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Review

Enzymes of Sphingolipid metabolism in *Drosophila melanogaster*

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Abstract. Sphingolipids are important structural components of membranes that delimit the boundaries of cellular compartments, cells and organisms. They play an equally important role as second messengers, and transduce signals across or within the compartments they define to initiate physiological changes during development, differentiation and a host of other cellular events. For well over a century *Drosophila melanogaster* has served as a useful model organism to understand some of the fundamental tenets of development, differentiation and signaling in eukaryotic organisms. Directed approaches to study sphingolipid biology in *Drosophila* have been initiated only recently. Nevertheless, earlier phenotypic studies conducted on genes of unknown biochemical function have recently been recognized as mutants of enzymes of sphingolipid metabolism. Genome sequencing and annotation have aided the identification of homologs of recently discovered genes. Here we present an overview of studies on enzymes of the de novo sphingolipid biosynthetic pathway, known mutants and their phenotypic characterization in *Drosophila*.

Key words. Drosophila; sphingolipids; molecular genetics; metabolism.

Introduction

For over a century, since their discovery in 1884, sphingolipids were primarily viewed as important structural components of cellular membranes. The importance of sphingolipid homeostasis was established by discoveries that demonstrated accumulation of glycosphingolipids in certain clinical disorders, for example accumulation of glucosylceramide in Gaucher's disease, a disease primarily of the macrophages [1]. Clinical symptoms and further characterization of other sphingolipid storage disorders in humans tied sphingolipid metabolism and turnover to normal function of the brain and other organs. The isolation of a sphingolipid auxotroph in the early 1980s set the stage for pioneering work of identifying genes and enzymes of de novo sphingolipid biosynthetic pathway in yeast [2]. As the de novo biosynthetic pathway of sphingolipids was being elucidated, it became apparent that these lipids were conserved across species and phyla. These studies in yeast also began to establish that sphingolipid metabolism is intimately tied to intracellular membrane transport and signaling. In the last two decades, many sphingolipids have emerged as signaling molecules. Sphingolipids have been implicated in a wide range of cellular mechanisms ranging from housekeeping functions of eukaryotic cells and organisms to distinctive roles in signaling, development and differentiation. Increasing attention has been paid to enzymes of sphingolipid metabolism in order to integrate the activity of these enzymes with signaling functions associated with their

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metabolites and to understand the regulation of the metabolic pathway. This has led to identification of proteins and enzymes involved in metabolism, transport and interconversion of sphingolipid metabolites. Various mechanisms have been described to control the flux of metabolites through this pathway. These include the existence of a sphingomyelin cycle that interconverts bioactive sphingolipid messengers such as ceramide, sphingosine and sphingosine 1-phosphate. The intracellular flux and concentration of sphingolipids is proposed to be maintained by the sphingolipid rheostat, which comprises several regulatory enzymes that interconvert various bioactive sphingolipids. Several experimental approaches and systems have been utilized to elucidate the functions of sphingolipids and enzymes of sphingolipid metabolism, and to identify crucial players involved in the regulation of sphingolipid metabolism, transport and function. In vitro approaches in mammalian tissue culture studies include the use of exogenous analogs, activators and inhibitors, intracellular expression of these enzymes and Ribonucleic acid interference (RNAi)-mediated targeting of enzymes. While in vitro studies provide a great deal of information in well defined and tightly controlled experimental settings, in vivo analyses provide greater insight into the role of proteins and metabolites in the context of development, differentiation, signaling and sustenance of life of an organism. In vivo attempts have included use of yeast, mouse, *Drosophila* and *Arabidopsis* as model organisms to gain insight into the physiological functions of enzymes and metabolites and complement in vitro studies [3–7]. In this regard, pioneering work in yeast has laid the foundation for studies on metabolism and metabolic pathways of sphingolipids [2, 3, 8, 9]. In recent years, efforts have been made in *Arabidopsis* to analyze sphingolipid metabolism and signaling in vivo [10–12]. *Drosophila* is emerging as an important model organism to study enzymes and metabolites involved in sphingolipid synthesis and function [13–16]. This review focuses on the use of *Drosophila* as a model organism for study of proteins involved in generation, metabolism and signaling by simple sphingolipids.

Drosophila as an in vivo model

Drosophila has served as an excellent genetic model system to dissect pathways involved in embryogenesis, development, differentiation, signaling, cell cycle and in recent times in cancer and metastases studies [17–23]. The post-genomic era has provided us with sequences of scores of genomes of higher eukaryotes. Increasingly sophisticated statistical analysis can now be applied to these sequences (as readily accessible programs) to predict gene families, their variations and evolution between and within organisms [18, 24–28]. The available information on sphingolipid metabolism in yeast makes it relatively easy to identify potential homologs in higher eukaryotes and evaluate their functions in sphingolipid metabolism. Both zwitterionic and acidic glycosphingolipids have been detected in *Drosophila* [29, 30]. Zwitterionic sphingolipids contain phosphoethanolamine linked to GlcNAC (N-acetyl glucosamine) residues. The acidic sphingolipids include those containing phosphoethanolamine and glucuronic acid or phosphoethanolamine and hexose and N-acetylhexose [29, 30]. The core structure of *Drosophila* glycosphingolipids consists of mannosylglucosylceramide (Manβ1-4Glcβ1-cer). Mutations in enzymes of glycosphingolipid biosynthesis such as *egghead* (encoding a β1,4-mannosyltransferase) and *brainiac* (encoding a glycosyltransferase) have been described. These mutants have similar and widespread phenotypes that affect several signaling pathways in embryonic development and oogenesis [29–32]. This review focuses on the enzymes of the canonical de novo sphingolipid biosynthetic pathway, a few related enzymes and proteins.

Traditionally, in *Drosophila*, phenotype-based classical genetic screens were the norm for identification of components of pathways [17, 21, 33–35]. Although classical genetic studies had already identified several developmentally required genes, their identities as enzymes of sphingolipid metabolism were established only recently [31, 36–42]. The ease of identifying homologs and the development of sensitive techniques such as mass spectrometry for identifying lipid molecules make reverse genetics an attractive approach for systematic in vivo analysis of enzymes of sphingolipid metabolism. Genome annotation, prediction of gene ontologies and identification of new enzymes of sphingolipid metabolism and signaling in other species have resulted in annotation of several genes as enzymes of sphingolipid metabolism in *Drosophila* [18, 43]. This has also bolstered recent attempts to apply reverse genetics approaches to obtain mutations in genes of interest.

