Elucidating the Lipoprotein-Mediated Mechanisms Involved in Dietary Fat Mobilization from the Gut

BY

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THESIS

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AGPAT	acyl-sn-glycerol-3-phosphate o-acyltransferases
ApoB	apolipoprotein B
ATGL	adipose triglyceride lipase
Bmm	brummer
CE	cholesterol ester
CM	chylomicron
DGAT	diacylglycerol acyltransferase
DG	diacylglycerol
Dhc	dynein heavy chain
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FA	free fatty acid
GC	gastric caeca
GPAT	glycerol-3-phosphate acyltransferases
HDLpp	high density lipophorin
Khc	kinesin heavy chain
LCFA	long chain fatty acid
LD	lipid droplet
LDL	low density lipoprotein
LDLpp	low density lipophorin
Lpp	lipophorin
Lpr1	lipophorin receptor 1
Lpr2	lipophorin receptor 2
LSD2	lipid storage droplet-2
LTP	lipid transfer particle
MCFA	medium chain fatty acid

MG	midgut
MT	microtubules
MTOC	microtubule organizing center
MTP	microsomal triglyceride transfer protein
NR	nile red
PLIN	perilipin
PM	plasma membrane
PV	proventriculus
TG	triacylglycerol
SHOT	shortstop
VLDL	very low density lipoprotein

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SUMMARY

Dietary fat in the form of triglycerides (TGs) is an important energy store in many animal cells. Because fat (oil) and water don't mix, elaborate mechanisms have evolved to store TGs in and retrieve TGs from lipid droplets (LDs, the cellular organelle that stores neutral lipids) within the polar cytoplasm. Research in the field of LD dynamics is growing tremendously, and new roles for LDs in lipid translocation at both the cellular and organismal levels are continually being discovered. Due to a growing understanding of the role of LDs in lipid homeostasis, abnormal LD dynamics are increasingly being implicated in human diseases such as obesity, diabetes and cardiovascular disease. Understanding the molecular mechanisms underpinning the complex processes of lipid translocation and LD dynamics will not only shed light on the cellular functions of LDs but also provide invaluable insights for designing targeted therapies to combat associated metabolic diseases. The data presented in this thesis establish critical spatial parameters governing lipid translocation: 1) the uptake of fatty acids (FAs) from the lumen of the gut and their storage as TGs in the form of LDs at the apical domain of the enterocyte, and 2) the subsequent incorporation of LD-derived FAs into lipoprotein particles that are eventually carried by the circulation to cells throughout the body.

Early descriptions of the insect fat processing machinery focused on points of divergence between animals and humans. In light of the differences between vertebrates and invertebrates, it suggested that the machinery responsible for handling dietary fat must have evolved independently. Thus, it came as somewhat of a surprise when molecular analyses established that key players in dietary fat packaging and transport in mammals are also conserved in animals. Over the last decade, there has been a shift in the use of key

model organisms (the worm *Caenorhabditis elegans*, the zebrafish *Danio rerio*, and the fly *Drosophila melanogaster*) from studies of growth and development toward genetic characterization of carbohydrate, sterol and lipid metabolic pathways. In Drosophila, the metabolic functions and organ systems responsible for dietary lipid uptake, storage and metabolism closely resemble their mammalian counterparts. As such, the Drosophila model system emerged as an excellent genetic model organism to study the gaps in our understanding of lipid uptake, storage and trafficking, particularly as it relates to LD dynamics.

Previous work in Drosophila suggested that the cytoskeleton plays an important role in lipid translocation. Specifically, knockdown of the cytoskeletal protein Spectrin in the larval fat body was found to result in the loss of small cortical LDs. The small cortical LDs in the fat body were found to be dependent on Spectrin for their association with the plasma membrane (PM). The positioning of the cortical LDs potentially implicates an active mechanism that aligns the lipid uptake machinery at the PM to LD formation. This led to the proposal of a model for "Coupled FA Transport," wherein FA transport into the cell is physically linked to the re-synthesis of TGs in the cytoplasm and subsequent incorporation into LDs. This model provides an explanation for how FAs are able to exist (and aggregate into LDs) in the polar environment of the cytoplasm; earlier working models of lipid translocation and LD formation had instead relied upon passive diffusion of FAs across the plasma membrane.

Initial discovery and characterization of Coupled FA Transport was accomplished using the larval fat body as a model system. Here, I present detailed findings on the spatial parameters governing fat uptake and transport in the enterocytes of the midgut, which

demonstrate a similar coupling phenomenon in the cytoplasm of these cells. Additionally, I present data suggesting that microtubules (MTs) and MT dependent motor proteins play important roles in coordinating the uptake of dietary fat and the polarization of LD populations within the enterocyte. I further propose an additional mechanism whereby FAs from the constitutive pool of LDs (formed from the Coupled FA Uptake step) are directed to the site of the export machinery responsible for the mobilization of fat out of the enterocyte via circulation; MTs and MT motor proteins also play critical roles in this targeting process. Continuation of these studies in Drosophila will enable us to probe the molecular mechanisms of the conserved apoB lipoprotein pathway, with possible implications for health and human disease.

CHAPTER I. Introduction

I.1 Lipid translocation overview

I.1.1 Why we need to study fat biology

Obesity and defects in the regulation of fat metabolism are growing problems on a global scale (Smith and Smith 2016). According to a recent survey in the United States, obesity has been identified as a national priority, with 73% of the population in favor of increasing investments to address the obesity crisis and other metabolic health concerns (Puricelli Perin et al. 2014). Many metabolic disorders, such as obesity, non-alcoholic fatty liver disease and atherosclerosis, result from the dysregulation of mechanisms involved in the breakdown, packaging and mobilization of dietary fat from the gut epithelium to target tissues like adipocytes (for storage) or muscles (to be used as energy)(Iskander et al. 2013; Trinh et al. 2016). The pathways mediating the breakdown and utilization of dietary fat have been studied extensively at the cellular level; however, the mechanism by which dietary fat is partitioned between storage and utilization as energy remains unclear (Coburn et al. 2000; Goldberg, Eckel, and Abumrad 2009; Iskander et al. 2013; Greenberg et al. 2011). If we are to develop targeted therapies to treat obesity and other metabolic disorders, we must first understand the molecular pathways that underpin the regulation of fat metabolism.

I.1.2 One way lipid translocation occurs is by a specialized protein class, ApoB-containing lipoproteins

Lipid metabolism involves the breakdown and storage of dietary fat. Triglycerides (TGs) are the major form of dietary fat, and the major storage form of fat found in the body.

This simple lipid consists of three fatty acid (FA) chains linked to a glycerol backbone, resulting in a neutral lipid (defined as a hydrophobic molecule that lacks charged groups). TGs are stored in the cytoplasm of the cell in specialized organelles known as lipid droplets (LDs). These fats may serve as a high-density energy reserves or as structural stores for the production of membrane lipids (Abumrad and Davidson 2012; Palm et al. 2012; Goldberg, Eckel, and Abumrad 2009). However, an overabundance of TGs can also become a liability; for example, increasing lipid concentrations can lead to lipotoxicity, resulting in stress on the cell and organism (Pol, Gross, and Parton 2014; Welte 2015b; Pilch and Liu 2011; Kühnlein 2012b). In fact, it is thought that specialized lipid storage mechanisms like LDs may have arisen in part to limit the toxicity caused by ingestion of large amounts of dietary fat (Abumrad and Davidson 2013; Welte, Box, and Hall 2009; Pol, Gross, and Parton 2014; Guo et al. 2009). A suite of specialized gene products has evolved to meet the unique requirements for uptake, storage and transport of ingested fats (Demignot, Beilstein, and Morel 2014; Giammanco et al. 2015). This class of specialized proteins, known as apolipoproteins B (apoB), function as acceptors of neutral lipids (TGs) and as organizing proteins that facilitate the transport of TGs intracellularly and between various tissues during lipid translocation (Canavoso et al. 2001; Panáková et al. 2005; Demignot, Beilstein, and Morel 2014; Davidson 2015). Lipid metabolism involves the breakdown and storage of dietary fat. Triglycerides (TGs) are the major form of dietary fat, and the major storage form of fat found in the body. This simple lipid consists of three fatty acid (FA) chains linked to a glycerol backbone, resulting in a neutral lipid (defined as a hydrophobic molecule that lacks charged groups). TGs are stored in the cytoplasm of the cell in specialized organelles known as lipid droplets (LDs). These fats may serve as a high-

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I.1.3 The process of lipid translocation occurs via apoB lipoproteins

ApoB-containing lipoproteins facilitate the balance between lipid uptake, storage and mobilization from intestinal cells (enterocytes) (Sieber and Thummel 2012). In both humans and animal models, the intestine (the gut in insects) is involved in the absorption of dietary fats and in the regulation of lipoproteins (Giammanco et al. 2015; Hussain, Shi, and Dreizen 2003; Kesaniemi, Miller, and Fisher 1990; Abumrad and Davidson 2012). If the coordinated regulation of lipid metabolism is disrupted, a range of severe metabolic disorders can result. A number of reports in humans have revealed that reduced levels of

apoB-containing lipoproteins are associated with fatty liver disease, intestinal lipid malabsorption, and defects in peripheral tissue function (Giammanco et al. 2015). In contrast, elevated levels of apoB-containing lipoproteins cause obesity, heart disease and diabetes. Elevated apoB levels correspond to increases in low-density lipoproteins (LDLs), which in turn result in abnormal accumulation of TGs in the form of LDs (Abumrad and Davidson 2012; Palm et al. 2012; Kühnlein 2012b, 2012a; Greenberg et al. 2011). Though the molecular mechanisms of dietary fat storage and transport from the intestine to downstream tissues have been extensively studied, there still exist large gaps in our understanding of (1) the biogenesis and regulation of LDs, for which there are several conflicting theories, and (2) the regulatory mechanisms by which arriving dietary fat is partitioned between storage in the cytosol (in LDs) or transport in apoB-lipoproteins.

Due to the critical role that apoB-lipoproteins play in lipid metabolism, there is a need to identify and better understand key components of the lipoprotein pathway. The subject of this thesis is the apolipoprotein B (ApoB) pathway, which mediates the harvesting of neutral fats from the diet, their uptake as FAs, their subsequent storage in the cytoplasm and their eventual incorporation into secreted lipoprotein particles that the blood carries to cells throughout the body.

I.1.4 Drosophila is a potentially useful model organism for addressing the gaps in the ApoB-lipoprotein pathway in mammals.

Drosophila melanogaster has served for decades as one of the most widely used model organisms for the study of developmental processes, and is an excellent genetic system by which to study the molecular mechanisms of metabolic disease (Bharucha 2009;

Baker and Thummel 2007; Schlegel and Stainier 2007). In fact, of the genes and gene products known to be involved in lipid translocation in mammals, 85% have putative homologs in Drosophila (Reiter et al. 2001). Furthermore, the tissues where fat metabolism occurs in mammals have counterparts in Drosophila (Trinh et al. 2016). For example, the fat body in insects is involved in energy metabolism and is the major site for lipid storage. The fat body is analogous to the liver and adipose tissue, where fat storage and fat metabolism occur in mammals (Nichol, Law, and Winzerling 2002; Palm et al. 2012; Canavoso et al. 2004, 2001; Lemaitre and Miguel-Aliaga 2013). Given the genetic conservation between humans and flies, it is reasonable to hypothesize that the molecular pathways may also be conserved. My dissertation will contribute to our understanding of fat uptake, storage and trafficking in mammals by studying how dietary fat is stored in and mobilized from enterocytes via the lipoprotein pathway in Drosophila.

I.2 Digestion and nutrient absorption occur in the intestine in mammals and the midgut in Drosophila

Dietary lipid translocation can be broken into four steps: (1) lipid digestion, (2) lipid uptake into the enterocyte, (3) intracellular packaging and secretion of particle, and (4) lipid transport into the circulation (Giammanco et al. 2015; Abumrad and Davidson 2012). First, digestion of lipids begins as soon as food is consumed; in mammals, digestion occurs as food enters the mouth, moves to the stomach, and then passes on to the small and large intestine (Leonard, Snodgrass, and Robertson 2010; Goldberg, Eckel, and Abumrad 2009; Jump and Clarke 1999). In Drosophila, food enters the esophagus and passes through distinct regions of the gut, from the foregut to the midgut, and then to the hindgut

(Marianes et al. 2013; Lemaitre and Miguel-Aliaga 2013). Second, dietary fats are taken up by intestinal cells. In mammals, this occurs in the small intestine; in Drosophila, this occurs in the midgut, a compartment of the gut that is physiologically equivalent to the mammalian stomach and small intestine (Giammanco et al. 2015; Coburn et al. 2000; Reiff et al. 2015; Buchon et al. 2013; Abumrad and Davidson 2012). Third, dietary fat in enterocytes is packaged intracellularly, in preparation for one of two fates: 1) incorporation into lipoproteins for export, or 2) incorporation into cytoplasmic LDs for storage (Demignot, Beilstein, and Morel 2014; Kühnlein 2012a). In mammals, lipids are packaged into TG-rich lipoprotein particles called chylomicrons in the small intestine (Su and Abumrad 2010; Abumrad and Davidson 2013). Chylomicrons have a central lipid core and an outer layer of phospholipids, free cholesterol and apolipoproteins (Giammanco et al. 2015). In Drosophila, dietary fats taken up by the midgut epithelial cells are packaged into apoB lipoproteins for translocation to downstream tissues or stored in the cytosol as LDs (Su and Abumrad 2010; Giammanco et al. 2015; Davidson 2015), (Butterworth, Emerson, and Rasch 1988). Fourth, lipids are transported to downstream tissues via circulation (Giammanco et al. 2015; Abumrad and Davidson 2012; Canavoso et al. 2001). In both mammals and Drosophila, lipid translocation depends on the circulatory system to mediate transport to downstream tissues (Van der Horst, Roosendaal, and Rodenburg 2009). Mammals have a closed circulatory system, in which blood is enclosed at all times within vessels. The majority of lipids absorbed from the diet enter the blood circulation through the lymphatic system, which facilitates transport of chylomicrons from the small intestine into circulation (Dixon 2010; Saba and Oridupa 2012). In contrast, Drosophila have an open circulatory systems in which blood (hemolymph) flows freely around tissues (Reiber and

McGaw 2009). In mammals, lipids are ultimately translocated to three primary tissue types: the small intestine, the liver, and the adipose tissue (Van der Horst, Roosendaal, and Rodenburg 2009; Reiber and McGaw 2009; Canavoso et al. 2001; Lemaitre and Miguel-Aliaga 2013). In Drosophila, lipids are translocated from the midgut to the brain, the fat body, imaginal discs, and many other tissues (Kühnlein 2012a).

I.2.1 Uptake of fatty acids by enterocytes: passive diffusion vs protein-facilitated fatty acid transfer

Dietary lipid metabolism begins when TGs are hydrolyzed by pancreatic lipases in the intestinal lumen to produce free FAs for uptake by enterocytes (Goldberg, Eckel, and Abumrad 2009). The mechanisms by which FAs are transported across the plasma membrane (PM) of the enterocyte are still up for debate. The classic view is that lipophilic FAs permeate and traverse the membrane by a 'flip-flop' mechanism, and ionization of FAs by the higher intracellular pH prevents back-diffusion of FAs (Abumrad and Davidson 2012; Pilch and Liu 2011). However, studies with isolated cells have shown that passive diffusion of BMIPP, a metabolic tracer for FA utilization, contributed less than 15% to the uptake of FAs (Coburn et al. 2000). The second proposed route of FA uptake is supported by studies that have shown FAs to be taken up selectively through the apical membrane, suggesting that FA processing is dependent on the cellular entry site (Abumrad and Davidson 2012; Mansbach and Gorelick 2007). When radiolabeled FAs were introduced into intestines in mice, the ratio of TG to phospholipid formed was 10-fold greater when FAs were added apically relative to basolaterally (Storch, Zhou, and Lagakos 2008; Abumrad and Davidson 2012). The selectivity of the apical membrane for FA uptake

suggests that FA processing and translocation into the enterocyte is regulated via a protein-mediated mechanism (Su and Abumrad 2009; Abumrad and Davidson 2012; Storch, Zhou, and Lagakos 2008; Coburn et al. 2000).

Several proteins have been implicated in the process of FA uptake; however, there are likely many others that play redundant roles but remain unidentified. Selective uptake and/or export of dietary lipids to the sub-apical domain of the enterocyte was shown to occur due to the action of long chain fatty acid (LCFA) transporters (Giammanco et al. 2015; Abumrad and Davidson 2012; Storch, Zhou, and Lagakos 2008). Among candidate transporters in the small intestine, apical membrane proteins such as fatty acid transport proteins (FATPs, like CD36) were suggested to play a role in regulating the uptake of fat in enterocytes (Coburn et al. 2000; Goldberg, Eckel, and Abumrad 2009; Nauli et al. 2006; Abumrad and Davidson 2012; Giammanco et al. 2015). However, experiments with CD-36 deficient mice showed no evidence of altered lipid absorption or lethality, suggestive of an as-yet uncharacterized mechanism that compensates for the loss of CD-36 and mediates proper FA uptake in enterocytes (Fig.1A)(Coburn et al. 2000).

Following lipid uptake across the apical membrane, FAs are transported into the endoplasmic reticulum (ER), where they are resynthesized into TGs. The intracellular transport of FAs has been proposed to involve fatty acid binding proteins (FABPs), which are present mainly in the cytoplasm (Pan and Hussain 2012). I-FABP, one of the FABP family proteins, has been shown to be confined to the small intestine in mammals, where it functions to extract FAs from the plasma membrane and deliver them to the lumen of the ER. However, I-FABP null mice showed no defects in the absorption of dietary fat or in levels of TG, suggesting that I-FABPs may not be essential for intracellular targeting of FAs

toward TG synthesis. It remains unclear how FAs, which are highly hydrophobic, traverse the cytoplasm to reach the ER membrane for re-esterification into TGs. It is known, however, that dietary FAs are ultimately packaged into chylomicrons as TGs (Abumrad and Davidson 2012; Van der Horst, Roosendaal, and Rodenburg 2009; Mansbach and Siddiqi 2010; Giammanco et al. 2015).

FAs are either directly incorporated into newly synthesized lipoproteins as TGs or are stored in cytoplasmic LDs as TGs. The events involved in partitioning FAs between the two paths remains to be fully elucidated. However, both of these pathways, eventually, lead to the mobilization of fat from the enterocyte to downstream target tissues via lipoproteins.

I.3 Lipid transport in mammals; chylomicron biogenesis and function

Chylomicron (CM) assembly is dependent on the activity of microsomal triglyceride transfer protein (MTP) to co-translationally incorporate apoB-48 (apoB isoform synthesized exclusively in the small intestine) into an immature (nascent) chylomicron particle (Mansbach and Siddiqi 2010; Hussain 2000; Giammanco et al. 2015). The nascent apoB-48 particle is co-translationally transported through the ER membrane; lipidation of the nascent CM, which is dependent on the activity of MTP, begins after its release into the ER lumen (Tso et al. 1984; Hussain, Shi, and Dreizen 2003; Hussain 2000).

After the nascent CM is released into the ER lumen, it fuses with key vesicular transport proteins that facilitate the acquisition of lipid cargo during transit from the ER to Golgi compartments (Hussain 2000; Giammanco et al. 2015). Two different models for CM maturation have been proposed. According to Tso et al., the assembly of very low-density lipoprotein (VLDL) and small nascent CM particles proceeds by two distinct, independent

pathways (the independent pathway model)(Tso et al. 1984). In this model, CM assembly is induced only upon infusion with high concentrations of fat (i.e., dietary fat ingestion), whereas VLDL assembly occurs during all dietary conditions. Alternatively, CMs have been proposed to mature through the core expansion model, in which TG-rich LDs fuse together with CMs to yield lipoproteins of various sizes. This model follows three sequential steps: formation of primordial lipoproteins, synthesis of TG-rich LDs, and, ultimately, biosynthesis of lipoproteins of various sizes driven by fusion (core expansion) of TG-rich LDs with primordial CMs. Despite our understanding from these models, some of the fundamental steps involved in CM assembly and maturation are still poorly understood. In particular, it remains unclear how FAs from the cytoplasmic pool of LDs contribute to LDs formed within the ER bilayer, which are ultimately incorporated into the maturing CM (Fig.1B).

Ultimately, mature CMs are loaded with dietary FAs, which have been re-esterified into TGs, secreted from the intestine and enter the blood circulation. As the chylomicron approaches the target tissue, it docks with lipoprotein lipase, which is located within the capillary (Cartwright and Higgins 2001). Lipoprotein lipase cleaves TGs to produce FAs, which then exit the blood vessel, traverse the extracellular space, and enter target cells such as adipocytes where they may ultimately be reincorporated into lipid droplets as TGs (Hussain 2000; Giammanco et al. 2015; Mansbach and Gorelick 2007). However, the mechanism by which FAs from the blood vessel lumen reach LDs in adipocytes is still not understood at all (Fig. 1C). Once CMs are depleted of most of their TGs, they become CM remnants, which are removed by the liver and digested for repackaging in VLDL particles (Hussain 2000; Giammanco et al. 2015; Mansbach and Gorelick 2007; Windler et al. 1996).

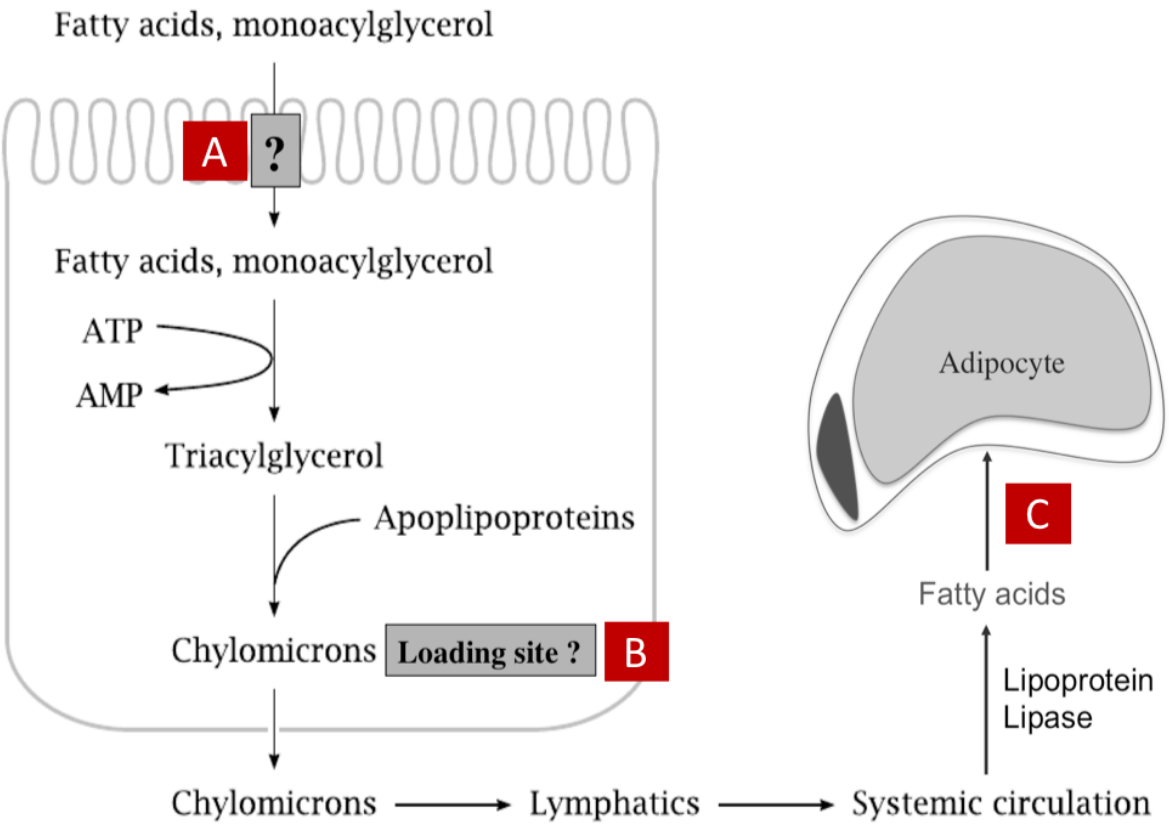

Figure 1. Illustration of some of the gaps in our understanding of the ApoB lipoprotein pathway in mammals. A) FAs cross the plasma membrane selectively through the apical membrane of enterocytes via an unknown protein-mediated mechanism, B) Chylomicron loading: FAs are packaged into chylomicrons as TGs in an MTP dependent process in an unknown endomembrane compartment through an unknown mechanism, C) TGs from circulating chylomicrons are cleaved into free FAs, that then exit the endothelial cell, enter adipocytes and incorporate into TG in adipocyte lipid drople through an unknown mechanism.

I.1.3 Intracellular lipid transport in Drosophila: Major pathways involved in LTP and LPP biology

As illustrated previously, there is a large degree of similarity in lipid biology overall between flies and mammals; however, there are two notable differences. First, in Drosophila, lipid translocation involves two homologs of ApoB, lipophorin (Lpp) and lipid transfer particle (LTP), with distinctly different functions. Mammals utilize a single ApoB lipoprotein, chylomicron (CM), for the export of fat from enterocytes into circulation (described in detail in Section 1.1.2) (Fisher and Ginsberg 2002; Cartwright and Higgins 2001). Second, in mammals, ApoB-containing lipoproteins are synthesized in enterocytes and are then secreted from the enterocyte into the circulation (Giammanco et al. 2015; Hussain 2000). However, in Drosophila, lipoproteins are synthesized in the fat body (analogous to the mammalian adipose tissue and liver) and are secreted into the hemolymph, wherein they traffic in the enterocyte to the site of lipid uptake (Palm et al. 2012; Canavoso 2003; Prasad et al. 1986)(Fig.2).

In Drosophila, Lpp and LTP together accomplish the role of the chylomicron in mammals. There is limited evidence to suggest that after their initial synthesis in the fat body, LTP is internalized in the enterocyte, while Lpp is thought to appear outside the enterocyte (Fig.2) (Palm et al. 2012; Canavoso 2003; Canavoso et al. 2001). Based on the subcellular localization of LTP, it was recently proposed that LTP functions in the midgut epithelium to load dietary lipids in the form of diacylglycerol (DG) onto Lpp particles located outside of the cell (Carvalho et al. 2010; Palm et al. 2012). In contrast to mammalian CM, which occupies various endomembrane compartments in its path in the

secretory pathway, LTP is likely to go through both the endocytic and secretory pathways for the entirety of its lifecycle.

Lpp has been shown to be the major hemolymph lipid carrier, responsible for transporting fat from the gut to target tissues downstream. Newly synthesized Lpp is released from the fat body as an underloaded lipoprotein (high density Lpp, HDLpp). HDLpp uses the circulatory system to reach the midgut to acquire dietary fat and circulates back to the fat body as a loaded lipoprotein (low density Lpp or LDLpp) (Palm et al. 2012; Canavoso 2003; Canavoso et al. 2001; Rodríguez-Vázquez et al. 2015; Prasad et al. 1986; Van der Horst, Roosendaal, and Rodenburg 2009). Similar to the gaps in our understanding of the lipidation and trafficking steps of CMs in mammals, it remains unknown in Drosophila how HDLpp is targeted to enterocytes for dietary fat uptake and how, upon return, the LDLpp is targeted to various tissues, such as muscle, oocytes, or the fat body for dietary fat utilization or storage (Fig.3).

