Mutation in Caenorhabditis elegans Krüppel-like factor, KLF-3 results in fat accumulation and alters fatty acid composition

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\textbf{ABSTRACT}

In vertebrates, adipose tissue stores energy in the form of fat. Fat storage is tightly controlled by and dynamically balanced with energy expenditure under physiological settings; the perturbation of fat in either excess (obese) or deficit (lipodystrophy) has devastating pathologic consequences in the fueling of homeostasis and organismal fitness. The process by which fat storage is coordinated through positive and negative feedback signals is still poorly understood. To address potential mechanisms underlying fat storage we study a Caenorhabditis elegans Krüppel-like transcription factor, Ce-klf-3 and demonstrate that klf-3 is a hitherto unrecognized key regulator of fat metabolism in C. elegans. The Ce-klf-3 is highly expressed during larval development and predominantly present in intestine: the site of fat digestion, absorption, storage, and utilization. We found a strong positive correlation between klf-3 expression and fat deposition in a worm’s intestine. Significantly, a klf-3 (ok1975) loss-of-function mutation, characterized by the deletion of a 1658-bp sequence spanning the 3′ end of exon 2 through to the 5′ end of exon 3 of klf-3, enhanced fat deposition in the intestine and caused severe defects in worm reproduction. Although klf-3 mutants seemed very similar to wild type worms in appearance and life span, 70% of mutants became semi-sterile, each producing 40–50 viable progenies, and the remaining 30% were rendered completely sterile toward adulthood. Notably, both mutant types displayed extensive deposition of fat in the intestine. Our study also demonstrates that klf-3 is critical for maintaining normal fatty acid composition by regulating genes involved in a fatty acid desaturation pathway. Strikingly, klf-3 mutant animals with impaired fatty acid β-oxidation pathway genes resulted in fat accumulation in the mutant worm. We present the first clear in vivo evidence supporting essential

\textbf{Keywords:}
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klf-3
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Introduction

Energy stored in the form of fat is a basic property universal to animals from Caenorhabditis elegans to humans, allowing organisms to continue life during fasting or starvation [1]. A complex multi-factorial trait driven by natural selection and food availability, fat storage is highly regulated by and dynamically balanced with energy consumption in physiological settings; its perturbation in either excess (obese) or deficit (lipodystrophy) has devastating pathologic consequences in the homeostasis and fitness of an organism. In humans, the obese state precursors: insulin resistance and impairs pancreatic islet $\beta$-cell function [2], two hallmarks of type 2 diabetes, a chief metabolic disease and a severe threat to the health of worldwide populations. Obesity also may result in reproductive deficiency [3] and cardiovascular disease [4]. Hence, understanding the cellular origins and regulatory mechanisms of fat storage in model organisms, such as C. elegans, should help unravel the molecular targets underlying its signal transduction, gene expression, and pathway coordination, yielding new approaches to therapeutic applications.

The C. elegans stores fat mainly in cells of its intestine, a derivative tissue of the developing layer of endoderm [5]. Prior genome-wide RNAi studies have uncovered a plethora of genes affecting lipid metabolism [6], which underscores the conserved nature of molecular mechanisms in fat storage in worms and mammals. It was found that the suppression of 305 genes reduced body fat, while the suppression of 112 genes either enhanced fat storage or enlarged fat-droplet size [6]. The products of these genes are metabolic enzymes, transcription factors, signaling modules, and nutrient transporters, reflecting a wide range of biochemical identities and pathway activities. However, the mechanisms by which these factors act either positively or negatively in the modulation of fat storage remain largely unexplored. Direct inactivation of those worm genes homologous to known mammalian lipid metabolism regulatory factors have demonstrated the existence of molecular players that serve as master switches at the level of gene transcription and/or signal transduction. Examples include the transcription factors SREBP and C/EBP, which cause a lipid-depleted phenotype when mutated [7]. The C. elegans $\Delta9$-desaturase $\text{fat-5}$, $\text{fat-6}$, and $\text{fat-7}$ genes are expressed in the intestine where they undergo strict regulation by a transcription factor, NHR-80, and maintain an optimum fatty acid composition in the worm [8]. In contrast to such positive regulators, little is known about the negative regulators of fat storage in regards to their mode of action and mechanisms of regulation. Several reports from mouse and cell culture studies have suggested that the differentiation of preadipocytes into adipocytes is regulated by a complex network of transcription factors which synchronize the expression of many proteins. These proteins are responsible for determining the shape of a mature fat-cell. Members of the Krüppel-like factor class, KLFs, form a subset of a broad class of proteins containing $\text{C}_{2}\text{H}_{2}$ zinc fingers, the most abundant motif in transcription factors. Although vertebrate KLF is involved in many physiological roles, few mammalian KLFs have been identified as key molecules in controlling adipocyte differentiation or adipogenesis though high levels of RNA or KLF proteins are found in adipocyte [9–12]. The aim of the present study is to understand the mechanisms of the klf regulatory network during C. elegans development.

