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LET-767 Is Required for the Production of Branched Chain and Long Chain Fatty Acids in *Caenorhabditis elegans*[*] [S]

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LET-767 from *Caenorhabditis elegans* belongs to a family of short chain dehydrogenases/reductases and is homologous to 17β-hydroxysterol dehydrogenases of type 3 and 3-ketoacyl-CoA reductases. Worms subjected to RNA interference (RNAi) of *let-767* displayed multiple growth and developmental defects in the first generation and arrested in the second generation as L1 larvae. To determine the function of LET-767 *in vivo*, we exploited a biochemical complementation approach, in which *let-767* (*RNAi*)-arrested larvae were rescued by feeding with compounds isolated from wild type worms. The arrest was only rescued by the addition of triacylglycerides extracted from worms but not from various natural sources, such as animal fats and plant oils. The mass spectrometric analyses showed alterations in the fatty acid content of triacylglycerides. Essential for the rescue were odd-numbered fatty acids with monomethyl branched chains. The rescue was improved when worms were additionally supplemented with long chain even-numbered fatty acids. Remarkably, *let-767* completely rescued the yeast 3-ketoacyl-CoA reductase mutant (*ybr159*Δ). Because worm ceramides exclusively contain a monomethyl branched chain sphingoid base, we also investigated ceramides in *let-767* (*RNAi*). Indeed, the amount of ceramides was greatly reduced, and unusual sphingoid bases were observed. Taken together, we conclude that LET-767 is a major 3-ketoacyl-CoA reductase in *C. elegans* required for the bulk production of monomethyl branched and long chain fatty acids, and the developmental arrest in *let-767* (*RNAi*) worms is caused by the deficiency of the former.

The nematode *Caenorhabditis elegans* has emerged as a valuable model organism for studying metabolism, storage, and function of lipids (1–4). The worm genome encodes a large number of proteins implicated potentially into lipid binding or metabolism (5). These include more than 270 nuclear hormone receptors (6, 7), which might interact with the putative products of more than 80 cytochrome P450s (8) and several short chain dehydrogenases (9).

Functional genomics and proteomics do not directly reveal substrate specificity, activity, and function of metabolic enzymes. In model organisms, such as *C. elegans*, their identification and functional annotation are typically achieved either (i) by classical biochemical genetics approach, where mutants are selected that fail to synthesize known metabolites, or (ii) by the reverse approach, where metabolites are identified based on their ability to complement mutations in a particular gene. A successful example of the first approach is delineating the synthesis of polyunsaturated fatty acids in *C. elegans* (10, 11), in which several mutants with abnormal fatty acid composition were isolated. The reverse complementation approach was used to identify a lipophilic fraction, which rescued reproductive development in dauer constitutive *daf-2* and *daf-9* mutants and contained the product of the cytochrome P450 DAF-9 dafachronic acid (12–14). Similarly, a sterol-related activity, which rescued reproductive development in dauers formed in the presence of methylated sterols, was identified and partially purified (15). The complementation approach is appealing because of the recent progress in analytical microanalysis. Moreover, this approach could be combined with RNAi,[2] a convenient method to inactivate a particular gene.

We aimed to isolate the biologically active sterols from *C. elegans* and delineate the biochemical pathway(s) of their synthesis. Sterols in worms regulate two basic processes, molting and dauer formation (2, 15). Currently, however, only a few active sterols, or their metabolizing enzymes, are known (2, 3, 14). A good candidate for an enzyme modifying sterols in *C. elegans* is encoded by *let-767*, which was previously identified in a genetic screen for essential genes on the third chromosome (16). LET-767, belongs to a family of short chain dehydrogenases/reductases, and it has four other paralogs in worms (Fig. 1*A*). LET-767 is closely related to human 17β-hydroxysterol dehydrogenase type 3 (17β-HSD), an enzyme required for the production of testosterone (17) (Fig. 1*C*). Based on mutant morphological phenotypes, it was proposed that *let-767* encodes a sterol-modifying enzyme that might be involved in the production of a molting hormone (18). Recently, it has been shown that LET-767 can catalyze the transformation of 4-an-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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[2] The abbreviations used are: RNAi, RNA interference; mmBCFA, monomethyl branched chain fatty acid; LCFA, long chain fatty acid; FAME, fatty acid methyl ester; MS/MS, tandem mass spectrometry; DDA, data-dependent acquisition; dsRNA, double-stranded RNA; 17β-HSD, 17β-hydroxysterol dehydrogenase type 3; KAR, 3-ketoacyl-CoA reductase; ER, endoplasmic reticulum; GFP, green fluorescent protein; TAG, triacylglyceride.

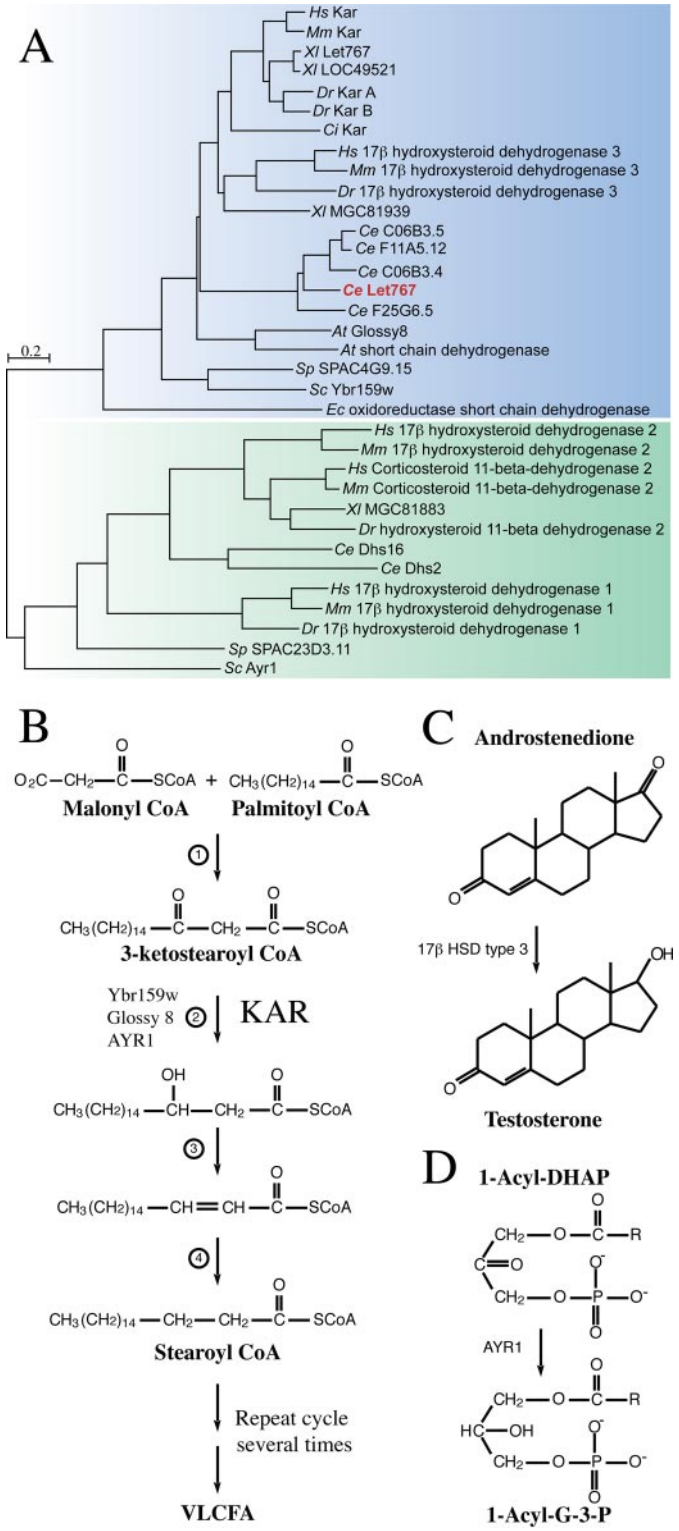

FIGURE 1. **Short chain dehydrogenases/reductases include ubiquitous members and can utilize a variety of substrates.** *A,* phylogenetic tree of the KAR subfamilies of short chain dehydrogenases/reductases. LET-767 is most closely related to human KAR and 17β-HSD type 3 (*blue background*); this subfamily is closely related to the AYR-1 family of 3-ketoacyl-CoA reductases (*green background*). *B,* some 3-ketoacyl-CoA reductases have been shown to be involved in the second step in the synthesis of LCFA, which is reduction of 3-ketoacyl-CoA to 3-hydroxyacyl-CoA. *C,* 17β-HSD is required for the production of the steroid hormone testosterone. *D,* family member AYR1 can continue on reduction of 1-acyl dihydroxyacetone phosphate in the synthesis of phosphatidic acid.

drostendione into testosterone and estrone into estradiol when the enzyme was expressed in HEK-293 cells and the cells were challenged with high concentrations of the substrates (19). However, in worm cells, in its native biochemical environment, LET-767 might have a different activity. We found that LET-767 is also homologous to another subfamily of conserved short chain dehydrogenases/reductases that are involved in fatty acid synthesis (Fig. 1, *A* and *B*) (20–26). Genetic and biochemical analyses have demonstrated that members of this subfamily are microsomal enzymes possessing 3-ketoacyl-CoA reductase (KAR) activity. These enzymes catalyze the second out of four reactions in the biosynthesis of long chain fatty acids (LCFA) with more than 16 carbon atoms (Fig. 1*B*). In this reaction, 3-hydroxyacyl-CoAs are produced by reducing 3-ketoacyl-CoAs, which are condensation products of malonyl-CoA and fatty acids fused by substrate-specific elongases (27) (in *C. elegans elo-1* to *-9*). It was demonstrated that the LET-767 homolog GL8 protein, the product of the *glossy8* gene in maize, is a component of the acyl-CoA elongase involved in the production of cuticular waxes that contain very long chain fatty acids (24, 25). The yeast strain bearing a deletion in *ybr159w* has a highly reduced content of LCFA (20, 21, 23). A more phylogenetically distant KAR is AYR1 (Fig. 1, *A* and *B*), which reduces 1-acyl dihydroxyacetone phosphate to 1-acyl glycerol 3-phosphate (Fig. 1*D*), a step in the production of phosphatidic acid (28). Interestingly, this enzyme might also have 17β-HSD activity (Fig. 1*C*) (29).