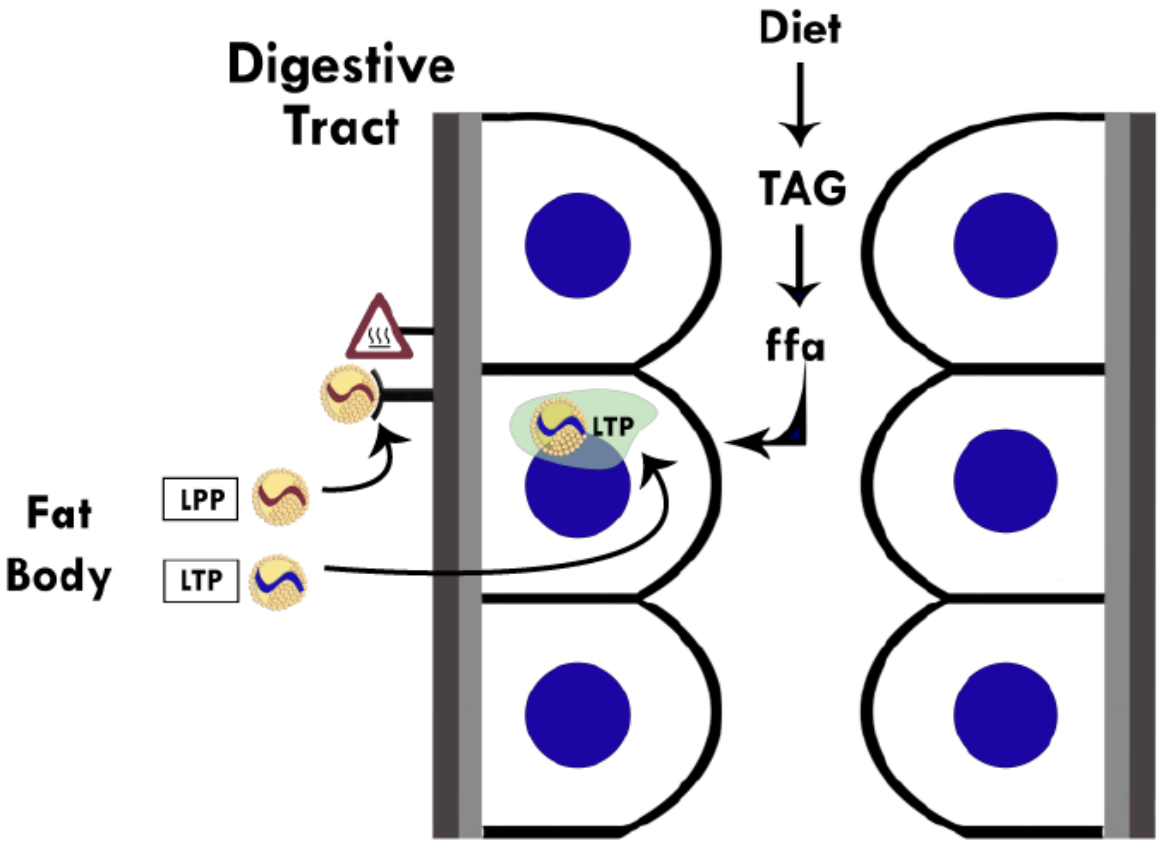

Figure 2. Illustration of fat translocation via ApoB lipoproteins in Drosophila. Drosophila lipophorin (Lpp) and lipid transfer particle (LTP) are synthesized and secreted from the fat body into circulation. LTP is internalized by the cell into an unknown endomembrane compartment to facilitate the export of fat from the gut to Lpp. Lpp remains as a hemolymph bi-directional shuttle transporting fat from LTP in the midgut to target tissues downstream . Loaded Lpp particle is detected by an unidentified receptor at the midgut. The fat movement between particles occurs via an unknown catalyst (red triangle).

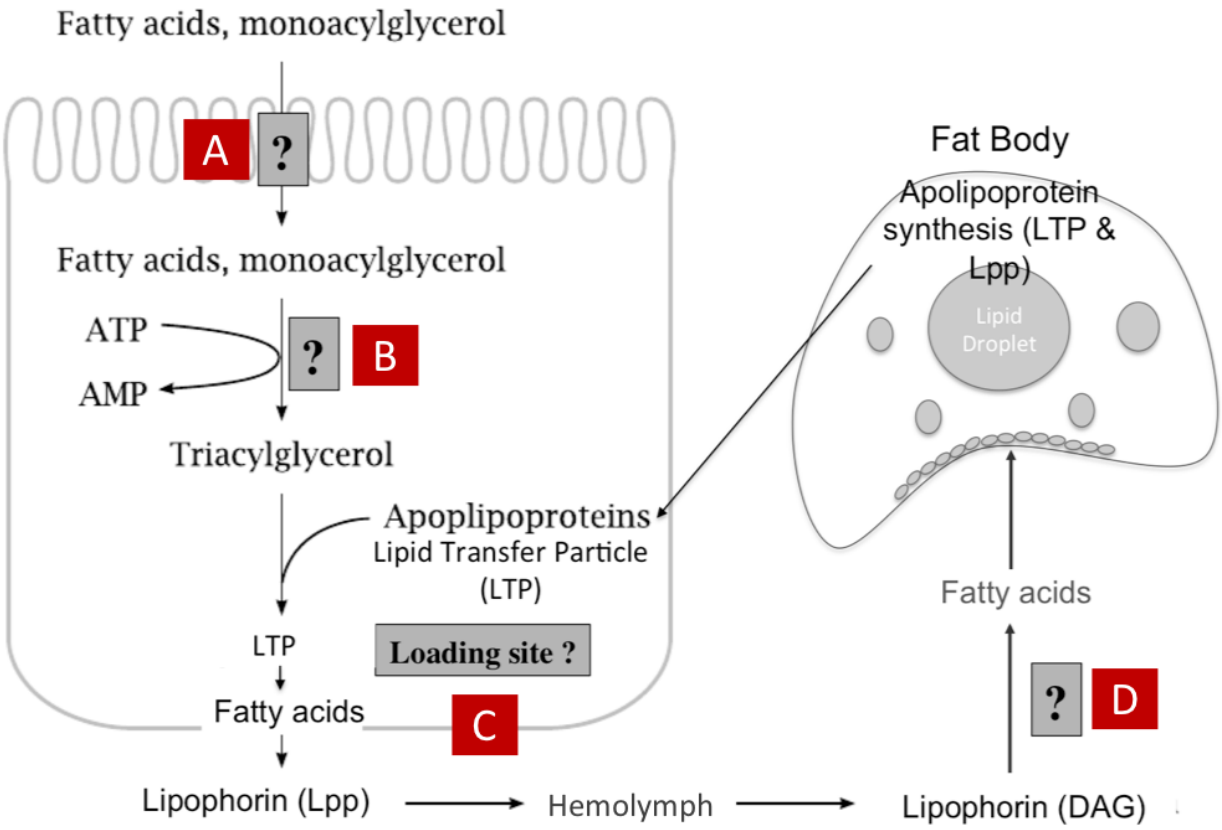

Figure 3. Illustration of the required steps of the ApoB lipoprotein pathway in Drosophila for which mechanisms are not fully understood. A) FAs cross the plasma membrane of enterocytes via an unknown mechanism, B) An unknown mechanism controls the distribution of FAs entering enterocytes, which have two paths (1) incorporation into lipoproteins for transport or (2) incorporation into cytoplasmic LDs for storage, C) Lipoprotein loading: FAs are packaged into LTP as TGs in the lumen of an unknown endomembrane compartment. In addition it is not clear how LTP and Lpp function together to mobilize fat from the enterocyte to blood, D) Diacylglycerides from circulating Lpp are cleaved into FAs, which are taken up and incorporated into LDs in the fat body by an unknown mechanism.

Early steps in lipid translocation in Drosophila closely resemble those in mammals, but key questions remain regarding passive or protein-mediated diffusion of FAs across the PM. Ultimately, FAs enter the cytoplasm of the enterocyte (Fig.3A) and are resynthesized into TGs for the loading of LTP. The next several steps of intracellular lipid translocation involve the re-esterification of FAs into TGs, TG incorporation into LDs, and their mobilization from LDs to lipoproteins. In mammalian enterocytes, the monoacylglycerol pathway (which is responsible for the biosynthesis of TGs in mammals) was shown to contribute up to 75% of the TGs to a lipid-rich CM for transport (Yang and Kuksis 1991; Prasad et al. 1986; Canavoso and Wells 2001). Using quantitative proteomics, it has been shown that the enzymes responsible for TG biosynthesis in mammals closely resemble those in Drosophila (Yen et al. 2008). In both organisms, the final and only committed step in the biosynthesis of TGs is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes that convert DGs into TGs (Wilfling et al. 2013; Yen et al. 2008). Newly synthesized TGs are deposited into cytoplasmic LDs or into nascent lipoproteins in the digestive tract (CMs in mammals; LTP in Drosophila) (Yen et al. 2008; Sieber and Thummel 2012; Wilfling et al. 2013).

The export of lipids from enterocytes in insects and humans differs in at least one important way (Prasad et al. 1986; Canavoso 2003; Palm et al. 2012). Unlike in mammals, the main lipid that is exported into circulation in Drosophila is DG (in mammals the dominant form is TG). However, lipids are loaded into LTP in the form of TGs. Limited evidence suggests that, in flies, the transfer of lipid from LTP to Lpp is directly tied to the conversion/de-esterification of TGs to DGs; in mammals, lipoproteins are loaded with TGs throughout translocation. The precise mechanism by which FAs are converted to DGs/TGs

or how TGs are deposited into LDs and DGs in lipoproteins (LTP & Lpp) are still unknown (Fig.3 B & C).

It is speculated that LTP catalyzes the transfer of DGs from the larval midgut to Lpp, but the reverse reaction has not been observed (Palm et al. 2012; Canavoso 2003). LTP promotes the transfer of lipids, mostly DG, from the enterocyte to an unloaded Lpp particle docked outside the cell in the hemolymph (Canavoso et al. 2004; Palm et al. 2012; Rodríguez-Vázquez et al. 2015). Once Lpp is loaded with lipids, it circulates and comes in contact with cells, allowing for the exchange of lipids. Lpp participates in multiple cycles of lipid loading (at the midgut) and unloading (at downstream target tissues) without degradation (Canavoso 2003; Rodríguez-Vázquez et al. 2015). Thus, Lpp operates as a bidirectional shuttle system for long range DG transport. However, the molecular mechanisms involved in the localization and selectivity between an unloaded and a loaded Lpp at the PM remain unclear (Palm et al. 2012).

At the molecular level, it has been proposed that circulating Lpp interacts with LTP via a Lpp-receptor complex, involving lipophorin (Lpp) receptor 1 and 2 (lpr1 and lpr2) (Prasad et al. 1986; Tsuchida and Wells 1990). Several recent studies have examined the mechanisms that mediate the transfer of fat from Lpp to imaginal discs and ovaries. It was shown that the recruitment of LTP to the PM, mediated by the Lpp receptor, is critical for initiating the transfer of neutral lipids (Rodríguez-Vázquez et al. 2015). Additional experiments have demonstrated that LTP also promotes the transfer of neutral lipids to Lpp from the midgut. In the absence of LTP and Lpp, fat mobilization is blocked, producing a striking phenotype of LD accumulation in the midgut (Palm et al. 2012; Canavoso 2003). Additionally, tissue specific knockdown of LTP in the fat body greatly reduced hemolymph

Lpp lipid levels, suggesting that LTP mediates the transfer of fat to Lpp. These data indicate that LTP and Lpp function together to mobilize fat from the gut (Palm et al. 2012; Canavoso 2003; Canavoso et al. 2001; Rodríguez-Vázquez et al. 2015; Prasad et al. 1986).

Further investigation is required to determine whether Lpr1 or Lpr2 mediates the interaction between LTP and Lpp at the plasma membrane of enterocytes to facilitate lipid transport (Rodríguez-Vázquez et al. 2015; Prasad et al. 1986; Tsuchida and Wells 1990). Seminal work by Culi and colleagues in 2015 revealed a strong LTP signal in discrete regions of the midgut, suggesting that the molecular mechanisms by which Lpp receptors mediate the transfer of lipid from LTP to Lpp is similar to that which occurs in imaginal discs and ovaries (Rodríguez-Vázquez et al. 2015; Tsuchida and Wells 1990; Buchon et al. 2013). However, it was found that neither Lpr1 nor Lpr2 are essential for facilitating the transfer of neutral lipids from LTP in the midgut to Lpp in the hemolymph, since *lpr1-, lpr2-* larvae showed no phenotype of LD accumulation in the midgut (Rodríguez-Vázquez et al. 2015; Parra-Peralbo and Culi 2011). These results indicate that other receptors may contribute to the targeting of Lpp to specific tissues during dietary fat uptake and mobilization. However, the specific mechanism by which LTP recruits Lpp to the plasma membrane of the gut and mediates the transfer of lipids to Lpp, as well as how a lipid-rich Lpp particle traffics to target tissues, are still not completely understood.

I.1.4 Intracellular lipid processing in Drosophila: Functions of lipid droplets in enterocytes

In mammalian enterocytes, LDs are thought to contribute to CM assembly through hydrolysis and re-esterification of their component TGs, similar to the process in

mammalian hepatocytes (Demignot, Beilstein, and Morel 2014). However, the extent to which LD dynamics contribute to the modulation of lipoprotein production in unclear (Wilfling et al. 2013; Demignot, Beilstein, and Morel 2014). Additionally, the accumulation and mobilization of cytosolic LDs has been shown to be dependent on lipid availability. An increase in the amount of TGs in cells results in the formation and accumulation of LDs (Walther and Farese 2009). On the other hand, during lipolysis, fatty acids and glycerol are liberated from LDs, resulting in a reduction in the size and number of LDs (Welte 2015b). Thus, cytosolic LDs serve as a transient storage compartment (Kühnlein 2012a).

During Drosophila larval development, observations have been made of dynamic accumulation and depletion of LDs in the fat body. It was noted that during oleic acid feeding, a number of other tissues displayed accumulation of LDs during the embryonic and larval stages; these include particular regions of the Drosophila gut, imaginal disc cells and oocytes (Guo et al. 2009; Welte 2015b; Kühnlein 2012b). Notably, in response to starvation conditions, animals shift away from TG storage in LDs, towards mobilization via lipoproteins (Reis, Van Gilst, and Hariharan 2010; Grönke et al. 2005).

Two key insights emerged from early studies in LD dynamics. First, one of the main functions of LDs is to protect the cell from toxic concentrations of FAs in the cytosol. Second, LDs are heterogenous in composition and function (Welte, Box, and Hall 2009; Demignot, Beilstein, and Morel 2014; Guo et al. 2009). Thus, LDs are beginning to emerge as highly dynamic organelles. Despite their importance in energy metabolism and disease, there is much that is still poorly understood about the synthesis, growth and mobilization of LDs in mammals and Drosophila.

I.2 Lipid droplets

I.2.1 What are lipid droplets?

LDs are ubiquitous cellular structures, found in prokaryotes, yeast, and throughout higher Eukarya, including plants and mammals (Demignot, Beilstein, and Morel 2014; Brasaemle 2007; Kühnlein 2012a; Abumrad and Davidson 2012; Welte 2015a). Recent discoveries have shed light on the diverse functions of LDs: they support membrane integrity, serve as storage organelles, protect cells from lipotoxicity when there is an unusually heavy lipid load, and function as the site of synthesis for several hormones (Guo et al. 2009; Kühnlein 2012a; Walther and Farese 2009). Interestingly, LDs are found to be most abundant in the fat body (Palm et al. 2012; Marianes et al. 2013). The loss of MTP in the fat body was found to block the synthesis and secretion of LTP and Lpp from the fat body, resulting in an increase of LDs in the anterior and posterior regions of the midgut (Palm et al. 2012). These experiments show that LDs may serve as an intermediate in the dietary lipid mobilization process, encompassing the transfer of neutral lipids from LDs to the loading of lipoprotein particles as they travel through the secretory pathway (Olofsson, Asp, and Boren 1999; Kühnlein 2012b).

LDs vary widely in size, from a diameter of 0.1μm to 100μm (Walther and Farese 2009; Wilfling et al. 2013). Similar to lipoproteins, LDs are bounded by a lipid monolayer that shields their hydrophobic contents from the polar cytoplasm (Hariri et al. 2018; Guo et al. 2009; Demignot, Beilstein, and Morel 2014; Walther and Farese 2009; Wilfling et al. 2013). However, the structure of LDs is unique among organelles – while most organelles are composed of an aqueous interior separated from the cytoplasm by a lipid bilayer, the interior of LDs consists of neutral lipids that are surrounded by a monolayer of

amphipathic lipids and proteins (Fig.4)(Kühnlein 2012a; Brasaemle 2007; Kühnlein 2012b). TG and cholesterol ester (CE) are the principal lipids that comprise the LD interior.

The LD monolayer is decorated with polar lipids as well as enzymes that function in lipid metabolism, signaling proteins, and cytoskeletal proteins (Walther and Farese 2009). These proteins play important roles in the function and structure of LDs, but little is known about their discrete functions (Brasaemle 2007; Kühnlein 2012b). Among the proteins that have been found to be associated with LDs are the PAT proteins (perilipin, adipose differentiation-related protein, tail-interacting proteins of 47 kDa (TIP47) and caveolins) which play important roles in lipid metabolism (Walther and Farese 2009; Londos et al. 2005). For example, PAT proteins that bind to LDs help to regulate the interaction between lipases and LDs in mammals, insects and fungi (Welte, Box, and Hall 2009; Londos et al. 2005).

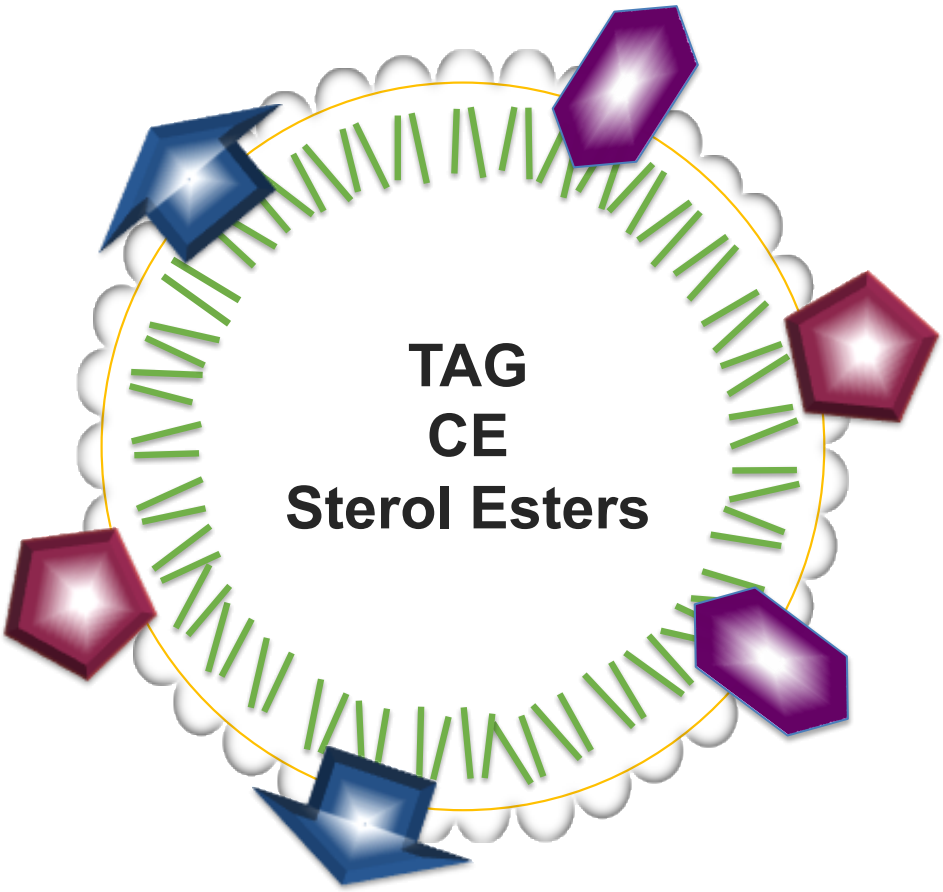

Figure 4. Artistic depiction of a lipid droplet (LD). The hydrophobic lipid core of the LD (yellow) consisting of triacylglycerol (TAG), cholesterol esters (CE) and sterol esters are encapsulated by a phospholipid monolayer (green). Geometric shapes represent the various proteins (e.g., perilipins) found at the LD surface like.

I.2.2 Lipid droplet biogenesis

In eukaryotes, there is substantial evidence that LDs are synthesized in the ER, where the machinery responsible for catalyzing lipid synthesis is found (Walther and Farese 2009). Among the most compelling suggestions that LDs originate in the ER is that fact that the lipid composition of the LD surface is similar to that of the ER bilayer (Ploegh 2007; Walther and Farese 2009; Ohsaki et al. 2008). In some cases, the ER membrane is found in close proximity to the LD, and several studies have uncovered a direct, tight association between LDs and the ER (Ohsaki et al. 2008; Robenek et al. 2006; Soni et al. 2009). However, these organelle associations and interactions are still poorly understood (Ohsaki et al. 2008; Wilfling et al. 2013). It has been proposed that the interactions between LDs and other organelles might be mediated by Rab GTPases (Guo et al. 2009). The Rab family of proteins are known to regulate the steps of membrane trafficking, including vesicle movement along actin and tubulin networks (Scott and Nilsson 2014; Guo et al. 2009). Additionally, several proteins found on the LD surface are known to be ER associated proteins, such as DGAT (Dvorak et al. 1992; Bozza et al. 1997; Robenek et al. 2004, 2006). The association between the ER and LDs may play an important role in the formation and growth of LDs, but there are still many unanswered questions.

Four models have been proposed for LD formation in the ER (Guo et al. 2009). (1) ER budding – here, LDs grow directly from the ER bilayer and detach from the organelle via budding. (2) Bicelle formation – in this model, it is proposed that lipid esters form "lenses" which are subsequently excised from the ER (Ploegh 2007; Walther and Farese 2009). (3) Vesicular budding – here, it is suggested that initial vesicle formation and subsequent filling with neutral lipids drives LD formation (Walther and Farese 2009; Hariri et al. 2018). (4)

Eggcup model – this model argues that LDs grow within a concave depression of the ER (Robenek et al. 2006).

The ER is presumed to play a major role in the synthesis of TGs and the regulation of LD growth (Guo et al. 2009; Kühnlein 2012a; Brasaemle 2007; Abumrad and Davidson 2012). De novo TG synthesis occurs in four discrete steps. In the first, glycerol-3-phosphate (G3P) 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 DG by the magnesium-dependent PA phosphatases (PAP1 activity). In the final step, DG is converted to TG by the enzyme diacylglycerol O-acyltransferases (DGAT) (Kühnlein 2012a; Walther and Farese 2009; Abumrad and Davidson 2012).

I.2.3 How do lipid droplets expand?

Once formed and separated from the ER, LDs grow through two proposed mechanisms (Wilfling et al. 2013; Guo et al. 2009; Hariri et al. 2018). In the first, it is proposed that LDs fuse with one another through a mechanism similar to that of vesicular fusion (Bostrom et al. 2007). Evidence in support of this comes from studies that have shown that vesicular fusion proteins are found associated with LDs. These include the 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). It has been shown that knockdown of SNARE results in a decreased rate of LD fusion, leading to smaller, more numerous LDs (Bostrom et al. 2005).

Interestingly, LD fusion seems also to depend on microtubules (MT). LDs in

fibroblast cells were observed in a time-lapse experiment to investigate whether the formation of complexes between LDs drives size increase (Bostrom et al. 2005). The cells were treated with nocodazole, a MT destabilizing drug, to examine the effects on LD growth. Exposure to nocodazole inhibited the formation of complexes between LDs, indicating that LDs increase in size by a process that is independent of TG biosynthesis and dependent on the MT system (Bostrom et al. 2005, 2007).

The second proposed mechanism suggests that LDs can expand by simultaneously acquiring triglycerides at their core and phospholipids at their surface (Demignot, Beilstein, and Morel 2014; Wilfling et al. 2013). The amphipathic monolayer of LDs is associated with proteins of diverse biochemical functions. Several studies have shown that some of these enzymes — such as the adipose triglyceride lipase (ATGL), methyltransferase-like 7B, along with several other TG synthesis enzymes re-localize to growing LDs from the ER after a rich fatty-acid diet (Schlegel and Stainier 2007; Leonard, Snodgrass, and Robertson 2010). The observed enzyme translocation would allow TG synthesis to occur directly at the surface of expanding LDs. It was recently proposed that the mechanism by which GPAT localizes from the ER to growing LDs involves direct connections between these organelles (Wilfling et al. 2013). In adipocytes, it has been demonstrated that LD growth occurs by the transferring of neutral lipids from a smaller LD to a larger LD through the ER-LD bridge (Wilfling et al. 2013).

I.2.4 Proteins associated with lipid droplets

Analysis of the Drosophila LD proteome revealed a surprising diversity of LD associated proteins, including most of those identified in mammalian studies. Of particular

note, several key enzymes involved in lipid metabolism (including FA synthases, acetyl-CoA carboxylases and Bmm lipase) were identified, along with several ribosomal (small GTPases) and mitochondrial proteins. These data are consistent with the results of freeze-fracture studies, which suggest that LDs interact with other organelles including the ER, endosomes, mitochondria, and peroxisomes (Walther and Farese 2009). Several of these proteins may be transiently 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 (Wilfling et al. 2013).

Several proteomic analyses have suggested that, at least in some cells, LDs with different protein compositions coexist (Wolins, Brasaemle, and Bickel 2006). Several groups have proposed that there may be several classes of LDs that differ according to size and surface protein composition. For example, Wilfling and colleagues suggested that mammalian cells contain small static LDs, which do not have TAG synthesis enzymes at their surface, and larger LDs, which do possess TAG synthesis enzymes (Wilfling et al. 2013). In Drosophila, it has been shown that the LSD2 protein is associated with the surface of small LDs (<4um in diameter) but absent from larger ones (>4um) (Bi et al. 2012). These and other lines of evidence support the hypothesis that distinct classes of LDs may be present in a cell, suggestive of possible underlying functional segregation.

I.3 The Drosophila larval midgut

The modeling of the gastrointestinal tract is possible in Drosophila because of the high degree of conservation between Drosophila and mammals. Developmentally, both the mammalian and Drosophila midgut are of endothelial origin, and, structurally, they consist

of an epithelial monolayer of cuboidal cells called enterocytes (Apidianakis and Rahme 2011). Furthermore, the compartmentalization of the gut into separate domains in mammals bears many similarities to that of Drosophila (Buchon et al. 2013).

