Published in final edited form as:

J Mol Biol. 2011 August 19; 411(3): 537–553. doi:10.1016/j.jmb.2011.06.011.

Regulation of Fat Storage and Reproduction by Krüppel-Like Transcription Factor KLF3 and Fat-Associated Genes in *Caenorhabditis elegans*

Jun Zhang¹, Razan Bakheet¹, Ranjit S. Parhar¹, Cheng-Han Huang², M. Mahmood Hussain³, Xiaoyue Pan³, Shahid S. Siddiqui⁴, and Sarwar Hashmi^{1,*}

¹Laboratory of Developmental Biology, Lindsley F. Kimball Research Institute, New York Blood Center, 310 East 67th Street, New York, NY 10065, USA

²Laboratory of Biochemistry and Molecular Genetics, Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA

³Department of Anatomy and Cell Biology, SUNY Downstate Medical Center, Brooklyn, NY, USA

⁴Section of Hematology/Oncology, Department of Medicine, University of Chicago Medical Center, Pritzker School of Medicine, Chicago, IL, USA

Abstract

Coordinated regulation of fat storage and utilization is essential for energy homeostasis, and its disruption is associated with metabolic syndrome and atherosclerosis in humans. Across species, Krüppel-like transcription factors (KLFs) have been identified as key components of adipogenesis. In humans, KLF14 acts as a master transregulator of adipose gene expression in type 2 diabetes and cis-acting expression quantitative trait locus associated with high-density lipoprotein cholesterol. Herein we report that, in *Caenorhabditis elegans*, mutants in *klf-3* accumulate large fat droplets rich in neutral lipids in the intestine; this lipid accumulation is associated with an increase in triglyceride levels. The *klf-3* mutants show normal pharyngeal pumping; however, they are sterile or semisterile. We explored important genetic interactions of *klf-3* with the genes encoding enzymes involved in fatty acid (FA) β -oxidation in mitochondria or peroxisomes and FA synthesis in the cytosol, namely acyl-CoA synthetase (*acs-1* and *acs-2*), acyl-CoA oxidase (*F08A8.1* and *F08A8.2*), and stearyl-CoA desaturase (*fat-7*). We show that mutations or RNA interference in these genes increases fat deposits in the intestine of *acs-1*, *acs-2*, *F08A8.1*, and *F08A8.2* animals. We further show that *acs-1* and *F08A8.1* influence larval development and fertility, respectively. Thus, KLF3 may regulate FA utilization in the intestine and reproductive tissue. We demonstrate that depletion of *F08A8.1* activity, but not of *acs-1*, *acs-2*, *F08A8.2*, or *fat-7* activity, enhances the fat phenotype of the *klf-3* mutant. Taken together, these results suggest that *klf-3* regulates lipid metabolism, along with *acs-1*, *acs-2*, *F08A8.1*, and *F08A8.2*, by promoting FA β -oxidation and, in parallel, may contribute to normal reproductive behavior and fecundity in *C.elegans*.

Keywords

β -oxidation; *C. elegans*; genetic interaction; Krüppel-like transcription factors; lipid metabolism

Introduction

Energy is required for living organisms to maintain homeostasis. In vertebrates, adipose tissue is the major site for storing energy in the form of fat or triglycerides (TGs). Free fatty acids (FAs) are taken up by adipocytes and stored as TGs, involving anabolic processes that are tightly controlled and dynamically balanced with energy consumption. The ability of metazoan animals to regulate fat storage is essential for their growth, development, reproduction, and environmental adaptation, and its deregulation can lead to cellular dysfunction.^{1–3} Due to its homeostatic nature, energy storage is coordinately regulated through signaling networks that integrate the biochemical pathways of fat deposition, mobilization, and utilization.⁴ Upon starvation, lipids stored in adipocytes are hydrolyzed into FAs and sent to other tissues, where FAs are taken up and converted into fatty acyl-CoAs. Breakdown of fatty acyl-CoAs, especially long-chain FAs, to yield acetyl-CoA and ATP through β -oxidation in mitochondria or peroxisomes is a key process of energy production.⁵ Mutations in the genes that encode the enzymes of this system underlie a range of lipid metabolic disorders.

Recently, there has been an increasing interest in understanding the role of mammalian Krüppel-like transcription factors (KLFs) in adipogenesis; 17 KLFs, which govern vital developmental and physiological processes, have been reported.^{6,7} A combination of cellular and knockout studies has identified KLF2, KLF3, KLF4, KLF5, KLF6, KLF7, KLF11, and KLF15 as crucial regulators of preadipocyte differentiation in mammals.^{8–12} In an exciting new discovery, the maternally expressed KLF14, which is associated with type 2 diabetes and the cis-acting expression quantitative trait locus of high-density lipoprotein cholesterol, is shown to act as a master transregulator of adipose gene expression.¹³ Because lipid metabolism, especially FA β -oxidation, is central to organismal energy homeostasis, understanding how its deregulation is linked to common disorders such as diabetes, obesity, and atherosclerosis constitutes an important field of biomedical investigations.

We have studied the functional role of KLFs in *Caenorhabditis elegans*, whose genome encodes three members—*klf-1* (F56F11.3), *klf-2* (F53F8.1), and *klf-3* (F54H5.4)—that contain three highly conserved C₂H₂ zinc fingers. These KLFs share the highest identity with members of mammalian KLFs in their C-terminal C₂H₂ zinc fingers and share little homology in their N-terminal regions. *klf-1* and *klf-3* are highly expressed during larval and adult development, and this expression is mainly observed in the intestine,^{14–16} a major endocrine system positioned close to sexual organs and engaged in nutrient sensing and energy metabolism.¹⁷ Depletion of *klf-1* activity by RNA interference (RNAi)^{14,16} and deletion of a ~1.6-kb segment of *klf-3(ok1975)* cause intestinal fat accumulation, along with a failure in reproduction.¹⁴ Suppression of *klf-3* by RNAi causes similar effects as its deletion mutant (Fig. 5j, Table 1). However, it remains unknown how this genomic lesion

causes the two phenotypes and whether the reproductive defects occur subsequent to metabolic perturbation or by directly affecting germline proliferation and/or development.

Reproduction is an energy-intensive process that is affected by the availability and metabolism of essential nutrients, including lipids. We showed previously that the expression of several genes key to FA metabolism (*acs-1*, *acs-2*, *F08A8.1*, and *F08A8.2*, involving mitochondrial/peroxisomal β -oxidation, and *fat-7*, a desaturase gene) was significantly altered in *klf-3(ok1975)* mutants.¹⁵ Thus, we hypothesize that the excessive fat deposits and severe fertility defects observed in *klf-3* mutants result from accumulative perturbation of FA metabolism, which causes gradual damage to reproduction in the course of worm development.

In this article, we have examined the hypothesis by characterizing the phenotypes of *klf-3* mutants through biochemical, genetic, and morphological analyses. We identified key components of the FA β -oxidation pathway as molecular targets regulated by *klf-3* and investigated their role in fat accumulation and reproduction. Significantly, we have uncovered a strong genetic interaction of *klf-3* with several genes encoding fat metabolic enzymes: acyl-CoA synthetase (*acs-1* and *acs-2*), acyl-CoA oxidase (*F08A8.1* and *F08A8.2*), and stearyl-CoA desaturase (SCD; *fat-7*). We show that inhibition of *acs-1*, an FA β -oxidation enzyme, by RNAi causes fat accumulation and results in embryonic lethality and early larval arrest. In contrast, the mutations in *acs-2* and *F08A8.1*, which reside in peroxisomes and mitochondria, respectively, lead to elevated levels of fat accumulation, while the mutation in *fat-7* causes alteration in FA composition. Using intercrossed double mutants or performing RNAi, our studies further reveal that depletion of *F08A8.1* activity, but not of *acs-1*, *acs-2*, *F08A8.2*, or *fat-7* activity, enhances the fat phenotype of the *klf-3* mutant. Our finding that *klf-3* interacts with *acs-1*, *acs-2*, *F08A8.1*, *F08A8.2*, and *fat-7* to modulate FA β -oxidation provides a novel insight for understanding obesity and relates obesity to reproductive abnormalities in vertebrates.

Results

The tissue specificity of *C. elegans klf-3* isoforms

Two nearly identical isoforms of *klf-3*—*klf-3a* and *klf-3b*—have been identified.¹⁵ Their protein products differ by 8 amino acids in their N-terminus but share the 301 C-terminal amino acids encoded by common exons[†]. A previous study of *klf-3* has described its expression and mutant phenotypes. Reporter gene studies showed that a 1-kb segment 5' terminal to the *klf-3a* ATG start codon directs a very strong intestinal expression.¹⁵ To determine the *in vivo* tissue expression of *klf-3b*, we established transgenic lines carrying the *klf-3b::gfp* fusion gene, which was driven by the cognate promoter, a 2.0-kb genomic sequence upstream of the first ATG codon (pHZ145; Fig. 1a). Similar to *klf-3a*, the *klf-3b* isoform is also expressed in the intestine during all larval and adult stages (Fig. 1c and d). These data suggested that the regulatory regions controlling the expression of both *klf-3a* and *klf-3b* isoforms reside in 2 kb of upstream genomic sequence from the 5'-terminus to

[†]<http://www.wormbase.org>

klf-3a. Due to their similar expression pattern and only a minor difference in the N-terminus, the proteins products of *klf-3a* and *klf-3b* could mediate potentially similar functions in fat storage.

