Starvation-Induced Stress Response Is Critically Impacted by Ceramide Levels in *Caenorhabditis elegans*

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ABSTRACT Our understanding of the cellular mechanisms by which animals regulate their response to starvation is limited, despite the strong relevance of the problem to major human health issues. The L1 diapause of *Caenorhabditis elegans*, where first-stage larvae arrest in response to a food-less environment, is an excellent system to study this mechanism. We found, through genetic manipulation and lipid analysis, that biosynthesis of ceramide, particularly those with longer fatty acid side chains, critically impacts animal survival during L1 diapause. Genetic interaction analysis suggests that ceramide may act in both insulin-IGF-1 signaling (IIS)-dependent and IIS-independent pathways to affect starvation survival. Genetic and expression analyses indicate that ceramide is required for maintaining the proper expression of previously characterized starvation-responsive genes, genes that are regulated by the IIS pathway and tumor suppressor Rb, and genes responsive to pathogen. These findings provide an important insight into the roles of sphingolipid metabolism, not only in starvation response, but also in aging and food-response-related human health problems.

KEYWORDS serine palmitoyltransferase; *Rb*; pathogen; *hyl-1*; *daf-16*

DURING evolution, organisms have developed complex mechanisms to adapt to food-deprived environments. Individual cells respond to starvation by modulating intracellular signaling to maintain basal cellular activities and survive long periods of starvation (Caro-Maldonado and Munoz-Pinedo 2011; Hardie 2011; Jonker *et al.* 2012). The study of an animal's response to starvation-induced stress is highly relevant to human health and medicine. For example, the study of signaling pathways and downstream events involved in the starvation response has had a major impact on the research of aging (Finch and Ruvkun 2001; Kenyon 2010) and obesity (Hoehn *et al.* 2009; Wells and Siervo 2011). Understanding the regulation of the starvation response is also closely related to our treatment of cancers (Levine and Puzio-Kuter 2010; Lee *et al.* 2012).

doi: 10.1534/genetics.116.194282
Manuscript received July 29, 2016; accepted for publication December 7, 2016; published Early Online December 14, 2016.
Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.194282/-/DC1.
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The nematode *Caenorhabditis elegans* presents a powerful model system for genetic and genomic analysis of the starvation response in animals. *C. elegans* responds to food deprivation by altering reproductive developmental growth at various larval stages (Fielenbach and Antebi 2008; Baugh 2013). When first larval stage (L1) animals encounter a food-free environment, they arrest development and reproductive growth (L1 diapause), and survive in this state for over 3 weeks. When nutrients are reintroduced, animals are capable of exiting the diapause and resuming larval development. Gene expression changes defined as the "starvation-induced transcriptome," and "refeeding induced transcriptome," have been systematically characterized (Baugh *et al.* 2009).

The roles of the insulin-IGF-1 signaling (IIS) and AMPK pathways in starvation are conserved among organisms ranging from yeast, worms, and flies, to mice (Baugh and Sternberg 2006; Narbonne and Roy 2009; Hardie 2011). A transient receptor potential vanilloid (TRPV) channel and microRNAs were shown to modulate L1 diapause by regulating the IIS-dependent or -independent pathways (Lee and Ashrafi 2008; Zhang *et al.* 2011b). The interneurons AIY and AIB provide systemic control of the starvation response during L1 arrest partly through amino acid sensation (Kang

and Avery 2009). We previously reported that tumor suppressor Rb critically impacts survival during L1 diapause by promoting the starvation-induced transcriptome, and repressing the refeeding induced transcriptome (Cui *et al.* 2013), which affect the activity of multiple regulatory pathways. Identifying and analyzing new factors involved in the process continues to be important for us to understand how different signals and regulatory pathways coordinately promote long-term survival of animals in response to starvation. In particular, the roles of lipid metabolites in the process have not been well characterized.

Ceramide has been studied for its role in apoptosis and the response to certain stresses such as anoxia, cytokines, toxins, and chemotherapeutic agents (Deng *et al.* 2008; Hannun and Obeid 2008; Menuz *et al.* 2009; Nikolova-Karakashian and Rozenova 2010; Mosbech *et al.* 2013; Cutler *et al.* 2014; Liu *et al.* 2014). Most of these studies were carried out in nutrient-rich conditions, while the roles of ceramide under fasting conditions in whole organisms remain to be investigated. In this study, we investigate the roles of ceramide biosynthesis in *C. elegans* survival during starvation-induced L1 diapause.

Materials and Methods

Strains

Strains were cultured and maintained at 20° unless specified otherwise. Strains *lin-35(n745)*, *lagr-1(gk327)*, *hyl-1(ok976)*, *hyl-2(ok976)*, *sptl-2(ok2753)*, and *sptl-3(ok1927)* were outcrossed five times to wild-type Bristol N2. The following strains were used in this study: *daf-16(mu86)*, *age-1(hx546)*, *asm-2(tm3746)*, *asm-3(tm2384)*, *sms-1(tm2660)*, *sms-2(tm2757)*, *sms-3(tm4022)*, *cgt-1(tm1027)*, *cgt-3(tm504)*, *sphk-1(ok1097)*, *asah-2(ok564)*, *cerk-1(ok1252)*, *unc-31(e928)*, *zIs356 [daf-16p::DAF-16a/b::GFP; rol-6]*, and *adIs2122* carrying *lgg-1:GFP*.

Plasmid construction and transgenic animals

For tissue-specific expression, promoters of *rgef-1* (pan-neurons), *ges-1* (intestine), *myo-2* (pharynx muscle), and *myo-3* (body wall muscle) were amplified from N2 genomic DNA, and cloned into pPD95.77. The *hyl-1* genomic DNA fragment from the translational start codon to the stop codon was amplified from N2 genomic DNA. It was then placed behind the specific promoter and followed by the *unc-54* 3′-UTR. Each DNA construct (50 ng/μl) was coinjected with the *sur-5*::*dsRed* (25 ng/μl) into *lagr-1(gk327)*; *hyl-1(ok976)* animals to create three or more extrachromosomal lines. The *myo-2P:hyl-1* and *myo-3P:hyl-1* constructs were combined to create muscle-specific expression lines.

L1 starvation survival assay and statistical analysis

The L1 starvation assay was done following the protocol described previously (Lee and Ashrafi 2008; Cui *et al.* 2013; Zhang *et al.* 2011b). Survival curves were drawn based on three or more independent experiments. To perform the basic starvation survival analysis, we simulated the survival rate of each genotype to 100 arbitrary "individual worms." The mean survival rate of individual replicates was calculated through the OASIS software available at http://sbi.postech.ac.kr/oasis (Yang *et al.* 2011). The average of the mean survival rate of all individual replicates for each strain was calculated. The mean survival rates of individual replicates were used to assess the difference between different strains or conditions. The statistical analyses (*P* value) to assess the difference between the mean survival rates were conducted using Student's *t*-test

RNAi by feeding

A *zip-2* feeding RNAi strain was obtained from the *C. elegans* ORF-RNAi library (Rual *et al.* 2004). Control RNAi was the L4440 RNAi feeding vector (Addgene; A. Fire, Stanford University School of Medicine, Stanford, CA) without any *C. elegans* DNA insert. Synchronized L1s were fed on *zip-2*-(RNAi) or control RNAi plates. The resulting gravid adults were bleached, and the L1 starvation survival of their progeny was measured.