P-element-mediated mutagenesis is a common method of randomly inserting engineered transposable elements to disrupt genes [44–63]. These transposable elements assist users to read off neighboring sequences and thus pinpoint their chromosomal location; can be excised, leading to reversion of effects of mutation; or can be improperly excised to generate deletion of neighboring sequences and thus produce an allelic series of mutants. They can even be manipulated to overexpress neighboring genes with the use of binary expression systems such as UAS-GAL4 systems. Another milestone in *Drosophila* is the standardization of techniques to target genes of interest by homologous recombination [64–66]. While, this approach is in its infancy, increased efficiency and ease of application should make it a popular tool to target gene mutations. Yet another method of obtaining mutants is

FlyBase 101

The *Drosophila* genome comprises of about 180 Mb of DNA of which 120 Mb is euchromatic chromosome. This DNA is encoded by the sex chromosomes X and Y, chromosomes 2 and 3 and a very small chromosome 4. The decoding of the *Drosophila* genome has been possible largely due a collaborative effort between the Berkeley Drosophila Genome Project and Celera Genomics. Such efforts have also resulted in sequencing more than 260,000 ESTs (expressed sequence tags) and information from these transcripts has aided the global structural annotation of the fly genome. It is now established that there are more than 13,000 protein-coding genes in *Drosophila* and in addition there are few hundred genes that code for tRNA, microRNA and other small RNAs. Detailed documentation of all the above information combined with archived information of about a century's worth of research in *Drosophila* has been the major focus of the database of the *Drosophila* community **FlyBase (http://flybase.bio.indiana.edu/)**. The FlyBase is getting closer to being a one-stop source for all information *Drosophila*!

Information can be retrieved in several formats, such as cyto-search, gene search, deficiencies, transposable elements etc. Typically structural annotation includes gene name, synonyms (including the CG {for **C**omputed **G**ene} number for each gene), chromosomal location, DNA scaffold and BACs of the region, stocks bearing chromosomes with deficiencies uncovering the region or duplications of the region, stocks with P-element insertions in the vicinity of the gene, transcript information, gene ontology links, findings from studies on the gene and mutants, if any, with their references. In addition, all new information pertaining to the gene is constantly updated.

MAP

The annotated data maintained at FlyBase has information of the gene location on the four chromosomes. The chromosomal location is identified by numbers 1–104. 1–20 for the X chromosome, 21–60 for the second, 61–100 for the third, 101–102 for the fourth, 103–104 for the Y (alternatively h1–h25 on the Y) chromosome. For the most part, these locations are identified as distinct band patterns clearly visible in chromosomal squashes prepared from the larval polytene salivary chromosomes. These numbers are further subdivided alphabetically based on minor band patterns. Map positions have played a very important role in application of genetic analysis in *Drosophila*; in addition, they are used for description of deficiencies and duplications and more recently have been of great use in correct and complete sequencing of the genome.

BALANCERS

The FlyBase also provides very useful information on balancers, chromosomes that carry a dominant visible marker and gross chromosomal rearrangements, thus preventing recombination during meiosis (meiotic recombination occurs only in females in *Drosophila*). These chromosomes thus facilitate the propagation of recessive mutations, especially lethal and semi-lethal ones, over successive generations without the aid of molecular analysis or selection.

DEFICIENCIES

Detailed information is also available on deficiency stocks (chromosomes with large segments of individual chromosome deleted, which prove very useful in mapping and in reverse genetic approaches to isolate mutations in specific genes.

P-ELEMENT INSERTION

Large scale genome-wide insertional mutagenesis has resulted in collections of thousand of lines where engineered P-elements have been inserted (largely) into the 5′ untranslated regions of genes. The precise site of insertion of these P-elements is established by performing 'plasmid rescue', a technique to circularize the DNA from the ends of the P-elements, including a piece of the adjacent genomic DNA. The adjacent DNA is then sequenced using primers from the foot of the P-element. In many instances the mere insertion of the P-element can disrupt the transcription and translation of the gene and produce a mutant. P-insertions can also have less severe or no effect on the transcription/translation of the gene. In these instances the P-elements can be mobilized using a fly strain carrying a modified transposase. Such mobilizations of P-elements are generally precise, but when performed in large numbers, a small but substantially recoverable number of them will result in 'imprecise excision' of the P-element and result in deletion of neighboring DNA. With a little more sophistication and PCR-based screening this method can be used to generate a series of directional and increasingly larger deficiencies and thus null mutations of the neighboring genes.

The FlyBase and *Drosophila* genome resource center also make it possible to obtain all available stocks such as mutant lines, P-insertion lines, balancers, deficiencies, all lines carrying accessory tools such as GAL4 driver lines, flies with FRT at the base of chromosomes to generate somatic mosaic for clonal analysis, FLP lines that initiate recombination between two FRT chromosomes and also all full-length and alternatively spliced ESTs. Where stocks are not available at the stock center, references are generally available for a possible source. Information on BACs mapping to a genomic location are also available, and these BACs can be obtained. BACs are very useful in the design and generation of gene targeting and genomic transgenic rescue constructs. Thus accessing the FlyBase makes it a one-stop center to obtain necessary information to elucidate the in vivo role of functional genes in *Drosophila*.

Link to the FlyBase reference manual including links to electronic version of Dan Lindsley's and Georgianna Zimm's 'Red Book', The Genome of Drosophila melanogaster
http://flybase.bio.indiana.edu/docs/lk/refman/refman-sections.html
http://flybase.bio.indiana.edu/docs/lk/refman/refman-G.html

Box 1. FlyBase 101.

random ethyl methane sulfonate (EMS) mutagenesis and subsequent transgenic rescue or Western analysis experiments to obtain either homozygous lethal or viable mutants, respectively [67, 68]. Although this technique is labor intensive, it provides an unbiased approach to obtain mutations in any gene of interest irrespective of whether it is viable or lethal. All these experimental applications benefit from *Drosophila* genome annotations maintained at FlyBase. Information is documented about the location, transposable elements, complementary DNA (cDNA), bacterial artificial chromosomes (BACs) and various *Drosophila* strains harboring deficiency and duplications of genes of interest (see box for more information). Thus, one could start with the identification of an annotated gene as a candidate and use available resources to generate mutants or undertake transgenic expression studies. Some of the enzymes of sphingolipid metabolism have been catalogued in recent studies [15, 16]. In one such study, 31 genes with homology to mammalian counterparts of enzymes of sphingosine phosphate and lysophosphatidic acid metabolism were identified, and RNA in situ hybridization of these genes was performed to evaluate their expression pattern during development in flies [16].

