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Sphingosine kinases and their metabolites modulate endolysosomal trafficking in photoreceptors

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Introduction

Sphingolipids are integral components of membranes, and several of them, such as ceramide, sphingosine, and sphingosine 1 phosphate (S1P), serve as second messengers regulating diverse processes, including growth, differentiation, apoptosis, and angiogenesis (Dickson et al., 2006; Hannun and Obeid, 2008; Maceyka et al., 2009). Enzymes involved in the generation and conversion of these sphingolipids are conserved across species, including Drosophila melanogaster (Acharya and Acharya, 2005; Futerman and Riezman, 2005). Sphingosine kinases are penultimate enzymes in the sphingolipid biosynthetic pathway that phosphorylate sphingosine to S1P, and two distinct isoforms have been identified in mammals, flies, worms, yeast, and plants (Kohama et al., 1998; Liu et al., 2000; Pitson et al., 2000a). Sphingosine kinases also phosphorylate dihydrosphingosine (DHS) and produce DHS 1 phosphate (DHS1P). A large volume of literature has established the participation of these kinases in many signaling pathways (Olivera and Spiegel, 1993; Hait et al., 2006; Alemany et al., 2007; Pyne et al., 2009). S1P not only acts as an intracellular messenger but also as an extracellular ligand for a family of G protein–coupled S1P receptors (S1P-GPCRs; Sanchez and Hla, 2004). By binding to these receptors, S1P regulates angiogenesis, cardiac development, and lymphocyte egress from the thymus. However, a clear distinction of the intracellular and extracellular roles of these kinases and S1P has not yet emerged. Drosophila lack S1P-GPCRs and, therefore, could serve as a good system to dissect intracellular functions of sphingosine kinases and their metabolites.

Drosophila phototransduction is a prototypic GPCR-signaling cascade that begins with the absorption of light by Rhodopsin followed by activation of $G_{\alpha q}$. $G_{\alpha q}$ activates NORPA, eventually leading to the gating of transient receptor potential (TRP) and TRP-like channels (Hardie and Raghu, 2001; Wang and Montell, 2007). Like many GPCRs, Rhodopsin 1 (Rh1) internalized membrane proteins are either transported to late endosomes and lysosomes for degradation or recycled to the plasma membrane. Although proteins involved in trafficking and sorting have been well studied, far less is known about the lipid molecules that regulate the intracellular trafficking of membrane proteins. We studied the function of sphingosine kinases and their metabolites in endosomal trafficking using Drosophila melanogaster photoreceptors as a model system. Gain- and loss-of-function analyses show that sphingosine kinases affect trafficking of the G protein–coupled receptor Rhodopsin and the light-sensitive transient receptor potential (TRP) channel by modulating the levels of dihydrosphingosine 1 phosphate (DHS1P) and sphingosine 1 phosphate (S1P). An increase in DHS1P levels relative to S1P leads to the enhanced lysosomal degradation of Rhodopsin and TRP and retinal degeneration in wild-type photoreceptors. Our results suggest that sphingosine kinases and their metabolites modulate photoreceptor homeostasis by influencing endolysosomal trafficking of Rhodopsin and TRP.