In this study, we provide new genetic insight into fat storage by identifying the C. elegans Krüppel-like factor 3, Ce-klf-3 (mua-1 or F54H5.4) as a hitherto unrecognized key regulator of fat metabolism in C. elegans. This was prompted by our recent finding that klf-1, a member of the KLF class in the worm, is involved in fat metabolism and in cell death and phagocytosis [13]. Here we show that klf-3 is notably distributed in the intestine conforming to its spatiotemporal expression during development and implying its role in intestinal fat metabolism. We demonstrate through detailed genetic and phenotypic analyses that the two alleles of klf-3 mutant klf-3 (ok1975) and klf-3 (rh160) carry different genomic deletions, each exhibiting distinctive loss-of-function phenotypes. A deletion in klf-3 (rh160) II mutants caused the majority of the animals to grow poorly and fail to reach adulthood. A molecular analysis of the klf-3 (rh160) allele (in this study) confirmed that the extensive genetic disruption that has occurred in this mutant worm affects few neighboring genes in addition to klf-3. Significantly and unexpectedly, the klf-3 (ok1975) allele, characterized by a 1658-bp deletion in the klf-3 gene that spans the 3’ end of exon 2 through the 5’ end of exon 3, manifests not only in severe reproductive defects but also in increased fat accumulation in the intestine. Moreover, our studies reveal that the multiple genetic components that participate in lipid metabolism pathways are deregulated in the absence of KLF-3 function. Taken together, the pleiotropic nature of the klf-3 mutation suggests a key physiological role of KLF-3 in the regulation of fat metabolism in C. elegans and sheds light on its human counterpart in disease–gene association.

Materials and methods

Nematode strains and culture conditions

All C. elegans strains used in this study were maintained and propagated at 20 °C on small petri plates containing nematode growth medium (NGM) seeded with Escherichia coli OP50 [14]. The WT strain N2 (Bristol) was used to create transgenic lines. The homozygous klf-3 (ok1975 and rh160) mutant alleles were obtained from C. elegans Genetics Center (Minneapolis, MN, USA), which is funded by the NIH National Center for Research Resources.

Stage-specific profile of the Ce-klf-3 mRNA transcript

Real-time quantitative RT-PCR (qRT-PCR) was used to profile the stage-specific expression of klf-3 in embryos, staged larvae, and adult worms. A synchronous population of all developmental stages was prepared as previously described [15]. 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 nematode growth media (NGM) plates, and allowed to develop into L2, L3, L4, and adult worms over 40 h. Total RNA was prepared from those worms using Trizol™ reagent (Invitrogen, Carlsberg, CA) according to the manufacturer’s protocol. The klf-3 cDNA was prepared from 2 μg of total RNA in a 50 μl volume reaction with forward 5′-CCACTACATCCAGGACG-3′ and reverse 5′-GGCTTCTTCTCCGCTGTC-3′, was used to amplify internal control, ama-1 transcripts [16]. An ABI Prism 7700 Sequence Detector (Applied Biosystems, Forster City, CA) was programmed for an initial step of 2 min at 50 °C, 15 min at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 58 °C, 45 s at 72 °C. The specificity of each amplicon was confirmed on agarose gel electrophoresis and the relative level of each transcript within a stage-specific cDNA preparation was calculated by the comparative Ct method (Applied Biosystems). The relative abundance of the transcript is presented as the ratio between klf-3 and ama-1.

In vivo site of klf-3 expression

To determine the in vivo expression site of klf-3, we made a klf-3::gfp translational fusion reporter construct that contained the 5′ putative promoter and the entire coding sequence covering its five exons. This sequence was PCR-amplified from WT DNA, digested with restriction enzymes, and cloned into the gfp reporter vector pPD95.75. The resulting construct pHZ122 (Fig. 1) was sequenced to confirm the WT sequence and correct fusion of klf-3::gfp. The pHZ122 plasmid was prepared using the Concert™ rapid plasmid miniprep system (Invitrogen), and then injected into the gonadal syncytium of individual adult hermaphrodites [17] at a concentration of 50 ng/μl. The pRF4 plasmid, which contains the dominant marker rol-6 (su1006) encoding a mutant collagen, was co-injected (80 ng/μl) to confer a visible roller phenotype to transgenic worms. The F3 roller worms were selected for observing klf-3::gfp expression. At least three independent transgenic lines were examined for each construct.

Characterization of klf-3 (ok1975) and klf-3 (rh160) mutant alleles

To determine the location and size of mutation in klf-3 (ok1975) and klf-3 (rh160) mutant alleles, genomic DNA was prepared from homozygous worms of each strain. A series of primers specific to either klf-3 or its flanking genes were designed to amplify the above genomic DNA by PCR (primer sequences will be provided upon request). The PCR products were then cloned into TOPO cloning vector (Invitrogen) and sequenced to pinpoint the exact breakpoints of gene deletion. To determine the effect of the mutation, RT-PCR, cloning, and sequencing were performed to characterize transcript expression of the affected genes in klf-3 (ok1975) and klf-3 (rh160) genetic backgrounds.