Insect and *Drosophila* sphingolipids

Like mammals, *Drosophila* membranes also contain sphingolipids [69, 70]. These include both the simple sphingolipids and the complex glycosphingolipids. *Drosophila* sphingolipids are important structural components of cellular membrane, as in mammals and other eukaryotic systems. Similar to other eukaryotic systems, they are also intimately associated with the phase separation of plasma membrane into raft like components [69]. Despite these similarities in function, there are some changes in the sphingolipid composition of dipterans compared to mammalian membranes [69, 71, 72]. There are some reports of sphingolipids containing phosphocholine ceramide (sphingomyelin) in insects [73, 74]. Most dipterans, including *Drosophila*, however, have phosphoethanolamine ceramide instead of sphingomyelin as a major constituent of membranes [69, 71, 72, 75]. Although *Drosophila* lacks sphingomyelin, basic components for the synthesis of sphingolipids exist in *Drosophila*. The major sphingoid bases in *Drosophila* and other dipterans are tetradecasphingenine (C14) and hexadecasphingenine (C16) [71, 76]. Glycosphingolipid analysis in *Calliphora vicina* reveals that tetradecasphing-4-enine (C14:1) constitutes about 54% of the base, while the hexadecasphing-4-enine (C16:1) is about 23%. The remaining 23% consists of C15 and C17 bases [72, 75, 77]. In contrast, C18 sphingoid base is the major constituent in mammals [78]. This is due to the preferential acyl-coenzyme A (CoA) recognition by the mammalian serine palmitoyltransferase enzyme [79, 80]. Furthermore, analysis of insect larval tissues showed that the amide-linked fatty acids are predominantly arachidic acid (C20:0), behenic acid (C22:0) and stearic acid (C18:0) [72, 77]. Recently, studies were undertaken to evaluate the lipid composition and analysis of rafts from embryos of *Drosophila*, and these studies also evaluated the sphingolipid composition [69]. Sphingolipids and phospholipids are shorter in chain length than those found in mammals. *Drosophila* sphingolipids are based predominantly on a tetradecasphingenine (C14). Arachidic acid is the predominant amide-linked fatty acid, followed by C22 and C18 fatty acids. Incidentally, the fatty acids of phosphoglycerolipids are also shorter than in mammals, the longest being C18. The existence of shorter sphingolipids and phosphoglycerolipids would predict lower melting temperatures, and membranes would remain fluid at lower temperature. Other studies have confirmed these findings subsequently [13]. An HPLC (high-performance liquid chromatography)-based method was recently developed for the analysis of free sphingoid bases in *Drosophila* wild-type flies, and compositions were compared with those extracted from some mutants of sphingolipid metabolism [70]. The predominant free sphingosine was tetradecasphingosine, followed in decreasing order by dihydrotetradecasphingosine, hexadecasphingosine and dihydrohexadecasphingosine. While the amounts of these sphingosines are dramatically reduced in a serine palmitoyltransferase mutant (*lace* mutant, see below), their relative levels, especially those of the dihydro-species, are increased in a mutant of sphingosine kinase (*SK2* mutant). The existence of shorter chain length sphingoid bases correlates well with the requirement of lower ambient temperatures for *Drosophila* survival (between 18– 20 °C). Although these efforts have begun to characterize the sphingolipid composition, comprehensive profiles of *Drosophila* sphingolipids and their metabolic pathways are far from being established. Since *Drosophila* has emerged as a very useful and powerful in vivo model system in several fields, the application of studies done in *Drosophila* could be extended to human situations with ease if one could compare and contrast the lipid composition and metabolic pathways of *Drosophila* and mammalian organisms. Comprehensive characterization of sphingolipids is therefore a prerequisite for useful comparisons between *Drosophila* and humans. In recent years, mutant and transgenic analyses have been carried out for some of the *Drosophila* enzymes. Sphingolipid profiles have also been generated in the context of some of the mutant flies. There is a need to continue these efforts and identify sphingolipid composition and distribution in greater detail for all the enzymes.