Caenorhabditis elegans total lipid extraction

Synchronized L1 larvae (30 hr after bleach treatment) of wild type Bristol N2, *sptl-2(lf)*, and *lagr-1(lf)*; *hyl-1(lf)*, were obtained using the same procedure as in L1 starvation survival assay. Around 900,000 L1s for each strain were collected as one experimental sample. Worm pellets were subjected to three cycles of freezing in liquid nitrogen and thawing followed by sonication. Lipid extraction was carried out in the presence of prespiked internal standards (Avanti Polar Lipids, Catalog#: LM6002) with 2:1 methanol and chloroform at 48° for 24 hr, followed by 15 min sonication at 37°, as described previously (Zhang *et al.* 2011a). The resulting samples were back-extracted with chloroform. After centrifugation, the lower organic phase was collected, washed once with artificial upper phase (chloroform/methanol/H_2O; 3:48:47), and dried under nitrogen gas.

Ceramide profiling by ESI-mass spectrometry

Lipid extracts were dissolved in isopropanol:hexane:100 mM ammonium acetate (58:40:2) with 1% formic acid added, and subjected to quantitative lipid analysis using a 4000 Q-Trap mass spectrometer (AB Sciex). Samples were infused at a flow rate of 8 μl/min using a syringe pump (Harvard Apparatus). Ceramides were detected using precursor ion scans in the positive mode for the 250.3 μ fragment of D17:1 sphingoid bases. For mutants and wild type control animals, the extracts from two different batches of culture were analyzed. The relative amounts of total ceramide species were calculated relative to the number of worms.

RNA isolation

Synchronized L1 worms were obtained as above. Animal samples were collected 30 hr after bleaching. Total RNA was isolated using Trizol (Invitrogen) as per the manufacturer's

protocol, and then treated with Turbo DNase (Ambion), followed by RNA cleanup using an RNeasy Mini Kit (Qiagen).

Affymetrix microarray analysis

Analyses were performed using Affymetrix Genechip Arrays for *C. elegans* as per the manufacturer's protocol at the Genomics and Microarray Core in the University of Colorado Denver. Biological replicates were analyzed in triplicate for wild type (Bristol N2) and *lagr-1(gk327)*; *hyl-1(ok976)* (*CerS (rf)*). Microarray analyses were performed with GeneSifter web-based software (VizX Labs), using the GC robust multi-array average (RMA) algorithm, and analyzed by applying a statistical *t*-test: $P < 0.01$ for *lagr-1(gk327)*; *hyl-1(ok976)* analysis with a threshold of twofold ratio of dysregulation. Gene lists were curated by cross-referencing with WormBase (http://www.wormbase.org, release WS230). When annotation indicated that a single probe correlated with multiple genes, all such genes were excluded from our final lists. Curated gene lists are included in Supplemental Material, Table S2A. The microarray gene expression data are available at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE84894).

Tissue enrichment analysis

Tissue enrichment analysis was carried out with the Tissue Expression Predictions for *C. elegans* program, version 1.0. (http://worm-tissue.princeton.edu/search/multi) (Chikina *et al.* 2009).

Gene ontology analysis

We performed gene ontology (GO) analysis by using the online tool (www.geneontology.org). Of the 272 ceramide-regulated genes, 213 genes had annotations to Biological Process GO terms. GO categories were retained if their Bonferroni-corrected *P* values were <5%. *P*-value was calculated by the binomial statistics.

Overlap analysis among different sets of genes

Four available datasets were used for overlap analysis with the list of ceramide-regulated genes identified in our microarray data analysis. (1) Two classes of *daf-16*-responsive genes were reported in Tepper *et al.* (2013). (2) *lin-35*/Rb regulated genes during L1 starvation were reported in Cui *et al.* (2013). (3) The FedUP and StarvUP gene lists obtained from www.wormbase.org were originally submitted by R. Baugh based on the published paper (Baugh *et al.* 2009) (http://www.wormbase.org/species/c_elegans/expression_cluster/WBPaper00032948:FedUp#0154-10; http://www.wormbase.org/species/c_elegans/expression_cluster/WBPaper00032948:StarveUp1#0154–10). (4) The *Pseudomonas aeruginosa* PA14 induced genes represent the pathogen-response genes (Troemel *et al.* 2006).

The hypergeometric probability test for statistical significance of the overlap between two sets of genes was calculated by using software provided by J. Lund accessible at http://nemates.org/MA/progs/overlap_stats.html. The total number of genes represents the overlap genes from the original data sets that the two sets of genes were generated from. The test gave rise to a representation factor (rf) and the probability (p) of finding an overlap of x genes. The representation factor is the number of overlapping genes divided by the expected number of overlapping genes drawn from two independent groups. A representation factor >1 indicates more overlap than expected of two independent groups, and a representation factor <1 indicates less overlap than expected. Expected overlap was determined by multiplying the number of genes dysregulated in data set 1 by the number dysregulated in data set 2, and then dividing by the total number of genes that were detectable (for microarray datasets) and present in both datasets.

Data availability

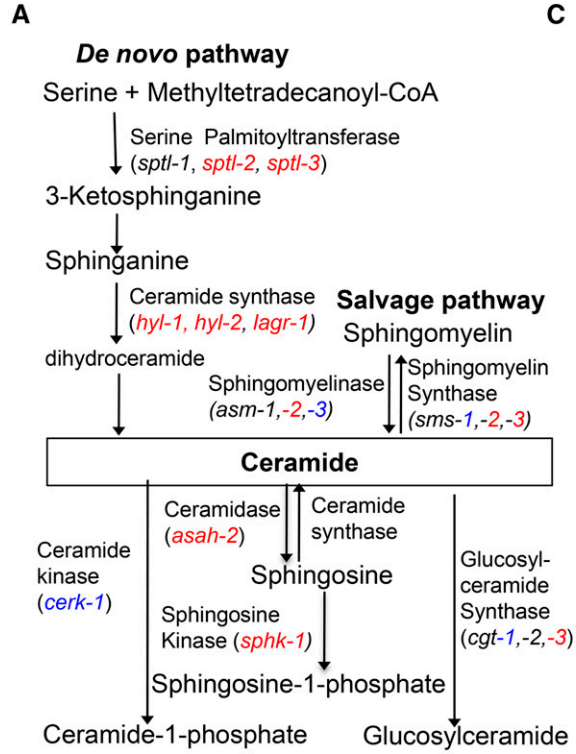
The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains are available upon request.

Results

Reducing ceramide synthesis severely impairs animals' ability to survive L1 starvation

To understand the role of ceramide in regulating animal starvation survival, we screened all available, viable, *C. elegans* mutants in the ceramide metabolism pathways for altered L1 starvation survival rates (Cui *et al.* 2013) (Figure 1A and Table S1), and found many such mutants were significantly short-lived during L1 starvation (Figure 1, A–C, Figure S1, A–C, and Table S1). Specifically, animals with mutations in the *de novo* ceramide biosynthesis pathway were sensitive to starvation-induced stress (Figure 1, B–C and Table S1). *sptl-2* and *sptl-3* encode two of the three serine-palmitoyltransferase (SPT) enzymes, and *hyl-1* and *hyl-2* encode two of the three ceramide synthase (CerS) enzymes (Deng *et al.* 2008) in *C. elegans*. Deletion mutants of each of these four genes resulted in significantly reduced L1 starvation survival rates (Figure 1, B and C). Although a deletion mutant of the third ceramide synthase gene, *lagr-1*, displayed no observable defect in L1 starvation survival, it enhanced the defect of the *hyl-1* mutant in a *lagr-1*; *hyl-1* double-deletion mutant (Figure 1, B and C). Because all deletions mentioned above truncate a key functional domain, they are most likely loss-of-function [referred to as (*lf*) hereafter] mutants.

