Published in final edited form as:

J Mol Biol. 2013 August 9; 425(15): 2641–2655. doi:10.1016/j.jmb.2013.04.020.

Regulation of Lipoprotein Assembly, Secretion and Fatty Acid β -Oxidation by Krüppel-Like Transcription Factor, *klf-3*

Jun Zhang¹, Sanya Hashmi¹, Fatima Cheema², Nafla Al-Nasser³, Razan Bakheet³, Ranjit S. Parhar³, Futwan Al-Mohanna³, Randy Gaugler², M. Mahmood Hussain⁴, and Sarwar Hashmi²

¹Developmental Biology, New York Blood Center, New York, NY 10065, USA

²Developmental Biology, Center for Vector Biology, Rutgers University, New Brunswick, NJ 08901, USA

³Department of Cell Biology Cardiovascular Unit, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

⁴Department of Cell Biology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA

Abstract

Lipid metabolism is coordinately regulated through signaling networks that integrate biochemical pathways of fat assimilation, mobilization and utilization. Excessive diversion of fat for storage is a key risk factor for many fat-related human diseases. Dietary lipids are absorbed from the intestines and transported to various organs and tissues to provide energy and maintain lipid homeostasis. In humans, disparity between triglycerides (TG) synthesis and removal, via mitochondrial β -oxidation and VLDL (very low density lipoprotein) secretion, causes excessive TG accumulation in the liver. The mutation in *Caenorhabditis elegans* KLF-3 leads to high TG accumulation in the worm's intestine. Our previous data suggested that *klf-3* regulates lipid metabolism by promoting fatty acid β -oxidation. Depletion of cholesterol in the diet has no effect on fat deposition in *klf-3* (*ok1975*) mutants. Addition of vitamin D in the diet, however, increases fat levels in *klf-3* worms. This suggests that excess vitamin D may be lowering the rate of fatty acid β -oxidation, with the eventual increase in fat accumulation. We also demonstrate that mutation in *klf-3* reduces expression of *C. elegans* *dsc-4* and/or *vit* genes, the orthologs of mammalian microsomal triglyceride transfer protein and apolipoprotein B, respectively. Both microsomal triglyceride transfer protein and apolipoprotein B are essential for mammalian lipoprotein assembly and transport, and mutation in both *dsc-4* (*qm182*) and *vit-5* (*ok3239*) results in high fat accumulation in worm intestine. Genetic interactions between *klf-3* and *dsc-4*, as well as *vit-5* genes, suggest that *klf-3* may have an important role in regulating lipid assembly and secretion.

Keywords

C. elegans; fatty acid β -oxidation; *klf*; lipoprotein assembly and secretion

Introduction

Dietary lipids that include triglycerides (TG), cholesterol esters, phospholipids and essential unsaturated fatty acids (FAs) are absorbed from the small intestines and transported to various organs and tissues to maintain lipid homeostasis. The excess of ingested food is largely converted into intracellular TG deposits in lipid droplets,¹ most prominent in mammalian adipose tissues and to a lesser degree in heart, muscle and liver. Most mammals strictly regulate lipid secretion and distribution to maintain normal levels of TG in adipocytes. TG is hydrophobic and cannot be transported in circulation; it is therefore assembled into a blend of lipid and protein termed lipoproteins, which can transport hydrophobic molecules. Apolipoprotein B (apoB), a structural protein, and microsomal triglyceride transfer protein (MTP), an endoplasmic resident chaperone, are essential for the assembly and secretion of TG-loaded lipoproteins.^{2–4} The assembly and secretion of apoB-containing lipoproteins requires MTP; inhibition of MTP has been shown to decrease lipoprotein secretion, increasing the accumulation of lipids in the liver.^{5,6} The *Caenorhabditis elegans dsc-4* encoding 892-residue protein is orthologous to the large subunit of the mammalian MTP.⁷ The *C. elegans dsc-4* has also been shown to support secreted apoB48 in Cos-7 cells.^{3,8} The *dsc-4 (qm182)* mutant suppresses the slow defecation⁹ and germline phenotypes⁷ of *clk-1 (e2519)* mutant. This suggests that lipoprotein assembly in intestine might be critical in germline development in the mutant defective in ubiquinone synthesis.

In mammals, there are two extensively studied apoBs; apoB100 is present in the liver, whereas apoB48 is localized in the intestine.^{10–12} Studies of apoB components of knockout mice have indicated that the assembly/secretion pathway of apoB-containing lipoproteins has a critical role in the transport of fat-soluble nutrients to the yolk sac.¹³ There are five *vit* (*vit-1*–*vit-5*) genes in *C. elegans* genome. These *vit* genes are lipid-binding protein precursor related to vertebrate vitellogenins and mammalian apoB-like genes.^{7,14,15} Although the role of *vit-1*, *vit-2* and *vit-3* genes in lipid transport is not clear, *vit-4* and *vit-5* are predicted to function as a lipid transport protein (Wormbase[†]). These genes are female specific and express in the *C. elegans* hermaphrodite intestine. The *vit-3*, *vit-4* and *vit-5* gene sequences are nearly identical and the effect of *vit-1*, *vit-2*, *vit-3* and *vit-4* RNA interference on wild types varies from no phenotype to embryonic lethality.^{16–19} Large-scale RNA interference screens, however, indicate that VIT-5 is required for embryogenesis and normal rates of postembryonic growth (17).

In previous studies, we have characterized a worm mutant *klf-3 (ok1975)* with substantial deletion in *klf-3* gene. We found that *klf-3* worm not only contained excess TG levels in its intestine but also developed defects in fertility.²⁰ We do not know precisely how excess

[†]www.wormbase.org

accumulation of TG can bring about fertility defect in the *klf-3* mutant. One possibility is that yolk protein, which serves as the nutrient for *C. elegans* developing oocytes, is not transported to the gonad. The *C. elegans* yolk is a lipoprotein particle complex composed of vitellogenin, TG, phospholipids and cholesterol.²¹ It is synthesized in the intestine of adult hermaphrodites secreted into the pseudocoelomic space.²² It is then endocytosed by receptors of the LDL receptor superfamily, on the surface of growing oocytes. Finally, it is taken up into vesicles (yolk granules) within the growing oocytes by a receptor-mediated endocytotic pathway. Yolk transport in vertebrate is similar to worms.²⁴ Since our finding that the level and localization of yolk protein in mutant oocytes and early embryos is similar to that in wild type, we suggest that fertility defects in *klf-3* mutant are not due to abnormality in yolk endocytosis.

The profound effect of *klf-3* mutation on the accumulation of TG suggests that *klf-3* functions to limit fat storage and plays a role in its mobilization to other tissues. Whether this observation is valid for the role of mammalian KLFs in these processes is not yet known. In human, disorders in lipid metabolism or an imbalance between lipogenesis and lipoprotein assembly and secretion can lead to hyperlipidemia and/or hepatic steatosis. In the current study, we observed a considerable reduction in the expression of *vit* and *dsc-4* genes in *klf-3* mutant. The possibility therefore exists whether the reduced expression of *vit* and *dsc-4*, the worm orthologs of mammalian apoB and MTP, respectively, could be a limiting factor for TG accumulation in the mutant worms. In the current study, we addressed two questions. First, we extended our studies on the role of *klf-3* in regulating β -oxidation. Second, we asked whether *klf-3* acts on *dsc-4* and/ or *vit-5* gene in the lipid secretory pathway.

