Novel Functions of Lipid-binding Protein 5 in Caenorhabditis elegans Fat Metabolism* 5

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The lipid-binding protein (LBP) family is conserved from Caenorhabditis elegans to mammals and essential for fatty acid homeostasis. RNAi-mediated knockdown of nine C. elegans lbp family members revealed that lbp-5 regulates fat accumulation. C. elegans LBP-5 bound directly to various fatty acids with varying affinities. lbp-5 expression in nhr-49(nr2041) worms was much lower than in N2 worms. Nhr-49 transcriptional activity also decreased with lbp-5 deletion, suggesting that they may work together as functional partners in fat metabolism. In support of this notion, LBP-5 translocated into nuclei, where it appeared to influence C. elegans NHR-49 target genes involved in energy metabolism. Interestingly, LBP-5 is required for stearic acid-induced transcription of NHR-49 target genes. Thus, this knowledge could help identify therapeutic targets to treat obesity and diseases associated with nematode-host interactions.

Fatty acids are essential molecules that act as energy suppliers, building blocks of membrane lipids, signaling molecules, and precursors for lipid mediators. Fatty acids are hydrophobic molecules that must be solubilized and transported within the cell by intracellular lipid chaperones known as fatty acid-binding proteins (FABP)2 (1–4). These low molecular mass (14–15 kDa) polypeptides are conserved from Caenorhabditis elegans to humans and are involved in fatty acid uptake, transport, and oxidation (5, 6). FABPs are abundant in tissues that either require large fluxes of fatty acids or have high demand for fatty acids as an energy source. Because fatty acid influx and efflux are essential for all tissues, FABPs are thought to be necessary for intracellular uptake and transport of fatty acids to target tissues. They are also involved in targeting fatty acids to specific metabolic pathways, regulation of gene expression, and cell growth (7).

In mammals, FABPs are members of the superfamily of intracellular lipid-binding proteins (LBP). To date, four subfamilies of intracellular LBPs with tissue-specific distribution have been identified (8–11). FABPs bind both saturated and unsaturated long-chain (≥14 C) FA with high affinity (12). The presence of intracellular FABPs increases the solubility of fatty acids and may create a larger cytosolic fatty acid pool that enhances substrate availability to fatty acid-metabolizing enzymes. As lipid chaperones, FABPs may actively facilitate the transport of lipids to specific compartments in the cell for fat storage (lipid droplets), signaling (endoplasmic reticulum), and membrane synthesis (6, 7). In so doing, they impact systemic energy homeostasis (13). Studies have also shown that FABPs (e.g. FABP4 and FABP5) can have synergistic functions in specific nutritional conditions, and deliver specific fatty acids to peroxisome proliferator-activated receptors (PPARs) for their transcriptional activation (14, 15). However, despite the fact that most tissues express several FABP isoforms, regulation of their tissue-specific expression and functions remain poorly understood.

A functional relationship exists between FABPs and PPARs in mammals. FABPs regulate the activity of PPARs, a process associated with lipid signaling in inflammation and cancer. Fatty acids, triglycerides, and phospholipids act as substrates to generate PPAR ligands, which are guided to their receptors by cytoplasmic FABPs. PPARs translate these lipid signals into responses that work to maintain energy homeostasis (14). For example, FABP1 (liver FABP) has been hypothesized to be involved in enterocyte lipid absorption, as well as hepatocyte lipid transport and lipoprotein metabolism. FABP1 delivers PPAR ligands to the nucleus to modulate PPAR-α and PPAR-γ gene expression (16). More examples have been reported for FABP4 and -5 (7, 15, 17).

FABPs are conserved in nematodes, where they appear to play critical roles in survival responses. Nematodes are one of the most abundant and ecologically diverse groups of multicellular organisms. Their parasitic forms cause human disease and economic damage to domestic animals and crops. FABPs in the nematode, which are known as LBPs, have attracted much research interest because of their potential roles in nutrient acquisition, manipulation of host tissues, and countering host defense reactions (18–20). Nonetheless, the exact function of individual LBPs in nematodes remains unclear. The C. elegans genome contains nine LBP genes, lbp-1 to lbp-9 (21). The function of these LBPs in fat metabolism or survival during the dauer stage of C. elegans development remains unknown.

