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The Role of ecdysone signaling in fat-body tissue remodeling and pupal metabolism

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THE ROLE OF ECDYSONE SIGNALING IN FAT-BODY TISSUE REMODELING AND PUPAL METABOLISM

by

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ABSTRACT

The Role of Ecdysone Signaling in Fat-Body Tissue Remodeling and Pupal Metabolism

by

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Holometabolous insects undergo an astonishing transition during their development. During metamorphosis, the larva dramatically changes form and becomes an adult fly. During this process obsolete larval tissues must be eliminated, while tissues required for further development are retained and often remodeled to meet the needs of the adult fly. Tissue remodeling is characterized by morphological changes of the cells in a tissue mass. In many cases, remodeling is characterized by dissociation of the tissue mass, releasing cells to move freely around the body cavity. This process is also common in wound healing and is a key feature of human disease processes such as metastasis and airway destruction in asthmatics. The detachment of remodeled cells requires proteases that can break down the extracellular matrix, which is responsible for the integrity of the tissue.

The larval fat body of *Drosophila* is an indispensable tissue required to fuel animal development, thus this tissue is retained and remodeled during the transition from a larva to an adult. In this dissertation I identify the functions of two important proteins in the signaling cascade responsible for the remodeling of the fat body, and I propose a model for the role of this cascade in the fat body for animal survival during

metamorphosis. I performed a detailed characterization of fat-body tissue remodeling and identified three distinct stages associated with remodeling (Nelliot *et al.*, 2006). Using several genetic techniques, I show that the hemocytes (*Drosophila* blood cells) are not required for fat body remodeling and that the process of fat body remodeling is tissue autonomous. I then outline a role for the 20-hydroxyecdysone (20E) signaling cascade in fat body tissue remodeling. Through expression of dominant negative forms of the 20E receptor (EcR) and mosaic analysis I have determined that signaling through the EcR and expression of the competence factor *βftz-f1* are both required for fat body remodeling. I have also identified the 20E signaling target gene *Matrix Metalloproteinase 2* (*MMP2*) as the protease required for remodeling of fat cells during metamorphosis. In addition the role of MMP2 in fat body remodeling, I show that proper expression of *MMP2* is required for animal survival. Also, through mutant analysis, I show that the other *Drosophila* Matrix Metalloproteinase, MMP1, is not involved in fat body remodeling. However, I do demonstrate a fat body specific role for MMP1 in the process of head eversion.

Overall, these results uncover another potential role for MMP2 in the fat body during metamorphosis. My experiments show that proper regulation of *MMP2* expression in the fat body is required for animal survival. In an attempt to explain the importance of MMP2 in the fat body, I propose a model where 20E signaling in the fat body modulates insulin signaling via its induction of *MMP2* expression. Matrix metalloproteinases are known to cleave Insulin-like Growth Factor Binding Proteins (IGF-BPs) and thus have a regulatory effect on insulin-like growth factor signaling in mammals (Fowlkes *et al.*, 1994). In *Drosophila*, IGF-BPs are involved in the protection

of the *Drosophila* insulin-like peptides (DILPS), the ligands for the Insulin Receptor (Arguier *et al*., 2008; Honegger *et al*., 2008). Thus, the binding of IGF-BPs to DILPS modulates insulin signaling. I propose that *MMP2* expression is required in the fat body to modulate insulin signaling during metamorphosis through the cleavage of IGF-BPs. With this model, *MMP2* expression would connect the role of 20E signaling in the fat body to the modulation of insulin signaling during metamorphosis.

In addition to my work on the regulation of fat body remodeling, I also explored the role of fat body 20E signaling in pupal metabolism. The fat body is the central tissue involved in the storage of nutrients and the sensing of nutrient availability. In this dissertation I show that 20E signaling in the fat body is required for animals survival during metamorphosis (Cherbas *et al*., 2003). I hypothesized that 20E signaling in the fat body is a critical factor in metabolic control of pupal development. Therefore I tested the role of 20E signaling in regulation of metabolic rate, as well as the acquisition and utilization of energy stores. Contrary to my hypothesis, I demonstrate that 20E signaling in the fat body does not affect pupal metabolism or animal's ability to attain proper size at pupariation. These data suggest that 20E signaling in the fat body is not required for proper metabolic function.

ACKNOWLEDGMENTS

First I wish to thank Dr. Deborah Hoshizaki for seeing potential in me, plucking me out of a sophomore biology class, and putting me straight to work in her lab. Her passion and enthusiasm for science inspired me to go to graduate school. I have enjoyed talking science with Dr. Hoshizaki so very much and I am grateful to her for her faith in my ability and her unconditional support of my ideas.

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nurtured. Andy is the pillar of encouragement that so many other graduate students and I have leaned on and I would like to sincerely thank him for his support.

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To the many undergraduates that have helped me complete this work, I extend my gratitude. Dan Bricker and Vineeta Jagtiani, thank you so much for your commitment to the project, your reliability and your friendship. I would especially like to thank Karol Zamora-Anderson, Jackie Villalta, and Maki Bernardo for helping me most recently. Your sunny dispositions have kept me from committing to complete cynicism. Maki and Karol, so much of this work would have fallen through the cracks without your attention to detail. Thank you so much for your hard work.

To my Mom and Dad, I will never be able to put into words how thankful I am for you. You have instilled in me the values of hard work and tenacity and it is these qualities that have enabled my success. Thank you so much for the incredible example that you

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LIST OF ABBREVIATIONS

20E	20-hydroxyecdysone
adgf-a	adenosine deaminase-groth-factor
APF	after puparium formation
BR-C	broad complex
crq	croquemort
dALS	drosophila acid labile subunit
dBlimp-1	drosophila B-lymphocyte-induced maturation protein 1
Diap1	drosophila inhibitor of apoptosis 1
DILP	drosophila insulin like peptide
dom	domino
ECM	extracellular matrix
EcR	ecdysone receptor
EcR-DN	dominant negative form of the ecdysone receptor
ETH	ecdysis triggering hormone
GFP	green fluorescent protein
hid	head involution defective
IGF	insulin-like growth factor
IGF-BP	insulin-like growth factor binding protein
Imp-L2	Imaginal morphogenesis protein-Late 2
InR	insulin receptor
IPC	insulin producing cell
JH	juvenile hormone
Lsp-2	larval serum protein 2
MARCM	mosaic analysis with a repressible cell marker
MMP	matrix metalloproteinase
MMP1-DN	dominant negative form of matrix metalloproteinase 1
PG	prothoracic gland
PI3K	phosphatidylinositol-3-kinase
ppl	pumpless
PTTH	prothoracicotropic hormone
RNAi	RNA interference
TAG	triacylglycerols
Tb	tubby
TIMP	tissue inhibitor of matrix metalloproteinases
TOR	target of rapamycin
UAS	upstream activation sequence

CHAPTER 1

INTRODUCTION

The larval stage in the life cycle of *Drosophila melanogaster* is a critical growth period characterized by continuous feeding and acquisition of sufficient energy stores to support metamorphosis and to fuel the metabolism of the young adult (Aguila *et al*., 2007). The insect fat body is the primary tissue involved in nutrient storage and thus has a central role in determining *Drosophila* growth and development. Large storage proteins (hexamerins), triacylglycerols (TAG), and glycogen are all stored in the fat body and can be mobilized as needed by the developing animal (reviewed by Beenakkers, 1969; Telfer and Kunkel, 1991; Thomassan and Mitchell, 1972; Gronke *et al*., 2005; Hoshizaki, 2005; Gutierrez *et al*., 2007; Hoshizaki and Gibbs, 2007). These critical aspects of fat body function make this tissue indispensable throughout development.

20E signaling and metamorphosis

During metamorphosis in holometabolic insects, dramatic changes in animal form are induced by the steroid hormone 20-hydroxyecdysone (20E) as the animal undergoes a complete transformation to give rise to the adult fly (Baehrecke 1996). In order to accomplish this transition, 20E binds to its nuclear hormone receptor, commonly known as the ecdysone receptor (EcR) to initiate transcription of target genes. The functional ecdysone receptor is composed of a heterodimer of two nuclear hormone receptors, Ultraspiracle and EcR (Koelle, 1991; Thomas *et al*., 1993; Yao *et al*., 1993; see Costantino *et al.*, 2008 for exceptions). The functional ecdysone receptor is responsible for initiating a genetic regulatory cascade that results in distinct stage and tissue specific developmental changes (Riddiford, 1996 and Thummel, 1995). 20E initiates a signaling

cascade that promotes the destruction of unnecessary larval tissues and the remodeling of other larval tissues required in the pupa and the adult. 20E signaling is required for the initiation of programmed cell death in the obsolete larval salivary glands and midgut, as well as the induction of cell proliferation and differentiation of the imaginal tissues and the gonads which are functional in the adult (Jiang *et al*., 1997; Lee et *al*., 2002a; Lee *et al*., 2002b; D'Avino and Thummel, 2000; Hackney *et al*., 2007). 20E signaling is also required for remodeling of the larval trachea and dentrites (Levine *et al*., 1995; Page-McCaw *et al*., 2003). The nutrients stored in the fat body are needed to fuel the development of the adult fly. Thus the larval fat body, unlike the larval salivary gland and midgut, is retained during pupal development. Instead of undergoing cell death during metamorphosis, 20E signaling directs a striking morphological change in the fat body resulting in the dissociation of individual cells from the tissue mass, a process known as tissue remodeling (Cherbas *et al*., 2003). During the larval stages, larval fat body cells are polygonal in shape and are tightly associated. During metamorphosis, remodeling of the fat body results in rounding and complete detachment of the cells from the tissue mass (Hoshizaki, 2005; Nelliot *et al*., 2006). The downstream 20E signaling target genes involved in fat body remodeling have not yet been discovered. However other tissue remodeling mechanisms have been described in mammalian systems and in *Drosophila.*

Tissue remodeling and matrix metalloproteinases

The process of tissue remodeling is a central theme in human disease processes such as wound healing, and tumor metastasis. During the wound healing process, degradation of the extracellular matrix (ECM) is required to remove damaged cells and initiate the signaling that promotes inflammation and regeneration of epithelial cells (Gill and Parks,

2008). Remodeling of the ECM also occurs during tumor cell metastasis. Much like the process of wound healing, degrading the ECM of a tumor cell clears the path for migration by releasing a cancerous cell from the tumor mass and activating signaling factors in the ECM that promote metastasis (Andreason *et al*., 1997; Nakahara *et al*., 1997; Xu *et al*., 2001).

Powerful genetic techniques, a rapid life cycle, and the extensive restructuring of tissues during metamorphosis make the fruit fly a valuable tool in the understanding of the molecular processes surrounding tissue remodeling. Proof of this principle can be found in studies that have used *Drosophila* to investigate the process of tumor metastasis. Specifically, it has been shown that expression of the matrix metalloproteinase, *MMP1*, promotes metastasis of brain tumors in *Drosophila* (Beaucher *et al*., 2007). Also, it was discovered that *MMP1* expression is induced by Jun N-terminal kinase signaling using *Drosophila* as a model system (Uhlirova and Bohmann, 2006). The investigation of MMP action during metastasis using *Drosophila* as a model organism is preferable to using a mammalian system because, while there are over 20 MMPs in mammals, there are only two MMPs (MMP1 and MMP2) in *Drosophila* (Sternlicht and Werb, 2001; Page-McCaw *et al*., 2003). This lack of redundancy allows for analysis of the specific roles played by each MMP.

In addition to their roles in metastasis, *Drosophila* MMPs are also involved in the restructuring of the larval tissues during metamorphosis. *Drosophila* MMPs are required for remodeling of the larval tracheae as well as the programmed cell death of the larval midgut (Page-McCaw *et al*., 2003).

20E signaling and fat body metabolism

Disruption of fat-body specific 20E signaling results in animal lethality (Cherbas *et al*., 2003). The cause of lethality resulting from blocking 20E signaling in the fat body has not yet been discovered. The role of the larval fat body as the nutrient storage depot of the developing animal suggests that 20E signaling might be involved in the metabolic function of this tissue. For example, 20E signaling in the fat body has been implicated in nutrient sensing, size control, and energy utilization. One mechanism in place to mobilize nutrients is the process of autophagy. Autophagy is a 20E inducible process which promotes amino acid mobilization in the fat body (Rusten *et al*., 2004). In terms of nutrient sensing and size control, there is evidence that suggests that insulin signaling and 20E signaling are somehow integrated in the fat body to control the final size of the animal (Colombani *et al*., 2005; Geminard *et al*., 2009). It has been proposed that 20E signaling in the fat body may produce factors that inhibit translational growth and suppress the production of insulin-like peptides (Colombani *et al*., 2005; King-Jones and Thummel, 2005). These processes in the fat body are likely to be critical for animal development and survival.

In this dissertation I present a detailed description of the process of fat body remodeling. I delineate the specific aspects of fat body remodeling that are controlled by 20E signaling. I present evidence that 20E initiates a signaling cascade in the fat body involving the competence factor ßFTZ-F1 and the matrix metalloproteinase (MMP2), both of which are required for the remodeling of the fat body tissue. I also demonstrate that the activity of matrix metalloproteinase (MMP1) is required in the fat body for proper development of the adult head. Additionally I explore the role of fat-body 20E

signaling in pupal metabolism in an effort to determine why 20E signaling is required in the fat body for animal survival. I hypothesized that 20E signaling may be required for pupal metabolism. Contrary to my hypothesis, I demonstrate that 20E signaling in the fat body is not required for pupal metabolic processes. Finally I present another model that may explain the importance of fat body remodeling in animal survival. With this model I propose that in addition to its role in fat body remodeling, MMP2 cleaves factors in the ECM that are required for proper insulin signaling in the fat body.

CHAPTER 2

FAT BODY REMODELING IN *DROSOPHILA MELANOGASTER*

Introduction

Insect metamorphosis is characterized by extensive tissue remodeling and proliferation of adult progenitor cells. In *Drosophila melanogaster*, part of this remodeling entails the destruction of larval tissues through a two-step steroid-hormone induced regulatory cascade that culminates in the coordinated induction of cell death genes (Thummel, 2001; Yin and Thummel, 2005). In sharp contrast, the larval fat body is remodeled by undergoing tissue dissociation, resulting in the redistribution of individual fat cells throughout the body of the pupa. Inhibition of fat-body remodeling is associated with pharate adult lethality and is likely to be essential for completion of pupal development (Cherbas *et al*., 2003; Chapter 3). In *D. melanogaster*, the detached fat cells persist throughout metamorphosis and are present in the newly eclosed adult as freely floating single, as well as, small clumps of cells (reviewed in Hoshizaki, 2005; Rizki, 1978; Rizki and Rizki, 1970). In the pupa and newly eclosed adult, the larval fat cells are the likely source of metabolic reserves (Aguila *et al*., 2007). In the young adult, the larval fat cells eventually undergo cell death to be replaced by the adult fat body, which arises from a distinct pool of progenitor cells (Hoshizaki *et al*., 1995).

In *Sarcophaga peregrina*, pupal hemocytes were thought to mediate fat-body dissociation through the production and storage of an inactive form of the cysteine protease Cathepsin B (Aronson and Barrett, 1978; Kurata *et al*., 1990, 1992a,b; Takahashi *et al*., 1993). It has been proposed that during metamorphosis, hemocytes interact with the fat body through a 200 kDa hemocyte-specific surface recognition

protein that triggers the localized release of Cathepsin B through an unknown mechanism. Cathepsin B in its activated form is thought to degrade the fat-body extracellular matrix (Hori *et al*., 1997; Kobayashi *et al*., 1991; Natori *et al*., 1999). Experimental data to support this model, however, has not been forthcoming; the putative 200 kDa surface recognition protein is myosin heavy chain derived from degraded larval muscle and not from pupal hemocytes (Hori *et al*., 1997; Natori *et al*., 1999). *In D. melanogaster*, hemocytes have also been implicated in fat-body remodeling based on genetic epistatic interaction between *croquetmort* (*crq*), which encodes a scavenger receptor found in hemocytes, and the fat-body gene *adenosine deaminase-growth-factor* (*adgf-a*) (Dolezal *et al*., 2005; Franc *et al*., 1996).

To begin to understand the final developmental fate of the larval fat body and its remodeling, and how this impacts on the physiological role of the fat cells in the pupa and young adult, I have begun by detailing the stereotypic changes that take place during fat-body remodeling in *D. melanogaster* and have directly tested the hypothesis that dissociation is hemocytes mediated.

Materials and Methods

Drosophila stocks and manipulations

The *UAS-GFPnls*, *UAS-GFPsyn*, *UAS-GFP T10*, *tub-gal80ts, dominok*^{08108}, and *croquemort*^{KG01679} stocks were provided by the *Drosophila* Stock Center, Bloomington IN, while the *Lsp2-Gal4*, *srpHemoGal4*, and *UAS-hid* lines were kindly provided by L. Cherbas, N. Perrimon, and R. Davis, respectively.

To identify *dom* mutant animals, a *domk*^{08108}; *Lsp2-GAL4 UAS-GFPnls/T(2;3) CyTb* stock was constructed. *Lsp2-Gal4*, *UAS-GFPnls* specifically marks fat-cell nuclei with

GFP. *Dom* mutant animals were selected as non-*Tb* animals and further examined for the characteristic blackened lymph glands of the *dom* mutant. Ablation of hemocytes by cell death was achieved by crossing *UAS-hid*; *tub-gal80ts/T(2;3) Cy Tb* to the hemocyte-specific driver *srphemo-Gal4*, *UAS-GFP*. *Tb* late third-instar larvae or white prepupae were selected, heat-shocked at 29°C, and incubated on wet filter paper until examined for loss of hemocytes and alterations in fat-body dissociation.

To identify *crq* mutant animals, $crq^{KG01679}$ was balanced over *T(2;3) Cy Tb* and non-*Tb* pupae examined.

Microscopy and imaging

Staged animals were rinsed in deionized water, and mounted on bridged slides in Gel/Mount (Biomedia). Both fluorescence and confocal imaging were carried out in the Department of Biological Sciences Imaging Center using a Zeiss Axioplan 2 microscope. Fluorescence images were captured with the Zeiss Axiocam using the Zeiss Axiovision software. LSM 510 software was used to procure the confocal images. All images were compiled in Corel Draw®.

Results

The remodeling of the fat body takes place during the early stages of metamorphosis. I have correlated this process with morphological events previously described in detail for the whole animal (see Figure 1) (Ashburner, 1989; Bodenstein, 1950; Riddiford, 1993; Robertson, 1936). To visualize the dissociation process, I expressed green fluorescent protein (GFP) specifically in the larval fat body using the Gal4/UAS system (Brand and Perrimon, 1993). Using fluorescent microscopy and confocal imaging, we have followed fat-body remodeling in live animals.

Prior to metamorphosis, the larval fat body is composed of single-cell layers of white, translucent cells, which form sheets of tissue floating in the hemolymph between the body wall and the midgut (Figure 1b(1)). The fat cells are flat and polygonal in shape, and appear tightly associated with each other (Figure 2a). At the end of larval development, an increase in the ecdysone titer initiates puparium formation; the larva ceases movement and contracts into a white shortened animal, i.e., the white prepupa. At this stage, the gross morphology of the fat body is unchanged; the fat body extends into the head area and fills most of the peripheral space between the body wall and the gut, and the fat cells remain tightly associated with each other (Figures 1b(2) and 2b). The fat body remains extended for a limited time (ca. 15– 30 min). Over the next 31/2 h, the larval cuticle hardens and tans to form the puparium cuticle (pupal case). These animals have yet to undergo apolysis (Ashburner, 1989), but during this time, the fat body begins to retract from the anterior region. Apolysis occurs from 4 to 6 h after pupariation formation (APF) as the animal separates its larval epidermis from the tanned puparium

cuticle (Figure 1b(3,4)). At this time, the fat cells begin to change shape and take on a slightly rounded appearance (Figure 2c). Completion of apolysis at 6 h APF marks the beginning of prepupal development and consistent with the observation of Rizki (1978), the fat body is completely retracted from the anterior region of the prepupa (Figure 1b(4)). I define white prepupae formation (0 h APF) through apolysis (6 h APF) as the retraction phase. In the next phase, during prepupal development, distinct changes are detected at the cellular level (see Figure 2).

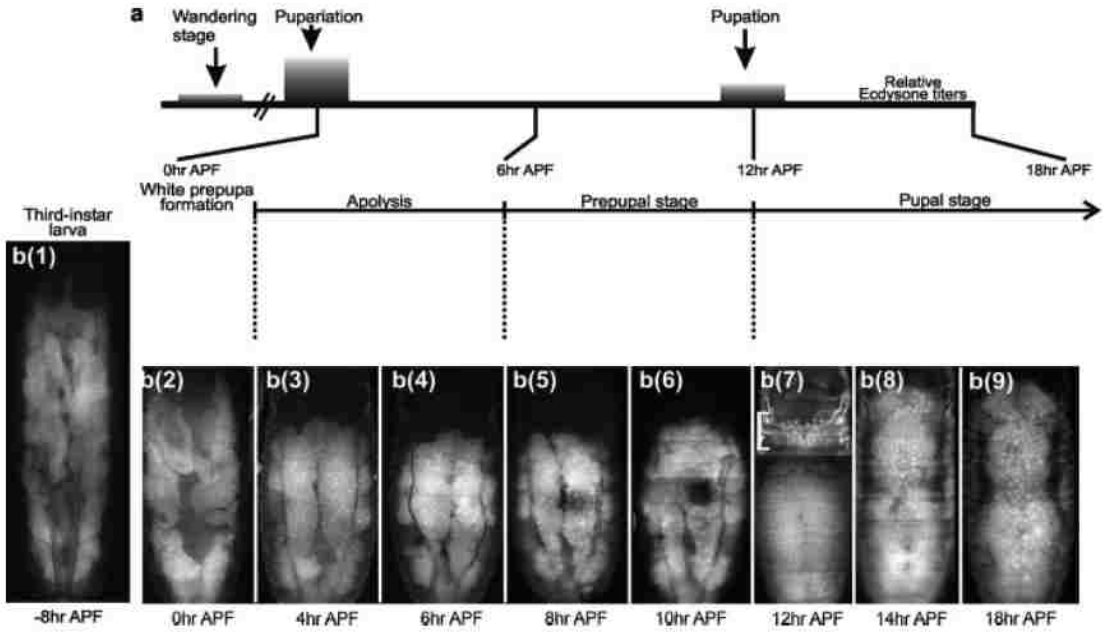

Figure 1. Fat-body remodeling in *D. melanogaster*. (a) Relative ecdysone titer from whole animals during the early stages of metamorphosis (based on Riddiford, 1993). b(1–9) Corresponding changes in fat-body morphology in whole-mount animals. b(1) Late third-instar larva. b(2) White prepupa. At puparium formation, the fat body retains its larval morphology and is extended throughout the body cavity. b(3) Early stage and b(4) Late stage apolytic animal. The fat body retracts from the anterior part of the animal. b(5) Early-stage and b(6) Late-stage prepupa. After completion of apolysis (6 h APF), the fat cells begin to round up (and see Fig. 2). b(7) Early-stage pupa. Immediately after pupation individual fat cells are easily detected entering the head capsule (bracket). b(8) 14 h pupa and b(9) 18 h pupa. Fat-body cells were visualized by GFP expression in living animals carrying *Lsp2-Gal4*, *UAS-GFPnls*. (APF is time after puparium formation at 25ºC.)