Results

KLF-3 protein distribution

Rabbit anti-KLF-3 antibodies were raised against recombinant KLF-3 protein and used the purified KLF-3 antibodies to stain embryos, larvae and adult animals. In the *klf-3* mutant animals, either the KLF-3 protein was undetected or its expression was very low (data not shown). In wild-type worm, KLF-3 protein was undetectable in embryos. However, KLF-3 expression was evident in all larval stages (Fig. 1a–c), as well as in adult (Fig. 1d). Microscopic observation of animals at larval and adult stages showed that level of KLF-3 expression was constant in each developmental stage. Throughout worm development, the anti-KLF-3 antibodies detected KLF-3 protein in the intestine, where it was most abundant (Fig. 1a–d). We also observed KLF-3 protein in both arms of the germline (Fig. 1e–g). In previous experiment, we observed intense expression of KLF-3:GFP (green fluorescent protein) translational construct along the length of intestine, and *gfp* expression was not detected in germline.²⁰

Mutation in *klf-3* causes reduced expression of *dsc-4*, *vit* and some *daf* genes

We have previously reported that *klf-3* mutant worms contain higher levels of TG. We therefore hypothesized that *klf-3* mutant may have high TG because normal secretion of lipids may have been disturbed in the mutant animals. The mammalian apoB and MTP are

necessary for lipid assembly and secretion. Therefore, we measured their *C. elegans* orthologs, *vit* and *dsc-4* genes in *klf-3* mutant, and compared their mRNA levels with wild-type animals. We found that the expressions of *dsc-4* (5-fold); *vit-1* (3-fold); *vit-2*, *vit-3* and *vit-4* (10-fold); and *vit-5* (15-fold) were significantly reduced in *klf-3* mutant (Fig. 2). This suggested that, perhaps, the reduced expression of *dsc-4* and/or *vit* genes affected the normal assembly and secretion of lipid in the mutant worms, thereby increasing TG accumulation. While our real-time PCR (RT-PCR) data show no change in the levels of *daf-12*, it indicates a substantial reduction (6-fold) in the mRNA levels of *daf-16*, the two genes involved in β -oxidation (Fig. 2).

***klf-3* and *dsc-4* are likely to act in a different pathway**

The *dsc-4* (*qm182*) mutants display 40% higher lipid contents than wild type in the intestine (Fig. 3c and e) similar to that seen in *klf-3* mutant (*ok1975*). Furthermore, KLF-3:GFP²⁰ and, as reported by Shibata *et al.*,⁷ *dsc-4:gfp* transcriptional and DSC-4:GFP translational construct are all expressed in the intestine throughout larval stages and adulthood. Further, their mutant *klf-3* (*ok1975*) and *dsc-4* (*qm182*) accumulate similar levels of fat. Because of these overlaps in the expression pattern of both *klf-3* and *dsc-4* genes and the fat phenotype of their respective mutants, we suspected that both genes might act in the same pathway to regulate fat deposition. To investigate this possibility, we constructed double mutants between the strains that were homozygous for the *klf-3* (*ok1975*) deletion and homozygous for *dsc-4* (*qm182*) and assayed for fat deposition in the progenies of *klf-3* (*ok1975*) and *dsc-4* (*qm182*) double mutant by Oil-Red-O staining. We found that the double mutant apparently accumulated approximately 35% more fat (Fig. 3d) than either *klf-3* (*ok1975*) or *dsc-4* (*qm182*) and 80% more fat accumulation (Fig. 3b–e) than wild type. This suggested that *klf-3* and *dsc-4* act additively to regulate fat storage.

dsc-4* is required for the normal expression of *klf-3

We also examined whether *dsc-4* is required for *klf-3* expression or vice versa. We examined the localization of the KLF-3 proteins in the intestine of *dsc-4* (*qm182*) mutant. We found that KLF-3 expression was diminished in *dsc-4* (*qm182*) mutant intestine (Fig. 1h–j), suggesting that *dsc-4* might positively regulate *klf-3*. The intensity and the level of KLF-3 expression were also diminished in the germline of *dsc-4* (*qm182*) mutant (Fig. 1k), suggesting that *dsc-4* may regulate *klf-3* in both intestine and germline.

Consistent with our observation that mutation in *klf-3* leads to reduction in the mRNA levels of *dsc-4* and *vit* genes, this suggests that lipoprotein assembly and secretion may have been disrupted in the mutant worm and rescuing the *klf-3* (*ok1975*) mutants by these proteins should bring the fat accumulation to a level comparable to wild-type worm. However, we found that restricted expression of *C. elegans* *dsc-4* or mammalian MTP in the intestine was weak and did not reduce the fat content in the *klf-3* mutant. Substantial *dsc-4* or MTP activity may be required to allow fat rescue in the intestine.

Eliminating *vit-5* activity has no effect on *klf-3* mutant fat phenotype

We examined the effects of *vit* mutation that would likely affect the lipoprotein assembly and secretion mechanisms. We examined *vit-1* (*ok2616*), *vit-2* (*ok3211*), *vit-3* (*ok2348*), *vit-4*

(*ok2982*) and *vit-5* (*ok3239*) mutants (kindly provided by *C. elegans* Genetics Center) for their fat phenotype. Prior to analyzing these mutants, we out-crossed each mutant at least 3× and confirmed the deletion allele by PCR followed by sequencing the PCR products. The deletion has removed extensive blocks of *vit* coding sequence in *vit* mutants[‡]. We found that *vit-5* (*ok3239*) mutant accumulated 40% higher fat than wild type (Fig. 4g and i) as indicated by Oil-Red-O staining and that all other *vit* mutants had a fat staining almost equal to or slightly greater (*vit-2* and *vit-4*) than wild type (Fig. 4a–e). Given that both *klf-3* (*ok1975*) and *vit-5* (*ok3239*) produce a fat phenotype, we asked whether *vit-5* can enhance or reduce the fat phenotype of *klf-3* mutant. We created *klf-3; vit-5* double mutant by standard genetic crosses and assayed for fat deposition in the mutant animals. We found that *klf-3; vit-5* double mutants accumulate similar amounts of TG as individual mutants (Fig. 4g and i), suggesting that they are targeting the same molecular pathway of TG control.

The intestinal fat content in *klf-3* mutant was reduced in 31% of worm ($N = 73$), indicating a partial rescue by *vit-5* expression in the intestine. These data support the interpretation that *klf-3* and *vit-5* in the intestine may control lipid mobilization.

Cholesterol deficiency in diet has no effect on fat deposition in *klf-3* (*ok1975*) mutant

In our study, we investigated whether cholesterol depletion or supplementation altered fat content or the brood size in the *klf-3* (*ok1975*) mutant. We found that cholesterol deficiency in the culture media had a suppressive effect on the growth and brood size of both wild-type and *klf-3* worms. On the other hand, cholesterol supplementation had a positive effect on the overall growth and development of wild-type worm but had no effect on the *klf-3* worm. We also found that elimination of cholesterol or its addition in the culture media had little effect on fat levels in the *klf-3* worm (Fig. 5a–e).

Vitamin D supplement in diet increases fat mass in *klf-3* mutant

Vitamin D has been known as an important regulator of FA β -oxidation. However, there have been conflicting reports about the effect of vitamin D on fat accumulation.^{25,26} To gain insight into the effect of vitamin D on fat accumulation and the mechanism underlying the distinctive effects of *klf-3* mutation in FA β -oxidation, we performed an analysis of fat level in both wild type and *klf-3* mutant fed on vitamin D incorporated *Escherichia coli* op50. We found that, after 30 min of feeding on vitamin D/ *E. coli* op50 (Materials and Methods), both wild-type and *klf-3* mutants increased their fat levels in the intestine. We suggest that feeding worm with vitamin D diet may have affected lipid catabolism, resulting in high fat accumulation in both wild-type and *klf-3* mutant worms (Fig. 6c and d). These data support an earlier study in mouse model where increase in fat mass has been correlated with increased vitamin D and reduced FA β -oxidation²⁵ but do not match with a clinical data suggesting low vitamin D predispose to fat accumulation.²⁶

Yolk transport is independent of *klf-3*

Yolk is essential for embryonic development, and lipids are major components of yolk granules in the oocytes.^{23,27} During their transport to the oocytes, yolk proteins are