Genetic and phenotypic analyses of klf-3 (ok1975) mutant

To dissect the loss-of-function of klf-3 (ok1975) mutant allele, the mutant strain was backcrossed three times using WT males according to a standard protocol [14] and maintained as homozygous. The presence of deletion after each crossing was confirmed by single-worm PCR and DNA sequencing. Individual homozygous mutant hermaphrodites were grown on plates at 20±1 °C and

Fig. 1 – (A) Amino acid sequence alignments of the C-terminal zinc finger domains of C. elegans KLFs proteins (CeKLF3, CeKLF2, and CeKLF1) with human KLF proteins (HsKLF1 and HsKLF7). Amino acid identity is marked with black. Asterisks denote the invariant zinc-chelating residues in the three zinc fingers and black diamonds indicates those DNA-contacting residues. (B) and (C) genomic organization of C. elegans klf-3a and klf-3b genes. Black boxes indicate exons and grey boxes 5′ and 3′UTR. The exon size in base pair (bp) is numbered under the box. Promoters and introns are indicated by a solid line above which the size of introns is numerated. (D) Diagram of the klf-3::gfp fusion construct, pHZ122. The construct contains the 1.0-kb upstream promoter and full-length coding sequence of klf-3 fused in frame with the gfp reporter.
their self-progeny used for experimentation. For morphological comparison between WT and mutants, living animals were observed under microscope using Nomarski differential interference contrast microscopy. To measure fertility, L1/L2 larvae were individually picked and transferred to fresh plates to allow self-fertilization. For this transgene rescue experiment the individual worms were of two possible genotypes: klf-3 (ok1975)/+; klf-3 (Ex): the Ex designates extrachromosomal array. The worms of both genotypes were screened for the presence of sterile or non-sterile animals over their reproductive periods. These worms were also tested for fat accumulation. If the worms produced ~200 worms during their reproductive periods (usually 5–6 days of their adulthood) and the presence of fat granules in their intestine were comparable to wild type they were considered rescued. The rescue was correlated with the presence or absence of the expression of klf-3 (klf-3::gfp construct) as well as their genotype (the presence and absence of klf-3 deletion) using single-worm PCR (fertile and nonfertile animals) with the corresponding gene-specific primers. The progenies of the heterozygous fertile or nonfertile roller worms were self-fertilized to obtain homozygous. We used single-worm PCR on roller mother to confirm their genotype and then the progenies of homozygous fertile or nonfertile roller worms were self-fertilized to ensure that these worms contained the rescue gene. Twenty L4 roller hermaphrodites from a successful mating plate were individually picked and transferred to fresh plates to allow self-fertilization. In brief: heterozygous mutant males were created by crossing rescue gene were then crossed, each with 12 heterozygous mutant males; 15 individual transgenic hermaphrodites expressing the transgene within the worms and thus successful crossings. In addition, single-worm PCR was performed on the roller worm to ensure that these worms contained the rescue gene. Twenty L4 roller hermaphrodites from a successful mating plate were individually picked and transferred to fresh plates to allow self-fertilization. For this transgene rescue experiment the individual worms were of two possible genotypes: klf-3 (ok1975)/+; klf-3 (Ex) or +/+; klf-3 (Ex): the Ex designates extrachromosomal array. The worms of both genotypes were screened for the presence of sterile or non-sterile animals over their reproductive periods. These worms were also tested for fat accumulation. If the worms produced ~200 worms during their reproductive periods (usually 5–6 days of their adulthood) and the presence of fat granules in their intestine were comparable to wild type they were considered rescued. The rescue was correlated with the presence or absence of the expression of klf-3 (klf-3::gfp construct) as well as their genotype (the presence and absence of klf-3 deletion) using single-worm PCR (fertile and nonfertile animals) with the corresponding gene-specific primers. The progenies of the heterozygous fertile or nonfertile roller worms were self-fertilized to obtain homozygous. We used single-worm PCR on roller mother to confirm their genotype and then the progenies of homozygous worms were tested for fertility or absence of fat accumulation.

**Fat staining and microscopic examination of lipid droplets**

Fat staining was performed with Sudan black (Sigma, St. Louis, MO) according to the published protocol [20] with minor modifications. In brief, mixed population, as well as non-starved L1 or adult klf-3 mutant worms were separately fixed in 1% paraformaldehyde in PBS separately, frozen at −70 °C for 30 min to overnight, washed, and incubated overnight in 1 ml of Sudan black solution (0.02% final concentration in propylene glycol). Then, the samples were washed twice with propylene glycol, mounted on a slide, and observed under a light microscope equipped with DIC optics. The control WT worms of similar age were treated in the same way for comparison. The Sudan black staining was performed separately on three different batches of synchronized mutant and wild type worm of similar age. Each batch containing approximately 300 worms were divided into three replicates. Electron microscope examination of worm thin sections was performed as previously described [21]. For electron microscopy many cross-sections from approximately 100 WT or mutant worms of same ages (L4 or young adult) were cut along

**Table 1 - List of genes predicted to participate in fatty acid synthesis, desaturation, elongation and β-oxidation pathways.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer pair sequences</th>
<th>Genes</th>
<th>Primer pair sequences</th>
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**Primers sequences used in RT-PCR analysis.**
the mid-body covering the mid-portion of intestinal tube and surface complex of hypodermis and cuticle. Approximately 50 sections each from WT and mutant worms were observed under microscope.

**Analysis for fatty acid composition**

A synchronized young adult population of both wild type (N2) and *klf-3 (ok1975)* mutant worms were grown on NGM plates seeded with *E. coli* OP50. Then the worms were washed off the plates with water, and rinsed 3 times. The worms were stored at ~80 °C. Fatty acid extraction and analysis were performed at the Fatty Acid Analysis Laboratory (University of Florida, Gainesville, FL). Fatty acids were extracted according to the method described by Sasser [22], and Brock et al. [8] with several minor modifications. Fatty acids as fatty acid methyl esters (FAMEs) were detected using an Agilent 6890 gas chromatograph with an FID. Fatty acids were identified using the Sherlock Microbial Identification System (MIS) version 4.5 with the EUKARY peak library and method version 3.71 (MIDI, Inc., Newark, DE). Peaks are the representative of nine measurements from three independent samples of mutant and wild type nematodes. Each sample contained three replicates.