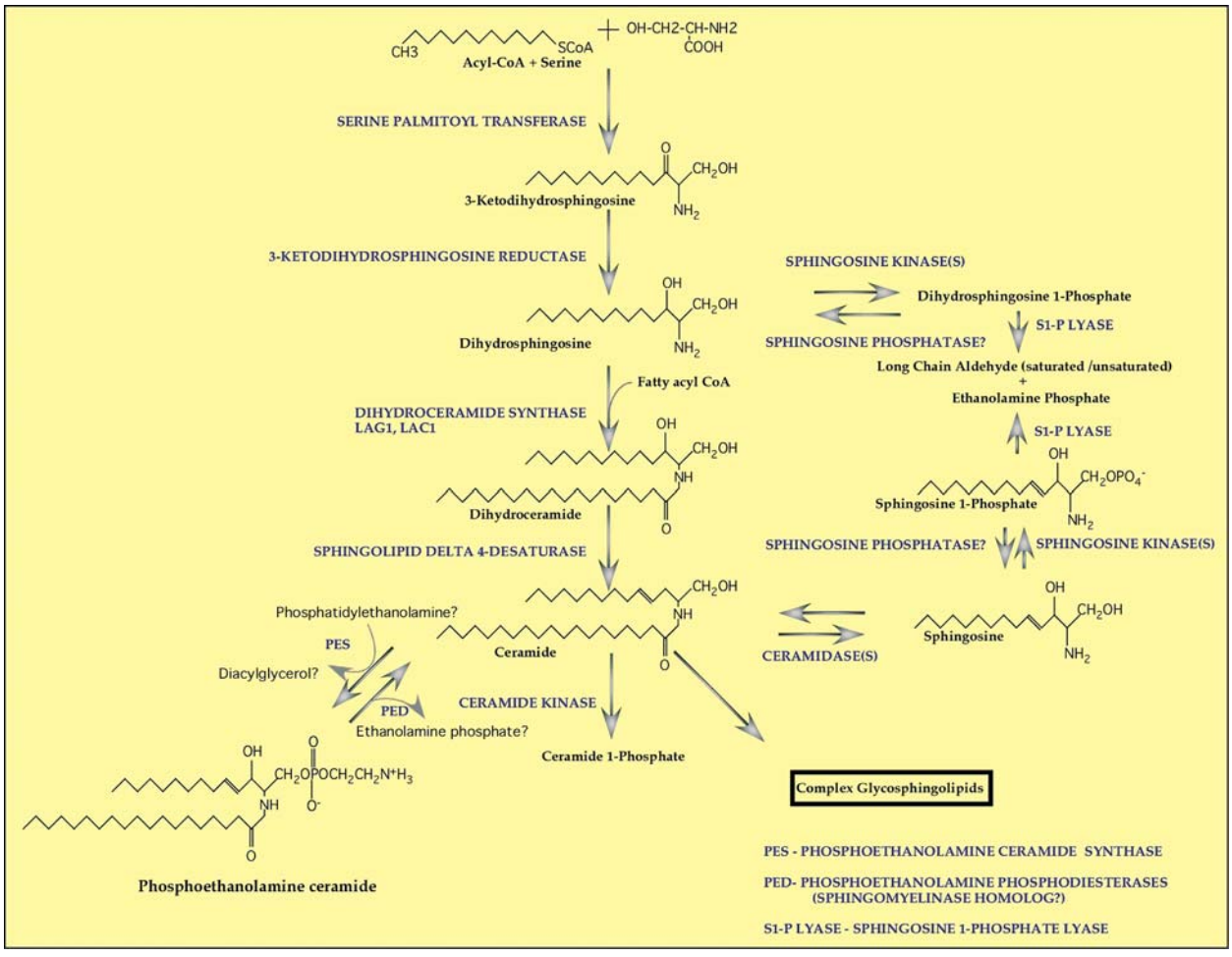

Figure 1. Plausible de novo sphingolipid biosynthetic pathway in *Drosophila*.

Enzymes of *Drosophila* sphingolipid metabolism

Some of the enzymes of the de novo sphingolipid biosynthetic pathway have been identified in *Drosophila* and several studies have highlighted the importance of these enzymes in development, differentiation, signaling and apoptosis [13–15, 31, 37, 39, 69, 70, 81, 82]. A canonical de novo sphingolipid biosynthetic pathway (modified to depict a potential *Drosophila* pathway) is depicted in figure 1. It begins with the condensation of acyl-CoA and serine and through a series of reduction, acylation and desaturation leads to the generation of ceramide. Sphingosine, sphingosine 1-phosphate and ceramide 1-phosphate are derived from ceramide.

Serine palmitoyltransferase (SPT)

The first step in the de novo sphingolipid biosynthetic pathway is the condensation of an acyl-coA with serine to generate 3-ketosphinganine [83, 84]. This is catalyzed by a rate-limiting enzyme in yeast and in mammals called serine palmitoyl CoA transferase, a pyridoxal phosphate (PLP)-dependent hetero-oligomer of LCB1 and LCB2 subunits. Both have been described as transmembrane proteins. The first genetic evidence for the in vivo significance of this enzyme came from studies in *Saccharomyces cervisiae* with the isolation of mutants that required external supply of phytosphingosine for growth and synthesis of complex sphingolipids [2, 85–88]. These mutants were then shown to be mutants for SPT activity. In yeast an additional protein termed TSC3p is an activator of this enzyme [89]. No mammalian or fly homolog has been identified for this protein, by homology search. *Drosophila* encodes genes for both LCB1 and LCB2 proteins.

The *Spt-1* gene encodes the LCB1 subunit of SPT. It is annotated as CG4016 in FlyBase and encodes a 468-amino acid (aa) protein. This gene is located at 49F4 on the right arm of the second chromosome. In a report of a genome-wide gene disruption project using engineered transposable elements, a fly line was obtained with a

P-element inserted into the coding region of *Spt-1* gene. These flies are homozygous viable and fertile. However, it remains to be investigated whether insertion of the P-element in the 5′-untranslated region of the cDNA affects its transcription and translation in the animal. This P-insertion can be used to generate small deficiencies that would delete the *Spt-1* gene, creating null alleles. Such null alleles can also be used to investigate its genetic interaction with the Lace protein described below. Mutations in the LCB1 subunit of the human *lcb1* gene lead to human hereditary sensory neuropathy type 1, a dominantly progressive neuropathy affecting the sensory and autonomic nervous system of the lower limbs [90]. Although it was initially thought to confer increased activity and hence the dominant phenotype, subsequent studies have argued that the mutation in itself suppresses SPT activity. The dominant effect could be due to a dominant negative effect of the mutated protein [91, 92].

The *lace* gene, annotated as CG4162, encodes for the LCB2 subunit of the SPT gene. It maps to 35D1-4 region of the left arm of the second chromosome. Its transcript encodes a protein of 597 aa. The *lace* mutant $lace^{l(2)k05305}$ is an insertion of a P-*lacW* transposable element 8–10 bp upstream of the transcription start site of the LCB2 subunit of the SPT gene [82]. The P-insertion interferes with the transcription of the gene and is a hypomorphic mutant of the lace gene. Null alleles generated by imprecise excision of this P-element insertion are homozygous lethal. Certain hetero-allelic combinations of independent excision lines do make it to adulthood and are thus considered hypomorphic allelic combinations. Hypomorphic alleles that yield adults have several defects in the adult external organs, for example appendages, eyes and bristles, thus demonstrating the importance of the gene in development and differentiation of adult structures in *Drosophila*. The Lace protein is required for proper development of various imaginal discs (epithelial in-folding in the larvae of holometabolous insects that develop into adult appendages such as legs, antennae, wings and so on, during metamorphosis from larval to adult forms – Gene Ontology Consortium definition). These aberrations of the discs are suppressed by increased mitogen-activated kinase kinase activity. *lace* mutants show increased cell death in some of the developing tissues in larvae. Tissues showing increased cell death are associated with activation of ectopic Janus kinase (JNK) pathway. An interesting feature of the *lace* mutant is that the degree of JNK activation varies from tissue to tissue and results in variable sensitivity to cell death in individual tissues. A noteworthy feature is that larval epidermis is least sensitive to the loss of lace activity [82].

It is interesting to note that in the genome-wide yeast two-hybrid analysis SPT-1 protein showed no interacting partners, while the Lace protein interacted with 13 proteins [93]. These include ribosomal proteins RpS27A; RpL31; CG4046, the cytosolic small ribosomal subunit protein; transporters such as G Vha13 (proton transporter) protein and CG6126 protein, an organic cation transporter; CG5802, a UDP-galactose transporter; alpha-glucosidase; hsc-70; and thioredoxin-like protein DmTrx-1. The biological relevance and the regulatory role of each of these interactions need to be validated. It is also worth mentioning that since tetradecasphingenine is the major sphingolipid in *Drosophila*, the SPT enzyme complex should recognize the C12 acyl-CoA (fig. 1). If a preference for C12 acyl-CoA can be biochemically demonstrated, the enzyme complex should be appropriately termed serine lauroyltransferase rather than serine palmitoyltransferase.

