormone-receptor complex to the chromosomes must induce the transcription of the early genes, while repressing the transcription of the late genes (Ashburner, 1972; Ashburner, 1973). Ashburner and Richards (1976)

suggested that early gene transcription products have to be made in sufficient quantity in order to compete with the ecdysone-receptor for binding sites on the DNA and thus induce late genes expression (Figure 4).

The Ashburner model has been supported over the years by molecular and genetic analyses of many early and late puffs. Several ecdysone response genes have also been identified outside of the early puff loci, suggesting that the hormone has a broader functional domain than originally thought (Hurban and Thummel, 1993). Currently, the search for new ecdysone-inducible genes is taking place at the genomic scale (Beckstead et al., 2005).

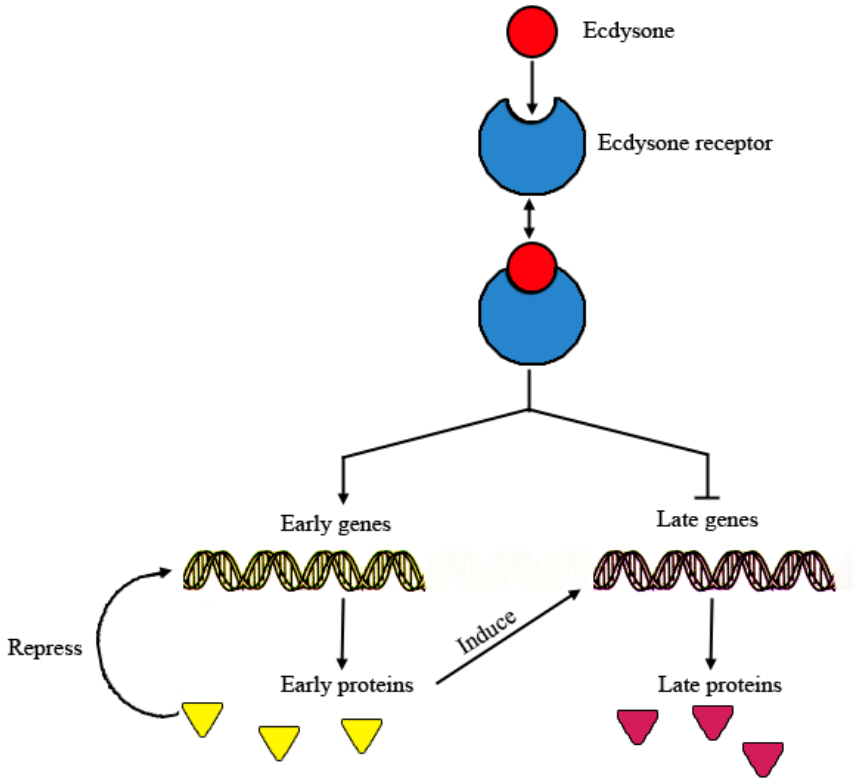


Figure 4. The Ashburner model. Once bound to its hormone receptor, ecdysone directly induces the expression of early puff genes and represses the transcription of late puff genes. As the proteins encoded by the early genes become abundant, they repress their own promoters and activate the expression of the late genes.

3.3. The 20-hydroxyecdysone receptor complex

What is most remarkable about the Ashburner model is that it was developed well before the discovery of the ecdysone receptor (EcR). The search for the ecdysone receptor led to several unsuccessful attempts to demonstrate that radioactively labeled ecdysone accumulates at the puff loci. Major advances were made with the discovery that ponasterone, a synthetic ecdysteroid with limited hormonal activity *in vivo,* which gains relatively high hormonal activity after iodination to form 26-[^{125}I]-iodoponasterone A. The radioactive iodine gives the

compound very high specific radiolabeling and therefore facilitates detection in the nucleus. By using labeled iodoponasterone as a ligand for the ecdysone receptor, Cherbas et al. (1988) showed that the radioligand accumulates in the nucleus and, specifically, at puff sites.

The *Drosophila EcR* gene was first isolated and characterized in 1991. The isolated EcR protein showed high specific binding to DNA and labeled active ecdysteroids. As expected, the protein was nuclear and was detected using anti-EcR monoclonal and polyclonal antibodies in all ecdysone target tissues. To increase the output of the ecdysone pulse, EcR generates an autoregulatory loop that activates its own transcription and further increases the levels of receptor protein in response to the hormone ligand (Koelle et al., 1991).

The EcR protein contains two highly conserved domains characteristic of steroid receptors: a DNA-binding domain (DBD), located at the N-terminus, and an ecdysteroid-binding domain, near the C-terminus, which constitutes the principal dimerization interface. All of the most conserved amino acids in the DNA-binding region of steroid receptors are present in EcR. Eight of the cysteine residues are involved in zinc coordination. The DBD of EcR comprises two highly conserved zinc fingers: the first zinc finger provides DNA-binding specificity, while the second zinc finger forms a weak dimerization interface that allows for protein-protein dimerization only in the presence of a target DNA molecule (King-Jones and Thummel, 2005).

In *Drosophila melanogaster*, the *EcR* gene spans 77 kb and encodes three splice variants, EcR-A, EcR-B1, and EcR-B2. These isoforms share a common

DNA binding domains but differ in their amino-terminal domains, which influence receptor activation and repression properties (Hu et al., 2003). EcR isoforms are expressed widely throughout development and display unique and complementary expression patterns. *EcR-A* is expressed in developing adult structures and is believed to be responsible for adult differentiation, while *EcR-B1* is expressed primarily in the larval cells and directs metamorphosis of larval tissues (Talbot et al., 1993; Truman et al., 1994). Little is known about the expression patterns and distribution of isoform EcR-B2. Nevertheless, Cherbas et al. (2003) showed that EcR-B2 is present in the larval fat body and epidermis at high titer. Mutants lacking both EcR-B1 and EcR-B2 die before entering metamorphosis (Schubiger et al., 1998), while *EcR-A* mutants show developmental arrest after head eversion (D'avino and Thummel, 2000).

All EcR isoforms exhibit similar binding affinities to ecdysone, thus suggesting that functional differences are not likely to be caused by differential affinities to the ligand. It then follows that functional dissimilarities among the three isoforms must be due to differences in transcription by the various N-terminal regions (Schubiger et al., 2003).

Although all EcR isoforms can individually bind to ecdysone, optimal ligand binding requires the addition of ultraspiracle (USP), which is a homolog of the mammalian retinoic acid X receptor. Unlike EcR, USP does not impart any activation function; rather, it acts as an obligatory allosteric effector for ligand binding by EcR (King-Jones and Thummel, 2005). The EcR-USP hormone complex binds directly to specific promoter sequences, called ecdysone response

elements (EcRE), and regulates the transcription of ecdysone-responsive genes (Schwedes and Carney, 2012). Crystal studies of the ligand-binding domain of the three isomers revealed that USP is required for forming a ligand-binding conformation, therefore suggesting that EcR alone cannot transcriptionally activate genes (Billas et al., 2003). Likewise, structural studies showed that the ligand-binding domain of USP is locked in an inactive conformation and that ecdysone becomes transcriptionally active only by binding to the EcR-USP heterodimer (Billas et al., 2001).

Target tissues interpret the ecdysone signal in a cell-type specific manner that is determined by the specific EcR isoform that makes up the nuclear complex, as well as the type of cofactors expressed in the tissue (Yamanaka et al., 2013). Because there are only two types of consensus half sites that are ubiquitously recognized by all nuclear receptors, target selectivity must rely primarily on the geometry and spacing of the half sites, and not just the specific DNA sequence. The EcR-USP heterodimer has been shown to mediate transcription via pseudo-palindromic response elements, which involve imperfect inverted repeats of the 5'-AGGTCA-3' motif, separated by 1 bp (Figure 5) (Devarakonda et al., 2003).

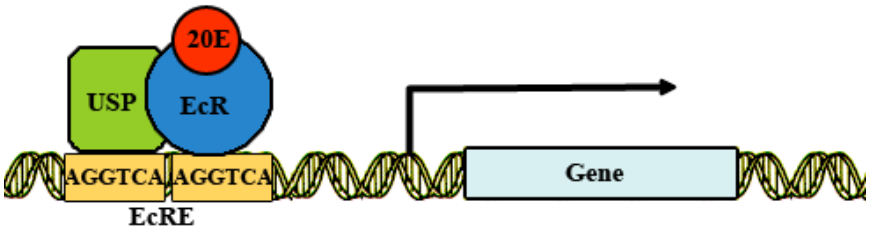


Figure 5. Ecdysone-induced genes are activated by EcR/USP heterodimer. During metamorphosis, the EcR/USP heterodimer mediates the action of 20-hydroxyecdysone at the transcription level through binding to palindromic response elements (EcREs), composed of repetitions of the motif 5'-AGGTCA-3', separated by 1 nucleotide.

3.4. Ecdysone signaling

During early metamorphosis, two successive 20E pulses occur: one at the end of the third instar larval stage and the other during the late prepupal stage (Figure 6) (Bond et al., 2011). The first 20E pulse occurs at 0 hours APF and induces the transcription of a set of primary response genes: *E74* from the 74EF puff (Burtis et al., 1990), *E75* from the 75B puff (Segraves and Hogness, 1990) and *BR-C* (*Broad-Complex*) from the 2B5 puff (DiBello et al., 1991). All three early genes encode transcription factors and are necessary for initiation of metamorphosis and puparium formation (Thummel, 1996). *E74,* a member of the *ets* protooncogene family, encodes two proteins, E74A and E74B, with an identical DNA binding domain (Burtis et al., 1990). *E74* transcripts are widely expressed at the beginning of metamorphosis and mutations in *E74A* or *E74B* are lethal during prepupal and pupal development, indicating an essential role for these genes during metamorphosis (Fletcher and Thummel, 1995). *E75* encodes three members of the nuclear receptor superfamily: E75A, E75B and E75C (Segraves and Hogness, 1990). Mutations in *E75* lead to a variety of phenotypes,

including embryonic head involution defects, larval molting defects and aberrant metamorphosis (Bialecki et al., 2002). The *BR-C* is a complex genetic locus that encodes several zinc finger proteins. Mutations in the *BR-C* gene are lethal during prepupal and pupal development, affecting larval and imaginal tissues (Kiss et al., 1988) and early and late ecdysteroid-inducible gene expression, suggesting a central role for this gene in transducing the ecdysteroid signal (Guay and Guild, 1991).

E74, *E75,* and *BR-C* are expressed only transiently, as their gene products repress their expression until they are reinduced by the second 20E high titer pulse, which occurs 10 hours APF and induces the prepupal-pupal transition, characterized by adult head eversion, leg and wing extension, larval salivary gland cell death (Woodard et al., 1994), and remodeling of the larval fat body (Bond et al., 2011). During the second 20E pulse, an additional gene, *E93*, is expressed. *E93* is a programmed-cell death gene that directs the elimination of several larval tissues, such as the midgut and salivary glands, during metamorphosis (Mou et al., 2012).

3.5. The orphan nuclear receptor: βFTZ-F1

As the first 20E pulse declines 3-8 hours after pupariation, the mid-prepupal genes, represented by a single puff at 75CD, are induced (Woodard et al., 1994). By mimicking the levels of ecdysone at pupariation, Richards (1976a) showed that the formation of the 75CD puff in larval salivary glands requires the addition of a high concentration of ecdysone, followed by hormonal withdrawal.

Addition of high concentrations of ecdysone to cultured salivary glands during mid-prepupal stages results in the regression of the 75CD puff, suggesting that this puff is rapidly repressed by ecdysone.

The 75CD puff is responsible for the synthesis of ßFTZ-F1, a member of the nuclear receptor superfamily of transcription factors (Lavorgna et al., 1993). The *ftz-f1* gene was first discovered by Ueda and colleagues (1990) in the course of studies of the transcriptional regulation of the pair-rule segmentation *fushi tarazu* (*ftz*) gene. *ftz* is a Hox gene that is expressed during embryonic development in a pattern of seven stripes. A specific element, called the zebra element, located in the promoter region of the *ftz* gene, is responsible for regulating this striped expression. FTZ-F1 was biochemically purified as a DNA-binding protein that interacts with the zebra element of the *ftz* transcriptional start site. Interestingly, the authors also found that the *ftz-f1* gene encodes two protein isoforms with different temporal expression patterns: αFTZ-F1, which is maternally supplied and is implicated in the transcriptional regulation of the *ftz* segmentation gene during embryogenesis (Yu et al., 1997), and βFTZ-F1, which is involved in regulating ecdysteroid titers at the prepupal-pupal transition (Yamada et al., 2000).

The *βftz-f1* gene sequence consists of an open reading frame of 816 codons, encoding a protein of 88 kDa (Lavorgna et al., 1993). This protein is expressed following the late larval ecdysone pulse, closely paralleling the expression pattern of the 75CD puff (Woodard et al., 1994). Interestingly, Richards (1976b) showed that early genes are not only induced by ecdysone but

they also later regress in its presence. As previously mentioned, this regression is inhibited by cycloheximide, thus indicating that a preceding period of protein synthesis and low ecdysone concentration is required for the reinduction of the early genes in competent prepupal glands. Woodard and colleagues (1994) demonstrated that βFTZ-F1 is the competence factor that makes the early genes competent so that they can appropriately respond to the prepupal ecdysone pulse. In accordance with their hypothesis, Woodard et al. (1994) showed that ectopic expression of *βftz-f1* in late third instar larvae results in enhanced levels of *BR-C*, *E74* and *E75* early gene transcription and premature induction of the *E93* gene. βFTZ-F1 is necessary but not sufficient for inducing the transcription of these genes, suggesting that ecdysone is also required for expression to occur.

The expression of *βftz-f1* is temporally restricted and this property is critical for βFTZ-F1's role as a competence factor. *βftz-f1* is repressed by ecdysone, thus ensuring that its expression is limited to the mid-prepupal stage, when the levels of ecdysone are low (Woodard et al., 1994). Expression of *βftz-f1* is promoted in part by two 20E-induced receptor proteins, DHR3 and DHR4. DHR3 is necessary but not sufficient to downregulate the expression of 20E-inducible early genes, indicating that this response is likely to be mediated by other negative regulators. DHR3 mutants show reduced expression of *βftz-f1* in prepupae (Lam et al., 1999). Three DHR3 binding sites have been indentified downstream from the start site of *βftz-f1* transcription, suggesting that DHR3 might regulate *βftz-f1*expression by binding directly to the gene's promoter region (Lam et al., 1997). DHR4 is a homolog of the vertebrate germ cell nuclear factor

(GCNF), a potential transcriptional repressor (Zhang and Dufau, 2004). Recent genetic analysis of the DHR4 locus has revealed that DHR4 expression patterns closely parallel that of DHR3, thus raising the possibility that these two orphan receptors might act together, in partially redundant manner, as transcriptional switches at the larval-prepupal transition (King-Jones et al., 2005).

Recent work by Agawa and colleagues (2007) has helped to further elucidate the mechanisms behind the temporal regulation of *βftz-f1* expression. They identified dBlimp-1, the *Drosophila* homolog of mammalian B lymphocyte-induced maturation protein-1, as an ecdysone inducible transcriptional repressor of *βftz-f1*. dBlimp-1 is a rapidly turned over protein that is expressed in response to the late larval pulse and acts as a transcriptional repressor by binding to the *βftz-f1* promoter. The transient nature of dBlimp-1 is important in determining the precise timing of *βftz-f1* expression.