[‡]<http://legacy.wormbase.org>

associated with cholesterol, which is distributed among the intracellular membranes of developing eggs after endocytosis.²¹ To examine if the sterility in the *klf-3* animals is due of unavailability of yolk to the developing oocytes, we used *C. elegans* strain DH1033 that contains *C. elegans* yolk protein gene *YP170* fused to a green fluorescent reporter gene (*YP170::GFP*). Previous characterization of DH1033 showed that, like endogenous yolk protein, the YP170::GFP protein is transported from the hermaphrodite intestine to the gonad for endocytosis by oocytes (23). If *klf-3* is required for endocytosis, it might be expected that there would be no uptake of YP170::GFP into mutant oocytes and GFP would accumulate in the pseudocoelom. Homozygous *klf-3* (*ok1975*) mutants carrying the *YP170::GFP* transgene were generated by standard genetic crosses, and GFP levels and distribution were compared to that in wild-type DH1033. As expected, in wild-type DH1033 hermaphrodites, GFP was observed in the adult intestine, late-stage oocytes and embryos (Fig. 7a). In homozygous *klf-3* (*ok1975*) mutants, YP170::GFP was also observed in the adult intestine, in late oocytes and in embryos (Fig. 7b). The level and localization of GFP in mutant oocytes and early embryos was similar to that in wild-type DH1033, and there was no apparent accumulation of GFP in the pseudocoelom. KLF-3 is, therefore, not required for yolk endocytosis. Thus, the germline defects as observed in *klf-3* (*ok1975*) mutant are not due to defects in yolk endocytosis.

Sterility is due to defects in both male and female germ cells

klf-3 (*ok1975*) mutant hermaphrodites are both sterile and semi-sterile. To determine the mating efficiency of *klf-3* animals, we generated a homozygous line of *klf-3* (*ok1975*) males. These males were then mated with wild-type or *klf-3* hermaphrodites. One out of 43 F1s from a mating between *klf-3* (*ok1975*) males and wild-type hermaphrodites was sterile, while 42 F1s were semi-sterile and each animal on average produced 88 progenies. Defective *klf-3* sperm cells appear to contribute to the sterile phenotype because mating with wild-type males suppresses the sterile phenotype of the *klf-3* (*ok1975*) mothers. We also found that 65% of all unmated *klf-3* mutants worms were sterile. The mating between wild-type male and *klf-3* (*ok1975*) hermaphrodites also increased the average brood size to 134 progenies per animal ($N = 45$). The semisterile F1 progenies of *klf-3* mutant mothers normally produce ~40 progenies.²⁰ All F1s from a cross between wild-type hermaphrodites and wild-type male were fertile ($N = 19$). Although frequency of breeding was low (1/6), homozygous *klf-3* hermaphrodites were able to cross with homozygous *klf-3* males, producing ~127 progenies, which suggests an inefficient mating between *klf-3* males and *klf-3* hermaphrodites. After two to three generations, however, males and hermaphrodite progenies obtained from this mating (homozygous male *versus* homozygous hermaphrodites) were unable to maintain their further generation (Fig. 8).

It has also been known that polyunsaturated fatty acids (PUFAs) are transported from intestine to the growing oocyte yolk, which is a lipoprotein complex. Loss of receptor-mediated endocytosis-2 LDL receptor, which mediates yolk endocytosis²³ and FA transport into oocytes, causes severe defects in sperm targeting. PUFAs function in oocytes to control directional sperm motility within the uterus.²⁸ We examined whether disruption in the ratio between PUFAs and saturated FAs²⁰ affected the directional sperm motility within the uterus. Although the breeding frequency was inefficient, we found almost the same number

of progenies, whether *klf-3* male mated with the wild-type worm or with *klf-3* hermaphrodites. This suggested that mating were successful. Our breeding experiment, however, is not sufficient to conclude whether or not the disruption in the ratio between PUFAs and saturated FAs affects sperm targeting in the *klf-3* mutant.

Discussion

We have previously reported that KLF-3 α GFP specifically expressed uniformly in the intestine but is absent from germline during *C. elegans* development.²⁹ In this study, we confirm the expression of KLF-3 protein in the intestine of all larval stages and adult worm as detected by antibody. *C. elegans* intestine is known to be a major site of fat metabolism.^{30,31} The presence of KLF-3 protein in the intestine is consistent with its regulatory function in fat metabolism.³² However, in addition to its expression in intestine, KLF-3 protein is also present in the germline and supports its important roles in both fat metabolism and germline development. Our previous examination of the *klf-3* (*ok1975*) sterile mutant indicated that both the germline and the oocyte region of the gonad arm were filled with small morphologically abnormal cells in the mutant worms. The semi-sterile *klf-3* worms also appeared normal in fertilization and egg laying, but their oogenesis became impaired after 40–50 oocyte-sperm fusion events.²⁰ The presence of KLF-3 in germline is consistent with the necessity of KLF-3 in the germline development. The difference between KLF-3 α GFP expression and detection of KLF-3 by antibody may indicate a difference in post-transcriptional regulation between the endogenous and KLF-3 α GFP proteins.

In mammals, the KLF proteins have been implicated in pre-adipocyte formation, adipogenesis and lipogenesis.^{33–39} However, their role in either FA β -oxidation or lipid secretion is not known. Previous work in our laboratory led to suggest that KLF-3 regulates multiple target genes and coordinately tunes several lipid metabolic pathways to block TG accumulation in the intestine. This is supported by our genetic studies on a *klf-3* (*ok1975*) deletion mutant, which shows a strong genetic interaction of *klf-3* with the genes encoding fat metabolic enzymes, that is, acyl-CoA (coenzyme A) synthetase (*acs-1* and *acs-2*), acyl-CoA oxidase (*F08A8.1* and *F08A8.2*) and stearoyl-CoA desaturase (SCD *fat-7*). These enzymes are predicted to function in pathways of FA β -oxidation in mitochondria or peroxisome and FA synthesis in cytosol.³¹

The increase in fat mass has been correlated with reduced FA β -oxidation and lipolysis in the adipose tissue in the transgenic mice over-expressing human (h) vitamin D receptor in adipocytes,²⁵ and these mice also developed obesity. The vitamin D receptor-null mutant mice have lean phenotype and lower plasma TG and cholesterol levels compared to wild-type mice. The rate of FA β -oxidation in these mice was higher than wild-type mice, suggesting high energy expenditure in the mutant mice and the importance of vitamin D in energy metabolism through the regulation of β -oxidation.⁴⁰ Based on the observation that *klf-3* mutants fed on vitamin D increased their fat deposit, we reasoned that excess of vitamin D may have lowered the rate of FA β -oxidation, resulting in increased fat accumulation. If this is true, then functional *klf-3* interaction with *C. elegans* vitamin D receptor, *daf-12*, may be required to mediate the effect of vitamin D on FA β -oxidation. However, our microarray data and subsequent RT-PCR analysis on *klf-3* (*ok1975*) mutant

did not show any alteration in *daf-12* gene expression (Fig. 2). Thus, increased fat content in the vitamin D fed *klf-3* worm is independent of *klf-3* interaction with *daf-12*, indicating that other pathways are involved in metabolic changes elicited by *klf-3* deficiency.

Further, to investigate the mechanism by which *klf-3* influence mitochondrial activity to modulate β -oxidation, we analyzed cellular mitochondrial content in *klf-3* (*ok1975*) mutant animals. We observed that *klf-3* deficiency reduced mitochondrial proliferation (data not shown). Thus, alteration in mitochondrial morphology is likely to affect its activity. Reduced mitochondria and abnormal mitochondrial morphology is associated with FOXO transcription factor; FOXO factor inhibits mitochondrial proliferation in adipose tissue of mice.⁴¹ However, our microarray and subsequent RT-PCR analysis of *klf-3* worm indicates a substantial reduction in the mRNA levels of *daf-16*, the *C. elegans* ortholog of mammalian FOXO transcription factor.⁴² This indicates that *klf-3* may interact with *daf-16* in fat metabolism, but its role in mitochondrial proliferation may be *daf-16* independent. As mitochondria are a center of energy production, it may be affected by changes in nutrient availability, enzymatic reaction and high fat accumulation. In vertebrate, increased lipogenesis may cause dual metabolic alterations: directly (through increased hepatic TG accumulation) and indirectly (through the inhibition of CPT-1 and blockade of FA entry into the mitochondria and reducing β -oxidation).⁴³ We did not notice any changes in *C. elegans* *cpts* expression in our RT-PCR analysis of *klf-3* mutant (data not shown), suggesting that *klf-3* deficiency may not have affected FA entry into mitochondria. We suggest that elimination of *klf-3* activity deregulates *dsc-4* and *vit* genes and thereby affecting lipid secretion pathway that results in high TG accumulation. That leads to mitochondrial dysfunction and reduced catabolism.

