LIPIDS, MEMBRANES, AND NEUROGENESIS:

A DEVELOPMENTAL CONTINUUM

by

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ABSTRACT

Lipids, including fats, waxes and sterols, are a group of naturally occurring cellular molecules that perform a diverse array of vital functions within every organism. Broadly, lipids directly or indirectly participate in signaling, act as building blocks within membranes, and function as highly efficient sources of energy. In all these roles, lipids can heavily influence the chemical activities that sustain life—processes collectively known as metabolism. Lipids are composed of fatty acids and particularly, the lipids that constitute biological membranes are composed of long chain fatty acids. Long chain fatty acids, in order to be used for energy generation, membrane biogenesis, or signaling within the cell, need to be activated by esterification to Coenzyme-A. Long chain Acyl CoA Synthase enzymes catalyze this important esterification reaction and hence act as key metabolic regulators of fatty acid metabolism within the cell. The membrane fatty acid composition of a cell determines the protein composition of biological membranes and can thus define the developmental fate of a cell as well as its membrane bending and migratory abilities. In this regard, Acyl CoA Synthases can also act as key developmental regulators.

In the current study, we present evidence for the role of Long chain Acyl CoA Synthases (ACSL): Bgm and Dbb in Drosophila embryogenesis. Particularly, maternally deposited *bgm* transcript is required for the processes of

cellularization and neurogenesis during Drosophila embryonic development. Rab-5 tagged endocytic vesicles are critical sources of membrane components during cellularization, and Bgm is required for the proper subcellular targeting of these vesicles. Neurogenesis also requires maternal expression of *bgm,* and abnormal neurogenesis in *bgm* mutants appears to be related to the early defect in cellularization. In addition, we also demonstrate that *bgm* and *dbb* are duplicated genes with partially diverged developmental expression patterns and are transcriptionally regulated by dorsoventral patterning genes. Lastly, we provide evidence for behavioral abnormalities in *bgm* and *dbb* mutant flies, thus making them attractive models of neurodegenerative disorders, which can be potentially used in large scale screens for diet and drug therapies.

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CHAPTER 1

INTRODUCTION

Lipids, including fats, waxes, and sterols, are a group of naturally occurring cellular molecules that perform a diverse array of vital functions within every organism. Broadly, lipids directly or indirectly participate in signaling, act as building blocks within membranes, and function as highly efficient sources of energy. In all these roles, lipids can influence the chemical activities that sustain life—processes collectively known as metabolism.

Within a cell, lipids can themselves act as signaling molecules or facilitate protein signaling. The signaling pathways requiring lipids binding as ligands to protein receptors comprise the lipid signaling pathways. Several different types of lipids can act as ligands. As examples, ceramides are signaling molecules required for regulation of insulin signaling, senescence, and cell death (Chasan et al., 1992). Ceramides are composed of a sphingosine (2-amino-4-octadecene-1,3-diol) head group and a fatty acid (a carboxylic **acid** with a long aliphatic tail); they bind to the ceramide receptor (Castro et al., 2014). Another lipid ligand derived from Sphingosine, Sphingosine-1-phosphate, is required for cell survival, cell migration, and inflammation (Mendelson et al., 2014). The Phosphotidyl inositol family of lipid second messengers includes inositol triphosphate (IP3) and

Diacylglycerol (DAG). These two ligands are required mainly for calcium signaling in muscles and for activation of Protein Kinase C (PKC) (Schink et al., 2013; Shisheva, 2013; Takasuga and Sasaki, 2013). Notably, all of these ligands are derived from lipids found in biological membranes (Goñi and Alonso, 1999). Several other lipid ligands, which are beyond the scope of discussion in this review, may or may not be derived from membrane lipids. Additionally, lipids can also influence signaling in an indirect manner. Several protein ligands are activated by the addition of acyl chains derived from fatty acids. As an example, the conserved developmental regulator Sonic hedgehog is activated by palmitoylation (Buglino and Resh, 2012; Ho and Scott, 2002). Most, although not all, Wnt ligands are also palmitoylated for their conserved and essential functions in development (Bartscherer and Boutros, 2008).

The second essential role of lipids in an animals' life history is as an essential source of energy. Most lipids are metabolized in either peroxisomes or mitochondria by the process of β–oxidation. When broken down, lipid constituent fatty acids generate significantly more energy than do carbohydrates or proteins (approximately 2.5 times more). The body also uses lipids to efficiently store unused energy in the form of lipid droplets.

The third essential role for lipids in all living organisms is as components of biological membranes—both cellular and subcellular. Indeed, the lipid composition of the membrane defines the protein components imparting unique protein signatures and unique properties to each type of membrane (Bogdanov et al., 2014; Poveda et al., 2014; Schmidt and Robinson, 2014). At the plasma

membrane, lipids form a selective barrier, which maintains a stable environment within the cell while also maintaining a pathway for communication with the external environment. As examples, plasma membrane receptors determine the signaling pathways that are active within a cell, and anionic transporters determine which energy sources will enter the cell (Bogdanov et al., 2014; Record et al., 2014). Lipids can also affect membrane bending properties (Stachowiak et al., 2013). At the cellular level, different membrane lipid compositions can affect changes in cell shape, allowing cells to adapt to the demands of developmental or homeostatic processes like morphogenesis, migration, and inflammation, to name a few (Bogdanov et al., 2014; Mendelson et al., 2014). At the subcellular level, certain lipids are essential for vesicle biogenesis, while others assist in trafficking between subcellular organelles (Martin, 2001; Osborne et al., 2001; Wurtzel et al., 2012). Thus, the lipid composition of membranes dictates a cell's energy state, developmental fate, and its ability to move and change shape in response to developmental or homeostatic demands. In this regard, some have suggested that the membrane roles of lipids cannot be overlooked in studies of any of their functions.

Membrane Lipids and Fatty Acids

Lipids are amphiphilic in nature, having both a hydrophilic end and a hydrophobic end. In forming a bilayer, with their polar ends pointing outwards and the nonpolar ends pointing inwards, membrane lipids assemble to form the plasma membrane that separates the interior of the cell from the exterior

environment. The hydrophobic (nonpolar) part of the lipid consists of long hydrocarbon chains that are derived from fatty acids (a carboxylic **acid** with a long aliphatic tail). There are four major classes of membrane lipids: phospholipids, sphingolipids, glycolipids, and cholesterol, defined on the basis of their polar head groups. Phospholipids are the predominant type in membranes; they are formed in a multistep reaction whereby dihydroxyacetone phosphate, a central metabolite in glycolysis, is reduced to *sn*-glycerol 3-phosphate using NADH (Pol et al., 2014). In the next step, long chain fatty acids are transferred from acyl-ACP to *sn*-glycerol 3-phosphate to form phosphatidic acid. Finally, depending on the lipid synthesized, a specific hydrophilic group is added to the phosphatidic acid (Pol et al., 2014). The hydrophobic chains of membrane lipids are derived from long or very long chain fatty acids.

These fatty acids have 14 or more carbons in their acyl chains and help membrane lipids span the membrane, either in pairs to form the lipid bilayer, or as singletons, spanning the membrane in its entirety to facilitate curving (Stachowiak et al., 2013). Importantly, the different fatty acids impart specific membrane bending or protein interaction properties to a membrane. Taken together, membrane lipids influence the developmental and physiological state of a cell, in large part due to their fatty acid composition.

Fatty acids (FAs) are classified based on carbon chain length as short, medium, long, or very long (Agostoni and Bruzzese, 1992). Short chain FAs are thought to function primarily as signaling molecules within the cell, medium chain FAs function primarily as energy sources, and long and very long chain fatty

acids as membrane components; however, these functions are not exclusive to each of these categories. As membrane components, long and very long chain FAs (LCFAs and VLCFAs, respectively) heavily influence signaling and energy generation by defining the membrane's protein composition as discussed earlier in this chapter. In order to be incorporated in to membrane lipids, LCFAs and VLCFAs need to be activated by esterification to Coenzyme A, and this reaction is catalyzed by Acyl CoA Synthases **(Figure 1.1)**.

Acyl CoA Synthases: Fatty Acid Activators

In order to be used for any catabolic, anabolic, or regulatory function within the cell, FAs must first be esterified to Coenzyme A (CoA) (Watkins and Ellis, 2012). This esterification activates fatty acids and allows their participation in enzymatic reactions. The esterification reaction is catalyzed by enzymes belonging to the Acyl CoA Synthetase/Synthase family. Acyl CoA Synthases (ACS) catalyze a two-step reaction that is ATP dependent and leads to the release of two AMP equivalents. In the first half-reaction, the FA substrate is adenylated, releasing inorganic pyrophosphate (PPi) (Watkins and Ellis, 2012):

$$\text{Fatty acid} + \text{ATP} \rightarrow \text{Fatty acyl}-\text{AMP} + \text{PPi}$$

The ubiquitous enzyme pyrophosphatase, which can be found in soluble, mitochondrial, peroxisomal, and other subcellular fractions, rapidly cleaves PPi, effectively preventing reversal of this reaction. In the second half reaction, CoA displaces AMP, forming a thioester bond to yield the activated FA (Young and

$$\text{Fatty acyl}-\text{AMP} + \text{CoA} \rightarrow \text{Fatty acyl-CoA}$$

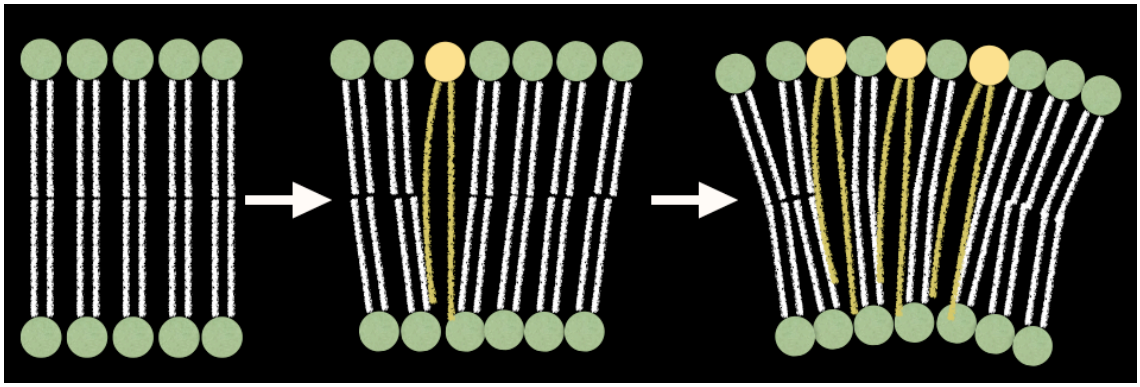

Figure 1.1: Lipids containing long/very long chain fatty acids induce curvature in membranes

Green circles in the above figure represent polar head groups, and white lines represent hydrophobic fatty acids in membrane lipids. Assembly of fairly short chained fatty acid containing lipids leads to a straight, noncurved lipid bilayer. Introduction of long/very long chain fatty acids or lipids containing these leads to a successive increase in membrane curvature with the increase in number of such lipids. The yellow circles represent polar head groups and yellow lines represent long/very long chain fatty acids in curvature-inducing lipids.

Anderson, 1974). ACS family members are homologous in sequence and identified based on the presence of two highly conserved motifs. Motif I is a 10 amino acid sequence typically located 200–300 residues from the N- terminus; it is an AMP-binding site defined by the consensus [YF]TSGTTGxPK. Mutations in this site decrease or abolish catalytic activity (Black and DiRusso, 2003). Motif II harbors the ACS catalytic domain. This motif shares homology with the ANL superfamily (**A**cyl-CoA synthetases, **N**onribosomal peptide synthetase, **L**uciferase) and contains 36–37 amino acids with four positions reserved for hydrophobic residues (TGDxxxxxxxGxxxhx[DG]RxxxxhxxxxGxxhxxx[EK]hE) (Stinnett et al., 2007). The conserved arginine at position 18 is always found approximately 260 residues downstream of Motif I. Results of site-directed mutagenesis experiments initially suggested that Motif II functions as a signature motif, determining the substrate specificity of the acyl-CoA synthetase family members, although this has been disputed. It is now understood that there are at least four residues outside of and distant from Motif II that are required for substrate specificity (Stinnett et al., 2007). Protein structural studies based on sequence and spectroscopy indicate the presence of a substrate binding “tunnel” that ensures FAs of only a certain chain length are activated by a specific ACS (Black et al., 1997; Soupene and Kuypers, 2008; Stinnett et al., 2007). Motif II, along with the four extra residues, forms the tunnel. There is limited flexibility in substrate activation as long chain ACSs can also sometimes activate very long chain FAs (Stinnett et al., 2007) (**Figure 1.2**). In mammals, 26 ACSs have been identified; of these, 13 activate long and/or very long chain fatty acids (>14

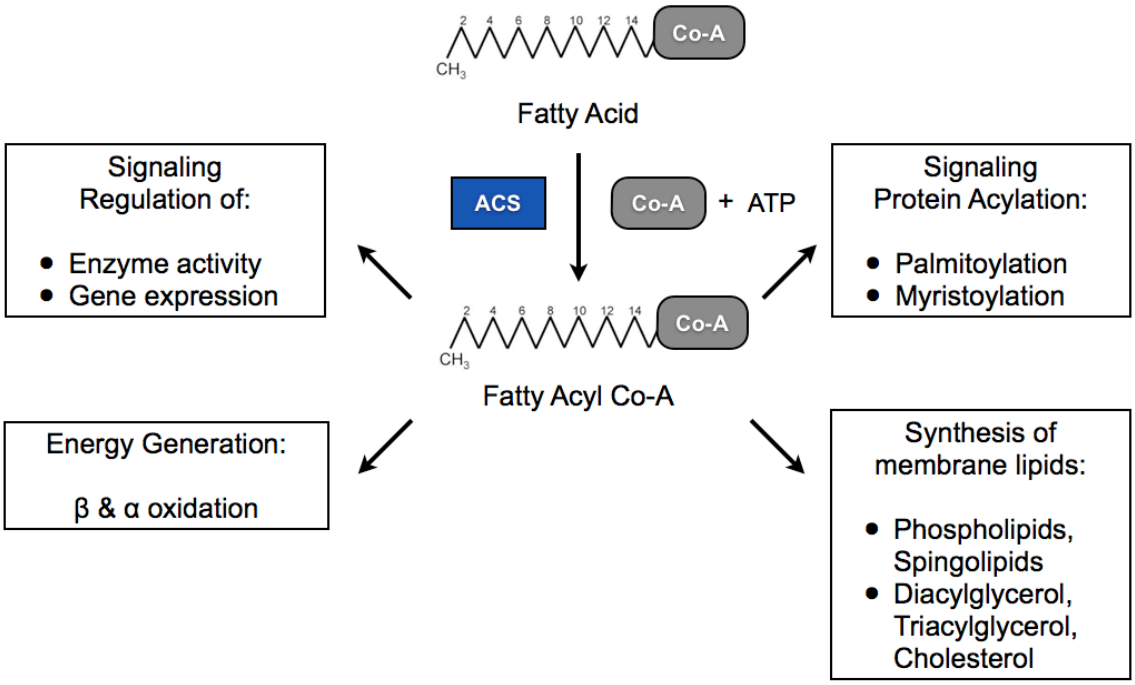

Figure 1.2: Acyl CoA Synthases are required for fatty acid functions

Fatty acids are activated by Acyl CoA Synthases (ACSs) in the presence of ATP. Activated fatty acids like palmitate and myristilate are used for protein acylation and thus facilitate signaling. Fatty acids are used for membrane lipid synthesis and thus facilitate membrane biogenesis. Lastly they act as efficient sources of energy and also regulate gene expression and enzyme activity.

carbons) (Mashek et al., 2007). These ACSs include members of the long-chain acyl-CoA synthase (ACSL) family and the very long chain acyl-CoA synthase (ACSVL) family (including members of the Bubblegum family [ACSBG]- the mammalian ACS homologous of the Drosophila long chain ACS Bgm). Finally, some ACSVLs are dual functioning, serving as fatty acid transporters (FATPs) in addition to their roles as acyl CoA synthases. Dual functioning ACSVLs are sometimes also designated as Solute Carrier Fatty Acid Transporters (SLCA) (Abumrad et al., 1998; Schaffer and Lodish, 1994).

Long and very long chain ACSs are partitioned in the cell. Both FATP1 and ACSL6 reside in the plasma membrane, ACSL4 resides in peroxisomes and the endoplasmic reticulum, and ACSL5 resides in the inner mitochondrial membrane (Gassler et al., 2007; Watkins and Ellis, 2012). As demonstrated by ACSL4, ACSL proteins can be present in multiple subcellular locations within a single cell; moreover, a single ACSL may vary in its subcellular location in different cell types (Küch et al., 2014). As examples, ACSL1 resides in the plasma membrane and in GLUT4 vesicles in adipocytes, on the endoplasmic reticulum in hepatocytes, and on mitochondria in epithelial cells (Mashek et al., 2007). ACSBG1 and ACSBG2 are found in the cytoplasm of COS-1 cells, mitochondria of neuronal cells, and microsomes of cells in the testis (Mashek et al., 2007). Cell-specific differences in ACSL localization may arise from splicing or protein-protein interaction differences that are specific to each tissue type. The differential subcellular localization of each of the ACSL/ACSVL/ACSBG isoforms points to cell type specific roles for ACSs. Consistent with this view is the

demonstration that mitochondrial-localized ACSL5 is a key regulator of mitochondrial energy generation and apoptosis (Gassler et al., 2007; Klaus et al., 2013).

Role of Acyl CoA Synthases in Fatty Acid Uptake

Cellular uptake of fatty acids derived from diet or synthesized by different tissues occurs either by passive diffusion or by facilitated transport. Because they are hydrophobic, it has long been thought that FAs move across the lipid bilayer and into a cell by either concentration-based flipping or passive diffusion. However, FA uptake studies deploying 1) saturation kinetics, *2*) protein-mediated plasma membrane transport inhibition, *3*) inhibition using nucleophilic fatty acid derivatives, and *4*) competitive inhibition sensitivities (Glatz et al., 2010) indicate that protein-mediated uptake is important at physiological concentrations of fatty acids, and evidence for passive diffusion only emerges when fatty acids concentrations are quite high and presumably nonphysiological (Abumrad et al., 1998, 1999). These studies have additionally identified several categories of fatty acid transporters. The integral and peripheral plasma membrane proteins that transport fatty acids into cells are known as “fatty acid transporter.” Included among these are fatty acid binding proteins (FABPs), fatty acid transport protein (FATPs), and fatty acid translocases (Glatz et al., 2010). Of these transporters, the FATP family predominates and is the focus of the discussion that follows.

The FATP family was discovered in 1994 when Schaffer and Lodish screened for adipocyte proteins that increase cellular uptake of fluorescent FA

analogs (Schaffer and Lodish, 1994). Two positives that emerged from that screen were cloned: ACSL1 and FATP1. In early studies of FATPs, it was found that these proteins shared several domains of sequence homology as well as their domain organization with well-characterized members of the acyl-CoA synthetase family. This discovery led to the recognition that FATPs are dual functioning—albeit bona fide members of the adenylate forming acyl-CoA synthetase superfamily, and specifically the very-long-chain acyl-CoA synthetase subgroup (Stahl, 2004).

Once transported inside the cell, FA retention likely depends on vectorial esterification, wherein exogenous FAs are esterified to acyl-CoAs upon uptake and channeled subsequently into downstream pathways (for signal transduction, energy production, and/or membrane biogenesis). Upon esterification, the once hydrophobic FAs are now negatively charged and thus unable to traverse the cell's hydrophobic lipid bilayer by flipping or by transporters, which are directional in their transport (Glatz et al., 2010).

In addition to cellular retention, there is also increasing evidence to suggest that the ACS activity of ACSL and ACSVL/FATP family members is necessary and/or sufficient to transport FAs into the cell. Several studies show that ACS activity alone is sufficient for FA transport, and expression of fatty acyl-CoA synthetases that are unrelated to the FATP-associated VLACS can enhance fatty acid uptake in Escherichia coli (Mangroo et al., 1995) and yeast (Tong et al., 2006). In other scenarios, ACS activity is necessary in conjunction with transporter activity to facilitate FA transport. In 3T3-L1 adipocytes, ACS1 is an

integral membrane protein which colocalizes and interacts with FATP1 (Hall et al., 2003). Overexpression of either of these ACS1 or FATP1 increases fatty acid uptake, while their concomitant overexpression has a synergistic effect on fatty acid uptake (Gargiulo et al., 1999). When FATP1 contains a mutation that abolishes ACS activity, its overexpression severely suppresses FA uptake. Similarly, overexpressing normal FATP4 enhances FA uptake in COS cells, but overexpressing a FATP4 mutant that lacks ACS activity abolishes these effects (Mashek et al., 2007) . Finally, overexpressing certain isoforms of either ACSL or FATP increases FA uptake despite the fact that ACSL1, ACSL4, ACSL5, and FATP4 are located only on intracellular organelles in the cells examined (Heimli et al., 2003; Marszalek et al., 2004; Mashek et al., 2006; Milger et al., 2006). These data suggest that FA uptake depends on inherent ACS activity of the cell and not on direct transport at the plasma membrane.

Role of Acyl CoA Synthases in Fatty Acid Channeling

Once inside the cell, it appears that ACSs channel FAs into specific downstream pathways. The existence of 13 ACSL, FATP, and ACSBG isoforms that all activate long-chain FAs has led to the suggestion that each has an independent role in channeling FA within cells and there are several lines of evidence supporting this supposition.

In cultured mammalian cells, gain-of-function and loss-of-function studies strongly implicate the different ACSLs in channeling FAs into specific metabolic pathways, consistent with differences in their subcellular residencies and

substrate preferences. As examples, ACSL1 overexpression in either NIH-3T3 fibroblasts or PC12 neurons increases oleic acid incorporation into Triacylglycerol (TAG) (Marszalek et al., 2004; Souza et al., 2002). In transgenic mice with heart-specific expression of ACSL1, TAG increases approximately 12-fold in the heart and choline glycerophospholipid increases 50% (Chiu et al., 2001). In contrast, overexpressing ACSL1 in rat primary hepatocytes increases oleate incorporation into phospholipid and diacylglycerol, while decreasing incorporation into cholesterol esters and having no effect on TAG (Li et al., 2006). In a study of ACSL3 knockdown in rat primary hepatocytes, decreased incorporation of labeled acetate into TAG and phospholipids was observed.

Altering the expression of FATPs similarly affects FA channeling. For example, FATP1 overexpression in HEK293 cells alters partitioning of both oleic acid (exogenously added) and FAs (synthesized *de novo* from acetate into cellular lipids) and increases cellular TAG content while decreasing cholesterol and sphingomyelin content (Hatch et al., 2002). In skeletal muscle, adenovirus-mediated overexpression of FATP1 increases the partitioning of oleate or palmitate into TAG and decreases β-oxidation; overexpression in mouse heart increases FA uptake and TAG content and causes a lipotoxic cardiomyopathy (Chiu et al., 2001). Conversely, in muscle from FATP1 null mice fed a high fat diet, the content of TAG, DAG, and acyl-CoA is lower than in wild-type controls. Knockdown of FATP1 in 3T3-L1 adipocytes reduces FA uptake with no changes in lipolysis, but knockdown of FATP4 instead, increases basal lipolysis (Mashek et al., 2007). Thus, the specific effects of individual ACSs support the notion that

different ACSLs or FATPs channel FAs to distinct metabolic fates and the role of each isoform varies based on the tissue type.

It is possible that the differential effects of different ACS isoforms in different tissues result from tissue specific protein interactions. These interactions may determine the fates of acyl-CoAs synthesized by ACSs and thus channel them according to the metabolic demands of a given cell type. There is, however, only limited evidence to support the role of protein-protein interactions in channeling of Acyl CoAs. Immunoprecipitation of endogenous FATP1 from 3T3-L1 adipocytes followed by mass spectrometry identified mitochondrial 2-oxoglutarate dehydrogenase (OGDH), a key enzyme in the tricarboxylic acid cycle. Additionally, FATP1 enhances OGDH activity in proteoliposomes, whereas FATP1 knockdown in 3T3-L1 adipocytes showed decreased OGDH and TCA cycle activity (Wiczer and Bernlohr, 2009). These data suggest that channeling of Acyl CoAs occurs via interactions of metabolic enzymes with ACSs, and these interactions heavily influence metabolic reactions by controlling substrate availability. However, it will be interesting to see if other FA channeling roles of ACSs require direct protein-protein interactions.

Long-chain Acyl-coenzyme A Synthetase's Role in Disease

Increases or decreases in ACSL isoforms under pathological conditions highlight the importance of optimal ACS function within an organism. Several ACS proteins have been implicated in a number of diseases; however, the

specific roles of these enzymes in disease conditions are completely understood only in a few cases.

Ichthyosis Prematurity Syndrome (IPS) is an autosomal recessive disorder characterized by premature birth, respiratory complications and dry, thickened, and scaly skin (Ichthyosis). The disease is caused by mutations in the gene encoding the FATP4 enzyme and a specific reduction in the incorporation of VLCFA into cellular lipids (Klar et al., 2009). The mutations identified are either nonsense mutations or missense mutations within the ATP binding pocket of FATP4. Studies of human IPS patients, as well as Fatp4 null mice, suggest that FATP4 plays a role in early epidermal development and that other ACS enzymes cannot compensate for a deficit of this enzyme.

Expression of the very long chain fatty acyl-CoA synthetase, ACSVL3, was found to be markedly elevated in clinical malignant gliomas in comparisons to normal glia (Pei et al., 2013). ACSVL3 levels correlated with the malignant behavior of human glioma cell lines with glioma cells propagating as xenografts. Direct ACSVL3 knockdown using RNA interference also inhibited glioma cell growth by 70% to 90%, while ACSVL3-depleted cells were less tumorigenic than control cells (Pei et al., 2009). It is now known that ACSVL3 maintains oncogenic properties of malignant glioma cells via up-regulation of Akt function (Pei et al., 2009).

Several mutations in the ACSL4 gene are known to be causative of X-linked mental retardation, a common cause for mental disability in young males. Deletions and missense mutations of the FACL4 gene are associated with

nonspecific mental retardation and with multisyndrome AMME complex disorder (Alport syndrome, Mental retardation, Midface hypoplasia, and Elliptocytosis). FACL4 is expressed in the human brain where it functions to activate polyunsaturated fatty acids (PUFA), like arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). In the mammalian brain, PUFAs function both as essential components of cellular membranes and as signaling molecules (Kantojärvi et al., 2011; Meloni et al., 2009; Yonath et al., 2011). It was proposed that defects in fatty acid metabolism due to the loss of FACL4 function can affect brain development. Cao et al. have shown that exposure to exogenous polyunsaturated fatty acids causes Caspase 3 dependent apoptosis in cell lines, and that this effect is abolished by overexpression of FACL4 (Cao et al., 2000). These data suggest that mutations in FACL4 cause nervous system damage via a direct cytotoxic effect of accumulated fatty acids. However, this hypothesis remains to be confirmed by in vivo studies.