Ceramides are essential to animal development. Depletion of ceramides by completely eliminating the key enzymes in the ceramide synthesis pathway results in larval lethality. Examples such as *sptl-1(RNAi)*, *hyl-1(lf)*; *hyl-2(lf)* double mutants and *cgt-1(RNAi)*; *cgt-3(RNAi)* double RNAi have been shown to cause strong larval lethal phenotypes (Menuz *et al.* 2009; Seamen *et al.* 2009; Nomura *et al.* 2011). The above tested mutants represent reduction, but not elimination, of the function at the corresponding enzymatic steps. Therefore, we refer to the *lagr-1(lf)*: *hyl-1(lf)* double mutants as *CerS(rf)*. The *sptl-2(lf)* and *CerS(rf)* mutants showed a significantly reduced survival rate at day 1 (Figure 1B), which was not due to embryonic lethality or early larval lethality.



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	Genotype	Mean survival (±SE) (Days)#	p-value*
Survival curves are presented in Fig.1B.	Wild type	19.4 ± 0.5	
	sptl-2(ok2753)	6.5 ± 0.5	1.40E-06[a]
	sptl-3(ok1927)	17.1 ± 0.4	0.05005[a]
	hyl-1(ok976)	13.3 ± 0.6	0.00191[a]
	hyl-2(ok1766)	15.2 ± 0.5	0.00216[a]
	lagr-1(gk327)	19.3 ± 0.6	0.90809[a]
	lagr-1; hyl-1	3.5 ± 0.3	2.65E-13[a]
	lagr-1; hyl-2	15.5 ± 0.6	0.00235[a]
Survival curves are presented in Fig.1D.	Wild type	19.4 ± 0.5	
	lagr-1; hyl-1	3.8 ± 0.3	1.98E-04[b]
	hyl-1; lagr-1; hyl-1 genomic DNA	17.0 ± 0.6	6.24E-06[c]
	hyl-1; lagr-1; rgef-1P:hyl-1	16.3 ± 0.7	1.30E-06[c]
	hyl-1; lagr-1; ges-1P:hyl-1	16.0 ± 0.5	8.07E-06[c]
	hyl-1; lagr-1; myo-3P:hyl-1+ myo-2P:hyl-1	11.5 ± 0.5	2.33E-05[c]

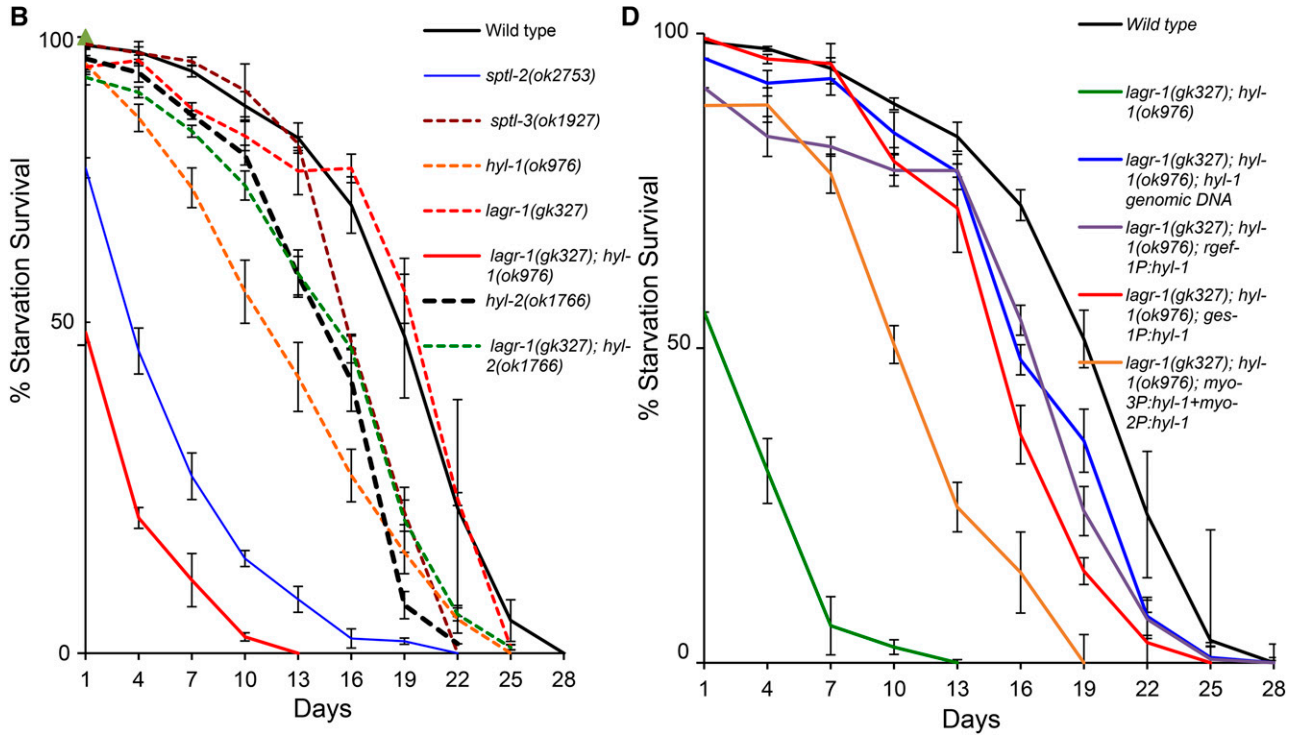


Figure 1 Ceramide functions to promote L1 starvation survival. (A) Simplified diagram to illustrate the *C. elegans* orthologs of genes involved in ceramide biosynthesis and metabolism. Deletion mutations of genes in red, but not genes in blue, displayed a significant defect in L1 starvation survival (see Table S1 for raw data and statistical analysis). Genes that are not highlighted with red or blue color are not included in L1 starvation study due to unavailable mutants, or the mutation of that gene resulting larval lethality. (B) Survival rates of ceramide synthesis reduction mutants [*lagr-1(gk327); hyl-1(ok976)* is referred as *CerS(rf)* in all figures]. Percentage survival is defined as the percentage of worms surviving to the third larval stage and beyond on NGM plates with OP50 bacteria after incubation in S-basal buffer in the absence of food for the indicated time. Data of each strain represent the mean of three or more independent biological replicates. Errors bars are the SEs at each time point indicated. (C) The mean survival rate of individual replicates was calculated through OASIS software available at http://sbi.postech.ac.kr/oasis (Yang *et al.* 2011). #The average of the mean survival rate of all

Tissue-specific expression suggests a nonautonomous role of the ceramide synthase

