

Defining *Caenorhabditis elegans* as a model system to investigate lipoic acid metabolism

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Antonela Lavatelli^{1,2}, Diego de Mendoza^{1,2}, and María Cecilia Mansilla^{1,2,*} 

From the ¹Laboratory of Microbial Physiology, Institute of Molecular and Cellular Biology of Rosario, CONICET, and the

²Department of Microbiology, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Rosario, Santa Fe, Argentina

Edited by Ursula Jakob

Lipoic acid (LA) is a sulfur-containing cofactor that covalently binds to a variety of cognate enzymes that are essential for redox reactions in all three domains of life. Inherited mutations in the enzymes that make LA, namely lipoyl synthase, octanoyltransferase, and amidotransferase, result in devastating human metabolic disorders. Unfortunately, because many aspects of this essential pathway are still obscure, available treatments only serve to alleviate symptoms. We envisioned that the development of an organismal model system might provide new opportunities to interrogate LA biochemistry, biology, and physiology. Here we report our investigations on three *Caenorhabditis elegans* orthologous proteins involved in this post-translational modification. We established that M01F1.3 is a lipoyl synthase, ZC410.7 an octanoyltransferase, and C45G3.3 an amidotransferase. Worms subjected to RNAi against M01F1.3 and ZC410.7 manifest larval arrest in the second generation. The arrest was not rescued by LA supplementation, indicating that endogenous synthesis of LA is essential for *C. elegans* development. Expression of the enzymes M01F1.3, ZC410.7, and C45G3.3 completely rescue bacterial or yeast mutants affected in different steps of the lipoylation pathway, indicating functional overlap. Thus, we demonstrate that, similarly to humans, *C. elegans* is able to synthesize LA *de novo* via a lipoyl-relay pathway, and suggest that this nematode could be a valuable model to dissect the role of protein mislipoylation and to develop new therapies.

Lipoic acid (LA) is a sulfur-containing cofactor derived from octanoic acid that is required for the function of several key multienzyme complexes involved in oxidative and one-carbon metabolism. It is also a potent antioxidant. Structurally, it is an eight-carbon fatty acid with a sulfur atom attached to each of carbons 6 and 8, which allow the molecule to switch between oxidized and reduced states. LA is found among prokaryotes and eukaryotes. Although LA was first purified and characterized in the early 1950s (1), the mechanisms of protein lipoylation in animals are the subject of ongoing investigations. This is, in part, because disorders in mitochondrial protein lipoylation result in grave metabolic defects and early death (2–4).

To date, six lipoate-dependent enzymatic complexes have been characterized (5). Four of them are α -ketoacid dehydrogenases (DH): pyruvate DH (PDH), oxoglutarate DH (ODH),

oxoadipate DH, and branched-chain α -ketoacid DH (BKDH). They are all composed of multiple copies of each of three catalytic subunits, a decarboxylase (E1), a dihydrolipoyltransferase (E2), and a dihydrolipoyl DH (E3) (5). The fifth complex, acetoacetyl DH, is highly homologous to PDH and shares the same architecture. The sixth complex is the glycine cleavage system (GCS). It is composed of four loosely associated proteins: a decarboxylase (P subunit), a dihydrolipoyl DH (L subunit, identical to E3s), tetrahydrofolate DH (T subunit), and the H subunit (5). LA is attached via amide bond to lysine residues present in conserved lipoylable domains (LDs) of E2 or H subunits, where it acts as a swinging arm transferring covalently attached reaction intermediates among the multiple active sites of the enzyme complexes (5). However, GCS shows several differences with DH complexes: E1 subunits use thiamine pyrophosphate as a cofactor, whereas P subunits require pyridoxal phosphate; H proteins lack the catalytic role of E2 subunits; and DH complexes catalyze irreversible reactions, whereas reactions catalyzed by the GCS are reversible.

Even though only six enzymatic complexes modified with LA have been described to date, the ways in which they become lipoylated can be incredibly diverse. Some species rely upon synthesis of the cofactor, others scavenge it from the medium, and some others use both pathways (6). In addition, in some organisms lipoylation pathways are segregated by organelles, as is the case in apicomplexans (6).

LA metabolism has been extensively characterized in *Escherichia coli*. This Gram-negative bacterium can lipoylate its proteins in a two-step reaction. First, an octanoyltransferase (LipB) transfers the octanoyl moiety attached to the acyl carrier protein (ACP), an intermediate of fatty acid biosynthesis, to the LDs (7–9) (Fig. 1). Then lipoyl synthase (LipA) catalyzes the insertion of the two sulfur atoms, using radical *S*-adenosylmethionine (SAM) chemistry (8, 10) (Fig. 1). In addition, *E. coli* is capable of attaching exogenous lipoate or octanoate via lipoate protein ligase (LplA). Both molecules are initially activated with ATP and then transferred to the LDs (11, 12) (Fig. 1).

Studies in our laboratory allowed us to understand how *Bacillus subtilis*, the Gram-positive model bacterium, lipoylates its proteins. *B. subtilis* lacks a LipB homologue but has three proteins similar to LplA. Only one of them, LplJ, functions as a lipoate ligase, whereas the others, LipM and LipL, function as an octanoyltransferase and an amidotransferase, respectively (13, 14). LipM specifically transfers octanoate from ACP to the H subunit of the GCS (GcvH), and then LipA inserts the sulfur

This article contains supporting information.

* For correspondence: María Cecilia Mansilla, mansilla@ibr-conicet.gov.ar.

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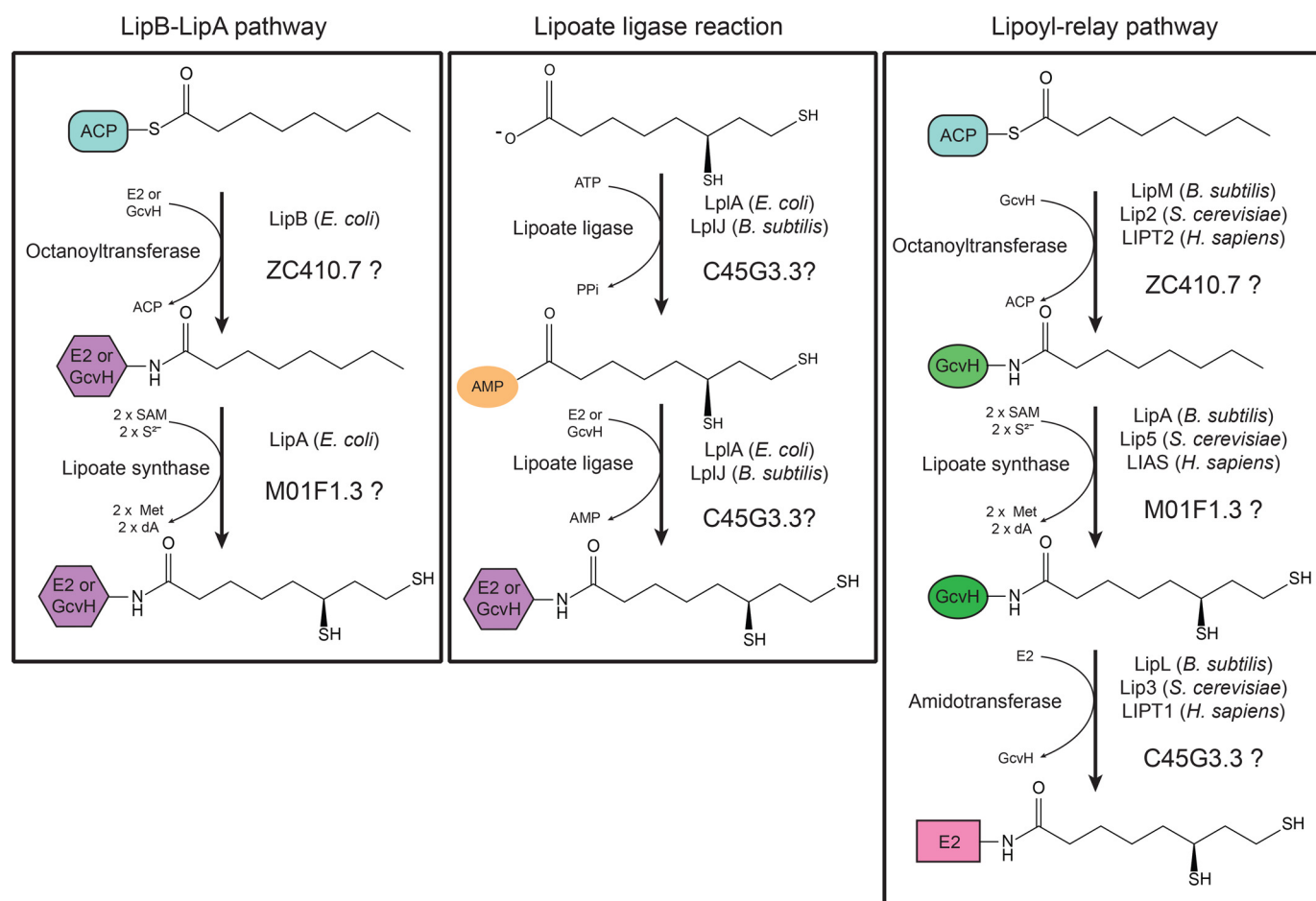