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Abbreviations used in this paper: DHS, dihydrosphingosine; DHS1P, DHS 1 phosphate; GMR, glass multimer reporter; GPCR, G protein–coupled receptor; IPP, inositol polyphosphate 1 phosphatase; Rh1, Rhodopsin 1; S1P, sphingosine 1 phosphate; TRP, transient receptor potential; UAS, upstream activating sequence; UPLC MS/MS, ultrafast liquid chromatography coupled to tandem mass spectrometry.
Figure 1. Targeted expression of Sk2 leads to photoreceptor degeneration and decrease in Rh1 and TRP levels. (A) 7-d-old photoreceptors overexpressing Sk1 show a mild effect on rhabdomeres, whereas photoreceptors overexpressing Sk2 show severe degeneration. The percentage of intact rhabdomeres for w1118 is 100%, for Sk1 overexpression is 80%, and for Sk2 overexpression is 4%. R1–R7 label the rhabdomeres of R1–R7 photoreceptor cells;
undergoes internalization and degradation after activation in *Drosophila* photoreceptors (Acharya et al., 2004; Xu et al., 2004; Orem et al., 2006; Chinchore et al., 2009). Perturbation of the endocytic regulation of Rh1 leads to retinal degeneration of photoreceptors (Alloway et al., 2000; Kiselev et al., 2000). In the endocytic pathway, some membrane proteins recycle from endosomes to the plasma membrane, whereas others are transported to lysosomes for degradation. The endolysosomal system is viewed as a mosaic of different membrane domains with different protein and lipid compositions (Mukherjee and Maxfield, 2000; Gruenberg, 2003). Although we know a lot about protein families that dictate trafficking and sorting, comparatively, less is known about lipid determinants involved in trafficking processes. Phospholipids, such as phosphatidylinositol 3-phosphate and its effector proteins, mediate endosomal receptor sorting and intraluminal vesicle formation (Odorizzi et al., 2000; Birkeland and Stenmark, 2004). Studies on Sec 14 and Sec 14–like phosphatidylinositol transfer proteins have revealed how this family of lipid-modifying proteins integrates lipid metabolism with signaling and membrane trafficking (Mousley et al., 2007; Bankaitis et al., 2010). Bis(monoacylglycerol)phosphate is rich in late endosomes and lysosomes and involved in the biogenesis, organization, and dynamics of these organelles (Kobayashi et al., 2002; Schulze et al., 2009). Diacylglycerol regulates protein transport from the Golgi to the cell surface in yeast and mammalian cells ( Kearns et al., 1997; Baron and Malhotra, 2002). Sterols are involved in endolysosomal trafficking by promoting the formation of specialized membrane domains (Pichler and Riezman, 2004). Among sphingolipids, glucosylceramide is involved in the sorting of melanosomal enzymes from lysosomal membrane proteins, and ceramide is required for the formation of exosomes (Groux-Degroote et al., 2008; Trajkovic et al., 2008).

In this study, gain- and loss-of-function analyses of Sk1 (*Drosophila* sphingosine kinase 1) and Sk2 sphingosine kinases in photoreceptors show that they modulate the endolysosomal trafficking of Rh1 and TRP. A relative increase in DHS1P over S1P enhances the lysosomal degradation of Rh1 and TRP and induces photoreceptor degeneration. Our results suggest that sphingosine kinases regulate photoreceptor homeostasis by maintaining a balance between DHS1P and S1P.

**Results and discussion**

**Targeted expression of *Drosophila* Sk2, but not Sk1, leads to the severe degeneration of photoreceptors**

Recently, we have reported on the functional significance of the sphingolipid-metabolizing enzymes ceramidase and ceramide kinase in *Drosophila* photoreceptors (Acharya et al., 2008, Dasgupta et al., 2009). In these and earlier experiments, we showed that ceramide level is important for photoreceptor viability and function (Acharya et al., 2003). Therefore, we decided to analyze enzymes and metabolites downstream of ceramide in this process. Sphingosine kinases catalyze the conversion of sphingosine to S1P, and two isoforms, Sk1 and Sk2, have been characterized in *Drosophila* (Herr et al., 2004). *Drosophila* Sk1 and Sk2 proteins are 39% identical and 63% similar to each other, and expression of either of these isoforms could functionally complement a yeast mutant deficient in Sk activity (Herr et al., 2004). Western blots of retinal extracts probed with antibodies to either Sk1 or Sk2 show that both are expressed in the eye (Fig. S1 A). We performed targeted overexpression of Sk1 and Sk2 in the *Drosophila* eye using a glass multimer reporter (GMR) Gal4 driver. Electron micrographs of photoreceptors show that expression of Sk1 does not disturb ommatidial architecture, whereas mild effects are seen on rhabdomere organization (Fig. 1 A). Expression of Sk2 leads to extensive photoreceptor degeneration: very few intact ommatidia are seen, rhabdomeres are lost, and cells are vacuolated. To ensure that the enzymatic activity of Sk2 is required for the observed degeneration, we generated transgenic flies expressing a catalytically inactive form of Sk2. Amino acid substitutions in a conserved diacylglycerol kinase catalytic domain of human sphingosine kinases (residues 270–288 of human Sphk2 [mammalian sphingosine kinase 2]) have been shown to result in catalytically inactive enzymes (Pitson et al., 2000b; Herr et al., 2004). We introduced substitutions (GSGN312–315 to DDDD) in the corresponding conserved domain of *Drosophila* Sk2 and generated transgenic flies. Targeted overexpression of this mutant form of Sk2 does not lead to photoreceptor degeneration (Fig. S1, B and C).