Taken together, functional genomic and bioinformatic annotation of LET-767 did not allow an unambiguous identification of its biochemical function and left open possibilities for several activities for this enzyme, including sterol modification, elongation of fatty acids, and production of phosphatidic acid. It is also conceivable that LET-767 possesses all these activities at the same time. Therefore, to uncover the genuine biological activity of LET-767, we isolated a lipid fraction from wild type worms, which complemented and rescued the developmental arrest in *let-767* (*RNAi*) animals. A complementation approach showed that monomethyl branched chain fatty acids (mmBCFA) are essential for the rescue of *let-767* (*RNAi*) phenotype. However, the developmental arrest was rescued to a higher extent if worms were supplemented with a mixture of mmBCFA and long chain fatty acids (LCFA) with a straight carbon chain. We also confirmed LET-767 has a 3-ketoacyl-CoA reductase activity by completely rescuing LCFA production in the deletion mutant of the yeast homolog YBR159W. Thus, we conclude that LET-767 is involved in the production of mmBCFA and LCFA, and the former have a vital role in the development.

EXPERIMENTAL PROCEDURES

Bioinformatics Analysis—First, a comprehensive tree of short chain dehydrogenases was constructed and then reduced to highlight the evolutionary relationship of the LET-767 group with the KAR family of dehydrogenases. Multiple sequence alignments of protein sequences, most closely related to LET-767, were carried out with ClustalX (30), and phylogenetic analysis was performed with the Phylip package (31). The tree was constructed with *protdist* and *fitch* from the Phylip package and bootstrapped for 1000 times.

Worm Strains—The following *C. elegans* strains were used: wild type Bristol N2; *dpy-17*(*e164*) *let-767*(*s2819*) *ncl-1*(*e1865*) *unc-32*(*e189*) *III; sDp3* (*III;f*) (strain BC4849 from Caenorhabditis Genetics Center); *unc-119*(*ed3*) *III* (strain DP38 from Caenorhabditis Genetics Center); *unc-119*(*ed3*)*; let-767*::*GFP* and *unc-119*(*ed3*)*; plet-767*::*GFP* (see below). *unc-119*(*ed3*)*; let-767*::*GFP* worms were obtained by ballistic transformation (32) with a construct containing a genomic fragment of *let-767* (plus 498 bp downstream and 87 bp upstream), which was subcloned into the NotI site of pDP#MM051 (33). GFP was introduced by Red ET (34). Initially, the genomic fragment was cloned into pBluescript SK+ with a NotI linker. An AgeI restriction site was introduced just before the Stop codon of *let-767*. Subsequently, this construct was linearized via the AgeI site, and GFP (*C. elegans* intron optimized version) was introduced by Red ET cloning. In the construct after recombination, the AgeI site is missing. This construct was also used to generate the *let-767* promoter reporter. For this, reverse primers (with AgeI site) starting 36 bp upstream from the *let-767* coding sequence, and from the first codon of GFP, were used. We kept the first 12 codons of *let-767* in the reporter because of a GATA box, which is probably necessary for intestinal expression (18). Subsequently, the NotI fragment was subcloned to the vector for ballistic transformation. All PCR sequences were verified by DNA sequencing.

For chemical complementation rescue of *let-767* (*s2819*), and to address functionality of LET-767::GFP, transformed lines with *let-767*::*GFP* not genomically integrated were selected. Males from those transformants were crossed to *dpy-17*(*e164*) *let-767*(*s2819*) *ncl-1*(*e1865*) *unc-32*(*e189*) *III; sDp3* (*III;f*). After self-fertilization of the progeny, in the second generation, Dpy Unc worms with GFP fluorescence were selected. From these worms several lines were established in which only LET-767::GFP-positive worms developed, the segregated non-GFP worms were *dpy-17*(*e164*) *let-767*(*s2819*) and arrested at early larval stages.

RNAi by Feeding—*let-767* and *elo-5* RNAi was performed in wild type N2, Bristol strain. *let-767* RNAi feeding construct was generated by cloning from 452 to 1088 of *let-767* with NotI linkers into the NotI site of pPD129.36 (L4440). Subsequently, this construct was used to transform HT115 *Escherichia coli* used for dsRNA production and feeding (35). For *elo-5* RNAi, bacteria provided by MRC GeneService from the Ahringer dsRNA feeding library (36) were used. Growing and induction of dsRNA producing bacteria were according to Ref. 37, with the following modifications: (i) instead of L3-L4 larvae, bleached embryos were put onto the plates; (ii) isopropyl 1-thio-β-D-galactopyranoside was directly mixed with the bacteria to ensure induction of dsRNA in chemical rescue experiments.

Fluorescent Nile Red was fed to RNAi worms after they reached the L4 larval stage on dsRNA plates. With this modification, Nile Red staining was performed according to Ref. 1. Light and fluorescent microscopy were as described in Ref. 15.

Yeast Strains—All yeast experiments were done in the background of *MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3–11,15* (strain W303-1A (38)). The deletional mutant *ybr159*Δ was generated as described in Ref. 23 using the *ybr159w*::*TRP1* disrupting fragment. *let-767* open reading frame was cloned into BamHI-HindIII sites of a modified YIplac128 with a *GALL* promoter (39). The EcoRV linearized construct was transformed into *ybr159*Δ, and LEU prototrophs were selected. For control purposes, strains transformed with the linearized empty vector alone were generated.

Heterologous Expression in Yeast and Fatty Acid Analyses—As a noninductive medium YP with 2% glucose and as an inductive YP with 2% galactose and 2% raffinose were used. Yeast cultures grown to saturation phase at 25 °C were used for fatty acid analyses. Yeasts were homogenized by bead beating and consequently extracted according to Ref. 40. The total lipid extracts were methanolized in 5% HCl/MeOH for 2 h at 70 °C, and fatty acid methyl esters (FAMEs) were extracted with hexane. Free fatty acids were obtained by saponification of FAMEs. Liquid chromatography-mass spectrometry analysis was carried out on a time of flight LCT mass spectrometer (Waters) interfaced to a Waters Alliance 2695 liquid chromatograph equipped with a Zorbax Eclipse XDB C8 column (150 × 4.6 mm). Fatty acids were eluted using the following gradients: 100% solvent A (90% methanol with 0.1% ammonium acetate in water) for 5 min, a gradient from 0% B (99% methanol with 0.1% ammonium acetate in water) to 100% B in 5 min, and 100% B for 35 min. The flow rate was 0.3 ml/min. Mass spectra were acquired from the *m/z* 100–1000 atomic mass units range in negative ion mode under the control of MassLynx software. Each measurement was performed in triplicate. Molar percentage of C26 was calculated as a percentage of the sum of C26:1 and C26:0 signals from the sum signals of all nonhydroxylated fatty acids detected (12:0, 14:1, 14:0, 16:1, 16:0, 18:2, 18:1, 18:0, 20:1, 20:0, 22:1, 22:0, 24:0, 26:1, and 26:0). Molar percentages of C26:0-OH and the sum of the rest of the hydroxylated fatty acids (16:0-OH, 18:1-OH, 20:1-OH, 22:0-OH, and 24:0-OH) were calculated in the same way from the sum signal of all hydroxylated fatty acids.

Extraction of Lipids and TLC Separation—Lipid extraction from *C. elegans* was performed according to Ref. 15. The amount per TLC was normalized to protein content and corresponded to 200 μl of pelleted worms. TAG spots were identified based on the *Rf* of synthetic standards. Preparative two-dimensional TLC was performed on Silica Gel 60 F_{254} 20 × 20-cm plates (Merck) using chloroform:methanol (24:1) as a first running system and *n*-hexane:diethyl ether:acetic acid (7:3:0.1) as a second running system. The plates were sprayed with primuline and visualized under UV light. Fluorescent spots were marked and scraped from the plate. Lipids were extracted from silica gel (41). Finally, lipids were dissolved in 50 μl of chloroform:methanol (1:1) and were either used in the rescue assays or analyzed by mass spectrometry. A fraction of glycosylceramides used to identify the glycosylceramide containing hydroxylated C17 sphingoid base was isolated similarly; however, the running system used for this separation was chloroform:methanol:water (45:18:3).

Determination of TAGs—TAGs quantification was done in a procedure similar to that described in Ref. 42. Briefly, a single 15-cm plate for the control and two plates for *let-767* (*RNAi*) (around 300 μl of pelleted worms) were used. After three washes with M9 medium followed by pelleting, the buffer was exchanged with bead beating buffer (25 mM Tris, 25 mM NaCl,

2 mM EDTA, pH 7.5), and worms were frozen at −80 °C. The worms were disrupted in a Mini-Beadbeater-8 with 0.7-mm zirconia beads (Biospec Products). In the homogenates total protein content was determined with the micro BCA protein assay kit (Pierce). The total amount of triglycerides was determined using the serum triglyceride determination kit (Sigma).

