Spectrin exerts multiple effects on lipid translocation pathways in *Drosophila melanogaster*

BY

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THESIS

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ATGL adipose triglyceride lipase	MCFA medium chain fatty acid
AKH adipokinetic hormone	MTP microsomal triglyceride transfer protein
Bmm brummer	NPC1L1 Niemann-Pick C1-like 1
CE cholesterol ester	NR nile red
DAG diacylglycerol	PLIN perilipin
Df deficiency	PM plasma membrane
DIC differential interference contrast	TAG triacylglycerol
EMS ethylmethane sulfonate	VLDL very low density lipoprotein
ER endoplasmic reticulum	
FATP fatty acid transport protein	
FFA free fatty acid	
GPAT glycerol-3- phosphate acyltransferases	
HDL high density lipoprotein	
HSL hormone sensitive lipase	
LCFA long chain fatty acid	
LD lipid droplet	
LDL low density lipoprotein	
Lpp lipophorin	
LSD1 lipid storage droplet-1	
LSD2 lipid storage droplet-2	
LTP lipid transfer protein	

SUMMARY

Over the past several years, significant progress has been made in understanding the cellular biology of lipid translocation. Unraveling the mechanisms underlying this complex process might provide useful knowledge for designing drugs that combat the metabolic diseases associated with it. The data I present in this thesis bring novel insights into the mechanisms of lipid translocation, specifically the transport of lipids across the plasma membrane, their incorporation into lipid droplets, their mobilization from lipid droplets and their transport between larval tissues.

Defects of the cytoskeletal protein Spectrin generate phenotypes that affect the plasma membrane, cell polarity and the secretory pathway. Data presented here suggest that Spectrin exerts unexpected multiple effects on lipid translocation pathways in the fat body. As a result of these studies, I propose a model of lipid uptake, in which the Spectrin tetramer connects the machinery of lipid uptake at the plasma membrane to that of lipid droplet growth in the cytoplasm. Additionally, I present data on lipid translocation through the midgut epithelium and show evidence that the lipid droplet associated protein, LSD2, plays an important role in lipid translocation. I propose a novel mechanism, in which LDS2 functions as a gatekeeper in translocating lipids to export machinery by directing their flow into pathways of lipid droplet formation. Furthermore, I present the results of a forward genetic screen, designed to identify novel candidate genes involved in the biological process of dietary lipid transport between tissues.

CHAPTER I. Introduction

I.1 Lipid translocation

Obesity is an alarming worldwide medical problem that has recently been recognized by the American Medical Association as a disease (Pollack, 2013). The Center for Disease Control and Prevention reports that the prevalence of obesity has reached pandemic proportions in the United States, where more than one third (35.7%) of the adults are currently obese (CDC, 2012). Because the disease is accompanied by profound changes in the physiological function of an organism, it has been associated with a variety of life-threatening pathologies including diabetes, atherosclerosis, liver malfunction and even cancer (Iskander *et al.,* 2013).

In order to develop novel methods in the prevention and treatment of these pathologies and of obesity, we first must understand the underlying molecular mechanisms that are associated with them. The cellular basis of obesity is excess of triacylglycerol (TAG) storage within adipocyte lipid droplets (LDs; Greenberg *et al.*, 2011). Elucidating the mechanisms of lipid droplet biogenesis, maintenance, storage and mobilization is therefore important for designing novel drugs that combat metabolic conditions, such as obesity. The changes in lipid droplet physiology and metabolism depend on the complex process of lipid translocation.

Lipid translocation is an intricate process that occurs both intracellularly and between various tissues. It involves multiple tissues and a multitude of different gene products, many of which are conserved in Drosophila and mammals. Intracellular lipid translocation occurs in four major steps (Fig. 1). Intracellular lipid translocation occurs as FFAs: 1) cross the plasma membrane of cells, 2) are packaged into complex

triacylglycerols (TAGs), which are composed of glycerol and three fatty acids, and incorporated into lipid droplets (LDs), 3) are mobilized by enzymes from LDs (via hydrolysis) and 4) are transported out of cells (Fig. 1). Two main classes of lipids are transported across the plasma membrane (PM) of cells: sterols and free fatty acids (FFAs). Complex lipids, such as TAGs, do not cross the PM of cells (Niot *et al.*, 2009).

It is not known whether the incorporation of FFAs into LDs is an obligatory step in intracellular lipid translocation or if FFAs can be directly transported out of cells (Fig.1 dotted line). It is possible that the two occur concurrently, that as FFAs cross the plasma membrane of cells, they may be both released immediately out of cells and packaged into TAGs and incorporated into LDs. This type of mechanism assures maximal absorption, while maintaining a low intracellular concentration, of FFAs. Because excess FFAs entry into cells can have detrimental effects, cells protect themselves from these effects by sequestering them as TAG within LDs (Greenberg *et al.*, 2011).

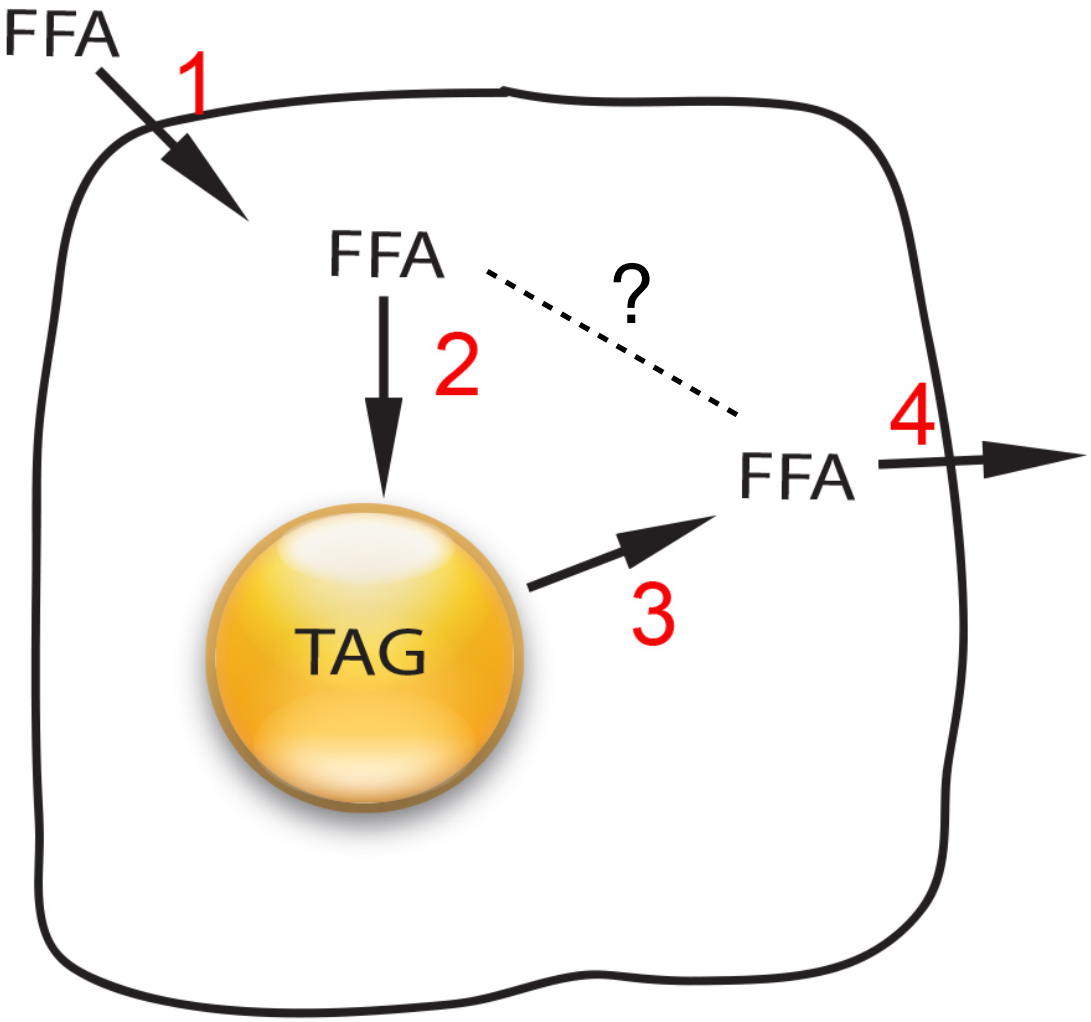

Figure 1. Illustration of intracellular free fatty acid translocation. 1) FFAs cross the plasma membrane of cells, 2) FFAs are incorporated into lipid droplets (LDs), 3) are mobilized from LDs, and 4) FFAs are exported out of cells. Dotted line represents the possibility that FFAs are exported out of the cell without being incorporated into LDs.

Lipid translocation occurs between tissues, through the circulatory system. Mammals have a closed circulatory system, in which the blood is constrained within blood vessels. As opposed to mammals, Drosophila have an open circulatory system, in which the blood (hemolymph) flows freely within tissues (Reiber and McGaw, 2009). Lipids are transported between tissues as lipoprotein particles (Fig. 2). In mammals, lipids are translocated between several tissues, three of which are: the small intestine, the liver and the adipose tissue. In Drosophila, two tissues between which lipid translocation occurs are the midgut and the fat body. The main mammalian tissue where lipids are packaged and stored as lipid droplets is the adipose tissue (Martin *et al.*, 2006), while in Drosophila, it is the fat body (Butterworth *et al.*, 1988).

Lipid translocation between tissues occurs in several steps. First, lipids are ingested from food. In mammals, food enters through the mouth, it passes the esophagus, to the stomach, to the small and large intestine (Thompson and Malagelada, 1981). In Drosophila, food enters the mouth hooks, and it passes through district regions of the gut (a long enclosed tube), from the foregut, to the midgut and the hindgut (Buchon *et al.*, 2013). Second, lipids are absorbed by intestinal cells. In mammals, this occurs in the small intestine and in Drosophila, in the midgut (Fig. 2). Third, lipids are packed into either lipoprotein particles or lipid droplet. In mammals, lipids are packaged into lipoprotein particles in the small intestine. In Drosophila, lipids are packaged into lipid droplets in the midgut. Fourth, lipids are transported to other tissues, such as the fat body or muscle tissue of Drosophila or the liver, adipose tissue and muscle in mammals (Fig. 2).

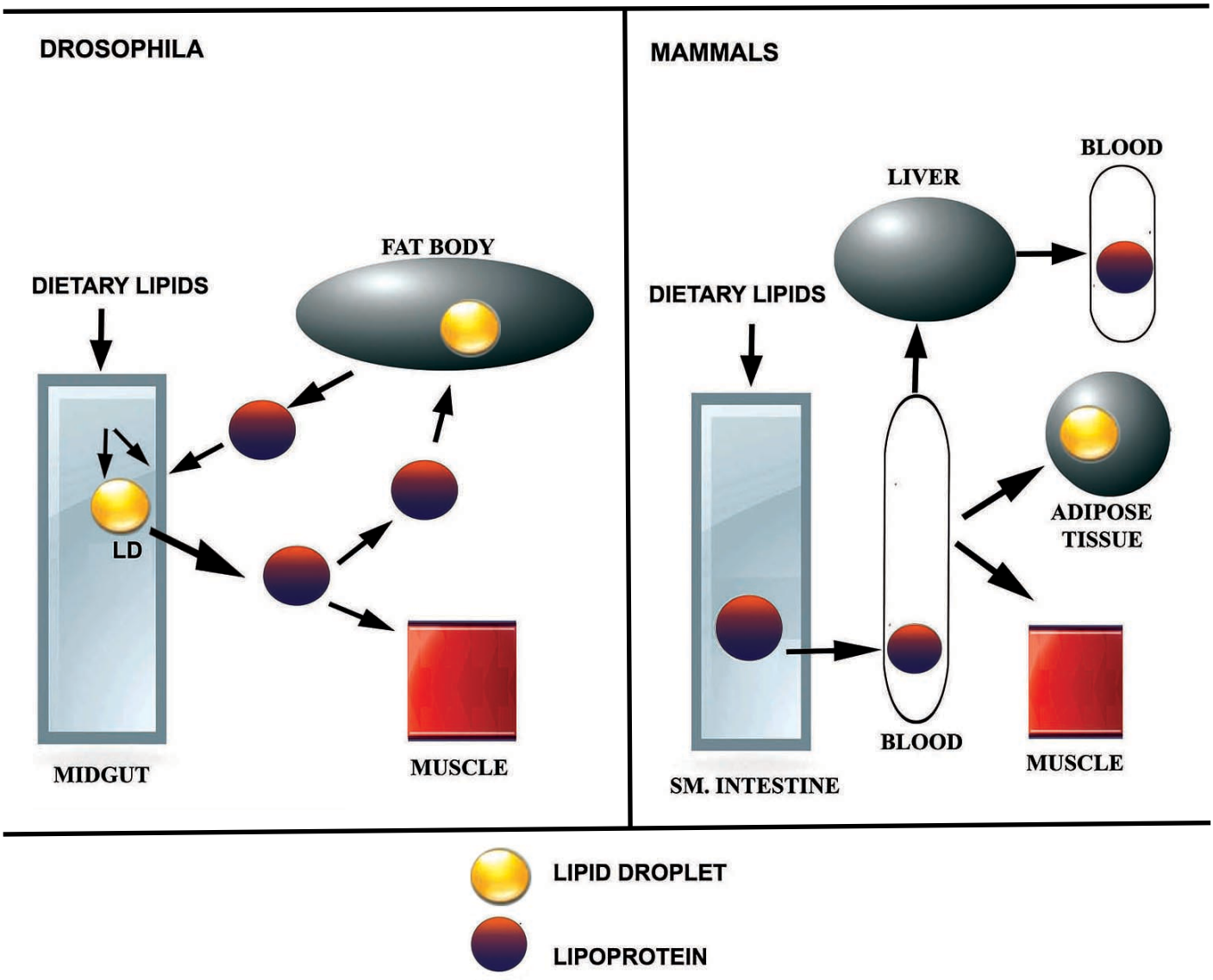

Figure 2. Lipid translocation between tissues: Drosophila vs. mammals. Dietary lipids are absorbed by the Drosophila midgut epithelium or the small intestine enterocytes in mammals. Lipoprotein particles are synthesized in the Drosophila fat body and in the mammalian small intestine and liver. Lipoproteins transport lipids through the circulatory system: in Drosophila, through an open circulatory system (hemolymph), and in mammals through blood vessels. In Drosophila, dietary lipids are either packaged into lipid droplets (LDs) in the midgut or are directly transferred onto lipoprotein particles for transport to other tissues. In contrast, in mammals, dietary lipids are packaged into lipoprotein particles in the small intestine.

I.2 The Drosophila larval fat body

The Drosophila larval fat body is a single-cell thick tissue composed of fat cells cells that function analogously to the mammalian liver, immune system and white adipose tissue, by having the necessary machinery for synthesizing and storing lipids, glycogen and proteins (Arrese *et al.*, 2010). First, similar to the mammalian white adipose tissue, the fat body stores dietary fats as energy reservoirs in the form of lipid droplets (LDs). The energy reservoirs are used to fuel the animal through metamorphosis and early adult periods (Butterworth *et al.*, 1988; Aguila *et al.*, 2007). Second, similar to the mammalian immune system, the fat body is the source of antimicrobial peptides, endocrine mediators of neuronal function and other enzymes that catalyze detoxification reactions (Lemaitre and Hoffmann, 2007; Kuhnlein, 2011). Third, similar to the mammalian liver, the fat body produces yolk proteins (analogous to mammalian liver lipases), larval serum proteins, metabolic enzymes (Sondergaard, 1993) and lipoproteins (Palm *et al.*, 2012).

During metamorphosis, the fat body undergoes an unusual transformation: it dissociates from an organized tissue in the larva to a loose association of individual fat cells in the pupa (Butterworth, 1972; Nelliot *et al.*, 2006). Through this process, the shape of fat body cells remains the same, but their size and energy reservoirs increase dramatically (Butterworth, 1972). Because young adults remain inactive for several hours until their wings expand and cuticle tans, sufficient nutrients must be stored in the fat body. Hence, the tissue is critically important throughout development, carrying out the uptake and storage of dietary fat.

I.3 The Drosophila larval midgut

The Drosophila gut shares similarities in anatomy and physiological function with the mammalian intestine (Rubin, 2007; Apidianakis and Rahme, 2011; Buchon *et al.*, 2013). The fruit fly is regarded as a good model system for several branches of research related to human physiology, such as intestinal infection and pathology (Apidianakis and Rahme, 2011; Buchon *et al.*, 2013), epithelial stem cell research (Casali and Batlle, 2009) and peristalsis (LaJeunesse *et al.*, 2010). Developmentally, both Drosophila and mammalian intestines are of endothelial origin and comprise an epithelial monolayer of cells called enterocytes (Tepass and Hartenstein, 1994).

Depending on position cues, the enterocytes exhibit various morphologies and functions such that the midgut is organized into several specialized regions (Filshie *et al.*, 1971). Ultrastructural analyses have revealed three regions of the midgut, composed of highly specialized cells: anterior, middle and posterior (Filshie *et al.*, 1971; Dimitriadis and Kastritsis, 1984). The anterior midgut is delimited by the proventriculus and the copper cell region. The middle midgut, termed the fly “stomach”, is located downstream of acid-producing copper cells that have a striking architecture of a deeply invaginated apical membrane covered with long microvilli and a basally located nucleus (Dubreuil, 2004; Dubreuil *et al.*, 1998; Dubreuil *et al.*, 2001; Yao & Forte, 2003). The cells of the posterior midgut have an extensively dilated basal extracellular labyrinth and a larger volume than that of anterior midgut cells. They have increased alkalinity and seem to absorb more fluid (Tripathi and Boulpaep, 1989) and lipids (Marianes and Spradling, 2013) than other regions.

Regionalization in gene expression and morphology suggest distinctive functions along the anterior-posterior axis of the digestive tract. First, the expression of the digestive enzymes *magro* (Sieber and Thummel, 2012) and *α-amylase* (Thompson *et al*., 1992) is restricted to specific regions of the midgut. Second, the midgut endocrine cells also acquire spatial differences in gene expression (Ohlstein and Spralding, 2006; Veenstra *et al*., 2008). Third, it has been proposed that the tissue displays a striking and extensive regionalization in which the anterior midgut mostly functions in macromolecule breakdown and the posterior midgut is mostly responsible for nutrient absorption.

I.4 Lipid droplets

I.4.1 What are lipid droplets?

For many years after their discovery in the nineteenth century, LDs have been perceived as having little functional relevance and have been referred to as: fat bodies, oil droplets, lipid bodies, spherosomes, liposomes, adiposomes or lifeless bodies suspended in the cytoplasm (Walther and Farese, 2012). Recently, however, LDs have emerged as complex, metabolically active, dynamic organelles that have diverse functions (Fujimoto *et al*., 2008; Walther *et al*., 2010). They act as key reservoirs for membrane integrity, sources of energy, substrates for the synthesis of several hormones and as safe shelters for toxic free fatty acids. Because almost all cells have the ability to synthesize LDs, they are ubiquitous organelles that exist in most organisms, ranging from bacteria to yeast, plants, and higher mammals.

The structure of LDs (illustrated in Fig. 3) is different from that of other organelles. While most organelles have an aqueous interior that is separated from the rest of the cytoplasm by a membrane bilayer, lipid droplets have a neutral lipid core surrounded by a monolayer of amphiphatic lipids and proteins (Farese and Walther, 2009). Lipid droplets are composed mostly of triacylglycerol (TAG) and of cholesterol ester (CE) (Athenstaedt and Daum, 2006). The lipid droplet surface contains polar lipids and several types of proteins, such as enzymes that function in lipid metabolism, proteins related to signaling, cytoskeletal proteins, and chaperones (Kuhnlein, 2012). Some of the most studied proteins that have been found associated with lipid droplets are: Perilipin, Adipose differentiation-related protein, tail-interacting proteins of 47 kDa (TIP47) and caveolins (Farese and Walther, 2009).

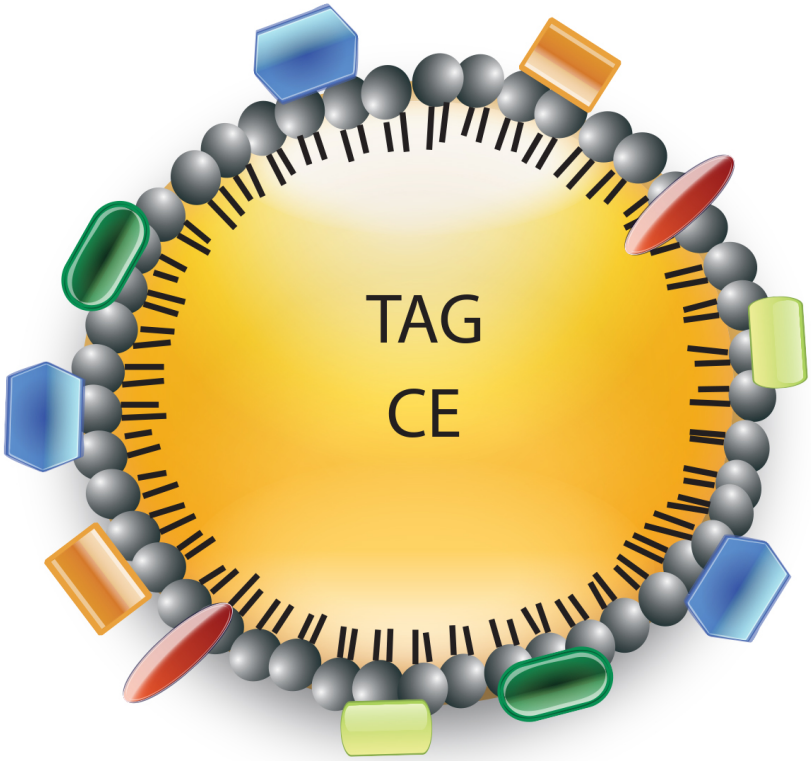

Figure 3. Illustration of a lipid droplet (LD). A phospholipid monolayer (grey) surrounds a hydrophobic lipid core of the LD (yellow). The hydrophobic core consists of triacylglycerol (TAG) and cholesterol esters (CE). A large diversity of proteins is found at the surface of LDs (colored geometrical shapes).

I.4.2 How are lipid droplets formed?

The mechanisms responsible for the synthesis, growth and mobilization of LDs are incompletely understood in both mammals and Drosophila. It is widely believed that LDs are synthesized in the ER, where the machinery responsible for neutral lipid synthesis is found (Guo *et al*., 2009). Evidence that they are formed in the ER comes from distinct sources. First, the LD surface is composed of a monolayer of phospholipids which is similar in lipid composition to that of the ER lipid bilayer (Ploegh, 2007). Second, several studies have shown that LDs are found tightly associated with the ER (Ohsaki *et al.*, 2008; Robenek *et al.*, 2006; Soni *et al*, 2009). Third, it has been found that several proteins found on the surface of lipid droplets are ER associated proteins too (Dvorak *et al.* 1992; Bozza *et al*. 1997; Robenek *et al*. 2004, 2005).

Four models of lipid droplet formation in the ER have been proposed (Farese and Walther, 2009). The first model, ER budding, suggests that lipid droplets grow from the ER bilayer and become detached from the organelle through a budding mechanism. The second model, bicelle formation, suggests that the lipid esters form lenses which are then entirely excised from the ER (Ploegh, 2007). The third model, vesicular budding, argues that vesicles form first and become filled with neutral lipids (Walther and Farese, 2009). The fourth model, the eggcup model, argues that lipid droplets grow within a concave depression of the ER (Robenek *et al.*, 2006). Which of the four mechanisms is correct remains to be elucidated.

LD synthesis involves the incorporation of FFAs into complex neutral lipids, such as TAGs. The machinery responsible for TAG synthesis is found in the ER (Guo *et al.*, 2009) and includes enzymes responsible for *de novo* TAG synthesis (Kuhnlein, 2012).

De novo TAG synthesis occurs in four steps catalyzed by multiple isoenzymes (Kuhnlein, 2012). In the first step, glycerol-3-phosphate is acylated by glycerol-3-phosphate acyltransferases (GPAT) to lysophosphatidic acid. In the second step, lysophosphatidic acid is acylated by acyl-sn-glycerol-3-phosphate O-acyltransferases (AGPAT) to phosphatidic acid. Third, phosphatidic acid is converted to DAG by the magnesium-dependent PA phosphatases (PAP1 activity). In the final step, DAG is converted to TAG by the enzyme diacylglycerol O-acyltransferases (DGAT; Kuhnlein, 2012).

I.4.3 How do lipid droplets expand?

Once formed, lipid droplets can grow through two proposed mechanisms. First, it has been proposed that LDs fuse through a mechanism similar to that of vesicular fusion (Bostrom *et al.*, 2007). Evidence for this type of event comes from studies which have shown that proteins involved in vesicular fusion processes are found associated with lipid droplets. These proteins are: NSF (N-ethylmaleimide-sensitive-factor), α-SNAP (soluble NSF attachment protein) and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins (Bostrom *et al.*, 2005). Knockdown of SNAREs decreases the rate of LD fusion, leading to smaller, more numerous LDs (Bostrom *et al.*, 2005). Interestingly, LD fusion seems to depend on microtubules and the motor protein Dynein (Bostrom *et al.*, 2005). The exact mechanism of fusion remains unknown.

Second, the organelles can expand by acquiring triglycerides at their core and phospholipids at their surface. As mentioned above, the amphiphatic monolayer of LDs

is associated with specific proteins that have many biochemical functions. Some of these proteins are enzymes involved in the synthesis, maintenance and mobilization of TAGs. Several studies have shown that enzymes, such as the adipose triglyceride lipase (ATGL), methyltransferase-like 7B and several TAG synthesis enzymes, relocalize to growing LDs from the ER after oleate induction (Soni *et al.*, 2009; Zehmer *et al.*, 2008, 2009; Wilfiring *et al.*, 2013). This translocation of enzymes allows TAG synthesis to occur directly at the surface of expanding LDs. It was recently proposed that the mechanism by which GPAT is able to localize from the ER to growing LDs involves membraneous connections between these organelles (Wilfiring *et al.*, 2013). It is not known whether these “ER-LD bridges” are present before oleate induction or if there is a specific machinery responsible for their assembly. This model, unlike the the fusion model, implies the requirement for phospholipid synthesis or transport during the growth of LDs.

I.4.4 Proteins associated with lipid droplets

The formation and maintenance of LDs is regulated by proteins associated with their surface. A wide variety of LD-associated proteins have been discovered and characterized by mass spectrometry-based proteome analyses. Discussed below are several of the known protein classes and their proposed functions.

PAT proteins:

PAT proteins, named after Perilipins, ADRP and Tail-interacting protein 47kDa are the most studied family of LD-associated proteins in mammals and flies. Mammals have five Perilipins (PLIN1-5; Greenberg *et al.*, 1991; Brasaemle *et al.*, 1997) and Drosophila

have two Perilipins, termed lipid storage droplet-1 (LSD1) and lipid storage droplet-2 (LSD2; Lu *et al.*, 2001; Beller *et al.*, 2006; Cermelli *et al.*, 2006). Perilipin 1 and ADRP are believed to be constitutively localized to the LD, rapidly degraded by proteasomes upon detachment and are believed to be targeted from the cytoplasm (Londos *et al.*, 1999; Wolins *et al.*, 2006). It has been suggested that PLIN2 (a.k.a. adipophilin, ADRP) is expressed ubiquitously, whereas PLIN1 expression is limited to adipocytes (Brasaemle *et al.*, 1997). The Drosophila counterparts, LSD1 and LSD2, are expressed during all developmental stages of the fly. Post-embryonic expression is particularly prominent in the lipid storing and processing tissues, the fat body and midgut respectively (Gronke *et al.*, 2003; Teixeira *et al.*, 2003).

It has also been proposed that the fly Perilipins have both opposite and redundant roles in lipid mobilization (Bi *et al.*, 2012). LSD2 seems to protect small lipid droplets from the lipolytic activity of the enzyme Brummer (Bmm), and LSD1 promotes the lipolytic activity of the enzyme Hormone Sensitive Lipase (dHSL) in large lipid droplets. LSD1 mutant flies have defects in lipolysis and develop adult obesity (Bi *et al.*, 2012). In contrast to the pro-lipolytic LSD1, LSD2 seems to protect TAG stores in a dose-dependent manner, as LSD2 mutants are lean and LSD2 over-expressors have increased fat accumulation (Gronke *et al.*, 2003). The function of LSD2 is further discussed in Chapter III.

The mammalian PLIN1 is a key component of LDs in adipocytes and is involved in stabilizing the storage of neutral lipids (Beller *et al.*, 2010). It was proposed that PLIN1 functions through the activity of the lipolytic effects of the adipokinetic hormone pathway (ADH), presumably involving its phosphorylation by protein kinase A (PKA). It has been

suggested that both PLIN1 (Beller *et al.*, 2010) and the Drosophila LSD1 (Bi *et al.*, 2012) are important for stimulated lipolysis, playing a role in stabilizing the lipase HSL (which translocates from the cytosol) to the LD surface. In mammals, it was shown in cell culture studies that the phosphorylation of PLIN1 is required for translocation of HSL (Sztalryd *et al.*, 2003) and that its expression alone is sufficient to reconstitute PKA-activated lipolysis (Tansey *et al.*, 2003).

Other Proteins

Interestingly, Drosophila LD proteome analyses identified a surprisingly large diversity of LD associated proteins, including most of those identified in mammalian studies (Beller *et al.,* 2006; Cermelli *et al.*, 2006). Particularly interesting is the identification of several key enzymes of lipid metabolism (such as fatty acid synthases, acetyl-CoA carboxylases and Bmm lipase) and of diverse ribosomal (small GTPases) and mitochondrial proteins. These data are in agreement with freeze-fracture studies which suggest that LDs interact with other organelles, including the ER, endosomes, mitochondria, and peroxisomes (Walther and Farese, 2012). Several of these proteins could be exchangeably associated with both the LD surface and with other organelles. For example, TAG synthesis enzymes seem to relocalize from the ER to the surface of growing LDs (Wilfiring *et al.*, 2013).

Another LD associated protein is caveolin. Caveolins were discovered as adipocyte scaffolding, integral membrane proteins of cell surface pits termed caveolae (Parton, 1996). They localize both at the PM and at intracellular LD surfaces. Although the precise function is not known, it has been suggested that they play a role in

regulating LD metabolism. For example, it has been proposed that caveolins facilitate lipolysis and fatty acid mobilization from lipid droplets by hormone sensitive lipase (Cohen *et al.*, 2003) and that it regulates lipid droplet expandability (Blouin *et al.*, 2010).

I.4.5 Are there different categories of lipid droplets?

Compelling proteomic analysis evidence suggests that at least in some cells, lipid droplets with different protein compositions coexist (Wolins *et al.*, 2005). It has been proposed by several authors that there are several classes of lipid droplets, differentiated by their size and surface proteins. For example, Wilfiring *et al.*, 2013, proposed that mammalian cells have both small static LDs, that do not have TAG synthesis enzymes at their surface, and larger LDs, that do contain TAG synthesis enzymes. In Drosophila, it has been shown that small LDs (<4um in diameter) have the protein LSD2 at their surface and large ones (>4um) do not (Bi *et al.*, 2012). These lines of evidence are in support of the hypothesis that distinct classes of LDs coexist in a cell.

I.5 Lipoproteins

I.5.1 Lipoproteins vs. lipid droplets

The molecular architecture of lipoproteins and LDs are very similar (Fig. 2 and Fig. 3). Both LDs and lipoprotein particles contain a neutral lipid core encased in a phospholipid monolayer decorated with specific surface proteins. Additionally, both particles are believed to be synthesized in the ER. What accounts for the difference between them is their surface proteins, size, function and their exact location in an organism. In terms of surface proteins, lipoprotein synthesis requires several obligate proteins,

apolipoproteins, and a chaperone, microsomal triglyceride transfer protein, MTP (Hussain *et al.*, 2012). LD synthesis, on the other hand, does not seem to require any of the known LD-associated proteins. In terms of size, LDs range from <1um to ~100um in diameter. Lipoproteins, however, are generally much smaller, ranging from < 20nm to 500nm in diameter (Walther and Farese, 2012). In terms of function, while LDs primarily store lipids, lipoproteins transport both dietary and endogenously synthesized lipids between different organs within the body. Finally, in terms of their exact location in an organism, most cells contain the machinery for LD biogenesis (Athenstaedt and Daum, 2006). On the other hand, only specialized cells (such as hepatocytes and enterocytes in mammals) have the proteins required for lipoprotein assembly (Goldstein and Brown, 2009). In Drosophila, only the specialized cells of the fat body synthesize lipoproteins (Palm *et al.*, 2012).

I.5.2 Lipoproteins: mammals vs. Drosophila

Lipoprotein-mediated lipid transport in mammals involves three major and different TAG-rich lipoproteins: chylomicrons, very low density lipoproteins (VLDL) and high density lipoproteins (HDL). These lipoproteins are converted into smaller lipoprotein remnants during the process of delivery of their lipid cargo. This process involves TAG hydrolysis by lipoprotein lipases. Chylomicrons are produced in the intestine. They transport dietary (exogenous) lipids to the liver, adipose tissue and skeletal muscles, becoming converted into chylomicron remnants (Fig. 5; Kindel *et al.*, 2010). VLDL particles are produced and secreted by the liver and transport endogenous (newly synthesized) lipids from the liver to adipose tissue through the bloodstream (Fig. 5). VLDL particles are

converted into intermediate density lipoproteins (IDL) and low density lipoproteins (LDL; Goldstein and Brown, 2009). LDL (a.k.a. “bad cholesterol”) transports lipid cargo from the liver to other cells of the body (Brown and Goldstein, 1986; Goldstein and Brown, 2009). HDL (a.k.a. “good cholesterol”) is produced in the liver and the small intestine. HDL serves as a lipid-scavenging protein, collecting excess lipids from cells of the body and delivering them to the liver through a process termed “reverse cholesterol transport” (Krieger, 1999). In the conversion process of VLDL to IDL and LDL, and chylomicron to chylomicron remnant, HDL donates lipids to the remnant lipoproteins (Zannis *et al*., 2004).

Drosophila have two lipoproteins: Lipophorin (Lpp) and Lipid transport protein (LTP). Both are synthesized and secreted by the fat body (Palm *et al*., 2012). Lpp is the only lipoprotein responsible for transporting lipids through the Drosophila circulation (Fig. 5; Palm *et al.,* 2012). The lipoprotein transports lipids in the form of mostly DAGs and its apolipoprotein is analogous to those of VLDL and of chylomicrons (Ryan, 1990; Soulages and Wells, 1994). The Lpp particle is secreted into the hemolymph as an underloaded, high density Lpp (HDLpp). HDLpp is carried by the hemolymph to the midgut, where it acquires dietary lipid cargo, becoming low-density Lpp (LDLpp), and it circulates back to the fat body, where it unloads its lipid cargo for storage (Fig. 5; Van der Horst *et al*., 2002). The insect Lpp operates differently from the mammalian lipoproteins because: 1) it functions as a reusable lipid shuttle (Van der Horst *et al*., 2002) and 2) it carries DAGs, as opposed to the mammalian lipoproteins, which carry TAGs (Rodenburg and van der Horst, 2005).

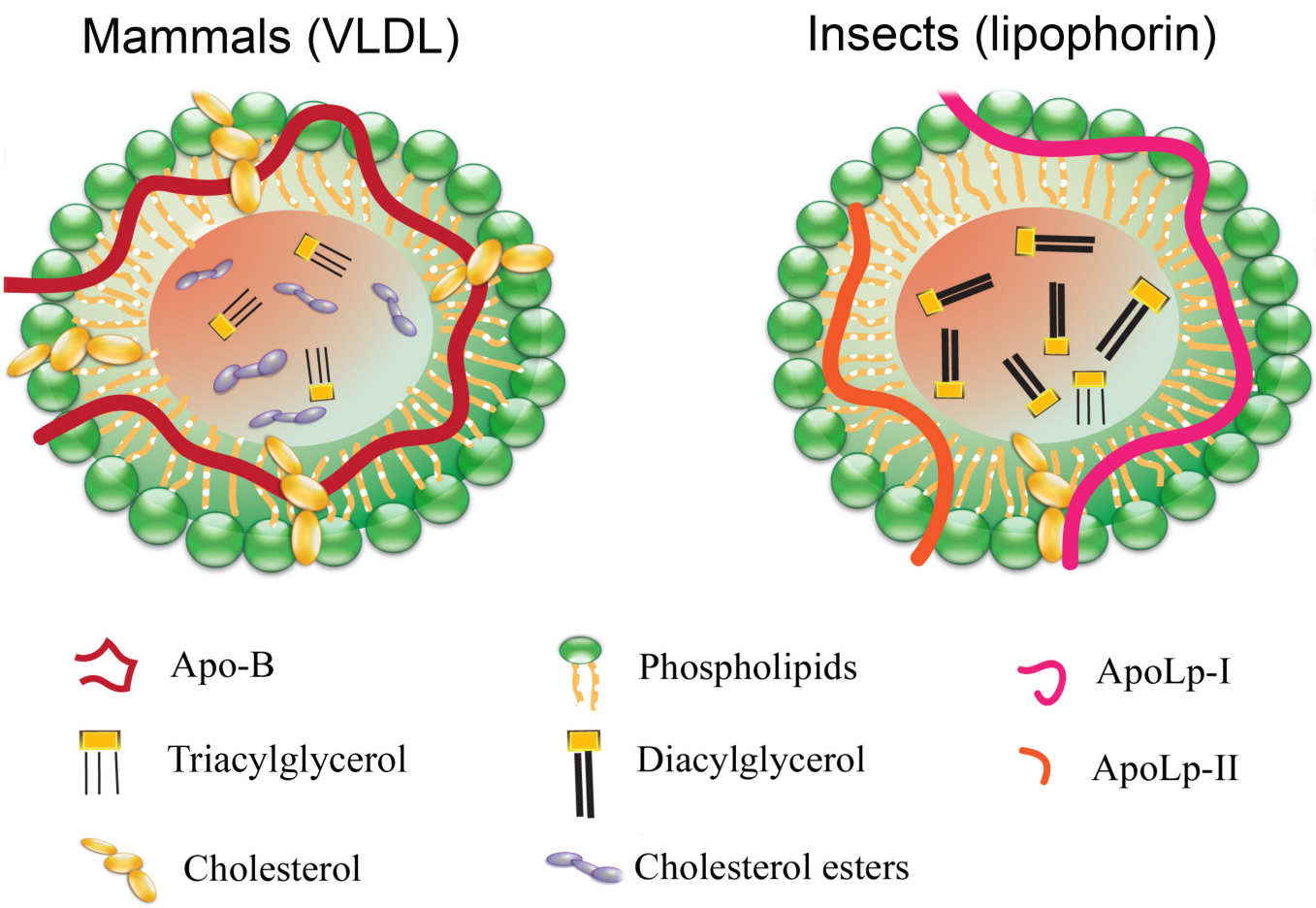

Figure 4. Illustration of lipoproteins in mammals (VLDL) and in insects (lipophorin). The structure of the two lipoproteins is very similar. Both have a neutral lipid core and are surrounded by a monolayer of phospholipids. The difference between the two is that: A) the mammalian VLDL has Apo-B at its surface and the insect Lpp has ApoLp-I and ApoLII; B) VLDL transports lipids in the form of TAGs and insect Lpp transports mostly DAGs throughout the body.