HYL-1 is expressed in the pharynx, intestine, and nervous system, while LAGR-1 is expressed in larval and adult pharyngeal muscle (Hunt-Newbury *et al.* 2007). The full-length *hyl-1* genomic DNA restored L1 starvation survival of the *CerS (rf)* mutants (Figure 1, C and D). We next examined whether ceramide synthases act mainly in a particular tissue for the L1 starvation survival function. Specifically, we asked whether expressing HYL-1 in one of the three major tissues (intestine, neurons, or muscle), using transgenes driven by tissue-specific promoters would be sufficient to restore the L1 starvation survival in the *CerS(rf)* animals. The expression of HYL-1 in either the intestine (*ges-1* promoter) or neurons (*rgef-1* promoter) could effectively rescue the defect of *CerS(rf)* animals (Figure 1, C and D). Expression of HYL-1 in the pharynx (*myo-2* promoter) and body wall muscle (*myo-3* promoter) partially restored the L1 starvation survival of *CerS(rf)* animals (Figure 1, C and D and Table S1). The partial rescue from muscle-specific expression of HYL-1 may be due to lower expression of HYL-1 or may be due to the inefficiency of ceramide export from muscle cells. These results at least suggest that ceramide synthesis in multiple tissues can protect *C. elegans* from starvation stress.

Reduction of very long fatty acyl chain ceramide levels correlates with reduced L1 starvation survival

Ceramides are produced from sphinganine and fatty acyl-CoAs by the actions of multiple CerS, each of which has a preference for a specific fatty acyl-CoA (Grosch *et al.* 2012). The *hyl-1(lf)* and *hyl-2(lf)* mutants displayed differential starvation survival phenotypes, with the *hyl-1* mutant being more sensitive to starvation stress. [The mean survival rate was 13.3 ± 0.6 for *hyl-1(lf)* and 15.2 ± 0.5 for *hyl-2(lf)*. *P* value for the difference of these two mutants by the Student's *t*-test is 0.034 (Figure 1B)]. We therefore quantified the major ceramide species in starved L1 worms of wild type, *sptl-2(lf)*, and *lagr-1(lf)*; *hyl-1(lf)* [*CerS(rf)*] mutants by electrospray ionization mass spectrometry (ESI-MS). The total ceramide levels of either *sptl-2(lf)* or *CerS(rf)* animals were significantly lower than those of wild-type animals (Figure 2A). Furthermore, it has been reported that *hyl-1(lf)* worms expressed significantly lower C25 and C26 ceramides, but more C21 and C22 ceramides compared to wild type (Menuz *et al.* 2009). These data suggest that levels of total ceramides, especially those with very long fatty acyl chains, promote survival of animals under starved conditions. Consistent with this hypothesis, disrupting the *acs-20* gene, which encodes an acyl-coA synthetase that has been shown to incorporate exogenous very long chain (C26:0) fatty acids into sphingolipids (Kage-Nakadai *et al.* 2010), significantly shortened starvation survival (Figure 2, B and C).

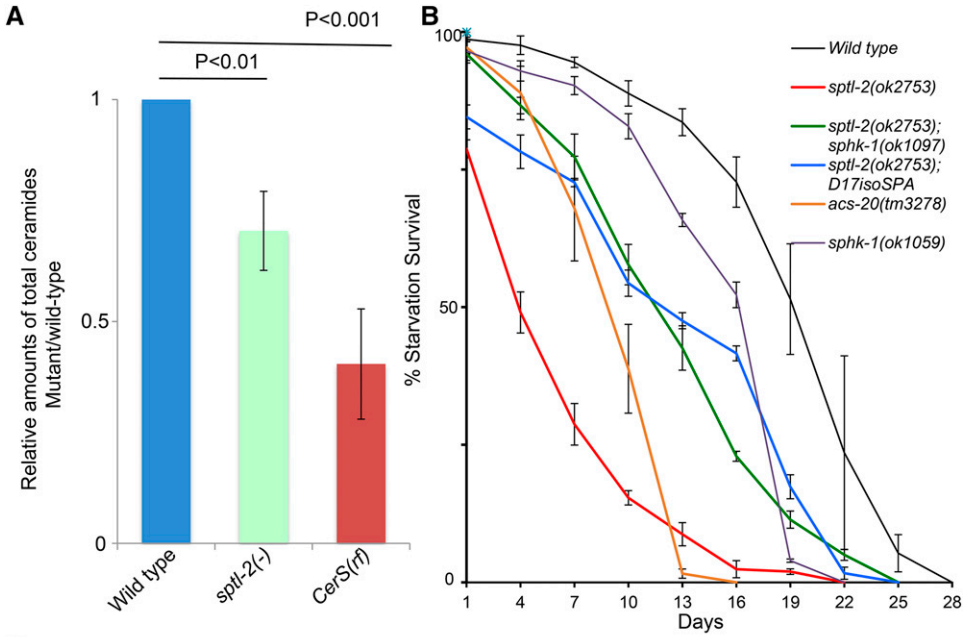
Dietary supplementation with sphingoid bases significantly rescues the reduced L1 starvation survival of sptl-2 (lf)

Short fatty acyl chain ceramides (C6 and C8) are soluble in DMSO, but are toxic to animals in high concentrations, and may not be physiologically relevant. The long-acyl chain ceramides (C16–25) are insoluble in either DMSO or aqueous solution, rendering dietary supplement analysis difficult. Indeed, we failed to rescue the L1 starvation survival of either *sptl-2(lf)* or *CerS(rf)* mutants with dietary supplementation of ceramides containing various lengths of fatty acyl chains. In contrast, the sphingoid bases (sphinganine), which are ceramide precursors and downstream products of the serine palmitoyltransferase, have better solubility in DMSO and aqueous solution. In *C. elegans*, the majority of sphingoid bases are derived from monomethyl branched-chain fatty acids (C15ISO and C17ISO) (Zhu *et al.* 2013). We then examined dietary supplementation with a custom synthesized d17iso-SPA (sphinganine), and found it was able to partially rescue the L1 starvation survival of *sptl-2(lf)* (Figure 2, B and C). This result may also suggest that intestinal ceramides play prominent roles in promoting starvation survival. Furthermore, *sphk-1(ok1097)*, a null allele of the sphingosine kinase that causes animals to lose the ability to convert sphingosine to sphingosine-1-phosphate, significantly rescued the L1 starvation survival of *sptl-2(lf)*, even though the *sphk-1(ok1097)* mutation alone causes only a modest reduction in L1 starvation survival (Figure 2, B and C). These data indicate that sphingosines, the ceramide precursors, are critical for L1 starvation survival, whereas sphingosine-1-phosphate may also have a modest role in the process.