The morphology of mitochondria and peroxisomes is maintained in the *klf-3* mutant

klf-3 protein expression is absent from both the mitochondria and the peroxisomes of intestinal cells (data not shown). Because the *klf-3* target proteins involved in FA β -oxidation reside and function in the mitochondria and/or peroxisomes, we have used electron microscopic analysis to investigate if the structure of these organelles was disrupted in the *klf-3(ok1975)* mutant. We prepared 75–100 ultrathin sections (65–70 nm) of a synchronized population of L4s or young adult *klf-3(ok1975)* and, for comparison, age-matched wild-type animals that, after processing, were observed under an electron microscope. We found that the morphology of the mitochondria and peroxisomes in *klf-3(ok1975)* mutant worms is indistinguishable at the ultrastructural level from that in wild type, suggesting that the *klf-3(ok1975)* mutation apparently does not alter the morphology of these organelles (Fig. 2a and b).

***klf-3* mutants exhibit normal dietary FA uptake but reduced catabolism**

C. elegans feeds on bacteria, takes up nutrients through pharyngeal pumping, and moves freely on nematode growth medium (NGM) seeded with OP50 (*Escherichia coli* strain) agar plate. We found that the locomotion span of *klf-3(ok1975)* mutant animals on the NGM OP50 plate, as well as their pharyngeal pumping, is quite comparable to wild-type animals. We measured the rate of pharyngeal pumping on 25 animals of each genotype and found that both wild-type and *klf-3(ok1975)* mutant animals pump pharyngeal muscles ~210 times per minute, whereas the wild-type N2 animals pump >200 times per minute.¹⁸ Thus, there is no apparent difference in pharyngeal pumping between the wild type and the *klf-3(ok1975)* mutant. However, a distinguishing feature of *klf-3(ok1975)* mutants is the presence of large lipid droplets in intestinal cells, which may be attributed to abnormal peroxisomal β -oxidation,¹⁹ resulting in the fat accumulation phenotype. Whether or not the excess deposition of fat in the intestine was due to increased food uptake and reduced catabolism, we assayed the nutrient uptake by following a fluorescent lipid dye accumulation protocol utilized in *C. elegans*,^{20,21} which allowed a comparison of the amounts of FA intake. Using fluorescence measurements, we determined the uptake and accumulation of BODIPY in the intestine of animals fed C1-BODIPY-C12, which is incorporated into endogenous fat, transported from the intestine to oocytes, and stains early embryos in the uterus.²²

The *klf-3(ok1975)* mutant animals and, for comparison, the wild-type animals were fed equal amounts of OP50 incorporated into BODIPY dye. After 10 min or 15 min of feeding, the animals were observed for fluorescence signal under a Zeiss Axioplan 2 fluorescence microscope, and the images of fat accumulation were recorded for a comparison of fat deposits between wild type and *klf-3(ok1975)* mutants. We found that *klf-3(ok1975)* mutants fed for 10 min or 15 min accumulated many more fat droplets, and these were also larger in size (Fig. 3d) than the fat droplets seen in the wild type (Fig. 3b) during the same feeding interval. The fluorescence signals weakened in wild-type animals, apparently as a result of FA oxidation, but the signal was retained in the mutant animals after 20 min of first

exposure. Thus, our data suggest that the high fat accumulation in *klf-3(ok1975)* mutants may be partly attributed to increased absorption and increased storage of FAs in intestinal cells. The presence of large lipid droplets in intestinal cells is not a consequence of altered feeding behavior or increased rate of dietary FA uptake, but these data imply that FA catabolism through β -oxidation could be altered in the *klf-3(ok1975)* mutants.

Total lipid profile demonstrates that the *klf-3(ok1975)* mutant exhibits increased levels of TGs

Next, we wanted to determine the type of lipids that show increased accumulation in *klf-3(ok1975)* mutants during development. We measured the total lipids in three major categories—TGs, phospholipids, and cholesterol (total lipids and corresponding esters)—in the synchronized larval and adult stages of *klf-3(ok1975)* mutants and compared them with the age-matched synchronized larval and adult stages of wild-type animals in two independent experiments. We found that the *klf-3(ok1975)* mutants at larval stage 4 and the adults contained a much higher level of TGs (~1.5-fold and ~2-fold increase, respectively) when compared to the similarly staged wild-type animals (Fig. 4a). The level of TGs continuously increased during larval development and reached its highest level in adult worms in the *klf-3(ok1975)* mutant animals. In contrast, no apparent difference in cholesterol (Fig. 4b), phospholipids (Fig. 4c), and cholesterol esters (Fig. 4d) was observed between wild-type worms and *klf-3(ok1975)* mutant worms. These results strongly indicate the specificity of TG accumulation in the *klf-3(ok1975)* mutants, with no significant alteration in the accumulation of phospholipids and cholesterol, and demonstrate that TGs are the major type of lipid stored in the mutants. These data also suggest that Oil Red O staining can be used as a simple and convenient marker for TG levels in the intestinal cells of *C. elegans*.

***klf-3* and *F08A8.1/F08A8.2* genes are likely to act in the same genetic pathway**

To investigate whether the *klf-3* and *F08A8.1* genes interact genetically, we characterized the *F08A8.1(ok2257)* mutant, which has a 1064-bp deletion that removes a portion of exon 3 to approximately half of exon 5 in the *F08A8.1* gene.¹ We studied 80 *F08A8.1(ok2257)* mutant animals and found that they showed normal growth and development profiles but produced fewer progeny than the wild-type animals. Moreover, *F08A8.1* mutant animals display a ~45% increase in fat accumulation in the intestine as compared to the wild type (Fig. 5a, b, and j). Therefore, the fat accumulation and reproductive defects are the shared phenotypes common to the *klf-3(ok1975)* and *F08A8.1(ok2257)* mutants.

We further tested genetic interactions between the deletion alleles of *klf-3(ok1975)* mutants and the deletion alleles of *F08A8.1(ok2257)* mutants. We constructed strains that were homozygous for *klf-3(ok1975)* and *F08A8.1(ok2257)* deletions, and assayed them for fat deposition phenotype in the progenies of the *klf-3(ok1975);F08A8.1(ok2257)* double mutant by Oil Red O staining. We have observed that the double mutant accumulated approximately 40% more fat (Fig. 5e and j) than either *klf-3(ok1975)* (Fig. 5d) or *F08A8.1(ok2257)* single mutants (Fig. 5b), and 85% more fat (Fig. 5j) than the wild-type animals. These phenotypes of the double mutant suggest that the *klf-3* and *F08A8.1* genes quite likely function in the same genetic pathway, and the deletion of *F08A8.1* in the

klf-3(ok1975) mutant background enhances fat accumulation in the double mutant. Since no mutations are available in the *F08A8.2* gene, we have tested the phenotype of the RNAi depletion of *F08A8.2* in wild-type animals, causing about a 20% increase in fat accumulation over wild-type animals (Fig. 5c and j). However, RNAi depletion of *F08A8.2* in the *klf-3(ok1975)* mutant background neither increased nor decreased the level of fat mass in the *F08A8.2* RNAi; *klf-3(ok1975)* mutant (Table 1); the fat mass remained the same as that of the *klf-3(ok1975)* single mutant. To test whether *F08A8.1* and *F08A8.2* both lost function to enhance the fat phenotype of the *klf-3* mutant, we injected *klf-3(ok1975)* mutant animals with double-stranded RNA (dsRNA) targeting both *F08A8.1* and *F08A8.2* transcripts. We found that RNAi depletion of both transcripts in the background of the *klf-3(ok1975)* mutant displayed a level of fat accumulation similar to that of the *F08A8.1;klf-3(ok1975)* double mutant.

We also examined *F08A8.1(ok2257)* mutant animals for their brood size to determine the role of the loss of function of *F08A8.1* in reproductive behavior (Table 1). We found that *F08A8.1* mutant adult hermaphrodites produced an average of 77 (N=86) viable progenies during their reproductive period, compared to an average of 255 (N= 60) viable progenies for wild type in the same period. We also found that, after producing 60–70 embryos, the *F08A8.1* hermaphrodites began to slow in egg-laying and eventually stopped egg-laying. These *F08A8.1* animals apparently have normal somatic gonad and germline development, but they developed egg-laying defects resulting in the accumulation of a large number of embryos in the uterus (Fig. 5f); about 15% of *F08A8.1(ok2257)* worms showed a “bag-of-worms” phenotype.²³ Although a majority (72%) of the *F08A8.1* worms still survived, about 28% (N=86) of these animals died as adults.

Next, we determined whether the loss of *F08A8.1(ok2257)* could alter the sterile or semisterile pheno-types of *klf-3(ok1975)* mutant animals. Therefore, we examined somatic gonad development, germline development, oocyte development, and overall reproduction in the *klf-3;F08A8.1* double mutant and found that, although germline proliferation, oocyte development, and somatic gonads appeared normal, the adult *F08A8.1;klf-3* animals resembled the phenotype of the *F08A8.1* single mutant, where a large number of developing embryos accumulated in the uterus (Fig. 5i). Furthermore, each of the individual double-mutant *F08A8.1(ok2257);klf-3(ok1975)* animals produced ~73 (N = 46) viable progenies as compared to the 47 progenies produced by the *klf-3(ok1975)* single-mutant animal (N=41), and none of the F1 progenies produced by the double-mutant animals was sterile (Table 1). These data suggest that *F08A8.1* mutation relieves the sterility defects of the *klf-3(ok1975)* mutant.

Since no mutation is available in the *F08A8.2* gene, we examined its loss of function by RNAi. RNAi depletion of *F08A8.2* in wild type or *klf-3(ok1975)* mutant background did not cause any significant effect on reproduction; both RNAi animals produced a normal number of progenies (Table 1). To determine whether *F08A8.1* and *F08A8.2* both function to increase overall reproduction or to suppress the phenotypes associated with reproduction in the *klf-3* mutant, we injected *F08A8.1* and *F08A8.2* dsRNA into the *klf-3(ok1975)* mutant. We found that F1 larvae from RNAi mother reached adulthood and produced an average of 63 progenies per animal (N=15) compared to about 47 progenies by *klf-3(ok1975)* single-

mutant animals (N=41) and 73 progenies by individual *F08A8.1;klf-3(ok1975)* double-mutant animals. However, 10% of embryos produced by *F08A8.1;klf-3(ok1975)* mutant animals died at ~ 100 cell stages. This suggests that suppression of the *klf-3* reproductive phenotype is enhanced in the double knockdown as compared to the genotype when activity of only one gene is inhibited.

