A branched-chain fatty acid is involved in post-embryonic growth control in parallel to the insulin receptor pathway and its biosynthesis is feedback-regulated in *C. elegans*

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Growth and development of multicellular organisms are controlled by signaling systems that sense nutrition availability and metabolic status. We report a novel and surprising factor in *Caenorhabditis elegans* development, the monomethyl branched-chain fatty acid C17ISO, a product of leucine catabolism. We show here that C17ISO is an essential constituent in a novel mechanism that acts in parallel with the food-sensing DAF-2 (insulin receptor)/DAF-16 (FOXO) signaling pathway to promote post-embryonic development, and that the two pathways converge on a common target repressing cell cycle. We show that C17ISO homeostasis is regulated by a SREBP-1c-mediated feedback mechanism that is different from the SREBP-1c-mediated regulation of common fatty acid biosynthesis, as well as by peptide uptake and transport. Our data suggest that C17ISO may act as a chemical/nutritional factor in a mechanism that regulates post-embryonic development in response to the metabolic state of the organism.

[Keywords: C17ISO; *daf-2*; FOXO; *elo-5*; SREBP; *cki-1*]

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In *Caenorhabditis elegans*, environmental cues regulate the decision between two post-embryonic developmental options: reproductive growth and an adaptive larval arrest, known as diapause. There are two distinctive types of larval arrest, L1 (first larval stage) diapause and dauer formation (Johnson et al. 1984; Riddle and Alberts 1997; Muñoz 2003). Dauer larvae, third-stage larvae (dL3) with distinct structures and behaviors suited for long-term survival, are induced at the L1 larval stage by unfavorable growth conditions. The food-sensing insulin/DAF-2 receptor-stimulated pathway that negatively controls activity of a FOXO transcription factor, DAF-16, plays a critical role in dauer formation (Vowels and Thomas 1992; Gottlieb and Ruvkun 1994; Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997; Gems et al. 1998; Lee et al. 2003). In contrast to dauer formation, the L1 diapause is characterized by a failure to initiate post-

embryonic development in the absence of food (Johnson et al. 1984) or at a high temperature (Muñoz 2003).

Fatty acids (FAs) are very diverse in their structures. Whereas the biochemistry and physiology of straight chain FAs have been analyzed extensively using animal models, nearly nothing is known about the homeostasis and functions of branched-chain FAs in animals. In fact, little is known about the roles of any FAs in coordination of animal growth with changes in environment or metabolic status. In the previous study, we described for the first time the essential physiological roles of unusual monomethyl branched-chain FAs (mmBCFA) in *C. elegans* (Kniazeva et al. 2004). We determined that *C. elegans* is capable of synthesizing long-chain mmBCFA and showed that the elongation enzyme ELO-5 is required for the biosynthesis of mmBCFAs C17ISO, C15ISO (Kniazeva et al. 2004). Inhibition of the endogenous production of mmBCFAs by *elo-5(RNAi)* results in various developmental defects in the intestine and gonads, as well as defects in egg laying in the first affected (P0) generation, with progeny (F1) arresting at the L1 stage (Kniazeva et al. 2004). Here, we investigate whether C17ISO plays a distinct regulatory role in the

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release from L1 diapause, whether it functions within the food-sensing insulin/DAF-2/DAF-16 pathway reported to play a role in L1 arrest (Baugh and Sternberg 2006; Fukuyama et al. 2006), and how C17ISO homeostasis is regulated. We also speculate on why this particular metabolite may reflect the nutritional balance within the organism.