The major site of digestion in fruit flies occurs in the midgut and functions much like the small intestine in mammals (Marianes et al. 2013). The midgut tissue is lined with digestive cells and hormone-producing cells that are strikingly organized into at least ten sub-regions marked by cell structure and composition occur in a defined order (Buchon et al. 2013; Marianes et al. 2013). The sub-regions of the midgut are differentiated along the anterior-posterior axis (Buchon et al. 2013). Structural analyses of the intestine have revealed three regions of the midgut, composed of highly specialized cells: the anterior, middle and posterior. The anterior portion of the midgut contains specialized enterocytes known as interstitial cells (Marianes et al. 2013). Food entering the anterior midgut is broken down via the action of digestive enzymes expressed specifically in the anterior midgut. The middle midgut, termed the fly "stomach", is located downstream of acid-producing copper cells (Dubreuil 2004). The posterior midgut contains cells that are large and flat that have a larger footprint than that of the anterior midgut cells (Marianes et al. 2013; Adams et al. 2000). These larger cells seem to absorb more fluid and lipids than other sub-regions of the midgut, leading to the proposal that the posterior midgut functions to absorb nutrients and the anterior midgut functions to break down macromolecules (Marianes et al. 2013).

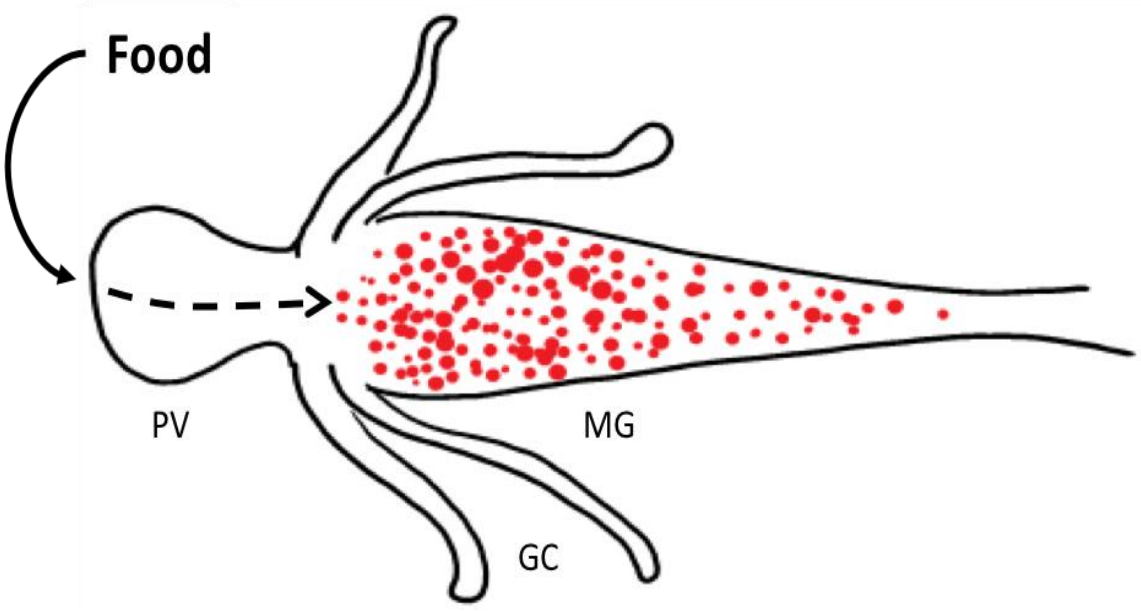

Figure 5. Schematic drawing of a dissected larval midgut. Food enters (arrow) through the lumen of the head like structure called the proventriculus (PV) to reach the anterior midgut (dashed arrow). The gastric caeca (GC) characterized by the four arm like projections, emerge just posterior to the PV linked to the anterior midgut region which contain enteroendocrine cells (enterocytes). The anterior midgut region shows high lipid content in the form of lipid droplets (red).

I.4 The Drosophila larval fat body

The larval fat body functions similarly to the mammalian liver, immune system and white adipose tissue, with respect to containing the necessary machinery for synthesizing and storing lipids, glycogen and proteins (Arrese and Soulages 2010). Energy stores in the fat body are mainly in the form of TGs in LDs which can be visualized using a neutral lipid stain (e.g., Nile red or Oil red O) (Arrese and Soulages 2010; Canavoso 2003). During metamorphosis, the fat body transitions from an organized tissue to a loose association of individual fat cells in the pupa (Arrese and Soulages 2010). Because young adults remain inactive for several hours (hrs) until their wings expand, the nutrients stored in the fat body must be sufficient to sustain the flies (Leonard, Snodgrass, and Robertson 2010; Arrese and Soulages 2010; Diaconeasa et al. 2013). Hence, this tissue is critically important during development, carrying out the uptake and storage of dietary fat coming from the gut (Palm et al. 2012). The fat body relies on the dynamic role of LDs to maintain lipid and energy homeostasis. Thus, the Drosophila fat body is an ideal system to study the lipoprotein pathway responsible for the storage or utilization of energy downstream of the gut (Arrese and Soulages 2010; Palm et al. 2012).

I.5 Defined spatial parameters underlying intracellular lipid transport

The existence of defined spatial parameters underlying the intracellular transport of FAs is just beginning to be appreciated. As mentioned previously, a novel model was recently proposed for coupled fatty acid transport in the larval fat body, in which the uptake of FAs into the cell is physically coupled to re-synthesis of TGs on the cytoplasmic side of the PM (Diaconeasa et al. 2013; Su and Abumrad 2009). This model seeks to explain

the disappearance of a novel population of cortical LDs in loss of function Spectrin mutants (Diaconeasa et al. 2013). Spectrin is believed to provide a submembrane scaffold that links the FA uptake machinery in the PM to the LD formation machinery (i.e., synthesis of TG) within the cell. The cortical LDs are intimately associated with the cytoplasmic face of the PM in the fat storage cells found in Drosophila larvae. This population of cortical LDs is hypothesized to function as a dynamic intermediate in the uptake and storage process which ultimately delivers incoming lipid cargo to a smaller number of large LDs found deeper in the cytoplasm of cells in the fat body.

It is speculated that spatial parameters are also key to the function of LTP. Intriguing work by Palm et al., has demonstrated that endocytosis of LTP at the basal end of the enterocyte is required for proper subcellular localization of LTP and for proper lipid mobilization from the gut (Palm et al. 2012). Subsequent to loading with dietary lipids, LTP returns to the basal cell surface where it surrenders its DG cargo to Lpp for broad distribution, suggesting that defined movement within the cell is critical to LTP function. LTP appears to maintain permanent residence at the enterocyte, where it goes through cycles of dietary fat loading and then unloading to Lpp. Together, these observations suggest a crucial role for the cytoskeleton in intracellular lipid transport.

I.5.1 The role of endocytic vesicles and cytoskeleton networks, like microtubules, in intracellular trafficking within the midgut epithelium

Endosomes (early, late and recycling) are involved in sorting and trafficking proteins throughout the cell (Matter and Mellman 1994). Members of the Rab family of small GTPases play a role in trafficking endocytic vesicles to their target membranes during

vesicle transport (Liu et al. 2008; Rodriguez-Boulan, Kreitzer, and Müsch 2005; Grosshans, Ortiz, and Novick 2006). Regardless of the cargo, vesicle trafficking between intracellular compartments is mediated by actin and MT cytoskeleton; meanwhile, targeted delivery is regulated by organelle specific Rab proteins (Granger et al. 2014). The meshwork of MTs regulates the efficiency of vesicle delivery in epithelial cells, in which MTs serve as tracks for transport via the motor proteins dynein and kinesin (Rodriguez-Boulan, Kreitzer, and Müsch 2005; Scott and Nilsson 2014; Mostov, Verges, and Altschuler 2000; Rogers and Gelfand 2000; Karcher, Deacon, and Gelfand 2002). Plus-end MT kinesin motors are involved in apical protein delivery, and minus-end MT dynein motors are involved in basolateral protein delivery (Gross et al. 2002; Karcher, Deacon, and Gelfand 2002; Rogers and Gelfand 2000; Hehnly and Stamnes 2007). In most of the cases that have been analyzed, the spatial arrangement of MT tracks, motors, coordinators and cargo is an important piece of intracellular trafficking. However, the role of endocytic vesicles and cytoskeleton networks involved in the intracellular trafficking of dietary lipids has not been completely elucidated.

An important theme is emerging in the field, that proteins present in endocytic vesicles may not simply be passive passengers within vesicles, but, rather, have the ability to direct intracellular motility and targeting (Gross et al. 2002; Karcher, Deacon, and Gelfand 2002; Rogers and Gelfand 2000; Hehnly and Stamnes 2007; Rodriguez-Boulan, Kreitzer, and Müsch 2005).

I.5.2 The contribution of microtubules in lipid metabolism

It is well understood that all eukaryotic cells are spatially organized. The proteins responsible for that organization are MTs, which are known to exist in all eukaryotic cells (Welte 2004; Rogers and Gelfand 2000; Karcher, Deacon, and Gelfand 2002; Khanal et al. 2016; Rodionov et al. 1998). In turn, the building blocks of MTs are known to be tubulin (Mostov, Verges, and Altschuler 2000; Hehnly and Stamnes 2007). The alpha(α) and beta(β) subunits of tubulin spontaneously form heterodimers. The tubulin heterodimers then polymerize to form MTs (Welte 2004). It has been well established that the array of polarized MTs provides a framework on which MT motors drive organelle transport towards the minus or plus-ends (Welte 2004; Tuma and Gelfand 1999).

Classical in vitro studies have examined the directed movement of pigment granules along MTs using Xenopus melanophores (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991; Rodionov et al. 1998). Interestingly, some pigment granules are LDs (Fig.6). Studies have shown MT disrupting drugs, like colchicine and nocodazole, to inhibit the shuttling motion of pigment granules (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991; Welte 2015b). However, in the absence of MT disrupting drugs, the dispersion of pigment granules occurred toward the plus-ends of MTs (Welte 2004; Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991; Rodionov et al. 1998). Taken together, these results show that MTs provide the tracks for the transport of lipid containing pigment granules and that MT polarity is what determines the direction of LD movement (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991).

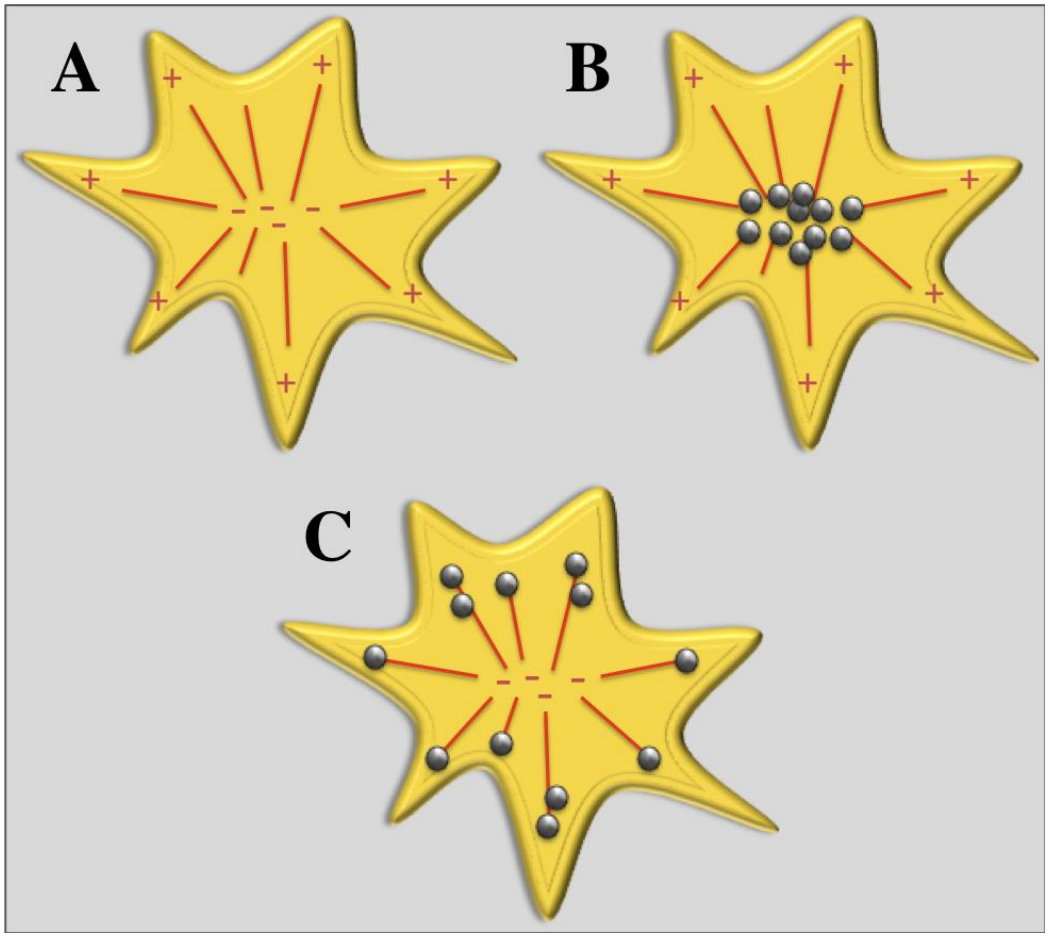

Figure 6. Lipid containing pigment granules in fish melanophores. A) In fish melanophores microtubules organized with minus-ends converging at the center of the cell, and the plus-ends out at the periphery, B) Upon stimulation lipid containing pigment granules (grey spheres) aggregate towards the minus-ends and C) disperse towards the plus-ends with the help of microtubule motor proteins.

I.5.3 Microtubule polarity in enterocytes in mammals and Drosophila

Most eukaryotic cells contain a MT dependent system of motility (Mallik and Gross 2004; Rogers and Gelfand 2000). To establish MT polarity, nucleation occurs at the MT organizing center (MTOC) or centrosome, where the minus-ends of MTs are stabilized by binding to the gamma-tubulin ring complex located at the centrosome (Mogensen and Tucker 1987; Mogensen, Tucker, and Stebbings 1989). As growing plus-end MTs elongate, they radiate away from the MTOC (Welte et al. 1998; Gross et al. 2000; Rogers et al. 2002).

Drosophila epithelial cells are thought to lack a MTOC (Mogensen and Tucker 1987). These non-centrosomal cells contain the MT nucleator, gamma-tubulin, along the apical plasma memberane. A recent study reported that two minus-end MT binding proteins, Patronin and Shortstop, are located at the apical membrane and are responsible for polarizing MTs along the apical-basal axis in the Drosophila follicle cell epithelium (Khanal et al. 2016). However, neither Shortstop nor Patronin were found to co-localize with gamma-tubulin, suggesting a unique role for these proteins. The knockdown of Patronin in follicle cells produced a disordered MT array. In addition Shortstop mutant with the null allele *shot* affected MT polarization (Khanal et al. 2016). These results revealed that Shortstop and Patronin function in the capture and stabilization of existing MT minus-ends, providing a scaffold for the polymerization of MTs.

I.5.4 Microtubules and microtubule motor proteins associated with LD motility: The role of dynein and kinesin motor proteins in Drosophila

The basic machinery for transport in eukaryotic cells has been shown to involve members of the dynein and kinesin superfamilies of motor proteins (Mallik and Gross

2004; Palacios and St Johnston 2002; Karcher, Deacon, and Gelfand 2002). Most molecules, including proteins, are too large to pass directly through membranes. Instead, large molecules are loaded into vesicles either at the PM, the ER or the Golgi (Rodriguez-Boulan, Kreitzer, and Müsch 2005; Scott and Nilsson 2014; Grosshans, Ortiz, and Novick 2006; Mostov, Verges, and Altschuler 2000; Rogers and Gelfand 2000). Once these endocytic PM vesicles are formed, motor proteins utilize the cytoskeleton to deliver the vesicle and their cargo to the destination. Different motor proteins are specialized to carry certain types of cargo along the cytoskeleton in one direction or the other (Rodriguez-Boulan, Kreitzer, and Müsch 2005; Grosshans, Ortiz, and Novick 2006). Dynein is a minus-end MT motor protein that travels towards the MTOC; meanwhile, kinesin, a plus-end MT motor protein, travels away from the MTOC (Welte 2004). The ability of these motors to transport such a wide variety of cargo is due to the structural domains of the motor itself. Motor proteins have two functional parts: a motor domain that binds reversibly to the cytoskeleton and converts chemical energy into motion, and the tail end of the protein that interacts directly with the cargo (Karcher, Deacon, and Gelfand 2002). Previously, in melanophores, kinesin and dynein were found to co-localize with pigment granules along MTs, suggesting MT motor proteins are involved in the dispersion and aggregation of pigment granules, respectively (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991; Rodionov et al. 1998). MT motor proteins were shown to move pigment granules along polarized MTs (Fig.6 A-C) (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991).

The movement of lipid droplets in Drosophila has been extensively studied in Drosophila embryos, where focus was directed towards characterizing the physical parameters associated with bulk movement of LDs during embryonic development (Fig.7)

(Gross et al. 2002; Welte et al. 1998). Experiments using embryos expressing kinesin-B-galactosidase fusion protein, which localizes to MT plus-ends, revealed LD movement to coincide with MT tracks that are arranged radially. Minus-ends point to the periphery; plus-ends point into the interior (Welte 2004; Gross et al. 2002; Welte et al. 1998).

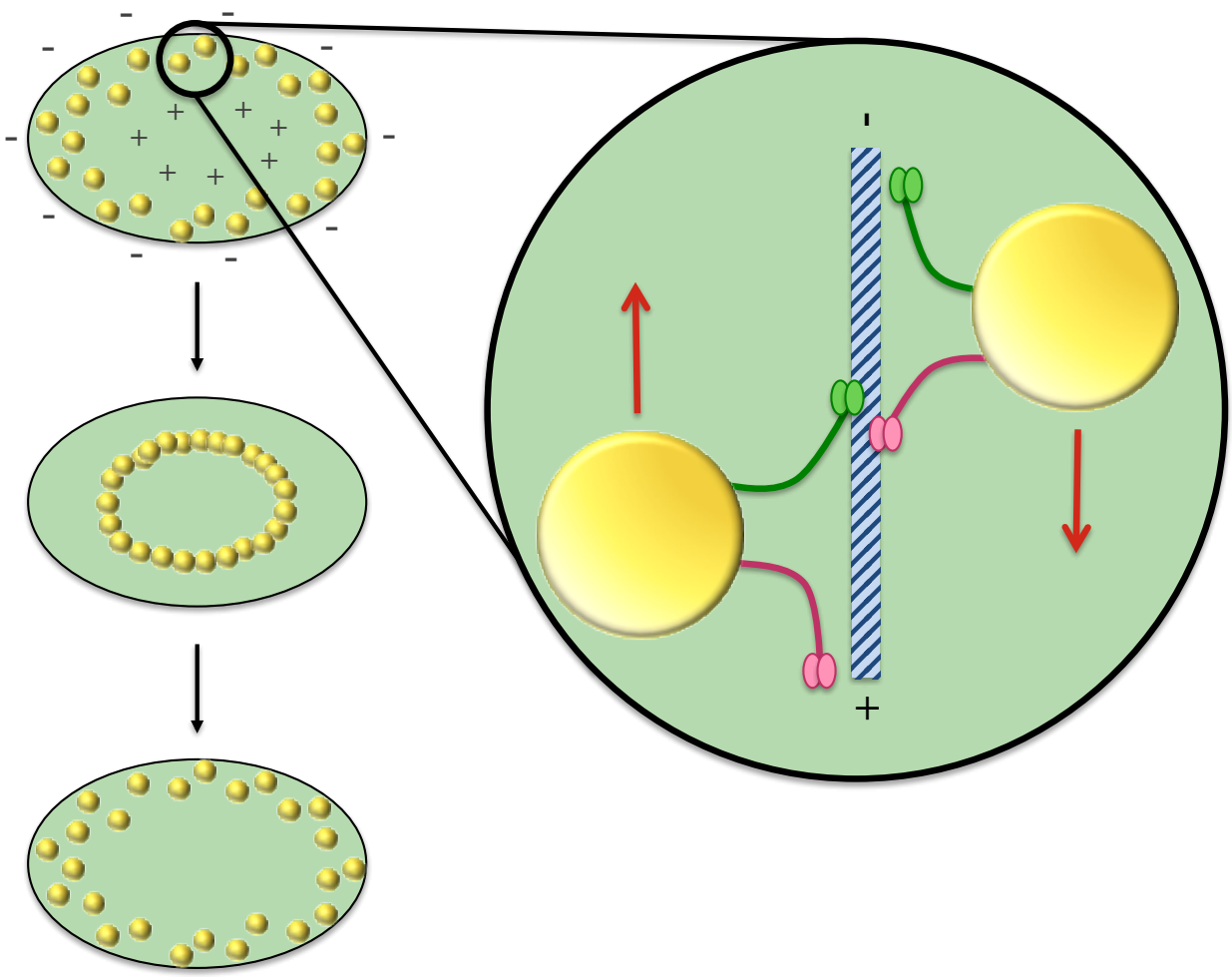

Figure 7. Lipid droplet movement in Drosophila embryos. A) Lipid droplet movement coincides with microtubule tracks that are arranged radially. Minus-ends of microtubules point to the periphery; plus-ends point into the interior, B) Lipid droplets move bidirectionally along microtubules, pulled by plus-end kinesin (green) and minus-end dynein (pink) active motors.

LDs are distributed throughout the periphery but then undergo net inward transport towards the MT plus-end (Welte 2004). Interestingly, in mutant embryos with two weak alleles of Dhc64C, a gene for heavy chain cytoplasmic dynein (a minus- end motor protein), LDs failed to redistribute towards the minus-ends (Gross et al. 2000). Hence, it was proposed that a switch in the net movement of LDs towards MT minus-ends occurred later in development, suggesting the involvement of motor proteins for the bidirectional movement of LDs (Gross et al. 2002; Kural et al. 2005). However, very few studies have examined LD transport at high spatial and temporal resolution to discern how motor proteins coordinate their movement (Welte 2004; Gross et al. 2002). Furthermore, there is no direct evidence for the coordination of LD movement to be motor protein dependent (Gross et al. 2000).

There are many uncertainties regarding how LD movement occurs during fat uptake and mobilization in Drosophila larval enterocytes, because no studies have been done in enterocytes. Studies of LD movement in Drosophila embryos might be indicative of LD movement in Drosophila enterocytes. Therefore, determining the spatial arrangement of LDs in relation to MT tracks and motor proteins could potentially provide some insight in understanding if and how MTs and MT motor proteins coordinate lipoprotein loading in enterocytes for fat mobilization.

CHAPTER II. New insights into the spatial parameters governing dietary fat uptake and transport

II.1 Abstract

Recent work by Diaconeasa and colleagues in the larval fat body has shed light on the proteins and spatial parameters governing FA uptake (Diaconeasa et al. 2013). Two proteins in particular have been implicated to play key roles in directing the flow of lipids into pathways of LD formation: β spectrin (discussed previously) and LSD2 (a fly Perilipin protein termed lipid storage droplet-2). It was shown that targeted knockdown of β spectrin or LSD2 in the larval fat body prevented the formation of a population of small LDs that are normally found at the cortex of fat cells. Additional analysis of third instar larvae by both DIC and GFP microscopy revealed that knockdown of β spectrin prevented the formation of LDs containing GFP-labelled LSD2. These data indicate that the cortical LDs in the fat body whose presence is spectrin-dependent are the same LDs that are LSD2-positive.

There are several important implications of the above results: (1) Active lipid uptake in the fat body and storage as small LDs is dependent on β spectrin and LSD2; (2) Cortical LDs in the fat body are intimately associated with the PM via β spectrin; and (3) The positioning of the cortical LDs potentially suggests an active mechanism that aligns LD formation with the lipid uptake machinery at the PM (Diaconeasa et al. 2013). Taken together, these results help establish a novel model for fat uptake at the fat body surface.

As previously mentioned, dietary fat in the form of DG is shuttled to the fat body from enterocytes by way of lipoproteins. The major circulating hemolymph lipoprotein, Lpp, docks with lipid transporters on the surface of the fat cell to facilitate the

internalization of FAs. The close association of cortical LDs with the PM obviates the need for incoming lipids to exist as free FAs in the aqueous intracellular environment (energetically unfavorable). Rather, the LD is immediately adjacent to the inner face of the PM so as to couple the incoming FAs to the LD machinery within the cell. This coupling phenomenon, in which β spectrin physically links lipid transporters on the PM to the cortical LD surface led me to ask whether this coupling phenomenon also exists in the midgut epithelial cells of Drosophila larvae.

Using the larval midgut as a genetic model system, I identified discrete spatial and temporal parameters that govern lipid uptake and the distribution of LDs in enterocytes. (1) The process of lipid uptake and LD formation in enterocytes may occur in a coupled process. (2) Under a low-fat diet, a constitutive population of small LDs are always present and are tightly concentrated at a site over the nucleus (supranuclear) closely associated with the PM. (3) The class of polarized LDs found in the supranuclear region are a transient intermediate dependent on the continuous input of dietary fat. (4) A high fat diet induces a dramatic increase in LD size and number throughout the cell, exposing an apparent difference between the rate of net lipid movement into the cell (lipid uptake and LD formation) and the rate of lipid mobilization out of the cell (LD unloading, lipoprotein packaging and directed export). (5) The initial site of LD formation and the terminal site of LDs exiting the cell occurs at the supranuclear domain of the cell. The supranuclear domain could potentially be the preferential site for LD formation and mobilization. Based on these results, I propose that the supranuclear site has a functional role in the process of lipid uptake, storage and mobilization via the lipoprotein pathway.

II.2 Fatty acid uptake and spatial regulation of lipid droplet biogenesis in the midgut

Recently it was found that *LSD2,* discussed above to be a critical protein for LD formation in the fat body, was also expressed in the midgut (Grönke et al. 2005; Diaconeasa et al. 2013). Further analysis revealed that larvae over-expressing LSD2 showed many small LDs in the midgut; in contrast, larvae with the knockdown of LSD2 in the midgut exhibited a loss of small LDs (Diaconeasa et al. 2013). Based on these results, the midgut serves as an important storage organ that seems to supports a mechanism for LD formation, storage and mobilization.

I hypothesized that the LSD2 positive LDs in the midgut would exhibit similar dynamics to the LSD2 positive LDs in the fat body. To discern if the LDs in the midgut belong to the same class of LSD2 positive LDs, I expressed an LSD2-GFP fusion protein in third instar larvae fed a standard diet of yeast paste using a midgut-specific Gal4 driver, MexII-Gal4. Midgut specific expression of LSD2-GFP revealed a population of small LDs that are also LSD2-positive, detectable by both DIC microscopy and GFP fluorescence in the anterior midgut (Fig. 8 A-C). This result suggests an association between LSD2 and LDs in the enterocyte. Additionally, I observed staining along the trunk of the gut and into the proximal regions of the gastric caeca. Only a subset of the epithelial cells in the gastric caeca accumulate small LDs, marked by LSD2-GFP (Fig.8 B, arrow). The LSD2-GFP fluorescence in the midgut formed a sharp boundary between cells accumulating LDs and those that did not. It is a striking result, where two adjacent cells show completely opposite phenotypes with respect to LD accumulation (Fig.8 B & C, arrows). The clear cell-to-cell heterogeneity with regard to LD accumulation between neighboring cells of the same cell

type suggests that the process of lipid uptake and LD formation in the midgut may be genetically controlled (i.e., proteins).