Figure 1. The LipB-LipA pathway, present in *E. coli*, is the simplest pathway characterized, because it only requires two enzyme activities to get all proteins lipoylated (octanoyltransferase and lipoate synthase). On the other hand, in the lipoyl-relay pathway found in Gram-positive bacteria, yeasts, and humans, two extra protein activities are required: GcvH, which is an obligate intermediary, and an amidotransferase, which allows lipoylation of E2 subunits after modification of GcvH. During lipoate uptake, lipoate ligases activate lipoate with an ATP molecule and then catalyze the transfer reaction to E2 or GcvH subunits. In the case of *B. subtilis*, this reaction is restricted to GcvH and ODH-E2.

atoms (15). Afterward, LipL is required to transfer the lipoyl moiety from GcvH to E2 subunits (13, 14) (Fig. 1). This kind of pathway, in which GcvH is an obligate intermediate during E2 lipoylation, is known as the “lipoyl-relay” pathway (16). Recently, a similar lipoyl-relay during exogenous lipoate utilization was described (17).

LA synthesis in the eukaryotes *Saccharomyces cerevisiae* and *Homo sapiens* seems to adhere to the lipoyl-relay model (16, 18), because it depends on four protein activities (Fig. 1). However, these organisms lack LA salvage mechanisms. Human patients with impaired protein lipoylation have abnormally elevated levels of lactate and suffer from severe respiratory deficiency and extreme muscle weakness. Reported clinical pictures of patients with mutations in the lipoyltransferase 1 (*LIPT1*) gene, coding for the amidotransferase, have normal levels of lipoylated GcvH, and hence of glycine levels, but undetectable PDH and ODH activities (19, 20). In contrast, patients that are deficient in *LIPT2* (the octanoyltransferase) or *LIAS* (the lipoate synthase) have elevated glycine levels and, in consequence, develop neurological disorders such as neonatal-onset epilepsy and encephalopathy (21–24). Presently, treatments for patients with LA deficiency are restricted to alleviating symptoms; therefore, further studies need to be done to develop possible therapies.

In this study, we use *Caenorhabditis elegans* as a model system to characterize protein lipoylation mechanisms because there is a strong conservation in molecular reactions between worms and mammals and the majority of human disease pathways are present in this organism (25).

Results

C. elegans is capable of synthesizing LA de novo

The standard laboratory diet of *C. elegans* is *E. coli* B strain OP50, which is able to synthesize LA. To test if *C. elegans* synthesizes LA or scavenges it from the bacterial diet, we fed the worms with *E. coli* K12 strains TM131 ($\Delta lipA \Delta lplA$) or TM136 ($\Delta lipB \Delta lplA$), which can neither synthesize LA nor take it from the environment (11, 12). When synchronized N2 worms were cultured with these *E. coli* mutant strains in a medium devoid of LA (M9sup, see “Experimental procedures”), they grew for many generations, suggesting that the animals can synthesize LA *de novo*. Indeed, when we probed total proteins from worms grown on TM131 with anti-LA antibodies, we detected two prominent bands corresponding to lipoylated proteins of apparent molecular mass of 54 and 50 kDa (Fig. 2). Moreover, no lipoylated proteins corresponding to the E2

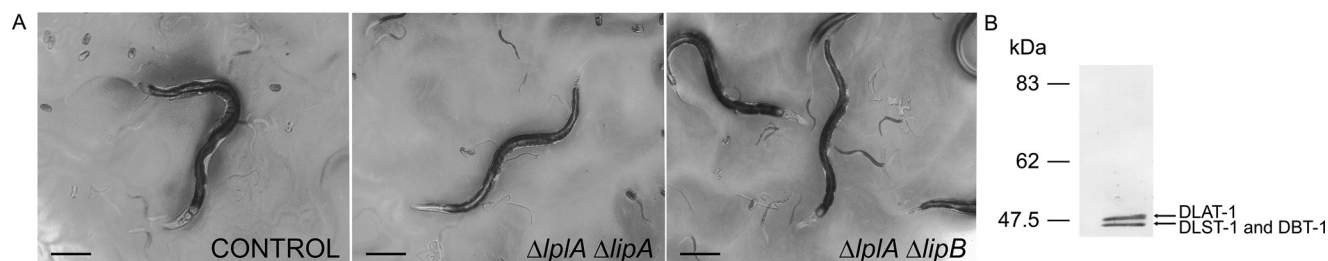


Figure 2. *C. elegans* development under depleted LA conditions. A, worms were grown in LA-free medium (M9sup) and fed with *E. coli* OP50 or strains unable to synthesize LA, $\Delta lplA \Delta lipA$ (TM131) and $\Delta lplA \Delta lipB$ (TM136). Pictures were taken on the fourth day of the second generation of worms grown on LA-depleted conditions. Scale bars represent 200 μm . B, protein extracts from nematodes fed with TM131 were analyzed by Western blotting using antibodies against LA. DLAT-1, DLST-1, and DBT-1 are the lipoylated E2 subunits of PDH, ODH, and BKDH, respectively.

subunits of *E. coli* PDH (52 kDa) and ODH (81 kDa) were detected in the protein blots, supporting the conclusion that the bacterial food is not a source of LA for *C. elegans*. Taken together, these data show that worms synthesize LA *de novo*.

To determine whether the *C. elegans* lipoylated proteins correspond to catalytic subunits of lipoate-dependent enzymatic complexes, we applied RNAi to knock down *dlat-1* and *dlst-1*, which are predicted to encode E2 subunits of PDH (53.4 kDa) and ODH (49.8 kDa), respectively. Knocking down *dlat-1* and *dlst-1* with RNAi resulted in reduction of the intensity of each of the lipoylated bands, indicating that the lipoylated proteins indeed correspond to the PDH and ODH complexes (Fig. S1). In agreement with previous reports, we determined that RNAi against *dlat-1* and *dlst-1* starting at the embryonic stage caused 100% of embryonic lethality or sterility, respectively (26, 27) (Fig. S1).

***M01F1.3* (RNAi) worms display growth and developmental defects**

To characterize the LA biosynthetic pathway in *C. elegans*, we first focused on the gene *M01F1.3*. This gene encodes a homolog of human LA synthase (LIAS), the enzyme that catalyzes the last step in the biosynthesis of the lipoyl cofactor, which is the attachment of two sulfhydryl groups to C6 and C8 of a hanging octanoyl chain. *M01F1.3* shares 41, 46, 59, and 58% identity with lipoate synthases from *E. coli*, *B. subtilis*, *S. cerevisiae*, and *H. sapiens*, respectively (Fig. S2). *M01F1.3* is a polypeptide of 354 amino acid residues with a molecular mass of 39.7 kDa and has two cysteine motifs that are conserved among LA synthases. One of them, CXXXCXXC, is located at positions 122 to 129 and is thought to bind an Fe-S center involved in the radical SAM reaction (28). The other, CXXXXCXXXXXC, which is unique to LA synthases and is involved in the coordination of a second Fe-S cluster, is located at positions 91 to 102 (29). As expected for a eukaryote organism, computational methods predict that *M01F1.3* has mitochondrial localization. The algorithm TargetP1 (30) predicts a transit peptide of 10 amino acid residues, whereas MitoProtII (31) predicts a longer transit peptide of 18 amino acid residues.

To deplete *M01F1.3*, we induced RNAi by feeding worms with bacteria expressing dsRNA. Because the commonly used dsRNA-producing bacterium, *E. coli* HT115, is a LA prototroph, we replaced its LA synthase gene (*lipA*) with a kanamycin resistance cassette. The resulting strain, AL100, is unable to synthesize LA but retains the ability to produce RNAi (Fig. S3).

RNAi was started early by letting embryos hatch on plates seeded with *E. coli* AL100 producing dsRNA. Knockdown of *M01F1.3* displayed no developmental defects in the first generation, which means that embryos hatched, went through the four typical larval stages (L1, L2, L3, and L4), and became adults able to reproduce. However, in the second generation, the RNAi treatment caused larval arrest at the L3 stage, as evidenced by the worm length and its vulva morphology (Fig. 3B). Western blotting analysis of total proteins from these arrested animals confirmed that they have reduced levels of protein lipoylation, implying that LA synthesis or protein lipoylation is essential for development (Fig. 3C).