**Enhanced lysosomal degradation of Rh1 and TRP in Sk2 overexpressors**

Because S1P is a known second messenger in GPCR signaling, we decided to test whether S1P targets any of the known phototransduction components. Analysis of the steady-state levels of various phototransduction components by Western analysis reveals that Rh1 and TRP levels are significantly reduced in these flies (Fig. 1, B–D). However, Rh1 and TRP levels are not reduced in transgenic flies overexpressing inactive Sk2 (Fig. S1 C). The levels of other phototransduction components, such as Gα, INAD, arrestin 2 (Arr2), and NinaC, are not affected as compared with Rh1 and TRP in functional Sk2 overexpressors (Fig. S2 A).

Two lines of experiments suggest that Rh1 and TRP are not arrested in the secretory pathway but traffic to the plasma membrane in flies overexpressing functional Sk2. Immuno-localization of Rh1 and TRP in retinae of newly eclosed Sk2
Figure 2. Overexpression of Sk2 in a car1 mutant restores Rh1 and TRP levels, and photoreceptor degeneration is suppressed. (A) Rh1 and TRP levels are recovered when Sk2 is overexpressed in a car mutant. (B) The percentage of expression of Rh1 and TRP in an Sk2 overexpressor (Sk2 OE) and an Sk2 overexpressor in a car mutant background compared with car mutant alone. n = 3; bars denote standard deviation. (C) Degeneration is suppressed in 7-d-old photoreceptors of the Sk2 overexpressor in a car mutant. 4% of rhabdomeres are intact in Sk2 overexpressors, whereas 88% are intact in Sk2 overexpressors in car. (D) S2 cells were stained with antibodies to Sk1 and cadherin, a plasma membrane marker. Overlay shows the colocalization of Sk1 with cadherin. S2 cells constitutively expressing GFP-tagged Lamp1 were stained with antibodies to Sk2. Overlay shows the colocalization of Sk2 with Lamp1-GFP.
overexpressors shows Rh1 and TRP staining in the rhabdomeres similar to control, suggesting that they are transported to the plasma membrane (Fig. S1 D). Moreover, although the level is low, a fully mature form of Rh1 is detected on blots in Sk2 overexpressors. Because GMR-Gal4 expression begins at the third instar larval stage in differentiating photoreceptor cells, it is possible that Sk2 overexpression affects photoreceptor development, leading to low Rh1 and TRP levels. To circumvent this, we used GMR-Gal4 expression in conjunction with Gal80ts. We raised flies at a permissive temperature at which Gal80 inhibits Gal4 used GMR-Gal4 expression in conjunction with Gal80ts. We raised instar larval stage in differentiating photoreceptor cells, it is expressors. Because GMR-Gal4 expression begins at the third low, a fully mature form of Rh1 is detected on blots in Sk2 overexpressors shows Rh1 and TRP staining in the rhabdomeres, it is seen by its colocalization with cadherin, a plasma membrane marker (Fig. 2 D). Sk2 shows cytoplasmic staining; additionally, we found that Sk2 can localize to lysosomal membranes, as seen by its colocalization with GFP-tagged Drosophila lysosome-associated membrane protein 1 (Lamp1), a lysosomal marker (Elwell and Engel, 2005). Noncolocalization experiments with markers for other organelles are shown in Fig. S3. The lysosomal localization of Sk2 could explain, in part, the accelerated degeneration of Rh1 and TRP upon its overexpression, suggesting that Sk2 could influence lysosomal degradation of proteins.