To further explore the spatio-temporal role of midgut-specific LSD2 to LD formation, I tested the effects of a high fat diet on LD formation and LSD2-GFP localization. Larvae fed a high fat diet of 10% oleic acid in yeast paste overnight (12 hrs) accumulated a massive quantity of LDs (the number and size of LDs increased) in the midgut, similar to observations in the fat body. Larger LDs no longer expressed LSD2-GFP signal from their surface (Fig. 8 F, asterisks), concomitant with their migration away from the apical surface of the PM and into the interior of the enterocyte (also consistent with previous work in the fat body). One interpretation of these data is that as LDs grow, they may reach a threshold that triggers dissociation of LSD2, presumably allowing the LD to dissociate from the PM and move deeper into the cytoplasm of the cell (Diaconeasa et al. 2013).

To better characterize the small LDs in the midgut, I used Oil Red O, a hydrophobic dye used to stain neutral lipids that has been employed extensively in studies with systems ranging in complexity from yeast to human fat tissue (Lin et al. 2018). Oil Red O staining of high fat-fed larvae, compared to controls fed standard yeast paste (Fig.8 G), revealed a striking shift in LD accumulation in the midgut epithelium after 4 hrs: while cells of the anterior midgut showed LD staining, the immediately adjacent cells of the gastric caeca lacked LDs altogether (Fig. 8 H, arrows). In addition, starvation experiments revealed an unexpected shift in the pattern of LD accumulation in the midgut (Fig.8 D & I). Larva removed from control yeast paste and starved overnight no longer showed LD accumulation in the cells of the anterior midgut; instead, small LDs began to accumulate in

cells of the gastric caeca (producing a full-sleeve pattern, Fig. 8 I & J) where previously there was little to no accumulation of LDs.

These findings together establish that cells in the midgut has the capacity for lipid uptake and LD synthesis; therefore, the absence of LDs in the gastric caeca observed under high-fat conditions implies that these cells may be able to selectively turn off the machinery for LD formation and degradation. On the other hand, cells in the gastric caeca could be thought to function differently than the rest of the midgut epithelium, where LDs begin to accumulate but only during starvation conditions. However, there is limited evidence for the function of the gastric caeca to speculate on its digestive function (Palm et al. 2012).

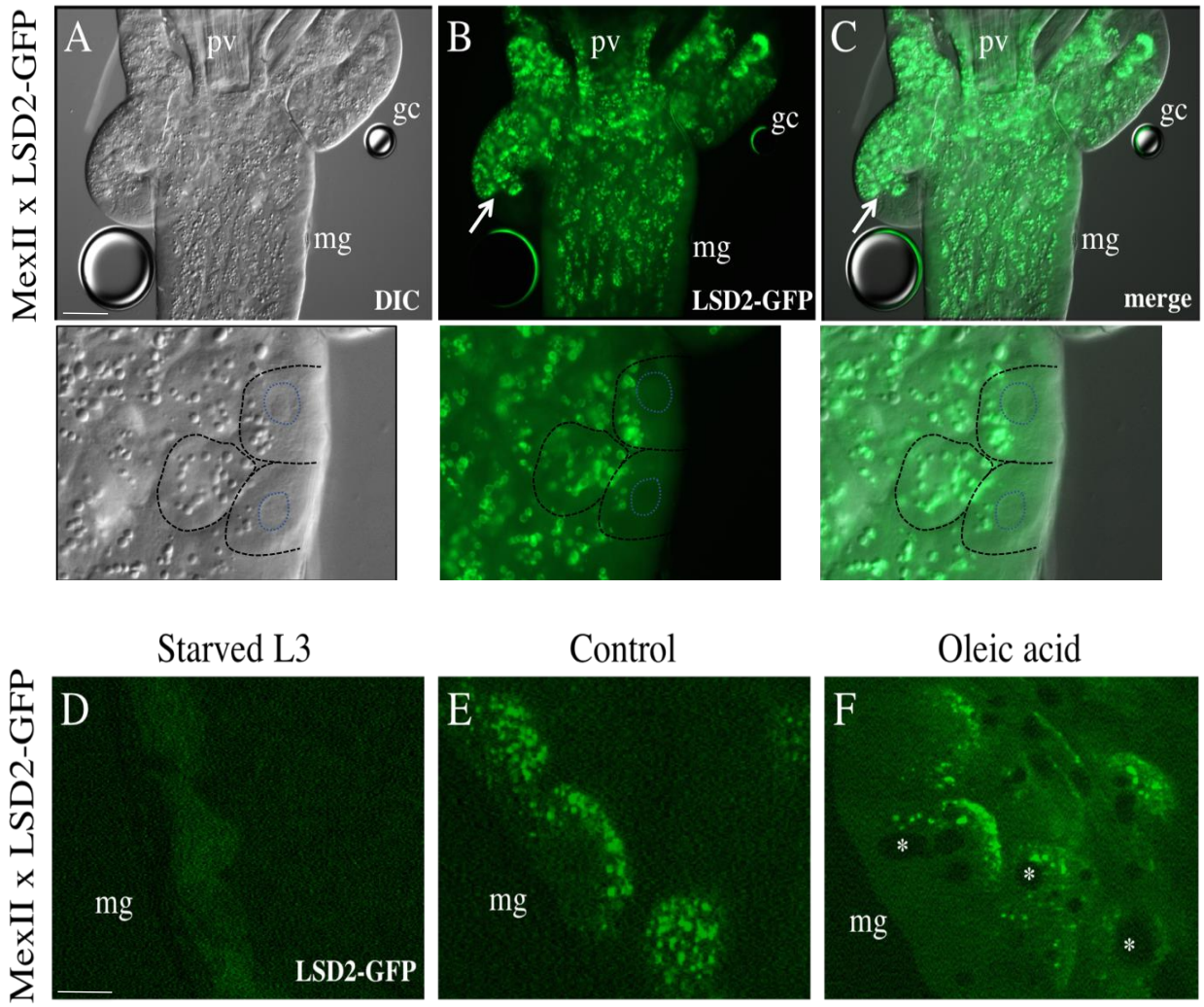

Figure 8. Lipid uptake and LD formation in the midgut correlates with LSD2 dynamics. 20X (A-H), 40X (I). Scale bar is 50 μm; mg=midgut, gc=gastric caecae, pv=proventriculus. Continued on next page

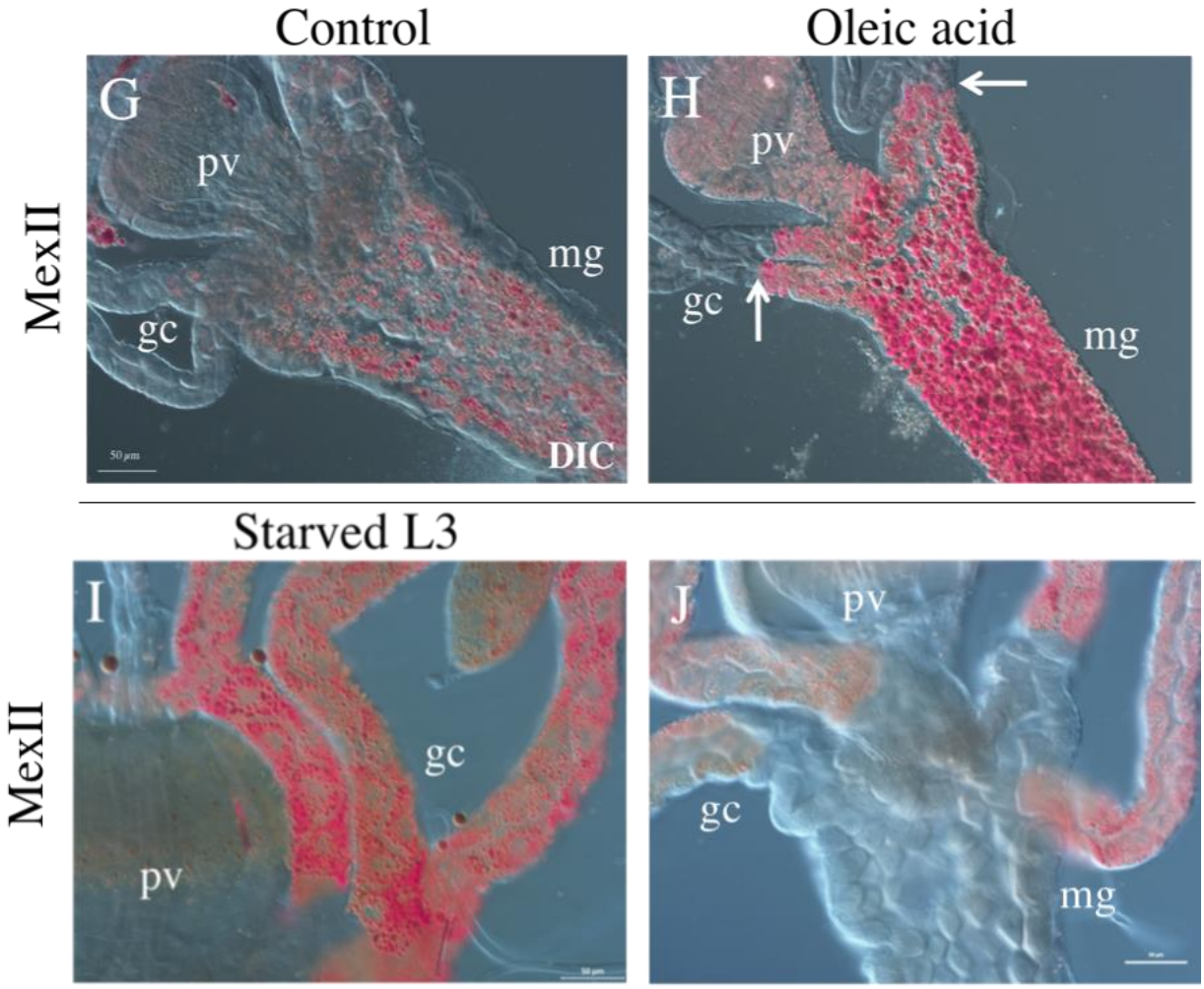

Figure 8. Lipid uptake and LD formation in the midgut correlates with LSD2 dynamics. Shown are dissected larval midgut tissues analyzed by DIC microscopy and GFP fluorescence of larvae expressing UAS-LSD2-GFP driven by a midgut specific MexII-Gal4. Larvae fed a standard yeast paste diet produced a population of small LDs that coincides with the DIC and GFP pattern (Top-down view, A-C). Expression of UAS-LSD2-GFP in the midgut produces a GFP border (arrows in B & C). Transverse view of anterior midgut expressing LSD2-GFP under starved(D), standard yeast paste diet (E) and high-fat diet(F). After a high fat diet, larger LDs were not labelled by LSD2-GFP (asterisks in F). Oil Red O staining shows a dramatic increase in LDs (H) and the LD border in the gastric caeca (arrows in H) Starved (12 hrs) larvae showed no signs of LDs in the midgut(D), instead LDs accumulated in the gastric caeca (I & J). 20X (A-H), 40X (I). Scale bar is 50 μm; mg=midgut, gc=gastric caecae, pv=proventriculus.

I.I.3 A constitutive population of lipid droplets is localized in a polarized manner in the larval midgut epithelium

The mechanism(s) by which the protein machinery responds to dietary changes to regulate the absorption and storage of lipids are still largely unknown. Specifically, I wondered whether there was a constitutive population of small LDs that is intimately associated with the PM, as is the case in the fat body. To better understand the baseline dynamics of LD accumulation and cellular localization, I analyzed cells in the anterior midgut under standard dietary conditions.

For these experiments, I used the wild-type fly strain, Oregon R (Ore^R). Every Ore^R larva fed a low-fat diet (control diet of yeast paste) accumulated a population of LDs that were relatively small in size and number (Fig. 9). Oil Red O staining of the midgut viewed from the transverse axis revealed a polar population of LDs that were tightly concentrated at a site between the nucleus and the apical PM, in the supranuclear compartment of the enterocyte (Fig.9 A-B). To further localize the LDs, I analyzed the same cells (under the same condition) from a top-down view, which revealed a tight pattern of LD accumulation in the central region of the cell; taken together, these views clearly show LD accumulation at the supranuclear compartment of the cell.

I further examined the midgut tissue of 3rd instar MexII larvae (another similar wild-type fly strain) fed a standard yeast paste diet using EM. EM analysis of a cross-section of the anterior midgut confirmed that a discrete population of small LDs was present in the supranuclear region (Fig. 10 A-B; the region is in between the nucleus and the inner face of the apical PM). This class of small LDs appears to be closely associated with the apical PM, while larger LDs tend to be found further from the PM (Fig. 10 A-B), reminiscent of

observations in the fat body (Diaconeasa et al. 2013). Additionally, the midgut cross-section showed cytoplasmic extensions, called microvilli. The microvillar surface of the enterocyte observed by EM provided a perspective on the morphology of the midgut cell that was not visible in the DIC images analyzed previously. The LDs appear to be closely associated with the PM instead of being directly in contact with the PM as previously hypothesized (Fig. 10 B, arrow).

Since every larva showed a similar pattern of LD accumulation in the anterior midgut after a standard yeast paste diet, I speculate that as FAs cross the PM, they are initially incorporated into LDs and stored, instead of being loaded directly onto LTP particles for transport out of the cells. Most commonly, LDs are characterized as sinks for fat storage when there is an overabundance of FAs to avoid lipotoxicity (Kühnlein 2012b; Abumrad and Davidson 2012; Welte 2015b). Therefore, I initially hypothesized that LD accumulation might not occur under the low-fat conditions of this study, possibly due to direct loading of incoming FAs onto LTP particles. However, these data suggest that even with very low levels of available fat, FAs follow a step-wise path in which they are first incorporated into LDs and stored (in the form of small, PM-associated LDs), rather than being loaded directly onto LTP particles for downstream transport. These results provide additional insights as to how dietary lipids from outside the cell may be incorporated into lipoproteins via LD intermediates inside the cell.

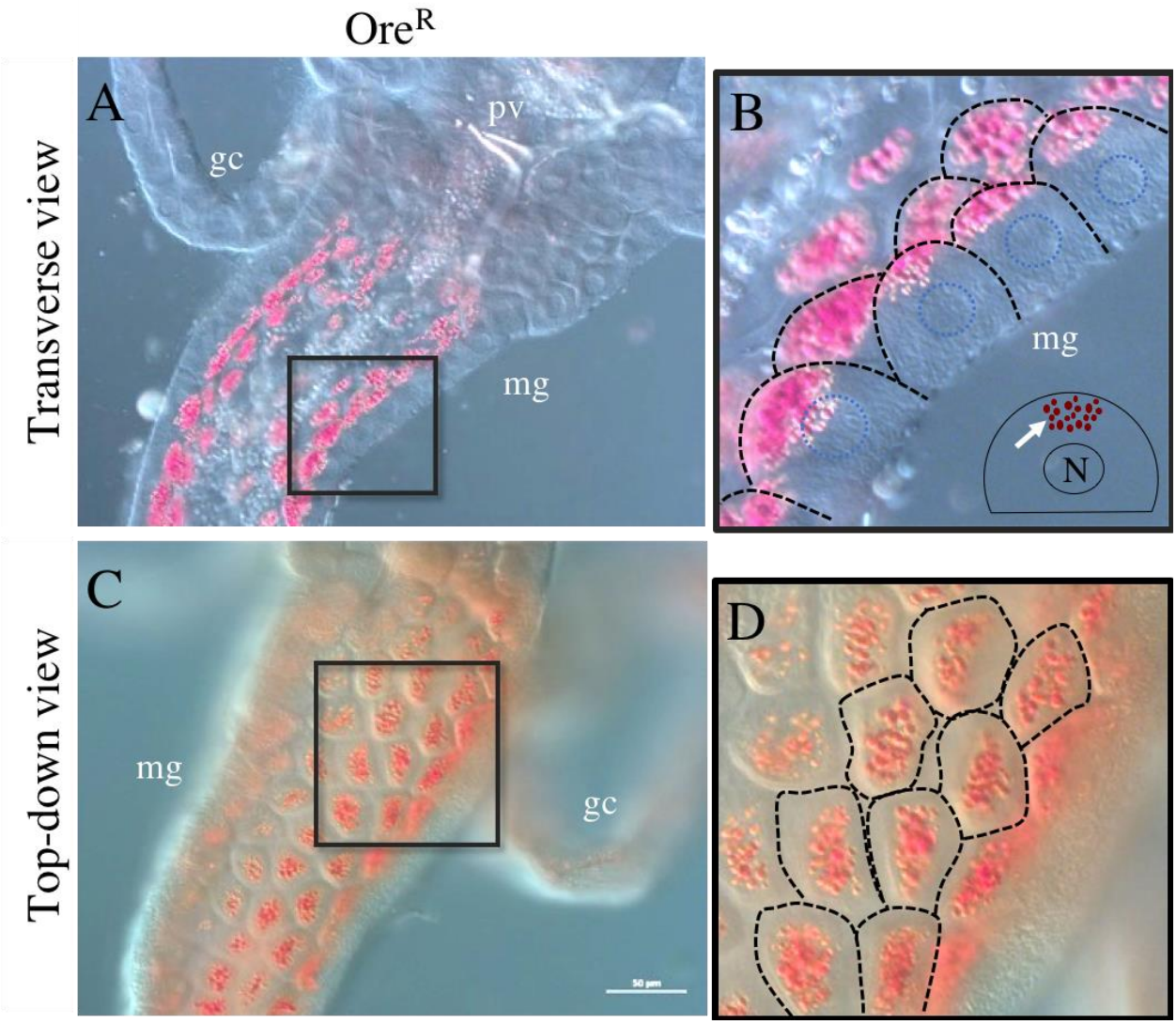

Figure 9. The spherical structures observed resulted from a standard yeast paste diet are lipid droplets (LD) (white arrow). Oil Red O stained images of the anterior zone of dissected midgut tissues of Ore^{R} larvae. These larvae always produce a population of small LDs that are tightly concentrated in the supranuclear compartment of the enterocyte. A) Transverse view of LD accumulation in the anterior zone of the midgut, B) Zoomed in view of transverse section with black dotted line outlining each enterocyte and blue dotted line bordering the nucleus, C) Top-down view, D) Zoomed in view of top-down section with black dotted line outlining the enterocyte and the nucleus is absent in this view. Scale bar is 50 μm; mg=midgut, gc=gastric caecae, pv=proventriculus.

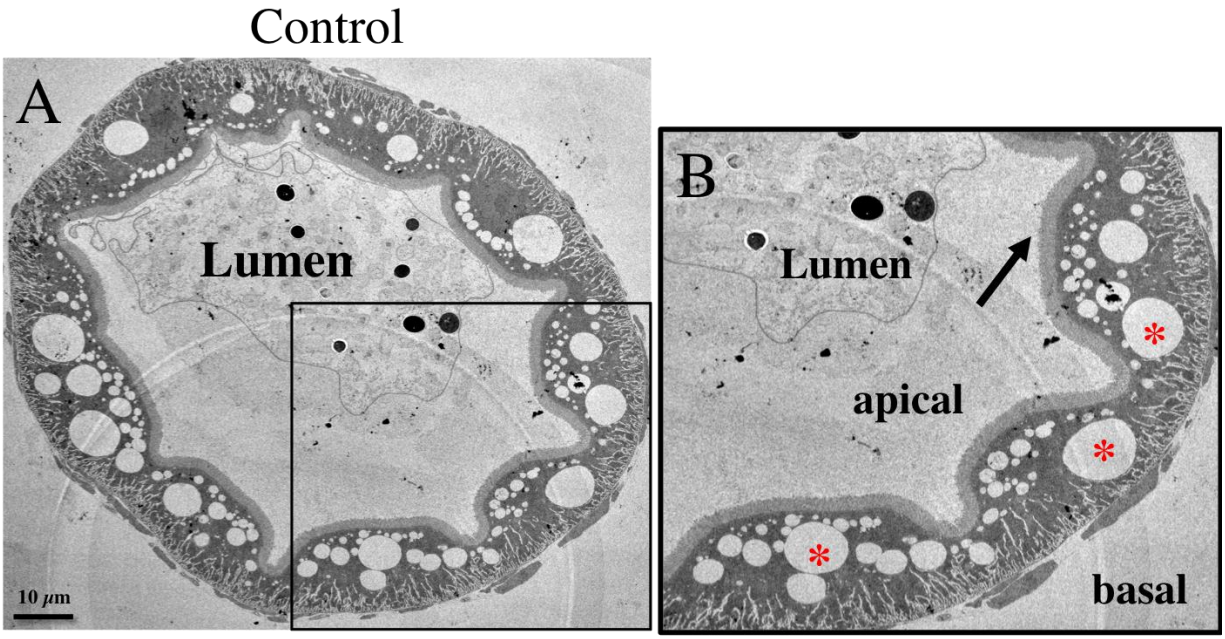

Figure 10. EM micrographs depicting the topography of the midgut of 2nd instar MexII larvae. Transverse views of the anterior midgut cross-section are shown in panels A and B. The population of white small "globular" structures seen in the apical region of the enterocyte are LDs. Small LDs in enterocytes are closely associated with the plasma membrane, meanwhile larger LDs are found deeper within the cells. Magnification 350X. Asterisk shows LDs found deeper towards the basal ends of the cell. Black arrow points to the microvillar surface of the enterocyte.

II.4 Evidence of a transient population of lipid droplets: Lipid can be readily mobilized

I next sought to further test the role of small LDs as intermediates between FA uptake and lipoprotein loading in the midgut epithelium. To address this, it is important to determine if the supranuclear population of LDs remains static in the cell or if it is readily mobilized. Since lipid mobilization from the gut is thought to involve TG hydrolysis from LDs and the re-synthesis in LTP (ultimately to be loaded as DG in Lpp for export), when there is no fat entering the cell, lipoprotein mediated lipid mobilization should cause a direct decrease in the LD population (Palm et al. 2012). Palm and colleagues previously demonstrated the inverse of this relationship, by showing that knockdown of LTP or Lpp resulted in LD accumulation in the midgut. To test my hypothesis, I starved wild-type (Ore^R) larvae that had been grown on control yeast paste medium for 24 hrs and used Oil Red O staining to analyze the change in LD abundance over time (0, 2, 4, 8, 16 and 24 hrs- not all time points are shown). The phenotypic effect was strikingly similar to that observed in the knockdown of β spectrin or LSD2 in the fat body, which prevents LD formation (Diaconeasa et al. 2013). Whereas LDs were clearly visible in the anterior midgut immediately following growth on normal yeast paste medium (Fig. 11A), within 4 hrs of starvation, LDs were no longer detectable in the anterior midgut (Fig. 11B). These results are consistent with those of the previous starvation experiments presented in this work (Section II.2, Fig 8D & I), and extend those findings by capturing the rapidity of the change in LD amount and demonstrating the transient nature of the supranuclear population of LDs.

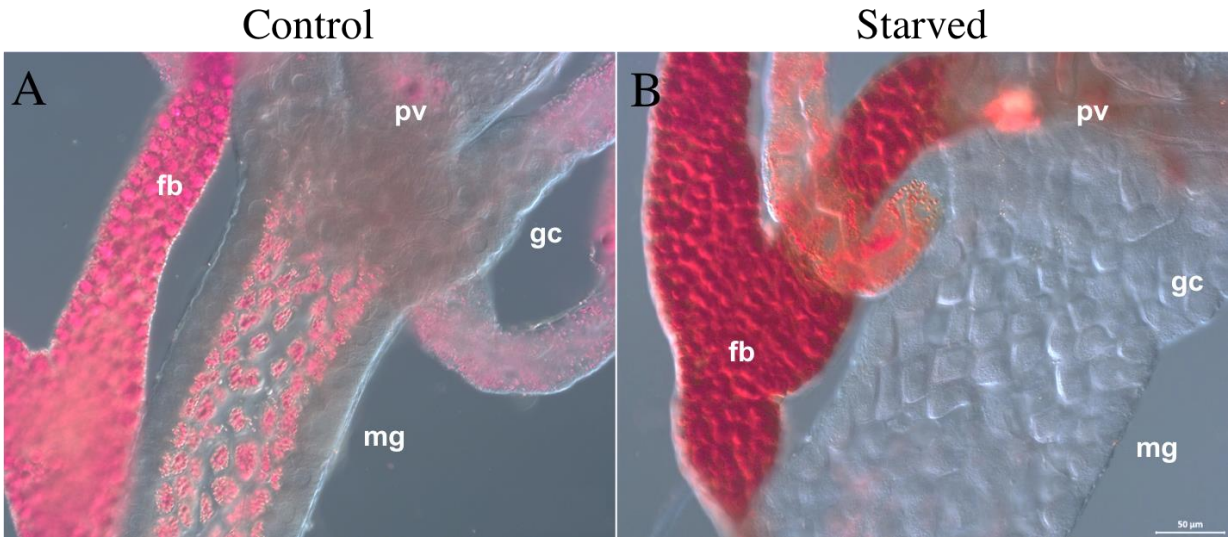

Figure 11. Oil Red O staining of LDs in dissected 3rd instar larvae of wild-type fed standard yeast paste (A) for a prolonged period (<24hrs). Basal level of LDs (A) is transient and chased out within 4hrs of starvation (B). Scale bar is 50 µm; mg=midgut, gc=gastric caecae, pv=proventriculus.

II.5 Lipid droplet accumulation exposes differential rates of lipid uptake vs. lipoprotein loading

The LD population in the midgut can be depleted under conditions of fat starvation, demonstrating the transient nature of these lipid structures (Section II.4). My previous observations of populations of supranuclear LDs as obligate intermediates in nearly every larva fed a standard yeast paste diet suggested to me that LD populations might be maintained as part of a steady-state of lipid flux. However, my earlier experiments with high-fat diets caused a strong accumulation of LDs in subpopulations of anterior midgut cells (Section II.2, Fig. 8F & H). Therefore, I wondered whether the rate of lipid uptake (represented by the accumulation of LDs) might be faster than the rate of lipid mobilization (represented by the disappearance of LDs). To test this possible rate disconnect between FA uptake and mobilization, I fed Ore^R larvae a high fat diet (yeast paste supplemented with 10% oleic acid) overnight and analyzed LD accumulation continuously (0, 2, 4, 8, 16, and 24 hrs- not all time points are shown) by Oil O Red staining. If the rate of lipid uptake and lipid mobilization can reach a steady state, then I would expect the excess LD accumulation observed previously after feeding with high-fat diet to equilibrate over time.

Control larvae fed a standard yeast paste diet accumulated few LDs in the cells of the anterior and posterior midgut, as expected (Fig. 12A). However, larvae fed a high fat diet showed massive LD accumulation through all time points, along with dispersed patterning of LDs across cell types and the loss of asymmetric distribution of LDs within each cell (Fig. 12B). Instead of maintaining a steady state, individual LDs exhibited a time-dependent increase in size, showing noticeable changes in size after 4 hrs and growing extremely large

after 24 hrs of continuous feeding. Furthermore, enterocytes that lack LDs in the standard condition began to accumulate LDs of various sizes (Fig.12 arrows).

The steady-state hypothesis described above seems to be incorrect, at least under conditions of a high fat diet, because the step of lipid uptake into the cell and the mobilization of lipids out of the cell are not equally efficient, which is supported by the massive increase in the magnitude and size of LDs in the midgut. Based on these results, it is clear that the capacity for FA uptake resulting in the formation and accumulation of LDs greatly outpaces LD mobilization (lipoprotein loading) and transport. I speculate on a possible evolutionary basis for this discrepancy later in this work (see Discussion).