***acs-1* is associated with fat accumulation, early larval development, and fecundity**

The *acs-1* gene encodes acetyl-CoA synthetase, which catalyzes the formation of acetyl-CoA from acetate and CoA and is predicted to function in peroxisomal β -oxidation.²⁴ Since the loss of function of *klf-3(ok1975)* also resulted in the reduced expression of *acs-1*, we investigated whether *acs-1* RNAi would alter fat storage or fecundity. We examined the effect of *acs-1* RNAi on the wild-type genetic background (as described in Experimental Procedures). Although the progenies of RNAi-treated worms were fertile, they showed reduced fertility (~30 progenies per worm). Moreover, the F1 progenies produced by *acs-1* RNAi worm showed a distinct early embryonic and larval arrest phenotype, with ~30% of F1 embryos arrested in the early stage (50-cell stage) of their development, whereas the other 70% of embryos hatched and developed to L1 but were arrested at L1/L2 stage. These phenotypes are similar to those reported in an earlier genomewide analysis.²⁵¹ We further tested whether the loss of function in *acs-1* enhanced the sterility caused by the *klf-3(ok1975)* mutation. The *acs-1* RNAi was carried out on the *klf-3(ok1975)* mutant by soaking L4 or young adult *klf-3(ok1975)* mutant animals in *acs-1* dsRNA. After 18–24 h of soaking, worms were individually transferred to fresh NGM plates and observed for reproduction under a light microscope. We found that RNAi depletion of *acs-1* in the *klf-3(ok1975)* mutant animals resulted in the synergism of phenotypes, resulting in larval arrest (60%), embryonic lethality (25%), and sterility (15%).

We then determined the effect of *acs-1* RNAi on fat content in the wild-type genetic background. We found that *acs-1* RNAi exhibited stronger staining (40%) with Oil Red O than the control wild-type worms (Fig. 6a and c). Given that *klf-3 (ok1975)* and *acs-1* RNAi both produce an increased fat uptake phenotype, we examined whether *acs-1* RNAi can enhance the fat phenotype of the *klf-3 (ok1975)* mutant. We performed *acs-1* RNAi on both wild-type and *klf-3(ok1975)* mutant worms and examined their fat uptake phenotype using Oil Red O staining. We found that RNAi depletion of *acs-1* had no effect on fat accumulation in *klf-3 (ok1975)* mutant animals (i.e., all *acs-1*-RNAi-treated strains had a ~40% fat mass increase over wild-type animals, indicating that *acs-1* activity is not necessary for the fat storage induced by the absence of *klf-3*).

Eliminating *acs-2* activity alleviates fertility defects in *klf-3* mutant animals

ACS-2 is a key enzyme (acetyl-CoA synthetase or the long-chain FA acyl-CoA ligase) of the β -oxidation pathway, and loss of its activity results in increased fat deposition in the *acs-2*(RNAi) worm.²⁴

Mutants in *acs-2(ok2457)* show an increased fat uptake by ~45%, as assessed by Oil Red O staining, when compared to the wild-type animals (Fig. 6d). The fertility of the *acs-2(ok2457)* mutant is quite similar to that of the wild type, as these mutant animals

produce almost the same number of progenies as the wild-type animals. To investigate whether mutation in *acs-2* enhances the fat phenotype of the *klf-3* mutant, we constructed the *acs-2(ok2457);klf-3(ok1975)* double mutant by standard genetic crosses and assayed these animals for fat uptake. We found that *klf-3;acs-2* mutant animals show an identical increase (44%) in fat mass when compared with the *klf-3* (45%) or *acs-2* single mutant (45%) (Fig. 6e), showing a lack of synergism in the fat accumulation phenotype of the double mutant as compared to the individual loss of function in the *klf-3(ok1975)* or *acs-2(ok2457)* single mutants (Table 1).

We next investigated the genetic interaction between *klf-3* and *acs-2* in reproductive behavior. A single *acs-2(ok2457)* mutant animal produces ~244 viable embryos ($N=61$) during its reproductive period compared to ~255 viable embryos ($N=60$) produced by wild-type worms during the same period. We scored the production of progeny in the *acs-2(ok2457);klf-3(ok1975)* double mutant (Table 1) and found that these double-mutant animals (*acs-2(ok2457);klf-3(ok1975)*) produced, averaging 89 viable embryos ($N=10$) compared to the *klf-3(ok1975)* single mutant (~47) but much lesser progenies than the *acs-2(ok2457)* single mutant (~244) (Table 1).

We examined the morphology of somatic gonad and germline development in the *acs-2(ok2457);klf-3(ok1975)* double mutant and found that germline development and somatic gonads were normal in the double-mutant animals. However, as opposed to *klf-3* or *acs-2* single mutants where vulval openings were normal and identical with wild-type worms (Fig. 6f–i), the vulva was not properly opened in the *acs-2(ok2457);klf-3(ok1975)* double mutant, likely due to altered vulval musculature as the vulval muscle in the double mutant appeared disorganized (Fig. 6i). Although the individual double-mutant worms produced an average of 85–90 viable embryos, further egg-laying stopped, resulting in the accumulation of a large number of developing embryos in the uterus. Many different factors can influence egg-laying ability. Horvitz and Sulston isolated many egg-laying-defective *C. elegans* mutants such as *unc-59*, *unc-83*, *unc-84*, *lin-2*, *lin-3*, and *lin-4*, where the vulva or other components of the egg-laying system are abnormal.²⁶ In these mutants, eggs are not laid properly and instead accumulate inside the body cavity. We have observed a similar effect in 5 out of 10 *acs-2;klf-3* double mutants, where eggs accumulated inside the body cavity and were not laid out. These data suggest an intermediate pattern of reproductive failure, resulting in abnormal vulval morphology and/or in other components of the egg-laying system, which could reflect the roles of *acs-2* in vulval development and egg-laying behavior.

The results described above raised the question on whether inhibition of *asc-1* and *acs-2* can suppress the phenotypes associated with reproduction in the *klf-3* mutant. Therefore, we injected *acs-1* and *acs-2* dsRNA into *klf-3(ok1975)* mutant animals. We found that all F1s from the RNAi-treated mother produced ~91 worms in the progeny ($N=10$) compared to 47 worms in the progeny of the *klf-3(ok1975)* mutant. Moreover, the *acs-1/acs-2(RNAi);klf-3(ok1975)* mutant affected the viability of 5% of the embryos produced by RNAi animals, but the other 95% of the embryos developed normally and reached adulthood, while *acs-1* RNAi on wild-type worm alone caused a significant number of larvae arrested at L1/L2 stages (60%), followed by embryonic lethality (25%) and sterility

(15%). We do not know whether the variable phenotypes associated with reproduction between *acs-1* RNAi alone, the *acs-2;klf-3* double mutant, and the *acs-1/acs-2*RNAi;*klf-3* mutant are the result of RNAi effect, or *acs-1* and *acs-2* have opposite roles in the *klf-3* mutant background.

***klf-3* controls FA desaturation**

SCDs play a key role in the synthesis of monounsaturated FAs from saturated ones. In our previous studies, we found that a decrease in the mRNA levels of SCD *fat-7* in *klf-3* mutant animals was associated with changes in the composition and ratio of FAs.¹⁵ Analysis of the *fat-7(wa36)* mutant revealed a single base-pair change that causes premature termination of the transcript and thus defines a loss-of-function allele.²⁷ The *fat-7* gene is expressed in the intestine, and the *fat-7(wa36)* mutant displays a normal life span with no obvious defect in reproduction. Mutants do not show altered fat deposition compared to wild type (Fig. 7), but *fat-7* RNAi reveals a change in FA composition.¹⁷ Our attempts to rescue the fat phenotype of the *klf-3* mutant by expressing FAT-7 in the intestine of the *klf-3* mutant failed (data not shown). However, we found that overexpression of FAT-7::GFP in wild-type animals apparently increased fat accumulation in transgenic worms, but careful examination of the stained worms revealed that the increased level of fluorescence in the intestine was primarily due to the green fluorescent protein (GFP) autofluorescence that can be blocked if proper excitation and barrier filters are used in fluorescence microscopy and if leakage of the GFP fluorescence is blocked in the Oil Red O observation channel. These results suggest that overexpression of FAT-7 may not result in an increased level of fat uptake in the intestine.

Next, we tested whether depletion of *fat-7* function affects fat accumulation in *klf-3(ok1975)* mutant animals by analyzing *klf-3(ok1975);fat-7(wa36)* double mutants. We found that eliminating *fat-7(wa36)* activity led to a similar fat increase (~40%) in the *klf-3(ok1975);fat-7(wa36)* double mutants, as compared to the *klf-3(ok1975)* single mutant (45%) (Fig. 8), although eliminating the *fat-7* activity does not improve worm fecundity, since the *fat-7;klf-3* worms were sterile or semisterile (data not shown). However, mutation in *klf-3* significantly down-regulates *fat-7* expression and alters FA composition, which signifies that, as a transcription factor, *klf-3* regulates β -oxidation in parallel with desaturation, in concert with other factors. This type of coordination is important for mammalian SCDs to control fat deposition and to maintain the proper level and composition of saturated and desaturated FAs.^{28,29}

Discussion

Fat metabolism is coordinately regulated through many signaling pathways that integrate fat deposition with its mobilization and utilization, and its essential regulatory mechanisms are likely conserved during the evolution of metazoan animals. The KLF family of transcription factors mediates diverse aspects of development, cellular proliferation and apoptosis, cell signaling, and differentiation, and affects, among others, the cardiovascular system, endothelial cell biology, respiratory system, hematopoietic system, reproductive system, digestive system, immune and inflammatory responses, and adipogenesis.^{6,16} Most recently, human KLF14 has been identified as a regulator linking obesity to type 2 diabetes.¹³ In

addition to the role of worm *klf-3* in reproduction, we speculate on its role in partially overlapping with that of human KLF14 in fat storage and metabolism. Thus, there are accumulating data suggesting the role of KLF family members in lipid metabolism across diverse organisms; however, it will be useful to utilize *C. elegans* to elucidate molecular pathways, as there are only three members of the KLF family in the nematode, and both classical and molecular genetics methods can be conveniently applied in *C. elegans*.