Adenocarcinomas with an invasive phenotype and enhanced proliferation of enterocytes show decreased levels of ACSL5; however, it is unclear if ACSL5 is causative of the disease (Gassler et al., 2003). ACSL5 is expressed in an ascending gradient along the crypt-villus axis of human small intestine with the highest expression level in enterocytes at the villus tip. It is thought that ACSL5 sensitizes enterocytes to TRAIL (TNF-Related Apoptosis-Inducing Ligand) derived apoptosis susceptibility by down-regulation of the antiapoptotic FLIP and up-regulation of TRAIL-R1 receptors on the cell surface. TRAIL shows a corresponding gradient expression pattern. In sporadic intestinal

adenocarcinomas, the ACSL5 gradient is lost along with its reduced expression. Thus, a functional correlation between the appearance of an adenocarcinoma phenotype in the absence of ACSL5-derived apoptosis susceptibility of enterocytes has been considered (Klaus et al., 2014).

Elongases are downstream of ACSs in the fatty acid biogenic pathway. They produce FAs with longer acyl chains by using CoA esterified products from ACSs. Elongase of very long chain fatty acids-4 (ELOVL4) is the only mammalian enzyme known to synthesize C28-C36 fatty acids (McMahon and Kedzierski, 2010). In humans, ELOVL4 mutations cause Stargardt Disease-3 (STGD3), a juvenile form of dominant macular degeneration (McMahon et al., 2007). Heterozygous Stgd3 mice that carry a pathogenic mutation in the mouse *Elovl4* gene demonstrate reduced levels of retinal C28-C36 acyl phosphatidylcholines (PC) and epidermal C28-C36 acylceramides (Barabas et al., 2013). Homozygous Stgd3 mice die shortly after birth with signs of disrupted skin barrier function. Targeted *Elovl4* expression, driven by an epidermal-specific involucrin promoter in homozygous Stgd3 mice, restores both epidermal Elovl4 expression and synthesis of the two missing epidermal lipid groups (McMahon et al., 2011). Transgene expression also restores skin barrier function and rescues the neonatal lethality of homozygous Stgd3 mice. These studies establish the critical requirement for epidermal C28-C36 fatty acid synthesis for animal viability.

Mutations in the peroxisomal ABCD1 transporter are responsible for X-linked Adrenoleukodystrophy (X-ALD) in humans (Fuchs et al., 1994; Mosser et

al., 1994). The ABCD1 transporter is a cassette ATP binding protein that as a dimmer transports fatty acids activated by ACSs into the peroxisomes. X-ALD affects 1 in 17,000 births and is equally distributed among ethnic populations. Mutations in the ABCD1 gene can either lead to a severe neurodegenerative phenotype affecting the central nervous system or to a milder form of the disease, affecting only the peripheral nervous system (Adrenomyeloneuropathy [AMN]. The cerebral form of X-ALD manifests itself in otherwise normal children at about 3–5 years of age. Symptoms include anxiety, temperamental issues, difficulty in reading, and sensory loss (Berger et al., 2014). Sadly, these symptoms quickly progress to paralysis and death. The genetic modifiers that determine the severity of the phenotype have not yet been identified. Reduced activity of the peroxisomal ACSL proteins ACSBG 1 and 2 and elevated VLCFA levels in fibroblasts of X-ALD patients have been found, suggesting that ACSs could function as a genetic modifier of the clinical severity of X-ALD (Jia et al., 2004; Moriya-Sato et al., 2000). However, murine models using either the ABCD1 transporter gene and the ACSBG 1 and 2 genes in combination or alone have been unsuccessful in recapitulating the cerebral form of X-ALD and only exhibit mild abnormalities associated with Adrenomyeloneuropathy. Thus it is not yet clearly understood how a dysfunction in fatty acid transport leads to neurodegeneration.

Together these disease studies suggest that any imbalance in the activity of ACSs can lead to severe pathological conditions affecting a variety of systems in the body. Thus, ACSs are critical metabolic regulators that play a significant

role in maintaining optimal physiology.

ACSs in Development

Although a fair amount of research has focused on the diseases that result from ACS malfunction, the developmental processes dependent upon this family of enzymes have remained largely unexplored. Indeed, given the importance of ACSs in fatty acid metabolism and given that so many life-threatening diseases result from ACS malfunction, it would be surprising if ACSs played no critical roles in development.

Limited data suggestive of developmental roles for ACSs are two-pronged and include expression studies documenting differential ACSL transcriptional regulation during development, as well as diet studies documenting the effects of polyunsaturated fatty acids in mother's milk on development. Expression studies have been useful in demonstrating that during 3T3-L1 adipocyte differentiation, *Acsl1* mRNA abundance increases 160-fold while other transcripts encoding other ACSs remain unchanged (Marszalek et al., 2004), suggesting that ACSL1 has an important role during adipocyte differentiation. In contrast, during the differentiation of PC12 neuronal cells, *Acsl1* and *Acsl3* mRNA levels remain unchanged, while that of *Acsl4*, *5*, and *6* increases significantly (Marszalek et al., 2004). Further, in mouse heart, *Acsl1* mRNA increases 4-fold postnatally, while *Acsl3* mRNA decreases and other ACSL isoforms do not change (de Jong et al., 2007). Additionally, in Drosophila we have shown that six long chain ACS isoform are expressed early on in embryogenesis (Scuderi et al., unpublished data). Of

these, only two (the *bubblegum* [*bgm*] and *double bubble* [*dbb*] long chain acyl CoA synthase genes, which are homologous to the mammalian ACSBG) are expressed in a ventrally restricted pattern, and their mRNA levels seem to be differentially regulated during development. Moreover, preliminary studies indicate that the expression of *bgm* and *dbb* is under the control of dorsoventral patterning genes (refer to Chapter 3).

A developmental role for ACSs has also been implicated in a very different line of research. Clinical studies suggest that polyunsaturated fatty acids such as Omega 3 and Omega 6, both long chain fatty acids, are critical for proper development of the human embryo. Absence or reduction of LCFAs in mother's milk, as well as in the diet of children, has been associated with an increased predisposition to neurodevelopmental disorders including Dyslexia, Dyspraxia and the Autism Spectrum Disorders (Ward, 2000; Young and Conquer, 2005). Additionally, each of these neurodevelopmental disorders is thought to result from disturbances in neuronal and/or glial membranes, which are rich in LCFAs and VLCFAs (Laycock et al., 2007). One reason why the developmental roles of long and very long chain fatty acids have not been explored systematically is the lack of a genetic model system that allows precise manipulations of small populations of fatty acids during activation of small, medium, or long chain fatty acids that will affect specific populations of fatty acids during development and thus provide a segue to their specific roles in development. To date, however, ACS knockout models in mice and flies have yielded only homozygous viable animals.

Drosophila ACSLs: A Genetic Model for Human Developmental Disorders

Drosophila melanogaster provides a powerful genetic model, one that is widely used to study development and the pathogenesis of human degenerative disorders. The high degree of conservation of fundamental biological processes and neural function between humans and flies, coupled with the broad repertoire of fly genetic approaches, makes Drosophila a powerful model system for understanding the basics of molecular and cellular pathology of the vertebrate systems. In addition, fly genomes have little to no redundancy in genetic pathways, thus making forward genetics much more efficient and successful. This is especially true with respect to ACS genes, six of which exist in the fly compared to 26 in mammalian systems. Other advantages of the Drosophila model system include its low cost, compact and sequenced genome, and amenability to large scale genetic and pharmacological screens. While the lipid composition in the insects is different from mammals, the components of lipid metabolism pathways are conserved. As in humans, lipid homeostasis in Drosophila is tightly linked to systemic integrity and development, and loss-of-function mutations affecting enzymes involved in Drosophila lipid metabolism manifest as developmental and degenerative changes in the fly. Significantly, fly models of several developmental disorders like Angelman syndrome, Rett syndrome, Neurofibromatosis Type 1, and Fragile X syndrome have recently been established (reviewed in Gatto and Broadie, 2011). These studies prove the existence of conserved pathways between flies and humans and indicate that

understanding fly development will go a long way in understanding human developmental processes. These investigations in Drosophila continue to provide the essential mechanistic understanding required to facilitate the conception of rational therapeutic treatments for developmental disorders.

There have been a few studies to explore the role of LCFAs/VLCFAs, lipids, and the enzymes responsible for their production in developmental processes using Drosophila as a model system. What follows is a brief overview of these studies.

There are three studies of Drosophila sperm that implicate very-long-chain fatty acids or their derivative phosphatidylinositol lipids in membrane biogenesis and/or its connection to contractile components during cell division. First, mutations in the gene *bond,* which encodes a Drosophila member of the family of very long chain fatty acid elongase (Elovl), block or dramatically slow cleavage-furrow ingression during early telophase in dividing spermatocytes (Szafer-Glusman et al., 2008). In *bond* mutant cells at late stages of division, the contractile ring detaches from the cortex and constricts or collapses to one side of the cell, and the cleavage furrow regresses.

Perhaps the best known Drosophila story of lipids in development is the *wunen* story. Lipid phosphate phosphatases (LPPs) are integral membrane enzymes that regulate the levels of bioactive lipids such as Sphingosine 1 Phosphate and lysophosphatidic acid. The two Drosophila LPPs, *Wunen (Wun)* and *Wunen-2 (Wun2),* have redundant roles in regulating the survival and migration of germ cells (Renault et al., 2010). *wun* and *wun2* function in the

central nervous system and other somatic tissues to repel germ cells, likely via generation of a lipid signal. *wun* and *wun2* also mediate germ cell–germ cell repulsion for germ cell dispersal to two embryonic gonads at the onset of germ cell migration. In addition, *wun2* is required in the germ cells for their survival and to perceive the signal. Besides its well-established role in germ cell migration, wun also regulates the function of septate junctions: in *wun* mutants, the integrity of septate junction in the trachea and the blood–brain barrier is lost (Renault et al., 2010).

Our studies of the *bubblegum* (*bgm*) and *double bubble* (*dbb*) ACSs contribute to the short list of developmental lipid stories in Drosophila. The Drosophila *bgm* gene, which encodes an ACSL, was identified in a P-element screen for genes that affect nervous system integrity. *bgm* mutants were found to exhibit subtle and incompletely penetrant defects in neuronal health. The similarities between *bgm* neurodegenerative phenotypes and human X-ALD are notable and perhaps not unexpected given that the fly and human genes responsible for this neurodegeneration are part of a single biochemical pathway (Min and Benzer 1999).

More recently we identified *double bubble* (*dbb*), a close homolog of *bgm* as a ventrally restricted transcript in an automated screen for transcriptionally regulated targets of early developmental signaling pathways (Simin, Scuderi et al., 2002). The sequence similarity of the *bgm* and *dbb* genes and their close proximity in the genome suggests that they are duplicated genes playing redundant or overlapping developmental roles. In order to analyze the functional

relationship between Bgm and Dbb, we generated *dbb* and *bgm dbb* mutant flies by homologous recombination. We demonstrated that *bgm dbb* exhibit a more fully penetrant and more severe neurodegenerative phenotype than do either of the single mutants; thus the closely related genes function in redundant fashion in neuromaintenance (Anna Sivatchenko, unpublished data). Intriguingly, upon closer examination, we observed developmental defects in our *bgm* and *bgm dbb* mutant lines, providing us with a previously unrecognized opportunity to study the developmental role of ACSLs in the fly.

Summary

In studies presented in this dissertation, I show that the maternally derived *bgm* is required for Drosophila embryonic development and survival. *bgm* is required for cellularization, and failures in this very early developmental process appear to have later widespread manifestations as well (Chapter 2); I also report studies of regulation of *bgm* and *dbb* gene expression (Chapter 3) and some behavioral studies relating to the neurodegenerative phenotype of *bgm* and *dbb* adult flies (Chapter 4).

References

Abumrad, N., Harmon, C., and Ibrahimi, A. (1998a). Membrane transport of long-chain fatty acids: evidence for a facilitated process. J. Lipid Res. *39*, 2309–2318.

Abumrad, N., Coburn, C., and Ibrahimi, A. (1999). Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. Biochem. Biophys. Acta *1441*, 4–13.

Agostoni, C., and Bruzzese, M.G. (1992). [Fatty acids: their biochemical and

functional classification]. Pediatric Med. Chir. *14*, 473–479.

Andersen, O.S., and Koeppe, R.E. (2007). Bilayer thickness and membrane protein function: an energetic perspective. Annu. Rev. Biophys. Biomol. Struct. *36*, 107–130.

Barabas, P., Liu, A., Xing, W., Chen, C.-K., Tong, Z., Watt, C.B., Jones, B.W., Bernstein, P.S., and Križaj, D. (2013). Role of ELOVL4 and very long-chain polyunsaturated fatty acids in mouse models of Stargardt type 3 retinal degeneration. Proc. Natl. Acad. Sci. U.S.A. *110*, 5181–5186.

Bartscherer, K., and Boutros, M. (2008). Regulation of Wnt protein secretion and its role in gradient formation. EMBO. Rep. *9*, 977–982.

Berger, J., Forss-Petter, S., and Eichler, F.S. (2014). Pathophysiology of X-linked adrenoleukodystrophy. Biochimie *98*, 135–142.

Black, P.N., and DiRusso, C.C. (2003). Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. Microbiol. Mol. Biol. Rev. *67*, 454–472, table of contents.

Black, P.N., Zhang, Q., Weimar, J.D., and DiRusso, C.C. (1997). Mutational analysis of a fatty acyl-coenzyme A synthetase signature motif identifies seven amino acid residues that modulate fatty acid substrate specificity. J. Biol. Chem. *272*, 4896–4903.

Bogdanov, M., Dowhan, W., and Vitrac, H. (2014). Lipids and topological rules governing membrane protein assembly. Biochim. Biophys. Acta *1843*, 1475–1488.

Buglino, J.A., and Resh, M.D. (2012). Palmitoylation of Hedgehog proteins. Vitam. Horm. *88*, 229–252.

Cao, Y., Pearman, A.T., Zimmerman, G.A., McIntyre, T.M., and Prescott, S.M. (2000). Intracellular unesterified arachidonic acid signals apoptosis. Proc. Natl. Acad. Sci. U.S.A. *97*, 11280–11285.

Castro, B.M., Prieto, M., and Silva, L.C. (2014). Ceramide: a simple sphingolipid with unique biophysical properties. Prog. Lipid Res. *54*, 53–67.

Chiu, H.C., Kovacs, A., Ford, D.A., Hsu, F.F., Garcia, R., Herrero, P., Saffitz, J.E., and Schaffer, J.E. (2001). A novel mouse model of lipotoxic cardiomyopathy. J. Clin. Invest. *107*, 813–822.

Coleman, R.A., Lewin, T.M., Van Horn, C.G., and Gonzalez-Baró, M.R. (2002). Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic

versus degradative pathways? J. Nutr. *132*, 2123–2126.

D'Souza, K., and Epand, R.M. (2014). Enrichment of phosphatidylinositols with specific acyl chains. Biochim. Biophys. Acta *1838*, 1501–1508.

Fuchs, S., Sarde, C.O., Wedemann, H., Schwinger, E., Mandel, J.L., and Gal, A. (1994). Missense mutations are frequent in the gene for X-chromosomal adrenoleukodystrophy (ALD). Hum. Mol. Genet. *3*, 1903–1905.

Gargiulo, C.E., Stuhlsatz-Krouper, S.M., and Schaffer, J.E. (1999). Localization of adipocyte long-chain fatty acyl-CoA synthetase at the plasma membrane. J. Lipid Res. *40*, 881–892.

Gassler, N., Schneider, A., Kopitz, J., Schnölzer, M., Obermüller, N., Kartenbeck, J., Otto, H.F., and Autschbach, F. (2003). Impaired expression of acyl-CoA-synthetase 5 in epithelial tumors of the small intestine. Hum. Pathol. *34*, 1048–1052.

Gassler, N., Roth, W., Funke, B., Schneider, A., Herzog, F., Tischendorf, J.J.W., Grund, K., Penzel, R., Bravo, I.G., Mariadason, J., et al. (2007). Regulation of enterocyte apoptosis by acyl-CoA synthetase 5 splicing. Gastroenterology *133*, 587–598.

Gatto, C.L., and Broadie, K. (2011). Drosophila modeling of heritable neurodevelopmental disorders. Curr. Opin. Neurobiol. *21*, 834–841.

Glatz, J.F.C., Luiken, J.J.F.P., and Bonen, A. (2010). Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol. Rev. *90*, 367–417.

Goñi, F.M., and Alonso, A. (1999). Structure and functional properties of diacylglycerols in membranes. Prog. Lipid Res. *38*, 1–48.

Hage Hassan, R., Bourron, O., and Hajduch, E. (2014). Defect of insulin signal in peripheral tissues: important role of ceramide. World J. Diabetes *5*, 244–257.

Hall, A.M., Smith, A.J., and Bernlohr, D.A. (2003). Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. J. Biol. Chem. *278*, 43008–43013.

Hatch, G.M., Smith, A.J., Xu, F.Y., Hall, A.M., and Bernlohr, D.A. (2002). FATP1 channels exogenous FA into 1,2,3-triacyl-sn-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells. J. Lipid Res. *43*, 1380–1389.

Heimli, H., Hollung, K., and Drevon, C.A. (2003). Eicosapentaenoic acid-induced

apoptosis depends on acyl CoA-synthetase. Lipids *38*, 263–268.

Ho, K.S., and Scott, M.P. (2002). Sonic hedgehog in the nervous system: functions, modifications and mechanisms. Curr. Opin. Neurobiol. *12*, 57–63.

Jia, Z., Pei, Z., Li, Y., Wei, L., Smith, K.D., and Watkins, P.A. (2004). X-linked adrenoleukodystrophy: role of very long-chain acyl-CoA synthetases. Mol. Genet. Metab. *83*, 117–127.

De Jong, H., Neal, A.C., Coleman, R.A., and Lewin, T.M. (2007). Ontogeny of mRNA expression and activity of long-chain acyl-CoA synthetase (ACSL) isoforms in Mus musculus heart. Biochim. Biophys. Acta *1771*, 75–82.

Kantojärvi, K., Kotala, I., Rehnström, K., Ylisaukko-Oja, T., Vanhala, R., von Wendt, T.N., von Wendt, L., and Järvelä, I. (2011). Fine mapping of Xq11.1-q21.33 and mutation screening of RPS6KA6, ZNF711, ACSL4, DLG3, and IL1RAPL2 for autism spectrum disorders (ASD). Autism Res. *4*, 228–233.

Klar, J., Schweiger, M., Zimmerman, R., Zechner, R., Li, H., Törmä, H., Vahlquist, A., Bouadjar, B., Dahl, N., and Fischer, J. (2009). Mutations in the fatty acid transport protein 4 gene cause the ichthyosis prematurity syndrome. Am. J. Hum. Genet. *85*, 248–253.

Klaus, C., Jeon, M.K., Kaemmerer, E., and Gassler, N. (2013a). Intestinal acyl-CoA synthetase 5: activation of long chain fatty acids and behind. World J. Gastroenterol. *19*, 7369–7373.

Klaus, C., Kaemmerer, E., Reinartz, A., Schneider, U., Plum, P., Jeon, M.K., Hose, J., Hartmann, F., Schnölzer, M., Wagner, N., et al. (2014). TP53 status regulates ACSL5-induced expression of mitochondrial mortalin in enterocytes and colorectal adenocarcinomas. Cell Tissue Res. *357*, 267–278.

Kniazeva, M., Shen, H., Euler, T., Wang, C., and Han, M. (2012). Regulation of maternal phospholipid composition and IP(3)-dependent embryonic membrane dynamics by a specific fatty acid metabolic event in C. elegans. Genes Dev. *26*, 554–566.

Küch, E.-M., Vellaramkalayil, R., Zhang, I., Lehnen, D., Brügger, B., Sreemmel, W., Ehehalt, R., Poppelreuther, M., and Füllekrug, J. (2014). Differentially localized acyl-CoA synthetase 4 isoenzymes mediate the metabolic channeling of fatty acids towards phosphatidylinositol. Biochim. Biophys. Acta *1841*, 227–239.

Laycock, R., Crewther, S.G., and Crewther, D.P. (2007). A role for the “magnocellular advantage” in visual impairments in neurodevelopmental and psychiatric disorders. Neurosci. Biobehav. Rev. *31*, 363–376.

Li, L.O., Mashek, D.G., An, J., Doughman, S.D., Newgard, C.B., and Coleman, R.A. (2006). Overexpression of rat long chain acyl-coa synthetase 1 alters fatty acid metabolism in rat primary hepatocytes. J. Biol. Chem. *281*, 37246–37255.

Liu, Z., and Huang, X. (2013). Lipid metabolism in Drosophila: development and disease. Acta Biochim. Biophys. Sin. (Shanghai) *45*, 44–50.

Mangroo, D., Wu, X.Q., and RajBhandary, U.L. (1995). Escherichia coli initiator tRNA: structure-function relationships and interactions with the translational machinery. Biochem. Cell Biol. *73*, 1023–1031.

Marszalek, J.R., Kitidis, C., Dararutana, A., and Lodish, H.F. (2004). Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth. J. Biol. Chem. *279*, 23882–23891.

Martin, T.F. (2001). PI(4,5)P(2) regulation of surface membrane traffic. Curr. Opin. Cell Biol. *13*, 493–499.

Mashek, D.G., and Coleman, R.A. (2006). Cellular fatty acid uptake: the contribution of metabolism. Curr. Opin. Lipidol. *17*, 274–278.

Mashek, D.G., McKenzie, M.A., Van Horn, C.G., and Coleman, R.A. (2006). Rat long chain acyl-CoA synthetase 5 increases fatty acid uptake and partitioning to cellular triacylglycerol in McArdle-RH7777 cells. J. Biol. Chem. *281*, 945–950.

Mashek, D.G., Li, L.O., and Coleman, R.A. (2007). Long-chain acyl-CoA synthetases and fatty acid channeling. Future Lipidol. *2*, 465–476.

McMahon, A., and Kedzierski, W. (2010). Polyunsaturated very-long-chain C28-C36 fatty acids and retinal physiology. Br. J. Ophthalmol *94*, 1127–1132.

McMahon, A., Jackson, S.N., Woods, A.S., and Kedzierski, W. (2007). A Stargardt disease-3 mutation in the mouse Elovl4 gene causes retinal deficiency of C32-C36 acyl phosphatidylcholines. FEBS Lett. *581*, 5459–5463.

McMahon, A., Butovich, I.A., and Kedzierski, W. (2011). Epidermal expression of an Elovl4 transgene rescues neonatal lethality of homozygous Stargardt disease-3 mice. J. Lipid Res. *52*, 1128–1138.

Meloni, I., Parri, V., De Filippis, R., Ariani, F., Artuso, R., Bruttini, M., Katzaki, E., Longo, I., Mari, F., Bellan, C., et al. (2009). The XLMR gene ACSL4 plays a role in dendritic spine architecture. Neuroscience *159*, 657–669.

Mendelson, K., Evans, T., and Hla, T. (2014). Sphingosine 1-phosphate signalling. Development *141*, 5–9.

Milger, K., Herrmann, T., Becker, C., Gotthardt, D., Zickwolf, J., Ehehalt, R.,

Watkins, P.A., Stremmel, W., and Füllekrug, J. (2006). Cellular uptake of fatty acids driven by the ER-localized acyl-CoA synthetase FATP4. J. Cell. Sci. *119*, 4678–4688.

Min, K.T., and Benzer, S. (1999). Preventing neurodegeneration in the Drosophila mutant bubblegum. Science *284*, 1985–1988.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu. Rev. Genet. *29*, 371–399.

Moriya-Sato, A., Hida, A., Inagawa-Ogashiwa, M., Wada, M.R., Sugiyama, K., Shimizu, J., Yabuki, T., Seyama, Y., and Hashimoto, N. (2000). Novel acyl-CoA synthetase in adrenoleukodystrophy target tissues. Biochem. Biophys. Res. Commun. *279*, 62–68.

Mosser, J., Lutz, Y., Stoeckel, M.E., Sarde, C.O., Kretz, C., Douar, A.M., Lopez, J., Aubourg, P., and Mandel, J.L. (1994). The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. Hum. Mol. Genet. *3*, 265–271.

Osborne, S.L., Meunier, F.A., and Schiavo, G. (2001). Phosphoinositides as key regulators of synaptic function. Neuron *32*, 9–12.

Pei, Z., Sun, P., Huang, P., Lal, B., Laterra, J., and Watkins, P.A. (2009). Acyl-CoA synthetase VL3 knockdown inhibits human glioma cell proliferation and tumorigenicity. Cancer Res. *69*, 9175–9182.

Pei, Z., Fraisl, P., Shi, X., Gabrielson, E., Forss-Petter, S., Berger, J., and Watkins, P.A. (2013). Very long-chain acyl-CoA synthetase 3: overexpression and growth dependence in lung cancer. PLoS ONE *8*, e69392.

Pol, A., Gross, S.P., and Parton, R.G. (2014). Review: biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites. J. Cell Biol. *204*, 635–646.

Poveda, J.A., Giudici, A.M., Renart, M.L., Molina, M.L., Montoya, E., Fernández-Carvajal, A., Fernández-Ballester, G., Encinar, J.A., and González-Ros, J.M. (2014). Lipid modulation of ion channels through specific binding sites. Biochim. Biophys. Acta *1838*, 1560–1567.

Record, M., Carayon, K., Poirot, M., and Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochim. Biophys. Acta *1841*, 108–120.

Renault, A.D., Kunwar, P.S., and Lehmann, R. (2010). Lipid phosphate phosphatase activity regulates dispersal and bilateral sorting of embryonic germ cells in Drosophila. Development *137*, 1815–1823.

Schaffer, J.E., and Lodish, H.F. (1994). Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell *79*, 427–436.

Schink, K.O., Raiborg, C., and Stenmark, H. (2013). Phosphatidylinositol 3-phosphate, a lipid that regulates membrane dynamics, protein sorting and cell signalling. Bioessays *35*, 900–912.

Schmidt, C., and Robinson, C.V. (2014). Dynamic protein ligand interactions—insights from MS. FEBS J. *281*, 1950–1964.

Shisheva, A. (2013). PtdIns5P: news and views of its appearance, disappearance and deeds. Arch. Biochem. Biophys. *538*, 171–180.

Soupene, E., and Kuypers, F.A. (2008). Mammalian long-chain acyl-CoA synthetases. Exp. Biol. Med. (Maywood) *233*, 507–521.

Souza, S.C., Muliro, K.V., Liscum, L., Lien, P., Yamamoto, M.T., Schaffer, J.E., Dallal, G.E., Wang, X., Kraemer, F.B., Obin, M., et al. (2002). Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. J. Biol. Chem. *277*, 8267–8272.

Stachowiak, J.C., Brodsky, F.M., and Miller, E.A. (2013). A cost-benefit analysis of the physical mechanisms of membrane curvature. Nat. Cell Biol. *15*, 1019–1027.

Stahl, A. (2004). A current review of fatty acid transport proteins (SLC27). Pflugers Arch. *447*, 722–727.

Stein, J. (2000). The neurobiology of reading difficulties. Prostaglandins Leukot. Essent. Fatty Acids *63*, 109–116.

Stinnett, L., Lewin, T.M., and Coleman, R.A. (2007). Mutagenesis of rat acyl-CoA synthetase 4 indicates amino acids that contribute to fatty acid binding. Biochim. Biophys. Acta *1771*, 119–125.