Rescue of RNAi by Complementation—For complementation rescue, hexane extracts (15), oils (commercial grade) and triolein, trilinolenin, testosterone (Sigma), fatty acid methyl esters (FAMEs) (mmBCFA *iso*-C15, *iso*-C17, *iso*-C19, *iso*-C21, C16, C18, C20, C22, and C24 (Sigma)) were used. FAMEs were dissolved in *n*-hexane as 20 mM stock solutions. Rescue of *let-767* (*RNAi*) was performed in 12- or 6-well plates on NGM agar, containing 0.1% tergitol (type Nonidet P-40; Sigma) in a procedure similar to the *elo-5* (*RNAi*) rescue described in Ref. 43, although 0.1% tergitol was added for better solubility. Lipids dissolved in appropriate solvent were poured onto the NGM agar, and solvents were evaporated in a laminar flow hood. FAMEs were at 100 μM final concentrations. Bacteria mixed with isopropyl 1-thio-β-D-galactopyranoside for dsRNA production were seeded onto the NGM agar. The same volume of solvent was used in simultaneous experiments, and the control was solvent without lipids. After an overnight dsRNA induction, second generation *let-767* (*RNAi*) embryos, obtained by hypochloride bleaching, were put onto the plates. Typically, 50–100 embryos were applied to each well. The maximum effect of the rescue was observed after 6 days. Rescue of *let-767* (*s2819*) was performed in a similar way. After hatching, the majority of LET-767::GFP positive worms was removed using a fluorescent dissecting microscope.

Mass Spectrometry—Mass spectrometric analysis was performed on a modified QSTAR Pulsar *i* quadrupole time-of-flight mass spectrometer (MDS Sciex) equipped with an automated nanospray chip ion source, NanoMate HD (Advion BioSciences). Ionization voltage was set to 1.05 kV and gas pressure to 0.1 p.s.i., and the source was controlled by the Chipsoft 6.3.2 software from the same company. The mass spectrometer was calibrated in MS/MS mode using a synthetic lipid standard 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine, as described previously (44). In all MS/MS experiments the analytical quadrupole Q1 was set to isolate and transmit precursor ions within the *m/z* range of 1 atomic mass unit if not specified otherwise.

TAGs were profiled by tandem mass spectrometry using the method of multiple neutral loss scanning driven by the data-dependent acquisition (DDA) as described previously (45). Analysis was performed for precursors within the range of *m/z* 750–950. In these experiments precursors were isolated by Q1 within the range of 2 atomic mass units and the collision energy was set to 40 eV. MS/MS spectra were acquired in the *m/z* range of 400–950 for 15 s per each fragmented precursor.

Phosphatidylethanolamines and phosphatidylcholines were profiled in positive ion mode by DDA-driven neutral loss scanning for *m/z* 141.04 and precursor ion scanning for the fragment with *m/z* 184.07, respectively (46). Analyses were performed for precursors in the *m/z* range of 600–860. MS/MS spectra were acquired in the *m/z* range of 100–860 for 20 s per fragmented precursor at the collision energy offset of 40 eV.

To profile ceramides and glycosylceramides, inclusion lists of prospective *m/z* were compiled and used to navigate DDA analyses of total lipid extracts. MS/MS spectra were acquired in the *m/z* range of 100 to 860 with an acquisition time of 40 s at the collision energy offset of 45 eV. For the quantitative analysis, tandem mass spectra were processed by the dedicated software LipidInspector as described in Ref. 45.

For the structural characterization of glycosylceramides, MS/MS spectra were acquired under operator control, whereas collision energy offset was ramped from 20 to 50 eV. Fragment ions were assigned based on their *m/z* determined with better than 10 ppm mass accuracy.

RESULTS

LET-767 (RNAi) Worms Display Multiple Growth and Developmental Defects—To deplete LET-767, we induced RNA interference by feeding worms with bacteria expressing dsRNA (35), because we intended to combine RNAi later with biochemical analysis and complementation studies. It was previously reported that *let-767* RNAi initiated at L3/L4 larval stages led to uncoordinated movement, slow growth phenotypes, and lethality (36). To obtain stronger phenotypes, RNAi was started earlier by letting embryos hatch on plates seeded with bacteria producing dsRNA. Already after reaching the L4/young adult stage, a strong difference emerged between the control and *let-767* (*RNAi*) worms (Fig. 2, *A–D*). *let-767* (*RNAi*) worms were uncoordinated, much thinner and transparent, and had visible morphological abnormalities. At low magnification in bright field, in wild type nonstarved worms, the intestine was seen as a dark thick stripe from head to tail (Fig. 2*A*). On the contrary, it was barely detectable in *let-767* (*RNAi*) animals (Fig. 2*B*). In *let-767* (*RNAi*) worms, embryos occupied a much larger part of the animal body. *let-767* (*RNAi*) worms had also egg-laying defects, and often their progeny developed inside the mothers. At high magnification, the intestine was seen to have lost the characteristic granulated appearance of normal worms (Fig. 2, compare *C* and *D*). Some granules were still detectable, although they were of an irregularly large size (Fig. 2*D*). The developed progeny were smaller, looked transparent, lacked normal gut granules, and arrested at L1/L2 larval stages (Fig. 2, compare *E* and *F*). Over time, these arrested larvae became more and more transparent, slowed down their movement, and died after 7–10 days.

The reduction in the number of gut granules in the first and second generations suggested that LET-767 might be involved in the storage of lipids. We then stained worms with Nile Red dye, an established marker for detection of lipid granules in the intestine (1). Indeed, in *let-767* (*RNAi*) worms, Nile Red staining was much weaker when compared with control animals (Fig. 2, *G–J*).

LET-767 Is Strongly Expressed in Intestinal Cells—The *let-767* (*RNAi*) phenotype indicated to the intestine as a primarily affected organ and suggested that major activity of the enzyme resided within intestinal cells. We further investigated if this notion was supported by *let-767* expression patterns and generated lines expressing GFP under the *let-767* promoter and full-length LET-767 tagged C-terminally with GFP. *let-767* expression was detected in embryos and throughout larval

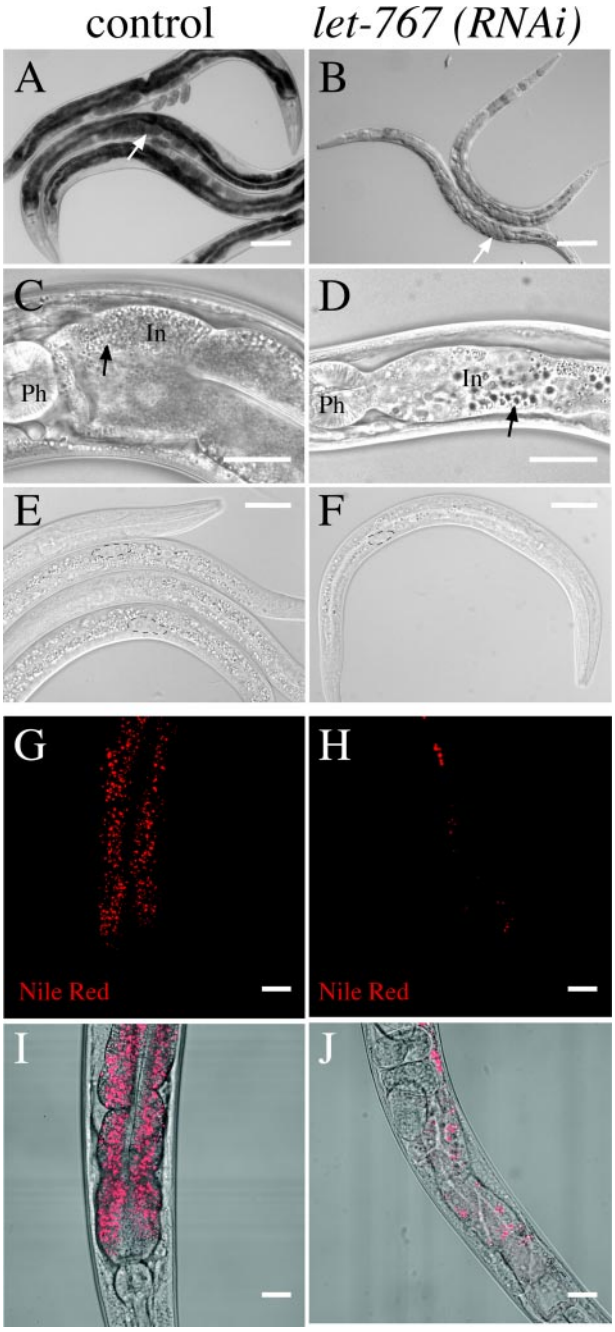

FIGURE 2. **Phenotypic analysis of *let-767* (*RNAi*) worms.** *A,* control adult worms, low magnification. *B, let-767* (*RNAi*) worms, low magnification. *A* and *B, arrows,* embryos. *C,* control adult worm, high magnification. *D, let-767* (*RNAi*) worm, high magnification; note that although the size of the pharynx (*Ph*) is similar, the intestine (*In*) is much thinner. *C* and *D, arrows,* granules. *E,* control larvae. *F, let-767* (*RNAi*) second generation arrested L1. *E* and *F, dotted outlines,* gonads. *G,* Nile Red staining, control worms. *H,* Nile Red staining, *let-767* (*RNAi*). *I,* bright field image of *G. J,* bright field image of *H. Scale bars, A* and *B,* 100 μm; *E* and *F,* 10 μm; *C* and *D*, *G–J*, 20 μm.