Alteration in the ratio of DHS1P to S1P leads to retinal degeneration in Sk2 overexpressors and wild-type photoreceptors

As previously mentioned in this paper, Sk’s phosphorylate sphingosine and DHS to S1P and DHS1P, respectively. Could any of these metabolites mediate the enhanced degradation of Rh1 and TRP and cause photoreceptor degeneration? We examined this possibility by feeding wild-type control flies (w1118) with either sphingosine, DHS, S1P, or DHS1P. We custom synthesized the tetradecasphinganine (the predominant sphingoid base in Drosophila) version of each of these lipids. It has been shown that lethality of lace alleles deficient in the de novo biosynthesis of sphingolipids can be rescued when flies are raised in food supplemented with sphingosine (Adachi-Yamada et al., 1999). The lace gene encodes the LCB2 subunit of serine palmitoyltransferase, the first and rate-limiting enzyme in sphingolipid biosynthesis. We tested the efficacy of the synthesized lipids by raising lace heterozygotes in food containing S1P, DHS, or DHS1P. Similar to sphingosine feeding, each of the three lipids could rescue lace lethality. Rescue with sphingosine, S1P, and DHS was almost complete with Mendelian segregation of the progeny, whereas rescue with DHS1P was partial (40% of the expected number of homozygous flies eclosed in lace heterozygotes fed DHS1P compared with control). Using the rescue of lethality as evidence for the functionality of these lipids, we raised w1118 in food supplemented with each of these lipids, and photoreceptors were processed by electron microscopy. Photoreceptors of w1118 raised in DHS1P show degeneration, whereas those fed sphingosine, DHS, or S1P do not show significant differences (Fig. 3 A). Degeneration is accompanied by a decrease in steady-state levels of Rh1 and TRP in w1118 fed DHS1P compared with other flies (Fig. 3, B and C). We observed that DHS-fed flies also show some decrease in Rh1, perhaps because it can be converted to DHS1P (Fig. 3, B and C).

To assess whether the DHS1P level was altered in Sk2 overexpressors, we measured levels of sphingosine, S1P, DHS,
compared with Sk1 (Fig. 3 D). Thus, the ratio of DHS1P to S1P is increased in Sk2 overexpressors, whereas that in Sk1 is comparable with w^{1118}. Results from feeding experiments and mass spectrometric measurements in Sk2 overexpressors show that an increase in DHS1P over S1P correlates with photoreceptor degeneration and lysosomal degradation of Rh1 and TRP.

Based on these results, we tested whether Sk1 photoreceptors could be induced to degenerate by raising Sk1 overexpressors and DHS1P in these overexpressors by ultrafast liquid chromatography coupled to tandem mass spectrometry (UFLC MS/MS). To detect endogenous DHS and DHS1P, substantial amounts of head extract were necessary. 4,000 crosses were set up for each overexpression, heads were cut, and sphingolipid-enriched fractions were prepared for mass spectrometry. Sk1 and Sk2 use both sphingosine and DHS as substrates and generate S1P and DHS1P. However, Sk2 overexpressors preferentially accumulate DHS1P compared with Sk1 (Fig. 3 D). Thus, the ratio of DHS1P to S1P is increased in Sk2 overexpressors, whereas that in Sk1 is comparable with w^{1118}. Results from feeding experiments and mass spectrometric measurements in Sk2 overexpressors show that an increase in DHS1P over S1P correlates with photoreceptor degeneration and lysosomal degradation of Rh1 and TRP.