Previously, we examined the role of *klf-3*, a member of the KLF family of transcription factors, by dissecting its *ok1975* mutant, showing that these animals accumulate high body fat, primarily in the intestine, and display reproductive defects in adult life.¹⁵ To reconcile with these seemingly unrelated phenotypes in *klf-3* mutants, we have investigated here the molecular genetic basis of *klf-3* regulation to explore how this fat accumulation intersects reproductive behavior. We identified a set of genes whose expression is significantly altered in *klf-3* mutant animals. KEGG pathway analyses showed that a majority of these genes encode metabolic pathway components, including FA β -oxidation and modification enzymes. In particular, the recognition of *F08A8.1*, *F08A8.2*, *acs-1*, *acs-2*, and *fat-7* as potential targets of KLF3 regulation prompted us to explore their genetic interactions, given their direct link to the lipid metabolic phenotypes. Through analyses of genetic interactions (summarized in Table 1), we demonstrate for the first time that KLF3 modulates FA β -oxidation and FA modification, two central biochemical processes in lipid metabolism. The deficiencies in many enzymes of FA β -oxidation and FA synthesis are recognized as important players of human lipid metabolic diseases. Hence, the present findings highlight the significance of *klf-3* as a transcriptional regulator of these enzymes in *C. elegans* and point to the occurrence of a conserved counterpart in vertebrates.

FA β -oxidation is a major energy source following fasting and occurs in both mitochondria and peroxisomes; its biochemical execution is likely conserved across worms and humans, given the presence of similar organelle-targeting signal sequences in both species.³⁰ The fat phenotype of the *klf-3(ok1975)* mutant animals is characterized by excessive fat deposition and large lipid droplet formation in the intestine. Here we show that TGs predominate in the accumulated lipid categories. Interestingly, these observations suggest that Oil Red O staining may provide a convenient marker for quantifying TG in the nematode intestine, and this notion can be tested in mammalian adipocytes also. In contrast, the total amount of phospholipids and the total amount of cholesterol (including its esters) are basically unaffected, regardless of whether *klf-3* is present or absent. Thus, our genetic and phenotypic investigations provided direct evidence that *klf-3* interacts with several FA metabolic enzyme genes, but clearly in a distinct manner.

Our data presented here (Table 1) support the view that a loss of *klf-3* activity dysregulated key lipid metabolism genes, including the β -oxidation genes *F08A8.1*, *F08A8.2*, *acs-1*, and *acs-2*, and the FA desaturation gene *fat-7*. Notably, *F08A8.1* and *F08A8.2* are orthologous to human peroxisomal acyl-CoA oxidase acting in three pathways: FA catabolism, polyunsaturated FA synthesis, and peroxisome proliferator-activated receptor signaling;³¹ acyl-CoA oxidase catalyzes the first rate-limiting step of the peroxisomal β -oxidation of FAs. The fat accumulation phenotypes of *F08A8.1* and *klf-3* mutant or *F08A8.2* RNAi are strikingly similar, suggesting a relationship between *klf-3* and *F08A8.1/F08A8.2*. Our

genetic analyses suggest that *klf-3*, *F08A8.1*, and *F08A8.2* act in the same pathway, and that the transcriptional regulation of *F08A8.1* and *F08A8.2* by *klf-3* contributes to the regulation of genes, which in turn contributes to β -oxidation. However, the role of *klf-3* in reproductive behavior is quite complex and needs further investigation. For instance, the reproductive defect of *klf-3* mutants is not enhanced by *F08A8.1* or *F08A8.1/F08A8.2* deletion; instead, we observed an additional egg-laying defect, perhaps caused by improper vulval development in *klf-3;F08A8.1* or *klf-3;F08A8.1/F08A8.2* animals. The mechanism underlying this observation is yet unclear. Possibly, the reduced expression of both *F08A8.1* and *F08A8.2* might account for the reproductive abnormalities associated with the *klf-3* (*ok1975*) mutant. With regard to how the reproductive defect caused by *klf-3* deletion relates to enhanced fat accumulation, our analysis suggests that the two phenotypes are interrelated and intrinsic to the *klf-3* mutant worms. Nonetheless, the control of reproduction and lipid metabolism during development is seemingly complex and may involve interactions with additional hitherto unknown pathway components, which may require further molecular and genetic analyses such as genetic mosaics, and determination of the focal tissue of *klf-3* and interacting genes.

In *klf-3* mutant animals, depletion or reduction of *acs-1* activity by RNAi results in high fat accumulation but does not alter the fat content in the intestine, implying that *acs-1* may not be required for fat accumulation. However, *acs-1* expression is reduced in the *klf-3* mutant,¹⁵ suggesting that similar to *F08A8.1* or *F08A8.2*, *acs-1* might act downstream of *klf-3* to promote β -oxidation. Because of their expression in two different organelles, these enzymes may function in two different pathways, and *klf-3* activity could be a potential factor in maintaining a basal transcription level of these enzymes. In addition to its enzymatic role in FA β -oxidation, *acs-1* gene may also function in early larval and embryonic development. Our analysis of the *acs-1* RNAi;*klf-3(ok1975)* mutant showed a variety of phenotypes, including larval arrest, embryonic lethality, and low fertility. Whether or not *klf-3* potentiates *acs-1* and whether or not the two cooperate in reproductive processes require further investigation.

As in mammals, the *acs-2* gene encoding *acs-2* activates FAs and converts them into acyl-CoA for lipid metabolism. The nematode *acs-2* apparently expresses in mitochondria and is positively regulated by the NHR-49 nuclear receptor.²⁴ Overexpression of *acs-2* has been reported to suppress the high-fat phenotype of the *nhr-49* mutant, whereas *fat-7* inhibits *acs-2* expression to prohibit fat consumption.²⁴ In contrast to these observations, we found a reversed situation (i.e., a marked increase in *acs-2* and a concurrent decrease in *acs-1*, *F08A8.1*, and *fat-7* mRNA levels in *klf-3* mutants).¹⁵ Hence, the high expression of *acs-2* and the low expression of *fat-7* in mutants may arise from a coordinated fine-tuning of fat metabolism. Thus, the relationship between *klf-3* and *acs-2* may be partly influenced by *fat-7*, whose decreased expression is expected to promote *acs-2* activation; *acs-2* expression was induced when *fat-7* expression was inhibited in the nuclear hormone receptor mutant *nhr-49(nr2041)*, indicating that *fat-7* blocks the expression of *acs-2* through *nhr-49* via an independent mechanism.

A specific group of enzymes is involved in the synthesis and modification of long-chain FAs through elongation and desaturation. Previously, we have shown that the composition and

ratio of long-chain FAs, such as C:18:0, C18:2w6c, or C20:2w6c, are altered in *klf-3* mutants, indicating defects in desaturation and elongation.¹⁵ Specifically, *klf-3* mutation causes a substantial decrease in *fat-7* expression, but a concurrent increase in *fat-3*, *fat-4*, *fat-5*, and *fat-6* expression. Prior studies indicated that in the nematode, *fat-7* suppression neither increases nor decreases fat mass,³² and *fat-7* mutation alone does not change FA composition.²⁸ However, *fat-7* RNAi showed a change in lipid composition.²⁴ In SCD-1 knockout mice, adiposity is reduced significantly.^{33,34} We have demonstrated here that fat increase in *klf-3;fat-7* double mutants is apparently identical with that in *klf-3* single mutants, but is greater than that in *fat-7* mutants (Table 1). These findings suggest that *fat-7* activity is not needed for fat accumulation in *klf-3* mutant animals and support the view that *klf-3* may regulate β -oxidation and desaturation in concert with other players. This type of coordination is crucial for mammalian SCDs to control fat deposition and to maintain the proper level and composition of saturated and desaturated FAs.^{28,29}

Dysfunction of mitochondrial FA β -oxidation is a well-established cause of genetic diseases, emphasizing the importance of this pathway and its tight control in energy homeostasis through balanced anabolism and catabolism. Until now, little is known about the *in vivo* mechanism of β -oxidation and its relationship with reproduction. The presence of high TGs as large droplets during adipocyte differentiation correlates with obesity in mammals.^{35,36} FA breakdown via β -oxidation occurs in peroxisomes and mitochondria, wherein FA mobilization and TG assimilation are balanced by a multistep process in the context of energy storage and redistribution.³⁷ *klf-3* protein is apparently not present in peroxisomes or mitochondria, and *klf-3(ok1975)* mutants show no apparent morphological abnormality in these organelles. Hence, *klf-3* is not involved in the structural integrity of these organelles. Possibly, *klf-3* exerts its role in the intestine via transcriptional regulation of β -oxidation enzymes and thus affects their availability to mitochondria or peroxisomes during FA breakdown for the utilization of stored energy (Fig. 8). Consistent with this view is the report that the intestine is the site of lipid metabolism, and that inactivation or down-regulation of *klf-3*, *F08A8.1*, *F08A8.2*, *acs-1*, or *acs-2* enhances lipid accumulation. Because a deficiency in the enzymes of FA β -oxidation underlies metabolic lipid disorders, the present findings highlight the significance of KLFs as key nodes of network formation. They may bear broad implications for human lipid metabolic disorders and offer a useful model system, enabling us to gain insights into the conserved regulatory mechanisms that are otherwise difficult to study in humans. We suggest that *klf-3* can function as either an activator or a repressor in the regulation of *F08A8.1*, *F08A8.2*, *acs-1*, *acs-2*, and *fat-7* linking lipid metabolism to reproduction in adult worms through their genetic interactions. Given the role of *klf-3* in modulating β -oxidation, identifying its counterpart in humans may entail the recognition of new molecular targets and lead to a potential therapeutic intervention for obesity and fat-linked metabolic syndromes.