In human, excessive TG accumulation in the liver results from an imbalance between TG synthesis and removal via mitochondrial β -oxidation and VLDL (very low density lipoprotein) secretion. Cholesterol is vital for cellular function and a precursor of steroid hormones, vitamin D and bile acids; its depletion is known to reduce the levels of LDL. ApoB is the main component of LDL. *C. elegans* does not synthesize cholesterol de novo. Thus, to grow them in laboratory, we supplement the nutrient agar with cholesterol. Hence, elimination of cholesterol under the condition of reduced secretion will further increase TG levels in *klf-3* mutant. However, under the present experimental condition, cholesterol depletion in the diet had little effect on the fat levels in the intestine of *klf-3* mutants. Our data suggest that either TG accumulation in *klf-3* worm is independent of cholesterol or the effect of cholesterol depletion on fat level was too little to detect in the mutant worm.

The *C. elegans* *dsc-4*, *vit* genes and *klf-3* all expressed in the intestine and their mutations result in increased fat accumulation, suggesting that these genes not only are involved but also may possibly interact during fat metabolism. Could these worm orthologs have similar function in lipid assembly and secretion as their mammalian counterpart? If so, then what is their relationship with *klf-3*? The first clue of *klf-3* interaction with *dsc-4* and *vit* genes came from our RT-PCR data that indicated a significant reduction of *dsc-4* and *vit* genes transcripts in the *klf-3* animal. One possible interpretation of this result is that normal function of *klf-3* is to promote both *dsc-4* and *vit* expressions in intestinal cells. *C. elegans* DSC-4 has been shown to support secretion of apoB48 in Cos-7 cells,^{3,8} suggesting that

worm ortholog of MTP and apoB may have a similar function. The recognition of *dsc-4* and all *vit* genes as potential targets of KLF-3 regulation prompted us to explore their genetic interactions, given the direct link to lipoprotein assembly and secretion pathway. We found that animal carrying a single mutation of *dsc-4* or *vit-5* or, to some extent, *vit-2* leads to fat increase compared to wild-type animals. This implies that these pathways cooperate to control specific function in lipid metabolism. We analyzed *dsc-4; klf-3* and *vit-5; klf-3* double mutants to study *klf-3* interactions with these genes. We noticed that the *klf-3; dsc-4* double mutants have twice as much fat as individual mutants. Perhaps a combination of defect in lipoprotein secretion less β -oxidation might explain higher fat in double mutants. Thus, both *dsc-4* and *klf-3* genes are likely to act together or in succession to promote lipoprotein secretion. Eliminating *vit-5* gene activity in *klf-3* mutant does not, however, alter fat content in *klf-3; vit-5* double mutant, implying that both genes function in the same pathway.

We also examined the underlying mechanism of fertility defects in *klf-3* mutant animal by breeding experiments. We found that *klf-3* males were much less efficient in producing cross progeny, suggesting that the male sperms were not 100% capable of fertilizing the oocytes. The semi-sterility in *klf-3* mutants also appeared to be due to defective oocytes and/or somatic gonad. This suggested that *klf-3* may have a role in either somatic gonad or oocyte development. Hence, the function of *klf-3* may not be restricted to hermaphrodite intestine. In fact, our present immunofluorescence assay (IFA) data showing the presence of KLF-3 protein in both intestine and germline attest that it functions in a process common to both the germline and intestines. Unlike *klf-3*, which is expressed in both hermaphrodite and male intestine, the vitellogenin genes are expressed in the adult hermaphrodite intestine but not in the male intestine.²² We tested if KLF-3 could function in the synthesis and/or endocytosis of the yolk protein to provide nutrient for the developing oocytes. To do this, we created a *vit-2:gfp; klf-3* transgenic line by introducing *vit-2:gfp* in *klf-3* mutant background. We found that the *gfp* expression patterns of *vit-2:gfp* in the *vit-2 Δ gfp; klf-3* transgenic line was indistinguishable from DH1033 (*vit-2 Δ gfp*) transgenes, suggesting that receptor-mediated endocytosis occurs normally in *klf-3* mutant. These data also demonstrate that fertility defect in the mutant worm was independent of yolk endocytosis from intestine to gonad. Thus, the morphologically abnormal oocytes and disorganized gonads in the *klf-3* animal may be due to insufficient or probably irregular nutrient available to developing oocytes. The high lipid stores probably created an extreme nutrient condition in the yolk, simultaneously a reduction of vitellogenin restricted essential component of yolk, affecting further oocyte development. Understanding the relationship between fat accumulation and the signals that regulate reproduction may provide insight into the significant changes in the utilization of stored lipids that may occur during reproduction. *klf-3* may have a specific role in the male worm, for instance, a regulatory function in male germline development. Further genetic and molecular analyses of KLF-3 will determine the functions of KLF-3 in germline in more detail. Some KLFs are known to be involved in reproduction. For instance, in mice, KLF-4 is expressed in both female and male reproductive tracts and performs important role in mouse reproductive functions.⁴⁴ Mouse testicular cells make intensive use of alternative polyadenylation of *klf-4* mRNA suggesting translational regulation of the *klf-4* message in spermatids.⁴⁵ Shibata *et al.* suggest that lipoprotein assembly by intestine might be critical

in germline development in *clk-1* mutants, which are defective in ubiquinone synthesis.⁷ Our observation that *klf-3* mutants store fat in the intestine, resulting in aberrant activity of *dsc-4* and *vit* genes, and show severe reproductive defects indicates that lipoprotein assembly and secretion by the intestine might be critical for normal reproduction.

Because *C. elegans* intestine is the site of fat metabolism and yolk synthesis, its normal digestive and metabolic activities are important for the growth and development of the worm. We propose that, under normal conditions, *klf-3* limit fat storage by promoting its secretion and that elimination of *klf-3* may deactivate or suppress the activity of *dsc-4* and *vit* genes, resulting in defective assembly and/or secretion response that contributes to mitochondrial dysfunction. The mitochondrial dysfunction may be responsible for reduced β -oxidation (Fig. 9). In human, mitochondrial function with regard to energy balance is important in normal physiology and cellular function and its dysfunction leads to insulin resistance. Our study provides an invertebrate model to explore the physiology of lipoprotein assembly and secretion. It substantiates the emerging picture of the evolutionary conservation between worm and mammalian lipid transport regulation.

Materials and Methods

Strains maintenance

All *C. elegans* strains used in this study were maintained and propagated at 20 °C unless otherwise noted. The wild-type strain was *C. elegans* variant Bristol, N2 cultured in small plates containing nematode growth medium (NGM) and seeded with the *E. coli* strain op50.⁴⁶ The other strains used were RB1603 *klf-3* (*ok1975*), MQ920 *dsc-4* (*qm182*), RB1982 *vit-1* (*ok2616*), RB2365 *vit-2* (*ok3211*), RB1815 *vit-3* (*ok2348*), RB2202 *vit-4* (*ok2982*), RB2382 *vit-5* (*ok3239*), and DH1033 (*bIs1*[*vit-2ΔGFP, rol-6; sqt-1* (*sc103*)]). All strains were provided by *C. elegans* Genetics Center (Minneapolis, MN), funded by the National Institutes of Health National Center for Research Resources. All mutant strains were separately backcrossed 3× using wild-type N2 (Bristol) strain males according to a standard protocol⁴⁷ and maintained as homozygous worms. The genomic deletion or mutation in each mutant strain used in this study was determined by nested PCR followed by sequencing the PCR products. We note that strain RB2382 listed in Wormbase² as *vit-5* (*ok3239*) mutant allele with a 700-bp deletion indeed matches with *vit-4* sequence with a 672-bp deletion. However, for this study, we retained the name *vit-5* as listed in Wormbase. After confirmation of the deletion, the individual homozygous mutant hermaphrodites were grown on plates at 20 °C and their self-progeny were used in subsequent analysis. For genetic crosses, a homozygous line of *klf-3* (*ok1975*) males was obtained by crossing wild-type male to homozygous *klf-3* (*ok1975*) hermaphrodites; the heterozygous males obtained from this mating were crossed to homozygous parents. The homozygous males and hermaphrodites were confirmed by single worm PCR. In each genotyping, we performed single worm PCR followed by sequencing the PCR products on 20 animals. We validated the genotype from ~20 animals from a homozygous double mutant.