Based on these results, we tested whether Sk1 photoreceptors could be induced to degenerate by raising Sk1 overexpressors.
we examined the effect of Sk2 overexpression in many phototransduction mutant backgrounds. Experiments with arr2, ninaE, inaC, ninaC, and trp mutants show that they do not suppress degeneration observed in Sk2 overexpressors (unpublished data). However, mutants in CalX partially suppress the degeneration (Fig. 4 B). In Drosophila photoreceptors, Ca2+ entry is critical for the activation and subsequent attenuation of signaling (O'Tousa, 2002). To accomplish dynamic changes in Ca2+ levels, a calcium extrusion mechanism mediated by Na+/Ca2+ exchangers counters TRP-dependent Ca2+ influx in cells. CalX is one such exchanger, and the calx mutant shows a transient response to light, a defect in signal amplification, and an increase in intracellular Ca2+ concentration (Wang et al., 2005).

Figure 4. Increasing DHS1P induces Sk1-expressing photoreceptors to degenerate, and photoreceptor degeneration in Sk2 overexpressors is suppressed in a CalX mutant. (A) Photoreceptors of Sk1 overexpressors degenerate when DHS1P is increased. In DHS1P-fed Sk1 flies, 32% of the rhabdomeres are intact compared with flies raised in solvent. (B) Photoreceptor degeneration in 7-d-old Sk2 overexpressors is suppressed in a calx mutant, suggesting altered calcium homeostasis is a likely cause of degeneration. 81% of the rhabdomeres are intact in Sk2 overexpressors in calx; however, many rhabdomeres are elongated in shape.
could not be detected by Western analysis in these deletion lines (Fig. 5, B and C). Sk1 mutant flies are viable and fertile at room temperature with no external morphological defects. We looked at the steady-state levels of TRP and Rh1 levels in sk1 and sk2. 1-d-old sk1 flies show low Rh1 and TRP. sk2 flies do not show considerable differences in their levels (Fig. 5, D–G). To test whether these mutants show alterations in their substrate or product metabolites, lipid fractions were subjected to UFLC MS/MS. As shown in Fig. 5 H, both sk1 and sk2 accumulate sphingosine and DHS. The ratio of DHS1P to S1P is increased in the Sk1 mutant because of the reduction in S1P level compared with Sk2 mutant and control flies. The Sk2 P-element mutant reported earlier is a hypomorph and not a null mutant because we could detect a transcript and a small amount of protein (unpublished data). The metabolite differences are not dramatic in the mutants as in the overexpressors because of the redundancy of the Sk1 and Sk2 function. This is also reflected in the observation that although single mutants are viable, Sk1 mutant shows trafficking defects and an altered DHS1P/S1P ratio

To determine whether endogenous Sk proteins modulate Rh1 and TRP trafficking by regulating DHS1P and S1P levels, we decided to analyze Sk1 and Sk2 mutants. A P-element mutant in Sk2 has been previously characterized; these mutant flies are viable and show impaired flight performance and diminished ovulation (Herr et al., 2004). Because Sk1 mutants were not available, we used ends-out homologous recombination–based gene targeting to generate Sk1-null mutant flies (Gong and Golic, 2003). The design of the targeting vector is shown in Fig. 5 A. A total of 1,500 lines were set up in the screen, and we recovered three independent targeting events wherein the endogenous gene was replaced with the deletion construct. Sk1 protein could not be detected by Western analysis in these deletion lines (Fig. 5, B and C). Sk1 mutant flies are viable and fertile at room temperature with no external morphological defects. We looked at the steady-state levels of TRP and Rh1 levels in sk1 and sk2. 1-d-old sk1 flies show low Rh1 and TRP. sk2 flies do not show considerable differences in their levels (Fig. 5, D–G). To test whether these mutants show alterations in their substrate or product metabolites, lipid fractions were subjected to UFLC MS/MS. As shown in Fig. 5 H, both sk1 and sk2 accumulate sphingosine and DHS. The ratio of DHS1P to S1P is increased in the Sk1 mutant because of the reduction in S1P level as compared with Sk2 mutant and control flies. The Sk2 P-element mutant reported earlier is a hypomorph and not a null mutant because we could detect a transcript and a small amount of protein (unpublished data). The metabolite differences are not dramatic in the mutants as in the overexpressors because of the redundancy of the Sk1 and Sk2 function. This is also reflected in the observation that although single mutants are viable, Sk1...
and Sk2 double mutants are lethal (unpublished data). In SphK1−/− mice, S1P tissue levels are normal, indicating that SphK deficiency could be compensated for by SphK2 (Allende et al., 2004). Only SphK1−/− and SphK2−/− double knockout has no measurable S1P levels (Mizugishi et al., 2005). It is also relevant to consider that S1P levels are likely regulated by the degrading enzymes, S1P lyase, and phosphatases.