Experimental Procedures

Nematode strains and culture conditions

All *C. elegans* strains used in this study were maintained and propagated at room temperature (~22 °C) as described previously, unless otherwise noted.¹³ The wild-type

strain was *C. elegans* variant Bristol N2 cultured in small (35 mm×10 mm; Falcon) plates containing NGM and seeded with *E. coli* OP50.¹⁷ Other strains—RB1603, *klf-3(ok1975)*, VC1785, *F08A8.1(ok2257)*, *acs-2(ok2457)*, and BX153 *fat-7(wa36)*—were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN), which is funded by the National Institutes of Health National Center for Research Resources. All mutant strains were separately backcrossed three times using wild-type N2 (Bristol) strain males according to the standard protocol³⁸ and maintained as homozygous strains. The genomic deletion in each mutant strain [*klf-3(ok1975)*, *F08A8.1(ok2257)*, *acs-2(2457)*, and *fat-7(wa36)*] used in this study was determined by sequencing with gene-specific primers and nested PCR products encompassing the transcription unit. Individual homozygous mutant hermaphrodites were grown on plates at 20 °C, and their self-progenies were used in subsequent experiments.

Construction and germline transformation of *klf-3::gfp* fusion reporters

There are two isoforms of *klf-3*—*klf-3a* (comprising 309 amino acid residues) and *klf-3b*¹⁴ (comprising 315 amino acid residues)—whose proteins differ by 8 amino acid sequences in the amino-terminal end. The promoter for the *klf-3* gene has been identified as a 1.0-kb segment from the 5' terminus to the *klf-3a* ATG start codon that directs intestinal expression.¹³ To study the expression pattern of the *klf-3b* isoform, we made a *klf-3b::gfp* reporter fusion construct that contained a probable *klf-3b* promoter segment beginning about –2.0 kb upstream of DNA sequences that extended to the end of the last exon of the *klf-3b* gene and fused to the *gfp* reporter cDNA in the *C. elegans* expression vector pPD95.67 (pHZ145; Fig. 1a). Previously, we have observed a significant reduction of *fat-7* mRNA level in the *klf-3(ok1975)* mutant. To determine whether the reduced expression of the *C. elegans fat-7* gene in *klf-3(ok1975)* mutant animals affects FA metabolic pathway and results in excessive fat accumulation in the intestine, we therefore tested whether providing *fat-7* overexpression in *klf-3(ok1975)* mutants should reduce the fat accumulation in the mutant worm.

Thus, we made a promoter construct (pHZ343; Fig. 1b) in which, 2 kb upstream, a probable promoter sequence from the *klf-1* ATG start codon was fused to the coding sequence of the *fat-7* gene in the pPD95.67 vector backbone. The *klf-1* promoter was chosen because it primarily directs intestinal expression like the *klf-3* promoter but avoids competition from the latter. Transgenic lines were generated through germline transmission. Briefly, 50–80 ng/μl test plasmid was mixed with 50 ng/μl pRF4 plasmid and injected into the young adult N2 hermaphrodites.³⁹ The F1 generation of injected animals was selected for roller phenotype and observed for *gfp* expression. Three independent transgenic lines were established, and at least 100 animals (consisting of all larval and adult stages from each line) were screened under a Zeiss Axioplan 2 imaging microscope. Images were captured with an AxioCam HRm camera and OpenLAB software. The *gfp* expression was consistent in all animals and between each transgenic line.

Fat staining and microscopic examination of lipid droplets

We examined several available methods that measure fat storage in *C. elegans*. These include Sudan black fixative-based staining,^{13,40,41} Nile red dye,^{42,43} or a standard assay for

mammalian adipocyte fat storage with Oil Red O staining.⁴⁴ We measured fat storage in the *klf-3(ok1975)* mutant or wild-type animals with the dye Oil Red O and compared the staining pattern with that of Sudan black or Nile red staining. We found that Oil Red O staining was much more consistent across animals of a given genotype than Sudan black or Nile red staining. Similar observations have been reported by Soukas *et al.*²⁰ Therefore, in the present study, we used Oil Red O staining to accurately measure fat mass in RNAi animals and in animals of various genotypes. Synchronized 100–120 L4 larvae (30 h after L1 hatch) from wild type, single mutants, and double mutants were separately collected by washing with phosphate-buffered saline (PBS) from NGM plates to a 2-ml Eppendorf tube. Similarly, 20–25 RNAi worms (egg-laying adults) and 150–200 of their progenies were separately collected by washing with PBS buffer. After collection, worms were washed twice and resuspended with 200 μ l of PBS. Worms were fixed in 1% formaldehyde in PBS for 1 h at room temperature, incubated overnight at -80°C , and thawed under a stream of running tap water, followed by addition of 1 ml of distilled water. Samples were mixed and collected by centrifugation. One milliliter of propylene glycol was added to the tube containing the sample and incubated at room temperature ($\sim 22^{\circ}\text{C}$) for 20–30 min on a gentle shaker. Worms were collected by centrifugation at ~ 5000 rpm, then 1 ml of Oil Red O stain (STORO100; American Master Tech Scientific, Lodi, CA) was added to the sample and incubated overnight at 4°C . After incubation, samples were brought to room temperature with gentle shaking and transferred to a glass well/wash plate (Pyrex plate, cat no. 71563; Electron Microscopy Sciences, Hatfield, PA). Samples were washed twice with propylene glycol, and 10–15 animals were transferred to a tiny drop of propylene glycol onto glass slides, mounted, sealed, and observed under a light microscope.

Quantitative analysis of fat deposition in intestinal cells

At least 60–70 animals from each genotype were observed using a $40\times$ objective microscope and imaged at a magnification of $400\times$. For quantitative analysis, we measured the integrated density of Oil Red O staining in 10–20 animals from each genotype using Photoshop CS3 (extended) software. Data are presented as mean $\pm$ standard deviation (Figs. 6, 7, and 8). Images of fat staining are representative of at least 60 animals.

Measurement of TG levels

Synchronized populations of larval and young adult animals were separately collected to measure the total TGs, phospholipids, and cholesterol of *klf-3* mutants, and we compared these to the age-matched N2 animals. Quantitative lipid biochemistry was performed using gas–liquid chromatography, as previously described.⁴⁵ A synchronous population of all developmental stages was prepared as previously described.¹⁷ Embryos were obtained by treating gravid hermaphrodites with sodium hypochlorite, and then hatched in water overnight to derive L1 larvae. The arrested L1 larvae were transferred onto NGM plates and allowed to develop into L2, L3, L4, and young adult worms over 36 h at room temperature ($\sim 22^{\circ}\text{C}$). Then 15,000 larval-stage animals (L1, L2, and L3) and 10,000 L4 animals (30 h after L1 hatch) and young adult animals (36 h after L1 hatch) were separately collected in 2-ml Eppendorf tubes by washing with PBS from the NGM plates and then by washing again two to three times with PBS to get rid of bacterial debris. Animals were pelleted in 100 ml of PBS and stored frozen at -80°C until needed. Wild-type and mutant animals were

separately homogenized in 1 ml of 1.0 mM ethylene glycol bis(β -aminoethyl ether) *N,N'*-tetraacetic acid, 1 mM Tris-HCl, and 1 mM MgCl₂. Then 3.75 ml of chloroform/methanol (2:1, vol/vol) was added, mixed, and incubated at room temperature with intermittent mixing. After 15 min, 1.25 ml of methanol was added, mixed, and incubated for 1 min. After addition of 1.25 ml of H₂O, all contents were thoroughly mixed and centrifuged for 10 min at 5000 rpm. The lower phase was collected with a Pasteur pipette, dried under nitrogen stream, and dissolved in 100 μ l of methanol. Ten microliters of samples was taken for TG measurements with a commercial kit (Thermo Trace Ltd., Melbourne, Australia). For lipid analysis, two independent experiments, each containing five separate samples of four larval stages (L1, L2, L3, L4) and young adults of both mutant and wild-type animals, were collected. The results were consistent between two samples.