Mammalian PPAR-α and C. elegans NHR-49, which is a homologue of mammalian HNF-4 (22), regulate the transcription of similar target genes including genes involved in fatty acid β-oxidation, lipid binding, and fatty acid desaturation (22–

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2 The abbreviations used are: FABP, fatty acid-binding protein; DAUDA, 11-((5-dansyl)amino)undecanoic acid; LBP, lipid-binding protein; TG, triglyceride; PPAR, peroxisome proliferator-activated receptor; SA, steric acid.
Regulation of C. elegans Fat Metabolism by LBP-5

24). Inhibition of either NHR-49 in C. elegans, or PPAR family members in mice, enhances fat storage (22, 25, 26). In addition, NHR-49 is a major regulator of fat-5, -6, and -7, and fat-7 has been reported to be regulated by at least five different transcriptional regulators (27). By convention, NHR-49 is also called a functional homolog of PPAR-α (22).

To further understand fat metabolism in C. elegans, which is essential for their survival throughout the life cycle, we identified the lbp-5 genes and characterized their regulation. Because lbp-5 knockdown caused the accumulation of large deposits of body fat, we focused on the role of LBP-5 regulation in fat metabolism. Here, we show that LBP-5 contributes to fat storage and fatty acid β-oxidation by cooperating with NHR-49 to modulate the expression of fatty acid metabolic genes.

EXPERIMENTAL PROCEDURES

Worm Strains—The Bristol N2 standard wild-type strain, the rrf-3 mutant, and lbp-5::gfp worms were obtained from the Caenorhabditis Genetics Center. lbp-5(tm1618) was obtained from the National BioResource Project (NBRP Japan), and nhr-49(nr2041) was kindly provided by Carl Johnson and Nemapharm Pharmaceuticals. The lbp-5(tm1618) and nhr-49(nr2041) mutant strains were performed by backcrossing six times, and cultured under standard laboratory procedures, as previously described (28). To create lbp-5 rescue worms, about 100 μg/ml of plasmid DNA containing the lbp-5 full gene::GFP construct (described below as LBP-5::GFP construct) was injected into lbp-5(tm1618) mutants. GFP expressing transgenic worms were selected from the progeny.

Single Worm PCR—To screen the progeny of heterozygotes, single worm PCR was performed using the following primers: lbp-5 sense, 5’-GCTAGAGTTTCGAGAAAG-3’, lbp-5 anti-sense, 5’-GTTGATTTTTGCAGAACGT-3’; nhr-49 sense, 5’-CTTCGTGATCAAGATTCCTTG-3’, nhr-49 anti-sense, 5’-GTCCTGATCGATGATCGAGAATGA-3’. A single adult worm obtained after egg laying (≈30 eggs) was picked and placed in a 5-μl drop of lysis buffer. Then the tubes were frozen at −70 °C for 15 min, incubated at 60 °C for 60 min, and then incubated at 95 °C for 15 min. After cooling to 4 °C in a PCR thermocycler, 2.5 μl of worm extract (template DNA) was added into each new Eppendorf tube for lbp-5 and nhr-49, and mixed with 22.5 μl of PCR master mixture to make a total volume of 25 μl for PCR. Aliquots of each reaction mixture were loaded onto 10% SDS-polyacrylamide gels along with a 1-kb standard marker.

RT-PCR and Gene Sequencing—First-strand cDNA was synthesized from RNA using the First Strand Synthesis kit (Amerham Biosciences). PCR was performed using Ex Taq polymerase (TaKaRa Korea Biomedical Inc.). The sequences obtained were aligned to the predicted sequence and analyzed with DNAMAN software (29). The data presented are the mean values (± S.D.) of three independent experiments for reactions performed in triplicate.