Rescue of the fat phenotype of *klf-3* (*ok1975*) mutant by germline transformation

To rescue the *klf-3* mutation by complementation, we created several rescue constructs in which 2-kb upstream sequence from *C. elegans klf-1* ATG start codon⁴⁸ was fused to the cDNA of *C. elegans dsc-4* or *vit-5* in *C. elegans* pPD95.67 vector backbone. To determine the functional homology between *C. elegans* and mammalian genes, we made two constructs in which the complete cDNA of mammalian MTP and apoB48 replaced the *C. elegans dsc-4* or *vit-5* genes in pPD95.67 vector. The cDNA clones of mammalian MTP and apoB^{3,8} were obtained from Hussain laboratory. The *klf-1* promoter was chosen over *klf-3* promoter because it primarily directs intestinal expression but avoids competition from the latter. Transgenic lines were generated through germline transmission. Briefly, 50–80 ng/μl of the test plasmid was mixed with 50 ng/μl of a selection marker, pRF4 plasmid, and injected into the early-adult *klf-3* (*ok1975*) mutant hermaphrodite gonads.⁴⁹ F1 generation of injected animal was selected for roller phenotype and observed for *gfp* expression. The F1 and subsequent F2 generations were tested for fat accumulation using Oil-Red-O staining as described below. The absence of fat staining in transgenes indicated that these worms were rescued.

Antibody production, immunostaining and microscopy

Full-length *Ce-klf-3* cDNA was amplified by PCR and cloned into the BamHI and SalI sites of the pET-28a expression vector (Novagen). The recombinant His tag *Ce-KLF-3* protein was expressed in *E. coli* strain HB101. Because the recombinant protein was essentially expressed in the form of inclusion bodies, it was therefore purified as follows: bacterial cells from a 500-ml culture were harvested and lysed by sonication in 40 ml of 50 mM Tris-HCl buffer, pH 8.0. The insoluble proteins were recovered by centrifugation at 12,000g for 15 min and re-sonicated in the same buffer one more time. After centrifugation, the pellet was suspended in 20 ml of 6 M urea in 50 mM Tris-HCl buffer, pH 8.0, and incubated at 37 °C for 10 min followed by centrifugation at 14,000 rpm for 20 min. The soluble fraction was then subjected to preparative separation using the Prep Cell (Bio-Rad, Hercules, CA) according to the manufacturers' instructions. Specific fractions containing the purified recombinant protein were identified by Western blot using antibodies against GST (glutathione *S*-transferase) and pooled. The KLF-3 protein was then cleaved from the GST fusion peptide by thrombin according to the manufacture's recommendation. A rabbit anti-serum was raised against the purified full-length KLF-3 recombinant protein. The anti-serum strongly reacted with the recombinant KLF-3 protein.

For the detection of KLF-3 proteins in *C. elegans* wild type [*klf-3* (*ok1975*) or *dsc-4* (*qm182*) mutant embryos], mixed larvae and adult worms, we used standard IFAs, which allow the determination of the specific locations of the KLF-3 native protein. The procedures for sample preparation and staining were essentially the same as described previously by Hashmi *et al.*^{50,51} In brief, *C. elegans* wild-type *klf-3* or *dsc-4* gravid hermaphrodites were washed with phosphate-buffered saline (PBS) and cut open to release the embryos. The embryos were then fixed in methanol/acetone using the freeze-cracking protocol,⁵² and the whole-mount fixation of the animals was performed using a modified protocol by Finney and Ruvkun.⁵³ The fixed embryos or the permeabilized whole worms were treated with 1% bovine serum albumin for 1 h before reaction with antibodies. The

embryos or mixed stage larvae and adult worms were incubated with a mixture of rabbit anti-KLF-3 antibodies at a dilution of 1:200. The samples were incubated with the antibodies at 4 °C overnight. After washing, we incubated the samples with fluorescein-isothiocyanate-conjugated goat anti-rabbit (1:250). Embryos were incubated with secondary antibodies for 2 h at 37 °C, while the larvae and adult worms were incubated at 4 °C overnight. Specimens were mounted on 2% agarose on glass slides with 15 µl of mounting medium, VECTASHIELD containing 4',6-diamidino-2-phenylindole (DAPI), and viewed under Axioskop 2 plus fluorescence microscope (Zeiss, Germany) using appropriate filter sets. The experiment was repeated once with substantial number of embryos, larvae and adult in each experiment.

RT-PCR analyses

A synchronized young adult population of both *klf-3* (*ok1975*) and wild-type (N2-Bristol) strains were separately grown at room temperature (22 ± 1 °C) on 10–12 NGM plates (15 mm × 60 mm) seeded with *E. coli* op50 bacteria. They were collected by washing off plates in PBS buffer to get rid of bacterial debris. Total RNA from approximately 15,000 young adult worms was prepared using Trizol™ reagent, and then the cDNA was prepared from 2 µg of total RNA in a 50-µl-volume reaction.²⁰ We used qRT-PCR to measure the expression level of target genes in wild-type and *klf-3* (*ok1975*) mutant worms with gene-specific primers, designed to amplify each test genes along with control primers to amplify 18S ribosomal RNA, *tbb-2* (β-tubulin) and *ubc-2* (ubiquitin-conjugating enzyme, E2) housekeeping genes. The specificity of each amplicon was confirmed by agarose gel electrophoresis, and the relative level of each gene transcript was calculated by the comparative Ct method (Applied Biosystems). The copy number of the transcript within cDNA preparations and the standard curves for test genes and the controls were drawn to allow the relative quantification of each gene expression in the *klf-3* mutant worm. The expression of transcripts in wild type *versus* *klf-3* (*ok1975*) mutant is presented as the mRNA abundance of each gene, relative to control genes.

Genetic analyses

To study epistasis, we selected *dsc-4* (*qm182*) and *vit-5* (*ok3239*) mutants to study their interaction with *klf-3* (*ok1975*). Double mutants between *klf-3* (*ok1975*) and *dsc-4* (*qm182*), as well as *klf-3* (*ok1975*) and *vit-5* (*ok3239*) mutants, were created by standard genetic crosses. The presence of double mutations was confirmed by single worm PCR followed by sequencing the PCR products. The double mutant animals were stained with Oil-Red-O staining as described below, and their staining was compared with the single mutants for fat accumulation. Animals were observed under Nomarski optics using a light microscope Nikon eclipse 80i, and photographs were taken with a digital camera Photometrics CoolSNAP cf (at a magnification of 400×).

Fat staining and microscopic examination of lipid droplets

We used Oil-Red-O staining to accurately measure fat mass in animals of various genotypes.³² A mixed population of 100–120 larvae (L1–L4) wild-type, single and double mutants were separately collected by washing with PBS from NGM plates to a 2-ml

ependorff tube. After collection, worms were washed twice and re-suspended with 200 μ l of PBS. Worms were fixed in 1% formaldehyde in PBS and kept frozen overnight at -8°C . After overnight incubation, the worms were passed through two freeze–thaw cycles. We added 1 ml of distilled water and mixed and pelleted the samples by centrifugation at 3000 rpm. Then, 1 ml of propylene glycol was added in the tube containing sample and left at room temperature ($\sim 20^{\circ}\text{C}$) for 20–30 min. Worms were pelleted again 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, followed by incubation at room temperature for 3–4 h with gentle shaking. After incubation time, samples were transferred to a glass well/wash plate (Pyrex plate, CAT no. 71563; Electron Microscopy Sciences, Hatfield, PA) and washed with propylene glycol. The stained worms were transferred to a 2% agarose pad on a glass slides, covered with coverslip and observed under Nomarski optics (Nikon eclipse 80i). Photographs were taken with a digital camera Photometrics CoolSNAP cf (at a magnification of $400\times$).