Results

C17ISO-deficient animals arrest at the early L1 stage similar to starvation-induced L1 diapause

elo-5 loss-of-function [*elo-5(lf)*] mutants cultured on plates containing mmBCFA-free bacteria (OP50 plates) fail to grow past the L3 stage, and this defect can be overcome by supplementing with a certain concentration of C17ISO (Supplemental Fig. S1A,B; Kniazeva et al. 2004). When the OP50 plates are supplemented with the same concentration of C13ISO, a likely precursor of C17ISO (Supplemental Material), the F1 animals grow to adults without obvious morphological or developmental defects (Supplemental Fig. S1). However, their progeny (F2) uniformly enter L1 diapause ($n > 1000$) (Fig. 1A), as was described for *elo-5(RNAi)* animals (Kniazeva et al. 2004). Rescue from the diapause can be achieved by ap-

plying the C17ISO supplement to the top of the bacterial lawn harboring arrested L1s (Fig. 1B; Kniazeva et al. 2004). These results suggest that C17ISO may carry out a critical function required for the initiation of post-embryonic development at L1.

We found that the growth response of the arrested animals to a gradient of supplemental C17ISO concentrations was nonlinear. The nearly all-or-none dosage response is illustrated in Figure 1C. The vast majority of animals that escaped the L1 arrest developed into normal fertile adults, regardless of the concentration of C17ISO (data not shown), indicating that the amount of C17ISO sufficient to reverse the L1 arrest in this experiment is also sufficient to provide normal reproductive growth.

There are notable similarities between the L1 diapause induced by starvation and the L1 diapause caused by depleting C17ISO: Both occur uniformly at the early L1 stage and both are reversible days after the arrest upon the addition of food and C17ISO, respectively. Using a GFP expressing *Escherichia coli* OP50-GFP (*C. elegans* Genetics Center) to trace the food, we determined that the developmental pause induced by C17ISO depletion is not caused by impaired food uptake or starvation (Supplemental Fig. S2).

Moreover, we found that a short-term food deprivation of C17ISO-deficient L1-arrested larvae, followed by supplying C17ISO-free food (OP50), allowed a complete bypass of the C17ISO requirement (data not shown; Materials and Methods). This effect may be due to metabolic or regulatory changes caused by dietary restriction.

We further determined the time of the onset of L1 arrest caused by C17ISO deficiency by examining M-cell divisions. Well-fed wild-type (wt) larva hatch with a single M cell that begins to divide 5–6 h post-hatching, giving rise to 16 cells at the end of L1 (Fig. 2A; Sulston and Horvitz 1977). During L1 diapause caused by starvation, the M cell does not divide (Baugh and Sternberg 2006). Using an *hlh-8::GFP* reporter expressed in the M-cell lineage (Harfe et al. 1998; Baugh and Sternberg 2006), we did not observe any *elo-5(RNAi)* larvae with more than one M cell on the third day of L1 arrest ($n > 800$) (Fig. 2B,C). This result indicates that C17ISO deficiency, like starvation, induces L1 diapause within 5 h after hatching, even in the presence of food. The stereotypical changes in seam cell shape, highlighted by the AJM-1 protein, which localizes to the dynamic adherens junction, have been shown to correlate with developmental stages during L1 progression (Fig. 2D; Knight et al. 2002). Using an AJM-1::GFP reporter transgenic line (Keoppen et al. 2001), we observed that the seam cell shapes in C17ISO-depleted L1-arrested animals corresponded to a stage ~3–5 h after hatching (Fig. 2E,F), similar to the ones observed in starved L1-arrested animals.

C17ISO acts in parallel to the DAF-2/DAF-16-mediated food-sensing pathway regulating post-embryonic growth and development

The similarity between the C17ISO-related L1 arrest and starvation-induced arrest raised the possibility that

Figure 1. C17ISO is required for post-embryonic development. (A) Progeny of the C17ISO-deficient *elo-5(lf)* adult uniformly entered L1 diapause (indicated by arrows). (B) The L1-arrested worms were rescued to full growth and propagation after adding C17ISO to the plates 2 d after the arrest. Arrowhead points to P0 animal surrounded by its progeny. (C) The C17ISO-depleted *elo-5(lf)* animals were maintained on OP50 plates supplemented with various concentrations of C17ISO from the time of hatching. (n) Number of animals counted in each experiment. Bars, 200 μ m.

