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Elongation and Desaturation of Fatty Acids are Critical in Growth, Lipid Metabolism and Ontogeny of *Caenorhabditis elegans*

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Recently, it was reported that a deficit in the mouse stearoyl-CoA desaturase 1 gene decreases biosynthesis and accumulation of fatty acid and revitalizes the β-oxidation of fatty acid. To examine the physiological role of fatty acid desaturase (FAT) and elongase (ELO)-gene transduction in ontogeny, fatty acid accumulation and individual lifespan, we performed bacteria-mediated RNA interference (RNAi) in the nematode *Caenorhabditis elegans*. Suppression of the expression of FAT-2 gene mRNA caused a drastic decrease in the amount of body fat and defects in egg-hatching. The amount of body fat was markedly decreased, and body size reduced, by down regulation of FAT-6 and FAT-7, whereas lifespan was drastically reduced. RNAi of the FAT-2 gene caused a remarkable increase of the β-oxidation-related gene expression and the DAF-16 transcriptional activity, whereas, ELO-2 RNAi caused a remarkable decrease in fatty acid biosynthesis-related gene expression. Additionally, RNAi of FAT-6 decreased the mRNA levels of the genes involved in fatty acid synthesis, and FAT-7 RNAi increased the mRNA levels of β-oxidation system genes. These results indicated that the elongation and desaturation of fatty acids are integral to various phenomena such as ontogeny and lifespan and play important roles in fatty acid accumulation and consumption.

Key words: fatty acid desaturase, fatty acid elongase, lipid metabolism, lifespan, ontogeny, RNA interference.

Abbreviations: ACC, Acetyl-CoA Carboxylase; ACS, Acyl-CoA Synthetase; DAF, Dauer Form Defective; DGAT, Diacylglycerol Acyltransferase; ECH, Enoyl-CoA Hydratase; ELO, Fatty Acid Elongase; FAS, Fatty Acid Synthase; FASN, Fatty Acid Synthase; FAT, Fatty Acid Desaturase; GPAT, Glycerol 3-Phosphate Acyl Transferase; HNF, Hepatocyte Nuclear Factor; NGM, Nematode Growth Medium; NHR, Nuclear Hormone Receptor; PGC, PPAR gamma Coactivator; PPAR, Peroxisome Proliferator-Activated Receptor; SCD, Stearoyl-CoA Desaturase; SREBP, Sterol Regulated Element Binding Protein.

In living creatures, sugars and proteins or lipids in food are resolved into acetyl-CoA and then finally converted to ATP via oxidative phosphorylation in mitochondria. However, in the case of an oversupply of food, energy in the form of acetyl-CoA is converted to fatty acids and then stored as triglycerides (*1*). Excessive accumulation of triglyceride by the body's adipocytes results in obesity. Although obesity has become a social problem and the leading cause of disease in many developed nations (*2*), the detailed molecular mechanisms that cause it remain unclear.

In mammals, various genes such as peroxisome proliferator-activated receptor (PPAR) (*3*), sterol regulated element binding protein (SREBP), hepatocyte nuclear factor (HNF)-4 (*4*, *5*) and PPAR gamma Coactivator (PGC)-1 (*6*) are known to be factors that regulate the uptake and metabolism of lipid. In recent years, it has become clear that the accumulation, biosynthesis and metabolism of lipids are controlled in *Caenorhabditis elegans* by nuclear hormone receptor (NHR)-49 (*7*, *8*), SBP-1, NHR-80, MDT-15 (*9–12*), which are also regulatory factors of mammalian lipid metabolism. This fact suggests that the mechanism of lipid metabolism is highly conserved in *C. elegans* and mammals.

Stearoyl-CoA desaturase (SCD)-1, a major component of mammalian desaturase that catalyzes stearic acid and generates oleic acid, is critically involved in fat accumulation under the control of these transcription factors (*13*). SCD-1 is expressed mainly in liver tissues, and is accumulated to especially high levels in white adipocytes.

It has recently been reported that SCD-1 production rises dramatically in obese-type mutant mice (*14*, *15*) fed high fat diets, and that SCD-1 cooperatively works with glycerol-3-phosphate acyl transferase (GPAT) (*16*) and diacylglycerol acyltransferase (DGAT) (*17*), suggesting that SCD-1 is closely involved in triglyceride composition. Furthermore, production of SCD-1 rises through the activation of SREBP-1c by oleic acid (*18*). Conversely, in SCD-1 knockout mice, deficiency of SCD-1 resulted in weight loss regardless of diet (*19–21*).

In SCD-1–deficient mice, expression of the genes encoding acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are involved in fatty acid biosynthesis, decreased, whereas the expression of acetyl-CoA synthetase (ACS) and enoyl-CoA hydratase (ECH),

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Table 1. **Primer sequences of FAT and ELO-2 genes for RNAi construct and real-time PCR.**

Gene	Sense	Antisense
(RNAi construct)		
FAT-1	CAGCTCGAGCACGGCTCCGGTCACCGG	CAGCTCGAGTGACTTTTCTGCCATGGG
FAT-2	CAGCTCGAGCAATCCCTGAGCACTGC	CAGCTCGAGAGTCTTCCTCAGTAACCC
FAT-3	CATGGATCCACAAGAATGCCTCCGGGC	CATGGATCCAATCCATCATCATGAAGC
FAT-4	CAGCTCGAGAGAGCAAGAGCATGAGCCCA	GCTCGAGATCCATCCATAAGTCC
FAT-5	CAGCTCGAGGCGATTATATCTAAACAG	CAGCTCGAGCAGCATCAGTATCCGTCC
FAT-6	CATGGATCCACGGCGGCCCAGAGACGC	CATGGATCCCAGTCCACTTGTGATGGC
FAT-7	CATGGATCCCTCAATACCTGTTCATGG	CATGGATCCCAGCATCTGTATCAGTCC
ELO-2	CATGGATCCCAAGTCCAGCAGCCACGC	CATGGATCCTCGACATCACAAAGGCCC
NHR-49	CATGGATCCTTGCGGAACCATGTGCCG	CATGGATCCCGCATATTCTCTTGTCCC
(Real time PCR primer)		
GPD-1	ATGTCGAAGGCCAACGTC	ACCACTTGAACCGTGTCC
ACS-2	CCACAGATTCTCATCACC	TGTCTGTCCCAAAGTCTC
ECH-1	ATAGAATTCATAGATCGTCTTCGG	TCTCAACGCGATGAGTAC
FASN-1	TTGAAGCTTCATGGGAGG	TTCTGAAGCTGAACATCC
DAF-16	CATGGATCCATCCAGATGCAAAGCCAG	GATTTTTCGAGTTGAGCC
SOD-3	TGCTGCAATCTACTGCTC	GAATGGGAGATCTGGGAG

*Bam*H1 site was added to primer for RNAi constructs of FAT-3, FAT-6, FAT-7, ELO-2, and NHR-49; *XHO*1 site was added to primer for RNAi constructs of FAT-1, FAT-2, FAT-4, and FAT-5.

which participate in β-oxidation of fatty acids, rose (*22*). In addition, expression of the gene encoding SCD-1 is reportedly controlled by factors other than PPAR-γ, a factor that regulates fatty acid consumption and SCD-1 production is restrained by leptin (*23*). These results suggest that SCD-1 occupies a central role in the accumulation of lipids.