**Analysis of genes involved in fatty acid metabolism**

Because of the high fat accumulation in *klf-3 (ok1975)* mutant we reasoned that *klf-3* might regulate genes involved in lipid metabolism. To test this premise, we identified 44 genes (Table 1) in the *C. elegans* genome predicted to participate in fatty acid synthesis, desaturation, elongation and β-oxidation pathways [23; www.kegg.com]. A synchronized adult population of both *klf-3 (ok1975)* and N2-Bristol (WT) were grown at room temperature (22 ± 1 °C) on NGM plate seeded with *E. coli* OP50 bacteria and collected by washing off plates in PBS, washed 3× in PBS buffer. As described above, the total RNA was prepared from those worms using Trizol™ reagent, and then the cDNA was prepared from 2 µg of total RNA in a 50 µl volume reaction. We used qRT-PCR to measure the expression level of each of forty genes in wt and *klf-3 (ok1975)* mutant worms with gene-specific primers designed to amplify each of the above genes along with control primers to amplify 18S rRNA, *tbb-2* (β-tubulin), and *ubc-2* (ubiquitin-conjugating enzyme, E2). The expression of transcripts in WT vs. *klf-3 (ok1975)* mutant is presented as the mRNA abundance of each gene relative to control genes.

**Results**

The *C. elegans* genome contains three Krüppel-like transcription factors

We identified three Krüppel-like transcription factors (*klfs*): *klf-1* (F56F11.3), *klf-2* (F53F8.1) and *klf-3* (mu-1 or F54H5.4), in the worm genome (http://www.wormbase.org), which are genuinely related as they all contain three highly conserved C-terminal C2H2 zinc fingers: *klf-1* and *klf-3* are both similar to human KLF1 and *klf-2* is very similar to human KLF7 (Fig. 1A), yet they display little homology in their N-terminal regions (not shown). In *C. elegans*, *klf-3* occurs in two isoforms differing in the 5′-coding region: *klf-3a* has five exons which encode a protein of 315aa, while *klf-3b* has six exons which encode a protein of 315aa (Figs. 1B, C). The ATG start codon of *klf-3a* begins approximately 1 kb downstream of the *klf-3b* ATG start codon. The spliced EST data available on Wormbase strongly supports that *klf-3a* and *klf-3b* use separate promoters. Our preliminary data on reporter gene expression also indicates that these two genes show differential gene expression (data not shown). In genome-wide screens, these worm KLFs did not demonstrate any role in fat regulation [6]. However, our recent studies showed that *klf-1* is involved in fat metabolism; *klf-1 RNAI* caused increased fat accumulation in the intestine of RNAi worm [13]. This finding prompted us to seek to determine if *klf-3* possesses a similar functional role and whether *klf-3* is the same gene mutated and genetically mapped as in *mu-1* (muscle attachment abnormal-1) [24].

The *klf-3* gene is expressed in all stages but is particularly elevated during the larval stages of development

Because the expression of *klf-3* has not been well characterized, we first determined the stage-specific pattern of its expression during development by measuring its mRNA levels in embryos, larvae, and adults. Using real-time RT-PCR, we obtained a reproducible estimate of the relative abundance of *klf-3* transcripts in various developmental stages (Fig. 2). The results presented indicate that *klf-3* is expressed throughout the lifespan of the worm but in varying abundances. The total amount of *klf-3* transcripts was lower in embryos and higher in developing larvae. The peak amount was seen in L1 larvae. After L1 growth, the *klf-3* level dropped slightly in the L2, L3, and L4 stages (Fig. 2). Since *C. elegans* increases in size from larval to adult stages after the final molting [25], these results suggest that the expression of *klf-3* during larval development is critical to the functional activity of this gene in these stages of development.

The intestine is the major site of *klf-3* gene expression

To determine the timing and location of *klf-3* expression in vivo, we established transgenic lines carrying the *klf-3::gfp* fusion gene, which was driven by a cognate promoter, a 1.0-kb genomic

*Fig. 2 – (A) Temporal expression pattern of the *klf-3* gene as determined by qRT-PCR. The levels of *klf-3* mRNA in each developmental stage were measured, using *ama-1* gene as an internal control. Total RNA samples used for cDNA synthesis were isolated from mixed-stage embryos, synchronized larvae, and adult populations, respectively. Note that *klf-3* transcript is low in embryos but increased steadily in the larval stages and decreased again in adult. Each experimental point was repeated at least twice.*
sequence upstream of the first ATG codon (Fig. 1D). As shown, klf-3::gfp expression first appeared in the early larvae which were still enclosed in the eggshell of the embryo (Fig. 3, I). During larval development, gfp fluorescence was frequently observed in the intestinal cells of developing larvae, young adults, egg-laying hermaphrodites, and male worm (Fig. 3, II–V). Gfp fluorescence was very strong and persisted even in very old adult worms. This pattern was consistently seen in all three transgenic lines. It appears that the expression of klf-3 in the intestine during larval development as well as in adults is genetically programmed, corroborating the mRNA data. These results indicate that the activity of klf-3 is primarily in the intestine, given that the intestine is a major site of fat metabolism, performing many vital functions in C. elegans such as food digestion, nutrient absorption, and energy storage [5–8].