Genetic analysis

To investigate the genetic interactions of *klf-3*(1975) with lipid utilization pathway genes, we selected *F08A8.1(ok2257)*, *acs-2(ok2457)*, and *fat-7(wa36)* mutants. Mutants in *F08A8.1(ok2257)* show fat phenotypes that are comparable to those of the *klf-3(ok1975)* mutant (this study). The *acs-2* gene encodes a mitochondrial acyl-CoA synthetase, which activates FA for transport into the mitochondrial matrix and subsequent β -oxidation. RNAi of *acs-2* resulted in worms with increased fat staining,⁴⁶ while *fat-7*(SCD) mutant worms do not accumulate fat (this study). We have shown that substantial deletion (~1.6 kb) in the *klf-3* gene down-regulates the expression of *F08A8.1*, *acs-1*, and *fat-7* genes but, at the same time, up-regulates the *acs-2* gene, indicating a differential transcriptional control mechanism.¹⁴ To study epistasis between *klf-3* and *F08A8.1*, *acs-2*, or *fat-7*, we constructed double mutants between *klf-3(ok1975)* and other mutations using standard genetic crosses. The presence of *klf-3(ok1975)* and other mutant alleles in the double mutant was confirmed by single-worm PCR. The double mutants were stained with Oil Red O staining, as described above, and their staining was compared with that of the single mutants for fat accumulation. We also examined double mutants for their brood size because *klf-3* mutant animals display reproductive defects. The brood size analysis on different genotypes was performed at least in two independent experiments. In each analysis, egg-laying on L4s (~30 h after L1 hatch) (Table 1) was monitored and recorded as described in Analysis of Fertility and Brood Size. Animals were observed under Nomarski optics using a Nikon Eclipse 80i light microscope, and photographs were taken with a Photometrics CoolSNAP cf digital camera (magnification of 400 $\times$).

dsRNA preparation and RNAi

To determine the genetic interaction between *klf-3* and *acs-1* or *F08A8.2*, we performed an RNAi assay to separately knock down *acs-1* or *F08A8.2* in the *klf-3(ok1975)* mutant worm. RNAi was performed by soaking synchronized L4 larvae in dsRNA^{24,47} or by microinjecting dsRNA into adult hermaphrodites. The full-length gene-specific cDNA was used as template for RNA synthesis, and dsRNA was prepared as described previously.²⁴ In brief, cDNA was first cloned into the vector pCR 4-TOPO and amplified with commercially available M13F and M13R (Invitrogen, Carlsbad, CA) primers. Then T3 or T7 RNA polymerase was used for single-stranded sense and antisense RNA syntheses using the MEGAscript High Yield Transcription Kit (Ambion, Inc., Austin, TX). In RNAi

experiments, two sets of 20–25 synchronized *klf-3(ok1975)* L4 larvae and, for comparison, age-matched wild-type N2 animals were separately soaked in 20 μ l of 1 $\times$ PBS containing *Ce-acs-1* or *F08A8.2* dsRNA (3 μ g/ μ l) and incubated at room temperature (22 °C). A similar number of synchronized L4 larvae soaked in PBS but without dsRNA served as controls. After 20–24 h of soaking, the animals from all treatments, including controls and *acs-1* RNAi, *acs-1* RNAi;*klf-3(ok1975)*, *F08A8.2* RNAi, and *F08A8.2* RNAi;*klf-3(ok1975)*, were separately transferred to individual NGM plates seeded with *E. coli* OP50. After recovery periods of 22–24 h, one set of RNAi animals and their F1 progenies from each treatment were separately transferred into a 2-ml Eppendorf tube containing PBS solution and then processed for fat staining, as described previously. After Oil Red O staining, 60–70 animals from each treatment were observed under Nomarski optics attached to a Nikon Eclipse 80i light microscope, and photographs were taken with a Photometrics CoolSNAP cf digital camera (magnification of 400 $\times$). Another set of RNAi animals was observed for progeny production, as described below. In brief, 25 RNAi animals were individually transferred every 24 h to fresh bacterially seeded NGM plates, and the hatched progenies were counted. Total brood size was determined by adding progeny produced across 2–3 days because RNAi effect begins to diminish after 2 days of RNAi treatment. Two independent but identical RNAi analyses were performed, and the results were consistent.

Analysis of fertility and brood size

To measure fertility, we individually transferred few L4 larvae from single *F08A8.1(ok2257)*, *acs-2(ok2257)*, and *fat-7(wa36)* hermaphrodites or from *klf-3;F08A8.1*, *klf-3;acs-2*, and *klf-3;fat-7* double-mutant animals onto OP50-seeded NGM plates, and we observed their reproduction and life span at 22 °C. When the animals began to lay eggs, the number of embryos or hatched larvae produced by each one of them was counted. Individual worms were transferred to fresh NGM plates every 24 h; eggs and larvae were scored over 5 days or until hermaphrodites stopped egg-laying. If a hermaphrodite did not produce any embryo in this period, it was considered sterile. If a hermaphrodite produced 30–40 viable embryos, it was considered semisterile. Life span was measured by a microscopic observation of the movement of the animals, which were occasionally touched by eyebrow attached to a needle and were considered alive if they were moved by a touch. Animals were monitored for 4–5 days under a light microscope and photographed using differential interference contrast (Nomarski) optics (magnification of 400 $\times$). Two independent analyses were performed.

Assay for FA uptake

To study fat uptake, we measured the fluorescence intensity in the intestine of worms fed BODIPY dye (no. D-3822; Molecular Probes).⁴⁸ A single colony of *E. coli* OP50 strain used for *C. elegans* diet was cultured in 25 ml of liquid LB medium overnight, pelleted, and then resuspended in 10 ml of S-Basal medium. BODIPY staining was performed as described previously,⁴⁹ with some modifications. In brief, C1-BODIPY-C12 was dissolved in dimethyl sulfoxide to make a 5 mM stock solution. The stock solution was diluted in S-Basal medium to 1 μ M, and 200 μ l of this solution was added to an equal amount of S-Basal OP50 solution, mixed, and applied to the surface of NGM (60 mm $\times$ 15 mm) plates. The plates were allowed to air dry. Synchronized populations of *klf-3(ok1975)* L4s and, for

comparison, N2 animals were washed off the *C. elegans* culture plate and rinsed two times with S-Basal medium, and the 25 L4 animals were separately transferred in replicates (a total of 50 for each group) onto NGM OP50 plates containing BODIPY dye. After 10–15 min of feeding, one set containing 25 animals of each group was transferred to a fresh NGM plate without bacteria. After 10 min on this plate, the animals were transferred to a drop of 0.2% azide solution on a slide, covered by a cover slip, and observed under a Zeiss Axioplan 2 imaging microscope. Pictures were taken with an AxioCam HRm camera and OpenLAB software. At least three independent experiments were performed.

Structure of mitochondria and peroxisomes in *klf-3(ok1975)* mutant worms

To examine the subcellular ultrastructure of organelles in *klf-3(ok1975)* and wild-type N2 animals, we utilized electron microscopy. Since mutation in *klf-3(ok1975)* caused alteration of *acs-1*, *acs-2*, *F08A8.1*, and *F08A8.2* gene expression in mitochondria or peroxisomes, we investigated whether the altered expression of these genes is associated with changes in the morphology of mitochondria and/or peroxisomes. We collected 75–100 synchronized populations of L4s or young adult *klf-3(ok1975)* and, for comparison, N2 animals by washing off the NGM plate, which was rinsed two times with PBS to get rid of bacterial debris. Postwashing, the animals were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature, rinsed in the same buffer several times, and postfixed in 1% osmium tetroxide solution. After dehydration in a series of increasing ethanol concentrations, worms were embedded in Poly/Bed Araldite media and cured for 24 h at 56 °C. Ultrathin sections (65–70 nm) were cut on an MT-XL ultramicrotome and stained with a uranyl acetate solution, followed by Reynold's lead citrate stain solution. Samples were observed under a Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (The Netherlands).

Acknowledgements

We thank the *Caenorhabditis* Genetics Center, University of Minnesota, for providing mutant strains. We thank Saima Limi for technical assistance, Yelena Oksov for electron microscopy, and Dr. Mohandas Narla for helpful comments on the manuscript.

Abbreviations used

KLF	Krüppel-like transcription factor
FA	fatty acid
TG	triglyceride
RNAi	RNA interference
SCD	stearoyl-CoA desaturase
NGM	nematode growth medium
dsRNA	double-stranded RNA
GFP	green fluorescent protein
PBS	phosphate-buffered saline

References

1. Bhathena SJ. Relationship between fatty acids and the endocrine system. *Biofactors*. 2000; 13:35–39. [PubMed: 11237196]
2. Agatha G, Hafer R, Zintl F. Fatty acid composition of lymphocyte membrane phospholipids in children with acute leukemia. *Cancer Lett*. 2001; 173:139–144. [PubMed: 11597788]
3. Ntambi M, Miyazaki M, Dobrzyn A. Regulation of stearoyl-CoA desaturase expression. *Lipids*. 2004; 39:1061–1065. [PubMed: 15726820]
4. Schwartz W, Woods C, Porte D Jr, Seeley J, Baskin DG. Central nervous system control of food intake. *Nature*. 2000; 404:661–671. [PubMed: 10766253]
5. Reddy JK, Hashimoto T. Peroxisomal beta-oxidation and peroxisome proliferators-activated receptor alpha: an adaptive metabolic system. *Annu. Rev. Nutr.* 2001; 21:193–230. [PubMed: 11375435]
6. Turner J, Crossley M. Mammalian Krüppel-like transcription factors: more than just a pretty finger. *Trends Biochem. Sci.* 1999; 24:236–240. [PubMed: 10366853]
7. van Vliet J, Crofts A, Quinlan G, Czolij R, Perkins C, Crossley M. Human KLF17 is a new member of the Sp/KLF family of transcription factors. *Genomics*. 2006; 87:474–482. [PubMed: 16460907]
8. Wu J, Srinivasan V, Neumann C, Lingrel JB. The KLF2 transcription factor does not affect the formation of preadipocytes but inhibits their differentiation into adipocytes. *Biochemistry*. 2005; 44:11098–11105. [PubMed: 16101293]
9. Li D, Yea S, Li S, Chen Z, Narla G, Banck M, et al. Krüppel-like factor-6 promotes preadipocyte differentiation through histone deacetylase 3-dependent repression of DLK1. *J. Biol. Chem.* 2005; 280:26941–26952. [PubMed: 15917248]
10. Birsoy K, Chen Z, Friedman J. Transcriptional regulation of adipogenesis by KLF4. *Cell Metab.* 2008; 7:339–347. [PubMed: 18396140]
11. Mori T, Sakaue H, Iguchi H, Gomi H, Okada Y, Takashima Y, et al. Role of Krüppel-like transcription factor 15 (KLF15) in transcriptional regulation of adipogenesis. *J. Biol. Chem.* 2005; 280:12867–12875. [PubMed: 15664998]
12. Sue N, Jack BH, Eaton SA, Pearson RC, Funnell AP, Turner J, et al. Targeted disruption of the basic Krüppel-like factor gene (*Klf3*) reveals a role in adipogenesis. *Mol. Cell. Biol.* 2008; 28:3967–3978. [PubMed: 18391014]
13. The MuTHER ConsortiumThe GIANT ConsortiumThe MAGIC InvestigatorsThe DIAGRAM Consortium. Identification of an imprinted master trans regulator at the KLF14 locus related to multiple metabolic phenotypes. *Nat. Genet.* 2011; 43:561–564. [PubMed: 21572415]
14. Hashmi S, Ji Q, Zhang J, Parhar R, Huang CH, Brey C, Gaugler R. A Krüppel-like factor in *Caenorhabditis elegans* with essential roles in fat regulation, cell death and phagocytosis. *DNA Cell Biol.* 2008; 27:545–551. [PubMed: 18680432]
15. Zhang J, Yang C, Brey C, Rodriguez M, Oksov Y, Gaugler R, et al. Mutation in *Caenorhabditis elegans* Krüppel-like factor, KLF-3 results in fat accumulation and alters fatty acid composition. *Exp. Cell Res.* 2009; 315:2568–2580. [PubMed: 19427851]
16. Brey C, Nelder M, Gaugler R, Hashmi S. Krüppel-like family of transcription factors: an emerging new frontier in lipid biology. *Int. J. Biol. Sci.* 2009; 5:622–636. [PubMed: 19841733]
17. White, J. The anatomy. In: Wood, WB., editor. *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY: 1988. p. 81-122.
18. McKay JP, Raizen DM, Gottschalk A, Schafer WR, Avery L. *Eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. *Genetics*. 2004; 166:161–169. [PubMed: 15020415]
19. Zhang O, Box C, Xu N, Le Men J, Yu J, Guo F, et al. Genetic and dietary regulation of lipid droplet expansion in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA.* 2010; 107:4640–4645. [PubMed: 20176933]
20. Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. Distinct transcriptional profiles of adipogenesis *in vivo* and *in vitro*. *J. Biol. Chem.* 2001; 276:34167–34174. [PubMed: 11445576]