So far, nine elongase (ELO) genes and seven fatty acid desaturase (FAT) genes are known to participate in *C. elegans* fatty acid metabolism. Their major metabolic pathways have been elucidated by previous studies (*24–26* and Supplementary Fig. 1). Mutations of the FAT-2, FAT-3, FAT-6 and FAT-7 genes lead to delayed onset of growth, and RNAi of ELO-2 causes miniaturization of body size and a decline in the rate of oviposition, as well as delayed growth, suggesting that fatty acid metabolism plays an extremely important role in the growth of *C. elegans* (*11*, *26–28*). Moreover, FAT-2 mutant worms are sterile because of severe defects in directional sperm motility (*29*), and lack of FAT-3 function results in abnormal movement because of defects in the neurotransmitters at cholinergic and serotonergic neuromuscular junctions (*30*, *31*). It has also been reported that lipid accumulation is restrained and β-oxidation activated in *C. elegans* by RNAi of FAT-7, a functional homolog of the mouse SCD-1 gene (*7*). Simultaneously, FAT-7 RNAi shortened the lifespans of FAT-7 mutants and caused growth delay and embryonic lethality. On the basis of these findings, we conclude that fatty acid metabolism is critically involved in the lifespan, reproduction and nervous function of *C. elegans*, as well as in body fat accumulation and growth. Furthermore, recent report revealed the fact that alternative metabolic pathway of fatty acid was rebuilt by changing gene expression involved in fatty acid metabolism, in case of disfunction of original fatty acid metabolic pathway (*32*).

To newly identify the gene involved in lipid accumulation of nematode and comparably analyze the mechanisms of fat metabolism, we introduced RNAi of FAT family and ELO-2 into wild type and mutant of *C. elegans*. Furthermore, we analyzed the physiological roles of these genes for ontogeny, morphology and individual lifespan of nematode in related to Dauer form defective (DAF)-2 signaling cascade (insulin/IGF-like signal).

MATERIALS AND METHODS

Worms and Culture—Wild-type *C. elegans* Bristol N2 (Caenorhabditis Genetics Center, Minnesota, USA), and mutant strains (National BioResource Project, Tokyo, Japan) were cultured at 20°C on Nematode growth medium (NGM) agar plated with *Escherichia* coli OP50 as previously described (*33*). The FAT-defective mutants used were FAT-2 (*tm789*), FAT-5 (*tm420*), FAT-6 (*tm331*) and FAT-7 (*tm326*).

Vector and Constructs—All RNA extracted from the mixed *C. elegans* population was used for cDNA synthesis by M-MLV reverse transcriptase (Takara, Otsu, Japan). PCR amplification was performed by using FAT-1 through FAT-7, ELO-2 and NHR-49 primers (Table 1), and DNA fragments were ligated into L4440 plasmid DNA. The primers were designed from DNA sequences according to the Wormbase database (www.wormbase.org).

Bacteria-mediated RNAi—Escherichia coli strain HT115 was transfected with RNAi plasmid and plated on NGM agar (RNAi-media) (*34*). After treatment of adult worms with 10% NaClO solution [10 N NaOH/NaClO (10 : 1)], eggs were collected and cultured overnight in S-basal (0.1 mol/l NaCl, 50 mmol/l Potassium Phosphate buffer) at 20°C until hatching. Hatched L1 larvae were then cultured on NGM RNAi-plates at 20°C.

Body Size Analysis—L1 larvae were bred on RNAi plates for 72 h. After the worms had been fixed with a paraformaldehyde (PFA) solution (0.4% PFA/10% EtOH/S-basal), they were photographed under a microscope (DMRXA HC RXA-6, Leica Microsystems, Inc.,

Bannockburn, IL, USA), and their individual lengths were measured.

Egg Laying and Hatching Analysis—The number of eggs laid by each worm was counted over a 6 h period beginning 72 h after the start of culture of the worms in the presence of the RNAi bacteria. After being counted, the eggs were cultured at 20°C for 2 days, and the number of hatched eggs was then counted.

Lifespan Analysis—On days 4 and 8 after the start of RNAi feeding, worms were treated with 0.5 mg/ml fluorodeoxyuridine (FUdR). Worms were transferred to new plates on each 4 days to analyze viability. Life or death of a nematode was judged by its response to tapping on the plate (*35*).

Transcriptional Activity of SOD-3—The promoter region of *SOD-3* gene (−994 to 1,120 ntd), was amplified by PCR using primers, sense; 5′-CATGGATCCTGCAGTGAT TCAGAGAGG-3′, and anti-sense; 5′-CATGGTACCGCAC AGGTGGC- GATCTTC-3′, and was subcloned into GFP expression plasmid pPD95.77 (*36*). This construct, Psod-3::gfp, was injected into *lin-15* mutant worm (*n765ts*) with *lin-15* expression plasmid, pDLH98, to have worm which stably expresses GFP in response to transcriptional activity of *SOD-3*. The Psod-3::gfp worm at L1 stage was bred on RNAi plate for 72 h, fixed in PFA solution, and was observed under fluorescent microscope (DMRXA, Leica).

Nile Red Assays—L1 worms of N2 and FAT mutant were cultured on RNAi media including 0.50 ng/l Nile Red (*37*). Seventy-two hours (for only N2 worms) or 84 h (for worms including FAT mutants) later, worms were collected, treated with 0.4% PFA solution, and observed under a fluorescence microscope (DMRXA, Leica).