**Two alleles of klf-3 mutant exhibit different deletions and phenotypes**

Before the phenotypic characterization of klf-3 (ok1975) and klf-3 (rh160) mutant alleles, we first determined their genomic abnormalities and expression of transcripts. We confirmed in the klf-3 (ok1975) allele a 1658-bp deletion spanning the 3′ end of exon 3 through to the 5′ end of exon 3 of klf-3, establishing that klf-3 is the only gene mutated in this strain (Fig. 4). In contrast, we identified a 2.5-kb deletion in the klf-3 (rh160) allele, which covers three genes from exon 3 in klf-3, to F54H5.3 and, the 5′ end of an uncharacterized F54H5.5 gene (Fig. 4) suggesting that an extensive genetic disruption in this mutant worm has occurred that affects other neighboring genes in addition to klf-3. The C. elegans wormbase (http://www.wormbase.org) indicates that F54H5.3 encodes a VAMP-associated protein; its RNAi causes a reduction in fat content and abnormal lipid metabolism [6]. Although both klf-3 (ok1975) and klf-3 (rh160) alleles are loss-of-function mutants, the phenotype of the klf-3 (ok1975) allele is not as severe as the klf-3 (rh160) allele in terms of survival, growth, development and movement. This is consistent with the finding that the latter carries a multi-gene deletion. Since the klf-3 (rh160) mutant phenotype is the result of multiple deletions, its detailed analysis will be reported elsewhere. Here, we focused on the functional and phenotypic alterations in the klf-3 (ok1975) mutant because the deletion there affects the klf-3 gene only and provides an advantage for genetic and phenotypic analyses by establishing the baseline of loss-of-function through a single gene alteration.

**The klf-3 mutant worms manifest abnormal morphology and severe reproductive defects**

To determine the morphology and phenotype of klf-3 (ok1975) mutant worms, we observed the growth and development of L1 larvae by growing them individually on NGM plates. We found that these L1 worms continued to develop to adulthood without obvious defects in pharyngeal pumping, intestinal contraction, and gross morphology. In a batch of 40 worms, 12 (30%) developed into sterile adults. In the adult stage, these sterile worms moved slowly and their intestines appeared very dark, despite an apparently normal lifespan. The remaining 28 mutant adult hermaphrodites (70%) each produced 40±10 (mean±standard error) viable progeny over 5 days before becoming sterile (progeny was scored every other day by transferring the hermaphrodite to a new plate). In comparison a WT hermaphrodite produced 262±12 viable embryos in the same period. Based on the two distinctive phenotypes, we classified those 12 and 28 worms as sterile (no progeny) and semi-sterile (reducing progenies), respectively.

After 5 days of observing their reproductive behaviors, both types of mutant hermaphrodites were transferred to a slide for further microscopic examination. Besides reproductive defects, we found various structural changes in live mutant worms. To
visualize the nuclei of germ cells or developing oocytes, worms were fixed and stained with DAPI. The typical patterns were seen in the germline and oocyte areas of normal worms but not in sterile mutants, where morphologically abnormal oocytes and disorganized gonads were evident (Fig. 4, II). In addition, few cells showed DAPI staining around the vulva (Fig. 4, III). The acridine orange (AO) staining [13] was negative in the germline area indicating that the morphologically abnormal cells were not apoptotic (data not shown). The oocyte region of the gonad arm was filled with small morphologically abnormal oocytes. The worms were fat in appearance with darkened intestines and degenerated gonads. In L4 and early adult semi-sterile worms, germ cells and oocytes appeared normal. The semi-sterile worms also appeared normal in fertilization and egg-laying, but their oogenesis became impaired after 40–50 oocyte–sperm fusion events. The degeneration of embryos began with the appearance of disorganized clumps of dead cells in the uterus (Fig. 4, IV). In some older egg-laying worms, gonadal muscle was also detached (Fig. 4, V). The gradual appearance of egg-laying defects in the semi-sterile mutant worms could be due to the gradual deterioration of certain klf-3 related activities.

**Mutant rescue**

In order to validate that the reproductive defects and fat accumulation observed in klf-3 (ok1975) mutant worms is due to the deletion of the klf-3 gene, we tested the klf-3::gfp (pHZ122) fusion genes bearing full KLF-3 protein-coding segments for the rescue of different aspects of the klf-3 (ok1975) mutant phenotype. There are three phenotypes of klf-3 (ok1975) mutant: complete sterility (0 progeny), semi-sterility (~50 progeny) and excessive fat accumulation. We found that pHZ 122 construct can directly express levels of KLF-3 proteins to rescue major aspects of klf-3 loss-of-function mutant phenotype. We found that 36 (n = 47) klf-3 (ok1975); klf-3::gfpEx transgenic animals were able to rescue the semi-sterile phenotype. The rescued worms were fertile and produced on average 196 viable progenies during their reproductive period (4–5 days of adulthood) (Table 2) and were positive for klf-3 (Ex) as indicated by gfp expression. Whereas 9 (n = 47) klf-3 (ok1975); klf-3::gfpEx transgenic animals remained completely sterile (0 progeny) during their reproductive period (Table 2). As expected, the klf-3 (ok1975); pRF4 (roller) Ex transgenic animals were sterile or semi-sterile. Although the expression levels of klf-3::gfp fusion genes, were high in multiple transgenic lines tested, this expression level may not be sufficient to rescue the complete