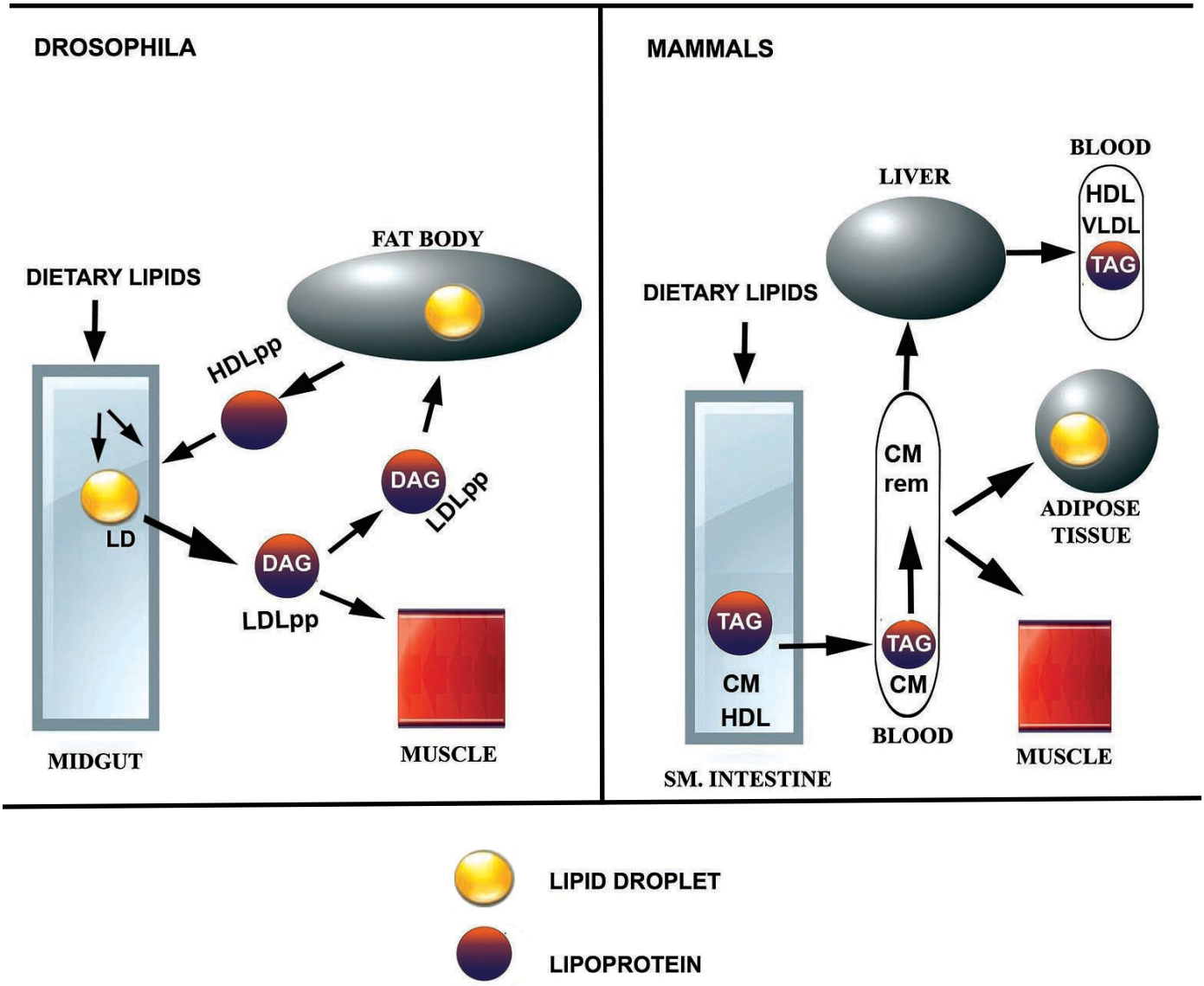

Figure 5. Lipid translocation via lipoproteins: Drosophila vs. mammals. The Drosophila lipophorin (Lpp) is secreted from the fat body as an underloaded high density Lpp (HDLpp). Dietary lipids are either packaged into lipid droplets (LD) in the midgut or are transferred onto lipoprotein particles in the form of mostly diacylglycerol (DAG) for transport to other tissues via low density Lpp (LDLpp). In contrast, in mammals, dietary lipids are packaged into lipoprotein particles in the small intestine in the form of triacylglycerols (TAGs). The lipoproteins are: chylomicrons (CM) and high density lipoprotein (HDL) particles. CM transport dietary (exogenous) lipids to the liver, adipose tissue and muscles, becoming converted into CM remnants in the blood. VLDL particles are secreted by the liver and transport endogenous (newly synthesized) lipids from the liver to adipose tissue through the bloodstream. HDL are also synthesized in the liver.They transport excess lipids from cells of the body to the liver.

I.5.3 Lipoprotein unloading and loading

The mechanism by which lipoproteins deliver lipid to cells is not entirely understood. From a mechanistic viewpoint, lipoprotein unloading can occur through two mechanisms. The two mechanisms are: receptor-mediated endocytosis and de-lipidation of lipoprotein particles extracellularly (independent of endocytosis). In both mammals and Drosophila, both types of lipoprotein unloading have been described.

It is well known that mammalian cells take up LDL by receptor-mediated endocytosis, in which LDL binds to a cell surface receptor and is endocytosed. After LDL is endocytosed, it is degraded in endosomes/lysosomes releasing cholesterol and FFAs in the cell (Brown and Goldstein, 1986; Goldstein and Brown, 2009). On the contrary, it was proposed that HDL delivers lipids to cells by both receptor dependent endocytotic (Rhainds *et al.,* 2004; Eckhardt *et al.*, 2006) and non-endocytotic mechanisms (Pittman *et al.*, 1987; Nieland *et al.*, 2005), involving scavenger receptors (Acton *et al.*, 1996; Trigatti *et al.*, 2000). The non-endocytotic mechanism would mediate lipid uptake without internalization of the HDL lipoprotein particle. In this type of mechanism, the mammalian HDL would release its lipid content after the hydrolytic activity of a lipase, and FFAs would be transported across the PM of cells.

Evidence regarding the mechanism of Lpp unloading at the fat body of Drosophila supports both mechanisms. First, similar to the vertebrate LDL, there is evidence that Lpp can be endocytosed by fat body cells (Dantuma *et al.*, 1998). If Lpp is endocytosed, the mechanism would be similar to that of LDL endocytosis and Lpp would be de-lipidated inside the cell. In contrast to LDL, which is degraded inside the cell after it is endocytosed, Lpp acting as a reusable shuttle would not be degraded in lysosomes. In

contrast to LDL, Lpp would be recycled back to the surface of the cell, returned to the hemolymph to be reloaded with incoming dietary lipids. Second, there is evidence that Lpp can unload its lipid cargo without endocytosis. The transferring of lipids from Lpp to fat body cells for storage involving the binding of the particle to a receptor (Canavoso *et al.*, 2001). Lipids would be hydrolyzed into FFAs outside the fat cells. The FFAs would then be transported across the PM either through active transporters or through passive diffusion. Which of the two occurs in Drosophila has not yet been determined.

In terms of lipoprotein loading, it has been suggested that lipid loading onto Lpp particles at the midgut epithelium occurs via the lipoprotein Lipid Transfer Particle (LTP; Palm *et al.*, 2012). Interestingly, it was recently proposed that LTP becomes internalized by the midgut epithelium and that it loads lipids in the form of DAG onto Lpp particles. Thus, the mechanism of lipid transfer between LTP and Lpp in Drosophila could be similar to that of lipid transfer between the mammalian HDL and CM and VLDL particles. The exact molecular mechanisms of lipoprotein loading/unloading remain elusive.

I.6 Lipid translocation: mammals vs. Drosophila

I.6.1 Lipid absorption

Two main classes of lipids are transported across the plasma membrane of cells: sterols (cholesterol, ergosterol and phytosterol) and free fatty acids (FFAs). Sterol uptake and transport are critical processes in both Drosophila and mammals, as cholesterol is universal for all eukaryotic plasma membranes and plays an important role in its structure by modulating membrane fluidity and permeability (Mouritsen *et al.*, 2004). As Drosophila are auxotrophic for dietary cholesterol (lacking the ability to synthesize sterols) and because cholesterol is a precursor for insect steroid hormones, such as ecdysone, its cellular uptake is critical (Carvalho *et al.*, 2010). Free fatty acids (FFAs) are an important cellular component, necessary to form precursors for membranes (Carvalho *et al.*, 2010). There are several classes of fatty acids (FA) differentiated by the length of the aliphatic tail: short chain fatty acids, having fewer than six carbons, medium-chain fatty acids (MCFA) having 6 to 12 carbons, long chain fatty acids (LCFAs) that have 13 to 21 carbons and very long chain (VLCFA) that have aliphatic tails longer than 22 carbons (Chan *et al.*, 2010).

Uptake of LCFA is believed to occur by two different mechanisms: diffusion (via “flip-flop”) and protein-mediated, facilitated transport (Ehehalt *et al.*, 2006). Three proteins that have a high affinity for LCFAs have been identified: the plasma membrane-associated fatty acid-binding protein (FABP; Stremmel *et al.*, 1985), the fatty acid transporter FAT/CD36 (Abumrad *et al.*, 1993), and the fatty acid transport protein (FATP) family (Schaffer *et al.*,1994). Although they have been extensively studied, their specific roles and mechanisms of function are not entirely understood. The human

FATP protein family has six isoforms, each of which displays distinct expression patterns (Stahl *et al*., 1999). FATP4 specifically is expressed in regions of the small intestine where fat absorption is known to occur, predominantly in the apical compartment, including the microvilli of the brush border membrane. *In vitro* mammalian studies have shown that FATP4 depletion leads to a decrease in LCFA uptake in enterocytes, suggesting that FATP4 is involved in the uptake of LCFAs from the intestinal lumen into the enterocyte (Stahl *et al*., 1999). Little is known about LCFA transport across the midgut epithelium in Drosophila. The only studied protein is one of the three fly isoforms, dFATP, which was shown to promote photoreceptor survival by regulating Rhodopsin-1 abundance in the retina (Dourlen *et al*., 2012). Interestingly, dFATP is also localized in the larval mid- and hindgut. This suggests that it could also play a role in LCFA uptake in the fly. This potential role of dFATP in nutritional uptake has not yet been addressed.

Previous work has suggested that sterol trafficking mechanisms are highly conserved in insects and vertebrates (Rodenburg and Van der Horst, 2005). It is well established that cholesterol uptake at the brush-border of the mammalian intestine involves membrane transporters, such as Niemann-Pick C1-like 1(NPC1L1), which is known to play an important role in the intestinal absorption and intracellular trafficking of dietary sterols (Wang and Song, 2012). The NPC1L1 transporter was identified as a molecular target for ezetimibe, a pharmacological agent that inhibits intestinal cholesterol absorption by inactivating the NPC1L1 protein (Garcia-Calvo *et al*., 2005). The two Drosophila NPC1 homologs, NPC1a and NPC1b, seem to have non-redundant and non-interchangeable roles in sterol trafficking and may function through

independent mechanisms (Voght *et al.*, 2007). Evidence suggests that while NPC1a is required for sterol transport in several tissues, NPC1b is essential for larval growth and plays a critical role in the early step of sterol absorption at the midgut epithelium (Voght *et al.*, 2007). The specific molecular mechanisms by which dietary cholesterol is absorbed and then transported into the hemolymph for utilization or storage remain poorly understood.

I.6.2 Lipid processing and mobilization

The second and third steps of lipid translocation involve lipid incorporation into lipid droplets (LDs) and their mobilization from LDs by enzymes (lipases for TAGs and hydrolases for sterols). Lipid droplet synthesis is discussed in Chapter I.4.2. Two models of lipolysis have been proposed for mobilization of FFAs: the complete hydrolysis of TAG to FFAs and glycerol (Weintrab and Tietz, 1973; Weintrab and Tietz, 1978; Tsuchida and Wells, 1988) and the formation of FFAs and monoacylglycerol (MAG) (Hoffman and Downer, 1979; Male and Storey, 1981). The enzymes involved in this process are discussed in Chapter I.6.4 from the perspective of lipid homeostasis.

I.6.3 Lipid homeostasis

Lipid homeostasis involves all of the steps of the lipid translocation process that play a role in maintaining the internal steady state of lipid in a cell. The homeostasis of lipid storage and the dynamics of LDs are controlled by the precisely tuned balance of lipolysis and lipogenesis (Greenberg *et al.*, 2011). Lipolytic regulation of LDs mainly occurs on the surface of lipid droplets through the accessibility of various lipases. Two mechanisms of lipolysis have been characterized in Drosophila: basal and stimulated (Gronke *et al.*, 2007). In basal lipolysis, under normal nutrient conditions, the mammalian homolog of the adipose triglyceride lipase (ATGL), Brummer (Bmm) lipase hydrolyzes TAGs/DAGs into FFAs. Under nutrient poor conditions, in stimulated (a.k.a hormone-stimulated) lipolysis, more lipases are recruited to LDs to release more FFAs (Wather and Farese, 2009). The LD-associated protein, Perilipin (PLIN1), becomes phosphorylated by PKA in response to the adipokinetic hormone (AKH/ADHR) pathway, which leads to the translocation of the hormone-sensitive lipase (HSL in mammals and dHSL in flies) from the cytosol to the LD surface. This assures the much needed rapid lipid mobilization under starvation conditions. It was also proposed that simultaneous loss of Bmm and AKHR causes extreme “obesity” and leads to defects in fly fat mobilization (Gronke *et al.*, 2007). This suggests that lipid mobilization is coordinated by two lipolytic systems, which are essential to keep the delicate balance of lipid homeostasis, and that Bmm lipase plays a role in both basal and stimulated lipolysis.

In terms of cholesterol homeostasis in mammals, a balance between uptake, absorption and *de novo* synthesis is sustained in enterocytes. This balance is regulated in part by the export of intestinal chylomicrons (Abumrad and Davidson, 2012). In

insects, sterol uptake is an essential requirement for proper development, since *de novo* synthesis does not occur. Evidence has suggested that the Drosophila nuclear receptor DHR96 is necessary to maintain the balance of lipid uptake and export, hence to regulate lipid homeostasis. It was shown that it is essential for TAG homeostasis in the midgut, where it is required for the breakdown of dietary fat (Sieber and Thummel, 2009). DHR96 mutants were sensitive to starvation and displayed decreased levels of TAG (Sieber and Thummel, 2009). Interestingly, it was further shown that DHR96 is not only required for maintaining TAG metabolism, but it is also required to maintain cholesterol homeostasis (Sieber and Thummel, 2012). The nuclear receptor was proposed to function through the dual enzymatic functions of the lipase magro, which is most similar to mammalian gastric lipase and LipA (which has both TAG lipase and cholesterol esterase activities). The authors proposed that the dual enzymatic function could arise from distinct regions of the Drosophila midgut, showing that while disruption of magro function specifically in the proventriculus blocked its TAG lipolytic activity without affecting cholesterol levels, disruption of its activity in the intestine affected both TAG and cholesterol homeostasis (Sieber and Thummel, 2012). These data provide interesting insights into lipid homeostasis, suggesting that region-specific enzymatic functions could correspond to lipid metabolic functions activities of nuclear receptors, such as DHR96. Moreover, the findings suggest a link between cholesterol levels, DHR96, and TAG homeostasis (Sieber and Thummel, 2012).

I.7 Spectrin and its function in vertebrates

Spectrin and its binding partner, Ankyrin, are ubiquitous components of the cytoskeleton found at specialized regions underneath the plasma membrane of animal cells. In humans, there are two α spectrin isoforms (Sahr *et al.*, 1990), four β spectrin isoforms and a larger isoform, known as β_V spectrin (β_H in Drosophila; Berghs *et al.*, 2000). The α and β spectrin isoforms function as tetramers underneath the plasma membrane and are composed mainly of many 106 amino acid, alpha helical, triple-helical repeats called Spectrin repeats (Bennett and Gilligan 1993). The individual Spectrin subunits form antiparallel αβ heterodimers, which then form a tetramer. α spectrin contains 20 repeats, an SH3 domain and a C-terminal EF-hand calcium-binding motif (Bennett and Baines, 2001). β spectrin has 17 spectrin repeats, an N-terminal actin-binding domain, and a C-terminal PH domain (Bennett and Baines, 2001). The actin binding domains of Spectrin are composed of two calponin homology domains (Banuelos *et al.*, 1998) and are located at the ends of the $(\alpha\text{-}\beta)_2$ tetramer (Fig. 6). Their positioning allows for Spectrin to form a hexagonal network (Byers *et al.*, 1985) by cross-linking actin filaments (Gardner and Bennett, 1987).

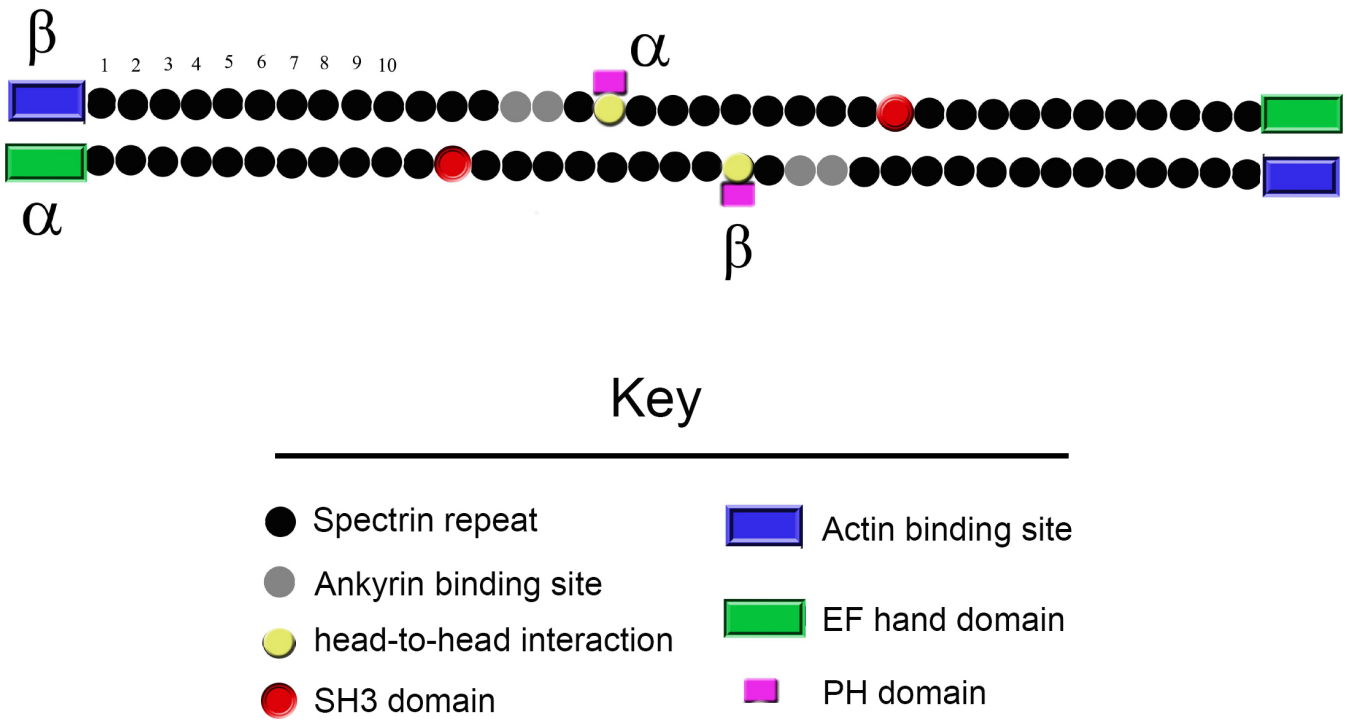

Figure 6. The spectrin $(\alpha\text{-}\beta)_2$ tetramer. The tetramer is composed largely of Spectrin repeats (black spheres). β spectrin has 17 repeats and: an N-terminal Actin binding domain (blue rectangle), an Ankyrin binding site (gray spheres) on repeats 14 & 15, and a PH domain (pink rectangle). α spectrin has 20 repeats and: a Src-Homology (SH3) domain (red sphere), and an EF-hand motif at its C-terminus (green rectangle). The yellow repeat represents the head-to-head interaction of α and β spectrin where tetramer formation occurs.

Three broad classes of Spectrin function have been proposed. First, Spectrin is thought to play an essential role in membrane structure by forming a cytoskeletal network underneath the plasma membrane. This role was proposed initially in red blood cells, where Spectrin forms the meshwork that provides their shape and stability. Evidence for this function is that point mutations in Spectrin cause hereditary defects of the erythrocytes (Dalaunay *et al.*, 1995). This role of Spectrin has been additionally proposed in neurons of both vertebrates and invertebrates. It was shown in *Caenorhabditis elegans* that β spectrin, encoded by the *unc-70* gene (Hammarlund *et al.*, 2000), is required for the physical integrity of neuronal processes and for neuronal mechanical strain resistance. Although neurons develop normally in β spectrin mutants, there are spontaneous breaks in neuronal processes (Hammarlund *et al.*, 2007). Additionally, it has also been shown in mice that βIV spectrin knockdown leads to structural defects at the nodes of Ranvier, where Spectrin specifically interacts with Voltage Gated Sodium Channels through binding to Ankyrin G (Lacas-Gervais *et al.,* 2004).

Second, in a long-standing hypothesis, Spectrin is thought to play a role in the polarity of assembly of interacting proteins, by capturing and stabilizing membrane proteins through Ankyrin (Drubin and Nelson, 1996; Dubreuil, 1996). Ankyrin links Spectrin to the anion exchanger in erythrocytes and interacts with various membrane proteins such as ion channels, calcium-release channels and cell adhesion molecules in non-erythrocytes (Bennett and Chen, 2001). Evidence for this type of mechanism comes from studies which found defects in membrane protein assembly at the plasma membrane. For example, Ankyrin B mutant mice had reduced accumulation of L1 family

cell adhesion molecules in pre-myelinated axons of long fibers (Scotland *et al.*, 1998). Additionally, knockdown of Ankyrin-G or β2 spectrin by RNAi in cultured bronchial epithelial cells resulted in loss of the lateral domain of the plasma membrane (Kizhatil and Bennett, 2004).

Third, relatively recent genetic studies in several model systems speculate that Spectrin plays an unconventional role in intracellular protein trafficking. Several studies have suggested that Spectrin and Ankyrin mutations interfere with the proper delivery of proteins to their normal sites of function. For example, it was shown in MDCK cells that the Na^+/K^+ ATPase, which is usually found on the plasma membrane, becomes blocked between the ER and Golgi in Ankyrin-R knockdown experiments (Stabach *et al.*, 2008). Additionally, it has been proposed that, in cultured epithelial cells, Ankyrin G is required for the exit of E-cadherin from the Golgi network and for its maintenance at the lateral membrane (Kizhatil *et al.*, 2007). Ankyrin G was also required for the transport of the cyclic nucleotide gated channels to the plasma membrane of rod outer segment in mouse retina experiments (Kizhatil *et al.*, 2009).

I.8 The role of α and β spectrin in Drosophila

In Drosophila, a single α spectrin isoform combines with either of two structurally distinct β spectrin isoforms, β and β Heavy (β_H) spectrin to produce $(\alpha\text{-}\beta)_2$ and $(\alpha\text{-}\beta_H)_2$ tetramers. Spectrin can be attached to the plasma membrane indirectly through Ankyrin (Dubreuil *et al.*, 1996) or independently of Ankyrin (Das *et al.,* 2006; 2008). Because the *spectrin* and *ankyrin* genes are conserved between vertebrates and invertebrates, it seems very likely that their functions are also conserved. The Drosophila α and β spectrin genes are required at the end of embryogenesis or beginning of larval stage (Dubreuil *et al.*, 2000). β spectrin mutant animals die before larval hatching and α spectrin mutants die just after hatching (Dubreuil *et al.*, 2000; Lee *et al.*, 1993).

It has also been shown that Spectrin is important for Drosophila development. Presynaptic Spectrin is required to maintain synapse stability at the neuromuscular junction (Pielage *et al.*, 2005). Loss of presynaptic Spectrin results in the elimination of synaptic cell-adhesion molecules in Spectrin knockdown mutants (Pielage *et al.*, 2005). Additionally, transgenic experiments in Drosophila indicated that Spectrin mutations known to cause the neurodegenerative disease Spinocerebellar ataxia type 5 (SCA5) may induce neurodegeneration and impair axonal transport in the fly (Lorenzo *et al.*, 2010).

Although Spectrin is essential in Drosophila, β spectrin knockdown in most tissues has no detectable effect on growth or viability (Mazock *et al.*, 2010). Knocking down β spectrin in the muscle, salivary gland, gastric cecae, midgut, brain, or prothoracic gland by dsRNA expression fails to produce a lethal phenotype despite a notable reduction in the abundance of β spectrin. On the other hand, β spectrin over-expression in most

tissues has dramatic effects on development or viability. Over-expressing β spectrin in various tissues led to larval lethal phenotypes whereas over-expressing α spectrin had no detectable effect (Mazock *et al.*, 2010). The effect of over-expressing β spectrin is dose-dependent. While over-expressing β spectrin using the UAS β95 transgene, at 25°C is lethal, over-expressing it at a lower temperature, 22°C, had no effect on viability. Interestingly, it has been shown that β spectrin plays an essential function in the Drosophila nervous system, where specific expression of the UAS β spectrin transgene, was sufficient to overcome the lethality of loss of function β spectrin mutation (Mazock *et al.*, 2010).

I.9 Spectrin and disease

Defects in both *spectrin* and *ankyrin* genes are responsible for neurodegenerative and hemolytic disorders. Spinocerebellar ataxia type 5 (SCA5) is a neurodegenerative disorder characterized by uncoordinated gait, limb and eye movements, slurred speech and swallowing difficulties (Ranum *et al.*, 1994). Hemolytic anemia, characterized by a reduction of erythrocytes in the blood, occurs as a result of defects in red blood cell membrane stability, such as hereditary elliptocytosis (HE), hereditary spherocytosis (HS) and hereditary poikiloerythrocytosis (HPP). HE is characterized by red blood cells that are elliptically shaped and HS by cells that are spherically shaped, as opposed to their normal concave-shape (Tse *et al.*,1990). HPP is a severe form of congenital hemolytic anemia and a subtype of homozygous hereditary elliptocytosis (Maillet *et al.*, 1996). It is caused by a molecular defect in Spectrin and it displays severe hemolytic anemia with abnormal heat instability of red blood cells (Agre *et al.*, 1982).

Mutations can cause disease through toxic gain-of-function effects or loss-of-function effects, dominant-negative effects on wild-type protein. Several diseases in humans have been proposed to arise as a result of haploinsufficiency, many of which have been associated with dominant-negative effects on both Spectrins and Ankyrins. For example, dominant alleles of human βIII spectrin caused SCA5 in an American kindred descended from President Lincoln's grandparents (Ikeda *et al.*, 2006). Because knockout mice that lack βIII spectrin have phenotypes that resemble symptoms of SCA5 patients, it has been proposed that these alleles function via a dominant negative effect on Spectrin function (Perkins *et al.*, 2010). As this speculation is based on overlap between SCA5 phenotypes and βIII spectrin knockouts, it is formally possible that the allele operates via a gain of function effect, and not via a dominant negative one. This possibility has not been addressed.

Using *Drosophila melanogaster* as a model system, I obtained novel insights into the function and genetics of Spectrin. Following up on data obtained by a former graduate student in our laboratory, Harper Mazock, Ph.D, I compare the effects of αβ Spectrin subunits knockdown with those of αβ over-expression and show that Spectrin exerts unexpected effects on lipid translocation pathways in the larval fat body.

CHAPTER II. Genetic studies of Spectrin in the larval fat body of *Drosophila melanogaster*: Evidence for a novel lipid uptake apparatus

II.1 Abstract

Spectrin defects generate phenotypes that affect the plasma membrane, cell polarity and the secretory pathway. Using the larval fat body as a genetic model system to further decode its function, I found that Spectrin exerts unexpected multiple effects on lipid translocation pathways. Specifically: 1) Loss of α spectrin in the fat body had a similar effect as loss of β spectrin (it eliminated the same population of small cortical lipid droplets and altered plasma membrane architecture, without affecting the viability of the organism); 2) Strong over-expression of β spectrin caused fat body atrophy and larval lethality; 3) Over-expression of β spectrin perturbed transport of dietary fat from the midgut to the fat body (due to blocking the secretion of the lipid carrier lipophorin (Lpp) from fat cells); 4) The toxic effects of β spectrin over-expression were ameliorated by co-expression of α spectrin. Because the effects of β spectrin knockdown are distinct from over-expression, I conclude that β spectrin exerts hypermorphic effects in the larval fat body and that it is not normally required for Lpp secretion. Additionally, because α knockdown had a similar effect as β spectrin knockdown, I suggest that the phenotypes observed with loss of function are due to loss of αβ spectrin functioning as a tetramer. Moreover, because co-expression of α with β led to rescue of the toxic effects caused by β over-expression, I propose that α regulates the function of β spectrin. Additionally, I suggest a novel model of αβ spectrin function where the tetramer connects lipid uptake at the plasma membrane to lipid droplet growth in the

cytoplasm. The data I present in this chapter bring novel insights into the mechanisms of both Spectrin function and of dietary lipid metabolism.

II.2 A discrete population of cortical lipid droplets in the larval fat body

II.2.1 The cortical lipid droplets are dependent on the αβ spectrin tetramer

It was previously shown that β spectrin knockdown in most Drosophila tissues has no detectable effects (Mazock *et al.*, 2010). Interestingly, β spectrin knockdown in the fat body results in subtle effects on plasma membrane (PM) morphology, without producing any effect on the growth or viability of the organism (Mazock Ph.D. thesis, 2010). Expression of UAS-mCD8-GFP (Lee and Luo, 1999) as a PM marker in the larval fat body (under control of Cg-Gal4; Asha *et al.,* 2003) produced a peculiar foamy pattern of fluorescence at the “ecto” surface of the tissue, the region of the tissue facing the hemolymph. This foamy pattern disappeared upon expression of dsRNA against β spectrin (Mazock Ph.D. thesis, 2010). The basis for the foamy pattern of ecto surface staining became clear after electron microscopy performed by Dr. Anthony Mahowald at the University of Chicago. The EM analysis showed that a dense population of small LDs is found at the cortex of the fat cells and that the LDs are surrounded by PM. Higher magnification views revealed a complex surface topology in which the PM seems to be closely associated with the cortical lipid droplets, where a very thin rim of cytoplasm between the LD surface and the surrounding PM was present. The EM pattern showed the foamy appearance of plasma membrane markers by fluorescence microscopy was due to negative staining of LDs within the zone of plasma membrane staining (Mazock Ph.D. thesis, 2010).

Since Spectrin functions as a tetramer of both α and β subunits, I hypothesized that the plasma membrane-associated LDs rely on αβ spectrin tetramer function, and not on β spectrin function alone. To test my hypothesis, I knocked down α spectrin in the fat body and analyzed the morphology of the plasma membrane with CD8-GFP at the ecto domain of the tissue. Interestingly, α spectrin knockdown had the same effect as β knockdown: it dramatically perturbed the plasma membrane architecture at the ecto surface and also eliminated the small cortical LDs. Shown in Fig. 7 are images of fat body tissues from 3rd instar larvae of control and α spectrin knockdown animals that express the PM marker, Cd8-GFP. The PM had a speckled pattern at its ecto surface, when observed with Cd8-GFP (Fig. 7). The animals, however, did not display any apparent defects survival, development or morphology. The analysis of α spectrin knockdown revealed that the cortical LDs can be easily assayed with the use of DIC microscopy. LDs have been thus far visualized by light, fluorescent and electron microscopy. The cortical LDs observed with DIC microscopy in control animals (Fig. 7 D) were absent in α spectrin knockdown animals (Fig. 7 H). The protein knockdown was efficient, as shown by anti α spectrin staining, which was nearly eliminated after RNAi (Fig. 7 C & G).

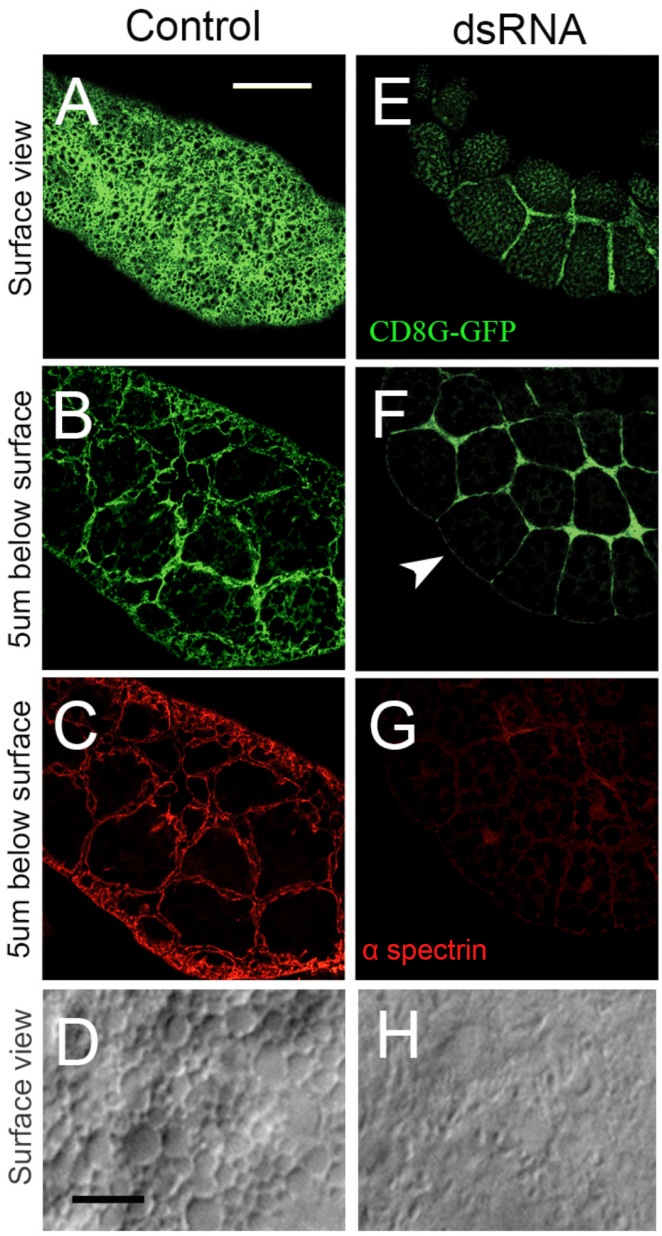

Figure 7. Loss of α spectrin leads to a similar phenotype to that of loss of β spectrin: 1) the plasma membrane marker CD8-GFP (green) produced a foamy pattern at the surface of wild type larval fat body (A). This pattern was lost after α spectrin knockdown and was replaced by a coarse speckled appearance (E). Deeper confocal sections revealed that the PM had a thin appearance at its ecto-domain facing the hemolymph after α spectrin knockdown (F, arrowhead) as compared with the convoluted PM of control (B); 2) the population of cortical LDs was also lost after knockdown. The cortical LDs are visible by DIC microscopy at the surface of fat body tissue of wild-type larvae (D) and are absent in α spectrin knockdown (H). The α spectrin knockdown was efficient as judged by anti-α spectrin staining (C&G). Bars = 50 μm (A), 10 μm (D).

Interestingly, targeted knockdown of α spectrin in most tissues of Drosophila produced effects similar to β spectrin knockdown. No detectable phenotypic effects were noticed after α spectrin was knocked down in the larval midgut, nervous system or the wing disc. The data presented here, showing that the parameters affected by β spectrin knockdown were similarly affected by knockdown of α spectrin indicate that αβ spectrin functioning as a tetramer may be responsible for the observed phenotypes in the larval fat body.

II.2.2 The cortical lipid droplets are transient

The cortical LDs are found underneath the PM at the ecto domain of the fat body. This domain faces the hemolymph, where Lpp lipoprotein particles are found. Lpp brings lipid cargo from the midgut to be stored in the fat body and also carries them from the fat body to other tissues to be used as energy (Palm *et al*., 2012). Therefore, the availability of Lpp particles at the ecto domain of the fat body tissue, where the cortical LDs are found, raises the possibility that the cortical LDs may be transient. To address this, I performed starvation experiments on 3rd instar larvae. I starved wild-type larvae for various amounts of time and analyzed the surface of the fat body with DIC microscopy. Interestingly, after 24 hrs of starvation, the cortical LDs were absent in starved animals (Fig. 8 B). The effect was similar to that of α or β spectrin knockdown in the fat body (Fig. 8 C). This data suggests that the class of cortical LDs are a transient intermediate and depend on the continuous input of dietary fat.