Studies of *βftz-f1* mutant phenotypes have helped elucidate the function of βFTZ-F1 during prepupal development. Mutations in *βftz-f1* severely disrupt the ecdysone signaling pathway at the onset of metamorphosis and result in prepupal lethality. *βftz-f1* mutants display defects in the prepupal-pupal transition, including adult head eversion, leg extension, and salivary gland cell death. In these mutants, ecdysone receptor levels remain unchanged, suggesting that *βftz-f1* does not confer competence by altering ecdysone receptor levels but rather by directly regulating early gene transcription (Broadus et al., 1999). Moreover, Bond et al. (2011) showed that premature expression of *βftz-f1* in the fat body of

third instar larvae results in premature fat body remodeling, while clones of *βftz-f1* null mutant fat body cells fail to undergo complete fat body remodeling. Taken together, these results suggest that *βftz-f1* is required for fat-body remodeling.

Recently, Bond et al. (2011) showed that expression of *EcR-DN* results in the downregulation of *MMP2* transcription, while premature *βftz-f1* expression results in early *MMP2* induction. These results implicate a novel role for βFTZ-F1 in the regulation of *MMP2* expression. Inspired by these findings, Bond et al. proposed that *MMP2* functions as a potential downstream target of βFTZ-F1-mediated 20E signaling.

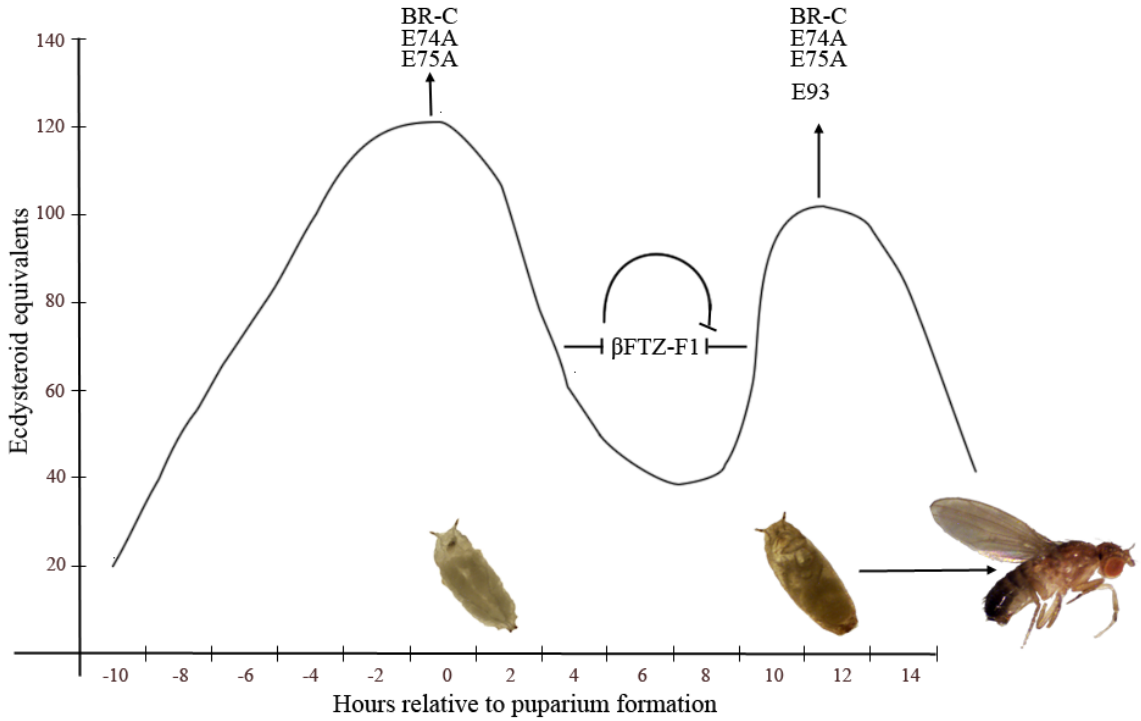


Figure 6. Ecdysone titers during *Drosophila* metamorphosis. During metamorphosis, two successive 20E pulses occur. The first 20E pulse occurs at 0 hours APF and induces the transcription of a set of primary response genes, including *BR-C*, *E74* and *E75*, which are necessary for initiation of metamorphosis and puparium formation. These early genes are only transiently expressed, as their gene products repress their expression and induce the subsequent transcription of a set of late genes in the prepupa. As the first 20E pulse declines, the nuclear receptor βFTZ-F1 is expressed and confers competence in the larval tissues to respond appropriately to the second 20E pulse. The second ecdysone pulse occurs 10 hours APF and it induces the prepupal-pupal transition, which involves adult head eversion, leg and wing extension and larval salivary gland cell death.

CHAPTER 4: THE LINK BETWEEN INSULIN SIGNALING AND HORMONALLY INDUCED AUTOPHAGY

Drosophila development provides a useful tool for studying the interface between cell growth, division and death. These three cellular processes are regulated via ecdysone signaling, insulin and insulin-like growth factor signaling

and autophagy (Tracy and Baehrecke, 2013). Even though recent studies have shed some light on the relationship between these developmental pathways, the coordination of steroid (Figure 7), insulin signaling and autophagy is still poorly understood. Elucidating what factors link these processes is not only important for understanding animal growth during normal development, but it is also critical for understanding numerous disease processes characterized by inappropriate regulation of insulin signaling and autophagy.

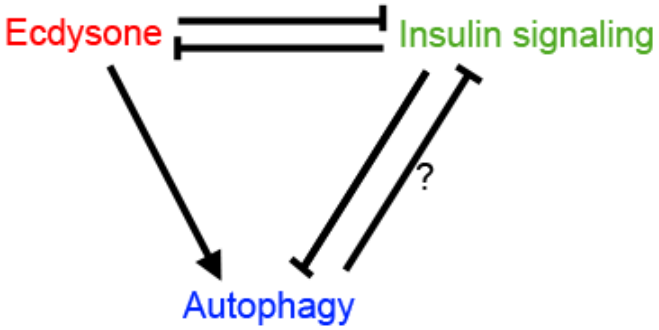


Figure 7. Relationship between ecdysone, autophagy and insulin signaling. Ecdysone and insulin signaling have antagonistic effects on each other; ecdysone promotes autophagy, while insulin signaling suppresses it. The mutual antagonistic relationship between ecdysone and insulin signaling regulates autophagy. As ecdysone levels increase, insulin signaling is inhibited, thus releasing insulin's inhibition on autophagy and further inducing autophagy. Conversely, increased insulin signaling inhibits autophagy through class I PI3K and TOR signaling and reduces ecdysteroid secretion. Whether autophagy itself regulates insulin signaling is still unclear. However, under certain circumstances, autophagy can act as a negative regulator of growth.

4.1. The larval fat body regulates insulin signaling

During fly development, a constant supply of nutrients is necessary to provide the energy required for growth, metabolism and survival. Eukaryotic cells have evolved a variety of strategies to adjust their metabolic activities in response to environmental stimuli, including changes in nutrient availability. As the

nutrient levels decrease, protein synthesis and cell cycle progression are rapidly suppressed, while nutrient uptake is upregulated to match the supply of nutrients. However, the mechanisms that coordinate these cellular processes are poorly understood (Neufeld et al., 2004).

Organisms modulate their growth according to nutrient levels. During the three larval stages of *Drosophila* development, the animal increases its size by ~200-fold (Church and Robertson, 1966). Reaching the proper body size is critical for surviving metamorphosis and enhancing reproductive success as an adult. In order to reach its proper size, the larva must pass three checkpoints. The first checkpoint, called the threshold size, occurs near the second to third instar transition phase and it determines whether the animal will become a metamorphic molt or remain a larval molt (Zhou et al., 2004). The second checkpoint is called the minimal viable weight, whereby the animal achieves sufficient body mass to complete larval and pupal development in the absence of nutrients (Bakker, 1959). The next checkpoint, called the critical size, occurs during the last larval stage. Reaching critical weight is key for the animal to pupate within a definite amount of time and enter metamorphosis (Nijhout, 2003; Bakker, 1959). If animals encounter poor nutrient conditions prior to achieving critical weight, their development will be stalled and 20E pulses will be delayed until the nutrient supply improves. However, if nutrients are still abundant after the animals have reached critical weight, they will continue to increase their body mass (Tennessen and Thummel, 2011).

The larval fat body, which functions as a hybrid of the mammalian adipose tissue and liver, is the major sensing organ for nutritional signals. Like mammals, fruit flies regulate their circulating sugar levels and store energy in the form of glycogen and lipids. Most of the nutrient sensing in *Drosophila* takes place via the insulin pathway. Three processes cooperate to regulate insulin signaling: the insulin/insulin-like growth factor signaling pathway (IIS), the phosphatidylinositol 3-kinase signaling pathway (PI3K) and the target of rapamycin signaling pathway (TOR). Located upstream of the insulin pathway, *Drosophila* insulin-like peptides (dILPs) act as insulin-like growth factors (IGFs). The genome of *Drosophila* encodes seven dILPs that are structurally similar but have dissimilar functions and are expressed in different tissues (Teleman, 2010). Three dILPs, 2,3, and 5, are secreted from the neurosecretory cells in the larval brain, suggesting that they have an endocrine function. These neurosecretory cells (NSCs) are functionally similar to pancreatic β cells and can secrete dILPs into the hemolymph (Rulifson et al., 2002). Ablation of the *dilp*-expressing NSCs in larvae results in a systematic growth defect (Colombani, 2003). Circulating dILPs bind to the insulin receptor (InR) and activate the highly conserved PI3K signaling cascade, which inhibits the dFOXO transcription factor, thus promoting autonomous cell growth (Oldham and Hafen, 2003). When the animal experiences nutrient deprivation, the PI3K pathway is shut off and dFOXO translocates from the cytoplasm to the nucleus, where it inhibits cell growth (Puig et al., 2003).

In a genetic screen test for growth modifiers, Colombani and colleagues (2003) identified *slimfast*, a gene that encodes a cationic amino-acid transporter

that is highly expressed in fat body cells. Remarkably, decreased expression of *slimfast* causes a whole-body growth defect similar to what was seen in animals raised under poor nutritional conditions. Mutations that disrupt the TOR pathway phenocopy *slimfast* loss of function mutations, while overexpression of S6K, a TOR downstream target, can partially rescue the growth defects caused by *slimfast* mutations. Conversely, reduction in S6K activity in the brain leads to behavioral phenotypes similar to those observed upon fasting (Wu et al., 2009). Taken together, these results suggest that the fat body monitors nutrient levels and coordinates growth via the TOR signaling pathway.

dILP2 secretion is the major target of nutrient-dependent fat body signaling. When *slimfast* or TOR activity is ablated in the fat body, dILP2 is not secreted and accumulates in the NSCs. Géminard et al. (2009) showed that the larval fat body couples the level of circulating dILP with amino acid levels by controlling dILP release via TOR. Thus, the fat body of fed animals must monitor insulin signaling by secreting an as yet unidentified factor that induces dILP release.

The larval fat body not only regulates dILP secretion, but also releases two insulin-like growth factor-binding proteins: dALS (*Drosophila* acid-liable subunit) and Imp-L2 (imaginal morphogenesis protein-late 2) (Colombani et al., 2003; Arquier et al., 2008). Imp-L2 acts as a negative regulator of insulin signaling, antagonizing the action of dILP2. Whereas overexpression of *Imp-L2* inhibits growth in a cell non-autonomous way and results in developmentally delayed, smaller flies, genetic loss of Imp-L2 function results in an increased

body size (Honegger et al., 2008). The function of dALS is, instead, still very controversial and requires further examination. Recently, Arquier et al. (2008) have proposed a dual effect of dALS that is dependent upon nutritional status. Under optimal nutritional conditions, overexpression of dALS results in the formation of more stable, but inactive, dILP/dALS/Imp-L2 trimeric complexes, leading to growth inhibition. These results seem to suggest that dALS sequesters and inactivates the insulin-like protein, thus acting as an ILP antagonist. However, under starvation conditions, dILP molecules become limiting and dALS overexpression can increase the half-life of circulating dILPs by protecting them from proteases. In this scenario, dALS functions as an agonist and promotes insulin signaling.

During periods of starvation, the fat body releases stored nutrients to ensure the survival of peripheral tissues. Under favorable environmental conditions, TOR signaling suppresses autophagy, thus preventing the breakdown of the energy stores. Conversely, when the animal becomes nutrient-deprived, TOR signaling is downregulated and this results in the autophagic degradation of the fat body and the release of nutrients to peripheral tissues (Figure 8) (Scott et al., 2004; Rusten et al., 2004).

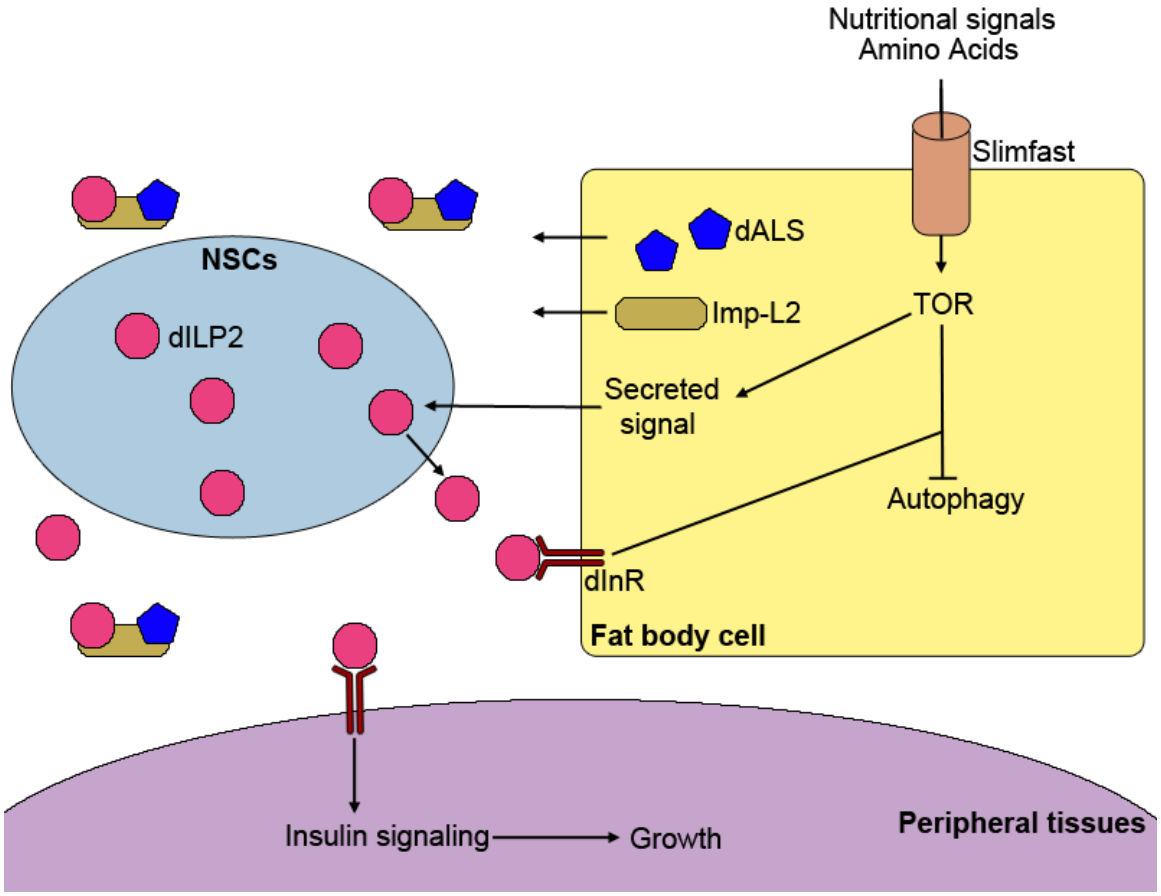


Figure 8. The larval fat body coordinates growth and provides an essential source of energy during starvation periods. Ingested nutrients, in particular amino acids, are sensed by the fat body cells via Slimfast, an amino acid transporter. These nutrients activate the TOR signaling pathway, which promotes the release of unknown factors that stimulate dILP2 synthesis in the neurosecretory cells in the animal's brain. dILP2 promotes growth and cell division in a cell-autonomous manner by binding to the insulin-like receptor (dInR). Moreover, the fat body cells release two inhibitors of ILP function: dALS and Imp-L2. dILP-2 binds directly to Imp-L2 but not to dALS and the three proteins form a stable complex that dampens insulin signaling.