Figure 2. C17ISO deficiency causes developmental arrest in early L1 independently from the DAF-2/DAF-16 food-sensing pathway. (A) The M-cell lineage during L1 development (data adapted from Sulston and Horvitz 1977). (B) *hlh-8::GFP* expression in a single M cell in the C17ISO-deficient L1-arrested *elo-5(RNAi)* larva. (C) *hlh-8::GFP* expression in the two divided M cells in a wild-type L1 growing larva. (D) The changes in the seam cell patterns during L1 (adopted with permission from Blackwell Publishing [© 2002; <http://www.blackwell-synergy.com>] from Knight et al. 2002). (E) AJM-1::GFP expression pattern at adherens junctions in a C17ISO-deficient L1-arrested *elo-5(RNAi)* larva corresponds to a stage about 3 h post-hatching (Knight et al. 2002). (F) AJM-1::GFP expression pattern in a wild-type growing L1 animal corresponding to about 12 h post-hatching. (G) The disconnected seam cell shapes, typical of animals ~6 h post-hatching (D), were observed in starved *daf-16(lf)* mutants. (H) The seam cell shapes in *daf-16(lf); elo-5(RNAi)* are similar to that in *elo-5(RNAi)* (E), and correspond to a stage about 3.3 h post-hatching (D).

C17ISO is part of the DAF-2/DAF-16 food-sensing pathway. We first determined that starved L1-arrested animals were not lacking C17ISO. Gas chromatography (GC) analysis of the FA composition in total lipid extracts indicated that C17ISO was present and that the overall FA composition in starved animals was similar to that in well-fed animals (data not shown). We then tested whether C17ISO plays a critical role in activating the DAF-2/DAF-16 signaling pathway. If so, C17ISO added to NGM agar plates lacking bacteria might activate DAF-2 and prevent L1 arrest caused by starvation (Supplemental Fig. S3A). A uniform L1 arrest under this condition was observed (Supplemental Fig. S3B), indicating that C17ISO is not likely to be a major activator of the DAF-2/DAF-16 pathway.

We then tested the possibility that C17ISO is required, but not sufficient for the activation of the DAF-2 pathway at the L1 stage. If C17ISO acts at a step upstream of DAF-16 but downstream from DAF-2 to activate the pathway, a *lf* mutation in *daf-18/Pten*, which antagonizes the *daf-2* activity (Ogg et al. 1997) and/or a *lf* mutation in *daf-16*, which is negatively regulated by *daf-2*, may suppress the L1 arrest caused by C17ISO deficiency. However, neither a *daf-18(lf)* nor a *daf-16(lf)* was observed to suppress the uniform L1 arrest phenotype

caused by *elo-5(RNAi)* (Supplemental Fig. S4C; data not shown). The inhibition of M-cell proliferation in starved larvae has been shown previously to be associated with DAF-16 activity, as M cells continue to divide in the food-deprived *daf-16(lf)* mutants (Baugh and Sternberg 2006). A completely penetrant M-cell division arrest was observed in *daf-16(RNAi); elo-5(RNAi)* animals ($n > 800$) (data not shown) just as in *daf-16(wt); elo-5(RNAi)* animals (Fig. 2B), indicating that the inhibition of M-cell division by C17ISO deficiency is independent of DAF-16 activity. Using the AJM-1::GFP reporter, we found that developmental arrest of seam cells in the C17ISO-depleted larvae was also DAF-16 independent, in contrast to the arrest in starved L1 larvae. In starved animals, *daf-16(lf)* partially released the developmental arrest in the seam cells, as indicated by the AJM-1::GFP marker (Fig. 2G). However, the seam cells in *daf-16(lf); elo-5(RNAi)* animals displayed a similar pattern to that in *daf-16(wt); elo-5(RNAi)* larvae (Fig. 2, cf. H and E), corresponding to ~3–5 h after hatching (Fig. 2D). These data indicate that C17ISO does not act within the DAF-2/DAF-16 pathway to regulate post-embryonic development. Consistent with this result, the C17ISO content does not change in the *daf-2(lf)* and *daf-16(lf)* mutants (data not shown). Additional analysis confirms that the DAF-2/DAF-16 pathway is functional in animals lacking C17ISO (Supplemental Material).