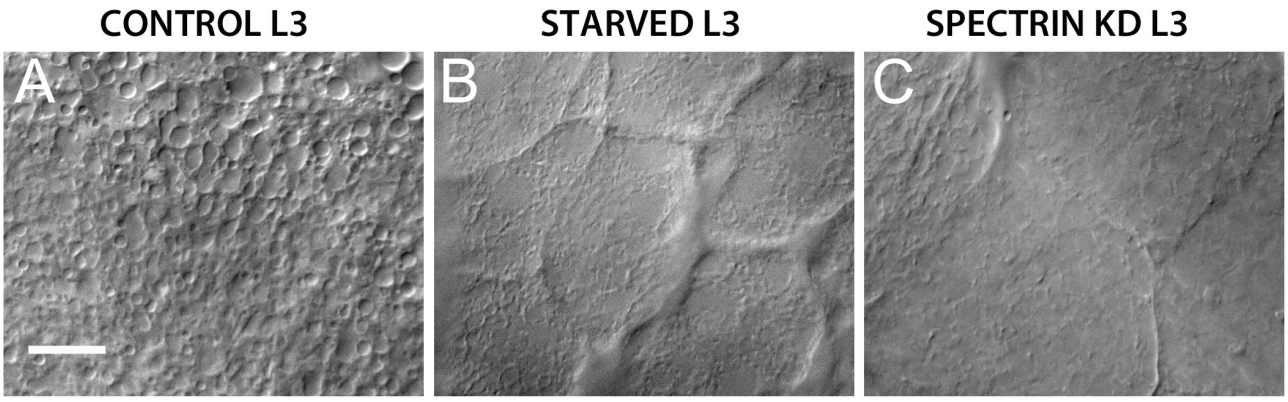

Figure 8. The population of cortical LDs is transient; they were lost from larval fat body during starvation. The population of small LDs seen in the cortical region of fat body from wild type 3rd instar larvae (A) were nearly absent after starvation (B) as found with β spectrin knockdown with dsRNA (C). Bar = 10 μm.

II.2.3 The cortical lipid droplets correspond to LSD2 bearing lipid droplets previously described

Two classes of LDs have been described in previous Drosophila studies. There is a population of small LDs (~1-4 um in diameter), which contain both fly Perilipin proteins, LSD1 and LSD2, on their surface. There is also a population of larger LDs (> 4 um) that express exclusively LSD1 (Bi *et al.*, 2012). To ask if the cortical LDs belong to either category, I expressed UAS-LSD2-GFP in the fat body and then analyzed the tissues of 3rd instar larvae with both DIC and GFP microscopy. Interestingly, UAS-LSD2-GFP specifically labelled the same population of cortical LDs that depends on the Spectrin tetramer (Fig. 9 A-C). Knockdown of LSD2 with RNAi in the fat body led to a significant reduction in the number of cortical LDs observed with DIC microscopy (Fig. 9 E). Because these results suggest that the LDs correspond to the LSD2 positive LDs previously described, I hypothesized that Spectrin knockdown could have an effect on LSD2. To test my hypothesis, I expressed both β spectrin dsRNA and UAS-LSD2-GFP in the fat body and performed anti β spectrin antibody staining on fat body tissues of 3rd instar larvae. The analysis revealed that knockdown of β spectrin eliminated the population of small LDs labeled with LSD2-GFP (Fig. 9 F & G). These data indicate that the class of cortical LDs that is Spectrin-dependent is the same as the class of LDs which contains LSD2.

Additionally, because it has been shown that LSD2 knockout Drosophila adult flies have a deficit in stored TAG and are sensitive to starvation (Gronke *et al.*, 2003), I asked if β spectrin knockdown led to a similar effect in the fat body. I performed starvation experiments on adult flies and found that β spectrin knockdown does not lead

to a similar effect. No starvation sensitivity was observed after Spectrin was knocked-down as compared with controls (Fig. 10). In conclusion, although β spectrin knockdown has no effect on starvation susceptibility, the cortical LDs that depend on the αβ spectrin tetramer function seem to correspond to same class of small LSD2 positive LDs that have previously been characterized.

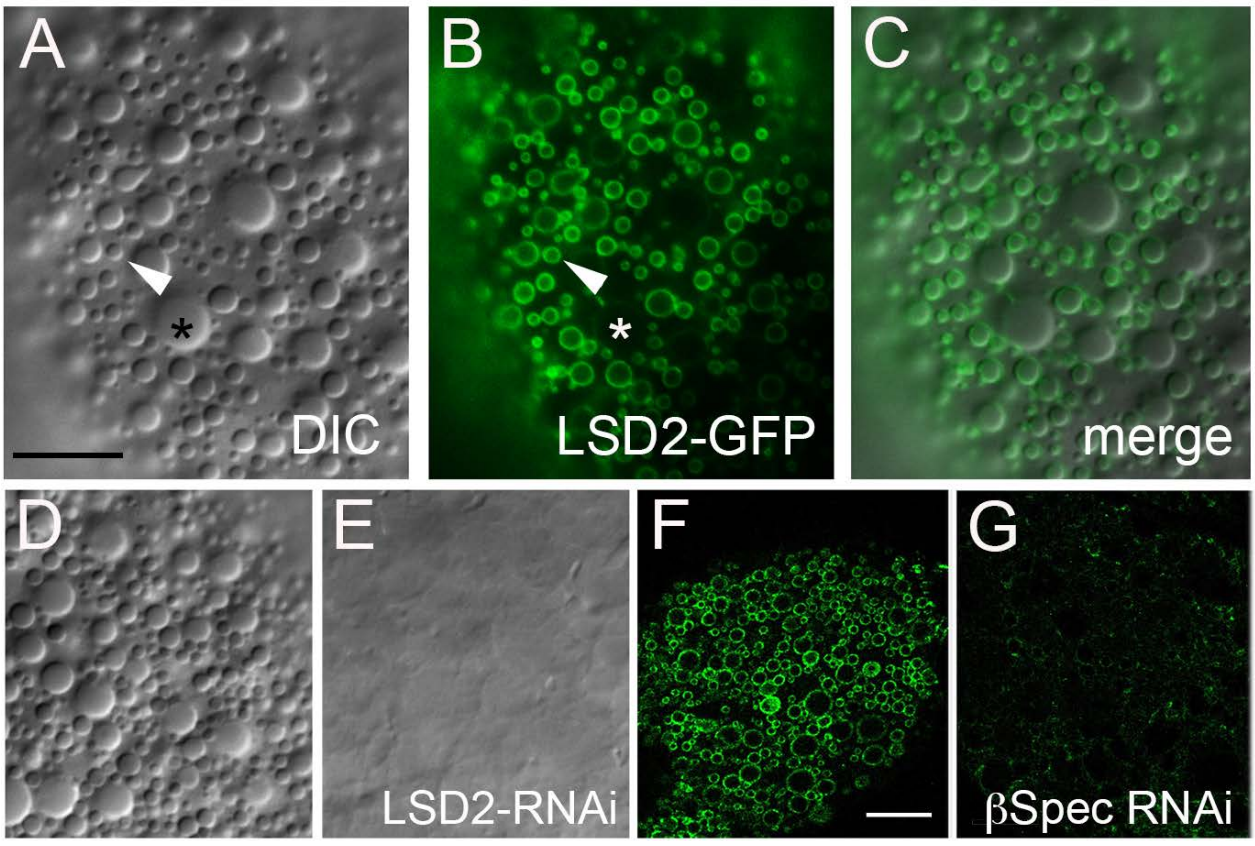

Figure 9. The small cortical lipid droplets are LSD2 positive. Lipid droplets at the ecto domain of fat body cells can be detected by DIC microscopy (A&D). Expression of UAS-LSD-2-GFP in the fat body (via Cg-Gal4) produces a pattern of small lipid droplets labeled on their surface (B) that coincides with the DIC pattern (merge in C). Larger lipid droplets (> 4 um) were not labeled by LSD-2-GFP (B, *). Knockdown of LSD-2 by RNAi resulted in disappearance of the cortical lipid droplets (E). Likewise, knockdown of β spectrin by RNAi eliminated the population of LSD-2-GFP labeled vesicles in the cortex (G). (F) Bars = 10 μm (A-E) or 20 μm (F-G).

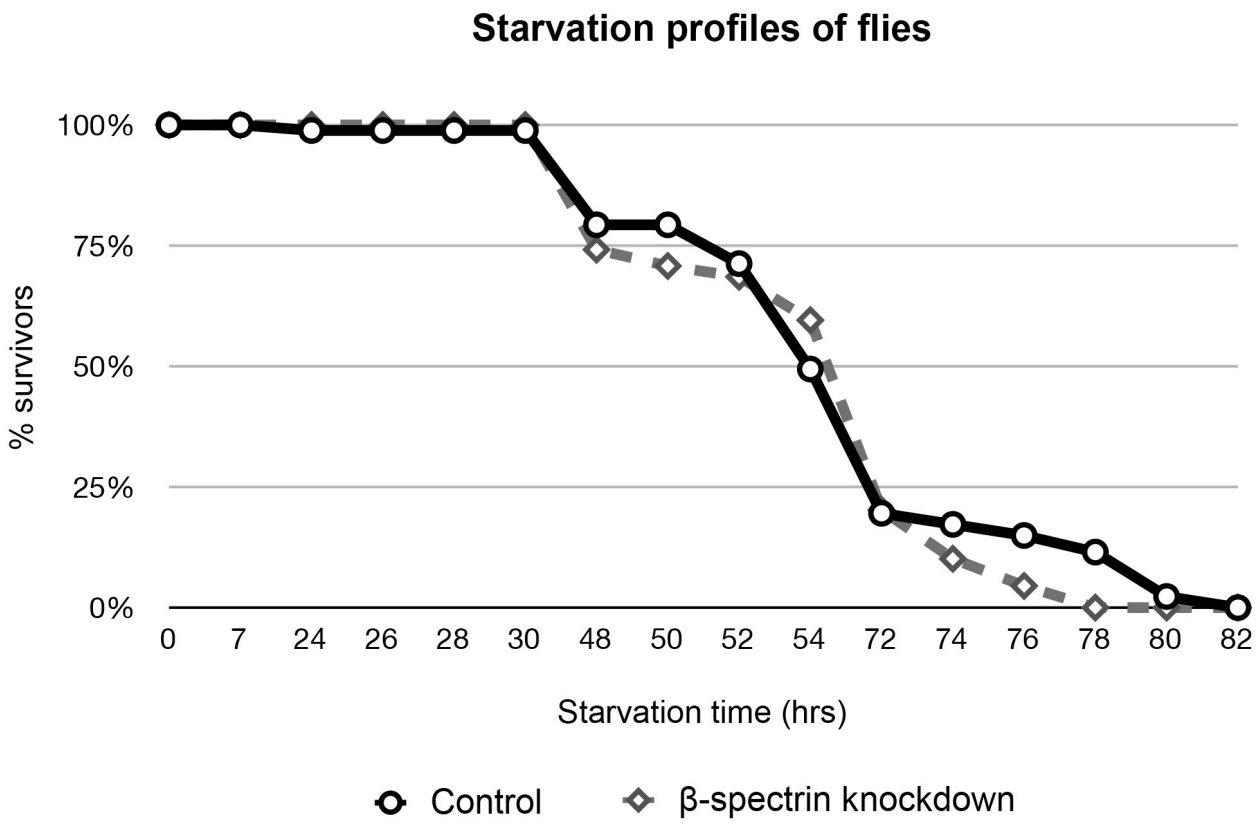

Figure 10. Starvation susceptibility profiles of control and β spectrin knockdown adults. Adult flies less than 24 hrs of age were transferred to agar vials containing water but no nutrients and kept at 25°C. 87 control (parental UAS and Cg-Gal4) and 89 β spectrin knockdown adults were analyzed. Starvation susceptibility was tabulated as percent survivors at indicated times. It was previously shown that increased susceptibility in LSD2 mutants reflects a diminution of TAG stores compared to wild type (Gronke *et al.*, 2003). No such change was observed here, suggesting that TAG stores were not altered. Thus, there appears to be effects of LDS2 beyond cortical LD formation.

II.3 SEM micrographs reveal novel insights into the surface topography of the larval fat body

The surface of the larval fat body of Drosophila is covered by a basement membrane. The basement membrane is a specialized extracellular matrix structure that plays an essential role in maintaining the integrity of organs and tissues (Srivastava *et al.,* 2007). It was previously suggested that the PM of the fat body of 3rd instar larvae forms "finger-like" structures that become invaginated as a result of the mechanical force exerted by the basement membrane (Mazock Ph.D. thesis, 2010). The model of the fat body surface morphology sought to account for the foamy pattern at the ecto domain observed with the PM marker CD8-GFP and for the presence of PM around the cortical LDs observed with TEM.

However, the model could not be validated. Because none of the TEM micrographs previously analyzed showed a plane of view where LDs were found in elongated finger-like structures, I recapitulated the analysis and further examined the fat body tissue of 3rd instar larvae with both TEM and SEM microscopy. After analyzing several specimens of fat tissue with TEM, I concluded that there was no indication that the PM of the fat body would form such elongated structures.

Remarkably, SEM microscopy brought a novel perspective into the morphology of the fat body, showing views of the tissue that resembled the fractal surface of a cauliflower (Fig. 11). Shown in Figure 9 are examples of fat body tissues from 3rd instar wild-type and β spectrin knockdown larvae at 1,000x magnification. Top views of the surface morphology show that the surface of the tissue seems to be covered with small globular structures, which give the fat body a "cauliflower" appearance. These globular

structures are absent after β spectrin knockdown. Interestingly, the structures are the same size as the cortical LDs, ranging from 1-8um in diameter (Fig. 11 A). They may be visible as a result of a difference in the electron intensity resulted from the electron interaction with the sample. SEM therefore provided information about the surface topography and composition. After β spectrin knockdown, few larger (>15um in diameter) globular structures are present at the fat body surface (Fig. 11 B). This suggests that the globular structures observed with SEM microscopy correspond to the cortical Spectrin-dependent LDs.

Additionally, the images shown in panels C and D, corresponding to internal views of the fat body tissue, do not provide evidence for the PM forming elongated structures which become invaginated as a result of the force exerted by the basement membrane. These data suggest that the previous model of PM morphology forming finger-like structures may be incorrect. Moreover, the data indicate and coincide with a subsequent model where the PM forms globular structures that surround the cortical LDs.

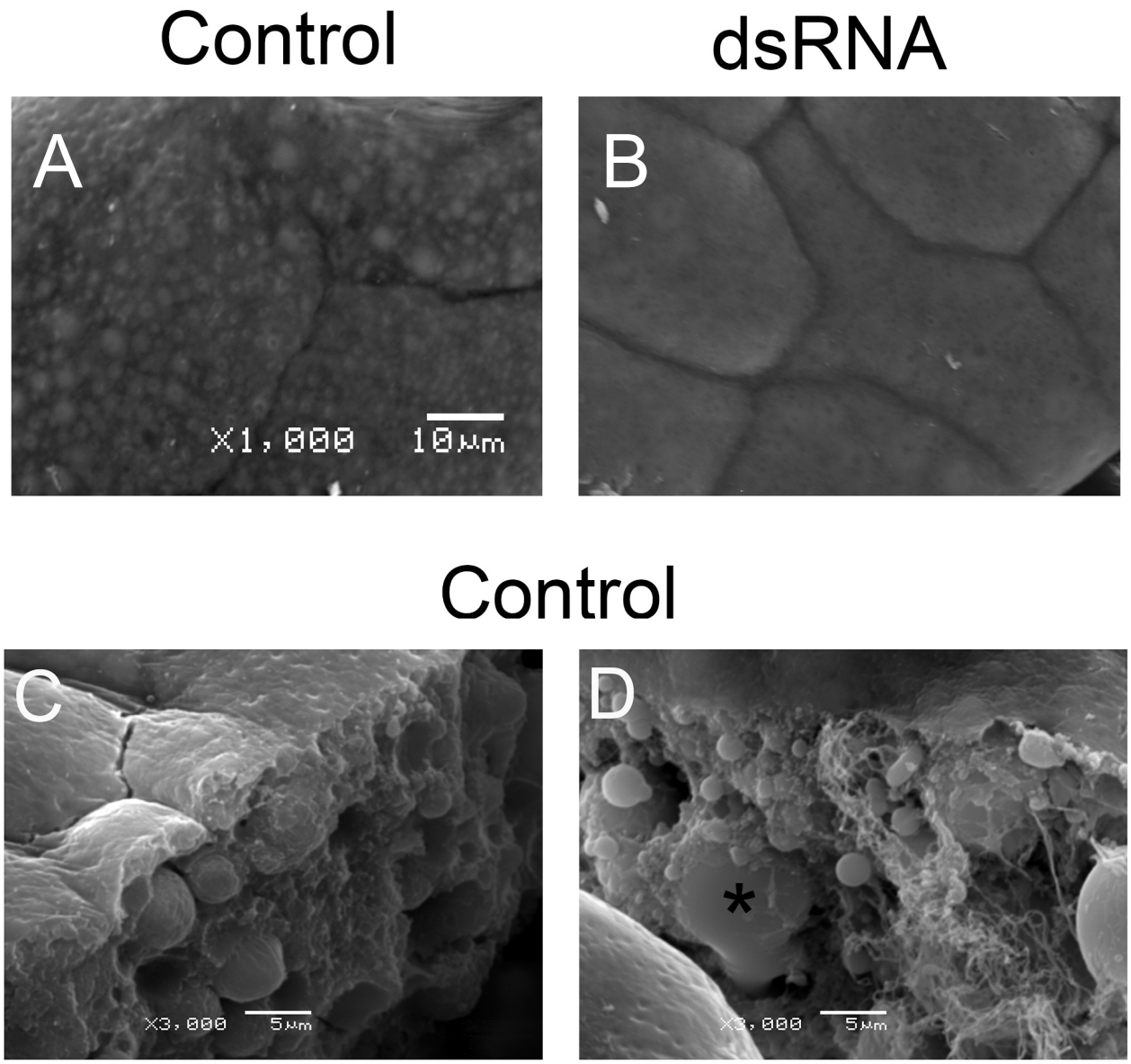

Figure 11. SEM micrographs depicting the topography of the fat body of 3rd instar larvae. Top views of the surface morphology are shown in panels A and B and sample views where the tissue has been ruptured are shown in panels C and D. The surface of the tissue seems to be covered with small “globular” structures which give the tissue a cauliflower-like morphology (A). The structures are absent after β spectrin knockdown (B). Shown in C & D are SEM views where the fat tissue has been ruptured at mag x3,000. Asterisk shows a LD found deeper in the cell. No indication of the presence of elongated structures was found.

II.4 Gain of function effects of β spectrin over-expression

According to their effect on function, mutations may be categorized as loss of function, gain of function or dominant negative. Hermann J. Muller termed mutations as amorph, hypomorph, hypermorph, antimorph and neomorph (Muller, 1932). A loss of function mutation is a mutation that results in reduced or abolished protein function. Both an amorphic and a hypomorphic mutation are loss of function mutations: amorphic causes complete loss of function and hypomorphic causes a partial loss of function. A gain of function mutation occurs when a mutation confers new or enhanced activity on a protein and a dominant negative mutation occurs when the gene product has a function opposite to that of wild-type gene product. Muller termed hypermorph, antimorph and neomorph as gain of function mutations, where a hypermorph causes an increase in normal gene function, an antimorph (a.k.a. dominant negative) acts by antagonizing the normal gene activity and a neomorph mutation causes a gain of gene function (Muller, 1932).

If the function of a protein cannot be studied using knockdown techniques, it can, in some cases, be studied by dominant negative mutations (Herskowitz, 1987). A dominant negative mutation, which has been termed an antimorph by Muller (Muller, 1932), inactivates the wild-type gene function in the presence of the endogenous protein. This type of mutation acts antagonistically to the function of the gene product. The finding that knockdown of α or β spectrin in several tissues had no detectable effects indicated that the function of Spectrin may be redundant. Another protein could take over its function when Spectrin is absent. To obtain insights into αβ spectrin

genetics, I compared the effects of Spectrin subunit over-expression with Spectrin knockdown in the larval fat body of Drosophila.

It has been shown that over-expressing β spectrin in several Drosophila tissue produces severe, larval lethal phenotypes at 25°C. At 22°C, over-expressing β spectrin does not affect the survival of the animal. Over-expression of α spectrin has no effect on survival at any temperature (Mazock *et al.*, 2010).

Because of these observations, I analyzed the effects of β spectrin over-expression on the PM morphology of the fat body at 22°C, where lethality was no longer observed. Interestingly, over-expression of the UAS β spectrin transgene, UAS-β-Spec95, in the fat body produced effects similar to Spectrin knockdown in the fat body. First, there was a concomitant loss of the foamy character of the plasma membrane as seen in control cells by fluorescence of the PM marker, CD8-GFP (Fig. 12 E & F). The PM retained the peculiar speckled pattern at its ecto surface, as was observed with Spectrin knockdown. Second, over-expression also resulted in a loss of cortical LDs from the surface of the fat body, as observed with DIC microscopy (Fig. 12 H). These two effects are identical to that of β spectrin knockdown. These observations suggests that at this level of expression, β spectrin over-expression in the fat body leads to dominant negative (due to loss of function) effects. Interestingly, antibody staining revealed a dramatic increase in β spectrin at the ecto surface of fat body cells (Fig. 12 C & G). This raises a possibility that the effects of β spectrin over-expression at the ecto domain could be due to its increased presence there.

At 25°C, the lethal effect of β spectrin over-expression was associated with a remarkable disappearance of the fat body, as observed with brightfield microscopy. After

several days of development, 2nd instar larvae displayed an abnormal empty appearance due to an almost complete disappearance of the fat tissue throughout the larval body cavity (Fig. 13). Control larvae exhibit fat body and gut tissue throughout the larval cavity which gives them a full appearance. These toxic consequences of β spectrin over-expression were first noted with Cg-Gal4 driven expression of UAS- β Spec95 at 25°C. When the level of transgene expression was reduced by lowering growth temperature to 22°C, fat body atrophy was no longer observed. It is noteworthy that these effects were not observed with α spectrin over-expression.

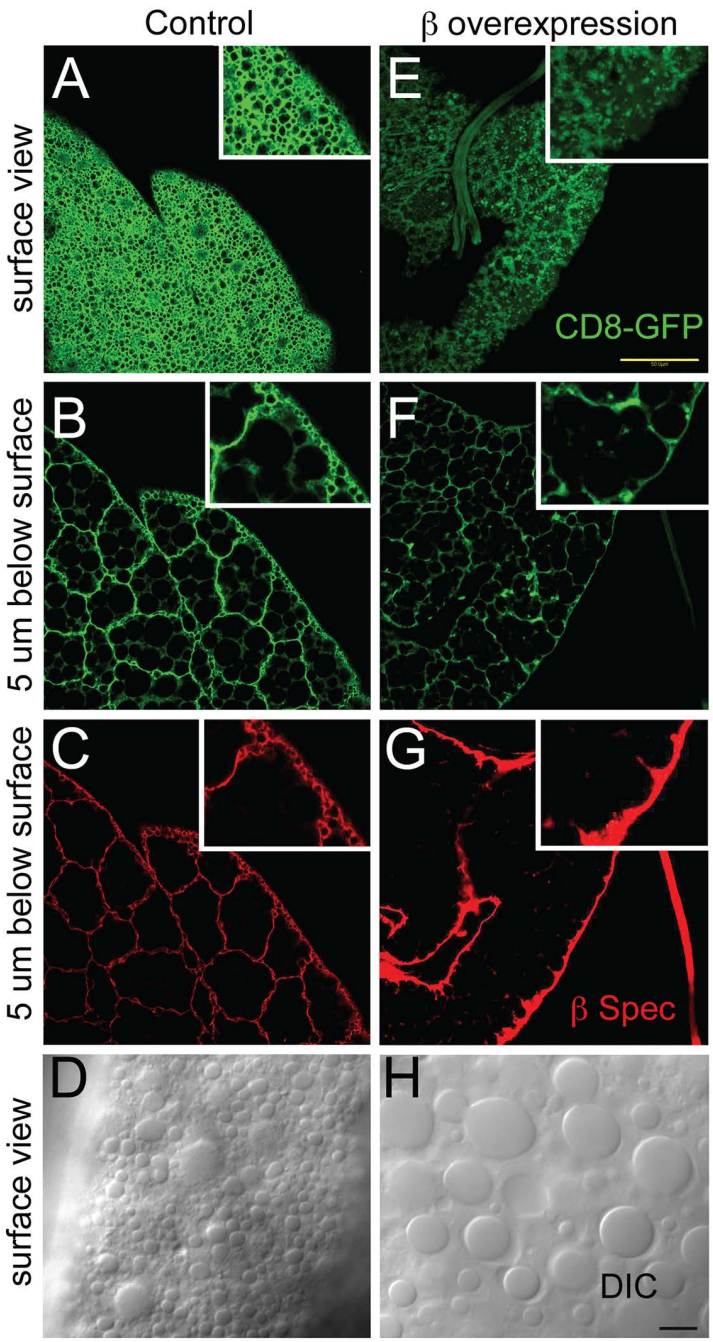

Figure 12. Over-expression of β spectrin in the larval fat body altered the surface CD8-GFP pattern and eliminated the population of small, cortical LDs. UAS-βSpec95 expression in fat body was driven by Cg-Gal4. Over-expression resulted in a dramatic increase of β spectrin antibody staining at the ecto domain (G) compared to wild type (C). There was also an increase in CD8-GFP intensity, making it necessary to lower the PMT setting during image capture (E,F) relative to controls (A,B). By lowering the PMT it was possible to see loss of the foamy pattern, which was replaced by a speckled pattern, similar to what was seen with β spectrin knockdown (Fig. 1). D,H: the cortical LDs (1-5 um) detected by DIC microscopy. Bar in E = 50 μm, in H = 10 μm.

Figure 13. Over-expression of β spectrin in the fat body caused fat body atrophy and lethality. Much of the space within a wild type larva is occupied by lobes of fat tissue (left, arrow). Larvae over-expressing β spectrin (UAS-β-spec95) at 25C ultimately die by 3rd instar, at which time the fat body has substantially atrophied, leaving larvae with large empty spaces (*).

II.5 A hypermorphic effect of β spectrin over-expression in the fat body

LDs can be detected by light microscopy using Oil Red O as a hydrophobic dye (Gutierrez *et al.* 2007). Oil Red O (Sudan Red 5B, Solvent Red) is a fat soluble dye commonly used for detecting neutral lipids, mostly TAGs. It has been extensively used as a lipid stain in studies using model organisms ranging from the simple unicellular yeast cell to the complex human fat tissue (Mehlen *et al.,* 2013). I used the stain as an alternative tool to analyze the distribution of LDs in the fat body of wild-type and β spectrin over-expression larvae. Oil Red O staining produced a pattern that appeared nearly solid in the fat body. The Spectrin-dependent cortical LDs at the ecto domain of the tissue could not be distinguished from larger LDs found deeper in the cytoplasm because of the high density of fat in the tissue (Fig.14 A, arrowhead).

Unexpectedly, β spectrin over-expression in the fat body had an effect on the midgut, and not the fat body. There was little effect on Oil Red O staining in the fat body, the tissue being intensely stained (Fig.14 C, arrowhead). However, over time, there was less detectable fat tissue, Figure 13. There was a dramatic increase in Oil Red O staining, which corresponded to an increase in abundance of LDs, in the midgut epithelium (Fig.14 C, arrow). The midgut of wild-type larvae was scantly stained with Oil Red O (A, arrow). In this view of the anterior midgut Oil Red O staining was detected from the base of the proventriculus to the middle midgut, which did not exhibit abnormal Oil Red O staining. The region of gastric caecae closest to the gut trunk also exhibited abnormal lipid accumulation. Lipid droplets were also visible in the midgut by DIC microscopy. They were relatively rare in control preparations of midgut (Fig.14 B), but

became conspicuous in both the anterior (Fig. 14 D) and posterior midgut of β spectrin over-expressers at 25°C and in the posterior midgut at 22°C (Fig. 15).

The accumulation of LDs in the midgut depends on the amount of β spectrin. At 25°C, it was lethal at second larval instar and led to accumulation of LDs in both the anterior and posterior midgut (Fig. 14 & 15). At 22°C, β spectrin over-expression had no effect on survival and led to accumulation of LDs only in the posterior midgut (Fig. 15). It is important to note that the abnormal accumulation of LDs in the midgut was not observed after loss of either α or β spectrin in the larval fat body. Additionally, α spectrin over-expression did not have an effect on LD presence in the midgut epithelium. Hence, the accumulation of LDs in the midgut resulted from exclusively β spectrin over-expression in the fat body. This suggests that because the over-expression phenotype is distinct from that observed with loss of function, and is dependent on the amount of β spectrin that is over-expressed, the abnormal accumulation of lipid in the midgut is a gain of function effect that is hypermorphic.

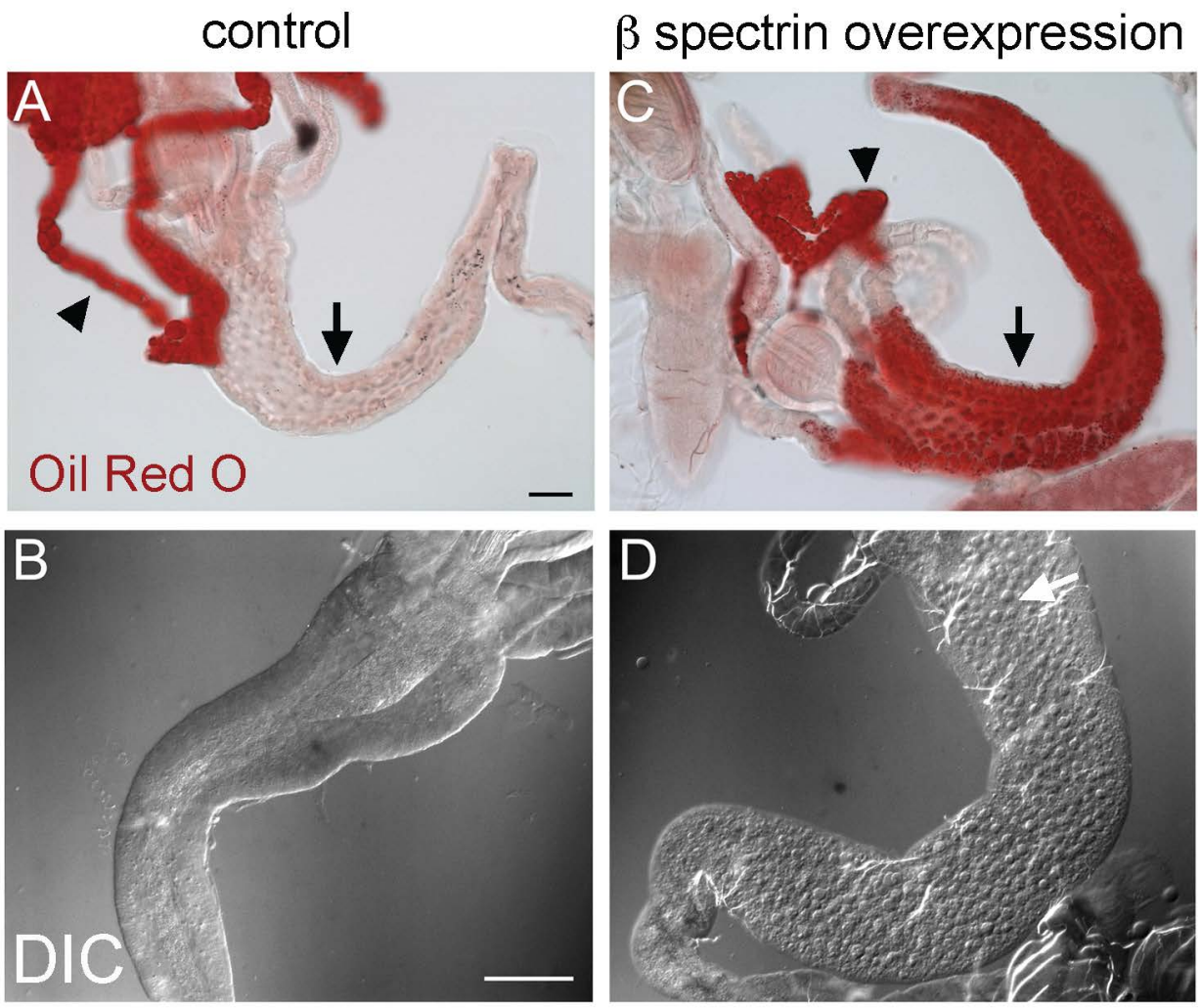

Figure 14. Oil Red O staining of lipid droplets in dissected preparations of wild-type and β spectrin over-expressing larvae. Most of the Oil Red O staining in wild-type was confined to the fat body (A, arrowhead) with only a trace of staining visible in the midgut epithelium (arrow). There was a dramatic increase in anterior midgut staining in larvae that over-expressed UAS-β-spec95 at 25°C (C). The change was also visible by DIC: lipid droplets were rarely detectable in controls (B) and there was a dramatic increase in lipid droplets in the anterior midgut (white arrow) upon UAS-β-spec95 over-expression (D). Bar is 50 µm (A,C) or 20 µm (B,D).

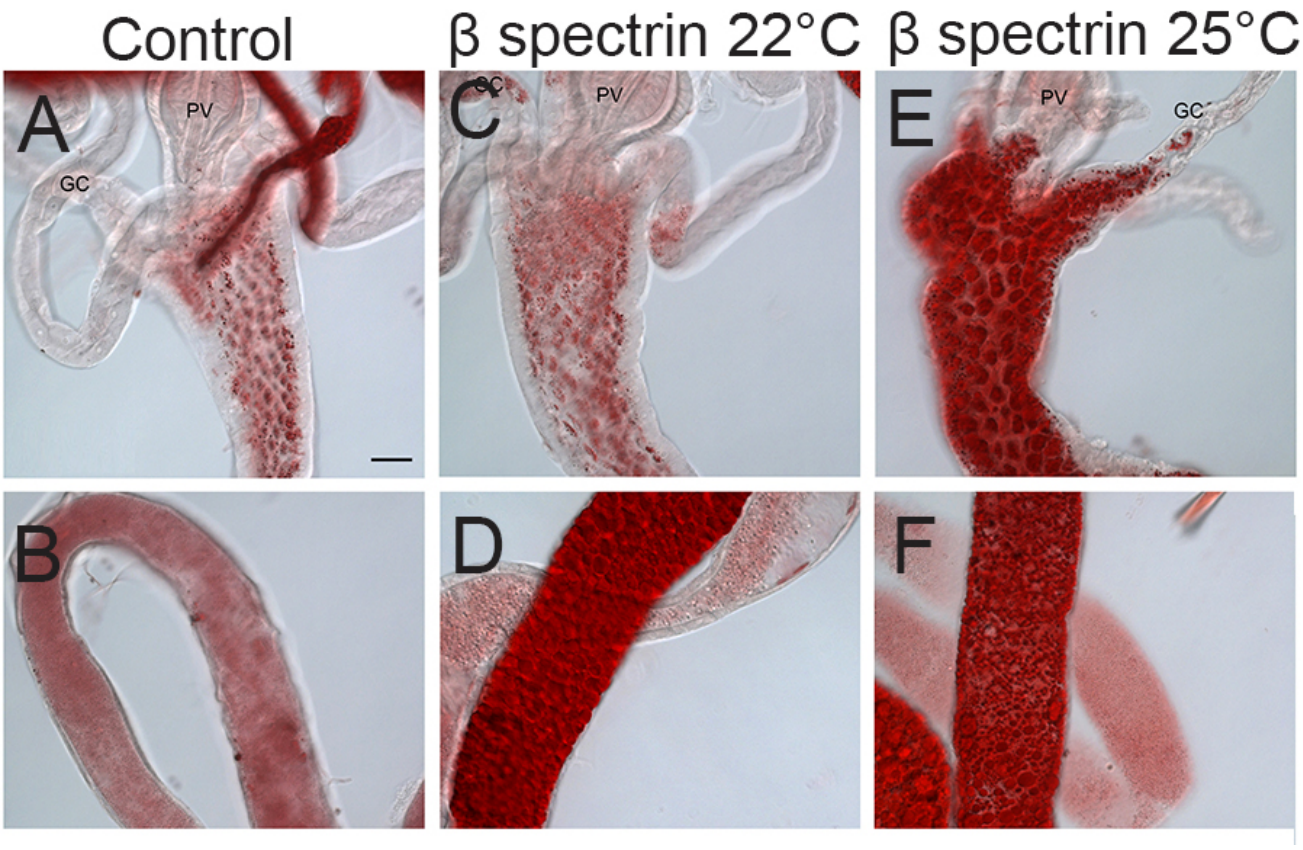

Figure 15. Oil Red O staining of lipid droplets in dissected preparations of wild-type and β spectrin over-expressing larvae shows that the extent of the accumulation of LDs in the midgut depends on the amount of β spectrin. When the level of expression was lowered by growth at a lower temperature, 22°C, there was no effect on survival but there was accumulation of LDs only in the posterior midgut (C). There was very little staining in the anterior (A) and posterior (B) midgut epithelium of wild-type larvae. There was a dramatic increase in both the anterior (E) and posterior (F) midgut staining in larvae that over-expressed UAS-β-spec95 at 25°C. Bar is 50 μm.

II.6 The midgut lipid accumulation phenotype is due to an effect on lipophorin secretion from the fat body

β spectrin over-expression in the fat body has a dramatic effect on midgut lipid droplet accumulation, which was similar to what has been previously observed after knockdown of lipophorin in the fat body by RNAi (Panakova *et al.*, 2005). Lipophorin is the lipoprotein secreted by the fat body, which transports dietary lipids from the midgut back to the fat body for storage (Panakova *et al.*, 2005). Because both β spectrin over-expression and lipophorin RNAi in the fat body led to accumulation of lipid droplets in the midgut, I hypothesized that over-expressing β spectrin interferes with lipophorin secretion from the fat body.

To test my hypothesis, I obtained a sample of the Lpp antibody from the group who characterized the lipoprotein (Eugster *et al.*, 2007) and performed anti-Lpp antibody staining experiments on larvae that over-expressed β spectrin in the fat body. If my hypothesis was correct, I expected to note an increase in the Lpp signal in the fat body and possibly a decrease in signal in the midgut. If my hypothesis was incorrect, the Lpp signal intensity would be similar to that of control larval preparations. Lpp was found at the fat body (Fig. 16 A) and the midgut of control larvae (Fig. 17). Lpp RNAi in the fat body eliminated the anti-Lpp signal (Fig.16 C & Fig. 17 G).