RNA Preparation and cDNA Synthesis—Batches of 400 worms were collected after culture on RNAi plates for 72 h, and RNA was extracted by the acid-GTC-phenol method (*38*). Genomic DNA was digested by treatment with DNase I (Takara) for 60 min at 37°C, and then the RNA was repurified by repeating the acid-GTC-phenol extraction. cDNA was synthesized by using M-MLV Reverse Transcriptase (Takara) and used for PCR.

Real-time PCR—Quantitative PCR analysis was performed with an ABI-7300 (Applied Biosystems, Foster City, CA, USA) analyzer and SYBR Premix Ex Taq reagent (Takara). Table 1 lists the primers used.

RESULTS

Morphological Changes Following FAT and ELO-2 RNAi—We first examined the morphogenic changes occurring in the nematodes as a result of RNA interference of the FAT and ELO genes. L1 worms were cultured on RNAi media targeted at FAT or ELO characteristics, and after 72 h incubation each individual's body length was measured. The body size of most RNAi larva was significantly shorter than RNAi-non-treated larva. RNAi-induced miniaturization was most remarkable in the ELO-2 RNAi—to as little as 80% of the length of the RNAi-control worm (Fig. 1). Moreover, a contraction in body length of around 5–15% was observed in RNAi-treated FAT-1–FAT-7 and NHR-49 worms

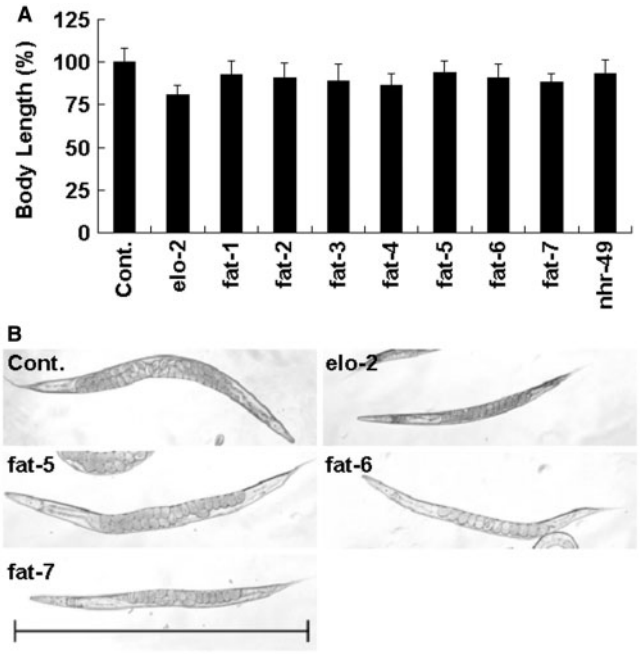

Fig. 1. **Morphological changes following FAT and ELO-2 RNAi.** (A) Nematode was cultured on RNAi-media for 72 h and body length was measured. Body length of each RNAi-treated worms is indicated, with the relative value for RNAi-control worm (Cont.) being 100%. Experiment was independently performed in triplicate. Error bars represent SD values. (B) Stereoscopic microphotograph of each RNAi-treated for L4440 (Cont.), ELO-2, FAT-5, FAT-6 and FAT-7. Bar indicates 1 mm.

(Fig. 1). We recorded an average ovipositional delay of several hours in ELO-2, FAT-2, FAT-6 and FAT-7 RNAi worms, as well as the individual miniaturization (data not shown). Additionally, FAT-2 RNAi caused a reduction of motility. On the other hand, RNAi of ELO-2 or other FAT family did not affect to the motility.

Effect of FAT and ELO-2 RNAi on Reproduction and Embryogenesis—Physiological effects on reproduction and embryogenesis were also observed among RNAi-treated worms. The number of eggs produced by each worm was measured over a 6-h period beginning after 72 h of culture on RNAi media. The number of eggs laid was markedly decreased in ELO-2 and FAT-2 RNAi worms (Fig. 2A). However, by treating the FAT-2 RNAi, worms contained a similar number of eggs as the RNAi-control; whereas the number of eggs visible inside each ELO-2 RNAi worm was clearly lower (Fig. 2B). Furthermore, when the shape of the eggs in the FAT-2 RNAi group was compared with that in the RNAi-control worm, many FAT-2 RNAi eggs exhibited heterogeneous forms and indistinct outlines. Therefore, it is conceivable that the eggs inside the FAT-2 RNAi worms experienced viviparous lethality as a result of some kind of abnormality. Besides miniaturization, RNAi worms of the FAT-6 group exhibited a slight decrement in egg-laying. We observed no particular influence either on the laying or hatching of eggs in the FAT-5 and FAT-7 RNAi groups (Fig. 2A and B).

To further examine the viability of the eggs, each plate was cultured for 2 more days and the number of hatched