The developmental arrest phenotype caused by *M01F1.3* RNAi in the second generation could not be rescued by supplying the treated worms with LA (Fig. 3D). This observation agrees with previous studies performed with other model organisms, such as yeast and mouse, where exogenous LA failed to be used for mitochondrial protein lipoylation (32–34). In addition, the same larval arrest phenotype in the second generation was obtained when RNAi treatment was performed by feeding the worms with an *E. coli* strain (HT115) able to lipoylate its proteins (Fig. S1) in peptone containing nematode growth medium (NGM) (Fig. 3E). This suggests that *C. elegans* lacks a LA salvage pathway.

We also failed to rescue the larval arrest by supplementation with acetate, succinate, and isovaleric acid (C5ISO), produced by the lipoylated enzymes PDH, ODH, and BKDH, respectively (Fig. 3F). The incapacity of metabolites produced by LA-dependent enzymes of rescuing larval arrest, pointed out that lack of protein lipoylation in *C. elegans* is responsible of a variety of not-yet precisely identified physiological abnormalities due to disruption of carbon and energy metabolism.

It has been reported that stronger RNAi phenotypes were obtained with *rrf-3* worms, which are hypersensitive to RNAi (35). Indeed, when we fed *rrf-3* with *M01F1.3* RNAi, a larval arrest phenotype was obtained in the first generation of treated worms. The treated worms grew to adults and had gonad arms healthy in appearance but had a much-reduced capacity for developing embryos. In contrast to control worms, on the fourth day of the experiment no larvae corresponding to the second generation could be seen with a low-magnification microscope. Furthermore, when treated worms were observed using high-magnification microscopy, it was evident that most of them were unable to develop embryos, whereas others had few embryos compared with control worms (Fig. 3, G–H). A typical

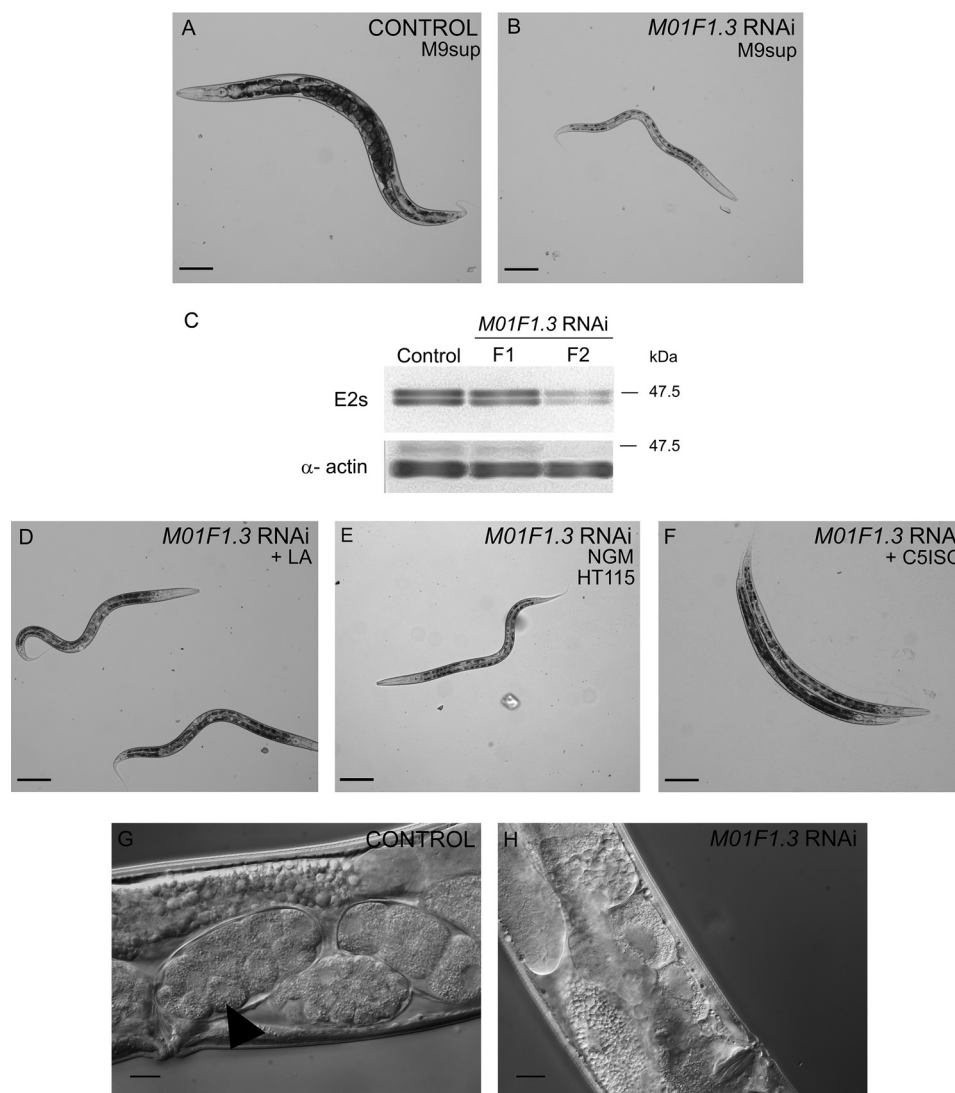


Figure 3. Depletion of *M01F1.3* causes a larval arrest phenotype. N2 worms grown in M9sup or with the addition of the indicated supplements and fed with *E. coli* AL100 (A, B, D, and F) or grown in NGM and fed with HT115 (E). Photographs were taken with 100× magnification and correspond to the fourth day of the second-generation animals. Scale bars represent 100 μ m. C, protein extracts from N2 control animals and from worms of the first and second generations treated with *M01F1.3* RNAi were analyzed by Western blotting using antibodies against LA. The same blot was probed with an antibody against actin to serve as loading control. G and H, Nomarski photographs taken on the fourth day of the experiment of *rrf-3* worms grown in M9sup fed with *E. coli* AL100. The triangle indicates a typical embryo before being laid. Scale bars represent 10 μ m.

embryo, which is ready to be laid when it reaches a 30-cell stage, is indicated with a triangle (Fig. 3G). In contrast, no embryo with cell divisions can be detected in *M01F1.3* (RNAi) worms (Fig. 3H). Thus, endogenous synthesis of lipoyl groups is essential for worm development and cannot be bypassed by LA synthesized by the mothers. Taken together, our results show that *M01F1.3* is an essential protein for larval development in *C. elegans*.

Reducing *M01F1.3* function by RNAi affects the synthesis of monomethyl branched-chain fatty acids

C. elegans produces sphingoid bases that are structurally distinct from those of other animals, because monomethyl branched-chain fatty acids (mmBCFAs) containing species have been reported (36). Moreover, the mmBCFAs C15ISO and C17ISO play an essential role in *C. elegans* development

because suppression of their biosynthesis results in larval arrest (37). Because BGDH, the enzyme that catalyzes the synthesis of the precursors of mmBCFAs, is a lipoate-dependent complex, we quantified fatty acid content by GC/MS in *M01F1.3* (RNAi) animals. We found that the percentage of C15ISO and C17ISO in worms treated with RNAi for two generations was significantly reduced (Fig. 4A). These worms show an increased incorporation of unsaturated fatty acids in their membranes ($61.38 \pm 11.21\%$ compared with $51.07 \pm 3.95\%$ in control worms), possibly to overcome the deficiency of the low melting point mmBCFAs (Table S1).