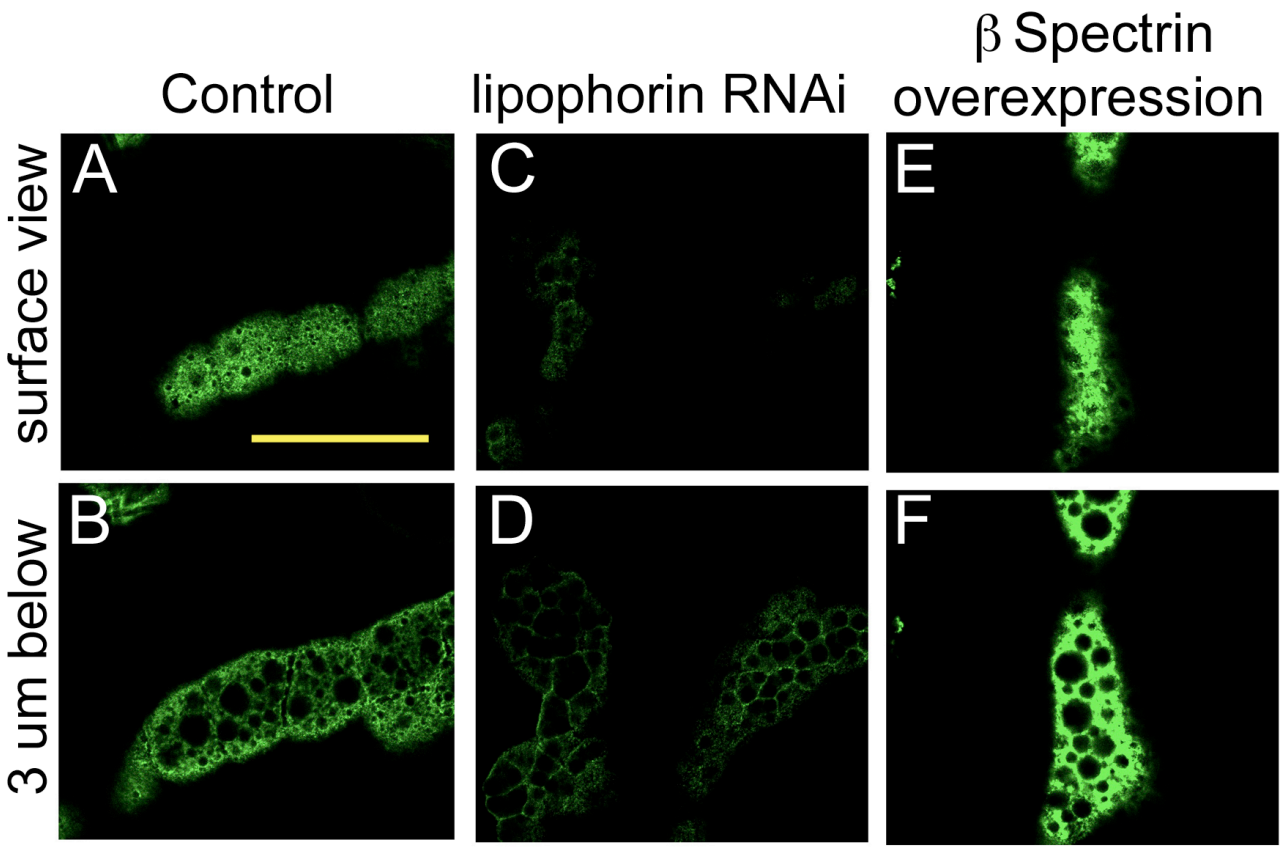

Figure 16. Effects of UAS-β-Spec95 over-expression and Lpp knockdown on anti Lpp antibody staining in the larval fat body. The staining pattern in control fat body (A & B) was greatly diminished after dsRNA knockdown of Lpp (C & D). Interestingly, Lpp staining became more intense upon β spectrin over-expression in fat body (E&F). Bar is 50 μm.

Consistent with my hypothesis, not only was the Lpp signal significantly higher in the fat body of larvae that over-expressed β spectrin (Fig. 16 E,F), it was completely absent from the midgut (Fig. 17 D). I analyzed the midgut tissue with both anti β_H spectrin (Fig. 17 B,E,H) and anti-β spectrin (see Fig. 20) antibody staining to control for the integrity of the tissue. Both staining experiments ensured that the absence of the Lpp signal at the midgut epithelium was not due to a trivial reason (e.g. dead tissue), but was due to the absence of the protein. This result supports my hypothesis that β spectrin over-expression interferes with Lpp secretion from the fat body. As Lpp is not secreted properly, it is not available to not pick up dietary fat from the midgut for transport back to the fat body. This then results in lipid accumulation at the midgut.

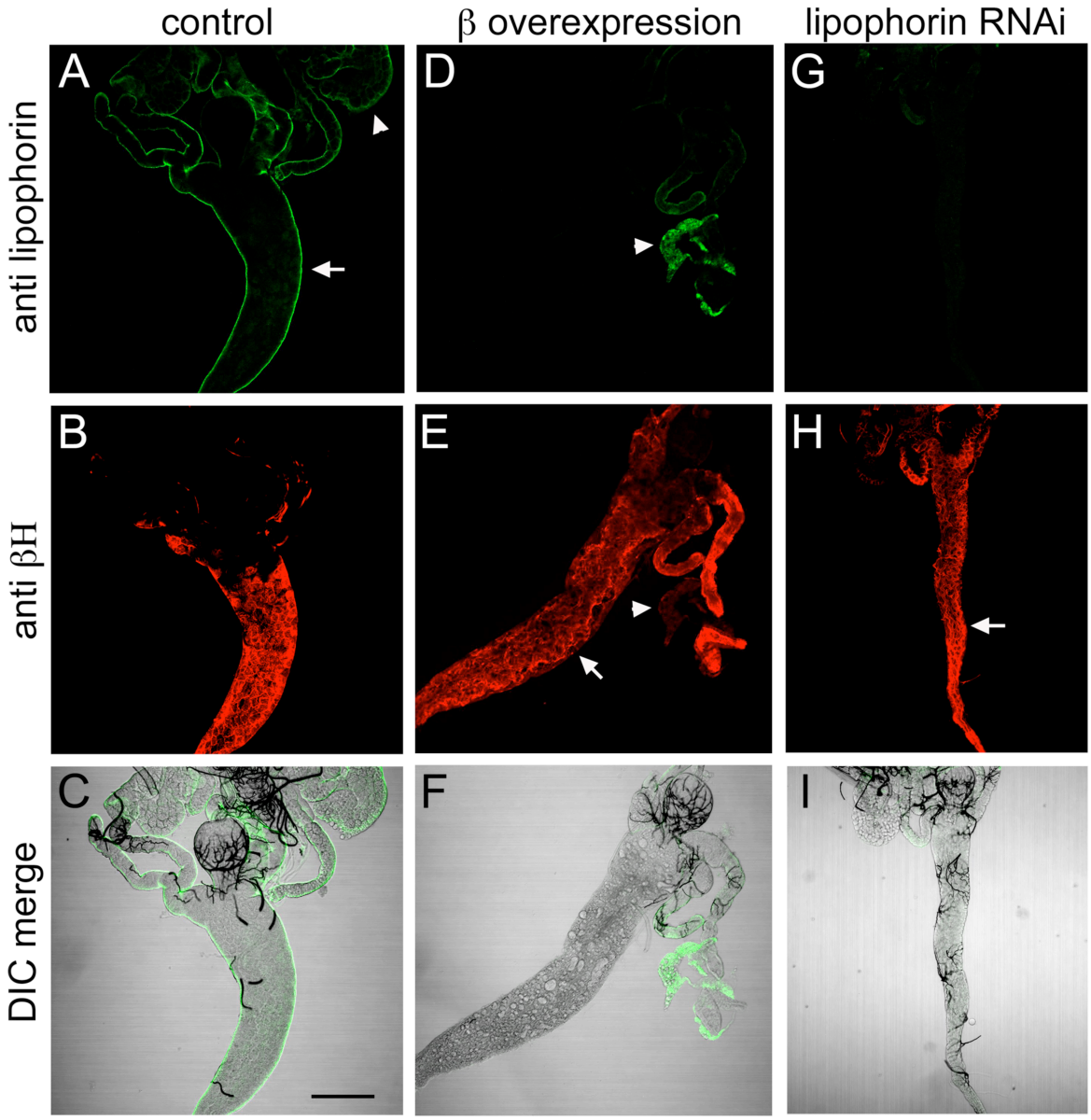

Figure 17. β spectrin over-expression in the larval fat body resulted in a loss of Lpp accumulation at the midgut. The effect was comparable to that observed with Lpp RNAi. Dissected preparations of larval midgut from wild type (A-C), β spectrin over-expresser in the fat body (Cg-Gal4 driven UAS-β-Spec95; D-F), or Lpp knockdown in the fat body (Cg-Gal4 drived UAS-Lpp RNAi; G-I) were stained with anti Lpp antibody. The Lpp antibody staining pattern on the surface of the midgut in wild type (Cg-Gal4 parent line, A) was eliminated by either β spectrin over-expression (D) or knockdown of Lpp (G). Anti β_H spectrin was used as a positive staining control (B,E,H). Fat body did not exhibit β_H spectrin staining above background (arrowhead, E) but did stain strongly for Lpp after β spectrin over-expression (arrowhead, D). Bar = 50 μm.

II.7 Hypermorphic effects of β spectrin over-expression are relieved by co-over-expression of α spectrin

Data gathered in the laboratory has shown that over-expression of α spectrin alone had no effect and over-expression of β spectrin had toxic effects in most tissues, including in the fly imaginal wing disc. High levels of β spectrin lead to failed wing development (Mazock *et al*., 2010). These results prompted me to address the potential consequences of over-expressing both subunits, as this became relevant to understanding of the origin of these effects.

Interestingly, I found that α spectrin co-expression with β spectrin ameliorated the toxic effects of over-expressing β spectrin alone in the developing wing. To analyze the effects of over-expressing both subunits, doubly heterozygous male flies that carried one copy of myc-tagged UAS-α Spec37 on chromosome II and one copy of myc-tagged UAS-β Spec95 on III were crossed to female flies homozygous for MS1096-Gal4 (cross described in methods). From this cross, I observed three classes of progeny: flies that displayed no wing defect, flies that had a severe defect and flies that retained a mildly cupped wing. I then analyzed the genotypes of the three classes of flies by western blotting and correlated them with the adult wing phenotypes (Fig. 18 D). F1 progeny with no wing defect corresponded to a class of progeny that inherited neither spectrin transgene (lane 4) or to flies that inherited only myc UAS-α Spec37 (lane 2). Flies with a severe wing defect were found to express only myc UAS-β Spec95 (lane 3). The class of flies with a mildly cupped wing shape corresponded to over-expression of both the α and β spectrin transgenes. Thus, I found that co-over-expression of α spectrin ameliorated the toxic effect of β spectrin over-expression in the wing disc.

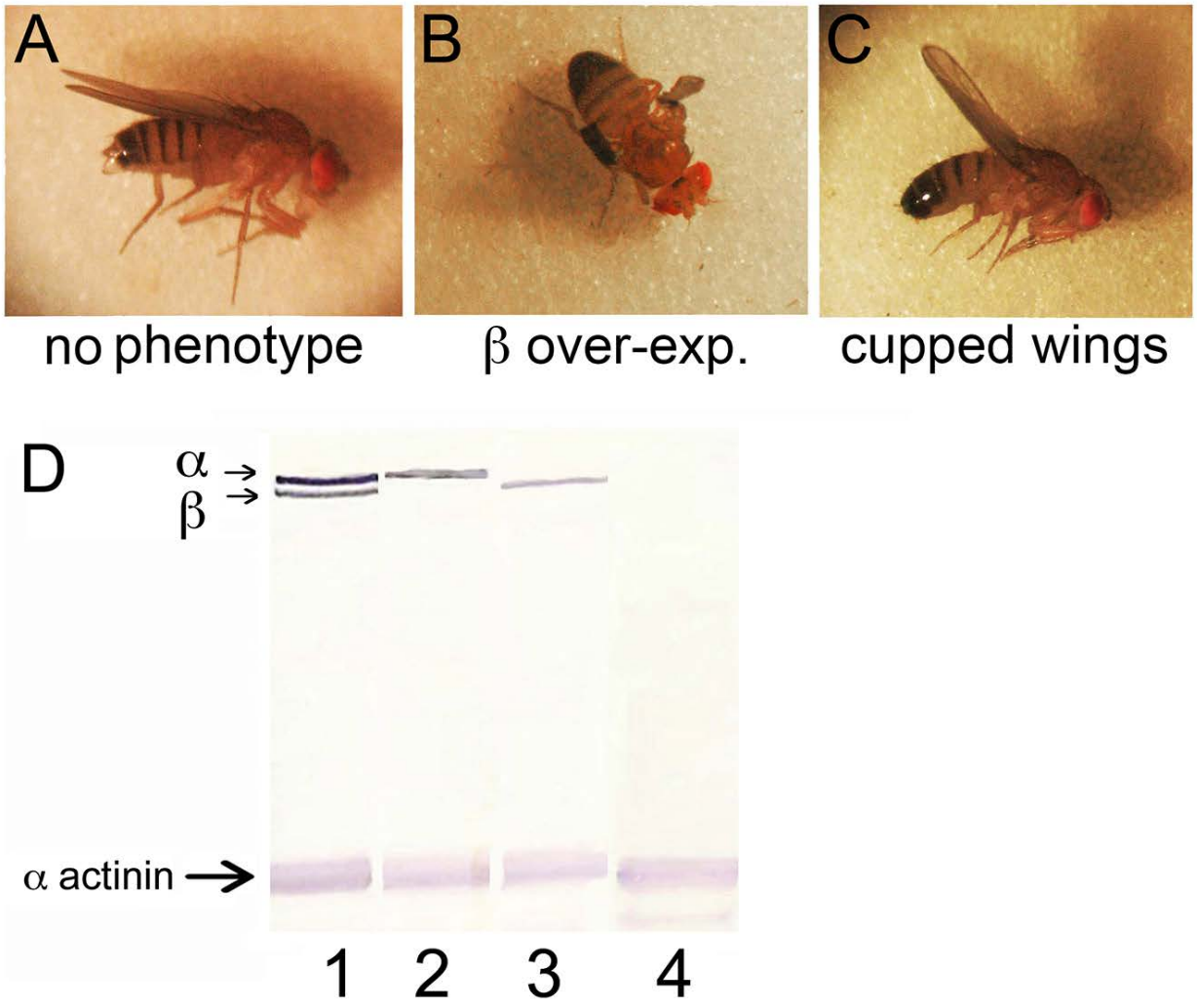

Figure 18. Rescue of β spectrin over-expression phenotype in wing by co-expression of α spectrin. Doubly heterozygous flies carrying UAS-α-spec37 and UAS-β-spec95 transgenes were crossed to MS1096-Gal4 to drive wing expression. Three classes of wing phenotypes were distinguished in the adult progeny (A-C). Western blot analysis with anti myc tag antibody detected the expected progeny classes expressing either transgene alone (lanes 2 and 3), both transgenes (lane 1), or neither (lane 4). When reared at 25°C adults expressing UAS-β-spec95 under control of MS1096- Gal4 produced a severe wing phenotype (B). Flies expressing neither transgene (A), or expressing just UAS-α-spec37 were indistinguishable with no detectable wing phenotype (no transgene shown). A third class of flies was observed in which wings had a mildly cuppedphenotype, corresponding to the class over- expressing both α and β spectrin (C). Thus α spectrin co-expression largely ameliorated the deleterious effect of β spectrin over-expression in the wing.

After finding that α spectrin ameliorated the toxic effect of β spectrin over-expression in the wing disk, I asked whether α co-expression could also rescue the lethal effect resulted from β spectrin over-expression in the fat body. To address this, I performed a similar experiment, where I produced doubly heterozygous male flies carrying one copy of myc-tagged UAS-α-Spec27 on chromosome II and one copy of myc-tagged UAS-β-Spec95 on III and crossed them to female flies homozygous for Cg-Gal4 at 25°C. Since, expressing UAS-β-Spec95 with Cg-Gal4 at 25°C leads to a toxic larval lethal effect, I analyzed the adult progeny from the rescue cross by western blotting. I rationalized that if α spectrin rescued the lethal effect of β, I would identify an adult fly that expressed both myc-tagged transgenes at 25°C. As predicted, I found that flies that over-expressed both α and β spectrin in the fat body survived to adulthood. As a follow-up, I performed additional experiments to ask whether co-expression of α with β spectrin would also rescue the accumulation of lipid droplets in the midgut and the loss of cortical LDs in the fat body. This time, I used another myc-tagged β spectrin transgene, UAS-β-Spec62 (described by Mazock *et al.*, 2010), which is X-linked, to be able to distinguish larvae that over-express β spectrin from those that do not. Expression of the transgene driven by Cg-Gal4 resulted in consistent early 2nd instar larval lethality at 25°C and of late 2nd instar lethality at 22°C. I crossed male progeny carrying a myc-tagged UAS-α-Spec37 transgene on chromosome III and Cg-Gal4 on II with females homozygous for UAS-β-Spec62, at both 25°C and 22°C. I initially chose 25°C to simplify identification of rescued 3rd instar larvae. As the male siblings did not express the X-linked transgene in this cross, they provided a useful control group that survived to adulthood. I found that, when I co-expressed UAS-α-Spec37 with UAS-β-

Spec62 (UAS- β-Spec62/Y; +/+; UAS-α-Spec37 /+ X +/+; Cg-Gal4/Cg-Gal4; +/+), lethality was overcome and all female progeny survived to adulthood (Fig. 14 A). Western blots confirmed that the female progeny expressed both the α and β spectrin transgenes (not shown). I also ruled out the possibility that rescue of the β spectrin over-expression phenotype was due to a trivial Gal4 dilution effect caused by introduction of the UAS-α spectrin transgene by co-expressing another UAS transgene (UAS-CD8-EGFP) together with UAS-β spectrin, with no change in outcome.

Subsequently, I also asked whether α spectrin co-expression with β spectrin rescues the accumulation of LDs in the midgut. To answer this question, I performed Oil Red O staining on male and female 2nd instar progeny at both 22°C and 25°C (Fig. 19). Interestingly, at 25°C, I observed partial rescue of the accumulation of LDs in the midgut. LDs were still present in the posterior midgut and the phenotype was similar to that of over-expressing β spectrin in the fat body at 22°C using the less potent UAS-β-Spec95 transgene (not shown). However, LDs were absent from the anterior midgut (Fig. 19 F). Thus at 25°C, co-expression of α with β spectrin partially rescued the accumulation of LDs in the midgut phenotype. Interestingly, at 22°C, α spectrin co-expression prevented the abnormal accumulation of lipid droplets in both the anterior and posterior midgut. I found that not only did α spectrin rescue the lethality caused by β spectrin over-expression, it also prevented the abnormal accumulation of LDs. Additionally, I note that the level of rescue depends on the amount of α spectrin present after over-expression.

A

spectrin overexpression:	β62	α	α + β62
larval survival	0/61	50/52	41/41
β spectrin staining intensity at plasma membrane	elevated	normal	elevated

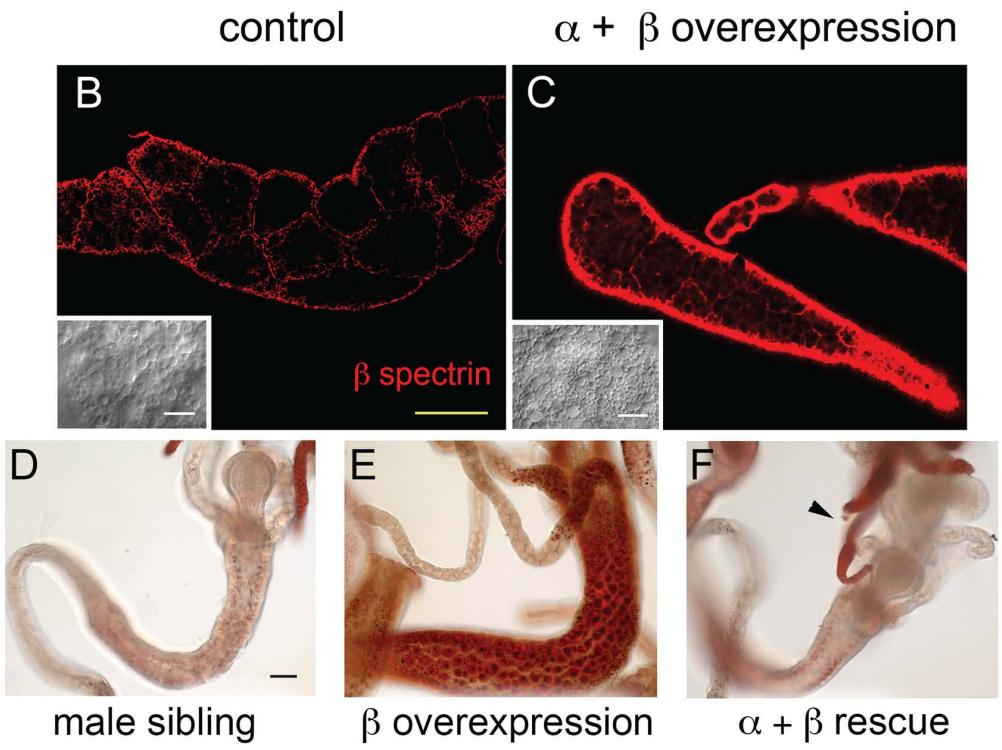

Figure 19. Rescue of the β spectrin over-expression phenotype in the fat body. Males carrying X-linked UAS-β-spec62 and heterozygous for autosomal UAS-α-spec37 (UAS-β-spec62/Y; UAS-α-spec37/+) were crossed to females homozygous for autosomal Cg-Gal4 (+/+; Cg-Gal4/Cg-Gal4) at 25°C. A. In control crosses, expression of UAS-β-spec62 alone at 25C resulted in 100% larval lethality at 2nd instar and expression of UAS-α spec37 alone had no effect on larva viability. However, in rescue crosses with α and β spectrin transgenes together, female progeny could be recovered as 3rd instar larvae and they were 100% viable to adulthood. These 3rd instar female progeny exhibited dramatically elevated β spectrin staining in the fat body (C)compared to their male siblings that did not express the β spectrin transgene (B, same microscope setting as in C). Insets show LDs in wild type fat body by DIC (B) and their return after rescue of β overexpression by α spectrin (C). D-F: Oil Red O staining of dissected 2nd instar larvae. The midgut LD accumulation observed with β spectrin over-expression alone (E) was not seen in larvae expressing both β and α spectrin (F), although lipid staining was apparent in the fat body (arrowhead). A male sibling from the rescue cross not expressing excess β spectrin is shown as a negative control (D). Bar = 50 µm.

The results suggest that the ratio of α to β spectrin is what accounts for the different degrees of rescue. As the intensity of the hypermorphic effects of β spectrin depend on the gene dose (transgene used and temperature), the extent of the rescue after α spectrin co-expression is gradual. The data I present here suggest that α spectrin regulates the function of β spectrin, in a dose dependent manner.

Additionally, I asked if the cortical LDs are restored at the fat body in rescue female larvae. I analyzed the surface of the fat body of male and female larvae, at both 22°C and 25°C, with DIC microscopy. At 22°C, the cortical LDs were restored to the fat body (Fig. 19 B & C insets) at 25°C, they were not. This result is consistent with the Oil Red O result in the midgut, showing partial rescue at 25°C (LDs present in the posterior midgut) and complete rescue at 22°C. These data strongly suggest that the progressive hypermorphic effects of β spectrin over-expression and their rescue with α spectrin depend on the ratio of α to β spectrin.

α spectrin over-expression may rescue the phenotypes of β over-expression because: a) α spectrin induces turnover of the excess β spectrin; b) α spectrin causes redistribution of excess β spectrin from the ecto domain or c) α spectrin modifies the activity of β spectrin. To understand which of these accounts for rescuing the phenotype, I performed anti β spectrin immunofluorescence experiments on larvae that co-expressed α with β spectrin in the fat body and asked where in the cell the excess β spectrin was found (e.g. at cell contacts, ecto domain, intracellularly). The immunofluorescent staining pattern seen with the antibody ruled out the first two possibilities (Fig. 19 B&C). Interestingly, in rescued females, β spectrin was accumulated at a high-density at the ecto domain of the fat body (Fig. 18 C). The β

spectrin antibody staining pattern was the same as that of larvae over-expressing β spectrin alone (Fig. 13 G). This was in contrast to the much weaker staining detected in control sibling males (Fig. 19 B, same microscope settings as 19 C).

Based on these striking results, I hypothesized that α over-expression rescues the phenotypes of β spectrin because it restores Lpp secretion from the fat body. To test my hypothesis, I performed anti-Lpp antibody staining experiments on male and female larvae that over-expressed both spectrin subunits (Fig. 20). Remarkably, I found that Lpp detection was restored in the midgut epithelium of larvae that over-expressed both subunits (Fig. 20 C). The data suggest that α spectrin exerts its effect by changing the activity, and not changing the amount or distribution, of β spectrin.

Additional evidence that support my conclusion that α spectrin regulates the hypermorphic effects of β spectrin by changing its activity was obtained genetically. I asked if lowering α spectrin gene dose while increasing β spectrin gene dose in the fat body of would lead to a more severe phenotype at 22°C. At this temperature, expressing the UAS β-Spec95 transgene in the fat body has no effect on survival (cross described in methods). The genotypes of the adult animals were described by western blotting. In this genetic background, survival of β spectrin over-expressers at 22°C was somewhat reduced (7/56 GFP+, TM3 F1 adults expressed myc-β spectrin) compared to other backgrounds where all of the β spectrin over-expressers were viable. None of the adult GFP+ , non-TM3 F1 adults (0/56) expressed myc-β spectrin, indicating that β spectrin over-expressers were not viable when the α-Specrg41 allele was present. Thus it appears that reducing the dose of α spectrin has a similar effect as increasing the dose of β spectrin.

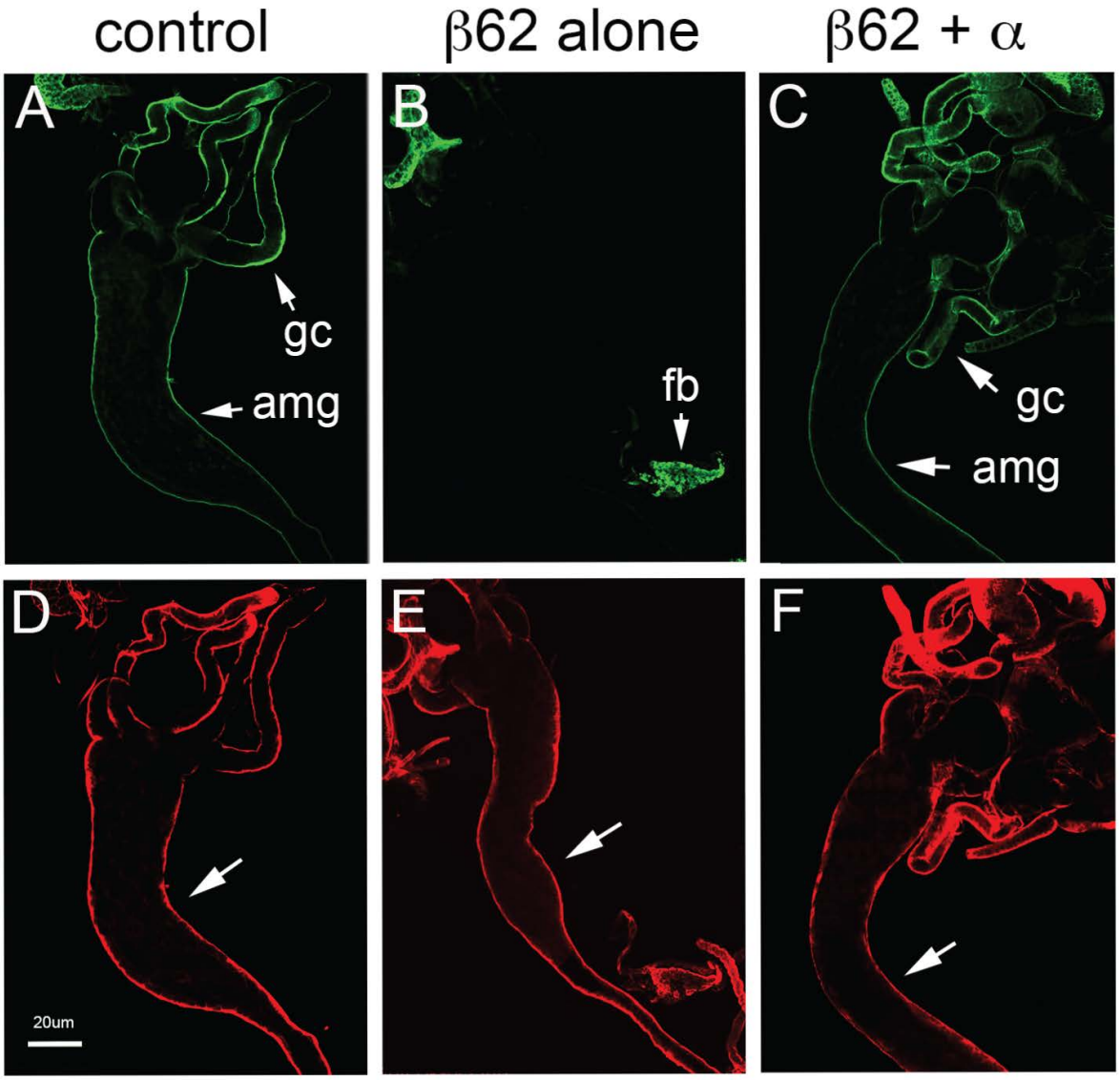

Figure 20. Over-expression of β spectrin alters the behavior of lipophorin. Dissected larvae expressing UAS-β-spec62 alone (B,E) or together with UAS-α-spec37 (C,F; as in Fig. 8) in the fat body (Cg-Gal4) were double labeled with anti-lipophorin antibody and FITC secondary antibody (A-C, green) and anti β spectrin antibody and TR secondary antibody as a staining control (D-F, red). Lipophorin antibody stained the outer surface of the anterior midgut (amg) and gastric caeca (gc) of wild type larvae (A). Midgut lipophorin staining was absent when β spectrin was over-expressed in the fat body (B), although it was still abundantly detected within the fat body (fb). Lipophorin staining of the midgut was restored in rescued larvae expressing both α and β spectrin transgenes (C). Bar = 20 μm.

CHAPTER III. Intracellular lipid translocation in the larval midgut: evidence that LSD2 is a regulator of lipid homeostasis

III.1. Abstract

Lipids (TAG and sterols) stored in lipid droplets become mobilized upon increased energy demand by hydrolysis into free fatty acids (FFA) and transport out of cells via lipoprotein particles. Two major insights emerged from the studies I present here. First, I show that increased levels of dietary FFAs lead to aberrant intracellular LD accumulation in the larval midgut. The size and number of LDs are directly influenced by the availability of dietary lipids. Interestingly, the LDs are efficiently mobilized by the midgut epithelium after the added FFAs are eliminated from diet. These data show that, unexpectedly, midgut cells store lipids in LDs more efficiently than export them via Lpp. Furthermore, I show that, unlike FFAs, complex TAGs are not efficiently absorbed by the midgut epithelium without the presence of a supplemental lipase. This finding suggests that complex TAGs are not efficiently absorbed unless they are hydrolyzed into less complex FFAs and that lipases play an important role in lipid processing in insects. Second, I show that over-expression of the LD associated protein, LSD2 leads to a substantial increase in the amount of LDs. It has thus far been proposed that LSD2 functions by preventing the access of lipases to the lipid droplet surface. I propose here that LSD2 is a regulator of lipid homeostasis and plays an important function in directing the flow of lipids into pathways of LD formation. I suggest that LSD2 acts as a gatekeeper of lipid translocation from LDs to LTP for export.

III.2 Perilipin function in mammals and Drosophila

PAT proteins, named after Perilipins, ADRP and tail-interacting protein 47kDa, are the most studied family of LD-associated proteins (Wather and Farese, 2009). Mammals have five Perilipins (PLIN1-5) and Drosophila have two, LSD1 and LSD2. Computational analyses identified that the Drosophila perilipins are related to all three proteins, Perilipin, ADRP, and TIP47 (Lu *et al.*, 2001). While both fly Perilipins are expressed during all ontogenetic stages, only *LSD2* is broadly expressed in various tissues (Gronke *et al.*, 2003). Northern blot and in situ analyses showed a strong enrichment of *LSD2* mRNA in early embryos, which reflects a maternal contribution. Continuous expression of *LSD2* was observed in two tissues known to function in lipid storage and lipid absorption, in the developing fat body and anterior midgut (Gronke *et al.*, 2003). LSD1 is found associated with most LDs and LSD2 is found exclusively on the surface of small LDs (Bi *et al.*, 2012). The functions of the two proteins have been regarded as both redundant and opposite. It has been suggested that LSD1 facilitates lipid mobilization and that LSD2 protects lipid droplets from lipolysis mediated by the Drosophila homolog of ATGL, Brummer (Bmm; Bi *et al.*, 2012).

Staining experiments led to contradictory reports of the sub-cellular localization of the mammalian PLIN2 (a.k.a adipophilin, ADPR). While several studies have reported a specific association of PLIN2 with LDs, others have presented evidence that PLIN2 is associated with ER membrane (Robenek *et al.*, 2006) or PM (Gao and Serrero, 1999; Gao *et al.*, 2000). PLIN2 was first identified as an early marker of adipocyte differentiation (Jiang *et al.*, 1992) and was characterized in milk-secreting mammary epithelial cells where it was found associated with milk lipid globule membranes (Heid *et*

al., 1996). PLIN2 is associated with lipid droplets in many cell types, such as muscle cells, adipocytes, hepatocytes, fibroblasts, endothelial cells and macrophage and is also found in droplets rich in cholesterol stored as steroid hormone in adrenocortical cells (Heid *et al.*, 1998).

PLIN2 is currently regarded as a marker of lipid accumulation in mammalian studies. While decreased expression has been associated with a decrease in TAG level and with protection against diet-induced obesity, adipose inflammation, and fatty liver disease (McManaman *et al*., 2013), increased expression of PLIN2 has been associated with an increase in LD synthesis. For example, in the mouse, it was found both *in vitro* and *in vivo*, that PLIN2 over-expression leads to increased TAG levels and LD accumulation (Bosma *et al*., 2012). Because PLIN2 levels were found increased during induction of mouse pre-adipocytes, it has been proposed that it plays a role in LD formation (Jiang and Serrero, 1992). Interesting, it has been reported by several diverse studies that PLIN2 expression is up-regulated after lipid induction. Several examples are: in human blood monocytes (Buechler *et al*., 2001), in peritoneal macrophages (Paul *et al*., 2008), lipid-laden macrophages in atherosclerotic lesions (Larigauderie *et al.,* 2004). Additionally, it was shown that long chain fatty acids (LCFA) stimulate PLIN2 mRNA expression level in several cell types (Dalen *et al*., 2006; Gao *et al*., 2000; Bindesboll *et al*., 2013). From a disease viewpoint, it has been proposed that PLIN2 plays an important role in foam cell (lipid-laden macrophages in atherosclerotic lesions) formation in atherosclerosis (Larigauderie *et al*., 2004; Nuotio *et al*., 2007; Paul *et al*., 2008), in cardiomyopathies, kidney diseases, hepatocyte steatosis, colon ischaemia, and at the border of organ infarcts (Straub *et al*., 2013).

In Drosophila, over-expression of LSD2 leads to an increase in TAG levels by 49%. This effect of LSD2 increase has been attributed to protein level increase specifically in the fat body (Gronke *et al.*, 2003). From a mechanistic viewpoint, studies in Drosophila have thus far suggested that the lipid droplet-bound protein regulates lipid metabolism by regulating the access of lipases to the LD surface. Specifically, it has been suggested that LSD2 either recruits or the prevents lipases to the LD surface (Bi *et al.*, 2012). Here I bring novel insights into the mechanism of LSD2 function, providing data that support a mechanism of function in LD homeostasis.

III.3 Added free fatty acids to diet are efficiently absorbed and incorporated into lipid droplets in the larval midgut epithelium

Using Oil Red O to detect neutral lipids, I found that a substantial amount of FFAs are absorbed and incorporated into LDs by the larval midgut enterocytes. Staining of the midgut epithelium of wild-type larvae fed a standard diet of yeast paste shows that LDs are relatively rare in both the anterior (Fig. 21 A) and posterior midgut (Fig. 21 B). As expected, because of the high density of LDs in fat body (FB) cells, Oil Red O produced a pattern there that appeared nearly solid red. Larvae fed a high fat diet of 10% oleic acid in yeast paste for several hours (shown here, 20 hrs) accumulated a remarkable amount of LDs in the midgut. Oil Red O staining indicated a dramatic increase in LD abundance in both the anterior (Fig. 21 C) and posterior (Fig. 21 D) midgut cells. The Oil Red O staining pattern of the midgut cells was solid red, similar to that of the fat body.

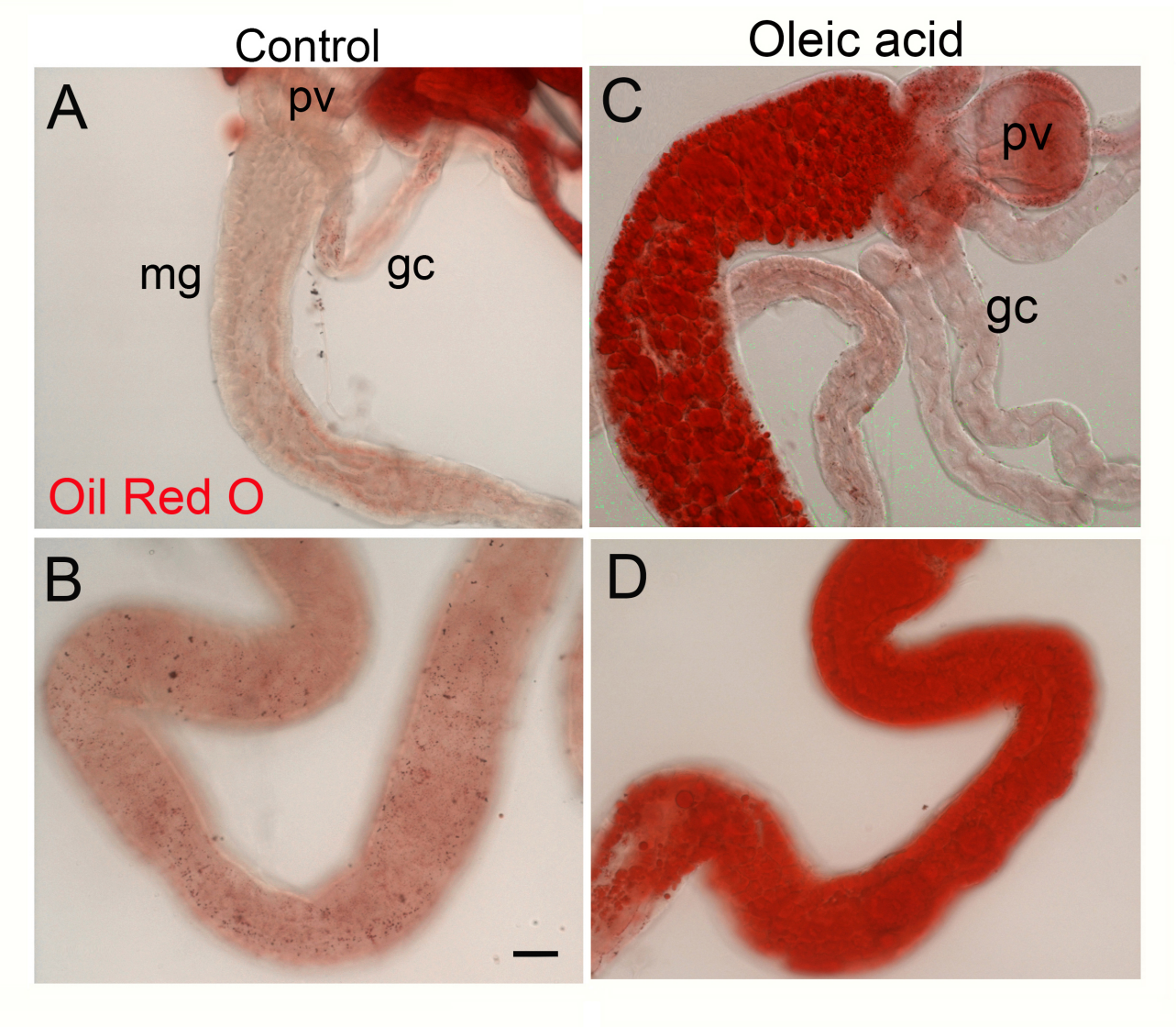

Figure 21. Larvae fed a high fat diet of 10% oleic acid in yeast paste accumulate lipid droplets in the midgut. Oil Red O staining of lipid droplets in dissected preparations of wild-type larvae fed a standard diet of yeast paste and a high fat diet of oleic acid in yeast paste. Larvae fed yeast paste have few lipid droplets in the anterior midgut (A) and posterior midgut (B). Larvae fed a high fat diet for several hours have an increased number of lipid droplets in the anterior (C) and posterior midgut (D). Scale bar is 50 μm; mg=midgut, gc=gastric caecae; pv=proventriculus.