development and adulthood (supplemental Fig. 1). LET-767::GFP fluorescence was stronger in the intestinal cells and much weaker in a number of cells in the pharynx, tail, and hypodermis (supplemental Fig. 1, *C–F*). Thus, the strong expression of *let-767* in intestinal cells correlated with the observed phenotypes. At the intracellular level, LET-767::GFP was mainly localized to a mesh-like pattern, most likely the ER, and also showed a prominent apical localization (supplemental

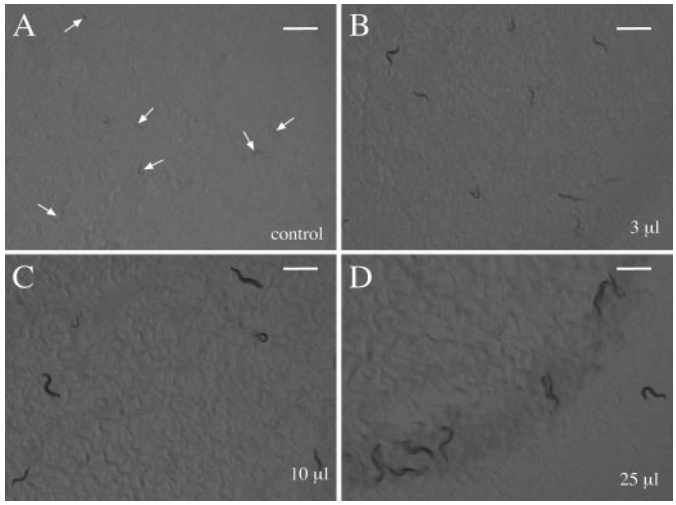

FIGURE 3. **Hydrophobic extract from nontreated worms rescues developmental arrest of the second generation *let-767* (*RNAi*).** *A, let-767* (*RNAi*)-arrested larvae (*arrows*) (no extract provided). Rescue of developmental arrest by a hydrophobic extract in a concentration-dependent manner: 3 μl (*B*), 10 μl (*C*), and 25 μl (*D*) of extract. *Scale bars,* 500 μm.

Fig. 1, *G* and *H*). The ER localization was expected for a 3-ketoacyl-CoA reductase because the elongation of fatty acids beyond 16 carbon atoms occurs in the ER (47).

LET-767 (RNAi) Developmental Arrest Is Rescued by TAGs from Nontreated Wild Type Worms—Based on the above, we reasoned that *let-767* (*RNAi*) phenotypes could be rescued by supplying the worms with either product(s) of LET-767 or molecules produced downstream in the pathway involving this enzyme. To test this, we performed complementation assays with hydrophobic lipid extracts from nontreated worms. Because the first generation of RNAi worms had multiple defects, we decided to identify the activity that could rescue the developmental arrest in the second generation. Indeed, the developmental arrest was rescued with a hydrophobic extract (hexane extract) from nontreated worms in a concentration-dependent manner (Fig. 3). It should be noted that this extract contains high amounts of acylglycerols but very low amounts of phospholipids or sphingolipids.

Next, we separated the hexane extract using preparative two-dimensional TLC and assayed the activity of individual spots (Fig. 4, *A* and *B*). We detected rescuing activity exclusively in the spots corresponding to TAGs (Fig. 4, *A* and *B*, *arrows*). Correspondingly, the amount of TAGs in *let-767* (*RNAi*) worms was 2.6 times lower than in wild type animals (Fig. 4*C*; 3.09 ± 0.47 *versus* 7.88 ± 1.25 μg TAGs per mg of total protein, respectively). TAGs are considered to represent a storage form of fatty acids. The rescue of *let-767* (*RNAi*) phenotype by feeding worms with TAGs and an overall decrease in TAG content in mutants indicated that LET-767 might be involved in the production of fatty acids, which corroborates with the homology of LET-767 to several KAR enzymes that are part of the fatty acid elongation machinery (Fig. 1).

Mass Spectrometric Quantification Revealed Altered Fatty Acid Composition of TAGs from Let-767 (RNAi) Worms—If the deficiency of TAGs/fatty acids *per se* caused developmental arrest of *let-767* (*RNAi*), addition of TAGs from any other source might rescue the worms. We supplied *let-767* (*RNAi*)-

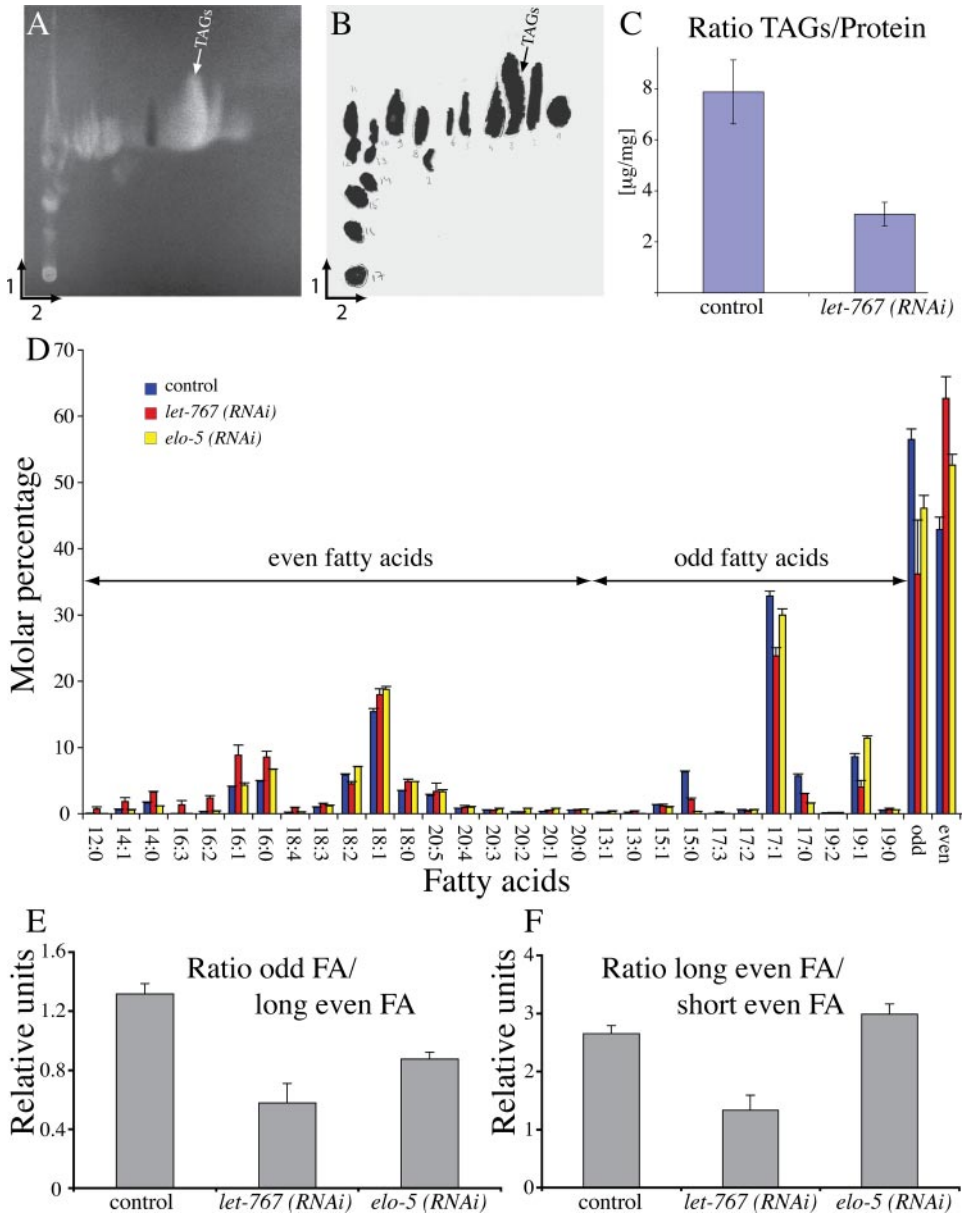

FIGURE 4. **Analysis of TAGs in *let-767* (*RNAi*).** *A,* two-dimensional TLC of the hydrophobic extract stained with primuline. *B,* individual spots were scratched and tested for rescuing activity on *let-767* (*RNAi*) worms; TLC origin, spot number 17; *arrows,* directions of the 1st and 2nd solvent systems (see "Experimental Procedures"); *C,* ratio of total TAGs to the total protein content. *D,* molar percentage of individual fatty acids. Normalization was performed by taking the sum of the peak areas of all detected fatty acids as 100%. *E,* ratio odd FA/long even FA. *F,* ratio long even FA/short even FA.

arrested larvae with TAGs from various natural sources, such as animal fats (butter and lard) and plant oils (from olive, sunflower, rapeseed, and peanut). Neither of them rescued the developmental arrest of *let-767* (*RNAi*) worms in the second generation (not shown). Thus, the rescue of *let-767* (*RNAi*) could not be achieved with whichever TAG was applied. Therefore, we aimed to determine the fatty acid composition of TAGs from *C. elegans* using mass spectrometry. Electrospray mass spectrometry readily detects TAGs as adducts with ammonium cations (45). Upon their collision-induced dissociation in a tandem mass spectrometer, ammonium adducts lose fatty acid moieties and ammonia as neutrals, whereas the net charge remains localized at the glycerol backbone. Therefore, the fatty acid composition of individual TAGs could be determined by considering the mass differences between the intact TAG ammonium adduct and corresponding diacylglycerol fragment ions (supplemental Fig. 2*A*). Quadrupole time-of-flight mass spectrometry allowed monitoring of the loss of multiple fatty acid moieties simultaneously and emulating the profile of TAG molecular species by analyzing total lipid extracts (45) (supplemental Fig. 2, *B* and *C*). With this method we acquired TAG profiles from control and *let-767* (*RNAi*) animals and determined the relative abundance of species having the same individual sum composition characterized by the total number of carbon atoms and the total number of double bonds in the fatty acid moieties (supplemental Fig. 2*C*). Mass spectrometry showed a significant and specific decrease in the relative abundance of several TAG species, such as 49:2, 51:2, 51:3, 52:3, 53:3, and 54:3 (supplemental Fig. 3). The molecular dissection of these TAGs by tandem mass spectrometry showed that they comprised mainly species containing fatty acids with odd numbers of carbon atoms. For example, TAG 49:2 contained up to 70% of TAG (15:0, 17:1, and 17:1); TAG 51:2 contained up to 50% of TAG (17:0, 17:1, and 17:1) and up to 25% of TAG (15:0, 18:1, and 18:1); and TAG 53:3 contained up to 70% of TAG (17:1, 17:1, and 19:1).