4.2. The antagonistic relationship between insulin signaling and ecdysone

While insulin signaling promotes growth, 20E signaling stimulates transitions between developmental stages. At onset of pupariation, basal levels of 20E produced from the PG inhibit insulin-mediated growth (Colombani et al.,

2005). In a recent gain-of-function screen test for genes promoting organ growth, Boulan et al. (2013) identified *bantam* - the first microRNA characterized in *Drosophila* that promotes systematic growth by repressing ecdysone release. Boulan et al. showed that repression of *bantam* activity is required for insulin signaling-mediated regulation of ecdysone production. This study suggests that high *bantam* activity in young larvae induces systemic growth by promoting the maintenance of low ecdysone titers. Conversely, *bantam* levels were found low in the PG of second and early third instar larvae, suggesting that reduced *bantam* activity in late PGs contributes to the generation of the ecdysone peak and stimulates entry into metamorphosis by inhibiting insulin-mediated growth.

Interestingly, fat-body-specific depletion of *EcR* is sufficient to suppress the inhibitory effects of 20E and induce growth. Although the actual mechanisms of action are still not known, it has been suggested that EcR attenuates insulin signaling, at least in part, via its interaction with the transcription factor Myc, which plays a key role in promoting growth. Ecdysone acts by inhibiting Myc in the fat body and this results in the non-autonomous suppression of growth (Delanoue et al., 2010). Intriguingly, TOR also regulates Myc expression (Teleman et al., 2008), hinting at a model whereby ecdysone and nutrient sensing signaling work together to control systemic growth.

In addition to suppressing systemic growth, ecdysone signaling in the fat body cells also suppresses insulin signaling at the level of PI3K activity. EcR activation was shown to interfere with PI3K activity, thereby causing the translocation of dFOXO into the nucleus, where it promotes the transcription of

4E-BP and *dDOR*. *4E-BP* is a transcription factor that is generally suppressed by insulin and that has been suggested to function as a metabolic break under conditions of environmental stress (Colombani et al., 2005; Teleman et al., 2005). *dDOR*, instead, encodes a transcriptional coactivator of EcR that is required for proper 20E signaling. Expression of *dDOR* is inhibited by insulin, implying that insulin signaling attenuates the ecdysone response. Conversely, ecdysone suppresses insulin signaling and this event stimulates dDOR expression, initiating a positive feed-forward loop in fat body cells, where ecdysone potentiates its own response (Figure 9) (Francis et al., 2010).

The precise mechanism by which ecdysone and insulin signaling antagonize each other is still not well defined. Bond et al. (2011) hypothesized that MMP2 is a potential downstream target of the βFTZ-F1-mediated, 20E signaling cascade, and that induction of *MMP2* expression by βFTZ-F1 and ecdysone, late in the prepupal period, leads to cleavage of the extracellular matrix of the cell, allowing for fat motility. In a previous study, Fowlkes et al. (1994) showed that MMPs degrade the insulin-like growth factor-binding protein-3, IGFBP-3, in the serum of pregnant rats and in human dermal fibroblasts. Bond et al. (2011) identified MMP2 as an ideal candidate for the downregulation of insulin signaling in the fat body during metamorphosis. By cleaving the dILP/dALS/Imp-L2 trimeric complex, MMP2 would leave the dILPs unprotected. This would lead to a decrease in insulin signaling, which would ultimately result in an increase in nutrient release during metamorphosis. Future studies are required to support this hypothesis and determine the exact target of MMP2.

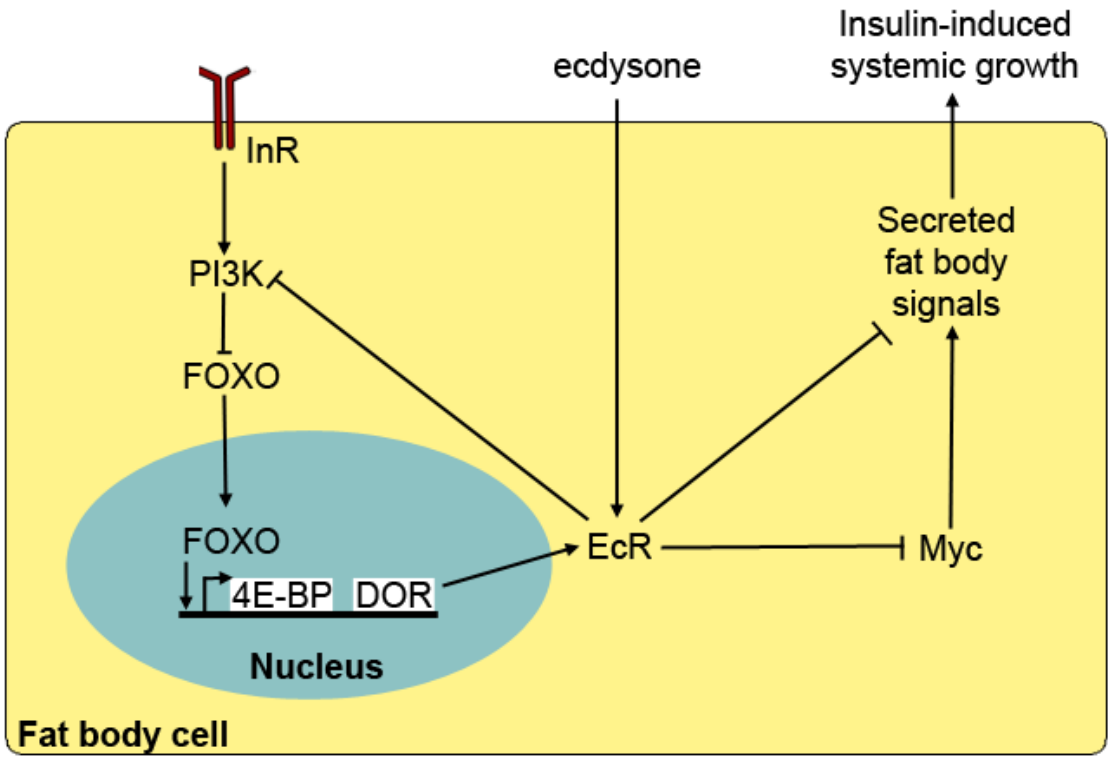


Figure 9. Communication between the insulin signaling pathway and the ecdysone receptor. EcR activation by 20E in the fat body inhibits insulin-induced systemic growth, in part, by suppressing Myc expression. Moreover, ecdysone-signaling cell-autonomously suppresses insulin signaling at the level of PI3K activity. EcR causes FOXO to translocate to the nucleus, where it regulates the expression of 4E-BP and DOR. DOR further stimulates ecdysone signaling, thus generating a positive feed-forward loop.

4.3. The process of autophagy and the autophagosome formation machinery

Once the larva achieves critical weight, the PG starts releasing low-titer pulses of 20E, preparing the animal to enter metamorphosis. At this time, the animal stops feeding, insulin signaling is shut off and the larval fat body cells initiate autophagy (Tennessen and Thummel, 2011). Through autophagy, nutrients are recycled from proteins, organelles and other cellular components of the cytoplasm to serve as an internal reserve of nutrients. During this catabolic

process, cytoplasmic constituents are engulfed by double-membrane vesicles known as autophagosomes, which subsequently fuse with lysosomes to form an autolysosome. The inner membrane of the autolysosome and its contents are degraded by hydrolases and recycled (Figure 10) (Scott et al., 2004).

The molecular mechanisms of autophagy were first characterized in *Saccharomyces cerevisiae*. Several genes involved in autophagy have been mainly identified via mutagenesis-based genetic screens and were given a unified nomenclature. To date, more than 31 autophagy-related (ATG) proteins have been found to be specifically involved in the process of autophagy (Mizushima, 2007).

In yeast, autophagosomes are generated from a novel perivacuolar structure called PAS (phagophore assembly site). Although an equivalent of the yeast PAS has not been identified in higher eukaryotes, it has been hypothesized that mammalian cells might also construct their autophagosomes from a pre-autophagosomal structure (Itakura and Mizushima, 2010). Autophagosome formation is a *de novo* process, in which membranes emerging from the PAS expand and enclose cytosolic cargos. Two ubiquitin-like (UBL) conjugation systems, Atg12 and Atg8, are involved in the process of vesicles expansion and completion. Both systems are composed of a ubiquitin-activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin-protein ligase enzyme (E3). Agt12 is a small ubiquitin-like protein that covalently binds to Atg5 and forms the Atg12-Atg5 conjugate. This new complex interacts with Atg16 and forms a oligomer. Even though the Atg12-Atg5-Atg16 complex is critical for autophagosome formation, little is currently known about its function (Mariño

and López-Otín, 2004). Recent studies have suggested that this complex exerts an E3 enzyme-like function and facilitates the formation of the Atg8-PE conjugate at the PAS (Suzuki et al., 2001). Analysis of *atg5*-deficient mouse embryonic stem cells has also revealed that Atg5 is important for membrane elongation and autophagosome formation (Mizushima et al., 2001).

Atg8, the ortholog of mammalian LC3, is conjugated to the lipid phosphotidylethanolamine (PE) and is involved in the second UBL system associated with autophagy. While Atg8-PE conjugates are present on both surfaces of the isolation membrane, where they are required for the expansion of the phagophore, some are left inside the autophagosome, delivered to the vacuoles and degraded by hydrolytic enzymes (Mariño and López-Otín, 2004). Once fused to the lysosome, GFP-Atg8 from the inner membrane of the autophagosomes is still observed in the lysosomal lumen, therefore making Atg8 a useful marker for autophagy (Mizushima et al., 2003). Despite extensive studies, however, little is known about the exact functions of Atg8. In yeast, Atg8 has been shown to determine the size of autophagosomes (Xie et al., 2008) and mediate the tethering and hemifusion of membranes *in vitro* (Nakatogawa et al., 2007). Moreover, Atg8 and LC3 are involved in cargo recognition (Noda et al., 2008).

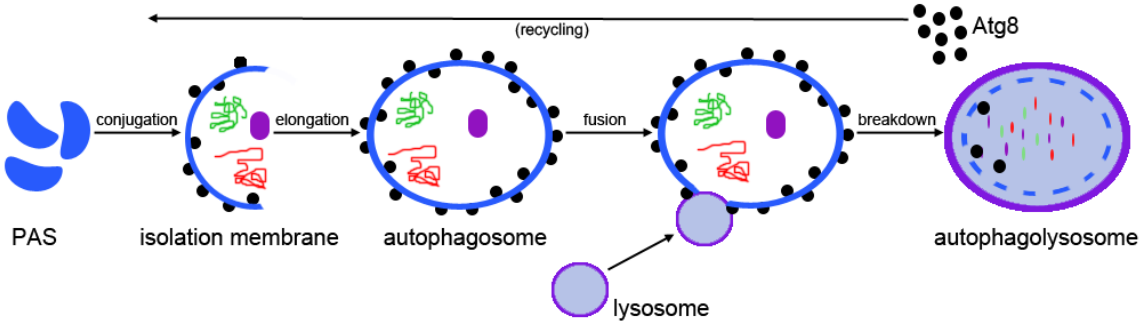


Figure 10. The process of autophagy. Macroautophagy is characterized by the formation of a cytosolic double-membrane vesicle, called the autophagosome, which is generated from the PAS. When autophagy is induced, cytoplasmic proteins and damaged organelles are surrounded by phagophores, which expand and close to form autophagosomes. These structures fuse with lysosomes to form autolysosomes, in which the cytoplasmic cargos are degraded by acid lysosomal/vacuolar hydrolases, together with the autophagosome inner membrane. Atg8 is a ubiquitin protein that is covalently conjugated to with the phosphatidylethanolamine on the autophagosome membrane.

4.4. Autophagy signaling complexes

Autophagy can be nutritionally, hormonally or developmentally induced. Under starvation conditions, cells can initiate autophagy to release the stored nutrients necessary to support the survival of the organism. Rusten et al. (2004) also showed that, as the animal prepares to enter metamorphosis, the late larval 20E pulse increases autophagy and reduces insulin signaling in the fat body cells. In fact, it was previously showed that premature induction of ecdysone signaling in the last larval stage of *Pieris brassicae* and *Mamestra brassicae* results in early-programmed autophagy of the midgut and fat body (Komuves et al., 1985; Sass and Kovacs, 1975).

Three signaling complexes/pathways are known to be involved in the development of autophagic responses. These include the TOR signaling pathway,

the ATG1 complex and components of the insulin/PI3K pathway, upstream of TOR. Inhibition of TOR by nutrient stress or the macrolide antibiotic rapamycin results in the induction of autophagy. TOR is an evolutionary conserved kinase that is thought to suppress autophagy under non-starvation conditions by regulating the expression of Atg8 and the activity of a complex containing the Ser-Thr kinase Atg1 (Scott et al., 2004). Atg1 is a protein kinase, whose catalytic activity is essential for autophagy. Under nutrient-rich conditions, TOR causes hyperphosphorylation of Atg13, thus preventing its association with Atg1. This event promotes the interaction between Atg1 and Atg11. The Atg1-Atg11 complex is involved in the induction of the cytoplasm-to-vacuole targeting (Cvt) pathway, which is required for the transport of hydrolases (Kamada et al., 2000). Under starvation conditions, Atg13 becomes dephosphorylated and interacts with Atg1. The Atg1-Atg13 complex triggers the generation of autophagosomes instead of Cvt vesicles.

In higher eukaryotes, autophagy can also be suppressed by inhibiting the activity of components of the insulin pathway upstream of TOR. For example, PI3K is a potent repressor of autophagy in mammalian cells and it catalyzes the formation of phosphatidylinositol-3,4,5-tris-phosphate (PIP_3) (Mariño and López-Otín, 2004). Inactivation of the PI3K pathways by mutation or by the expression of the tumor suppressor PTEN, a negative regulator of insulin/PI3K signaling, results in autophagy (Scott et al., 2004). The level of PIP_3 is negatively regulated by the PTEN tumor suppressor protein and expression of a lipid phosphatase-deficient form of PTEN results in the inhibition of autophagy (Arico et al., 2001).

CHAPTER 5: HYPOTHESES AND AIM OF STUDY

The goal of my experiment is to elucidate the mechanism by which *βftz-f1*, 20E signaling and MMP2 regulate insulin signaling and autophagy in the larval fat body. I hypothesize that MMP2, in addition to functioning in fat body remodeling, also downregulates insulin signaling during metamorphosis, therefore triggering nutrient release by autophagy. By cleaving either or both dALS and Imp-L2, MMP2 would expose dILPS to the action of proteases, thus downregulating insulin signaling. The dual activity of MMP2 would ultimately result in nutrient release by autophagy in the fat body and this would guarantee the survival of the starving organism during metamorphosis. Moreover, I hypothesize that βFTZ-F1 is involved in promoting autophagy in the fat body during metamorphosis, possibly by regulating *MMP2* expression. To investigate whether βFTZ-F1 and MMP2 are involved in hormone-mediated autophagy and insulin signaling, I performed mosaic analyses in *βftz-f1* and *MMP2* mutants and looked for the presence of autophagosomal structures.