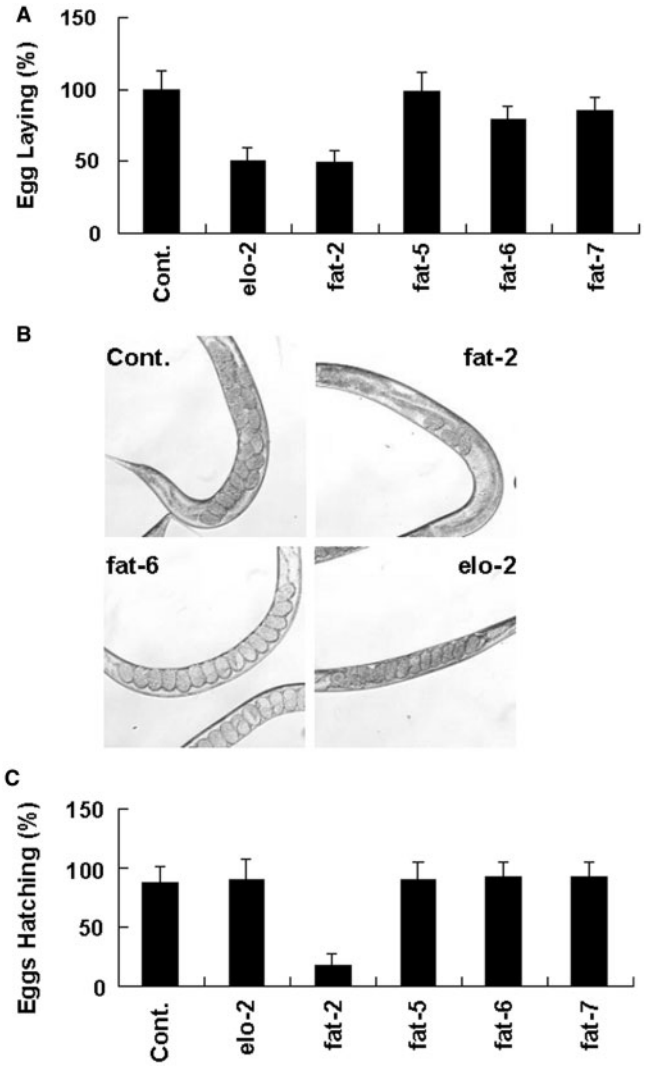

Fig. 2. **Effect of FAT and ELO-2 RNAi on reproduction and embryogenesis.** (A) Nematodes were moved to another plate after 72 h of incubation on RNAi media and the number of eggs measured after 8 h. Experiment was performed independently three times. Error bars indicate SEM values. (B) Abdominal macrophotographs of each worms of RNAi-treated for L4440 (Cont.), FAT-2, FAT-6 and ELO-2. (C) Eggs obtained in (A) were cultured and the population that hatched 48 h later was measured. Experiment was performed independently three times. Error bars indicate SEM values. Cont., Control RNAi (L4440).

eggs counted. More than 90% of the eggs from ELO-2 and FAT-5–FAT-7 RNAi worms hatched normally. However, hatchability was extremely low—less than 20%—in the FAT-2 RNAi group (Fig. 2C). Additionally, some worms among the hatched ones died (data not shown).

Influence on Lifespan of FAT and ELO-2 RNAi—We next examined the influence of RNAi on the lifespans of the FAT and ELO groups. After 4 or 8 days culture of worms on RNAi-media, worms were treated with 0.5 mg/ml FUdR and their viability observed everyday.

An apparent extension of lifespan was found in the ELO-2, FAT-3 and FAT-4 RNAi groups compared with

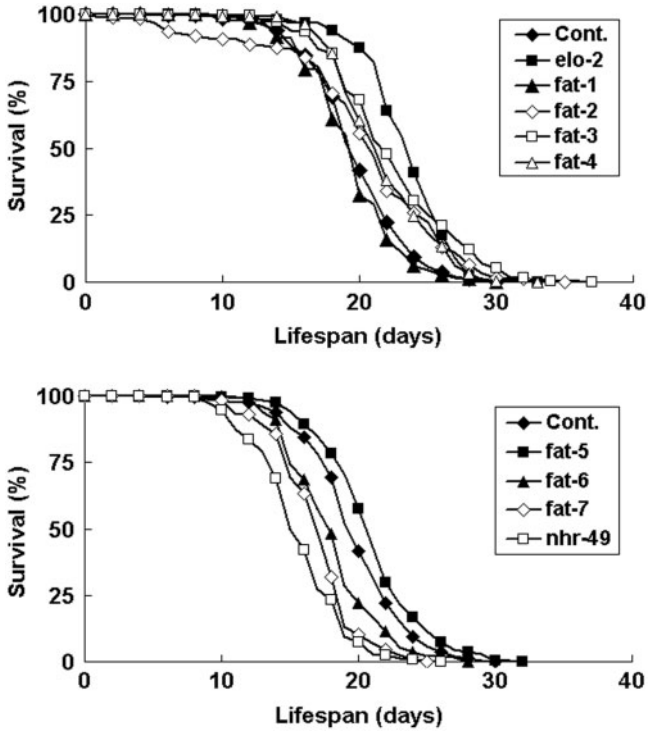

Fig. 3. **Influence on lifespan of FAT and ELO-2 RNAi.** (A) Life or death of nematode was judged by the response to tapping on the plate (*33*). For each RNAi-treated worms, the survival rate of adult worms was calculated and a survival curve drawn. Each RNAi-procedure for L4440 (Cont.), ELO-2, FAT-1, FAT-2, FAT-3, FAT-4, FAT-5, FAT-6, FAT-7 and NHR-49 are indicated in the small rectangles.

that of the RNAi-control worm (Fig. 3). The effect was most strongly seen in the ELO-2 group; its lifespan was 20% longer than that of the RNAi-control worm.

In contrast, a life-shortening effect of RNAi has already been reported in a FAT-7 population (*17*). FAT-6 and FAT-7 are functional homologs, and we found that the lifespans of FAT-6 worms were also conspicuously shortened by RNAi (Fig. 3), as occurred with FAT-7. NHR-49 is known as the transcriptional control element for FAT-5, FAT-6 and FAT-7 genes. The lifespans of RNAi worms in the NHR-49 were more markedly shortened than those of the FAT-6 RNAi and FAT-7 RNAi groups. The average lifespan in the NHR-49 RNAi cohort was shortened to about 80% of that of the RNAi-control worm.

Although, FAT-2 RNAi did not affect to lifespan, the survival rate of the FAT-2 cohort was lower than that of the RNAi-control worm after about 10 days of culture, but the survival rate was comparatively high after 20 days. RNAi had almost no effect on the lifespans of nematodes in the FAT-1 and FAT-5 groups.

Influence on DAF-16 Activity of FAT and ELO-2 RNAi—Using a Psod-3::gfp transgenic worm, promoter activity of *SOD-3* in each RNAi worms was analyzed. The Psod-3::gfp nematode was bred on RNAi plate for 72 h, and was observed under fluorescence microscope (DMRXA, Leica) after fixation by PFA. As a result, although lifespan was not change with a FAT-2 RNAi