C17ISO acts in a DAF-2/DAF-16-independent pathway to repress cyclin-dependent kinase inhibitor CKI-1

The similarity in the early onset of the L1 diapause caused by starvation and by C17ISO deficiency implies that the two mechanisms affect common regulators of post-embryonic growth and development. We evaluated the expression of the *cki-1* gene during L1 diapause in C17ISO-deficient animals. *cki-1* encodes a cyclin-dependent kinase inhibitor that was shown to be required for cell cycle exit during development and to be strongly expressed in seam cells during L1 arrest caused by starvation (Hong et al. 1998; Baugh and Sternberg 2006). A *daf-16*-null mutation was shown to significantly reduce the *cki-1* expression in seam cells of starvation-induced L1-arrested larvae (Baugh and Sternberg 2006). To examine the expression of *cki-1* in C17ISO-deficient larvae, we induced the L1 arrest by feeding *elo-5(RNAi)* to transgenic animals carrying a *cki-1::GFP* reporter. While a low level of *cki-1::GFP* was detected in seam cells in control animals (Fig. 3A,B), a significant increase in the expression was observed in seam cells of the *elo-5(RNAi)*-induced L1-arrested larvae (Fig. 3C). A prominent expression of the reporter was also observed in seam cells in *daf-16(lf); elo-5(RNAi)* L1-arrested larvae at a level similar to that in the *elo-5(RNAi)* L1 larvae (Fig. 3E), indicating that the increase in *cki-1* expression in C17ISO-deficient L1 animals is *daf-16* independent. Based on the above results, we propose that C17ISO plays an essential role in the initiation of post-embryonic growth in parallel to the DAF-2/DAF-16 pathway.

Figure 3. C17ISO acts in parallel to the DAF-2/DAF-16 pathway to repress *cki-1* expression in seam cells. (A–E) Images of L1 animals of various genotypes and culture conditions expressing an integrated *cki-1::GFP* reporter in seam cells, as indicated. The animal in A is committed to growth, the animal in C was among progeny of animals that were exposed to *elo-5(RNAi)* for a lifetime, it is L1-arrested. The GFP expression in seam cells was prominent in L1 animals arrested by deficiency of mmBCFA or by starvation, but not in developing L1, irrelevant to the *daf-16* function. Bars, 15 μ M for all images.

Because the physiological consequences of depleting C17ISO are similar to that of food starvation, the two mechanisms may regulate more common genes in addition to *cki-1*.

The expression of ACS-1, the enzyme involved in the mmBCFA synthesis, is feedback regulated by C17ISO

The critical role of C17ISO suggests the importance of factors contributing to mmBCFA homeostasis. In a search for genes involved in feedback regulation of mmBCFA biosynthesis, we previously identified *acs-1* as a gene significantly up-regulated in *elo-5(RNAi)* animals deficient in mmBCFAs (Kniazeva et al. 2004). *acs-1* encodes a very long-chain acyl-CoA synthetase that plays an essential role in mmBCFA biosynthesis and activation (Supplemental Material; Kniazeva et al. 2004). An *acs-1Prom::GFP* reporter is expressed in intestinal cells and canal-associated neurons (CAN) in L4 and adult animals (Fig. 4A; Kniazeva et al. 2004). We found that the GFP expression from this reporter was drastically increased in *acs-1(RNAi)* animals maintained with the C13ISO supplement (and therefore deficient for C17ISO) (Fig. 4B). In contrast, the increase in GFP expression was absent in the *acs-1(RNAi)* worms maintained with C17ISO supplementation (Fig. 4C), which were visually indistinguishable in growth and morphology from those supplemented with C13ISO. Thus, C17ISO, not C13ISO, negatively regulates *acs-1* expression.