Using the same pool of acquired tandem mass spectra, we estimated relative changes in the abundance of individual fatty acids among all detectable TAGs (Fig. 4*D*). In this way, we determined the relative abundance of all TAG fragments, corresponding to the neutral loss of each of 69 detectable fatty acid moieties. TAGs from *let-767* (*RNAi*) worms contained, on average, 25% less fatty acids with an odd number of carbon atoms compared with TAGs from the wild type animals. At the same time, the relative abundance of fatty acids with even numbers of carbon atoms increased in *let-767* (*RNAi*) worms, compared with the wild type. The most significant decrease was observed for the abundance of 15:0, 17:0, and 19:1, whereas the even-numbered 16:0, 16:1, 16:2, 16:3, and 14:0 were most significantly increased.

Altogether, mass spectrometric profiling of TAGs in *let-767* (*RNAi*) and wild type animals revealed that significant changes

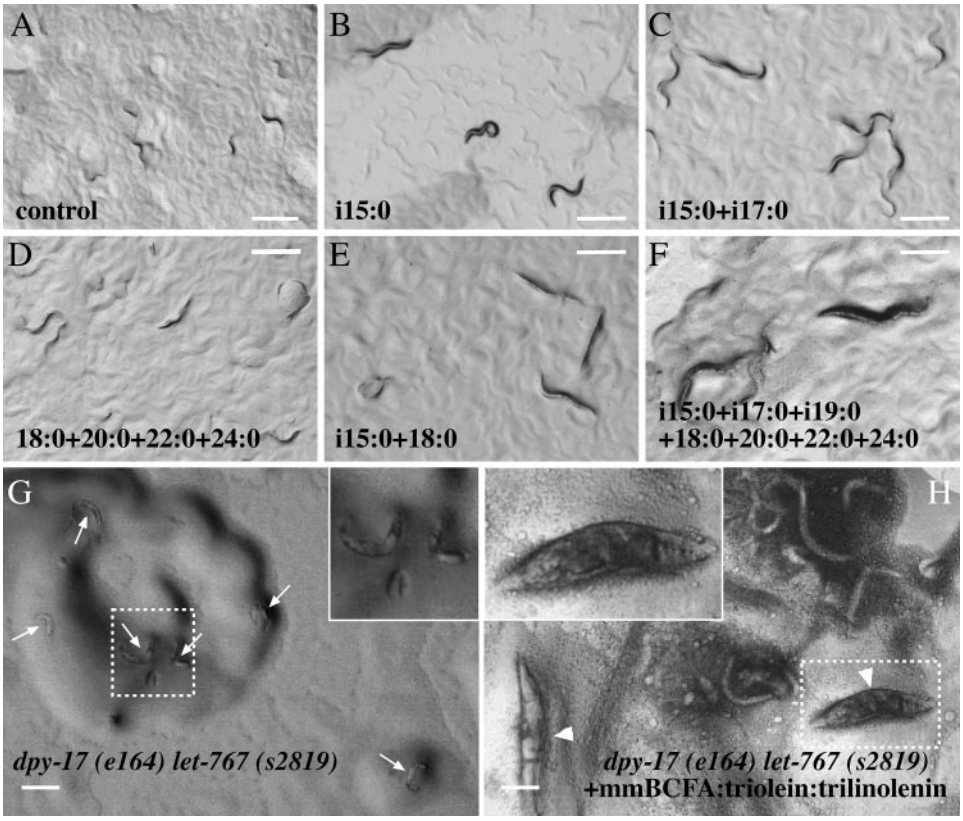

FIGURE 5. **Rescue of *let-767* (*RNAi*) and *let-767* (*s2819*) by supplementing with different fatty acids.** *A–F,* rescue of *let-767* (*RNAi*)-arrested second generation larvae by indicated mmBCFA and LCFA. *G,* arrested *dpy-17*(*e164*) *let-767* (*s2819*) worms (*arrows*). *H,* partial rescue of *let-767* (*s2819*) to adults (*arrowheads*) with a mix of mmBCFA with triolein and trilinolenin, compare bigger worms with the ones in *G*. *Insets* (*G* and *H*), high magnifications of the *marked areas*. *Scale bars, A–F,* 200 μm; *G* and *H,* 100 μm.

occurred, both in relative abundance of individual TAG species and in the total fatty acid composition of TAGs. The content of TAG species consisting of odd-numbered fatty acids was decreased and led us to the hypothesis that exactly these species (present in abundance in the wild type) might be responsible for rescuing the RNAi phenotype. Odd-numbered fatty acids have been detected in a variety of species and are common in bacteria (48). Their hydrocarbon chains can be straight or branched. Branched odd-numbered fatty acids consist of a main chain containing an even number of carbons, which bears a methyl group (branch) at ω (*iso*) or ω-1 position (*anteiso*) (48). Thus, the decreased abundance of odd-numbered fatty acids in RNAi animals might be associated with lower content of straight or branched chain fatty acids, or both. The first possibility seemed less probable because worms were fed on *E. coli*, which do not produce branched chain fatty acids (43), but contain an abundance of odd-numbered fatty acids (49).

The relative reduction in the total content of odd-numbered fatty acids detected in TAGs was relatively modest (around 25% (Fig. 4*D*)). To estimate the significance of this reduction, we analyzed TAGs of worms fed with dsRNA bacteria for *elo-5*, a specific elongase required for the production of mmBCFA in *C. elegans* (43). It was shown that RNAi of *elo-5* strongly reduced mmBCFA *iso*-C15 and *iso*-C17 content (43). Indeed, in TAGs from *elo-5* (*RNAi*) worms, the content of 15:0 and 17:0 fatty acids was reduced by 20- and 4-fold, respectively. Overall fatty acid composition in TAGs from *elo-5* (*RNAi*) worms was similar to the *let-767* (*RNAi*) profile (Fig. 4*D*). There was a significant reduction in the ratio "odd-numbered" FA/"long even-numbered" FA, also similar to *let-767* (*RNAi*) (Fig. 4*E*). This is consistent with the notion that LET-767, similarly to ELO-5, might be involved in the production of mmBCFA.

Total reduction of TAGs and therefore of fatty acids suggested that LET-767 directly, or indirectly, was involved in the synthesis of fatty acids. We wanted to address whether the effect was specific for the odd-numbered fatty acids or whether the elongation of even long chain fatty acids was also affected. To quantify the efficiency of LCFA synthesis, we compared the ratio of the content of even-numbered LCFA (more than 16C) to even-numbered short chain fatty acids (16C or less). This ratio was decreased in *let-767* (*RNAi*), although it was not changed in *elo-5* (*RNAi*) (Fig. 4*F*). Therefore, LET-767 is involved in the production of both mmBCFA and LCFA.

Rescue of let-767 (RNAi) Developmental Arrest by Supplementation with Fatty Acids—We next reasoned that if the deficiency of mmBCFA plays critical role in *let-767* (*RNAi*)-arrested animals, supplementing them with mmBCFA might rescue the arrest. Therefore, animals were then fed with particular species of mmBCFA (*iso*-15:0, *iso*-17:0, and *iso*-19:0), mixtures of mmBCFA, and a combination of mmBCFA and LCFA (18:0, 20:0, 22:0, 24:0) as well as LCFA alone (the indicative examples are shown in Fig. 5, *A–F*). The rescue was followed by two traits: the exit from the larval arrest and the further growth of larvae. The addition of any mmBCFA was sufficient for the exit from *let-767* (*RNAi*) developmental arrest (Fig. 5*B*; shown for *iso*-15:0). However, the rescued larvae grew better if the worms were supplemented with a mixture of two or more different mmBCFAs (Fig. 5*C* and not shown). This rescue could be further enhanced if the mmBCFA were complemented with LCFA with an even number of carbon atoms, either individually, *e.g.* 18:0 (Fig. 5*E*), or in mixtures (Fig. 5*F*). However, LCFA alone had little or no effect (Fig. 5*D*). It was previously suggested that LET-767 might produce steroid hormones, such as testosterone (19). To check whether steroids might also be important for *let-767* (*RNAi*) developmental arrest, we supplemented the worms with testosterone alone or in combination with fatty acids. However, we could not detect any additional effect (data not shown). Thus, the rescue experiments pointed out that the deficiency of mmBCFA was most important for *let-767* (*RNAi*) developmental arrest, although LET-767 is also involved in the production of LCFA.