The serine palmitoyltransferase 2-subunit gene of *Leishmania* was knocked out, and these organisms are viable. They are completely deficient in de novo sphingolipid biosynthesis and internally lack sphingolipids. They show differentiation defects and upon entering the stationary phase die while accumulating extensive multivesicular tubular structures [94, 95]. Interestingly, *Leishmania* that are deficient in phosphoglycan persist in hosts without causing the disease, and those deficient in ether phospholipids and glycosylinositolphospholipids are virulent as amastigotes, effectively inhibiting macrophage activation [96].

3-Ketodihydrosphingosine (3-ketosphinganine) reductase

The second enzyme in the pathway is 3-ketodihydrosphingosine reductase, an NADPH requiring dehydrogenase. In this reaction 3-ketodihydrosphingosine is converted to dihydrosphingosine by the NADPH-dependent oxidoreductase, a member of the short chain dehydrogenase/reductase family [97]. Over 60 enzymes are found in both prokaryotic and eukaryotic cells. The enzymes display between 15 and 30% identity, and the family is characterized by an YXXXK motif in the catalytic site. These proteins also bear a Rossman fold that borders the NADPH-binding domain. In yeast the reaction is catalyzed by TSC10 enzyme, a member of the short-chain dehydrogenase and reductase family. Two members of this family of dehydrogenase/reductase show homology to the TSC10 protein in *Drosophila*, but a cognate NADPH-reductase is yet to be definitively identified.

Dihydroceramide synthase

Although the next step catalyzed by dihydroceramide synthase has been characterized as an acyl-CoA dependent sphinganine acyltransferase (dihydroceramide synthase), the identity of this gene is not yet reported. In

yeast, studies have indicated that loss of *Lag1* and *Lac1* genes result in almost no ceramide synthesized and accumulation of abnormal sphingolipid products. It is now generally agreed that activities of LAG1 and LAC1 are required for normal enzymatic activity of ceramide synthase [98–103]. Two annotated genes in *Drosophila* show homology to the yeast *Lag1* and *Lac1* and are briefly described here.

CG3576 has been annotated as the fly *Lag1* homolog. It is localized to 5E3-4 of the X chromosome. The protein is predicted to have 400 amino acids and includes a thin-layer chromatography (TLC) domain (TRAM, LAG1 and CLN8 homology domain), a homeobox domain and transmembrane domains. Several lines with P-element insertions in the gene are available. Some of these P-element insertions lead to lethality during the larval phase of development [104].

A less homologous second gene is annotated as CG11642. It is localized to the X chromosome at 1D2. It encodes for a 368-aa protein with a putative signal recognition particle (SRP)-dependent cotranslational membrane-targeting sequence, a TLC domain and transmembrane domains. This protein lacks a recognizable homeodomain but contains a coiled-coiled region. A line with P-element insertion in the vicinity of the gene is available from the stock center. This insertion line is viable and fertile as homozygote. Although this P-insertion could serve as an excellent starting point for evaluating this gene, it is not clear if its insertion alone disrupts either the transcription or translation of the gene. The two *Drosophila* proteins share about 20% identity and 40% similarity in amino acid sequence.

While it is not known if Lag1 and Lac1 proteins are only a part of the ceramide synthase complex, or regulatory subunits or are the sole components, their activity is certainly required for CoA-dependent acylation of sphinganine to dihydroceramide in *Saccaharomyces cerevisiae*. It remains to be addressed if this is true in mammalian sphingolipid metabolism and in *Drosophila*.

Dihydroceramide desaturase

The next step in the metabolic pathway is the desaturation of dihydroceramide to ceramide by the desaturase enzyme. It is annotated as CG9078 and encodes a 321-aa protein sphingolipid delta 4 desaturase. It is also known as degenerative spermatocyte and infertile crescent. It maps to 26A on the left arm of the second chromosome. Phenotypic analysis of this gene was first initiated by the identification of a semi-lethal mutant in a screen for male sterility [105]. In a subsequent study (before characterization of the protein as a desaturase), the sequence of the gene was described and the phenotype was further elaborated [37]. This mutant was called *degenerative spermatocyte* (*des*). The protein was proposed to be required for initiation of meiosis in spermatogenesis. In *des* mutant testes, the primary spermatocytes mature to the right size but subsequently degenerate without initiating meiotic chromosome condensation. The females are apparently normal. The gene alternatively transcribes transcripts α, β and γ. While the *des* mutants show no difference in levels of α and β transcripts, they have very low levels of γ transcript in the spermatocytes of mutant flies. Genomic DNA encoding only for γ transcript rescues the semi-lethality and male sterility. The authors proposed that the protein is required for initiation of meiosis during spermatogenesis and may be required for interactions between primary spermatocytes and surrounding somatic cells. Lethal alleles of this gene have also been reported [106]. In this study affinity-purified antiserum against a recombinant Des-1 protein was used to localize Des-1 protein throughout male meiosis. Along with antibodies against tubulin and DNA staining with Hoechst 33258, they followed the fate of Des-1 protein throughout male meiosis. Initially the Des-1 was loosely associated with the developing meotic spindle. The distribution overlapped with the distribution of mitochondria. During meiosis, the mitochondria align themselves along the microtubule bundles of the spindle apparatus. The pattern of Des-1 protein paralleled that of mitochondria along the microtubule bundles. Like mitochondria, no Des-1 protein was seen at the astral microtubule. In later stages Des-1 protein localized to structures called Nebenkern, a microscopically identifiable mitochondrial derivative. Its co-localization along microtubule bundles and mitochondria and presence of six-potential membrane spanning domains raised the possibility that Des-1 may act as part of an anchoring mechanism that links membrane-bounded cellular compartments to components of the cytoskeleton. However, with the identification of the protein as an authentic delta 4-desaturase, it is likely that failure to convert dihydroceramide to ceramide could account for some of the phenotype instead of only a physical interaction of the protein with components of spermatocyte differentiation process. A comprehensive analysis of gene transcripts, proteins and the embryonic lethal phenotype of null mutants will reveal more about this intriguing gene.

Ceramidases

Enzymes with ceramidase activity catalyze the conversion of ceramide to sphingosine. Depending on the pH optima of these enzymes, they have been categorized as acid, neutral and alkaline ceramidases. In mammals, a hetero-oligomeric enzyme catalyzes acid ceramidase activity. This enzyme has been implicated in sphingolipid catabolism and has been localized to the lysosomes. No acid ceramidase homolog has been identified in *Droso-*

phila. However, two *Drosophila* homologs of genes with ceramidase activity in mammals have been identified. CG1471 (Cdase) has been shown to have neutral ceramidase activity. The second *Drosophila* gene, called brainwashing, is homologous to a recently characterized mouse gene that acts on long chain ceramides in tissue culture studies [107].

