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Sply regulation of sphingolipid signaling molecules is essential for *Drosophila* development

Deron R. Herr[1], Henrik Fyrst[2], Van Phan[1], Karie Heinecke[2], Rana Georges[1], Greg L. Harris[1,*] and Julie D. Saba[2,*]

[1]Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, CA 92182-4614, USA
[2]Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr Way, Oakland, CA 94609, USA
*Authors for correspondence (e-mail: jsaba@chori.org and gharris@sunstroke.sdsu.edu)

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SUMMARY

Sphingosine-1-phosphate is a sphingolipid metabolite that regulates cell proliferation, migration and apoptosis through specific signaling pathways. Sphingosine-1-phosphate lyase catalyzes the conversion of sphingosine-1-phosphate to ethanolamine phosphate and a fatty aldehyde. We report the cloning of the *Drosophila* sphingosine-1-phosphate lyase gene (*Sply*) and demonstrate its importance for adult muscle development and integrity, reproduction and larval viability. *Sply* expression is temporally regulated, with onset of expression during mid-embryogenesis. *Sply* null mutants accumulate both phosphorylated and unphosphorylated sphingoid bases and exhibit semi-lethality, increased apoptosis in developing embryos, diminished egg-laying, and gross pattern abnormalities in dorsal longitudinal flight muscles. These defects are corrected by restoring *Sply* expression or by introduction of a suppressor mutation that diminishes sphingolipid synthesis and accumulation of sphingolipid intermediates. This is the first demonstration of novel and complex developmental pathologies directly linked to a disruption of sphingolipid catabolism in metazoans.

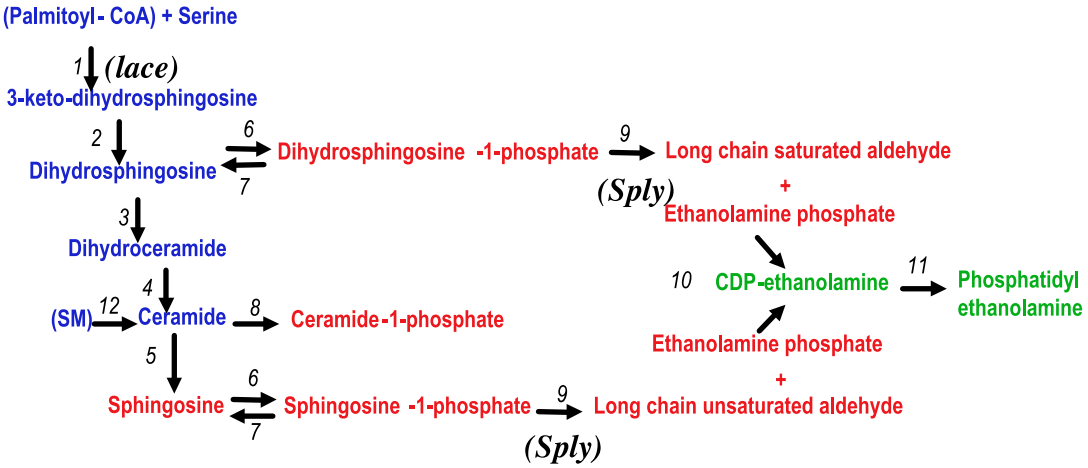
Key words: Sphingosine-1-phosphate, Sphingolipids, Sphingosine phosphate lyase, Muscle, *Drosophila*, Serine palmitoyltransferase, Sphingolipidoses, Reproduction, Apoptosis

INTRODUCTION

Sphingolipids are a complex and ubiquitous class of membrane lipids found in most eukaryotic cells. The polar metabolites of membrane sphingolipids, including sphingosine, sphingosine-1-phosphate (S-1-P), ceramide (Fig. 1) and others, have been implicated in signaling pathways which regulate cell death, survival, differentiation and migration in multi-cellular organisms (Hannun et al., 2001; Merrill et al., 2001; Prieschl and Baumruker, 2000; Shayman, 2000; Spiegel and Milstien, 2000; Pyne and Pyne, 2000). As these processes are also required for embryogenesis, sphingolipid signaling could play a role in various aspects of animal development. Recent studies in a variety of developmental model organisms support this notion. In particular, several lines of evidence support a role for S-1-P and the enzyme responsible for its catabolism, sphingosine-1-phosphate lyase (SPL) in development. Mice that lack the S-1-P receptor endothelial differentiation gene 1 (Edg1) exhibited embryonic lethality and failure of vascular maturation (Liu et al., 2000). The zebrafish mutant *miles apart* (*mil*), which contains a mutation in the S-1-P receptor Edg5 demonstrated cardia bifida, a cardiac developmental defect caused by failure of proper migration of cardiomyocyte precursors to the midline (Kupperman et al., 2000). *Dictyostelium* SPL, which was isolated in a screen for resistance to cisplatin, was found to be important for normal development in this organism, and mutants lacking the enzyme demonstrated abnormalities of fruiting bodies, apical spores and slug migration (Li et al., 2000; Li et al., 2001). The exact molecular mechanisms responsible for these defects remain to be elucidated.

There is evidence that sphingolipid signaling also influences some aspects of *Drosophila* development. The *lace* gene encodes a mutant subunit of *Drosophila* serine palmitoyltransferase, the first enzyme in the de novo pathway of sphingolipid synthesis (Fig. 1). Previous work has implicated *lace* in the regulation of MAPK/ERK signaling (Adachi-Yamada et al., 1999). 'Strong' alleles of *lace* were found to be lethal, whereas a heterozygous allelic combination resulted in viable mutants that displayed various developmental defects of eye, wing and limb structures, ascribed to an increase in apoptosis in imaginal disc cells. These developmental defects were ameliorated by addition of sphingosine to the growth media, indicating a requirement for long-chain bases or downstream sphingolipid metabolites in the developmental processes required for formation of these adult structures. *Drosophila* Jun N-terminal kinase (JNK; BSK – FlyBase) activation was increased in the *lace* mutant, leading to increased apoptosis and indicating that some aspect of sphingolipid synthesis is normally required to inhibit DJNK and stimulate cell survival.

In addition to these findings, recent studies performed



Step	Enzyme	*Drosophila* Homolog	Experimental Evidence
1	Serine palmitoyltransferase	CG 4162	(Adachi-Yamada, 1999)
	Serine palmitoyltransferase	CG 4016	None
2	3-keto dihydrosphingosine reductase	NGI	-
3	Ceramide synthase	NGI	-
4	Sphingosine desaturase	CG 9078	(Ternes, 2002)
5	Ceramidase	CG 1471	(Yoshimura, 2002)
6	Sphingosine kinase	CG 2159	None
	Sphingosine kinase	CG 1747	None
7	S-1-P phosphatases	NHI	-
8	Ceramide kinase	CG 16708	None
9	Sphingosine phosphate lyase	CG 8946	This study
10	Phosphoethanolamine cytidylyltransferase	CG 5547	None
11	CDP-ethanolamine phosphotransferase	CG 6016	None
12	Acid sphingomyelinase	CG 3376	None
	Neutral sphingomyelinase	CG 12034	None

Fig. 1. *Drosophila* homologs of sphingolipid metabolism. De novo synthesis of sphingolipids (blue). Sphingolipid degradative pathway (red). Phospholipid salvage pathway (green). NGI, no gene identified; NHI, no homolog identified. Palmitoyl-CoA is placed in parentheses to indicate the possibility that *Drosophila* serine palmitoyltransferase may use a short-chain fatty acyl-CoA substrate.

in *Drosophila* S2 cells demonstrate that sphingolipid intermediates are required for regulation of fatty acid biosynthesis through their ability to inhibit the cleavage of *Drosophila* sterol regulatory element binding protein (SREBP) (Dobrosotskaya et al., 2002; Seegmiller et al., 2002). SPL and a product of the reaction catalyzed by SPL, ethanolamine phosphate, appear to be required for regulation of SREBP cleavage, possibly through conversion of ethanolamine phosphate to phosphatidylethanolamine. Although the effect of SREBP dysregulation on *Drosophila* development was not explored, these findings raise the possibility that SPL could exert biological effects by modulating the levels of upstream (sphingolipid) and/or downstream (phospholipid) intermediates.