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**Table 2 - Klf-3 gene rescue the reproductive and fact accumulation defects of klf-3 (ok1975) mutant.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sterile</th>
<th>Semisterile</th>
<th>Fertile</th>
<th>Progeny^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>klf-3 (ok1975); klf-3::gfpEx (N = 47)</td>
<td>0</td>
<td>0</td>
<td>36</td>
<td>196 (173–210)</td>
</tr>
<tr>
<td>klf-3::gfpEx (N = 52)</td>
<td>0</td>
<td>52</td>
<td>0</td>
<td>170 (153–198)</td>
</tr>
<tr>
<td>klf-3 (ok1975); roller (pRF4)Ex (N = 37)</td>
<td>16</td>
<td>21</td>
<td>0</td>
<td>35 (26–48)</td>
</tr>
</tbody>
</table>

^a Average number of progenies produced by each worm over 4 days at 20 °C±1 °C. The numbers of a worm (N) are the combined total of two separate rescue assays. The klf-3::gfpEx rescued the semi-sterile phenotype but did not rescue the sterile phenotype of the klf-3 (ok1975) mutant. The klf-3::gfpEx also rescued the fat accumulation phenotype of klf-3 (ok1975) mutant. klf-3::gfpEx as well as klf-3 (ok1975); roller (pRF4)Ex transgenes served as controls. The Ex designates extrachromosomal array. Rescue assays were performed twice.
sterility of the klf-3 mutant. Alternatively, there are other factors involved that cause the complete sterility in the mutant worm. Further quantitative analyses of different klf-3::gfp constructs with varying promoter lengths will resolve these issues. We also observed the fat accumulation in 25–30 klf-3 (ok1975); klf-3:: gfpEx transgenic animals by Sudan black staining. We found that these worms displayed significantly low fat content than klf-3 (ok1975) mutant. The results clearly indicate that reproductive defect and excessive fat build up in the klf-3 (ok1975) mutant worm was the result of a disruption in the normal function of klf-3.

The klf-3 mutant worms accumulate abnormally high fat contents

Given the intestinal expression of klf-3 and the appearance of fat in klf-3 (ok1975) mutants, we ask whether the klf-3 deletion caused the fat accumulation phenotype. We examined the accumulation of fat in mutant worms through Sudan black staining and observation under light microscope. While normal control worms showed the typical low fat content (Fig. 5A, I), we found extensive buildup of fat deposits in the intestines of all mutant worms. Although fat accumulation was seen in young larvae, the buildup of fat was particularly pronounced in the L4 and adult stages (Fig. 5A, II, III). This finding suggests that the effect of the klf-3 deletion on fat content is incremental with the growth of mutant worms to adulthood. We further examined many sections of the similar regions of both WT and the mutant worms under electron microscope. Again, we found that in all samples normal WT worms had relatively small and few lipid droplets in their intestinal walls (Fig. 5B, panel I) whereas all the sections from klf-3 (ok1975) mutant worms displayed extensive accumulation of large lipid droplets in both the hypodermis and the intestinal wall (Fig. 5B, II, III) as is evidenced by Hall [21] and Petrov and Rachubinski [26]. Thus, we conclude that ablating the function of klf-3 gene results in severe reproductive defects and extensive fat deposition. Fig. 5B is the representative of at least 60 cross-sections of WT or mutant worm.

Fatty acid composition is altered in klf-3 (ok1975) mutants

The klf-3 (ok1975) mutant accumulates a large amount of fat in its intestine. We anticipated that the accumulation of abnormally high fat contents resulted from the alteration of fatty acid (FA) composition in mutant worms. We utilized GC analysis of the total lipids to measure the FA composition of both mutant klf-3 (ok1975) and wild type (N2) worms grown under standard culture conditions and feeding on E. coli OP50 bacteria. Our analysis revealed that an alteration in the long chain fatty acid composition had occurred in the mutant worms along with an increase in stearic acid (C18:0) and linoleic acid (C18:2w6c) (Fig. 6). We also noticed a slight increase in unusual fatty acids, C20:2w6c in the mutant worms (Fig. 6). In addition, a reduction in palmitic acid (C16:0) was noticed in the mutant when compared to the wild type worm. In C. elegans, the pathway for unsaturated fatty acid

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**Fig. 5** – (A) Fat storage and morphological appearance of klf-3 (ok1975) mutant worms. (I) Low fat content in WT L4; (II) extensive fat accumulation in klf-3 (ok1975) larvae; and (III) enhanced Sudan black staining in klf-3 (ok1975) adult hermaphrodite. All photographs were taken using Nomarski optics (400× magnifications). (B) Electron micrographs of thin sections of mutant and WT worms. (I) Few small lipid droplets (star) are present in the WT worm; (II) the mutant worm bearing large lipid droplets (star); and (III) another section of mutant worm showing large lipid droplets. Hy, denotes hypodermis and Int, intestine. The horizontal scale bar is 2 μm.
The fatty acid composition in \textit{klf-3 (ok1975)} mutant is compared to \textit{C. elegans} (N2) wild type strain. Gas Chromatography (GC) profiles: retention time at X-axis; intensity of signal is shown at Y-axis. The arrows point to the peaks corresponding to stearic acid (C:18.0) and linoleic acid (C18:2\text{w6c}) in both wild type and \textit{klf-3 (ok1975)} mutant. Note that these peaks are higher in \textit{klf-3} mutant than wild type worm. Arrowhead indicates a slightly lower peak of palmitic acid (C:16.0) in \textit{klf-3} mutant. GC analysis were performed on 9 samples, each of \textit{klf-3 (ok1975)} mutant and adult population of wild type (N2) strain collected in 3 different dates.