MATERIALS AND METHODS:

Drosophila stocks and care

The stock genotypes used for this experiment were:

1. *UAS-dBlimp-1*
2. *UAS-Timp*
3. *ywhsFlp; pmCherryAtg8a; Act>CD2>GAL4, UAS-nlsGFP/TM6B*

Stocks were cultured in plastic bottles and vials on standard *Drosophila* culture medium, made with agar, malt, extract corn syrup, yeast, cornmeal, propionic acid and methyl 4-hydroxybenzoate (Tegosept). After addition of adult flies, bottles and vials were supplemented with dry yeast and stored at 25°C at 50% humidity. To collect virgin flies, stock bottles were cleared and sexed after 8 hours of incubation at 25°C or after 18 hours of incubation at 18°C.Virgin female and male flies were separated into different vials to block mating and were collected until they were ready to be crossed.

Drosophila crosses and induction of cell clones

Virgin females of *ywhsFlp; pmCherryAtg8a; Act>CD2>GAL4* (">" indicates an FRT site)*, UAS-nlsGFP/TM6B* were crossed with males of *UAS-dBlimp-1* or *UAS-Timp.* The transgene-bearing chromosome is maintained over the multiply inverted balancer chromosome, *TM6B*. This balancer chromosome

carries both the *Tubby (Tb)* dominant marker, which confers a tubby body phenotype (Figure 11), and a recessive lethal allele. Balancer chromosomes allow for the maintenance of lethal mutants as balanced heterozygotes by preventing viable recombination between homologues. One-day egg lays were heat shocked at 37°C for 15-18 min to induce FLP recombinase expression and consequently generate mosaic flies, expressing both the wild-type and transgenic genotypes (either *TIMP* or *dBlimp-1*) within one population of cells. *TIMP* and *dBlimp-1* pupae were distinguished by the absence of the tubby phenotype. The expression of the transgene in a given cell was determined by the presence of the green fluorescent protein (GFP) in the nucleus. After heat shock, animals were stored at 25°C.

Figure 11. Phenotype of pupae heterozygous for the *Tubby* mutation. *Left*: homozygous wild-type pupa; *right*: heterozygous pupa for the *Tb* mutation. The *TM6B* balancer chromosome carries the *Tb* dominant mutation, which results in a short and stout larva, and eventually pupa.

The *GAL4-UAS* and *FLP-FRT* systems

The *GAL4-UAS* system is a useful tool that regulates the spatial expression of targeted genes. First developed by Brand and Perrimon (1993), this bipartite genetic system makes use of the yeast regulator protein GAL4, a transcription factor induced by galactose and derived from *Saccharomyces cerevisiae.* The yeast GAL4 protein controls the transcription of two divergently transcribed genes, *GAL10* and *GAL1,* by binding to four similar 17 basepair sequences via a $Zn(II)_2Cys_6$ binuclear cluster (Giniger et al., 1985; Pan and Coleman, 1990). These sites are referred to as the Upstream Activating Sequences (*UAS*) element and they are essential for the transcriptional activation of genes under the control of GAL4.

The *UAS* responder and the GAL4 driver are maintained as separate parental lines. When transcriptionally inactive females carrying a *UAS* responder (e.g. *UAS GFP*) are mated to transcriptionally inactive males carrying a GAL4 driver (e.g. *cg-GAL4*), progeny containing both elements of the system are produced (Figure 12). Transcription of the gene of interest requires the presence of both elements of the *GAL4-UAS* system. If only one element of the system is present, the transcription of the downstream genes fails to occur (Duffy 2002).

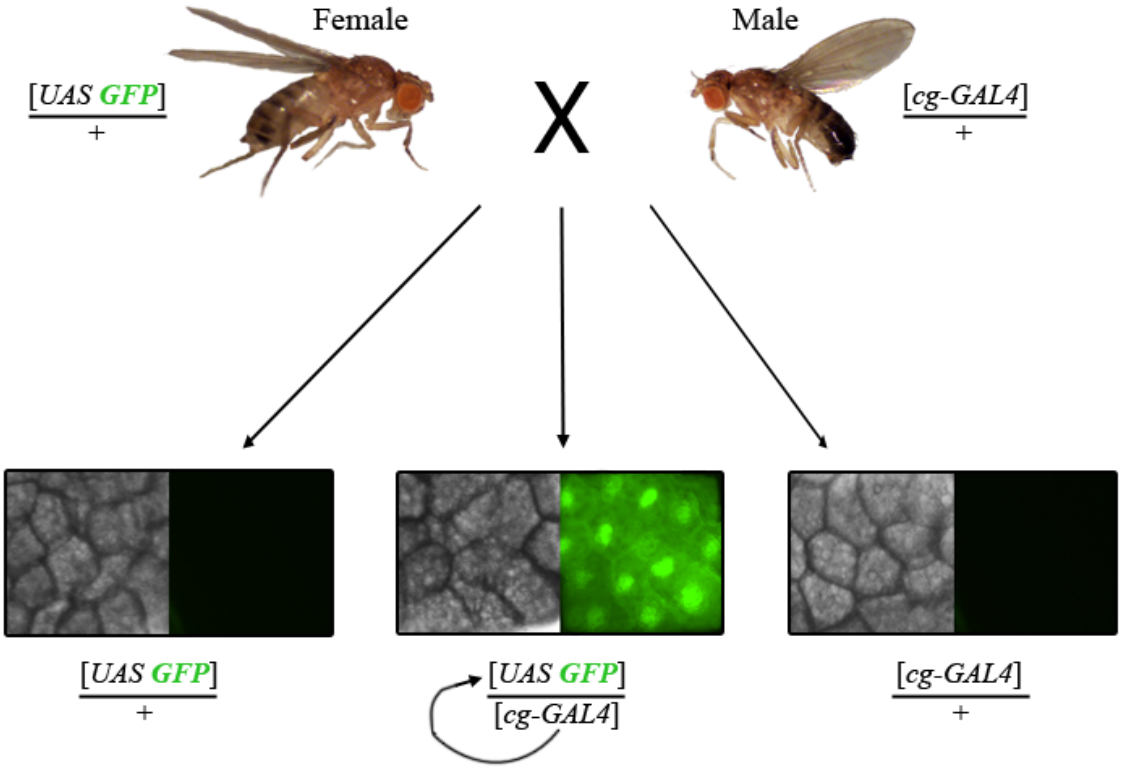


Figure 12. The *GAL4-UAS* system in *Drosophila*. Heterozygous females carrying the *UAS GFP* reporter are mated to heterozygous males carrying the fat-body specific GAL4 driver, *cg-GAL4*. Only the F1 that contains both elements of the system results in the fat-body specific expression of GFP.

The only drawback of the *GAL4-UAS* system is that the temporal expression of the reporter cannot be fully regulated: the *UAS* transgene is expressed *whenever* there is GAL4 present. One can refine the temporal regulation of the *GAL4-UAS* system by combining it with the *FLP-FRT* technique. In this modification of the bipartite system, the gene of interested is placed under the control of a ubiquitous promoter, such as an actin (*Act*) promoter. In order to render transgenic expression silent, a terminator cassette flanked by FLP recognition sites is placed between the *UAS* promoter and the target gene. In the presence of the flippase enzyme, recombination between the

two FLP recognition sites takes place, resulting in the excision of the termination cassette and the expression of the gene of interest. Low level, transient expression of flippase is triggered by using a heat shock promoter, so that recombination will only take place in a few cells. As these cells replicate, they will give rise to clones in which the gene of interest is ectopically expressed (Elliott and Brand 2008; Perrimon 1998) (Figure 13).

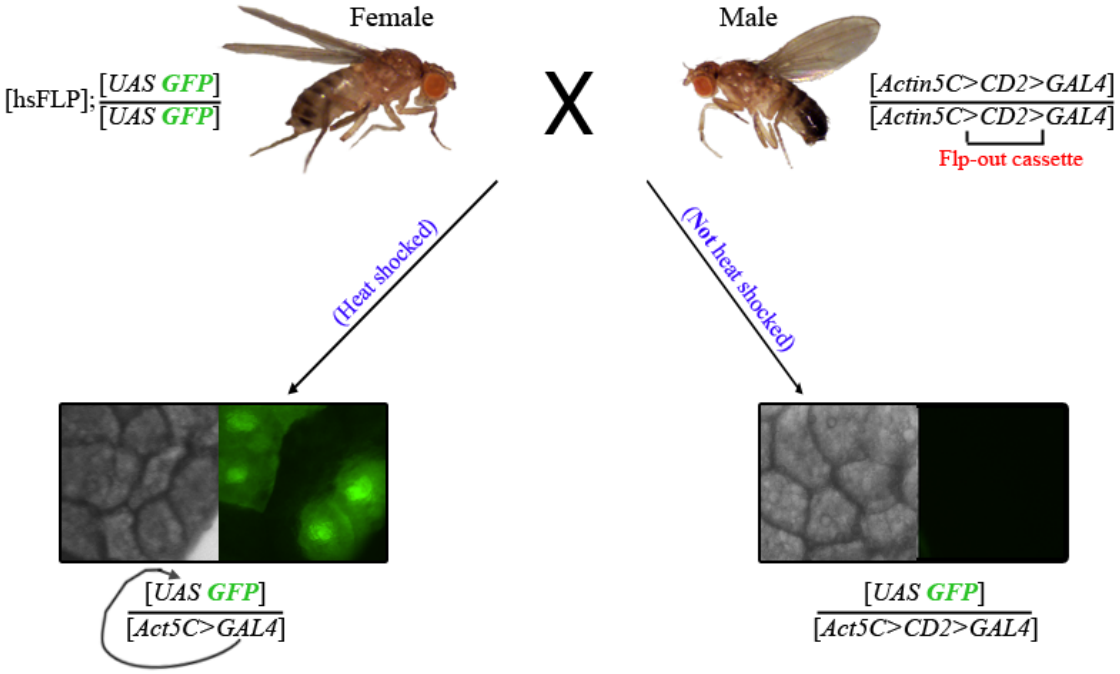


Figure 13. GAL4 regulation via the *FLP/FRT* system. In this modified version of the GAL4/UAS system, the presence of the FLP-out cassette prevents the *Actin5C* promoter from inducing GAL4 expression. Expression of the responder in response to GAL4 is mediated by the activation of the enzyme flippase, which catalyzes the removal of sequences between FLP recognition target sequences (symbolized as ">"). Flippase expression is under the control of a heat-shock inducible promoter. Thus, a mild heat shock applied to the animal during development activates flippase in some of the cells. This enables the expression of the reporter genes only in those cells in which flippase is expressed.

Sample preparation

Prepupae from the crosses were staged at 0 h APF and incubated on wet filter paper at 25°C until a specific time point. 0 h APF prepupae were identified by their immobility, untanned cuticle and everted anterior spiracles. Animals were aged to multiple time-points (0, 2, 4, 6, 8, 10 and 12 h APF) before larval fat bodies were dissected from them and examined for presence of autophagic mCherry-Atg8a puncta. Fat body cells from aged animals were isolated using a stereo microscope, rinsed in 1X phosphate buffered saline (PBS) and mounted on a single microscope slide. Nuclei of unfixed cells were stained for 5 min with 20ug/mL 4',6-diamidino-2-phenylindole (DAPI) and then washed with PBS. The mounted tissue was gently pressed between the glass slide and a cover slip to reduce the depth of field.

Epifluorescence microscopy

An epifluorescence microscope was used to determine the presence of GFP and mCherry-Atg8a in the fat body cells. In a fluorescence microscope, a high-pressure mercury vapor or xenon lamp supplies an intense UV radiation that passes through an excitation filter, which transmits only those wavelengths that match the excitation spectrum of the fluorochrome. Fluoresced light then passes through the dichromatic beam splitter and the barrier filter, which allows passage of only the wavelengths of fluorescence. As a result, the viewer sees only those structures that are fluorescing. Epifluorescence microscopy often suffers from

autofluorescence (fluorescence signals from unlabeled objects) and low resolution due to out-of-focus objects (Spector and Goldman, 2006).

Fluorescence images were captured using a Nikon TE20000-U equipped with a mercury vapor fluorescent lamp and a Roper Coolsnap HQ digital camera. To visualize the stained nuclei, DAPI was excited with ultraviolet light and detected using a blue/cyan filter (excitation: 340-380nm, emission: 441-488nm). mCherry-Atg8a autophagic puncta were examined using a Texas red filter cube (excitation: 540-580nm, emission: 593-668nm), while GFP fluorescent signals were viewed using a blue emission filter (excitation: 453-486nm, emission:504-547nm). The exposure times used to detect DAPI, mCherry and GFP were 800, 2000 and 200 msec, respectively. The image software MetaVue^{TM} was used to capture images of the fat body using primarily the 40x objective. Images were false colored and their brightness and contrast were enhanced using MetaVue^{TM}. Layered merges were created using Photoshop CS5.

Stereomicroscopy

Images depicting the cell cycle stages and the *Tubby* phenotype were captured using an Olympus SZ40 stereo microscope, equipped with a PixeLINK camera. Stereo microscopes, also called dissecting microscopes, provide a three dimensional view of the specimen by focusing on the same point from slightly different angels. One drawback of stereomicroscopy is the low magnification (usually below 100x), when compared to compound microscopes. However, stereo microscopes allow for a longer working distance, thus permitting work to

be done on the specimen while it is being observed through the microscope (Burgess et al., 1990). Captured images were enhanced using Photoshop CS5 to improve the images' contrast and brightness.

Statistical analysis on the number of autophagosomal puncta

The number of puncta in GFP-expressing and wild-type cells was manually calculated using the multi-point selections tool on ImageJ. For each hour, values were recorded using the collected images of seven different animals. For each animal, the number of puncta from one GFP-expressing cell and a wild-type cell was determined. Any punctuated red fluorescing object was included in the count, regardless of its size or intensity of fluorescence. Collected data was analyzed on SPSS using a Split Plot/Repeated Measure Design.

RESULTS

Qualitative analysis of the role of *βftz-f1* in regulating autophagy during metamorphosis.

Drosophila fat body cells undergo extensive autophagy at the onset of puparium formation. In order to study the role of *βftz-f1* in regulating autophagy in the *Drosophila* fat body during metamorphosis, I established several mosaic fly lines by crossing *ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B* females with *UAS-dBlimp-1* males. All fat body cells expressed the red fluorescing molecule mCherry fused to the autophagosomal marker Atg8a, while only the transgenic cells that expressed dBlimp-1, and thus had reduced *βftz-f1* levels, expressed a nuclear green fluorescent protein, nlsGFP, as a clonal marker. Animals expressing the repressor protein dBlimp-1 were viable and showed no obvious phenotypic defects or growth delay (data not shown).

The process of autophagy was marked by the presence of several small, punctuated structures distributed throughout the cytosol of fat body cells. Atg8a marks both immature autophagosomes, as well as mature autophagolysosomes. Once autophagosomes form completely by the fusion of both tips of the PAS, Atg8a mainly localizes to the inner membrane surface but still remains detectable (DiAntonio et al., 2012). The size, number and distribution of puncta varied significantly throughout the beginning of metamorphosis and therefore I avoided developing a strict set of criteria for the identification of the autophagosomal puncta. Red autofluorescing background was often present and was easily

identifiable by the reduced fluorescence as well as the lack of punctuated structures (Figure 14).

Between 0 and 4 h APF, Atg8a-positive autophagosomes were observed in both transgenic and control cells (Figure 15). As previously mentioned, mCherryAtg8a puncta were distributed diffusely throughout the cell and did not assume a fixed morphology. No correlation in the morphology of autophagosomal puncta was detected between wild-type and transgenic cells.