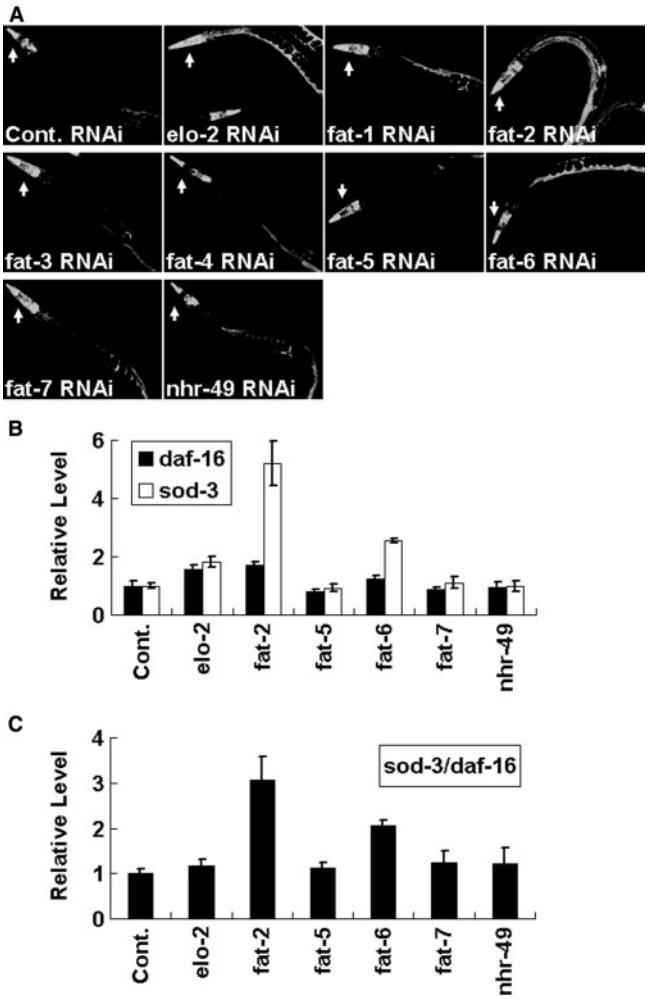

Fig. 4. **Influence on DAF-16 activity of FAT and ELO-2 RNAi.** (A) The Psod-3::gfp worms were cultured on each RNAi-media for 72 h. A fluorescence microphotograph using an L5 filter (Leica) was taken. (B) RNA was prepared from RNAi-influenced worms of FAT, ELO-2 and NHR-49 groups, and real-time PCR was performed. Value of gene expression is shown on the vertical axis, with a relative value for Control RNAi (L4440) (Cont.). Horizontal axes list the genes subjected to RNAi. Experiments were performed in triplicate. Error bars indicate SEM values. Expression of DAF-16, a representative stress response factor, and *SOD-3*, the main target gene of DAF-16. (C) Relative expression ratio of *SOD-3* to DAF-16 in each RNAi-worms.

worm, the fluorescence intensity remarkably increased (Fig. 4A). And, fluorescence slightly increased in an ELO-2 RNAi worm. However, fluorescence intensity was not influenced in FAT-3, FAT-6, FAT-7 and NHR-49 RNAi worms in which duration of life has changed.

To confirm these results, qRT-PCR was performed. RNA was prepared from RNAi-influenced worms of FAT, ELO-2 and NHR-49 groups, and real-time PCR was performed. We used a representative nematode lifespan-controlling gene, DAF-16 and a target gene regulated by DAF-16, *SOD-3*. As a result, as well as GFP promoter assay, an expression of *SOD-3* rose by FAT-2 RNAi conspicuously (Fig. 4B). In addition, by comparison of an expression level of *SOD-3* to that of DAF-16, transcription factor activity of DAF-16 rose to about three times of Control RNAi by FAT-2 RNAi (Fig. 4C). Expression level of both DAF-16 and *SOD-3* increased to double of Control RNAi in ELO-2 RNAi; however, the transcription factor activity of DAF-16 was same to that of Control RNAi (Fig. 4B and C). Interestingly, FAT-6 RNAi by which lifespan was reduced did not affect to expression of DAF-16, whereas transcriptive activity of DAF-16 increased to about two times (Fig. 4B and C). The expression of DAF-16 and *SOD-3* and the transcription factor activity of DAF-16 were the same as Control RNAi in FAT-5, FAT-7 and NHR-49 RNAi (Fig. 4B and C). These expressions were not different from those of Control RNAi in FAT-1, FAT-3 and FAT-4 RNAi too (data not shown).

Changes in Adiposity Caused by Fat Mutation and FAT and ELO RNAi—At first adiposity was compared between FAT mutants, FAT-2 (*tm789*), FAT-5 (*tm420*), FAT-6 (*tm331*) and FAT-7 (*tm326*), and FAT RNAi worms, FAT-2, -5, -6 and FAT-7. Adipose accumulation was observed under fluorescence microscope (DMRXA, Leica) after each worms were bred on RNAi plate for 84 h, and having fixed in PFA.

In comparison with the RNAi-control, there was drastic change of adiposity was found in FAT-2, FAT-6 and FAT-7 RNAi worms (Fig. 5A). In contrast, there was not a change of adiposity in FAT-2 and FAT-5 mutant, and in addition, only few decreases were detected in FAT-6 and FaT-7 mutant. Therefore adiposity in FAT and ELO-2 RNAi worms were observed next.

Lipid accumulation clearly decreased in ELO-2 RNAi worms (Fig. 5B). However, the location of the adipose tissue and the degree of decrease varied slightly with the kind of gene affected by RNAi. For example, in the FAT-2 RNAi cohort, a large amount of FAT was present in the area of the intestine, with a decrease in the presence of small fatty granules, whereas the amount of systemic fat decreased with equal distribution in the ELO-2, FAT-6 and FAT-7 RNAi groups (Fig. 5A and B). We observed fat accumulation in NHR-49 RNAi worms, as reported previously (*7*) (Fig. 5B). Marked adiposity was absent in each of the FAT-1, FAT-3, FAT-4 and FAT-5 RNAi cohorts.

Next, expression of fatty acid metabolism-related gene was analyzed by qRT-PCR. RNA was prepared from RNAi-influenced worms of FAT, ELO-2 and NHR-49 groups, and real-time PCR was performed. The genes related to fatty-acid metabolism that we used were FASN-1, ACS-2 and ECH-1.

As a result, FASN-1 and ECH-1 expression was suppressed in the RNAi ELO-2 cohort, and only ACS-2 expression was significantly increased (Fig. 5C). In the FAT-2 RNAi worms, suppression of ACS-2 expression and induction of FASN-1/ECH-1 were observed.

These results were different from the following known results. As was previously reported in the case of FAT-7 RNAi (*7*), the introduction of FAT-6 RNAi, which is a homolog of FAT-7, increased the expression of ACS-2 and ECH-1 (Fig. 5C). In addition, Expression of FASN-1 decreased in FAT-6 RNAi worms. This result was not same as that for the FAT-6 and FAT-7 RNAi groups.