A total of approximately 60–70 animals from each genotype were observed using $40\times$ objectives and imaged at a magnification of $400\times$. For quantitative analysis, we measured the integrated density of Oil-Red-O staining in 10 animals from each genotype using Photoshop CS3 (extended) software. Data are presented as mean $\pm$ standard deviation. Images are representative of at least 60 animals.

***In vivo* analysis of yolk endocytosis/transport**

To test whether the fertility defects in *klf-3* mutant is caused by reduced yolk transport to developing oocytes, we used transgenic strain, DH1033 (*bIs1[*vit-2*ãGFP, *rol-6*; *sqt-1* (*sc103*)]*) expressing YP170 proteins [obtained from *Caenorhabditis* Genetic Center (23)]. *klf-3* (*ok1975*) homozygous males were crossed with an integrated line of DH1033 expressing YP170ãGFP, and the progeny obtained was observed for changes in the expression pattern of YP170ãGFP. Oil-Red-O staining was performed to measure the fat contents in DH1033; *klf-3* (*ok1975*) animals. The DH1033 strain is useful to study the yolk transportation from the intestine to the oocytes, allowing an *in vivo* analysis of secretion and endocytosis. Two independent experiments were performed yielding similar results.

Effect of cholesterol on fat deposition

In mammals, cholesterol is a major constituent of lipoprotein: reducing its intake leads to reduced levels of LDL. We tested whether *klf-3* mutant worms growing on cholesterol-free or cholesterol-supplemented media show any effect on their fat accumulation. Single adult worms from wild-type and *klf-3* (*ok1975*) mutant worms were separately placed on a modified NGM media⁴⁷ (for purity, instead of agar, we used agarose in the medium) containing $1\times$ cholesterol, $10\times$ cholesterol and on modified NGM plates without cholesterol. Thirty L1 larvae from their F1 generation were transferred again onto fresh NGM or cholesterol-restricted NGM culture plates and examined for their viability and reproduction. Following reproduction, we collected and stained their F2 generations with Oil-Red-O stain. Worms were observed under Nomarski optics attached to a light microscope (Nikon eclipse 80i). The experiments were repeated at least twice.

Fat deposition in response to vitamin D incorporated diet

To investigate the underlying mechanism of defective β -oxidation, we measured the fat levels in both wild type and *klf-3* mutant fed on vitamin D incorporated bacterial food. A stock solution (10 μ M) of 25-dihydroxy vitamin D3 (active form) (D3-D1530-Sigma) was prepared. We then diluted 50 μ l of the stock solution in 50 μ l of water. We then added 50 μ l of this solution to plates seeded with *E. coli* op50 and left it at room temperature until dried. Synchronized larval stages (L1/L2) of both wild-type and *klf-3* worms were separately fed on normal NGM media seeded with vitamin D incorporated *E. coli* op50 bacteria. After 30 min of feeding, worms were washed off the plates with M9 buffer and transferred to NGM plates without bacteria. After another 30 min, the worms were again washed off the plates with M9 buffer and fixed in acetone/methanol, using Finney and Ruvkun's modified freeze-thaw method.⁵³

The worms were then stained for 15–30 min with freshly made Nile Red solution⁴³ from a 0.5 mg/ml stock of Nile Red in acetone (2 μ l of stock Nile Red solution to 1 ml of M9). They were washed with M9 buffer and transferred onto 2% agarose pad to observe under the spinning disk confocal microscope (LSM 510 Zeiss confocal microscopy; Germany). Two independent experiments were performed and results were consistent between experiments.

Acknowledgement

We thank Walter Conca for his valuable suggestion and *C. elegans* Genetics Center (funded by the National Institutes of Health National Center for Research Resources), Minneapolis, MN, for providing strains. This work was supported in part by Rose M Badgeley Trust, New York. Funding was provided by King Abdulaziz City for Science and Technology Biotechnology Initiative, National Comprehensive Plan for Science, Technology and Innovation grant no. 11-BIO2074-20, Saudi Arabia.

Abbreviations used

MTP	microsomal triglyceride transfer protein
apoB	apolipoprotein B
PUFA	polyunsaturated fatty acid
FA	fatty acid
IFA	immunofluorescence assay
NGM	nematode growth medium
PBS	phosphate-buffered saline
DAPI	4',6-diamidino-2-phenylindole
RT-PCR	real-time PCR

References

1. Martin S, Parton RG. Lipid droplets: a unified view of a dynamic organelle. *Nat. Rev., Mol. Cell Biol.* 2006; 7:373–378. [PubMed: 16550215]

2. Bakillah A, Zhou Z, Luchoomun J, Hussain MM. Measurement of apolipoprotein B in various cell lines: correlation between intracellular levels and rates of secretion. *Lipids*. 1997; 32:1113–1118. [PubMed: 9358438]
3. Rava P, Ojakian GK, Shelness GS, Hussain MM. Phospholipid transfer activity of microsomal triacylglycerol transfer protein is sufficient for the assembly and secretion of apolipoprotein B lipoproteins. *J. Biol. Chem.* 2006; 281:11019–11027. [PubMed: 16478722]
4. Gordon DA, Jamil H, Sharp D, Mullaney D, Yao Z, Gregg RE, Wetterau J. Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc. Natl Acad. Sci. USA*. 1994; 91:7628–7632. [PubMed: 8052632]
5. Jamil H, Dickson JK Jr, Chu CH, Lago MW, Rinehart JK, Biller SA, et al. Microsomal triglyceride transfer protein, specificity of lipid binding and transport. *J. Biol. Chem.* 1995; 270:6549–6554. [PubMed: 7896791]
6. Hussain MM, Shi J, Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* 2003; 44:22–32. [PubMed: 12518019]
7. Shibata Y, Branicky R, Landaverde IO, Hekimi S. Redox regulation of germline and vulval development in *Caenorhabditis elegans*. *Science*. 2003; 32:1779–1782. [PubMed: 14657502]
8. Rava P, Hussain MM. Acquisition of triacylglycerol transfer activity by microsomal triglyceride transfer protein during evolution. *Biochemistry*. 2007; 46:12263–12274. [PubMed: 17924655]
9. Branicky R, Shibata Y, Feng J, Hekimi S. Phenotypic and suppressor analysis of defecation in *clk-1* mutants reveals that reaction to changes in temperature is an active process in *Caenorhabditis elegans*. *Genetics*. 2001; 159:997–1006. [PubMed: 11729148]
10. Kane JP, Hardman DA, Paulus HE. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl Acad. Sci. USA*. 1980; 77:2465–2469. [PubMed: 6930644]
11. Krishnaiah KL, Walker LR, Borensztain J, Schonfeld G, Getz GS. Apolipoprotein B variant derived from rat intestine. *Proc. Natl Acad. Sci. USA*. 1980; 77:3806–3810. [PubMed: 6933436]
12. Sparks CE, Marsh JB. Metabolic heterogeneity of apolipoprotein B in the rat. *J. Lipid Res.* 1981; 22:519–527. [PubMed: 7240977]
13. Higgins DG, Bleasby AJ, Fuchs R. CLUSTAL V: improved software for multiple sequence alignment. *Comput. Appl. Biosci.* 1992; 8:189–191. [PubMed: 1591615]
14. Spieth J, Nettleton M, Zucker-Aprison E, Lea K, Blumenthal T. Vitellogenin motifs conserved in nematodes and vertebrates. *J. Mol. Evol.* 1991; 32:429–438. [PubMed: 1904098]
15. Mann CJ, Anderson TA, Read J, Chester SA, Harrison GB, Kochl S, et al. The structure of vitellogenin provides molecular model for the assembly and secretion of atherogenic lipoproteins. *J. Mol. Biol.* 1999; 285:391–408. [PubMed: 9878414]
16. Maeda I, Kohara Y, Yamamoto M, Sugimoto A. Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Curr. Biol.* 2001; 11:171–176. [PubMed: 11231151]
17. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, et al. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature*. 2003; 421:231–237. [PubMed: 12529635]
18. Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, et al. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature*. 2003; 424:277–283. [PubMed: 12845331]
19. Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, Hirozane-Kishikawa T, et al. Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res.* 2004; 10B:2162–2168. [PubMed: 15489339]
20. 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]
21. Matyash V, Geier C, Henske A, Mukherjee S, Hirsh D, Thiele C, et al. Distribution and transport of cholesterol in *Caenorhabditis elegans*. *Mol. Biol. Cell.* 2001; 12:1725–1736. [PubMed: 11408580]