Szafer-Glusman, E., Giansanti, M.G., Nishihama, R., Bolival, B., Pringle, J., Gatti, M., and Fuller, M.T. (2008a). A role for very-long-chain fatty acids in furrow ingression during cytokinesis in Drosophila spermatocytes. Curr. Biol. *18*, 1426–1431.

Takasuga, S., and Sasaki, T. (2013). Phosphatidylinositol-3,5-bisphosphate: metabolism and physiological functions. J. Biochem. *154*, 211–218.

Tong, F., Black, P.N., Coleman, R.A., and DiRusso, C.C. (2006). Fatty acid transport by vectorial acylation in mammals: roles played by different isoforms of rat long-chain acyl-CoA synthetases. Arch. Biochem. Biophys. *447*, 46–52.

Ward, P.E. (2000). Potential diagnostic aids for abnormal fatty acid metabolism in a range of neurodevelopmental disorders. Prostaglandins Leukot. Essent. Fatty Acids *63*, 65–68.

Ward, J.D., Mullaney, B., Schiller, B.J., He, L.D., Petnic, S.E., Couillault, C., Pujol, N., Bernal, T.U., Van Gilst, M.R., Ashrafi, K., et al. (2014). Defects in the C. elegans acyl-CoA synthase, acs-3, and nuclear hormone receptor, nhr-25, cause sensitivity to distinct, but overlapping stresses. PLoS ONE *9*, e92552.

Watkins, P.A., and Ellis, J.M. (2012). Peroxisomal acyl-CoA synthetases. Biochim. Biophys. Acta *1822*, 1411–1420.

Wiczer, B.M., and Bernlohr, D.A. (2009). A novel role for fatty acid transport protein 1 in the regulation of tricarboxylic acid cycle and mitochondrial function in 3T3-L1 adipocytes. J. Lipid Res. *50*, 2502–2513.

Wurtzel, J.G.T., Kumar, P., and Goldfinger, L.E. (2012). Palmitoylation regulates vesicular trafficking of R-Ras to membrane ruffles and effects on ruffling and cell spreading. Small GTPases *3*, 139–153.

Yonath, H., Marek-Yagel, D., Resnik-Wolf, H., Abu-Horvitz, A., Baris, H.N., Shohat, M., Frydman, M., and Pras, E. (2011). X inactivation testing for identifying a non-syndromic X-linked mental retardation gene. J. Appl. Genet. *52*, 437–441.

Young, G., and Conquer, J. (2005). Omega-3 fatty acids and neuropsychiatric disorders. Reprod. Nutr. Dev. *45*, 1–28.

Young, O.A., and Anderson, J.W. (1974). Properties and substrate specificity of some reactions catalysed by a short-chain fatty acyl-coenzyme A synthetase from seeds of Pinus radiata Biochem. J. *137*, 423–433.

CHAPTER 2

LONG CHAIN ACYL CoA SYNTHASE: A NECESSITY TO MEMBRANES AND NEUROGENESIS

Introduction

Lipids, one of the four macromolecules of the cell, perform a diverse array of extremely vital functions within every organism. Broadly, lipids directly or indirectly participate in signaling pathways, act as building blocks within membranes, are highly efficient sources of energy compared to carbohydrates, and lastly directly or indirectly affect the availability of vitamins in the body, thus affecting biochemical reactions and metabolism.

As components of biological membranes, the lipid composition of the membrane defines the protein components of the membrane under consideration and thus, lipids impart to each type of membrane in an organism unique protein signatures and unique properties. At the plasma membrane, lipids form a selective barrier that maintains a stable environment within the cell while also maintaining a pathway for communication with the external environment. The plasma membrane protein composition specifies the signaling pathways that are active within a cell as well as the energy sources that enter the cell. Depending on the type of lipids present, each membrane has a unique shape and also the

unique ability to change its shape in response to the demands of morphogenesis, migration, and the immune response, to name just a few biological processes (Bogdanov et al., 2014; Poveda et al., 2014; Schmidt and Robinson, 2014; Stachowiak et al., 2013). The lipid composition of a cell is very sensitive to the physiological state of the organism, and this sensitivity imparts a dynamic ability to the cell, in terms of energy sources used and the signaling pathways activated. This ability allows a cell and/or organism to adapt and survive in a changing environment. Thus, the role of lipids as building blocks for membranes is the most influential as it affects all aspects of a cell's physiology (Abumrad et al., 1999).

Lipids are amphiphilic: having both a hydrophilic end and a hydrophobic end. The hydrophobic chains of membrane lipids are derived from long or very long chain fatty acids which have 14 or more carbons in their acyl chains (Agostoni and Bruzzese, 1992). These help membrane lipids span the membrane either in pairs to form the lipid bilayer, or singly in the case of very long chain fatty acids, which can span the membrane in its entirety to facilitate curving. Importantly, the different fatty acids impart specific membrane bending or protein interaction properties to a membrane (Stachowiak et al., 2013). In contrast, short chain FAs are thought to function primarily as signaling molecules within the cell and medium chain FAs function primarily as energy sources; however, all these functions may not be exclusive to each class of FAs (Agostoni and Bruzzese, 1992).

Irrespective of their function, in order to participate in catabolic, anabolic,

or regulatory functions within the cell, FAs need to be esterified to Coenzyme A (CoA) (Watkins and Ellis, 2012). This esterification activates fatty acids and makes them energetically efficient as substrates in enzymatic reactions. The esterification reaction is catalyzed by the enzymes belonging to the Acyl CoA Synthetase/Synthase (ACS) family. ACSs catalyze a two-step reaction that is ATP dependent and leads to the formation of Acyl-CoA and two AMP equivalents (Watkins and Ellis, 2012). In mammals, 26 ACSs have been identified; of these, 13 activate long and/or very long chain fatty acids (>14 carbons) (Mashek et al., 2007). These ACSs include the members of the long-chain acyl-CoA synthetase (ACSL) family and members of the very long chain acyl-CoA synthetase (ACSVL) family, as well as members of the BGM family, which are mammalian ACSs homologous to the Drosophila long chain ACS Bgm (ACSBG). Some ACSVLs are dual functioning, serving as both fatty acid transporters (FATP) and acyl CoA synthases (Abumrad et al., 1998; Schaffer and Lodish, 1994). In addition to roles in esterification and transport, ACSs appear to facilitate the channeling of fatty acids to specific processes in the cell by associating with the specific enzymes/proteins. Moreover, the specific downstream pathways vary in a tissue-specific manner (reviewed in Mashek et al., 2007). Taken together these data indicate that each ACS isoform has a specific function and each isoform is different and important enough for it to be conserved through evolution.

Several ACSs and their downstream effectors in fatty acid biosynthesis have been implicated in a number of diseases. Ichthyosis Prematurity Syndrome (IPS) is an autosomal recessive disorder characterized by premature birth;

respiratory complications; and dry, thickened, and scaly skin (Ichthyosis). The disease is caused by mutations in the gene encoding the FATP4 enzyme and a specific reduction in the incorporation of VLCFA into cellular lipids (Klar et al., 2009). Expression of the very long chain fatty acyl-CoA synthetase, ACSVL3, was found to be markedly elevated in clinical malignant gliomas in comparisons to normal glia (Pei et al., 2013). ACSVL3 levels correlated with the malignant behavior of human glioma cell lines with glioma cells propagating as xenografts. Direct ACSVL3 knockdown using RNA interference also inhibited glioma cell growth by 70% to 90% (Pei et al., 2009). Mutations in the ACSL4 gene are known to be causative of X-linked mental retardation, a common cause for mental disability in young males. Deletions and missense mutations of the FACL4 gene are associated with nonspecific mental retardation. Mutations in FACL4 cause nervous system damage via a direct cytotoxic effect of accumulated fatty acids. (Kantojärvi et al., 2011; Meloni et al., 2009; Yonath et al., 2011) Adenocarcinomas with an invasive phenotype and enhanced proliferation of enterocytes show decreased levels of ACSL5; however, it is unclear if ACSL5 is causative of the disease (Gassler et al., 2003). A functional correlation between the appearance of an adenocarcinoma phenotype in the absence of ACSL5-derived apoptosis susceptibility of enterocytes has been considered (Klaus et al., 2014).

Elongases are downstream of ACSs in the fatty acid biogenic pathway. They produce FAs with longer acyl chains by using CoA esterified products from ACSs. Elongase of very long chain fatty acids-4 (ELOVL4) is the only

mammalian enzyme known to synthesize C28-C36 fatty acids (McMahon and Kedzierski, 2010). In humans, ELOVL4 mutations cause Stargardt Disease-3 (STGD3), a juvenile form of dominant macular degeneration (McMahon et al., 2007). Targeted *Elovl4* expression, driven by an epidermal-specific involucrin promoter in homozygous Stgd3 mice, restores both epidermal Elovl4 expression and rescues the phenotype.

Mutations in the ABCD1 transporter, which is downstream of ACSs in the peroxisomal fatty acid metabolic pathway, are known to be causative of X-linked Adrenoleukodystrophy (X-ALD) in humans (Fuchs et al., 1994; Mosser et al., 1994). The ABCD1 transporter is a cassette ATP binding protein that as a dimmer, transports fatty acids activated by ACSs into the peroxisomes. X-ALD affects 1 in 17,000 births and is equally distributed among ethnic populations. Mutations in the ABCD1 gene can either lead to a severe neurodegenerative phenotype affecting the central nervous system or to a milder form of the disease, affecting only the peripheral nervous system (Adrenomyeloneuropathy [AMN]). The cerebral form of X-ALD manifests itself in otherwise normal children at about 3–5 years of age. Symptoms include anxiety, temperamental issues, difficulty in reading, and sensory loss (Berger et al., 2014). Sadly, these symptoms quickly progress to paralysis and death. The genetic modifiers that determine the severity of the phenotype have not yet been identified. Reduced activity of the peroxisomal ACSL proteins ACSBG 1 and 2 and elevated VLCFA levels in fibroblasts of X-ALD patients have been found, suggesting that ACSs could function as a genetic modifier of the clinical severity of X-ALD (Jia et al.,

2004; Moriya-Sato et al., 2000).

ACSLs are thus quite clearly critical metabolic regulators and promising targets for the treatment of several diseases. Although a considerable amount of research has been done on diseases that result from ACS malfunction, surprisingly there are only a handful of studies that focus on the role of these enzymes in developmental processes. Given that so many diseases affecting different tissues result from ACS malfunction and knowing how important ACSs are in fatty acid metabolism, it seems likely that these enzymes must play critical roles in developmental processes as well. Moreover, the multiple roles of LCFAs/VLCFAs within membranes make them particularly interesting from a developmental perspective. Development entails extensive membrane remodeling, widespread cell migrations and movements, a hierarchy of signaling cascades, and most importantly energy to drive each of these processes. Each of these functions is heavily influenced by LCFA/VLCFAs in the membranes and within the cellular cytoplasm, and it would be surprising if ACSLs played no critical roles in development.

Limited data suggestive of developmental roles for ACSs are two-pronged and include expression studies documenting differential ACSL transcriptional regulation during development as well as diet studies documenting the effects of polyunsaturated fatty acids in mother's milk on development.

Several clinical studies have suggested that polyunsaturated fatty acids such as Omega 3 and Omega 6 are critical for the development of the human embryo. Absence or reduction of LCFAs in mother’s milk or in the diet of children

has been associated with an increased predisposition to neurodevelopmental disorders including Dyslexia, Dyspraxia, and Autism Spectrum disorders (Ward, 2000; Young and Conquer, 2005). Additionally, each of these neurodevelopmental disorders is thought to result from disturbances in neuronal and/or glial membranes that are rich in LCFAs and VLCFAs (Laycock et al., 2007). All these pieces of information make it impending to explore the role of LCFAS and ACSLs in development.

Expression studies have been useful in demonstrating that during mammalian and invertebrate development, many ACSL isoforms are differentially expressed, many of them during key developmental events (Marszalek et al., 2004; Scuderi et al., unpublished data, refer to Chapter 3).

One reason why the developmental roles of long and very long chain fatty acids have not been explored systematically is the lack of a genetic model system that allows precise manipulations of small populations of fatty acids during development. It is likely that mutations in Acyl CoA synthases responsible for activation of small, medium, or long chain fatty acids will affect specific populations of fatty acids during development and thus provide a segue to their specific roles in development. To date, however, ACS knockout models in mice and flies have yielded only homozygous viable animals (Heinzer et al., 2003).

Drosophila embryos, lacking the long chain acyl CoA synthases Bgm and Dbb, are the ideal genetic system to explore this subject. In addition to the excellent set of genetic tools available for study in *Drosophila melanogaster*, the fly embryo provides us with an excellent *in vivo* system that allows experimental

manipulation and microscopic observation. In this dissertation, I explore the developmental role of the highly conserved Drosophila proteins Bgm and Dbb during embryogenesis in Drosophila. I find that embryos derived from *bgm* homozygous females suffer an incompletely penetrant lethality very early in embryonic development. This lethality is due to a failure in cellularization. Loss of maternal *bgm* also leads to defects in neuromusculature and behavioral abnormalities in first instar larvae. At the molecular level, it appears that the Bgm ACS is required for vesicular targeting within the cell, and this failure in vesicular trafficking leads to a failure in cellularization.

Materials and Methods

Fly strains and genetic analysis

Wild type Canton S flies, mutant flies with the bgm^{1} null allele, the *dbb* null allele, and *bgm dbb* flies carrying both null alleles were used for all the experiments. The bgm^{1} as well as the *dbb* maternal zygotic stocks are homozygous semiviable or viable, respectively, and can be maintained as such. Flies lacking maternal *bgm* were generated using standard genetic techniques where F1 progeny from a cross of bgm^{1} females with Canton S males were collected. Zygotic *bgm* mutants were generated by crossing bgm^{1} / CyO females with bgm^{1} / CyO males and collecting F1 progeny with no GFP expression in them. The CyO balancer used carries a GFP transgene, thus marking any flies containing the balancer.

Immunocytochemistry

Embryos were collected at the appropriate stage after incubation at 25 C. Embryos were dechorionated and fixed using standard fixation methods except, when using Phalloidin. Ethanol was used instead of methanol for the divitellization step when Phalloidin staining was to follow. Standard immunostaining techniques staining techniques were used including the Rapid Staining Procedure (unpublished by Nipam Patel) in some cases. Primary mouse monoclonal antibodies were obtained from DSHB and used at these concentrations: Singleminded (1:300), Neurotactin (1:10), BP102 (1:200), Anti-Futch/mAb22c10(1:100), Anti-Robo (1: 300). Anti-Rab 5 antibody generated in Rabbit was obtained from Abcam and used at 1:250 dilution. Secondary antibodies against mouse or rabbit primaries were tagged with Alexa 488 or 594 in most cases. These were obtained from Jackson Laboratories and used at 1:300 dilution. Phalloidin(Abcam) tagged with Alexa fluor 488 or 594 was used to stain filamentous actin.

Microscopy

Live time lapse imaging of embryos was done by placing the embryos, after dechorionation, in halocarbon oil in a hydrated chamber. DIC or phase contrast or Fluorescence (when imaging transgenic flies) microscopy was used to capture images. Standard fluorescence microscopy and Confocal microscopy was done on the Olympus FV1000 microscope in the University of Utah microscopy core.

Larval assays

Peristalsis assay: 1st instar larvae which were about to hatch were observed under halocarbon oil using Phase contrast or Fluorescence microscopy(when transgenic GFP present). The time taken for one peristaltic wave to reach from the posterior end of the larva to the anterior end was measured as described by (Gjorgjieva et al., 2013). Nod assay: 2nd instar larvae were placed in a drop of water on a juice plate at 25 C. The number of head nods/ minute were measured. These measurements were repeated thrice per animal. Roll Over assay: 3rd instar larvae were placed dorsal side down on a juice plate at 25 C and the time taken to roll over so that the dorsal side is up, and move away was measured (Varnam et al., 1996). Each animal was tested using the assay three times and each measurement is an average of three these three measurements. The number of animals for each experiment and assay was determined based on requirements for statistical significance. This averaged between 10-50 animals for each experiment.

Statistical analysis

The error bars in all graphical representations in the present study represent standard error of mean (SEM). The significance values were calculated using standard statistical methods. Students t test, paired t tests, one tailed or two tailed t tests were used depending on the hypothesis for a given experiment. ANOVA was used to calculate the significance values following the t tests.

Results

Maternal bgm expression is required for embryogenesis

To determine whether defects in lipid metabolism lead to defects in Drosophila development, we assessed the consequences of loss-of-function for the homologous ACS genes *bgm* and *dbb*, alone and in combination. Lethal stage analysis of *bgm, dbb,* and *bgm dbb* maternal-zygotic null animals revealed that whereas *dbb* homozygotes derived from *dbb* homozygous females are fully viable, *bgm* and *bgm dbb* maternal-zygotic nulls suffer an incompletely penetrant lethality that is confined to the embryonic stage of development. ~40% of both *bgm* and *bgm dbb* maternal-zygotic nulls suffer embryonic death, indicating that loss of *bgm* is sufficient to cause lethality in both *bgm* and *bgm dbb* animals, and *bgm*, but not *dbb*, plays an essential developmental role in Drosophila embryogenesis.

Embryonic transcription profiles for *bgm* and *dbb* are largely overlapping, although only *bgm* is deposited maternally (**Figure 2.1B**). Given that only *bgm* is required for embryogenesis, we postulated that *bgm*-dependent lethality results from a strictly maternal effect. Standard genetic methods were employed to generate embryos lacking either maternal or zygotic *bgm* only. Consistent with our hypothesis, we found that only embryos deficient in maternal *bgm* (either as bgm^{1} homozyotes or bgm^{1} in Trans to deficiency) suffer an embryonic lethality (**Figure 2.1C)**. In both cases lethality occurred in 40% of embryos, confirming previous reports that that bgm^{1} allele is a null (Min and Benzer 1999; Sivatchenko

and Letsou, in prep). Ubiquitous expression of UAS: *bgm*-FLAG using a Tubulin:Gal4 driver rescued embryonic lethality in embryos derived from *bgm* homozygous females (**Figure 2.1D**).

The Bgm ACSL is required in cellularization for targeting of Rab 5 and Rab 11 vesicles to the growing furrow canals

To identify the developmental process that requires *bgm*, we visualized development in live embryos (**Figure 2.1A**). Using time lapse microscopy, we documented failures in cellularization in 40% of embryos derived from *bgm* females. Embryos which successfully undergo cellularization complete embryogenesis and eventually hatch into larvae. However, embryos that fail to complete cellularization, also fail to progress in embryogenesis and abort development soon after. Therefore, failed cellularization accounts for all the embryonic lethality observed in lethal stage analyses (**Figure 2.2A**). Moreover, although *bgm*-dependent lethality is incompletely penetrant, it results from a block at a single embryonic stage very early in development. This is only the second demonstration of a specific defect attributable to loss-of-function of a single very long chain fatty acidy acyl-coA synthase (Kniazeva et al., 2012). In Drosophila, cellularization occurs via multiple well-characterized steps (Lecuit, 2004). After 14 cycles of nuclear division, the 6000 zygotic nuclei move to the periphery of the embryo, immediately adjacent to the embryo plasma membrane. Columnar membrane structures called furrow canals ingress between aligned

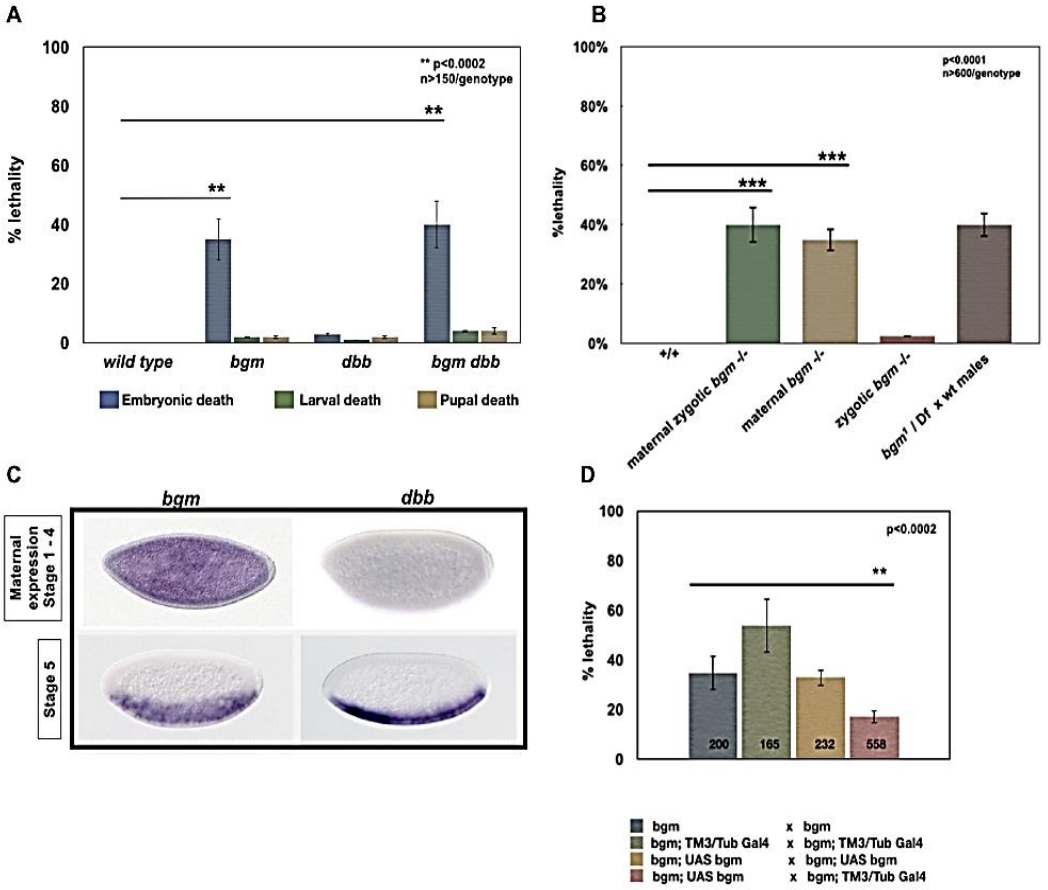

Figure 2.1: *bgm* is required maternally for embryogenesis.

A. Lethal stage analysis of wild type and maternal zygotic loss of function mutants in *bgm, dbb,* and *bgm dbb*.

B. Analysis of embryonic lethality in wild type embryos and embryos lacking either maternal zygotic, maternal, or zygotic *bgm.* Embryos derived from a crossing of bgm^{1}/Df females with wild type males were also analyzed.

C. Maternal and Zygotic expression patterns of *bgm* and *dbb* transcripts.

D. Rescue of embryonic lethality in bgm^{1} embryos using a UAS:*bgm* FLAG transgene driven by a ubiquitous Tubulin::GAL4 driver.

nuclei. Membrane addition via vesicle fusion increases furrow canal length. After about 60 minutes, bilateral furrow canals fuse at the base of the nuclei; thereby each nucleus is enclosed within a contained cellular space. In *bgm*-deficient embryos, all steps preceding the extension of furrow canals occur normally. Nuclei move to the periphery of the embryo and align, and furrow canal formation initiates. Once initiated, however, furrow canals fail to extend (**Figure 2.2A**).

Next we used the junction protein Armadillo/B-catenin to monitor furrow progression during cellularization. At the onset of cellularization, Armadillo is localized in an apicolateral position with respect to the nuclei. In contrast, with completion of cellularization 3 hours after egg lay (AEL), Armadillo constitutes adherence junctions that are localized bilaterally along the furrow canals as they help the newly formed lateral membranes to be held tightly together. Spaghetti squash (Sqh)/Nonmuscle Myosin-2 and filamentous Actin (F-Actin) produce the contractile force initially needed for extension of furrow canals and later for the fusion of the basal membranes. In congruence with their functional requirement, both these components are localized apicolaterally at the beginning of cellularization and basally with respect to the nuclei towards the completion of cellularization.

In *bgm*-deficient embryos, Armadillo and F-Actin fail to localize appropriately on completion of cellularization; instead, the proteins are distributed in a diffuse pattern apically, which is reminiscent of earlier stages of cellularization (**Figure 2.2B, 2.2C**). Mislocalization does not merely represent a delay in cellularization because these proteins fail to localize appropriately in

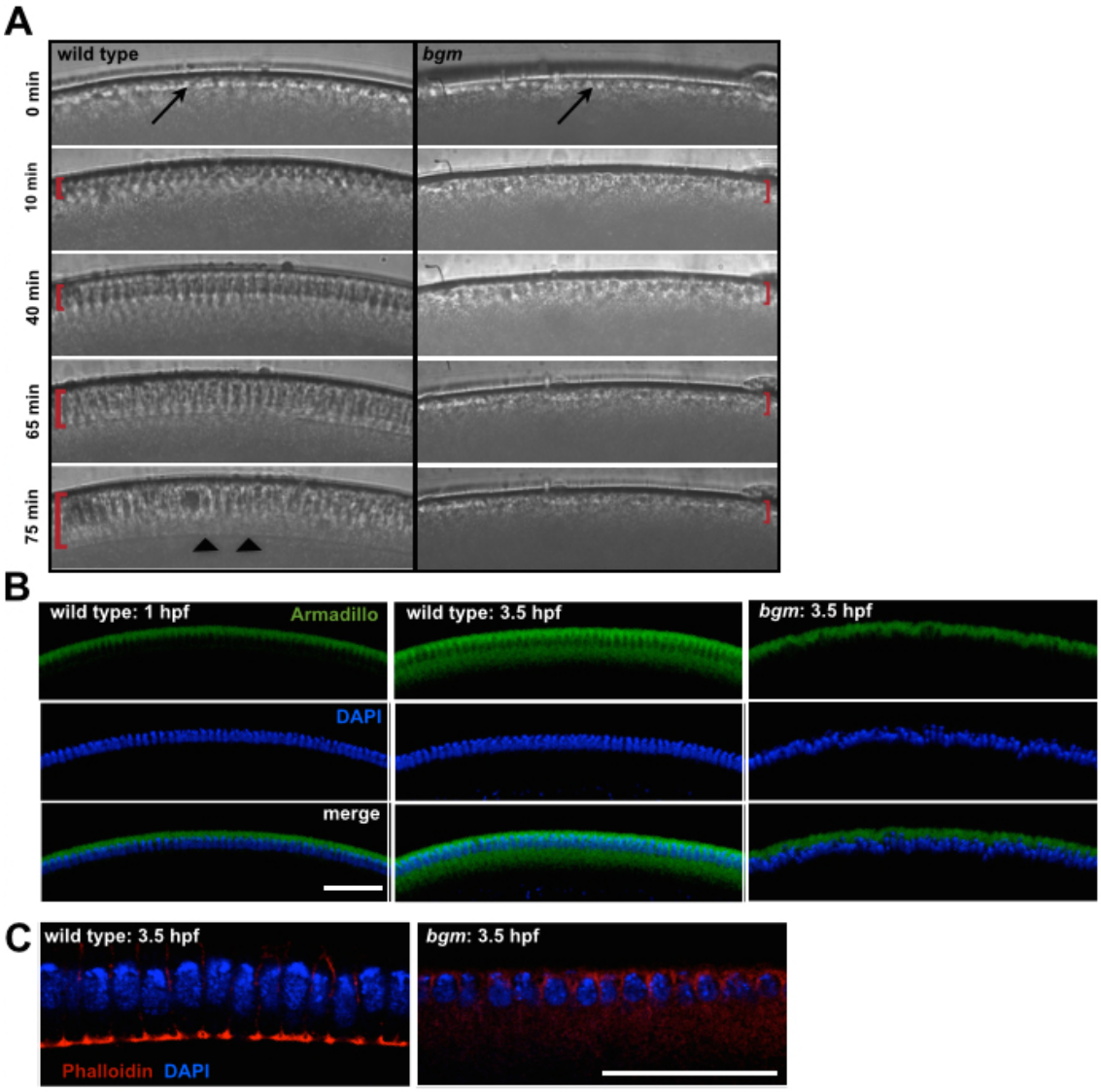

Figure 2.2: The Bgm LACS is required for cellularization during embryogenesis.