Construction of Double Mutant by Genetic Crosses—To create an lbp-5(tm1618);nhr-49(nr2041) mutant, N2 males were crossed with homozygous mutant lbp-5(tm1618) hermaphrodites at the L4 stage. The F1 heterozygotes were crossed again with homozygous mutant nhr-49(nr2041) hermaphrodites at the L4 stage, and these F1 heterozygotes were self-mated to obtain lbp-5(tm1618);nhr-49(nr2041) progeny. Progeny of the heterozygotes were screened by single worm PCR for lbp-5(tm1618);nhr-49(nr2041) recombinants. Single worm PCR was performed using primers: lbp-5 sense, 5’-GCTAGAGTTTCGAGAAAG-3’, lbp-5 anti-sense, 5’-GTTGATTTTTGCAGAACGT-3’; nhr-49 sense, 5’-CTTCGTGATCAAGATTCCTTG-3’, and nhr-49 anti-sense, 5’-GTCCTGATCGATGATCGAGAATGA-3’.

Sudan Black B Staining—For Sudan Black staining of fat stored in the worm body, well fed worms were fixed in 1 ml of 1% paraformaldehyde in PBS by rocking at room temperature for 15 min, subjected to three freeze-thaw cycles, and dehydrated sequentially in three different concentrations of ethanol (25, 50, and 70%). The worms were stained overnight in a 50% saturated solution of Sudan Black B (Sigma, number s2380) in 70% ethanol, rehydrated, and photographed (30).

Triglyceride (TG) Content Analyses—To obtain a synchronously growing population, eggs were prepared by treating a population of C. elegans with NaClO/KOH solution. The eggs were allowed to hatch overnight in 5-basal buffer at 20 °C to obtain a highly synchronous population of L1-stage animals. Nematodes were then shifted to nematode growth medium plates covered with Escherichia coli OP50 and allowed to grow until the L4 larval stage. Worms were then harvested by three consecutive washes in ice-cold M9 buffer to separate nematodes from bacteria. Nematodes were flash frozen in liquid nitrogen and stored at −80 °C until use. Approximately 200 μl of worms (without water) for each sample were ground in a nitrogen-chilled mortar and pestle, then gathered in a reaction tube and kept on ice. PBS (200 μl) was added to the worm powder, and the extracts were sonicated three times and then centrifuged for 7 min at 12,000 × g at 4 °C to remove the insoluble matter. Protein concentrations were measured by Bradford assay. Fat content was determined with a commercially available serum TG determination kit (Sigma, TR0100). At least two sample sets were independently obtained and each sample was measured for TG content twice.

Construction of LBP-5::GFP and Transformation of C. elegans—The lbp-5 gene and the 1.91-kb upstream regulatory sequence were subcloned into the pPD 95.79 GFP vector using standard molecular biology protocols: 5’ sense primer, ATCTGAGTTTCGAGAAAG, and 3’ antisense primer, GGGTACCTTTCGAGAAAG, and then transformed into C. elegans OP50. To obtain LBP-5::GFP transgenic worms, C. elegans OP50 was microinjected with LBP-5::GFP pRF4 (100 μg/ml) and pRF4 (50 μg/ml) into wild-type N2 animals, we selected heritable roller lines. Transgenic worms expressing translational fusions of LBP-5::GFP were analyzed to assess nuclear localization.

Quantitative RT-PCR Measurements—To determine the relationship between cycle number (Ct) and mRNA levels, primers were calibrated using serial dilutions of cDNA and genomic DNA. Quantitative RT-PCR were performed with an MJ Research Chromo4 Detector using the QuantiTect SYBR
Regulation of C. elegans Fat Metabolism by LBP-5

Green PCR kit as described by the manufacturer (Qiagen) using 200 ng of cDNA per sample in a total volume of 20 μl. The percent relative expression was determined using the ΔΔCt method, and an average of the expression of the reference gene, actin, was used to control for template levels. Each experiment was performed in triplicate.