\textbf{klf-3 regulates genes involved in fatty acid metabolism pathway}

The fat phenotype of \textit{klf-3 (ok1975)} mutants suggests that \textit{klf-3} plays a key role in fat regulation and that its deletion may interfere with fatty acid synthesis, composition or metabolism related signal transduction [28; \url{www.kegg.com}]. To test this hypothesis, we used qRT-PCR to assess the expression of a panel of genes (Table 1) involved in lipid metabolism pathways in \textit{klf-3 (ok1975)} mutants and compared their expression to wild type worms. We found that a substantial deletion in the \textit{klf-3} coding sequence produced a dramatic effect on multiple genes involved in the fatty acid \(\beta\)-oxidation (mitochondrial \(\beta\)-oxidation and peroxosomal \(\beta\)-oxidation) pathway. In addition, a mutation in \textit{klf-3} also resulted in dramatic changes in essential genes involved in fatty acid desaturation metabolic pathways [28] (Fig. 7).

Upon detailed examination of the RT-PCR data we observed that the 7 known genes predicted to function in mitochondrial or peroxosomal \(\beta\)-oxidation pathways: \textit{acs-1}, \textit{acs-2}, \textit{ech-6} (T05G5.6, \textit{ech-9}, \textit{F08A8.1}, \textit{F08A8.2} and \textit{T08B2.7}, altered expression in \textit{klf-3 (ok1975)} mutants. Fatty acids in the form of Acyl-CoA molecules are broken down in mitochondria and/or peroxisomes to generate acetyl-CoA. The observed alteration in the expression of the genes that facilitate this breakdown could interrupt the process of \(\beta\)-oxidation, ultimately leading to the accumulation of fat in \textit{klf-3} mutants.

Through further analysis we identified the increased expression of 7 fatty acid desaturases: \textit{fat-1}, \textit{fat-3}, \textit{fat-4}, \textit{fat-5}, and \textit{fat-6}, (Fig. 7), and the decreased expression of desaturase, \textit{fat-7}, and elongase, \textit{elo-2}, in the mutant worm. The \textit{C. elegans} \textit{fat-5}, \textit{fat-6}, and \textit{fat-7} genes encode \(\Delta9\)-desaturases that catalyze the biosynthesis of mono-unsaturated C16:1 and C18:1 fatty acids from saturated C16:0 (palmitic acid) and C18:0 (stearic acid) fatty acids [27]. The \textit{fat-5} encodes a palmitoyl-CoA desaturase, which specifically acts on palmitic acid (C16:0), while \textit{fat-6} and \textit{fat-7} genes encode stearoyl-CoA desaturases (SCD), which preferentially desaturate stearic acid (C18:0) (Fig. 8).

The \textit{fat-3} encodes a \(\Delta6\)-desaturases and is required for synthesis of C20 fatty acids. In \textit{klf-3 (ok1975)} worms we saw a significant increase in \textit{fat-3} expression (Fig. 7) and, marginal increase in the amount of C20:\text{w6c} (Fig. 6). With the exception of \textit{fat-2}, the altered expression of the fat genes in our RT-PCR screen is consistent with data from lipid analysis which indicates a change in C18 and C20 fatty acid composition in \textit{klf-3} mutant worms (Fig. 6).

Conceivably, increased expression of enzymes will increase consumption of their substrates, possibly leading to the formation of unsaturated fatty acids. Deletions in the \textit{klf-3} gene also affected two important enzymes, acetyl-CoA carboxylase (ACC; \textit{pod-2}) and fatty acid synthase (FAS; \textit{fasn-1}) which are involved in fatty acid synthesis pathways (Figs. 7 and 8). Acetyl-CoA carboxylase catalyses the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, while FAS catalyzes a series of multi-step chemical reactions through which FAS uses one acetyl-coenzyme A (CoA) and seven malonyl-CoA molecules to synthesize a 16-carbon palmitic acid. Overall, our results suggest the possible involvement of KLF-3 in the control of fatty acid metabolism pathways. It is possible that KLF-3 selectively acts on \(\beta\)-oxidation and on fatty acid desaturation pathway components to regulate their activity and integrate their crosstalk into a fat metabolism network.

\section*{Discussion}

Here we present a detailed characterization of the worm \textit{klf-3} gene whose molecular properties and biological functions have not been understood prior to this study. We showed that \textit{klf-3}, together with \textit{klf-1} and \textit{klf-2}, form a small gene family that falls into the superfamily of Krüppel-like transcription factors which is highly conserved and broadly expressed across metazoan lineages [29,30] including humans [23,31]. These KLFs bind to CACCC elements and GC-rich regions of DNA and mediate the activation or repression of transcription, performing diverse roles in proliferation, differentiation, and development. The KLF-3 protein, like its two cousins, contains a high amino acid sequence identity in the C-terminal DNA binding C\(_{3}\)H\(_{2}\) zinc finger domains typical of all KLF members.
This molecular signature connects worm KLFs to a core set of transcriptional regulators and, has thus led us to several basic questions concerning the function of \textit{klf-3} gene. Is \textit{klf-3} expressed specifically in the intestine and does it function in intestinal fat metabolism? If so, what is the mode of action of \textit{klf-3} in this regulatory function and how important is this molecular mechanism to the worm in the context of organismal physiology? Our genetic and phenotypic analyses of both WT and mutant \textit{klf-3} genes have provided several lines of evidence suggesting that KLF-3 acts as a negative regulator and plays an essential role in the fat metabolic network of \textit{C. elegans}.