Deficiency in LA affects *C. elegans* resistance to oxidative stress and lifespan

LA is a critical component of the antioxidant network because of its ability to regenerate other antioxidants such as

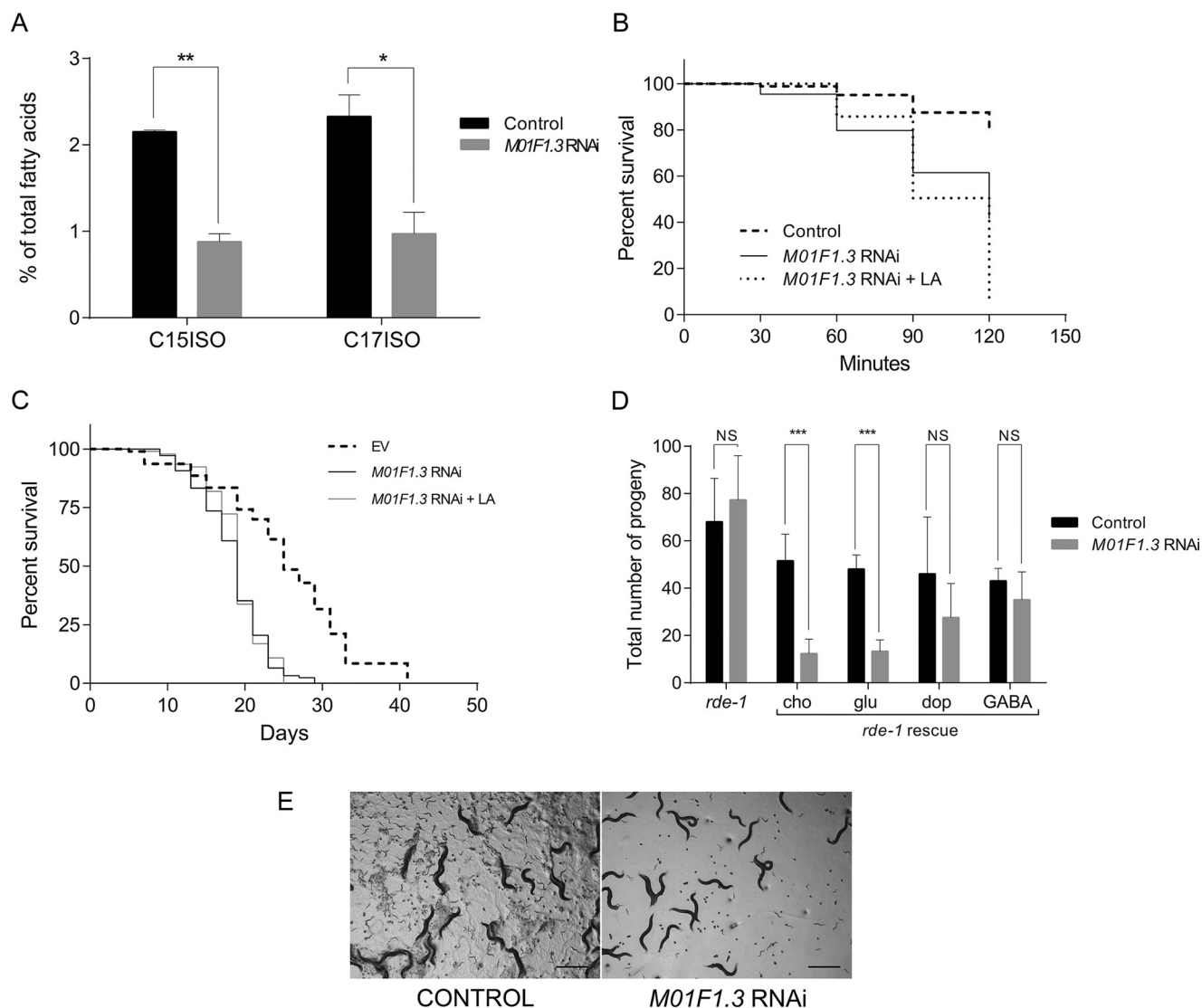


Figure 4. Characterization of *M01F1.3* RNAi treatment. A, comparison of the content of mmBCFAs between N2 treated (subjected to *M01F1.3* RNAi) and control (stage L3) worms. Bar graph represents the content of mmBCFAs. Each bar is the mean $\pm$ S.D. from three independent experiments. * $p < 0.05$; ** $p < 0.005$. B, control and *M01F1.3* RNAi *rrf-3* worms were transferred as young adults to plates containing 15 mM Fe(II) sulfate. Viability was scored every 30 min and represented as percent survival of worms. C, *rrf-3* worms were grown in M9sup plates and fed with *E. coli* Δ *lplA* Δ *lipA* (AL103) strain transformed with either the empty vector (control, dashed line) or the *M01F1.3* dsRNA-producing vector (black solid line). *M01F1.3* RNAi treatment was also performed in the presence of 25 μ M LA (gray solid line). Worms were transferred to fresh plates when necessary to avoid generation mixes. 50 worms were used for each treatment in each assay. Data were collected from three independent experiments. D, four young adult worms subjected to RNAi treatment with *E. coli* AL100 strain were transferred to fresh plates and permitted to lay eggs for 4.5 h. Progeny was scored after 2 days. Each graph is the mean $\pm$ S.D. from four independent experiments. *** $p < 0.001$; NS, no significant difference between counted progeny from control and treated worms. Worm strains used were WM28 (*rde-1*, insensitive to RNAi treatment), XE1581, XE1582, XE1474, and XE1375 (sensitive to RNAi treatment in cholinergic, glutamatergic, dopaminergic, and GABAergic neurons, respectively). E, pictures taken with 25 $\times$ magnification on the fourth day of RNAi treatment in strain XE1581. Scale bars represent 1 mm.

vitamin E and C, increase intracellular GSH level, and provide redox regulation of protein function and transcription (38). Therefore, defects in protein lipoylation alter the capacity of organisms to react to oxidative species, as evidenced by reduced levels of GSH in erythrocytes from heterozygous LIA5 defective mice (32). Consistently with this crucial role in redox regulation, *rrf-3* young adults depleted of *M01F1.3* by RNAi died faster than control worms when exposed to Fe (II), which generates the highly reactive hydroxyl radical (39) (Fig. 4B). Although reduction of oxidative stress by LA dietary supplementation has been demonstrated in diabetic patients and animal models (38), exogenous added LA did not rescue the lower resistance to Fe (II) of RNAi-treated worms (Fig. 4B). It should

be noted, however, that the positive effect of LA supplementation in model animals and humans had been accomplished in conditions of normal levels of lipoylated proteins. According to theories proposed to explain the process of aging, accumulation of free radicals would considerably decrease longevity. Indeed, treatment with some antioxidants result in oxidative stress resistance and increased lifespan in *C. elegans* (40, 41). Because we have observed that *M01F1.3*-depleted animals have reduced antioxidant capacity, we expected that reactive oxygen species would accumulate faster than in control worms. In agreement with these observations, the lifespan of *rrf-3* worms was shortened by *M01F1.3* RNAi treatment, even if the plates were supplemented with LA (Fig. 4C).

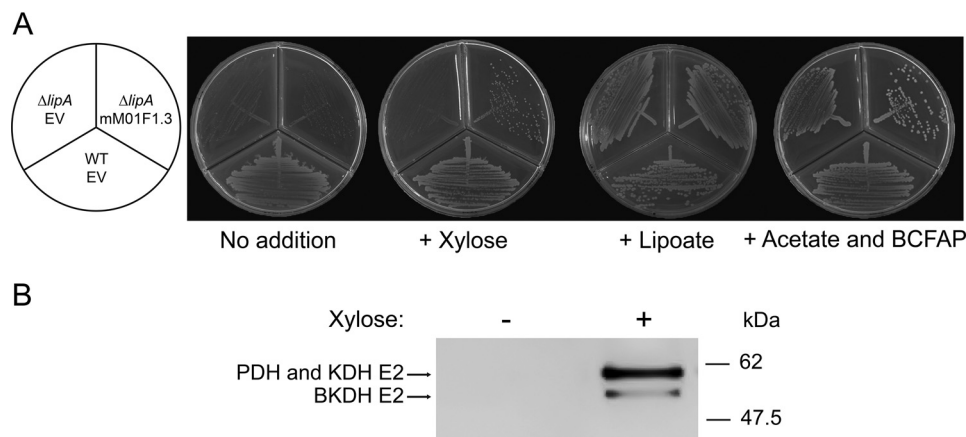


Figure 5. M01F1.3 complements a *lipA* mutant in *B. subtilis*. A, growth of *B. subtilis* WT (JH642) or $\Delta lipA$ (CM37) strains transformed with either the empty vector (EV) or pAL14, a plasmid with the cDNA coding for mature M01F1.3 under a xylose-inducible promoter (mM01F1.3). Strains were streaked onto minimal medium plates with the supplements indicated. Plates were incubated at 37 °C for 2 days. B, protein extracts from strain CM37 transformed with pAL14 and grown with or without xylose were analyzed by Western blotting with antibodies against LA, BCFAP, branched-chain fatty acid precursors.

Altogether, these results support the idea that worms are unable to ligate exogenous LA to their proteins and that *de novo* synthesized LA attached to their cognate proteins appears to be critical for survival and reproduction in an ever-changing environment.