22. Kimble J, Sharrock WJ. Tissue-specific synthesis of yolk proteins in *Caenorhabditis elegans*. Dev. Biol. 1983; 96:189–196. [PubMed: 6825952]
23. Grant B, Hirsh D. Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. Mol. Biol. Cell. 1999; 10:4311–4326. [PubMed: 10588660]
24. Schneider WJ. Vitellogenin receptors: oocyte-specific members of the low-density lipoprotein receptor supergene family. Int. Rev. Cytol. 1996; 166:103–137. [PubMed: 8881774]
25. Wong KE, Kong J, Zhang W, Szeto FL, Ye H, Deb DK, et al. Targeted expression of human vitamin D receptor in adipocytes decreases energy expenditure and induces obesity in mice. J. Biol. Chem. 2011; 286:33804–33810. [PubMed: 21840998]
26. LeBlanc ES, Rizzo JH, Pedula KL, Ensrud KE, Cauley J, Hochberg M, Hillier TA. Associations between 25-hydroxyvitamin D and weight gain in elderly women. J Womens Health (Larchmt). 2012; 21:1066–1073. [PubMed: 22731629]
27. Hall DH, Winfrey VP, Blaeuer G, Hoffman LH, Furuta T, Rose KL, et al. Ultrastructural features of the adult hermaphrodite gonad of *Caenorhabditis elegans*: relations between the germ line and soma. Dev. Biol. 1999; 212:101–123. [PubMed: 10419689]
28. Kubagawa HM, Watts JL, Corrigan C, Edmonds JW, Sztul E, Browse J, Miller MA. Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment *in vivo*. Nat. Cell Biol. 2006; 8:1143–1148. [PubMed: 16998478]
29. Sherwood DR, Butler JA, Kramer JM, Sternberg PW. FOS-1 promotes basement-membrane removal during anchor-cell invasion in *C. elegans*. Cell. 2005; 121:951–962. [PubMed: 15960981]
30. McKay RM, McKay JP, Avery L, Graff JM. *C. elegans*: a model for exploring the genetics of fat storage. Dev. Cell. 2003; 4:131–142. [PubMed: 12530969]
31. Brock TJ, Browse J, Watts JL. Genetic regulation of unsaturated fatty acid composition in *C. elegans*. PLoS Genet. 2006; 2:997–1005.
32. Zhang J, Bakheet Parhar RS, Huang C-H, Hussain MM, Pan X, et al. Regulation of fat storage and reproduction by Krüppel-like transcription factor KLF-3 and fat associated genes in *Caenorhabditis elegans*. J. Mol. Biol. 2011; 411:537–553. [PubMed: 21704635]
33. Oishi Y, Manabe I, Tobe K, Tsushima K, Shindo T, Fujiu Nishimura G, et al. KLF5 is a key regulator of adipocyte differentiation. Cell Metab. 2005; 1:27–39. [PubMed: 16054042]
34. Wu J, Srinivasan SV, Neumann JC, 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]
35. 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]
36. Birsoy K, Chen Z, Friedman J. Transcriptional regulation of adipogenesis by KLF4. Cell Metab. 2008; 7:339–347. [PubMed: 18396140]
37. Sue N, Jack BHA, Eaton SA, Pearson RCM, Funnell APW, Turner J, et al. Targeted disruption of the basic kruppel-like factor gene (*klf3*) reveals a role in adipogenesis. Mol. Cell. Biol. 2008; 28:3967–3978. [PubMed: 18391014]
38. Nagare T, Sakaue H, Matsumoto M, Cao Y, Inagaki K, Sakai M, et al. Overexpression of KLF15 transcription factor in adipocytes of mice results in down-regulation of SCD1 protein expression in adipocytes and consequent enhancement of glucose-induced insulin secretion. J. Biol. Chem. 2011; 286:37458–37469. [PubMed: 21862590]
39. Mori T, Sakaue H, Iguchi H, Gomi H, Okada Y, Takashima Y, et al. Role of Krüppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. J. Biol. Chem. 2005; 280:12867–12875. [PubMed: 15664998]
40. Wong KE, Szeto FL, Zhang W, Ye H, Kong J, Zhang Z, et al. involvement of the vitamin D receptor in energy metabolism: regulation of uncoupling proteins. Am. J. Physiol.: Endocrinol. Metab. 2009; 296:E820–E828. [PubMed: 19176352]
41. Nakae J, Cao Y, Oki M, Orba Y, Sawa H, Kiyonari H, et al. Forkhead transcription factor FoxO1 in adipose tissue regulates energy storage and expenditure. Diabetes. 2008; 57:563–576. [PubMed: 18162510]

42. Ogg S, Paradis S, Gottlieb S, Patterson G, Lee L, Tissenbaum H, Ruvkun G. The Fork head transcription factor DAF-16 transduces insulinlike metabolic and longevity signals in *C. elegans* . Nature. 1997; 389:994–999. [PubMed: 9353126]
43. Ashrafi K, Chang FY, Watts JL, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. Nature. 2003; 421:268–272. [PubMed: 12529643]
44. Godmann M, Kosan C, Behr R. Krüppel-like factor 4 is widely expressed in the mouse male and female reproductive tract and responds as an immediate early gene to activation of the protein kinase A in TM4 Sertoli cells. Reproduction. 2010; 139:771–782. [PubMed: 20051481]
45. Godman M, Kromberg I, Mayer J, Behr R. The mouse Krüppel-like factor 4 (*Klf4*) gene: four functional polyadenylation sites which are used in a cell-specific manner as revealed by testicular transcript analysis and multiple processed pseudogenes. Gene. 2005; 361:149–156. [PubMed: 16185820]
46. Sulston, J.; Hodgkin, J. Methods. In: Wood, WB., editor. The nematode *Caenorhabditis elegans*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1988. p. 587-606.
47. Brenner S. The genetics of *Caenorhabditis elegans* . Genetics. 1974; 77:71–94. [PubMed: 4366476]
48. Hashmi S, Ji Q, Zhang J, Parhar R, Huang C-H, 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]
49. 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]
50. Hashmi S, Britton C, Liu J, Guiliano DY, Oksov Y, Lustigman S. Cathepsin L is essential for embryogenesis and development of *Caenorhabditis elegans* . J. Biol. Chem. 2002; 277:3477–3486. [PubMed: 11707440]
51. 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]
52. Finney M, Ruvkun G. The unc-86 gene product couples cell lineage and cell identity in *C. elegans* . Cell. 1990; 63:895–905. [PubMed: 2257628]
53. Miller DM, Shakes DC. Immunofluorescence microscopy. Methods Cell Biol. 1995; 48:365–394. [PubMed: 8531735]

Fig. 1.