To get a better understanding of these effects, I asked whether the LDs detected by Oil Red O staining were a result of an increase in number, size or both. Individual LDs of the midgut epithelium are not easily identified by Oil Red O staining after the addition of oleic acid to diet. The staining pattern is solid red, similar to that of the fat storage organ, the fat body. Because of this, I analyzed the LD morphology with both DIC microscopy and Nile Red staining.

Nile Red, similar to Oil Red O, is a lipophilic dye that has been used extensively in fixed tissue preparations for detecting of neutral lipids (Greenspan *et al.*, 1985). I performed Nile Red staining on midgut tissues of larvae fed oleic acid for various times. Interestingly, there was a substantial increase in both number and size of LDs after only 4 hrs of oleic acid feeding (Fig. 22). Very few, small LDs were found in control preparations in both the anterior (Fig. 22 A & B) and posterior midgut (Fig. 22 G & H). The LDs increase gradually in size after oleic acid feeding, growing from ~ 1um in diameter to ~8um after 4 hrs (Fig. 22 C, D, I, J) and to extremely large > 20um after 20 hrs of continuous feeding (Fig. 22 E, F, K, L). After 4 hrs of feeding, it seems that the LDs increase in both number and size as a result of oleic acid feeding. I can not distinguish whether the added FFAs are incorporated first into existing LDs, or if they first get incorporated into de novo synthesized droplets, which then fuse to form larger LDs, or both. I suggest that these occur simultaneously: FFAs are efficiently absorbed by the epithelium and get incorporated into both existing and de novo synthesized LDs. Therefore, I conclude that the size of LDs positively correlates to the amount of FAs that is absorbed by the epithelium, increasing gradually after longer times of oleic acid feeding. The same result was observed with linoleic acid feeding (not shown).

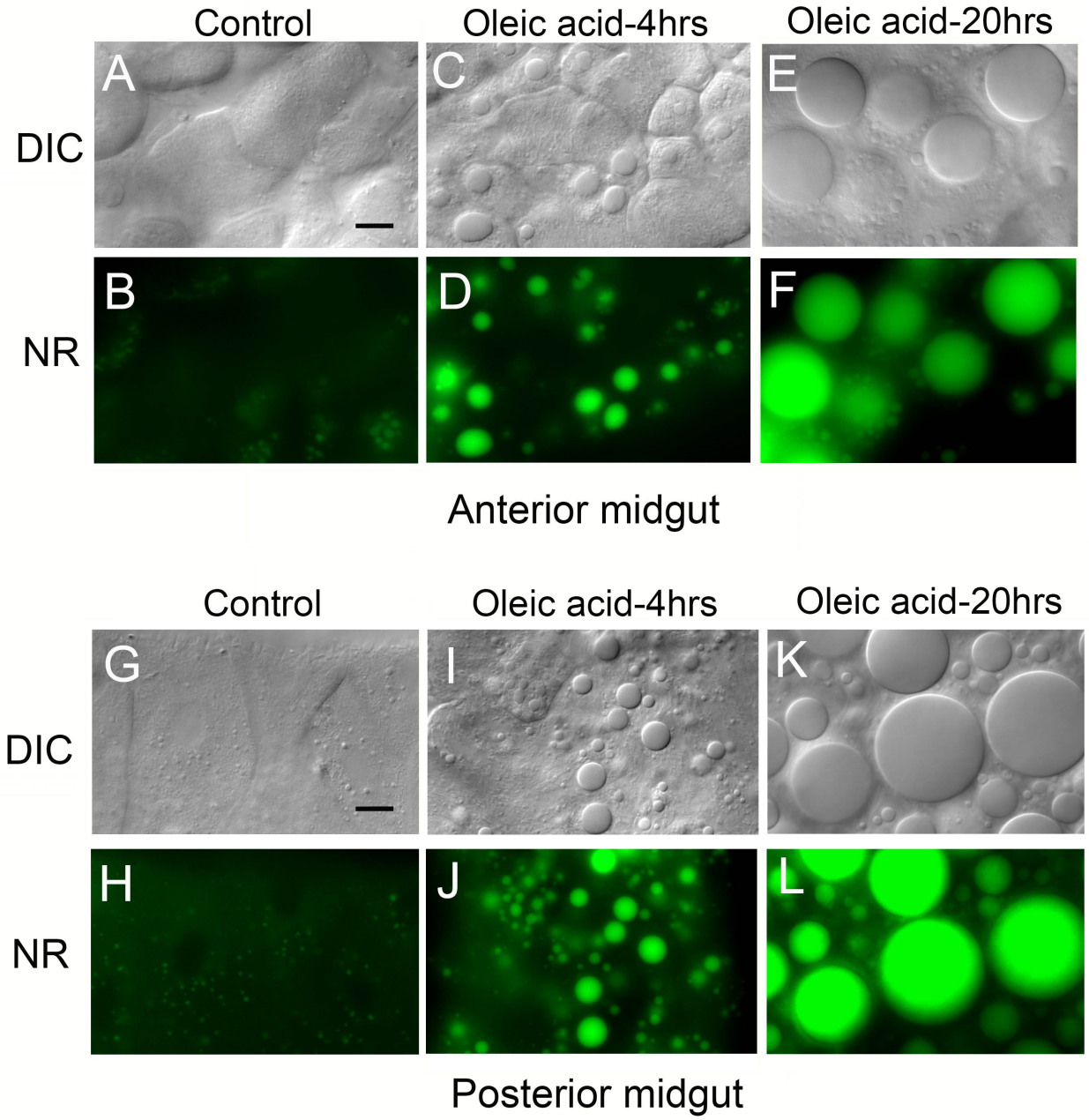

Figure 22. Added dietary FFAs leads to an increase in number and size of lipid droplets in the midgut epithelium. Shown here are representative images of dissected midgut tissues taken with DIC and microscopy after Nile Red (NR) staining. Larvae fed yeast paste have few, small lipid droplets in the anterior (A & B) and posterior (G & H) . The LDs increase gradually in size after oleic acid feeding, growing from ~ 1um in diameter to ~8um after 4 hrs of oleic acid feeding. Shown are DIC and Nile Red staining of anterior (C & D) and posterior (I & J) midgut. After 20 hrs of continuous feeding, the LDs are extremely large, > 20 µm in diameter, in both anterior (E & F) and posterior (K & L) midgut. Scale bar is 10 µm.

Interestingly, although both the anterior and posterior regions of the midgut show a remarkable capacity to absorb FFAs and incorporate them into LDs, they do so at distinct rates. I found that LDs accumulate initially in the posterior midgut. After 1.5 hrs of oleic acid feeding, very few LDs were present in the anterior midgut (Fig. 23 C). However, LDs accumulated in the posterior midgut (Fig. 23 D). After 16 hrs of continuous oleic acid feeding, LDs accumulated in both anterior (E) and posterior (F) midgut. The data shown here suggest that the regions of larval anterior and posterior midgut differ in rate of lipid accumulation.

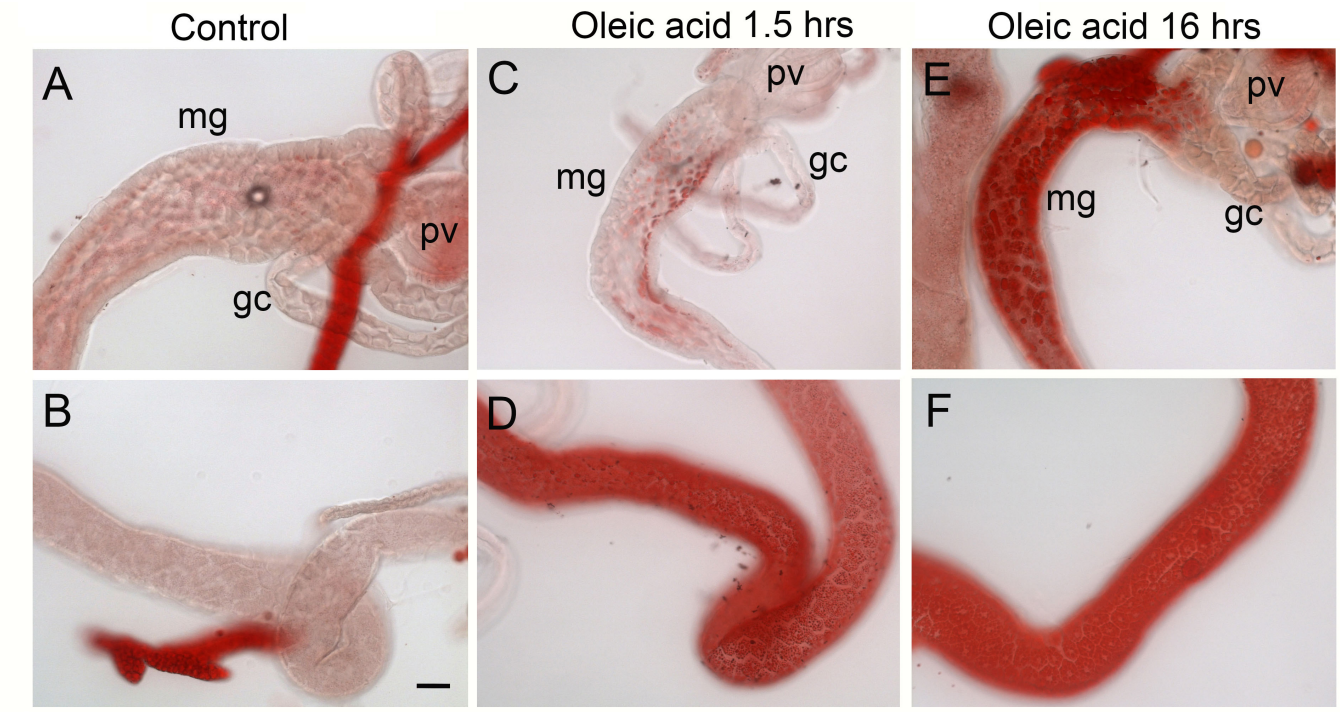

Figure 23. The accumulation of lipid droplets after oleic acid feeding starts in the posterior midgut. Oil Red O staining of lipid droplets in dissected preparations of larvae fed a yeast paste diet (A,B) and a diet of 10% oleic acid in yeast paste for 1.5 hrs (C,D) and for 16hrs (E,F). Larvae fed oleic acid for 1.5 hrs have an increased number of lipid droplets in the posterior midgut (D). After 16 hours of continuous oleic acid feeding, both the anterior and posterior midgut accumulate lipid droplets (E,F). Scale bar is 50 µm; mg=midgut, gc=gastric caecae; pv=proventriculus.

III.4 Added complex lipids, such as triacylglycerols, are not as efficiently absorbed by the midgut epithelium

After finding that FFAs are promptly absorbed and incorporated into LDs, I asked whether the same occurs with more complex lipids, such as TAGs. To address this, I performed feeding experiments on larvae by adding a 10% concentration of coconut, canola, sesame or peanut oils to yeast paste. I then performed Oil Red O staining experiments after 20 hrs of continuous larval feeding (Fig. 24). Oil Red O staining experiments showed that after oil feeding, there was an increase in the amount of LDs in the midgut epithelium. However, the increase in LD abundance, was not nearly as dramatic as that resulted after FFA addition. This result suggests that food oils, which are mostly composed of TAGs, are not efficiently absorbed and processed by the midgut epithelium of Drosophila larvae (Fig. 24). Oil droplets in the lumen of the gut, that were not absorbed by the epithelial cells, were readily distinguishable from the LDs (Fig. 24, arrow) found in epithelial cells.

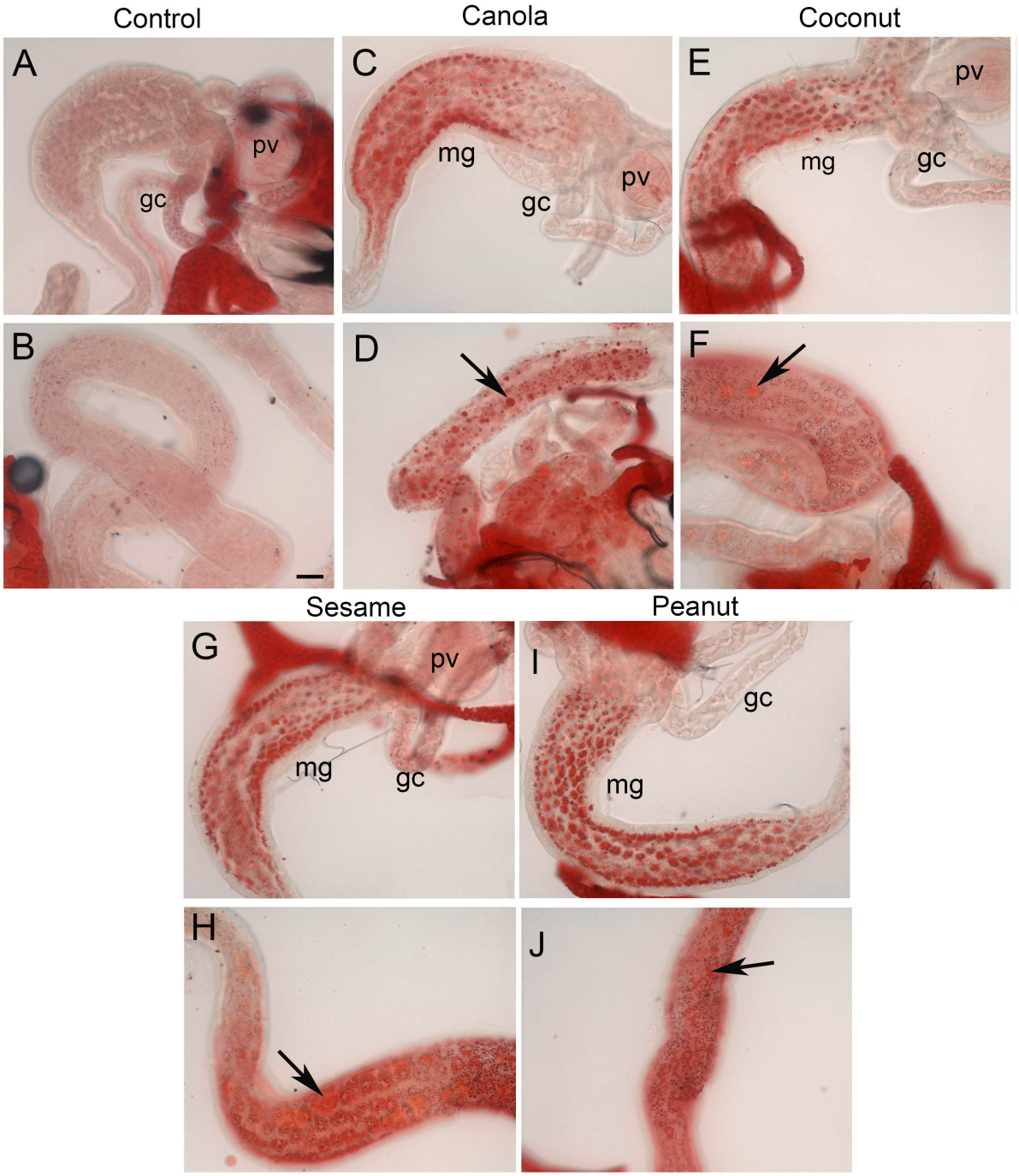

Figure 24. Larvae fed a high fat diet of 10% food oils in yeast paste accumulate a small amount of LDs in the midgut. Oil Red O staining of lipid droplets in dissected preparations of larvae fed diet of yeast paste (A,B) and 10% food oils in yeast paste (C-J). Larvae fed10% canola (C,D), coconut (E,F), sesame (G,H) or peanut oil (I,J) accumulate few LDs in the midgut. The arrows represent oil droplets found in the lumen of the gut. Scale bar is 50 µm; mg=midgut, gc=gastric caecae, pv=proventriculus.

It has been proposed in mammalian studies that enterocytes absorb FFAs and not complex TAGs (Niot *et al.*, 2009). Because of this, I asked if lumenal gastric lipase was rate-limiting in the TAG uptake process in the Drosophila larval midgut epithelium. I therefore added 10-40 units porcine lipase to yeast paste containing 10% olive oil and analyzed the effect on midgut LDs after 20 hrs of feeding with Oil Red O staining. After the lipase was added, the epithelial cells efficiently absorbed the lipids found in olive oil. Oil Red O staining experiments showed that while LDs were not present in the midgut epithelium of larvae fed olive oil, they accumulated in the midgut of larvae fed olive oil and gastric lipase in both the anterior (Fig. 25 E) and posterior midgut (Fig. 25 F). The extent of the accumulation of LDs was similar to that of added FFAs. This finding suggests that the complex lipids found in olive oil, mostly TAGs, became hydrolyzed by the gastric lipase into FFAss, crossed the PM of midgut epithelial cells and became incorporated into cytoplasmic LDs. This data raises the possibility that Drosophila may metabolize lipids more efficiently in nature if more lipase would be present in the lumen of the larval midgut.

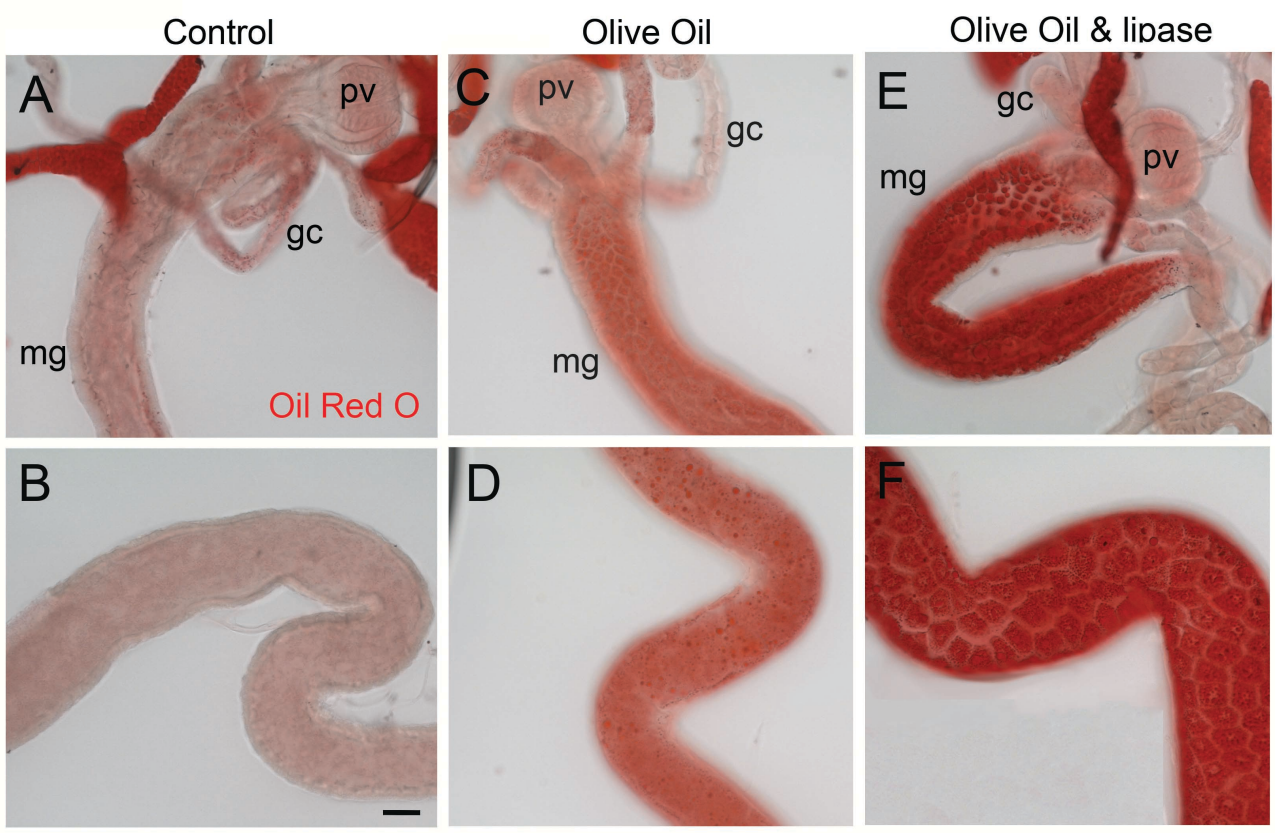

Figure 25. Larvae fed a high fat diet of 10% various oils in yeast paste do not accumulate lipid droplets in the midgut because insufficient gastric lipase is available to hydrolyze TAGs. Oil Red O staining of lipid droplets in dissected preparations of larvae fed diet of yeast paste (A,B), larvae fed olive oil in yeast paste (C, D) and larvae fed a diet of units lipase in olive oil and yeast paste (E, F). While larvae fed a diet of 10% olive oil in yeast paste for several hours do not accumulate lipid droplet in the midgut, larvae fed lipase in olive oil do (E, anterior midgut; F, posterior midgut). micron bar is 50 µm; mg=midgut, gc=gastric caecae; pv=proventriculus.

III.5 Added free fatty acids to diet are efficiently mobilized from lipid droplets in the midgut epithelium

After finding that FFAs were efficiently absorbed and incorporated in LDs, I asked if they were also efficiently mobilized after a chase period in the absence of added FFAs. I fed larvae 10 % oleic acid in yeast paste for 20 hrs and then switched the diet to a standard diet of yeast paste for an additional 24 hrs. I performed Oil Red O staining to analyze the abundance of LDs. Larvae fed oleic acid and chased with yeast paste had very few LDs in both the anterior (Fig. 26 E) and posterior midgut (Fig. 26 F). This discovery indicates that the LDs accumulated as a result of added dietary FFA were efficiently mobilized by these cells. Added FFAs are not only efficiently absorbed and incorporated into LDs, but are also efficiently mobilized in the midgut epithelium. Similar results were obtained with as little as 9 hours of chase time (not shown).

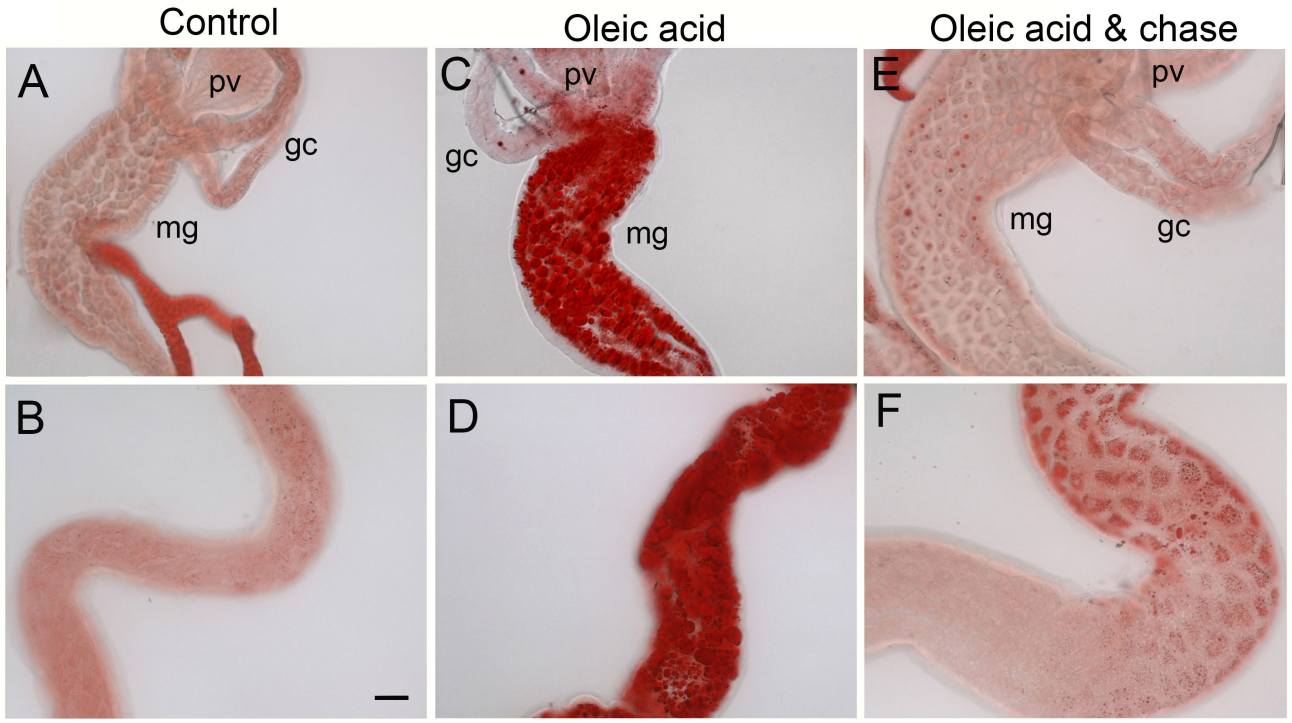

Figure 26. Added FFAs to diet are efficiently mobilized by the midgut epithelium. Larvae fed a high fat diet of 10% oleic acid in yeast paste for 20 hours were switched to a regular yeast paste diet for 24 hrs. Oil Red O staining of lipid droplets in dissected preparations of wild-type larvae fed a yeast paste (A, anterior midgut; B, posterior midgut), larvae fed oleic acid (C, anterior midgut; D, posterior midgut) and larvae fed oleic acid and switched to a diet of yeast paste (E, anterior midgut; F, posterior midgut). As shown previously, larvae fed yeast paste have few lipid droplets (A,B) in the the midgut and larvae fed oleic acid for several hours accumulate lipid droplets in the midgut (C,D). Larvae switched to a yeast paste diet after oleic acid feeding have few lipid droplets in the midgut (E,F). Scale bar is 50 μm; mg=midgut, gc=gastric caecae; pv=proventriculus.

III.6 LSD2 promotes lipid droplet biogenesis in midgut epithelial cells

Knowing that the *LSD2* gene is expressed in the midgut (Gronke *et al.*, 2003) I wanted to visualize the intracellular localization of LSD2 *in vivo*. Expression of an LSD2-EGFP fusion protein was targeted to the larval midgut by using the Gal4/UAS system in conjunction with a midgut-specific Gal4 driver, MexII-Gal4. Expression of UAS-LSD2-GFP driven by MexII-Gal4 resulted in an increase in LD number. The LDs were detected with both DIC microscopy and GFP fluorescence (Fig. 27 C,D) in larvae over-expressing LSD2. They were absent in the parallel preparations of control larvae not expressing UAS-LSD2-GFP (Fig. 27 A).

Additionally, I analyzed the effects added FFAs have on the appearance of LDs present in the midgut epithelium as a result of LSD2 over-expression. Nile Red staining experiments were performed on LSD2 over-expressing larvae fed oleic acid in yeast paste for 20 hrs. Interestingly, I found that the lipid droplets increase significantly in size and that as they do, they lose the LSD2-GFP signal from their surface (Fig. 27 E,F). The effect oleic acid had on the size of LDs of LSD2-GFP over-expressors was similar to that of wild type control larvae.

Oil Red O staining verified that these LSD2 positive structures were filled with lipids (Fig. 28). Preparations from control larvae showed wild-type staining in the anterior (Fig. 28 A, C) and posterior midgut (Fig. 28 B, D). Larvae over-expressing LSD2 showed a dramatic increase in Oil Red O staining in both the anterior (E) and posterior midgut (F). These results were further validated by Nile Red staining (not shown). Thus, LSD2 over-expression in the midgut leads to accumulation of LDs.

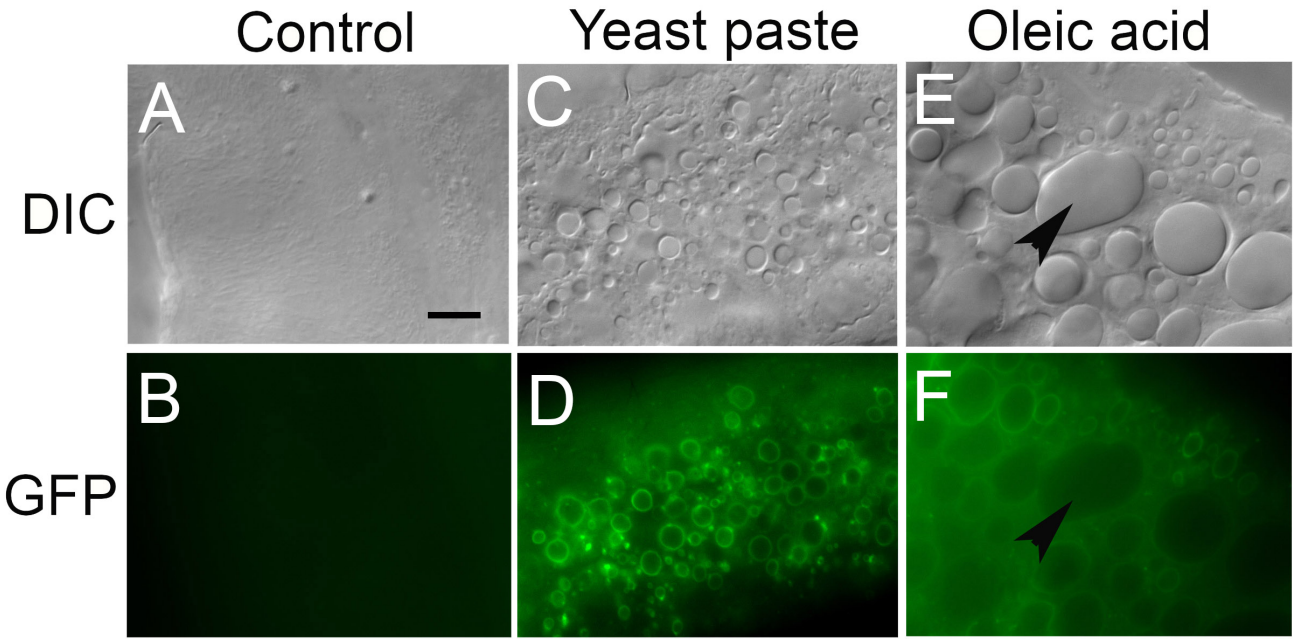

Figure 27. LSD2 over-expression in the midgut leads to the appearance of small spherical structures which appear to be lipid droplets. Shown are dissected midgut tissues taken DIC microscopy and GFP fluorescence of midguts from larvae expressing UAS-LSD2-GFP in the midgut with MexII-Gal4. Larvae over-expressing LSD2 have spherical structures in the midgut that are visible with both DIC and GFP in the midgut of larvae fed a common diet of yeast paste (C, D) and larvae fed a high oleic acid diet (E, F). The structures lose the GFP signal as they increase in size after oleic acid feeding (E,F arrowhead). These structures are not present in control preparations (A, B). Scale bar is 10 μm.

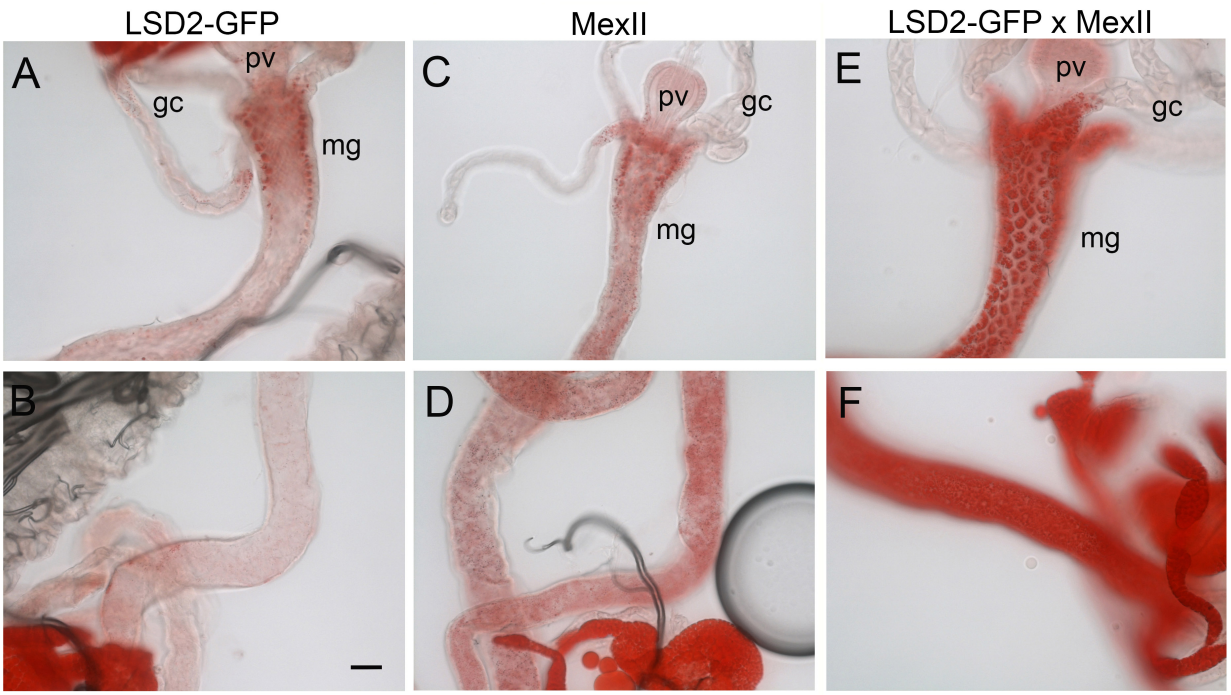

Figure 28. The spherical structures observed resulted from LSD2 over-expression are lipid droplets. Oil Red O staining images of dissected midgut tissues of larvae expressing UAS-LSD2-GFP in the midgut with MexII-Gal4. UAS-LSD2-GFP and MexII-Gal4 parental lines were used as control. Control larvae have few lipid droplets in the midgut (A-D). Larvae over-expressing LSD2/Plin2 have many small lipid droplets in the midgut (C, D). Scale bar is 50 µm; mg=midgut, gc=gastric caecae, pv=proventriculus.

III.7 Evidence that the midgut epithelium undergoes adaptation after addition of free fatty acids to diet

I define adaptation as the sum of cellular responses, in the form of physiological, morphological and functional changes in lipid metabolism (lipid uptake, storage and mobilization), that occur in the midgut epithelium as a result of dietary changes. To further characterize the effect oleic acid feeding has on midgut LDs, I performed Oil Red O staining on larvae after various times of oleic acid feeding. To accurately analyze the effect, I classified the staining intensity into three levels (Fig. 29 B). Level 1 is the lowest level of Oil Red O staining. This level of staining is observed in midgut preparations of larvae fed a standard yeast paste diet. Level 2 staining is a mid Oil Red O staining level. Level 2 is significantly higher than Level 1. The epithelial cells contain many, but are not completely filled with, LDs. Level 3 is the highest amount of staining. The cells appear completely filled with LDs and the Oil Red O staining pattern has a nearly solid red appearance, similar to that of the fat body tissue. I counted the number of larvae that showed the three levels of Oil Red O staining and calculated the percentages of larvae found in each category after various times of oleic acid feeding (Fig. 29). I completed the analysis by comparing the percentages of larvae representing each Oil Red O staining level (Fig. 29). Interestingly, after prolonged feeding (>27 hrs), a lower percentage of larvae had the highest level of Oil Red O staining intensity. After 3.5 hrs of oleic acid feeding, 8% of larvae accumulated level 3 staining, and after 14 hrs, 85 % of larvae did. The highest percentage of larvae accumulate the highest amount of LDs after 14-20 hrs of oleic acid feeding. After 41 hrs of continuous oleic acid feeding, 28 %

of larvae showed level 3 Oil Red O staining. Because of these findings, I suggest that Drosophila larvae may undergo adaptation after oleic acid feeding.