SREBP-1c homolog SBP-1 mediates the regulation of acs-1 expression by C17ISO

We then tested whether *sbp-1*, which encodes the SREBP-1c homolog in *C. elegans* (McKay et al. 2003), and

nhr-49, encoding a nuclear hormone receptor, play roles in C17ISO-mediated regulation of *acs-1* expression. Both transcription factors were selected from our screen for genes potentially sensitive to mmBCFA deficiency (Kniazeva et al. 2004). *sbp-1* has been shown to regulate mmBCFA biosynthesis as well as the expressions of *elo-5* and *elo-6* (Kniazeva et al. 2004), while *nhr-49* has been shown to play a role in straight-chain FA homeostasis (Kniazeva et al. 2004; Van Gilst et al. 2005a,b). In *acs-1(RNAi)* animals supplemented with C13ISO, a suppression of *sbp-1*, but not *nhr-49*, by RNAi eliminated the stimulating effect of C17ISO deficiency on *acs-1Prom::GFP* expression (Fig. 4D,E).

We further used real-time RT-PCR (qPCR) to quanti-

Figure 4. *acs-1* expression is up-regulated by deficiency of C17ISO but not C13ISO, and this regulation requires transcription factors *sbp-1* and *cbp-1*. (A–E) Images of worms expressing GFP from the *acs-1Prom::GFP* reporter transgene. RNAi treatment and mmBCFA supplementations are indicated. A moderate expression of the reporter in a wild-type background (A) increased dramatically following the depletion of C17ISO [*acs-1(RNAi)* + C13ISO] (B). (C) The increase was abolished in the experiments where C13ISO supplementation was replaced with C17ISO. RNAi of *sbp-1* (D), but not *nhr-49* (E), inhibited the *acs-1* up-regulation. Bars, 100 μ M. (F) Results of a quantitative RT-PCR analysis of the endogenous *acs-1* gene expression in C17ISO-deficient animals. The levels are relative to a wild-type sample. (G–L) Images showing the GFP expression from either the *elo-5Prom::GFP* or the *acs-1Prom::GFP* transgene in wild-type or RNAi-treated animals as indicated. RNAi of *cbp-1* inhibits the steady state *elo-5* expression as well as the up-regulation of *acs-1* by mmBCFA deficiency.

tatively measure the mRNA level of the endogenous *acs-1* gene under the tested conditions. C17ISO-deficient animals were obtained by culturing *elo-5(lf)* mutants in the presence of C13ISO supplementation. The result indicates that the deficiency of C17ISO, but not C13ISO, caused an approximate sevenfold increase in the *acs-1* mRNA level and the increase was dependent on *sbp-1* but not on *nhr-49* (Fig. 4F). These results indicate that the end product of mmBCFA biosynthesis, C17ISO, negatively regulates *acs-1* expression through modulating SBP-1 activity.

CBP-1, but not MDT-15, is involved in SBP-1 mediated regulation of mmBCFA biosynthesis

In mammals and *C. elegans* a transcription mediator, ARC105 (worm homolog MDT-15), cooperates with SREBP-1c (SBP-1) for their functions in FA synthesis (Taubert et al. 2006; Yang et al. 2006). We found MDT-15 is unlikely involved in mmBCFA biosynthesis, as no significant changes in levels of branched-chain FA were detected in *mdt-15(RNAi)* animals (Fig. 5A,B). We also tested *cbp-1*, which encodes the *C. elegans* homolog of mammalian CBP/p300, another cofactor capable of binding to SREBP-1c in mammals (Yang et al. 2006). Previous reports showed that CBP-1 is a critical transcriptional cofactor in promoting cell differentiations (Shi and Mello 1998; Eastburn and Han 2005), but its role in FA metabolism has not been analyzed. We found that *elo-5Prom::GFP* expression was down-regulated and the response of *acs-1Prom::GFP* expression to the C17ISO deficiency was abolished in *cbp-1(RNAi)* animals (Fig. 4G–J; Supplemental Fig. S7C,D). *cbp-1(RNAi)* animals were also severely deficient in mmBCFAs as well as in polyunsaturated 20-carbon straight-chain FAs (C20 PUFA) (Fig. 5C). These *cbp-1(RNAi)* effects on mmBCFA production were similar to that of *sbp-1(RNAi)* (Fig. 4), suggesting that CBP-1, but not MDT-15, collaborates with SBP-1 in regulating mmBCFA biosynthesis.