Although S1P has been well studied, less is known about the effects of DHS1P. An elevated level of DHS1P has an anti-proliferative effect in yeast (Kim et al., 2000). Recent experiments in mammalian cells show opposite effects of DHS1P and S1P on transforming growth factor β/Smad signaling (Bu et al., 2006). Despite being closely related enzymes, mouse SphK1 and SphK2 have opposite effects on cell survival: SphK1 is antiapoptotic, whereas Skph2 promotes apoptosis (Maceyka et al., 2005). Similar to mammalian cells, our observations in Drosophila support the idea that although the two kinases have some redundant functions, they can localize to distinct cellular compartments, and localized production of their metabolites could cause distinct changes in the lipid microenvironment that either alter the structure of cargo molecules or alter their behavior to trigger differential turnover. Although we have studied Rh1 and TRP using photoreceptors as a model, it is reasonable to hypothesize that these metabolites would similarly affect the endolysosomal trafficking of other membrane proteins. Addressing how these lipids interact with other lipids and protein families involved in trafficking and sorting will improve our understanding of the participation of lipids in endolysosomal homeostasis.

Materials and methods

Fly stocks and husbandry

Drosophila stocks were raised on standard maize meal agar and maintained at 25°C unless otherwise mentioned. Sk1[35Sphingosine], car1, and lacek[5432] were obtained from the Bloomington Stock Center. Upstream activating sequence (UAS)–Sk2 flies expressing Gal80T and control flies were generated at 18°C and shifted at the adult stage after 1 d to 30°C. For heat shock experiments, Sk2 transgenics were crossed to Gal80-Tp flies, and adult flies were heat shocked at 37°C. To prepare antibodies, flies were collected before and after heat shock, and adult flies were heat shocked at 37°C for 40 min and then subjected to SDS-PAGE and Western blotting. Antibodies to Arf2, InaD, Gß/2a, and Arr2, were a gift from J. Sisson (University of Texas, Austin, TX). Affinity-purified goat polyclonal antibody to cadherin was obtained from Santa Cruz Biotechnology, Inc., rabbit polyclonal antibody to Rab5 was obtained from Abcam, and mouse monoclonal antibody to V5 was obtained from Invitrogen. The Rhodopsin monoclonal antibody (4C5) and TRP monoclonal antibody (B3F6) were obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa’s Department of Biological Sciences. The antibodies were used at the following dilutions: anti–Rhodopsin, 1:5; Sk2 at 1:10; V5 at 1:750; cadherin at 1:25; Lavalamp (LSM 510; Carl Zeiss, Inc.) using a 40× objective. For localization of Sk1, images were acquired on a confocal microscope (LSM 510; Carl Zeiss, Inc.) and incubated in secondary antibody at room temperature for 2–4 h. Antibodies were washed extensively and mounted using Vectashield. S2 cells expressing Lamp1-GFP were a gift from R. Vale and J. Engel (University of California, San Francisco, San Francisco, CA). The antibody to Lavalamp was a gift from J. Sisson (University of Texas, Austin, TX). Affinity-purified goat polyclonal antibody to cadherin was obtained from Santa Cruz Biotechnology, Inc., rabbit polyclonal antibody to Rab5 was obtained from Abcam, and mouse monoclonal antibody to V5 was obtained from Invitrogen. The Rhodopsin monoclonal antibody (4C5) and TRP monoclonal antibody (B3F6) were obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa’s Department of Biological Sciences. The antibodies were used at the following dilutions: anti–Rhodopsin, 1:5; Sk2 at 1:10; V5 at 1:750; cadherin at 1:25; Lavalamp (LSM 510; Carl Zeiss, Inc.) using a 40× objective. For localization of Sk2, stained sections were visualized with a 100× objective using an imaging system (Axioplan; Carl Zeiss, Inc.) using a camera (ORCA-ER; Hamamatsu) and Axiovision 4.5 software (Carl Zeiss, Inc.).