In this study, we demonstrate that normal sphingolipid catabolism is required for *Drosophila* development. We identified the *Drosophila* SPL homolog *Sply* and showed that this gene is developmentally regulated. Furthermore, we demonstrated the biochemical and physiological characteristics of $Sply^{05091}$ loss-of-function mutants. $Sply^{05091}$ homozygotes exhibited pattern abnormalities in the dorsal longitudinal flight muscles (DLM) of the adult thorax and were flightless. Mutant larvae exhibited diminished survival, with the most pronounced effect occurring during the first larval instar. Egg-laying was diminished in homozygous $Sply^{05091}$ adults, and enhanced apoptosis in the region of the embryonic genital disc was observed. Some improvement in flight performance and reduction in phosphorylated long chain bases (LCBPs) occurred after treatment with the sphingosine kinase inhibitor D,L-*threo*-dihydrosphingosine (D,L-*threo*-DHS). Normalization of muscle morphology, viability, flight and reproductive function were achieved by genetic $Sply^{05091}$ reversion. Interestingly, introduction of a single hypomorphic allele of *lace*, resulting in diminished de novo synthesis of sphingolipids, could correct the $Sply^{05091}$ mutant phenotype. Finally, we demonstrated that introduction of $Sply^{05091}$ into a homozygous *lace* background greatly improves viability by blocking sphingolipid catabolism and increasing available sphingolipid intermediates. Taken together, our results show that the primary mechanism responsible for the *Sply* mutant phenotype is the accumulation of sphingolipid signaling molecules, and that tight regulation of sphingolipid metabolite levels is essential for *Drosophila* development.

MATERIALS AND METHODS

Drosophila stocks

Wild-type Canton-S (BL-1), $Sply^{05091}$ (BL-11393), $lace^{2}$ (BL-3156) and $lace^{k05305}$ (BL-12176) lines were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). General fly husbandry was performed as described (Sullivan et al., 2000). The P-element insertion line KG6148 was a gift of the P-element Screen/Gene Disruption Project of the Bellen/Rubin/Spradling laboratories.

Cloning of *Drosophila* SPL homologue

The *Drosophila melanogaster* genomic database (http://flybase.bio.indiana.edu) was searched for predicted proteins using mouse (Accession Number AAH26135) and human (Accession Number XP_166113) SPL sequences. DNA homology searches were

performed via the Berkeley *Drosophila* Genome Project web site (http://www.ncbi.nlm.nih.gov) using the BLAST search program. One computed gene (CG8946) was identified that corresponded to a predicted SPL gene. Subsequently, two ESTs were identified which contained open reading frames that corresponded to the two predicted splice variants. The open reading frame contained in LP04413 was amplified using primers LPEcoRI5 (5′-TGGAATTCGATGCGTC-CGTTCTCCGGCAGC-3′) and LPXhoI3′ (5′-CTCCTCGAGTCTAT-TTCTGGCTGGGAGT-3′) and was cloned into the yeast expression vector, pYES2 (see below), at *Eco*RI and *Xho*I restriction sites. This construct was transformed into a *dpl1Δ* strain using the lithium acetate method (Ito et al., 1983).

Saccharomyces cerevisiae strains and growth conditions

Wild-type yeast used in this study were of strain JK9-3d (*leu2-3,112 ura3-52 rme1 trp1 his4 HML*a) (Heitman et al., 1991). The yeast strain JSK386 (*dpl1Δ*) is an isogenic derivative of strain JK9-3d in which the *DPL1* gene has been replaced by a G418-resistant marker (Kim et al., 2000). Strains JS204 and JS205 are derivatives of JSK386 which contain the *Drosophila* ESTs LP04413 and GH13783 respectively in expression vector, pYES2 (Invitrogen, Carlsbad, CA). pYES2 is a yeast expression vector containing the *URA3* gene (which provides transformants the ability to grow in media without uracil), and an Ampicillin resistance marker and origin of replication functional in *Escherischia coli*. Genes expressed using this system are regulated under the control of the *GAL1,10* promoter, which allows expression in the presence of galactose and not in the presence of glucose. Cells were grown in minimal or uracil$^-$ media containing either 20 g glucose or galactose per liter, as indicated.

Functional complementation in yeast

Strains of interest were grown to saturation in liquid culture for 2-3 days. They were then resuspended in minimal medium, placed in the first row of a 96-well plate and diluted serially from 1:2 to 1:4000 across the plate. The cultures were normalized for OD_{600}=2 and template inoculated onto a control plate and a plate containing 50 μM sphingosine, obtained from Sigma Chemical Company (St Louis, MO). Sphingosine enriched plates were made with minimal media containing 0.0015% NP40 and 50 μM D-*erythro*-sphingosine. At this concentration of NP40, no effects on cell viability are observed. Plates were incubated at 30°C for 2 days and assessed visually for differences in growth.

SPL assays

SPL assays of yeast extracts from strains expressing *Drosophila* sequences LP04413 and GH13783 were performed as previously described using a [^{3}H] labeled C_{18}-dihydrosphingosine-1-phosphate substrate, obtained from American Radiolabeled Chemicals (St Louis, MO) (Saba et al., 1997; Van Veldhoven and Mannaerts, 1991). In this method, SPL activity is measured by determining the conversion of radiolabeled C_{18}-dihydrosphingosine-1-phosphate substrate to long chain aldehyde product. To assess the ability of homozygous *Sply^{05091}* versus wild-type flies to degrade endogenous LCBPs, an HPLC method was developed and employed to examine extracts of wild-type and homozygous *Sply^{05091}* adults. Endogenous LCBPs were first isolated as described under ‘Analysis of *Drosophila* Sphingolipids’, and the lipid extract from 15 mg of homozygous *Sply^{05091}* flies were dried down using nitrogen gas. Lipids were resuspended in SPL reaction buffer and incubated for various time points at 37°C. Lipids were reisolated, derivatized with *o*-phthalaldehyde and analyzed by HPLC, as described below. Activity was determined by measuring the percent degradation of endogenous LCBPs in comparison with standards incubated in the absence of protein extracts.

Expression of *Sply*

For northern analysis, full-length probes were labeled by random priming with [γ-^{32}P] dGTP. Hybridization was carried out under standard conditions against an RNA blot prepared from total RNA of *Drosophila* embryos. *RpL32* is a constitutively expressed ribosomal gene used as a loading control.

The ribonuclease (RNase) protection assay was performed by hybridizing a radiolabeled antisense riboprobe to 20 μg of total RNA obtained from staged *Drosophila* as indicated. Unhybridized probe was digested with 60 μg/ml RNase A and 150 u/ml RNase T1 for one hour at 37°C. Samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated and run on a 10% polyacrylamide gel.

In situ hybridization was performed with a digoxigenin-labeled probe (Roche catalog number 1 175 025) and hybridized to fixed embryos at various stages essentially as described (Tautz and Pfeifle, 1989).

Analysis of *Drosophila* sphingolipids

Flies (100 mg) of were homogenized in 6 ml of ice-cold methanol/water 1:1 (vol:vol) with a Potter-Elvehjem homogenizer with a loose pestle followed by a tight pestle until the pestle moved smoothly. Extract was further homogenized by tip sonication for three times 20 seconds. Extract was spun at low speed and supernatant was removed and dried down in speed vac. Extract was resuspended in 500 μl of methanol containing 0.1 M potassium hydroxide and incubated for 1 hour at 37°C. After incubation, the extract was dried down in speed vac. Extract was resuspended in 500 μl of 50% methanol containing 0.1% glacial acetic acid and applied to a C18E STRATA solid phase extraction column. C18E STRATA column was washed with 50% methanol containing 0.1% glacial acetic acid followed by a wash with 100% methanol containing 0.1% glacial acetic acid. Lipids of interest were eluted with methanol/10 mM ammonium acetate, 9:1 (vol:vol). Lipids were dried down in speed vac. and *o*-pthaladehyde labeled for HPLC analysis as previously described (Kim et al., 2000).

Lethal phase analysis

One hundred embryos from the indicated lines were collected and observed at each developmental stage. Viability is expressed as the percentage of flies that survived through the indicated stage.