mmBCFA biosynthesis is regulated by protein uptake and transport

Since leucine is a precursor of C17ISO, we investigated additional links between protein and mmBCFA homeostasis. *pep-2* encodes an essential oligopeptide transporter, which is involved in absorption of digested proteins by intestinal cells in *C. elegans* (Supplemental Fig. S7E,F; Nehrke 2003; Meissner et al. 2004). GC analysis showed that *pep-2(RNAi)* caused a drastic reduction of mmBCFAs, along with a significant reduction in the levels of polyunsaturated FAs (PUFAs) (Fig. 5D). Since supplementing *pep-2(RNAi)* animals with C13ISO did not significantly restore C17ISO levels (data not shown), we examined the expression of GFP driven by promoters of mmBCFA biosynthetic genes, *elo-5*, *acs-1*, and *sbp-1* in *pep-2(RNAi)* animals (Fig. 5E–J). A decrease in the expression of *elo-5Prom::GFP* was observed (Fig. 5E,F). Therefore, transcriptional down-regulation of *elo-5* may

Figure 5. *pep-2(RNAi)* and *cbp-1(RNAi)*, but not *mdt-15(RNAi)*, significantly influence mmBCFA biosynthesis. (A–D) GC analysis of FA content in total lipid extracts obtained from RNAi-treated strains as indicated. Arrowheads point to the C15ISO and C17ISO peaks. Drastic decreases in mmBCFA biosynthesis were observed in animals treated with *pep-2(RNAi)* and *cbp-1(RNAi)*, but not animals with *mdt-15(RNAi)*. By comparison, all three RNAi treatments affected the fractions of C20 PUFA. (E–J). GFP images showing the expression of the *elo-5Promoter::GFP* or *sbp-1Promoter::GFP* or *acs1Promoter::GFP* transgene in the wild-type or *pep-2(RNAi)* strain as indicated. *pep-2(RNAi)* causes a significant reduction of the expression of *elo-5::GFP* but not the expression of *sbp-1::GFP* or *acs1::GFP*. Bars, 100 μ M.

at least in part be responsible for mmBCFA deficiency in *pep-2(RNAi)*. It has recently been shown that leucine deprivation in mammals causes down-regulation of genes related to the FA biosynthesis by reducing mRNA and protein levels of SREBP (Guo and Cavener 2007). Thus, it is possible that impaired peptide transport may mimic essential amino acid deficiency and may affect *elo-5* transcription through regulating *sbp-1*, although our GFP reporter analysis suggests that this potential regulation on *sbp-1* would be more likely through a post-transcriptional mechanism (Fig. 5G,H). The fact that wild-type animals left without food for 2 d have an essentially normal mmBCFA composition (data not shown) suggests that it is not a deficiency of dietary amino acids per se, but the imbalance in these metabolites that is associated with the dramatic down-regulation of mmBCFA. It implies that levels of mmBCFAs largely depend on coordinated lipid and protein homeostasis, rather than on nutrient deprivation.

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pendent growth control and the physiological roles of branched chain FAs in animals, including humans.

Materials and methods

Worm strains and assays in general

The wild-type strain was N2 Bristol. A deletion strain *elo-5(gk208)* was obtained from the *C. elegans* knockout consortium. The following alleles including transgenic genes were obtained from the *Caenorhabditis* Genetic Center or from the laboratory indicated in the reference: *hlh-8::GFP(ayIs7 IV)*, *ajm-1::GFP(jcIs1 IV)*, *cki-1::GFP*, *daf-2(e1370)III*, *daf-16(mgDf50) I*, *daf-18(nr2037)* (Gil et al. 1999), and *mals113(cki-1::GFP)* (Baugh and Sternberg 2006), and *sbp-1::GFP* (McKay et al. 2003). *elo-5(gk208)*, *acs-1Prom::GFP*, *elo-5Prom::GFP*, and *elo-6Prom::GFP* were described previously (Kniazeva et al. 2004).