Fat storage has a pivotal role in the natural selection and evolution of metazoans. It offsets food shortage, a constant threat to animal survival (except humans of recent evolution) \cite{32}, and is most likely to have arisen first in the gut, the most ancient organ \cite{6,7}. The intestine-specificity of KLF-3 and its identification as a

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Deregulation of genes for lipid metabolism in the \textit{klf-3 (ok1975)} mutant. The level of expression of multiple genes (designated at bottom) on the \textit{klf-3} mutant background was measured by real-time PCR. Lines at top of each bar represent standard error of the measurement. Abundance of individual gene is expressed as relative to WT at scale “1”. The bars above “1” represent up-regulated, while the bars below “1” represent down-regulated genes.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Schematic presentation of the FA desaturation in \textit{C. elegans}. The genes involved in FA desaturase steps are taken from Van Gilst et al. \cite{28}. The red box represents genes that are up-regulated while the green box represents genes that are down-regulated in \textit{klf-3 (ok1975)} mutant as detected by qRT-PCR. The expression of \textit{fat-2} (grey box) remained unchanged. Black box represents fatty acids altered in \textit{klf-3 (ok1975)} mutant and was easily detectable by GC.}
\end{figure}
key factor in fat regulation reinforces the early origin and adaptation of this genetic mechanism in lipid metabolism and energy homeostasis. This notion is also corroborated by the presence and function of its cousin KLF-1 in the intestine, albeit KLF-1 is more widely expressed with roles in fat metabolism as well as in other cellular processes in C. elegans [13]. Given the still incompletely defined and multi-factorial nature of fat storage [1], it is not surprising that both KLF-3 and KLF-1 have escaped RNAi screenings of fat regulation factors [6]. Accordingly, our studies define KLF-3 as the first of the KLF members now known to directly result in excessive fat deposition and big fat-droplet formation upon genetic mutation. The results support the stipulation that KLF-3 has a significant role in modulating the activity of key metabolic and signaling pathways, which collectively manifest in a negative regulatory mechanism for fat storage and lipid metabolism.

Regulation of fat storage involves a complex array of signaling pathways which govern food intake, nutrient transport, and metabolite flow in a highly concerted manner in both worms and humans [20,33]. In worms as in mammals, SCDs play key roles in keeping a proper level and ratio of saturated to unsaturated fatty acids [34,35], acting in the intestine and under the strict control of the transcription factor NHR-80. A fat-5/fat-6/fat-7 triple mutant produces sterile adults with imbalanced fatty acid composition, reduced movement, and early death, while an NHR-80 mutation down-regulates all of them [8]. Although desaturases have no linkage to the lifespans of worms [8] or mammals [35], a change in the ratio of saturated to monounsaturated fatty acids contributes to shortened lifespan in worms [28]. We found that in spite of greatly reduced fertility in klf-3 mutant worms, they lived nearly as long as wild type worms [36]. Thus, alterations in fat regulation or fatty acid composition do not reduce life span as has been reported in an earlier study [36].

Our quantitative RT-PCR analysis showed that the expression of genes devoted to the regulation of fatty acid desaturation and saturated fatty acid ß-oxidation pathways [28] were changed in klf-3 (ok1975) mutants. We observed substantial increases in fat-1, fat-3, fat-4, fat-5, and fat-6 expression but a significant decrease in the expression of fat-7 suggesting klf-3 could differentially regulate these fat genes in the maintenance of proper enzymatic activities via balancing actions. Whether klf-3 and NHR-80 function cooperatively to form a negative-positive feedback loop in the same pathway or independently in different pathways needs further investigation. KLF-3 maintains the balance of saturated and monounsaturated fatty acids by regulating the expression of fatty acid Ñ-desaturase genes, fat-1, fat-3, fat-4, fat-5, and fat-6 and fat-7, which in turn catalyze the biosynthesis of monounsaturated C16:1 and C18:1 fatty acids from saturated C16:0 and C18:0 fatty acids [27]. An imbalance in fatty acid saturation has been linked to numerous fatty acids from saturated C16:0 and C18:0 fatty acids [27].

When compared to their mammalian counterparts, the observations made concerning the expression and actions of worm KLF-3 provide insight into the regulation of fat storage and adiposity in several ways. First, they link KLF to an essential negative regulatory mechanism of fat storage in the intestine as the major site of early origin [6,7]. This makes the worm intestine a useful model in future studies to address the positive and negative impact of neuroendocrine signals on lipogenesis and fat deposition as in mammals [50]. Second, they connect KLF-3 expression to larval development and reveal the significant pathogenic effects of fat storage on germ cell function and worm reproduction. This causal link presents an example when looking into parallel conditions in humans, namely,
obesity-associated reproductive deficiency [3] and gestational diabetes [51]. Third, they relate the regulatory function of KLF-3 to the desaturases and β-oxidation signaling pathway that control fatty acid metabolism. Given the universal nature of these three genetic modules in animals, the newly uncovered aspects of fat regulation may identify conserved organismal features and direct research avenues to therapeutic applications.

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**References**