Blocking M01F1.3 neuronal expression reduces worm brood size

To determine in which tissue the expression of M01F1.3 is required, we performed tissue- and neuronal-specific gene knockdown in derivatives of *rde-1* worms, which are insensitive to RNAi treatment (42). RNAi-mediated abrogation of M01F1.3 in muscle, intestine, hypodermis, GABAergic, and dopaminergic neurons did not produce larval arrest or embryonic phenotype. Nevertheless, after inhibition of M01F1.3 expression in glutamatergic and cholinergic neurons, the worm brood size was significantly reduced (Fig. 4, D–E), thus suggesting that protein lipoylation in these neurons is important for either synthesis or release of major neurotransmitters.

M01F1.3 fully rescues *LipA* deficiency in *B. subtilis* and *E. coli*

M01F1.3 shares substantial sequence identity with bacterial and eukaryotic lipoate synthases (Fig. S2). *B. subtilis* and *E. coli* *lipA* strains, deficient in lipoate synthase, cannot grow in minimal medium unless provided with LA or the metabolites produced by the lipoylated enzymes. If M01F1.3 has lipoate synthase activity, its heterologous expression in *lipA* mutants of model bacteria might rescue its deficiency. Strikingly, we observed that M01F1.3 expression completely rescued the growth of a *B. subtilis* *lipA* strain in a defined minimal medium (Fig. 5A). The *B. subtilis* *lipA* complemented strain recovered the ability to lipoylate its proteins, as confirmed by Western blotting analysis, directly demonstrating that LA synthesis is reestablished by the *C. elegans* enzyme (Fig. 5B). Consistent with this notion, M01F1.3 also rescued the growth and lipoylation ability of the *E. coli* strain *lplA* double mutant (Fig. S5).

Thus, based on the molecular characteristics of the M01F1.3 gene, the growth phenotype of RNAi-treated worms and their protein lipoylation pattern, and the ability of M01F1.3 to

complement *lipA* bacterial mutants, we demonstrated that M01F1.3 is a genuine lipoyl synthase and renamed it as LipA.

ZC410.7 encodes an octanoyltransferase

The *C. elegans* gene ZC410.7 encodes a protein of 250 amino acids in length that has 36, 37, and 38% identity with LipB, LIP2, and LIPT2, the octanoyltransferases from *E. coli*, *S. cerevisiae*, and *H. sapiens*, respectively (Fig. S5). The worm protein is predicted to be a mitochondrial enzyme and contains the conserved lysine residue (Lys-157) necessary for the transferase activity. This gene has been annotated as *lpl-1* because it was supposed to encode a lipoate ligase. Octanoyltransferases, amidotransferases, and lipoate ligases are members of the same enzyme family because they have a homologous catalytic module. Lipoate ligases contain an accessory domain involved in activation of lipoate with ATP. The absence of this accessory domain precisely allows differentiating between octanoyl or amidotransferases and ligases. Noteworthy ZC410.7 lacks the typical carboxylic lipoate-activating domain found in the lipoate ligases.

Knockdown of ZC410.7 in N2 worms produced larval arrest in the second generation, similar to the phenotype observed in animals fed with *lipA* RNAi (Fig. 6A). Thus, *C. elegans* ZC410.7 is a good candidate to carry out octanoyltransferase activity, an essential process for lipoyl assembly.

We tested if ZC410.7 could functionally replace the yeast Lip2 octanoyltransferase. Cells of $\Delta lip2$ mutants can grow using a fermentative carbon source, such as glucose (which bypass the lack of lipoylated proteins), but are unable to grow on a respirable carbon source, such as glycerol, because of the essentiality of PDH and ODH enzyme activities (Fig. 6B). We found that growth of $\Delta lip2$ yeasts in a glycerol-containing medium was completely rescued by a plasmid that allows expression of ZC410.7 (Fig. 6B). In addition, Western blotting analysis of $\Delta lip2$ mutants complemented with ZC410.7 showed restoration of protein lipoylation (Fig. 6C), strongly indicating that the *C. elegans* protein was indeed an octanoyltransferase. Finally, we found that expression of the worm protein in a *B. subtilis* mutant unable to synthesize and ligate LA failed to restore

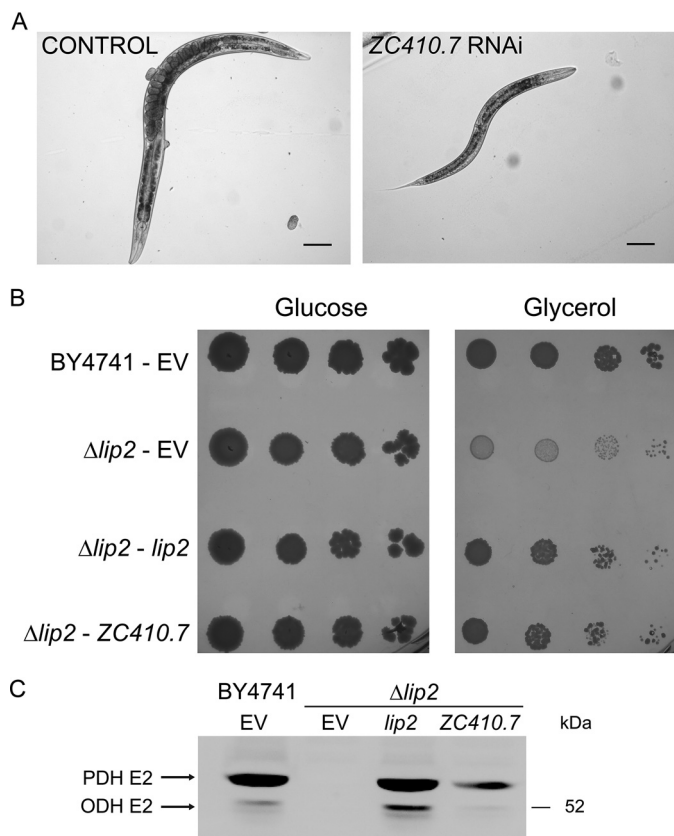


Figure 6. Characterization of ZC410.7. A, N2 worms grown in M9sup and fed with *E. coli* $\Delta lipA$ strain (AL100). Photographs were taken with 100 $\times$ magnification and correspond to the fourth day of the second-generation animals. Scale bars represent 100 μ m. B, yeast strains were grown overnight in synthetic glucose medium lacking uracil, and upon standardization by A_{600} , dilutions were spotted in complex medium plates containing glucose or glycerol as carbon sources. Parental strain BY4741 and *lip2* mutant were transformed with the empty vector (EV) as positive and negative controls, respectively. The *lip2* mutant was transformed with plasmid p426-GPD containing a WT copy of *lip2* or *C. elegans* ZC410.7 coding sequence. C, protein extracts obtained from these cultures were incubated with antibodies against LA.

protein lipoylation even in the presence of exogenous octanoate (Fig. S6). This result rules out the possibility that ZC410.7 has octanoyl ligase or octanoyl-CoA-protein transferase activity.

In summary, we show here that although ZC410.7 has been annotated as a *C. elegans* lipoate ligase, this protein is an octanoyltransferase, and rename it as LIPT2.

C45G3.3 encodes the worm amidotransferase

C45G3.3 is composed of 289 amino acids. It has significant sequence similarity with bacterial lipoate ligases LplA and LplJ (34 and 32%, respectively) and with the amidotransferases from yeast (Lip3, 32%) and humans (LIPT1, 40%) (Fig. S7). *C. elegans* C45G3.3 has a conserved lysine (Lys-141) present in the active site of the members of the cofactor transferases enzyme family, which includes lipoyl and biotinyl ligases, octanoyl, and amidotransferases (43). Because C45G3.3 lacks a lipoate-activating accessory domain, the *C. elegans* protein could be an amidotransferase rather than a ligase. Confirming this prediction, we found that C45G3.3 could rescue the growth of yeast $\Delta lip3$ in a glycerol-supplemented medium (Fig. 7A). Furthermore, lipoy-

lation profiles were restored in yeast $\Delta lip3$ complemented with C45G3.3 (Fig. 7B).

When we expressed C45G3.3 in a *B. subtilis* *lipM* *lplJ* double mutant, which neither synthesize nor ligate LA, cells were unable to utilize free LA provided in the medium (Fig. 7C), indicating that this protein is devoid of lipoyl-protein ligase activity. However, its expression restored the ability of this mutant to grow in minimal medium when provided with octanoate (Fig. 7C). This resembles the observed behavior of yeast Lip3 expression in *E. coli* *lipB* mutants because of a moonlighting octanoyl-CoA-protein transferase activity of amidotransferases (44). All together, these results confirm that C45G3.3 is indeed an amidotransferase, and we renamed it as LIPT1.