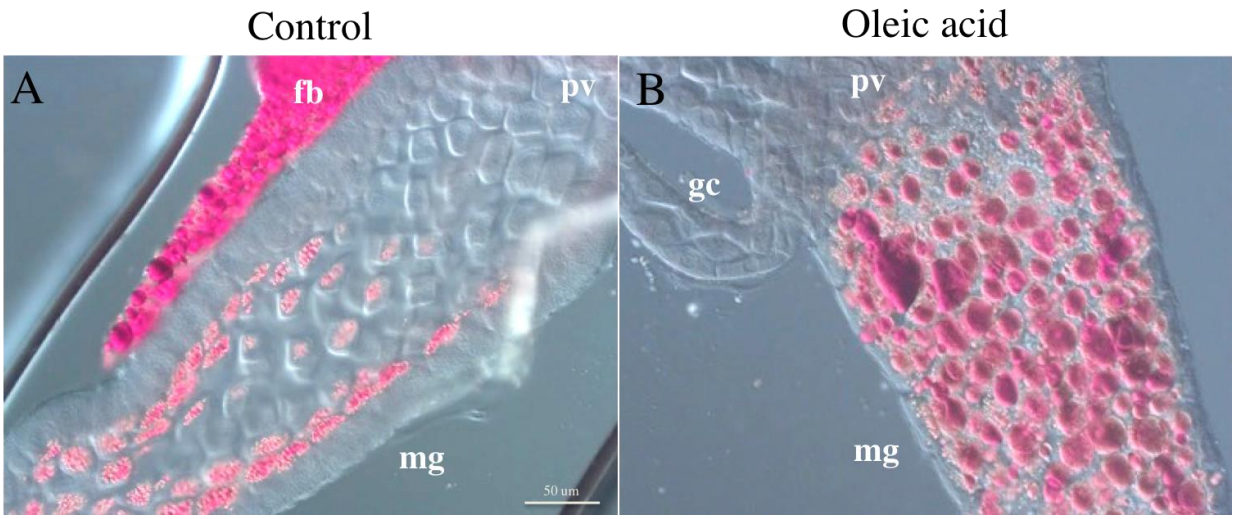

Figure 12. Larvae fed a high fat diet of 10% oleic acid in yeast paste accumulate LDs in the midgut. Oil Red O staining of LDs in dissected preparations of wild-type Ore^R larvae fed a diet of standard yeast paste (A) and high fat diet of oleic acid in yeast paste (B). Larvae fed a high fat diet overnight have an increased number of LDs in the anterior midgut region(B). Scale bar is 50 μm; mg=midgut, gc=gastric caecae, pv=proventriculus.

II.6 The supranuclear region of the cell is the same site where lipid droplets initially appear and disappear in enterocytes

As discussed above, LDs accumulate in a polarized manner at the supranuclear domain of the enterocyte (for example, see Fig. 8E, Fig. 9), demonstrating the concerted spatial organization of LD storage. It was previously shown in the fat body (Diaconeasa 2014) that FA uptake was physically coupled to LD synthesis and maintenance at the apical PM (Diaconeasa et al. 2013). Considering that the apical domain of the enterocyte faces the lumen of the gut, where FAs are taken up selectively through the apical membrane, I wondered whether FA uptake and LD synthesis is also a coupled process in the midgut. I hypothesized that the apical PM and the supranuclear domain of larval enterocytes in Drosophila play essential roles in facilitating FA uptake and maintaining newly synthesized LDs at the supranuclear region of the cell.

To test this hypothesis, I first wanted to establish unequivocally that the supranuclear region of the cell is the site of new LD synthesis. While my earlier observations were consistent with this interpretation (Section II.3), those experiments only captured a snapshot in time, so I could not rule out the possibility that the LDs observed in the supranuclear region were initially synthesized at another site in the cell. To directly observe de novo LD formation, I performed starvation – re-feeding experiments and monitored nascent LD formation by Oil Red O staining. Control larvae were fed a standard yeast paste diet resulted in basal levels of LD accumulation (as expected). Larvae were then starved for 24 hrs, leading to a complete clearance of the LDs (Fig.13 A & B). Starved larvae were then re-fed a standard yeast paste diet and stained quickly after initiating feeding (1.5 hrs) to detect formation of new LDs. I observed a pattern of nascent LDs forming in the

same supranuclear region as LDs observed previously (Fig. 13C), suggesting that the supranuclear site is the site of de novo LD synthesis in addition to LD storage, as hypothesized.

During lipid mobilization, TGs stored within LDs are proposed to be loaded onto LTP for export out of the basal end of the cell (Palm et al. 2012). I wondered whether the spatial parameters governing LD synthesis and accumulation in the supranuclear region of the enterocyte also applied to LD disappearance during lipoprotein loading. In mammals, lipoproteins (CMs) have been proposed to acquire lipid cargo by fusing nascent LDs in the ER lumen with nascent ApoB particle to form a CM. Premature CMs mature as additional lipids are acquired during transit through the secretory pathway from the ER to Golgi compartments (Demignot, Beilstein, and Morel 2014). However, it is not clear where CMs intersect with cytoplasmic LDs to facilitate CM loading for the mobilization of FAs from cytoplasmic LDs. It is possible that the coordinated localization of LDs at a single, supranuclear site within the cell would support efficient LD mobilization. I therefore hypothesized that LD localization to the supranuclear site creates a region of discrete LD-LTP interactions, which spatially links this population of LDs with LTP for lipid loading and export out of the enterocyte.

In my previous attempts to explore LD dynamics (presented in Section II.4, and above in this section), I was unable to effectively visualize the steps of LD disappearance, since LDs were completely cleared within 4 hrs of starvation after feeding larvae a standard yeast paste diet. I reasoned that a higher initial level of LDs might permit a clearer picture of the steps of LD disappearance. In an attempt to better visualize the spatial dynamics of LD disappearance (i.e., lipoprotein loading), larvae were fed a high fat diet

(10% oleic acid) to elevate baseline LD levels and then starved for 4 hrs (Fig.13 D & E). As expected, feeding on a high fat diet resulted in a massive accumulation of LDs of varying size that were dispersed throughout the enterocyte (Fig. 13D). During the chase period (4 hr starvation), I observed a gradual shift in the localization and size of the LD population – LDs decreased in size and became increasingly concentrated in the supranuclear region of the cell (Fig. 13E), as observed under standard dietary conditions.

These observations clearly illustrate the existence of a tight spatial control over LD localization during LD processing. During starvation after high fat feeding, LD depletion was not observed at random sites throughout the cell; rather, there was a clear and reproducible return of LD localization to the supranuclear site of the cell. This preference for maintaining LDs in the supranuclear region of the enterocyte is consistent with a functional role for the supranuclear site not only in lipid uptake, but also in lipid mobilization, and is consistent with several other studies in mammals and flies (Cartwright and Higgins 2001; Demignot, Beilstein, and Morel 2014), will be discussed in detail in the Discussion.

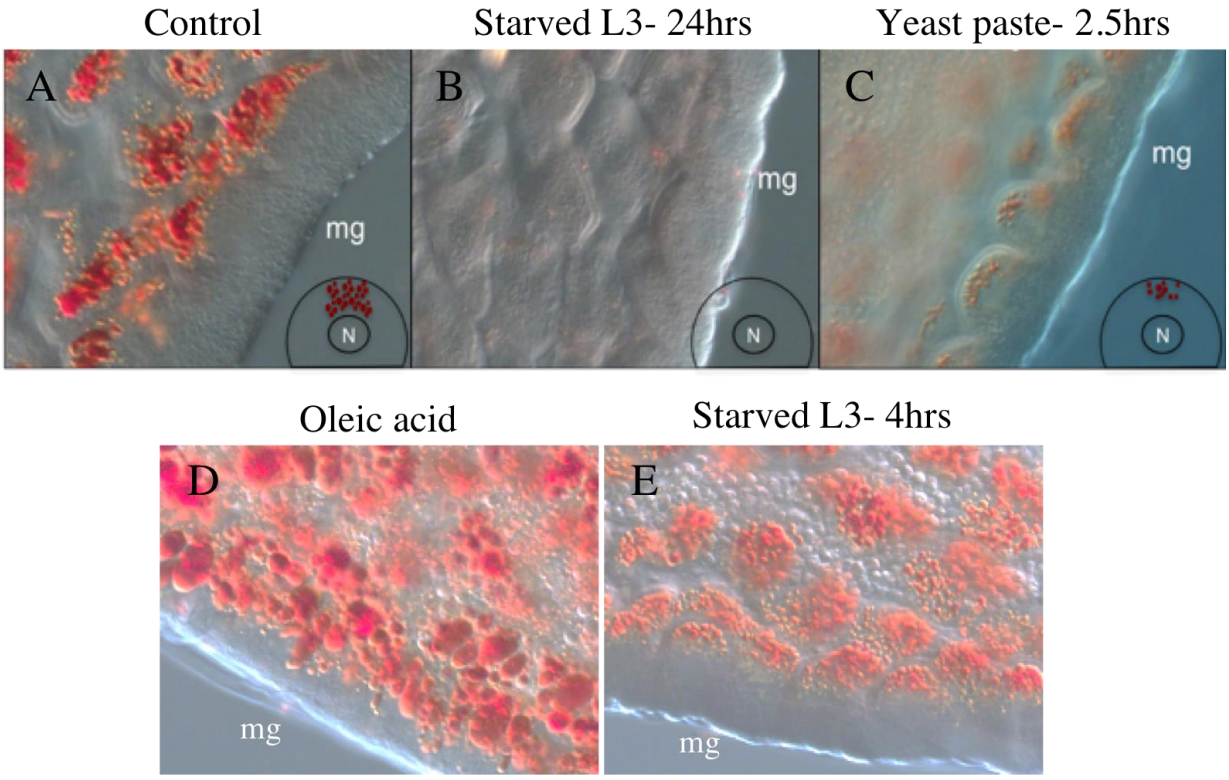

Figure 13. Refeeding and starvation experiments indicate that the same supranuclear site is where LDs first appear and also where excess accumulated fat exit the cell. A) Ore^{R} control flies fed a standard yeast paste diet showed basal levels of LDs, which were chased out completely after 24hrs of starvation (B). Starved larvae were re-fed a standard yeast paste diet for 2.5hrs (C). The hazy pattern of premature LDs in the cells (C) are emerging in the same space as the supranuclear population of LDs in the control . D) Ore^{R} flies fed a 10% oleic acid diet mobilized the large LDs during a 4hrs starvation period. E) During the chase period the remaining LDs appeared to become increasingly concentrated in the supranuclear region of enterocytes. mg=midgut.

II.7 Spatial regulation of LTP

Recent studies suggest that inter-organelle membrane contact sites are important centers for spatially compartmentalizing lipid metabolic reactions (Hariri et al. 2018). When I examined LD accumulation in the midgut under standard dietary conditions, LDs were always present and tightly concentrated in the supranuclear compartment of enterocytes (for example, see Fig. 9). Additionally, EM analysis of larval midguts showed small LDs to be subjacent to the PM of the enterocyte (larger LDs appear to dissociate from the apical PM and are found deeper within the cells, Fig. 10). I also present evidence that the supranuclear region of the cell is the last site where LDs are observed, supporting the conclusion that the apical domain may be the site of LD unloading and lipoprotein loading (Fig. 13). Taken together, it is plausible that the supranuclear site has a functional role in the process of lipid uptake, storage and mobilization via the lipoprotein pathway. However, the spatial regulation of the lipoprotein pathway within the enterocyte, specifically, the parameters governing the movement of LTP, remains unclear.

II.7.1 The intracellular localization of Rab proteins, regulators of the endocytic pathway in the midgut

As the molecular machinery driving LD metabolism in each cell type continues to be elucidated, regulatory proteins known to play roles in various other cellular processes are emerging as key players in LD dynamics. Chief among these are the GTPases belonging to the Rab protein family, which appear to be important molecular switches used in the regulation of intracellular trafficking and storage of lipids (Scott and Nilsson 2014). Rab proteins are evolutionarily conserved and exist in diverse species, from mammals,

nematodes, insects, plants to yeast, with a high degree of sequence identity which points to Rab proteins as conserved key regulators of cellular events.

There has been at least one proteomics-based report of each of the known Rab proteins (Rab1, 5, 7 and 18) being associated with an isolated LD (Scott and Nilsson 2014). Rab proteins have been widely used as markers to identify specific sub-compartments of the endocytic pathway. The intersection of the endocytic pathway and the supranuclear population of LDs is where I suspected lipoprotein loading to take place. Since LTP is speculated to be contained within an endomembrane compartment as it shuttles TGs from LDs to Lpp outside the cell (Palm et al. 2012), this model helps to illustrate the current thinking of how the lipoprotein pathway may be spatially regulated. I used midgut specific MexII-Gal4 to drive expression of Rab1, 5, or 7 to visualize possible intersection of the endocytic pathway with the tightly concentrated population of LDs in the enterocyte (Fig.14). Rab5 is typically used to mark early endosomes, Rab7 for late endosomes, and Rab11 for recycling endosomes; I observed three distinctly different patterns for each. Rab5 showed high expression only at the basal end of the enterocyte. Rab5 has been implicated in multiple intracellular trafficking processes, none of which suggests an interaction with LDs (Scott and Nilsson 2014). Rab11 showed high expression lining the apical domain of the PM. Similar to Rab5, Rab11 has not been implicated in any suspected interaction with LDs. However, Rab7 appeared to co-localize with the supranuclear population of LDs (Fig. 14 B). After investigating more closely the cells expressing Rab7, I observed high Rab7 expression in the apical subregion of the cell closely associated with the PM and with the negatively-staining LDs (Fig. 14 D). Given the known function of Rab proteins in endosome trafficking, and LTP's predicted location within an endomembrane

compartment, this interpretation would suggest that LTP be carried to the supranuclear domain by the endocytic pathway. It is hypothesized that LTP may localize to the apical domain of the enterocyte and may further lend support to the model for LTP lipoprotein loading in the supranuclear region of the cell.

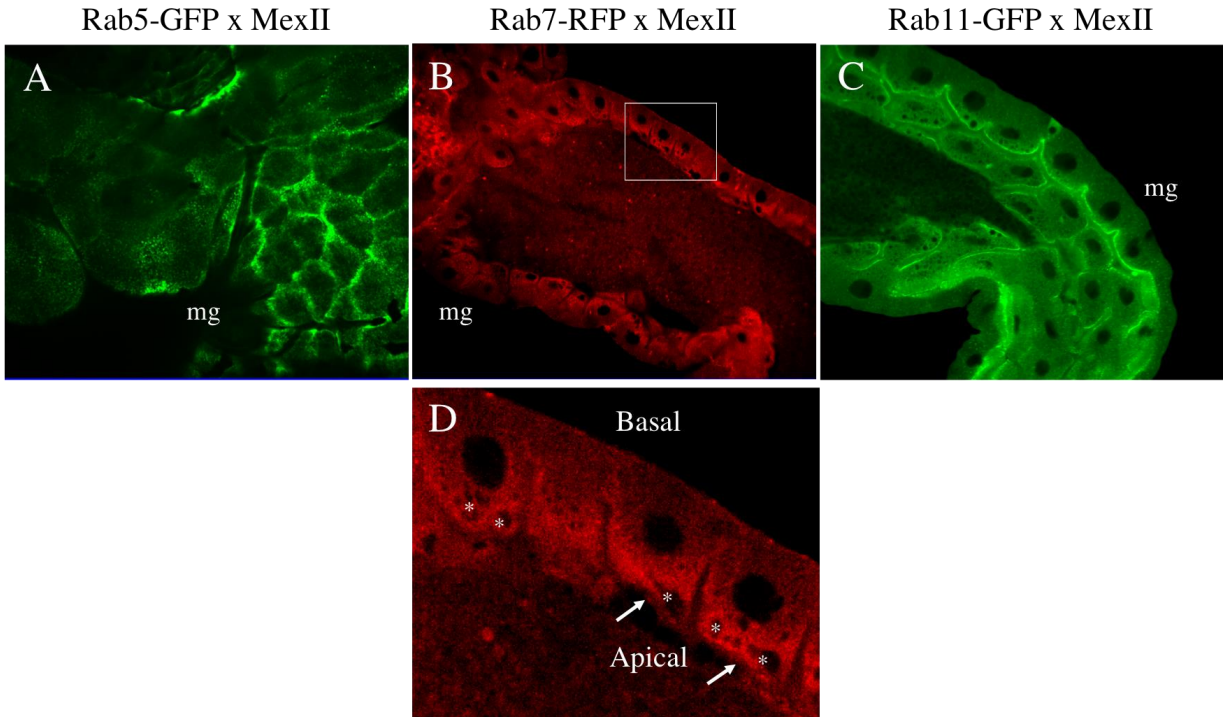

Figure 14. Intracellular localization of Rab proteins in the midgut. Midgut specific MexII-Gal4 and UAS-Rab5, 7 and 11 proteins used to visualize the intersection of the endocytic pathway with the supranuclear population of LDs in the midgut. A) Rab5-GFP marks early endosomes, B & D) Rab7-RFP marks late endosomes and C) Rab11-GFP marks recycling endosomes. D) Rab7 is detected at higher levels at the apical domain of the enterocyte (arrows) colocalizing with LDs (white asterisks) (other Rabs not shown) mg=midgut

CHAPTER III. Intracellular dietary fat translocation in the larval midgut: evidence that microtubules and microtubule dependent motor proteins contributes to the spatial regulation of lipid droplet accumulation and mobilization in enterocytes

III.1 Abstract

The intracellular site of LD accumulation may be related to the spatial distribution of the lipoprotein pathway. Several major insights emerged from the studies I presented in Chapter II. First, I suggest that a substantial amount of FAs are absorbed and incorporated into LDs in the larval midgut enterocytes by an unknown mechanism. Second, in wild-type larvae fed a standard diet, LDs accumulated in a highly organized, polarized manner and this tight localization was maintained at the supranuclear compartment of the cell. The asymmetric distribution of LDs in the supranuclear region of the enterocyte observed in vivo constitutes a novel observation in Drosophila that provides a new level of insight into the spatial regulation of LD synthesis. Third, starvation experiments revealed that LDs are readily mobilized within 4 hrs of starvation, from their site of temporary storage in the supranuclear region of the cell to the site of LTP loading. I speculate that the rapid mobilization of FAs is accomplished by direct FA transfer between LDs and LTP within the cell, facilitated by a close physical association. Fourth, my finding that the small GTPase Rab7-YFP, a late endosomal marker, localized to the supranuclear domain of larval enterocytes suggests that endosomal trafficking to the supranuclear region of the cell may be a critical function associated with intracellular lipid translocation . More broadly, the asymmetric distribution of both LDs and Rab7 endosomes to the apical domain is suggestive of an efficient intracellular transport system, such as the microtubule (MT)

network, that positions these cellular organelles/ structures in close proximity with one another.

An additional insight from the work presented in Section II was my observation that the initial site where LDs appear in the cell is also the last site where LDs are observed before they disappear during starvation – the supranuclear region of the cell. While not conclusive with respect to establishing the site of LD unloading and LTP loading, these results do suggest that the supranuclear site is important during lipid trafficking. In light of the observed apical localization of Rab7 endosomes, these findings suggest a possible role for Rab7 endosomes in FA trafficking via the lipoprotein pathway. I speculate that endocytic vesicles (Rab7) might utilize the cytoskeleton array of MTs for the intracellular trafficking of dietary lipids. Irrespective of a dedicated role for Rab7, several of my findings support a role for MTs in lipid translocation and LD dynamics; therefore, I next focused on understanding the role of the MT array in coordinating intracellular lipid trafficking in Drosophila enterocytes.

III.2 Evidence supports a role for microtubules in mediating the spatial organization of lipid droplets

There is growing evidence supporting an important role for MTs in enterocytes. In Drosophila epithelial cells, unlike in mammals, nucleation of microtubules does not occur via a microtubule organizing center (MTOC). Instead, MTs are nucleated by PM-associated proteins at the apical surface of cells (Mogensen, Tucker, and Stebbings 1989). This finding is compatible with a more recent study by Khanal and colleagues, in which follicle epithelial cells in Drosophila displayed a pattern of MTs organized linearly where one end of the MT

is at the apex of the cell (minus-end) with the other at the basal end (plus-end)(Khanal et al. 2016). Furthermore, Khanal and coworkers showed that the minus-end MT binding proteins Patronin and Shortstop (Shot) act in parallel at the apical domain to polarize MTs (Khanal et al. 2016). These findings revealed a new mechanism linking epithelial cell polarity to the MT cytoskeleton to direct membrane trafficking.

Based on these results, and my own results with Rab7 localization (discussed above), I hypothesized that the MT cytoskeleton might be organized along the apicobasal axis of enterocytes, and might contribute to the polarized population of LDs in the cell. To initially explore the distribution of MTs in midgut epithelial cells, I used a midgut specific MexII-Gal4 to drive expression of UAS-Nod-GFP, a marker of MTs (Cui et al. 2005) (Fig. 15 A). Nod-GFP larvae produced a MT patterning with high fluorescence evident in the sub-apical region of the cell, overlaying with the supranuclear population of LDs (Fig. 15 B & C).

To reproduce the MT pattern seen in MexII X Nod-GFP, I next attempted to visualize MTs directly with α-Tubulin-GFP, as well as with an additional marker of MTs, Jupiter-GFP. Unfortunately, Jupiter-GFP failed to produce a fluorescent signal, and low levels of α-Tubulin-GFP did not offer much insight into the distribution of MTs in enterocytes. We used a different α-Tubulin reporter line, UAS-Maple-GFP, to attempt to mark MTs in the midgut epithelium. Midgut specific expression of α-Tubulin-GFP revealed a fluorescent signal showing high expression in the sub-apical region of the cell, overlaying with the negatively stained supranuclear population of LDs (Fig.16 A & B, red arrows) consistent with my UAS-NOD-GFP results (Fig.15). To further define the organization of cytoplasmic MTs, I utilized a Z-stack approach to permit a 3-D visualization of the anterior midgut. In a transverse view of the anterior midgut, using a cross section of a combined Z-stack (made with

confocal GFP images), I observed uniformly aligned MTs parallel to the apicobasal axis (Fig.16 C & D, white arrow) similar to the MT distribution in Drosophila follicle epithelial cells described by Khanal et al (Khanal et al. 2016).

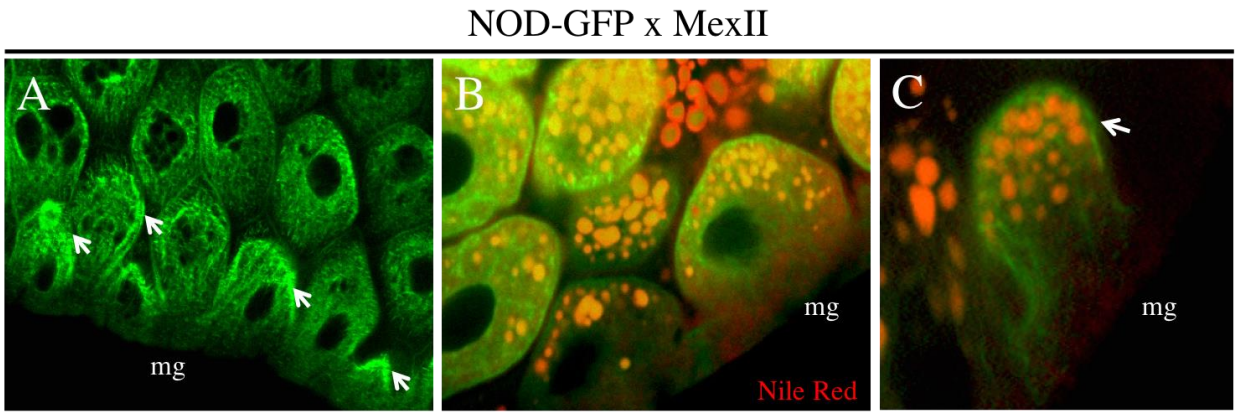

Figure 15. LDs in the supranuclear region of enterocytes overlap with a microtubule pattern that extends from the supranuclear region to the basal cytoplasm. Shown here are representative mages of dissected midgut tissues taken with fluorescence microscopy before and after Nile Red (NR) staining. Larvae fed yeast paste diet have few, small LDs in the supranuclear region of the cell. A & B) Midgut specific MexII-Gal4 and UAS-NOD-GFP, a microtubule marker, produce a filamentous microtubule patterning with high concentrations of microtubules (white arrows) in the supranuclear subregion of the cell. Shown are NR staining of LDs that accumulate in the supranuclear region of the cell (B & C). Scale bar= 50 µm, mg=midgut

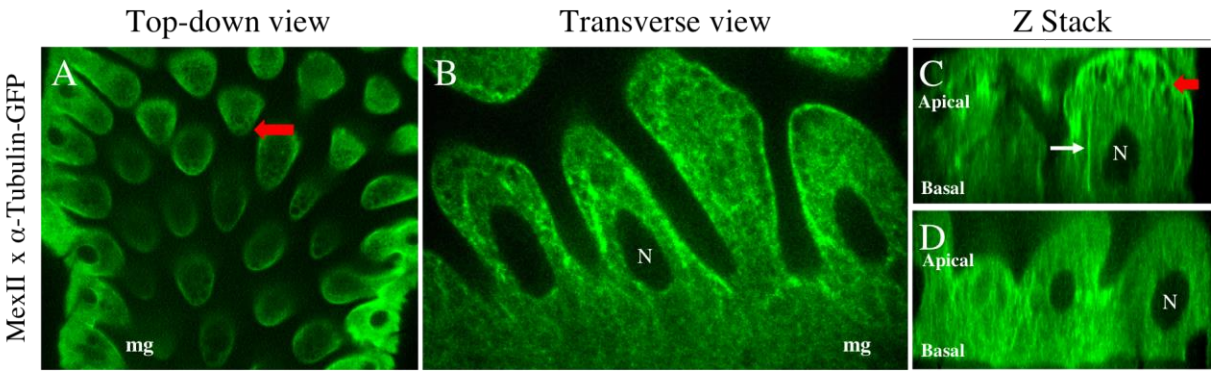

Figure 16. Microtubule (MT) array in enterocytes are aligned parallel to the apical-basal axis. Shown are dissected larval midgut tissues analyzed by GFP fluorescence of larvae expressing UAS- α-Tubulin-GFP (A) & UAS-Maple-GFP (another α-Tubulin line; B-D) driven by a midgut specific MexII-Gal4. Larvae fed a standard yeast paste diet produced a polarized population of LDs, shown by the negative staining of spherical structures (red arrows), closely associated with the apical plasma membrane (A-C). Over-expression of α-Tubulin produced elongated cone shaped cells in the midgut. Transverse view and Z-stacks of cells in the midgut shows high expression levels at the apical region of the cell (B-D). Z-stacks show a MT distribution that is uniformly aligned along the apical-basal axis (white arrow). N= nucleus, mg= midgut

The current model of MT polarity in follicle epithelial cells involves Patronin and Shortstop, minus-end MT binding proteins, which act to polarize MTs along the apicobasal axis (Khanal et al. 2016). To investigate if these proteins exhibit similar polarity (marking along the apical PM) as described in follicle epithelial cells, I expressed UAS-Patronin-GFP specifically in the midgut and examined fluorescence patterns (Fig.17). Midgut specific expression of Patronin-GFP produced a speckled pattern with high signal localized to the apical membrane of the cell (Fig.17 C). Since Patronin has been established to bind at the minus-end of the MT structure, these results indicate that MTs are oriented with minus-ends apically, and plus-ends basally in the enterocyte. Furthermore, these results suggest that MTs and their associated proteins are present and highly abundant at the supranuclear subregion of the cell, in close proximity to the supranuclear population of LDs. The tight co-localization of MTs and LDs suggested a possible role for MTs (and, specifically minus-end MT motor proteins) in maintaining the asymmetric LD distribution within the cell.