A. Analysis of furrow canal progression during cellularization in wild type and *bgm*^{1} embryos using time lapse phase contrast microscopy.

B. Localization of Armadillo (Green) and blastoderm nuclei(Blue) in wild type embryos (0 hours post fertilization (hpf) and 3.5 hpf) and *bgm*^{1} embryos (3.5 hpf).

C. Localization of filamentous actin and Spaghetti Squash (Nonmuscle myosin)(Red) along with blastoderm nuclei (Blue) in wild type and *bgm*^{1} embryos 3.5 hpf.

bgm-deficient embryos even after an extended time period, and the embryos ultimately abort their development. These data confirm and extend results from our phase contrast studies indicating that *bgm* embryos suffer from an incomplete furrow extension that leads subsequently to a failure in cellularization.

The extension of furrow canals during cellularization requires enormous amounts of membrane addition, the majority of which is recycled from the embryonic plasma membrane using the endocytic machinery of the embryo. Rab 5 and Rab 11 GTPases, important members of the endocytic pathway, have been shown to be necessary to the process of furrow extension during cellularization (Lecuit, 2004). We have illustrated in the present study that 40% of *bgm* embryos fail to complete cellularization due to a failure in furrow extension. Given these data, we hypothesized that vesicle biogenesis and/or vesicle targeting is impaired in *bgm*-deficient embryos, and this leads to a failure in cellularization in 40% of the embryos. We tested this hypothesis by immunostaining of wild type and *bgm* embryos using antibodies directed against the Rab-5 protein to visualize vesicles. We observed a 2-fold increase in the number of membranous particles positive for Rab-5 in *bgm*-deficient embryos as compared to wild type. Additionally, we observed enlarged Rab-5 positive compartments in the *bgm*-deficient embryos, but not in their wild type counterparts. We suspect the later to be large vesicular bodies, also observed by Sheckman et al. (Wuestehube et al., 1996) in their yeast vesicle targeting mutants. Lastly, we observed that whereas the cellularization zone in wild-type embryos is divided into areas of dense (basally) and sparse (apically) vesicle

distributions, such a partition of vesicles is absent in the cellularization zone in *bgm* embryos. We see the above defects in 65% of *bgm* embryos. These data imply that in the absence of the *bgm* gene product, proper targeting of Rab-5 tagged vesicles does not occur, and this prevents the extension of furrow canals leading to a failure of cellularization. Similar experiments to visualize the localization of the Rab-11 GTPase are currently underway.

Maternally deposited bgm is necessary for neuromuscular development in the embryo

Bgm is clearly essential for embryogenesis, yet only 40% of *bgm* maternal zygotic null animals suffer embryonic lethality, and the remaining 60% animals survive to adulthood. Data in the following sections demonstrate that although 60% of *bgm* homozygous animals do not suffer from embryonic lethality, they do demonstrate developmental abnormalities that do not result in lethality. Thus, 100% of *bgm* homozygous embryos suffer from developmental defects to different degrees of severity. Bgm expression throughout embryogenesis, combined with cellularization defects in a fraction of the embryos that survived, led us to speculate that loss of *bgm* might have additional developmental consequences (**Figure 2.3**). Additionally, during lethal stage analyses, we noticed that although all hatched larvae survived to adulthood, they were sluggish in their movements in comparisons to wild type. We used three motor assays to characterize and quantify these effects. First, we quantified the rate of

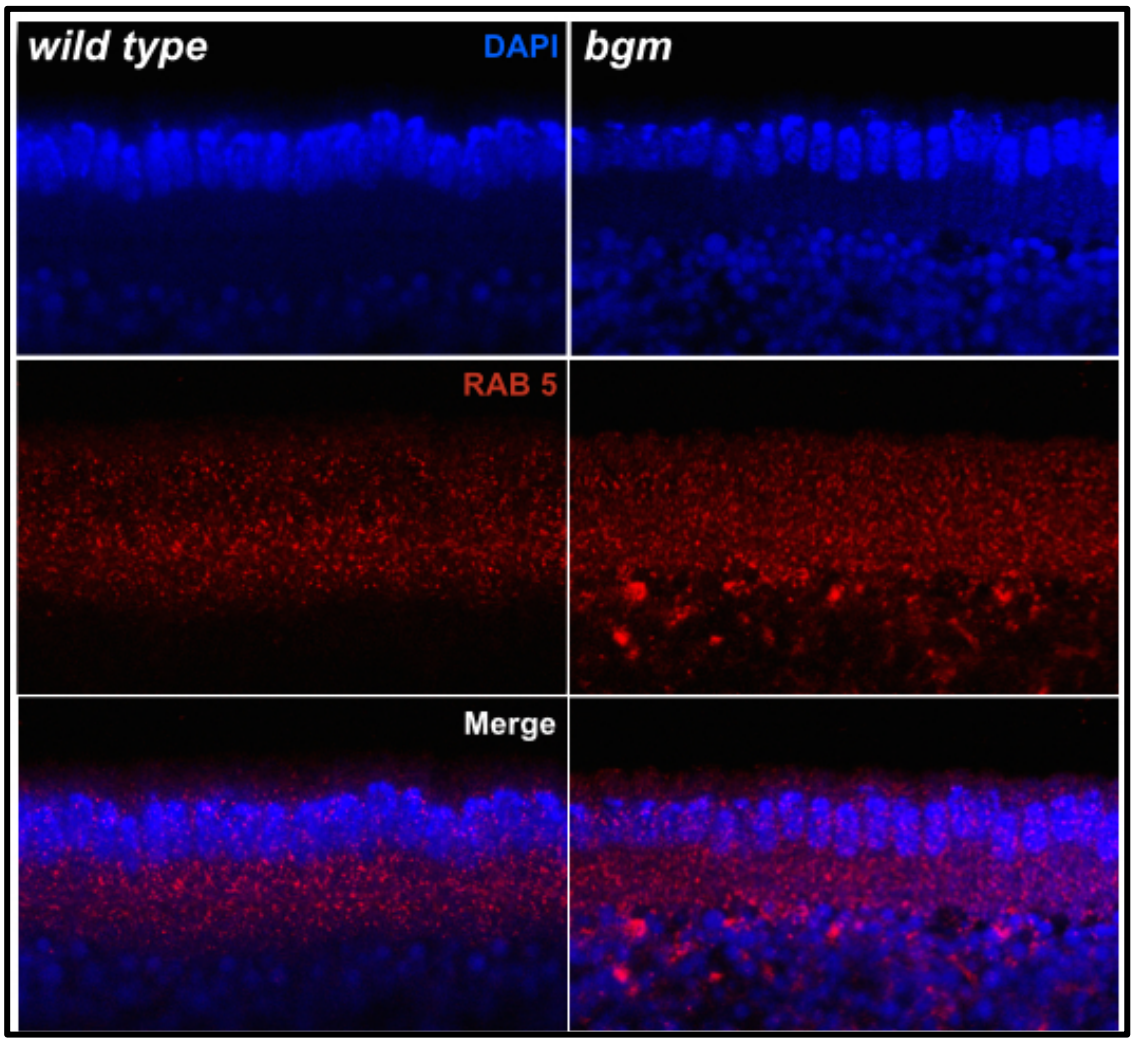

Figure 2.3: Rab 5 vesicles are mistargeted in *bgm*^{1} mutants.

Rab 5 marked vesicles (Red) are partitioned into a clear area of sparse (apical) and dense (basal) distribution with respect to the nuclei (Blue) in wild type cellularizing embryos. This differential distribution of vesicles is lost in *bgm*^{1} embryos, along with an increase in total vesicle number accompanied by an increase in large vesicular bodies. We observe all of these vesicular defects in all affected embryos and hence, they appear to be related to each other and to the primary causative event.

peristalsis at the time of hatching of wild type and *bgm*-deficient animals. In hatching wild type 1st instar larvae, one peristaltic wave takes less than 1 second to reach from the most posterior to the anterior most segments. However, in case of *bgm* larvae, the movement is asynchronous and variable and takes a significantly longer time (5–10 seconds) to propagate across the animal (n = 50/genotype). Second, we measured head nods per minute in 2nd instar larvae. *bgm* larvae performed approximately 40 nods/minute compared to wild type larvae, which performed 100 nods/minute (n = 50/genotype, **Figure 2.4B**). Third, we measured the time taken by L3 larvae to roll over and move away after placing them with their dorsal side down. We find that while wild type L3 larvae take about 5–7 seconds to roll over, *bgm* larvae take 15–20 seconds to do the same (n = 100/genotype, **Figure 2.4C**). Thus, all three of the above assays at each larval stage demonstrate that loss of *bgm* during larval development leads to a 50% loss of motor function.

These data made us ask the obvious question: are these severe motor impairments caused by defects in the neuromusculature of the larvae or do they result from a defect elsewhere in the system? Based on microscopic analysis during the peristalsis assay, we find that the nervous system of larvae is severely compromised (data not shown).

We see that neuromuscular and behavioral defects in *bgm* maternal zygotic mutant larvae persist throughout larval development **(Figure 2.4A**). Maternally expressed *bgm* is required for early embryonic development, while zygotic expression of *bgm* seems to be required for maintenance of the adult

nervous system (Sivachencko et al., unpublished). We wondered if the larval phenotypes in *bgm* maternal zygotic mutants are of maternal or zygotic origin. Also, do they arise due to a developmental abnormality, or are they early signs of neurodegeneration that we and Benzer et al. (Min and Benzer, 1999) have seen in the adult *bgm* flies? Interestingly, we find that based on preliminary experiments, the behavioral and neuromuscular defects in *bgm* 1st instar larva arise from maternal loss of *bgm* **(Figure 2.4D)**, whereas preliminary data suggests that the behavioral defects observed in 2nd and 3rd instar *bgm* larvae arise from loss of zygotic *bgm* (data not shown).

The early embryonic phenotype in *bgm* embryos, where we see defects in cellularization and finally a failure to complete embryogenesis, arises from a loss of maternal *bgm* expression **(Figure 2.1C).** Given that the larval motor defects in *bgm* mutants are also maternal in nature, it is highly likely that these behavioral defects arising from defects in the neuromusculature have an embryonic origin. We tested this hypothesis by using distinct markers to look at different stages of neuronal development in the embryo. We began our analysis at the earliest stage of neuronal development in the embryo and worked our way through later developmental stages. Neuroblasts first delaminate and appear in the ventral region of the embryo at Stage 9 and express the Singleminded (SIM) protein. Upon immunostaining Stage 9 embryos with an antibody against Singleminded, we found 1) the arrangement of neuroblasts in *bgm* embryos was highly disorganized and 2) the neuroblasts were reduced in number by 25–30% (180–185 neuroblasts) compared to wild type embryos (225 neuroblasts). On further

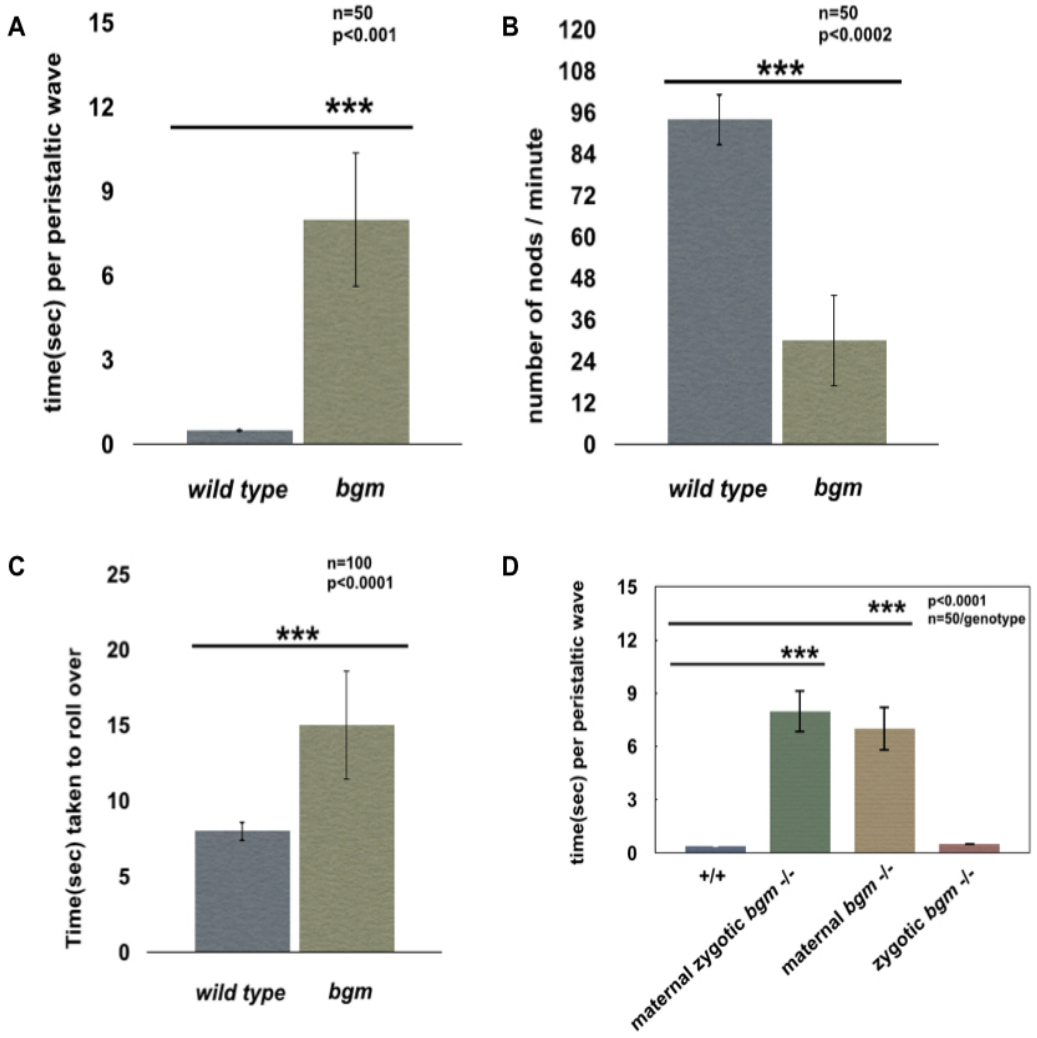

Figure 2.4: Early larval movement defects in *bgm*^{1} embryos are maternal in origin.

A. Measurement of time taken in seconds for a single propagation of the peristaltic wave in wild type and *bgm*^{1} 1st instar larvae.

B. Measurement of head nods/minute in wild type and *bgm*^{1} 2nd instar larvae.

C. Measurement of time taken in seconds to roll over in wild type and *bgm1* 3rd instar larvae.

D. Measurement of time taken in seconds for propagation of a peristaltic wave in wild type 1st instar larvae and *bgm*^{1} *1*st larvae lacking either maternal zygotic, maternal only, or zygotic *bgm,* respectively.

analysis, we find that defects in the nervous system—including absence of neuronal cell bodies and missing and/or mistargeted axons—continue to appear through later developmental stages. Immunostaining with an antibody against the protein Roundabout (Robo) at stage 13 and the antibody BP102 at stage 17, reveals severe defects in the axonal tracts of the central nervous system (**Figure 2.5A'**). Specifically, in the stage 17 CNS of *bgm* embryos we observe breaks in the longitudinal fascicles as well as the commissures. In addition the commissures are frequently disorganized and cross over incorrectly. Thus we do not see the ladder like pattern in the CNS of *bgm* embryos, which is typically observed in wild type embryos at this stage. Upon staining the stage 17 peripheral nervous system using the mAb22c10/anti-Futch antibody, we find that in *bgm* embryos a large number of neuronal cell bodies are missing or mislocalized, in addition to defasciculation of the axon bundles arising from the four sets of cell bodies in each segment (**Figure 2.5B**). Both the CNS and PNS of *bgm* embryos have severe defects, and the phenotype is variably expressed. The number of segments affected as well as the severity of the axonal defects in each segment varies between *bgm* embryos. Upon quantification, a total of approximately 45% of *bgm* embryos (n = 300/genotype for each assay) show the above described phenotypes in the CNS and PNS. Significantly, we observe that the nervous system defects (**Figure 2.5A)** are always accompanied by defects in the musculature (**Figure 2.5C**). All neurons in the CNS and PNS arise as a result of mitotic cell divisions of the neuroblasts, and so any defects in neuroblast formation or division should result in missing or mislocalized neurons. Absence

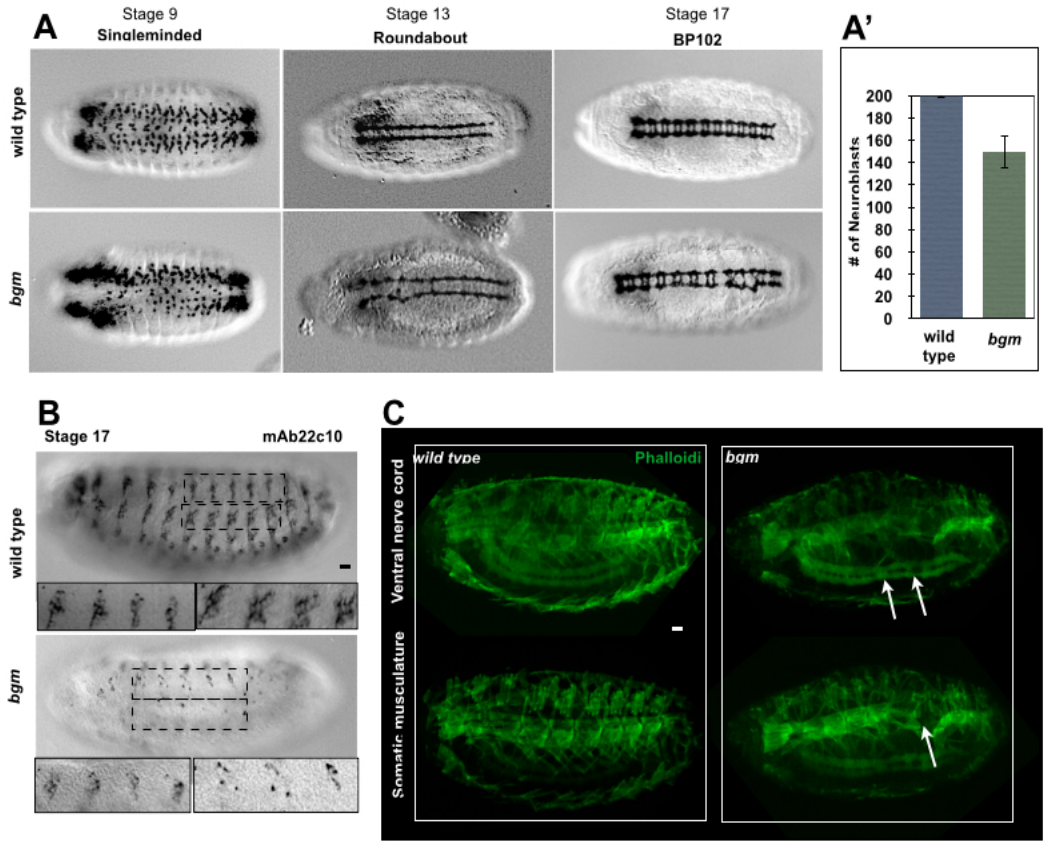

Figure 2.5: Movement defects in *bgm*^{1} 1st instar larvae have an embryonic origin.

A. Wild type and *bgm*^{1} embryos at stage 9, 13, and 17, respectively, stained using antibodies against Singleminded, Roundabout, and BP102.

A' Measurement of neuroblast number in stage 9 wild type and *bgm*^{1} embryos stained with an antibody against Singleminded.

B. Analysis of defects in the peripheral nervous system of wild type and *bgm*^{1} stage 16 embryos using an anti-Futch antibody (mAb22c10).

C. Analysis of neuromuscular defects in wild type and *bgm*^{1} stage 17 embryos by staining filamentous actin in these tissues. Filamentous actin has been stained using phalloidin (Green). Ventral nerve cord seen in slightly ventralized views and the somatic musculature in lateral views.

or ectopic location of neurons may result in disorganization of the newly formed axonal architecture. We tested the possibility of mitotic phenotypes being correlated to the axonal phenotypes by colabeling embryos with the neuronal nuclear marker Singleminded and the F-Actin stain Phalloidin to mark the axons. DAPi was used as a general nuclear stain. Upon analysis, we found that reduced and disorganized neuronal nuclei in *bgm* embryos spatially coincide with a disorganized and/or damaged axonal architecture. It appears that in the absence of a given set of neurons, the nervous system of *bgm* embryos is unable to develop normally and results into a disorganized axonal architecture, presumably as the nervous system tries to compensate for the missing neurons. However, these data are correlative and not definitive. Live imaging or fate mapping of neuroblasts will be required to confirm the above described results. Neuroblasts in the Drosophila embryo are specified during the process of gastrulation, which follows immediately after completion of cellularization. Cellularization requires maternal expression of *bgm,* and the process of gastrulation requires many maternally expressed genes in addition to zygotic expression. Thus, it remained to be determined whether the defects in neuroblast formation and neuronal architecture arise from maternal and/or zygotic loss of *bgm.* In order to distinguish between the two possibilities, we employed standard genetic methods to generate embryos lacking either maternal or zygotic *bgm* only. Loss of maternal *bgm* accounts entirely for the neuromuscular defects (**Figure2.6A**) observed in the *bgm* maternal zygotic mutants while zygotic loss of *bgm* has no significant effect (**Figure 2.6B**). In our close examination of embryos derived

from *bgm* females, we find that 40% embryos fail to complete cellularization and abort embryogenesis. However, a subset of the remaining 60% embryos show huge cells with incompletely formed cell membranes as seen using the membrane stain Neurotactin **(Figure 2.6C).** Additionally, during our time lapse imaging experiments to study the process of cellularization, we find that a small percentage of *bgm* mz embryos undergo only a partial failure in cellularization (data not shown). These defects in cellularization are localized to a small area of the embryo, and they do not cause the embryos to abort development. It is possible that these subtle defects in cellularization lead to the neuronal defects observed in *bgm* embryos.

Discussion

In the present study we have demonstrated the requirement of maternally deposited *bgm* transcripts for furrow extension during Drosophila cellularization. Additionally, membrane vesicles, which are known to be absolutely critical to furrow extension, have been shown to be mislocalized in bgm^{1} mutant embryos. These data suggest that the failure in cellularization is due to defects in vesicle targeting. The extension of furrow canals during cellularization requires enormous amounts of membrane addition. This membrane is only partially synthesized *de novo* with the majority of it recycled from the embryonic plasma membrane via endocytosis (Strickland and Burgess, 2004). Endocytosed vesicles harbor the GTPase Rab-5 and these Rab-5 vesicles are then targeted to and fuse with the recycling endosome (RE). Membrane components that will

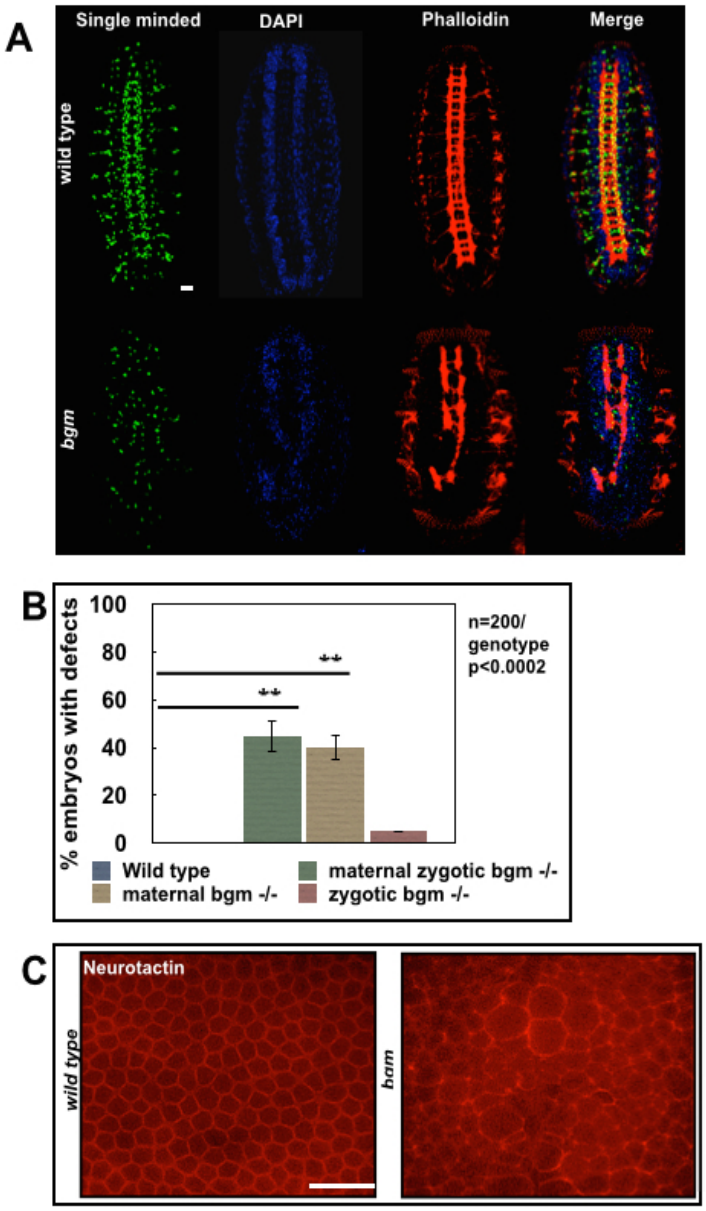

Figure 2.6: Defects in the embryonic nervous system of *bgm1* embryos have a maternal origin.

A. Coimmunostaining of neuroblasts (Singleminded, Green) and axonal tracts (Phalloidin, Red) to determine origin of axonal defects.