Adult flight performance

Two- to 7-day-old adult flies were released into a top-lit Plexiglas chamber. Flight behavior was scored as follows: upward flight, 3; lateral flight, 2; downward flight, 1; flightless, 0 (Vigoreaux et al., 1993). Average flight scores were compared using a two-tailed Student’s *t*-test.

Adult and larval microscopy

Preparation of tissue, staining, mounting and visualization was performed using standard techniques (Sullivan et al., 2000). Thoraces from adult flies were dissected, fixed with formaldehyde and osmium tetroxide, and embedded in EPON. These blocks were then cut into 1 μm sections, stained with Methylene Blue and Azure II, and viewed with a Lieca DMIRBE microscope.

Larvae were filleted during the third instar, pinned with the dorsal cuticle down, and eviscerated to allow an unobstructed view of the body wall muscles. The tissue was fixed with 4% formaldehyde, permeabilized in 100% acetone and stained with fluorescein-conjugated phalloidin. (Molecular Probes catalog number F-432.)

Electron microscopic analysis of DLMs was performed on adults essentially as described (O’Donnell and Bernstein, 1988).

Hemithoraces were visualized essentially as described (Fyrberg et al., 1994). Briefly, adult flies were frozen in liquid nitrogen, bisected with a razor blade and dehydrated in an ethanol series. The cuticles were then cleared with methyl salicylate to allow visualization of the muscles with a Leica DMIRBE microscope under polarized light.

Fluorescence microscopy

Embryos (0-24 hours) were prepared and fixed using standard

techniques (Rubin Manual) and stained with the indicated primary antibody or assayed for apoptosis using a TUNEL-based staining method (in situ cell death detection kit, Roche catalog number 1 684 795). Incorporation of fluorescein was assessed with a Leica DMIRBE epifluorescence microscope and an upright Leica TCS-NT confocal laser scanning microscope.

Antibodies and fluorescent reagents were as follows: polyclonal rabbit anti-*Drosophila* myosin heavy chain (Kiehart and Feghali, 1986) 1:1,000. Polyclonal rabbit anti-DMEF2 (Lilly et al., 1995) 1:10,000. Secondary antibody was a fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) 1:1,000.

Genetics

The precise excision of the ry^+ PZ P-element was performed by introducing transposase allele Δ2-3 into insertion line BL-11393. In the subsequent generation, the transposase was removed and the second chromosome was balanced over CyO. Offspring of these flies that lacked the P-element were selected by scoring for loss of ry^+. Homozygous lines were generated, assayed for restoration of flight behavior, and assessed for precise excision by PCR if indicated. Lines homozygous for the $Sply^{05091}$ allele and the $lace^{k05305}$ allele were generated by meiotic recombination. $Sply^{05091}$ and $lace^{k05305}$ mutations were introduced in trans and balanced in the next generation. Flies carrying the $lace^{k05305}$ allele were selected by presence of w^+. Presence of $Sply^{05091}$ was verified by PCR. The $Sply^{27A}$ allele was generated by mobilization of the P-element in insertion line KG6148. Briefly, transposase was introduced into KG6148 flies for one generation. Offspring were screened for loss of the P-element, bred to homozygosity and assayed for reduced flight performance.

RESULTS

Identification and expression of a *Drosophila melanogaster* sphingosine phosphate lyase

A candidate *Drosophila* SPL gene (*Sply*) located on the right arm of chromosome II, position 53F8-12 was identified from the Berkeley *Drosophila* Genome Project database of predicted proteins based on high degree of similarity (49% identical and 68% similar allowing conservative substitutions) to the human SPL sequence (Fig. 2A). This similarity is particularly striking in a putative pyridoxal phosphate-binding site. Two full-length cDNA clones (LP04413 and GH13783) corresponding to the

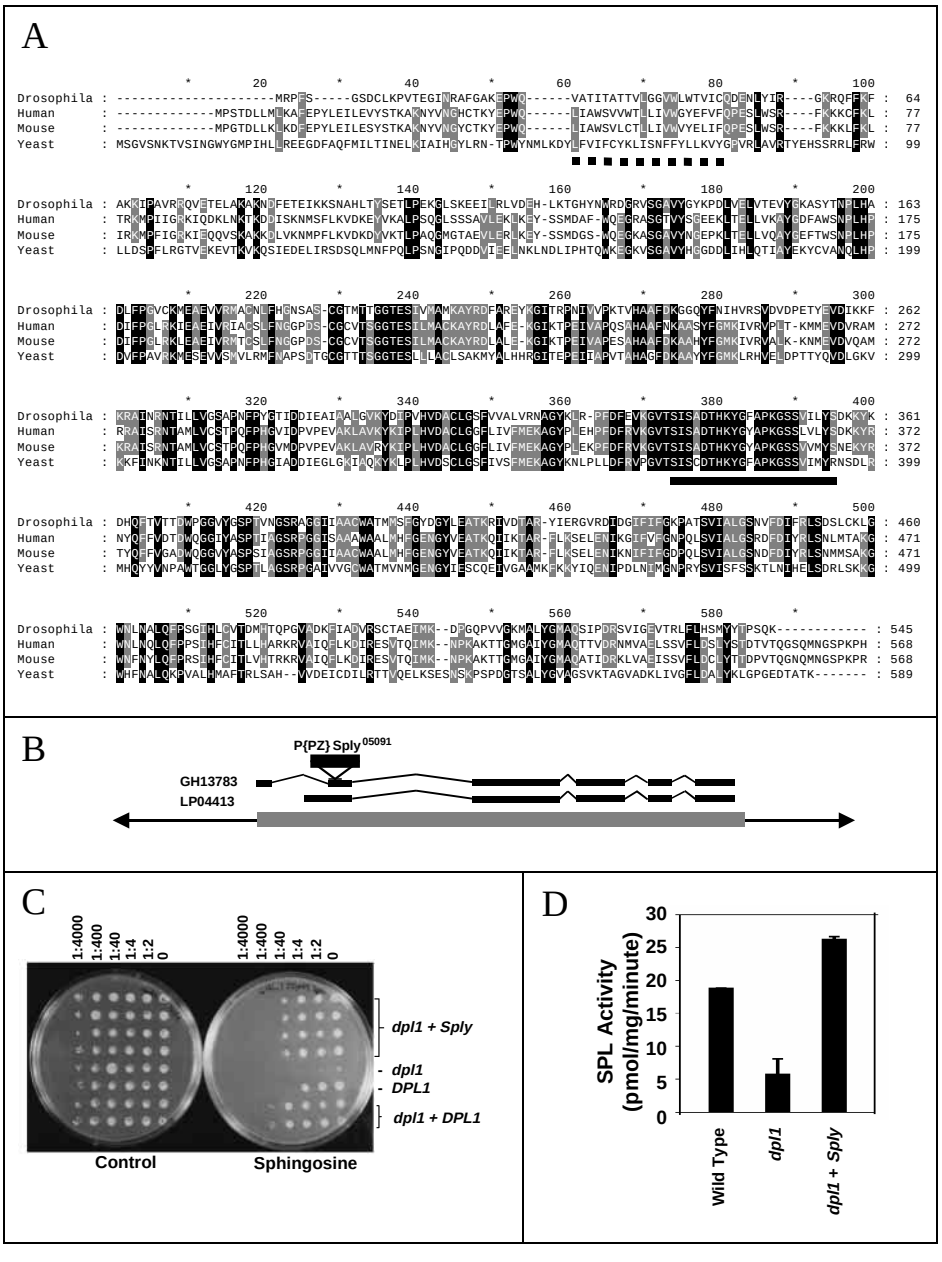
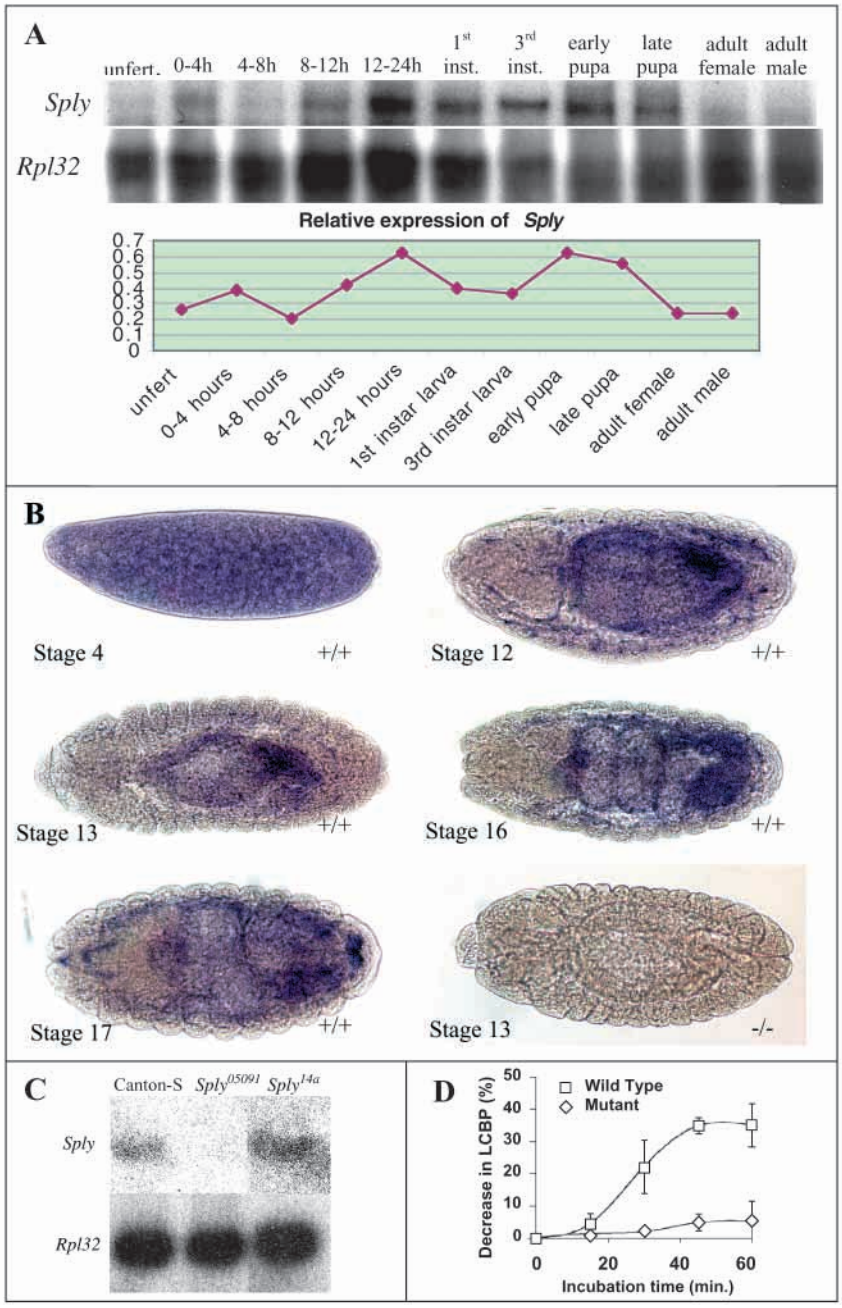