Change in IIS pathway significantly affects starvation survival of CerS(rf) animals

Previous studies have indicated that the IIS pathway critically regulates L1 starvation survival (Baugh and Sternberg 2006; Lee and Ashrafi 2008; Zhang *et al.* 2011b). A loss-of-function (*lf*) mutation of *unc-31/CAPS* and a reduction-of-function (*rf*) allele of *age-1*/PI3K were shown to extend L1 starvation

individual replicates for each strain is presented here. *The statistical analyses (*P* value) to assess the difference between the mean survival rates were conducted using Student's *t*-test. [a,b,c]*P* values indicate the significance of the difference from wild type in (B) ([a]*P*), wild type in (D) ([b]P) and *lagr-1(gk327); hyl-1(ok976)* mutant animals in (D) ([c]*P*), respectively. (D) Starvation survival rate of the *CerS(rf)* L1 mutant animals carrying extrachromosomal arrays expressing the wild-type *hyl-1* gene driven by its own promoter, and three other tissue specific promoters [intestine (*ges-1*), pan-neurons (*rgef-1*), and muscle (*myo-2/myo-3*)]. Transgenic animals were scored on the basis of the expression of the *sur-5P:dsRed* coinjection marker with a Leica fluorescence microscope. Percentage survival is defined as the percentage of animals surviving to the third larval stage and beyond on food after L1 worms were starved in S-basal buffer for the indicated time. The average from multiple independent transgenic lines for each genotype is reported with the SEM for each time point (±SEM). The starvation survival data for wild-type animals are the same as that presented in (B). Raw data and the statistical analysis data for individual starvation survival experiments for (B) and (D) are presented in Table S1.



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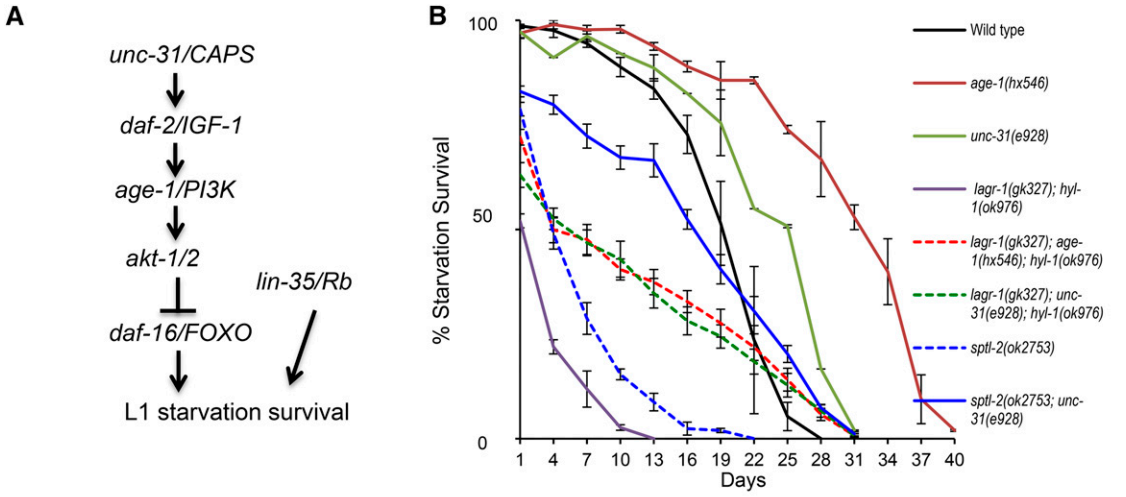
Genotype	Mean survival (±SE)	p-value*
Wild type	19.4 ± 0.5	NA
sptl-2(ok2753)	6.5 ± 0.5	1.40E-06[a]
sphk-1(ok1097)	15.6 ± 0.5	0.0107[a]
sptl-2(ok2753); sphk-1(ok1097)	12.8 ± 0.6	1.62E-07[b]
sptl-2(ok2753); d17iso-SPA	12.9 ± 0.7	4.62E-06[b]
acs-20(tm3278)	9.8± 0.3	0.00213[a]

Figure 2 L1 starvation survival is reduced when ceramide levels are decreased, but it can be partially rescued with sphingoid base supplementation. (A) Ceramide levels are decreased in both *sptl-2(lf)* and *lagr-1(lf)*; *hyl-1(lf)* double mutants [referred to *CerS(rf)*]. Total ceramide levels of mutants relative to that of wild type are shown in columns. Errors bars represent SE at each time point indicated. *P* values were calculated by Student's *t*-test. (B) Survival rates of wild type, *sptl-2(lf)* ± dietary supplementation, *sptl-2(lf)*; *sphk-1(ok1097)*, and two *acs-20* mutants. The *sptl-2(lf)* defect was significantly suppressed by both dietary supplementation of 250 nM iso-branched d17iso-sphinganine (d17iso-SPA) and *sphk-1(ok1097)* mutation. Percentage survival is defined as the percentage of animals surviving to the third larval stage and beyond on food after L1 worms were starved in S-basal buffer for the indicated time. The starvation survival data for wild type and *sptl-2(lf)* are the same as that presented in Figure 1B. (C) The mean survival rate of individual replicates was calculated through OASIS software. The average of the mean survival rate of all-individual replicates for each strain is presented here. *The statistical analyses (*P* value) to assess the difference between the mean survival rates were conducted using Student's *t*-test. [a,b]*P* values indicate the significance of the difference from wild type ([a]*P*) and *sptl-2(ok2753)* mutant animals ([b]*P*), respectively. Raw data and detailed statistical analysis data for individual starvation survival experiments for figure 1B are presented in Table S1.

survival (Lee and Ashrafi 2008; Zhang *et al.* 2011b) (Figure 3, A and B), while *lf* mutations in *daf-18/Pten* and *daf-16/FOXO*, both negative regulators of the pathway, shortened L1 starvation survival and lifespan (Baugh and Sternberg 2006; Kenyon 2010; Cui *et al.* 2013) (Figure 3A). We thus analyzed the genetic interactions of *CerS* with *unc-31*, *age-1*, and *daf-16*. We found that the starvation survival defects associated with both *CerS(rf)* mutants were partially but significantly suppressed by both *unc-31(lf)* and *age-1(rf)* mutations (Figure 3, B and C). The survival rates of *CerS(rf)*; *unc-31(lf)* and *CerS(rf)*; *age-1(rf)* mutants were more than threefold higher than that of *CerS(rf)*, but more than onefold lower than that of *unc-31(lf)* and *age-1(rf)*. These partial suppression data suggest that ceramide may potentially function both upstream of the IIS pathway and through IIS-independent mechanisms. To further examine their functional relationship, we combined the *sptl-2(lf)* allele with a *daf-16(lf)* allele, and found that the mean and maximum L1 starvation survival rates of this strain were significantly lower than that of either single mutant (Figure 3, C and D). A similar functional relationship is also observed in *sptl-2(lf)*; *lin-35/Rb(lf)* double mutants (Figure 2D and Figure 3C). Therefore, neither the IIS pathway nor Rb is the sole major target of ceramide for L1 starvation survival.