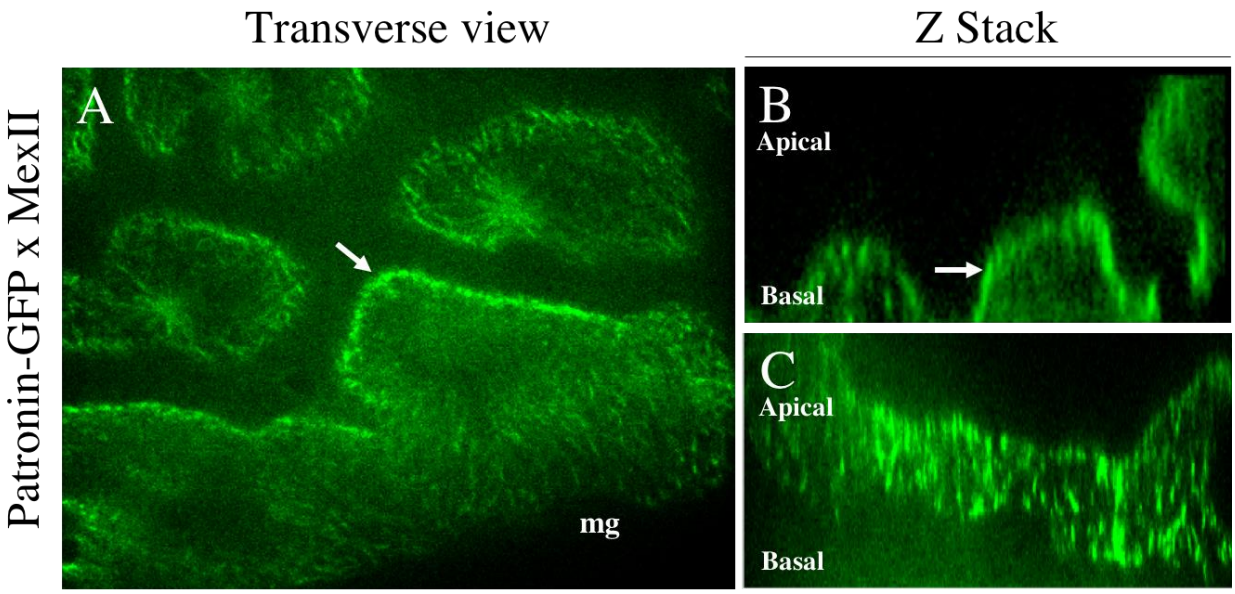

Figure 17. Patronin-GFP, a minus-end MT binding protein, is localized along the apical PM. Shown are dissected larval midgut tissues analyzed by GFP fluorescence of larvae expressing UAS- Patronin-GFP driven by a midgut specific MexII-Gal4. Transverse view and Z-stacks of cells in the midgut shows a bright fluorescent outline of the cell (arrows) along the apical PM (A & B). Expression of Patronin-GFP shows the protein producing a speckled pattern along the apical PM (C). mg= midgut

III.3 The functional association between microtubules and lipid droplets

The overall apical-basal polarization of MTs in larval enterocytes raised the question of whether polarized MTs are truly essential for polarized trafficking and localization of LDs and lipoproteins. I noticed that overexpression of UAS-α-Tubulin-GFP in the midgut resulted in a dramatic change in cell shape (based on fluorescence results), transitioning to a more elongated cone shaped cell (Fig. 16 A). Previous work with UAS-α-Tubulin-GFP in neurons has revealed an MT sliding action that that pushes the apical membrane outward resulting in cellular elongation (Lu et al. 2013).

The obvious dramatic change in cell shape caused me to wonder about the effect on LD distribution in enterocytes overexpressing α-Tubulin. To visualize the effects of cell shape on LD distribution, I fed larvae a standard yeast paste diet and analyzed the pattern of Oil Red O staining by DIC microscopy. Oil Red O staining revealed a very tight association of LDs at the sub-apical tip of the cone-shaped cell instead of the supranuclear domain like in wild-type larvae (further explained on pg.74-75) (Fig.18 C & F). One interpretation of this dramatic phenotype is that α-Tubulin overexpression drives membrane expansion (causing elongation of the enterocyte into a cone shaped cell) and, as a result, LDs are *indirectly* pulled along with the expanding membrane due to their tight association with the apical PM, independent of MTs. However, the extraordinarily tight localization of the LDs observed upon α-Tubulin overexpression suggested a *direct* role for MTs (and/or associated MT minus-end motor proteins) in maintaining LD accumulation in the distal tips of elongating microtubules in the enterocyte.

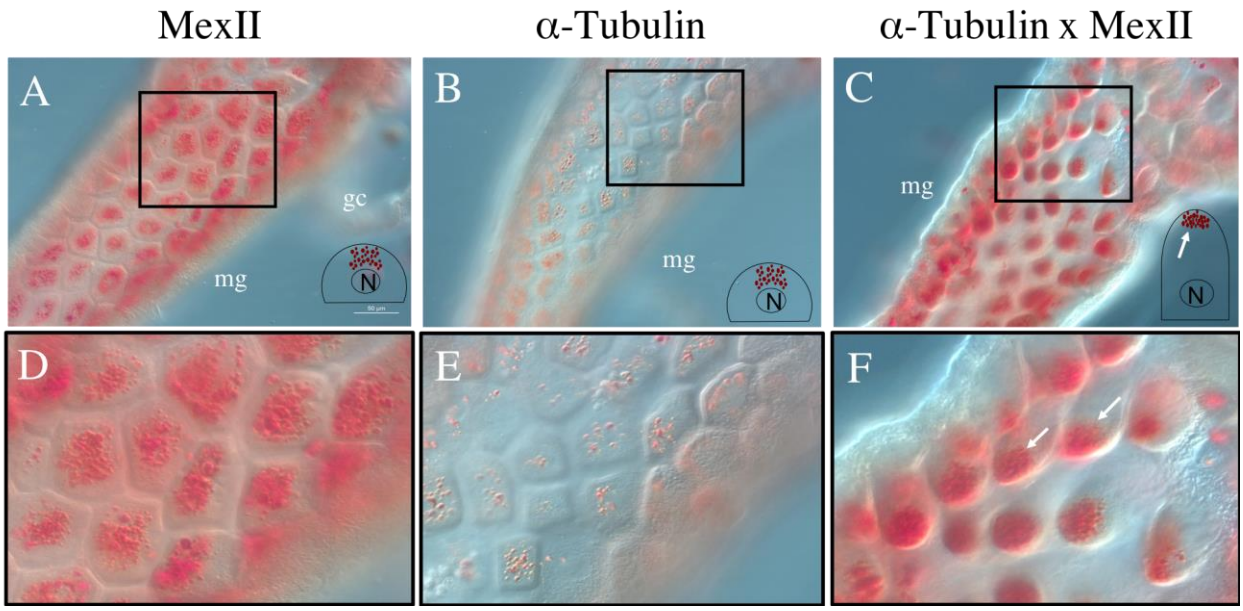

Figure 18. Midgut specific α-tubulin overexpression in enterocytes results in cell elongation (the apex of the cell extends further from the basal end) and accumulation of lipid droplets at the extreme apical tip of the cell. Oil Red O stained midgut tissues of controls, Mex^2 and α-Tubulin, show cuboidal shaped cells (Top-down view; A-B & zoomed in view D-E). Larvae overexpressing α-tubulin produced a cone shaped cell shape (Top-down view; C & zoomed in view F) where LDs appear to be intimately associated with the cytoplasmic side of the apical plasma membrane (arrows). Scale bar is 50 μm; mg= midgut

III.3.1 Insights into LD dynamics and localization from MT overexpression

The LD localization to the sub-apical tip of the enterocyte may be directly mediated by MTs and/or MT binding proteins and is consistent with current knowledge regarding the dynamics of MTs and their associated motor proteins. In particular, knockdown of Dynein, a minus-end MT motor protein, in Drosophila embryos has been shown to disrupt LD localization during embryogenesis (Gross et al. 2000; Welte 2015b). In addition, my earlier results showing apical localization of Patronin are suggestive of a possible functional role for this minus-end MT binding protein in maintaining MT polarity and possibly LD localization to the apical domain of the cell. Beyond supporting these direct observations, the unique phenotype of the MexII x α-Tubulin overexpressing fly line provided a useful research tool to further investigate the dynamics of LD synthesis and mobilization at the apical PM, as described in Section II.6 (Fig.13).

To further explore a direct relationship between MTs and LD synthesis/breakdown, I performed starvation- re-feeding experiments and monitored LD localization by Oil Red O staining in the α-Tubulin overexpressing line. My previous work in Section II (Fig. 13) suggested that the supranuclear site of the cell is the site of new LD synthesis (and, possibly, LD unloading and mobilization) in the midgut enterocytes of wild-type larvae. I was curious about the effect of very tight LD localization (caused by α-Tubulin overexpression) on LD mobilization and formation. Larvae fed a standard yeast paste diet showed basal levels of LDs tightly associated with the apical PM (Fig.19 A & D), as expected. However, upon starvation, these larvae showed incomplete depletion of apical LDs, even after 24 hrs of starvation. While a large portion of the cells in the midgut showed complete mobilization of LDs out of the cell, a subset of the cells in the anterior midgut retained a

small population of LDs at the sub-apical tip of the enterocyte (Fig.19 B & E). This contrasted with LD mobilization in wild-type Ore^R larvae, which were completely chased out within 24 hrs of starvation (Section II, Fig.13 B), suggesting that the factors controlling the tight localization may interfere with downstream mechanisms responsible for LD unloading and mobilization (explored further in Discussion).

To observe de novo LD formation in α-Tubulin overexpressing enterocytes, starved larvae were re-fed a standard yeast paste diet for 2.5 hrs, resulting in a diffuse staining of nascent LDs which were found in the same supranuclear space as the lipid droplets in the control (Fig. 19 C & F). Initial LD formation in these elongated α-tubulin expressing cells was found to occur at the very distal tip of the cell during re-feeding, and LDs continued to accumulate only at that site, consistent with my earlier observations in Ore^R wild-type flies (Section II, Fig. 13). These results further support that the sub-apical domain (closely associated with the PM) has the same functional role in the process of lipid uptake and mobilization in α-Tubulin overexpressing enterocytes as in Ore^R wild-type cells.

Beyond providing additional insight into LD dynamics, the dramatic elongated phenotype of the α-Tubulin overexpressing cells permitted a finer level of spatial insight into the precise site of LD localization. During starvation and re-feeding, the LDs in α-Tubulin overexpressing enterocytes could be clearly pinpointed to the sub-apical tip of the enterocyte, immediately adjacent to the apical PM, whereas in my earlier observations in wild-type enterocytes the much shorter cell length allowed only for an approximate determination of localization to a less well-defined "supranuclear domain" between the apical PM and the nucleus. Thus, the previously defined supranuclear region may not be the most accurate description of the site of LD formation or mobilization via LTP. Instead, it

seems that the site of LD synthesis and mobilization moves along with the point of anchorage of MT minus-ends at the apical PM. I therefore speculate that polarized MTs and MT directed motor proteins contribute to the positioning of LDs within the enterocyte.

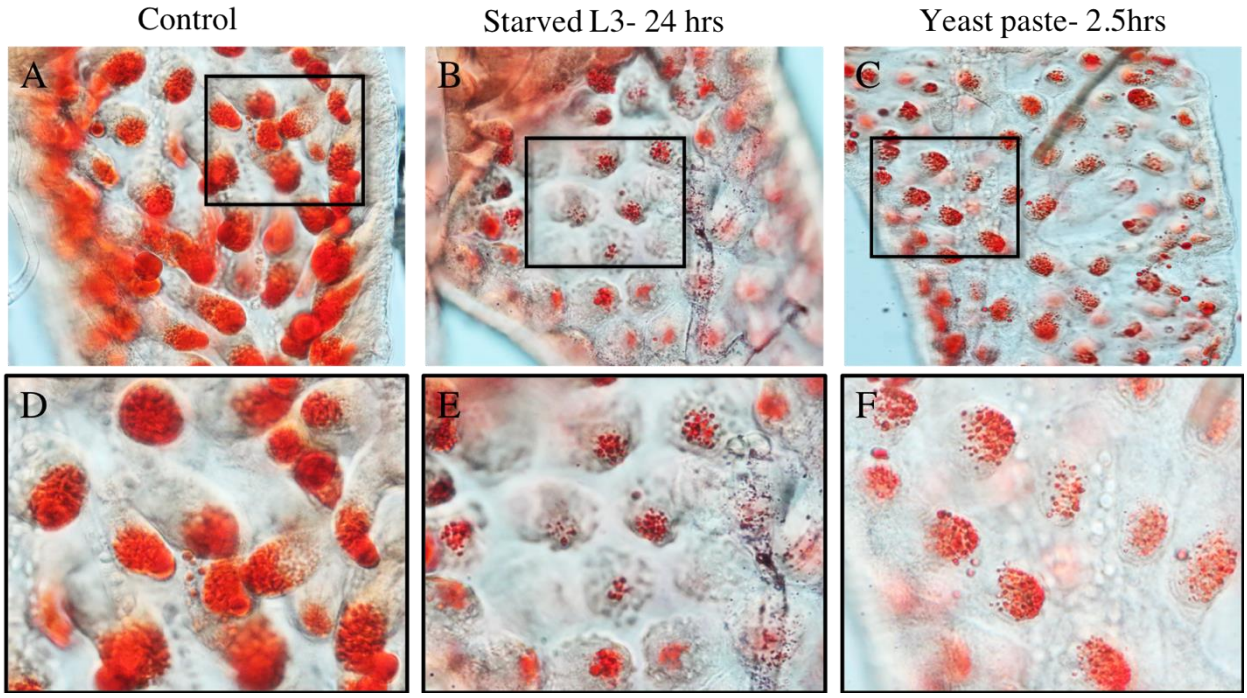

Figure 19. Refeeding and starvation experiments indicate that the site where LDs first appear and where LDs exit the cell occurs at the same site. 3rd instar larvae over-expressing α-Tubulin in the midgut fed a standard diet showed basal levels of LDs accumulate at the distal tip of these elongated cells (A & D). Larvae starved for 24hrs did not completely chase out all LDs; the remaining LDs appeared to become increasingly concentrated at the very apical tip of the cell (B & E). Starved larvae were re-fed for 2.5hrs accumulated LDs at the same apical tip of the cell, closely associated with the apical PM (C & F). mg=midgut

III.3.2 Insights into LD dynamics from MT disruption

If the spatial organization of LDs in enterocytes is MT dependent, then disrupting MTs should lead to disruption of the highly polarized population of LDs in the supranuclear region. To further investigate the contribution of MTs to the asymmetric patterning of LDs, I exposed Ore^R larvae to MT depolymerizing drugs and analyzed the effects on LD patterning using DIC and fluorescence microscopy. Many studies exploring the effects of MT assembly in cells, in vitro and in vivo, have relied on a small list of effective drugs to disrupt MTs polymerization. Among these drugs, colcemid, colchicine and nocodazole are most commonly used in cell biology experiments (Tuma and Gelfand 1999; Rodionov, Gyoeva, and Gelfand 1991; Rodionov et al. 1998; Yang, Ganguly, and Cabral 2010). Colcemid and nocodazole, mitotic inhibitors, bind to MT plus-ends to prevent the elongation process and thereby promote MT depolymerization. Colchicine inhibits the polymerization of MTs by binding directly to free tubulin subunits (Yang, Ganguly, and Cabral 2010). It has been established that exposure of animals to MT destabilizing drugs for over 4 hrs results in complete lethality.

First to establish that the MT destabilizing drugs were being internalized during in vivo exposure, I assessed rate of development and lethality. Late-second to early-third instar wild-type Ore^R larvae were placed onto fresh agar plates with 1%, 0.5% or 0.25% drug and compared for viability and rate of development; larvae were assessed for lethality every hour. The goal was to identify a time of exposure at which MT functionality is compromised, but not completely lost resulting in lethality. Once a time point was reached, larvae were collected, dissected and analyzed by Oil Red O staining to see the effects of the drug on the supranuclear population of lipid droplets.

Larvae fed 1%, 0.5% or 0.25% colcemid showed no signs of lethality. In addition, Oil Red O staining of the midgut showed no changes in LD distribution relative to no drug exposure. In contrast, larvae treated with colchicine for 4 hrs showed a significant delay in motility and eventually resulted in lethality. Oil Red O staining of dissected 2nd instar larvae showed a reduction in LD accumulation and dramatically disturbed LD distribution (Fig.20 A). The few LDs that were observed no longer localized to the supranuclear domain of the enterocyte; instead, LDs were observed throughout. These data suggest that by disrupting MTs (using colchicine), the polarized organization of LDs in enterocytes is lost.

I next asked whether the loss of LD formation and distribution observed upon exposure to colchicine could be reversed. Surviving larvae from each drug treatment were removed from colchicine plates, re-fed with standard drug-free yeast paste, and analyzed by Oil Red O staining to test if the effects of MT destabilization on LD distribution could be reversed. I found that within 2 hrs of re-feeding on standard yeast paste in the absence of MT destabilizing drug, LDs began to reappear, tightly localized at the apex of the cell. This result further supports the notion that MTs are important in maintaining an accumulation of LDs at the supranuclear region of the enterocyte.

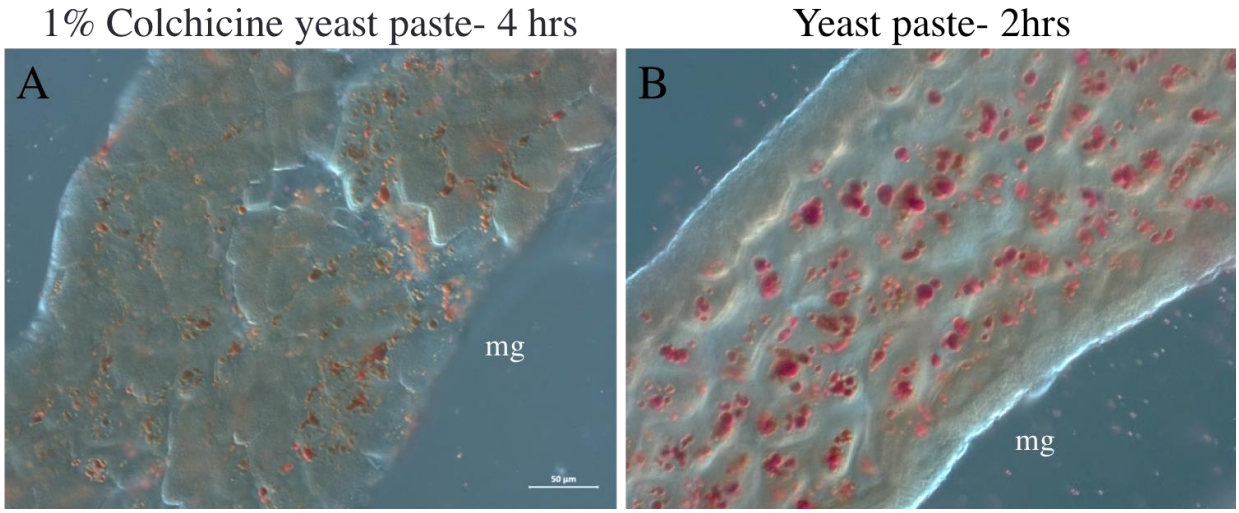

Figure 20. Colchicine (MT destabilizing drug) feeding dramatically perturbed the LD distribution and blocked further dietary fat uptake, which was reversed by re-feeding standard yeast paste diet. Ore^{R} larvae were treated with 1% colchicine yeast paste for 4 hrs(left) and then re-fed for 2 hrs with standard yeast paste diet (right). Colchicine feeding appeared to block further dietary fat uptake and disrupted the supranuclear patterns of LDs (A). The 2 hr chase saw a recovery of lipid uptake with numerous and large LDs accumulating at the supranuclear region again (B). Scale bar is 50 µm; mg=midgut

An important caveat to note when considering the implications of the above results is that exposing cells to MT destabilizing drugs like colchicine may have global effects on cell morphology. Therefore, it is difficult to assess whether the drastic disruption of LD organization observed is an indirect effect of overall disrupted cellular integrity, or if it is due to a direct loss of interaction between LDs and MTs and/or MT binding proteins. To determine the direct effects of colchicine on enterocyte morphology, midguts of Ore^R larvae were dissected and incubated in fly ringer solution with 0.5% colchicine for 15 minutes. After incubation with colchicine, the distribution of LDs originally found tightly concentrated in the supranuclear region of the cell (Fig.21 A & C) became dispersed randomly throughout the enterocyte (Fig.21 B & D). I also tested the toxic effects of colchicine in a live/dead assay to determine if the dispersed distribution of LDs was an indirect result of cell death. The live/dead cell assay showed that cells exposed to colchicine at these concentrations had not undergone cell death, confirming that the loss of polarized localization of LDs within the enterocyte upon colchicine treatment was likely due to MT disruption.

Taken together, it is clear that disruption of MTs has severe effects on LD accumulation and localization. In addition, the effects of MT destabilizing agents are reversible (i.e., when MTs recover, so do the polarized population of LDs) lending further support to the role of MTs in forming and maintaining LDs at the supranuclear region of the cell. Though these results are striking, it is nonetheless difficult to separate the loss of LD localization from the overall disrupted integrity of cell structure upon treatment with colchicine. However, in light of the results of the previous sections, these findings provide support for the involvement of MTs in LD formation and localization in enterocytes.

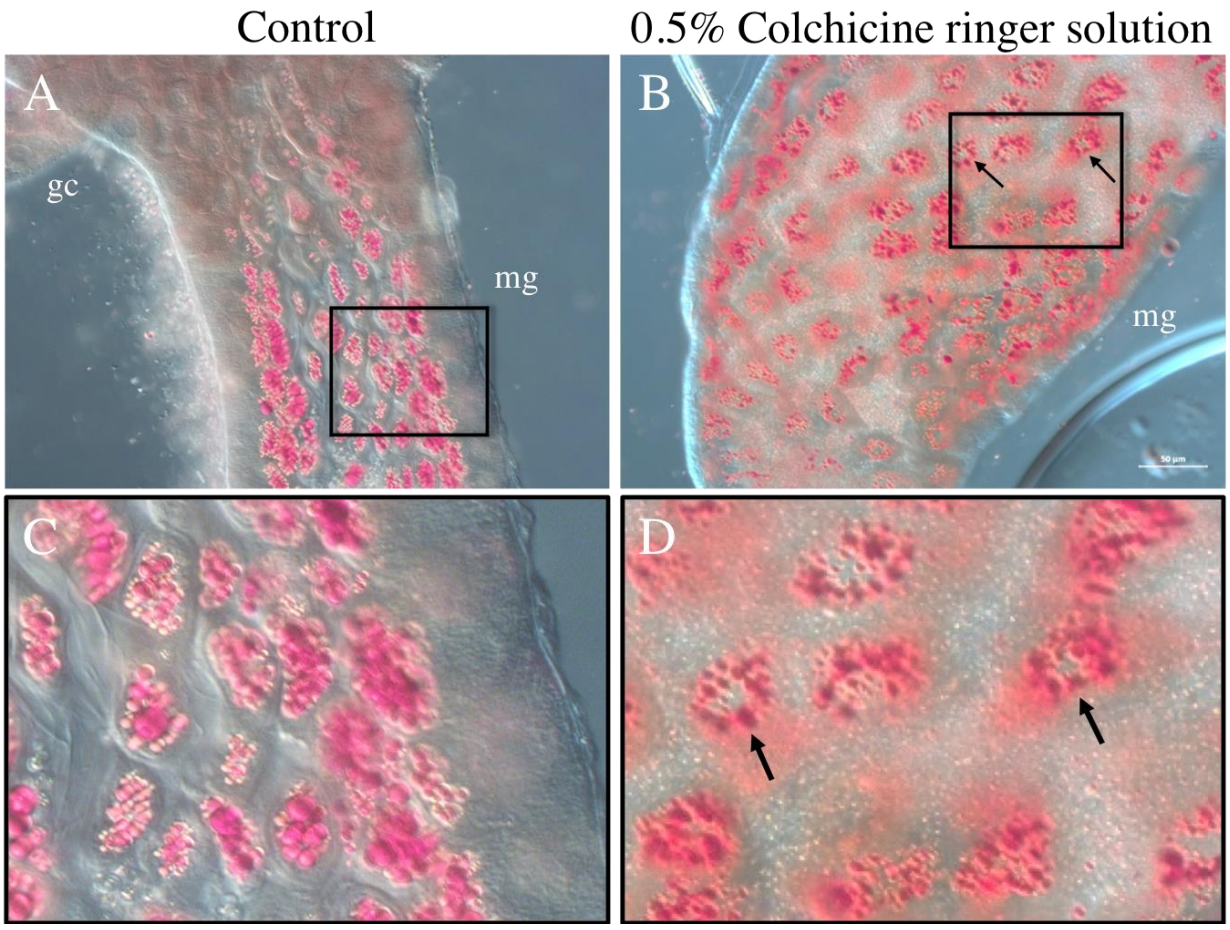

Figure 21. Treatment with colchicine led to dispersal of LDs. Ore^{R} larvae were dissected and incubated in fly ringer solution (left) and fly ringer solution with 0.5% colchicine (right) for 15 mins. The 0.5% colchicine treated larvae produced LDs that are no longer concentrated above the nucleus at the supranuclear region of the cell (A & C), they appear to sink in the basal direction surrounding the nucleus (arrows) (B & D). Scale bar is 50 µm; mg= midgut, gc=gastric caecae

III.4 Contribution of microtubule motor proteins – evidence of direct role for microtubules in lipid droplet localization

It is well documented that MT motor proteins Kinesin and Dynein contribute to the spatial organization of the cell by moving many types of cargo along the MT cytoskeleton (Karcher, Deacon, and Gelfand 2002; Rogers and Gelfand 2000; Khanal et al. 2016; Welte 2004). Furthermore, previous work showed that the knockdown of cytoplasmic dynein or kinesin in Drosophila embryos results in the loss of LD trafficking, demonstrating a direct relationship between LDs and MT motor proteins (Kural et al. 2005; Welte 2004). Based on these results, and my above findings implicating MTs in LD dynamics and localization, I hypothesize that MT motor proteins play a role in maintaining LD localization in enterocytes. In order to determine if kinesin (a plus-end MT motor protein) and dynein (a minus-end MT motor protein) play roles in lipid trafficking, I used a UAS line dsRNA directed against each of the plus and minus-end MT motor proteins. Dissected larval midgut cells were then examined using Oil Red O to analyze the effects on LD distribution by DIC microscopy. I hypothesized, specifically, that dynein heavy chain (Dhc) is responsible for the maintenance of LDs at the apex of the cell, while Kinesin-1 (Khc) mediates the transfer of fat from LDs to LTP, and further facilitates the movement of LTP basally to transfer fat to Lpp.