21. Mak Y, Nelson S, Basson M, Johnson D, Ruvkun G. Polygenic control of *Caenorhabditis elegans* fat storage. *Nat. Genet.* 2006; 38:363–368. [PubMed: 16462744]
22. Grant B, Hirsh D. Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell.* 1999; 10:4311–4326. [PubMed: 10588660]
23. Sundaram M, Greenwald I. Suppressors of a *lin-12* hypomorph define genes that interact with both *lin-12* and *glp-1* in *Caenorhabditis elegans*. *Genetics.* 1993; 135:765–783. [PubMed: 8293978]
24. Van Gilst MR, Hadjivassiliou H, Jolly A, Yamamoto KR. Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in *C. elegans*. *PLoS Biol.* 2005; 3:301–312.
25. Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, Brehm M, et al. Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature.* 2005; 434:462–469. [PubMed: 15791247]
26. Horvitz HR, Sulston J. Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics.* 1980; 96:435–454. [PubMed: 7262539]
27. Brock TJ, Browse J, Watts JL. Genetic regulation of unsaturated fatty acid composition in *C. elegans*. *PLoS Genet.* 2006; 2:997–1005.
28. Ntambi JM. The regulation of stearoyl-CoA desaturase (SCD). *Prog. Lipid Res.* 1995; 34:139–150. [PubMed: 7480063]
29. Dobrzyn P, Dobrzyn A, Miyazaki M, Cohen P, Asilmaz E, Hardie DG, et al. Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. *Proc. Natl Acad. Sci. USA.* 2004; 101:6409–6414. [PubMed: 15096593]
30. Gurvitz A, Langer S, Piskacek M, Hamilton B, Ruis H, Hartig A. Predicting the function and sub-cellular location of *Caenorhabditis elegans* proteins similar to *Saccharomyces cerevisiae* beta-oxidation enzymes. *Yeast.* 2000; 17:188–200. [PubMed: 11025529]
31. Osumi T, Hashimoto T, Ui N. Purification and properties of acyl-CoA oxidase from rat liver. *J. Biochem.* 1980; 87:1735–1746. [PubMed: 7400120]
32. Watts JL, Browse J. A palmitoyl-CoA-specific 9. *Biochem. Biophys. Res. Commun.* 2000; 272:263–269. [PubMed: 10872837]
33. Cohen P, Friedman JM. Leptin and the control of metabolism: role for stearoyl-CoA desaturase-1 (SCD-1). *J. Nutr.* 2004; 134:2455S–2463S. [PubMed: 15333742]
34. Cohen P, Ntambi JM, Friedman JM. Stearoyl-CoA desaturase-1 and the metabolic syndrome. *Endocr. Metab. Disord.* 2003; 3:271–280.
35. Faust IM, Johnson PR, Stern JS, Hirsch J. Diet induced adipocyte number increase in adult rats: a new model of obesity. *Am. J. Physiol.* 1978; 235:E279–E286. [PubMed: 696822]
36. Rodeheffer S, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells *in vivo*. *Cell.* 2008; 135:240–249. [PubMed: 18835024]
37. Reddy K, Mannaerts GP. Peroximal lipid metabolism. *Annu. Rev. Nutr.* 1994; 14:343–370. [PubMed: 7946524]
38. Sulston, J.; Hodgkin, J. Method. In: Wood, WB., editor. *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY: 1998. p. 587–606.
39. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics.* 1974; 77:71–94. [PubMed: 4366476]
40. Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* 1991; 10:3959–3970. [PubMed: 1935914]
41. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science.* 1997; 277:942–946. [PubMed: 9252323]
42. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G. The Forkhead transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature.* 1997; 389:994–999. [PubMed: 9353126]
43. Ashrafi K, Chang Y, Watts L, Fraser G, Kamath S, Ahringer J, Ruvkun G. Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature.* 2003; 421:268–272. [PubMed: 12529643]

44. Mullaney BC, Blind RD, Lemieux GA, Perez CL, Elle IC, Faergeman NJ, et al. Regulation of *C. elegans* fat uptake and storage by acyl CoA synthase-3 is dependent on NR5A family nuclear hormone receptor *nhr-25*. *Cell Metab.* 2010; 12:398–410. [PubMed: 20889131]
45. Soukas A, Kane A, Carr E, Mello A, Ruvkun G. Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. *Genes Dev.* 2009; 23:496–511. [PubMed: 19240135]
46. Pan X, Hussain FN, Iqbal J, Feuerman MH, Hussain MM. Inhibiting proteasomal degradation of microsomal triglyceride transfer protein prevents CCl₄-induced steatosis. *J. Biol. Chem.* 2007; 282:17078–17089. [PubMed: 17405876]
47. Hashmi S, Zhang J, Oksov Y, Lustigman S. The *Caenorhabditis elegans* cathepsin Z-like cysteine protease, Ce-CPZ-1, has a multifunctional role during the worms' development. *J. Biol. Chem.* 2004; 279:6035–6045. [PubMed: 14630920]
48. Tabara H, Grishok A, Mello CC. RNAi in *C. elegans*: soaking in the genome sequence. *Science.* 1998; 282:430–431. [PubMed: 9841401]
49. You J, Kim J, Raizen M, Avery L. Insulin, cGMP, and TGF- β signals regulate food intake and quiescence in *C. elegans*: a model for satiety. *Cell Metab.* 2008; 7:249–257. [PubMed: 18316030]

Fig. 1.

Tissue-specific expression of *klf-3* in the intestine. (a) A translational fusion construct was created by the fusion of a 2-kb promoter region upstream of the *klf-3b* ATG and its full coding sequence consisting of six exons in-frame with the *gfp* reporter (pHZ145). (b) A 1-kb promoter region upstream of *klf-1* ATG and the full-length cDNA of *fat-7* were fused in-frame with the *gfp* reporter (pHZ343). Exons are indicated as shaded black boxes, and gray boxes indicate 5' and 3' untranslated regions. Promoters and introns between exons are indicated by a continuous line. (c) Images of *klf-3b::gfp* expression in the adult worm. Transgenic lines of *C. elegans* carrying the pHZ145 construct for the *klf-3::gfp* fusion gene were generated as described previously. As shown, *klf-3b::gfp* expression (green fluorescent) is seen in intestinal segments covering the midbody and the tail region of a (c) *C. elegans* larva and a (d) young adult worm, but is absent from the gonadal region (gray area adjacent to green fluorescent are) and the vulval region (arrow). For clarity, GFP expression image is merged with differential interference contrast images. Transgenic worms were observed and paragraphed using an Axioskop 2 Plus fluorescent microscope with appropriate filter sets (magnification of 400×).

Fig. 2.

Ultrastructural analysis of the *klf-3(ok1975)* mutant worm and N2 worms. Although the *klf-3(ok1975)* mutant worm shows large lipid droplets, the morphologies of both peroxisomes and mitochondria are indistinguishable between (a) wild-type N2 and (b) *klf-3(ok1975)* mutant. Few small lipid droplets are present in the wild-type worm, with the *klf-3(ok1975)* mutant worm bearing large lipid droplets. P, peroxisome; M, mitochondria; L, lipid droplet. Horizontal scale bar represents 2 μm.

Fig. 3.

Large lipid droplets caused by defective β -oxidation. Bright-field and BODIPY images of L4 wild type (a and b) and the same developmental stage of the *klf-3(ok1975)* mutant (c and d) are shown. The presence of large lipid droplets in *klf-3* mutant worms is indicated by arrows. Briefly, 25 synchronized L4 larvae (30 h after L1 hatch) of *klf-3(ok1975)* or N2 larvae were separately transferred onto NGM OP50 plates containing BODIPY dye. After 5–10 min of feeding, animals were transferred onto a microscopic slide, and fluorescent signals in BODIPY-fed worms were observed with fluorescein isothiocyanate excitation and barrier filter sets using a 40 $\times$ objective Zeiss Axioplan 2 imaging microscope. There was no difference in fluorescence signal between 5 min and 10 min of feeding. Animals were imaged as L4 larvae at a magnification of 400 $\times$ using an AxioCam HRm camera and OpenLAB software attached to an Axioskop 2 Plus fluorescent microscope. At least two independent experiments were performed.