Starting from the anterior fat body and progressing toward the posterior, individual fat cells begin to lose their tight associations with each other (Figure 2d). This phase is referred to as the disaggregation stage. Upon completion of prepupal development and in response to a brief rise in the ecdysone titer that peaks at ca. 12 h APF (Figure 1a), the animal undergoes pupation, an event that is marked by the eversion of the head capsule (Handler, 1982; Sliter and Gilbert, 1992). Following this transition, anterior fat-body cells become spherical and begin to physically detach from each other (Figure 2e). After head eversion, fat cells are readily visible as individual cells that are propelled into the head region by abdominal muscular contractions (Figure 1b(7)). As the head capsule fills with cells, the remaining fat cells detach in a progressive wave in the anterior to posterior direction. By 14 h APF, the fat cells are freely packed in the open interior space of the pupa (Figure 1b(8)).

The anterior fat cells autofluoresce when viewed at the wavelength designed to visualize DAPI-stained material (359/461 μm absorption/ emission) (see Figure 3). I have used this observation to follow the anterior fat cells and find that there is little mixing of fat cells during disassociation such that the anterior fat cells fill the head capsule and contribute primarily to the fat cells in the anterior half of the animal (see Figure 3).

The detailed description of fat-body remodeling provides necessary information to test the hypothesis that fat-body dissociation in *D. melanogaster* is hemocyte mediated. To test the role of hemocytes, I genetically ablated these cells and looked for disruption of cell detachment from the fat body in the early pupa. The hemocyte population is made up primarily of plasmatocytes, which are phagocytic cells that differentiate into pupal macrophages during metamorphosis and are thought to be important for phagocytosis of

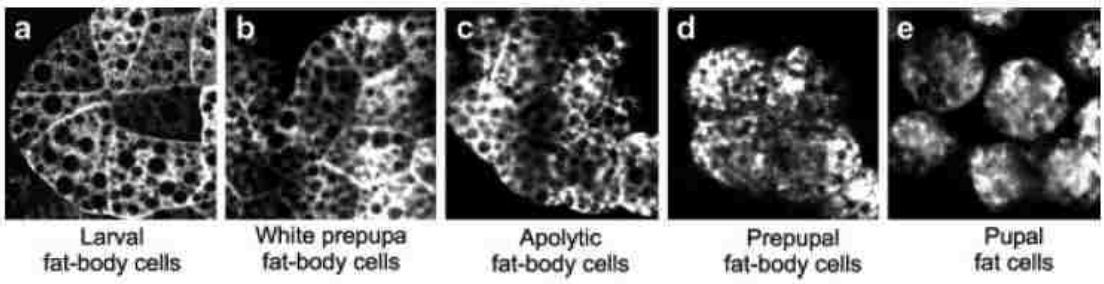

Figure 2. Changes in fat-cell shape during fat-body remodeling. During the disassociation of the fat body, the fat cells undergo stereotypic cell-shape changes where the flat, polygonal larval fat cells are transformed into spherical cells that are detached from each other. Groups of fat cells from (a) third-instar larva; (b) white prepupa; (c) apolytic animal; (d) prepupa; and (e) early-stage pupa. Fat-body cells were visualized by confocal microscopy in whole-mount animals carrying *Lsp2-Gal4*, *UAS-GFP T10*. GFP protein is localized to the cytoplasm. The lipid droplets are visible as vesicles lacking GFP.

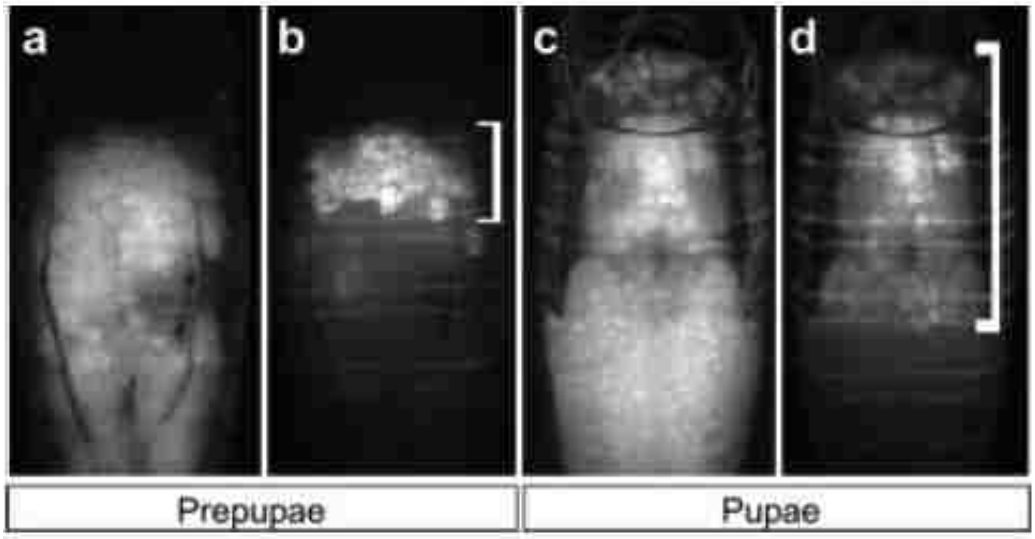

Figure 3. Fat-body remodeling does not result in extensive mixing of cells. Fat cells in whole-mount prepupa (a,b) or pupa (c,d) were detected either by GFP (*Lsp2-Gal4*, *UAS-GFP T10*) in the larval fat cells (a,c) or by autofluorescence (anterior fat-body cells, bracket in b,d).

apoptotic cells (Meister and Lagueux, 2003). At the onset of pupation, plasmatocytes are intimately associated with the fat body and accumulate at the boundaries between the cells (see Figure 4). This association is consistent with the phagocytic nature of the macrophages and their role in the degradation and removal of extracellular matrix. I genetically ablated the hemocytes by using the *domino* (*dom*) mutation, which causes massive cell death of hemocytes in the lymph glands of third-instar larvae (Braun *et al*., 1997). The loss of hemocytes was confirmed by visual examination of dissected mutant larvae (data not shown). To assess whether fat-body remodeling has taken place, live animals were examined for detachment of cells from the anterior fat body and normal displacement into the head capsule. In early-stage *dom* pupae, the fat body undergoes normal remodeling although in many cases the head capsule ruptures during head eversion, allowing individual fat cells to be released throughout the anterior half of the animal (Figure 5a,b). These data suggest that fat-body dissociation does not depend upon hemocytes.

Because the surviving *dom* mutant larvae die shortly after the prepupal/pupal transition, it is possible that the detachment of fat cells is a general defect associated with the dying animal. I, therefore, employed a second strategy to ablate the hemocytes by ectopically expressing the cell death gene *head involution defective* (*hid*) (Grether *et al*., 1995). The loss of hemocytes was established by the absence of GFP positive hemocytes (Figure 5c compared to 5f) and the anterior fat cells were visualized by their autofluorescence (Figure 5e,g). Loss of hemocytes did not affect the normal detachment of the anterior fat cells from the fat body nor their redistribution into the head capsule (Figure 5f,g).

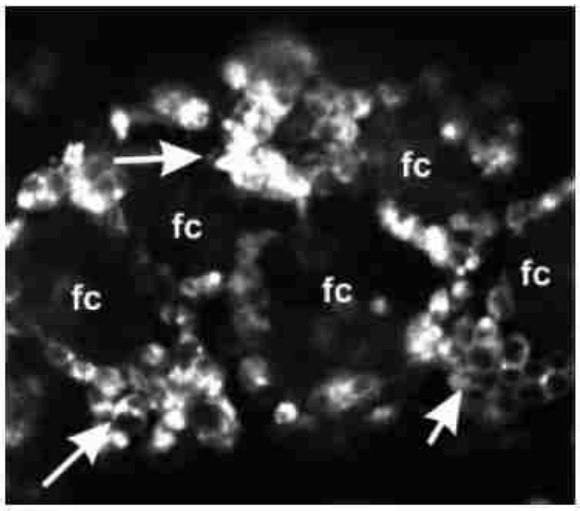

Figure 4. Hemocytes are intimately associated with the fat body during early metamorphosis. Fat cells (fc) are surrounded by hemocytes (arrows). Hemocytes were visualized in a late prepupa by expression of the hemocyte driver *srpHemo-Gal4*, *UAS-GFP*.

Dolezal *et al*. (2005) have supported the idea that fat body dissociation in *D. melanogaster* is a hemocyte mediated event based on genetic studies of the fat body gene *crq* and the hemocyte gene *adgf-a*. The *adgf-a* mutant has a pleiotrophic phenotype culminating in pupal lethality. The larvae have elevated levels of adenosine and deoxyadenosine, exhibit multiple melanotic tumors, and have an increase in the number of hemocytes. The fat body undergoes an aberrant disintegration in the larva which Dolezal *et al*. suggest is premature metaphoric fat-body dissociation (compare Dolezal, Figure 1b to Figure 1b(7)). In support of this suggestion, they report that aberrant fat-body disintegration in the larva is blocked in the *adgf-a*, *crq* double mutant even though there is a decrease in lamellocytes. On the basis of these observations, interaction between fat body and hemocytes, through *crq* and *adgf-a*, respectively, are thought to be necessary for normal metamorphic fat-body dissociation. I have directly tested *crq* for a role in fat-body dissociation and find $crq^{KG01679}$ mutant pupae undergo normal fat-body remodeling (see Fig. 6). I conclude that fat-body disintegration in *adgf-a* mutant larva is not a reflection of premature metamorphic fat-body remodeling but might be due to an acute tissue response to elevated levels of adenosine and deoxyadenosine that is mediated through the Croquemort receptor.

Discussion

In this chapter, I have presented a detailed description of fat-body remodeling in *D. melanogaster* and several lines of evidence that lead to the conclusion that fat-body remodeling in *D. melanogaster* is independent of hemocytes. Supporting this conclusion are the observations that in *Plodia interpunctella* (Indian meal worm), exposure of isolated fat body to the steroid-hormone ecdysone induces dissociation in a dose

dependent manner (Oberlander, 1976), while tissue-specific disruption of ecdysone signaling in the fat body of *D. melanogaster* inhibits the fat-body disassociation (Cherbas *et al*., 2003; Chapter 3). Thus, in *D. melanogaster* fat-body remodeling is likely to be hormone-regulated and cell autonomous.

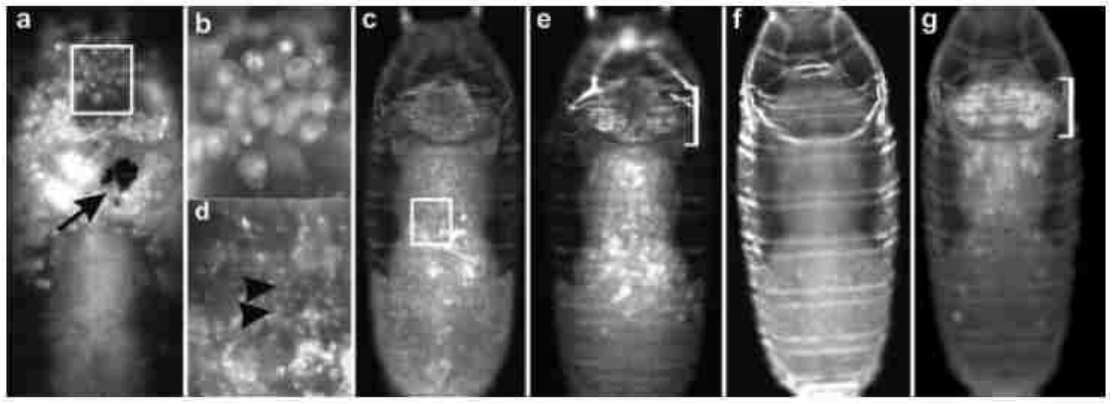

Figure 5. Fat-body remodeling does not require hemocytes. (a) In the *dom* mutant pupa, the fat cells have detached from the fat body and are present as individual cells. Note the blackened lymph glands (arrow) characteristic of dom larvae. Fat cells are marked by expression of *Lsp2-Gal4*, *UAS-GFsyn*. (b) Higher magnification of inset in (a). (c–e) Early-stage *srphemo-Gal4*, *UAS-GFP* pupa. (c) Hemocytes visualized by the GFP expression. (d) Higher magnification of inset of (c) (arrowheads, hemocytes). (e) Individual fat cells have detached from the fat body and are distributed into the head capsule (bracket). (f,g) Heat-shocked treated *srphemo-Gal4*, *UAS-GFP/UAS-hid*+; +/*tub-Gal80*^{ts} early-stage pupa. (f) Hemocytes are absent. (g) Individual fat cells have detached from the fat body and are distributed into the head capsule (bracket). Anterior fat cells in (e) and (g) are visualized by their autofluorescence.

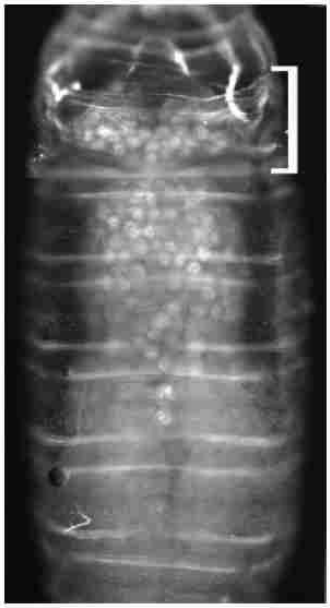

Figure 6. Fat-body dissociation does not require Croquemort receptor. In a *cqr*^{KG01679} pupa fat-body cells detach are distributed into the head capsule (bracket). Fat cells are detected by their autofluorescence.

CHAPTER 3

20E SIGNALING AND ßFTZ-F1 ARE REQUIRED FOR FAT-BODY TISSUE REMODELING

Introduction

In holometabolous insects, the developmental stages of the organism are marked by dramatic changes in animal form as the larva undergoes a complete transformation during pupal development to give rise to the adult. This pupal metamorphic stage is characterized by the destruction or remodeling of most larval tissues and the proliferation, differentiation, and organogenesis of adult tissues (Bainbridge and Bownes, 1981; Bodenstein, 1950; Roberson 1936). Because the pupa does not feed, the energy resources acquired during the preceding larval stage are critical for fueling proliferation (Britton and Edgar, 1998). The fat body is the central energy storage organ of the larva, and plays an important role as an endocrine organ and in monitoring the nutritional status of the organism (Martin *et al*., 2000; Colombani *et al*., 2003; King-Jones and Thummel, 2005; Geminard *et al*., 2009). During metamorphosis, the larval fat body is refractive to cell death and, unlike the majority of larval tissues, is not destroyed. Instead, it is transformed from sheets of polygonal cells to individual free-floating cells (Hoshizaki, 2005; Nelliot et al., 2006). The individual larval fat cells serve as an energy reservoir to fuel pupal development and persist into the adult (Butterworth, 1972; Hoshizaki, 2005; Aguila *et al*., 2007). In the immature adult prior to feeding, the larval fat cells continue to serve as an energy source (Aguila *et al*., 2007) and are critical for gonadal development in the feeding adult (Aguila *et al*., in prep).

The transformation of the larva to an adult during metamorphosis is developmentally regulated by pulses of the steroid hormone 20-hydroxy-ecdysone (20E), which binds to

its nuclear hormone receptor, commonly known as the ecdysone receptor (EcR). The functional ecdysone receptor is actually a heterodimer composed of two nuclear hormone receptors, Ultraspiracle and EcR (Koelle, 1992; Thomas *et al*., 1993; Yao *et al*., 1993; see Costantino *et al.*, 2008 for exceptions). The functional ecdysone receptor is responsible for initiating a genetic regulatory cascade that results in distinct stage-specific developmental changes (Thummel, 1995; Riddiford, 1996). This is accomplished by 20E mediated gene transcription. Studies of the larval salivary gland puffing patterns provided the first evidence for an ecdysone regulatory hierarchy (Ashburner, 1972; Ashburner, 1974; Thummel, 2002). Late in the third larval stage a peak in the 20E titer directly initiates transcription of the primary response genes (also called the "early" genes), leading to puparium formation, which marks the beginning of metamorphosis (Richards, 1981; Riddiford, 1993). The primary response gene products repress their own expression and induce the subsequent transcription of a set of genes known as the secondary response genes (also called the "late" genes). As the 20E titer drops, the mid-prepupal genes are induced (Thummel 1996). One such mid-prepupal gene encodes the competence factor ßFTZ-F1. ßFTZ-F1 is a nuclear hormone receptor, and its expression is required in order for some tissues to respond to a subsequent peak of 20E (Broadus *et al*., 1999; Woodard *et al*., 1994; Yamada *et al*., 2000; Fortier *et al*., 2003). At approximately 10 hours after puparium formation, a second pulse of 20E sets the primary and secondary transcription mechanism back into action inducing developmental events such as head eversion (Handler, 1982; Sliter and Gilbert, 1992; reviewed by Riddiford, 1993). ßFTZ-F1 and 20E are also required for other developmental transitions such as

progression through the larval molts and induction of the third-instar larval wandering phase and the prepupal-pupal transition (Yamada *et al*., 2000; Fortier *et al*., 2003).

The 20E-signaling pathway triggers cascades of gene transcription that control distinct tissue-specific changes. During metamorphosis, 20E signaling induces programmed cell death in some larval tissues (such as the salivary glands and the midgut) and cell proliferation and differentiation in others (such as the imaginal tissues and the gonads) (Jiang *et al*., 1997; Lee et *al*., 2002a; Lee *et al*., 2002b; D'Avino and Thummel, 2000; Hackney *et al*., 2007). Larval fat-body tissue remodeling is also thought to be regulated by 20E signaling (Oberlander, 1976; Cherbas *et al*., 2003). These tissue-specific developmental programs are made possible by expression of different Broad Complex (BR-C) isoforms, and EcR isoforms (Restifo and Merrill, 1994, Talbot *et al*., 1993, Thummel 1996). The roles that these transcription factors play in programmed cell death have been extensively investigated. Regulation of salivary gland cell death by 20E has been particularly well characterized. EcR, BR-C, E74 and E93 are all required for salivary gland cell death (Jiang *et al*., 2000; Lee *et al*, 2000; Kucharova-Mahmood *et al*., 2002; Lee *et al*., 2002b ; Lee *et al*., 2002; Yin and Thummel, 2005). In contrast, E74 is not required for the death of the larval midgut (Lee *et al*., 2002). ßFTZ-F1 is required for the late prepupal induction of BR-C, E74A, E75A, E93 (Broadus *et al*., 1999) and diap2 (Jiang *et al*., 2000) and is therefore also required for larval salivary gland and midgut cell death. ßFTZ-F1 is not sufficient for transcription of these genes however, suggesting that ßFTZ-F1 requires active 20E signaling for expression to occur (Woodard *et al*., 1994; Murata *et al*., 1996; Kawasaki *et al*., 2002).

The temporal control of *ßftz-f1* expression is critical to its proper function as a competence factor. *ßftz-f1* expression only occurs after a decline in the titer of 20E. This expression profile is due in part to the induction of *ßftz-f1* by DHR3 (Lam *et al*., 1997; Lam *et al*., 1999; White, *et al*., 1997). *DHR3* is a 20E target gene whose product binds the *ßftz-f1* promoter and activates transcription of the *ßftz-f1* gene (Lam *et al*., 1997; Kageyama *et al*., 1997; White *et al*., 1997). DHR3 action on the *ßftz-f1* promoter is attenuated by the early-gene product E75B (White *et al*., 1997). E75B binds DHR3 and presumably represses the DHR3 activation of the *ßftz-f1* gene (White *et al*., 1997). Another orphan nuclear receptor, DHR4, is also required for maximal levels of *ßftz-f1* as an activator of the *ßftz-f1* gene and, along with DHR3, may be a redundant activator of *ßftz-f1* expression. Recent work by Agawa *et al*. (2007) has helped to further elucidate the molecular mechanism behind control of *ßftz-f1* expression. dBlimp-1 is a rapidly turned-over 20E-inducible gene product and has been identified as a regulator of *ßftz-f1* expression. dBlimp-1 binds to the *ßftz-f1* promoter and acts as a transcriptional repressor (Agawa *et al*., 2007). In addition to the coordinated effects of DHR3, E75B and DHR4, the transient nature of the dBlimp-1 protein likely results in the tight regulation of *ßftz-f1* expression (Agawa *et al*., 2007).

ßftz-f1 mutant phenotypes have helped to elucidate the role of ßFTZ-F1 in *Drosophila* development. ßFTZ-F1 is necessary for larval molting, cuticle formation, and the prepupal to pupal transition (Yamada *et al*., 2000, Broadus *et al*., 1999, Fortier *et al*., 2003). Tissue specific defects such as failure of larval salivary gland cell death (Broadus *et al*., 1999; Yamada *et al*., 2000), and leg and wing extension (Fortier *et al*., 2003) occur in *ßftz-f1* mutants. Salivary gland cell death, leg and wing disk extension, and larval fat-

body remodeling occur at the prepupal/pupal transition (Yamada *et al*., 2000; Broadus *et al*., 1999; Fortier *et al*., 2003; Nelliot et al., 2006). As previously noted, both remodeling and cell death are developmental events that are dependent upon 20E signaling (Oberlander, *et al*., 1976; Cherbas *et al*., 2003; Levine *et al*., 1995). While it has been shown that the ecdysone receptor is required for fat body remodeling (Cherbas *et al*., 2003), the downstream targets of 20E signaling responsible for specifying the developmental decision to remodel have not been described for the larval fat body. To gain a better understanding of the genetic control of fat body remodeling during pupal development, and the underlying mechanisms controlling fat body dissociation, we carried out a detailed study of fat body dissociation *in vivo* and in *ex vivo* organ cultures, testing the role of ßFTZ-F1 as a key regulator of this process. I found that the redistribution of individual fat cells in the pupa occurs during early pupal ecdysis and that ßFTZ-F1 is sufficient to induce fat-body dissociation in the presence of 20E. Determining the genetic cascade required for the initiation of *Drosophila* fat body remodeling might pave the way for development of a tractable model system that can be used to understand mammalian processes such as metastasis and wound healing. Furthermore, understanding the molecular mechanisms responsible for sparing the larval fat body from apoptosis may provide insight for potential ways to prevent cellular death.

Materials and Methods

Fly stocks

The *UAS-GFPgap* stock (referred to in the text as *UAS-GFP*)was provided by the *Drosophila* Stock Center, Bloomington IN. The *UAS-EcR-DN* (*UAS-EcR-F645A*), *UAS-βftz-f1* (LA276), *βftz-f1*hypomorphic mutants (*Df(3L)Cat*^{DH104}/*ftz-f1*^{17}) and *UAS-dBlimp1*

lines (*UAS-dBlimp1*^{225}, *UAS-dBlimp1*^{226}, *UAS-dBlimp1*^{227}) were generously provided by L. Cherbas, J. Merriam, C. Woodard and G. Call, respectively.