III.4.1 Dynein knockdown

If MT motor proteins are the driving force(s) behind the polarized localization of LDs in enterocytes, then knockdowns of minus-end or plus-end motor proteins should disrupt the supranuclear population of LDs. In turn, disrupted LD localization should lead

to impaired fat mobilization intracellularly and to downstream tissues. For the knockdown of dynein, I predicted several outcomes: 1) if Dhc is absolutely required for coupling lipid uptake to LD formation within the cell, no LDs will be observed, 2) if Dhc is required only for LD polarization, LDs are predicted to be present in wild-type abundance but dispersed throughout the cell, rather than being only apically localized and 3) if dynein is not required for LD formation or localization, then LDs should remain polarized as in wild-type enterocytes. To test the effects of dynein knockdown on LD formation and localization in the midgut, I drove expression of UAS-RNAi dynein heavy chain (Dhc64C) in the midgut and analyzed LDs by Oil Red O staining via DIC microscopy. Strikingly, larvae fed a standard yeast paste diet showed a complete absence of LDs in the midgut (Fig.22). The complete absence of LDs in the dynein knockdown suggests that the minus-end MT binding protein may play a role in directing FA uptake or in facilitating the synthesis of LD precursors at the supranuclear region of the enterocyte.

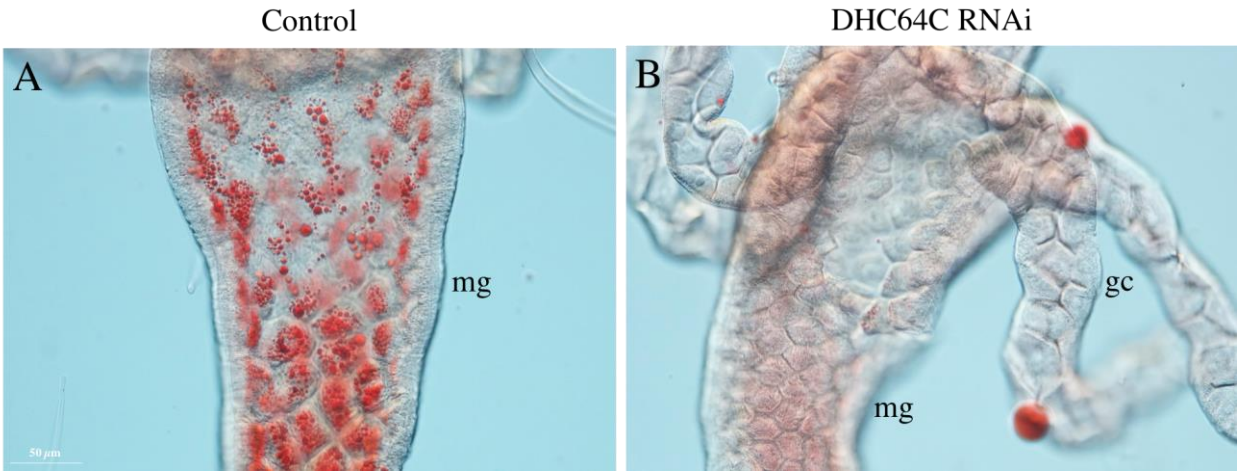

Figure 22. Dynein knockdown in the midgut produced an absence of LDs in the midgut. Shown are dissected larval midgut tissues analyzed by DIC microscopy of larvae expressing UAS-RNAi Dhc64C driven by a midgut specific MexII-Gal4. Larvae fed standard yeast paste diet produced a basal level of LDs in MexII controls (A) but midgut specific knockdown of dynein (Dhc64C RNAi) produced no LD phenotype in the midgut(B). Scale bar is 50 µm; mg= midgut, gc=gastric caecae

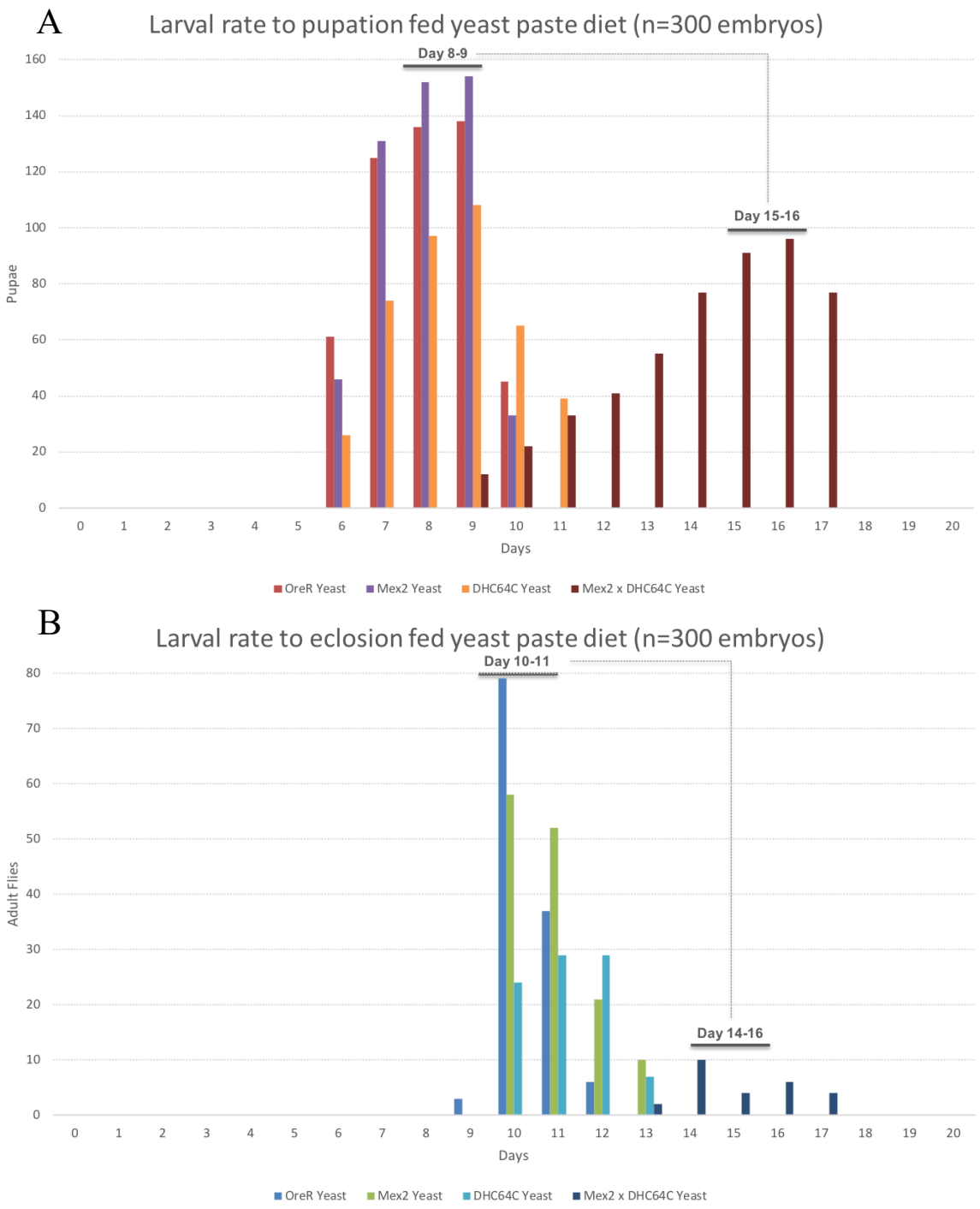

Figure 23. Ore^{R}, MexII, Dhc64C and UAS-RNAi Dhc x MexII-Gal4 larvae fed a regular yeast paste diet, exposed a correlation between the presence of LDs and normal rate of development.

A) Larval rate to pupation, controls peaked day 8-9 and mutant larvae peaked day 15-16,
B) Larval rate to eclosion, controls peaked day 10-11 and mutant larvae peaked day 14-16.

I next wanted to verify that the observed effects on lipid uptake and LD localization from knockdown of Dynein were developmentally relevant. Larvae expressing dynein RNAi in the midgut, parent lines (MexII & Dhc64C) and wild-type Ore^R were fed a standard yeast paste diet and tracked daily to measure larval rate to pupation, rate to eclosion, and lethality. Larval rate to pupation was calculated from four experiments accounting for 300 embryos for each fly line in total. Whereas wild-type and parent lines peaked in the number of pupae formed between Day 7 and 9, UAS-RNAi Dhc64C larvae began to pupate starting at Day 9 and peaked at Day 15, a delay of almost one week (Fig. 23 A). For larval rate to eclosion, which is the act of larva emerging from their pupal case as an adult fly, control fly lines peaked between Day 10 and 12, while UAS-RNAi Dhc64C larvae peaked between Day 14 and 16 (Fig.23 B). Finally, the rate of lethality is almost 8-fold higher in UAS-RNAi Dhc64C larvae than in control lines.

Since no LDs were observed in the midgut, it is difficult to discern from this experiment alone the contribution of dynein to lipid uptake, LD formation, or supranuclear LD localization. The result suggested that the RNAi construct is very strong, resulting in on/off control of expression. To parse the contribution of dynein to each of the above steps, I attempted to modulate expression of the RNAi construct using the temperature-dependence embedded in the Gal4/UAS system. In flies, Gal4 activity is minimal at 16^{o}C and maximal at 29^{o}C. I hoped that at a temperature permissive for dynein expression (low temp., low activity of UAS-RNAi Dhc64C), I might be able to observe at least some FA uptake and LD formation in the enterocyte. Subsequent shift to a temperature restrictive for dynein expression (high temp.) would then allow me to characterize the effects of dynein knockdown specifically on LD movement and localization (Fig.24).

However, LDs did not accumulate at any temperature for UAS-RNAi Dhc64C larvae (Fig.24 B), suggesting the subtle change in temperature might not have had an effect on the larvae. This result led me to question the integrity of the UAS-RNAi Dhc64C line – were the observed effects on LD accumulation and localization due specifically to knockdown of dynein or due to an indirect effect? Collaborators had previously shown that dynein is critical in the brain, and expressing UAS-RNAi Dhc64C in the larval nervous system led to complete pupal lethality. Therefore, as a positive control to verify the specific and activity of UAS-RNAi Dhc64C for dynein, I drove expression of UAS-RNAi Dhc64C in larval brain neurons using elav-Gal4 (neuron-specific). My results confirmed that knockdown of dynein in larval brain neurons resulted in complete pupal lethality, validating the activity of the UAS-RNAi Dhc64C line.

As an alternative approach to isolate the contribution of dynein to FA uptake/LD formation and LD polar localization, I wondered if there was a threshold for FA uptake in dynein RNAi larvae. If I could overcome the lipid uptake defect by feeding UAS-RNAi Dhc64C larvae a high fat diet, I might be able to test the contribution of dynein to downstream steps in lipid translocation. Feeding UAS-RNAi Dhc64C x MexII-Gal4 larvae a high fat diet resulted in the re-emergence of LDs in the midgut, but dispersed throughout the cell rather than localized to the apical PM (Fig.25 C-F). This result is consistent with a role for dynein in mediating the asymmetric distribution of LDs at the supranuclear region of the cell.

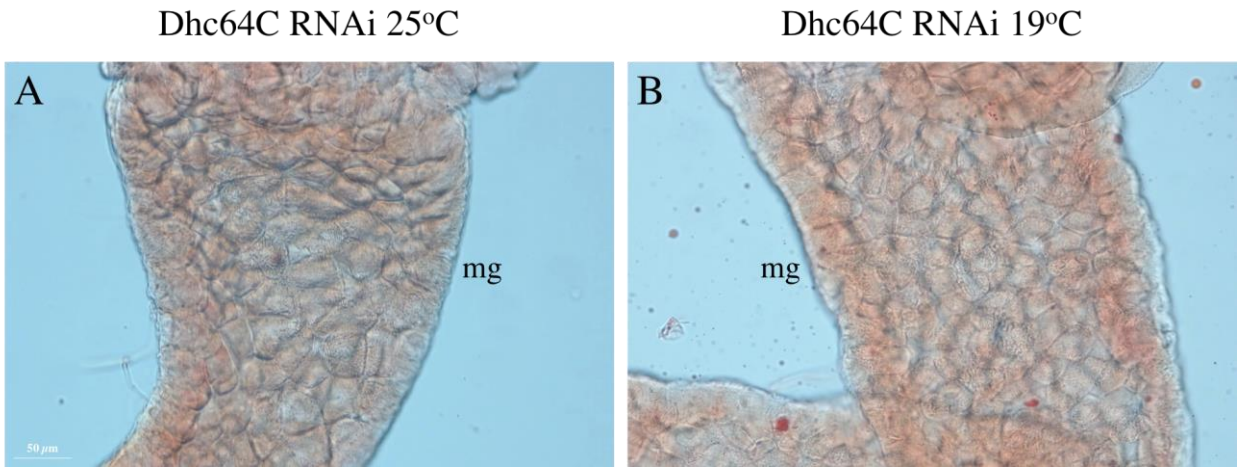

Figure 24. Dhc RNAi expression is lowered by growth at a lower temperature, 19°C. Larvae expressing UAS-RNAi Dhc64C driven by a midgut specific MexII-Gal4 fed standard yeast paste diet were stained with Oil Red O and analyzed by DIC microscopy. There was very little to no staining in larvae at 25°C (A) and 19°C (B). The change in temperature was apparently not sufficient to trigger a decrease in the Dhc RNAi activity in the midgut. Scale bar is 50 μm; mg= midgut, gc=gastric caecae

In addition, feeding on a high fat diet ameliorated several of the developmental defects associated with the dynein knockdown (Fig.26). Whereas larval rate to pupation for control lines showed a one-day delay when fed a high fat diet compared to standard yeast paste, UAS-RNAi Dhc64C larvae fed a high fat diet showed a two-day shift towards the wild-type rate to pupation (Fig.26 A). In observing the larval rate to eclosion, UAS-RNAi Dhc64C larvae fed a high fat diet exhibited a shift towards the wild-type developmental rate (peaking at Day 14-16) along with a two-fold increase in viability (relative to feeding on standard yeast paste) (Fig.26 B). These significant shifts in the rate of development demonstrate that a high fat diet can partially rescue severe developmental deficiencies associated with a compromised function of dynein. Surprisingly, despite the loss of polarized distribution of LDs in the enterocytes, many dynein knockdown flies fed a high fat diet survived to adulthood. This may suggest that some functional dynein is still able to properly localize with a subpopulation of LD to support proper development. Alternatively, since LDs are dispersed throughout the cell in the dynein knockdown, a small population of LDs may "randomly" be found in the supranuclear region to support a viable rate of lipoprotein loading.

These results suggest a role for the MT minus-end binding protein, dynein, in the collective process of LD accumulation and localization to the supranuclear subregion of the cell. These results help support a functional link between the MT cytoskeleton and LD formation and accumulation in enterocytes. Moreover, these results establish that dynein's role in lipid translocation is developmentally and biologically relevant. More work remains to be done to fully understand the specific contribution of dynein to the individual steps of LD formation, and maintenance of LD localization.

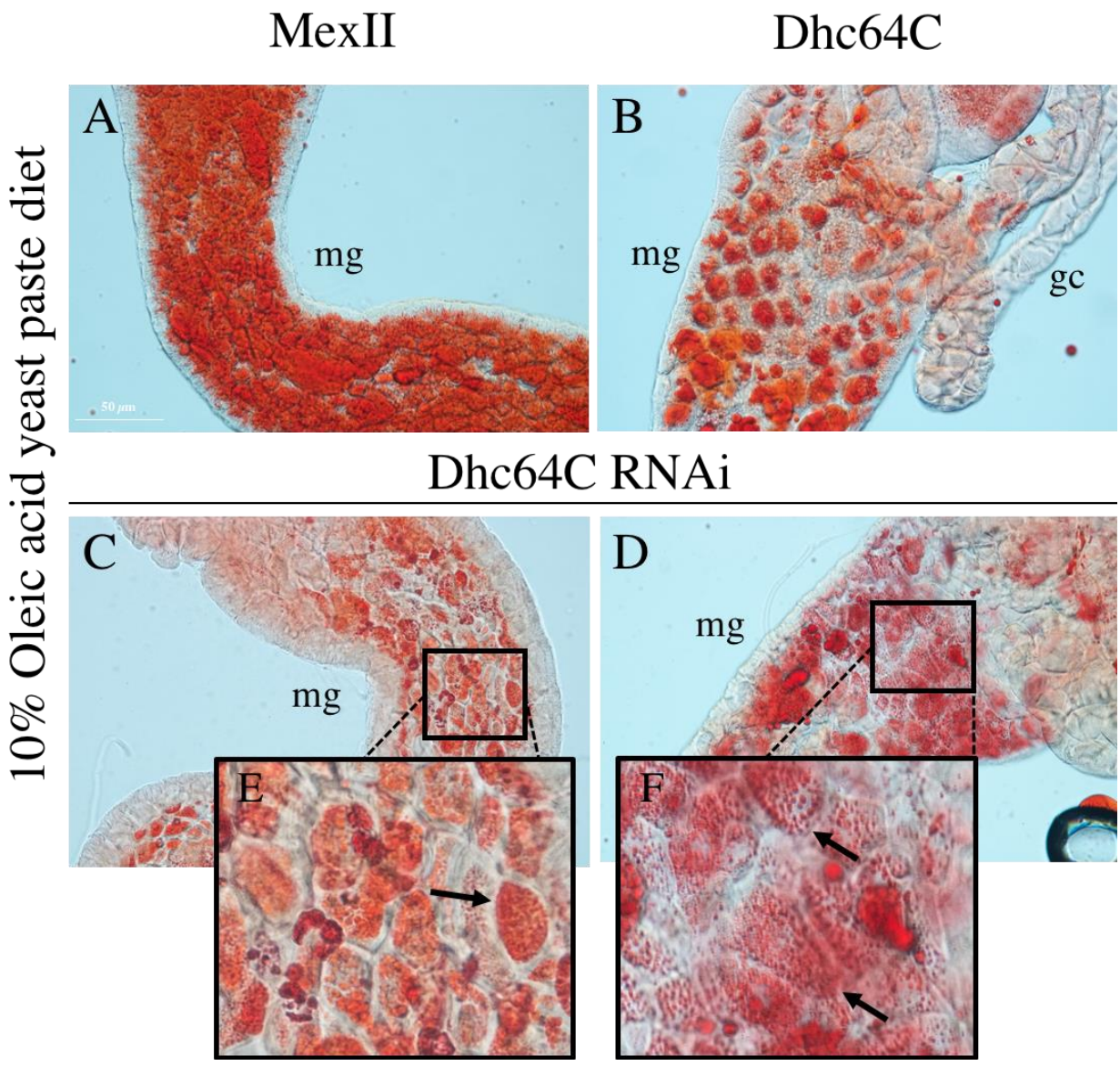

Figure 25. UAS-RNAi Dhc64C larvae fed a high fat diet resulted in the re-emergence of LDs in the midgut. Larvae fed a high fat diet of 10% oleic acid in yeast paste at room temperature were dissected and stained with Oil Red O and analyzed using DIC microscopy. Stained midgut tissues of controls, MexII and Dhc64C, show a dramatic accumulation of LDs (A & B). UAS-RNAi Dhc64C larvae fed a high fat diet showed an elevated level of LDs (C & D) and a dispersed distribution of LDs throughout the cell (arrows). Scale bar is 50 μm; mg=midgut, gc=gastric caecae

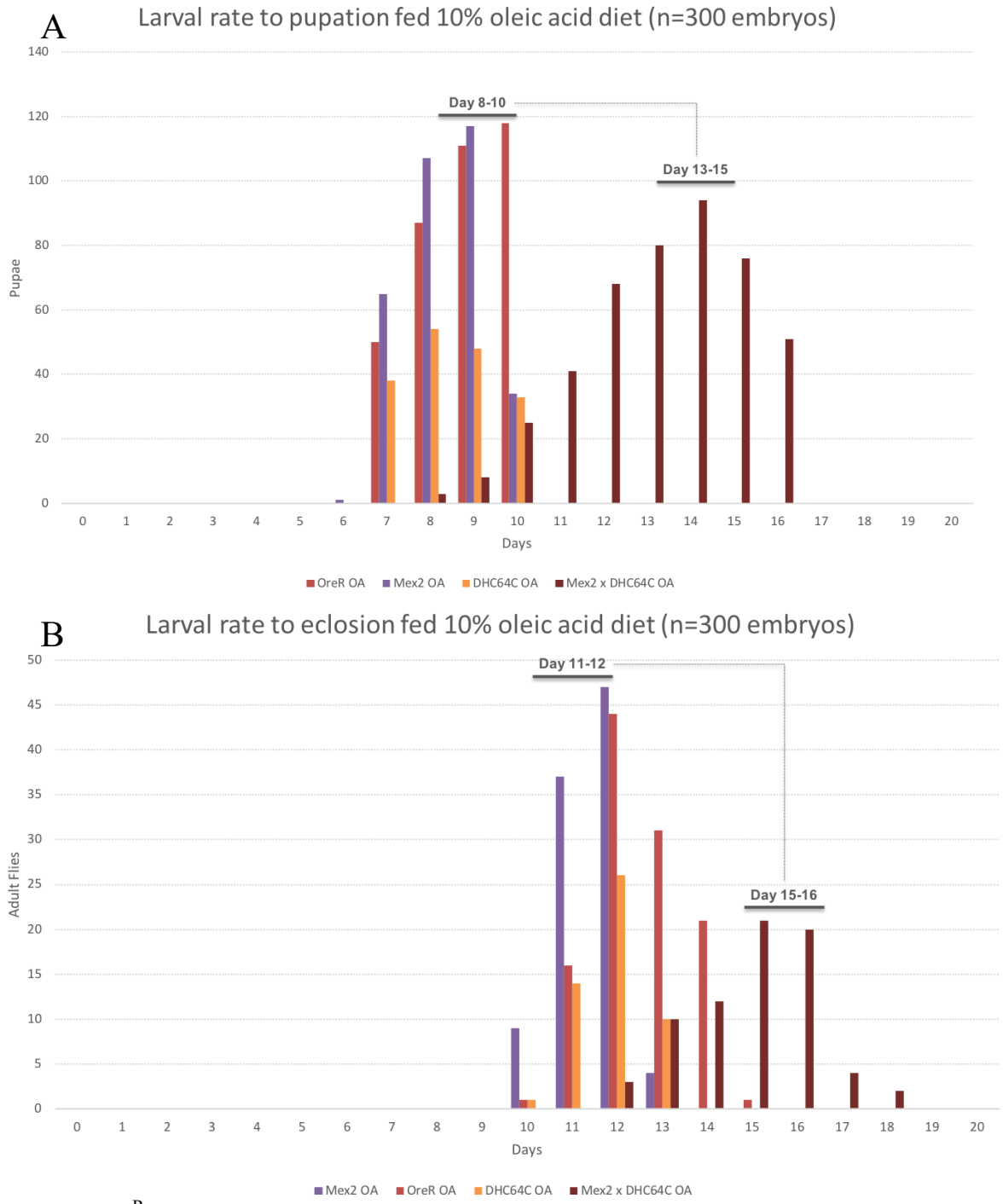

Figure 26. Ore^{R}, MexII, Dhc64C and UAS-RNAi Dhc x MexII-Gal4 larvae fed a high fat diet consisting of 10% oleic acid (OA) yeast paste diet, exposed a correlation between the presence of LDs and normal rate of development. Despite the toxicity of OA diet on controls as seem by a delay in their rate to pupation, mutant larvae showed a faster developmental rate by almost 2 days.

A) Larval rate to pupation, controls peaked day 8-10 and mutant larvae peaked day 13-15,
B) Larval rate to eclosion, controls peaked day 11-12 and mutant larvae peaked day 15-16.

III.4.2 Effect of kinesin knock down

Previous research has shown that LTP is found in endocytic vesicles, partially colocalizing with Rab5 in wing imaginal discs(Rodríguez-Vázquez et al. 2015). Earlier in this work, I presented data showing that Rab 7, a marker for late endosomes, colocalizes with the supranuclear population of LDs in the enterocyte (Fig. 14 D). Given that Kinesins are known to function as plus-end MT motor proteins, and that the export of lipids via LTP occurs at the basal end of the enterocyte (plus-end of MTs), I wondered if kinesin might be involved in transporting loaded LTP towards the basal end of the cell for the mobilization of lipids out of the cell. I therefore hypothesized that knockdown of kinesin would result in abnormal LD accumulation at the supranuclear region. If LTP loading occurs at the supranuclear region of the cell, LTP will need to move basally to facilitate the transfer of fat to Lpp (docked outside the basal end of the enterocyte); without kinesin present, LTP would be unable to mobilize fat from enterocytes resulting in an accumulation of LDs throughout the cell. To determine the role of kinesin in LD distribution and trafficking, I drove midgut specific knockdown of kinesin from the kinesin-1 family, UAS-RNAi Khc73 (highly expressed in the midgut), and analyzed LD distribution using Oil Red O and DIC microscopy. When control and UAS-RNAi Khc73 larvae fed a standard yeast paste diet were analyzed using DIC, I observed that knockdown of kinesin resulted in an abnormal LD accumulation throughout the midgut (Fig.27 C), similar to the phenotype seen in Ore^{R} control larvae fed a high fat diet (Fig.12 B). As expected, knockdown of kinesin showed no defects in lipid uptake or LD formation; instead, UAS-RNAi Khc73 larvae showed complete loss of supranuclear LD localization and exhibited dramatic accumulation of LDs throughout the cell. Based on our current understanding of lipid translocation, the best

interpretation of this result may be that impaired fat mobilization out of the enterocyte is responsible for the observed accumulation of LDs. However, in the absence of direct evidence, I cannot rule out that the kinesin knockdown may be exhibiting accelerated FA uptake/ LD formation. Preliminary rate of development results showed no significant delay or lethality in the rate of pupation or eclosion, suggesting that the mobilization of lipid out of enterocytes is sufficient for normal development to occur. Thus, kinesin appears be important for balancing LD unloading with FA uptake/ LD formation, but may not be absolutely be required for lipid export.

Together the results of this section help support key roles for MTs and the MT motor proteins dynein and kinesin in mediating several of the key steps in intracellular lipid translocation. In the Discussion, we will outline the key results from Section II and III that support each step in our model for intracellular lipid translocation, and highlight unknowns and future experiments that will facilitate a more complete understanding of eukaryotic lipid translocation.

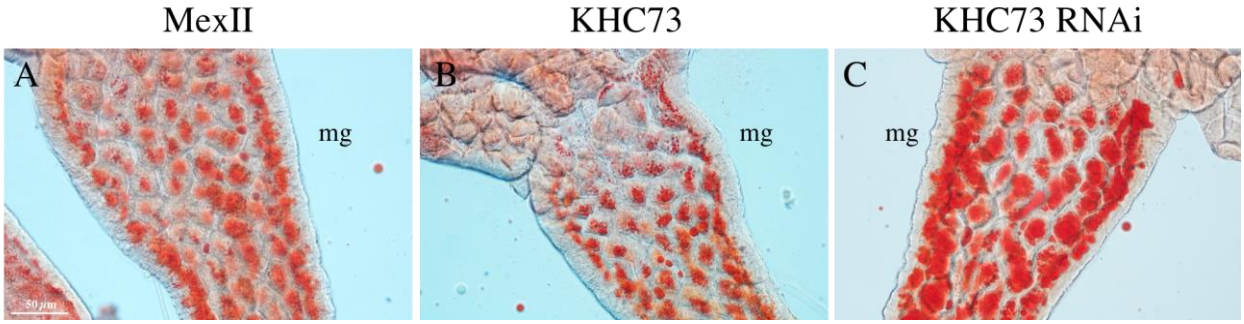

Figure 27. UAS-RNAi Khc73 resulted in an abnormal LD accumulation throughout the cell. Shown are MexII-Gal4 larvae (control) and larvae expressing UAS-RNAi Khc73 driven by a midgut specific MexII-Gal4 fed standard yeast paste diet were stained with Oil Red O and analyzed by DIC microscopy. A) Control larvae showed basal levels of LD accumulation in the midgut, consistent with previous results. B) UAS-RNAi Khc73 larvae produced an abnormally high level of LD accumulation. Scale bar is 50 μm; mg= midgut, gc=gastric caecae

CHAPTER IV. Discussion

IV.1 Summary of results

The overall goal of this thesis was to characterize the steps in dietary lipid uptake and lipid translocation, particularly as it relates to LD dynamics in enterocytes of the *Drosophila melanogaster*. Via targeted knockdowns, visualization techniques (Oil Red O assay and fluorescent proteins) and dietary perturbations (starvation, low fat diet, high fat diet) my findings have advanced our understanding of lipid uptake and translocation.