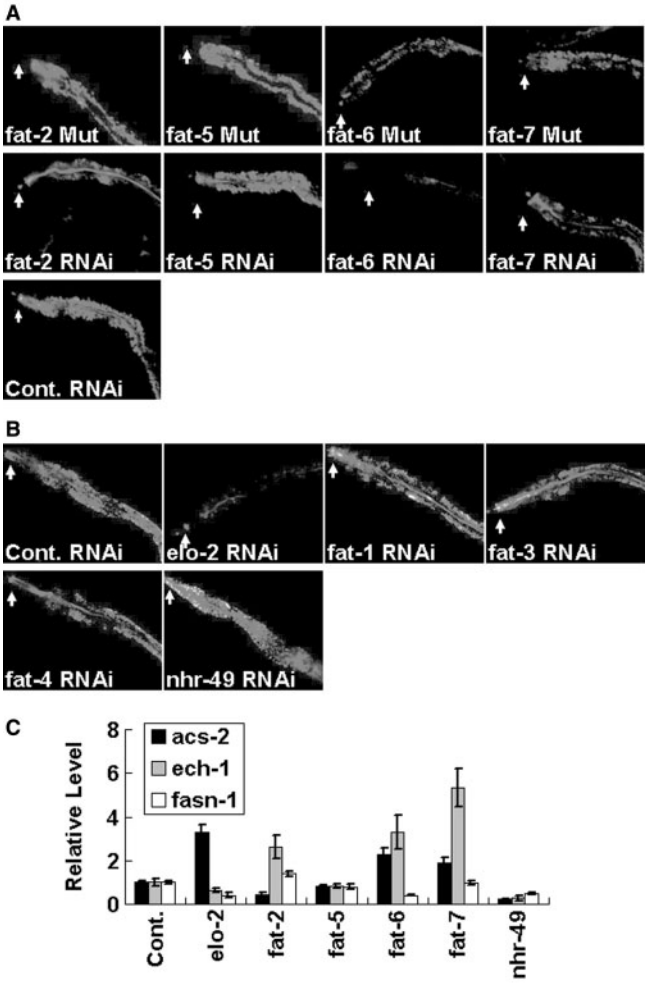

Fig. 5. **An adiposity and fatty acid metabolism-related gene expression in FAT and ELO-2 RNAi and FAT mutants.** (A) FAT-2 (*tm789*) (FAT-2 mutant), FAT-5 (*tm420*) (FAT-5 mutant), FAT-6 (*tm331*) (FAT-6 mutant), and FAT-7 (*tm326*) (FAT-7 mutant) were bred on Control RNAi media containing 0.5 μg/ml Nile Red. N2 worms were bred on Control RNAi media (Cont. RNAi) or RNAi media (FAT-2 RNAi, FAT-5 RNAi, FAT-6 RNAi, FAT-7 RNAi) containing 0.5 μg/ml Nile Red. Nematodes were collected after 84 h of incubation. A fluorescence microphotograph using an N3 filter (Leica) was taken. (B) Worms were bred on RNAi media containing 0.5 μg/ml Nile Red. Nematodes were collected after 72 h of incubation. White arrow indicates pharynx. (C) RNA was prepared from RNAi-influenced worms of FAT, ELO-2 and NHR-49 groups, and real-time PCR was performed. Value of gene expression is shown on the vertical axis, with a relative value for Control RNAi (L4440) (Cont.). Horizontal axes list the genes subjected to RNAi. Experiments were performed in triplicate. Error bars indicate SEM values. Expression analysis of ECH-1, ACS-2 and FASN-1 which contribute to fatty acid biosynthesis and metabolism.

As it was previously reported about ACS-2 and ECH-1 (*7*), expression of FASN-1 was also inhibited in the NHR-49 RNAi worm cohort (Fig. 5C). Additionally, expression pattern of these genes in the FAT-2, FAT-6 and FAT-7 mutants are not same to that of RNAi worms (data not shown).

Influence of FAT and ELO-2 RNAi on FAT mutants—To further analyze the role of lipid metabolism in body size, growth and reproduction of nematodes, RNAi of FAT-1 through FAT-7, ELO-2 and NHR-49 was introduced into mutant strains, specifically into FAT-5 (*tm420*), FAT-6 (*tm331*) and FAT-7 (*tm326*). Worms were cultured for 84 h on RNAi-media.

The FAT-5 mutant developed normally, grew and laid eggs, as did the WT strain (N2), following the introduction of Control RNAi. On the other hand, delayed growth and decreased egg number were observed in FAT-6 and FAT-7 mutants compared with those of the WT strain (Fig. 6 and Table 2).

We found newly the following facts. RNAi of FAT-2 and ELO-2 caused clear growth delay, downsizing in the FAT-5, FAT-6 and FAT-7 mutants. In addition, RNAi of ELO-2 caused ovipositional dropoff and RNAi of FAT-2 caused strong embryonic lethal in the FAT-5, FAT-6 and FAT-7 mutants. In addition, only ELO-2 RNAi of FAT-5 mutant caused fetus death slightly.

Furthermore, growth completely stopped in FAT-6 and FAT-7 mutants under the influence of RNAi of these genes (Fig. 6 and Table 2). However, with FAT-5 RNAi, obvious miniaturization, growth delay and ovipositional decline were found in the FAT-6 mutant, but not in the FAT-7 mutant. Only a slight delay in growth was observed in FAT-5 RNAi of the FAT-7 mutant group. Although growth retardation, miniaturization and fetal death occurred with FAT-6 and FAT-7 RNAi of the FAT-5 mutant, it was not a substantial change. In addition, no apparent change from the use of FAT-1, FAT-3, FAT-4 and NHR-49 RNAi was observed in any mutant.

DISCUSSION

Our results demonstrated that the FAT and ELO genes have various physiological functions in ontogenesis and lipid metabolism of the nematode *C. elegans*. We noted a markedly miniaturized body size, extremely low number of eggs and decreased fatty accumulation in nematodes from RNAi of ELO-2, an elongase of palmitic acid. However, RNAi of palmitic acid desaturase FAT-5 had almost no effect on the worms' physical size, oviposition and fatty accumulation.