Fig. 2. *Sply* encodes the *Drosophila* sphingosine-1-phosphate lyase. (A) CLUSTALW alignment of Sply. The predicted protein product of *Sply* is 49% and 43% identical and 68% and 60% similar to human and yeast SPL protein sequences respectively. The broken line indicates the putative transmembrane region. The unbroken line indicates a consensus pyridoxal phosphate-binding motif. (B) *Sply* gene organization. Open reading frames GH13783 and LP04413 and location of transposon insertion (P{PZ}Sply^{05091}) are indicated. (C) Overexpression of *Sply* in a *Saccharomyces cerevisiae* SPL mutant restores sphingosine resistance. The LP04413 and GH13783 cDNAs were cloned into yeast expression vector pYES2 and transformed into a yeast SPL mutant strain (*dpl1*), as described in the Materials and Methods. The transformed strain (*dpl1 + Sply*) was compared with wild type (*DPL1*) and SPL mutant overexpressing endogenous yeast SPL (*dpl1 + DPL1*) strains in a sphingosine resistance assay. Dilutions of saturated cultures for each strain are indicated above. (D) Expression of *Sply* in a *Saccharomyces cerevisiae* SPL mutant restores SPL enzyme activity. Whole cell extracts of *Saccharomyces cerevisiae* wild type, SPL mutant (*dpl1*) and SPL mutant overexpressing *Sply* (*dpl1 + Sply*) strains were analyzed for SPL activity.

Sply locus were identified in a search of the Berkeley *Drosophila* Genome Project EST database (Rubin et al., 2000). The two clones are predicted based on alternative 5′ exon usage (Fig. 2B). These clones were obtained and their integrity confirmed by sequence and restriction analysis. In order to evaluate whether *Sply* encodes a functional SPL, the cDNAs were re-cloned into yeast expression vector pYES2 in which gene expression is driven by a galactose-inducible promoter. These constructs were transformed into a *dpl1Δ* strain, in which the sole endogenous *Saccharomyces cerevisiae* SPL gene has been deleted (Saba et al., 1997). The *dpl1Δ* strain is unable to catabolize LCBPs, and it cannot proliferate on media containing low concentrations of D-*erythro*-sphingosine. *Sply* overexpression complemented the sensitivity of the *dpl1Δ* strain to 50 μM D-*erythro*-sphingosine (Fig. 2C). Furthermore, whole-cell extracts of *dpl1Δ* strains overexpressing *Sply* demonstrate restoration of SPL enzyme activity (Fig. 2D).

RNase protection of RNA from staged wild-type *Drosophila* indicates that *Sply* expression is developmentally regulated. Transient expression is observed in the early embryo which is due in part to maternal contribution. Transcription declines until 8-12 hours of embryogenesis, increases thereafter, and peaks in the late embryo. A second peak of expression is observed early in metamorphosis, followed by reduction to basal levels after eclosion of adult flies in both males and females (Fig. 3A). In situ hybridization reveals that *Sply* expression is localized to the developing gut primordium during embryogenesis. Lack of staining in the $Sply^{05091}$ mutant confirms both specificity of the probe and lack of gene expression in our mutant model (Fig. 3B).



Characterization of a *Sply* P-element insertional mutant

Flies from the Berkeley *Drosophila* Genome Project gene disruption project (Spradling et al., 1995) were identified that harbor a transposon within the *Sply* open reading frame (designated $Sply^{05091}$). This transposon is located at nucleotide +269 relative to the start site of the larger transcript, LP04413, which places it within the coding region of both predicted transcripts (Fig. 2B). Northern analysis of total RNA obtained from $Sply^{05091}$ homozygotes confirmed an absence of *Sply* expression (Fig. 3C). Based on the result of the Northern analysis, in situ data and the presence of the P-element in the coding region near the translational start site, we consider the $Sply^{05091}$ to be a 'null' allele. The sphingolipids of

Fig. 3. *Sply* expression. (A) *Sply* expression is developmentally regulated. *Sply* mRNA was quantified by RNase protection, as described in the Materials and Methods. Relative expression was determined using ImageQuant software and standardized to the intensity of a ribosomal protein subunit (*RpL32*) transcript. (B) In situ hybridization of wild-type embryos shows that *Sply* has strong, transient expression in the syncytial blastoderm (stage 4) that declines to undetectable levels after cellularization. At stage 11-12, *Sply* mRNA reappears in the midgut/hindgut rudiments where the developing gut is undergoing extensive reorganization. This gut expression persists for the duration of embryogenesis. The absence of any detectable staining in $Sply^{05091}$ mutants under identical conditions demonstrates probe specificity and reaffirms that there is no *Sply* expression in this line. (C) Expression of *Sply* in wild-type (Canton-S), homozygous *Sply* mutant ($Sply^{05091}$) and homozygous *Sply* revertant ($Sply^{14a}$) lines. RNA was obtained from 0- to 24-hour-old embryos. *RpL32* is again used as a loading control. (D) Degradation of endogenous LCBPs. Extracts of wild-type (square) and *Sply* mutant (diamond) adult flies were analyzed for the ability to degrade *Drosophila* endogenous phosphorylated long chain bases.