Discussion

Despite the well-known role of LA as a protein-bound coenzyme in bacteria and unicellular eukaryotes, the information available about protein lipoylation in multicellular organisms is still scarce. To help fill this gap, we decided to determine how *C. elegans* assembles LA in its proteins. Depleting M01F1.3 or ZC410.7 generated a developmental arrest of the worms, demonstrating that lipoylated proteins are essential for their normal life cycle. In agreement with these observations, there are not submitted mutants deficient in the candidate genes involved in LA metabolism in either worm strain collection (*Caenorhabditis* Genetics Center or National Bioresource Project for the Experimental Animal “Nematode *C. elegans*”).

According to protein databases (UniProtKB/Swiss-Prot and WormBase), five lipoylable proteins are present in the worm: PDH, ODH, BKDH, and two orthologs of human and bacterial GcvHs (GCSH-1 and GCSH-2, Fig. S8), all of them involved in important metabolic routes. The detrimental developmental defects in *M01F1.3* RNAi assays are indicative that lipoate-dependent enzymes, such as PDH and ODH required for entry of carbon into the Krebs cycle and progression of carbon through the cycle, are essential for *C. elegans* development. Indeed, under conditions that favor reproductive growth, various insulin-like peptides responding to environmental and internal stimuli indirectly activate this pathway and power global anabolism by providing energy from intensive oxidative phosphorylation and building blocks for biosynthetic reactions (45). In agreement with the essentiality of the Krebs cycle for *C. elegans* reproductive growth, inhibition of lipoylation of the E2 subunits of PDH and ODH by down-regulation of *M01F1.3*, *dlat-1*, or *dlt-1* by RNAi produces multiple developmental and embryonic defects (Fig. 3 and Fig. S1). We also found that down-regulation of *M01F1.3* by RNAi resulted in a decrease in mmBCFAs synthesis (Fig. 4A). The E2 subunit of BKDH, dihydrolipoamide branched-chain transacylase E2 (DBT-1), has to be lipoylated to generate branched-chain acyl-CoAs, which in turn are used to produce the mmBCFAs C15ISO and C17ISO. It has been demonstrated that mmBCFA-derived glycosyl ceramides are required for postembryonic development in *C. elegans*. Further analysis indicated that these mmBCFA-derived glycosyl ceramides promote intestinal target of rapamycin complex 1 (TORC1) activity (36). *C. elegans* loss-of-function mutants in the *dbt-1* gene have deficiency in mmBCFAs, and

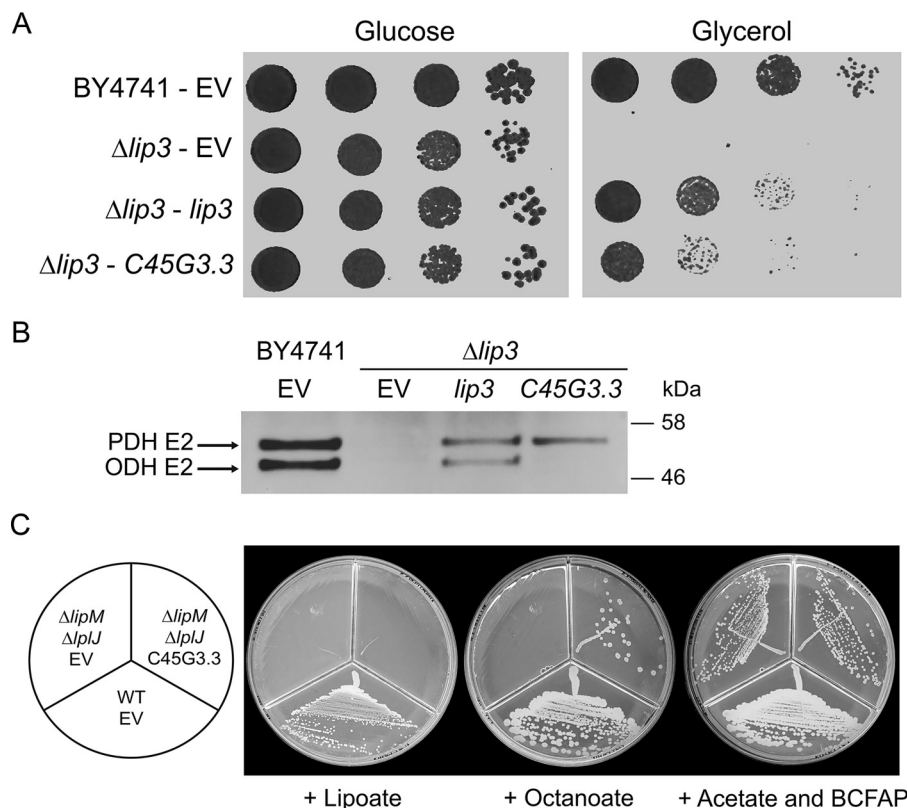


Figure 7. C45G3.3 expression functionally complements yeast and bacterial mutants. A, yeast strains were grown overnight in synthetic glucose medium lacking uracil, and upon standardization by A_{600} , dilutions were spotted in complex medium plates containing glucose or glycerol as carbon sources. Parental strain BY4741 and *lip3* mutant were transformed with the empty vector (EV) as positive and negative controls, respectively. *lip3* mutant was transformed with plasmid p426-GPD containing a WT copy of *lip3* or *C. elegans* C45G3.3 coding sequence. B, protein extracts obtained from these cultures were incubated with antibodies against LA. C, growth of *B. subtilis* WT (JH642) or $\Delta lipM$ $\Delta lipJ$ (NM65) strains transformed with either the empty vector (EV) or pAL18, a plasmid that allows expression of *C. elegans* C45G3.3 protein under the control of a xylose-inducible promoter. Strains were streaked onto minimal medium plates containing xylose and casamino acids vitamin-free, with the indicated supplements. Plates were incubated at 37°C for 2 days. BCFAP, branched-chain fatty acid precursors.

their larval arrest is partially suppressed by supplementation with these fatty acids (46). Nevertheless, supplementation with C5ISO, a mmBCFA precursor product of BKDH, failed to rescue the phenotype of *M01F1.3* RNAi-arrested larvae. This result was somehow expected because, as discussed above, LA is also required for key steps in central metabolism of *C. elegans* and for protecting the worms from oxidative stress. In addition, abnormal oxoglutarate levels might also be affecting the activity of oxoglutarate-dependent oxygenases, for instance, prolyl hydroxylases (47). In line with this possibility, it has been described that *C. elegans* mutants devoid of HIF-prolyl hydroxylase activity, which is required for regulation of hypoxia-inducible factors, show egg-laying defects (48, 49).

Supplementation with exogenously provided LA was ineffective to rescue the larval arrest of *M01F1.3* RNAi-treated animals. Furthermore, carrying out RNAi experiments in a rich medium such as NGM, which contains peptone and hence LA, or using the lipoate prototroph HT115 as the dsRNA-producing bacteria generated the same larval arrest phenotype as obtained when using LA auxotroph strains and a medium depleted of the cofactor. These results strongly suggest that *C. elegans* lacks a salvage pathway and cannot utilize the LA in its free form or bound to peptides. This is consistent with reports showing that feeding mouse mothers with a diet supplemented with LA was inefficient to allow growth of knockout *Lias*

embryos (32) and that administration of LA to human patients with mutations in *LIAS* failed to reverse the metabolic defects (21).

Using a powerful complementation approach designed to rescue well-characterized bacteria or yeast defective in lipoylation with three putative *C. elegans* genes involved in LA synthesis or scavenging, we elucidated the essential mechanism of protein lipoylation of the worms. We propose the lipoyl-relay pathway depicted in Fig. 1. First, the octanoyltransferase LIPT2 (ZC410.7), initiates lipoyl group assembly by transference of the octanoyl moiety from ACP to the octanoyl acceptor proteins, presumably GCSH-1 or GCSH-2 proteins. Then these octanoylated proteins become substrates for sulfur insertion by the SAM radical enzyme LipA (M01F1.3). Finally, the lipoyl moieties are transferred by the amidotransferase LIPT1 (C45G3.3) to the E2 subunits of the 2-oxoacid dehydrogenases, which are required for key steps in central metabolism.

In conclusion, we determined that *C. elegans* is capable of synthesizing LA *de novo* via a lipoyl-relay pathway and that LA deficiency is responsible for developmental defects in worms. We also identified the enzymes involved in the process, all homologs to human proteins involved in LA synthesis. The close analogy of the human lipoate metabolism defects to those of worms with deficiency in protein lipoylation could provide a powerful tool for dissecting the diverse roles of LA in a

multicellular organism under a variety of conditions, and hence, new therapies could flourish.