Broadly, this work has helped to fill in the gaps in our understanding of lipid translocation in two major ways. First, I was the first to establish that there are spatial parameters governing lipid uptake, LD formation and LD maintenance in the enterocyte. My results suggest that FA uptake and LD formation occur in a coupled process in the supranuclear region of the cell. My work was able to effectively parse FA uptake/LD formation from LD unloading/mobilization out of the enterocyte, revealing a possible difference between the rate of FA entry into the cell and the rate of FA mobilization out of the cell. A second major achievement of the experiments described here was identifying roles for the MT cytoskeleton and MT motor proteins in lipid translocation and LD dynamics. Not only did MTs colocalize with the polarized population of LDs in enterocytes, but changes in MT abundance affected LD localization. Furthermore, MT motor proteins dynein and kinesin were shown to be important for initial FA uptake/LD formation and lipid mobilization out of the cell, respectively.

Beyond considerations specific to LD dynamics, this work has some important higher-level implications for our understanding of cell polarity and the role of MTs in enterocytes. My data suggests that MTs may be important for apical-basal trafficking and

localization of lipid cargo in enterocytes. In addition, I report the first *in vivo* analysis of the details of MT distribution, polarity and dynamics in Drosophila enterocytes. I will discuss these results and the following conclusions in the context of my 4-step model (Section IV.2) for lipid translocation in enterocytes.

IV.2 Model overview and key contributions of my results

IV.2.1 Step 1: Fatty acid uptake and initial lipid droplet formation

In Drosophila, dietary fat processing begins when TGs are hydrolyzed by a lipase in the gut lumen to produce FAs. Little is known about the relationship between FA uptake and LD synthesis in Drosophila. In mammals, following FA uptake, it is speculated that FAs transport to the ER where they are esterified into TGs. Newly synthesized TGs are stored as nascent LDs in the ER, and also contribute to a population of cytoplasmic LDs (Yen et al. 2008; Sieber and Thummel 2012; Wilfling et al. 2013). FAs from both luminal and cytoplasmic LD populations are loaded onto lipoproteins for mobilization of fat out of the cell (Demignot, Beilstein, and Morel 2014; Abumrad and Davidson 2012). The relationship between FA uptake and cytoplasmic LD formation in enterocytes is still being explored in both mammals and insects. However, my results with Drosophila provide clues as to how FA uptake is related to LD formation (Fig.28 A).

Three key findings of my work advance our understanding of FA uptake and LD formation. First, the tight co-localization between LSD2 and LDs at the apical membrane of the enterocyte is consistent with previous work implicating LSD2 in FA uptake and LD formation in the fat body. A finding with respect to LSD2 was its apparent preference for association with smaller LDs located nearer the apical PM, while it did not show evidence of association with larger LDs (Diaconeasa Thesis 2014). Second, the more clearly defined sub-apical region (supranuclear) of the enterocyte is the site of new LD synthesis. The obvious and reproducible polar localization of LDs, demonstrated most clearly by starvation and re-feeding experiments, establish that the sub-apical region of the cell is the site of LD formation. Third, FA uptake and LD formation depends on the minus-end MT

motor protein dynein. Dynein had previously been implicated in LD trafficking in Drosophila embroys by Welte and colleagues (Welte 2015b). My work here suggests that dynein may be important for FA uptake and LD formation in the enterocyte, and that the absence of dynein can lead to severe developmental delays and increased lethality. These data add to our working model of FA uptake and LD formation: LSD2, possibly in a dynein-dependent fashion, mediates contact at the apical PM to align and couple the lipid uptake machinery directly to LD formation within the enterocyte.

An intriguing area for further exploration is the relationship between Dynein and LSD2. To investigate a possible role for dynein in maintaining proper LSD2 localization at the apical PM during lipid uptake/LD formation, one could test the effect of knocking down dynein on the localization of LSD2. This would require expression of both the dynein knockdown (UAS-RNAi Dhc64C) and LSD2-GFP in the midgut using the MexII-Gal4 driver. This experiment would ideally be facilitated with a temperature-sensitive UAS-RNAi Dhc64C larvae (Fig.24). This would allow FA uptake/LD formation and normal LSD2 localization to occur at low temperatures (low activity of UAS-RNAi Dhc64C). LSD2-GFP localization could then be followed using fluorescence microscopy during the shift to high temperatures (higher activity of UAS-RNAi Dhc64C). As an alternative to bypass the FA uptake/LD formation defect of the dynein knockdown, this experiment could be conducted with high-fat feeding. Although LD polar localization would be disrupted in the absence of dynein, analysis of the distribution of LSD2-GFP in relation to the pool of nascent LDs just formed at the supranuclear region should still be feasible. If dynein is responsible for localizing the lipid uptake machinery involving LSD2 at the apical PM, knocking down dynein should lead to disrupted distribution of LSD-GFP. If LSD2-GFP localization is

unaffected in the dynein knockdown, that would suggest that dynein mediates its effects on FA uptake/LD formation through as-yet-unidentified factors at the apical PM.

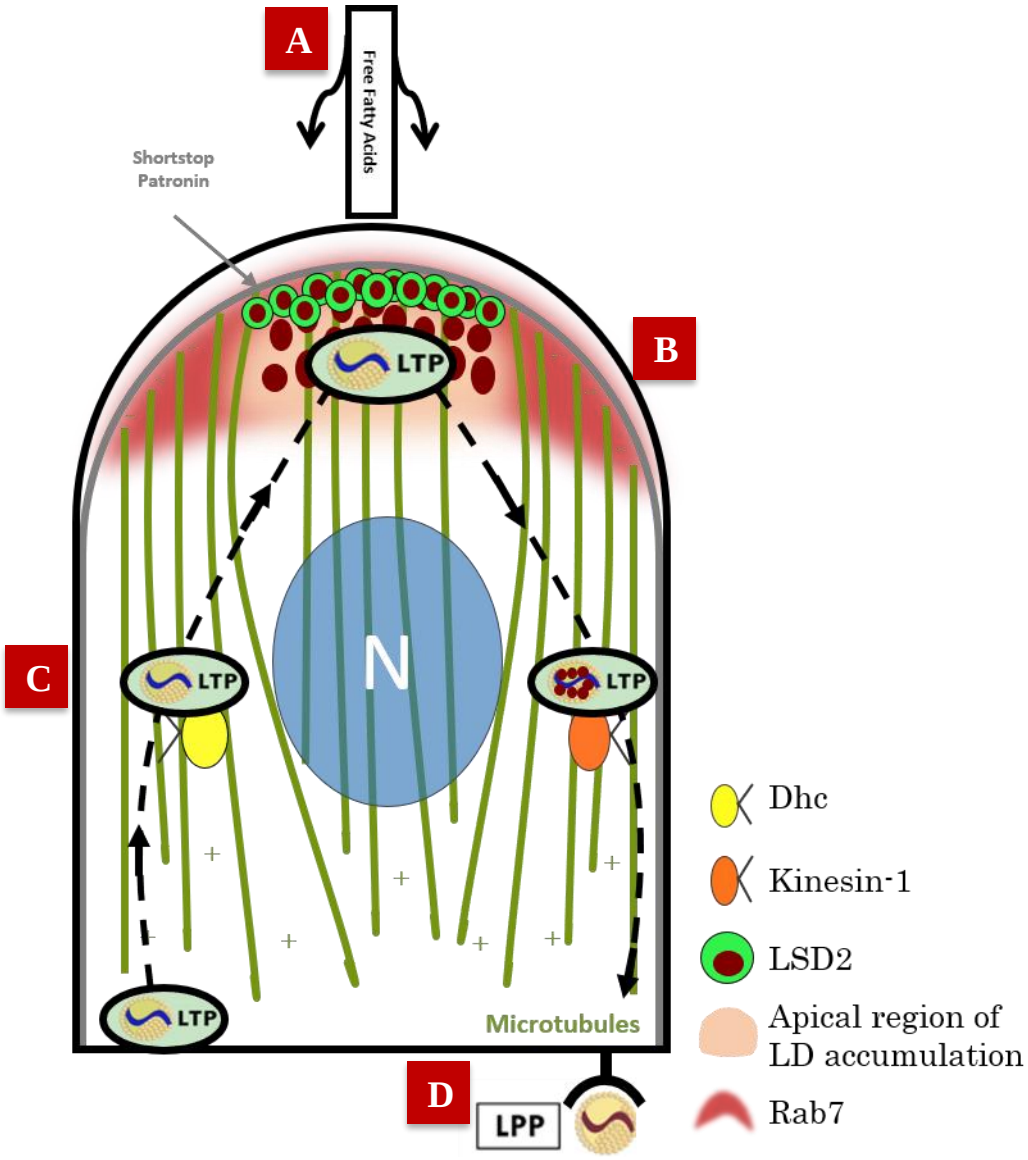

Figure 28. Current model of the ApoB lipoprotein-mediated mechanisms involved in dietary fat mobilization from the gut. A) Fatty acid uptake B) LD formation and maintenance at the supranuclear domain of the cell, C) LD mobilization is facilitated via LTP. Unloaded LTP particle in an unknwon endomembrane space (geen oval) enters through the basal end of the enterocyte and traffics towards the site of LDs. As a loaded LT P particle, it shuttles down to the basal end wherer D) LTP lipid transfer to LPP.

IV.2.2 Step 2: Lipid droplet maintenance at the apical domain of the cell

My work here is the first to describe localization of LDs to the supranuclear domain of the enterocyte. The results discussed above (Step 1) show that the sub-apical region is the site of initial LD formation; the observations here (1) establish that LDs are maintained in the sub-apical tip of the enterocyte, and (2) identify players that control the apical polarization of LDs. The conclusion that the sub-apical tip of the enterocyte is the site at which LDs are actively maintained followed from observations on the effects of dietary perturbations on LD localization. After starvation (which resulted in depletion of LDs) or high-fat feeding (which resulted in an increase in LDs that are distributed throughout the cell), restoration of normal dietary lipid levels was accompanied by LDs re-localizing to the sub-apical tip. The return of LDs to the this specific region of the enterocyte during steady-state implied an active mechanism for controlling LD localization (Fig.28 B).

The involvement of the MT cytoskeleton and dynein in maintaining the apical localization of LDs was consistent with previous results showing the involvement of MTs in LD trafficking in Drosophila embryos (Welte 2015b). Overexpression of α-tubulin resulted in a much tighter LD localization at the sub-apical tip, while destabilization of MTs with colchicine disrupted LD localization. However, since perturbations to the MT cytoskeleton had drastic effects on cell morphology and integrity, it was difficult from these experiments alone to discern a direct role for MTs in controlling LD localization. Nonetheless, the elongation in cell shape resulting from α-tubulin overexpression permitted an additional level of spatial resolution, which revealed LDs to accumulate near the minus-end of MTs (intimately associated with the apical PM) rather than just appearing to localize to the supranuclear region of the cell.

Testing dynein's role in maintaining the apical localization of LDs was more challenging that initially expected, since: (1) knockdown of dynein may have impaired FA uptake, and (2) temperature shift did not change expression. However, by feeding larvae a high-fat diet, I was able to bypass the defect of the dynein knockdown in FA uptake/ initial LD formation, and observed an accumulation of LDs within the cell. The complete loss of LD apical polarization observed under these conditions argues for a role for dynein in controlling LD localization. However, the loss of apical LD localization did not have a strong effect on the rate of development or lethality. This suggests either that LD localization to the sub-apical tip is not a strict requirement for lipid mobilization out of the enterocyte, or that a sufficient number of LDs localized to the sub-apical tip in the mutant to support normal development.

As described earlier, in analyzing the co-localization of LSD2 and LDs, I noticed that the intensity of LSD2-GFP signal appeared to be inversely proportional to LD size. While smaller LDs tended to be located nearer the sub-apical tip and showed a high degree of spatial overlap with LSD2, larger LDs showed little co-localization with LSD2 and tended to be located further from the sub-apical region. This was further supported by EM results, which captured the same inverse relationship between LD size and proximity to the apical PM. While far from conclusive, these observations hint at an intriguing size-dependent aspect that may be involved in the dynamics of LD localization, in which LD size drives dissociation from the apical PM simultaneous with loss of interaction with LSD2. This mechanism could also inform our understanding of how feeding on a high-fat diet leads to LD accumulation throughout the cell.

To further understand the possible size-driven dissociation of LDs from the apical PM, it could be informative to develop a more quantitative perspective of the relationship between LD size and distance from the apical PM. One could measure LD diameter and distance from the apical PM, in both Oil Red O stained samples and by EM, determine if there is a direct relationship between LD size and distance from the PM. This analysis would also reveal if there is an LD size threshold beyond which association with the PM (via LSD2) is no longer observed. To directly test the hypothesis that the growth of apical LDs causes dissociation from the PM, leading to the population of larger LDs located deeper in the cell's interior, a pulse-chase analysis using radio-labeled lipids could be undertaken. If labeled lipids were initially observed in smaller LDs near the apical PM and were later observed in larger LDs located further from the apical PM, this would suggest that smaller LDs do in fact *become* the larger PM-dissociated LDs, consistent with the LD-size dissociation hypothesis described above. In addition, following labeled lipids throughout lipid translocation could inform our understanding of subsequent steps involved in lipid mobilization out of the cell.

IV.2.3 Step 3: Lipid droplet mobilization (lipid droplet unloading and LTP loading)

Mobilization of lipid out the cell requires unloading of these LDs and loading of LTP with the resulting TGs, and subsequent trafficking to the basal end of the cell for export. I hypothesize that LTP must therefore access LDs at their site of accumulation, in the sub-apical region of the enterocyte. Several lines of indirect evidence may support the hypothesis that LD unloading and LTP loading occur at the sub-apical region of the cell (Fig.28 C). First, under conditions of normal lipid availability, LDs are observed *only* at the sub-apical region; therefore, the unloading of LDs is presumed to also occur at the sub-apical region. Given the energetically unfavorable prospect of unbound lipids moving to the basal end of the enterocyte, LTP loading likely occurs concomitant with LD unloading at the sub-apical tip of the cell. Even under conditions of abnormally tight apical localization of LDs (due to α-tubulin overexpression), lipid mobilization out of the cell was able to proceed, providing further support that LD unloading and LTP loading may occur at the sub-apical tip the cell. The basis for the slight reduction in the rate of LD depletion in the tubulin overexpression line is unclear at this point. I speculate that the tight localization of LDs or the elevated levels of tubulin throughout the cell could be interfering with the physical access of LTP to the sub-apical tip.

Additional insights into the spatial parameters guiding lipid mobilization were learned from the experiments with high-fat feeding followed by starvation. High-fat feeding resulted in a loss of localization of LDs to the sub-apical tip, and LDs were distributed throughout the enterocyte. Subsequent starvation resulted in depletion of LDs non-randomly, suggesting that LDs are broken down in a concerted, spatially-regulated manner. Additionally, the last site at which LDs were observed to accumulate was at the sub-apical

region of the cell. Two possible scenarios could account for LD unloading under these conditions: (1) a process in which LD unloading/LTP loading proceeds from the basal end towards the apical end of the cell, or (2) a model in which LDs are broken down at the apical end of the cell, and basal LDs are continually pushed towards the apical end (possibly via the action of MTs and MT-associated proteins). Distinguishing between these two scenarios is difficult, but given my other observations presented above, I favor the second model. Future experiments with radio-labeled lipids could shed light on this open question. A pulse with labeled lipids near the end of high-fat feeding should in principle lead to a population of labeled LDs nearest the apical end of the cell. Following the labeled LDs at discrete time points during starvation could help distinguish between the bottom-up model and the top-down model (favored). If the labeled LDs were observed to persist in the apical region throughout the duration of starvation, this would suggest that LD unloading occurs from the bottom-up. However, if labeled LDs were depleted early during starvation, this would imply that LD unloading occurs at the apical end of the cell, consistent with my other results.

The observations discussed above provide good evidence that LD unloading occurs at the sub-apical region, and indirectly suggest that LTP loading may occur at the same site. While direct evidence of co-localization between LTP and LDs is still lacking, several of my results hint at parameters that may guide the localization of LTP in the cell. First, I detected Rab7, a marker of late endosomes, at the apical end of the enterocyte. The relevance of lipid loading into LTP is only weakly suggestive. Limited evidence indicates that LTP is endocytosed at the basal end of the enterocyte (after trafficking from its site of synthesis in the fat body) and resides within an endomembrane compartment in the enterocyte. Thus,

the detection of Rab7 endosomes at the sub-apical region of the cell suggests a possible mechanism by which endosome-bound LTP might access LDs in the sub-apical region. Second, I observed that knockdown of kinesin led to a dramatic accumulation of LDs within the enterocyte, similar to the effect of high-fat feeding. Due to the high volume of LDs in the enterocyte, the polarized distribution of LDs is no longer observed. The function of kinesin in trafficking intracellular cargo towards the plus-ends of the cell is well described (Karcher, Deacon, and Gelfand 2002; Mallik and Gross 2004; Rodionov, Gyoeva, and Gelfand 1991). Thus, it is intriguing to speculate that accumulation of LDs in the kinesin knockdown is due to defective lipid mobilization out of the cell, caused by an inability to traffic loaded LTP from the site of LD unloading at the sub-apical tip towards the basal end for lipid transfer to Lpp.

IV.2.4 Step 4: LTP lipid transfer to Lpp

Observations made by others and myself in the larval midgut *in vivo* have clearly demonstrated the dynamic accumulation and depletion of LDs in the enterocyte. The last step in intracellular lipid translocation, as outlined in this work, is the transfer of lipid from LTP to Lpp. Upon trafficking of loaded LTP to the basal end of the cell (possibly mediated by kinesin), lipid is transferred to the extracellular shuttle lipoprotein Lpp (docked outside of the cell in the hemolymph) (Fig.28 D). However, the dominant form of lipid in LTP is TG, but the main form of lipid that is ultimately mobilized out of the enterocyte and into circulation is DG. It has been shown that LTP catalyzes the transfer of DGs to Lpp, but the reverse reaction has not been observed. Limited evidence suggests that the transfer of lipid from LTP to Lpp is directly tied to the conversion of TGs to DGs. In mammals, lipids are maintained as TGs throughout translocation. Despite extensive research, the precise mechanism by which TGs are converted to DGs during lipid mobilization remain unknown.

IV.2.5 Overview of proposed model for lipid translocation in enterocytes

Lipid translocation begins when FAs are taken up at the apical end of the cell, in a process that we believe is coupled to the synthesis of LDs on the cytoplasmic side of the membrane. While a dedicated FA transporter has yet to be identified, LSD2 and dynein appear to play important roles in uptake and LD formation. There is some evidence to suggest that LSD2 mediates contact between the PM and the nascent LD, while the precise role for dynein during uptake is less clear. FA uptake and LD formation involves the first of two re-esterification events during lipid translocation (FAs are re-esterified into TGs), but the precise mechanism is unknown.

Upon LD formation, LDs are maintained at the apical membrane of the cell in a manner that is dependent upon dynein. Evidence suggests that LD localization at the apical pole of the cell is maintained at least in part by association with the MT minus-end, likely through dynein, and not only by association with the apical PM. Spectrin is required for association of cortical LDs with the PM (Diaconeasa et al. 2013), while spectrin-associated proteins shortstop and patronin play an indirect role in LD localization by polarizing MTs along the apical-basal axis of the cell (Khanal et al. 2016). There is some limited evidence to suggest that the localization of LDs at the apical membrane is highly dynamic, as LDs appear to dissociate from the PM in a size-dependent manner and move further from the PM.

There is some circumstantial evidence to suggest that the sub-apical region of the cell is both the site of LD unloading and the site of LTP loading. Since LDs are never observed at a location other than the supranuclear region (under normal conditions), it is assumed that the sub-apical region is also the site of LD unloading. And since it is

energetically unfavorable for lipids to exist as free TGs in the polar cytoplasm, it is thought that LD unloading happens concomitant with LTP loading. Thus, LTP loading with TGs is believed to occur at the apical end of the cell.

In a parallel pathway, LTP is synthesized in the Drosophila fat body and is trafficked to the enterocyte, where it is speculated that it is internalized via endocytosis (Palm et al. 2012). Within the cell, I speculate that endosomal unloaded-LTP is trafficked to the apical end of the cell (possibly in a Rab-dependent fashion) for LTP loading. I further speculate that LTP may acquire TGs from LDs by passing alongside and physically coming into contact with LDs. Once loaded with TGs, it is possible that a loaded LTP is trafficked to the basal end of the cell in a kinesin-dependent fashion, to deliver its TGs to Lpp.

Lpp, also synthesized in the fat body, remains in the hemolymph, outside of the basal membrane. Lipid transfer is uni-directional (from LTP to Lpp), and involves a conversion from TG to DG. Once loaded with lipid in the form of DG, loaded-Lpp shuttles its cargo to downstream tissues, including the fat body, imaginal discs, and wings.

IV.3 Future experimental directions

IV.3.1 Technical limitations of current approach

A major strength of this work was being able to perturb individual steps in lipid translocation and study them in isolation. This permitted me to identify specific players that are important for each step. However, a limitation in my analyses was an inability to pull apart each of these larger steps (uptake, LD maintenance, and LD mobilization) into their component sub-steps. For example, the experimental techniques used in this research did not allow me to distinguish between FA uptake and LD formation. Similarly, I was unable to separate lipid mobilization into discrete steps: LD unloading and LTP loading (assumed to take place simultaneously), LTP trafficking to the basal end of the enterocyte, and lipid transfer to LPP across the basal membrane.

This is in part a technical limitation, since many of my analyses were dependent on detecting the physical LD (by staining and microscopy, EM, etc.). In my current analyses, the appearance of the LD is indicative of lipid uptake, and the disappearance of the LD represents all the steps of mobilization. However, I have begun piloting experiments with finer analyses such as mass spectrometry-based lipidomics, which may permit detection of lipids in free-, protein-bound, and LD forms as they are trafficked intracellularly.

For example, current analyses do not permit a clear distinction between FA uptake and LD formation. Therefore, it is possible that knocking down dynein impairs only LD formation and not FA uptake (i.e., lipids could be accumulating intracellularly but are undetectable by my current techniques). A lipidomics approach would allow me to analyze whole lipid levels in mutant and WT enterocytes, which might be able to pinpoint the defect in lipid translocation associated with the dynein knockdown.

IV.3.2 Potential future approach to identifying novel players in lipid translocation

Despite the advances in our understanding of lipid translocation highlighted by this work and the work of many others, gaps still remain. To discover novel components contributing to this lipoprotein pathway, an EMS mutagenesis screen could be performed to identify mutants with attenuated fat mobilization. A crossing scheme for a X-chromosomal would enable a screen for X-linked lethal mutations.

Additionally, it has been shown that knocking down LTP or Lpp in larvae leads to a striking accumulation of LDs in the gut (Palm et al. 2012). This suggests that disruption of any of the individual genes involved in lipid mobilization in the normal lipoprotein pathway could result in LD accumulation, a striking phenotype which could be screened using a light or DIC microscope. Larvae with the mutations of interest would be expected to produce a phenotype similar to OreR larvae fed a high fat diet, showing massive accumulation and dispersed patterning of LDs in the midgut. Given what is already known about the lipoprotein pathway, potential classes of candidate genes discovered in the above screen could include: (1) lipases, (2) fat transport proteins, (3) lipophorin receptors and (4) LD synthesis/maturation enzymes.

CHAPTER V. MATERIALS AND METHODS

V. 1 Fly stocks:

The wild-type fly line Ore^R was obtained from the Bloomington Stock Center. The UAS-LSD2-GFP was previously described (Diaconeasa Thesis 2014). The midgut *MexII*-Gal4 driver, UAS-Nod-GFP, UAS-*Khc73*-RNAi and were obtained from the Bloomington Stock Center (Bloomington, IL). The dsRNA lines for Dhc64C, UAS-*Jupiter*-GFP, UAS- α-*Tubulin*-GFP, UAS-*Maple*-GFP and UAS-*Patronin*-GFP were a gift from Dr. Vladimir Gelfand at Northwestern University. UAS-Rab5-GFP, UAS-Rab11-GFP and UAS-Rab7-RFP were also from Bloomington Stock Center.

V. 2 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 captured using a spinning disk confocal microscope (Olympus, Center Valley, PA) with a 30× oil immersion objective used Andor iQ3 software. Images using Nikon TU-2000 inverted microscope equipped with a Perfect Focus system (Nikon) and Coolsnap CCD camera (Roper Scientific) driven by Metamorph software. A 100-W halogen light source was used for fluorescence excitation to minimize photobleaching and phototoxicity. Montages were assembled using Photoshop CS 4.0 (Adobe Systems, San Jose, CA). The Z axis reconstruction of midgut was produced from a Z series of images using NIS Elements Viewer. Lipid droplets were analyzed by differential interference contrast (DIC) using a

Zeiss Axioskop microscope. Images were captured using an Axiocam camera and AxioVision software.

V. 3 Oil Red O staining

Larval midgut tissues were dissected and fixed in 4% paraformaldehyde for 10 min. Specimens were then rinsed twice with *Drosophila* Ringer solution, incubated for 25 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 Ringer solution. Stained material was then transferred to a slide with Vectashield mounting medium.

V.4 Electron Microscopy

Drosophila third instar larvae were prepared in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at room temperature. After 10 minutes specimens were put on ice for an additional 50 minutes. Following a brief rinse with buffer, the tissues were postfixed with 2% OsO_4 for 2 hr at 4°, followed by staining with uranyl acetate overnight at 4°. The tissues were dehydrated in ethanol and embedded in Dow epoxy resin 332/732 plastic, using propylene oxide as a transitional fluid. Thin sections were stained with 1% uranyl acetate and lead citrate and examined in a Technai F30 electron microscope at 300 kV.

V. 5 Nile Red staining

For staining intracellular lipid droplets, larval midguts 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.

V.6 Colchicine feeding

Ore^R flies were kept in chambers at 22^oC with apple juice agar plates and fed standard yeast paste. Late second to early third instar larvae were collected and placed onto a new apple juice agar plate with a solution of the drug mixed with yeast paste. The drug was dissolved in water to make the following working solutions of the drug: 1%, 0.5% and 0.25%. Approximately 0.30-0.40 grams of yeast paste was thoroughly mixed with 30-40ul of the working solutions of the drug to create a final concentration of 10%.

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