Fatty Acid Feeding Experiments—Fatty acid sodium salts were obtained from Sigma and stored in the dark. For each experiment, a fresh 10 mM stock solution was prepared by dissolving fatty acids in ethanol. Nematode Growth Medium agar was prepared with the addition of 0.1% tergitol (Nonidet P-40). Agar was cooled to 45–50 °C, and the fatty acid stock solution was added slowly with stirring for 1 min. Agar solution was poured immediately onto plates, which were covered and dried in the dark for 24 h. Plates were then seeded with E. coli and allowed to dry for 2 days in the dark at room temperature before the addition of embryos. Embryos were prepared by alkaline hypochlorite treatment of adult nematodes to obtain a semi-synchronized population of early embryos. Eggs were placed on agar media containing fatty acids at a concentration of 80 μM: palmitic acid (C16:0), oleic acid (Δ9, 18:1), stearic acid (18:0), arachidonic acid (C20:4, Δ9, 12, 15), linoleic acid (C18:2, Δ9, 12), and α-linolenic acid (C18:3, Δ9, 12, 15).

After phenotypic analysis by Sudan Black staining of adult worms, the worms were washed off the plates using double-distilled water and centrifuged gently. The water was removed and the worm pellets were frozen for qRT-PCR analysis (22, 25).

Statistical Analyses—Data were presented as the mean ± S.D. Statistical significance was determined using a Student’s t test (SPSS 16.0, SPSS Inc.). p values of <0.05 were taken to indicate statistical significance.

RESULTS

Identification of Fatty Acid-binding Domain in C. elegans LBP Family Members—Sequences for the lbp-1 to lbp-9 genes were obtained from WormBase. The amino acid sequences of these genes were identified using the National Center for Biotechnology Information data base and published literature (21–23). Amino acid alignment of all nine LBP members revealed that all of them possess a similar fatty acid-binding domain (18 amino acid length; supplemental Fig. S1). Although some amino acids are highly conserved, the LBP sequences are generally diverse. DNA sequence-based cluster analysis grouped the lbp genes such that lbp-1–4 and lbp-5–8 were categorized into discrete groups, whereas lbp-9 remained unclustered (supplemental Fig. S2A). The sequence identity between the LBP family members is shown in supplemental Fig. S2B.

Deletion of lbp-5 Causes Fat Accumulation in C. elegans—To determine which lbp genes are important for maintaining homeostasis of fatty acid metabolism, we tested the effects of RNAi-mediated depletion of each LBP family member on C. elegans fat accumulation. Bacteria carrying each lbp RNAi construct (supplemental Table S1) were fed to the rrf-3 mutant worms, and then the differential fat accumulation was examined by Sudan Black staining. Our data demonstrate that lbp-5(RNAi) worms exhibited the highest fat accumulation, suggesting that lbp-5 is important for fat metabolism in C. elegans (supplemental Fig. S3A). To confirm the function of lbp-5 in fat homeostasis, we obtained an lbp-5(tm1618) mutant containing a 940-bp deletion within the 5′ UTR and coding region, as well as a 20-bp insertion within the 3′ UTR (supplemental Fig. S3B, NBRP). Fat accumulation in this mutant was detected by Sudan Black staining and compared with that of the nhr-49(nr2041) single mutant and the lbp-5(tm1618); nhr-49(nr2041) double mutant worms. Although the overall phenotype of the lbp-5(tm1618) mutant was similar to the wild-type N2 worms, this mutant exhibited elevated fat storage that was similar to the nhr-49(nr2041) and lbp-5(tm1618); nhr-49(nr2041) mutants (Fig. 1A). Sudan Black staining showed that lbp-5(tm1618) mutant worms accumulated fat granules in the intestine, similar to nhr-49(nr2041) mutant worms (Fig. 1A). Changes in fat were also determined by measuring the total TG in each strain. The lbp-5(tm1618), nhr-49(nr2041), and lbp-5(tm1618); nhr-49(nr2041) worms showed much higher TG content than wild-type N2 worms (Fig. 1B). To ensure that fat accumulation is caused by deletion of lbp-5, we performed a lbp-5 rescue experiment on lbp-5(tm1618) mutant worms. The results showed that the gfp-fused lbp-5 genome sequence transgene rescued the high-fat phenotype (supplemental Fig. S3C). These data confirm the functional contribution of LBP-5 to fat homeostasis.
Regulation of C. elegans Fat Metabolism by LBP-5