Drosophila contain C_{14} and C_{16} sphingosine and dihydrosphingosine LCBs (D.R.H., H.F., V.P., K.H., R.G., G.L.H. and J.D.S., unpublished). Extracts of wild-type and mutant flies were compared for their ability to degrade endogenous *Drosophila* LCBPs in vitro. Extracts of *Sply*^{05091} mutants failed to catabolize endogenous LCBPs, whereas extracts of wild-type flies degraded endogenous *Drosophila* LCBPs, indicating that the *Sply* gene product is responsible for LCBP catabolism in this organism (Fig. 3D).

To determine whether loss of *Sply* expression affects the levels of *Drosophila* endogenous LCBs and corresponding LCBPs, the sphingolipid profile of homozygous *Sply*^{05091} flies was evaluated and compared with wild-type controls. Homozygous *Sply*^{05091} adults demonstrated an eightfold increase in LCBs and a 20-fold increase in LCBPs when compared with wild type (Table 1), indicating significant derangement of sphingolipid metabolism. This accumulation of LCBs and LCBPs was observed in homozygous *Sply*^{05091} mutants as early as hours 12-18 of embryogenesis, correlating with the onset of *Sply* expression.

Homozygous and heterozygous *Sply*^{05091} flies were examined for evidence of anatomical, developmental and functional abnormalities. Flies heterozygous for *Sply*^{05091} were indistinguishable from wild type. Initial evaluation of flies homozygous for the *Sply*^{05091} allele revealed no obvious defects in external anatomical structures at embryonic, larval or adult stages. However, adult mutants were almost uniformly flightless, with 91% of the mutant population scoring zero (in comparison with 4% wild-type flies) in a standard flight performance assay (Table 1). Despite the severity of the flight defect in *Sply*^{05091} homozygotes, the function of other muscle groups, including the jump and leg muscles did not appear to be affected (data not shown). Moreover, evaluation of the giant fiber neuromuscular pathway by electrophysiological analysis indicated that this pathway remained functionally intact and was not responsible for the observed flight defect (D.R.H., H.F., V.P., K.H., R.G., G.L.H. and J.D.S., unpublished).

Sply^{05091} homozygotes demonstrate abnormal flight muscle morphology

To investigate further the etiology of *Sply*^{05091} flight defects, adult mutants were sectioned through the thorax, and muscles were examined by light microscopy (Fig. 4). These studies revealed a reduction in the number of muscle fibers comprising the DLMs required for flight. Whereas the thoraces of wild-type flies invariably contained six symmetrical pairs of fibers, *Sply*^{05091} homozygotes exhibited a general pattern of missing fibers, asymmetry and hypertrophy of remaining fibers. Quantitative analysis of DLM fibers revealed a reduction from six per hemithorax in wild type to an average of 4.15 per hemithorax in the mutants (Table 1). Microscopic analysis of hemithoraces illuminated with polarized light confirmed the abnormal muscle configuration while demonstrating that muscle insertions were not affected (Fig. 5).

Sply^{05091} mutation does not disrupt muscle ultrastructure, template formation or embryonic muscle fusion

To determine the origin of the DLM defect, adult myocyte ultrastructure and larval and embryonic muscle development were investigated. Examination of Dmef2 expression in myoblast nuclei of nascent muscle fibers of early wild type and mutant embryos revealed no appreciable differences in muscle organization (Fig. 6A). Thus, myoblasts appear to successfully migrate from somites to correct sites in mutant embryonic segments. Similarly, analysis of myosin heavy chain expression in 0- to 24-hour wild-type and mutant embryos revealed no gross changes in the organization of the developing mutant muscle fibers when compared with wild type (Fig. 6B), indicating that myocyte fusion was not impaired.

Table 1. Biochemical and biological characteristics of mutant models of sphingolipid metabolism

	Strain							
Characteristic	Canton-S	*Sply*^{05091}	*Sply*^{14a}	*lace*$^{k05305/2}$	*lace*^{k05305}	*lace*$^{k05305/+}$, *Sply*^{05091}	*lace*^{k05305}, *Sply*^{05091}	*Sply*^{05091}+1 mM D,L-*threo*-DHS
$C_{14/16}$ LCBs (nmol/100 mg)	2.71±0.28	24.22±1.73	5.30±0.59	0.15±0.01	0.07	12.67±1.93	5.75±0.42	136% *
$C_{14/16}$ LCBPs (nmol/100 mg)	0.30±0.09	6.38±0.44	1.02±0.33	0.08±0.04	0.08	4.06±0.64	1.88±0.17	81% *
Average flight score	2.60±0.032	0.40±0.036	1.70±0.074	1.62±0.14	N.D.	1.41±0.063	0.56±0.13	0.62±0.057
Number of DLM fibers/hemithorax	6.00±0.00	4.15±0.21	5.97±0.089	5.94±0.030	4.82±0.53	5.13±0.26	5.81±0.14	N.D.
Average number of eggs/day	44.5±3.28	15.8±2.98	43.4±3.43	N/A	N/A	52.9±4.03	N/A	N.D.
Developmental lethality (%)	20	66.5	27	N.D.	91*	20	61*	N.D.

Adult wild-type flies and the indicated models of sphingolipid metabolism were analyzed for total phosphorylated (LCBPs) and unphosphorylated (LCBs) long-chain base levels, flight performance, number of DLM per hemithorax, fecundity (egg-laying) and % mortality prior to completion of metamorphosis. The latter represent the results of three separate experiments for a total of 300 individuals for each group. Flight performance and LCB/LCBP levels were also determined in *Sply*^{05091} homozygous flies treated with the sphingosine kinase inhibitor, D,L-*threo*-DHS. LCB/LCBP levels in inhibitor-treated flies are given as percentage of untreated controls; these determinations were obtained in a separate experiment, and baseline sphingolipid levels were not comparable between the two experiments. Canton-S is wild type. *Sply*^{05091} indicates the homozygous *Sply*-null mutant. *lace*^{2} and *lace*^{k05305} are recessive lethal alleles of serine palmitoyltransferase. *Sply*^{14a} indicates the homozygous *Sply*^{05091} revertant. All biochemical, flight and fiber count data were obtained from mixed-age adults. Values are as indicated ±s.e.m.

*Developmental lethality for *lace*^{k05305} and *lace*^{k05305}, *Sply*^{05091} were calculated as reduction from % expected homozygous offspring of heterozygous parents.
N.D., not determined.

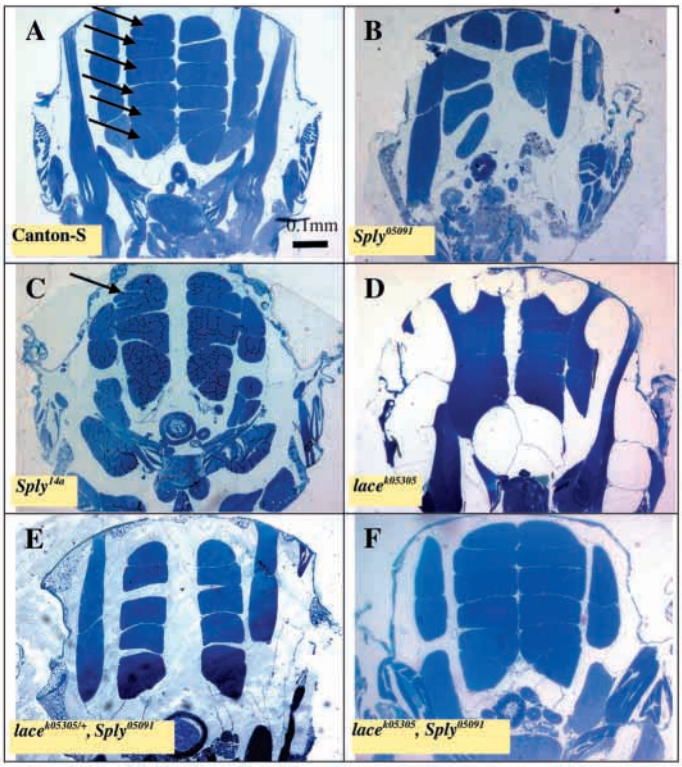


Fig. 4. Thoracic cross-sections. The Canton-S control fly (A) demonstrates the invariant array of six symmetrical pairs of DLMs (arrows). *Sply*^{05091} homozygotes often have missing fibers as represented by this sample (B) that has only four fibers in the left hemithorax and two on the right. Excision of the transposon restores the normal complement of DLMs in most cases, but occasional aberrancies were found, as shown in this *Sply*^{14a} homozygote (C). This thorax has an extra (seventh) fiber on the left side (arrow). Thoraces of *lace*^{k05305} homozygotes generally presented with a normal number of DLMs (D), but the morphology was often distorted, owing to the presence of large vacuolar structures that displaced them from their normal positions towards the midline. The addition of either one (E) or two (F) copies of *lace*^{k05305} in the *Sply*^{05091} homozygous background restored normal musculature in most cases. With a single copy of *lace*^{k05305}, however, DLMs were generally smaller than wild-type fibers.