Microscopy and imaging

Staged animals were rinsed in deionized water, and mounted on bridged slides in Gel mount (Biomedia). Both fluorescence and confocal imaging were carried out in the School of Life Sciences Imaging Center using a Zeiss Axioplan 2 microscope. Fluorescence images were captured with the Zeiss Axiocam using the Zeiss Axiovision software. LSM 510 software was used to procure the confocal images. All images were complied in Corel Draw®.

Time-lapse imaging of fat body remodeling was carried out at Mount Holyoke College using the BioRad MRC 600 laser scanning confocal microscope (Nikon inverted Diaphot base) according to protocols detailed in Fortier *et al*. (2003). Still images were compiled in Corel Draw®.

Organ ex vivo culture assay

Fat bodies from late third instar larvae expressing either *UAS-GFP*;*Lsp2-Gal4*, or *UAS-GFP*; *Lsp2-Gal4*/*UAS-βftz-f1* were dissected in 1X DPBS (52 mM l^{-1} NaCl; 40 mM l^{-1} KCl; 10 mM l^{-1} Hepes; 1.2 mM l^{-1} $MgSO_4$; 1.2 mM l^{-1} $MgCl_2$; 2 mM l^{-1} Na_2HPO_4; 0.4 mM l^{-1} KH_2PO_4; 1 mM l^{-1} $CaCl_2$; 45 mM l^{-1} sucrose; 5 mM l^{-1} glucose, pH 7.2) and placed in 200µl Schneider Media (Sigma) in tissue culture chambers at 25°C. 20-hydroxyecdysone (20E) (Sigma) was diluted to a 10^{-3} M solution by dissolving in 100% ethanol. A final working solution of 10^{-5} M 20E was made by dilution of the 10^{-3} M directly into the Schneider Media and was used during the experiment. Fat bodies were incubated for 8 hours with or without 10^{-5} M 20E (also in ethanol) and were imaged

using the methods described above. The experiment was repeated with animals not expressing GFP (*Lsp2-Gal4*). These animals were stained with Sytox® Green Live/Dead Assay (Invitrogen) and imaged as described above.

RNA isolation and cDNA synthesis

Animals were collected at the white prepupal stage, placed on moist filter paper in a Petri dish, and left to develop to the desired stage (8 to 16 hours APF) at 25ºC. When the desired stage was reached, 4 to 5 animals were dissected and the fat bodies were placed in 30 µl of PBS. 300 µl of TriZol (Life Technology) was then added to the tubes containing fat bodies and PBS and the tissue was homogenized. The sample was then transferred to a 2ml Phase Lock Gel-Heavy microfuge tube (Eppendorf), and centrifuged at 12,000 g for 10 min at 4ºC. After centrifugation, the aqueous phase was transferred to a new tube and 160 µl of isopropanol was added. The RNA was allowed to precipitate overnight at -20ºC. The precipitated sample was centrifuged at 12,000 g for 20 minutes. After centrifugation, the supernatent was removed by pipette. The remaining pellet was washed with 500 µl of 75% ethanol, centrifuged for 10 minutes and the supernatant was removed by pipette. The pellet was air dried and resuspended in 5 µl of Rnase free water. RNA concentration was determined by Nandrop. cDNA was synthesized from the RNA samples using the Invitrogen First-Strand cDNA sythesis kit.

Quantitative RT-PCR

Primers were synthesized by IDT (Integrated DNA Technologies) and designed from sequences from Flybase using the program on the IDT website (www.idtdna.com).

Sequences:

ß-actin

Forward: 5'-TCTACGAGGGTTATGCCCTT-3'

Reverse: 5'-GCACAGCTTCTCCTTGATGT-3'

E93

Forward: 5'-ACATTCATCAGCACGAGAGT-3'

Reverse: 5'-GAGTCCATCGATGTCATTTT-3'

qRT-PCR was performed using PerfeCta™ SYBR® Green Supermix, ROX (Quanta Biosciences) according to the manufacturer's instructions on an Applied Biosystems cycler using the following program: 95°C 2 min, 40 cycles of 95°C for 15s, 56.7°C for 30s, and 72°C for 30 s, and 1 cycle of standard melt curve at the end of the program. Primer titrations and standard curves were generated by qRT-PCR to test primer efficacy. *E93* expression was normalized to *ß-actin* and the expression of experimental samples (*Lsp2-Gal4*; *UAS-ßftz-f1*) was compared to control samples (w^{1118}) of the same stage using the ΔΔ-Ct method.

MARCM

Progeny from *yw hs-flp/yw hs-flp; cg-Gal4*, *UAS-GFP/CyO*; *FRT2A, tubulin-gal80/TM6B*, *Tb* females crossed to *yw hs-flp*/+; *FRT2A, ftz-f1*^{19}/*TM6B*, *Tb* males were heat shocked at 37°C for 30 min 2 to 4 hours after egg lay to induce FLP recombinase and generate clones of mutant fat cells expressing GFP. These animals were placed at 25°C after heat shock. After head eversion, animals clonally expressing GFP were selected and imaged as described above.

Results

Fat body maturation during pupal development

The remodeling of the larval fat body during metamorphosis is divided into three stages: retraction, disaggregation, and detachment (Nelliot *et al*., 2006). The final step, detachment, is associated with the prepupal/pupal transition and the redistribution of individual fat cells into the head capsule and body cavity. We carried out a time-lapse analysis of fat body remodeling by marking the larval fat cells with green fluorescent protein (GFP) using a *Lsp2-Gal4;UAS-GFP* stock (Nelliot *et al*., 2006). *Lsp2-GAL4* is a fat-cell specific driver that initiates expression in the fat body during the third instar (Andres *et al*., 1993; Cherbas *et al*., 2003). The distribution of the fat cells into the anterior portion of the animal was complete within 43 min (+/- 2.5 min at 23ºC) of head eversion. Head capsule eversion is accomplished by abrupt body movements that occur during the late prepupal stages. The late prepupal body contortions are part of the pupal ecdysis behavior, which consists of air bubble translocation and abdominal contractions resulting in separation of the pupal cuticle from the larval cuticle (Kim *et al*., 2006). Ecdysis is an event that is initiated by the release of the Ecdysis Triggering Hormone (ETH) from the Inka cells (Zitnan *et al*., 1999; Zitnan *et al*., 2007). After ecdysis-mediated head eversion, pulsatile abdominal contractions function to push the detached fat cells into the head capsule (Figure 7). At first glance one could make the assertion that shearing forces produced from the muscle contractions and the moving air bubble could be responsible for the detachment of the fat body cells. Closer inspection of the process revealed that the fat cells are detached prior to head eversion (data not shown). Thus the

process of fat body remodeling occurs before head eversion, and it is ecdysis-related muscular contractions that propel the detached fat cells into the head capsule.

The role of 20E signaling in fat body remodeling

20E signaling drives the destruction of many larval tissues during metamorphosis as well as the remodeling of other tissues that do not undergo programmed cell death. Previous work suggest a role for 20E signaling in fat body remodeling (Cherbas *et al.* 2003). I carried out a detailed characterization of fat body remodeling in animals in 20E signaling is disrupted in the fat body. Again I used the fat body specific *Lsp2-GAL4* driver to drive expression of *UAS-GFP* and *EcR-DN* simultaneously. Animals were analyzed at 4 hours AFP and 8 hours APF, time points corresponding to completion of fat body retraction and disaggregation, respectively (Figures 8 and 9). Disruption of 20E signaling did not affect fat body retraction (Figure 8, h) Disaggregation of the fat body did however was inhibited when 20E signaling was blocked (Figure 8, j). On a cellular level, I found that disruption of 20E signaling prevents normal cell shape changes associated with remodeling. The fat body cells remained flat and attached after apolysis, a time when disaggregation should occur. The cells remain firmly attached at 12 hours APF (Figures 8 and 9) and complete detachment was not observed at any later time point. I conclude that fat-body specific 20E signaling through EcR is required for the disaggregation and detachment stages of remodeling, whereas fat body retraction occurs independently of 20E signaling in the fat body.

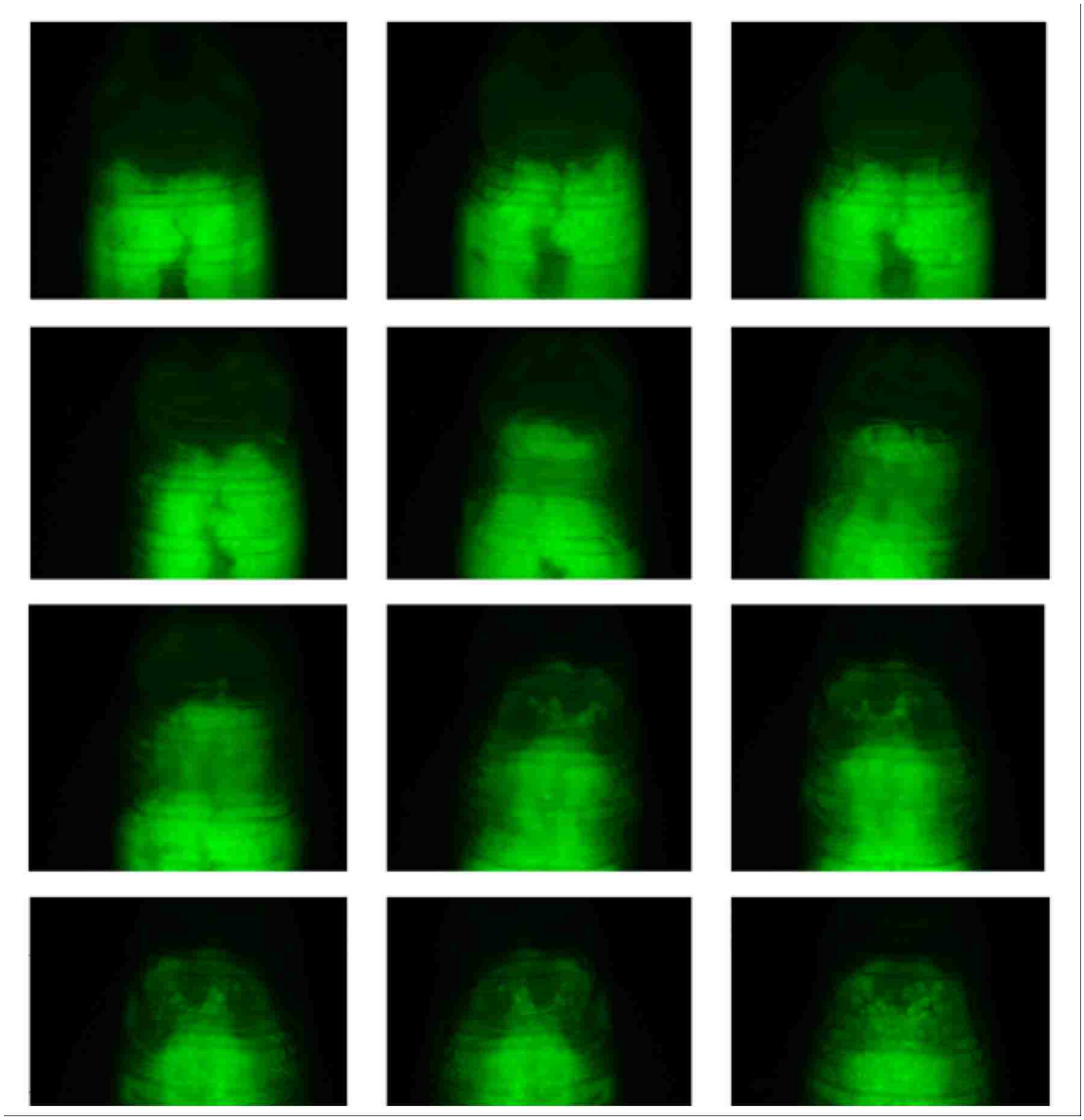

Figure 7. Still images from time-lapse movie of fat body remodeling. Animals expressing GFP in the fat body tissue (*UAS-GFP*; *Lsp2-Gal4*) were collected as prepupae and imaged during the process of head eversion and subsequent fat cell population of the head capsule. After the head has everted, the detached fat cells are pushed into the head capsule by the muscular contractions during ecdysis.

Wild Type

20E Signaling Blocked

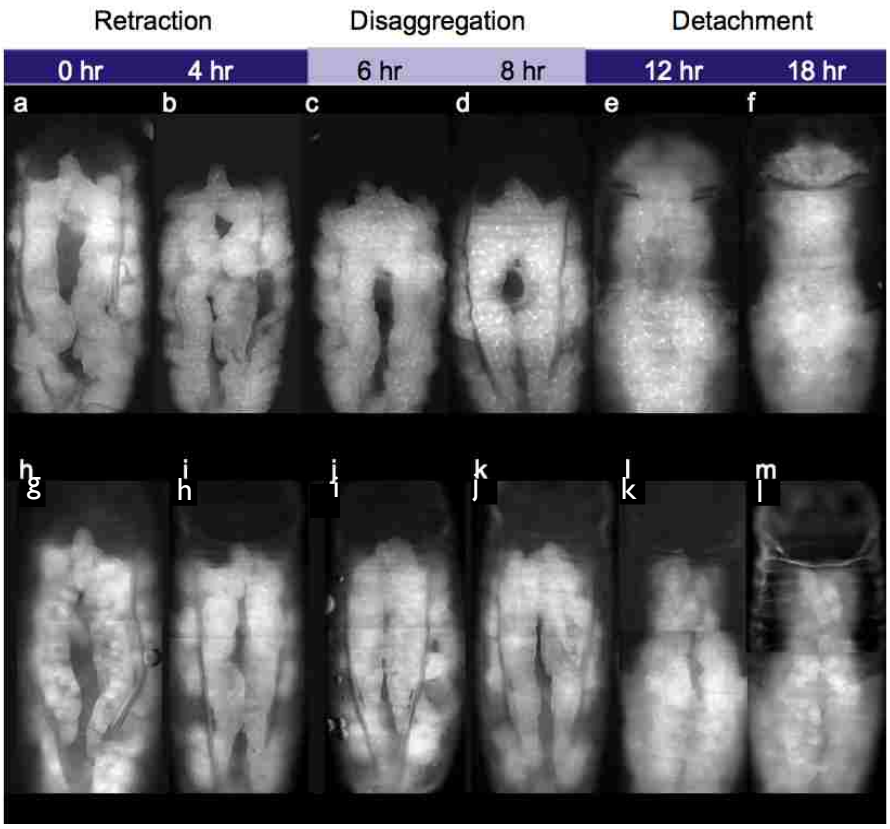

Figure 8. Fat body disaggregation and detachment requires EcR. (a-f) Wild type fat body remodeling. Animals expressing GFP specifically in the fat body (*UAS-GFP*; *Lsp2-Gal4*). (g-l) Fat body remodeling is blocked in animals expressing the dominant negative form of EcR (*UAS-EcR-DN*,*UAS-GFP*; *Lsp2-Gal4*). Developmental time points marked in hours after pupariation.

Premature expression of βFTZ-F1 in the larval fat body induces premature fat body remodeling

βFTZ-F1 plays a fundamental role in head eversion through the release of ETH from the Inka cells (M.E. Adams, personal communication) resulting in the movement of the abdominal air bubble (Fortier *et al*., 2003) and separation of the larval cuticle from the pupal cuticle. Animals mutant for *ßftz-f1* also display many defects at the prepupal/pupal transition (Broadus *et. al*., 1999; Yamada *et al*., 2000; and Fortier *et al*., 2003). Fat-body remodeling also occurs at this critical developmental time. Thus, I tested whether *ßftz-f1* might also be involved in the process of fat body remodeling.

To determine whether expression of *ßftz-f1* is sufficient to drive fat-body remodeling, I prematurely expressed *ßftz-f1* in the fat body using the *Lsp2-Gal4* driver (Figure 10). Fat body remodeling occurred prematurely in these animals (Figure 11, B). Fat-body disaggregation occurred by 4 hours APF (Figure 11, B) and fat cell detachment occurred by 6 hours APF (Figure 11, C). In comparison, wild-type disaggregation occurs at 6 to 8 hours APF and detachment is complete by 12 hours APF (Figure 8, e and 9, e). Thus, *ßftz-f1* is sufficient to promote fat-body remodeling *in vivo*.

Premature expression of βftz-f1 and a subsequent increase in the 20E titer is sufficient to induce fat body remodeling

According to the established hierarchical 20E-signaling cascade, the late genes are turned on during the pupal pulse of 20E, after the system is reset by the competence factor βFTZ-F1 (Broadus *et al*., 1999). Presumably, these 20E response genes are required for fat body remodeling. Based on quantitative RT-PCR analysis, endogenous

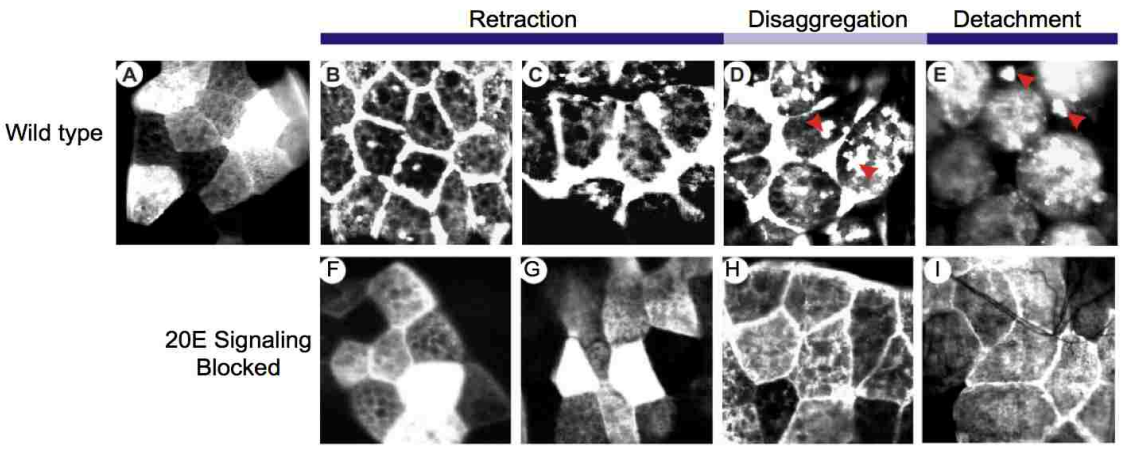

Figure 9. Fat-body disaggregation and detachment require 20E signaling. (A-I) Confocal imaging of *in vivo* fat bodies. (A-E) Animals expressing (*UAS-GFP*; *Lsp2-Gal4*). (F-I) Animals expressing (*UAS-EcR-DN*, *UAS-GFP*; *Lsp2-Gal4*).

βftz-f1 expression is detected in the fat body prior to the high titer 20E pulse that initiates pupariation (Craig Woodard, personal communication). Subsequently, at the onset of the pupariation 20E titer, fat body remodeling occurs. In animals prematurely expressing *βftz-f1* in their fat bodies, fat body remodeling is completed in the early prepupa, up to 8 hours earlier than wild type fat body remodeling. As with wild type fat body remodeling, precocious remodeling does not occur immediately after premature expression of *βftz-f1* is initiated, at the third instar. Instead, I observed that fat body detachment does not occur until sometime after the white prepupal pulse of 20E (Figure 11). I hypothesized that fat cell detachment would occur when *βftz-f1* is ectopically expressed only after a subsequent increase in the titer of 20E (see Figure 10). To test this hypothesis I carried out an *ex vivo* organ culture experiment. Fat body explants from third instar larvae prematurely expressing *βftz-f1* were cultured in Schneiders insect media and subjected to a single exposure of 20E (see Materials and Methods). After eight hours of incubation, the fat body explants subjected to 20E underwent remodeling and the cells completely detached from one another (Figure 12). Cells of fat body explants cultured without 20E remained attached to one another. These data suggest that expression of *βftz-f1* along with a subsequent increase in the 20E hormone titer is induces fat-body remodeling. If both 20E signaling and *βftz-f1* are indeed necessary, one would expect that premature expression of *βftz-f1* would be insufficient to induce in fat body remodeling when *EcR-DN* is expressed. In agreement with this logic I found that co-expression of *βftz-f1* and *EcR-DN* in the fat body failed to rescue fat body remodeling (Figure 13). Therefore, *βftz-f1* is sufficient to induce fat body tissue remodeling provided a functional EcR receptor is available. These results together with my tissue culture results lead me to

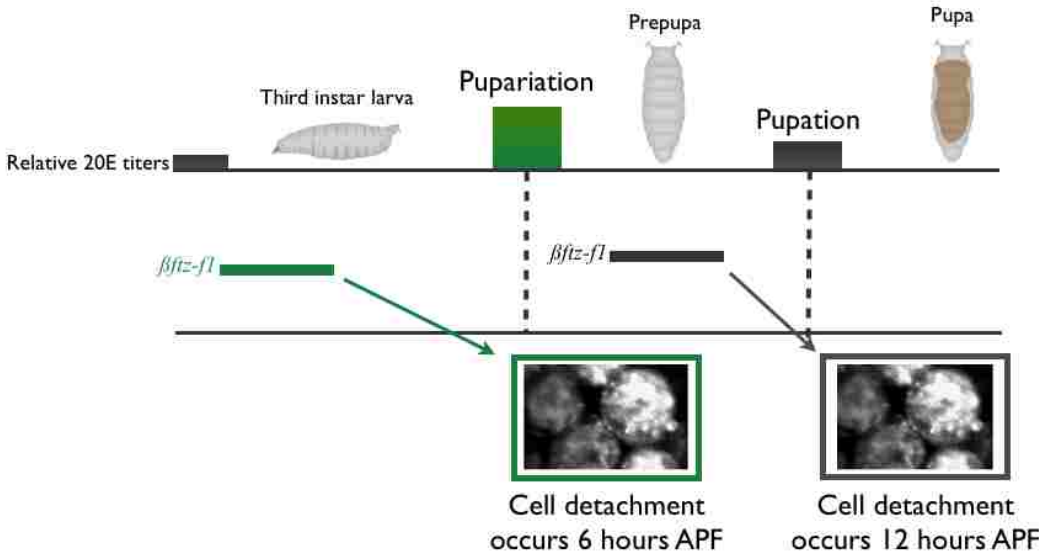

Figure 10. Fat cell detachment occurs when *βftz-f1* after a subsequent 20E titer. Wild type fat body remodeling is completed after the pupation pulse of 20E (endogenous expression of *βftz-f1* is shown in grey). Premature expression of *βftz-f1* (in green) results in premature fat body remodeling after the pupariation pulse of 20E.