Transcriptional profiles of the CerS(rf) mutant during L1 starvation

To learn more about functions downstream of ceramide in regulating L1 diapause, we compared the global gene-expression profiles of the *CerS(rf)* mutant and wild type animals using high-density oligonucleotide microarrays. Our microarray data analysis revealed that at a $P < 0.01$ and twofold



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Genotype	Mean survival (±SE) (days)#	p-value
Wild type	19.4 ± 0.5	
age-1(hx546)	30.5 ± 0.9	8.62E-05[a]
unc-31(e928)	23.6 ± 0.7	0.0245[a]
lagr-1(gk327); hyl-1(ok976)	3.5 ± 0.3	2.65E-13[a]
lagr-1; age-1; hyl-1	11.5 ± 1.0	2.98E-13[b]
lagr-1; unc-31; hyl-1	11.0 ± 1.0	2.77E-10[b]
sptl-2 (ok2753)	6.5 ± 0.5	1.40E-06[a]
sptl-2; unc-31	16.8 ± 1.0	1.67E-10[c]
lin-35(n745)	8.3 ± 0.4	1.47E-06[a]
lin-35(n745); sptl-2(ok2753)	4.4 ± 0.2	0.00435[c]
		0.000168[d]
daf-16(mu86)	7.0 ± 0.2	6.35E-05[a]
daf-16(mu86); sptl-2(ok2753)	4.5 ± 0.2	1.65E-05[c]
		4.49E-07[e]

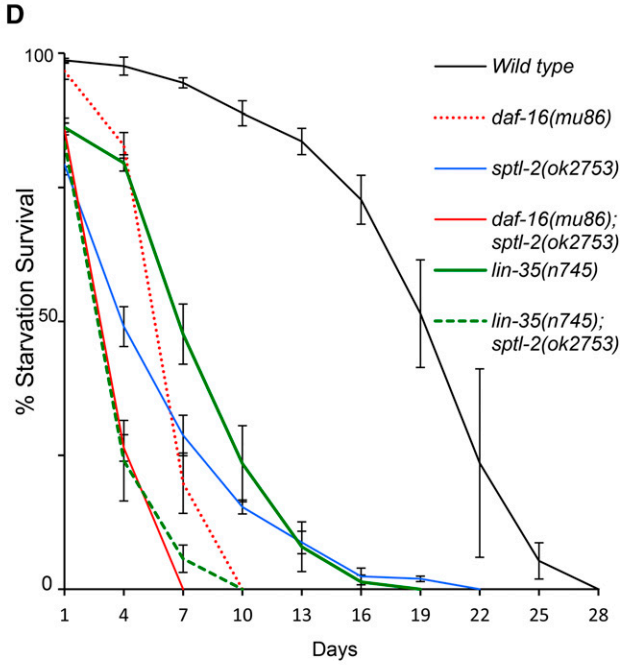

Figure 3 Ceramide may regulate L1 starvation survival by affecting both the Rb and IIS pathway-dependent and -independent functions. (A) A simplified diagram of the Rb and IIS pathway and their relationship with L1 starvation survival. (B) Survival curves showing that the L1 starvation survival defect of the *CerS(rf)* mutant and the *sptl-2(lf)* mutant was partially rescued by *unc-31(lf)* and *age-1(rf)*. Percentage survival is defined as the percentage of animals surviving to the third larval stage and beyond on food after L1 worms were starved in S-basal buffer for the indicated time. Errors bars represent SE at each time point indicated. The starvation survival data for wild type, *CerS(rf)*, and *sptl-2(lf)* are the same as that presented in Figure 1B. (C) Table of mean survival and *P*-values for survival curves presented in (B) and (D). # The average of the mean survival rate of all individual replicates for each strain is presented here. The combination of *sptl-2(lf)* with *daf-16(lf)* or *lin-35/Rb(lf)* resulted in more severe defects than each single mutant. [a,b,c,d,e]*P* values indicate the significance of the difference from wild type ([a]*P*), *CerS(rf)* ([b]*P*), *sptl-2(ok2753)* ([c]*P*), *lin-35(n745)* ([d]*P*), and *daf-16(mu86)* ([e]*P*) respectively. (D) Survival curves showing the defect of *sptl-2(lf)* was enhanced by either *daf-16(lf)* or *lin-35/Rb(lf)*. % Survival is defined as the percentage of animals surviving to the third larval stage and beyond on food after L1 worms were starved in S-basal buffer for the indicated time. Errors bars represent SE at each time point indicated. The starvation survival data for wild type and *sptl-2(lf)* are the same as that presented in Figure 1B. Raw data and statistical analyses for individual starvation survival experiments for Figure 3 are presented in Table S1.

cut-off, 272 genes were dysregulated in the *CerS(rf)* mutant (Table S2A). Of the 272 dysregulated genes, 77% (208/272) were upregulated, and 33% (64/272) were downregulated in the *CerS(rf)* mutant during L1 diapause. Tissue enrichment predications suggested that upregulated genes are enriched in the intestine, and downregulated genes are enriched in neurons (Figure 4A). GO analysis revealed that "immune response," "response to defense," and "response to stress" descriptors were highly enriched among upregulated genes (Table S3).

Ceramide affects the expression of daf-16/FOXO- and Rb-regulated genes

We further analyzed the functional relationship between ceramide biosynthesis and the IIS pathway by comparing our microarray data with other relevant transcriptional profiles. The comparison between *CerS(rf)* affected genes and *daf-16/FOXO* responsive genes (Murphy *et al.* 2003; Tepper *et al.* 2013) identified 139 overlapping genes, which is 2.5-fold higher than expected by chance ($P < 1.66\ e^{-30}$) (Figure 4B, Table S2, B and C, and Table S4). Among these 139 genes, 92 are repressed by *daf-16/FOXO*, and 47 are upregulated by *daf-16/FOXO*. Of the 92 overlapping genes that are repressed by *daf-16/FOXO*, 87 were upregulated in the *CerS(rf)* mutant, which is 4.1-fold higher than expected by chance ($P < 7.39\ e^{-33}$) (Table S2C and Table S4). These results indicated that there are a significant number of genes affected by both ceramide and the IIS-DAF-16 pathways, suggesting that *ceramide* acts in part through IIS to affect DAF-16 targets, which is consistent with the suggestion from above genetic interaction data that ceramide may partially function upstream of the IIS pathway to regulate L1 starvation survival.

Rb has been shown to play an important role in regulating the starvation-responsive transcriptome (Cui *et al.* 2013). We thus compared gene expression between *CerS(rf)* and *Rb(lf)* mutations. A total of 107 genes significantly changed their expression in both ceramide synthase and *lin-35/Rb* mutants, which is 5.5-fold higher than expected by random chance ($P < 6.77\ e^{-52}$), and equal to 39% of the total dysregulated genes in *CerS(rf)* (Figure 4C, Table S2D and Table S4). Of the overlapping genes, 98% (104/107) were changed in the same directions in both mutants during L1 starvation, with 85% of these genes (89/105) upregulated in both mutants, suggesting that ceramide and Rb normally repress the expression of these genes (Table S2D). Further comparisons revealed that 59% (63/107) of the overlapping genes between *CerS(rf)* and *Rb(lf)* mutants were *daf-16/FOXO*-responsive genes (Table S2E). Of these, 73% (46/63) were upregulated in *daf-16/FOXO(lf)*, *CerS(rf)*, and *Rb(lf)* mutants (Table S2E), suggesting that ceramide, *lin-35/Rb*, and *daf-16/FOXO* repress common targets in response to starvation stress during L1 diapause.