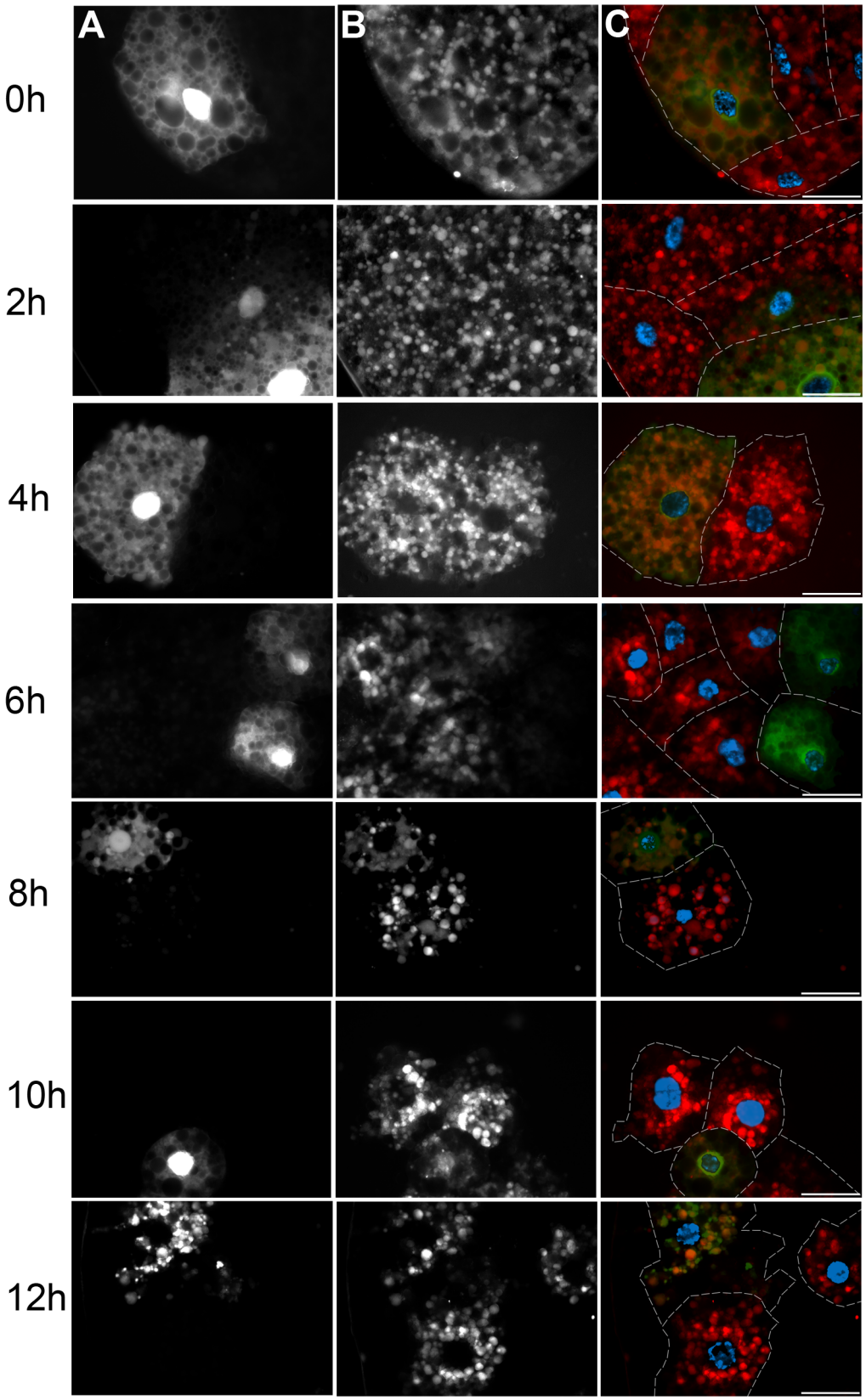
βftz-f1 is generally expressed at 6 h APF (Woodard et al., 1994). At this time, the fat body tissue was still intact, even though some cells were starting to adopt a more rounded shape. The data collected at 6 h APF showed great variability. While some animals displayed no difference in the number of puncta between wild type and GFP-expressing cells (Figure 16:A), other animals showed complete loss of autophagy in the transgenic cells (Figure 16:B). Furthermore, I also observed scenarios in which the GFP-expressing cells displayed a significant reduction in the number of puncta (data not shown). Overall, puncta size remained mostly unvaried and wild type cells exhibited fewer puncta as compared to previous hours.

At 8 h APF, most of the larval fat cells had assumed a spherical shape and some single cell clones had entered the disaggregation phase. However, GFP-expressing clones that were found in larger clusters often failed to remodel and maintained their larval morphology. Surprisingly, about 50% of the cells expressing GFP displayed cytoplasmic localization of nls-GFP (data not shown).

Transgenic cells showed a marked reduction in the number of puncta, which remained unvaried in size (Figure 17).

At 10 h APF, most individual fat cells had entered the detachment phase, during which they were redistributed into the head capsule and body cavity. Almost all transgenic cells displayed cytoplasmic GFP fluorescence and, strikingly, the GFP signal did not diffuse throughout the cytoplasm but instead appeared as punctuated structures that were mostly localized around the nucleus (data not shown). GFP-expressing clones that were found in large clusters failed to remodel and disaggregate from the rest of the tissue. Transgenic cells exhibited a marked reduction in the number of puncta and sometimes complete loss of autophagy. Puncta morphology and distribution remained unvaried (Figure 18).

At 12 h APF, all wild type fat cells were spherical and detached from one another, making it arduous to find two cells of opposite genotypes in close proximity. Conversely, larger cell clusters of GFP-expressing cells that had failed to remodel were often observed. Punctuated, cytoplasmic nls-GFP signals were present in all transgenic cells. GFP-expressing cells exhibited a slight decrease in the number of puncta, which appeared unvaried in size, though they seemed to be mostly localized around the nucleus (Figure 19).


A
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0h
2h
4h
6h
8h
10h
12h

Figure 14. Inactivation of *βftz-f1* expression results in the inhibition of autophagy in the *Drosophila* fat body between 0 and 12 h APF.
Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* between 0 and 12 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Mosaic flies were generated by FLP/FRT-mediated recombination (A) Fluorescence microscopy of the indicated strain showing GFP-expressing transgenic cells with reduced *βftz-f1* expression. (B) Fluorescence microscopy of the indicated strain showing mCherry-Atg8a puncta, a marker for autophagy. (C) Merged images, showing GFP-expressing transgenic cells (false colored green), autophagosomal mChrerry-Atg8a puncta (false colored red) and DAPI-stained nuclei (false colored blue). Images were edited using Photoshop CS5. Scale bar is 50 μm.

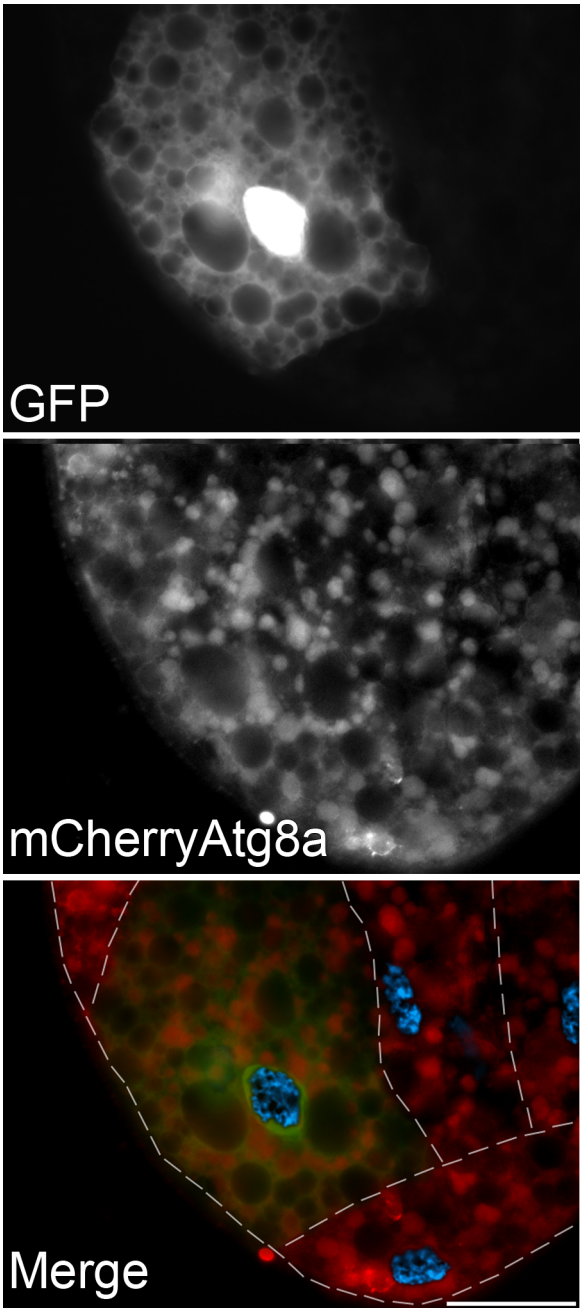
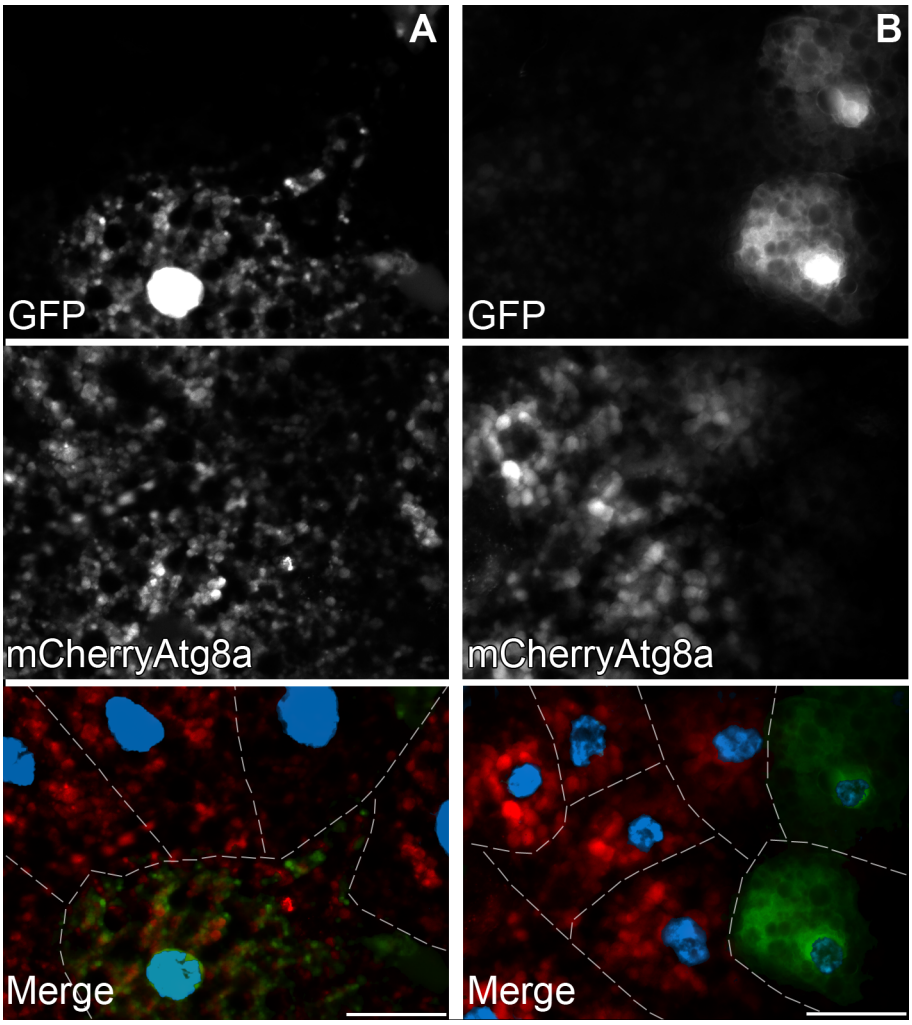

GFP
mCherryAtg8a
Merge

Figure 15. *βftz-f1* is not requited for autophagy in the larval fat body at 0 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* at 0 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the white prepupal stage and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. No remarkable difference in the number of puncta between transgenic and control cell was observed. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.


A
B
GFP
GFP
mCherryAtg8a
mCherryAtg8a
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Merge

Figure 16. *βftz-f1* is required for autophagy in the larval fat body at 6 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* at 6 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 6 h APF and were then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. (A) no remarkable difference in the number of puncta between transgenic and wild type cells, (B) complete loss of autophagy in GFP-expressing cells. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

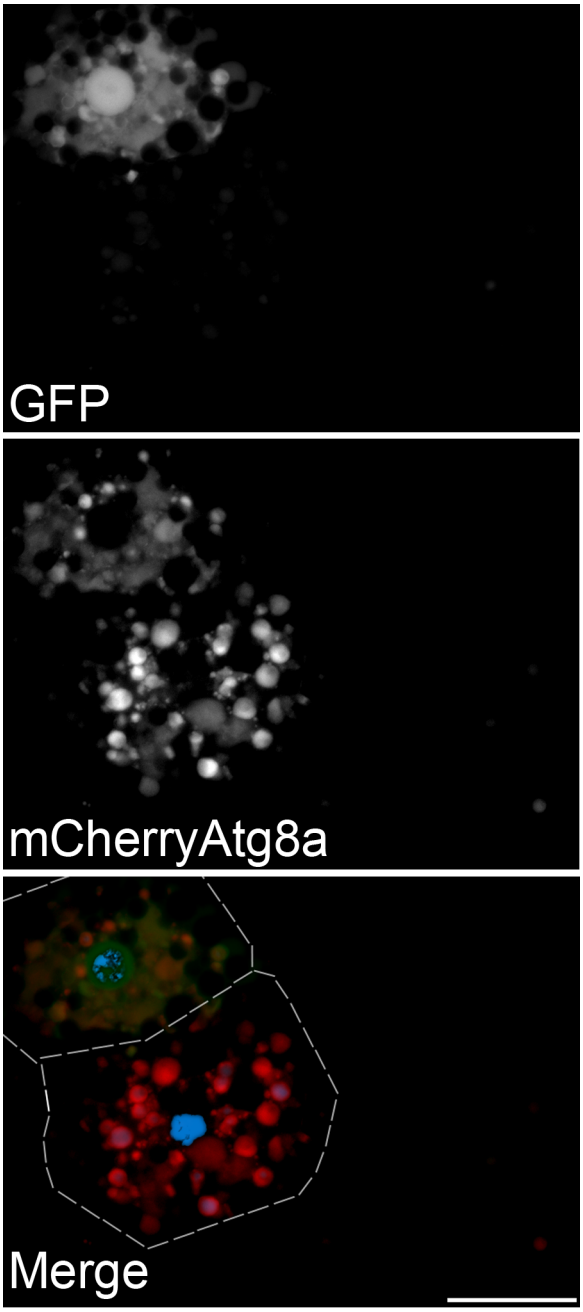
GFP
mCherryAtg8a
Merge

Figure 17. *βftz-f1* is required for autophagy in the larval fat body at 8 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* at 8 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 8 h APF and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. Transgenic cells exhibited marked reduction in the number of puncta. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