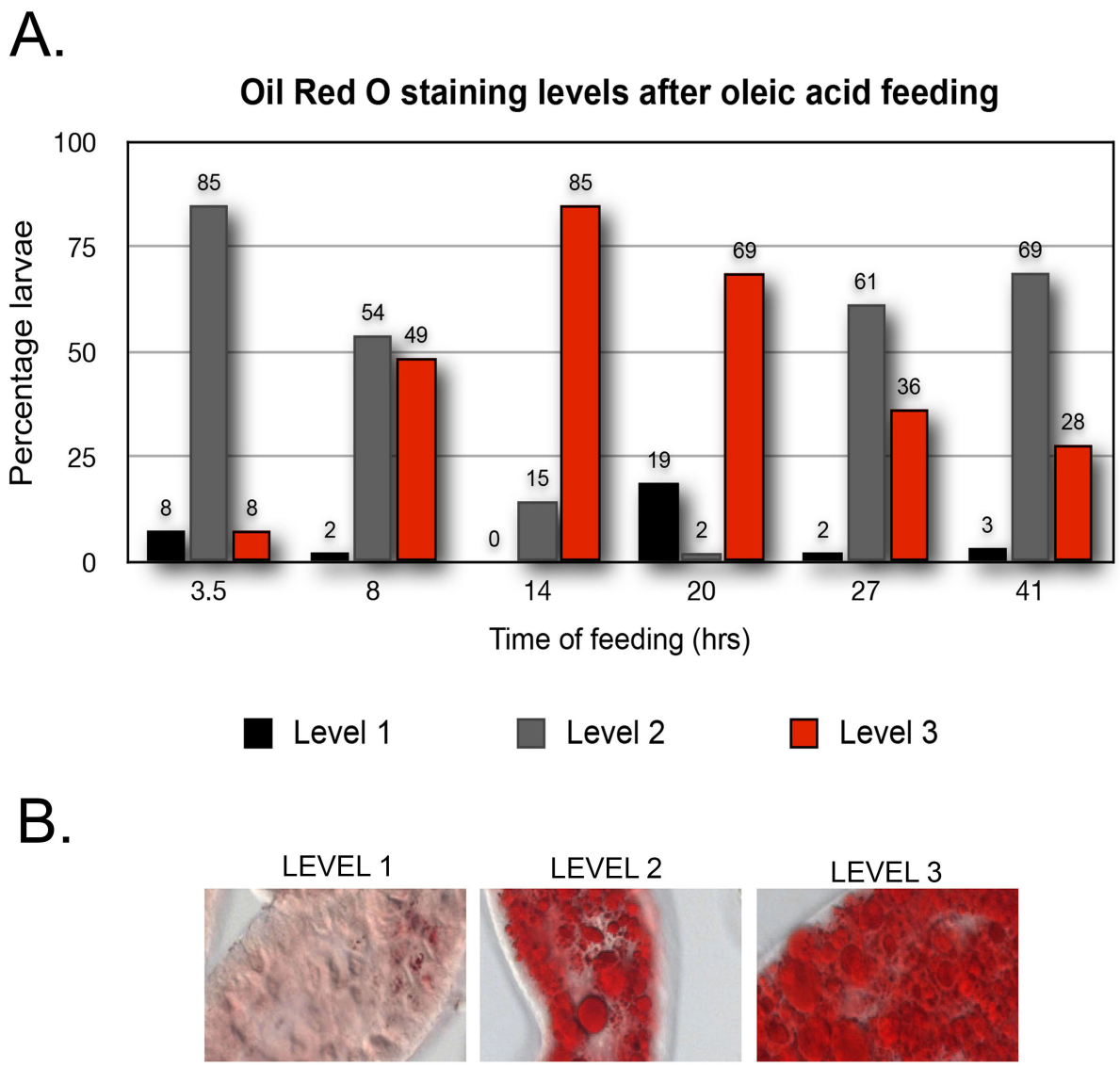

Figure 29. Larvae fed oleic acid for prolonged periods of time (<27hrs) seem to undergo adaptation. Larvae were fed oleic acid for various hrs and scored according to the Oil Red O level present in the midgut. Chart in A represents the percentages of larvae that show the three levels of staining after various times of oleic acid feeding. Representative images of Oil Red O levels are shown in B. A smaller percentage of larvae accumulate lipid droplets after prolonged feeding than after several hours of oleic acid feeding.

CHAPTER IV. Discussion

IV.1 Genetic studies of Spectrin in the larval fat body of *Drosophila melanogaster*: Evidence for a novel lipid uptake apparatus

Spectrin and its binding protein, Ankyrin, have long been associated with several genetic diseases, such as spinocerebellar ataxia and anemia. Most recently, it has been proposed that they exert unconventional effects, in addition to their effects on plasma membrane structure and function, in the secretory pathway, on intracellular traffic (Kizhatil *et al.*, 2007, 2009; Ayalon *et al.,* 2008; Stabach et al; 2008; Clarkson *et al.*, 2010; Tjota *et al.,* 2011; Lorenzo *et al.*, 2010; deMatteis & Morrow, 2000). Here, I used a genetic approach to further elucidate the contribution of α and β spectrin to the architecture of the plasma membrane in the larval fat body. Unexpectedly, I found that spectrin exerts multiple effects on lipid translocation pathways.

Previous analysis showed that the PM of the fat body surrounds a class of small LDs found at the cortex of the fat cells and that these small LDs were dependent on β spectrin (Mazock, 2010). Because of these observations, it was believed that the PM forms elongated “finger-like” structures that contain small LDs and become invaginated due to the pressure exerted by the basement membrane at the ecto domain of fat cells. Following up on these observations, I further studied the effects Spectrin exerts in the fat body by analyzing the effects of α spectrin knockdown, α spectrin over-expression and β spectrin over-expression. Additionally, I further studied the morphology of the fat body surface using TEM and SEM microscopy.

Two major insights emerged from these studies. First, regarding the cortical LDs, I showed that they correspond with the previous LDs characterized by their small size and distinct LSD2 composition relative to other larger LDs present in the same cells (Bi *et al.*, 2012). I also showed that the cortical LDs rely not only on β spectrin, but also on α spectrin, and I propose that Spectrin functioning as a tetramer is required in the larval fat body. Second, the effects resulting from the perturbations of Spectrin have thus far been believed to occur through loss of function, or in several cases, through dominant negative effects. I show here that β spectrin exerts hypermorphic effects on intracellular traffic in the Drosophila larval fat body. These results establish for the first time that β spectrin hypermorphic effects, that are distinct from loss of function, can also be disruptive to secretory traffic.

IV.1.1 A model for lipid uptake in the fat body

The uptake of dietary lipids is critical to the development of Drosophila. The lipids are stored as energy reservoirs to be used as nutrients and fuel the animal through metamorphosis and survival through the late larval and early adult periods. The fly Lpp, which is related to the mammalian lipoproteins (such as chylomicrons and VLDL), plays the critical role of transporting dietary lipids from the digestive tract to the fat body for storage (Panakova *et al.*, 2005; Palm *et al.,* 2012). The mechanism of Lpp function is not entirely understood. It is however established that Lpp traverses the hemolymph to the midgut to acquire dietary lipid cargo (mostly diacylglycerol and esterified sterols) and it circulates back to the fat body to be unloaded and recycled. As it is unloaded, Lpp can be endocytosed by fat body cells (e.g. Dantuma *et al.*, 1998) or it can unload its

lipid cargo without endocytosis (Canavoso *et al.*, 2001). Whether only one out of the two or both proposed mechanisms occur, lipid cargo has to cross the lipid bilayer (of the PM or of the endosome) and lipids have to be packaged into the hydrophobic core of cytoplasmic LDs.

Data presented in chapter II shows that: 1) targeted knockdown of either α or β spectrin leads to loss of small cortical LDs from the fat body; 2) the cortical LDs disappear after starvation, which suggests they are a transient intermediate in the transport of dietary fat to larger LDs found deeper in the cell; 3) the cortical LDs correspond to the LSD2 bearing LDs described previously; 4) knockdown of LSD2 eliminates the cortical LDs; 5) knockdown of β spectrin eliminates the population of small LSD2 bearing LDs. These lines of evidence along with the EM evidence showing that the cortical LDs are intimately associated with the PM (Mazock Ph.D. thesis, 2010) suggest that a lipid uptake apparatus exists at the ecto domain of the fat body cells and that this apparatus depends on the function of α2β2 spectrin tetramers. The model I propose accounts for the unloading of Lpp that is docked outside the fat cells without the need of endocytosis.

EM analysis showed that the small cortical LDs seem to be intimately associated with the PM over most of their surface. Only a thin rim of cytoplasm was observed between the LD surface and the PM (Mazock Ph.D thesis, 2010). This seems energetically unfavorable because while the cytosol is hydrophilic, the LD core is hydrophobic. Thus, the intimate association between cortical LDs and the PM raises the possibility that an active mechanism is linking the PM bilayer and the phospholipid monolayer of the LDs together. The cortical LDs are dependent on Lpp activity

(Panakova *et al*., 2005) and are transient, being lost after starvation. Because of this, the cortical LDs seem to be a PM intermediate in the lipid uptake process. I propose that a lipid translocation apparatus is found at the PM of fat cells, where the α2β2 spectrin tetramer connects the process of lipid uptake from extracellular Lpp with that of lipid storage by stabilizing lipid transporters at the PM and by binding to LD-associated proteins (such as LSD2) at the surface of the cortical LDs. This specific linkage would enable the direct translocation of non-polar lipid molecules from outside the cell to the LD interior, bypassing the need of their passage through the polar cytoplasm.

Additionally, data showing that the cortical LDs disappear as a result of starvation raises the possibility that they may serve as intermediates in the lipid uptake process at the PM. Relatively little is known about the origin of LDs. It is believed that they are synthesized within the leaflets of the ER (Martin and Parton, 2006). The data presented here suggest that the cortical LDs may be arise from a progenitor in the ER with LSD2 at their surface. As LSD2 is known to associate primarily with small (<4 um) LDs, I propose that as the cortical LDs bud off of the ER, they become in close association with PM either through a direct or indirect interaction of LSD2 with the Spectrin tetramer, forming a stable complex. The presence of LSD2 on the LDs could trigger the initial association with Spectrin on the PM and the two surfaces ultimately come together into a tightly associated complex. The cortical LDs could grow until they reach a threshold that triggers dissociation of LSD2 (Fig. 30, bottom), allowing the LD to dissociate from the plasma membrane, presumably making it available for fusion with larger lipid droplets deeper in the cytoplasm.

Two interesting observations were reported in mammalian studies by Gao and Serrero (1999): 1) PLIN2 was associated with the PM, a physical location where FAs uptake occurs and 2) expression of PLIN2 increased resulted in increased FA uptake in transfected COS-7 cells. As these findings seemed to be consistent with a putative role of PLIN2 in FA transport, it was speculated that PLIN2 is involved in carrier-mediated FA influx into cells (Gao and Serrero, 1999). These observations are consistent with my proposed model of lipid uptake apparatus at the PM of the larval fat body ecto domain of Drosophila. No studies thus far have taken into account the location of LDs in relation to the PM. It should be addressed whether PLIN2 is associated with LDs at the PM and whether such a lipid uptake apparatus at the PM exists in mammals as well.

The apparatus of lipid uptake at the PM seems to be dispensable. I showed here that perturbing the cortical LDs leads to no detectable effects on the organism. Contrary to loss of Lpp, loss of either α or β spectrin in the fat body does not lead to accumulation of LDs in the midgut epithelium. This suggests that lipid uptake at the fat body continues and that an alternative mechanism mediates the unloading of Lpp particles and the transferring of their contents into the fat cell when the lipid uptake apparatus at the PM is compromised. Three possibilities may account for this: 1) endocytosis of Lpp may facilitate the transfer of the cargo to larger LDs found deeper in the cytoplasm; 2) an independent pathway of Lpp unloading to larger LDs (either via endocytosis or not) may occur; 3) dietary lipids may be directed to an alternate site, such as oenocytes, which were previously shown to be a Lpp destination during starvation (Gutierrez *et al.*, 2007).

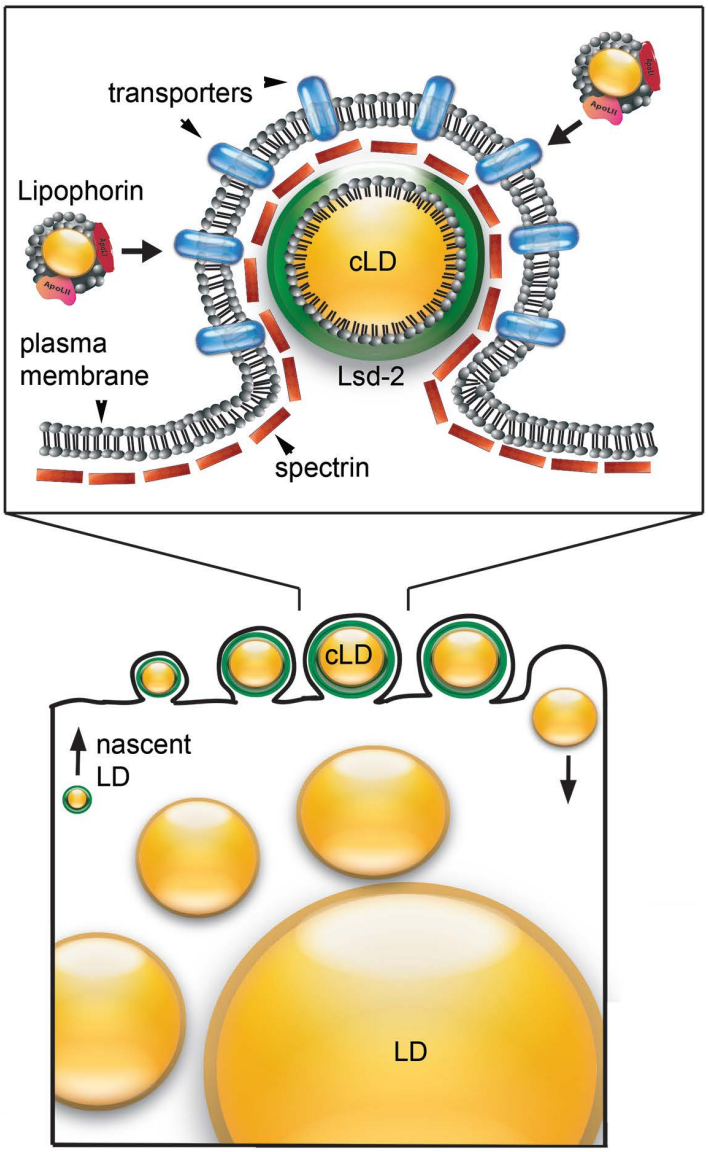

Figure 30. Model for lipid uptake at the fat body surface. Cortical lipid droplets (cLD) in the larval fat body are intimately associated with the plasma membrane in surface protuberances (top). We propose that this structure represents a lipid uptake apparatus that carries lipid cargo from extracellular Lpp to the hydrophobic core of the cytoplasmic LD. The PM is uniformly lined with αβ spectrin (red rectangles) and the surface of the small cortical LDs is covered with the perilipin LSD2 (green). These proteins seem to be part of a stable complex that links lipid transport proteins in the PM to the cLD. Nascent LDs are thought to arise from the ER membrane. The presence of LSD2 on these LDs could directly or indirectly trigger initial association with spectrin on the PM and ultimately to zip the two surfaces together into a tightly associated complex. LD growth may proceed until the LD reaches a size that causes dissociation of LSD2, allowing it to fall away from the PM and fuse with larger LDs in the cytoplasm.

IV.1.2 A model for the morphology of fat cell surface

The morphology of fat cells is relevant when considering the mechanisms of lipid uptake, storage and mobilization. Because the large and rigid hydrophobic LCFAs and cholesterol esters are unable to easily cross the lipid bilayer, efficient lipid cargo uptake at the PM could involve transport through lipid transporters. I propose that lipid transporters could be located at specialized membrane domains that contain the LD-associated protein LSD2 and Spectrin.

The SEM images depicting the fat body surface and the TEM images previously described (Mazock Ph.D. thesis, 2010) suggest that the PM of fat cells forms protuberances that are specialized membrane domain structures. The presence of the structures at the ecto domain of the fat body gives the tissue a cauliflower-like appearance, as observed with SEM. Interestingly, the structures seem to resemble inverted caveolae, which are flask-shaped invaginations of the PM composed of several proteins, most of which are caveolins (Parton, 1994). Caveolae are extremely abundant in mammalian adipocytes, where they may bind to fatty acids and may also associate with LDs. It has been proposed that the structures may function in lipid regulation, playing an important role in regulating lipid trafficking to lipid droplets (Parton and Simons, 2007). It seems possible then that the surface protuberances of the PM and caveolae have similar roles in fat cells. Just as caveolins may function as scaffolding proteins within caveolar membranes in adipocytes, the Spectrin-LSD2 (and possibly other proteins) complex may concentrate and stabilize particular fat transporters and other proteins at the PM in specialized domains. The nature and protein composition

(possible lipid enzymes, transporters and other proteins) of the PM domains found at the ecto domain of the fat body tissue should be studied in further detail.

Whether the morphology of the ecto domain of the fat tissue directly influences the formation of the stable complexes of proteins at the PM, or vice-versa, is not known. I suggest that the distinct morphology of the protuberances is influenced by the availability of both incoming dietary lipids and of lipid transporters. Because this distinct morphology is peculiar to third instar larvae, an increase in surface area of the fat tissue may account for the specialized morphology of the PM and for the configuration of this lipid uptake apparatus at the PM. As both the PM structures and cortical LDs disappear as a result of starvation (which implies a transient nature), I believe that the cauliflower-like morphology influences the dynamic nature of the lipid processing mechanisms. Thus, the PM morphology, along with its protein composition, of fat cells may be important factors in the processes of lipid uptake and storage. These suggestions should be addressed in further research.

IV.2 Distinguishing gain of function and loss of function effects of Spectrin mutations

The second major insight that emerged from this study is that β spectrin over-expression produces gain of function effects on intracellular traffic, that are not observed with loss of function. I showed here that over-expression of β spectrin does not appear to exert its effect as a dominant negative (antimorphic) and that the phenotypes are a direct consequence of wild-type β spectrin abundance. β spectrin over-expression leads to: 1) loss of cortical LDs from the fat body and 2) accumulation

of LDs in the posterior midgut. When expressed at higher levels, β spectrin over-expression leads to: 1) lethality, 2) accumulation of LDs in both the anterior and posterior midgut, 3) defect in Lpp secretion from the fat body. Interestingly, I also mentioned that these parameters were not altered by β spectrin knockdown (lethality, midgut lipid accumulation, Lpp secretion). As these over-expression phenotypes do not result after β spectrin loss of function, I suggest that they are gain of function, being caused by an increase rather than a decrease in β spectrin function.

Additionally, the finding that the severity of the phenotype is dependent on the amount of wild-type β spectrin suggests that the gain of function phenotype could be hypermorph. I describe this as a hypermorphic mutation because it occurs as a result of an increase in wild-type gene dose, where the phenotype is worsened by increasing the gene product and vice versa. Another piece of data in accordance with this speculation is that when β spectrin was over-expressed in a lower α spectrin background, it led to increased severity of the phenotype resulted from β spectrin over-expression. Alternatively, the phenotypes resulted from β spectrin over-expression could be neomorphic, where the protein gains a novel function. This is unlikely because changing the wild-type gene dose has no effect on the phenotype of a neomorph (Muller, 1932). Because of this and because of my findings, I suggest that the effects of β spectrin over-expression are hypermorphic.

Unlike β spectrin over-expression, α spectrin over-expression in the fat body has no effect on plasma membrane morphology, lipid accumulation in the midgut or survival rate (not shown). Interestingly, similar to over-expression, loss of α spectrin in the fat body also has no detectable effects on the organism. Because of this, α spectrin seems

not prone to hypermorphic effects in the Drosophila fat body. Because the hypermorphic effects are specific to β spectrin, where the best-characterized functional sites are found (Ankyrin-binding, Actin-binding, PH domain), it is possible that one of these sites acquires an unconventional behavior. Which functional site(s) is(are) responsible for the peculiar hypermorphic effects of β spectrin should be addressed in future studies.

IV.3 Future perspectives: the mechanism by which α spectrin regulates the function of β spectrin

The evidence presented here shows that β spectrin over-expression exerts hypermorphic effects on Lpp secretion from the larval fat body and that these effects are eliminated when α spectrin is over-expressed with β spectrin. This data therefore uncovers a novel link between Spectrin over-expression and secretion of Lpp from the fat body. Three questions that should be addressed in future studies are: 1) does β spectrin over-expression affect all secreted markers in the fat body or just Lpp?, 2) which, if any, of the known functional sites of β spectrin is(are) responsible for the hypermorphic effects on secretion? and 3) which functional sites of α spectrin is(are) responsible for the rescue?

Answering these questions would provide useful information in further understanding the nature of the effects Spectrin exerts on lipid translocation pathways in the fat body. Additionally, identifying the functional site(s) of α spectrin that is(are) responsible for rescuing the toxic effects would provide a step into further understanding the exact mechanism of Spectrin function. It is possible that one, or several, of the known α spectrin functional domains rescue the toxic effect. On the other hand, it is also

possible that none of the known α spectrin functional domains, but other domains, could rescue Lpp secretion. Finding the specific repeat(s) and domain(s) of α spectrin that are sufficient for rescuing Lpp secretion from the fat body might provide useful insight into understanding the process of Lpp secretion and might lead to identifying novel functional domains of Spectrin. This can be addressed by over-expressing specific regions of α spectrin with β spectrin in the fat body and analyzing the effects with the techniques described in this thesis.

IV.4 Intracellular lipid translocation in the larval midgut: evidence that LSD2 is a regulator of lipid homeostasis

IV.4.1 The anterior and posterior midgut accumulate lipid droplets at distinct rates

Larvae fed a high fat diet of 10% oleic acid in yeast paste for several hours (shown here, 20 hrs) accumulated a vast amount of LDs in the midgut. Oil Red O staining indicated a dramatic increase in LD abundance in both the anterior (Fig. 23 C) and posterior (Fig. 23 D) midgut cells. This finding is consistent with the suggestion that LCFA adsorption is thought to be thermodynamically favorable and extremely fast (Zakim *et al.*, 2000; Hamilton *et al.*, 2002). Evidence presented here shows that the anterior and posterior midgut accumulate LDs at distinct rates after oleic acid feeding. LDs accumulate first in the posterior midgut and second in the anterior midgut (Fig. 23).

The distinct rates of accumulation of lipids in the anterior and posterior midgut could be due to a difference in gene expression. It has already been determined that the three major regions of the midgut have specialized structures and distinct ion gradients,

which correlate with distinct functions (Dimitriadis and Kastritsis, 1984; Dimitriadis, 1991; Dubreuil, 2004; Dubreuil *et al*., 1998; Dubreuil *et al*., 2001). It has also been shown that the posterior midgut cells have a larger volume than of anterior midgut cells, and that the digestive enzyme magro (Sieber and Thummel, 2012) and a-amylase (Thompson *et al*., 1992) are restricted to specific regions of the midgut. Additionally, a recent study by Marianes and Spradling (2013) proposed that the midgut displays a striking extensive regionalization into ten parts, each of which differ in their morphology, physiology and gene expression. The regional variation of the midgut is further supported by the evidence I present here, which shows that the posterior accumulates lipids at a faster rate than the anterior midgut.

IV.4.2 Evidence that Drosophila larvae undergo adaptation

The data in chapter III shows that the midgut of Drosophila metabolizes a considerable amount of FFAs. The FFAs are absorbed, incorporated into LDs, and mobilized from LDs. This seems problematic, because the presence of a vast amount of FFAs in cells can be toxic (Andrade *et al*., 2005). Additionally, fewer LDs seem to be present in the midgut epithelium after prolonged (>27 hrs) oleic acid feeding. Because of these data, I speculate that two types of adaptation mechanisms may occur at the midgut epithelium. Adaptation can be thought of as cellular responses occurring through mechanisms involving change in gene expression. The cellular responses can be any physiological, morphological and functional changes in lipid metabolism (lipid uptake, storage and mobilization) that occur in response to dietary changes.

First, midgut cells could adapt to the presence of a vast amount of FFAs in the diet by up-regulating the machinery responsible for lipid uptake and storage. A considerable amount of FFAs were absorbed, incorporated into LDs and mobilized efficiently by the midgut epithelium after oleic acid feeding. This occurred without any adverse repercussions for survival. Because it is known that high concentrations of FFAs may be detrimental to cells, absorbing lipids at a high rate may be an unfavorable cellular process. Hence, I propose that this type of cellular response could increase the likelihood that the animal survives under poor nutritional conditions. Additionally, lipid packaging into LDs may be an initial cellular defense response against potential lipotoxicity. Thus, I propose that midgut cells may undergo adaptation, responding to added dietary FFAs by up-regulating the machinery responsible for absorption and storage of lipids. Along these lines, I speculate that one of the molecules up-regulated as a result of the increased presence of dietary FFAs is LSD2, which would induce LD biogenesis and growth. Evidence that supports this comes from mammalian studies which found that PLIN2 expression was induced upon FFA induction (Bosma *et al.*, 2012). I therefore speculate that LSD2 could be up-regulated after a high fat diet in Drosophila as well. It would be interesting to investigate the effects added dietary fats have on gene regulation *in vivo*.

Second, midgut cells could adapt to prolonged (>27 hrs) exposure to added dietary FFAs by up-regulating the cellular machinery responsible for lipid mobilization from LDs and lipid export. I presented evidence that the extent of LD accumulation decreases after it reaches a plateau. I used Oil Red O staining intensity to categorize the amount of LDs in the midgut after oleic acid feeding. The highest percentage of

larvae that accumulated the highest amount of LDs was observed after 14-20 hrs of feeding. After more than 27 hrs of feeding, a significantly lower percentage of larvae showed the highest amount of LDs in the midgut. This suggests that the lipids are mobilized more efficiently than they are stored in LDs after prolonged feeding. I suggest that the system switches from a bias toward lipid uptake and storage in the midgut to a bias toward lipid export via lipoprotein packaging. This may involve up-regulation of genes encoding proteins involved in lipid mobilization pathways, such as lipases, lipoproteins (LTP, Lpp) and others. If they occur, these adaptation responses could increase the likelihood that the animal survives under changing nutritional conditions.

It is possible that, in the wild, fruit flies have acquired the remarkable capacity to absorb a vast amount of dietary nutrients, when available. This adaptability would then maximize the absorption of essential sterols and FFAs, which display numerous fundamental biological functions. As wild type larvae do not accumulate LDs in the midgut after a standard diet, they seem to adapt to a high fat diet by storing as much energy as possible in the midgut. Interestingly, the midgut is not normally regarded as a storage organ, but as a absorptive/digestive one. Nevertheless, molecular mechanisms responsible for this adaptation should be studied. The expression of genes responsible for the metabolic fate of dietary lipids should be determined, as a better understanding of the mechanisms leading to lipid-dependent intestinal adaptation could provide new therapeutic approaches and, ultimately, decrease prevalence of fat mediated diseases. Knowing what genes become up-regulated/down-regulated as a result of a high fat diet could lead to the discovery of novel candidate genes and could ultimately lead to the designing of new drugs.

IV.5 A model of LSD2 function

It has been reported that LSD2 over-expression leads to an increase in whole organism TAG levels in Drosophila and this effect has been attributed to LSD2 protein level increase specifically in the fat body (Gronke *et al.*, 2003). However, the Gal4 line used to drive expression of PLIN2/LSD2 in the fat body, *Adh*, is highly expressed in midgut cells as well (Fisher *et al.*, 1988). The authors attribute the effects to increased protein level specifically in the fat body without taking into account the level of LSD2 in the midgut. I suggest here a different interpretation, that the increase in TAG level by 49% may be ascribed to increase in LD biogenesis in both the midgut and the fat body. From a mechanistic viewpoint, LSD2 could promote LD biogenesis indirectly by inhibiting TAG hydrolysis, perhaps by physically excluding the TAG lipase enzymes from the LD surface. This type of mechanism was proposed in human embryonic cells where it was suggested that PLIN2 expression increases the cellular TAG stored in LDs by decreasing the association of ATGL and/or other lipase enzymes with LDs (Listenberger *et al.*, 2007). This mechanism where LSD2 protects LDs from lipolysis has been proposed in most Drosophila studies thus far (Gronke *et al.*, 2003; Teixeira *et al.,* 2003; Bi *et al.*, 2012). The data I present here do not entirely support this type of mechanism because LSD2 over-expression leads to a significant increase in the number of LDs. If LSD2 operated only by protecting LDs from hydrolysis enzymes, its over-expression should result in an increase in the size of LDs, not their number. I therefore favor the alternative models of LSD2 function described below.

IV. 5.1 LSD2 operates as a gatekeeper of lipid export

Because the processes of LD formation and lipid mobilization for loading onto LTP particles for transport out of the cell may be interconnected, and because LSD2 induces LD synthesis, I propose that LSD2 plays a novel role in lipid homeostasis. After FAs cross the plasma membrane, they can be either incorporated into LDs (via synthesis of TAGs) and temporarily stored in midgut epithelial cells, or they can be incorporated into DAGs and loaded directly onto LTP particles for transport out of the cells. It seems unnecessary for FAs to obligatorily become incorporated into TAGs in LDs just to be later mobilized back into FAs to be transported out of the cell. It would be more energy efficient for FAs to be loaded directly onto LTP particles and then onto Lpp particles for transport to the fat body. I therefore suggest that both incorporation into LDs and loading onto LTP particles can occur simultaneously and that LSD2 acts as a gatekeeper of lipid loading onto LTP by directing the flow of lipids into pathways that incorporate them into LDs (Fig. 31).

It is possible that LSD2 could function specifically at the step where DAGs are converted into TAGs. Incoming dietary FAs are first incorporated into DAGs and then into TAGs. Additionally, incoming FAs are transported between Drosophila tissues in DAGs and are stored intracellularly in LDs in the form of TAGs. Therefore, LSD2 could act as a gatekeeper of lipid loading onto LTP, directing the flow of lipids into pathways that incorporate them into LDs, specifically by inducing TAG synthesis or activating enzymes involved in TAG synthesis. At this step of intracellular lipid processing, LSD2 may play a role in controlling whether DAGs become loaded onto LTP or become

converted into TAG and packaged into LDs. If this mechanism is accurate, then LSD2 would inhibit lipid export from the midgut epithelium via LTP and Lpp.

Therefore, it should be asked in future studies whether LSD2 over-expression leads to decreased availability of DAG to be loaded onto lipoprotein particles and exported out of the midgut epithelium. To answer this question, the lipid composition of fat body and midgut tissues from LSD2 over-expression larvae could be identified by mass spectrometry and compared with that of wild-type larvae. If a lower amount of dietary lipids are exported from the midgut epithelium of LSD2 over-expression larvae to the fat body, then the hypothesis that LSD2 functions as a gatekeeper in lipid loading onto lipoprotein particles may be true. In this case, the fat body of LSD2 over-expression larvae may contain fewer lipids than the fat body of wild-type larvae. On the contrary, if no difference in the amount of lipids stored in the fat body is observed, then the hypothesis may not be correct. Additionally, to ask whether over-expressing LSD2 might change the lipid composition of circulating Lpp, the amount of circulating Lpp in the hemolymph loaded with DAG may be quantified by density gradient centrifugation and shotgun mass spectrometry, as previously described (Palm *et al.*, 2012).

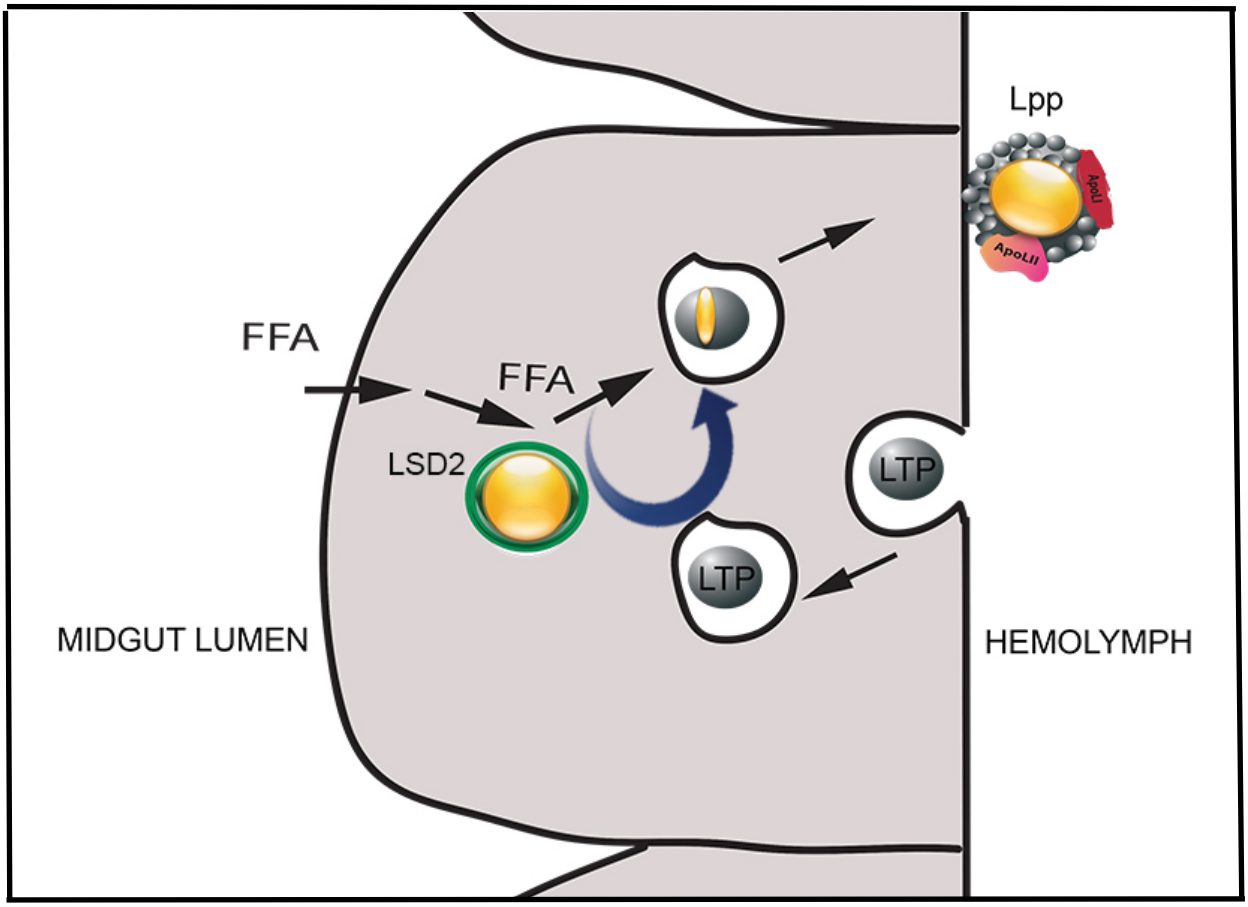

Figure 31. Model for LSD2 function in the larval midgut. LSD2 (green) found on the surface of LDs (yellow sphere) operates as a gatekeeper of lipid loading onto LTP (gray sphere) for loading to Lpp. LTP is internalized, picks up lipids (yellow lens) and loads them onto Lpp particles found outside the cell. FFAs can either be incorporated into TAG and packaged into LDs, or they may be incorporated into DAG and loaded onto LTP particles. This process may be competitive and LSD2 may operate as a gatekeeper of lipid translocation onto LTP particles, directing incoming FFAs to LD synthesis pathways.

IV. 5.2 LSD2 may induce lipid droplet synthesis at distinct ER domains

LSD2 could promote LD synthesis by stimulating the incorporation of FFAs into TAGs. This type of mechanism has been proposed in atherosclerosis. It was found that over-expression of human PLIN2 along with inhibition of acyl-coA synthetase decreased the levels of TAG in human macrophages (Larigauderie *et al.*, 2006). It has been suggested that PLIN2 is a marker of lipid accumulation, as the cellular amount of PLIN2 reflects the mass of neutral lipids stored within cells (Brasaemle *et al.*1997). Over-expression of PLIN2 in cultured cells increased the content of TAG by stimulation of LD formation (Imamura *et al.*, 2002). However, the exact mechanism of how this occurs has not yet been proposed.

Others have observed in freeze fracture studies that the mammalian LSD2, PLIN2, is found enriched at specialized sites of the ER membrane (Robenek *et al.*, 2009). Because of this observation and because of my finding that over-expressing LSD2 in the larval midgut leads to an increase in the number of LDs, I propose that LSD2 may also be located at specialized regions of the ER, where it may promote LD synthesis. LSD may stimulate the incorporation of FFAs into complex TAGs (Fig. 32) by activating TAG synthesis enzymes. It should be addressed in future studies whether LSD2 is found associated with the ER or whether it is exclusively associated with LDs. Immunogold labeling could be used to address this possibility.

IV. 5.3 LSD2 may recruit triacylglycerol synthesis enzymes from the ER to the surface of expanding lipid droplets

The data presented here suggest that as LDs increase in size after oleic acid feeding, they lose LSD2 from their surface, I speculate that LSD2 may play a role not only in LD synthesis, but also in LD expansion. A recent breakthrough emerged in cell culture studies. It was shown that TAG synthesis enzymes relocalize from the ER onto expanding LDs and mediate LD growth (Wilfling *et al.*, 2013). Because it was recently proposed in mammalian adipocyte *in vitro* studies that another Perilipin, PLIN1, moves between the ER and LDs during lipid synthesis (Skinner *et al.*, 2013), I propose that LSD2 may also relocate between the ER and LDs, playing a role in LD expansion (Fig. 32). LSD2/PLIN2 could recruit TAG synthesis enzymes from the ER to the surface of expanding LDs. Each of these proposed mechanism of function should be throughly tested in future studies.