To investigate the biochemical properties of LBP-5, we produced recombinant LBP-5 fusion protein (supplemental Fig. S4). This protein was validated by N-terminal sequencing followed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) analysis (supplemental Fig. S4C). The structural analysis using tandem mass spectrometry confirmed the identity of our recombinant LBP-5 protein (supplemental Fig. S4D). Theoretical analysis of the amino acid secondary structure revealed that LBP-5 is a β-sheet-rich protein. This prediction was confirmed by circular dichroism (CD) spectra in the far UV region (190–250 nm; Fig. 2A). The fatty acid binding activity of purified recombinant LBP-5 was also examined using fluorescent lipid analogues, such as 11-(5-dansyl)amino)undecanoic acid (DAUDA), whose emission spectrum is altered by protein binding (32). A titration curve was produced by incubating fixed amounts (1 μM) of LBP-5 with differing quantities of DAUDA (Fig. 2B). The fluorescence emission of DAUDA was sensitive to polarity, and its intensity was increased and shifted to a shorter emission wavelength following LBP-5 binding. For example, the peak emission of DAUDA in the presence of buffer alone was 535 nm; however, the emission shifted to 470 nm and became more intense upon addition of LBP-5 (Fig. 2C). This phenomenon is called blue shift. This observed blue shift to 470 nm of DAUDA in the presence of LBP-5 suggests a binding site that is highly apolar or has unusual ligand orientation constraints.

To test the binding properties of recombinant LBP-5 for various ligands, we screened a panel of natural, non-fluorescent fatty acids and other hydrophobic molecules by testing their ability to competitively interfere with LBP-5-DAUDA binding. Polyunsaturated fatty acids (e.g. arachidonic acid and α-linolenic acid) were also included in these assays (supplemental Fig. S5 and Table S2). Binding of LBP-5 to DAUDA was disrupted by the addition of these selective fatty acids; however, the efficiency of competitive displacement varied considerably (supplemental Table S2). For instance, progressive addition of stearic acid (0.5, 1, 2, 4, and 8 μM) reversed the fluorescence shift caused by LBP-5-DAUDA binding, presumably by displacing DAUDA from the LBP-5 binding site (Fig. 2C), resulting in free DAUDA and a fluorescence shift back to 535 nm. Competitive inhibition was also observed for oleic acid, lauric acid, pentadecanoic acid, palmitic acid, arachidonic acid, and α-linolenic acid (supplemental Fig. S5 and Table S2). Fluorimetric titration of LBP-5 with DAUDA (Fig. 2B) produced a dose-dependent increase in relative fluorescence intensity, which is consistent with the presence of a single DAUDA binding site (n = 0.7) on each LBP-5 monomer with an apparent dissociation constant (Kd) equal to 33 nM (Fig. 2D). Taken together, these data show that our recombinant LBP-5 protein binds to various fatty acids with different affinities.

Biochemical Characterization of Recombinant LBP-5 Protein—To visualize LBP-5 localization, we generated a transgenic strain expressing LBP-5-GFP fusion protein under the regulatory strain expressing LBP-5-GFP fusion protein under the genetic strain expressing LBP-5-GFP fusion protein under the
control of the lbp-5 promoter. The spatio-temporal expression pattern of LBP-5 was assessed in C. elegans. LBP-5-GFP was observed in the intestine during all larval stages (Fig. 3A). This fusion protein was also detected in adult nematodes (Fig. 3B). Expression in individual seam cells was often highly punctate (Fig. 3B), and strong GFP expression was observed in these cells in the L4 larval and adult stages (Fig. 3, C and D). GFP expression was observed during embryogenesis and in all subsequent larval and adult stages (Fig. 3, E and F). LBP-5 protein was expressed widely in many different cell types, indicating that LBP-5 most likely plays important roles throughout the entire worm body. In particular, LBP-5 expression is high within the hypodermis and intestine, which are major sites of fat accumulation.