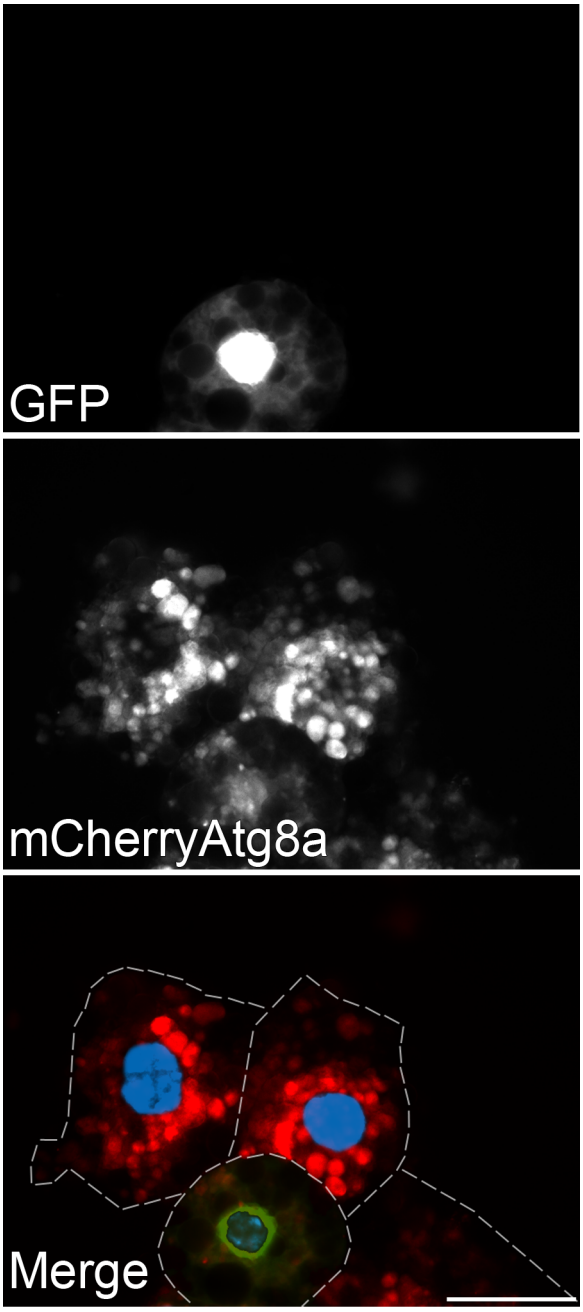
GFP
mCherryAtg8a
Merge

Figure 18. *βftz-f1* is required for autophagy in the larval fat body at 10 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* at 10 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 10 h APF and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. Transgenic cells displayed a significant reduction in the number of puncta as compared to wild-type cells. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

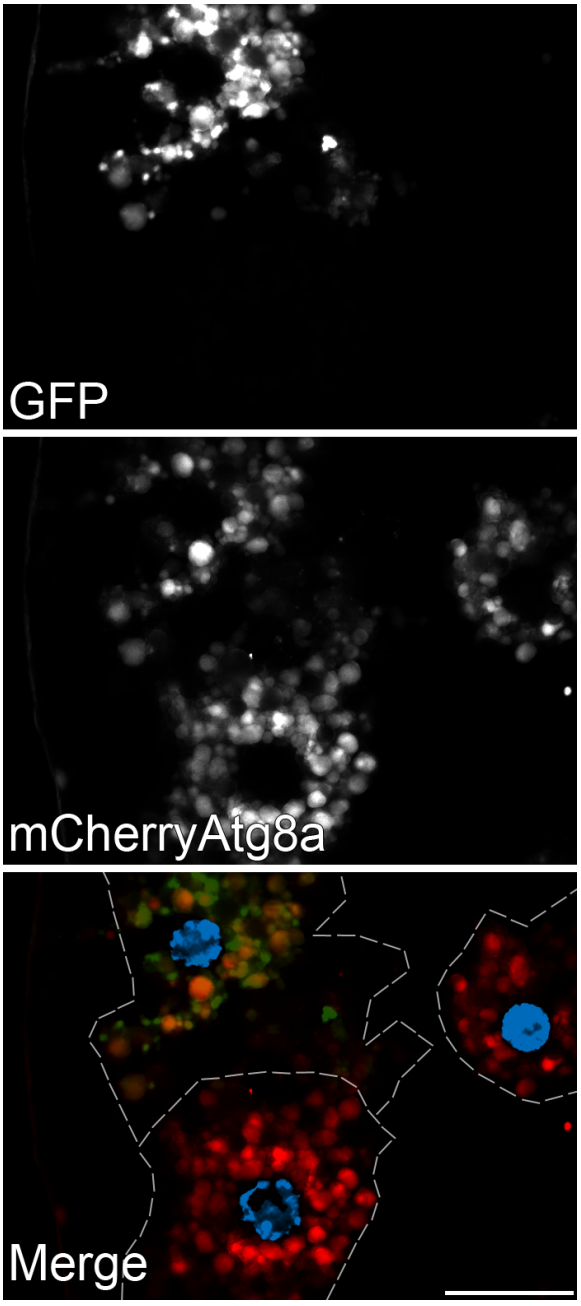
GFP
mCherryAtg8a
Merge

Figure 19. *βftz-f1* is required for autophagy in the larval fat body at 12 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *dBlimp-1* at 12 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-dBlimp-1/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 12 h APF and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. Transgenic cells exhibited a slight reduction in the number of puncta as compared to wild type cells. nls-GFP signal appeared cytoplasmic and punctuated. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

Quantitative analysis of the role of *βftz-f1* in regulating autophagy during metamorphosis.

Overall, the number of autophagic puncta in cells with normal *βftz-f1* expression seemed to increase in response to the rising 20E titers. As the first 20E peak decreased, around 4-6 h APF, the number of puncta in each cell was also greatly reduced. Conversely, during the second 20E high titer pulse at 10-12 h APF, the number of puncta seemed to slowly rise.

Until 4 h APF, no statistically significant difference in the mean number of puncta was detected in the mosaic cells of 7 fruit flies. Starting from 6 h APF, the number of puncta in the transgenic cells markedly decreased as compared to the number of puncta in the wild-type cells (Figure 20). The distribution of individual puncta scores at 6 h APF revealed that there is significant variability in the number of puncta in the GFP-expressing cells (Figure 21). However, starting from 8 h APF, the mean number of puncta in the transgenic cells clearly departed from the mean number of puncta in the GFP expressing cells. The mean number of puncta at 8, 10, and 12 h APF in the transgenic cells was 45.71 (S.E.= 8.454), 29.57 (S.E.= 6.834) and 28.29 (S.E. 5.743), respectively. Conversely, the mean number of puncta in the wild-type cells at 8, 10, and 12 h was 88.57 (S.E.= 5.702), 72.14 (S.E.= 9.384) and 73.29 (S.E.= 5.834), respectively (Figure 20).

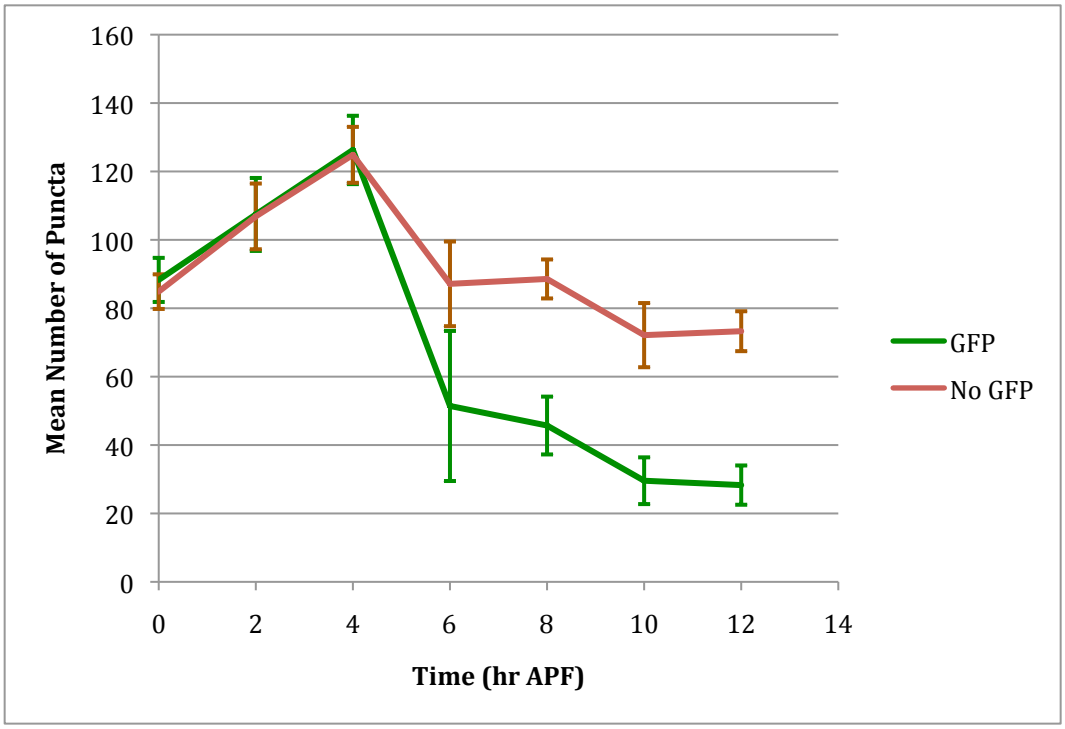

Figure 20. Mean differences in the number of puncta between wild type cells (no GFP) and cells with reduced *βftz-f1* expression (GFP-expressing cells) during metamorphosis. For each time point, scores were collected from seven different fruit flies and analyzed using a split-plot repeated measure design on SPSS. Standard error bars are shown.

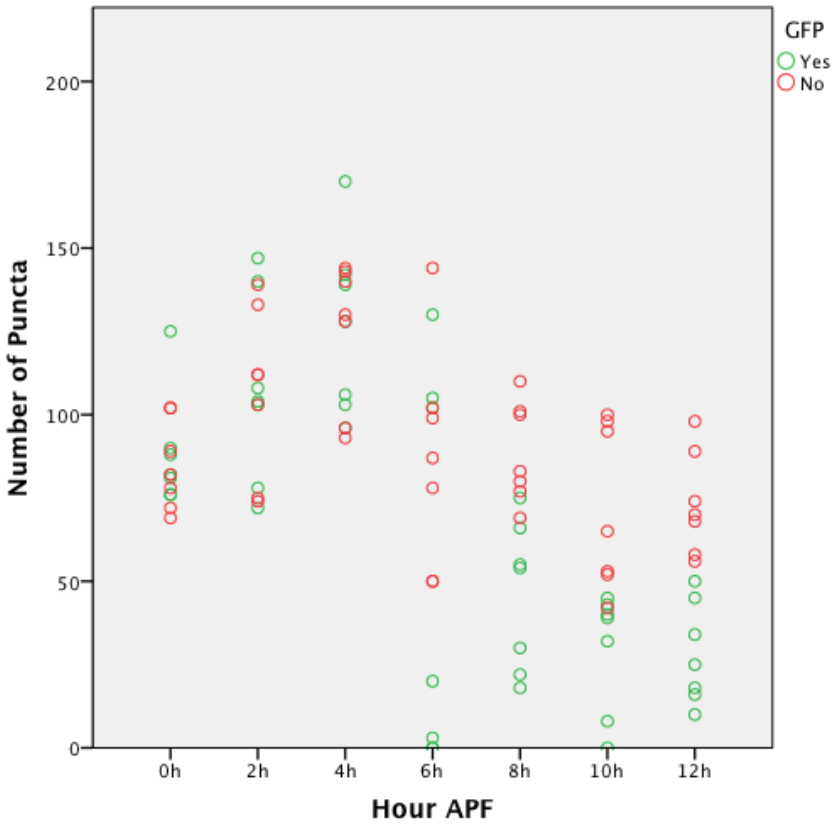

Figure 21. Puncta score distribution in wild type cells (no GFP) and cells with reduced *βftz-f1* expression (GFP-expressing cells) during metamorphosis. Individual scores were collected from 7 different animals. At 6 h APF, the puncta scores from the GFP-expressing cells were spread out roughly equally throughout the entire range. Starting from 8 h APF, the scores from the wild-type and GFP-expressing cells clearly departed from each other.

Qualitative analysis of the role of MMP2 in regulating autophagy and insulin signaling during metamorphosis.

Basal levels of *MMP2* are expressed in the fat body throughout development. The prepupal pulse of 20E, in combination with prior expression of *βftz-f1*, upregulates *MMP2* expression around 8 h APF. At this time, large quantities of MMP2 protein are able to overcome the inhibitory action of TIMP, thus allowing fat-body remodeling to occur. MMP2 has also been previously

identified as an ideal candidate for the down-regulation of insulin signaling and the upregulation of autophagy during metamorphosis. By cleaving the IGFBPs, MMP2 would expose the dILPs to the action of proteases naturally present in the hemolymph. This would ultimately result in a decrease of insulin signaling and an increase of nutrient release via autophagy – a process that is required for animal survival during metamorphosis (Bond et al., 2011).

In order to assess the role of MMP2 in regulating autophagy in the *Drosophila* fat body during metamorphosis, I established several mosaic fly lines by crossing *ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B* females with *UAS-TIMP* males. Similarly to the mosaic analysis performed with *UAS-dBlimp-1*, all fat body cells expressed mCherry-Atg8a, while only the *TIMP* expressing transgenic cells with reduced MMP2 activity expressed nls-GFP. Animals expressing the repressor protein TIMP were viable and showed no obvious phenotypic defects or growth delay (data not shown).

Throughout the beginning of metamorphosis, the number of puncta in the *TIMP* expressing transgenic cells did not considerably deviate from the number of puncta in the wild-type cells (Figure 22). Between 0 and 4 h APF, Atg8a-positive autophagosomes are observed in both transgenic and control cells (Figure 23). Fat cells appear polygonal and still attached. No correlation in the morphology of autophagosomal puncta was detected between wild-type and transgenic cells. mCherry-Atg8a puncta were extremely abundant and distributed diffusely throughout the entire cytoplasm.

Like the mosaic cells with reduced *βftz-f1* expression, most of the fat cells with repressed MMP2 activity also failed to assume a spherical appearance at 6 h APF and the fat body tissue was still intact. Inhibition of MMP2 activity at this time did not lead to a marked reduction in the number of mCherry-Atg8a-positive puncta. Moreover, the number and morphology of the puncta remained comparable to those observed at previous hours (Figure 24).

Between 8 and 12 h APF, the nls-GFP signal of transgenic cells increasingly began to form punctuated structures outside of the nucleus. Most transgenic fat body cells retained the polygonal morphology and failed to disaggregate and consequently translocate into the head capsule. Repression of MMP2 activity did not result in a cell autonomous inhibition of autophagy in the GFP-expression cells. In fact, no changes in the morphology, localization and number of puncta were detected between the two cell types. However, at 12 h APF, the larval fat tissue seemed to become less autophagically active, as fewer puncta were detected in both cell types as compared to previous hours (Figure 25).