CG1471 the *Drosophila* neutral ceramidase homolog localizes to 99F on the third chromosome. Biochemical characterization of the *Drosophila* neutral ceramidase was first reported by Ito and colleagues [108]. When the protein is overexpressed in S2 cells, it is continuously secreted into the medium. The enzyme hydrolyzes various N-acylsphingosine and not other sphingolipids such as galactosylceramide, sphingomyelin or GM1. It has a pH optimum of 6.5–7.5. RNAi-mediated deletion of this protein does not affect cell viability or ceramide levels, although the enzymatic activity is lost. Recently, it has been shown that targeted expression of this enzyme rescues retinal degeneration in a subset of *Drosophila* phototransduction mutants [13].

The annotated brainwashing gene is CG13969 and localizes to 38B2-3 region of the left arm of second chromosome. A P-element insertion was isolated in this gene and was subsequently mobilized to generate brainwashing (*bwa*) mutants. These mutants have been reported to show fusion of the beta lobes in the central brain [107]. In a communication to the FlyBase, the authors report incomplete penetrance of this phenotype in subsequent generations. Thus it remains unclear if the fused phenotype results from the loss of the gene. It would be interesting to see if there are any genetic interactions between this ceramidase and the alkaline ceramidase localized on the third chromosome. Although in vivo expression of the mammalian homolog of brainwashing specifically acted on long chain ceramides, it nevertheless acted on other ceramide substrates in vitro.

A neutral ceramidase homolog has been identified in the slime mold *Dictyostelium discoideum* based on sequence identity to the neutral ceramidase family of genes. However, the protein encoded by the gene has acidic pH optimum and its knockout eliminates ceramidase activity at acidic pH. The loss of this activity has no apparent phenotypic consequence in the animal [109].

Ceramide metabolism and membrane turnover in *Drosophila* photoreceptors

Mutations in genes encoding for proteins of Drosophila phototransduction cascade, a prototypic guanine nucleotide-binding protein-coupled receptor signaling system, lead to retinal degeneration and have been used as models to understand human degenerative disorders [110–114]. Modulation of the sphingolipid biosynthetic pathway rescues retinal degeneration in *Drosophila* mutants [13]. Targeted expression of *Drosophila* neutral ceramidase rescues retinal degeneration in arrestin and phospholipase C mutants. Decreasing flux through the de novo sphingolipid biosynthetic pathway by losing one copy of the LCB2 subunit of the rate-limiting enzyme SPT, as in *lace* heterozygotes, also suppresses degeneration in these phototransduction mutants. Both genetic backgrounds modulate the endocytic machinery because they suppress defects in a dynamin mutant. Suppression of degeneration in arrestin mutant flies expressing ceramidase correlates with a decrease in ceramide levels. These effects of ceramidase were likely due to facilitation of a downstream endocytic process. This has now been demonstrated in a sensitized mutant background to better visualize the effects of targeted expression of ceramidase. $ninaE^{I17}$ are null mutants of rhodopsin (Rh1), the light transducing G-protein-coupled receptor. Rhodopsin not only serves as a transducer of light signal but also has a critical role during differentiation of photoreceptors. The process of phototransduction is initiated in a specialized microvillar region of highly dense plasma membrane called rhabdomere. The organization of this rhabdomeric membrane commences during pupal development and rhodopsin in conjunction with members of the Rho family of proteins organizes an actin-rich terminal structure called rhabdomeric terminal web [115–117]. The rhabdomere terminal web organizes the differentiating plasma membrane into rhabdomere and directs proper signaling and trafficking during its biogenesis. In the absence of rhodopsin this structure is not well established, and rhabdomere is disorganized. The ill-formed rhabdomeric membrane is then withdrawn and assimilated into the cell. The photoreceptor cells subsequently undergo disuse atresia. Thus, rhodopsin null mutant photoreceptors provide an extremely sensitized background to evaluate whether ceramidase expression facilitates membrane turnover. Indeed, expression of ceramidase facilitates the dissolution of improperly organized and involuting rhabdomeric membranes [81]. Ceramidase expression also facilitates the endocytic turnover of tagged, inducible Rh1. Although ceramidase expression aids the removal of internalized rhodopsin, it does not affect the turnover of Rh1 in photoreceptors maintained in dark, where Rh1 is not activated and thus has a slower turnover and a long half-life. Therefore, the phenotypic consequence of ceramidase expression in photoreceptors is caused by facilitation of endocytosis. This study provides mechanistic insight into the sphingolipid biosynthetic pathway-mediated modulation of endocytosis and suppression of retinal degeneration.

Sphingosine kinase

The laboratories of Harris and Saba have analyzed the primary structure of the sphingosine kinase (*SK*) genes

and have described phenotypic characterization of one of the mutants [14, 70]. Two *Drosophila* genes, named *SK1* and *SK2,* have been annotated. The two *Drosophila* genes are closer in structure to murine *SPHK2* than *SPHK1* and show a slightly greater degree of sequence conservation amongst them. While SK1 is localized to 10B13 on the X chromosome, the *SK2* gene is on the third chromosome at 63A3-5. The two genes encode functional sphingosine kinases since they complement a yeast mutant of sphingosine kinase and show in vitro ability to phosphorylate sphingosine, dihydrosphingosine and phytosphingosine. Most of the SK activity is recovered from cytosolic fractions of a cell extract, although activity is detected in membrane fractions as well. The two genes are ubiquitously expressed, with highest levels detected in pupae and embryos. *SK2* has consistently higher expression than *SK1*.

A mutant line called $SK2^{KG05894}$, a P-element insertion in the vicinity of the 5′-untranslated end of the cDNA was identified, and Northern analysis and in situ hybridization showed no message was being transcribed in this mutant animal [14, 104]. These mutant animals are homozygous viable. However, they show increased levels of sphingolipid metabolites throughout their life cycle, reduced flight ability and decreased fertility. Although gross structural abnormalities are not detected in the muscles of these flies, they are flightless. Mutant females retained eggs longer in their abdomen, which resulted in egg enlargement due to increase in the size of the ovaries in these eggs. Egg laying is decreased 2-fold compared to wild-type controls. The flies are viable probably due to functional redundancy between the two genes. Although a certain extent of compensation is expected by the presence of the *SK1* gene in these mutant flies, it is not complete, since the *SK2* mutant animals still show a 1.8-fold increase in the basal levels of long chain bases (LCBs) and have defects in reproduction and muscle function.