As a result of gene expression analysis in ELO-2 RNAi worm, an expression rise of ACS-2, restraint of ECH-1 (namely, suppression of β-oxidation) and activation of DAF-16 were newly observed. It has been known that ACS-2 works as one of the starvation responsive factors (*8*, *12*), and that DAF-16 is activated by starvation. These results suggest that the energy state in the ELO-2 RNAi worm cohort may be lower than that of the RNAi-control worm, leading to ovipositional decrease and an extension of lifespan. It is well understood that reproduction requires an extreme amount of energy in many creatures, and that nutritional factors strongly influence reproductive potential. Furthermore, because the lifespan of everything from yeasts to mammals can be extended by promoting a low-energy habit, it is conceivable that life extension by ELO-2 RNAi depends on the falloff of energy levels. A marked decrement in lipid accumulation was observed in the ELO-2 RNAi cohort. Our results indicated that ELO-2 RNAi caused the remarkable reduction of mRNA expression of FASN-1

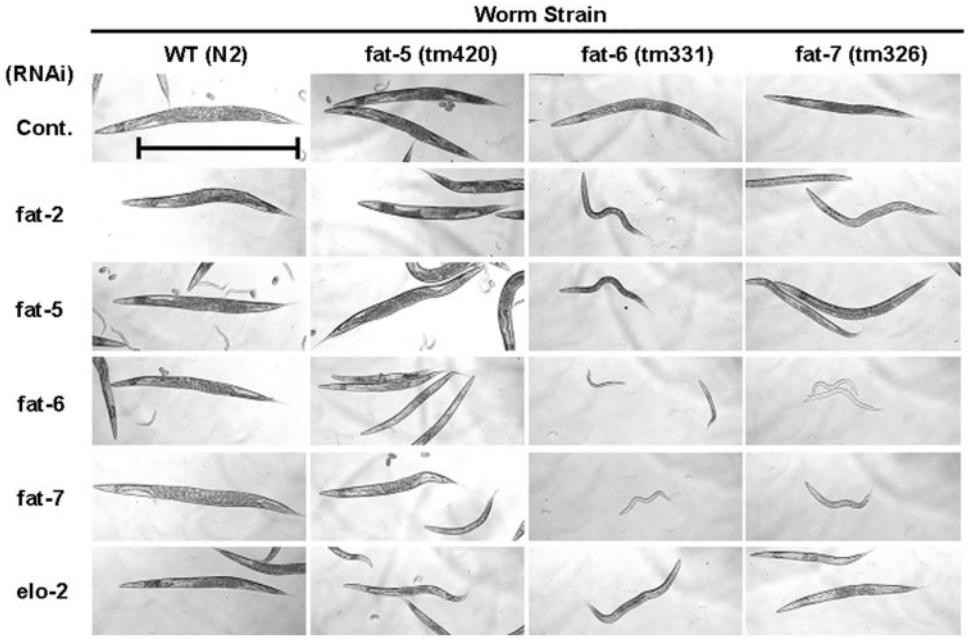

Fig. 6. **Influence of FAT and ELO-2 RNAi on FAT-5, FAT-6 and FAT-7 mutants.** FAT-5 (*tm420*), FAT-6 (*tm331*) and FAT-7 (*tm326*) were used as FAT mutants. After 84 h of culture on RNAi-media (vertical axis), worms were collected and observed. Cont., Control RNAi (L4440). Bar indicates 1 mm.

Table 2. **Influence of FAT and ELO-2 RNAi on FAT-5, FAT-6, and FAT-7 mutants.**

RNAi	FAT-5 (tm420)	FAT-6 (tm331)	FAT-7 (tm326)
Cont.	None	Gro per WT (N2)	Gro per WT (N2)
FAT-1	None	None	None
FAT-2	Gro, Small, Stronge Emb	Gro, Small, Stronge Emb	Gro, Small, Stronge Emb
FAT-3	None	None	None
FAT-4	None	None	None
FAT-5	None	Gro, Small, Rep	Gro
FAT-6	Gro, Small, Weak Emb, Rep	Arrest	Arrest
FAT-7	Gro, Small, Weak Emb	Arrest	Arrest
ELO-2	Gro, Small, Weak Emb, Rep	Gro, Small, Rep	Gro, Small, Rep
NHR-49	None	None	None

FAT-5 (*tm420*), FAT-6 (tm331) and FAT-7 (*tm326*) were used as FAT mutants.
Phenotypes observed in each RNAi-treated cohort are indicated. Gro, slow growth; Emb, embryonic lethal; Rep, replication defect; Arrest, growth arrested. None, no difference between controls; Cont., Control RNAi (L4440).

gene, resulted in a decrease of adiposity. Because expression pattern of ACS-2, ECH-1 and FASN-1 in ELO-2 RNAi worm is different from that in FAT-7 RNAi worm (*7*) or SCD-1 knockdown mouse (*19–22*), it is thought that a decrease of adiposity by ELO-2 RNAi occurs with a mechanism unlike a known mechanism. We can speculate whether this is because FASN-1 was functionally inhibited by the accumulation of palmitic acid.

In this study, we newly found that RNAi of FAT-2, an oleic acid desaturase, caused a large decrease of lipid accumulation. Furthermore, ovipositional falloff and a marked increase in fetal death was observed with FAT-2 RNAi, but no changes in lifespan or form appeared. An individual body size only slightly decreased, too. As a result of gene expression analysis of a FAT-2 RNAi worm, an expression of ECH-1 increased, and the expression of ACS-2 decreased, whereas the expression of FASN-1 did not change. These results do not accord with a known result of FAT-7 RNAi worm (*7*) or SCD-1 knockdown mouse (*19–22*), and thereby, suggest that a decrease of adiposity by ELO-2 RNAi occurs with a mechanism unlike a known mechanism. Additionally, downregulation of ACS-2 expression and activation of β-oxidation in FAT-2 RNAi were possibly caused by some factor not related to starvation. Interestingly, transcription factor activity and expression of DAF-16, a starvation-inducible gene, increased significantly with FAT-2 RNAi. The reasons for these phenomena are unclear, but we think that RNAi of FAT-2 affects intracellular localization as well as expression of DAF-16, because the transcription factor activity of DAF-16 increased markedly. Judging from the fact that an expression of one of the starvation responsive factors

ACS-2 was inhibited, FAT-2 RNAi is possibly the phenomenon that mimics mammalian insulin resistance.

Although slight downsizing and a slight decrease in oviposition rate were observed following RNAi of FAT-6 and FAT-7 from stearic acid desaturase in *C. elegans*, there was no extreme change as with ELO-2 RNAi. Although slightly smaller body sizes and a slight decrease in oviposition rate were observed following RNAi of FAT-6 and FAT-7 from stearic acid desaturase in *C. elegans*, the apparent effects were not as major as those found in the ELO-2 RNAi cohort. These results indicated that physical downsizing and ovipositional decline are changes specific to ELO-2 RNAi, and thus it is possible that palmitate elongation plays an important role in normal growth and egg-laying in the nematode.