Experimental procedures

Growth conditions

E. coli and *B. subtilis* strains were routinely grown in lysogeny broth (LB) (50) at 37 °C. In nutritional studies, *E. coli* was grown in M9 minimal medium (50) supplemented with 5 mM acetate and 5 mM succinate. Complementation of *E. coli* strains with worm genes expressed from plasmids with an arabinose-inducible promoter was tested in LB containing 0.2% arabinose. Spizizen salts (51), supplemented with 0.5% glycerol and 0.01% each tryptophan and phenylalanine, were used as the minimal medium for *B. subtilis*. Different supplements, including 0.8% xylose, 50 nM DL- α -LA, 0.5 mM octanoic acid, 5 mM sodium acetate, 0.005% casamino acids vitamin free, and 0.1 mM of each mmBCFA precursor (isobutyric acid, isovaleric acid, and 2-methylbutyric acid) were added as needed. In experiments involving gene expression under the control of the xylose-inducible promoter (*P_{xyLA}*), 0.5% glycerol was used as a carbon source instead of glucose. Antibiotics were added to the media at the following concentrations: 50 μ g ml⁻¹ ampicillin, 10 μ g ml⁻¹ tetracycline, 25 μ g ml⁻¹ kanamycin, 50 μ g ml⁻¹ spectinomycin, 0.5 μ g ml⁻¹ erythromycin, and 12.5 μ g ml⁻¹ lincomycin.

Yeast strains were grown at 30 °C in rich media consisting of 1% yeast extract, 2% peptone, and either 2% glucose or 3% glycerol. Selection and growth of transformants were performed on glucose synthetic medium (0.67% yeast nitrogen base with ammonium sulfate and 2% glucose), with supplements added according to Sherman *et al.* (52), lacking uracil. 2% agar was added for preparation of solid plates.

Worms were grown at 20 °C in either NGM (53) or M9 supplemented with 5 mM acetate, 5 mM succinate, and 5 μ g ml⁻¹ cholesterol (M9sup). C5ISO 100 mM or LA 25 μ M were added to the growth medium when indicated. Worms were fed with *E. coli* OP50 for maintenance, or specific *E. coli* strains as specified in each experiment.

Genetic and molecular biology techniques

E. coli strains were transformed using the calcium chloride procedure (54). Transformation of *B. subtilis* was accomplished by the method of Dubnau and Davidoff-Abelson (55).

Worm cDNA was obtained using TRI REAGENT® (Molecular Research Center) and Direct-zol™ MiniPrep Kit (Zymo Research). All *C. elegans* genes were amplified using worm cDNA as a template. *B. subtilis* chromosomal DNA was obtained using a Wizard® Genomic DNA Purification Kit (Promega). For yeast gene amplification, one BY4741 colony was boiled for 10 min in NaOH 50 mM, and 5 μ l of this was used as a template for PCR reaction. PCR was carried out using Q5 high fidelity DNA polymerase and appropriately designed primers from Genbiotech or Invitrogen. Plasmids were extracted using the Promega Wizard® Plus SV Minipreps-DNA Purification System. Restriction enzymes and ligases were from Promega and New England Biolabs. DNA

sequencing was performed by the DNA Sequencing Facility of the University of Maine.

Strains and plasmids construction

Several *C. elegans* strains were obtained from *Caenorhabditis* Genetics Center: Bristol N2, NL2099 (*rrf3* (*pk1426*)), XE1581 (*lin-15B*(n744) X; *eri-1*(mg366) IV; *rde-1*(ne219) V; *wpSi10* [*Punc-17::rde-1:SL2:sid-1,Cbunc-119(+)*] II), XE1582 (*lin-15B*(n744) X; *eri-1*(mg366) IV; *rde-1*(ne219) V; *wpSi11*[*Peat-4::rde-1:SL2:sid-1, Cbunc-119(+)*] II), XE1474 (*lin-15B*(n744) X; *eri-1*(mg366) IV; *rde-1*(ne219) V; *wpSi6*[*Pdat-1::rde-1:SL2:sid-1, Cbunc-119(+)*] II), and WM28 (*unc-32*(e189) III; *rde-1*(ne219) V).

Bacterial strains and plasmids used in this work are listed in Table 1.

A *B. subtilis* strain with a deletion of the *lipA* gene was constructed by gene replacement with a spectinomycin resistance determinant through a double cross-over event. For this purpose, a 396-bp fragment, corresponding to an upstream region of the gene, was PCR-amplified from JH642 chromosomal DNA with oligonucleotides LipHind and LipBam (Table 2), ligated into pCR2.1-TOPO, and then cloned into XbaI and BamHI sites of pBluescriptSK(+) to render pSK469. The spectinomycin resistance determinant, which was obtained by digesting pJM134 with *EcoRV*, was inserted into the SmaI site of pSK469. Then, a 535-bp fragment containing the 3' end of *lipA* and part of the downstream gene was PCR-amplified with primers LipA3up and Lip5fill and cloned into ClaI and HincII sites of the previously generated plasmid. The plasmid obtained, pCM24, was linearized with ScaI and used to transform strain JH642. Transformants were selected for spectinomycin resistance. The resulting strain was named CM37.

To construct HT115 derivatives defective in LA metabolism, we carried out generalized transduction using P1vir phage (63). The kanamycin resistance cassette disrupting *lipA* gene in TM131 was moved to HT115. The resulting strain, AL100, was able to lipoylate its proteins only when grown in presence of LA (Fig. S3). To confirm that AL100 strain was still able to produce interference, we performed a *let-767* RNAi experiment because its resulting phenotype is very well-characterized in literature (64). Indeed, we obtained uncoordinated, transparent, and morphologically abnormal worms as described.

To construct an HT115 derivative unable to synthesize and ligate LA, the kanamycin and chloramphenicol resistance cassettes from QC145, disrupting *lplA* and *lipB* genes, respectively, were moved to HT115. The resulting strain, AL103, failed to grow on M9 even when supplemented with LA (data not shown).

A DNA fragment coding for the mature M01F1.3 protein was amplified using oligonucleotides mlipAsRBSSma, which includes a *B. subtilis* ribosome binding site, and mlipAREVXba. The resulting 1070-bp fragment was inserted into the SmaI and XbaI sites of vector pLarC1, which allows expression of mM01F1.3 under a xylose-inducible promoter (60). The resulting plasmid was named pAL14.

For expression of mature M01F1.3 in *E. coli*, the DNA fragment was amplified using oligonucleotides mlipAcRBSXba,

Protein lipoylation in *C. elegans*

Table 1

Strains and plasmids used in this work

Relevant characteristics ^a		Source or reference
Strains		
<i>E. coli</i>		
OP50	<i>ura</i> [−]	(53)
TM131	<i>rpsL lipA150::Tn1000dKm lplA148::Tn10dTc</i>	(12)
TM136	<i>rpsL lipB182::Tn1000dKm lplA148::Tn10dTc</i>	(11)
QC145	<i>lplA::FRT::Km lipB::FRT::cat</i>	(56)
HT115	F [−] , mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase) Tc ^r	(57)
AL100	HT115 <i>lipA150::Tn1000dKm</i>	This study
AL103	HT115 <i>lplA::FRT::Km lipB::FRT::cat</i>	This study
<i>B. subtilis</i>		
JH642	<i>trpC2 pheA1</i>	Laboratory stock
CM37	JH642 <i>lipA::Sp</i> ^r	This study
NM65	JH642 <i>lplJ::Sp</i> ^r <i>lipM::Km</i> ^r	(13)
<i>S. cerevisiae</i>		
BY4741	<i>S. cerevisiae</i> MATa <i>his3 leu2 met15 ura3</i>	EUROSCARF
Δ <i>lip2</i>	<i>S. cerevisiae</i> MATa <i>his3 leu2 met15 ura3 lip2</i>	(58)
Δ <i>lip3</i>	<i>S. cerevisiae</i> MATa <i>his3 leu2 met15 ura3 lip3</i>	(58)
Plasmids		
pBluescriptSK(+)	<i>E. coli</i> cloning vector, Amp ^r	Stratagene
pBAD33	Arabinose-inducible expression vector	(59)
pCR2.1-TOPO	TOPO TA cloning vector, Km ^r	Thermo Fisher Scientific
pSK469	pBluescriptSK(+) containing a region upstream <i>lipA</i>	This study
pJM134	Integrational vector; Sp ^r	Perego, unpublished
pCM24	pBluescriptSK(+) containing <i>lipA</i> regions interrupted with spectinomycin cassette	This study
pNM48	pJM134 containing <i>lplJ</i> interrupted by a spectinomycin cassette	(13)
pLarC1	pHPKS containing <i>PxyIA</i> and <i>xylR</i> , MCL ^r	(60)
p426-GPD	Vector for constitutive protein expression in yeast driven by glyceraldehyde 3-phosphate dehydrogenase promoter. Amp ^r	(61)
pAL6	p426-GPD containing C45G3.3 coding sequence	This study
pAL10	p426-GPD containing WT copy of <i>lip2</i> gene	This study
pAL11	p426-GPD containing WT copy of <i>lip3</i> gene	This study
pAL14	pLarC1 containing the mature coding sequence of <i>M01F1.3</i>	This study
pAL15	Mature coding sequence of <i>M01F1.3</i> cloned in pBAD33	This study
pAL18	Coding sequence of C45G3.3 cloned in pLarC1	This study
pAL23	p426-GPD containing ZC410.7 coding sequence	This study
pAL24	Coding sequence of ZC410.7 cloned in pLarC1	This study
L4440	Vector for production of dsRNA molecules. Amp ^r	(62)

^aAmp^r, Tc^r, Cm^r, Km^r, MCL^r, and Sp^r denote resistance to ampicillin, tetracycline, chloramphenicol, kanamycin, macrolides, and spectinomycin, respectively.