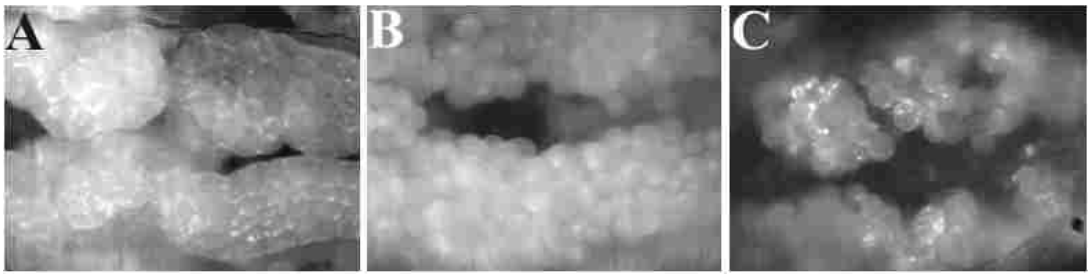

Figure 11. βFTZ-F1 can induce premature fat-body remodeling. (A) Wild type 4 hour APF whole mount animal expressing (*UAS-GFP*; *Lsp2-Gal4*). (B,C) Animals expressing (*UAS-GFP*; *Lsp2-Gal4/UAS-βftz-f1*). (B) 4 hours APF. (C) 6 hours APF.

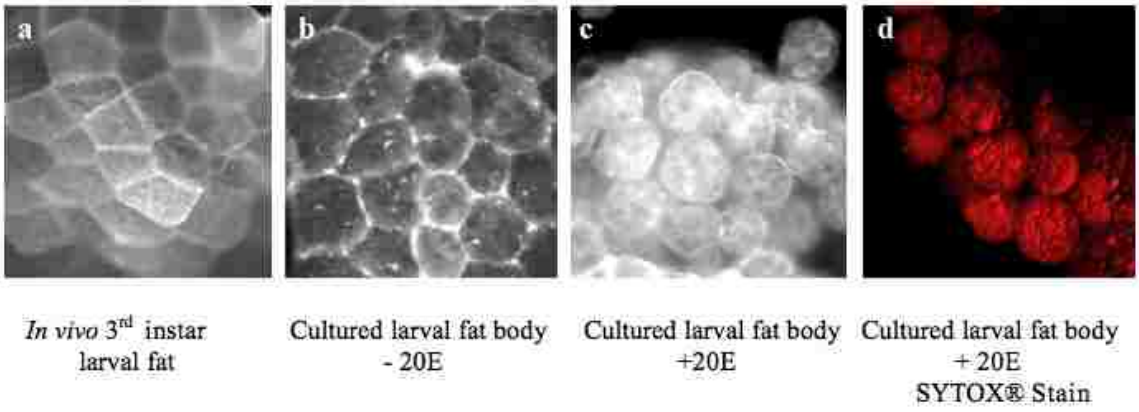

Figure 12. ßFTZ-F1 and 20E together are sufficient to promote fat body remodeling. (a-d) Larval fat body from wandering 3rd instar larvae misexpressing *ßftz-f1* (a-c) *UAS-GFP*;*Lsp2-Gal4*/*UAS-ßftz-f1* (d) *Lsp2-Gal4*/*UAS-ßftz-f1*. (a) Larval fat body imaged from a live animal (b-d) dissected fat body explants (a-b) Fat body not remodeled *in vivo* (a) or fat body explants without addition of 20E. (c, d) Fat remodeling occurs when explants cultured with 10^{-5} M 20E for 8 hours. (d) Stained with SYTOX® after incubation with 20E. The red staining indicates that the cells are not necrotic.

conclude that *ßftz-f1* expression in the fat body is sufficient to induce fat body remodeling after a subsequent round of signaling occurs through. These data suggest that *ßftz-f1* and 20E signaling are both necessary for fat body tissue remodeling.

Premature expression of ßftz-f1 in the fat body is sufficient to induce expression of the primary response genes

The role of ßFTZ-F1 as a competence factor necessary for the re-induction of ecdysone-regulated genes is well established. The presence of ßFTZ-F1 protein is necessary for the induction of a novel set of 20E response genes in addition to the re-induction of primary 20E response genes during the prepupal to pupal transition (Broadus *et al*., 1999). I hypothesized that the *ßftz-f1*-dependent 20E-response genes expressed at the prepupal/pupal transition are involved in fat-cell detachment. According to this hypothesis, when *ßftz-f1* is prematurely induced at mid-third instar in the fat body the following high-titer pulse of 20E (at pupariation) should allow for premature induction of *ßftz-f1* dependent 20E-response genes. To begin to identify the 20E signaling cascade likely to be involved in fat body remodeling, I tested whether E93 expression was prematurely induced by expressing *ßftz-f1* in the third instar larval fat body. *E93* is a primary response gene whose expression is directly induced by 20E (Baehrecke and Thummel, 1995; Lee *et al*., 2000). In wild type animals, E93 is expressed in the fat body at 12 hours APF (Baehrecke and Thummel, 1995). E93 expression coincides with the detachment stage of fat body remodeling and it is a known regulator of other tissue specific developmental changes; thus it could potentially be involved in the process of fat body remodeling. To test this hypothesis I performed quantitative RT-PCR on fat body explants prematurely expressing *ßftz-f1*. I found when *ßftz-f1* was prematurely expressed

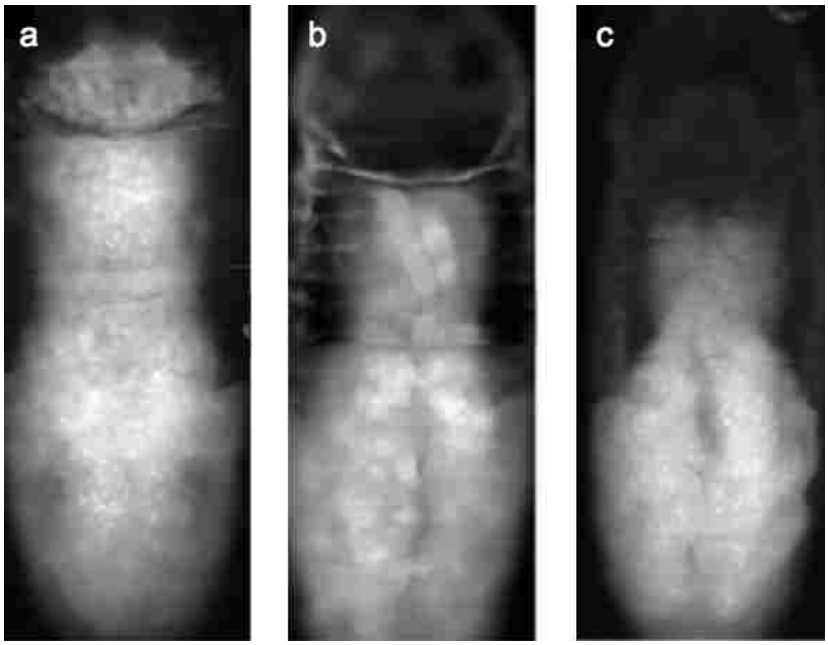

Figure 13. Induction of fat-body remodeling by BFTZ-F1 requires 20E signaling.

(A-D) Animals staged to post head eversion (12-14 hours APF). (A) Fat body has been remodeled in wild type animals (*UAS-GFP*; *Lsp2-Gal4*) (B) Fat body remodeling is disrupted when the *EcR-DN* is expressed in the fat body (*UAS-GFP*,*UAS EcR-DN*; *Lsp2-Gal4*). (C) Fat body remodeling is also disrupted when *ßftz-f1* and *EcR-DN* are coexpressed in the fat body (*UAS-GFP*, *UAS-EcRF-DN*;*Lsp2-Gal4*/*UAS-ßftz-f1*).

in the fat body, expression of *E93* increased over four fold (Figure 14). I also discovered that *E93* is expressed endogenously in wild type animals during the prepupal stages (0-6 hours APF). Since fat body remodeling does not occur in the wild type animal until sometime after these prepupal time points, these data suggest that E93 is not sufficient to induce fat body remodeling. Although there is insufficient evidence to suggest the E93 is involved in fat body remodeling I have shown that premature expression of *ßftz-f1* in the fat body results in premature induction of the 20E primary response gene E93. Thus I conclude that premature expression of *ßftz-f1* results in premature induction of 20E signaling in prepupal fat bodies.

ßftz-f1 mutant analysis

Experiments described above demonstrate that premature expression of *ßftz-f1* is sufficient to induce precocious fat body remodeling. In an effort to determine whether *ßftz-f1* is necessary for fat body remodeling, I conducted a *ßftz-f1* mutant analysis. Unfortunately, *ßftz-f1* null mutants do not survive the larval stages of development (Broadus *et al*., 1999); therefore I examined *ßftz-f1* hypomorphs (*ßftz-f1*^{17}/*Df(3L)CatDH*^{104}, herein referred to as *ßftz-f1* mutants, for defects in fat body remodeling. Although some *ßftz-f1* mutants survive larval development and pupariate, these animals are developmentally arrested at various stages during metamorphosis (Broadus *et al*., 1999). In some cases, developmental arrest occurs at the prepupal/pupal transition along with defects in the muscle-driven movements and loss of head eversion. In other cases, arrest occurs later in pupal development after head eversion (Fortier *et. al*., 2003). To monitor the development of the fat body in this mutant, I took advantage of the observation that anterior fat-body cells autofluoresce (Nelliot *et al*., 2006).

Detachment and redistribution of the fat cells into the head capsule was detected if head eversion occurred in the *ßftz-f1* mutant. However, in the absence of head eversion, the fat body is confined within the body cavity making it difficult to determine whether the fat body had undergone remodeling (Figure 15). Thus, due to the hypomorphic nature of the *ßftz-f1* mutants we were unable to determine if *ßftz-f1* is required for fat body remodeling utilizing this strategy. Presumably if there exists enough *ßftz-f1* in the hypomorph mutant to induce head eversion, there may be enough *ßftz-f1* expression to induce fat body remodeling.

Mosaic analysis with a repressible cell marker

Because the phenotypic analysis of the *ßftz-f1* hypomorph was inconclusive I chose to carry out a Mosaic Analysis with a Repressible Cell Marker (MARCM) experiment (Lee and Luo, 2001; Luo, 2007). The MARCM technique induces mitotic recombination, which results in *ßftz-f1* mutant clones in all tissues of the animal while marking the fat cells with GFP. This strategy allowed me to examine the effects of the *ßftz-f1* null allele, $ftz\text{-}f1^{19}$ (Broadus et al., 1999) in the fat body tissue. Because only a small number of cells within each tissue are affected, this strategy also avoids the complication that results from complete loss of ßFTZ-F1 protein in the entire animal via a null mutation.

Because fat body remodeling is cell autonomous, (Figure 12) I predicted that individual cells mutant for *ßftz-f1* would not undergo the cell shape changes associated with fat body remodeling. On averaged I observed 6 cloned cells when I induced mitotic recombination in MARCM progeny. Of these cloned cells some cells occurred singly and some occurred in clusters of 3 or more marked cells. Presumably, the single cells were the result of a single cell division event after recombination. The marked clusters on

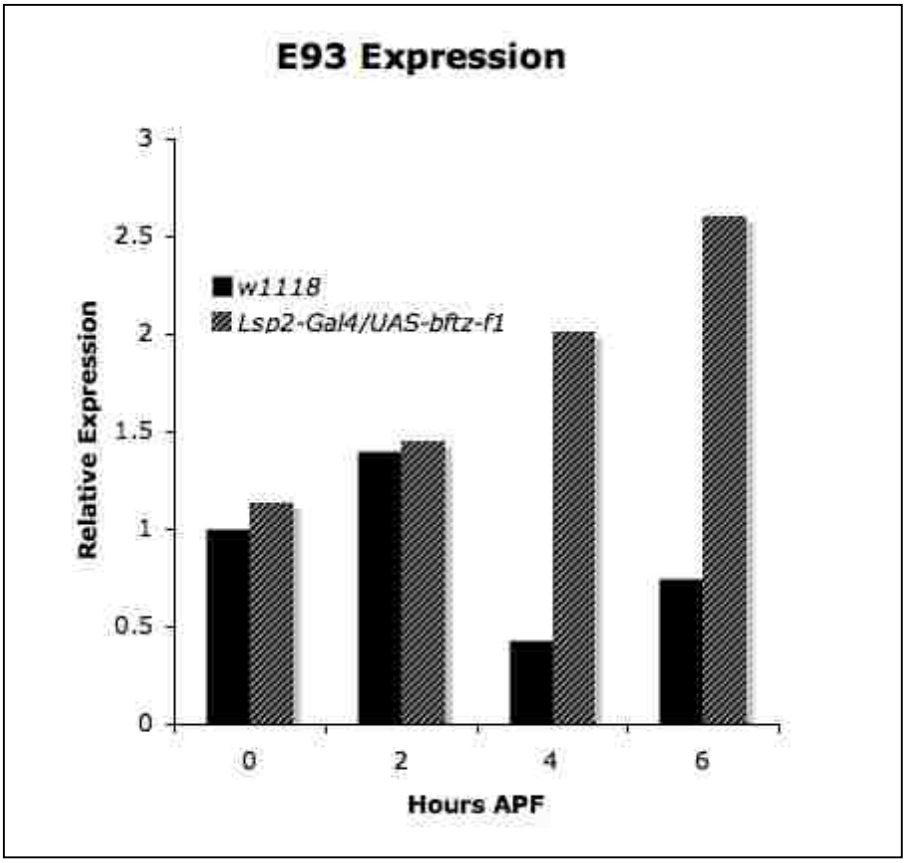

Figure 14. ßFTZ-F1 induces *E93* expression in the fat body. Relative expression of *E93* in animals misexpressing *ßftz-f1* (*Lsp2-Gal4/UAS- ßftz-f1*) was compared to wild type expression levels (w^{1118}). *E93* expression was normalized to *ß-actin*.

the other hand may have resulted from an earlier recombination event, which was followed by two or more cell divisions. Marked cells found in clusters of three or more appeared irregular in shape. These cells did not appear round; instead they maintained their polygonal-larval morphology, having sharp corners and very little rounding (Figure 16). Surprisingly, the majority of the singular marked cells appeared rounded in shape and detached. The amount of rounding seemed to coincide with the number of cloned cells neighboring one another. Clones with a high number of adjacent wild type cells appeared round. In contrast, cloned cells amongst cloned neighbors appeared increasingly irregular in shape, with the highest irregularity corresponding to cloned cells completely surrounded by other cloned cells. Due to the lack of complete rounding in *ßftz-f1* MARCM clones, I conclude that *ßftz-f1* is required for fat body remodeling. I also conclude that the process of fat body remodeling is not cell autonomous. Cells mutant for *ßftz-f1* do not necessarily lack the required factors necessary for remodeling. Instead fat body remodeling is a *tissue* autonomous event where wild type cells of the tissue may participate in the remodeling of mutant cells.

Repression of ßftz-f1 expression via dBlimp-1 results in a block in fat body remodeling

As a final attempt to determine the role of ßFTZ-F1 in fat body remodeling, I took advantage of dBlimp-1, a known repressor of *ßftz-f1I.* I drove expression of *UAS-dBlimp-1* in the fat body using the fat body dirvers *cg-Gal4* and *Lsp2-Gal4*. Three transgenic lines of *UAS- dBlimp-1* were tested. Two of the lines (*UAS-dBlimp-1*^{225} and *UAS-dBlimp-1*^{226}) resulted in death before pupariation when crossed to the *cg-Gal4* driver. Animals expressing either of these transgenes failed to complete the third instar molt. These lines also showed reduced viability when crossed to *Lsp2-Gal4*. However,

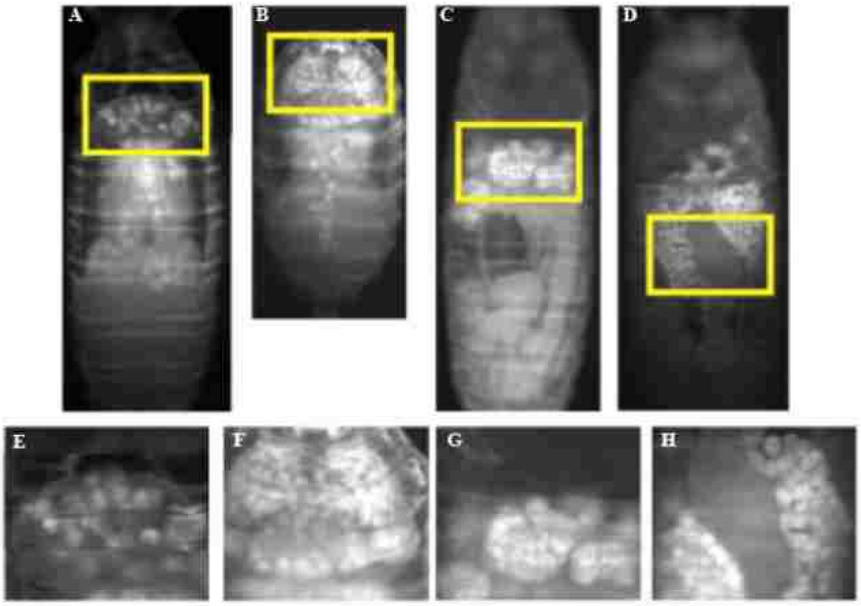

Figure 15. *ßftz-f1* hypomorphic mutation does not disrupt fat-body remodeling. (A-D) Animals imaged under the DAPI filter to visualize autofluorescent fat cells. At 14 hours APF the fat body has remodeled in all animals and detached fat cells are found in the head region (A) Control (*Lsp2-Gal4*) (E close up of A). (B) Control (*ßftz-f1*^{ex17}*/TM6*) (F close up of B). (C) *ßftz-f1*hypomorph mutant (*ßftz-f1*^{ex17}*/Df*) 12 hours APF (G close up of C). (D) *ßftz-f1*hypomorph mutant (*ßftz-f1*^{ex17}*/Df*) 14 hours APF (H close up of D).

when *UAS-dBlimp-1*^{227} was crossed to *cg-GAL4* animals pupariated, occasionally survived the prepupal stages, and underwent head eversion. As predicted, these animals did not undergo fat body remodeling (Figure 17). These data, in addition to my mosaic analysis, lead me to conclude that *ßftz-f1* is necessary for fat-body remodeling.

Discussion

Fat body remodeling is a tissue autonomous process and is dependent upon 20E signaling

Previous studies have shown that fat body remodeling is a tissue autonomous process (Cherbas *et al*., 2003; Nelliot *et al*., 2006). The idea that fat-body remodeling may require fat-cell specific 20E signaling has been put forth by Cherbas *et al*., (2003) and has been further investigated here. I found that only certain aspects of fat body remodeling, namely disaggregation and detachment, require a functional EcR in the fat body. I therefore conclude that the disaggregation and detachment phases of fat body remodeling are dependent upon fat-cell specific 20E signaling. It is likely that the retraction of the fat body is not a cell autonomous event. Perhaps the abdominal contractions characteristic of ecdysis behavior are responsible for the movement of the fat body tissue into the posterior portion of the animal. Future studies are necessary to implicate Ecdysis Triggering Hormone and prepupal ecdysis in fat body tissue retraction.

Other aspects of fat body remodeling may also be initiated by ETH. In wild type animals, fat body cells are completely detached prior to head eversion, thus it is unlikely that the shearing forces generated at the time of head eversion cause fat cell detachment. I have observed the abdominal pulses of ecdysis after head eversion, and with each contraction of the abdomen individual fat cells are propelled into the head capsule.

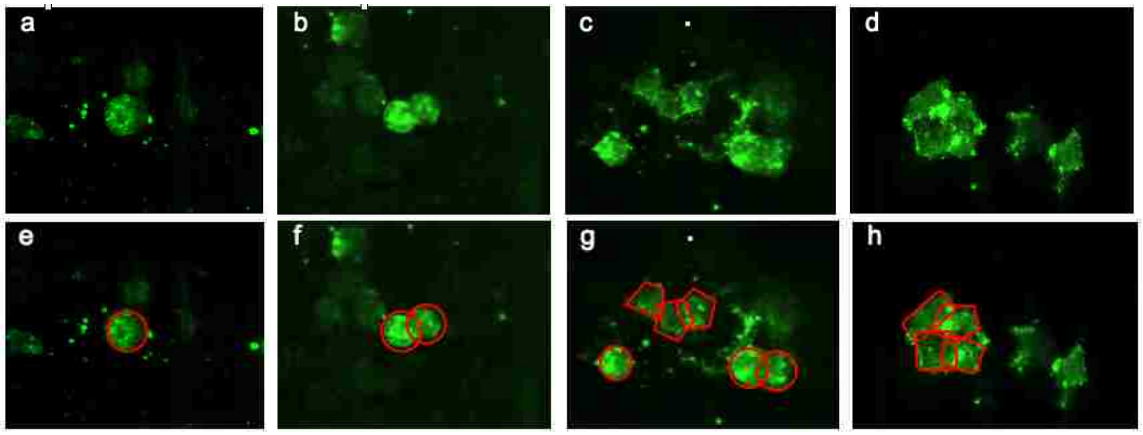

Figure 16. Fat-body remodeling is a tissue autonomous process which requires ßFTZ-F1. Animals of the genotype *yw hs-flp*; *cg-Gal4*, *UAS-GFP*; *FRT2A*, *ftz-f1*^{19}/*FRT2A*, *ftz-f1*^{19} were generated by heat shock induced recombination and selected based on the presence of GFP. Animals were imaged after head eversion. (a-h) Confocal images. (e-h) cells in cluster outlined for emphasis.

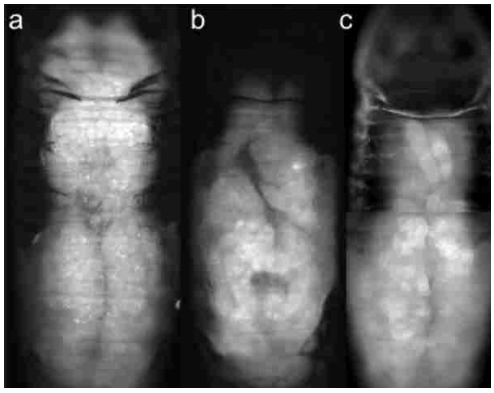

Figure 17. Expression of *ßftz-f1* is required for fat-body remodeling. Animals staged to 12 hours APF. (a) Wild type (*cg-Gal4, UAS-GFP*). (b) Animal expressing *dBlimp-1* in the fat body (*cg-Gal4*, *UAS-GFP*; *UAS-dBlimp1*). (c) Animal expressing *EcR-DN* in the fat body (*cg-Gal4, UAS-GFP*/*UAS-EcR-DN*).

Fat cells that maintain their larval morphology when EcR-DN is expressed in the fat body, however, are not pushed into the head by the abdominal contractions, leading me to conclude that the body movements of ecdysis are not involved in the detachment phase of fat body remodeling. The ecdysial abdominal contractions do, however, appear to be involved in the redistribution of detached fat cells into the anterior portion of wild type animals.