LBP-5-GFP was detected in large intestinal nuclei (Fig. 3A), and as punctate staining in the nuclear region of seam cells (Fig. 3B). These data provide additional evidence for our hypothesis that LBP-5 may translocate from the cytoplasm into the nucleus to carry fatty acids. The observed nuclear localization (Fig. 3) indicates that LBP-5 may play a role in transporting fatty acids into the nucleus. This data also suggests the possible existence of a nucleus-cytoplasm shuttling system for translocation of LBP-5 in response to changes in nutrient conditions (e.g. fatty acids).

**LBP-5 Is Required for NHR-49 Target Gene Activity—**To examine the functional relationship between NHR-49 and lbp gene expression, we measured lbp-5 expression in an nhr-49 mutant. lbp-5 expression analyzed by qRT-PCR in nhr-49(nr2041) worms was much lower than in N2 worms (1[nhr49] versus 6.35[N2]; Fig. 4A). Consistent with this, the lbp-5-gfp reporter expression was also reduced significantly in nhr-49(RNAi) animals, indicating that NHR-49 appears to be required for controlling lbp-5 expression (Fig. 4B).

By analogy to the case of interaction between FABPs and PPAR in mammals, we postulated that LBP-5 may also influence NHR-49 target genes. To test this hypothesis, we explored the potential of LBP-5 to influence NHR-49 target genes by comparing the expression of NHR-49 target genes in both nhr-49(nr2041) and lbp-5(tm1618) mutant worms to that in N2 worms. NHR-49 target genes were identified by assessing the effect of the nhr-49 mutation on 19 genes involved in energy metabolism (23) by qRT-PCR. Supplemental Fig. S6, A and B shows that both nhr-49(nr2041) and lbp-5(tm1618) mutant worms exhibited decreased expression of NHR-49 target genes involved in mitochondrial β-oxidation, including acs-2 and acs-15, which are members of the fatty acyl-CoA synthetase family, carnitine palmitoyltransferase (cpt-5), and acyl-CoA dehydrogenase (acd-8). However, genes predicted to function in peroxisomal β-oxidation were up- and down-regulated in the nhr-49(nr2041) mutant. For instance, enoyl-CoA hydratase (ech-9) expression was increased, whereas C48B4.1 and F59F4.1, two acyl-CoA oxidase genes, were down-regulated (supplemental Fig. S6B). Moreover, expression of three genes involved in fatty acid desaturation, fat-5, fat-6, and fat-7, was down-regulated, whereas palmitic acid elongase (elo-2) was up-regulated (supplemental Fig. S6C). Finally, genes involved in gluconeogenesis, namely succinate dehydrogenase (sdha-2) and isocitrate lyase/malate synthase (gel-7), displayed decreased expression in nhr-49(nr2041) mutant worms (supplemental Fig. S6D). Collectively, of the 19 putative NHR-49 target genes, 14 genes showed a similar expression pattern with different levels of alteration in lbp-5(tm1618) and nhr-49(nr2041) mutant worms, whereas the remaining five genes do not respond to nhr-49 deficiency (supplemental Fig. S7). In general, alterations in gene expression were larger in nhr-49(nr2041) than in lbp-5(tm1618) for NHR-49 target genes. To summarize this expression data, we constructed a model of NHR-49 regulation of these genes and biological processes (Fig. 5A).

Interestingly, we observed induction of elo-2 and ech-9 genes by nhr-49 deficiency (supplemental Fig. S6) and gain-of-function (GOF) experiments (Supplemental Figs. S2 and S3). To confirm these results, we created lbp-5(tm1618);nhr-49(nr2041) double mutant worms and found that this induction of these two genes by nhr-49 deficiency was abolished (supplemental Fig. S6, B and C). LBP-5 most likely regulates elo-2 through other unknown signals. Thus, given that expression of NHR-49 target genes decreased with lbp-5 deletion, we conclude that LBP-5 may be required for NHR-49 target gene activity.