B. Analysis of neuronal defects using BP102 staining in wild type Stage 16 embryos and *bgm1* stage 16 embryos lacking either maternal zygotic, maternal, or zygotic *bgm.*

C.Membrane staining in stage 9 wild type and *bgm 1* embryos using anti-Neurotactin (Red) to demonstrate the presence of huge cells resulting from partial acellularization in *bgm1* embryos.

impart apicobasal polarity to the newly assembled cellular membranes postcellularization are also delivered to the RE via vesicular traffic from the Golgi. Here, in the RE, vesicles are repackaged with a defined set of cellular components intended for specific cellular compartments. The reconstituted vesicles contain GTPases in the vesicular membrane, and these GTPases function to 1) assign a specific identity to each set of vesicles and 2) target them to specific cellular compartments. One particular class of vesicles harbors the Rab-11 GTPase on the vesicle surface, and fusion of these Rab 11 vesicles at the growing end drives the rapid extension of furrow canals during cellularization (Lecuit and Pelissier, 2003). The Rab-11 GTPase directs these vesicles to the furrow canals and thus allows furrow extension. However, it is not understood as to how Rab 5 tagged vesicles are specifically targeted to the RE and the Rab 11 tagged vesicles from the RE are targeted to the growing furrow canals. Data presented in the current study demonstrate the requirement of the Bgm Long chain Acyl CoA synthase for targeting of Rab-5 vesicles during cellularization. Molecules acting as “Identifier signals” for subcellular vesicular targeting have been largely elusive to cell biologists, and our research is an important first step towards the discovery of such molecules. Based on the data presented in this study, we hypothesize that activated long chain fatty acids themselves or via interaction with specific proteins provide the identifier signal that directs Rab5 vesicles to the recycling endosome and Rab 11 vesicles to the growing furrow canals. In the absence of the Bgm ACS, these activated LCFAs are missing and so is the identifier signal.

Ours is only the second study to show the requirement of an Acyl CoA synthase in a specific developmental process. Kniazeva et al. have demonstrated the requirement of *C. elegans* Acsl-1 in cytokinetic processes during mitotic cell divisions in *C. elegans* embryogenesis. Additionally, a few studies in Drosophila have implicated enzymes required for lipid biogenesis and modification in cytokinesis of spermatocytes (Routt and Bankaitis, 2004). Thus there is emerging evidence for the role of Long chain fatty acids and enzymes required for their biogenesis in cytogenetic processes. We also provide evidence for the role of the Bgm ACSL in neurogenesis. The maternal requirement of *bgm* for the processes of neuroblast formation and axonogenesis suggests that these defects are related to a partial failure in cellularization. It is possible that failure in cellularization leads to a failure in proper cell fate specification, thus leading to defects in neurogenesis (**Figure 2.7)**. However, experiments to obtain direct evidence to prove this hypothesis are underway. It is also possible that there is an independent requirement for LCFAs and the Bgm ACS for neurogenesis and axonogenesis. In either case, our findings are significant in light of the many clinical studies suggestive of a requirement of LCFAs and VLCFAs in neuronal development. Several studies have implicated an imbalance in LCFAs and VLCFAs in neurodevelopmental disorders like Dyslexia, Dyspraxia, Schizoaffective disorder, and Autism Spectrum Disorders (ASD) (Das, 2013; Schuchardt et al., 2010). All of these disorders have overlapping neurological symptoms accompanied by a dysfunctional immune response (Ward, 2000). Neuronal and glial membranes are very rich in LCFAs and VLCFAs and hence,

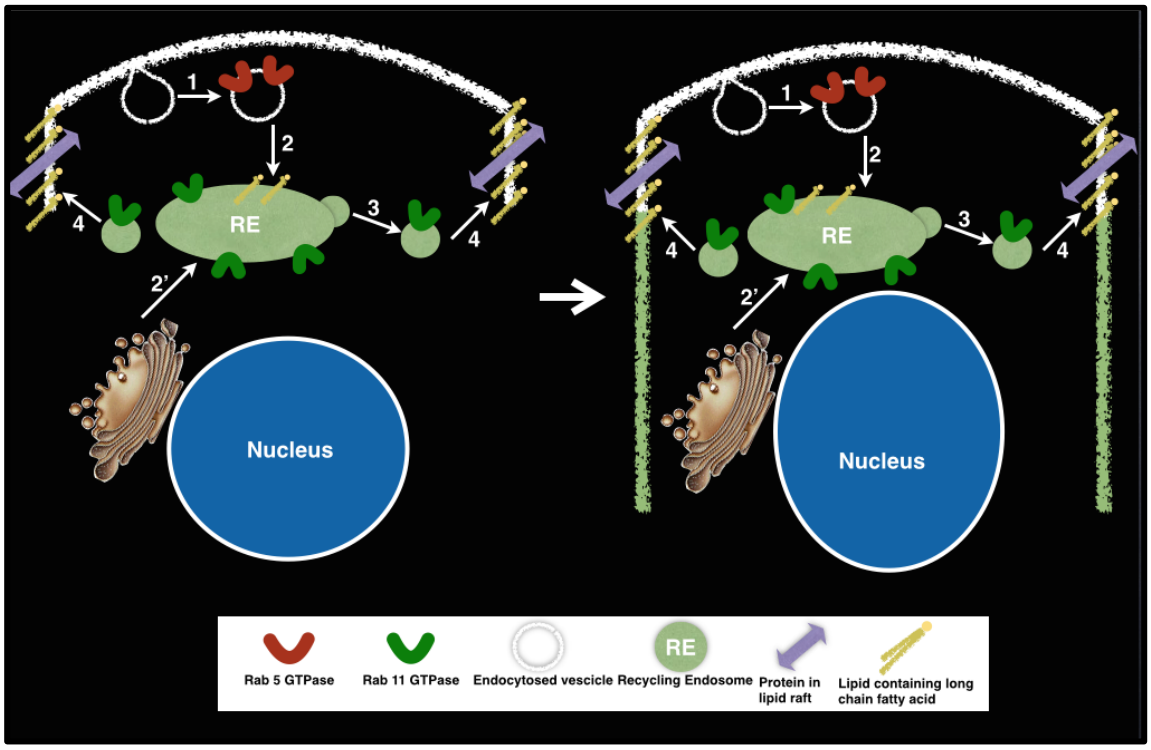

Figure 2.7: Model for the role of Bgm ACS and Long Chain Fatty Acids in cellularization and vesicle targeting.

Endocytosed vesicles from the apical surface of the embryo are tagged with Rab5 GTPase(1). These vesicles are targeted towards the recycling endosome (2, RE) and use LCFAs or LCFA associated proteins as a signal to identify the RE compartment. New membrane components from the Golgi apparatus are also delivered to the RE(2'). Reconstituted vesicles from the RE are tagged with Rab11 GTPase and targeted towards the growing furrow canals during cellularization (3). LCFAs or LCFA associated proteins in the growing furrow canals are identified by Rab 11 GTPase, causing the Rab11 vesicles to fuse with the furrow canals.

the nervous system is very sensitive to any changes in fatty acid metabolism (Laycock et al., 2007). In addition, a large number of signaling molecules required by the immune system are lipid based. These findings in association with clinical studies, are very suggestive of a suboptimal lipid metabolism in patients with neurodevelopmental disorders. Our findings are a significant first step towards a successful genetic model to understand the mechanistic role of LCFAs/VLCFAs and ACSLs in neuronal development. Further research in this direction will provide promising avenues for targeted treatment strategies, which are unavailable at the moment.

References

Abumrad, N., Harmon, C., and Ibrahimi, A. (1998a). Membrane transport of long-chain fatty acids: evidence for a facilitated process. J. Lipid Res. *39*, 2309–2318.

Abumrad, N., Harmon, C., and Ibrahimi, A. (1998b). Membrane transport of long-chain fatty acids: evidence for a facilitated process. J. Lipid Res. *39*, 2309–2318.

Abumrad, N., Coburn, C., and Ibrahimi, A. (1999). Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. Biochim. Biophys. Acta *1441*, 4–13.

Agostoni, C., and Bruzzese, M.G. (1992). [Fatty acids: their biochemical and functional classification]. Pediatr. Med. Chir. *14*, 473–479.

Andersen, O.S., and Koeppe, R.E. (2007). Bilayer thickness and membrane protein function: an energetic perspective. Annu. Rev. Biophys. Biomol. Struct. *36*, 107–130.

Barabas, P., Liu, A., Xing, W., Chen, C.-K., Tong, Z., Watt, C.B., Jones, B.W., Bernstein, P.S., and Križaj, D. (2013). Role of ELOVL4 and very long-chain polyunsaturated fatty acids in mouse models of Stargardt type 3 retinal degeneration. Proc. Natl. Acad. Sci. U.S.A. *110*, 5181–5186.

Bartscherer, K., and Boutros, M. (2008). Regulation of Wnt protein secretion and its role in gradient formation. EMBO Rep. *9*, 977–982.

Berger, J., Forss-Petter, S., and Eichler, F.S. (2014). Pathophysiology of X-linked adrenoleukodystrophy. Biochimie *98*, 135–142.

Black, P.N., and DiRusso, C.C. (2003). Transmembrane movement of exogenous long-chain fatty acids: proteins, enzymes, and vectorial esterification. Microbiol. Mol. Biol. Rev. *67*, 454–472, table of contents.

Black, P.N., Zhang, Q., Weimar, J.D., and DiRusso, C.C. (1997). Mutational analysis of a fatty acyl-coenzyme A synthetase signature motif identifies seven amino acid residues that modulate fatty acid substrate specificity. J. Biol. Chem. *272*, 4896–4903.

Bogdanov, M., Dowhan, W., and Vitrac, H. (2014). Lipids and topological rules governing membrane protein assembly. Biochim. Biophys. Acta *1843*, 1475–1488.

Buglino, J.A., and Resh, M.D. (2012). Palmitoylation of Hedgehog proteins. Vitam. Horm. *88*, 229–252.

Cao, Y., Pearman, A.T., Zimmerman, G.A., McIntyre, T.M., and Prescott, S.M. (2000). Intracellular unesterified arachidonic acid signals apoptosis. Proc. Natl. Acad. Sci. U.S.A. *97*, 11280–11285.

Castro, B.M., Prieto, M., and Silva, L.C. (2014). Ceramide: a simple sphingolipid with unique biophysical properties. Prog. Lipid Res. *54*, 53–67.

Chiu, H.C., Kovacs, A., Ford, D.A., Hsu, F.F., Garcia, R., Herrero, P., Saffitz, J.E., and Schaffer, J.E. (2001). A novel mouse model of lipotoxic cardiomyopathy. J. Clin. Invest. *107*, 813–822.

Coleman, R.A., Lewin, T.M., Van Horn, C.G., and Gonzalez-Baró, M.R. (2002). Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? J. Nutr. *132*, 2123–2126.

D'Souza, K., and Epand, R.M. (2014). Enrichment of phosphatidylinositols with specific acyl chains. Biochim. Biophys. Acta *1838*, 1501–1508.

Das, U.N. (2013a). Autism as a disorder of deficiency of brain-derived neurotrophic factor and altered metabolism of polyunsaturated fatty acids. Nutrition *29*, 1175–1185.

Fuchs, S., Sarde, C.O., Wedemann, H., Schwinger, E., Mandel, J.L., and Gal, A. (1994). Missense mutations are frequent in the gene for X-chromosomal adrenoleukodystrophy (ALD). Hum. Mol. Genet. *3*, 1903–1905.

Gargiulo, C.E., Stuhlsatz-Krouper, S.M., and Schaffer, J.E. (1999). Localization of adipocyte long-chain fatty acyl-CoA synthetase at the plasma membrane. J. Lipid Res. *40*, 881–892.

Gassler, N., Schneider, A., Kopitz, J., Schnölzer, M., Obermüller, N., Kartenbeck, J., Otto, H.F., and Autschbach, F. (2003). Impaired expression of acyl-CoA-synthetase 5 in epithelial tumors of the small intestine. Hum. Pathol. *34*, 1048–1052.

Gassler, N., Roth, W., Funke, B., Schneider, A., Herzog, F., Tischendorf, J.J.W., Grund, K., Penzel, R., Bravo, I.G., Mariadason, J., et al. (2007). Regulation of enterocyte apoptosis by acyl-CoA synthetase 5 splicing. Gastroenterology *133*, 587–598.

Gatto, C.L., and Broadie, K. (2011). Drosophila modeling of heritable neurodevelopmental disorders. Curr. Opin. Neurobiol. *21*, 834–841.

Gjorgjieva, J., Berni, J., Evers, J.F., and Eglen, S.J. (2013). Neural circuits for peristaltic wave propagation in crawling Drosophila larvae: analysis and modeling. Front Comput. Neurosci. *7*, 24.

Glatz, J.F.C., Luiken, J.J.F.P., and Bonen, A. (2010). Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol. Rev. *90*, 367–417.

Goñi, F.M., and Alonso, A. (1999). Structure and functional properties of diacylglycerols in membranes. Prog. Lipid Res. *38*, 1–48.

Hage Hassan, R., Bourron, O., and Hajduch, E. (2014). Defect of insulin signal in peripheral tissues: important role of ceramide. World J. Diabetes *5*, 244–257.

Hall, A.M., Smith, A.J., and Bernlohr, D.A. (2003). Characterization of the Acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. J. Biol. Chem. *278*, 43008–43013.

Hatch, G.M., Smith, A.J., Xu, F.Y., Hall, A.M., and Bernlohr, D.A. (2002). FATP1 channels exogenous FA into 1,2,3-triacyl-sn-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells. J. Lipid Res. *43*, 1380–1389.

Heimli, H., Hollung, K., and Drevon, C.A. (2003). Eicosapentaenoic acid-induced apoptosis depends on acyl CoA-synthetase. Lipids *38*, 263–268.

Heinzer, A.K., McGuinness, M.C., Lu, J.-F., Stine, O.C., Wei, H., Van der Vlies, M., Dong, G.-X., Powers, J., Watkins, P.A., and Smith, K.D. (2003). Mouse models and genetic modifiers in X-linked adrenoleukodystrophy. Adv. Exp. Med. Biol. *544*, 75–93.

Ho, K.S., and Scott, M.P. (2002). Sonic hedgehog in the nervous system: functions, modifications and mechanisms. Curr. Opin. Neurobiol. *12*, 57–63.

Jia, Z., Pei, Z., Li, Y., Wei, L., Smith, K.D., and Watkins, P.A. (2004). X-linked

adrenoleukodystrophy: role of very long-chain acyl-CoA synthetases. Mol. Genet. Metab. *83*, 117–127.

De Jong, H., Neal, A.C., Coleman, R.A., and Lewin, T.M. (2007). Ontogeny of mRNA expression and activity of long-chain acyl-CoA synthetase (ACSL) isoforms in Mus musculus heart. Biochim. Biophys. Acta *1771*, 75–82.

Kantojärvi, K., Kotala, I., Rehnström, K., Ylisaukko-Oja, T., Vanhala, R., von Wendt, T.N., von Wendt, L., and Järvelä, I. (2011). Fine mapping of Xq11.1-q21.33 and mutation screening of RPS6KA6, ZNF711, ACSL4, DLG3, and IL1RAPL2 for autism spectrum disorders (ASD). Autism Res. *4*, 228–233.

Klar, J., Schweiger, M., Zimmerman, R., Zechner, R., Li, H., Törmä, H., Vahlquist, A., Bouadjar, B., Dahl, N., and Fischer, J. (2009). Mutations in the fatty acid transport protein 4 gene cause the ichthyosis prematurity syndrome. Am. J. Hum. Genet. *85*, 248–253.

Klaus, C., Jeon, M.K., Kaemmerer, E., and Gassler, N. (2013a). Intestinal acyl-CoA synthetase 5: activation of long chain fatty acids and behind. World J. Gastroenterol. *19*, 7369–7373.

Klaus, C., Kaemmerer, E., Reinartz, A., Schneider, U., Plum, P., Jeon, M.K., Hose, J., Hartmann, F., Schnölzer, M., Wagner, N., et al. (2014). TP53 status regulates ACSL5-induced expression of mitochondrial mortalin in enterocytes and colorectal adenocarcinomas. Cell Tissue Res. *357*, 267–278.

Kniazeva, M., Shen, H., Euler, T., Wang, C., and Han, M. (2012a). Regulation of maternal phospholipid composition and IP(3)-dependent embryonic membrane dynamics by a specific fatty acid metabolic event in C. elegans. Genes Dev. *26*, 554–566.

Küch, E.-M., Vellaramkalayil, R., Zhang, I., Lehnen, D., Brügger, B., Sreemmel, W., Ehehalt, R., Poppelreuther, M., and Füllekrug, J. (2014). Differentially localized acyl-CoA synthetase 4 isoenzymes mediate the metabolic channeling of fatty acids towards phosphatidylinositol. Biochim. Biophys. Acta *1841*, 227–239.

Laycock, R., Crewther, S.G., and Crewther, D.P. (2007). A role for the “magnocellular advantage” in visual impairments in neurodevelopmental and psychiatric disorders. Neurosci. Biobehav. Rev. *31*, 363–376.

Lecuit, T. (2004). Junctions and vesicular trafficking during Drosophila cellularization. J. Cell. Sci. *117*, 3427–3433.

Lecuit, T., Pelissier, A., and Chauvin, J.P. (2003). Trafficking through Rab 11 endosomes is required for cellularization during Drosophila embryogenesis. J. Cell. Sci. *109*, 51-57.

Lécuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., and Krause, H.M. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell *131*, 174–187.

Li, L.O., Mashek, D.G., An, J., Doughman, S.D., Newgard, C.B., and Coleman, R.A. (2006). Overexpression of rat long chain acyl-coa synthetase 1 alters fatty acid metabolism in rat primary hepatocytes. J. Biol. Chem. *281*, 37246–37255.

Liu, Z., and Huang, X. (2013). Lipid metabolism in Drosophila: development and disease. Acta Biochim. Biophys. Sin. (Shanghai) *45*, 44–50.

Mangroo, D., Wu, X.Q., and RajBhandary, U.L. (1995). Escherichia coli initiator tRNA: structure-function relationships and interactions with the translational machinery. Biochem. Cell Biol. *73*, 1023–1031.

Marszalek, J.R., Kitidis, C., Dararutana, A., and Lodish, H.F. (2004). Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth. J. Biol. Chem. *279*, 23882–23891.

Martin, T.F. (2001). PI(4,5)P(2) regulation of surface membrane traffic. Curr. Opin. Cell Biol. *13*, 493–499.

Mashek, D.G., and Coleman, R.A. (2006). Cellular fatty acid uptake: the contribution of metabolism. Curr. Opin. Lipidol. *17*, 274–278.

Mashek, D.G., McKenzie, M.A., Van Horn, C.G., and Coleman, R.A. (2006). Rat long chain acyl-CoA synthetase 5 increases fatty acid uptake and partitioning to cellular triacylglycerol in McArdle-RH7777 cells. J. Biol. Chem. *281*, 945–950.

Mashek, D.G., Li, L.O., and Coleman, R.A. (2007). Long-chain acyl-CoA synthetases and fatty acid channeling. Future Lipidol. *2*, 465–476.

McMahon, A., and Kedzierski, W. (2010). Polyunsaturated very-long-chain C28-C36 fatty acids and retinal physiology. Br. J. Ophthalmol. *94*, 1127–1132.

McMahon, A., Jackson, S.N., Woods, A.S., and Kedzierski, W. (2007). A Stargardt disease-3 mutation in the mouse Elovl4 gene causes retinal deficiency of C32-C36 acyl phosphatidylcholines. FEBS Lett. *581*, 5459–5463.

McMahon, A., Butovich, I.A., and Kedzierski, W. (2011). Epidermal expression of an Elovl4 transgene rescues neonatal lethality of homozygous Stargardt disease-3 mice. J. Lipid Res. *52*, 1128–1138.

Meloni, I., Parri, V., De Filippis, R., Ariani, F., Artuso, R., Bruttini, M., Katzaki, E., Longo, I., Mari, F., Bellan, C., et al. (2009). The XLMR gene ACSL4 plays a role in dendritic spine architecture. Neuroscience *159*, 657–669.

Mendelson, K., Evans, T., and Hla, T. (2014). Sphingosine 1-phosphate signalling. Development *141*, 5–9.

Milger, K., Herrmann, T., Becker, C., Gotthardt, D., Zickwolf, J., Ehehalt, R., Watkins, P.A., Stremmel, W., and Füllekrug, J. (2006). Cellular uptake of fatty acids driven by the ER-localized acyl-CoA synthetase FATP4. J. Cell. Sci. *119*, 4678–4688.

Min, K.T., and Benzer, S. (1999). Preventing neurodegeneration in the Drosophila mutant bubblegum. Science *284*, 1985–1988.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu. Rev. Genet. *29*, 371–399.

Moriya-Sato, A., Hida, A., Inagawa-Ogashiwa, M., Wada, M.R., Sugiyama, K., Shimizu, J., Yabuki, T., Seyama, Y., and Hashimoto, N. (2000). Novel acyl-CoA synthetase in adrenoleukodystrophy target tissues. Biochem. Biophys. Res. Commun. *279*, 62–68.

Mosser, J., Lutz, Y., Stoeckel, M.E., Sarde, C.O., Kretz, C., Douar, A.M., Lopez, J., Aubourg, P., and Mandel, J.L. (1994). The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. Hum. Mol. Genet. *3*, 265–271.

Osborne, S.L., Meunier, F.A., and Schiavo, G. (2001). Phosphoinositides as key regulators of synaptic function. Neuron *32*, 9–12.

Pei, Z., Sun, P., Huang, P., Lal, B., Laterra, J., and Watkins, P.A. (2009). Acyl-CoA synthetase VL3 knockdown inhibits human glioma cell proliferation and tumorigenicity. Cancer Res. *69*, 9175–9182.

Pei, Z., Fraisl, P., Shi, X., Gabrielson, E., Forss-Petter, S., Berger, J., and Watkins, P.A. (2013). Very long-chain acyl-CoA synthetase 3: overexpression and growth dependence in lung cancer. PLoS ONE *8*, e69392.

Pol, A., Gross, S.P., and Parton, R.G. (2014). Review: biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites. J. Cell Biol. *204*, 635–646.

Poveda, J.A., Giudici, A.M., Renart, M.L., Molina, M.L., Montoya, E., Fernández-Carvajal, A., Fernández-Ballester, G., Encinar, J.A., and González-Ros, J.M. (2014). Lipid modulation of ion channels through specific binding sites. Biochim. Biophys. Acta *1838*, 1560–1567.

Record, M., Carayon, K., Poirot, M., and Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. Biochim. Biophys. Acta *1841*, 108–120.

Renault, A.D., Kunwar, P.S., and Lehmann, R. (2010). Lipid phosphate

phosphatase activity regulates dispersal and bilateral sorting of embryonic germ cells in Drosophila. Development *137*, 1815–1823.

Routt, S.M., and Bankaitis, V.A. (2004). Biological functions of phosphatidylinositol transfer proteins. Biochem. Cell Biol. *82*, 254–262.

Schaffer, J.E., and Lodish, H.F. (1994). Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell *79*, 427–436.

Schink, K.O., Raiborg, C., and Stenmark, H. (2013). Phosphatidylinositol 3-phosphate, a lipid that regulates membrane dynamics, protein sorting and cell signalling. Bioessays *35*, 900–912.

Schmidt, C., and Robinson, C.V. (2014). Dynamic protein ligand interactions--insights from MS. FEBS J. *281*, 1950–1964.

Schuchardt, J.P., Huss, M., Stauss-Grabo, M., and Hahn, A. (2010). Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. Eur. J. Pediatr. *169*, 149–164.

Shisheva, A. (2013). PtdIns5P: news and views of its appearance, disappearance and deeds. Arch. Biochem. Biophys. *538*, 171–180.

Soupene, E., and Kuypers, F.A. (2008). Mammalian long-chain acyl-CoA synthetases. Exp. Biol. Med. (Maywood) *233*, 507–521.

Souza, S.C., Muliro, K.V., Liscum, L., Lien, P., Yamamoto, M.T., Schaffer, J.E., Dallal, G.E., Wang, X., Kraemer, F.B., Obin, M., et al. (2002). Modulation of hormone-sensitive lipase and protein kinase A-mediated lipolysis by perilipin A in an adenoviral reconstituted system. J. Biol. Chem. *277*, 8267–8272.

Stachowiak, J.C., Brodsky, F.M., and Miller, E.A. (2013). A cost-benefit analysis of the physical mechanisms of membrane curvature. Nat. Cell Biol. *15*, 1019–1027.

Stahl, A. (2004). A current review of fatty acid transport proteins (SLC27). Pflugers Arch. *447*, 722–727.

Stein, J. (2000). The neurobiology of reading difficulties. Prostaglandins Leukot. Essent. Fatty Acids *63*, 109–116.

Stinnett, L., Lewin, T.M., and Coleman, R.A. (2007). Mutagenesis of rat acyl-CoA synthetase 4 indicates amino acids that contribute to fatty acid binding. Biochim. Biophys. Acta *1771*, 119–125.

Strickland, L.I., and Burgess, D.R. (2004). Pathways for membrane trafficking during cytokinesis. Trends Cell Biol. *14*, 115–118.

Szafer-Glusman, E., Giansanti, M.G., Nishihama, R., Bolival, B., Pringle, J., Gatti, M., and Fuller, M.T. (2008a). A role for very-long-chain fatty acids in furrow ingression during cytokinesis in Drosophila spermatocytes. Curr. Biol. *18*, 1426–1431.

Takasuga, S., and Sasaki, T. (2013). Phosphatidylinositol-3,5-bisphosphate: metabolism and physiological functions. J. Biochem. *154*, 211–218.

Tong, F., Black, P.N., Coleman, R.A., and DiRusso, C.C. (2006). Fatty acid transport by vectorial acylation in mammals: roles played by different isoforms of rat long-chain acyl-CoA synthetases. Arch. Biochem. Biophys. *447*, 46–52.

Varnam, C.J., Strauss, R., Belle, J.S., and Sokolowski, M.B. (1996). Larval behavior of Drosophila central complex mutants: interactions between no bridge, foraging, and Chaser. J. Neurogenet. *11*, 99–115.

Ward, P.E. (2000). Potential diagnostic aids for abnormal fatty acid metabolism in a range of neurodevelopmental disorders. Prostaglandins Leukot. Essent. Fatty Acids *63*, 65–68.

Ward, J.D., Mullaney, B., Schiller, B.J., He, L.D., Petnic, S.E., Couillault, C., Pujol, N., Bernal, T.U., Van Gilst, M.R., Ashrafi, K., et al. (2014). Defects in the C. elegans acyl-CoA synthase, acs-3, and nuclear hormone receptor, nhr-25, cause sensitivity to distinct, but overlapping stresses. PLoS ONE *9*, e92552.

Watkins, P.A., and Ellis, J.M. (2012). Peroxisomal acyl-CoA synthetases. Biochim. Biophys. Acta *1822*, 1411–1420.

Wiczer, B.M., and Bernlohr, D.A. (2009). A novel role for fatty acid transport protein 1 in the regulation of tricarboxylic acid cycle and mitochondrial function in 3T3-L1 adipocytes. J. Lipid Res. *50*, 2502–2513.

Wuestehube, L.J., Duden, R., Eun, A., Hamamoto, S., Korn, P., Ram, R., and Schekman, R. (1996). New mutants of Saccharomyces cerevisiae affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. Genetics *142*, 393–406.

Wurtzel, J.G.T., Kumar, P., and Goldfinger, L.E. (2012). Palmitoylation regulates vesicular trafficking of R-Ras to membrane ruffles and effects on ruffling and cell spreading. Small GTPases *3*, 139–153.