ßFTZ-F1 and 20E signaling are both necessary and sufficient for fat-body remodeling

The competence factor ßFTZ-F1 is necessary for many major developmental transitions in *Drosophila,* including the prepupal to pupal transition (Broadus *et al*., 1999; Yamada *et al*., 2000; Fortier *et al*., 2003). The final stage of fat-body remodeling is also completed at the time of the prepupal to pupal transition (Nelliot *et al*., 2006). In addition to its role in the major developmental transitions, ßFTZ-F1 has also been shown to be required for tissue-specific developmental changes (Broadus *et al*., 1999; Yamada *et al*., 2000; Fortier *et al*., 2003). Thus it seemed likely that ßFTZ-F1 would be a key player in fat-body tissue remodeling. Indeed, I found that premature expression of ßFTZ-F1 in the fatbody resulted in premature fat-body remodeling. The timing of this phenotype pointed to the need for a pulse of 20E hormone in addition to the presence of ßFTZ-F1. Using the *Lsp2-GAL4* driver, expression of *GAL4* (and thus *UAS-ßftz-f1*) was initiated by early third instar. Fat body remodeling was not complete in these transgenic animals until after the pulse of 20E that initiates puparium formation. I hypothesized that by expressing ßFTZ-F1 I provided the fat body with premature competence, but that 20E hormonal signaling was still required to achieve further gene expression and thus developmental progression. My *ex vivo* organ culture experimental results confirmed this

hypothesis. Cultured fat body explants prematurely expressing ßFTZ-F1 completed fat-body remodeling only when 20E was added to the culture media. Additionally, fat bodies concurrently expressing the dominant negative form of EcR (EcR-DN) and ßFTZ-F1 did not undergo fat body remodeling.

In addition to the ability of ßFTZ-F1 to promote fat-body remodeling I showed that expression of ßFTZ-F1 was essential for the process. Although hypomorphic mutant analysis was inconclusive, I was able to achieve repression of *ßftz-f1* by expressing *dBlimp-1* in the fat body. Repression of *ßftz-f1* by dBlimp-1 disrupted fat-body remodeling. All fat cells maintained their larval morphology and failed to disaggregate and detach. From these data, I conclude that expression of *ßftz-f1* in the fat body is required for fat-body remodeling.

Due to the fact that *ßftz-f1* null mutants do not survive embryogenesis, I was not able to directly observe the affect of a ßFTZ-F1 null mutation in whole animals at the prepupal/pupal transition. Instead I employed the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique to examine clones of mutant cells. I generated clones of cells that were null mutant for ßFTZ-F1 and marked with GFP. Interestingly, clones that were found in clusters (3 or more adjacent cells) did not show significant cell shape change while single cell clones were round and had achieved disaggregation. The multiple cell clone clusters maintained their larval morphology, suggesting that in these cells ßFTZ-F1 is required for fat body remodeling.

The confounding results obtained from the single cell clones may be explained by a model that takes into account the potential participation of adjacent fat cells in the remodeling of their neighboring cells. The remodeling of other larval tissues, such as the

tracheae for example, requires expression of a protease to breakdown the extracellular matrix (ECM) holding the tissue together (Page-McCaw *et al*., 2003). These proteases are either secreted into the ECM or anchored to the cell membrane. The larval fat body, like the tracheae, may also require the action of a protease. One could envision a model involving a protease present at the membrane of a wild type fat cell that degraded ECM as a means of remodeling the tissue. A membrane bound protease would not only have the potential to remodel the cell that it is directly bound to but it also could potentially have an effect on other cells in the immediate vicinity. This mechanism would allow for protease action on cells that are not able to express the protease. Perhaps *ßftz-f1* is required in the fat cell for expression of a protease that degrades ECM. When cells are mutant for *ßftz-f1* (as is the case for the MARCM cloned cells) they would not produce the protease and thus would not have the potential to remodel any ECM. If wild type cells are also present (as in the unmarked cells of MARCM animals) these cells would display the protease at the membrane and ECM around that particular cell would be remodeled, even ECM that is shared with other mutant cells. This mechanism would enable the remodeling of mutant cells by adjacent wild type cells in the fat body tissue mass (Figure 18).

Expression of ßFTZ-F1 in the fat body induces expression of E93

In an effort to demonstrate that 20E signaling cascade is prematurely initiated when *ßftz-f1* is prematurely expressed in the fat body I performed quantitative RT-PCR and obtained a transcriptional profile for the 20E inducible gene *E93*. I found that *E93* transcription was indeed induced when *ßftz-f1* was prematurely expressed in the fat body. I also discovered that *E93* was endogenously expressed during the early prepupal stages

Figure 18. Proposed model for tissue autonomous fat-body remodeling. *ßftz-f1* mutant cloned cells in green, wild type cells in beige. Wild type cells are capable of expression of a protease that degrades ECM while the mutant cells are not. This protease is present at the membrane (in purple) of the wild type cell and degrades ECM that the wild type cell shares with the mutant cell. Thus, the mutant cell's ECM is remodeled.

of development. This premature expression of *E93* rules out the possibility that this protein is sufficient to induce fat body remodeling. I cannot rule out the possibility that E93 may be involved in fat body remodeling in wild type animals. Fat cell detachment occurs just prior to head eversion at a time when *E93* is endogenously expressed in the fat body (Baehrecke and Thummel 1995). Perhaps at these later stages of prepupal development additional factors are present that work in concert with *E93* to achieve fat body remodeling.

CHAPTER 4

ECDYSONE INDUCED MMP2 IS REQUIRED FOR FAT BODY TISSUE REMODELING

Introduction

The metamorphosis of *Drosophila melanogaster* is characterized by a complete transformation of structure and morphology. During this period of intense change the larval tissues either undergo programmed cell death or remodel to generate the tissues of the adult fly. Obsolete larval tissues such as the salivary gland and the midgut undergo programmed cell death during metamorphosis (Jiang *et al*., 2002; Lee *et al*., 2000; Kucharora-Mahmood *et al*., 2002; Lee *et al*., 2002a; Lee *et al*., 2002b), while other tissues such as nervous and adipose tissue are retained and remodeled (Levine *et al*., 1995; Butterworth, 1972; Hoshizaki, 2005; Cherbas *et al*., 2003; Nelliot *et al*, 2006). During metamorphosis, the larval fat body undergoes a stereotypic transformation in morphology, a process which is regulated by 20E signaling and the competence factor ßFTZ-F1 (Cherbas *et al*., 2003; Hoshizaki, 2005; Nelliot *et al*., 2006; Chapter 3). During the larval stages, the fat body cells are polygonal in shape and remain attached to one another. As metamorphosis progresses, the fat cells begin to disaggregate, and lose their tight associations. At the time of the prepupal to pupal transition, the fat cells completely detach from one another and exist as spherical free floating cells in the body cavity.

It has been proposed that in *Sarcophaga peregrina* the hemocytes induce fat body remodeling by triggering the release of the protease Cathepsin B in the fat body (Hori *et al*., 1997; Kobayashi *et al*., 1991; Natori *et al*., 1999). In *Drosophila melanogaster*, the mechanism responsible for fat body remodeling is tissue autonomous and does not rely

on the action of the hemocytes (Nelliot *et al*., 2006; Chapter 3). The role of Cathepsin B expression and other cellular events necessary for fat body remodeling has not yet been identified. Remodeling of the fat body requires dissociation of the larval fat cells and destruction of the extracellular matrix (ECM) used to maintain the integrity of the tissue. A class of protease, the matrix metalloproteinases (MMPs) are involved in the degradation of the ECM and are required for remodeling of many mammalian and *Drosophila* larval tissues (Page-McCaw, 2008; Page-McCaw *et al*., 2007). Thus the MMPs are excellent candidates for fat body remodeling.

The MMPs are characterized by their conserved Met residue and zinc ion at the active site of the enzyme (Bode *et al*., 1993). In general, MMPs cleave components of the extracellular matrix (ECM) such as collagen and laminin (Page-McCaw, 2008). This cleavage of ECM components can clear space between cells and thus increase mobility of cells (Sternlicht and Werb, 2001). MMPs also cleave signaling molecules residing inside the ECM. One example of this is the cleavage of Insulin-like growth factor binding proteins (IGF-BPs) (Fowlkes *et al*., 1994). Cleavage of the IGF-BPs leads to increased availability of Insulin like growth factor (IGF). Increased availability of IGF enhances growth and proliferation.

Drosophila have two MMPs, (MMP1 and MMP2) (Llano *et al*., 2000; Llano *et al*., 2002; Page-McCaw *et al*., 2003). MMP1 is a secreted protein, and MMP2 has a GPI anchor and is membrane associated (Page-McCaw *et al*., 2003; Llano *et al*., 2002). The two *Drosophila* MMPs have the canonical MMP structure but are not orthologs of any of the 24 mammalian MMPs (Page-McCaw *et al*., 2003). MMP1 and MMP2 are each required for distinct aspects of tissue remodeling and programmed cell death during

metamorphosis. MMP1 expression is up-regulated during metamorphosis in tissues undergoing 20E signaling-induced cell death (Lee *et al*., 2002). MMP1 is required for larval trachea remodeling, while MMP2 is required for programmed cell death of the larval midgut (Page-McCaw *et al*., 2003). Both MMPs are required for head eversion and dendrite remodeling (Page-McCaw, 2008).

MMP1 and *MMP2* transcripts are most abundant during the early pupal stages, but expression of both MMPs is detected at all developmental stages (Llano *et al*., 2002; Page-McCaw *et al*., 2003). Expression of *MMP1* is induced at the time of head eversion in the early pupa, a period corresponding to a low 20E titer. The expression profile of MMP1 is induced in the salivary gland when BR-C and E93 (components of the 20E signaling cascade) are defective, thus it is likely that expression of *MMP1* is regulated by 20E.

The catalytic activity of MMPs is inhibited by TIMP (tissue inhibitor of Metalloproteases), which blocks MMP activity by occupying the active site of the protease. There is one endogenously expressed *TIMP* gene in the *Drosophila* genome (as compared to four in vertebrates) (Wei *et al*., 2003). *TIMP* expression is detected throughout development but expression declines at the time of head eversion (Godenschwege, *et al*., 2000; Page-McCaw *et al*., 2003; Lee *et al*., 2003). As with the MMPs, expression of *TIMP* is downregulated when components of the 20E-signaling cascade are disrupted (Godenschwege, *et al*., 2000; Page-McCaw *et al*., 2003; Lee *et al*., 2003). Also, expression of *TIMP* is significantly decreased in the dying larval salivary gland (Lee *et al*., 2002).

My work has demonstrated that the process of fat-body remodeling is a tissue autonomous event requiring *ßftz-f1* and 20E signaling. Mosaic *ßftz-f1* mutant analysis revealed that fat body remodeling was not disrupted in *ßftz-f1* mutant clones which were adjacent to wild type cells. These data led me to propose a model for 20E mediated fat body remodeling. With this model I proposed that a 20E inducible protease is anchored to the fat-cell membrane and is responsible for the degradation of the ECM thus achieving fat body remodeling. Also, due to the location of this factor in the membrane, I proposed that this protease would be capable of remodeling the cell that it is anchored to in addition to neighboring cells, thus producing a tissue autonomous event. Here I report MMP2 is the 20E inducible protease required for fat body remodeling. This study also identifies a fat-body specific role for MMP1 in head eversion.

Materials and Methods

Fly stocks

The *Lsp2-Gal4* stock was provided by L. Cherbas. *UAS-MMP1*, *UAS-MMP2*, *UAS-TIMP*, *UAS-MMP1-DN*, $MMP1^{W439}$*/CyO, arm-GFP*, $MMP1^{Q273}$*/CyO, arm-GFP*, *MMP1-2/CyO, arm-GFP*, $MMP2^{W307}$*/CyO, arm-GFP* and *MMP2Df/CyO, arm-GFP* were all generously provided by P. Mc-Caw. Homozygous MMP mutants were selected by the absence of GFP, as were the mutant progeny from the crosses of: $MMP1^{W439}$*/CyO* crossed to *MMP1-2/CyO, arm-GFP,* $MMP1^{Q273}$*/CyO, arm-GFP* crossed to *MMP1-2/CyO*, and $MMP2^{W307}$*/CyO, arm-GFP* crossed to *MMP2Df/CyO, arm-GFP*.

Microscopy and imaging

Staged animals were collected as white prepupae, placed on wet filter paper in a Petri dish at 25°C, aged appropriately, then rinsed in deionized water, and mounted on bridged

slides in Gel mount (Biomedia). Both fluorescence and confocal imaging were carried out in the School of Life Sciences Imaging Center using a Zeiss Axioplan 2 microscope. Fluorescence images were captured with the Zeiss Axiocam using the Zeiss Axiovision software. LSM 510 software was used to procure the confocal images. All images were complied in Corel Draw®.

Animals expressing *UAS-GFP; Lsp2-Gal4*/*UAS-MMP1-DN* were collected as white prepupae, placed on wet filter paper in a Petri dish at 25°C, and aged to 120 hours APF. Aged animals were examined by light microscopy on a Zeiss Stemi 2000-C microscope. A Canon A620 digital camera and Canon Zoom Browser EX software were used to procure the images. Again the images were compiled in Corel Draw®.

RNA isolation and cDNA synthesis

Animals were collected at the white prepupal stage, placed on moist filter paper in a Petri dish, and left to develop to the desired stage (8 to 16 hours APF) at 25ºC. When the desired stage was reached, 4 to 5 animals were dissected and the fat bodies were placed in 30µl of PBS. 300µl of TriZol (Life Technology) was then added to the tubes containing fat bodies and PBS and the tissue was homogenized. The sample was then transferred to a 2ml Phase Lock Gel-Heavy microfuge tube (Eppendorf), centrifuged at 12,000 g for 10 min at 4ºC. After centrifugation, the aqueous phase was transferred to a new tube and 160µl of isopropanol was added. The RNA was allowed to precipitate overnight at -20ºC. The precipitated sample was centrifuged at 12,000 g 20 minutes. After centrifugation, the supernatent was removed by pipette. The remaining pellet was washed with 500µl of 75% ethanol, centrifuged for 10 minutes and the supernatant was removed by pipette. The pellet was air dried and resuspended in 5µl of Rnase free water.

RNA concentration was determined by Nandrop. cDNA was synthesized from the RNA samples using the Invitrogen First-Strand cDNA sythesis kit.

Quantitative RT-PCR

Primers were synthesized by IDT (Integrated DNA Technologies) and designed from sequences from Flybase using the program on the IDT website.

Sequences:

ß-actin

Forward: 5'-TCTACGAGGGTTATGCCCTT-3'

Reverse: 5'-GCACAGCTTCTCCTTGATGT-3'

MMP2

Forward: 5'-AGCAATCCGGAGTCTCCAGTCTTT-3'

Reverse: 5'-TGGAGCCGATTTCGTGATACAGGT-3'

qRT-PCR was performed using PerfeCta™ SYBR® Green Supermix, ROX (Quanta Biosciences) according to the manufacturer's instructions on an Applied Biosystems cycler using the following program: 95°C 2 min, 40 cycles of 95°C for 15s, 58.2°C for 30s, and 72°C for 30 s, and 1 cycle of standard melt curve at the end of the program. Primer titrations and standard curves were generated by qRT-PCR to test primer efficacy. MMP2 expression was normalized to *ß-actin* and the expression of experimental samples (*Lsp2-Gal4*/*UAS-EcR-DN*) was compared to control samples (w^{1118}) of the same stage using the ΔΔ-Ct method.

Results

Misexpression of MMP2 in the fat body results in premature tissue remodeling

The process of fat-cell detachment presumably involves a protease that can cleave substrates present in the ECM that hold the cells together. The detachment phase of fat body remodeling occurs at the time of the prepupal to pupal transition (Nelliot *et al*., 2006). MMP expression occurs at the time of fat body remodeling, and previous reports have shown that the MMPs are required for midgut and trachea remodeling (Llano *et al*., 2002; Page-McCaw *et al*., 2003); therefore I set out to test the role of MMPs in fat-body tissue remodeling. I first tested whether *Drosophila* MMPs were sufficient to promote premature fat-body remodeling by misexpressing the two proteases in the fat body using the Gal4/UAS system. UAS-*MMP1* and *UAS-MMP2* were each individually expressed specifically in fat body using the *Lsp2-Gal4* driver (Page-McCaw *et al*., 2003; Cherbas *et al*., 2003). I expected premature fat body remodeling to occur during the third instar larval stage as this is the time that expression of the MMPs would be initiated by the *Lsp2-Gal4* driver (Cherbas, *et al*., 2003).

Misexpression of *MMP1* did not lead to premature fat-body remodeling. Fat cells expressing *Lsp2-Gal4/UAS-MMP1* maintained their associations with their neighboring cells and displayed no cell rounding (Figure 19). Misexpression of *MMP2,* however, did result in premature fat-body remodeling. Dissected fat cells from third-instar larvae were free floating, spherical and resembled wild-type remodeled fat cells (Figure 20).

Additionally, misexpression of MMP1 or MMP2 resulted animal death prior to the wandering stage of the third-instar. From these data I conclude that MMP1 does not induce fat body remodeling, while MMP2 is sufficient to promote fat body remodeling in the third instar larvae. Also, expression of MMP1 or MMP2 in the fat body during the third larval instar results in animal death before the wandering stage.

Misexpression of TIMP in the fat body results in a block in head eversion and prepupal death

To further explore the role of MMP2, I expressed the inhibitor of MMPs (*TIMP*) in the fat body. Endogenous *TIMP* expression declines in whole animals at the time of fat body remodeling (Page-McCaw *et al*., 2003). It is likely a decline in *TIMP* expression is necessary in the fat body if MMP2 action is required for fat-body remodeling. If a decrease in *TIMP* expression is required, I expected to see a block in tissue remodeling when *TIMP* was misexpressed in the fat body. Indeed, when *UAS-TIMP* was expressed in the fat body via the *Lsp2-Gal4* driver, the fat body did not appear to undergo any remodeling (Figure 21). Animals in which *TIMP* was misexpressed were developmentally arrested before head eversion occurred. Thus it is difficult to distinguish between a defect in fat-cell detachment and disruption of detachment due to the premature death of the animal.

Some aspects of fat body remodeling occur during the early prepupal stages of development. Fat body disaggregation is the stage of fat-body remodeling where the fat cells lose their tight associations with each other (Nelliot *et al*., 2006). Fat bodies misexpressing *TIMP* fail to disaggregate (Figure 21). The fat cells maintain their larval morphology up until the time of animal death when *TIMP* is expressed specifically in the fat body. Therefore, from these data I conclude that fat-body disaggregation requires the action of MMPs.

MMP mutant analysis

Page-McCaw *et al*. (2003) have published an extensive characterization of both *MMP1* and *MMP2* mutants. Through their analysis the roles of MMPs in head eversion,

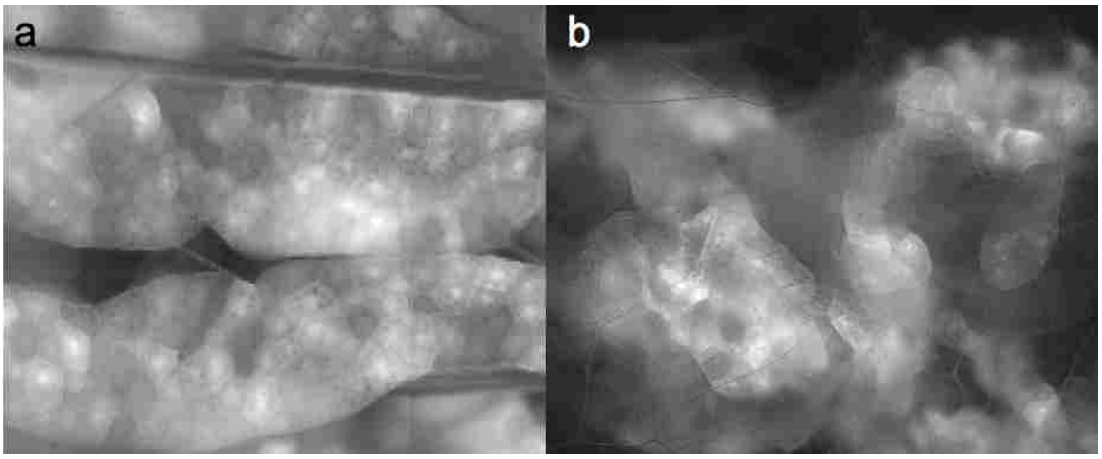

Figure 19. MMP1 cannot induce premature fat-body remodeling. (a) Wild-type third-instar fat body. Animal expressing GFP specifically in the fat body (*UAS-GFP*; *Lsp2-Gal4*). (b) Third instar fat body expressing *MMP1* and GFP (*UAS-GFP*; *Lsp2-Gal4/UAS-MMP1*).

midgut programmed cell death, and tracheal remodeling have been determined. Although *MMP* mutants infrequently pupariate, a small percentage of both *MMP1* and *MMP2* mutants do go on to evert their heads and make the prepupal to pupal transition. I took advantage of these weaker alleles generated by Page-McCaw *et al*. (2003) and conducted a mutant analysis, paying special attention to fat body morphology during the prepupal to pupal transition. Two mutant alleles for *MMP1* were examined either as homozygotes or in combination with an *MMP1* deletion ($MMP1^{Q273}/MMP1^{Q273}$, $MMP1^{Q273}/MMP1^{2}$, $MMP1^{W439}/MMP1^{W439}$, see Materials and Methods section for details). The autofluorescent property of the fat cells was utilized and live animals were imaged using a DAPI filter (Nelliot *et al*., 2006). All *MMP1* mutant animals that progressed through the prepupal to pupal transition completed the fat body remodeling program, resulting in detached free floating cells in the pupa (Figure 22).

I also examined one mutant allele of *MMP2* both as a homozygote and in combination with a deletion ($MMP2^{W307}/MMP2^{W307}$, and $MMP2^{W307}/Df$, see Materials and Methods section for details). In agreement with my hypothesis, both genotypes of *MMP2* mutant displayed a complete block in fat-body remodeling. Fat cells from *MMP2* mutant animals maintained their larval morphology throughout the life of the animals (Figure 22) showing no sign of disaggregation or cell detachment.

Misexpression of *MMP2* results in premature fat-body remodeling while mutations in the *MMP2* gene result in a block in fat body remodeling. From these data I conclude that *MMP2* is required for fat-body remodeling. In contrast, *MMP1* misexpression and mutations in the *MMP1* gene have no effect on fat-body remodeling. Thus I conclude that *MMP1* is not involved in the process of fat-body remodeling.

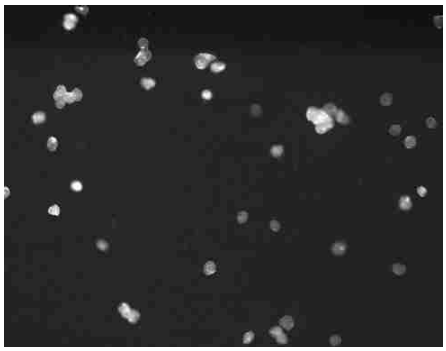

Figure 20. MMP2 can induce premature fat-body remodeling. Dissected third instar fat body from animal expressing (*UAS-GFP*; *Lsp2-Gal4*/*UAS-MMP2*).