**LBP-5 Is Required for Stearic Acid-induced Transcriptional Activity of NHR-49 Target Genes—**Because LBP-5 can selectively bind fatty acids (supplemental Fig. S5) and exhibit regu-
FIGURE 5. A summary diagram showing the regulatory effects of NHR-49. A, the different boxes represent genes involved in *C. elegans* mitochondrial β-oxidation, peroxisomal β-oxidation, fatty acid desaturation/elongation, and gluconeogenesis. qRT-PCR was used to measure the expression of these NHR-49 target genes in N2 and *nhr-49*(*nr2041*) worms (see supplemental Fig. S7). The red dashed line represents down-regulation and the green arrowhead represents up-regulation. B, logarithmic plot of the qRT-PCR gene expression levels of NHR-49 target genes involved in fatty acid desaturation/elongation in N2, *lbp-5*(*tm1618*), *nhr-49*(*nr2041*), and *lbp-5*(*tm1618*);*nhr-49*(*nr2041*) worms fed with stearic acid. A detailed graph showing the actual relative expression level of NHR-49 targets involved in fatty acid desaturation/elongation in N2 worms fed with stearic acid compared with control. C, gene expression levels of NHR-49 targets involved in gluconeogenesis in N2, *lbp-5*(*tm1618*), *nhr-49*(*nr2041*), and *lbp-5*(*tm1618*);*nhr-49*(*nr2041*) worms fed with stearic acid as assessed by qRT-PCR. Actin served as an internal control. At least three independent experiments were performed. Error bars indicate the standard deviation.
We hypothesized that LBP-5 may transport fatty acids (e.g. stearic acid) into and within the nucleus to influence NHR-49-mediated transcriptional activation of its target genes involved in lipid and glucose catabolism. To test this hypothesis, we investigated the selectivity of fatty acid(s) for LBP-5-induced NHR-49 transcriptional activity by performing feeding experiments with five different fatty acids including oleic acid, palmitic acid, stearic acid, and polyunsaturated fatty acid (arachidonic acid and α-linolenic acid) in N2, lbp-5(tm1618), nhr-49(nr2041), and lbp-5(tm1618) nhr-49(nr2041) worms. To ensure that these fatty acids were taken up by the worms, we measured changes in fatty acid content by GC-MS after feeding. As anticipated, the relative content of each fatty acid in the worms increased after feeding (supplemental Fig. S8). Next, we measured NHR-49 target gene expression in worms fed each fatty acid. Note that LBP-5 is also required for stearic acid-mediated alteration of NHR-49 target genes. That is, stearic acid caused a significant induction of fat-5 (1.71-fold), fat-6 (1.67-fold), fat-7 (1.72-fold), gei-7 (1.41-fold), and sdha-2 (1.98-fold) expression in N2 worms, whereas elo-2 expression decreased (0.58-fold; Fig. 5, A and C). However, this stearic acid-mediated induction effect was abolished in lbp-5(tm1618), nhr-49(nr2041), and lbp-5(tm1618) nhr-49(nr2041) mutant worms, suggesting that LBP-5 is critical for this regulation (Fig. 5, B and C). These results also demonstrate that LBP-5 is required for stearic acid-mediated gene regulation of NHR-49 target genes involved in fatty acid desaturation/elongation and gluconeogenesis. It is notable that feeding the various fatty acids resulted in induction of aca-2, F59F4.1, gei-7, sdha-2, fat-6, and fat-7 expression, as well as inhibition of aca-15, ech-9, aca-1, and elo-2 expression, a typical regulatory function of NHR-49 (supplemental Fig. S9).