Fig. 4.

Mutation in *klf-3(ok1975)* is associated with high TG content. Lipid content for *klf-3(ok1975)* mutant and wild-type animals. Levels of TGs are higher in the *klf-3(ok1975)* mutant than in wild-type animals (a). TG levels increased with each developmental stage. However, there were no significant differences in the levels of total cholesterol (b), phospholipids (c), and cholesterol esters (d). Error bars indicate standard deviations. Briefly, total lipid content, comprising TGs, phospholipids, and cholesterol, was measured in four larval stages and young adults of *klf-3(ok1975)* mutant animals and compared with the same developmental stages of wild-type animals. For lipid analysis, two independent experiments were performed. Each experiment contained five samples consisting of four larval stages and young adults of both mutant and wild-type animals. The results were consistent in two independent experiments.

Fig. 5.

Epistatic relation between *klf-3(ok1975)* and other lipid utilization pathway genes. Genetic interactions between *klf-3* and *F08A8.1* or *F08A8.2* genes were assessed by fat mass measurements and reproductive parameters. Fat uptake assay using Oil Red O staining: (a) wild-type animal showing a very low fat mass; (b) elevated staining seen in *F08A8.1* and (c) *F08A8.2* RNAi animals; (d) *klf-3(ok1975)* mutant animals; and (e) *F08A8.1;klf-3(ok1975)* double-mutant animals generated by standard genetic crosses, fixed, and stained with Oil Red O. The intensity of red staining was clearly higher than either *F08A8.1* or *klf-3* single mutant. Egg-laying phenotype in *klf-3;F08A8.1* double mutants. Light microscopic images showing (f) wild-type N2; (g) *F08A8.1* mutant that accumulates a large number of developing embryos in the gonad (continuous line); (h) *klf-3* mutant with a defect in germline development (arrows mark the position of abnormal germline; mutant hermaphrodites display a deteriorated gonad, as shown by the continuous lines); (i) *F08A8.1;klf-3* double mutant laying few normal eggs initially but later exhibiting egg-laying arrest resulting in the accumulation of a large number of developing embryos in the gonad (continuous white line). Fecundity analysis of different genotypes was performed in two independent experiments. In each analysis, progeny production on 25–30 L4 larvae (~30 h after L1 hatch) was monitored and recorded (Table 1). (j) Quantitative analysis of fat mass indicates an almost similar fat mass density in *klf-3(ok1975)*, *F08A8.1(ok2257)*, single-mutant, or *F08A8.2* RNAi animals, but a substantial increase over wild-type animals. The *klf-3;F08A8.1* double-mutant animals show a high percentage of fat mass than either single mutant. Data are presented as percent increase or decrease in fat mass in various genotypes over wild-type animals. Each data point is expressed as mean±standard deviation.

Fig. 6.

Interaction of *acs-1* or *acs-2* genes with *klf-3* in terms of fat uptake and reproductive behavior. Oil Red O staining of (a) a wild-type animal that displays a very low fat mass, (b) a *klf-3(ok1975)* mutant, (c) *acs-1* RNAi animals, (d) *acs-2* mutant animals, and (e) a *klf-3;acs-2* double mutant shows a substantial fat increase over wild-type animals. We also examined reproductive phenotype in *klf-3;acs-2* double mutants. Light microscopic images showing (f) wild type. (g) The *klf-3(ok1975)* mutant shows a defect in germline development (arrows mark the position of the abnormal germline, and mutant hermaphrodites show a deformed gonad, as represented by the continuous lines). (h) The *acs-2(ok2457)* mutant has normal growth, development, and reproduction. (i) The *klf-3(ok1975);acs-2(ok2457)* double mutant accumulated a large number of developing embryos in the gonad. Other phenotypes included disorganized vulval muscles (black arrow) and abnormal vulval opening (white arrow). Experimentally, in brief, we performed *acs-1* RNAi by soaking synchronized L4 larvae in dsRNA to knock down *acs-1* in wild-type and *klf-3(ok1975)* mutant animals. Two sets of 20–25 synchronized *klf-3(ok1975)* L4 mutant larvae with age-matched wild-type (N2 strain) animals were separately soaked in 20 μ l of 1 $\times$ PBS containing *acs-1* dsRNA (3 μ g/ μ l) incubated at 25 $^{\circ}$ C. In controls, a similar number of synchronized L4 larvae are soaked in PBS but without dsRNA. After 20–24 h of soaking, the animals from all treatments, including controls, *acs-1* RNAi, and *acs-1* RNAi;*klf-3(ok1975)*, were separately transferred to individual NGM plates seeded with *E. coli* OP50. After 5–6 h, one set of RNAi animals of each treatment was separately transferred into 2-ml Eppendorf tubes containing PBS solution and stained. Another set of RNAi animals was observed for reproductive phenotype. We generated *klf-3(ok1975);acs-2(ok2457)* double-mutant animals by standard genetic cross. To measure fat mass between genotypes, we stained the animals with Oil Red O, as described previously. For each genotype, a total of 60 animals were observed with a 40 $\times$ objective microscope and imaged at a magnification of 400 $\times$. Images are representative of 60 animals. Two independent experiments were performed to assess the fecundity of mutants. In each analysis, progeny production on 25–30 L4s (~30 h after L1 hatch) was monitored and recorded. To measure brood size, we individually transferred 25 RNAi animals onto fresh

NGM plates containing OP50 every 24 h and counted the hatched progeny. Total brood size was determined by adding progeny produced across 4–5 days. Two independent but identical RNAi analyses were performed.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 7.

Interaction of the *fat-7(wa36)* mutant with *klf-3(ok1975)* in fat uptake. Oil Red O staining of (a) wild-type N2 strain showing normal low fat uptake; (b) high fat accumulation in the *klf-3(ok1975)* mutant; (c) *fat-7(wa36)* mutant animal showing a very low fat accumulation comparable to that of wild type; and (d) *klf-3(ok1975);fat-7(wa36)* double mutant showing a fat phenotype similar to that of the *klf-3(ok1975)* mutant but much higher than that of the *fat-7(wa36)* mutant animal. Animals were observed under Nomarski optics attached to a Nikon Eclipse 80i light microscope, and photographs were taken with a Photometrics CoolSNAP cf digital camera (magnification of 400×). (e) Quantitative analysis of fat mass indicates high fat mass density in *klf-3(ok1975)* but not in *fat-7(wa36)* animals, while *klf-3(ok1975);fat-7(wa36)* animals show fat increase similar to that of the *klf-3(ok1975)* single mutant. Data are presented as percent increase or decrease in fat mass in various genotypes over wild-type animals. Each data point is expressed as mean±standard deviation.

Fig. 8.

Schematic drawing of gene interactions and their role in fat accumulation and reproduction. Model for *klf-3*-mediated regulation of fat accumulation and reproduction. *klf-3* integrates the expression of FA β -oxidation pathway components. Transcriptional regulation of *F08A8.1*, *F08A8.2*, *acs-1*, and *acs-2* by *klf-3* contributes to FA β -oxidation. Through an independent pathway, *klf-3* modulates FA desaturation by enhancing the expression of *fat-7*.

Table 1

Fecundity and reproduction phenotypes in various genes in the FA metabolism pathway

Genotype	Average number of progeny of a hermaphrodite	Phenotype	
		Reproduction	Fat mass
Wild type	255±24		
<i>klf-3</i> (RNAi)	58±18	Sterile and semisterile progeny	More fat mass than wild type
<i>klf-3(ok1975)</i>	47±11	Sterile and semisterile progeny	More fat mass than wild type
<i>acs-1</i> RNAi	30±8	30% of embryos arrested (early embryos); 70% of embryos arrested at larval L1/L2 growth	More fat mass than wild type
<i>acs-2(ok2457)</i>	244±22	No apparent reproductive abnormality	More fat mass than wild type
<i>acs-2</i> (RNAi)	96±12	7% of embryos arrested (early embryos)	More fat mass than wild type
<i>acs-1/acs-2</i> (RNAi)/N2	46±7	35% of embryos arrested (early embryos)	More fat mass than wild type
<i>acs-1/acs-2</i> (RNAi)/ <i>klf-3(ok1975)</i>	91±10	5% of embryos arrested (early embryos)	More fat mass than wild type
<i>klf-3(ok1975);acs-2(ok2257)</i>	89±12	Egl (egg-laying defect); large number of eggs in the uterus	Fat mass equal to either single mutant
<i>F08A8.1(ok2257)</i>	77±12	Egl (egg-laying defect); reduced brood size	More fat mass than wild type
<i>F08A8.1(ok2257);klf-3(ok1975)</i>	73±18	Somatic gonads appear normal, but the double mutant shows egg-laying defects	More fat mass than either single mutant
<i>F08A8.2</i> RNAi	209±19	No apparent reproductive abnormality	More fat mass than wild type
<i>F08A8.2</i> RNAi/ <i>klf-3(ok1975)</i>	225±29	No apparent reproductive abnormality	Fat mass equal to the <i>klf-3(ok1975)</i> mutant
<i>F08A8.1(ok2257);F08A8.2</i> RNAi; <i>klf3(ok1975)</i>	163±22	10% of embryos die during the early stages of embryogenesis	Fat mass equal to the <i>F08A8.1;klf-3</i> double mutant
<i>fat-7(wa36)</i>	205±28	No apparent reproductive abnormality	Fat mass equal to wild type
<i>fat-7(wa36);klf-3(ok1975)</i>	74±10	Semisterile, sterile	Fat mass equal to the <i>klf-3</i> single mutant