Fat-body specific expression of a MMP1 dominant negative protein results in a block in head eversion

MMP1 misexpression in the fat body and mutant analysis lead to the conclusion that MMP1 is not required for fat body tissue remodeling. The phenotype resulting from misexpression of *TIMP* however does suggest a role for MMP1 in the fat body. Animals expressing *TIMP* in the fat body consistently die during the early prepupal stages. Fat body remodeling occurs 6 to 8 hours after animals expressing *TIMP* die. Therefore, blocking fat-body remodeling via inhibition of *MMP2* cannot be considered as a cause for prepupal death in animals expressing *TIMP* in the fat body. Thus, an unknown event requiring MMPs in the fat body during the prepupal stages of development is critical for animal survival. In an effort to determine if MMP1 could be specifically required in the fat body for progression through prepupal development, I expressed a dominant negative form of *MMP1* (*UAS-MMP1-DN*) in the fat body. As expected by the results obtained from the mutant analysis, animals expressing *MMP1-DN* in their fat bodies undergo the process of fat body remodeling (Figure 23) with the exception that the fat does not get distributed throughout the head region. Closer inspection of these animals revealed that the head capsule was absent. In fact, these animals continue to develop during metamorphosis but never underwent head eversion. Wings, legs, and eye pigments appeared at the anterior portion of the animal where the head would normally develop (Figure 24). Development continued with most of the animals getting stuck inside the pupal case after the operculum opened. From these data I conclude that *MMP1* expression is required in the fat body in order for head eversion to occur. Perhaps MMP1 is released from the fat body and remodels the head capsule.

Expression of EcR-DN results in a downregulation of MMP2 expression

Previous work has shown that 20E signaling is required for fat body remodeling (Cherbas *et al*., 2003, Chapter 3). In this chapter I presented data which shows that MMP2 is also required for fat body remodeling. These results suggest a link between 20E signaling and expression of *MMP2*. Previous work has shown that *MMP1* is a 20E regulated gene (Lee *et al*., 2002), but a link between *MMP2* expression and 20E signaling has not yet been made. Thus I set out to determine whether *MMP2* expression is dependent upon 20E signaling in the fat body. Control animals (w^{1118}) and animals expressing *UAS-EcR-DN* specifically in their fat bodies were staged to 8, 10, 12, 14 and 16 hours APF. Fat bodies were removed and RNA was isolated from each stage and genotype. Quantitative RT-PCR was performed and *MMP2* expression relative to ß-actin was determined from each sample. Fat bodies expressing *EcR-DN* showed a consistent reduction in *MMP2* expression from 8 hours APF through 14 hours APF, culminating in a 90% decrease in *MMP2* expression at 14 hours APF as compared to controls (Figure 25).

Misexpression of *MMP2* results in premature fat body remodeling. Animals mutant for *MMP2* do not undergo fat body remodeling, a phenotype also described when 20E signaling is blocked in the fat body. Blocking EcR function in the fat body results in a dramatic reduction in *MMP2* expression. Therefore I conclude that *MMP2* is the 20E inducible gene whose product is the protease required for fat body remodeling.

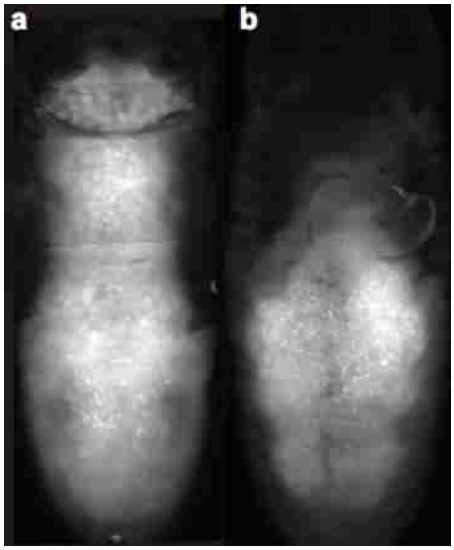

Figure 21. Blocking MMP1 and MMP2 disrupts fat-body remodeling. (a) Wild type remodeled fat body. Animal expressing (*UAS-GFP*; *Lsp2-Gal4*) imaged at 14 hours APF. (b) Fat body remodeling blocked in animal expressing (*UAS-GFP*; *Lsp2-Gal4*/*UAS-Timp*). Animal imaged at 14 hours APF

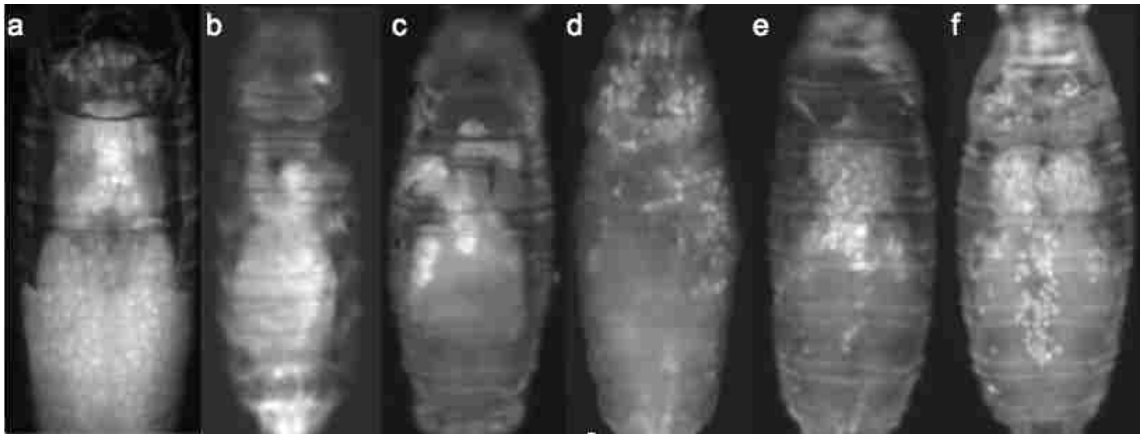

Figure 22. *MMP2* is required for fat-body remodeling. (a-f) Animals staged to 14 hours APF and fat cells detected by autofluorescence. (a) Control (*Lsp2-Gal4*). (b-c) *MMP2* mutants. (b) $MMP2^{W309}$ (c) $MMP2^{W309}/MMP2^{Df}$ (d-f) *MMP1* mutants. (d) $MMP1^{W439}$ (e) $MMP1^{Q273}$ (f) $MMP1^{Q273}/MMP1\text{-}2$.

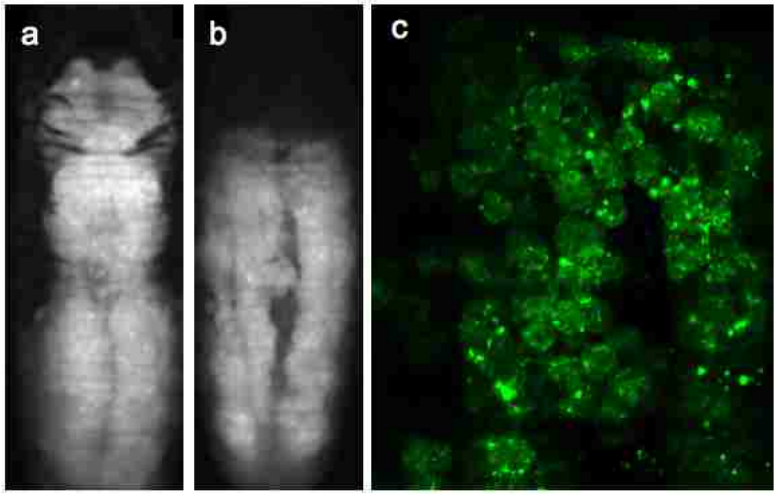

Figure 23. *MMP1* expression in the fat body is required for eversion of the head capsule. (a-c) Animals expressing GFP in the fat body imaged at 14 hours APF. (a) Wild type fat body remodeling and head eversion. Animal expressing *UAS-GFP*; *Lsp2-Gal4*, fluorescent image. (b, c) Head eversion blocked in animal expressing *UAS-GFP; Lsp2-Gal4*/*UAS-MMP1-DN*. (b) Fluorescent image. (c) Close up on fat cells, confocal image.

Discussion

MMP2 is an 20E inducible gene and is required for fat body remodeling

Previous results have demonstrated that fat-body specific 20E signaling is required for fat-body remodeling and animal survival (Cherbas *et al*., 2003; Chapter 3). Here I have presented data which suggest that *MMP2* is a 20E-inducible gene whose product is required for fat-body remodeling. Intriguingly, expressing *MMP2* in the fat body during the third instar results in premature fat body remodeling and animal death. Likewise, blocking 20E signaling in the fat body (and thus decreasing *MMP2* expression in the pupa) results in animal death. Thus it appears that proper expression of *MMP2* is required for animal survival. It is known that MMPs are involved in cleaving signaling factors in the ECM of mammalian cells (Sternlicht and Werb, 2001). It is possible that MMPs are also involved in cleavage of signaling molecules in *Drosophila* as well. Preventing cleavage of signaling molecules by blocking MMP2 action in the fat body could very likely produce global developmental effects. Likewise, aberrant cleavage of important signaling molecules in the fat body by inducing *MMP2* expression prematurely could relay signals to the entire animal that would disrupt normal animal development.

Misexpression of MMP1 in the fat body results in third-instar larval death

I have shown that MMP2 is required for fat body remodeling while MMP1 has no role in the process. However, animals misexpressing either *MMP1* or *MMP2* in the fat body during the third instar stage die before wandering is initiated. The only notable defect in fat bodies prematurely expressing *MMP1* was the diminished intensity of GFP signal. Perhaps the decrease in GFP expression is a result of induction of cell death in the fat body. In the larval salivary gland it has been reported that *MMP1* expression is

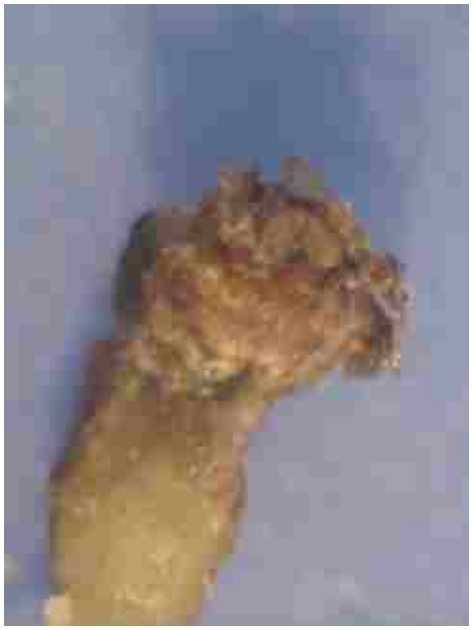

Figure 24. MMP1 is not required in the fat body for development of adult structures other than the adult head. Animal expressing *UAS-GFP; Lsp2-Gal4*/*UAS-MMP1-DN* dissected from pupal case 120 hours APF. Wings, legs and some eye pigments are visible at the anterior portion of the animal where the head should be.

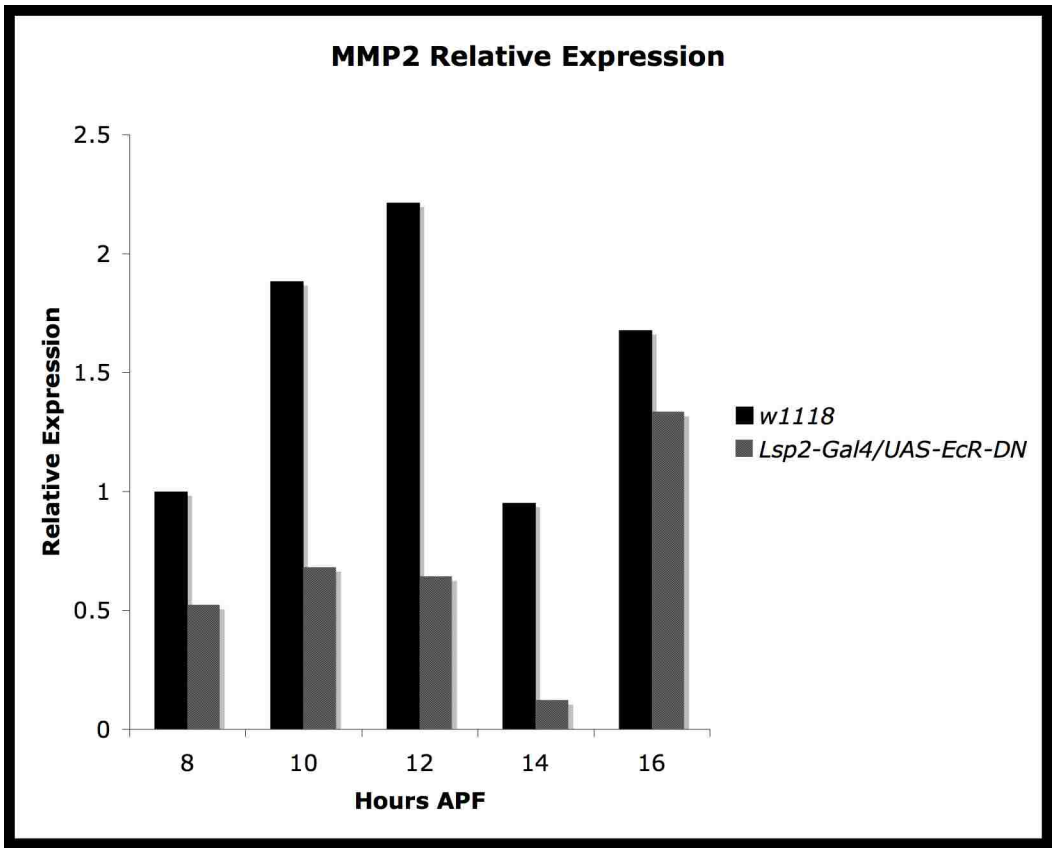

Figure 25. 20E signaling is required for induction of *MMP2* expression in the fat body. Relative expression of *MMP2* in dissected fat bodies expressing *UAS-EcR-DN*, *UAS-GFP*; *Lsp2-Gal4* was compared to wild type expression levels (w^{1118}) at several time points. *MMP2* expression was normalized to *ß-actin*. Hours refer to hours APF.

induced during 20E mediated cell death (Lee, *et al*., 2002). A specific role for MMP1 during cell death has not yet been described. *MMP1* expression may be initiated during programmed cell death as a means to clear away the ECM of the dying cell. MMP1 is a secreted protease, therefore it is likely to have global effects when expressed from a single tissue. Perhaps misexpression of *MMP1* in the fat body floods the body capsule with MMP1, which in turn begins degrading ECM of other larval tissues. This global ECM degradation could trigger cell death, thus leading to animals dying during early third instar. Future experiments should be conducted in order to investigate whether cell death is occurring in the fat body and other tissues when *MMP1* is misexpressed in the fat body.

Blocking action of MMP1 specifically in the fat body results in loss of head eversion

I have shown that blocking MMP1 action in the fat body by expression of *MMP1-DN* or *TIMP* blocks in the head eversion. This phenomenon has been reported for *MMP1* mutants (Page-McCaw *et al*., 2003) but has not been attributed specifically to the fat body until now. Page-McCaw *et al*. (2003) suggest that the event of head eversion most likely involves extracellular matrix remodeling and MMP1 may be required for this event. My data suggest that MMP1 may be secreted from the fat body in order to accomplish this ECM remodeling during head eversion.

The fat body plays many important roles in the overall physiology of the fruit fly. The fat body is responsible for the production of growth factors that support proliferation of imaginal tissue (Hoshizaki, 2005). The fat body is also a reported nutrient sensing tissue responsible for relaying information regarding the developing animals nutritional

status (Colombani *et al*., 2003, Geminard *et al*., 2009). Future studies are required to determine whether MMPs in the fat body are involved in these signaling events.

CHAPTER 5

THE ROLE OF 20E SIGNALING IN *DROSOPHILA* PUPAL METABOLISM

Introduction

The larval stage of *Drosophila melanogaster* is a critical period characterized by continuous feeding to support the growth of the larva and the acquisition of sufficient energy stores to support metamorphosis and to fuel the rapid development of the gonads in the immature adult (Aguila, unpublished data). The adult fly does not grow, thus the final size of the adult is determined, to a first approximation, at the end of larval development (Tu and Tatar, 2003; Gefen *et al*., 2006; reviewed by Mirth and Riddiford, 2007). Two hormones, 20-hydroxyecdysone (20E) and Juvenile Hormone (JH) have central roles in controlling the length of the larval stages. In the presence of JH, 20E will initiate a larval molt (Riddiford, 1993). During the third-larval instar a minor rise in the 20E titer induces wandering, where upon the larva leaves the food in search of a place to pupariate. Approximately 24 hours after wandering has been induced, JH is absent and a major 20E peak triggers pupariation. The animal ceases wandering and forms a shortened puparium, thus marking the beginning of metamorphosis (Riddiford, 1993). The timing of the wandering phase and puparium formation is critical because it is at this stage the animal no longer feeds and animal commits to metamorphosis. The developmental decision between feeding vs. initiation of metamorphosis is critical because it determines the length of time committed to feeding and the final amount of energy stored (Gefen *et al*., 2006). Longer feeding periods result in larger adults and thus might influence fecundity. Larger females have higher fecundity in many animals including *Drosophila* (Stearns, 1992). Larger size, however, requires longer larval feeding time and thus requires a delay in reproduction. Therefore, a fundamental

evolutionary trade-off between size and fecundity vs. time to reproduction exists. This trade-off highlights the need for a balance between duration of larval feeding and growth, with the initiation of metamorphosis. From these studies the concept of critical weight has emerged.

Critical weight is the larval weight at which a series of physiological events are initiated which trigger pupariation. As originally defined (Nijhout and Williams, 1974), critical weight is the mass at which JH secretion stops. After cessation of JH secretion, the period of time necessary to clear JH is termed the Interval for Continued Growth, or ICG (Davidowitz and Nijhout, 2005). During this period the larva continues to feed and grow. After JH has been cleared the hormones necessary to promote pupariation can be secreted (Nijhout and Williams, 1974; Fain and Riddiford, 1976). This is accomplished via secretion of Prothoracicotropic hormone (PTTH) (Roundtree and Bollenbacher, 1986). PTTH stimulates secretion of the 20E precursor α-ecdysone from the prothoracic gland (Ciancio *et al*., 1986; Mirth and Riddiford, 2007). The conversion of α-ecdysone to 20E (the active form of the hormone) is catalyzed in the peripheral tissues (Huang *et al.,* 2008). Once critical weight is reached, the larva ceases to feed and the animal forms a puparium in response to 20E (Riddiford, 1993; Mirth and Riddiford, 2007). In the best-characterized example, *Manduca sexta*, secretion of PTTH is regulated by light and causes visible changes in larval appearance, so the changes in PTTH levels can be easily monitored (Bowen *et al*., 1984). In *Drosophila melanogaster* changes in hormone levels are difficult to follow because *D. melanogaster* is over three orders of magnitude smaller than *M. sexta*, and there are no visual markers of hormone secretion. Thus, most studies of "critical weight" in *D. melanogaster* measured the "minimum weight for viability"

(Davidowitz, *et al*., 2003), the smallest mass at which larvae can undergo metamorphosis to adulthood.

Although the precise mechanism that allows for cross-talk between tissues that sense nutritional status to give rise to the hormonal establishment of critical weight has not been established, the determinates of critical weight in *Drosophila* are likely to involve the insulin signaling pathway (Brogiolo *et al*., 2001; Colombani *et al*., 2005; Mirth *et al*., 2005; Caldwell *et al*, 2005). *Drosophila* Insulin-like peptides (DILPs) are used to monitor the nutritional status of the animal, and insulin signaling controls the rate of cellular growth and division through a phosphatidylinositol-3' kinase (PI3K) signal transduction cascade (Hafen 2003; Mirth and Riddiford, 2007). The PI3K signaling cascade can affect energy supplies by promoting glycogen synthesis and suppressing dFOXO, a transcription factor that is a negative regulator of cell growth (Taniguchi *et al*., 2006). PI3K signaling also affects growth by activating the TOR pathway (Taniguchi *et al*., 2006). TOR signaling promotes growth by increasing translation, ribosome biosynthesis (Wullschleger *et al*., 2006) and uptake of amino acid transporters in the fat body (Hennig *et al*., 2006).

DILPs are released from the insulin producing cells (IPCs) in the brain and travel through the hemolymph and bind the InR receptor in two key nutrient sensing tissues, the prothoracic gland (PG) and the fat body (Britton *et al*., 2002; Caldwell *et al*., 2005; Colombani *et al*., 2005; Mirth *et al*., 2005). The prothoracic gland produces ecdysteroid and is likely a size-sensing organ (Colombani *et al*., 2005; Mirth *et al*., 2005). Several reports suggest that cross-talk between 20E signaling and the insulin signaling pathway is involved in coordinating developmental timing with growth and thus could be a major

factor in determining body size. Specifically, it has been proposed that insulin signaling in the PG gland can induce secretion of α-ecdysone. The interplay between these pathways is likely to coordinate developmental timing with growth and thus could be a major factor in determining final body size (Caldwell *et al*., 2005; Mirth *et al*., 2005; Colombani *et al*., 2005). Genetic manipulation of the PG can induce precocious or delayed release of α-ecdysone precursors, resulting in premature or delayed pupariation and consequently smaller or larger adults, respectively (Britton *et al*., 2002; Mirth *et al*., 2005; Caldwell *et al*., 2005). Similar results have been described by Colombani *et al*., (2005) but were associated with changes in overall growth rates, not developmental delays. Discrepancies between these studies are likely to lie in differences in how insulin signaling was manipulated in the PG and possibly differences in the food. In any event, the ability of the PG to normally act as a size-sensing organ is likely to be due to its response to DILPs and the nutritional status of the animal (Colombani *et al*., 2003).

The larval fat body has a central role in metabolism and is the most likely tissue transducing the PG's response to nutritional status, perhaps by monitoring the accumulation of energy stores or amino acid levels via TOR signaling (Colombani *et al*., 2003; Hwangbo *et al*., 2004). The insect fat body is the primary tissue involved in nutrient storage. Large storage proteins (hexamerins), triacylglycerols (TAG), and glycogen are all stored in the fat body and can be mobilized as needed by the developing animal (reviewed by Beenakkers, 1969; Telfer and Kunkel, 1991; Gronke *et al*., 2005; Hoshizaki, 2005; Gutierrez *et al*., 2007). In terms of nutrient sensing and size control, the insect fat body has also been recognized for its endocrine function in the production of growth factors (Martin *et al*., 2000; Kawamura *et al*., 1999) and might very well produce

other unidentified peptides that interact with the PG to monitor size (Colombani *et al*., 2005). There is evidence that insulin signaling and 20E signaling are somehow integrated in the fat body to control the final size of the animal (Colombani *et al*., 2005; Geminard *et al*., 2009). Colombani *et al*. (2005) report that disruption of 20E signaling in the fat body results in a decrease in pupal size, while Cherbas *et al*. (2003) have reported pupal lethality but describe no differences in size or developmental timing. Because the fat body is the primary nutrient storage tissue, it is likely the central site involved in monitoring nutritional status. Indeed 20E is known to induce autophagy (a process which promotes amino acid mobilization) in the fat body (Rusten *et al*., 2004). It is feasible that a lack of nutrient availability in the fat body could result in cessation of development and animal lethality due to the fat body's central role in monitoring nutritional status.