Yonath, H., Marek-Yagel, D., Resnik-Wolf, H., Abu-Horvitz, A., Baris, H.N., Shohat, M., Frydman, M., and Pras, E. (2011). X inactivation testing for identifying a non-syndromic X-linked mental retardation gene. J. Appl. Genet. *52*, 437–441.

Young, G., and Conquer, J. (2005). Omega-3 fatty acids and neuropsychiatric disorders. Reprod. Nutr. Dev. *45*, 1–28.

Young, O.A., and Anderson, J.W. (1974). Properties and substrate specificity of some reactions catalysed by a short-chain fatty acyl-coenzyme A synthetase from seeds of Pinus radiata. Biochem. J. *137*, 423–433.

CHAPTER 3

DROSOPHILA *bgm* AND *dbb*-DUPLICATED GENES DISPLAYING PARTIAL DIVERGENCE IN DEVELOPMENTAL EXPRESSION AND COREGULATED BY DORSAL PATHWAY GENES

Introduction

Genetic analysis has allowed subdivision of the genetic loci involved in dorsoventral (DV) patterning into three conserved signaling pathways defined by the Epidermal Growth Factor Receptor (EGFR), Toll/Dorsal, and Decepentaplegic (Dpp), each of which transmits positional information through the localization of an extracellular morphogen. As development proceeds, sequential activities of these signaling cascades refine the positional identity of cells, giving rise to unique molecular and morphological characteristics for each domain along the dorsoventral axis.

The EGFR signaling cascade patterns the oocyte prior to fertilization. The TGF alpha-like ligand encoded by *gurken* provides the dorsalizing signal to the EGF receptor encoded by torpedo. Thus, the most proximal follicle cells adopt a dorsal cell fate (Neuman-Silberberg and Schüpbach, 1993; Schejter and Shilo, 1989). The signal generated by Gurken and Torpedo is transmitted subsequently through the Ras pathway; however, the events downstream of Ras are not

completely understood (Brand and Perrimon, 1994). The dorsalizing signal represses transcription of the Dorsal/Toll signaling component Pipe (Sen et al., 1998), thereby restricting activity of the Dorsal/Toll signaling pathway to the ventral-most regions of the embryo.

The Dorsal/Toll pathway is a maternal signaling cascade that interprets the DV signal initiated in oogenesis. Eleven dorsal group mutants and the negative regulator cactus were identified in genetic screens by their dramatic loss of DV positional identity (Anderson and Nüsslein-Volhard, 1984a; Morisato and Anderson, 1995). In the absence of any one dorsal-group gene, cells at all DV positions differentiate into dorsal epidermis, while ventral epidermis, neuroectoderm, and mesoderm fail to develop. Partial loss of function alleles leads to preferential loss of the ventral-most pattern elements (Anderson et al., 1985). The DV pattern is based on a nuclear gradient of the maternal morphogen Dorsal, the Drosophila member of the NFκB family. dorsal mRNA and protein are uniformly distributed in the embryo, but the protein is selectively imported into the nucleus in a ventral to dorsal gradient (Roth et al., 1989; Steward, 1987). Proper generation of this gradient requires the upstream activities of the eleven dorsal-group genes and cactus. Three somatic genes pipe, *nudel,* and *windbeutel* are transcribed in follicle cells and localize to the embryonic vitelline space where they initiate an activation cascade (Hong and Hashimoto, 1995; Manseau and Schüpbach, 1989; Sen et al., 1998). Ventrally restricted signaling by *pipe, nudel,* and *windbeutel* activates the Easter protease, which in turn leads to the localized proteolytic cleavage of the Spatzle ligand (Chasan et al., 1992; Schüpbach and

Roth, 1994; Smith and DeLotto, 1994). Cleaved and activated Spatzle activates the Toll receptor, allowing Toll to transduce the ventral signal intracellularly. Toll shares homology with the vertebrate interleukin-1 receptor (Gay and Keith, 1991). Toll protein is uniformly distributed in the embryonic membrane; thus, restriction of the Spatzle ligand is essential for localized receptor activation (Hashimoto et al., 1991). A complex between two intracellular components of the pathway, Tube and Pelle, is presumably formed at sites of receptor activation (Towb et al., 1998). The serine threonine kinase Pelle interacts with the novel protein Tube via death domains present in both proteins, then signals to disrupt the inhibitory complex between Dorsal and its antagonist, the IKB homolog Cactus (Galindo et al., 1995; Letsou et al., 1991; Norris and Manley, 1992; Shen and Manley, 1998; Xiao et al., 1999). Similar to mammalian IKB, phosphorylation of Cactus marks it for ubiquitin-mediated degradation and releases the NFKB transcription factor, thus allowing it to enter ventral nuclei and regulate transcription (Beg et al., 1993).

Subdivision of the DV axis into distinct domains of transcriptional readout occurs in response to defined thresholds of Dorsal activity (Huang et al., 1997; Rusch and Levine, 1996). By acting as both transcriptional activator and repressor, Dorsal specifies domains distinguished at the molecular level by the expression of zygotic marker genes and at the morphological level by the cell fates that these domains predict. High levels of Dorsal in ventral-most cells pattern the mesoderm through the activation of transcription factors, Twist and Snail (Boulay et al., 1987; Thisse et al., 1987, 1988, 1991). Expression of Twist

and Snail is restricted to ventral cells by the inability of the low affinity binding sites in their promoters to be activated in more lateral regions (Pan et al., 1991). Intermediate levels of nuclear Dorsal activate neurogenic fate markers including *rhomboid*, *short gastrulation*, and *single minded* (Ip et al., 1992). Dorsal simultaneously restricts dorsal fates by repression *of decapentaplegic (dpp), zerkneult (zen),* and *tolloid (tld)* in ventral and lateral domains (Ip et al., 1992; Shimell et al., 1991). The inherent activator functions of Dorsal are transformed into repressor capabilities by interactions with additional cofactors (Dubnicoff et al., 1997; Jiang et al., 1992; Valentine et al., 1998). Together, Dorsal and its cofactors bind to neighboring sites in the promoter of the target gene and repress transcription. The absence of dorsal activity in dorsal-most cells permits expression of *dpp, zen, and tld*, allowing them to fix the limits of dorsal ectoderm and amnioserosa cell fates.

The Dpp ligand is a member of TGF-alpha superfamily of growth factors sharing the most extensive homology with vertebrate BMP 2A and BMP-4 (Wozney et al., 1988). Transcriptional repression of *dpp* by Dorsal restricts *dpp* transcripts to the dorsal 40% of the blastoderm where it specifies dorsal cell fates (Irish and Gelbart, 1987; Wharton et al., 1993).

Few Targets of Signaling Have Been Isolated Genetically

Taken together, the genetically defined pathways that specify dorsoventral patterning comprise signaling molecules and their attendant transcription factors, but very few of the targets of signaling have been identified to date. These target

genes are essential to our understanding of fate specification as they are the biological effectors of signaling. Identification and characterization of these effector targets will further our understanding of how they contribute to the determination of cell fates and in a broader context will provide insight into the developmental signals present in eukaryotes as a whole. Our limited understanding of the effectors of signaling may be due to our inability to identify genes with redundant functions, genes with both maternal and zygotic components, and genes with specialized developmental roles through classic genetic approaches. The forward genetic screens that successfully identified signaling components of patterning relied on mutations that would cause embryonic lethality and additionally resulted in some visible abnormality of the larval cuticle (Anderson and Nüsslein-Volhard, 1984b; Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984). Overall, only 25% (450) of the 1800 lethal mutations generated caused embryonic death, and only 13% (580) of the embryonic lethal mutations caused visible alterations in the morphology of the larval cuticle (Wieschaus, 1996). In light of these numbers, as well as the relative paucity of effector molecules isolated in genetic screens, it appears likely that screening criteria were too stringent to identify most target genes.

At the level of transcriptional activation, the linear signaling pathway expands to regulate a diverse set of biological effectors. Effector molecules are expected to encode a wide range of biological functions. Some, such as structural proteins, enzymes, and cell adhesion molecules may directly generate the final differentiated state. Others, such as transcription factors and cofactors,

may serve as intermediate regulators in the differentiation process. These genes may share overlapping or interacting functions that make them difficult to isolate through classic genetic methods. In addition, because they are at the branch point of a linear signal, effector genes are expected to display only a subset of the characteristics that contribute to phenotypes displayed by the pathway as a whole; thus, phenotypes that arise from mutations in target genes will range from strong to nonexistent.

Finally, genes with redundant functions are also likely to be missed in genetic screens since both genes of the pair must be mutated simultaneously to disrupt shared functions. The Drosophila genome encodes 13,601 genes and based on sequence similarity alone nearly half of these are designated as duplicated genes (Adams et al., 2000; Rubin, 2000). Coupled with the genetic observation that only one third of loci mutate to a detectable phenotype, the high number of duplicated genes suggests that many nonmutable genes share redundant functions. However, at this point genome analysis cannot reveal functional requirements for duplicated genes. Additional approaches will be needed to distinguish duplicated genes that function redundantly from those that share sequence similarity but do not have overlapping functional requirements.

Few examples of functionally redundant genes have been reported in Drosophila. In these case studies, functional redundancy has been addressed in pairs of genes in which one partner was isolated genetically and the other was identified subsequently by sequence similarity. Examples include *engrailed* and *invected* (Coleman et al., 1987), *knirps* and *knirp-like* (González-Gaitán et al.,

1994), and *buttonhead* and *D-spl* (Schöck et al., 1999). These related genes share similarities in sequence and expression profile, and in all cases the molecularly identified partner displays only a subset of the phenotypes presented by their genetically isolated counterparts. Duplicated genes with equivalent roles in development are expected to be more difficult to detect genetically.

Effectors of DV Patterning Remain to Be Identified

Very few effectors or downstream targets of DV patterning have been identified to date. And taken together theses effector genes represent only a subset of the phenotypes that would result from complete loss of DV patterning, thus revealing many gaps in our knowledge of DV effectors. For example the transcription factor encoded by *zen* is a target of DPP signaling; however, *zen* mutants display a weak ventralized phenotype, and amnioserosa fates are lost while the dorsal ectoderm remains intact (Wakimoto et al., 1984). Thus, whereas Dpp patterns the dorsal domain in its entirety, transcriptional regulation through *zen* alone is not sufficient to provide all dorsal fates. Importantly, very few transcriptionally regulated targets of zen have been identified, and none of them display mutant phenotypes corresponding to their role in DV patterning (Hirose et al., 1994; Rusch and Levine, 1997; Tatei et al., 1995). Similarly, twist and snail are downstream targets of the Dorsal signaling pathway. However, both genes encoded are transcription factors, and very few cytological effectors downstream of this pair of genes have been identified. One example is folded gastrulation, which codes for a receptor that transduces the ventralizing signal into ventral

cells and is responsible for inducing the cytological changes that lead to ventral furrow formation during gastrulation (Costa et al., 1994). *fog* mutants display a failure to invaginate only in a subset of ventral furrow forming cells, and the effectors inducing changes in other cells still need to be identified (Dawes-Hoang et al., 2005).

Conservation

One of the themes in developmental biology that has emerged over the past decade is that a small number of signaling pathways have been evolutionarily conserved, and these pathways are exploited repeatedly in numerous developmental and life history contexts within the same organism, as well as across species. As an example, Dorsal/Toll signaling mediates immune responses to microbial infection in flies, plants, and mammals (Anderson, 2000). Similarly studies in Xenopus have identified a BMP signaling pathways required for DV patterning analogous to Dpp signaling in Drosophila (Ferguson, 1996).

Signaling pathways have evolved various mechanisms to generate unique cellular responses at different developmental stages in different organisms. Modulation of the signal by the use of multiple ligands, receptors, differential endocytosis, receptor turnover, and crosstalk with other signaling pathways have been demonstrated to allow signaling pathways to elicit the correct biological response for each developmental event. As effectors of signaling, target genes are directly responsible for generating different biological responses. For example, the EGFR signaling pathway has been studied as a potential target for

drug development, but because the core components are reused in so many distinct processes, emphasis has been placed on molecules involved more peripherally in signaling. Essentially, the emphasis is now on identifying effectors of signaling rather than the upstream drivers of signaling, with the hope for effective and localized responses to drug treatments. In this regard, identification and characterization of effectors that modulate specific subsets of signals represent an important next step.

Expression-based Reverse Genetic Approach to Identify Effectors of DV Patterning

In order to identify effectors of DV patterning, we assayed expression profiles early in development. The rationale for the screen being that if an embryonic transcript is expressed early on in development and has a restricted pattern of expression, it must have a critical function at that time and place in development. We used a high-throughput robotic screen for the identification of genes with spatially restricted RNA in situ expression patterns during the 0–4 hours of embryonic development (Simin et al., 2002). Among the 778 sequence-selected genes from a 0–4 hour embryonic cDNA library, some had established roles in pattern formation such as the pair-rule gene odd-skipped and the dorsoventrally restricted genes *neuralized* and delta. In addition, we discovered a number of novel genes with spatially restricted patterns of expression in the early embryo, like the dorsally restricted gene *scylla*, the *charybde* homologue. Our studies revealed *scylla* and *charybde* to be duplicated genes with essential (but

redundant) functions as effectors of the Dpp/Zen pathway in head involution (Scuderi et al., 2006). Our studies of *scylla* and *charybde* helped validate our approach to identification of biologically significant cell fate determinants in expression screens. A yet another previously unidentified dorsoventrally restricted gene that emerged from the screen was the *U5F9* or *CG4500* transcript, which exhibited ventral expression in the blastoderm stage and in gastrulating Drosophila embryos (Simin et al., 2002).

Material and Methods

RNA in situ hybridization

cDNA clones (LD28132 and GM14009), corresponding to *dbb* and *bgm*, respectfully, were obtained from BDGP. cDNA inserts were amplified from bacterial cultures diluted 1:50 in ddH2O by the polymerase chain reaction (PCR). Primers, SP6 and T7, specific for the pOT2 plasmid were used to amplify cDNA. Full-length antisense probes labeled with digoxigenin-UTP were generated using 1ug of the amplified reaction product as template in an in vitro transcription reaction. RNA in situ hybridization to whole-mount Drosophila embryos and third instar larvae was performed as described (Tautz and Pfeifle, 1989). For detection, embryos and dissected larvae were incubated in alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim) diluted 1:2000, followed by incubation with chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). The color reaction was terminated with a series of PBT washes with subsequent rinse in 100% methanol. Embryos and

larvae were cleared for 24 h in 80% glycerol, mounted on glass slides, and then scored using light microscopy. Embryos were classified by developmental stage: preblastoderm and blastoderm (stages 0–5), early gastrula (stages 6–8), and germ band extended (stages 9–11) (Campos-Ortega and Hartenstein 1997). Images were captured on an Zeiss Axioscope microscope using digital camera (AxioCam).

Northern blot analysis

Nucleic acid manipulations were performed according to standard protocols (Sambrook et al., 1989). For northern blot analyses, total RNA was isolated from developmentally staged embryos, larvae, and adults. Approximately 4 µg/lane were separated on denaturing 1% formaldehyde-agarose gels. Fractionated RNA was transferred to nylon membranes and immobilized by UV crosslinking. Hybridization with random-primed radiolabeled probes was performed by standard methods.

Cryosectioning protocol for Drosophila embryos

Fix and perform in situ hybridization (RNA or Protein). Saturate embryos in sequential steps in sucrose + gelatin solution, with the final saturation being 7.5% sucrose+ 15% gelatin. Saturation/equilibration is when the embryos sink at the bottom of the tube of sucrose + gelatin solution. Start a set up with couple mug warmers and a few WET paper towels on top. Place one mug warmer under a dissecting scope. Place the plastic mould on the wet paper towel. Add about 25–

50 ul of gelatin solution containing about 10–15 stained embryos. Have a couple forceps, the embryo containing vials of gelatin and extra gelatin solution heating on the other mug warmer. Line up the embryos in the vertical direction towards the front end of the mould (this is Side A). The easiest way to do this is take as little gelatin as possible. Drag embryos as you align them vertically towards the front of the mould, i.e., towards side A. Drag the next set of embryos towards Side A but behind the front row of embryos. This will prevent embryos from being disturbed.

Once you have a decent number of embryos aligned vertically, remove the mould from the mug warmer and set it down to cool. Once gelatin has solidified, gradually add small amounts of warm gelatin solution on top and fill up the mould to the top. Do not fill up with hot gelatin all at once, or the gelatin at the bottom will melt and all the alignment will be lost. Once fairly solid, place the mould overnight at -20 C. When ready to section, set the cryosection at -30 C at least 2 hours in advance. Have the cryosection blade and chucks inside the cryochamber. Have liquid N2, OCT, and compressed CO2 at hand. The following should be done inside the Cryo chamber as much as possible. Remove the gelatin block from the plastic mould carefully without breaking it. Cut the plastic mould with a blade if needed to remove the gelatin block neatly. Hold the block with a forcep and wave it very close to the surface of Liquid N2 but not within it. Expose all sides of the block to Liquid N2 in this manner. Once fairly solid and frozen, dip the block briefly into the Liquid N2 solution. Place a drop of OCT on the chuck and quickly place the frozen gelatin block on the OCT. Apply more

OCT on the chuck surrounding the gelatin block to provide support. Make sure Side A is facing towards you and is not stuck to the chuck because that is the part containing embryos and needs to be sectioned. Spray with compressed CO2 and allow solidifying again inside the Cryo chamber.

When solid, cut out extra parts of the gelatin block without disengaging it from the chuck. Make it a nice square to obtain neat ribbons. Adjust the section thickness to 10 uM. Adjust the angle of the chuck so that it is perpendicular to the blade. Adjust the glass over the blade so that it is completely aligned with the blade and not in front or back. Clean blade and the glass with ethanol and Kim wipes. Go ahead and section! If everything goes well, you should get neat ribbons that can be picked up by just touching a room temperature slide on top of the ribbon lightly. Keep these slides in the refrigerator overnight and cover with an aqueous mounting medium and coverslip the next day. Allow to dry and they are ready for microscopy.

Results

Acyl CoA synthases in Drosophila melanogaster

Examination of the nucleotide sequence revealed *U5F9* to encode a long chain fatty acyl CoA Synthase (ACSL), which had not yet been characterized at the functional level. The predicted mRNA size of 2069 bp corresponded well with the 2.4 kb transcript detected in northern blot analyses. Database searches allowed us to uncover five Drosophila homologs of *U5F9* in Drosophila melanogaster, which are also predicted to encode Acyl CoA synthases based on

their sequence (work done by previous graduate students: Anne Schuderi and Anna Sivachenko). However, the most closely related homolog was the gene *bubblegum* (*bgm*), and its protein sequence is 43% identical and 62% similar to that of U5F9 at the amino acid level. Homology extended across the entire length of the protein sequence (data not shown). Additionally, although all homologs of U5F9 identified are ACSs, only *bgm* and *U5F9* encode long chain Acyl CoA Synthases. *U5F9* was later named double bubble *(dbb*) based on its close homology with *bgm. bgm* and *dbb* map only 7.6 kb apart from one another on chromosome 2L in the Drosophila genome. Hence in silico analysis suggests that *bgm and dbb* are duplicated genes with a common ancestor **(Figure 3.1A)**.

In order to determine if any of the other ACS genes share the ventrally restricted spatial expression pattern with *dbb*, we performed mRNA in situ hybridization using RNA probes against some of the ACS genes **(Figure 3.1B)**. Most of the ACS genes are ubiquitously expressed at 2 hrs AEL, when the *dbb and bgm* mRNAs are ventrally restricted. Their shared pattern of expression suggests that *dbb and bgm* are regulated by a common mechanism(s), at least at this stage of development and that both these genes have a potential role in mesodermal patterning.

bgm and dbb are duplicated genes with differential gene expression during development

To gain insight into the evolutionary dynamics of the *bgm and dbb* gene pair, we performed BLAST alignments between the D.melanogaster genomic

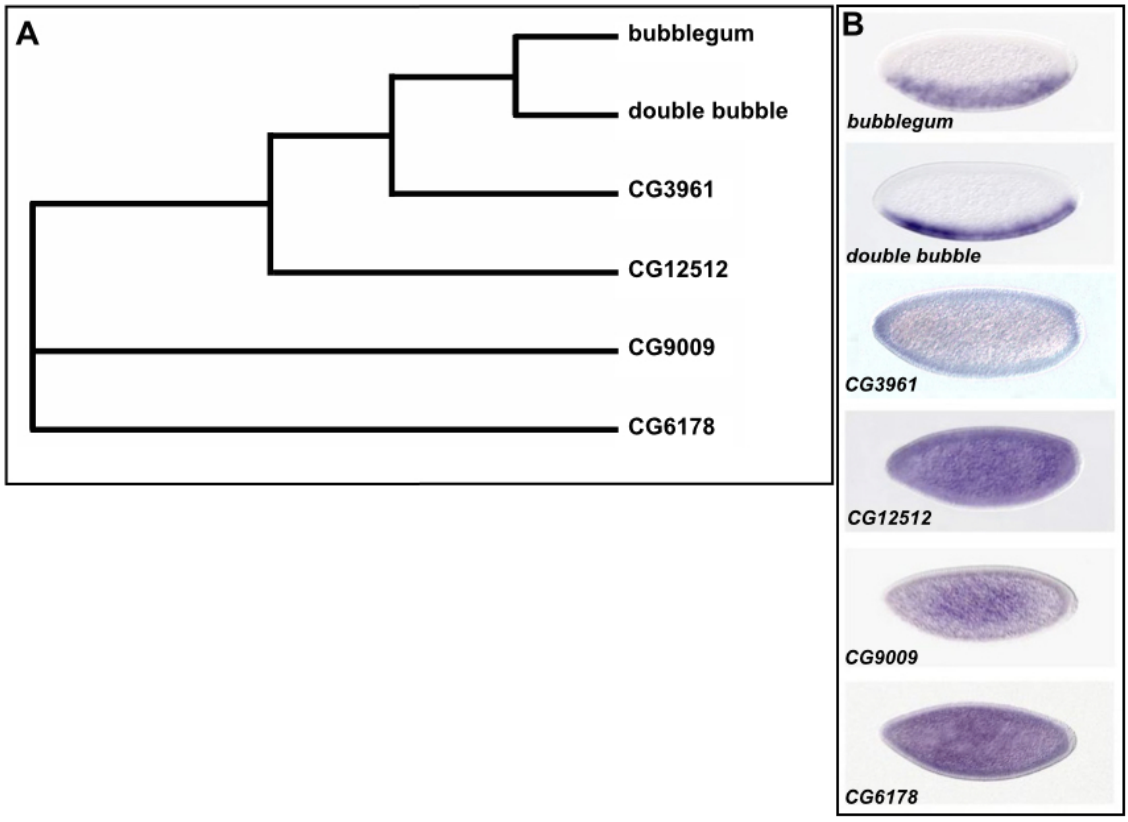

Figure 3.1:Predicted Acyl CoA Synthases in Drosophila Melanogaster

A. Phylogenetic tree of Acyl-CoA synthases in D. melanogaster.

B. *bgm* and *dbb* transcripts show overlapping expression profiles at stage 5 in Drosophila embryos. Note ubiquitous expression at Stage 5 of the genes encoding other Acyl CoA Synthases.

region, to which *bgm and dbb* are localized and the genomes of ancestral Drosophila species, as well as to other insect genomes (refer to Sivatchenko thesis). We found that all Drosophilidae possess both the *bgm/dbb* gene pair and that in all species the genes are located in close proximity to one another. Furthermore, we have seen remarkable conservation of the exon-intron structure *of bgm and dbb* orthologs across all the Drosophila species. The analysis of other insect genomes revealed that A.gambiae, A. aegypti, N. vitripennis, and T. castaneum have one *bgm* ortholog. The genomic structure of the bgm group gene in Anopheles was similar to Drosophila *bgm*. These observations suggest that the ancestral gene is most homologous to the D. Melanogaster *bgm*, that *dbb* is the newly acquired gene, and that the gene duplication event has probably occurred in Drosophilidae lineage. Because *bgm and dbb* have different exon-intron structures that are well conserved in ancestral species, the duplication event must be very old. Indeed, we have identified close homologues of *bgm and dbb* as far back into the speciation tree as in D. Pseudoobscura, suggesting that the duplication took place earlier than 43 million years ago. The *bgm and dbb* exonic sequences have high sequence similarity; however, the *dbb* single intronic sequence appears to be newly acquired as it appears neither in the *bgm* genomic sequence nor in any other gene in Drosophila or other species. Thus the *dbb* gene most likely arose by retrotransposition and postduplication; the gene acquired a new intronic sequence.

As a first step in studying the gene regulatory relationship between *bgm* and *dbb* genes, we used developmental Northern blots to visualize temporal

distribution of transcripts at critical stages in development. Consistent with the lack of maternally derived *dbb* expression in situ, transcripts were expressed at very low levels in RNA samples from 0–2 hr embryos. The peak of expression occurred at 2–8 hours after egg lay (AEL), concurrent with the onset of zygotic transcription. In subsequent stages *dbb* was expressed at low levels and was ultimately undetectable in larval stages. A second smaller peak of expression was observed during pupal development. Low level expression was observed in adults. The temporal expression profile of *bgm* was distinct from that of its homolog dbb. Expression of *bgm* peaked between 0–2 hr AEL, suggesting maternal depositions of the *bgm* transcript. The expression of *bgm* mRNA was reduced between 2–16 hrs of embryogenesis, indicating low zygotic expression during embryogenesis. The *bgm* mRNA expression peaked again during larval and adult stages with reduced expression in pupal stages. These data suggest an overlapping as well as complementary requirement of *the bgm* and *dbb* transcripts during development **(Figure 3.2A**).