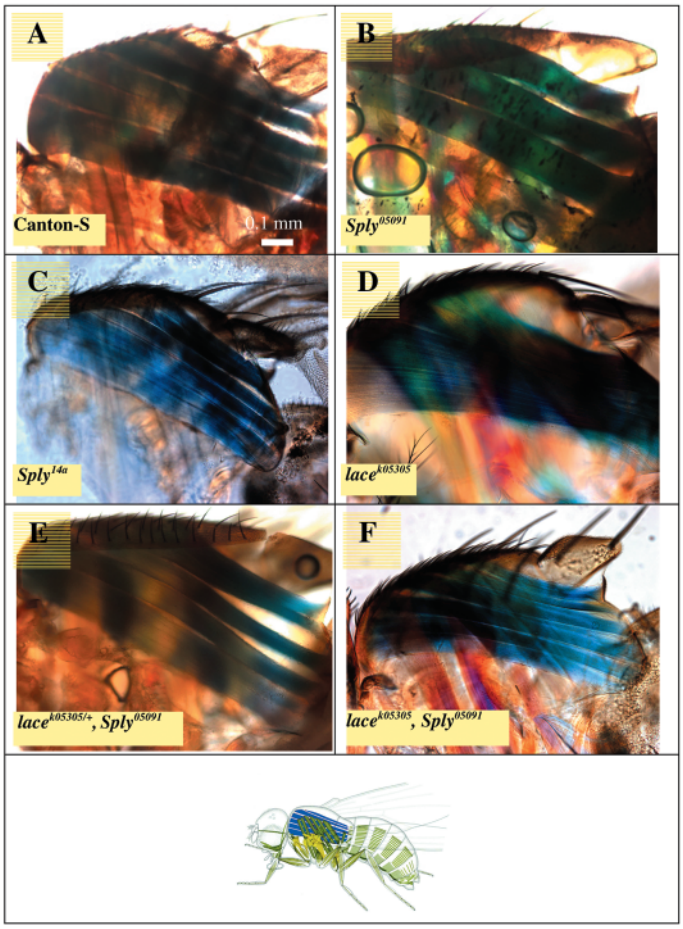


Fig. 5. Bisected thoraces viewed with polarized light. Canton-S control flies (A) display the stereotyped configuration of six DLMs (blue). Seventy-nine percent of the hemithoraces from *Sply*^{05091} homozygotes have a reduced number of fibers. This representative hemithorax (B) has only three fibers, which exhibit compensatory hypertrophy. Excision of the transposon reduces the occurrence of aberrant fiber count to 13% as shown in this *Sply*^{14a} homozygote (C). *lace*^{k05305} homozygotes generally (76%) have a normal complement of DLMs; however, occasionally only one large fiber is present as shown (D). A single copy of *lace*^{k05305} reduces the occurrence of aberrant hemithoraces in the *Sply*^{05091} homozygotes to 63% (E), and 92% of *Sply*^{05091}, *lace*^{k05305} double mutants are normal (F). A DLM schematic shown below [adapted, with permission, from Hartenstein (Hartenstein, 1993)].

To determine whether the DLM defect observed in *Sply*^{05091} adult homozygotes occurred because of lack of template structures required for their formation during metamorphosis, T2 dorsal oblique muscles (DOMs) were evaluated in mutant larvae (Fig. 6C). Late-stage mutant larvae exhibited no alterations in number and/or size of DOMs. Therefore, it appears that the mutant muscle defect is restricted to DLMs and affects the adult muscle configuration subsequent to myoblast fusion events during metamorphosis. Despite this defect, the ultrastructure of the DLMs that are present in the *Sply*^{05091} mutants generally appears to be intact, as evidenced by transmission electron microscopy (Fig. 6D).

Sply^{05091} homozygotes demonstrate decreased fecundity, semi-lethality and increased apoptosis in embryos

The number of offspring resulting from homozygous *Sply*^{05091} crosses was about 10% of the number observed in wild-type crosses. This loss of progeny could result from diminished egg-laying and/or diminished survival of embryos and larvae. Analysis of egg-laying indicated that fecundity of the mutants was about one third that of control flies (Table 1). This outcome could be the result of diminished male and/or female fertility. To distinguish between these possibilities, both male and female *Sply*^{05091} homozygotes were mated to wild-type flies, and egg-laying was measured in comparison with wild-type pairs and homozygous mutant pairs. Numbers of eggs produced were significantly diminished in crosses of both male and female mutant flies with wild-type mates (data not shown), indicating that the effect on fecundity was not gender specific. Additionally, crosses between *Sply*^{05091} homozygous males and females resulted in progeny with an overall survival (from

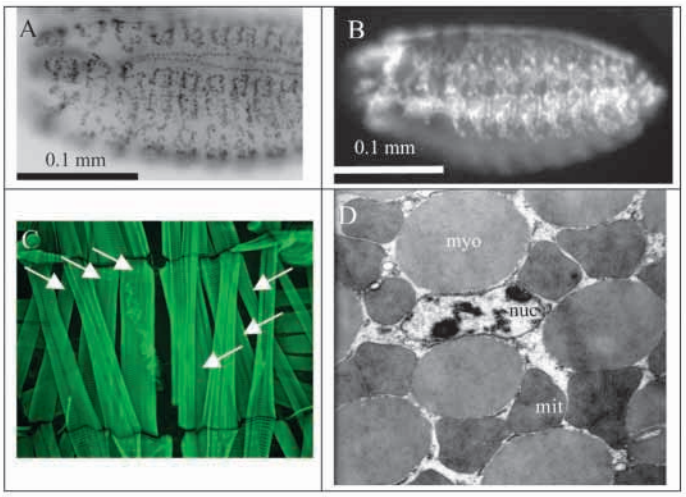


Fig. 6. Muscles appear to develop normally in *Sply*^{05091} homozygous embryos and larvae. Immunofluorescent staining with α-Mef2 (A) and α-myosin heavy chain (B) suggests that myoblasts differentiate, migrate, and fuse normally to produce a well-patterned array of embryonic muscles. Larval somatic musculature appears normal (C), most notably with respect to the dorsal oblique muscles (white arrows), which serve as templates for the adult dorsal longitudinal muscles. (Note that dorsal acute muscle 1 was ablated to facilitate visualization of DOMs.) Furthermore, transmission electron microscopy (D) reveals that persistent adult DLMs have intact myofibrils (myo) and structurally normal mitochondria (mit) and nuclei (nuc).

egg to adulthood) of 33.5%, compared with an 80% survival rate in wild-type flies. Lethality in the *Sply*^{05091} mutants was high during larval stages (46%, compared with 3% in wild type), with the majority of larval death occurring during the first larval instar. Less severe effects were observed during metamorphosis (22% lethality, compared with 1% in wild type), and no appreciable differences in survival were noted during embryogenesis. *Sply*^{05091} mutant embryos were examined by in situ TUNEL assay, and patterns of apoptosis were compared to those of wild-type controls (Fig. 7). *Sply*^{05091} mutant embryos demonstrated a pronounced enhancement of apoptosis compared with wild-type controls, especially in a specific region of the posterior pole near the developing genital disc.