Ceramide is important for maintaining starvation-induced gene expression dynamics

To gain further insight into how ceramide impacts starvation-response related gene expression, we further compared the transcriptome of *CerS(rf)* mutant animals during L1 diapause with previously described FedUP and StarvUP genes (Baugh *et al.* 2009). FedUP genes are expressed at higher levels when animals hatch in the presence of food, and were proposed to promote reproductive growth. In contrast, StarvUP genes are expressed at higher levels when animals hatch in the absence of food (starvation), and were proposed to support an animal's survival during starvation. Of the total 272 genes dysregulated by *CerS(rf)*, we found 95 genes (35%) are FedUP genes (Figure 4D, Table S2F, and Table S4), and 90 of these 95 genes were upregulated in the *CerS(rf)* mutant, indicating that ceramide functions to repress these genes during L1 diapause in wild type. Furthermore, 33 of the genes (12% of the total 272 genes) dysregulated by *CerS(rf)* were StarvUP genes (Figure 4D, Table S2G, and Table S4). Of these 33 genes, 20 were downregulated in the *CerS(rf)* mutant, indicating that ceramide promotes the expression of these genes during L1 diapause in wild type. Therefore, like *Rb(lf)*, *CerS(rf)* alters the "starvation transcriptome" toward a "feeding transcriptome" (Cui *et al.* 2013).

Many pathogen-inducible genes are repressed by ceramide during L1 diapause

GO analysis of the 272 dysregulated genes in the *CerS(rf)* mutant showed that the "immune response," in particular the "innate immune response," is the most enriched GO category based on *P*-value (Table S3). Therefore, we compared our *CerS(rf)* microarray datasets with pathogen-responsive gene datasets. Specifically, when the dataset of *CerS(rf)* was compared against the 4-hr exposure to the *P. aeruginosa* strain PA14 (Troemel *et al.* 2006), we identified 63 dysregulated genes in *CerS(rf)* that were PA14_4 hr-responsive genes, which is dramatically greater than expected by chance. Of these 63 genes, 59 were upregulated in both the *CerS(rf)* mutant and PA14-induced gene list (Figure 4E, Table S2H, and Table S4). These results suggest that ceramide repressed the expression of these pathogen-inducible genes for the benefit of starvation survival. A role of repressing a large number of pathogen- and toxin-inducible genes during L1 diapause was also identified for *Rb* in a previous report (Cui *et al.* 2013). As described earlier, nearly half of the *CerS(rf)* affected genes were found to overlap with *Rb(lf)* affected genes (Figure 4A and Table S2D). These shared activities support the idea that repressing pathogen-inducible genes is vital for protecting starvation survival. Many pathogen-inducible genes encode proteins with antimicrobial or detoxification roles (Hoeven *et al.* 2012). While they function to protect animals against various environmental threats, they are likely to be harmful to the starvation-induced response for long-term survival. However, our data do not exclude the possibility that these genes are simply unresponsive to starvation stress; they are not necessarily repressed to enhance starvation survival.

Among the 272 genes dysregulated in *CerS(rf)* mutants, 12 are listed in a compendium of 934 predicted *C. elegans* transcription factors (referred to as wTF2.0) (Reece-Hoyes *et al.* 2005) (Table S5). Of the nine upregulated transcription factors, *zip-2*, encoding a bZIP transcription factor, was upregulated by threefold in *CerS(rf)* mutants at $P < 0.0025$. A previous study indicated the role of *zip-2* in regulating an early response to *P. aeruginosa* infection in *C. elegans*, and that the induction of 25 *P. aeruginosa* infection response genes is ZIP-2 dependent (Estes *et al.* 2010). We found that 18 out of these 25 genes were highly induced in starved L1 animals of the *CerS(rf)* mutant (Table S6), suggesting that

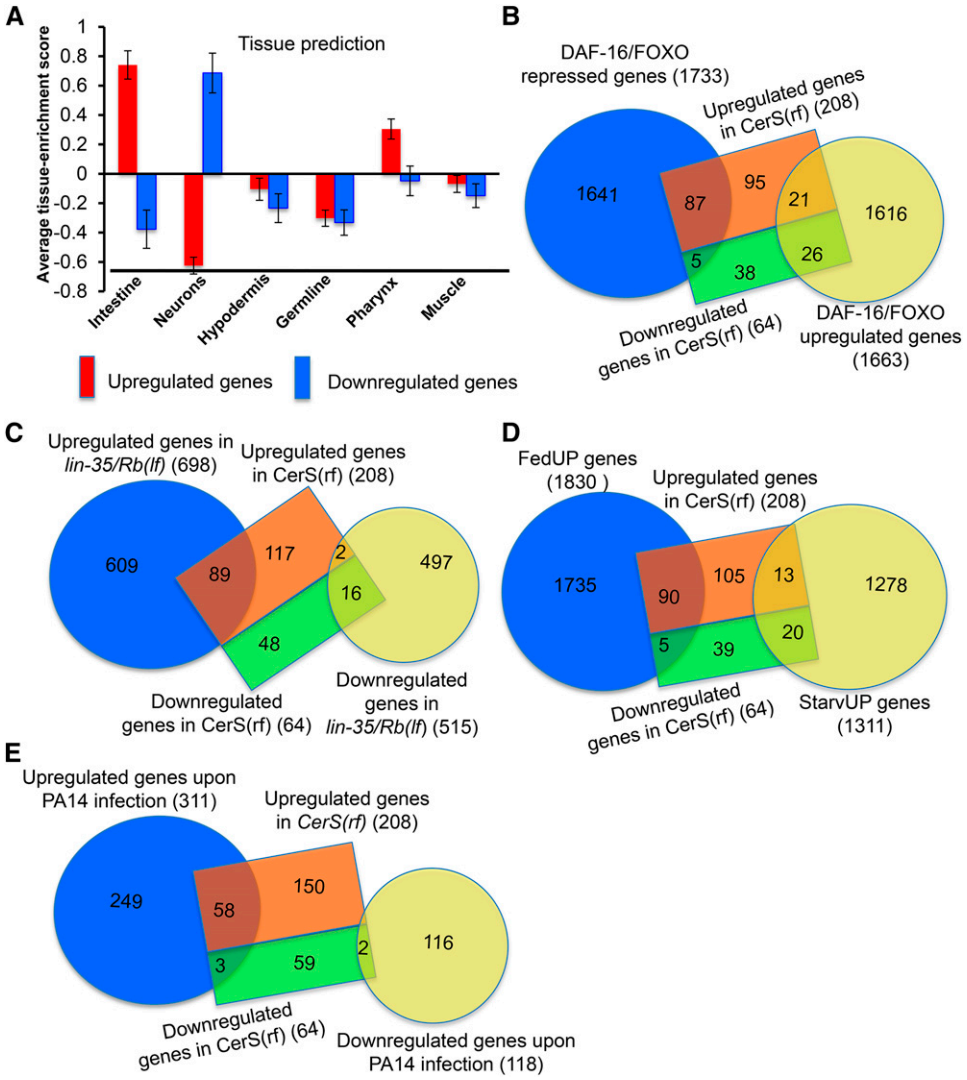

Figure 4 Ceramide synthases regulate the expression of genes regulated by the IIS-pathway, Rb, feeding, starvation condition, and genes induced by pathogen. (A) Tissue enrichment analysis. Tissue enrichment scores were calculated by the online program Tissue Expression Prediction for *C. elegans* (Chikina *et al.* 2009). (B) Diagram showing the overlap between dysregulated genes in CerS(rf) and DAF-16/FOXO responsive genes (Tepper *et al.* 2013). The lists of overlapping genes are included in Table S2, B and C. The statistical significance of the overlap is presented in Table S4. (C) Diagram showing the overlap between dysregulated genes in CerS(rf) and lin-35/Rb (Cui *et al.* 2013). The list of overlapping genes is included in Table S2D. The statistical significance of the overlap is presented in Table S4. (D) Diagram showing the overlap between dysregulated genes in CerS(rf) and FedUP genes or StarvUP genes (Baugh *et al.* 2009). The lists of overlapping genes are included in Table S2, F and G. The statistical significance the overlap is presented in Table S4. (E) Diagram showing the overlap between dysregulated genes in CerS(rf) and previously identified pathogen (*P. aeruginosa*, PA14)-inducible genes (Troemel *et al.* 2006). The list of overlapping genes is included in Table S2H. The statistical significance of the overlap is presented in Table S4.