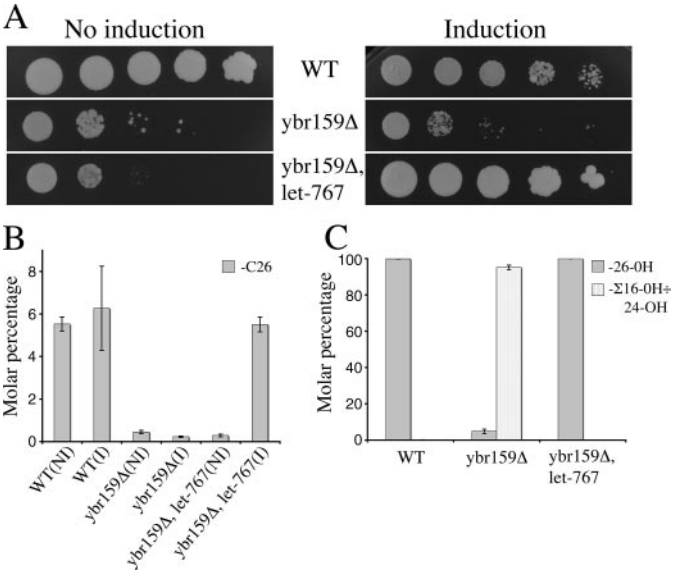

FIGURE 6. **Rescue of *ybr159*Δ by expression of *let-767*.** *A*, growth of *ybr159*Δ at 37 °C is rescued upon expression of *let-767*. *B*, molar percentage of C26 fatty acids from total fatty acids. *C*, relative distribution of hydroxylated fatty acids. *NI*, noninduced; *I*, induced; *WT*, wild type.

To independently confirm that the deficiency of mmBCFA and LCFA was critical for the observed phenotype in *let-767* (*RNAi*) worms, we used a genetically isolated mutant of the *let-767* (*s2819*) allele which, similarly to *let-767* (*RNAi*) animals, arrested as L1 larva. To obtain homozygous *let-767* (*s2819*), we produced a strain carrying LET-767::GFP in the mutation background (see "Experimental Procedures"). Because the *let-767*::*GFP* rescuing construct was not stably integrated, it was only inherited by a fraction of the total progeny. Thus, *let-767* (*s2819*) homozygous animals were identified based on the absence of LET-767::GFP fluorescence and arrested as L1 (Fig. 5*G*). Similar to the *let-767* (*RNAi*) rescue experiments, mmBCFA mixed with triglycerides triolein and trilinolenin could rescue *let-767* (*s2819*) worms (Fig. 5*H*). However, the rescued worms could not develop into normal adults. The grown up worms had distorted morphology and could not produce progeny. In general, *let-767* (*RNAi*) worms were easier to rescue than *let-767* (*s2819*) animals, probably because of the hypomorphic nature of RNAi knockdown.

Taken together, rescue of *let-767* developmental arrest by fatty acids and high homology of LET-767 to known 3-ketoacyl-CoA reductases indicated that this enzyme is involved in the synthesis of both mmBCFA and LCFA. The rescue of *let-767* (*RNAi*) developmental arrest by mmBCFA alone depicts their importance for development.

LET-767 Fully Rescues 3-Ketoacyl-CoA Reductase Deficiency in S. cerevisiae—YBR159W is a close homolog of LET-767 in yeast (Fig. 1) and is a major 3-ketoreductase of the elongase system required for very long chain fatty acid synthesis (20, 23). The *ybr159*Δ mutant is viable, albeit with strongly impaired growth rate, especially at higher temperatures (*e.g.* 37 °C) (23) (Fig. 6*A*). It lacks fatty acid with 26 carbon atoms and accumulates hydroxyacyl elongation intermediates (23) (Fig. 6, *B* and *C*). If LET-767 has a 3-ketoacyl-CoA reductase activity, its heterologous expression in *ybr159*Δ might reduce the mutant traits. Strikingly, we observed that LET-767 expression completely restored *ybr159*Δ growth (Fig. 6*A* and supplemental Fig. 5). The content of C26 LCFA and the distribution of hydroxylated fatty acids in the rescued lines were undistinguishable from the wild type controls (Fig. 6, *B* and *C*), indicating that LET-767 can fully replace YBR159W. These results strongly suggest that, similar to YBR159W, LET-767 is a genuine 3-ketoacyl-CoA reductase.

let-767 (RNAi) Affects Sphingolipid Profile in Worms—Considering LET-767 activity, we anticipated that *let-767* (*RNAi*) should also affect the biosynthesis of other lipid classes containing mmBCFA and LCFA. Sphingolipids in *C. elegans* contain a single branched chain C17 sphingoid base, 15-methyl-2-aminohexadec-4-en-1,3-diol (50). Conceivably, *iso*-15:0 mmBCFA, produced both by ELO-5 and LET-767, is the precursor of this sphingoid base. We analyzed by MS/MS ceramides and glycosylceramides in extracts from *let-767* (*RNAi*) and *elo-5* (*RNAi*) (Fig. 7). The C17 sphingoid base containing ceramides showed a significant decrease in both *let-767* (*RNAi*) and *elo-5* (*RNAi*) (Fig. 7*A*), whereas the content of glycosylceramides with C17 sphingoid base was slightly decreased only in *elo-5* (*RNAi*) (Fig. 7*A*). Interestingly, in both RNAi extracts we could detect ceramide species containing a sphingoid base with a C16 chain (Fig. 7*C*; MS/MS data of *elo-5* (*RNAi*) not shown).

In cerebrosides from *let-767* (*RNAi*), we identified a new hydroxylated sphingoid base (Fig. 7*D*), which was not detected in the wild type control. The identification of a new sphingoid base in the extract from *let-767* (*RNAi*) supports the notion that LET-767 is involved in the synthesis of mmBCFA because these bases could emerge as side products synthesized in the absence of mmBCFA (for C16 base) and when LET-767 substrate accumulated (for hydroxy C17 base).

DISCUSSION

Fatty acids are common building blocks in complex lipids, and their chain length, saturation, and hydroxylation affect the molecular properties of produced lipids, thus determining their structural and signaling roles. In addition, free fatty acids alone can act as signaling molecules, although in the form of TAGs, they are used for energy storage. Therefore, the study of fatty acids biosynthesis facilitates our understanding of their functional role. Several genes involved in fatty acid synthesis have been identified and functionally characterized in *C. elegans* (11, 43, 51–53). Most probably, in *C. elegans*, short chain fatty acids (up to 16C) are synthesized *de novo* by a canonical type I system that consists of a megaenzyme- fatty-acid synthase (*fasn*) and acetyl-CoA carboxylase (*pod-2*) (51). Furthermore, fatty acids with longer chains are produced by several specific elongases (*elo* genes) required for the initial condensation step (*reaction 1* in Fig. 1*B*). Here we propose that the consequent step of reduction of the 3-ketoacyl-CoA (*reaction 2* in Fig. 1*B*) is carried out by the short chain dehydrogenase LET-767. Several lines of evidence support the notion that LET-767 is the 3-ketoacyl Co reductase as follows: (i) it is homologous to both budding yeast YBR159W and human KAR; (ii) total decrease in the amount of fatty acids stored in the form of TAGs in *let-767* (*RNAi*); (iii) rescue of *let-767* (*RNAi*) and *let-767* (*s2819*) by exogenously added TAGs and fatty acids; (iv) identification of a hydroxy-