Western analysis

Fly heads were dissected from 7-d-old flies grown at 25°C were decapitated under anesthesia, and their heads were dissected, fixed, and processed as previously described (Acharya et al., 2003).

Immunohistochemistry

Drosophila Sk1 (as a Kpn1–Not1 fragment) and SK2 (as a Kpn1–Ecor1 fragment) were cloned into pMTV5 HisA, and stable S2 cells expressing Sk1 and Sk2 were generated. Sk1 staining was performed on S2 cells, whereas Sk2 staining was performed on S2 cells constitutively expressing GFP-tagged Drosophila lamp1 under the control of an actin promoter. S2 cells were stained as described previously (Acharya et al., 2008; Dasgupta et al., 2009). In brief, S2 cells on polylysine-coated coverslips were fixed with 4% paraformaldehyde and blocked in PBS containing 0.2% Triton-X-100 and 5% BSA for 2 h. The cells were stained with primary antibody in blocking buffer overnight at 4°C, washed with PBS, and stained with secondary antibody for 1 h at room temperature. Cells were washed and coverslips were mounted using Vectashield (Vector Laboratories). Immunofluorescent staining of adult eyes was performed following protocols described previously (Walther and Pichaud, 2007). Retinae were dissected in 4% paraformaldehyde and blocked in 10% normal goat serum in PBS supplemented with 0.3% Triton X-100. Eyes were incubated overnight at 4°C with primary antibody in blocking solution. The eyes were washed and incubated in secondary antibody for 2–4 h. Antibody to room temperature for 2–4 h. Antibodies were washed extensively and mounted using Vectashield. S2 cells expressing Lamp1-GFP were a gift from R. Vale and J. Engel (University of California, San Francisco, San Francisco, CA). The antibody to Lavalamp was a gift from J. Sisson (University of Texas, Austin, TX). Affinity-purified goat polyclonal antibody to cadherin was obtained from Santa Cruz Biotechnology, Inc., rabbit polyclonal antibody to Rab5 was obtained from Abcam, and mouse monoclonal antibody to V5 was obtained from Invitrogen. The Rhodopsin monoclonal antibody (4C5) and TRP monoclonal antibody (B3F6) were obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa’s Department of Biological Sciences. The antibodies were used at the following dilutions: anti–Rhodopsin, 1:5; Sk2 at 1:10; V5 at 1:750; cadherin at 1:25; Lavalamp (LSM 510; Carl Zeiss, Inc.) using a 40× objective. For localization of Sk2, stained sections were visualized with a 100× objective using an imaging system (Axioplan; Carl Zeiss, Inc.) using a camera (ORCA-ER; Hamamatsu) and Axiovision 4.5 software (Carl Zeiss, Inc.).

Western analysis

Fly heads were dissected from 7-d-old flies (unless otherwise specified), homogenized in sample buffer, boiled, and subjected to SDS-PAGE and Western analysis. For detection of Rh1 and TRP, the samples were incubated at 37°C for 40 min and then subjected to SDS-PAGE and Western blotting. Antibodies to Arf2, InaD, Gß/2a, and inositol polyphosphate 1 phosphatase (IP1) were gifts from C. Zucker (Columbia University, New York, NY), and antibody to NinaC was a gift from C. Montell (Johns Hopkins University, Baltimore, MD).