In this chapter, I have tested several hypotheses in an effort to determine whether 20E signaling in the fat body may affect animal development and survival through a metabolic mechanism. I disrupted 20E signaling in the larval fat body and measured changes in animal size, accumulated energy stores, and the expenditure of energy stores during pupal development. I found that 20E signaling in the fat body is necessary for completion of pupal development but is not required for larval nutrient accumulation or pupal expenditure of energy stores. Moreover I determined that 20E signaling in the fat body alone is not sufficient to induce whole animal size defects.

Materials and Methods

Fly stocks

Lsp2-Gal4 and *UAS-EcR-DN*^{F645A} were provided by L. Cherbas. *ppl-Gal4* and *cg-Gal4* were provided by M. Pankratz and C. Dearolf respectively.

Microscopy

Animals were collected as white prepupae, placed on wet filter paper in a Petri dish at 25°C, and aged to 90 hours APF. Aged animals were examined by light microscopy on a Zeiss Stemi 2000-C microscope. A Canon A620 digital camera and Canon Zoom Browser EX software were used to procure the images. The images were compiled in Corel Draw®.

Dry weights

Ten to twelve animals of each genotype were collected as white prepupae and dried over night at 60ºC. Dried animals were weighed on a Cahn C-30 microbalance. The dry weights of all animals of a particular genotype were averaged together to give the weights represented in Figure 27.

Protein, carbohydrate and lipid content

Animals were collected at the white prepupal stage and either frozen immediately or placed on wet filter paper in a Petri dish and allowed to age at 25°C to 90 hours APF. 2 animals for each stage and genotype were homogenized in 60µl of lysis buffer (1%NP-40, 0.5% deoxycholic acid, 0.1% Triton-X 100, 100 mM NaCl, 0.1 mM $CaCl_2$ 2 mM $MgCl_2$, pH 7.6). Hydrolases were heat killed by incubating at 70°C for 5 minutes, then samples were centrifuged at 12,000 g for 2 minutes. Supernatants were diluted as necessary in lysis buffer.

Triacylglyceride levels were measured using a serum triglyceride kit (Sigma). Protein BSA standard levels were quantified using the bicinchinonic acid (BCA) method (Smith *et al*., 1985). Carbohydrates were digested with *Rhizopus* amyloglucosidase (Sigma) and glucose levels were quantified using a blood glucose kit (Pointe Scientific).

Metabolic rates

Pupal metabolic rates were measured using flow-through respirometry. Groups of 5 pupae from each stage/genotype were transferred to a 1 ml glass-aluminum respirometry chamber. Dry CO_2-free air was pumped through the chambers at 50 ml per minute to a Li-Cor LI-6262 infrared CO_2 sensor (Li-Cor). Metabolic rates were calculated from the release of CO_2 into the air stream. Data acquisition and analysis were performed using Datacan V software (Sable Systems, Las Vegas, NV, USA).

Results

Disruption of 20E signaling in the fat body results in pupal lethality

Disruption of 20E signaling in the fat body was achieved by expression of *UAS-EcR-DN*, a dominant-negative allele of the Ecdysone Receptor (EcR) which is one of the two subunits of the active 20E heterodimer receptor (Cherbas *et al*., 2003). Tissue-specific expression was directed using the Gal4/UAS system (Brand and Perrimon, 1993). I utilized three *Gal4* drivers, *cg-Gal4* , *Lsp2-Gal4* and, *ppl-Gal4* to express *UAS-EcR-DN* in the fat body. *Cg-Gal4* is expressed throughout larval development in the fat body and hemocytes starting from the first instar and persisting through pupal development (Asha *et al*., 2003; Evans *et al*., 2009). *Lsp2-Gal4* expression begins during the third instar and continues throughout pupal development (Andres *et al*., 1993; Cherbas *et al*., 2003). *ppl-Gal4* expression has been described as a fat body-specific driver (Zinke *et al*., 1999). Although *ppl* gene expression is restricted to the fat body (Zinke *et al*., 1999), my data show that expression of the *ppl-Gal4* driver was not exclusive to the fat body (Figure 26). In addition to the lack of fat body specificity found with the *ppl-Gal4* expression pattern, fat body expression of *ppl-Gal4* did not persist into pupal development. Disruption of

20E signaling throughout larval fat body development via expression of *UAS-EcR-DN* using *cg-Gal4* resulted in late pupal lethality (Figure 26). These results are in agreement with Cherbas *et al*. (2003) in which disruption of 20E signaling in the fat body at third instar also caused pupal lethality. Expression of EcR-DN directed by *ppl-Gal4* also caused lethality, but this occurred much earlier, during the larval stages; thus the phenotype observed in these progeny cannot be attributed to loss of 20E signaling in the fat body alone.

From these data I conclude that EcR signaling in the fat body is essential for pupal development. Despite published reports, these data suggest that 20E signaling in the fat body is not necessary for determination of critical weight. Larvae with 20E signaling blocked in their fat body tissue pupariated normally, a process that would be disrupted if critical weight determination were not functional. Since lethality of animals expressing EcR-DN does not occur until later in metamorphosis, I set out to determine if other fat body relevant processes were disrupted in these animals.

Blocking 20E signaling in the fat body does not affect energy storage

During the pupal stages vast structural changes occur that would appear to depend upon stored energy accumulated in the fat body during the larval stage. Therefore, the larval fat body is likely to have a central role in pupal metabolism. Thus, in searching for a fat body specific process that may be necessary for animal survival, I set out to test the role of 20E signaling in various aspects of fat body pupal metabolism.

I first tested the hypothesis that 20E signaling in the fat body is necessary for proper nutrient storage. I reasoned that animals which have 20E signaling blocked in the fat

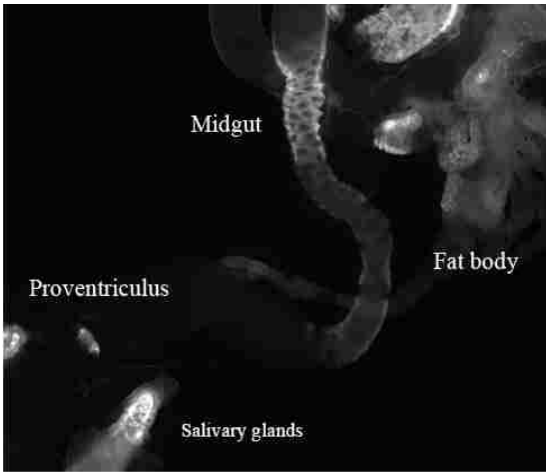

Figure 26. *ppl-gal4* expression is not fat body specific. *ppl-gal4* was crossed to a stock carrying *UAS-GFP,* and third instar larvae were dissected and photographed using fluorescent microscopy. *ppl-gal4* directed expression of GFP in many tissues including the proventriculus, the salivary gland, the midgut and the fat body.

body might not be capable of storing enough nutrients to make it through metamorphosis, thus resulting in pupal death. If animals are not capable of storing nutrients I would expect to see less energetic storage in the experimental animals as compared to controls at the onset of metamorphosis. In keeping with this logic, I expected animals with 20E signaling blocked in the fat body would also be smaller in size if energy storage was defective. To generate the 20E signaling blocked animals, *cg-Gal4/CyO* animals were crossed to *UAS-EcR-DN*. The *cg-Gal4*/*UAS-EcR-DN* (experimental) and *CyO/UAS-EcR-DN* (sibling controls) animals were collected at pupariation. The dry weights of animals were measured at pupariation and no significant difference was observed (Figure 28). Next, I measured the nutrient stores of 20E signaling blocked animals at the end of larval development (at pupariation). The amount of proteins, carbohydrates, and triglycerides present at pupariation were determined (Figure 29) and, as expected from the dry weight data, I did not observe a significant difference in carbohydrate, lipids or protein content in experimental animals as compared to sibling controls. At pupariation, the 20E signaling blocked animals and the sibling controls had accumulated the same amounts of carbohydrates, proteins and triglycerides and presented equivalent dry weights as their sibling controls. Thus, I conclude that 20E signaling in the in the larval fat body is not required for nutrient storage, accumulation.

Blocking 20E signaling in the fat body does not affect energy utilization

After ruling out the possibility that 20E signaling is involved in nutrient storage, I next explored the idea that 20E signaling might be involved in regulation of metabolic rate. Metabolic rates of holometabolic insects, including *Drosophila melanogaster*, decline during early metamorphosis, then begin to increase in the late pupal stage

Figure 27. 20E signaling is required in the fat body for animal survival. (a) Wild type (*cg-Gal4*) animal just before eclosion. (b) Animal expressing *UAS-EcR-DN/cg-Gal4* dies before eclosion.

(Merkey, 2008 and Figure 30). I speculated that this lull in metabolic rate might be an important mechanism for the conservation of energy stores, allowing them to last throughout metamorphosis. If 20E signaling blocked animals are unable to utilize nutrients we should observe a decrease in metabolic activity. Alternatively, if 20E signaling is required to reduce metabolism, then the animal might use resources too rapidly and die of starvation.

I tested these hypotheses by using flow-through respirometry (Gibbs *et al*., 1997, 2001, 2003). Animals which have 20E signaling blocked in the fat body were not defective in overall metabolic rate during metamorphosis. They had the same metabolic rate as controls when they began metamorphosis and underwent the typical metabolic depression at day one and metabolic increase at days 3-4 (Figure 29). From these data I conclude that 20E signaling in the fat body does not affect overall metabolic rate during metamorphosis.

I next explored the idea that 20E signaling may affect energy utilization. There are three potential energy sources (lipids, carbohydrates and proteins) available to fuel metamorphosis. I hypothesized that animals with 20E signaling blocked in the fat body might use the three energy sources differently. Perhaps the experimental animals preferentially use carbohydrates, for example. If animals that express EcR-DN in the fat body are unable to use the proper amount of carbohydrates, lipids or proteins they might exhibit developmental defects during metamorphosis leading to an inability to eclose.

To test this hypothesis, I collected animals at the white prepupal stage and aged them to immediately before eclosion (90 hours APF) and I measured carbohydrate, protein and lipid levels. I found no evidence of preferential macronutrient utilization in

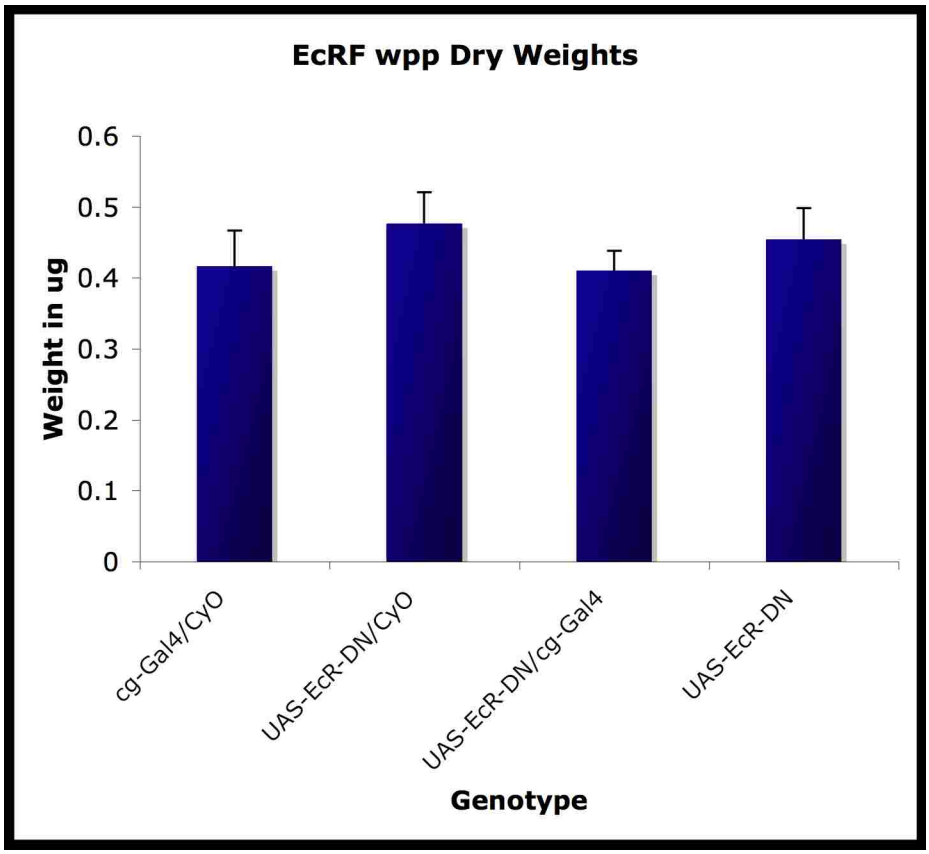

Figure 28. Blocking 20E signaling in the fat body does not affect animal size at pupariation. Animals were collected at pupariation, dried at 60º C overnight and weighed on a microbalance. Animals Expressing EcR-DN in the fat body had similar dry weights to control animals (*cg-Gal4*, *UAS-EcR-DN/CyO* and *UAS-EcR-DN*).

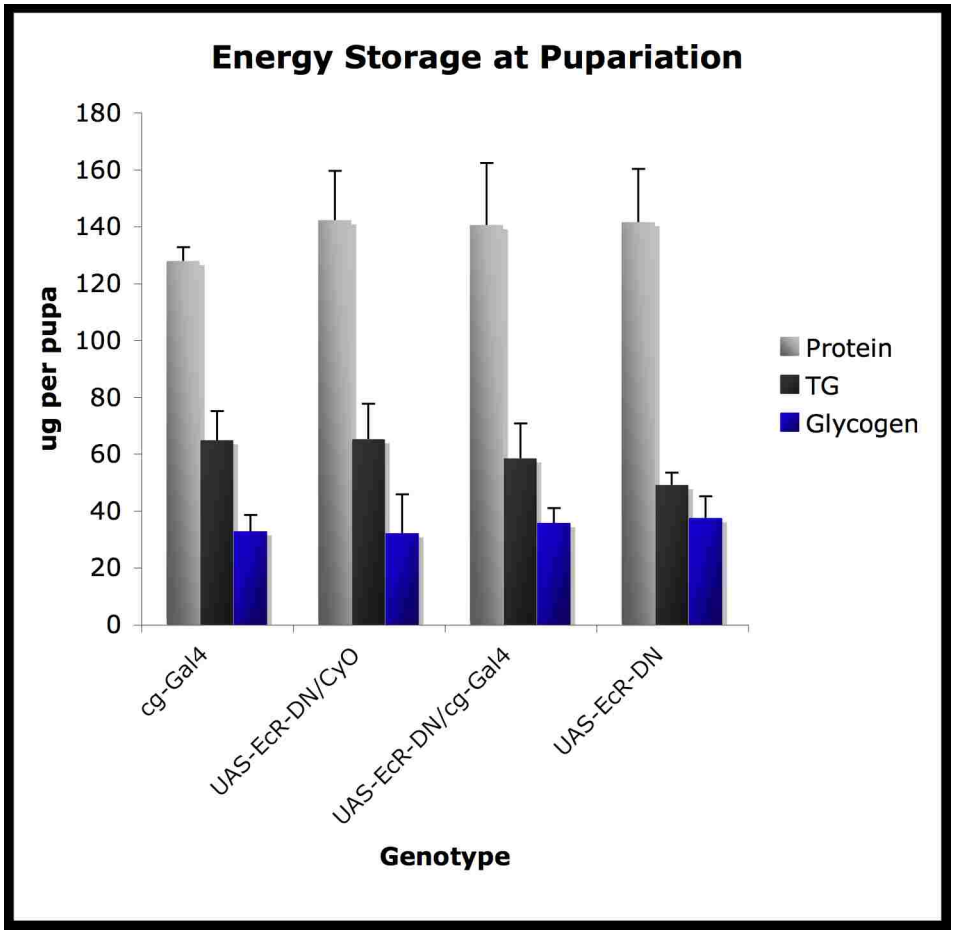

Figure 29. 20E signaling in the fat body is not required for accumulation of nutrient stores. Animals were collected at pupariation, homogenized and protein, triglyceride (TG) and glycogen levels were determined. Animals expressing EcR-DN in the fat body (*UAS-EcR-DN/cg-Gal4*) accumulated similar amounts of energy stores as controls (*cg-Gal4, UAS-EcR-DN/CyO,* and *UAS-EcR-DN*).

experimental animals (Figure 31).

From these data I conclude that 20E signaling in the fat body does not have a role in pupal nutrient utilization or larval nutrient acquisition. Lack of 20E signaling did not affect any measurement of size, energy storage or energy consumption, thus death of 20E blocked animals is not caused by failure to store nutrients or utilize them correctly.

Discussion

Previous studies have implicated fat-body specific 20E signaling in size control. Colombani *et al*. (2005) report that blocking 20E signaling in the fat body results in a larger animal at puparium formation. Final animal size is determined at puparium formation; therefore it is the growth rate and length of growth period during the larval stage that dictates adult animal size (Mirth and Riddiford, 2007). According to Colombani *et al*. (2005) 20E signaling in the fat body regulates the rate of growth during the larval stage. These reports conflict with the data presented here. I have demonstrated that blocking 20E signaling in the fat body does not alter metabolism and has no effect on animal size entering metamorphosis. These discrepancies may be explained by examining the genetic tools utilized in the individual studies. Colombani *et al*. (2005) used a Gal4 driver derived from the fat body specific gene *pumpless* (*ppl-gal4*) to direct expression of an RNAi construct specific to the EcR transcript (*UAS-EcRi*). Although this driver is derived from a fat body specific gene, the expression pattern of the driver is not fat body specific during the larval stages (Figure 26) and expression is not detectable during the pupal stages. In contrast, we employed *cg-Gal4* (expressed in hemocytes and fat body specifically) and *Lsp2-Gal4* (expressed in fat body specifically) to drive expression of EcR-DN. Phenotypes could significantly differ between knockdown via

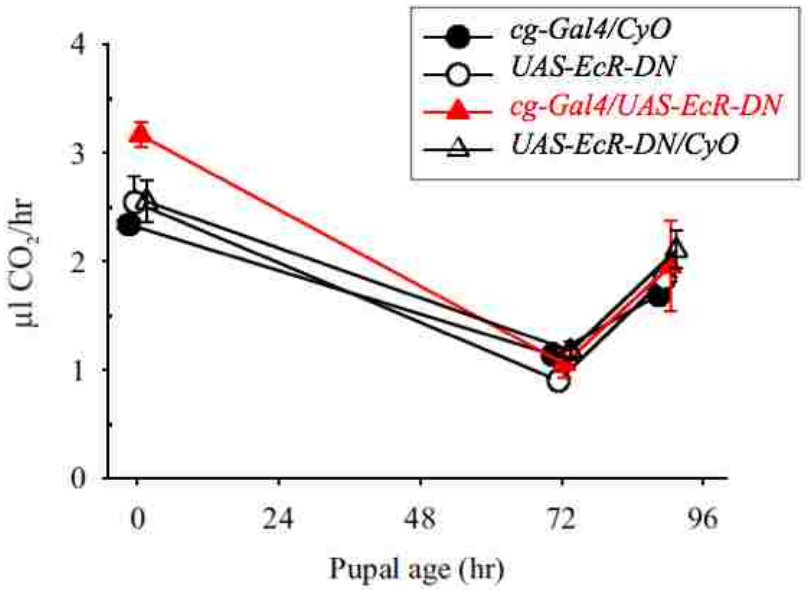

Figure 30. 20E signaling in the fat body is not required for regulation of metabolic activity during metamorphosis. Animals at pupariation, 72 hours APF, and 90 hours APF were placed in the respirometer and their metabolic rates were determined from CO2 release. Animals expressing EcR-DN in the fat body (*cg-Gal4*/*UAS-EcR-DN*) had similar metabolic rates as control animals (*cg-Gal4*/*CyO*, *UAS-EcR-DN*, and *UAS-EcR-DN/CyO*) at all three time points.

RNA interference versus expression of a dominant negative form of a receptor. However, employing the cg-Gal4 driver to express *UAS-EcRi* results in a phenotype that is consistent with what has been reported for expression of *UAS- EcR-DN* (Cherbas *et al*., 2003; Chapter 3). Therefore it is unlikely that these two different responders could produce these disparate results. Instead it is likely that the expression of *ppl-Gal4* in tissues other than the fat body produces the size defects observed when *UAS-EcRi* is expressed.