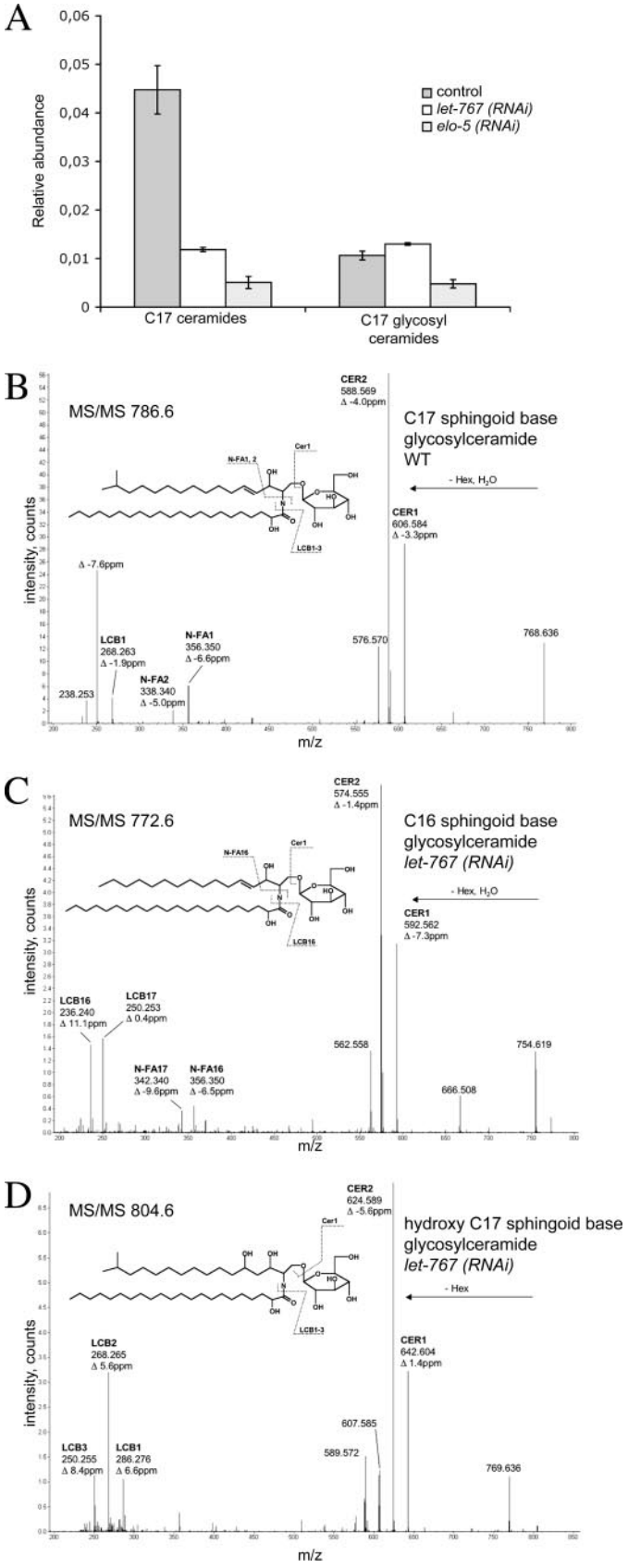

FIGURE 7. **Mass spectrometric analyses of ceramides and glycosylceramides in *let-767* (*RNAi*) and *elo-5* (*RNAi*).** *A*, relative abundance of ceramides and glycosylceramides with branched chain C17 sphingoid base 15-methyl-2-aminohexadec-4-en-1,3-diol. Peak intensities were normalized to the sum of peak areas of all phosphatidylethanolamines and phosphatidylcholines species detected in the same lipid extract (see supplemental Fig. 4). *B*, MS/MS spectrum of a glycosylceramide precursor with *m/z* 786.6 having a C17 sphingoid base, which was isolated from wild type worms (*inset*, chemical structure of the glycosylceramide with assigned fragment ions for the C17 sphingoid base- *LCB*, ceramide– *Cer* and N-amide– *N-FA*); Δ stands for the difference between the measured and calculated masses in ppm. *C*, MS/MS spectrum of a glycosylceramide precursor with *m/z* 772.6 that contained a C16 sphingoid base and was isolated from *let-767* (*RNAi*) (*inset*, chemical structure C16 sphingoid base glycosylceramide with assigned fragment ions for the C16 sphingoid base, *LCB16*, and the corresponding fragment containing acidamide, *N-FA16*). Accordingly, assignments for the isobaric C17 sphingoid base containing glycosylceramide are shown. *D*, MS/MS spectrum of 15-methyl-2-aminohexadec-4-en-1,3,5-triol C17 sphingoid base containing glycosylceramide (*m/z* 804.6) detected in *let-767* (*RNAi*) (an *inset*, chemical structure of the glycosylceramide with fragment ions of 3-OH C17 sphingoid base, *LCB1–3*). MS/MS spectra in *B–D* were acquired in positive ion mode.

lated sphingoid base in *let-767* (*RNAi*), similar to 3-OH acyl intermediates accumulated in the yeast 3-ketoacyl-CoA reductase mutant *ybr159*Δ; and finally, (v) full complementation of fatty acid production in the yeast mutant *ybr159*Δ. We could not exclude that, in addition to 3-ketoacyl-CoA reductase activity, LET-767 might also be involved in another activity (*e.g.* production of steroids proposed earlier (18, 19)), which, however, does not lead to the developmental arrest caused by depletion of LET-767, because addition of fatty acids is sufficient to overcome it.

Specificity in the synthesis of fatty acids is usually achieved at the level of elongases (27). Accordingly, in *C. elegans*, ELO-1 and ELO-2 are involved in the elongation of long chain saturated and polyunsaturated fatty acids (10, 54) and ELO-5 and ELO-6 in the elongation of mmBCFA (43). We propose that LET-767 can work in the process of elongation of both even-numbered LCFA and mmBCFA (Fig. 8). Thus, upon depletion of LET-767 by RNAi, the content of both LCFA and mmBCFA was reduced as monitored by mass spectrometric analysis of stored TAGs. The involvement of LET-767 in the production of LCFA is supported by the observed increase in abundance of LCFA precursors 14:0, 16:0, and 16:1 and an overall decrease in the index long fatty acid/short fatty acid. Further evidence comes from the expression of *let-767* in yeast *ybr159*Δ deficient in the elongation of fatty acids. Expression of LET-767 completely restored C26 synthesis and eliminated the hydroxylated intermediates accumulated in the mutant. The mmBCFA in *let-767* (*RNAi*) was most severely affected, as could be shown from the relative distribution of these fatty acids in the TAGs. However, complementation of *let-767* (*RNAi*) with LCFA and mmBCFA allowed us to differentiate the role of these two classes of fatty acids in the developmental arrest caused by LET-767 depletion. Interestingly, blocking the synthesis of either LCFA or mmBCFA by interfering with both *elo-1* and *elo-2* or *elo-5*, respectively, leads to developmental arrest in *C. elegans* (10, 43). We note that, although adding LCFA alone had little or no effect in complementing the arrest caused by *let-767* depletion, it enhanced the rescue effect of mmBCFA. Nevertheless, supplementation with mmBCFA alone effectively rescued the *let-767* (*RNAi*) phenotype. Therefore, an insufficient amount of mmBCFA is most critical for the development when LET-767 is depleted.

LET-767 is present in a number of cells with the strongest expression in the intestine. This underlines the importance of

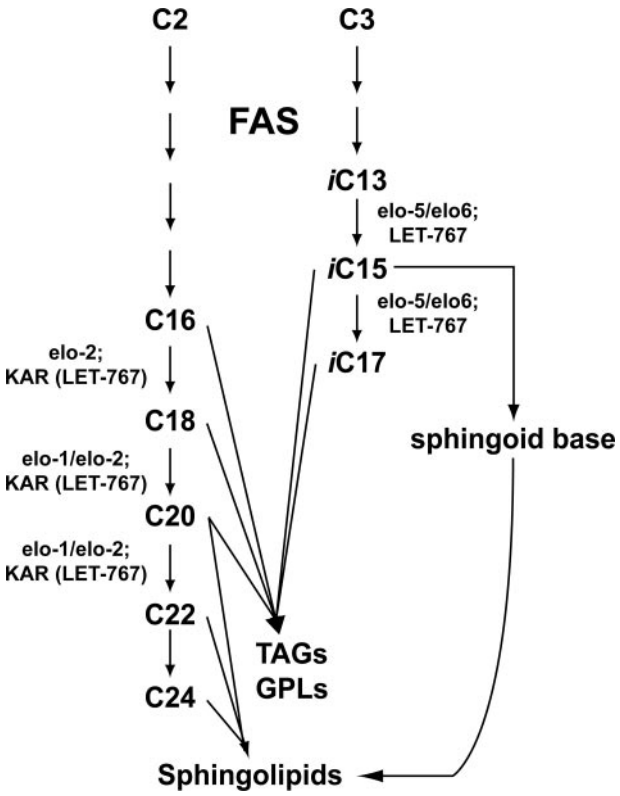

FIGURE 8. **Elongation of fatty acids in *C. elegans*.** The steps in which LET-767 is required are indicated.

intestinal cells for the production of storage lipids. The *C. elegans* genome encodes for at least four other paralogs of LET-767 (C06B3.5, C06B3.4, F11A5.12, and F25G6.5, Fig. 1), which also might possess 3-ketoacyl-CoA reductase activity. In contrast to *let-767*, these four short chain dehydrogenases/reductases are expressed at very low levels (55, 56), and their RNAi has no pronounced effect. LET-767 is important for the synthesis of the bulk of mmBCFA and LCFA, and other KARs might have more specific functions in *C. elegans*. For example, microarray studies suggest that two of them, C06B3.4 and F11A5.12, act downstream of *daf-16* and, therefore, might be important for the regulation of the life span in *C. elegans* (57).

Developmental arrest in *let-767* (*RNAi*) is probably because of effects on lipids containing, or produced from, mmBCFA and LCFA (*e.g.* TAGs, phospholipids, and sphingolipids). We found that the total quantity and molecular composition of TAGs were severely altered in *let-767* (*RNAi*). Therefore, the deficiency of storage lipids could be one of the reasons for the developmental arrest in *let-767* (*RNAi*). Stalling of fatty acid synthesis in *let-767* (*RNAi*) also severely affects the worm sphingolipids. The reduced content of ceramides with *iso*-C17 branched sphingoid base and the appearance of unusual ceramides in *let-767* (*RNAi*), must also be important for the developmental arrest. The C16 sphingoid base found in *let-767* (*RNAi*) and *elo-5* (*RNAi*) is probably produced from myristoyl-CoA, if the amount of *iso*15-co-A is not sufficient. An unusual ceramide with a hydroxylated sphingoid base appeared only in *let-767* (*RNAi*). Appearance of this base might be similar to the accumulation of 3-OH fatty acids in yeast mutants of LET-767 homolog YBR159W (23). Synthesis of this aberrant ceramide in worms can be explained by the accumulation of the LET-767 substrate during the elongation of mmBCFA, a 3-keto isopentadecanoyl-CoA, which probably is further used by a serine palmitoyltransferase. Taken together, the developmental arrest of *let-767* (*RNAi*) larvae originates from both perturbed energy storage, insufficient quantities, and the altered molecular profile of basic cellular lipids, such as sphingolipids.

The usual way to study the activity of an enzyme with unknown function involves *in vitro* experiments, where the purified enzyme is reacted with different substrates followed by the identification and quantification of the products. Alternatively, we took an unbiased approach where, by complementing RNAi of *let-767* with lipophilic extracts from nontreated worms, we were able to identify mmBCFA and LCFA as the crucial downstream products of this enzyme. Later, we confirmed the 3-ketoacyl-CoA reductase activity of LET-767 by expressing and fully rescuing the yeast 3-ketoacyl-CoA reductase mutant *ybr159*Δ. One of the advantages of the complementation approach is that it not only provides the information on the product (function) of the investigated enzyme, but it also allows the identification of the metabolic pathway, in which the enzyme is involved. In fact, several classes of small molecules could be identified by this approach as follows: (i) direct product(s) of the enzyme, (ii) molecules that are downstream in a biosynthetic pathway, and (iii) indirect activators of the pathway. However, several issues should be considered when analyzing rescue experiment outcomes as follows: the availability and stability of the direct products of the enzyme, and the possibility that it is multifunctional and produces several molecules with different chemical properties. For instance, as a 3-ketoacyl-CoA reductase LET-767 should produce 3-hydroxyacyl-CoA, whereas mmBCFA are downstream final products. Because of the instability of the intermediate-CoA compounds, lipophilic extracts contain very low amounts, and the rescue is achieved mostly by the final product of the pathway. The complementation approach could become a powerful tool for studying metabolic enzymes and pathways because, in recent years, sheer functional data on morphological phenotypes, caused by either RNAi or deletion mutants of *C. elegans* genes, have been accumulated (see Wormbase website). It could be particularly fruitful to combine RNAi with emerging metabolomics technologies. Because of the recent progress in high-throughput analytical screening (58) the complementation approach can be applied in a high-through put genome-wide analysis providing a direct link between mutations, metabolites, and their regulation and function.