Genetic reversion of the *Sply*^{05091} mutation restores normal muscle configuration

To verify the importance of *Sply* in mediating the semi-lethality, egg-laying defects and flight muscle phenotype of the mutant line, the transposon in the *Sply*^{05091} locus was mobilized in *Sply*^{05091} homozygotes following introduction of an active transposase. Precise excision of the transposon was subsequently confirmed by PCR and DNA sequence analysis. A homozygous revertant line (*Sply*^{14a}) was generated as described in the Materials and Methods, and was found to express *Sply* mRNA at levels equivalent to those in wild type (Fig. 3C). As shown in Figs 4 and 5, *Sply*^{14a} demonstrated reversion of the muscle fiber morphology defect, and flight performance was largely restored (Table 1). Additionally, apoptosis in the revertant embryo was diminished in comparison with *Sply* mutants (Fig. 7). The appearance of the specific cluster of TUNEL-positive cells was <1% (n=197), 48% (n=160) and 72% (n=324) in Canton-S, *Sply*^{14a} and *Sply*^{05091} in stage 12-15 embryos, respectively. Phenotypic reversion correlated with normalization of LCB and LCBP levels in revertant extracts (Table 1).

The *Sply*^{05091} muscle defect is suppressed by reducing sphingolipid intermediates

To investigate the possibility that the *Sply*^{05091} muscle phenotype was caused by accumulation of LCBPs, an inhibitor of sphingosine kinase, D,L-*threo*-DHS, was introduced to the growth media of mutant and wild-type flies. Flies were grown on the supplemented media, and F2 progeny were examined. When wild-type flies were grown on media supplemented with 10 μM D,L-*threo*-DHS, no deleterious effects were observed. *Sply*^{05091}mutants grown on this media demonstrated a slight but significant improvement in flight performance. To determine whether the flight improvement coincided with a restoration of LCBP levels, LCB/LCBP levels were analyzed in mutants and controls grown on D,L-*threo*-DHS. LCBP levels in *Sply*^{05091} homozygotes grown in the presence of sphingosine kinase inhibitor were reduced by approximately 20% (Table 1). Similarly, LCBP levels in wild-type flies were reduced by 20% from normal levels (data not shown). Because it appeared that significant depletion of LCBP intermediates would be difficult to achieve using pharmacological means, a genetic approach was taken to more effectively block the accumulation of sphingolipid intermediates in *Sply*^{05091} homozygotes.

Assuming that the mutant phenotypes are caused by an accumulation of LCB/LCBPs, we predicted that diminishing SPT activity in the *Sply*^{05091} homozygote would suppress the *Sply*^{05091} phenotype by reducing production of sphingolipid intermediates. Accordingly, a *lace*^{k05305} hypomorphic allele was introduced onto the *Sply*^{05091} chromosome by genetic recombination, thus generating a *Sply*^{05091}, *lace*^{k05305}/+ line. *Sply*^{05091}, *lace*^{k05305}/*Sply*^{05091}, *lace*$^{+}$ flies exhibited reversion of the abnormal muscle patterning (Figs 4 and 5), and flight performance was substantially improved (Table 1). Additionally, the pattern of embryonic apoptosis appeared

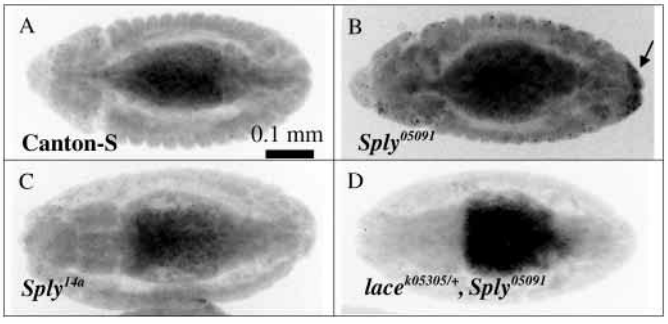


Fig. 7. TUNEL stain. Stage 12-15 embryos were assessed for apoptotic cell death. Canton-S control embryos (A) show background staining in the developing gut. *Sply*^{05091} homozygotes (B) consistently show an overall increase in TUNEL-positive cells with a notable cluster at the posterior tip (arrow). The frequency of appearance of this cluster was substantially reduced by both excision of the P-element (C) and by introduction of a single copy of *lace*^{k05305} (D).

similar to that of the wild type (Fig. 7). Phenotypic reversion correlated with a marked reduction of the LCBs and LCBPs (Table 1).

Loss of *Sply* expression suppresses the hypomorphic *lace* phenotype

Inheritance of two $lace^{k05305}$ hypomorphic alleles was reported to be almost completely lethal, whereas a heterozygous allelic combination ($lace^{k05305}/lace^{2}$) yields flies that frequently survive but manifest severe developmental phenotypes leading to eye, bristle and wing abnormalities (Adachi-Yamada et al., 1999). We predicted that the *lace* mutant phenotype is due to diminished levels of sphingolipid intermediates. Furthermore, we reasoned that inhibiting sphingolipid catabolism in *lace* mutants might allow sufficient accumulation of trace sphingolipids obtained through the diet to ameliorate developmental defects induced by the lack of crucial sphingolipid intermediates. To address this possibility, a *Drosophila* line homozygous for both the $Sply^{05091}$ and $lace^{k05305}$ alleles was generated. Significantly, the presence of the $Sply^{05091}$ allele increased the recovery of *lace* homozygotes from 9% to 39% of that expected by independent assortment. Furthermore, the introduction of $Sply^{05091}$ fully suppressed the eye, bristle and wing phenotypes in the resulting flies (Fig. 8). In accordance, sphingolipid intermediates were substantially increased in this line, in comparison with $lace^{k05305}$ mutants and $lace^{2}/lace^{k05305}$ heterozygotes (Table 1).

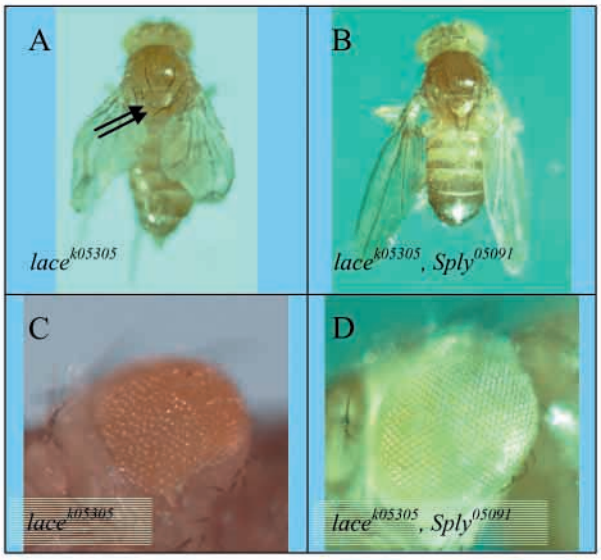


Fig. 8. Genetic rescue of $lace^{k05305}$ by $Sply^{05091}$. The $lace^{k05305}$ allele is almost completely lethal and the few homozygotes that do survive to adulthood have a lifespan of less than 1 week and manifest pronounced morphological defects (A,C). Wings are notched and often fail to inflate, bristles are missing (arrows), and the eyes are often rough with irregular ommatidia. The addition of the $Sply^{05091}$ allele (B,D) greatly improves viability, and external morphology is indistinguishable from wild-type flies.

DISCUSSION

Mutations that result in failure or dysregulation of sphingolipid synthesis or catabolism are directly responsible for a number of human diseases, including hereditary sensory neuropathy type 1 and the group of lysosomal storage diseases called the sphingolipidoses (Bejaoui et al., 2001; Dawkins et al., 2001; Gable et al., 2002). A large body of evidence now indicates that sphingolipid metabolites and enzymes of sphingolipid metabolism play important roles in regulating cell migration, stress response, survival, differentiation, senescence, apoptosis, receptor signaling and endocytosis in eukaryotic cells. These findings suggest molecular mechanisms by which sphingolipids may affect animal physiology and contribute to disease states. Whereas animal models of sphingolipid metabolism have been paramount in the study of the pathophysiology and treatment of sphingolipidoses, only a few animal models have been identified or generated that facilitate investigation of the sphingolipid degradative pathway, its role in physiology, and its potential role in disease.