Expression pattern and subcellular localization of KLF-3 protein in wild-type *C. elegans*. Rabbit anti-KLF-3 antibody was used to localize KLF-3 proteins in *C. elegans* embryos, mixed larvae and adult worms by IFAs. (a and b) Strong signal of KLF-3 protein is observed along the length of intestine of early larva; (c) L4 larva; (d) young adult (e) in germline; (f) DAPI-stained germline is shown to visualize the nuclei; (g) merged image of DAPI-stained germline is merged with fluorescein isothiocyanate for clarification, *dsc-4* deficiency affects intensity of expression but not the subcellular localization of KLF-3 proteins. The purified KLF-3 antibodies were used to stain larval and young adult stages of *dsc-4* (*qm182*) mutant, (a–g) Strong signal of KLF-3 protein was observed in the intestine and germline of wild-type animals but diminished in (h and i) early larva, (j) in L4 larva and (k) and in the germline of *dsc-4* mutant. Images were observed and photographed using Axioskop 2 plus fluorescent microscope with appropriate filter sets (at a magnification of 400×). Images are the representative of ~50 animals.

Fig. 2. Deregulation of genes for lipoprotein assembly and secretion in the *klf-3* (*ok1975*) mutant. The level of expression of multiple genes (designated at the bottom) on the *klf-3* mutant background was measured by RT-PCR. Lines on top of each bar represent standard error of the measurement. Abundance of individual gene is expressed as relative to wild type at scale “1”. The bars above 1 represent up-regulated genes, while the bars below “1” represent down-regulated genes.

Fig. 3.

Genetic interaction between *klf-3* (*ok1975*) and *dsc-4* (*qm182*): genetic interactions between *klf-3* and *dsc-4* genes were assessed by fat mass measurements. Fat assay using Oil-Red-O staining (a) wild-type animal showing low fat mass, (b) high fat deposit in the intestine of *klf-3* (*ok1975*) mutant and (c) high fat deposit in the intestine of *dsc-4* (*qm182*) mutant. (d) We generated *dsc-4* (*qm182*); *klf-3* (*ok1975*) double mutant animals by standard genetic crosses, fixed and stained these mutants with Oil-Red-O. The intensity of red staining was clearly higher in *klf-3*; *dsc-4* double mutant than either *dsc-4* or *klf-3* single mutant. Animals were observed under Nomarski optics attached to a light microscope Nikon eclipse 80i, and photographs were taken with a digital camera Photometrics CoolSNAP cf (at a magnification of 400×). (e) Quantitative analysis of fat mass in *klf-3* mutant, *dsc-4* and *klf-3*; *dsc-4* double mutant. Images are the representative of approximately 50 animals. Data are presented as percent increase or decrease in fat mass in various genotypes over wild-type animals. Each data point is the mean $\pm$ standard deviation.

Fig. 4.

Deficiency in *vit-2* and *vit-4* genes causes minor effects on fat mass but causes significant increase in fat mass in *vit-5* mutant. In *vit* mutant animals: Oil-Red-O staining was used to accurately measure fat mass in animals carrying *vit* gene mutation. (a) Low fat mass in wild type animal; (b) low fat mass in *vit-1* (*ok2616*) mutant; (c) a slight increase of fat mass in *vit-2* (*ok3211*) mutant; (d) low fat mass in *vit-3* (*ok2348*) mutant; (e) a slight increase in fat mass in *vit-4* (*ok2982*) mutant; (f) high fat deposit in the intestine of *klf-3* (*ok1975*) mutant; (g) high fat deposit in the intestine of *vit-5* (*ok3239*) mutant. (h) We generated *vit-5*

(*ok3239*); *klf-3* (*ok1975*) double mutant animals by standard genetic crosses, fixed and stained these mutants with Oil-Red-O. The intensity of staining was slightly higher in *vit-5*; *klf-3* double mutant than either *vit-5* or *klf-3* single mutant. Animals were observed under Nomarski optics attached to a light microscope Nikon eclipse 80i, and photographs were taken with a digital camera Photometrics CoolSNAP cf (at a magnification of 400×). (i) Quantitative analysis of fat mass in wild type, *klf-3*, *vit-1*, *vit-2*, *vit-3*, *vit-4* and *vit-5* single mutant and *vit-5*; *klf-3* double mutants. Fat mass indices indicate high fat mass in *klf-3*, *vit-5* single mutant and *klf-3*; *vit-5* double mutants. A similar fat mass density in *vit-2* and *vit-4* mutants but a slight increase over wild-type animals. Data are presented as percent increase or decrease in fat mass in various genotypes over wild-type animals. Each data point is the mean $\pm$ standard deviation.

Fig. 5.

Cholesterol deficiency in diet has no effect on fat deposition in *klf-3* (*ok1975*) mutant: Oil-Red-O staining was used to accurately measure fat mass in wild-type and *klf-3* (*ok1975*) mutants fed with cholesterol, (a) Wild-type animal; (b) *klf-3* mutant fed on media without cholesterol; (c) *klf-3* mutant fed on regular NGM media with cholesterol (1×); (d) *klf-3* mutant fed on NGM media with cholesterol supplement (10×). Animals were observed under Nomarski optics attached to a light microscope Nikon eclipse 80i, and photographs were taken with a digital camera Photometrics CoolSNAP cf (at a magnification of 400×). (e) Quantitative analysis of fat mass in wild type, *klf-3* and *klf-3* mutant fed on two different concentration of cholesterol and cholesterol-free MGM media. Fat mass indices indicate high fat mass in *klf-3* mutant grown on cholesterol or cholesterol-free media. Data are presented as percent increase or decrease in fat mass in various genotypes over wild-type animals. Each data point is the mean $\pm$ standard deviation.

Fig. 6.

Vitamin D supplement in diet increased fat mass in *klf-3* mutant: synchronized larval stages (L1/L2) of both wild-type and *klf-3* worms were separately fed on normal NGM media seeded with vitamin D incorporated *E. coli* op50 bacteria. After 30 min of feeding, worms were washed off the plates and stained with freshly made Nile Red solution. After washing, worms were transferred onto 2% agarose pad and observed under spinning disk confocal microscope, (a) Wild-type animal showing low staining; (b) *klf-3* animal; (c) vitamin D fed wild-type animal showed increased staining than unfed wild-type animal; and (d) intensity of red staining increased in *klf-3* animal fed with vitamin D. Two independent experiments were performed, and results were consistent between experiments.

Fig. 7. *klf-3* is not involved in yolk transport: we used transgenic strain DH1033 (*bIs1*[*vit-2::GFP*, *rol-6*; *sqt-1* (*sc103*)] expressing YP170 proteins to study the secretion and endocytosis of yolk proteins in *klf-3* (*ok1975*) mutant. (a) VIT-2::GFP was observed in the adult intestine, as well as in the two proximal latestage oocytes (continuous line) and embryos; (b) similar expression pattern was observed in VIT-2::GFP; *klf-3* (*ok1975*) mutant.

- (a)** *klf-3 (ok1975)* male X Wild-type hermaphrodite
 ↓
 43 F1s
 1 sterile + 42 semi-sterile (average 88 progenies per animal)
- (b)** Wild-type male X *Klf-3 (ok1975)* hermaphrodite
 ↓
 Fertile (average 134 progenies per animal)
- (c)** Wild-type male X Wild-type hermaphrodite
 ↓
 Fertile (average 237 progenies per animal)
- (d)** *klf-3 (ok1975)* male X *klf-3(ok1975)* hermaphrodites
 ↓
 1fertile (127 progenies); 5 non fertile

Fig. 8.

Fertility test: (a) *klf-3* males × wild-type hermaphrodite; (b) wild-type male × *klf-3* hermaphrodites; (c) wild-type male × wild-type hermaphrodite; (d) *klf-3* male × *klf-3* hermaphrodites.

Fig. 9.

Model for the regulation of lipid metabolism by *klf-3*. *klf-3* may control lipid metabolism in the intestine and muscle. In the intestine, it may up-regulate *dsc-4* and *vits* to increase lipoprotein assembly and secretion and mobilize lipids to provide energy to other tissue. It may also increase mitochondria to enhance β -oxidation and utilization of fat. As a preferred source, we also observed that *dsc-4* mutants have lower *klf-3* expression more likely indicating the availability of fat in the circulation might affect KLF-3 expression in the intestine and muscle.