Unless otherwise specified, *C. elegans* strains were maintained under standard conditions on NGM agar with *Escherichia coli* OP50 as a food source at 20°C. The *daf-2(e1370)* animals carry a reduction-of-function (rf), or partial loss-of-function (lf) allele and become dauers at 20°C. To prevent the dauer formation, parental worms were initially maintained at the permissive temperature, 15°C, and switched to 20°C at the L4 or young adult stage to inactivate the gene. All experiments and assays started from plating eggs released from parental animals by bleaching onto corresponding plates. Unless specified differently, all assays were done at least three times and a set of three to five plates containing 100–150 animals were analyzed for each condition tested.

FA supplements

C13ISO, C15ISO, or C17ISO (Sigma, Matreya, or Larodan) were dissolved in 100% DMSO at 10 mM concentration and mixed with overnight bacterial culture to 1 mM final concentration prior to spotting plates. The bacteria/supplements were spotted onto NGM agar plates. The plates were left to dry with the lids on. Control plates were prepared by mixing DMSO into overnight bacterial suspension to a final concentration of 10% prior to spotting plates. Experimental and control plates for a given assay were prepared with a single source of FA- or DMSO-supplemented bacterial food.

*Maintenance of the *elo-5(lf)* homozygous mutant*

The homozygous lethal *elo-5(lf)* strain is maintained on NGM plates seeded with *E. coli* OP50 mixed with supplementary 1 mM C17ISO dissolved in 10% DMSO (final concentration in bacterial suspension) or on NGM plates seeded with a mixture of OP50 and mmBCFA-producing bacteria *Stenotrophomonas maltophilia*.

Generation of mmBCFA-depleted animals

elo-5(lf) adults maintained on *S. maltophilia* were bleached and the released eggs were placed on *E. coli* OP50 or HT115 seeded plates. Alternatively, wild-type or mutant strains were subjected to *elo-5(RNAi)* or to *acs-1(RNAi)* without mmBCFA supplements. “HT115” refers to *E. coli* strain HT115 transformed with the empty pPD129.36 RNAi vector.

Generation of C17ISO-depleted adults

elo-5(lf) adults maintained on *S. maltophilia* were bleached and the released eggs were placed on *E. coli* OP50 or HT115 seeded

plates supplemented with C13ISO. Worms of the first generation (P0) displayed superficially normal growth and development.

Generation of C17ISO-depleted L1 arrested animals

elo-5(gk208; lf) adults (P0) maintained on *S. maltophilia* or C17ISO supplements were bleached and the released eggs (F1) were placed on OP50 or HT115 plates supplemented with C13ISO. Three days after plating, adult F1s were bleached to release eggs (F2) on the plates according to experimental tasks. Without C17ISO supplements, the eggs (F2) gave rise to the C17ISO-depleted L1-arrested larvae.

To generate C17ISO-depleted L1 arrest larvae in other mutant backgrounds, P0 worms were subjected to *elo-5(RNAi)* without any mmBCFA supplements from the time of hatching. This treatment resulted in gradual mmBCFA depletion in the P0 generation. The adults were maintained on the same plates or bleached, and eggs (F1) were plated onto fresh plates prepared according to the experimental tasks. The resulting F1 generation was C17ISO-depleted and L1-arrested.