Feeding of sphingolipids to flies

Tetracaponsphinoglycine (long-chain base of 14-carbon length) was obtained from Matreya. DHS, DHS1P, and S1P with a 14-carbon long-chain base upstream of the start codon and 454 bp downstream of the stop codon. A 3,081-bp genomic fragment (extending from -3,103 to -222 of the start codon) was inserted into the BsiW1–AscI site of the vector as the upstream homology arm, and another 3,04b genomic fragment (454–3,453) was inserted into the Acc651–Not1 site of the pwx25.2 vector. The targeting construct was injected into w[1;70G10c5][2];[y1;70FLP] flies, and the resulting mosaic flies were crossed to P(hs-9C-rx);[2];[y1;70FLP];[y1;70FLP] flies. The resulting red-eyed progeny were screened by Western analysis for loss of Sk1 protein, and three independent targeted alleles were recovered.
were custom synthesized at the Lipidomics Synthetic Core (A. Bielawska and Z. Szulc, Medical University of South Carolina, Charleston, SC). Sphingolipid and DHS were fed at a final concentration of 300 µM, whereas their phosphates were fed at 150 µM. Standard fly food containing 20% methanol/sphingosine-containing food and fixed, and their eyes were processed by electron microscopy.

Estimation of sphingolipids by mass spectrometry

The mass spectrometric standard mix was obtained from Avanti Polar Lipids, Inc. Sphingolipid-enriched fractions were prepared from head extracts of 1,000 flies for each condition following the protocol described in Merrill et al. (2005). Frozen fly heads were homogenized in 500 µl methanol/ chloroform (2:1) using a Teflon homogenizer in a microcentrifuge tube. The homogenate was transferred to a glass tube, and 1.5 ml methanol/chloroform (2:1) was added followed by 500 µl of water and vortexed. The homogenate was sonicated in a water bath–type sonicator for 20 min and incubated overnight at 37°C. To the extract, 1 ml of water and 500 µl chloroform were added, vortexed, and centrifuged at 1,000 rpm for 10 min at room temperature. The organic phase was collected and dried under nitrogen. The extracts were dissolved in 2 ml of synthetic upper (methanol/water/chloroform, 94:9.6:6) and applied to a preconditioned SepPak C18 column for solid-phase extraction (Waters Corporation). The column was washed with 4 ml of water, and lipids were extracted in 4 ml methanol followed by 4 ml methanol/chloroform. The samples were dried under nitrogen and redissolved in the requisite amount of chloroform/methanol (1:1). For sphingolipid analyses, liquid chromatography was performed on the Prominence UFLC system (Shimadzu Co.). Separation was achieved on an ACQUITY UPLC HSS T3 column (100 x 2.1-mm internal diameter) maintained at 60°C. The mobile phase was composed of 5-mM HCOONH4 in H2O containing 0.1% HCOOH and 5-mM phosphoric acid (solvent A) and 5-mM HCOONH4 in MeOH containing 0.1% HCOOH and 5-mM phosphoric acid (solvent B) with a flow rate of 400 µl/min. The column temperature was at 60°C. Mass spectrometric detection was performed on a mass spectrometer (AB SCIEX QTRAP 5500; AB SCIEX Japan, Ltd.) with an electrospray ionization source set in both positive and negative modes. Quantification was performed with a scan time of 50 ms per transition with the following selected reaction monitoring: sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); S1P d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingosine d14:1 (244.2/196.2), d16:1 (272.2/224.2), and d17:1 (286.2/238.2); DHS d14:1 (246.2/198.2), d16:1 (274.2/226.2), and d17:1 (288.2/240.2); and d with being the number of carbon atoms in the long-chain base of each sphingolipid. The values for the mammalian fibroblasts are sphingo-