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REFERENCES

1. Ashrafi, K., Chang, F. Y., Watts, J. L., Fraser, A. G., Kamath, R. S., Ahringer, J., and Ruvkun, G. (2003) *Nature* **421,** 268–272
2. Entchev, E. V., and Kurzchalia, T. V. (2005) *Semin. Cell Dev. Biol.* **16,** 175–182
3. Kurzchalia, T. V., and Ward, S. (2003) *Nat. Cell Biol.* **5,** 684–688
4. McKay, R. M., McKay, J. P., Avery, L., and Graff, J. M. (2003) *Dev. Cell* **4,** 131–142
5. The *Caenorhabditis elegans* Sequencing Consortium (1998) *Science* **282,** 2012–2018
6. Sluder, A. E., and Maina, C. V. (2001) *Trends Genet.* **17,** 206–213
7. Van Gilst, M., Gissendanner, C. R., and Sluder, A. E. (2002) *Crit. Rev. Eukaryotic Gene Expression* **12,** 65–88
8. Nelson, D. R. (1999) *Arch. Biochem. Biophys.* **369,** 1–10
9. Kallberg, Y., Oppermann, U., Jornvall, H., and Persson, B. (2002) *Protein Sci.* **11,** 636–641
10. Kniazeva, M., Sieber, M., McCauley, S., Zhang, K., Watts, J. L., and Han, M. (2003) *Genetics* **163,** 159–169
11. Watts, J. L., and Browse, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 5854–5859
12. Gill, M. S., Held, J. M., Fisher, A. L., Gibson, B. W., and Lithgow, G. J. (2004) *Aging Cell* **3,** 413–421
13. Held, J. M., White, M. P., Fisher, A. L., Gibson, B. W., Lithgow, G. J., and Gill, M. S. (2006) *Aging Cell* **5,** 283–291
14. Motola, D. L., Cummins, C. L., Rottiers, V., Sharma, K. K., Li, T., Li, Y., Suino-Powell, K., Xu, H. E., Auchus, R. J., Antebi, A., and Mangelsdorf, D. J. (2006) *Cell* **124,** 1209–1223
15. Matyash, V., Entchev, E. V., Mende, F., Wilsch-Brauninger, M., Thiele, C., Schmidt, A. W., Knolker, H. J., Ward, S., and Kurzchalia, T. V. (2004) *Plos Biol.* **2,** e280
16. Stewart, H. I., O'Neil, N. J., Janke, D. L., Franz, N. W., Chamberlin, H. M., Howell, A. M., Gilchrist, E. J., Ha, T. T., Kuervers, L. M., Vatcher, G. P., Danielson, J. L., and Baillie, D. L. (1998) *Mol. Gen. Genet.* **260,** 280–288
17. Geissler, W. M., Davis, D. L., Wu, L., Bradshaw, K. D., Patel, S., Mendonca, B. B., Elliston, K. O., Wilson, J. D., Russell, D. W., and Andersson, S. (1994) *Nat. Genet.* **7,** 34–39
18. Kuervers, L. M., Jones, C. L., O'Neil, N. J., and Baillie, D. L. (2003) *Mol. Genet. Genomics* **270,** 121–131
19. Desnoyers, S., Blanchard, P. G., St-Laurent, J. F., Gagnon, S. N., Baillie, D. L., and Luu-The, V. (2007) *J. Endocrinol.* **195,** 271–279
20. Beaudoin, F., Gable, K., Sayanova, O., Dunn, T., and Napier, J. A. (2002) *J. Biol. Chem.* **277,** 11481–11488
21. Rossler, H., Rieck, C., Delong, T., Hoja, U., and Schweizer, E. (2003) *Mol. Genet. Genomics* **269,** 290–298
22. Moon, Y. A., and Horton, J. D. (2003) *J. Biol. Chem.* **278,** 7335–7343
23. Han, G., Gable, K., Kohlwein, S. D., Beaudoin, F., Napier, J. A., and Dunn, T. M. (2002) *J. Biol. Chem.* **277,** 35440–35449
24. Xu, X., Dietrich, C. R., Delledonne, M., Xia, Y., Wen, T. J., Robertson, D. S., Nikolau, B. J., and Schnable, P. S. (1997) *Plant Physiol.* **115,** 501–510
25. Xu, X., Dietrich, C. R., Lessire, R., Nikolau, B. J., and Schnable, P. S. (2002) *Plant Physiol.* **128,** 924–934
26. Puyaubert, J., Dieryck, W., Costaglioli, P., Chevalier, S., Breton, A., and Lessire, R. (2005) *Biochim. Biophys. Acta* **1687,** 152–163
27. Leonard, A. E., Pereira, S. L., Sprecher, H., and Huang, Y. S. (2004) *Prog. Lipid Res.* **43,** 36–54
28. Athenstaedt, K., and Daum, G. (2000) *J. Biol. Chem.* **275,** 235–240
29. Vico, P., Cauet, G., Rose, K., Lathe, R., and Degryse, E. (2002) *Yeast* **19,** 873–886
30. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) *Nucleic Acids Res.* **31,** 3497–3500
31. Felsenstein, J. (1989) *Cladistics* **5,** 164–166
32. Wilm, T., Demel, P., Koop, H. U., Schnabel, H., and Schnabel, R. (1999) *Gene* (*Amst.*) **229,** 31–35
33. Maduro, M., and Pilgrim, D. (1995) *Genetics* **141,** 977–988
34. Zhang, Y., Muyrers, J. P., Testa, G., and Stewart, A. F. (2000) *Nat. Biotechnol.* **18,** 1314–1317
35. Timmons, L., and Fire, A. (1998) *Nature* **395,** 854
36. Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., and Ahringer, J. (2003) *Nature* **421,** 231–237
37. Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000) *Nature* **408,** 325–330
38. Thomas, B. J., and Rothstein, R. (1989) *Cell* **56,** 619–630
39. Oelschlaegel, T., Schwickart, M., Matos, J., Bogdanova, A., Camasses, A., Havlis, J., Shevchenko, A., and Zachariae, W. (2005) *Cell* **120,** 773–788
40. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
41. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497–509
42. Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., and Ristow, M. (2007) *Cell Metab.* **6,** 280–293
43. Kniazeva, M., Crawford, Q. T., Seiber, M., Wang, C. Y., and Han, M. (2004) *Plos Biol.* **2,** E257
44. Ekroos, K., and Shevchenko, A. (2002) *Rapid Commun. Mass Spectrom.* **16,** 1254–1255
45. Schwudke, D., Oegema, J., Burton, L., Entchev, E., Hannich, J. T., Ejsing, C. S., Kurzchalia, T., and Shevchenko, A. (2006) *Anal. Chem.* **78,** 585–595
46. Brugger, B., Erben, G., Sandhoff, R., Wieland, F. T., and Lehmann, W. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2339–2344
47. Voet, D., Voet, J. G., and Pratt, C. W. (2006) *Fundamentals of Biochemistry*, 2nd Ed., pp. 627–681, John Wiley & Sons, Inc., New York
48. Kaneda, T. (1991) *Microbiol. Rev.* **55,** 288–302
49. Ekroos, K., Chernushevich, I. V., Simons, K., and Shevchenko, A. (2002) *Anal. Chem.* **74,** 941–949
50. Chitwood, D. J., Lusby, W. R., Thompson, M. J., Kochansky, J. P., and Howarth, O. W. (1995) *Lipids* **30,** 567–573
51. Rappleye, C. A., Tagawa, A., Le Bot, N., Ahringer, J., and Aroian, R. V. (2003) *BMC Dev. Biol.* **3,** 8
52. Brock, T. J., Browse, J., and Watts, J. L. (2006) *Plos Genet.* **2,** e108
53. Lesa, G. M., Palfreyman, M., Hall, D. H., Clandinin, M. T., Rudolph, C., Jorgensen, E. M., and Schiavo, G. (2003) *J. Cell Sci.* **116,** 4965–4975
54. Beaudoin, F., Michaelson, L. V., Hey, S. J., Lewis, M. J., Shewry, P. R., Sayanova, O., and Napier, J. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6421–6426
55. Baugh, L. R., Hill, A. A., Claggett, J. M., Hill-Harfe, K., Wen, J. C., Slonim, D. K., Brown, E. L., and Hunter, C. P. (2005) *Development* (*Camb.*) **132,** 1843–1854
56. Hill, A. A., Hunter, C. P., Tsung, B. T., Tucker-Kellogg, G., and Brown, E. L. (2000) *Science* **290,** 809–812
57. Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H., and Kenyon, C. (2003) *Nature* **424,** 277–283
58. Schwudke, D., Hannich, J. T., Surendranath, V., Grimard, V., Moehring, T., Burton, L., Kurzchalia, T., and Shevchenko, A. (2007) *Anal. Chem.* **79,** 4083–4093