L1 arrest reversal through food deprivation

The C17ISO-depleted L1-arrested animals were obtained from the *elo-5(gk208)* parents as indicated above. The larvae were cultured on food-free NGM plates supplemented with DMSO, C13ISO, or nothing for 2 d. Afterward, a small amount of OP50 paste was added to all the conditions. Animals were evaluated the next day for growth using dissection and high-resolution light microscopy. Lipid extracts were prepared from propagating animals and the FA composition was analyzed by GC. The result indicated that these starvation-rescued animals did not produce more C17ISO (data not shown). The *elo-5(lf)* genotype of the rescued animals was verified by PCR.

Strains and conditions for RNAi treatment

RNAi feeding strains used in this study were obtained from the *C. elegans* whole-genome RNAi feeding library (J. Ahringer, MRC Geneservice). The RNAi feeding control strain was *E. coli* HT115 transformed with the empty pPD129.36 vector. All RNAi strains used were verified for the correct inserts by sequencing the isolated plasmids. RNAi plates were prepared as described in Kamath et al. (2001). When RNAi plates included mmBCFA supplements, two controls were used: (1) plates with the RNAi strain alone and (2) plates with the same RNAi strain supplemented with 10% DMSO.

Analysis of growth response to concentrations of C17ISO

Worms maintained on *S. maltophilia* plates were collected, rinsed in water, and bleached. The eggs were plated onto OP50 plates supplemented with 1 mM C13ISO in DMSO. The hatched animals grew to adults depleted of C17ISO. On day 3 post-hatching, adults were collected, rinsed with water, and the eggs were plated on OP50 plates supplemented with different concentrations of C17ISO. The eggs were placed outside a bacterial lawn so that L1 animals that were damaged or ill from the treatment, and thus unable to move toward the food, were not scored. The animals were observed for the following 4 d, during which the structure and developmental stages were recorded. Aliquots of C17ISO in DMSO (50 µL) were mixed with 450 µL of OP50 bacteria before spotting plates to obtain final concentrations of 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, and 1.0 mM. (Note: The actual concentration of C17ISO on a plate can vary due to

the limited solubility of C17ISO.) Error bars reflect variations within three biological replicates used in the experiment.

GFP expression analysis

GFP fluorescence was observed using a Zeiss Axioplan microscope. The images were captured with a Hamamatsu ORCA-ER digital camera.

Animals were anesthetized on agar pads in a drop of 10 mM (0.7%) sodium azide (Sulston and Hodgkin 1988) and analyzed immediately. Specimens were scored within 5 min after preparing the slides. For comparative analysis of GFP expression, experimental and control animals were mounted on the same slide. GFP images were obtained with the fixed settings.

Developmental stage and morphology evaluation

Growth and development was monitored using a Leica MZ6 dissecting scope equipped with a Canon PowerShot A620 digital camera, and using Nomarski optics on a Zeiss Axioplan microscope.

GC analysis

Worms were washed off the plates with water, rinsed three to four times, and the pellet was frozen at -80°C . Lipid extraction and FA methyl esters were prepared as described (Miquel and Browse 1992). GC was performed on a HP6890N (Agilent) apparatus equipped with a DB-23 column ($30\text{ m} \times 250\text{ }\mu\text{m} \times 0.25\text{ }\mu\text{m}$) from Agilent. Each experiment was repeated at least five times.

Quantitative RT-PCR

The C17ISO-depleted adults were obtained from the *elo-5(lf)* parents, maintained as described above. The eggs were released by bleaching and plated on *HT115*, *sbp1-(RNAi)*, and *nhr-49(RNAi)* supplemented with C13ISO. Wild-type animals maintained on HT115 supplemented with C13ISO from the time of hatching were used as a normal reference. Three days after plating the eggs, samples of total RNA were prepared from corresponding mixed populations of adults, eggs, and L1-arrested larvae according to the RNeasy protocol (Qiagen). q-PCR was done according to SYBR-protocol (Sigma). *rpl-21* mRNA was used as an internal reference. Three biological and six technical replicates were used for each sample. Average and standard deviation were calculated in Excel. Relative quantification analysis was performed using the $2^{-\Delta\Delta\text{CT}}$ Method (Livak et al. 2001).

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