DISCUSSION

The data presented in this study reveal novel functions of LBP-5 in C. elegans. First, among the lbp family members, only lbp-5(RNAi) worms displayed significant fat accumulation (supplemental Fig. S3A). This was confirmed by the lbp-5(tm1618) mutant, suggesting that lbp-5 is important for fat metabolism and storage in C. elegans. Second, fat accumulation peaked as lbp-5 expression reached its lowest level in dauer larvae (supplemental Fig. S10), which are in a special dormant state (33–35). This suggests that lbp-5 expression is functionally linked to fatty acid energy metabolism and fat storage (34). Because recent studies have shown that Nile Red staining of live nematodes often does not correlate with actual fat stores (36–38), we performed Sudan Black staining of fixed worms to examine fat accumulation. We also confirmed our results by measuring TG content (Fig. 1). Structural analysis of recombinant LBP-5 by examination of CD spectra (Fig. 2A) revealed a β-sheet-rich structure that is conserved in C. elegans LBP-5 and mammalian FABPs (39). This indicates that the fatty acid binding and transfer properties are also likely to be conserved. Our binding assay results using DAUDA support this conclusion by showing that LBP-5 binds differentially to fatty acids with different chain lengths and saturation conditions, and may also function in fatty acid transport. Interestingly, the effects of ω-3 and ω-6 fatty acids on NHR-49 were independent of LBP-5 (supplemental Fig. S9).

It appears that LBP-5 and NHR-49 have a functional partnership in regulation of energy metabolism. Several lines of evidence support this notion. First, LBP-5 was localized in both the cytoplasm and nucleus (Fig. 3). Second, lbp-5 gene expression was heavily dependent on the presence of NHR-49 (Fig. 4). Third, NHR-49 target genes involved in mitochondrial β-oxidation appeared to be influenced by lbp-5, because expression of most of the NHR-49 target genes was reduced when lbp-5 gene expression was disrupted (Fig. 5 and supplemental Fig. S6). This is consistent with the observation that inhibition of either nhr-49 or lbp-5 in C. elegans lead to increased fat storage (22, 25, 26). Our stearic acid binding assay results support that stearic acid-stimulated NHR-49 target gene expression is also dependent on the presence of LBP-5 (Fig. 5, B and C).

It is worth noting that in the stearic acid-mediated LBP-5 and NHR-49 activation models (Fig. 6), the effect of NHR-49 transcriptional activity on the fatty acid desaturation genes (fat-5, -6, and -7) was more significant than on those related to mitochondrial and peroxisomal β-oxidation enzymes (data not shown). In addition, stearic acid feeding also caused a dramatic increase in gei-7 and sdha-2 expression. Thus, it appears that LBP-5 may have dual functions as an SA carrier and an important mediator of SA-induced expression of NHR-49 target genes. Taken together, our data revealed a dual role for LBP-5 (e.g. stearic acid carrier and a functional partner of NHR-49) in regulating fat metabolism in response to fatty acid ligands such as stearic acid. However, further study is needed to determine how these two proteins work together to regulate genes involved in lipid metabolism.

This newly explored functional partnership between LBP-5 and NHR-49 may prevent the storage of “bad” fatty acids. One probable mechanism underlying the regulatory function of this
Regulation of C. elegans Fat Metabolism by LBP-5

proposed partnership is that specific ligands, such as stearic acid, may bind to LBP-5 and then enter the nucleus to stimulate NHR-49 transcriptional activity (Fig. 6). In support of this, stearic acid stimulates LBP-5-dependent induction of polyunsaturated fatty acid biosynthesis while also functioning as a polyunsaturated fatty acid precursor. Consequently, up-regulation of the fat-5, -6, and -7 genes may help maintain stearic acid levels in feeding conditions by converting saturated fatty acids into unsaturated fatty acids, which are more suitable for fat storage and thereby prevent saturated fat accumulation.

In conclusion, our study introduces a novel functional relationship between the key regulators, LBP-5 and NHR-49, of fatty acid metabolism in C. elegans (Fig. 6). This partnership may regulate target genes during fatty acid metabolism and transport to organelles in response to changes in physiological conditions (e.g. dauer state). Because the fundamental mechanisms of lipid metabolism and transport are likely to be evolutionarily conserved, understanding the function of LBP-5 is useful for predicting the functions of mammalian FABPs. Such work may help identify therapeutic targets for a range of associated disorders including obesity, diabetes, and atherosclerosis. Furthermore, our work can be applied to identification of anti-nematodal agents for pathogenic plant host nematodes (e.g. pinewood nematode) (40).

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REFERENCES