In order to assess the spatial relationship between *bgm* and *dbb*, we examined mRNA expression in staged whole-mount embryos **(Figure 3.2B)**. Indeed, our analysis of mRNA expression localization revealed that *bgm* mRNA is maternally deposited as well as zygotically expressed, confirming the results obtained from Northern blot analysis. When zygotic expression begins in the embryo, both *bgm* and *dbb* exhibit overlapping, ventrally restricted expression patterns. This is in contrast to the transcripts corresponding to the other acyl-CoA synthetases in Drosophila that retain ubiquitous expression in blastoderm-stage

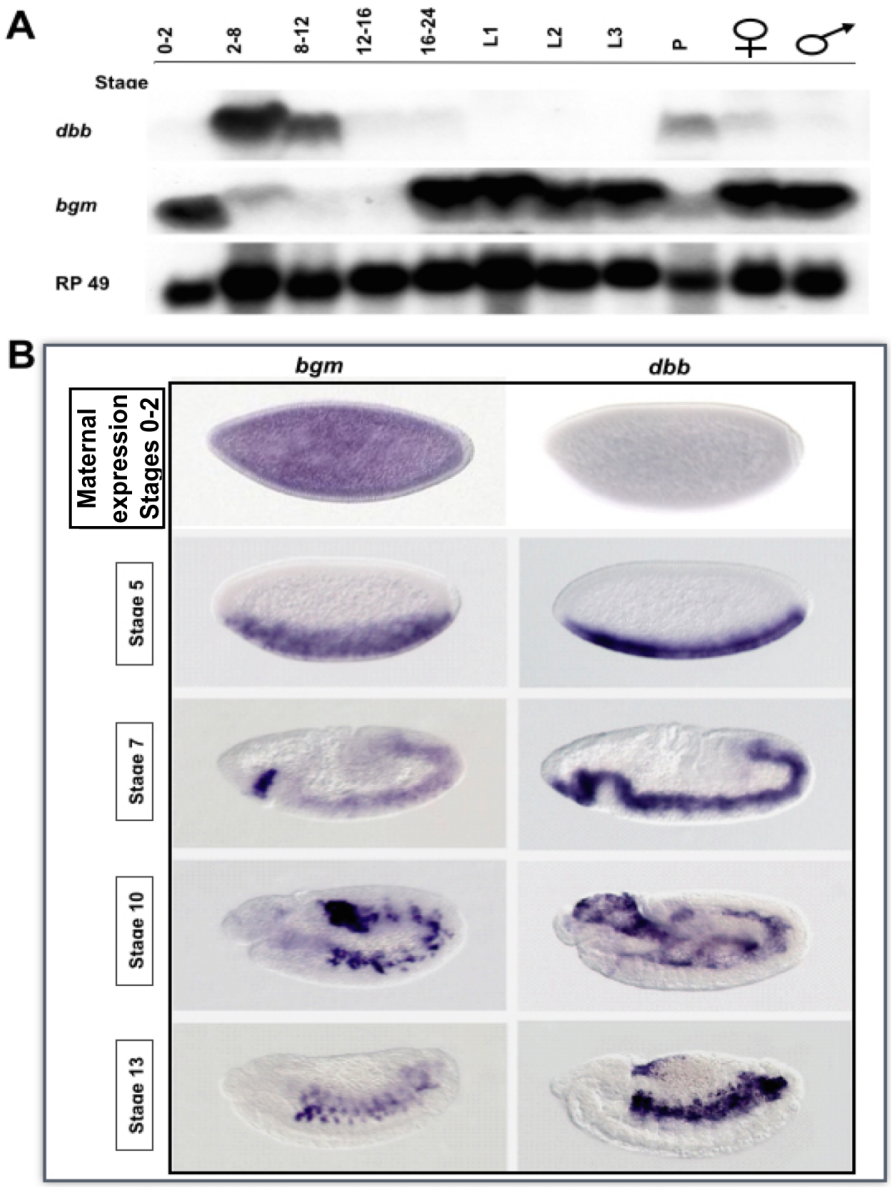

Figure 3.2: Overlapping yet divergent expression patterns of *bgm* and *dbb* during embryonic development.

A. Northern blot analysis of *dbb and bgm.* Developmental northern blots were hybridized with a radiolabeled probe for *dbb or bgm.*

B. Spatial distribution of *bgm* and *dbb* transcripts during embryogenesis. Stages are shown according to Hartenstein and Ortega 1997. *bgm* transcripts are deposited maternally, whereas *dbb* is expressed zygotically. Stage 5 expression patterns of *bgm and dbb* are overlapping, however the expression patterns diverge into distinct mesoderm derivatives at later stages.

(Experiments contributed by Anne Schuderi)

embryos. It is only in later embryonic stages that the *bgm and dbb* gene expression profiles diverge, the most striking differences being their relative expression levels in mesodermal derivatives. The overlapping as well as complementary spatiotemporal expression patterns, as well as the difference in transcript levels of *bgm and dbb* at different developmental stages, suggest that changes in their regulation have allowed the duplicated *bgm and dbb* genes to evolve divergent developmental roles. It is possible that this pair of genes is under the control of both shared and distinct regulatory mechanisms for transcription. Possibly, the initial zygotic transcription of both genes is controlled by a common signaling pathway, with slightly overlapping and differential mechanisms of regulation coming into play as the embryo progresses through embryonic development.

Molecular epistasis

To place *dbb* within the developmental context of the well-characterized Dorsal pathway that specifies ventral fates, expression of *dbb* was examined in embryos lacking DV fate cues. In embryos derived from dorsal-deficient females, *dbb* expression is absent. Conversely, in ventralized embryos, the extent of *dbb* expansion is proportional to the ventralizing strength of the mutant. The *dbb* expression domain expands from the ventral-most region of the embryo into lateral regions in embryos derived from females homozygous for a hypomorphic allele of *cactus (cact011).* In contrast *dbb* expression expands to lateral as well as dorsal regions in embryos derived from females harboring a dominant

ventralizing (constitutively active) allele of Toll. Together, these data indicate the Dorsal transcription factor is necessary and sufficient for *dbb* transcription **(Figure 3.3A)**.

We next tested whether *dbb* is transcriptionally regulated by patterning genes downstream of Dorsal signaling. The expression of transcription factors Twist (Twi) and Snail (Sna) is directly regulated by Dorsal, and the genes encoding these proteins represent the first zygotic targets of signaling in the Dorsal pathway. We assessed *bgm and dbb* mRNA expression in twist, snail, and twist snail double mutants in hybridization studies in situ **(Figure 3.3B)**. We find that in both twist and snail single mutants there is reduced *dbb* mRNA expression as compared to wild-type embryos, while in the twist snail double mutants there is a complete loss of *dbb* mRNA expression. Thus, *dbb* transcription is controlled by both Twist and Snail and transcriptional inputs from both Twist and Snail were required for wild type expression levels of *dbb.* Analogous *bgm* experiments are ongoing, but preliminary studies indicate a similar pattern of regulation. The transcriptional regulation of *dbb* (and presumably *bgm*) by Twist and Snail places the Bgm and Dbb ACSs in a key position enabling them to impact mesoderm patterning. To further define their mesodermal expression domains, we examined transcription in cryosections of embryos at different stages of embryogenesis, starting at stage 5 (2 hrs AEL) when zygotic expression begins and the ventral furrow forms through stage 9, when germ band elongation initiates. We observed both *bgm and dbb* mRNAs in each of the 18 ventral-most cells that undergo apical constriction and

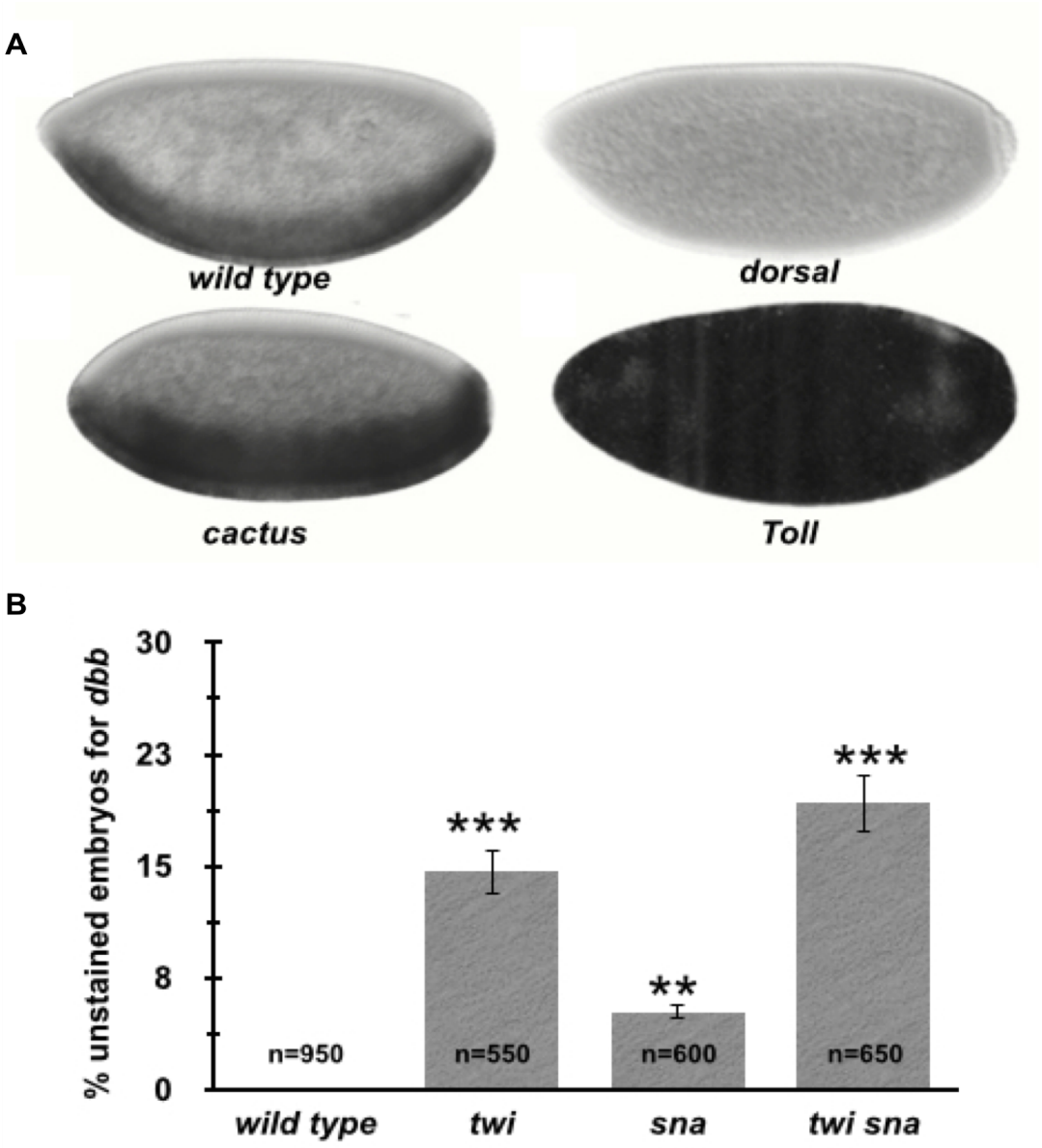

Figure 3.3: *dbb* gene expression is regulated by the Dorsal pathway genes.

A. RNA in situ hybridization of stage 5 mutant embryos. Ventrally localized wild-type expression of *dbb* mRNA is lost in *dorsal* mutants, and partially or fully expanded in *cactus* and Tl^{10b} mutants.

B. Graphical representation of the loss of *dbb* mRNA expression in *twist, snail,* and *twist snail* mutant embryos shows partial reduction in the *twi and sna* single mutants and a complete loss in *twi sna* double mutants.

(Experiments contributed by Anne Schuderi)

invagination during ventral furrow formation. Whereas *bgm* transcripts are distributed throughout the cell, *dbb* mRNAs appear to be restricted to the apical portion of these cells. Restricted subcellular expression of mRNAs is rare, but is usually associated with localized translation. Such a machinery is able to provide an immediate supply, with respect to time, of the specific protein at a given subcellular location (Besse and Ephrussi, 2008). Hence, it is intriguing to speculate that Dbb ACS activity is required specifically at the site of apical constriction in the ventral furrow forming cells. We have not observed any embryonic lethality associated with zygotic loss of *bgm or dbb*, either alone or in combination (Chapter 2); however, it is possible that the phenotype is very subtle and has thus been undetected. Given the very specific expression pattern of *dbb* mRNA, we are conducting precise and careful experiments to determine any role of *dbb* in development of the ventral furrow. For this analysis, we will examine ventral furrow formation in live and fixed *bgm, dbb*, and *bgm dbb* mutant embryos using time lapse microscopy and high resolution techniques like Scanning Electron Microscopy. Our expectation is that defects, if they exist will be subtle enough to not result in lethality, but may result in delayed and/or improper ventral furrow formation **(Figure 3.4)**.

Discussion

The *bgm/dbb* gene pair represents the second pair of duplicated genes (after *scylla* and *charybde*) that we identified based on an asymmetric pattern of DV axis expression. As was the case for *scylla* and *charybde*, both *bgm* and *dbb*

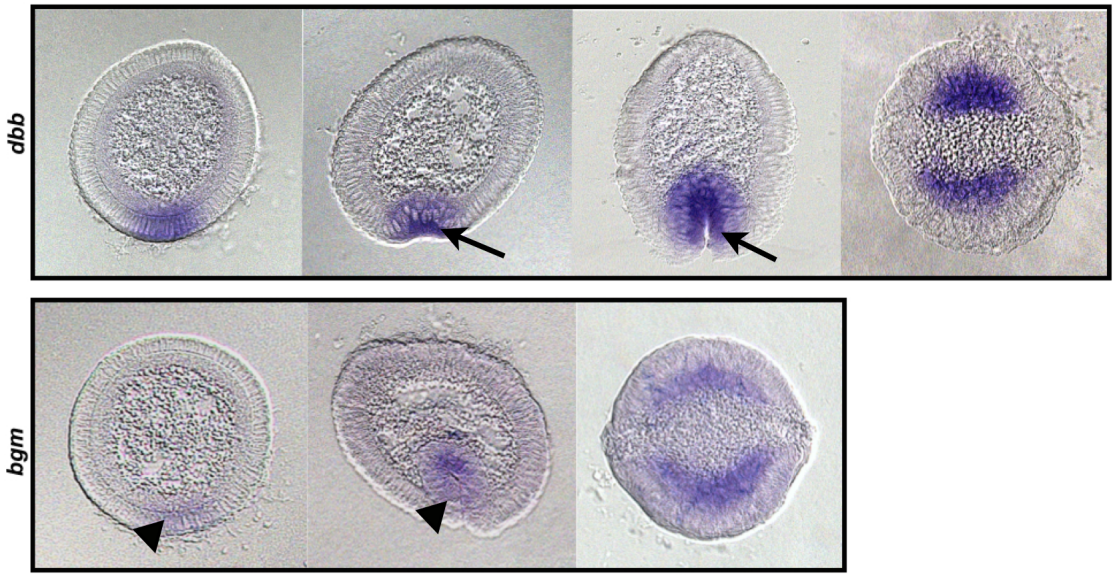

Figure 3.4: *dbb* and *bgm* mRNA localization in the ventral furrow forming cells.

Cryosections of wild type embryos at different stages of gastrulation stained for *dbb* and *bgm* transcripts. *dbb* transcripts are expressed in the ventral furrow forming cells starting at stage 5, and the mRNA is localized to the apical region of the cells, as shown by arrows. *bgm* transcripts are also present in the ventral furrow forming cells, albeit in a diffused fashion, as shown by arrowheads.

are regulated by the Dorsal signaling pathway, although their expression is restricted ventrally (rather than dorsally), and thus one or both may have roles in effecting ventral cell specific fates. mRNA localization is a widespread posttranscriptional mechanism for targeting protein synthesis to specific cellular sites. It is involved in the generation of cell polarity, asymmetric segregation of cell fate determinants, and germ cell specification. Lecuyer et al. employed a high-resolution fluorescent in situ hybridization procedure to comprehensively evaluate mRNA localization dynamics during early Drosophila embryogenesis.

Of the 3370 genes analyzed, 71% of those expressed encode subcellularly localized mRNAs. Tight correlations between mRNA distribution and subsequent protein localization and function indicate major roles for mRNA localization in nucleating localized cellular machineries (Lécuyer et al., 2007). Apical restriction of *dbb* transcripts in the invaginating cells of the ventral furrow is particularly intriguing, and we are currently investigating whether this localization facilitates Dbb function in ventral furrow formation. Based on other examples of subcellular RNA localization (Cody et al., 2013; Gaspar, 2011; Jansen and Niessing, 2012), it is possible that *dbb* mRNA localization to the apical side of invaginating cells assists in rapid translation and localization of the Dbb ACS and thereby facilitates rapid lipid-dependent remodeling of membranes at the cells' constricting surface. The invaginating ventral furrow consists of a 18 cell wide stripe along the ventral midline of the blastoderm (Foe, 1989). In a brief 10 min, these cells constrict apically and invaginate as a unit, forming a tube within the ventral portion of the embryo. In *twist* and *snail* mutants,

the ventral furrow does not form, and consequently mutants fail to develop mesoderm derivatives. The ventral furrow defects in *twist* and *snail* mutants represent the earliest visible requirements for zygotic transcription (Ferguson and Anderson, 1991). In fact, the window for transcription and translation of *twist* and *snail* targets is only 30–40 min, making it likely that target genes will encode effectors directly and that these effectors will correspond to modulators rather than structural proteins themselves (Simpson, 1983). Accordingly, both integrin subunit PS2a and Xanthine Dehydrogenase (rosy) are transcribed in cells that become the furrow (Leptin et al., 1989). An enzyme that catalyzes the activation of membrane components, such as *dbb,* also fits this prediction and fits the profile of an effector of DV patterning.

Despite 60% sequence similarity (40% identical) of *dbb and bgm*, differences in their spatiotemporal expression profiles suggest that their functions have diverged. In this regard, results from northern blot studies are indicative of complementary roles in development. Peaks of *dbb* expression correspond to the troughs of *bgm* expression. Moreover, our observation that *dbb* transcripts accumulate apically at the site of constriction in invaginating cells of the ventral furrow tempts us to speculate that the developmental regulation of *dbb* in the embryo is important for the rapid membrane remodeling that takes place during gastrulation and possibly again later in embryogenesis, while *bgm* functions predominate later in larval and adult stages of the animal's life history. Comparisons of ventral furrow formation in wild-type, *dbb,* and *bgm* mutant embryos, required as a first step in testing potential mechanisms of *dbb and bgm*

function in ventral furrow formation, are ongoing.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of Drosophila melanogaster. Science 287, 2185–2195.

Anderson, K.V. (2000). Toll signaling pathways in the innate immune response. Curr. Opin. Immunol. 12, 13–19.

Anderson, K.V., and Nüsslein-Volhard, C. (1984a). Information for the dorsal—ventral pattern of the Drosophila embryo is stored as maternal mRNA. Nature 311, 223–227.

Anderson, K.V., Bokla, L., and Nüsslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. Cell 42, 791–798.

Anderson, K.V., Schneider, D.S., Morisato, D., Jin, Y., and Ferguson, E.L. (1992). Extracellular morphogens in Drosophila embryonic dorsal-ventral patterning. Cold Spring Harb. Symp. Quant. Biol. 57, 409–417.

Beg, A.A., Finco, T.S., Nantermet, P.V., and Baldwin, A.S. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol. Cell. Biol. 13, 3301–3310.

Belvin, M.P., and Anderson, K.V. (1996). A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annu. Rev. Cell Dev. Biol. 12, 393–416.

Besse, F., and Ephrussi, A. (2008). Translational control of localized mRNAs: Restricting protein synthesis in space and time. Nature Reviews Mol. Cell Biol. 9, 971–980.

Boulay, J.L., Dennefeld, C., and Alberga, A. (1987). The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers. Nature 330, 395–398.

Brand, A.H., and Perrimon, N. (1994). Raf acts downstream of the EGF receptor to determine dorsoventral polarity during Drosophila oogenesis. Genes Dev. 8, 629–639.

Chasan, R., Jin, Y., and Anderson, K.V. (1992). Activation of the easter zymogen

is regulated by five other genes to define dorsal-ventral polarity in the Drosophila embryo. Development 115, 607–616.

Cody, N.A.L., Iampietro, C., and Lécuyer, E. (2013). The many functions of mRNA localization during normal development and disease: from pillar to post. Wiley Interdiscip Rev. Dev. Biol. 2, 781–796.

Coleman, K.G., Poole, S.J., Weir, M.P., Soeller, W.C., and Kornberg, T. (1987). The invected gene of Drosophila: sequence analysis and expression studies reveal a close kinship to the engrailed gene. Genes Dev. 1, 19–28.

Costa, M., Wilson, E.T., and Wieschaus, E. (1994). A putative cell signal encoded by the folded gastrulation gene coordinates cell shape changes during Drosophila gastrulation. Cell 76, 1075–1089.

Dawes-Hoang, R.E., Parmar, K.M., Christiansen, A.E., Phelps, C.B., Brand, A.H., and Wieschaus, E.F. (2005). folded gastrulation, cell shape change and the control of myosin localization. Development 132, 4165–4178.

DeLotto, Y., and DeLotto, R. (1998). Proteolytic processing of the Drosophila Spätzle protein by easter generates a dimeric NGF-like molecule with ventralising activity. Mech. Dev. 72, 141–148.

Dubnicoff, T., Valentine, S.A., Chen, G., Shi, T., Lengyel, J.A., Paroush, Z., and Courey, A.J. (1997a). Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. Genes Dev. 11, 2952–2957.

Eberl, D.F., and Hilliker, A.J. (1988). Characterization of X-linked recessive lethal mutations affecting embryonic morphogenesis in Drosophila melanogaster. Genetics 118, 109–120.

Ferguson, E.L. (1996). Conservation of dorsal-ventral patterning in arthropods and chordates. Curr. Opin. Genet. Dev. 6, 424–431.

Ferguson, E.L., and Anderson, K.V. (1992a). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the Drosophila embryo. Cell 71, 451–461.

Ferguson, E.L., and Anderson, K.V. (1992c). Localized enhancement and repression of the activity of the TGF-beta family member, decapentaplegic, is necessary for dorsal-ventral pattern formation in the Drosophila embryo. Development 114, 583–597.

Foe, V.E. (1989). Mitotic domains reveal early commitment of cells in Drosophila embryos. Development 107, 1–22.

Galindo, R.L., Edwards, D.N., Gillespie, S.K., and Wasserman, S.A. (1995). Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in Drosophila embryos. Development 121, 2209–2218.

Gaspar, I. (2011). Microtubule-based motor-mediated mRNA localization in Drosophila oocytes and embryos. Biochem. Soc. Trans. 39, 1197–1201.
Gay, N.J., and Keith, F.J. (1991). Drosophila Toll and IL-1 receptor. Nature 351, 355–356.

González-Gaitán, M., Rothe, M., Wimmer, E.A., Taubert, H., and Jäckle, H. (1994). Redundant functions of the genes knirps and knirps-related for the establishment of anterior Drosophila head structures. Proc. Natl. Acad. Sci. U.S.A. 91, 8567–8571.

Hashimoto, C., Gerttula, S., and Anderson, K.V. (1991). Plasma membrane localization of the Toll protein in the syncytial Drosophila embryo: importance of transmembrane signaling for dorsal-ventral pattern formation. Development 111, 1021–1028.

Hirose, F., Yamaguchi, M., and Matsukage, A. (1994). Repression of regulatory factor for Drosophila DNA replication-related gene promoters by zerknüllt homeodomain protein. J. Biol. Chem. 269, 2937–2942.

Hong, C.C., and Hashimoto, C. (1995). An unusual mosaic protein with a protease domain, encoded by the nudel gene, is involved in defining embryonic dorsoventral polarity in Drosophila. Cell 82, 785–794.

Huang, A.M., Rusch, J., and Levine, M. (1997). An anteroposterior Dorsal gradient in the Drosophila embryo. Genes Dev. 11, 1963–1973.

Ip, Y.T., Park, R.E., Kosman, D., Yazdanbakhsh, K., and Levine, M. (1992a). Dorsal-Twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. Genes Dev. 6, 1518–1530.

Ip, Y.T., Park, R.E., Kosman, D., Bier, E., and Levine, M. (1992b). The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo. Genes Dev. 6, 1728–1739.
Irish, V.F., and Gelbart, W.M. (1987). The decapentaplegic gene is required for dorsal-ventral patterning of the Drosophila embryo. Genes Dev. 1, 868–879.

Jansen, R.-P., and Niessing, D. (2012). Assembly of mRNA-protein complexes for directional mRNA transport in eukaryotes—an overview. Curr. Protein Pept. Sci. 13, 284–293.

Jiang, J., Kosman, D., Ip, Y.T., and Levine, M. (1991). The dorsal morphogen

gradient regulates the mesoderm determinant twist in early Drosophila embryos. Genes Dev. 5, 1881–1891.

Jiang, J., Rushlow, C.A., Zhou, Q., Small, S., and Levine, M. (1992). Individual dorsal morphogen binding sites mediate activation and repression in the Drosophila embryo. EMBO J. 11, 3147–3154.

Lécuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T.R., Tomancak, P., and Krause, H.M. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell 131, 174–187.

Leptin, M., Bogaert, T., Lehmann, R., and Wilcox, M. (1989). The function of PS integrins during Drosophila embryogenesis. Cell 56, 401–408.

Letsou, A., Alexander, S., Orth, K., and Wasserman, S.A. (1991). Genetic and molecular characterization of tube, a Drosophila gene maternally required for embryonic dorsoventral polarity. Proc. Natl. Acad. Sci. U.S.A. 88, 810–814.

Manseau, L.J., and Schüpbach, T. (1989a). cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the Drosophila embryo. Genes Dev. 3, 1437–1452.

Manseau, L.J., and Schüpbach, T. (1989b). The egg came first, of course! Anterior-posterior pattern formation in Drosophila embryogenesis and oogenesis. Trends Genet. 5, 400–405.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo. Annu. Rev. Genet. 29, 371–399.

Myers, E.W., Sutton, G.G., Delcher, A.L., Dew, I.M., Fasulo, D.P., Flanigan, M.J., Kravitz, S.A., Mobarry, C.M., Reinert, K.H., Remington, K.A., et al. (2000). A whole-genome assembly of Drosophila. Science 287, 2196–2204.

Neuman-Silberberg, F.S., and Schüpbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165–174.

Norris, J.L., and Manley, J.L. (1992). Selective nuclear transport of the Drosophila morphogen dorsal can be established by a signaling pathway involving the transmembrane protein Toll and protein kinase A. Genes Dev. 6, 1654–1667.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature 287, 795–801.

Pan, D.J., Huang, J.D., and Courey, A.J. (1991). Functional analysis of the Drosophila twist promoter reveals a dorsal-binding ventral activator region. Genes Dev. 5, 1892–1901.

Perrimon, N., and Perkins, L.A. (1997). There must be 50 ways to rule the signal: the case of the Drosophila EGF receptor. Cell 89, 13–16.

Roth, S., Stein, D., and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell 59, 1189–1202.

Rubin, G.M. (2000). Biological annotation of the Drosophila genome sequence. Novartis Found. Symp. 229, 79–82; discussion 82–83.

Rusch, J., and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the Drosophila embryo. Curr. Opin. Genet. Dev. 6, 416–423.

Rusch, J., and Levine, M. (1997). Regulation of a dpp target gene in the Drosophila embryo. Development 124, 303–311.

Schejter, E.D., and Shilo, B.Z. (1989). The Drosophila EGF receptor homolog (DER) gene is allelic to faint little ball, a locus essential for embryonic development. Cell 56, 1093–1104.

Schneider, D.S., Jin, Y., Morisato, D., and Anderson, K.V. (1994). A processed form of the Spätzle protein defines dorsal-ventral polarity in the Drosophila embryo. Development 120, 1243–1250.

Schöck, F., Purnell, B.A., Wimmer, E.A., and Jäckle, H. (1999). Common and diverged functions of the Drosophila gene pair D-Sp1 and buttonhead. Mech. Dev. 89, 125–132.

Schüpbach, T., and Roth, S. (1994). Dorsoventral patterning in Drosophila oogenesis. Curr. Opin. Genet. Dev. 4, 502–507.

Scuderi, A., Simin, K., Kazuko, S.G., Metherall, J.E., and Letsou, A. (2006). scylla and charybde, homologues of the human apoptotic gene RTP801, are required for head involution in Drosophila. Dev. Biol. 291, 110–122.

Sen, J., Goltz, J.S., Stevens, L., and Stein, D. (1998). Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. Cell 95, 471–481.

Shen, B., and Manley, J.L. (1998). Phosphorylation modulates direct interactions between the Toll receptor, Pelle kinase and Tube. Development 125, 4719–4728.