The cause of death when 20E signaling is blocked in the fat body is unclear. Evidence of cross-talk between 20E and insulin signaling (Colombani *et al*., 2003) led me to hypothesize that larval energy storage or energy utilization during metamorphosis might be disrupted. Animals might enter with insufficient energy stores, consume the energy too rapidly and starve, consume it too slowly to produce enough ATP to fuel tissue restructuring, or use the "wrong" type (lipid, carbohydrates, protein) of fuel. My data does not support the hypothesis that 20E signaling in the fat body affects pupal metabolism, thus we cannot attribute the eclosion phenotype to a metabolic defect. An alternative model may involve the *Drosophila* hepatocyte-like cells, the oenocytes. It has been documented that the fat body regulates the accumulation of lipid droplets in the oenocytes (Gutierrez, *et al*., 2007). When the oenocytes are unable to perform fatty acid metabolism, defects in adult eclosion occur (Gutierrez *et al*., 2007). TOR signaling is the nutrient sensing mechanism implicated in the export of lipids from the fat body to the oenocytes (Colombani *et al*., 2003; Gutierrez *et al*., 2007). The possibility of 20E signaling and TOR signaling interacting in the process of lipid release from the fat body is especially appealing in light of the finding that both signaling cascades may contribute

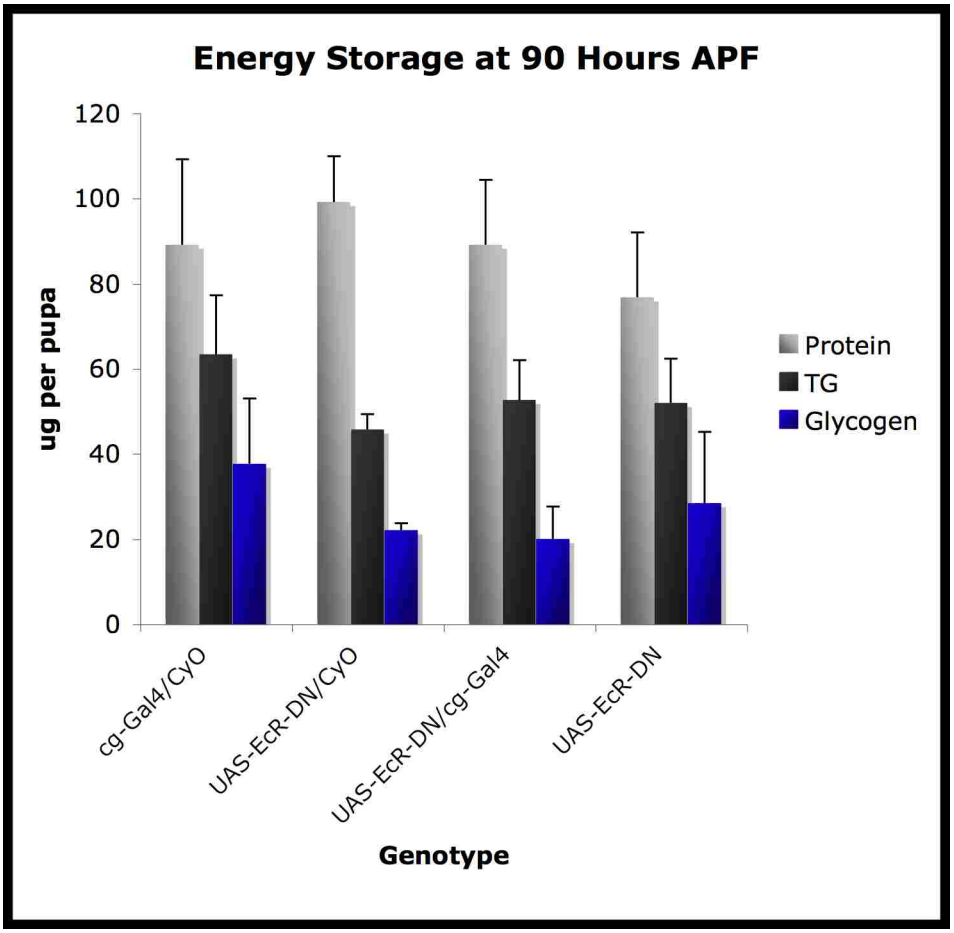

Figure 31. 20E signaling is not required for utilization of nutrient stores during metamorphosis. Animals were collected at pupariation and incubated at 25º C for 90 hours. Staged animals were then homogenized and protein, triglyceride (TG) and glycogen levels were determined. Animals expressing EcR-DN in the fat body (*UAS-EcR-DN/cg-Gal4*) utilized similar amounts of protein, TG and glycogen as control animals (*cg-Gal4*, *UAS-EcR-DN/CyO*, and *UAS-EcR-DN*).

to the process of autophagy in the fat body (Rusten *et al*., 2004). Our experimental results cannot rule out a defect in fat body lipid exportation to the oenocytes, a process which presumably would result in pupal lethality just prior to eclosion. Further investigation of 20E signaling and lipid export may provide insight into the role of fat body specific 20E signaling and eclosion.

CHAPTER 6

DISCUSSION

In this dissertation I have detailed the gross morphological changes that occur during fat body remodeling. I have demonstrated, by a number of different techniques, that the hemocytes are not involved in the process of fat body remodeling. I have expanded on the data published by Cherbas *et al*. (2003) and have determined the processes of disaggregation and detachment do require 20E signaling while retraction of the fat body does not. I have determined that the competence factor *ßftz-f1* is also required for fat body remodeling and that the 20E signaling cascade is required for the induction of *MMP2* expression. I have also uncovered a fat body specific role for MMP1 during the process of head eversion.

In an effort to determine why blocking 20E signaling in the fat body results in lethality, I tested the ability of animals expressing EcR-DN in the fat body to undergo various aspects of metabolism. I suspected that blocking 20E signaling in the fat body would lead to metabolic defects during metamorphosis. Contrary to my hypothesis, my data do not suggest a role for 20E signaling in fat body metabolism. While my experimental data have not provided insight into the reason why 20E signaling in the fat body is required for animal survival, my research has uncovered other potential mechanisms that might explain this observation. I explore these possibilities in the discussion presented here.

In this discussion, I propose a role for 20E signaling and MMP2 in the regulation of Insulin signaling. Additionally, I propose that βFTZ-F1 is required in the fat body to induce expression of MMP1 which is required for eversion of the head capsule.

Fat body MMP1 controls the process of head eversion

While investigating the role of MMPs in the degradation of ECM during fat body remodeling, I uncovered an unexpected role for MMP1 in the fat body. Expression of a dominant negative form of MMP1 (MMP1-DN) specifically in the fat body blocks the process of head eversion. MMP1 is a secreted protease, thus it might have non-cell autonomous effects. Therefore, MMP1 secreted from the fat body may have the ability to affect other tissues. It has been suggested that MMP1 might be required for the remodeling necessary to promote head capsule eversion (Page-McCaw *et al*., 2003). My data lend support to this hypothesis. Furthermore, my data suggest that *MMP1* expression specifically in the fat body is required for head eversion to occur. To confirm that this function of MMP1 is indeed fat body specific, expression of *UAS-MMP1-DN* in other tissues should be performed. If the head eversion phenotype described above cannot be recapitulated by blocking MMP1 action in other tissues than it can be concluded that this is a fat body specific funtion of MMP1

Regulation of MMP1 expression

I have shown that *MMP2* expression is induced by 20E signaling (Figure 25). This was done by qRT-PCR after I noticed that the *MMP2* mutant phenotypes recapitulated the phenotype observed when EcR-DN was expressed in the fat body. *MMP1* has also been described as a possible 20E inducible gene (Lee *et al*., 2002). The phenotype observed when MMP1-DN is expressed in the fat body, however, does not recapitulate EcR-DN phenotypes. Unlike animals expressing *MMP1-DN*, animals expressing *EcR-DN* do evert their heads. This suggests that signaling through EcR is not necessary to promote activity of MMP1 in the fat body. Interestingly, I found that blocking

expression of *ßftz-f1* in the fat body does result in a block in head eversion (Figure 32). Head eversion was disrupted when *ßftz-f1* expression was blocked by expression of *UAS-dBlimp-1* in the fat body and hemocytes via the *cg-Gal4* driver. These results indicate that either *ßftz-f1* in the fat body can directly initiate head eversion, or that *ßftz-f1* in the hemocytes is required for head eversion. In line with the first possibility, there are predicted ßFTZ-F1 binding sites upstream of the *MMP1* gene (Figure 32). Expression profiles of fat body MMP1 in wild type and *ßftz-f1* mutant animals should to be performed in order to begin to distinguish between these possibilities. If *MMP1* expression is disrupted in fat bodies lacking *ßftz-f1* this will provide evidence in support of the idea that *MMP1* expression is directly regulated by *ßftz-f1*.

20E signaling and the regulation of insulin signaling in the fat body

Previous reports have demonstrated that disruption of 20E signaling in the fat body results in animal lethality (Cherbas *et al*., 2003). Evidence of cross-talk between 20E and insulin signaling (Colombani *et al*., 2003) led me to hypothesize that larval energy storage or energy utilization during metamorphosis might be disrupted, leading to animal lethality when EcR-DN is expressed in the fat body. My data do not support this hypothesis, thus I cannot attribute the lethality phenotype to a metabolic defect. Instead my research has lead me to propose a model involving 20E mediated expression of *MMP2* in the control of insulin signaling in the fat body.

The regulation of insulin signaling is tightly controlled. Down-regulation of insulin signaling during starvation promotes the release of nutrients from the fat body, a process which promotes animal survival (Hafen, 2004). Two *Drosophila* insulin-like growth factor-binding proteins (IGF-BPs) are required to modulate DILP (*Drosophila* insulin-

like peptides) availability. Expression of the IGF-BPs (*dALS* and *Imp-L2*) is required to form trimeric complexes with the DILPs. This complex protects circulating DILPs and can inhance insulin signaling (Arguier *et al*., 2008; Honneger *et al*., 2008).

The insulin signaling pathway, which is located upstream of TOR, has the ability to upregulate TOR signaling, thus resulting in a block in nutrient release (Mirth and Riddiford, 2007). Upstream of both insulin signaling and TOR signaling is 20E signaling, which has the ability to downregulate Insulin signaling in the fat body (Runsten *et al*., 2004) through an unknown mechanism. Therefore, 20E signaling has the ability to affect nutrient release through the modulation of TOR signaling through the insulin signaling cascade in the fat body. Blocking 20E signaling in the fat body results in late pupal lethality a phenotype closely resembling that which happens when lipid exportation to the oenocytes is blocked. This phenotype underscores the role that 20E signaling has in insulin signaling control.

In this dissertation I have shown that 20E signaling in the fat body is required for expression of *MMP2*. In mammalian systems, matrix metalloproteases (MMPs) are known to cleave IGF-BPs (Fawlkes *et al*., 1994). Recall that IGF-BPs in *Drosophila* function to protect circulating DILPs and therefore have the ability to upregulate Insulin signaling. The ability MMPs to cleave IGF-BPs may help to explain some of the phenotypes I have described in this dissertation.

Perhaps the expression of *MMP2* in the fat body serves to fine-tune the insulin signaling response during metamorphosis by cleaving the IGF-BPs. For all practical purposes, metamorphosis can be thought of as a time of starvation. The pupa does not feed, thus, proper timing of nutrient release from the fat body is likely to be very

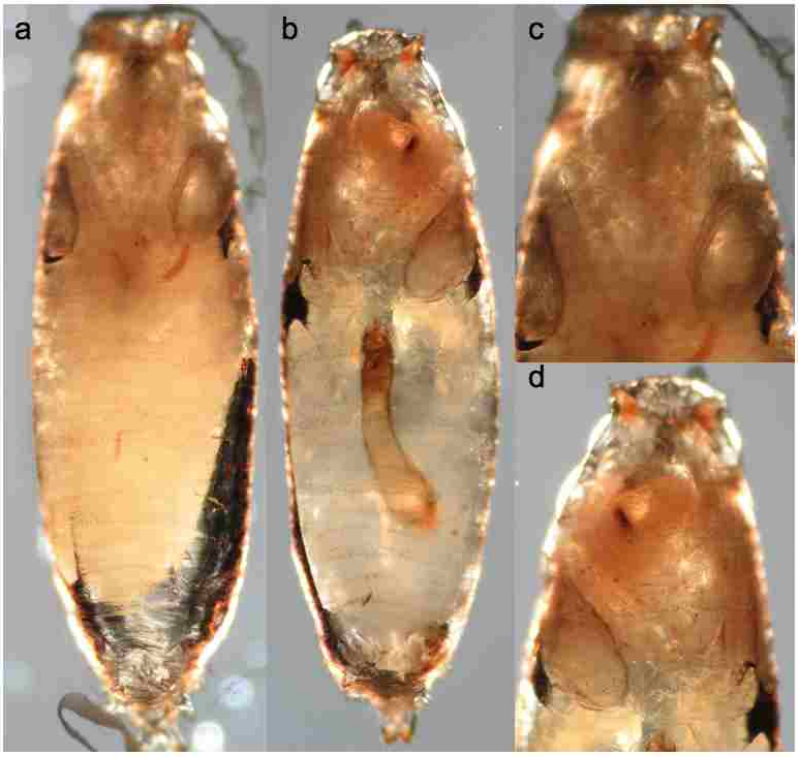

Figure 32. Expression of *ßftz-f1* in the fat body is required for head eversion. (a-d) Animals expressing *cg-Gal4*;*UAS-dBlimp-1*. (c) close up of (a), (d) close up of (b). Head eversion has not occurred in (a-d) and in (b) the midgut has not undergone programmed cell death.

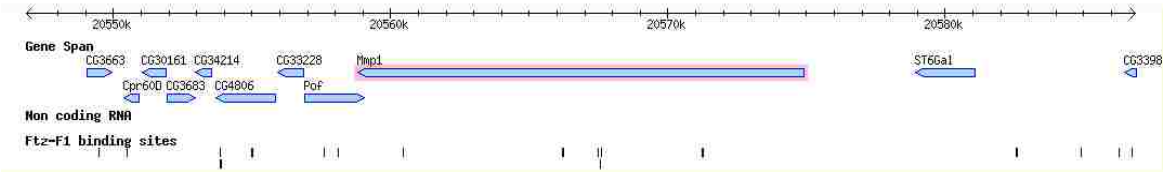

Figure 33. ßFTZ-F1 binding sites upstream of the *MMP1* gene. Binding sites for ßFTZ-F1 on and around the *MMP1* gene were determined using the Flybase g-browse program. The ßFTZ-F1 annotation of the *D. melanogaster* genome was generously provided by R. Anderson.

important to the development of the adult fly during metamorphosis. It is known that during times of starvation, *Drosophila* IGF-BPs are upregulated, while secretion of DILPS is decreased (Honegger *et al*., 2008; Geminard *et al*., 2009). This increase in IGF-BP should result in protection of the small amount of DILPS circulating in the animal (Arguier *et al*., 2008; Honegger *et al*., 2008). Protection of DILPS results in an increase in insulin signaling and thus a decrease in nutrient release (Arguier *et al*., 2008). Blocking the release of nutrients by up-regulating Insulin signaling during metamorphosis could be detrimental to the developing animal. Therefore, there must be a mechanism in place to down-regulate insulin signaling in the fat body during metamorphosis. MMP2 is an ideal candidate for the down-regulation of this potentially devastating increase in insulin signaling during metamorphosis. Pulses of 20E during metamorphosis initiate the transcription of *MMP2*. MMP2, in addition to remodeling the fat body tissue, may cleave the IFG-BPs. This cleavage would leave the DILPS unprotected, thus a decrease in insulin signaling should ensue. This decrease in insulin signaling would result in an increase in nutrient release which may be important to animal survival during metamorphosis (Figure 34).

This model could explain some of the phenotypes detailed in this dissertation. Previously I proposed that 20E signaling in the fat body was required for the release of lipids from the fat body to the oenocytes and a lack of this process could result in pupal lethality. Indeed it has been shown that blocking lipid mobilization to the oenocytes from the fat body results animal lethality (Gutierrez *et al*., 2007). In pharate adults, blocking 20E signaling directly in the fat body results in lethality at the same stage (Figure 27). MMP2 regulation of insulin signaling could be involved in this process. When 20E

signaling is blocked in the fat body, *MMP2* expression is also decreased. According to my model, this would block cleavage of IFG-BPs from DILPs and thus result in protection of DIPLS, increased insulin signaling and a net block in nutrient mobilization. Such a block in nutrient mobilization would result in a lack of lipid export to the oenocytes and thus animal lethality. Therefore, perhaps the animals expressing EcR-DN in their fat bodies die due to their inability to express *MMP2* which results in insulin signaling and a block in nutrient mobilization (Figure 35).

The opposite effects could be elicited in the fat body when *MMP2* is prematurely expressed. Recall that expression of *MMP2* in third-instar fat bodies results in animal death before the wandering stage. According to my model, overexpression of *MMP2* would result in cleavage of the DILP/IGF-BP complex resulting in unprotected DILP and downregulation of insulin signaling. A downregulation in insulin signaling in the third instar would result in an increase in nutrient mobilzation. The third instar is a time of growth and storage. Blocking growth and storage and increasing nutrient mobilization during the third instar by downregulation of Insulin signaling could cause serious defects in the attainment and sensing of critical size. If the animal cannot store enough nutrients to attain critical size the animal will not pupariate, a process that would lead to animal death. In line with this logic, I have shown that upregulation of *MMP2* expression during third instar results in an inability to pupariate and the animal dies. Interestingly, animals prematurely expressing *MMP2* in the fat body do not initiate the wandering stage. The animals continue to feed until they ultimately die. These animals are not visibly small in size. This behavior suggests that the animal continues to feed because it is does not have the ability to sense its attainment of critical size. This defect in size sensing could be due

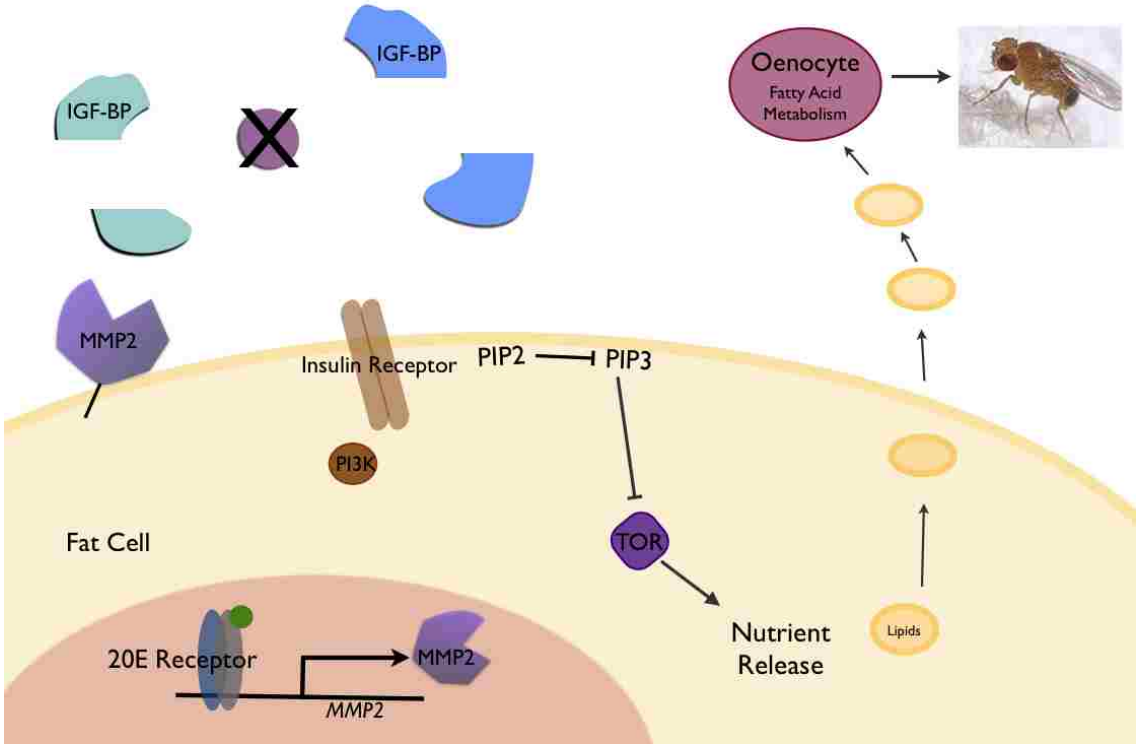

Figure 34. Proposed role of MMP2 in insulin signaling during metamorphosis. The ecdysone receptor initiates transcription of *MMP2*. MMP2 cleaves the IGF-BPs thus releasing the DILP. Release of IGF-BPs from the DILP results in a lack of protection of the that blocks insulin signaling. Blocking insulin signaling represses TOR which allows for nutrient release from the fat body. Lipids are exported to the oenocytes, allowing fatty acid metabolism and further development to occur.

to an increase in insulin signaling, which leads to increased mobilization and depletion of nutrient stores.

Determining whether MMP2 can modulate Insulin signaling is the first step in determining whether the protease can interact with IGF-BPs. Assaying the initiation of insulin signaling can be qualitatively performed with relative ease in *Drosophila.* A special GFP construct containing a Plecstrin homology domain is recruited to the membrane upon conversion of PIP2 to PIP3 in the insulin signaling cascade (Britton *et al*., 2002). Thus the movement of GFP to the membrane is indicative of Insulin signaling initiation. Comparing the Insulin signaling activity in animals with disrupted or premature expression of *MMP2* to wild type controls may provide evidence for the role of MMP2 in insulin signaling.

In conclusion, the process of fat body remodeling, while fascinating in and of itself, coincidentally may be occurring in concert with signaling events that are required for animal development and survival. The mechanism responsible for relaying nutritional signals from the fat body to the rest of the animal has been the subject of intense research and debate. The dual roles of the matrix metalloproteinase MMP2 in the fat body may prove to be the link between fat body physiology and animal survival.

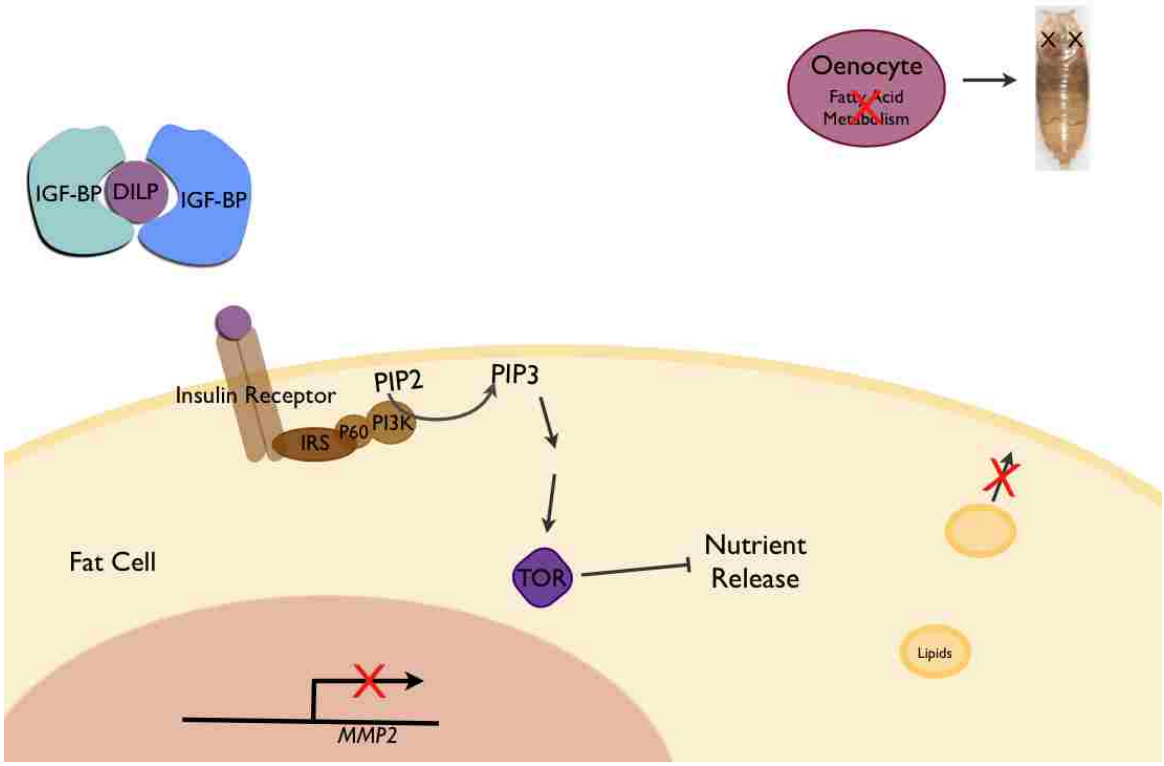

Figure 35. Proposed role of 20E signaling in the modulation of insulin signaling in the fat body. Blocking the ecdysone receptor disrupts expression of *MMP2*. MMP2 cannot cleave the IGF-BPs thus DILPs remain protected. Protection of DILPs results in an increase in insulin signaling. Insulin signaling activates TOR which blocks nutrient release. Lipid export to the oenocytes is blocked. Fatty acid metabolism cannot occur in the oenocytes which results in late pupal lethality.

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