No marked miniaturization and abnormal reproduction were found following FAT-6 and FAT-7 RNAi imposition, however, lipid accumulation fell and the expression of genes involved in lipid metabolism varied; induction of β-oxidation and suppression of fatty acid synthesis were observed. From this result, we newly suggested that FAT-6 potentially play a similar role to that of FAT-7 which was already reported (*7*). Furthermore, a similar variation of gene expression related to lipid metabolism has been observed in the SCD-1 knockdown mouse (*19–22*).

SCD-1 is a functional homolog of FAT-6 and FAT-7, indicating that stearate desaturation may play a primary role in lipid accumulation in both nematodes and mammals. Because of the variation in localization and imbalance of expression in the FAT-6 and FAT-7 groups, we conjecture that some individual gene expression exists between FAT-6 RNAi and FAT-7 RNAi processes. The relative expression of *SOD-3*/DAF-16 in FAT-7 RNAi was similar to that of the RNAi-control, whereas the relative *SOD-3* level in FAT-6 RNAi was higher than that of control, suggesting that life shortening by RNAi of FAT-6 and FAT-7 may be controlled with a mechanism independent of the DAF-2 signaling cascade. This result suggests that FAT-6 and FAT-7 possibly have both of common function and independent function. However, RNAi of NHR-49—a transcription regulator of FAT-6 and FAT-7—caused a marked decrease in lifespan compared to that caused by FAT-6 and FAT-7 RNAi, and the adipose accumulation pattern is completely opposite to that of NHR-49 RNAi and the RNAi of FAT-6/FAT-7. This indicates that stearate biosynthesis itself may play some important physiological role.

Because we know that the expression of FAT-7 rises in FAT-6 mutants, and that of FAT-6 rises in FAT-7 mutants (*11*), it is conceivable that FAT-6 and FAT-7 play supplemental roles for each other. On the other hand, in our results with FAT-6 RNAi of the FAT-6 mutant and FAT-7 RNAi of the FAT-7 mutant, growth completely stopped simultaneously (Table 2). Because there is high homology (>80%) between FAT-6 and FAT-7, it is suggested that the other gene expression is inhibited by RNAi of FAT-6 or FAT-7 (*39*). Therefore, our results suggest that expression of both genes were remarkably inhibited by having introduced RNAi into FAT-6 and FAT-7 mutants. Because introduction of FAT-5 RNAi caused a different phenotype between FAT-6 mutant and FAT-7 mutant, it is possible that FAT-6 and FAT-7 have an individual function.

Most phenotypic and gene expression observed in the RNAi-control strain were absent in the FAT-5 RNAi and FAT-5 mutant groups, whereas markedly smaller bodies and growth suppression, reduced egg production, and minor increases in fetal death rates were observed in the FAT-5 mutant following RNAi of FAT-6 or ELO-2, suggesting that FAT-5 plays a role in moderating the dysfunction of FAT-6 and ELO-2. Overall, we think that the expression of genes involved in fatty-acid metabolic system is controlled so as to make up for their mutual functions.

Because RNAi of FAT-1, FAT-3 and FAT-4 did not affect at all to growth, morphogenesis and adiposity of mutants FAT-6, FAT-7 and FAT-5, it is thought that ontogenesis and adiposity are not almost influenced by a suppression of individual PUFA synthase, such as FAT-1, FAT-3 and FAT-4, one by one.

Combining these findings, we conclude that FAT and ELO contribute to various physiological phenomena such as duration of life, body length, growth and egg production, as well as fat metabolism. In particular, this work suggests that the pathway that leads to FAT-2 from ELO-2 via FAT-6 and FAT-7 occupies a very important position both physiologically and metabolically. Above all, the physiological function of FAT-6 and FAT-7 resembles that of SCD-1 of the mouse, and we conjecture that SCD is the main gene that acts in animal lipid accumulation. At the same time, the regulatory system of fat metabolism in the nematode resembles that of mammals, suggesting that the nematode can be used as a model animal for the investigation of obesity. It was recently reported that expression and localization of GPAT and DGAT, proteins involved in triglyceride synthesis, are coordinately regulated with *scd* genes (*16*, *17*). Functional analysis of FAT and ELO in triglyceride synthesis is currently occurring. In this report, we focused on only fatty acid metabolism. However, it is known that fatty acids distribute in a different composition in phosphatide (PC, PE, PS, PI, etc.) (*40*), and therefore, the effect to fatty acid composition should be analyzed in the future.

In addition, our result could present a new knowledge from a standpoint of lifespan study. Our result indicated that individual duration of life did not agree with activity of DAF-16 at all in RNAi worms of FAT-2, FAT-6, FAT-7 and NHR-49, except an ELO-2 RNAi worm. Because FAT-7 is located in a downstream of DAF-16 signaling cascade (*41*), it is possibly thought that fatty acid metabolism plays a role as a portion of lifespan regulatory mechanism.

In this report, the comparison experiments with mutant and RNAi, of FAT-2, FAT-5, FAT-6 and FAT-7, confirmed the fact that RNAi let a drastic change of adiposity. Previously, it was reported that FAT-6 and FAT-7 do complementation of each other's expressions (*32*). Our observation also confirmed that the level of expression of FAT and ELO-2 remarkably changes by RNAi of FAT and ELO-2 (data not shown). Furthermore, in FAT mutant, it was recently reported that plural different course is rebuilt instead of original fatty acid metabolic pathway (*32*). Because potential new fatty acid

metabolic pathway was rebuilt in FAT mutant, it is thought that adipose accumulation recovered to some extent. On the contrary, it is thought that a change of extreme adiposity was observed because a disfunction of FAT and ELO-2 is not supplemented in RNAi worms.

These results lead us to believe that fatty acid metabolism, and particularly the desaturation of fatty acid, plays an important role in the uptake and consumption of fatty acid, and that these mechanisms may be widely conserved from nematodes to mammals. Therefore, *C. elegans* is potentially useful as a model animal for understanding the mechanism of obesity or lifestyle-related disease and for screening of an anti-obesity drug. Finally, a FAT-2 RNAi individual can be used for analysis of insulin resistance, and therefore, the change of phosphorylation and acetylation level of DAF-16 is currently being analyzed.

Supplementary data are available at *JB* Online.

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