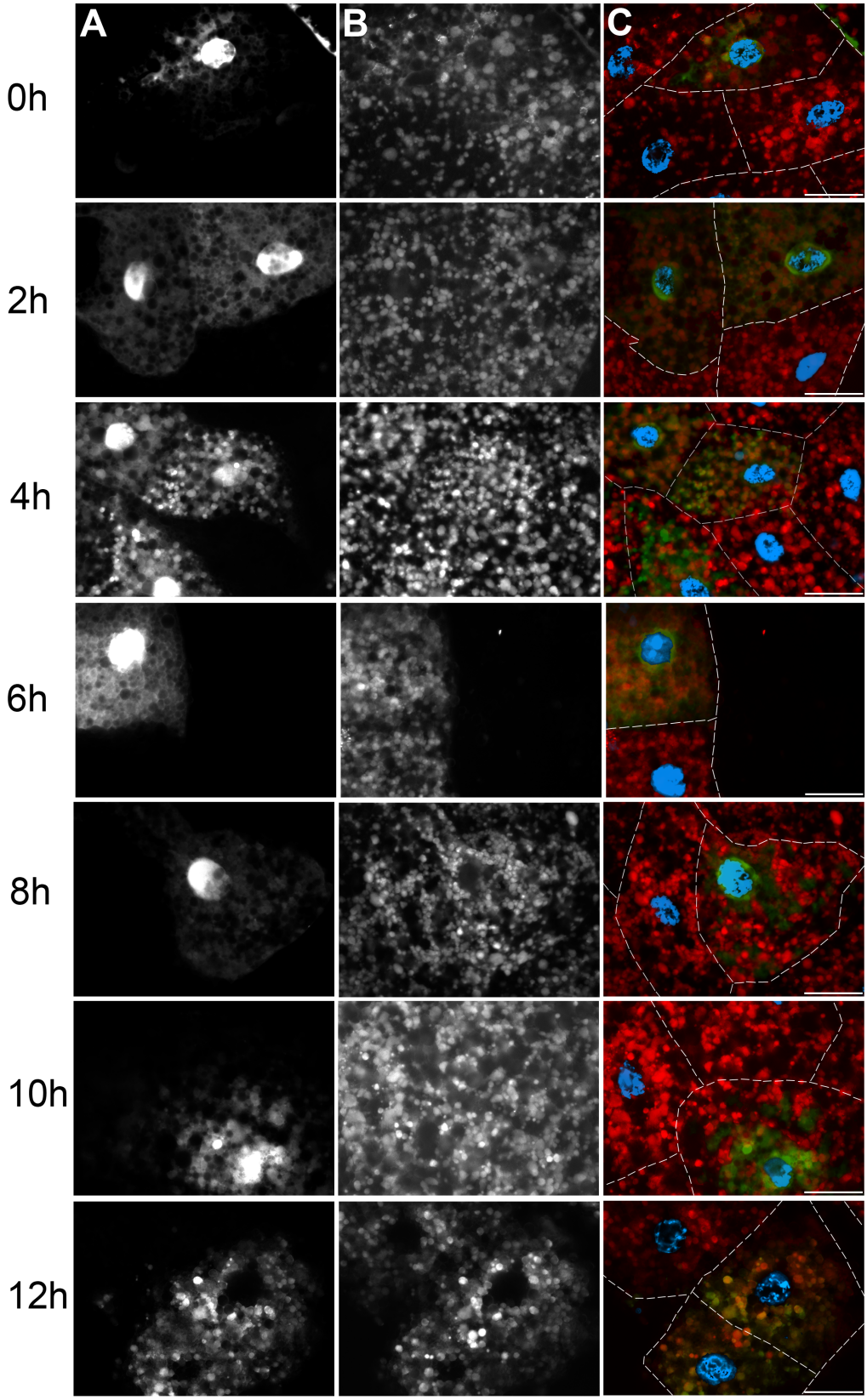
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Figure 22. Repression of MMP2 function does not result in the inhibition of autophagy in the *Drosophila* fat body between 0 and 12 h APF.
Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *TIMP* between 0 and 12 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-TIMP/+)*. Mosaic flies were generated by FLP/FRT-mediated recombination. (A) Fluorescence microscopy of the indicated strain showing GFP-expressing transgenic cells with reduced MMP2 activity. (B) Fluorescence microscopy of the indicated strain showing mCherry-Atg8a puncta. (C) Merged images, showing GFP-expressing transgenic cells (false colored green), autophagosomal mChrerry-Atg8a puncta (false colored red) and DAPI-stained nuclei (false colored blue). Images were edited using Photoshop CS5. Scale bar is 50 μm.

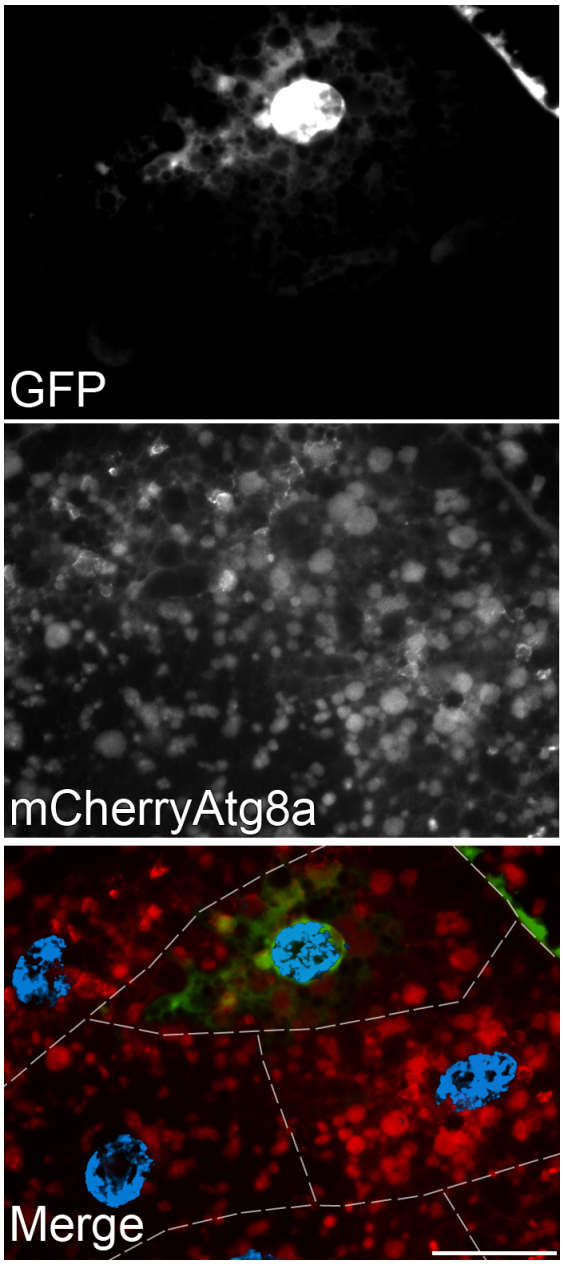
GFP
mCherryAtg8a
Merge

Figure 23. MMP2 is not required for autophagy in the larval fat body at 0 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *TIMP* at 0 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-TIMP/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the white prepupal stage and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. No remarkable difference in the number of puncta between transgenic and control cell was observed. Fat body cells retained larval fat body morphology. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

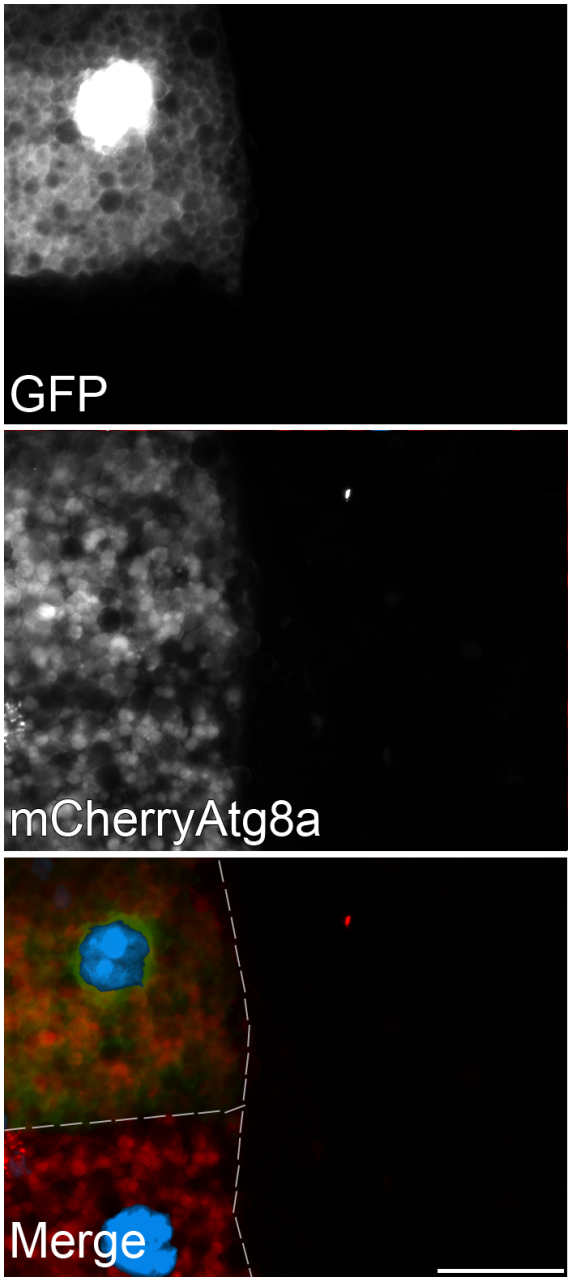
GFP
mCherryAtg8a
Merge

Figure 24. MMP2 is not required for autophagy in the larval fat body at 6 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *TIMP* at 6 h APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-TIMP/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 6 h APF then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. No remarkable difference in the number of puncta between transgenic and control cell was observed. Most fat body cells remained polygonal and failed to remodel. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

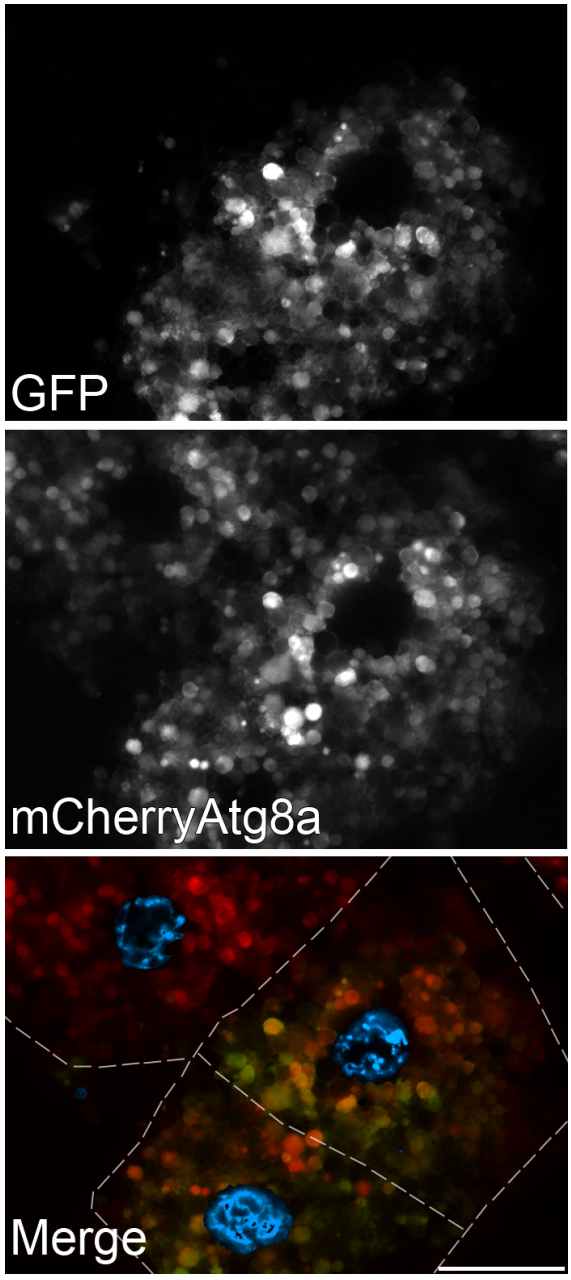
GFP
mCherryAtg8a
Merge

Figure 25. MMP2 is not required for autophagy in the larval fat body at 12 h APF. Fluorescence microscopy of Atg8a-labelled fat body containing single-cell flip out clones (GFP positives) overexpressing *TIMP* at APF (*ywhsFlp; pmCherryAtg84; Act>CD2>GAL4, UAS-nlsGFP/TM6B; UAS-TIMP/+)*. Postmitotic clones of cells in mosaic fat bodies were generated via heat shock at 24 h after egg laying. Larvae were incubated at 25°C to the prepupal stage until 12 h APF and then dissected. Dissected fat body tissue was stained with DAPI and imaged unfixed. No remarkable difference in the number of puncta between transgenic and control cell was observed. Most fat body cells retained larval fat body morphology and failed to disaggregate and detach. Images were pseudo colored and merged using Photoshop CS5. Scale bar is 50 μm.

Quantitative analysis of the role of MMP2 in regulating autophagy and insulin signaling during metamorphosis.

The distribution of puncta followed the rise and fall of ecdysone during metamorphosis. Around 4 h APF, fewer puncta were observed in both wild-type and GFP expressing cells and the number of puncta slowly increased starting from 6 h APF. Throughout the beginning of metamorphosis, mean differences in the number of puncta between the two cells types were not determined to be statistically significant (Figures 26-7).

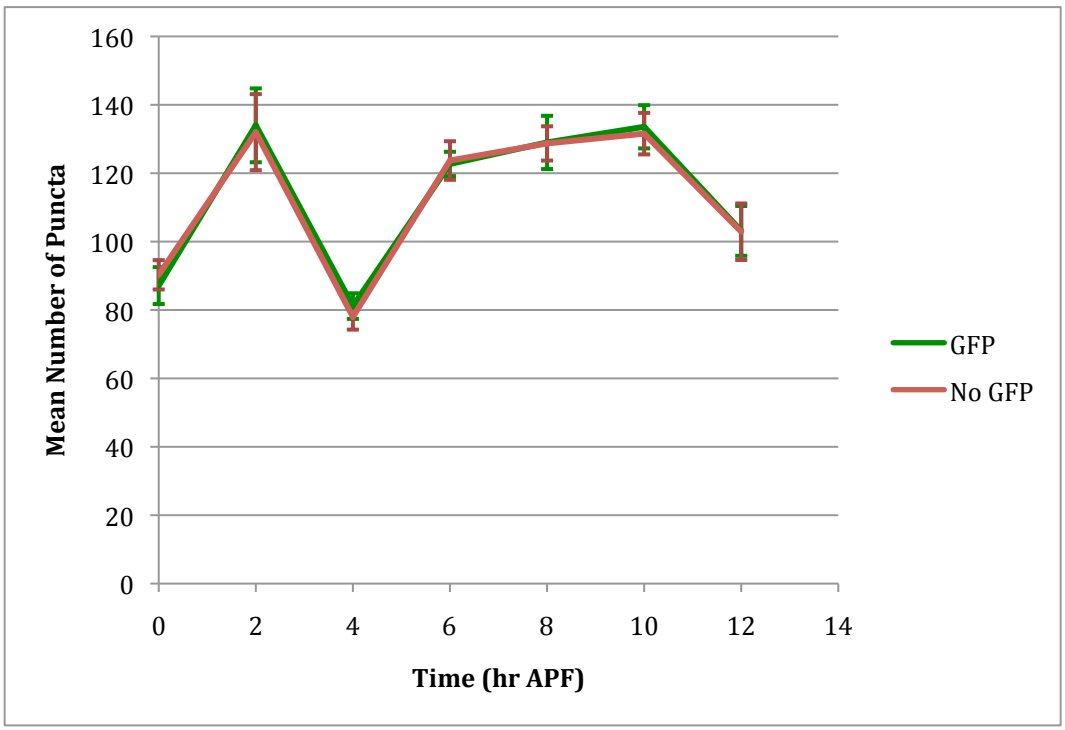

Figure 26. Mean differences in the number of puncta between wild type cells (no GFP) and cells with reduced MMP2 activity (GFP-expressing cells) during metamorphosis. For each time point, scores were collected from seven different fruit flies and analyzed using a split-plot repeated measure design on SPSS. Standard error bars are shown.