Ceramide kinase

Ceramide is converted to ceramide-1-phosphate by ceramide kinase. It belongs to a new family of lipid kinases distinct from sphingosine and diacylglycerol kinases. A human ceramide kinase was first cloned and described [118]. Homologs have been identified in *Drosophila*, *Caenorhabditis elegans* and plants; however, no such gene has been found in yeast. It has been proposed that ceramide kinase may be involved in terminating pro-apoptotic signals of ceramide.

The *Drosophila* homolog of ceramide kinase, CG16708, maps to 82F11-83A1 on the right arm of the third chromosome. During development there is a strong maternal contribution of this protein. It is detected in hindgut from stages 11 to 13 and shows a speckled pattern in all regions of the midgut by stage 15. In addition, very specific expression of this message is detected in the posterior spiracle specific anlagen, embryonic spiracle and embryonic hindgut. Based on SAGE (serial analysis of gene expression) analysis this is one of the genes indicated in the autophagic cell death of the salivary gland of larvae during pupariation of *Drosophila* [119].

A recent study described a truncating mutation in a ceramide kinase like gene that resulted in autosomal recessive retinitis pigmentosa [120]. Although its ceramidase kinase activity is yet to be evaluated, it has a high degree of sequence conservation with the ceramide kinase gene from other organisms. These mutants are expected to accumulate ceramide and contribute to the pathogenesis of retinitis pigmentosa. Further work will have to be undertaken to substantiate these findings and delineate the role of the sphingolipid biosynthetic pathway in the pathogenesis of retinitis pigmentosa in these patients.

A ceramide kinase mutant in *Arabidopsis* called the accelerated cell death 5 mutant shows enhanced disease symptoms during pathogen attack and apoptotic-like cell death, dependent on defense signaling late in development [10]. Phosphorylated C2 ceramide suppresses plant programmed cell death, while C2 ceramide induces it. In fact, not only do plants contain components of the sphingolipid signaling pathway, but recent studies indicate that they might participate in phenomena unique to plants [11].

Ceramide transfer protein

Sphingolipids are synthesized vectorially. The hydrophobic core of sphingolipids is synthesized in the endoplasmic reticulum (ER) [4, 121–123]. Ceramides synthesized in the ER are transported to the Golgi, where they are further modified for the biosynthesis of complex sphingolipids. The transport of ceramide from the ER to Golgi is proposed to be mediated by two pathways – a major ATP-dependent non-vesicular, cytosol-requiring pathway and an ATP-independent minor pathway [124, 125]. Recently, a ceramide transfer protein (CERT) was identified as a key component in the ATP-dependent major pathway [126]. CERT was identified as the factor defective in LY-A cells, which can synthesize ceramide in the ER but are genetically impaired in delivering ceramide to the Golgi. Consequently, this mutant cell line is defective in sphingomyelin synthesis and resistant to the toxic effects of lysenin, a toxin initially isolated from the earthworm *Eisenia foetida* [127]. It binds to sphingomyelin in cell membranes and causes lysis [128]. Cells lacking sphingomyelin are therefore resistant to it. CERT is a cytosolic protein that contains a START domain predicted to form a lipid-binding pocket (a putative domain for catalyzing lipid transfer), a pleckstrin homology (PH) domain, similar to PH domains that target proteins to the

Golgi, and an FFAT motif that is predicted to anchor it to ER/Golgi. CERT homologs have been found in *Drosophila* and *C. elegans*. The Drosophila CERT homolog CG7207 encodes a 601-aa protein. Like the mammalian protein, it contains an N-terminal PH domain (between residues 40 and 134) and a C-terminal START domain (between residues 370 and 596). Yeast two-hybrid analysis suggests interaction with two proteins, including CG10971, a protein, implicated in cytoskeleton organization and biogenesis.

Sphingomyelin synthase

Sphingomyelin synthase generates sphingomyelin by transferring a phospholcholine moiety from phosphatidylcholine to ceramide. The products of the reaction are diacylglycerol and sphingomyelin. Thus, this enzyme is not only capable of regulating the concentration of sphingomyelin and ceramide but can also influence intracellular concentrations of diacylglycerol, another important second messenger in the cell. Using a functional cloning strategy, two groups have recently identified a family of integral membrane proteins that could function as spingomylelin synthase [129, 130]. A host of structurally related proteins that could function in the synthesis of closely related sphingolipids have also been identified [130]. It would be interesting to see if these members have overlapping functions or are specific to unique substrates. A genome search in organisms whose genomic sequencing were available was performed. No sphingomyelin synthase homologs are found in *Drosophila*. However the authors' analysis show sphingomyelin synthase-related *Drosophila* proteins termed *Dm* CSS3β (CG32380 localized at 65F7-9 on the third chromosome), *Dm* CSS1α (CG11438), *Dm* CSS1β (CG11426, both these genes are located around 78E4 on the third chromosome) and *Dm* CSS2 (CG31717 located on the second chromosome at 31B3). Members of the 3β family show the closest sequence similarity to bona fide mammalian sphingomyelin synthases SMS1 and SMS2. Since *Drosophila* membranes contain little or no sphingomyelin but rather are composed of its close relative phosphoethanolamine ceramide, it remains to be seen if this enzyme serves to synthesize phosphoethanolamine ceramide.

Sphingomyelinases

In vitro studies in higher eukaryotes have indicated the existence of several sphingomyelinases based on their pH optima [131]. Acid sphingomyelinases have been ascribed a function in sphingomyelin catabolism. This gene is alternatively spliced to generate a secretory form of the acid sphingomyelinase [132]. *Drosophila* has traditionally been described as lacking sphingomyelin, but instead has phosphoethanolamine ceramide, where a phosphoethanolamine replaces phosphocholine in sphingomyelin. Several acid sphingomyelinase homologs have been identified in *Drosophila*. At least three genes show a high degree of sequence similarity, and these encode for several alternatively transcribed genes. One molecular study of these transcripts has examined their expression pattern [69].

CG3376 localizes to 60C4 on the left arm of the third chromosome and encodes a protein of 708 aa. Structural annotation depicts it as a metallophosphoesterase. Three transcripts have been described coding for proteins of 322, 735 and 708 aa. CG3376 transcripts are found in stage 14 pharynx, esophagus and tracheal dorsal trunk and in stage 15 in pharynx, esophagus and trachea.

CG15533 is at 99F4 on 3R and encodes a 692-aa protein. This gene shows maternal contribution in the embryo and is ubiquitously found in stages 5–16.

CG15534 is located adjacent to CG15533 on 3R, encoding a 666-aa protein. CG15534 encoded by an adjacent acid sphingomyelinase-like gene is expressed in two symmetrical patches in clypeolabrum between stages 11 and 13 of embryonic development.