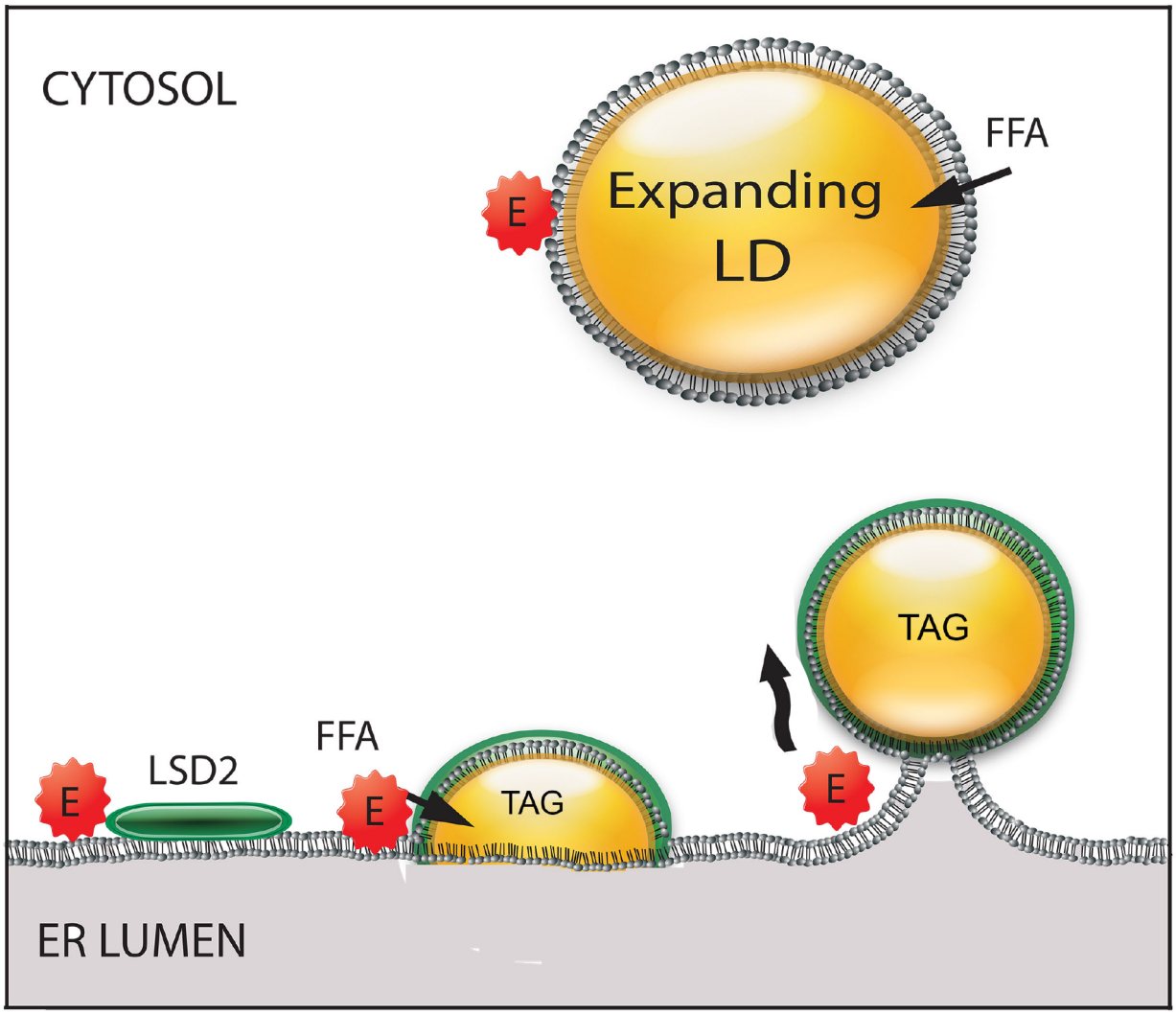

Figure 32. Proposed mechanisms of LSD2 function at specialized sites of the ER. LSDs could play a role in:1) LD synthesis and 2) LD expansion. In this cartoon, TAG synthesis enzymes (E, red) and LSD2 (green) are located at specialized ER regions. The presence of LSD2 triggers (activates) the enzymes to convert FFAs into TAGs, which become incorporated in between the ER leaflets. As the emerging LD grows in size, LSD2 triggers the relocalizing of TAG synthesis enzymes from the ER to the LD surface (wavy arrow). As the LD buds off of the ER, it contains TAG synthesis enzymes on its surface which further convert FFAs to TAG, leading to LD expansion.

IV.6 Future perspective

IV.6.1 LSD2 may function in cholesterol metabolism

The mechanism of LSD2 function illustrated in Fig. 31 shows that LSD2 may function as a gatekeeper of FFA loading onto LTP particles by directing the flow of lipids into pathways that incorporate them into LDs. An important aspect of this proposed role of LSD2 is its potential role in intracellular cholesterol translocation. Although several studies have shown that LSD2 over-expression results in extra TAG accumulation (Bi *et al.*, 2012; Gronke *et al.*, 2003), its effects on cholesterol metabolism have not yet been considered. I suggest that, as LDs are composed of both TAG and CE, LSD2 may operate as a gatekeeper not only in FFAs, but also in cholesterol ester translocation onto LTP particles. If LSD2 induces LD biogenesis by directing the incorporation of FFAs into TAG, leading to LD synthesis, it should be addressed if the same occurs for cholesterol. FFAs may act as a solvent for cholesterol esters in the cell and LSD2 may play a role in directing the flow of both FFAs and cholesterol into LD synthesis pathways. I also suggest that LSD2 over-expression in Drosophila leads to modifications in the expression of other genes controlling cholesterol metabolism, favoring the processes of cholesterol uptake and storage over that of export. This implication is important, as insects do not synthesize sterols, solely relying on their uptake from the diet (Carvalho *et al.*, 2010). Future studies should address the possibility that LSD2 knockdown/over-expression may affect cellular cholesterol processing.

IV. 6.2 LSD2 may function through the regulation of LXRs and PPARs

As a result of the evidence presented in this thesis, I speculate that the mechanism of LSD2 function in the Drosophila larval midgut does not exclusively involve the recruitment or the regulation of lipases, as previously suggested (Bi *et al.,* 2012). I proposed a mechanism of LSD2 function and hypothesized that LSD2 over-expression in Drosophila leads to modifications in the expression of other genes controlling lipid (TAG and cholesterol) homeostasis. It would be interesting to study the effects LSD2 knockdown/over-expression may have on the regulation of other genes.

One gene that may be up-regulated in the presence of LSD2 is NPC1b, the Drosophila homolog of Niemann-Pick C1-like (NPC1L1). It has been suggested that NPC1b promotes an early step in sterol absorption from the larval midgut epithelium (Voght *et al.*, 2007). As it is well accepted that the mammalian NPC1L1 protein is important for intestinal absorption of dietary sterols (Garcia-Calvo *et al.*, 2005) and because insects lack the ability to synthesize sterols relying on dietary uptake, this protein could be up-regulated as a result of an increase in LD biogenesis.

Additionally, several other genes encoding molecules known to be involved in both PLIN2 function and lipid metabolism could be up-regulated along with LSD2. For example, liver X receptors (LXR) and peroxisome proliferator-activated receptors (PPARs) are Thyroid Hormone Receptor-like nuclear receptors. LXRs are members of the nuclear receptor superfamily activated by intermediates in cholesterol synthesis that regulate many target genes involved in both cholesterol and FA metabolism in liver, macrophages and intestine (Kotokorpi *et al.,* 2010). It has been shown that the human *plin2* gene contains several potential LXR response elements (LXREs) and moreover

that LRX directly regulates human PLIN2 expression by binding to LXREs (Kotokorpi *et al.*, 2010). Interestingly, changes in the Drosophila LXR homolog, DHR96, protein levels have the same effects as changes in LSD2 levels: DHR96 knockdown leads to sensitivity to starvation and decrease in TAG levels and DHR96 over-expression leads to increase in TAG levels and LD accumulation (Sieber and Thummel, 2009). Moreover, DHR96 possibly binds cholesterol and coordinates both TAG and cholesterol homeostasis in Drosophila (Sieber and Thummel, 2012). Because a connection between PLIN2, LXR, LD synthesis has been suggested in mammals, it would be interesting to address if the same occurs in Drosophila. Specifically, is LSD2 activated by nuclear receptors (such as DHR96 and others) to function in TAG and cholesterol homeostasis? Does the *lsd2* promoter have any DHR96 response elements? Are other nuclear receptors involved in lipid homeostasis by activating LSD2 expression?

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors closely related to LXRs, that function as transcription factors and play essential roles in the regulation of lipids, by up-regulating genes involved in lipid and lipoprotein metabolism (Desvergne *et al.*, 1999). Interestingly, it was shown that transcription of the *plin2* gene is regulated by PPARα in various cell types (Targett-Adams *et al.*, 2005). Because mammalian *plin2* has nuclear hormone receptor response elements (NHREs) in its promoter and because PPAR activation directly stimulated PLIN2 expression (Eisinger *et al.*, 1993; Vosper *et al.*, 2001; Edvardsson *et al.*, 2006), it would be interesting to address whether the same occurs with the fly LSD2. Specifically, is *lsd2* transcription regulated by PPARs?

Furthermore, the transcription factor SREBP-1 (Sterol Regulatory Element-Binding Protein) is required for both cholesterol and FA biosynthesis and uptake, as it becomes activated by PLIN2. SREBPs are attached to ER and nuclear envelope membranes and become activated as a result of low levels of cellular sterols, being cleaved and translocated to the nucleus where they bind to specific sterol regulatory DNA sequences and up-regulate the synthesis of enzymes involved in sterol biosynthesis (Wang *et al.*, 1994). In hepatocytes, lipogenic gene expression is up-regulated under the control of SREBP-1 and the number of LDs rich in TAG grows simultaneously with PLIN2 expression (Edvardsson *et al*., 2006). Similar effects have been described for PLIN1. It has been shown that the levels of SREBP-1 and TAG are reduced in adipocytes of PLIN mutant mice and that PLIN over-expression leads to TAG accumulation and activation of SREBP-1 (Takahashi *et al.*, 2013). Interestingly, in Drosophila, loss of *dSREBP* results in a transcriptional deficit of fatty acid synthesis genes, reduced fatty acid content, and larval lethality (Kunte *et al.,* 2006). Because of this, a feedback loop type of mechanism where SREBP-1 stimulates gene expression (e.g.PLIN) and TAG synthesis, leading to LD formation has been proposed (Takahashi *et al*., 2013). Because of these findings, it may be possible that the fly LSD2 triggers SREBP activation, which stimulates lipogenic gene expression, leading ultimately to an increase in number of LDs.

IV. 7 Does β_H play a role in lipid translocation in the midgut epithelium?

It has been previously shown that αβ tetramers are located at the basolateral domain of epithelial cells and that tetramers of α and of the unconventional large β_H spectrin are confined to the apical domain (Dubreuil *et al.* 1997, Thomas and Kiehart 1994, Thomas *et al.* 1998). Shown in Fig. 33 are images of β_H spectrin immunofluorescent staining and of LDs observed with DIC microscopy of the midgut epithelium, which indicate that the apical domain of the cells is not only the region where β_H spectrin is localized, but it is also the cell domain where LDs are found. This is interesting because it raises the possibility that, similar to the function β spectrin plays at the ecto domain of the fat body, β_H spectrin could play a role in lipid translocation at the apical domain of the midgut. Specifically, β_H spectrin could connect lipid uptake machinery (lipid transporters) at the plasma membrane to that of lipid droplet growth in the cytoplasm. This possibility should be addressed in future studies.

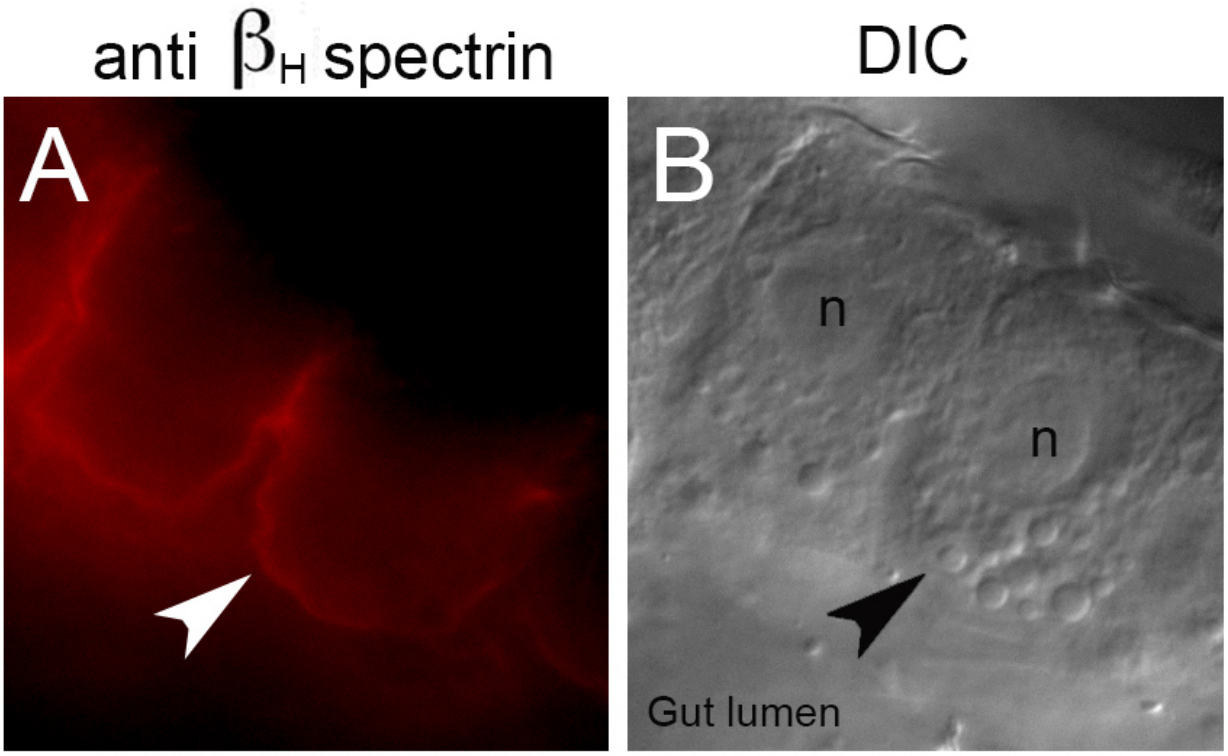

Figure 33. Localization of β_H spectrin by fluorescence microscopy and of LDs by DIC microscopy in the larval midgut. Dissected preparations of midgut tissues were labeled with rabbit anti-β_H spectrin. β_H-spectrin is enriched at the apical domain and is also present on the lateral surface of midgut cells (A). LDs are observed at the apical domain of the midgut (B). Arrowheads depict the apical domain of cells; n=nucleus.

CHAPTER V. A forward genetic screen to identify novel genes involved in dietary lipid transport from the midgut to the fat body

V.1 Rationale and approach

Forward genetic screens are an extremely powerful experimental approach for identifying unknown candidate genes involved in biological pathways and Drosophila is one of the most valuable model organisms for this purpose. The fruit fly is a good model system because it is: 1) simple, 2) cheap, 3) conserved. First, Drosophila is valuable for its genetics as it has only four pairs of chromosomes (three autosomes and one sex chromosome), containing approximately 13,600 genes (Adams *et al.*, 2000). Additionally, males do not show meiotic recombination, which facilitates genetic studies. Second, Drosophila is easy to work with, is cheap to maintain, has short developmental stages, and has features that can provide easy to score phenotypes. Third, the fruit fly is valuable as a model system because many fundamental biological processes, such as development and metabolism, seem to be conserved between flies and vertebrates (Bier, 2005).

The biological process I am interested in studying is the transport of dietary lipids between tissues. Drosophila is a valuable model organism for studying this process because the fruit fly possesses tissues involved in lipid translocation that are similar to those of vertebrates. As described in Chapter I, two main tissues involved in dietary lipid transport are the fat body and the midgut. Dietary lipids are absorbed by the midgut epithelium and are transported to the fat body for storage. The fat body performs similar functions to the vertebrate liver, immune system and white adipose tissue. It is the organ where lipids are stored in LDs and is the site where lipoproteins are synthesized

(Palm *et al.*, 2012). The midgut is analogous to the mammalian gastrointestinal tract. It is the site where dietary lipids (and other nutrients) are chemically digested, absorbed, and mobilized.

Dietary lipid transport from the midgut to the fat body is essential. Hampering of the process by knocking down genes encoding for each of the three proteins mentioned above (Lpp, LTP and MTP) leads to larval lethality (Palm *et al.*, 2012). This process may be essential in Drosophila because the fly is a sterol auxotroph that relies on incoming dietary sterols for development (Carvahlo *et al.*, 2010). As sterols are required for development, interfering with the transport machinery from the midgut to the fat body leads to lethality. Because the lipid transport process is not entirely understood and because it is essential, identifying novel candidate genes that encode for products involved in lipid transport between tissues is an important step in unraveling novel pathways involved in this process.

A powerful experimental technique to use in order to identify genes that function in lipid transport is performing a forward genetic screen. A forward genetic screen involves introducing mutations in a genome and screening potential mutants for a phenotype of interest. In this particular screen, the phenotype of my interest was accumulation of LDs in the midgut. It has been previously shown that interfering with lipid transport to the fat body leads to accumulation of LDs in the midgut (Palm *et al.*, 2012). This phenotype occurred as a result of: 1) β spectrin over-expression in the fat body (Chapter II); 2) Lpp RNAi in the fat body (Palm *et al.*, 2012); 3) MTP RNAi in the fat body (Palm *et al.*, 2012); 4) LTP RNAi (Palm *et al.*, 2012). Additionally, accumulation of LDs is observed as a result of an increase in dietary lipid content (Chapter III).

Two components are required for a successful screen: 1) a feasible screening method; 2) a way to introduce mutations. Mutations can be introduced in various ways. But, in this specific case, two approaches are discussed: the use of deficiency kits provided by the Bloomington stock centre and exposing of the organism to a chemical mutagen, ethylmethane sulfonate (EMS). For a simple screening method, Oil Red O staining or Nile Red feeding can be used, as described below.

V.2 A simple screening method: Oil Red O staining and Nile Red feeding

Oil Red O has been successfully used as a lipid stain in research studies in model organisms ranging from the simple unicellular yeast cell to complex human tissue (Mehlem *et al.*, 2013). The dye is very effective in dissected, fixed tissue preparations. Because of this, Oil Red O can only be used in dead animals and can not be used as a screening method to recover a living animal. The importance of this is discussed in Section V.3.

Nile Red, similar to Oil Red O, is a lipophylic dye that has been extensively used in fixed tissue preparations for the detection of neutral lipids (Greenspan *et al.*, 1985). Interestingly, unlike Oil Red O, the dye can be used in visualizing fat storage LDs in live animals. Nile Red was used as a screening method via feeding in *C. elegans* (Ashrafi *et al.*, 2003). It has never been used in Drosophila this way. Here, I describe Nile Red feeding of wild-type larvae and of mutant larvae that accumulate LDs in the midgut.

Nile Red feeding of wild-type Drosophila larvae

Wild-type larvae that were fed Nile Red in yeast paste for one hour, displayed a bright red fluorescent signal in the gut. Unexpectedly, the dye did not cross the plasma membrane and was not incorporated into lipid droplets of living larvae. This was unexpected because the dye did label LDs found in the *C. elegans* gut (Ashrafi *et al*., 2003). No other Drosophila tissue showed a red fluorescent signal. Larvae fed Nile Red in yeast paste and chased with a diet of yeast paste alone for an additional 24 hours did not display the red signal in the gut. The only dim signal observed was due to autofluorescence from yeast that was present in the gut (Fig. 34 A, 35 B & C).

Nile Red feeding of Drosophila larvae that accumulate LDs in the midgut

Larvae that were known to have accumulation of LDs in the midgut were fed Nile Red in yeast paste and chased with yeast paste for an additional 24 hours. The larvae displayed a striking, bright red fluorescent signal at the midgut epithelium. The Nile Red dye seemed to label the plasma membrane of the larval gut. This was observed in the midgut of: β spectrin over-expressors in the fat body, Lpp RNAi in the fat body and MTP mutants (Section V.3.2). Shown in Figure 34 are images of living larvae fed Nile Red in yeast paste for one hour and chased for another 24 hrs. The Nile Red signal disappears from the midgut of wild-type larvae (A). A bright red fluorescent signal was present in the midgut of β spectrin over-expressors (C) and Lpp RNAi larvae (E). Figure 34 shows that not only was the Nile Red staining retained in the midgut of larvae that accumulate LDs but also that the effect is visible in un-dissected preparations. Additionally, Figure 35 shows the pattern of Nile Red staining of dissected preparations of midgut tissues of

wild-type and of β spectrin over-expressors. After 24 hrs of chase, wild-type larvae did not show a fluorescent signal in the midgut (B & C). However, larvae that accumulated LDs in the midgut, displayed a striking red signal (E & F).

Based on the observations that Nile Red staining of the PM occurred specifically in larvae that accumulate LDs in the midgut, I conclude that Nile Red feeding is a useful tool for detecting mutants with disrupted transport of dietary lipids. This may occur because the stain is detecting FAs (or other lipids) that may be located inside fat transporters found in the PM of epithelial cells. If dietary lipids are not exported from the midgut epithelium of mutants, leading to accumulation of LDs, then FAs may be trapped inside fat transporters found in the PM. The Nile Red may then be detecting the FAs, which occupy the transporters. Thus, Nile Red staining may not detected in wild-type larvae because the FAs are properly exported out of epithelial cells via Lpp and the fat transporters are constantly freed of lipids. Importantly, not only is this phenotype easily distinguished from that of wild-type larvae, it is also visible in living larvae. Until this assay was developed, it was necessary to dissect larvae to detect the phenotype, which is incompatible with screening.

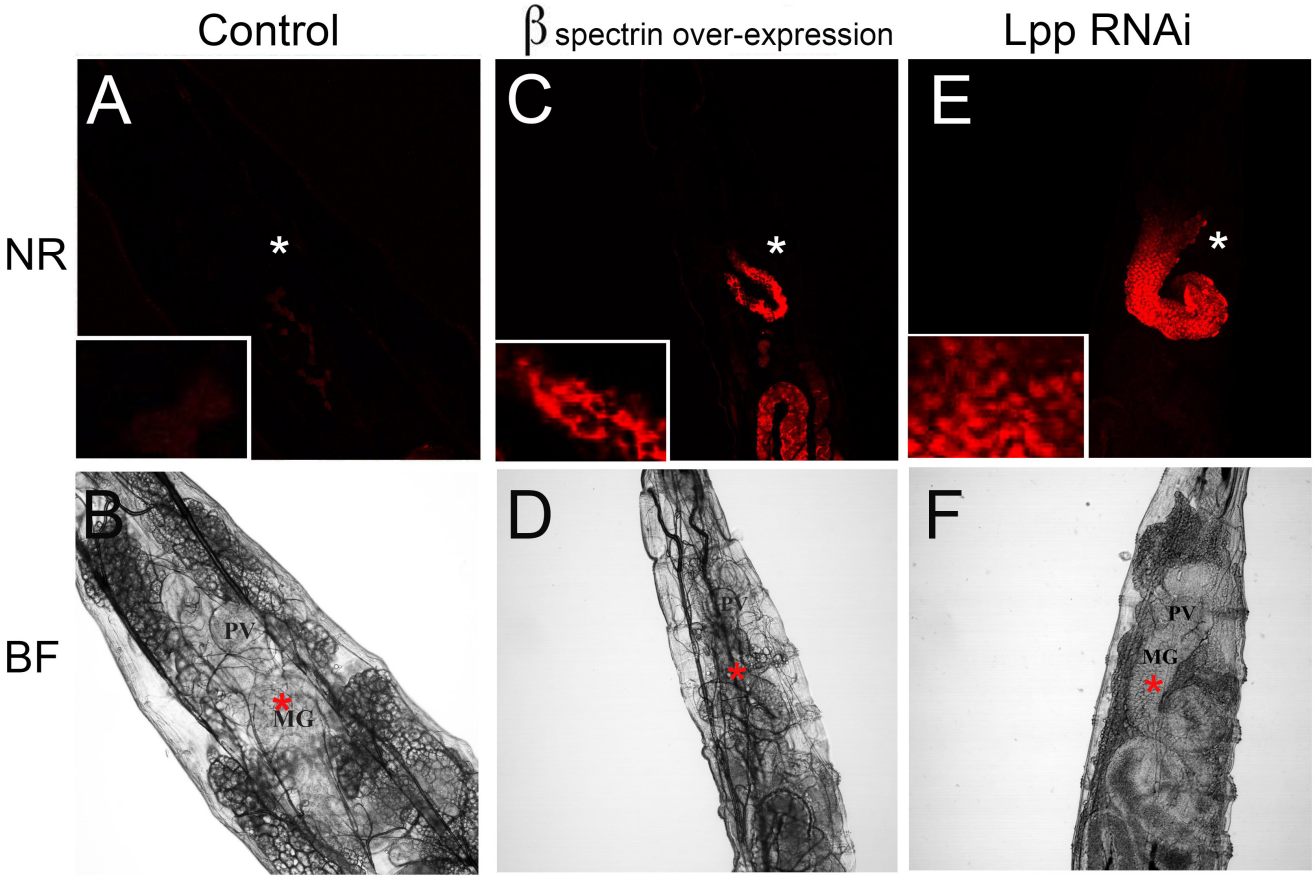

Figure 34. Larvae that accumulate LDs display Nile Red staining in the midgut epithelium. Larvae were fed Nile Red in yeast paste for 1hr and chased with yeast paste for another 24hrs. They were placed on slides and analyzed on the confocal microscope with fluorescence (top panels) and brighfield (bottom) microscopy. Top panels show that while control larvae do not display a Nile Red (NR) signal in the midgut (A, white asterisk), β-spectrin over-expression (C, white asterisk) and Lpp RNAi larvae (E, white asterisk) show a dramatic signal. The white and red asterisks do not perfectly correspond to one another because it is easier to observe the NR staining of the midgut.

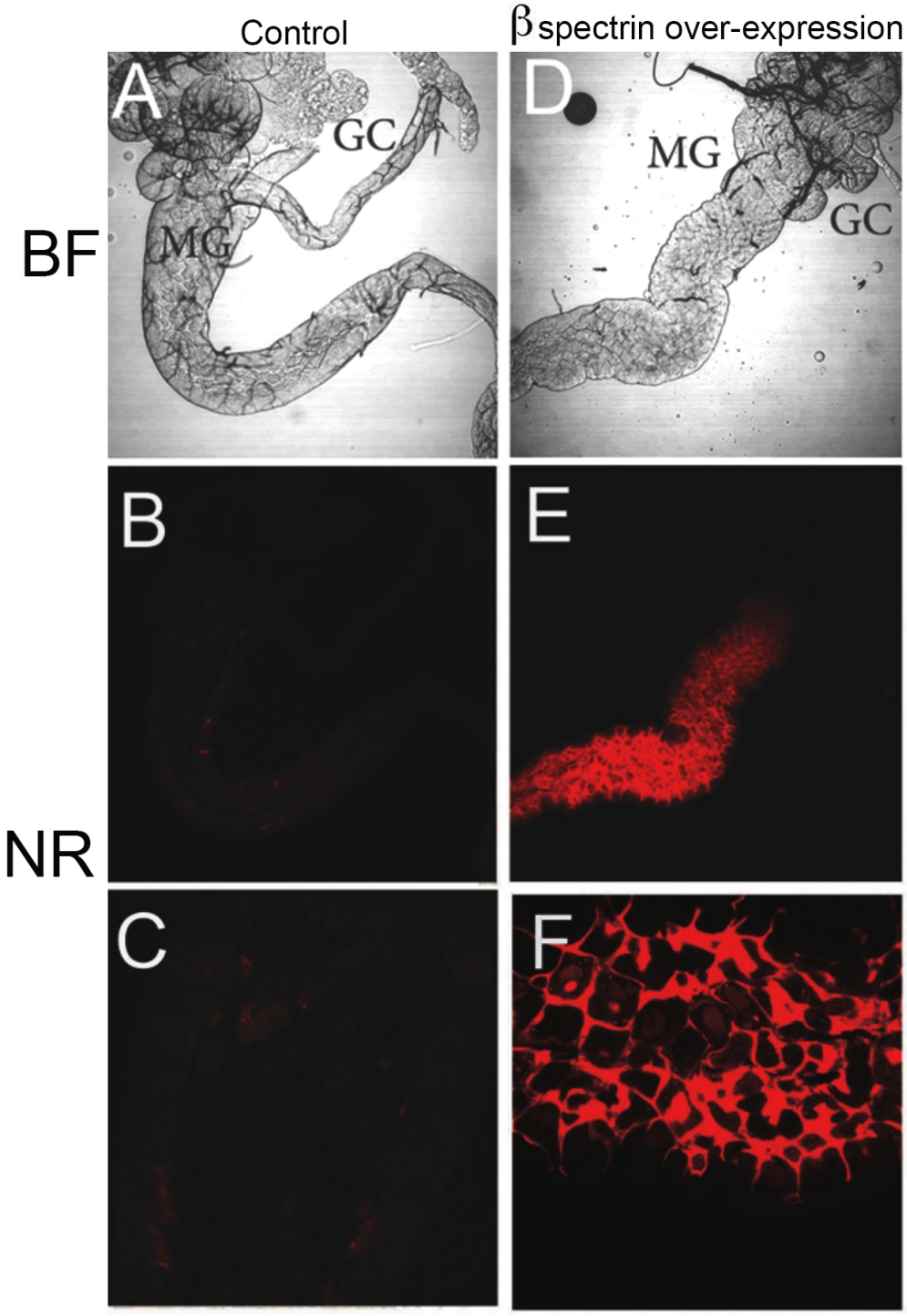

Figure 35. Dissected preparations of wild-type control and β spectrin over-expression larvae after Nile Red feeding. Larvae were fed Nile Red (NR) in yeast paste for 1hr and chased with yeast paste for another 24hrs. They were dissected and fixed in formaldehyde, placed on slides and analyzed on the confocal microscope with fluorescence and brightfield (BF) microscopy. While control larvae do not display a NR signal in the midgut (B & C), β spectrin over-expressors (E & F) show a dramatic NR signal. MG=midgut; GC= gastric cecae.

V. 3 A way to introduce mutations: the use of deficiency kits vs. EMS mutagenesis

The second component of a successful screen is a way to introduce mutations. In this specific forward genetic screen, designed to identify novel genes involved in dietary lipid transport from the midgut, two approaches are taken into consideration: 1) the use of deficiency kits and 2) the use of a chemical mutagen, ethyl methanesulfonate (EMS). If the first approach is used, deficiency kits, the recover of a living candidate mutant is not required. In this case, larvae can be screened for the accumulation of LDs in the midgut via Oil Red O staining. On the other hand, if the second approach is used, EMS mutagenesis, the recovery of living candidate mutants is needed. Here is where Nile Red feeding can be used as a screening assay. This is the only screening method currently available for identifying accumulation of LDs in the Drosophila larval midgut *in vivo*.

Deficiency kits are collections of fly stocks, each carrying a chromosomal deletion, or deficiency (Hiensinger *et al.,* 2004). The deficiency (Df) kits provide complete coverage of the Drosophila genome and are available at the Bloomington stock centre (Cook *et al*., 2012). The deletions were created by either the Flp/FRT system, where the yeast Flipase (Flp) induces recombination between FRT-carrying insertions (Thibault *et al*., 2004) or by P-element transposase in the presence of trans-heterozygous P-element insertions (Parks *et al*., 2004). Dfs have been successfully used in identifying genes required for embryonic development (Thomas *et al*., 1988). Because the deficiency fly stocks cover the entire genome, the kits can be used efficiently for screening large chromosomal regions for genes involved in the process of lipid transport

from the midgut. If Df kits are used in this screen, Oil Red O staining can be used as a screening method.

Using the chemical mutagen ethyl methanesulfonate (EMS) is the most popular strategy used for introducing mutations in Drosophila (St. Johnston, 2002). It has become a standard approach and has been successfully used in classic forward genetic screens. Nusslein-Vollhard and Wieschaus won the Nobel prize for their pioneering work after performing an EMS screen to identify genes that control patterning of the Drosophila embryo. This work ultimately led to the elucidation of many fundamental mechanisms that control development (Nusslein-Vollhard and Wieschaus 1980). The mutations EMS induces are usually point mutations that disrupt gene function by causing missense or nonsense mutations. Using EMS mutagenesis, combined with an assay involving Nile Red feeding could therefore be an efficient approach in identifying novel genes involved in lipid transport from the midgut to the fat body.

There are strengths and weaknesses to both approaches. First, using Df kits does not require the recovery of a living animal. As recovery of a living candidate mutant can be challenging, Df kits could be a more effective approach to use. Second, as many stocks can be screened simultaneously, the Df kits could accelerate the screening process. Third, unlike EMS, Df kits provide a safe method to introduce mutations. EMS is a dangerous mutagenic and carcinogenic chemical that readily evaporates. Hence, appropriate measures must be taken when used to avoid contamination. Fourth, and probably the most important advantage of using Df kits, is that the mutated chromosome region would be known, facilitating identification of gene(s) responsible. Df

kits, which cover the genome with a minimum of fly lines, were specifically designed for the purpose of facilitating mapping.

To decide which approach to use, I tested whether using Df kits would be an adequate approach to use, by analyzing two heterozygous fly lines. Because Df lines from Df kits have one functional copy of a gene, I tested whether loss of one functional copy of the *Lpp* or *MTP* gene would also lead to accumulation of LDs in the midgut. If it did so, then using Df kits in this forward genetic screen could potentially be adequate, and vice versa. I am providing evidence here that using Df kits is not an effective method to use because heterozygous larvae fail to develop the phenotype of interest.

The first fly line I analyzed was obtained from the Bloomington stock centre (#16302). The line disrupts the *Lpp* gene by the insertion of a 3' half transposable P-element; flies of this stock carry one functional copy of the *Lpp* gene (Bellen *et al.*, 2004). The second line I tested here was an *MTP* mutant line, named $mtp^{\Delta ex1}$, which was created by imprecise excision of EP[GE10702] and harbors a 559 bp deletion (Palm *et al.*, 2012). Homozygous $mtp^{\Delta ex1}$ animals arrest in the first larval instar and heterozygotes survive to adulthood (Palm *et al.*, 2012). As previously mentioned, complete loss of either the *Lpp* or *MTP* genes leads to accumulation of LDs in the midgut (Palm *et al.*, 2012).

V.3.1 Loss of one functional copy of the *Lpp* or *MTP* gene does not lead to a phenotype with Nile Red or Oil Red O staining

The two lines specified above were tested for accumulation of lipid droplets in the midgut using Oil Red O staining and Nile Red feeding. Figure 36 shows Oil Red O

staining of lipid droplets in dissected preparations of wild-type and *mtp*$^{\Delta ex1}$ heterozygous larvae. The Oil Red O staining pattern of the midgut epithelium of larvae that have one functional copy of each gene is the same as that of wild-type larvae. On the other hand, complete loss of either *MTP* or *Lpp* leads to accumulation of lipid droplets in the midgut.

Additionally, Figure 37 shows the Nile Red feeding pattern in *mtp*$^{\Delta ex1}$ heterozygous (act-GFP positive) and *mtp*$^{\Delta ex1}$ homozygous (act-GFP negative) larvae. While homozygous larvae show the dramatic intensity of Nile Red labeling in the midgut, the heterozygous ones do not. These data indicate that having only one functional copy of the *Lpp* or *MTP* gene seems to provide enough functional protein for transporting lipids out of midgut cells, hence leading to no phenotype and that Df kits would probably not be an effective strategy to use because other heterozygous larvae may fail to develop the phenotype of my interest.

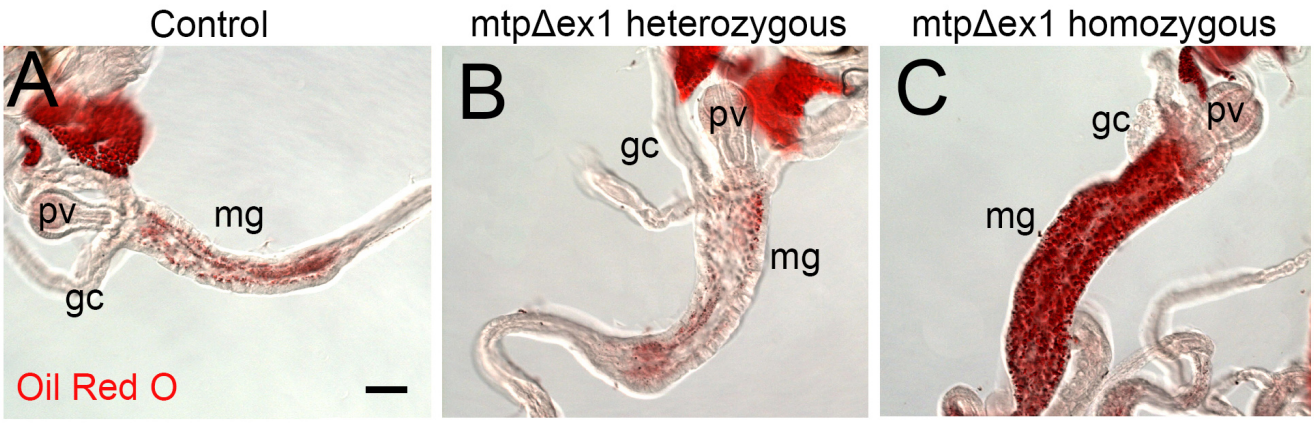

Figure 36. *mtp*$^{\Delta ex1}$ heterozygous larvae do not accumulate lipid droplets in the midgut epithelium. Oil Red O staining of lipid droplets in dissected preparations shows that both wild-type (A) and *mtp*$^{\Delta ex1}$ heterozygous larvae (B) have few lipid droplets in the anterior midgut. On the contrary, *mtp*$^{\Delta ex1}$ homozygous larvae have a significant accumulation of lipid droplets in the midgut (C). Scale bar is 50 µm; mg=midgut, gc=gastric caecae; pv=proventriculus.

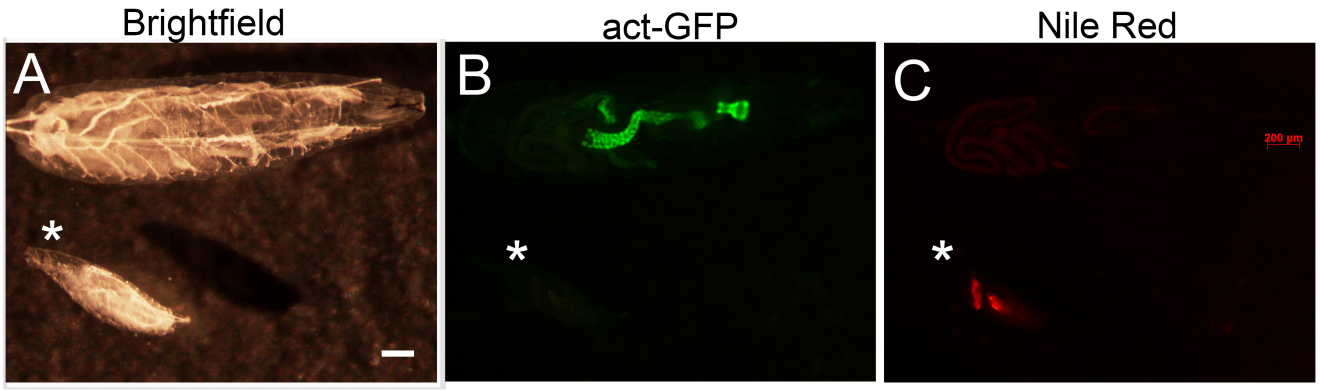

Figure 37. *mtp*$^{\Delta ex1}$ heterozygous larvae do not display Nile Red staining in the midgut epithelium. *mtp*$^{\Delta ex1}$ heterozygous (top larva in A; act GFP positive in B) and *mtp*$^{\Delta ex1}$ homozygous larvae (larva on the bottom in A; act-GFP negative in B) were fed Nile Red in yeast paste for 1hr and chased with yeast paste for another 24hrs. *mtp*$^{\Delta ex1}$ heterozygous do not have a signal in the midgut (C). On the contrary, *mtp*$^{\Delta ex1}$ homozygous larvae show a dramatic, intense signal in the midgut (C, asterisk). Scale bar is 200 µm.