Shimell, M.J., Ferguson, E.L., Childs, S.R., and O'Connor, M.B. (1991). The Drosophila dorsal-ventral patterning gene tolloid is related to human bone morphogenetic protein 1. Cell 67, 469–481.

Simin, K., Scuderi, A., Reamey, J., Dunn, D., Weiss, R., Metherall, J.E., and Letsou, A. (2002). Profiling patterned transcripts in Drosophila embryos. Genome Res. 12, 1040–1047.

Smith, C.L., and DeLotto, R. (1994). Ventralizing signal determined by protease activation in Drosophila embryogenesis. Nature 368, 548–551.

Steward, R. (1987). Dorsal, an embryonic polarity gene in Drosophila, is homologous to the vertebrate proto-oncogene, c-rel. Science 238, 692–694.

Tatei, K., Cai, H., Ip, Y.T., and Levine, M. (1995). Race: a Drosophila homologue of the angiotensin converting enzyme. Mech. Dev. 51, 157–168.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98, 81–85.

Thisse, B., el Messal, M., and Perrin-Schmitt, F. (1987). The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. Nucleic Acids Res. 15, 3439–3453.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., and Perrin-Schmitt, F. (1988). Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. EMBO J. 7, 2175–2183.

Thisse, C., Perrin-Schmitt, F., Stoetzel, C., and Thisse, B. (1991). Sequence-specific transactivation of the Drosophila twist gene by the dorsal gene product. Cell 65, 1191–1201.

Towb, P., Galindo, R.L., and Wasserman, S.A. (1998). Recruitment of Tube and Pelle to signaling sites at the surface of the Drosophila embryo. Development 125, 2443–2450.

Valentine, S.A., Chen, G., Shandala, T., Fernandez, J., Mische, S., Saint, R., and Courey, A.J. (1998). Dorsal-mediated repression requires the formation of a multiprotein repression complex at the ventral silencer. Mol. Cell. Biol. 18, 6584–6594.

Wakimoto, B.T., Turner, F.R., and Kaufman, T.C. (1984). Defects in embryogenesis in mutants associated with the antennapedia gene complex of Drosophila melanogaster. Dev. Biol. 102, 147–172.

Wall, N.A., and Hogan, B.L. (1994). TGF-beta related genes in development. Curr. Opin. Genet. Dev. 4, 517–522.

Wharton, K.A., Ray, R.P., and Gelbart, W.M. (1993). An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the Drosophila embryo. Development 117, 807–822.

Wieschaus, E. (1996). Embryonic transcription and the control of developmental pathways. Genetics 142, 5–10.

Wieschaus, E., Nusslein-Volhard, C., and Kluding, H. (1984). Krüppel, a gene whose activity is required early in the zygotic genome for normal embryonic segmentation. Dev. Biol. 104, 172–186.

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M., and Wang, E.A. (1988). Novel regulators of bone formation: molecular clones and activities. Science 242, 1528–1534.

Xiao, T., Towb, P., Wasserman, S.A., and Sprang, S.R. (1999). Three-dimensional structure of a complex between the death domains of Pelle and Tube. Cell 99, 545–555.

CHAPTER 4

DROSOPHILA ACYL CoA SYNTHASE MUTANTS DISPLAY MOTOR DISABILITIES DUE TO PROGRESSIVE NEURODEGENERATION

Introduction

Defects in fatty acid synthesis or distribution can have widespread effects on an organism. One specific tissue that is particularly susceptible to these defects is the nervous system. Potentially fatal human neurodegenerative diseases such as Tay Sachs, Zelweger Syndrome, and Adrenoleukodystrophy (ALD) exemplify this (Moser, 1997; Schmitt et al., 2014).

Adrenoleukodystrophy (ALD) is a rare but oftentimes fatal progressive neurodegenerative disease. The most common form of the disease is X-linked (X-ALD); it occurs equally in all ethnic groups with an estimated incidence of 1:17,000. X-ALD is a clinically heterogeneous disorder, exhibiting incomplete penetrance and variable expressivity (Moser et al., 2005). The most severe form of X-ALD is cerebral ALD, which affects about 45% of all patients (Moser, 1997). Demyelination in the central nervous system (CNS) constitutes the major burden of cerebral ALD accompanied by a treatable adrenal dysfunction—Addison's disease. The disorder is diagnosed as progressive neurological dysfunction in previously healthy boys most commonly between the ages of 4 and 8 years old.

Affected children often present with school failure, hyperactivity, visual and hearing loss, cognitive impairment, and finally progression to paralysis and death. A less severe form of the disease, Adrenomyeloneuropathy [AMN], occurs in ~35% of X-ALD patients. In AMN patients, demyelination is confined to the peripheral nervous system (PNS). The gene responsible for X-ALD encodes a peroxisomal ATP-binding transporter (ABCD1) (Mosser et al., 1993). The ABCD1 protein localizes to the peroxisomal membrane, where it functions to transport very long chain fatty acids (VLCFAs) into peroxisomes for their degradation. Indeed all X-ALD patients, including asymptomatic carriers, show elevated levels of VLCFAs in plasma, brain, and adrenal glands4, but whether this accumulation is causal to the neurodegeneration seen in ALD patients is not clear (Igarashi et al., 1976; Moser, 1997). Attempts to resolve the mechanism of neurodegeneration as well as the clinical heterogeneity of ALD in animal models have met with very limited success.

Acyl-CoA synthetases (ACSs) function immediately upstream of ABCD transporters and have been thought to be involved in ALD disease pathology. *bubblegum (bgm)*, a Drosophila long chain acyl-CoA synthetase (ACSL), was isolated in a genetic screen for neurodegenerative mutants (Min and Benzer, 1999). *bgm* mutant males exhibit neurodegeneration and elevated levels of certain VLCFAs indicative of a link between the *bgm* phenotype in flies and ALD in humans (Min and Benzer, 1999). However, *bgm* mutant flies recapitulate only some aspects of ALD and thus are far from perfect as an ALD disease model. Most of the ABC transporter or ACSVL synthetase gene studies in fly or mouse

models have not contemplated the huge potential for redundancy within both gene families. We have shown that a far more profound phenotype, and thus a better ALD disease model, is found in animals harboring mutations both in Bgm and its close homolog, Double bubble (Dbb), encoded by the *dbb* gene (unpublished from Anna Sivachenko). The high degree of conservation of fundamental biological processes in humans and flies, coupled with a broad repertoire of fly genetic approaches, makes Drosophila a powerful model system for understanding the molecular and cellular pathology of the nervous system (Celotto and Palladino, 2005; Kretzschmar, 2005; Nichols, 2006).

To study the functional relationship between *bgm* and its homologue *dbb* in the maintenance of adult CNS integrity, Sivatchenko et al., (Sivachenko and Letsou, in prep) analyzed brain morphologies in 18-day old wild type, single and double mutant flies. Histological and statistical analyses, of bgm^{1}, dbb^{KO} (both phenotypic nulls) and bgm^{1} dbb^{KO} brain sections revealed optic lobe and retinal degeneration. Degenerative changes were much stronger in the bgm^{1} mutant than in the dbb^{KO} mutant; however, the double mutant had a significantly worse phenotype compared to the wt and both single mutant flies. Our analysis indicates that these abnormalities occur after retinal differentiation is complete, and this is reminiscent of the human ALD phenotype where neurodegeneration affects previously normal children.

One of the major focal points of our research is identification of potential routes for therapy. This can be addressed either by diet intervention or by identification of potential drug therapies to target the ACS biochemical pathway.

For this, we need to conduct large-scale diet and drug screens and thus require a phenotype that is more easily scored than brain morphology. Also importantly, having documented severe eye abnormalities in *bgm dbb* double mutants, we seek to determine if the neurodegeneration is global or whether it is restricted to the eye. In order to address these questions, I used three different behavioral assays that have been previously reported to be optimally representative of neuronal abilities in the fruit fly.

Materials and Methods

Negative geotaxis assay

Flies are singly placed in an empty fly vial and tapped to the bottom. The time taken by each fly to reach a marked position at the top of the vial is measured. The experiment is repeated thrice for each animal.

CO2 recovery assay

Flies are exposed to a specific dose of CO2 while placed on the diffuser, and the time taken to recover, i.e., to move and climb the vial is measured. The experiment is done with single flies and is performed only once/day with each animal due to the increased recovery times upon repeated exposures.

Results

In both, a negative geotaxis (climbing ability) and CO2 recovery assays, we observed significantly reduced abilities in 15-day old *bgm dbb* and *bgm* flies

compared to similarly aged wt flies **(Figure 4.1)**. These differences in behavior between the wild type and mutant flies were only observed at a higher temperature of 25 C and not at 22 C. Thus these phenotypes are indicative of a temperature sensitive motor dysfunction. The *Dbb* ACSL does not seem to significantly contribute to this phenotype. Thus these data demonstrate that there is widespread neurodegeneration in the *bgm* and *bgm dbb* ACSL mutants which affects the motor behavior of these animals. Additionally, the behavioral defects in *bgm dbb* double mutant flies are comparable to those seen in *bgm* flies, suggestive of the fact that loss of *bgm* accounts for the behavioral defects in the double mutants and *dbb* has an accessory role. Hence, only *bgm* or *bgm dbb* flies will be accessed for the treatment of neurodegeneration in drug screens.

In order to further validate the behavioral phenotypes of our ACSL mutants in terms of neurodegeneration we assessed whether the progressive pattern of neurodegeneration observed in the eye and brain phenotypes, is also evident in the behavior of *bgm dbb* double mutant flies. As a first step we performed these studies only in wild type and *bgm dbb* mutant flies because the double mutants are comparable to *bgm* mutant flies in terms of the behavioral phenotypes and *dbb* mutants have no significant behavioral defects. We used the Negative Geotaxis assay to determine if progressive neurodegeneration affects behavior. We find that wild type flies perform consistently well starting from day 0 to day 15 in our behavioral assays. However, while *bgm dbb* mutants are comparable to wild type flies from day 0 to Day 7, they begin showing signs of motor dysfunction starting at day 7 or 8 approximately. We also see significantly more death in the

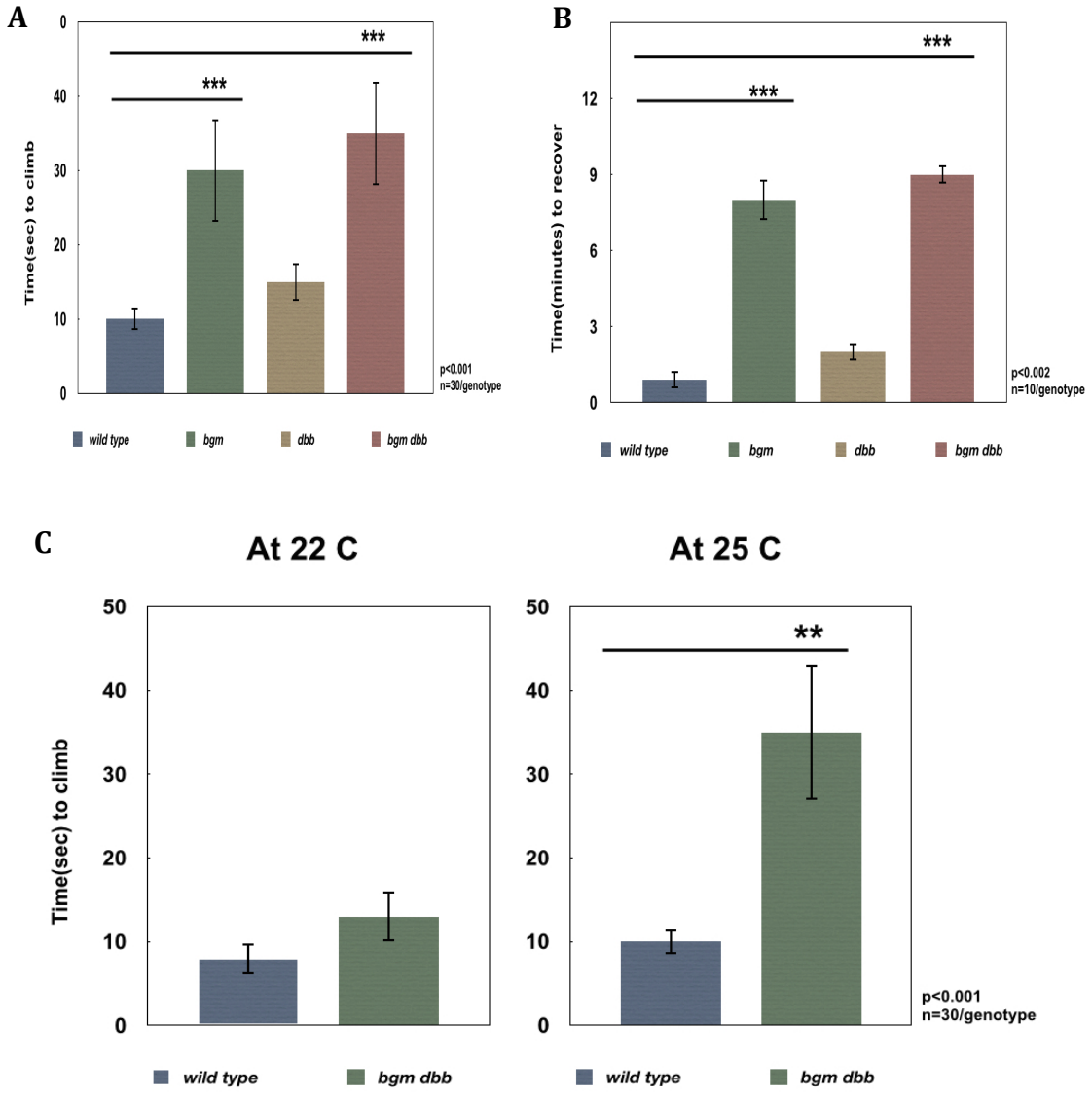

Figure 4.1:Behavioral defects in 15-day old *bgm* and *bgm dbb* adult flies at 25 C.

A. Graphical representation of time taken(seconds) to climb a vial 25 C by wild type, *bgm, dbb,* and *bgm dbb* 15-day old flies during the Negative Geotaxis assay.

B. Graphical representation of time taken(minutes) to recover at 25 C by wild type, *bgm, dbb,* and *bgm dbb* 15-day old flies during the CO^2 recover assay.

C. Temperature sensitive negative geotaxis behavior of *bgm dbb* mutants.

double mutant flies compared to wild type starting at day 7. The behavioral phenotype however, does not worsen from day 8 to day 15. It is known that the development of the adult Drosophila nervous system is complete at the time of eclosion. Since the *bgm dbb* flies appear comparable to wild type for the first 6 days posteclosion and show behavioral abnormalities starting at day 8, these phenotypes are suggestive of the onset of neurodegeneration at day 8 **(Figure 4.2)**. Significantly, the results suggest that neurodegeneration in the *bgm dbb* flies begins at day 8 at the latest, as opposed to the previously reported retinal neurodegeneration occurring at day 18.

Discussion

In summary, we have shown that *bgm* and *bgm dbb* mutants exhibit widespread neurodegeneration, as shown earlier by Sivachenko et al.(Sivachenko and Letsou, in prep) in the retina and as evidenced by behavioral abnormalities discussed in the current study. The behavioral phenotypes are also temperature sensitive. This is suggestive of a role of the Bgm ACSL or their fatty acid products in neuronal or glial membranes, and increased temperatures presumably make these membranes more susceptible to damage thus leading to behavioral abnormalities (Sepp and Auld, 2003). Lastly, neurodegeneration, as assessed from the behavioral phenotypes, appears to begin much earlier than the previously reported retinal degeneration. We begin to see behavioral abnormalities in *bgm dbb* flies at day 7/8 posteclosion, whereas retinal degeneration in our laboratory, as well as by Min and Benzer (Min and Benzer,

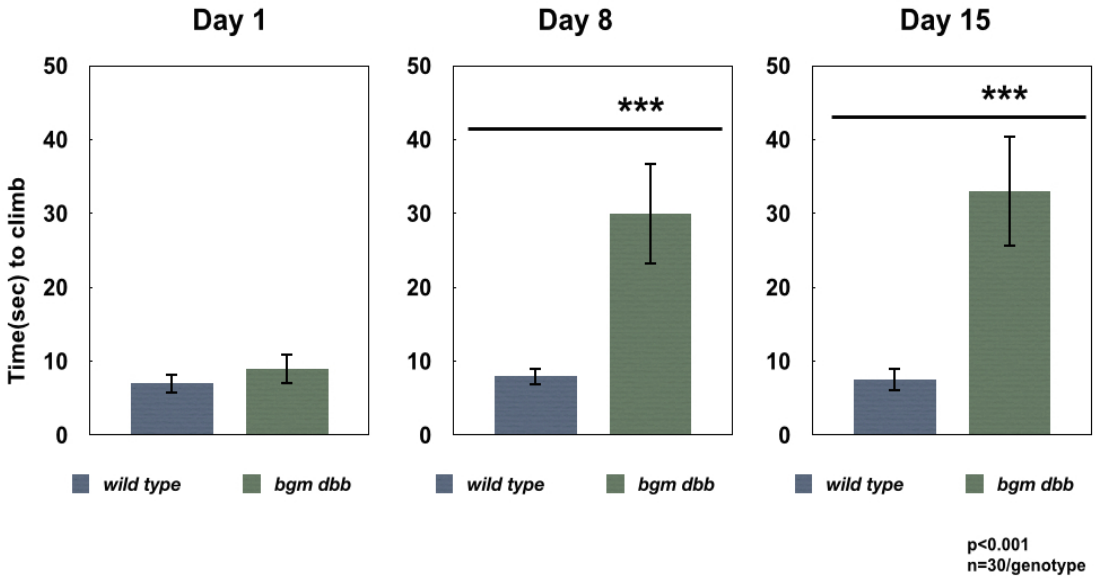

Figure 4.2: Neurodegeneration starts at Day 8 in *bgm dbb* mutants.

Graphical representation of the performance of Day 1, Day 8, and Day 15 wild type and *bgm dbb* flies in a Negative Geotaxis assay at 25 C. Mutant flies show signs of neurodegeneration starting at Day 8 post eclosion.

1999), has been reported only at day 18. It will be interesting to see if the neurodegeneration that presumably results in behavioral abnormalities at day 7/8 also affects eye and brain tissues.

Lastly, we have shown that behavioral abnormalities indicative of widespread neurodegeneration do occur in the *bgm* and *bgm dbb* flies and these phenotypes can be used to assess effects of remedial treatments in drug screens and diet studies. When taking up large scale drug screens or diet studies, it will be helpful, however, to automate the process and access the effects of diet and drugs on a large number of flies under highly controlled conditions. Automation will help us reduce environmental and experimental variations, which can drastically affect behavior. Additionally, the phenotypes observed in our ACSL mutants are variably expressed and are reminiscent of human ALD patients. Automation will help us assess phenotypes and treatment effects in a large number of flies, thus providing high statistical power and will allow us to focus on the most promising treatment alternatives.

References

Celotto, A.M., and Palladino, M.J. (2005). Drosophila: a “model” model system to study neurodegeneration. Mol. Interv. *5*, 292–303.

Igarashi, M., Schaumburg, H.H., Powers, J., Kishmoto, Y., Kolodny, E., and Suzuki, K. (1976). Fatty acid abnormality in adrenoleukodystrophy. J. Neurochem. *26*, 851–860.

Kretzschmar, D. (2005). Neurodegenerative mutants in Drosophila: a means to identify genes and mechanisms involved in human diseases? Invert. Neurosci. *5*, 97–109.

Min, K.T., and Benzer, S. (1999). Preventing neurodegeneration in the Drosophila mutant bubblegum. Science *284*, 1985–1988.

Moser, H.W. (1997). Adrenoleukodystrophy: phenotype, genetics, pathogenesis and therapy. Brain *120 (Pt 8)*, 1485–1508.

Moser, H.W., Moser, A.B., Frayer, K.K., Chen, W., Schulman, J.D., O'Neill, B.P., and Kishimoto, Y. (1998). Adrenoleukodystrophy: increased plasma content of saturated very long chain fatty acids. 1981. Neurology *51*, 334-343.

Moser, H.W., Raymond, G.V., and Dubey, P. (2005). Adrenoleukodystrophy: new approaches to a neurodegenerative disease. JAMA *294*, 3131–3134.

Mosser, J., Douar, A.M., Sarde, C.O., Kioschis, P., Feil, R., Moser, H., Poustka, A.M., Mandel, J.L., and Aubourg, P. (1993). Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature *361*, 726–730.

Nichols, C.D. (2006). Drosophila melanogaster neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. Pharmacol. Ther. *112*, 677–700.

Schmitt, F., Hussain, G., Dupuis, L., Loeffler, J.-P., and Henriques, A. (2014). A plural role for lipids in motor neuron diseases: energy, signaling and structure. Front Cell Neurosci. *8*, 25.

Sepp, K.J., and Auld, V.J. (2003). Reciprocal interactions between neurons and glia are required for Drosophila peripheral nervous system development. J. Neurosci. *23*, 8221–8230.

CHAPTER 5

SUMMARY AND PERSPECTIVES

In Chapter 2 of the present study we have demonstrated the requirement of maternally deposited *bgm* transcripts for the processes of cellularization and neurogenesis. Specifically, *bgm* is required for the proper subcellular targeting of Rab 5 endocytic vesicles that are critical to furrow extension during Drosophila cellularization. The extension of furrow canals during cellularization requires enormous amounts of membrane addition, which in most part is supplied by endocytic vesicles (Strickland and Burgess, 2004). However, it is unclear as to how these vesicles are targeted to specific subcellular compartments that finally lead to membrane addition at the growing furrow canal. Data presented in the current study demonstrate the requirement of the Bgm Long chain Acyl CoA synthase for the proper targeting of Rab-5 vesicles during cellularization. This is significant, as the search for identifier signals for subcellular targeting has continued for long and our research is an important first step towards the discovery of such molecules. Based on the data presented in this study, we hypothesize that activated long chain fatty acids themselves or via interaction with specific proteins provide the identifier signal that directs Rab5 vesicles to the recycling endosome and Rab 11 vesicles to the growing furrow canals. In the

absence of the Bgm ACS, these activated LCFAs are missing and so is the identifier signal.

Importantly, ours is only the second study to show the requirement of an Acyl CoA synthase in a specific developmental process. Kniazeva et al. have demonstrated the requirement of *C.elegans* Acsl-1 in cytokinetic processes during mitotic cell divisions in *C.elegans* embryogenesis. Additionally, a few studies in Drosophila have implicated enzymes required for lipid biogenesis and modification in cytokinesis of spermatocytes (Routt and Bankaitis, 2004). Thus there is emerging evidence for the role of Long chain fatty acids and enzymes required for their biogenesis in cytogenetic processes. This is intriguing, because thus far, in spite of lipids being important structural molecules within a cell, only proteins have been implicated in a majority of processes relating to cytokinesis. Thus, ours is one of the first few studies in an emerging field to study the role of lipids and long chain fatty acids in cytogenetic processes. These studies can have far reaching developmental implications because cell divisions in early embryogenesis define the embryonic and finally, the organismal body plan. Subtle defects or alterations in the properties of membranes may be overlooked during embryogenesis but may be manifested as developmental disorders at later stages, when cellular demands in terms of signaling and energy requirements are much more complex. Such a model of a developmental scar probably explains the neuronal abnormalities that we see in *bgm* mutant embryos and may provide a model for genetic predisposition to neurodevelopmental disorders. What follows is a discussion of the role long chain fatty acids in

neuronal development.

In Chapter 2, we also provide evidence for the role of the Bgm ACSL in neurogenesis. Loss of maternal *bgm* transcript leads to reduced number of neuroblasts as well as defects in axonogenesis. In addition to *bgm* embryos, which suffer a complete failure in cellularization and thus suffer lethality, we also observe the presence of partially cellularized *bgm* embryos that do not suffer lethality. These data, along with a maternal requirement of *bgm* for neuroblast formation, suggest that the neurogenesis defects in *bgm* embryos may arise from a partial failure in cellularization. It is possible that partial failure in cellularization leads to a failure in proper cell fate specification, thus leading to defects in neurogenesis. However, experiments to obtain direct evidence proving this hypothesis are currently underway. It is also possible that there is an independent requirement for LCFAs and the Bgm ACS for neurogenesis and axonogenesis. In either case, our findings are significant in light of the many clinical studies suggestive of a requirement of LCFAs and VLCFAs in neuronal development. Several studies have implicated an imbalance in LCFAs and VLCFAs in neurodevelopmental disorders like Dyslexia, Dyspraxia, Schizoaffective disorder, and Autism Spectrum Disorders(ASD) (Das, 2013; Schuchardt et al., 2010). Most of these disorders have overlapping neurological symptoms accompanied by a dysfunctional immune response (Ward, 2000). Neuronal and glial membranes are very rich in LCFAs and VLCFAs and hence, the nervous system is very sensitive to any changes in fatty acid metabolism (Laycock et al., 2007). In addition, a large number of signaling molecules

required by the immune system are lipid based (Mendelson et al., 2014). These findings, in association with clinical studies, are very suggestive of a suboptimal lipid metabolism in patients with neurodevelopmental disorders. Our findings are a significant first step towards a successful genetic model to understand the mechanistic role of LCFAs/VLCFAs and ACSLs in neuronal development. Further research in this direction will provide promising avenues for targeted treatment strategies, which are unavailable at the moment.

In Chapter 3, our studies of the developmental expression patterns of the homologous *bgm* and *dbb* ACSL genes provide interesting perspectives regarding the duplication of these genes. In addition, the transcriptional regulation of this gene pair by the dorsoventral patterning genes of the Dorsal pathway makes them interesting candidates as effectors of Dorsoventral signaling. In addition, the specific localization of the *dbb* transcript at apical margins of invaginating ventral furrow cells may be indicative of its role in rapid membrane remodeling required, first for the apical constriction of invaginating ventral furrow cells and later for cell movements driving morphogenesis in the Drosophila embryo. The absence of a strong phenotype, associated with the apical localization of *dbb* mRNA, could be due to the presence of a yet unidentified ACSL, which functions redundantly along with Dbb. However, as potential effectors of dorsoventral patterning, it is not surprising that *bgm* and *dbb* mutants present with no phenotypic manifestations with regards to their ventrally restricted expression pattern in the prospective mesoderm. The absence of a phenotype could be due to an unidentified redundancy or the possibility of a very

subtle unidentified phenotype itself. Both of these possibilities are worth pursuing, and experiments in the lab are underway to further explore these venues of investigation.

In Chapter 4 we have presented preliminary evidence of behavioral defects in Drosophila mutants of Bgm and Dbb ACSs. These data provide a segue towards easily scored phenotypes to be used for drug screens and nutritional intervention aimed at finding potential therapies for neurodegenerative diseases.

Overall, we present a comprehensive study of the developmental regulation and roles of long chain acyl CoA synthases in Drosophila. We have provided compelling evidence for the role of Bgm ACS as a key developmental regulator during Drosophila embryonic development. Given the high degree of conservation between the Drosophila and mammalian ACSs, including Bgm and Dbb, our studies will play a significant role in uncovering the role of ACSs and their associated long and very long chain fatty acids in human development and disease.