In this study, we identify the *Drosophila melanogaster Sply* gene and demonstrate the importance of *Sply* expression in maintenance of *Drosophila* viability, reproduction and muscle development. *Sply* encodes a functional SPL that is capable of metabolizing dihydrosphingosine-1-phosphate and phytosphingosine-1-phosphate substrates, as shown by in vitro biochemical studies employing the former substrate and functional complementation in yeast strains that accumulate primarily the latter. Furthermore, this enzyme is responsible for catabolism of the endogenous LCBPs of *Drosophila*. This conclusion is supported by the finding that extracts from wild-type flies but not homozygous $Sply^{05091}$ mutants are able to degrade this substrate in vitro, and by the lipid profile of homozygous $Sply^{05091}$ mutants which demonstrate early, sustained and severe accumulation of endogenous LCBPs (and, to a lesser extent, accumulation of LCBs) in vivo.

Drosophila SPL expression is developmentally regulated, with earliest expression occurring at 8-12 hours of embryogenesis (after loss of maternal load), a time frame consistent with the accumulation of sphingolipid intermediates in $Sply^{05091}$ late embryos. Our data are in agreement with a more extensive developmental analysis presented by the Yale *Drosophila* Developmental Gene Expression Timecourse Database (http://genome.med.yale.edu/Lifecycle/). The LCBP levels observed in the homozygous $Sply^{05091}$ mutants are on the same order of magnitude (10- to 20-fold higher than wild-type levels) as those observed in the *Saccharomyces cerevisiae dpl1Δ* strain, in which the sole endogenous SPL gene is deleted. The temporal pattern of LCBP accumulation suggests that LCBP synthesis is minimal in early embryogenesis.

We observed a significant defect in the DLM muscle configuration of adult homozygous $Sply^{05091}$ mutants. This pattern abnormality appears to originate during metamorphosis and increases in severity with age. During *Drosophila* metamorphosis, most larval muscles undergo histolysis and are replaced by adult muscles formed entirely from myoblasts derived from the imaginal discs. However, DLMs are unique in that they are formed by a fusion event between myoblasts derived from the wing disc and larval DOMs, which do not histolyse during pupal stages, but undergo a splitting event, giving rise to six fusion-competent templates per hemithorax (Roy and VijayRaghavan, 1998). Embryonic muscle organization, larval muscle patterning, and persistence of

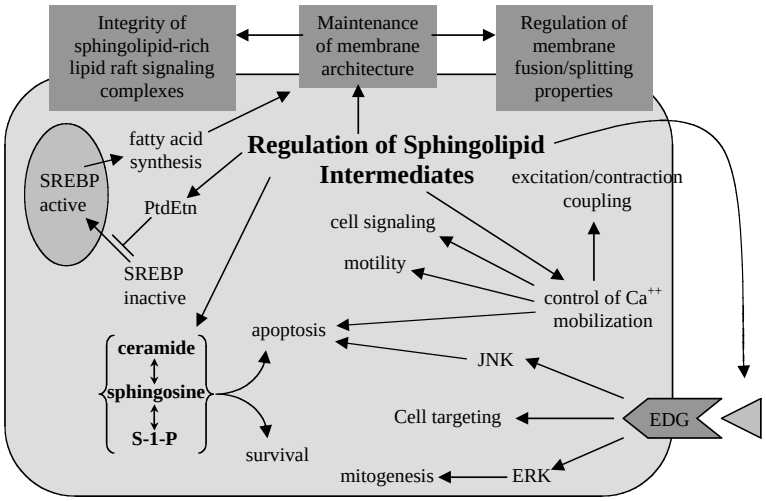


Fig. 9. Possible mechanisms of sphingolipid action during *Drosophila* development. Sphingolipids have a diverse repertoire of cellular effects depending on cell type. This is due to the diversity of downstream effectors, including both intracellular targets and G-protein-coupled receptors, and to the apparently antagonistic actions of different sphingolipids. The ability to rapidly interconvert these lipids provides a convenient 'rheostat' to regulate cell fate (reviewed by Pyne and Pyne, 2000). In addition, sphingoid phospholipids are important components of the plasma membrane. Their long, generally saturated fatty acid moieties increase membrane rigidity and are highly concentrated in specialized rafts that serve as structural elements and have been implicated in a number of signal transduction cascades. This figure summarizes only a few of the possible mechanisms by which sphingolipid intermediates may be acting during development.

DOM templates remained intact in *Sply*^{05091} mutants. Our preliminary analysis of pharate adults shows a similar but less severe pattern of missing fibers than in older flies. Furthermore, shortly after eclosion a small subset of fibers demonstrate hallmarks of degeneration, including myofibrilar disorganization and mitochondrial disruption, coincident with the appearance of large vacuoles in the myoplasm (D.R.H., H.F., V.P., K.H., R.G., G.L.H. and J.D.S., unpublished). In addition, *Sply*^{05091} larvae appear flaccid and have greatly decreased locomotion relative to controls, suggesting that muscle function is compromised prior to the patterning defect. This may be due to the effect that sphingosine has been shown to have on calcium mobilization in muscle cells (Sabbadini et al., 1992). Thus, there is a patterning defect in *Sply* mutant pupae, and the muscle fibers that do form are susceptible to degeneration. There are a number of cellular events downstream of these molecules that may be responsible for the pathological sequela of this sphingolipidosis, including the disruption of sphingolipid-regulated signaling pathways, loss of targeting cues for migrating myoblasts (Spiegel et al., 2002) or a pathologic compromise in normal membrane architecture (Fig. 9).

The defects of LCB/LCBP metabolism, embryonic and larval survival, muscle development and integrity, flight performance, reproductive capacity and developmentally regulated apoptosis characteristic of the *Sply*^{05091} homozygote were largely ameliorated in the homozygous *Sply*^{05091} revertant, *Sply*^{14a}. The failure of complete phenotypic reversion is probably due to mutation(s) in *Sply* regulatory regions that may have occurred as a result of P-element transposition. To confirm that the observed phenotypes were due to the *Sply* locus and not a second site mutation, a second allele was generated by imprecise excision of a P-element near *Sply* in a different genetic background. This allele (*Sply*^{27A}) does not complement the flight behavior of *Sply*^{05091} reaffirming that the absence of *Sply* is responsible for the muscle defects.

The notable accumulation of LCBPs and LCBs in the *Sply*^{05091} homozygote was markedly reduced, and the muscle phenotype was greatly improved by replacing one wild-type *lace* allele with a *lace*^{05305} allele. This is consistent with a model in which the accumulation of sphingolipid intermediates is responsible for the observed muscle defect. However, not all features of the *Sply*^{05091} homozygote phenotype were completely abrogated by a partial block in de novo synthesis. This could be explained by the failure to normalize LCB/LCBP levels completely in the double mutant, or it is possible that LCB/LCBP accumulation is only partially responsible for the *Sply*^{05091} homozygote phenotype. We cannot discount the possibility that one or both of the products of the reaction catalyzed by *Sply*, ethanolamine phosphate and long-chain aldehyde, could influence *Drosophila* development, either through their influence on SREBP cleavage, or by some other mechanism which remains to be elucidated.

Conversely, we found that the semi-lethality of *lace* hypomorphs was abrogated by introduction of a block in sphingolipid degradation. This finding supports the notion that certain sphingolipid intermediates play a crucial role(s) in regulating *Drosophila* eye, limb and wing development. Furthermore, the finding of severe phenotypes that are corrected by normalization of LCBs/LCBPs in *Sply* and *lace* double mutants indicates that maintaining tight regulation of sphingolipid intermediates is crucial to the developing animal. The spatial expression of *Sply* during embryogenesis suggests that the phenotypes observed in *Sply*^{05091} mutants are due to the global derangement of sphingolipids in the developing organism rather than by localized mechanisms. Further studies are under way to determine what specific mechanisms are disrupted by this sphingolipidosis.

These pathologies are consistent with the roles that sphingolipid intermediates play in signaling, and the toxicity induced by abnormal accumulation of these lipids in mammalian cells (Linn et al., 2001). The future identification of additional genes and mutants of sphingolipid metabolism in *Drosophila* will provide powerful genetic resources to predictably manipulate specific sphingolipid intermediates and to elucidate the roles of these signaling molecules during development and in cell function.

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