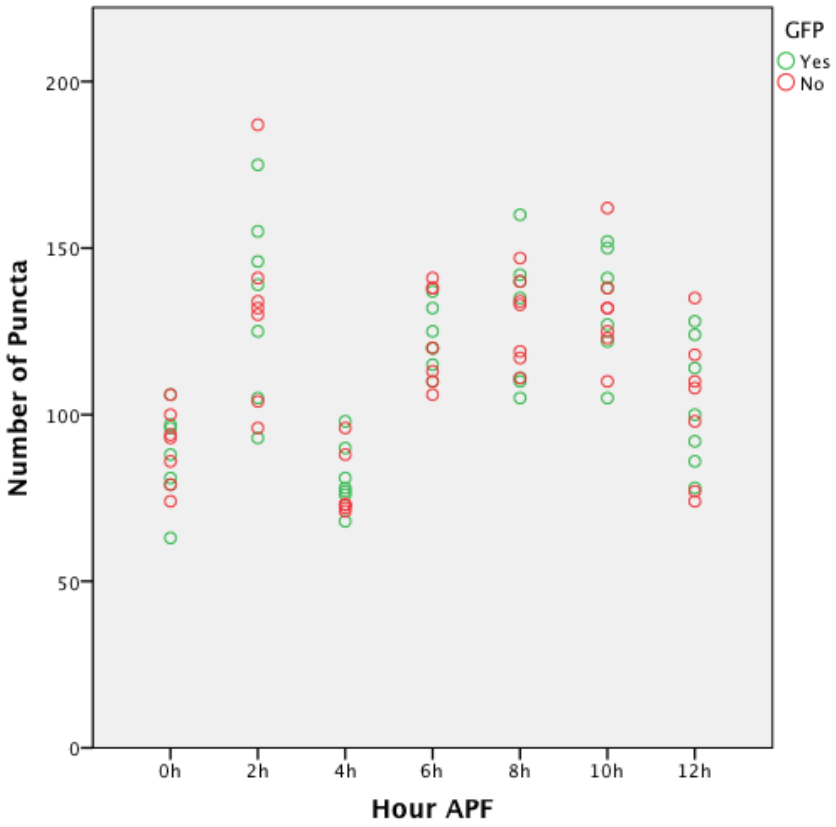

Figure 27. Puncta score distribution in wild type cells (no GFP) and cells with reduced MMP2 activity (GFP-expressing cells) during metamorphosis. Individual scores were collected from 7 different animals. Throughout the beginning of metamorphosis, the puncta scores collected from both wild type and GFP-expressing cells overlapped.

DISCUSSION

βftz-f1 promotes autophagy in the *Drosophila* fat body during metamorphosis

Autophagy is an evolutionarily conserved catabolic process that involves the vacuolar targeting and degradation of bulk cytoplasm. Autophagy likely evolved in single-cell eukaryotes to provide an energy source in response to nutrient deprivation (Scott et al., 2004). In yeast, Tor1 and Tor2 function as nutrient sensors, directly linking cellular responses and nutrient levels. In the presence of nutrients, these proteins mediate a variety of starvation responses, including protein synthesis, nutrient import, cell cycle arrest and autophagy. Thus, autophagy occurs in the context of cellular reorganization aimed at providing temporary survival during periods of starvation (Jacinto and Hall, 2003).

In times of low nutrient availability and/or reduced hormonal and growth factor signaling, many metabolic processes, including ribosome biogenesis, are down-regulated to conserve energy and restrict unwarranted cell growth and division (Boglev et al., 2013). This property could potentially explain the loss of GFP nuclear localization in the remodeling transgenic cells. It is possible that as the cells being to remodel and upregulate autophagy, nuclear import is downregulated and the rate of diffused export from the nucleus is faster than NLS-directed import.

In higher eukaryotes, besides promoting cell survival against apoptosis, extensive autophagy can also cause cell death. During *Drosophila*

metamorphosis, many obsolete larval structures are degraded mainly by autophagic cell death. For example, autophagy, sometimes functioning in parallel with caspases, governs cell death in the midgut and salivary glands (Berry and Baehrecke, 2007; Denton et al., 2007). Conversely, the remodeling of the fat body involved a type of autophagy that appears to play a more significant role in cell survival rather than cell death. A balancing crosstalk occurs between autophagy and caspase activity in this tissue: the inhibition of autophagy induces caspase activity, and the inhibition of caspases induced autophagy (Liu et al., 2013).

The developmental induction of autophagy in the fat body occurs in response to ecdysone. In *Drosophila*, 20E induces autophagy by two means. First, the ligand-receptor complex, 20E-EcR-USP, triggers a transcriptional cascade that inhibits TOR complex 1, TORC1. TORC1 is a central inhibitor of autophagy that prevents autophagosome initiation by phosphorylating the ULK1 protein kinase, a key initiation of the autophagic process (Rusten et al., 2004). Second, the 20E primary response gene *E93* mediates ecdysone-induced autophagy in the salivary glands and the midgut by upregulating the transcription of the *Atg* genes, as well as that of many genes that are responsible for caspase activity, including the caspases *Dronc* and *Drice* and the death activators *Reaper* and *Hid* (Tian et al., 2013; Liu et al., 2013; Yin and Thummel, 2005).

Via mosaic analysis, I demonstrated the *βftz-f1* is cell autonomously required for the induction of autophagy in the fat tissue during metamorphosis. Starting from 6 h APF, cells overexpressing *dBlimp-1* showed a marked reduction in the number of puncta as compared to wild-type fat cells. Moreover, the number

of autophagosomal puncta in some of the transgenic cells seemed to progressively increase from 6 h to 12 h APF. Preliminary studies performed by Perez (2014) on the expression of *βftz-f1* in the fat body of animals overexpressing *dBlimp-1* revealed that *βftz-f1* expression was significantly lower (by 23%) than the expression in wild type fat body cells at 6 h APF. Interestingly, *βftz-f1* expression was at wild-type levels in the 7 h APF prepupae overexpression *dBlimp-1*. These results suggest that overexpression of *dBlimp-1* does not fully repress *βftz-f1* expression but rather it results in slightly delayed and reduced *βftz-f1* expression. This would explain why a slow increase in the number of puncta was observed over time after the initial suppression of autophagy at 6 h APF by dBlimp-1.

A recent study performed in the fat body of the silkworm, *Bombyx mori*, revealed that RNAi knockdown of *Br-C*, *E74*, *HR3* and *βftz-f1* in the 20E-triggered transcriptional cascade resulted in the downregulation of *Atg* genes. Using the MATINSPECTOR program, Tian and colleagues also identified potential binding sites of Br-C, E74, HR3 and βFTZ-F1 in the promoter regions of 12 *Atg* genes (Tian et al., 2013). Even though E93 was known to predominantly transduce 20E signaling to regulate autophagy by affecting a subset of *Atg* genes, the role of *βftz-f1* in regulating autophagy had never been investigated before. These results suggest that, in *Drosophila*, βFTZ-F1 might also play a similar role as in the silkworm and promote autophagy by directly inducing *Atg* gene expression.

Another finding published by the same research group revealed a novel role for *E93* in regulating caspase activity and autophagy in the fat body. Using

TUNEL staining and transmission electron microscopy (TEM), Liu et al. (2014) demonstrated that mutations of *E93* inhibit both autophagy and caspase activity in the fat body of third instar larvae, entering the late wandering stage. Conversely, *E93* overexpression causes precocious and enhanced autophagy in the fat body during the larval-prepupal transition. Curiously, the authors refer to the larval fat body at these times as in the process of "remodeling", even though fat body remodeling, as defined by Nelliot et al. (2006), does not begin until 4 h APF, when the retraction phase occurs and the fat body detaches from the anterior region of the prepupa.

Because βFTZ-F1 has been shown to regulate *E93* expression at 12 h APF (Hoang, 2011), these results would confirm our findings and further suggest that βFTZ-F1 promotes autophagy in part by regulating *E93* expression. However, the role of *E93* in regulating autophagy in the fat body still remains dubious. While it is well known that the fat body of starving and late third-instars undergoes autophagy (Scott et al., 2004; Rusten et al., 2004), the role of E93 in mediating this process is very controversial. Lam (2011) detected numerous lysosomes in the third-instar larval fat tissue from *E93* mutant animals via fluorescence and TEM analyses. Moreover, Baehrecke and Thummel (1995) showed that low levels of transcript are detectable in the fat body of prepupae and that these levels increase only in response to 20E at 12 h APF. Taken together, these results contradict Liu's findings and suggest that *E93* does not regulate the autophagic activity of the fat body of third instar larvae. A mosaic analysis of *E93* repressed

fat body cells at the onset of metamorphosis would help clarify the role of *E93* in autophagy.

MMP2 does not promote autophagy by suppressing insulin signaling in the *Drosophila* fat body during metamorphosis

The process of autophagy is regulated through multiple signaling pathways. The insulin signaling pathway is known to be a potent repressor of autophagy in mammalian cells. In higher eukaryotes, starvation-induced autophagy is suppressed by components of the insulin/class I phosphoinositide (PI) 3-kinase (PI3K), including the insulin receptor, PI3K, and Akt. Activated PI3Ks phosphorylates inositol lipids, generating a variety of secondary messengers, including PIP_3. The phosphoinositide-interacting domain of the cytoplasmic protein kinase Akt binds to PIP_3 and this interaction initiates a signaling cascade that stimulates protein synthesis, lipid storage, glucose import and glycogen synthesis (Britton et al., 2002). The level of PIP_3 is negatively regulated by the tumor suppressor PTEN, which dephosphorylates PIP_3. Expression of a lipid phosphate-deficient form of PTEN results in the suppression of autophagy, due to a failure to antagonize the PI3K pathway (Arico et al., 2001).

In *Drosophila*, developmentally programmed autophagy and subsequent cell death occur in tissue remodeling during metamorphosis and are in part controlled by the hormone 20E (Riddiford, 1993). During larval development, fat body cells accumulate proteins, lipids and produce growth factors via insulin signaling (Kawamura et al., 1999). As the animal enters metamorphosis, these

nutrients must be utilized and the fat body cells remodel, losing organelles and depleting stored metabolites via autophagy (Britton and Edgar, 1998).

The regulation of insulin signaling is tightly controlled. Two *Drosophila* insulin-like growth factors-binding proteins, dALS and Imp-L2, regulate insulin signaling by forming a trimeric complex with dILPs (Arquier et al., 2008). The function of the dILP/dALS/Imp-L2 complex is controversial: while some studies suggest that the complex enhances insulin singling by protecting dILPs from the action of proteases (Jones and Clemmons, 1995; Jones et al., 1993; Mohan et al., 1995; Andress and Birnbaum, 1992; Conover and Powell, 1991; Chen et al., 1994), other studies suggest that the complex prevents dILPs from binding to the insulin receptor, and consequently downregulates insulin signaling (Kalus et al., 1998; Rechler, 1993; Oh et al., 1993).

Arquier and colleagues (2008) proposed a model that reconciles these seemingly opposing roles of IGFBPs. Under starvation conditions, dILPs become limiting and the trimeric complex acts to prolong the half-life of the circulating dILPs by protecting them from degradation. Conversely, under optimal nutritional conditions, the complex acts to prevent dILPs from binding to the insulin receptor.

In mammalian systems, MMPs are known to cleave IGFBPs (Fawlkes et al., 1994). To begin to understand the molecular mechanisms of autophagy and insulin signaling in *Drosophila*, I explored the role of MMP2 as a possible regulator of these mutually antagonistic processes. By cleaving the dILP/dALS/Imp-L2 complex, MMP2 would fine–tune the insulin signaling

response by leaving the dILPs unprotected, thus causing a decrease in insulin signaling. This would ultimately result in an increase in nutrient release, which is key for animal survival during metamorphosis.

Via mosaic analysis, I demonstrated that the inhibition of MMP2 function does not lead to a decrease in autophagy in the fat tissue during metamorphosis. These results do not support the hypothesis that MMP2 promotes autophagy by cleaving the dILP/dALS/Imp-L2 complex. However, these findings do not preclude the possibility that other factors might link insulin signaling and autophagy in the remodeling fat body during metamorphosis. Even though most of the molecular mechanisms still remain unknown, emerging evidence is validating the crosstalk between insulin signaling and autophagy. In the last decade, several insulin-like growth factor-binding proteins have been shown to be directly involved in cell death and animal survival. Specifically, multiple lines of investigation have validated IGFBP-3 as an inducer of autophagy and apoptosis (Lee et al., 2005). Even though the mechanisms by which IGFBP-3 induces these cellular processes are still unknown, recent studies have suggested a role for IGFBP-3 in promoting breast cancer progression. Grkovic et al. (2013) demonstrated that IGFBP-3 binds to GRP79, an endoplasmic reticulum protein, and stimulates autophagy, thus promoting the survival of breast cancer cells challenged by nutrient starvation and hypoxia.

Problems encountered

Fluorescence imaging of the fat body proved to be more difficult than anticipated. Pre-exposure of the fat tissue to blue fluorescent light resulted, for unknown reasons, in severe autofluorescence when the tissue was then exposed to green fluorescent light. This made puncta detection extremely difficult. Surprisingly, pre-exposing the tissue to green fluorescent light first and then exposing it to blue fluorescent light did not produce the same artifact and was used as a technique to prevent autofluorescence. Moreover, I was able to attenuate autofluorescence by partially blocking the emitted blue light.

DAPI staining also posed some problems. Interestingly, the chromosome-specific fluorescent stain would often bind to unidentified structures in the cytoplasm of remodeling fat body cells. These structures would often resemble the autophagic puncta in shape and size but the two types of puncta were often not co-localized. Conversely, non-remodeled transgenic cells only exhibited faint nuclear staining after 8 h APF. It is possible that the stain failed to successfully penetrate the cell and nuclear membranes of non-remodeled fat cells, while remodeling fat cells allowed for better membrane penetrability to DAPI.

Dissection of the fat body became progressively more difficult after 6 h APF. As the fat body cells begin to remodel, they change shape and detach from one another. Frequently, the non-remodeled transgenic cells would remain attached, while the wild type fat cells would detach from the rest of the tissue and individually migrate. This made mosaic analysis very tedious, as this technique relies on the vicinity of both cell types. For later time points, numerous

dissections had to be repeated before I was able to fortunately see cells with opposite genotypes in the same field of view.

Another problem encountered was the inability to accurately count the number of puncta within each cell. Due to the lack of a confocal microscope, autophagic puncta that were not into focus appeared as blurry dots that sometimes blended into the autofluorescing background, making their detection impossible. Thus, I believe that the number of puncta that I have counted represents an underestimation of the actual number of puncta within each cell type.

Drawbacks and future directions

This study proposes a novel role for *βftz-f1* in autophagy regulation. To further support this finding, additional experiments must be performed. First, quantifying *Atg* expression in *βftz-f1* repressed cells would further support the hypothesis that *βftz-f1* regulates autophagy by upregulating *Atg* expression. Second, it is necessary to quantify *βftz-f1* expression in the transgenic fly lines at 8, 10 and 12 h APF by qPCR-RT. Unfortunately, due to the unstable nature of the βFTZ-F1 protein, identifying a potential inhibitor of βFTZ-F1 activity that completely abrogates *βftz-f1* expression becomes a challenge. Previous attempts at knocking down *βftz-f1* expression have not been successful. Bond et al. (2011) tried to block *βftz-f1* expression by RNA interference, however qPCR-RT analysis revealed that this approach had failed to reduce the levels of *βftz-f1* transcripts. Moreover, they showed that *βftz-f1* null mutant animals fail to reach metamorphosis and die during embryonic development. Beckstead et al. (2001)

identified bonus, a *Drosophila* homolog of TIF1 proteins, as an inhibitor of *βftz-f1*—dependent transcription. By binding via an LxxLL motif to the AF-2 activation domain present in the ligand binding domain of βFTZ-F1, bon behaves as a transcriptional inhibitor *in vivo*. Immunohistochemical staining of numerous tissues revealed that bon is a nuclear protein expressed during embryogenesis and the larval stages in most cells, including fat body, imaginal discs, salivary glands, brain, gut, Malpighian tubes and trachea. During the second instar stage, *bon* levels are low. Prior to pupariation, *bon* is upregulated in late third instar larvae and this event correlates with high titer pulses of ecdysone. It is possible that *bon* overexpression during metamorphosis might successfully downregulate *βftz-f1* expression.

Even though my mosaic analysis did not support the hypothesis on the dual role of MMP2 in the fat body, a more quantitative study would help clarify the role for MMP2 in regulating atuophagy. For example, qPCR-RT could be performed to directly quantifying *Atg* gene expression in flies overexpressing *TIMP*. Moreover, my results do not preclude the possibility that other 20E-regulated factors might regulate the crosstalk between insulin signaling and autophagy during metamorphosis.

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