Because larvae that have one functional copy of the *Lpp* or *MTP* gene did not show a lipid transport phenotype with Oil Red O staining, I asked whether sensitizing the lipid transport process would have an effect. To address this, I increased the dietary fat content of both wild-type and larvae that have one functional copy of the *Lpp* and *MTP* genes and compared the rate of lipid accumulation in the midgut. The rationale of feeding larvae a high fat diet (of 10% oleic acid in yeast paste) was that it could lead to a similar effect as that of not having any functional copy of the *Lpp* or *MTP* gene. My hypothesis was that larvae require a specific amount of Lpp and that this amount depends on the concentration of incoming dietary lipids. This implies that while having only one copy of the *Lpp* or *MTP* gene may not be enough to put a strain on the process of lipid translocation under normal conditions, it might lead to a detectable effect under high dietary fat concentrations.

Additionally, the data presented in Chapter III shows that the midgut epithelium of wild-type larvae is able to not only efficiently uptake a vast amount of FFAs and incorporate them into lipid droplets but also to efficiently mobilize them. Wild-type larvae fed oleic acid and chased with a standard diet of yeast paste mobilize the added FFAs completely. Afterward, Oil Red O staining experiments showed that the pattern of lipid droplets in the midgut was wild-type. To address the possibility that larvae that have one functional copy of the *Lpp* or *MTP* gene would show a phenotype, I also asked if they recover at a slower rate than wild-type larvae. Both the rate of LD accumulation and the rate of recovery after LD accumulation were observed in deficient larvae and compared with those of wild-type larvae after oleic acid feeding.

Four consequences were possible. First, larvae having one functional copy of the *Lpp* or *MTP* gene would accumulate LDs in the midgut at a similar rate to that of wild-type larvae. Second, the larvae could recover at a similar rate to that of wild-type larvae. These two outcomes would indicate that one functional copy of the gene provides enough mRNA which would be translated into a sufficient amount of Lpp particles to keep up with transporting lipids from the midgut even under sensitizing, high dietary fat, conditions. Third, larvae having one copy of the *Lpp* or *MTP* gene could accumulate lipid droplets at a faster rate than wild-type larvae. Fourth, the larvae could recover from high fat feeding at a slower rate than wild-type. These latter two possibilities would indicate that one functional copy of either gene does not yield sufficient Lpp function to keep up with transporting of added dietary lipids from the midgut.

To address the rate of LD accumulation in the midgut after a high fat diet, larvae having one functional copy of the *Lpp* or *MTP* gene and wild-type larvae were fed 10% oleic acid in yeast paste for various amounts of time. Oil Red O staining was performed and its intensity was recorded for each preparation. For representative images of Oil Red O levels, refer to figure 29, Chapter III. The chart in figure 38 shows the percentage of $mtp^{\Delta ex1}$ heterozygous and control larvae that had the highest Oil Red O staining intensity. The number and percentage of larvae observed in each category are shown in table I. The data show that after a high lipid diet, both wild-type and larvae having one functional copy of the *Lpp* or *MTP* gene accumulate LDs in the midgut at a similar rate.

Table I Percentage of wild-type and *mtp*$^{\Delta ex1}$ heterozygous larvae showing three levels of Oil Red O staining in the midgut epithelium after oleic acid feeding.

Time of feeding	Level 1		Level 2		Level 3		# Larvae	
	Control	*mtp*$^{\Delta ex}$	Control	*mtp*$^{\Delta ex}$	Control	*mtp*$^{\Delta ex}$	Control	*mtp*$^{\Delta ex}$
3.5 hrs	7.5%	12.5%	85%	81.2%	7.5%	6.2%	34	32
8 hrs	2.7%	3.4%	35.1%	37.3%	62.2%	59.3%	37	59
20 hrs	0%	0%	33.3%	30.4%	66.6%	69.6%	72	56
27 hrs	2.3%	5.4%	61.4%	56.8%	36.4%	37.7%	44	37

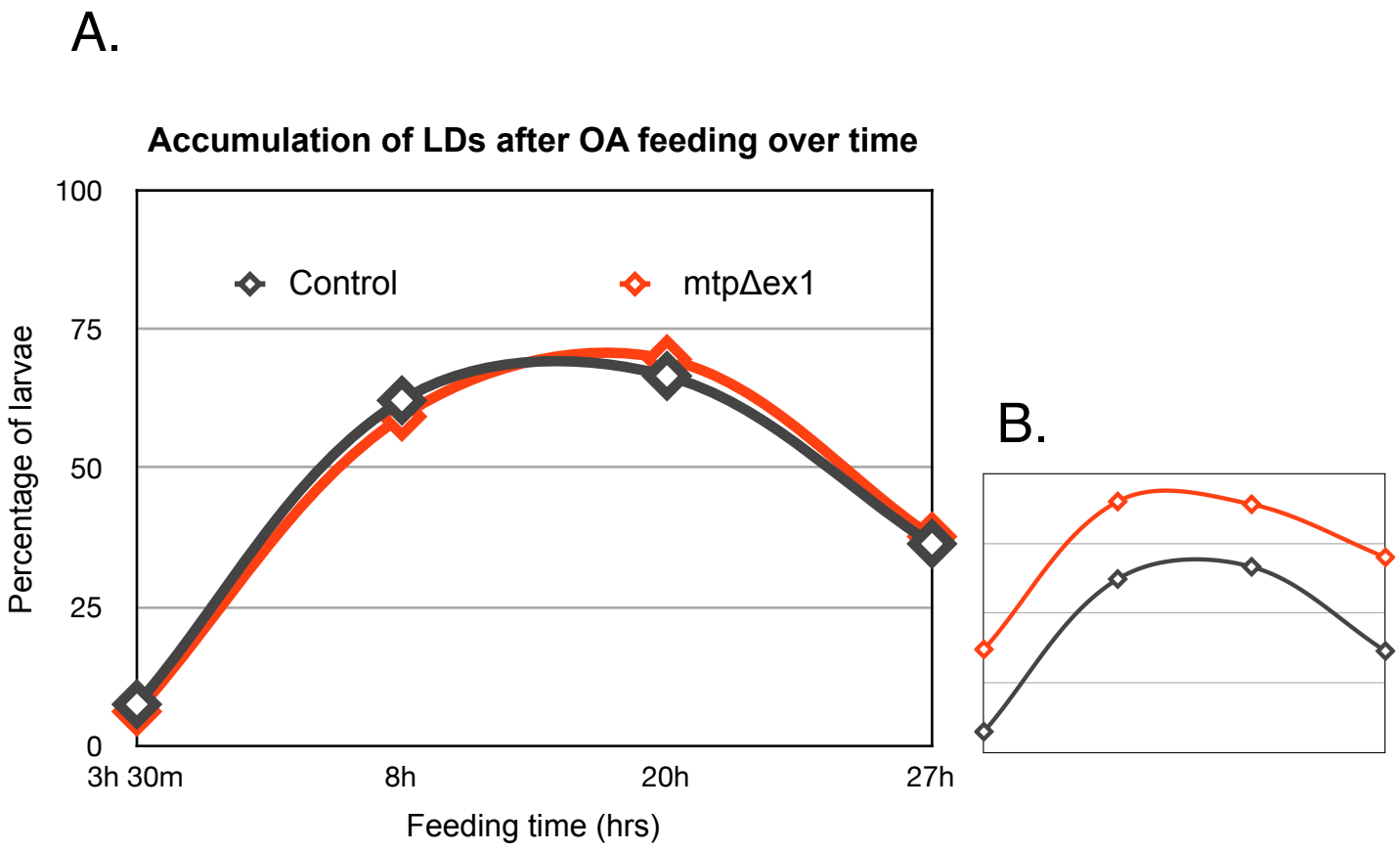

Figure 38. Accumulation of lipid droplets after oleic acid feeding over time in the midgut epithelium of wild-type and *mtp*$^{\Delta ex1}$ heterozygous larvae. Larvae were fed a diet of 10% oleic acid in yeast paste for various times. Oil Red O staining was then performed and recorded as Level 1 (lowest level) Level 2 (middle) and Level 3 (highest level of staining). Shown here in A is the actual percentage of larvae that have the highest level of accumulation of lipid droplets (Level 3). The chart in B is an example of the percentage that was expected.

V.3.2 After a high fat diet, wild-type larvae and larvae having one functional copy of the *Lpp* or *MTP* gene recover at a similar rate

The data presented in Chapter III, Figure 26 shows that wild-type larvae are able to mobilize all of the lipid droplets in the midgut resulted after high fat diet. Because the rate of accumulation of lipid droplets in the midgut was not different in $mtp^{\Delta ex1}$ heterozygous larvae, the rate of clearance was also tested and compared to that of wild-type larvae. $mtp^{\Delta ex1}$ heterozygous and wild-type larvae were fed 10% oleic acid in yeast paste and chased with yeast paste for various amounts of time. Oil Red O staining was performed and its intensity was recorded for each preparation. The results, presented in table II, show that the percentages of wild-type and $mtp^{\Delta ex1}$ heterozygous larvae having LDs in the midgut after oleic acid feeding and chase is similar.

Table II Percentage of wild-type and *mtp*$^{\Delta ex1}$ heterozygous larvae showing three levels of Oil Red O staining in the midgut epithelium after various oleic acid feeding and yeast paste chase times.

Time of feeding	**Time of chase**	**Level 1**		**Level 2**		**Level 3**		**# Larvae**	
		Control	*mtp*$^{\Delta ex1}$	Control	*mtp*$^{\Delta ex1}$	Control	*mtp*$^{\Delta ex1}$	Control	*mtp*$^{\Delta ex1}$
16 hrs	7 hrs	30.5%	63%	52.5%	35.5%	16.9%	1.4%	59	90
20 hrs	3 hrs	0%	0%	46.9%	57.1%	53%	42.8%	49	77
20 hrs	5.5 hrs	21.8%	17%	76.4%	78.7%	1.8%	4.2%	55	47
20 hrs	8 hrs	52.8%	37.7%	43.4%	62.2%	3.7%	0%	53	53
21 hrs	3 hrs	16%	3.5%	58%	58.8%	26%	37.6%	50	90

The lines of evidence presented here demonstrate that there is no detectable effect on lipid transport from the midgut of *mtp*$^{\Delta ex1}$ heterozygous larvae, even after a high fat diet. The hypothesis described above seems to be incorrect because after decreasing the gene dose there seems to be no detectable reduction in gene function. Therefore, the single functional allele of *Lpp* or *MTP* may have produced enough protein to allow proper lipid transport. Additionally, there may be up-regulation in the remaining functional allele. These data suggest that using Df kits as a way to introduce mutations may not be effective in this particular screen. However, because only two Df lines were tested, Df kits cannot be ruled out as a way to introduce mutations. Df kits may be an effective strategy in a genetically sensitized background. For example, another screen could be designed to test the Df lines in a *Lpp* heterozygous background. Under these circumstances, the deficient larvae may accumulate LDs in the midgut.

V.4 The screening approach

EMS mutagenesis was used to produce mutations. The forward genetic screen was designed to specifically identify X-linked genes required for the transport of dietary lipids from the larval midgut to other tissues. The flies used in the screen were C(1)Dx females and isogenic Oregon^{R} (OrR) males. C(1)Dx females have a compound-X, or attached-X, chromosome, where two X chromosomes are joined to a common centromere. The compound-X assures that the two chromosomes do not segregate during meiosis and are jointly passed on to one gamete (Novitski, 1954). These females also have one Y chromosome from their fathers and produce two classes of viable offspring: females who have the maternal compound-X chromosome along with a

paternal Y, and males with a maternal Y chromosome along with a paternal X (Novitski, 1954). This fly stock is frequently used to study X-linked genes because when an attached-X female is mated with a male carrying any X-linked mutation, the male progeny receive the mutant X chromosome and the females receive the attached-X chromosome. Therefore, in genetic crosses with attached-X females, the X-linked inheritance is opposite to normal, the X-linked gene in the male being passed on from father to son, instead of mother to son. Males from an isogenic OrR stock were used. Isogenic males (every individual is genetically identical) were used to ensure that no pre-existing DNA polymorphisms may appear in candidate mutants, which would make mapping and recovery of any newly induced mutations more difficult (St. Johnston, 2002).

The F1 screen was performed by crossing EMS mutagenized OrR males with C(1) Dx virgin females (Fig. 39). Four classes of progeny were possible: 1) females that inherited the compound X chromosome from the mother and the X chromosome from the father (do not survive), 2) males that inherited two Y chromosomes, one from the mother and one from the father (do not survive), 3) females that inherited the compound X chromosome from the mother and the Y chromosome from the father, 4) males that inherited the Y chromosome from the mother and the X chromosome from the father (Fig. 39). The rationale was that this cross would lead to the recovery of hemizygous male progeny that may have a mutagenized X chromosome (Fig. 39, 4th category). The larval progeny were collected and screened by Nile Red feeding, as described above. If the phenotype of interest was observed, living animals were recovered and set aside to develop to adulthood.

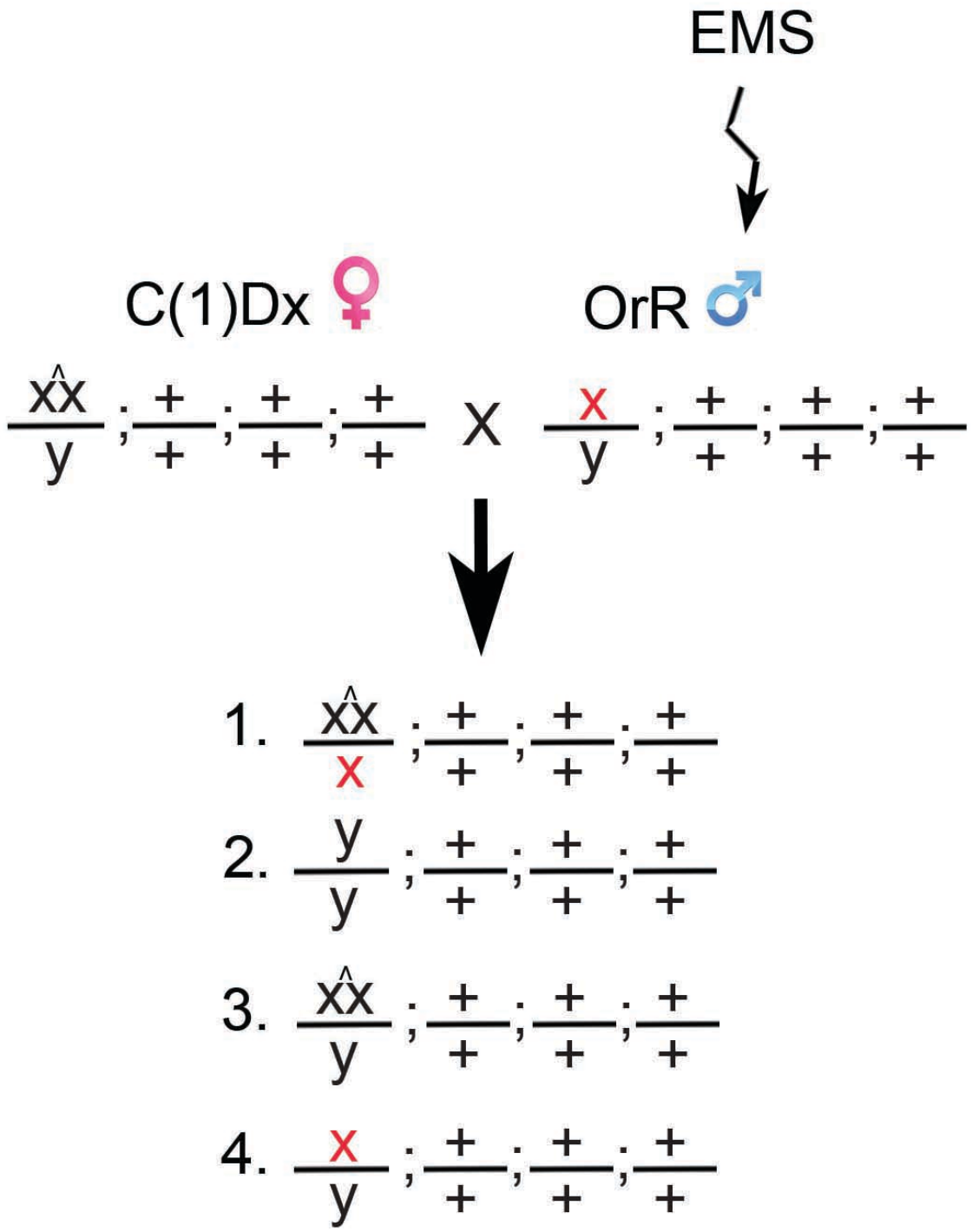

Figure 39. Diagram of genetic cross of the forward screen. A C(1)DX females that have an attached-X chromosome and a Y chromosome are crossed with EMS mutagenized OrR male. XY inheritance is often opposite to normal: the X chromosome is passed from father to son and the Y chromosome is passed from mother to daughter. The four possible genotypes of F1 progeny are depicted. # 1 and #2 are nonviable. The red X depicts a potential mutated chromosome.

Additionally, besides the desired outcome, detection of hemizygous male progeny that have a mutagenized X chromosome, three other outcomes were possible. First, because the complete loss of function of either of the *LTP, Lpp* or *MTP* genes leads to larval lethality (Palm *et al.*, 2012), it was expected that a high percentage of candidate mutants recovered from this screen may not survive to adulthood. Second, it was possible that some larvae may display Nile Red staining without having a disruption in transport of dietary lipids from the midgut. These larvae were referred to as “false-positives”. Third, it was also possible that some larvae do have a perturbed transport of dietary lipids without displaying Nile Red staining in the midgut epithelium. These larvae were referred to as “false-negatives”.

Several rounds of the screen were performed and a total of approximately 7,900 F1 larvae were screened. Out of 7,900 larvae, 207 were screened positive for Nile Red staining in the midgut (table III). As expected, a large number of the candidate mutants that showed the phenotype did not survive to adulthood. After several rounds of the screen had unsuccessful outcomes, changing few of the parameters of the screen led to the recovery of three candidate mutants (table III). The parameters that were changed were: 1) the temperature was changed from 25°C to 22°C and 2) the chase time was changed from 24 hrs to 26 hrs. The reason the screen was successful under these conditions is not known. It may be that these conditions are ideal for this particular forward screen. However, it is also possible that it was an incidental occurrence that the screen worked.

After the adult candidate mutants were recovered, they were tested for the presence of a heritable X-linked mutation. This is because EMS treatment induces mutations on all chromosomes of the mutagenized sperm, not only on the X chromosome. Additionally, because Drosophila's response to mutagenesis triggers DNA repair mechanisms, which can occur in the embryo (not during spermatogenesis), a high percentage of F1 flies are genetic mosaics (St. Johnston, 2002). In F1 screens the germline of a candidate mutant may not therefore transmit the responsible mutant allele (the mutation would not be fully penetrant), which would result in "false positive" mutants. Hence, to test whether the mutation was passed on to the next generation, the recovered adult males were crossed with C(1)Dx virgin females. The larval progeny were then re-tested with Nile Red feeding under the same conditions. Three candidate mutants showed a heritable mutation. Shown in figure 40 are examples of Nile Red fed larval progeny that had inherited the phenotype and examples that did not.

Table III. Approximate number of larvae screened and selected in the forward genetic screen

Date	Approx # Ls screened	# Ls picked	# Candidate mutants recovered
3/5/13	700	16	0
3/7/13	900	15	0
4/10/13	800	11	0
4/12/13	1400	15	0
4/16/13	600	17	0
4/18/13	400	23	0
4/19/13	900	43	0
5/8/13	300	19	0
5/9/13	600	15	0
5/10	700	22	2
5/11/13	600	11	1
Total	7900	207	3

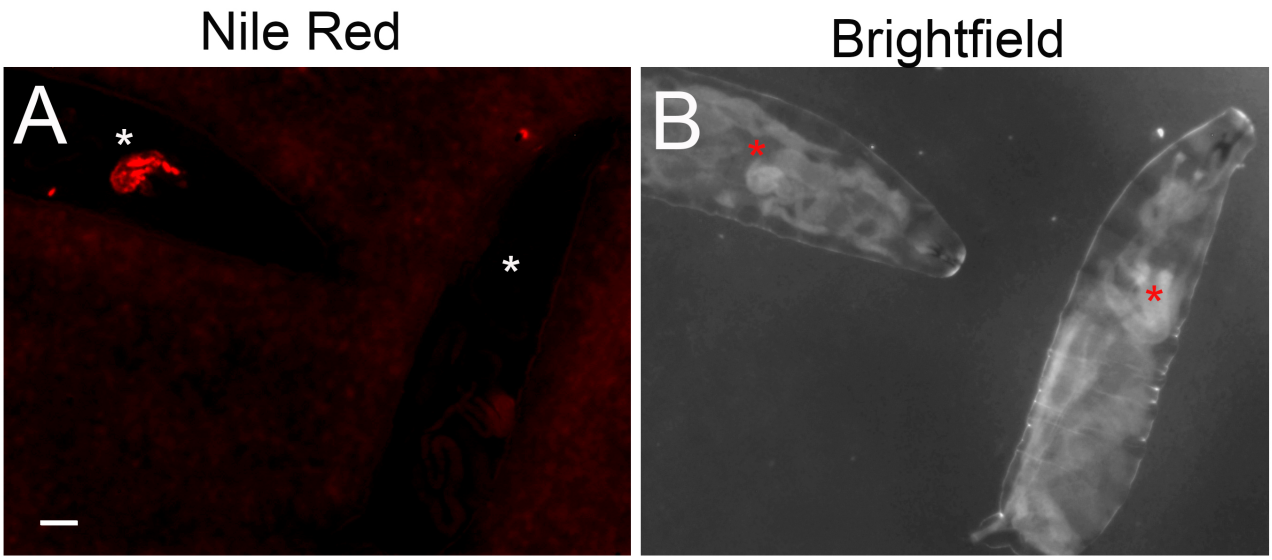

Figure 40. Larval progeny from candidate mutants display the midgut phenotype after Nile Red feeding. Shown here are two larvae: one that displays the NR midgut phenotype (left) and one that does not (right). The white/red asterisk represents a landmark of the midgut. Scale bar is 200 µm.

After noting that the phenotype was heritable, the males were tested three additional times for the presence of an inheritable linked mutation. The larval progeny were screened for the phenotype with Nile Red feeding and yeast paste chase and allowed to grow to adulthood. The percentages of male and female progeny that did and did not display the phenotype were recorded. If the mutation was on the X chromosome, the progeny showing the phenotype with Nile Red feeding should be males. Shown in table IV below are: the number of progeny that showed the Nile Red phenotype (termed Nile Red positive), the number of progeny that did not show the Nile Red phenotype (termed Nile Red negative) scored as males and females. The three candidate mutants were named RD6, BD3 and BD4.

The preliminary data is shown in table IV, which shows the number and sex of the progeny that accumulated LDs. Almost all of the progeny of the candidate mutants that displayed Nile Red staining in the midgut epithelium were males. The female progeny that had staining in the midgut were false-positives and the males that did not display staining in the midgut were false negatives. These results suggest that the forward genetic screen worked, leading to the recovery of three candidate mutants, which have an X-linked mutation.

Table IV. Number of Nile Red-positive and Nile Red-negative of larval progeny

Mutant	Nile Red Positive		Nile Red negative	
	♂	♀	♂	♀
RD6	13	1	6	9
BD3	4	0	6	6
BD4	5	1	0	6

V.5 Identifying the affected X genes of candidate mutants

Identifying the affected X-linked genes of mutants requires two steps. The first step is finding out whether the mutation is dominant or recessive. To address this, each of the mutant males could be crossed with OrR virgin females and the resulting F1 female progeny would be analyzed for the presence of Nile Red staining in the midgut epithelium. If the phenotype is observed in female progeny, then the mutation seems to be X-linked dominant. If the female progeny do not have the phenotype, then the mutation must be X-linked recessive. The second step is to map the mutation.

Because EMS induces random mutations within the genome, identifying the genes that are responsible for the accumulation of lipid droplets in the midgut could be a challenging process. To map mutant alleles, a step-wise strategy can be designed to determine the region on the chromosome and ultimately the actual gene locus. Mapping can be achieved with various molecular strategies, such as high-throughput genome sequencing, inverse PCR, deletion mapping (Cook et al., 2012), complementation testing and many others, used separately or in combination.

The time and effort required to identify the molecular nature of the EMS mutations could be drastically reduced by using either deficiency (Df) kits or collections of duplications as mapping strategies. Both Df and duplication lines cover most of the X-chromosome and are readily available at the Bloomington Stock Center. The stock center provides 92 Df fly stocks, each of which have gene deletions for the X chromosome, covering 98% of genes. Additionally, the stock center provides 378 stocks of Dp(1;Y) chromosomes, each of which have duplicated chromosomal regions

generated by attached-XY chromosomes with inversions in the X chromosome (Cook *et al.*, 2010).

To use Df kits as a mapping strategy, each recovered candidate mutant male from the EMS screen could be crossed with virgin females from each of the 92 Df lines. The F1 progeny would then be analyzed for accumulation of lipid droplets in the midgut. Presence, or worsening, of the phenotype would indicate that the mutations could be located within the region covered by the deficiencies. The rationale is that Df lines would fail to complement loss-of-function mutations located in the same chromosomal region of the deletion. Absence of the phenotype would exclude the group of genes uncovered by the deficiency, suggesting that the defects are in a different chromosomal region.

Alternatively, to use collections of chromosomal duplications as a mapping strategy, each recovered candidate mutant male could be crossed with virgin females from each of the 378 available Dp(1;Y) lines. The F1 progeny would then be analyzed for accumulation of lipid droplets in the midgut. Contrary to the rationale applied for Df lines, in this case, the ability of chromosomal duplications to rescue the accumulation of lipid droplets in the midgut would be analyzed. Specifically, Dp(1;Y) lines could complement loss-of-function mutations located in the same chromosomal region of the deletion. Rescue, partial or complete, of the phenotype would indicate that the mutations are likely be located within the region covered by the duplication.

Because both Df and Dp(1;Y) lines cover most of the X-chromosome, using them through complementation testing may be an efficient mapping strategy as a first step in identifying the unknown genes (Cook *et al.*, 2010; Cook *et al.*, 2012). The narrowed down chromosomal region could then be confirmed by using P{SUPor-P} P-element

insertion set for the X chromosome. The P-element insertion lines contain mapped single transposon insertions and are available at the Bloomington stock center. Additionally, the chromosomal region can then be further narrowed down using the UAS/Gal4 system. Specific UAS-RNAi fly lines can be obtained or created, if not readily available, and used to systematically knockdown the function of the multiple potential candidate genes found within the chromosomal region of interest.

In final consideration, I showed here that the systematic forward genetic screen composed of EMS mutagenesis and Nile Red feeding can be successfully used to identify novel mutations that affect lipid translocation in Drosophila larvae. Additional rounds of the forward screen and identification of the candidate genes obtained from the screen should be performed in future studies. Because many known human disease genes have Drosophila homologues (Fortini *et al.*, 2000; Reiter *et al.*, 2001; Bier, 2005), the forward genetic screen could lead to unraveling molecular mechanisms in lipid translocation that may be relevant to understanding lipid translocation in humans.

CHAPTER VI. Materials and methods

VI. 1 Fly stocks:

The double-strand RNA (dsRNA) line carrying two autosomal β spectrin–specific inserts (UAS- β-SpecdsRNA) was obtained from Dr. Graeme Davis (University of California, San Francisco, CA; Pielage *et al.*, 2005). The UAS-dsRNA lines for Lsd-2 (stock number 34617) and brummer (stock number 25926), the wing *MS1096*-Gal4 driver, the fat body and hemocyte *Cg*-Gal4 driver, the midgut *MexII*-Gal4 driver, UAS-*DSRed* and Lpp deficient line 16302 were obtained from the Bloomington Stock Center (Bloomington, IL). The dsRNA lines for lipophorin (stock number 28946) and α spectrin (stock number 31209) were obtained from TRIP at Harvard Medical School. The myc-tagged UAS α- and β-spectrin transgenes were previously described (Mazock *et al.,* 2010). The MTP mutant line, $mtp^{\Delta ex1}$, was created by imprecise excision of EP[GE10702] and harbors a 559 bp deletion (Palm *et al.*, 2012). The Lpp fly line, carrying one functional copy of the *Lpp* gene, created by the insertion of a 3' half transposable P-element (Bellen *et al.,* 2004) obtained from the Bloomington Stock Centre (stock #16302). The wild-type fly line OrR was also obtained from the Bloomington Stock Centre.

VI.2 Antibodies

Rabbit anti-β spectrin serum (KCar; Dubreuil and Yu, 1994) was used for immunofluorescence and rabbit anti-β spectrin serum (337; Byers *et al.*, 1989) was used for Western blots. Myc-tag specific antibody 9E10 was from Sigma-Aldrich (St. Louis, MO). Immunofluorescent staining was carried out as previously described (Dubreuil *et al.*, 2000) using Texas Red– (Zymed, South San Francisco, CA) or Cy3-

labeled secondary antibodies (Invitrogen, Carlsbad, CA). Guinea pig anti-lipophorin was a gift from Dr. Suzanne Eaton at the Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany (Eugster *et al.,* 2007). Affinity-purified anti-β_H spectrin was described previously (Dubreuil *et al.*, 1997).

VI. 3 Microscopy

Larval tissues were dissected and fixed as previously described (Dubreuil *et al.,* 2000) and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were captured using a FV500 confocal microscope (Olympus, Center Valley, PA) with a 40× Plan-Apo oil immersion objective and Fluoview 2.1 software. Images were saved as “Experiments” in Fluoview and were converted to jpeg format. Montages were assembled using Photoshop CS 4.0 (Adobe Systems, San Jose, CA). The Z axis reconstruction of fat body was produced from a Z series of images using the Reslice feature in ImageJ (Abramoff *et al.,* 2004). Lipid droplets were analyzed by differential interference contrast (DIC) using a Zeiss Axioskop microscope. Images were captured using an Axiocam camera and AxioVision software.

VI.4 Electron Microscopy-SEM

Drosophila third instar larvae were prepared for scanning electron microscopy (SEM). Larvae were fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.2 for four hours at room temperature. They were then washed three times for 10 minutes with phosphate buffer and then treated with 2% osmium tetroxide for 30 minutes. After they were washed three more times for 10 minutes with phosphate buffer, the samples were

dehydrated in a graded series of ethanol concentrations (25%, 50%, 50%, 75%, 95% and 100%) each for 30 min. Following critical-point drying, the larvae were mounted on stubs with double sticky tape, coated with gold and taken to the SEM for imaging: JEOL 5600 LV digital scanning electron microscope working at 280 Å.

VI. 6 Oil Red O staining

Larval tissues were processed as described by Gutierrez et al. (2007). Briefly, larvae were dissected and fixed in 4% paraformaldehyde in phosphate buffered saline for 10 min. Specimens were then rinsed twice with *Drosophila* Ringers solution, incubated for 20 to 30 min in Oil Red O stain (6 ml of 0.1% Oil Red O in isopropanol and 4 ml distilled water: prepared fresh and passed through a 0.45-µm syringe filter), and rinsed twice with Ringers solution. Stained material was then transferred to glycerol mounting medium.

VI.7 Antibody staining

Larvae were dissected in 4% paraformaldehyde and fixed for 10-30 min (depending on the tissue- for midgut tissues, 10 min and for fat body-30 min). Following fixation, the larvae were rinsed with Ringers solution and let sit for 10 min. They were then permeabilized with 1% Triston X 100 for 10 min. Blocking was in incubation solution (TBSTS) for 10 min. Antibody incubations were also performed in TBSTS. The Primary antibody was incubated overnight at 4C and the secondary for 2 hrs at room temperature. Primary and secondary antibodies were removed using 4x10 minute TBST solution washes. Tissues were mounted in Vectashield mounting medium.

VI. 8 Nile Red staining

For staining intracellular lipid droplets, larvae were dissected in 4% paraformaldehyde and fixed for 10 min. They were rinsed with Ringers solutions 2 x and then incubated in Nile Red staining working solution. The working solution was diluted 1:1,000 in Ringers sol (from 100mg/ml stock in ethanol). The tissues were incubated with the dye for 30min at room temperature. They were then washed with Ringers solution 2x and transferred on slides.

VI.9 Nile Red feeding

Larvae were fed Nile Red in yeast paste for 1 or 2 hrs. 10 μl of the working solution of Nile Red (100mg/ml in ethanol) was added to 0.1g yeast paste. The solution was mixed well in yeast paste and fed to larvae on apple juice plates.

VI.10 Western blotting

Western blots were performed with alkaline phosphatase–coupled secondary antibodies (Zymed) and stained with bromochloroindolyl phosphate as previously described (Dubreuil and Yu, 1994). One fly/sample was used. Each fly was added to 40 µL of 1x Laemmli Sample Buffer that contained 2-mercaptoethanol (LSB), boiled for 2 min boiled for 3 minutes and then centrifuged at 4C. The samples were run on an SDS-PAGE gel and analyzed by Western blotting. Incubation with the primary myc antibody was performed overnight at 4C and with secondary for 2hrs at room temperature.

VI.11 Fly crosses

VI. 11.1 Rescue crosses- Wing phenotype:

Homozygous autosomal stocks with insertions of the myc-epitope-tagged α and β spectrin UAS transgenes were crossed to *MS1096*-Gal4 females, either singly or together. For rescue crosses, UAS *α-Spec37* males were first crossed with UAS-*β-Spec95* females. Doubly heterozygous F1 males were then crossed with *MS1096*-Gal4 virgin females at 25osC. Individual adult fly progeny were scored for transgenes present by western blotting with anti-myc and anti α-actinin antibodies (loading control).

VI. 11.2 Rescue cross- Fat body and lipid transport defects

Crosses between UAS-*β-Spec62/ Y ; +/+* males and +/+; *Cg-Gal4/Cg-Gal4* females resulted in 100% lethality in F1 females *(*UAS-*β-Spec62/+; Cg- Gal4/+)* but not males *(+/ Y; Cg-Gal4/+).* For rescue crosses, UAS α-*Spec37* males were first crossed with UAS-β-*Spec62* females (both parents homozygous). F1 males (UAS-*β-Spec62/ Y*; UAS α-*Spec37 / +)* were then crossed with *Cg-Gal4/ Cg-Gal4* virgin females for fat body expression. Individual F1 flies were analyzed on western blots to verify transgene expression, as above.

VI. 11. 3 Gal4 dilution cross: To ask if the rescue of the β spectrin over-expression phenotypes by α spectrin over-expression was simply due to dilution of Gal4 activity by a second UAS transgene, UAS-*β-Spec62* / UAS-*β-Spec62* females were crossed with UAS-*CD8 GFP/* UAS-*CD8 GFP* males. F1 males (UAS-*β-Spec62/ Y*; UAS-*CD8 GFP / +)* were then crossed with *Cg-Gal4/ Cg-Gal4* virgin females for fat body expression.

GFP-positive F1 female progeny were tested for viability (expression of UAS-*β-Spec62* alone was known to be lethal in the absence of UAS α-*Spec37* co-expression).

VI.11.4 Effect of α spectrin gene dose on β spectrin over-expression phenotype

The viability of flies over-expressing UAS β-*Spectrin* at 22°C was tested in flies that were heterozygous for a null allele of α spectrin (α*Specrg41*). Homozygous *Cg-Gal4,* UAS-*CD8- GFP* virgin females were crossed with UAS *β-Spec95* males at 22oC. F1 virgin females *(X / X; Cg-Gal4,*UAS *CD8-GFP / +; UAS-β-Spec95/ +) w*ere then crossed with α*Specrg41*/TM3 males. Individual F1 larvae were scored for the presence of *GFP* fluorescence to select for the presence of the *Cg-Gal4,* UAS-*CD8-GFP* chromosome*.* Larvae were grown to adulthood then selected for the presence or absence of the α*Spec*rg41 chromosome by scoring the *Sb*-marked balancer chromosome. Finally individual flies were analyzed on western blots to test for UAS-*β-Spec95* transgene expression.

VI.12 Genetic screen procedure

100 1-day-old isogenic X OrR males were collected and aged for 3 more days before mutagenesis. C(1)Dx virgin females collected for 6 days before mutagenesis cross (goal was about 200). On the day of mutagenesis, males were transferred to an empty vial for 1 hour to dehydrate. Mutagenesis buffer = 10 mM Tris pH 7.5 + 1% sucrose; neutralization solution for deactivating ems = 10% sodium thiosulfate made fresh; ems diluted in 10 ml buffer to concentration of 25 mM; 3ml of ems solution added to a cotton-plugged vial with 2 kim wipes compressed into bottom. Males were added to this vial

after dehydration and left in hood overnight at room temp. Next day, males transferred to a 2nd plugged vial with kim wipes and without ems for 30 minutes. They were then transferred to standard food vial for 2 hours. Finally, males were crossed with C(1)Dx virgin females and placed into collection chamber for larval collection. Plates were changed every day for 3 additional days. The F1 larval progeny were collected and screened for accumulation of lipid droplets in the midgut. Larvae were transferred one by one onto apple juice plates, Nile Red in yeast paste was then added to the plate. After two hours, larvae were washed with dH_2O using a nitex cloth and an Eppendorf test tube and then were transferred to a new plate. The excess water was absorbed from the plate and yeast paste was added. The plate was kept at 25°C. After 24 hrs, the larvae were washed using the same approach and transferred with dH2O to a big plastic plate for screening under the fluorescent dissecting microscope. The screening process took approximately 1 hr/ 1,000 larvae. Larvae that showed a phenotype were recovered using a pasteur pipette and transferred to an apple juice plate. They were then transferred to a vial after 2 days to develop to adulthood. The ideal conditions of detecting the lipid droplets accumulated in the midgut were: larvae fed a 10% Nile Red dye in yeast paste for 2 hrs and switched to a diet to yeast paste for 24 hrs at 25°C and larvae fed a 10% Nile Red dye in yeast paste for 2 hrs and switched to a diet to yeast paste for 26 hrs at 22°C.

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