Two genes that are similar in sequence, *nSMase1* and *nSMase2*, show neutral sphingomyelinase activity in mammals [133, 134]. A neutral sphingomyelinase homolog exists in *Drosophila*, and this is the only homolog of the identified neutral sphingomyelinases in mammalian studies. In mammals, this gene has been described as both a neutral sphingomyelinase and a lyso-platelet activating factor phospholipase [135, 136]. The *Drosophila* gene CG12034 is located on the left arm of the third chromosome at 63B14. It encodes a 442-aa transmembrane protein. Amino acids 6–271 are described as an exonuclease/endonuclease/phosphatase family domain that is shared by proteins such as AP endonuclease, DNAse 1, synaptojanin and nocturnin. P-insertions in the vicinity of this gene have recently been reported but have not been characterized. Its biochemical properties and role in sphingolipid metabolism are still open to studies.

Since *Drosophila* does not synthesize sphingomyelin, it remains to be seen if any of these sphingomyelinases do exhibit preference for sphingomyelin. Hence, enzymatic characterization needs to be undertaken for all putative sphingomyelinases in *Drosophila*. It would be interesting to evaluate if any of these sphingomyelinases are secretory or digestive, used for digestion of ingested sphingomyelin and also if they possess the esterase activity to hydrolyze phosphoethanolamine ceramide, the fly substitute for sphingomyelin. In this context it is worth noting that cholesterol constitutes at best less than 3% of total sterols in *Drosophila*, and they lack all enzymes required for the synthesis of cholesterol. Instead, *Drosophila* has ergosterol and lanosterol. Nevertheless, adult *Drosophila*

utilise ingested cholesterol for modification of hedgehog protein, which plays a critical role in *Drosophila* development and differentiation. Thus, even though *Drosophila* does not synthesize cholesterol, it has retained proteins capable of identifying and utilizing it for post-translational modification of hedgehog and probably as yet unidentified substrates.

Sphingosine phosphate lyase

Sphingosine phosphate lyase (S1P lyase) catalyzes a pyridoxal phosphate-dependent reaction that cleaves the C2-3 bond aldolytically to yield ethanolamine phosphate and a long chain base. Work in *Dictyostelium* has indicated that S1P lyase is important in development, since mutants of S1P lyase show defective fruiting bodies and apical spores and abnormal slug migration [137, 138]. A *Drosophila* sphingosine phosphate lyase called *Sply* was identified by homology at cytological location 58 on the right of the second chromosome [15]. The authors also identified a P-element insertion within the coding exon of both splice variants of this gene. Complementation studies in yeast indicate that the *Sply* gene of *Drosophila* was an authentic sphingosine phosphate lyase, since it complements the deficiency of the yeast S1P lyase gene. The P-insertion compromises the function of the gene severely. No RNA is detected by either in situ hybridization or by Northern analysis in homozygous mutant flies. Moreover, there is a global increase in the amounts of LCBs and phosphorylated LCBs in these mutant animals. There is an 8-fold increase in (LCBs and about 20-fold increase in LCB phosphates (LCBPs). The mutant flies are partially lethal, and survivors have a specific defect in the dorsal longitudinal flight muscle. Although the development and anatomical arrangement of individual muscle fibers are not affected, mutants on average have fewer pairs of muscle fibers and may show missing fibers, asymmetric arrangement and hypertrophy. Lethality is also complicated by decreased fertility of mutants. Most of these defects are reversed by precise excision of the P-insertion, indicating that P-element-mediated disruption of the gene is responsible for the phenotype in these mutant animals. The increased LCBs and LCBPs are responsible for many of the observed phenotypes. The levels of these intermediates can be decreased by decreasing the gene copy number of the rate-limiting enzyme of the sphingolipid biosynthetic pathway, serine palmitoyltransferase (*lace* heterozygote). *lace* heterozygotes decrease LCB and LCBP levels, and this results in reversion in abnormal muscle pattern and improved performance in flight test of the *sply* mutants. Conversely, loss of both copies of the *Sply* gene results in increased survival of a certain hetero-allelic combination of *lace* mutants that barely survive.

Conclusions

Phenotypic characterization of mutants of enzymes of sphingolipid metabolism is limited to a few, as noted in the preceding paragraphs. It is apparent, even from these early studies, that the de novo sphingolipid biosynthetic pathway is not a simple linear sequence of enzymatic reactions responsible for generating the products ceramide, sphingosine, sphingosine 1-phosphate and complex sphingolipids. This is evident from phenotypes observed in mutants of sphingolipid metabolism studied so far. While even hypomorphic mutants of *lace* have wide ranging and nearly lethal phenotype, *des1* mutants have observable phenotype only in the males. It is conceivable that with further genetic epistatic experiments and sophisticated analytical methods, wider interplay between existing metabolic pathways and presence of branched pathways leading to newer sphingolipid metabolites will be revealed. For example, a recent study uncovered a link between sphingolipid and fatty acid metabolism, demonstrating that the sphingolipid biosynthetic pathway is involved in the palmitate-mediated suppression of SREBP cleavage and regulation of fatty acid metabolism in *Drosophila* S2 cells [139–141]. In mammals the SREBP family of proteins regulate fatty acid and cholesterol metabolism. In *Drosophila* the SREBP protein acts similarly to the SREBP1 family of proteins in regulating fatty acid metabolism and unlike the SREBP2 family, which predominantly modulates sterol metabolism. Cleavage of SREBP is required for its transcriptional regulation of fatty acid and cholesterol metabolism. RNAi-based protein degradation of enzymes involved in sphingolipid and phosphoethanolamine metabolism resulted in interference with palmitate-mediated inhibition of suppression of SREBP cleavage.

Although it is increasingly appreciated that sphingolipids and enzymes of sphingolipid metabolism have a critical function in *Drosophila*, a great deal needs to be learned about cellular distribution and metabolic routes of sphingolipid biosynthesis. We need to understand the spatial localization and sequential changes in the flux of metabolites through this pathway in different situations. Understanding the anatomic and the temporal distribution of sphingolipids and their flux is vital for correlating metabolism with cellular events. An equally important task is to integrate sphingolipid metabolism with activity and subcellular localization of enzymes. Characterization of biosynthetic enzymes, elucidation of mutant phenotypes and genetic interaction studies will further assist in vivo structure-function studies of sphingolipid metabolites. These efforts will determine critical components that regulate synthesis, breakdown and transport of sphingolipids. Information obtained from in vivo studies combined with incisive information obtained from in vitro experiments will have wider ramifications.

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