the increase in *zip-2* expression may partially mediate the impact of *CerS(rf)* on the expression of pathogen response genes, which implicates a negative role of ZIP-2 in the impact of ceramide on L1 starvation survival. This negative role is also consistent with our further genetic analysis of the *zip-2* gene (Figure S2). However, the genetic data also suggest that *zip-2* may promote other cellular processes needed for starvation survival (Figure S2), which is consistent with the impact of *zip-2(lf)* on the expression of a large number of genes (Estes *et al.* 2010).

Discussion

In this study, we described a strong impact of ceramide deficiency on animal survival during L1 diapause. Ceramide is an important secondary signaling molecule generated in response to multiple extracellular stimuli, such as DNA damage, cytokines, and growth factors, to regulate multiple cellular events like apoptosis, cell senescence, the cell cycle, and differentiation (Hannun and Obeid 2008). While studies using cell culture systems have contributed the most to our current understanding of the roles of ceramide in these cellular processes, the analysis of ceramide function in stress responses using animal models has been limited. Our findings suggest that ceramide may function as a secondary messenger of the food-deprivation signal upstream of multiple stress response pathways.

We showed that three ceramide synthases have differential roles in the L1 starvation response. *hyl-1* has the most prominent role in starvation survival, whereas *lagr-1* has the weakest role, based on single and double mutant analyses (Figure 1). It has been shown that *hyl-1* is mainly responsible for synthesizing ceramides containing very-long-fatty-acyl chains (C25/C26) (Menuz *et al.* 2009). There are six mammalian CerS enzymes (CerS1–6) that vary in their spatiotemporal expression patterns and their abilities to produce ceramides with different chain lengths (reviewed by Grosch *et al.* 2012; Mullen *et al.* 2012). Equilibrium between very-long and long-chain ceramides is also thought to be important for normal cellular physiology (Grosch *et al.* 2012).

Further study is needed to identify the chain-length dependence of ceramide target proteins in order to explain the molecular basis for how different ceramide species play differential roles in animal starvation survival.

Our analysis of ceramide-regulated genes during L1 diapause, and previous study on the function of *Rb* in the process, indicated that gene expression dynamics induced by starvation share common features, as well as possess distinct features from that induced by other environmental stresses, such as pathogen-induced responses. The IIS pathway has been shown to play prominent roles in animal responses to many different stresses (Baugh and Sternberg 2006; Evans *et al.* 2008). However, unlike in other stressed situations when animals need to counter toxic threats, food or nutrient deprivation presents a distinct physiological challenge to cells and tissues, and thus demands unique changes in gene regulation. Our previous studies of miRNA functions also indicate sharply different roles of major miRNA functions in the intestine for starvation survival compared to that during pathogen responses (Zhang *et al.* 2011b; Kudlow *et al.* 2012).

A previous study showed the impact of ceramide biosynthesis on mitochondrial functions in stress response under well-fed conditions (Liu *et al.* 2014). We also previously reported that *Rb* regulates the L1 starvation response partly by promoting the expression of many mitochondrial respiratory chain (MRC) proteins (Cui *et al.* 2013). However, we found that the expression of most of these MRC proteins are not significantly affected by *Cer(rf)* mutations (Table S2A), suggesting that, under starved conditions, the impact of ceramide on mitochondrial function may be limited.

The benefit of ceramide on starvation survival would seem to be consistent with a potential positive role of ceramide molecules in lifespan extension. Many known lifespan regulators, such as *daf-16* and *mir-71*, play positive roles in both lifespan extension and L1 starvation survival (Baugh and Sternberg 2006; Kenyon 2010; Pincus *et al.* 2011; Zhang *et al.* 2011b; Boulias and Horvitz 2012). However, a previous publication showed that *C. elegans* strains with mutations in both *hyl-1* and *lagr-1* genes, like our *CerS(rf)* mutants, displayed autophagy-dependent lifespan extension under well-fed conditions (Mosbech *et al.* 2013). It has also been shown that, while the *hyl-2(lf)* mutant was sensitive to anoxia, the *hyl-1(lf)* mutant was resistant to anoxia (Menuz *et al.* 2009). These findings suggest that the poor L1 survival rate of *CerS (rf)* animals is not due to a general sickness, or a nonspecific sensitivity to all stresses. In addition, these observations also raise an interesting question regarding whether, under well-fed conditions, ceramide has roles that are distinct from roles observed under starved conditions. It has also been previously reported that ceramide inhibits insulin sensitivity in mammals under nutrient-rich conditions, which potentially renders individuals at risk for diabetes and cardiovascular disease (Chavez and Summers 2012). More investigation is clearly needed to learn about the mechanisms underlying the benefits and detriments of ceramide under both starved and well fed conditions.

The appropriate level of autophagy is required for optimal starvation survival of *C. elegans* (Kang and Avery 2009). To investigate whether an abnormal level of autophagy contributes to the poor starvation response of mutants in the ceramide synthesis pathway, we examined the level of autophagic activation in *sptl-2(lf)* animals. There was no significant difference in the autophagic response between *sptl-2(lf)* and wild-type animals during L1 starvation (Figure S3), suggesting that *sptl-2(lf)* affects L1 starvation survival mainly through autophagy-independent mechanisms.

Ceramide has been previously suggested to function as a tumor-suppressor lipid (Morad and Cabot 2013). Our study reveals that ceramide promotes animal starvation survival. Previous studies also showed that tumor suppressor genes, *daf-16/Foxo*, *daf-18/Pten*, and *lin-35/Rb* are required for promoting *C. elegans* starvation survival (Baugh and Sternberg 2006; Cui *et al.* 2013). Starvation or fasting has recently been investigated in cancer therapy (Naveed *et al.* 2014; Cangemi *et al.* 2016), as tumor cells are thought to be more susceptible to starvation due to the Warburg effect (Iansante *et al.* 2015). Results from this study and previous work using *C. elegans* may suggest that cancer cells, that commonly harbor mutations in tumor suppressor genes, are highly sensitive to starvation stress.

Acknowledgments

We thank S. Mitani and the Caenorhabditis Genetics Center (CGC) [supported by National Institutes of Health (NIH) P40 OD010440] for strains and materials; M. Kniazeva, H. Zhu, A. Sewell, and S. Fechtner for assistance; A. Sewell for editing; and W. Wood and Han laboratory members for discussions. This study was supported by the Howard Hughes Medical Institute and NIH (RO1GM37869).

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Communicating editor: M. V. Sundaram