Table 2

Oligonucleotide primers used in this work

Primer Name	Sequence (5'-3') ^a
LipHind	CGTGTAAGCTTCGATTTTATGATATAATTC
LipBam	ATTAGGATCCATTCCGGGCTTTCTGAG
LipA3up	GCAATCGATGCCCGTTCAACAAC
LipAfill	CGCCAGTTAAACCTGGCGTTTGG
mlipAsRBSSma	TACCCGGAAGGAAGGTCTTAAATGGCAGCCACAAAGAAAAAACC
mlipAREVXba	CGTCTAGATTAATTTTGTCTATTTCTCAACACATTTTTC
mlipAcRBSSba	ATTCTAGAGAAGGAGATATACCATGGCAGCCACAAAGAAAAAACC
M01F1.3_Hind_REV	CGAAGCTTATTAATTTTGTCTATTTCTCAACAC
C45G3.3sRBSSma	TACCCGGAAGGAAGGTCTTAAATGTGTTCTATATCACG
C45G3.3REVXba	AATCTAGACTAAAGATCCGGCGAGAAT
C45G3.3BamFOR	TGGGATCCTATGTGTTCTATATCACGC
C45G3.3EcoREV	AGGAATTCCTAAAGATCCGGCGAG
lip2BamFOR	CAGGATCCATGTCGAGGTGATTTCGC
lip2stHinREV	ATAAGCTTGCTCACGGATTCTTTTCAAAATATTGACG
lip3BamFOR	GCGGATCCATGTCTATGATGCTAAGTAATTGGG
lip3stHinREV	GCAAGCTTTATGTGTAAGTGTCAATATTCTCC
ZC410.7bBamFOR	TTGGATCCATGCGTCTCTCTCAC
ZC410.7bHinREV	ATAAGCTTTTAACTAGAAACAACATTTCG
ZC410.7bsubSma	ATTCTAGAGAAGGAGATATACCATGATGCGTCTCTCTCAC
ZC410.7bXbaREV	AATCTAGATTAAGTAAAGAAACAACATTTCG

^aRestriction sites are underlined.

which includes an *E. coli* ribosome binding site, and M01F1.3_Hin_REV. The resulting 1070 bp fragment was inserted into XbaI and HindIII sites of vector pBAD33 (59) rendering plasmid pAL15, which therefore allows expression of mM01F1.3 under an arabinose-inducible promoter.

The coding sequence of C45G3.3 was amplified using oligonucleotides C45G3.3sRBSSma, which includes a *B. subtilis*

ribosome binding site, and C45G3.3REVXba. The resulting 902-bp fragment was inserted into the SmaI and XbaI sites of vector pLarC1 (60), rendering plasmid pAL18.

The coding sequence of C45G3.3 was amplified using oligonucleotides C45G3.3BamFOR and C45G3.3EcoREV. The 889-bp fragment obtained was inserted into BamHI and EcoRI sites of vector p426-GPD, which allows constitutive protein

expression in *S. cerevisiae* (61). The resulting plasmid was named pAL6.

The coding sequence of ZC410.7 was amplified using oligonucleotides ZC410.7BamFOR and ZC410.7HinREV. The 770-bp fragment obtained was cloned into BamHI and HindIII sites of vector p426-GPD, rendering plasmid pAL23.

To express ZC410.7 in *B. subtilis*, the encoding gene was amplified using oligonucleotides ZC410.7bsubSma, which includes a *B. subtilis* ribosome binding site, and ZC410.7bXbaREV. The resulting 787-bp fragment was inserted into the SmaI and XbaI sites of vector pLarC1 (60), rendering plasmid pAL24.

lip2 and *lip3* sequences were amplified from *S. cerevisiae* BY4741 genomic DNA. The pair of oligonucleotides used were lip2BamFOR and lip2HinREV for *lip2* sequence, and lip3BamFOR and lip3HinREV for *lip3*. The resulting DNA fragments were cloned into BamHI and HindIII sites of p426-GPD rendering plasmids pAL10 and pAL11, respectively.

Immunoblotting analyses

B. subtilis strains were grown overnight in minimal medium supplemented with acetate and mmBCFA precursors at 37°C. The cells were resuspended in fresh media of the same composition, with or without xylose, and cultured at 37°C. A 1-ml aliquot of each culture was harvested after 22 h of growth. The samples were centrifuged and the pellets were washed with buffer (20 mM Tris-HCl, pH 8.0, and 150 mM NaCl). They were resuspended in 180 μ l of lysis buffer (50 mM Tris-HCl, pH 8.0, and 1 mM PMSF) per A_{600} unit. Resuspended cells were disrupted by incubation with lysozyme (100 μ g ml⁻¹) for 15 min at 37°C, followed by 5 min of boiling in the presence of loading buffer.

C. elegans worms were collected with M9 buffer (53), centrifuged, washed three times, and resuspended in 500 μ l of M9 buffer with 1 mM PMSF. Samples were sonicated and protein content was measured according to the method of Lowry (65). Samples were boiled in the presence of loading buffer.

S. cerevisiae protein extracts were obtained based on the protocol published by Sattlegger *et al.* (66). Briefly, yeast cells were grown overnight in synthetic glucose medium lacking uracil, collected, and resuspended in breaking buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 1 mM PMSF). The cells were broken with an equivalent volume of glass beads by vortexing 8 times for periods of 30 s alternating with 30 s on ice. The extract obtained was centrifuged for 5 min at 1000 $\times$ g and the supernatant was boiled with loading buffer.

All samples were fractionated by SDS-PAGE on 12% polyacrylamide gels. Proteins were electroeluted to a nitrocellulose membrane, and lipoylated E2 subunits were detected using rabbit anti-lipoate antibody and a secondary anti-rabbit IgG conjugated to alkaline phosphatase or horseradish peroxidase (Bio-Rad). When the last antibody was used, the bands were visualized by use of the ECL Plus Western Blotting Detection System (GE Healthcare). In samples from RNAi experiments, the same blots were probed with an antibody against actin to serve as a loading control (α -actin, Santa Cruz Biotechnology).

RNAi by feeding

The RNAi feeding vectors were obtained from the *C. elegans* whole genome Ahringer RNAi feeding library (Source Bio-Science). The RNAi feeding strains were *E. coli* HT115, AL100, and AL103, transformed with either L4440 empty vector or with dsRNA-producing constructs.

Feeding RNAi experiments were done as described (64) with modifications. Briefly, the cells were grown overnight in LB with the corresponding antibiotics, collected, washed, and resuspended in M9 supplemented with acetate and succinate. Induction was performed overnight at room temperature in liquid culture containing 1 mM isopropyl 1-thio- β -D-galactopyranoside and ampicillin. The cells were collected, mixed with 1 mM 1-thio- β -D-galactopyranoside, and spotted, either on M9sup or NGM plates with ampicillin. Bleached embryos or synchronized L1s were placed on the bacterial spots.

Oxidative stress assays

Ten worms subjected to RNAi treatment were transferred as late L4 or young adults to agar plates supplemented with 15 mM iron (II) sulfate and scored for survival every 30 min. Plates were prepared the day before the experiment. Six experiments were performed independently in triplicate.

Longevity assays

Lifespans were assayed at 20°C. Data were collected from three independent trials, using an initial number of 50 worms/treatment/trial. The worms were grown in M9sup and fed with strain AL103 transformed with either the empty vector or the *M01F1.3* dsRNA-producing vector. Adult worms were transferred to fresh plates when necessary to avoid generation mixes. Worms were considered dead when they lacked pharyngeal pumping and failed to move when touched with a platinum wire. Worms were considered "censored" if they exhibited protruding vulva or internal hatching, or if they crawled off the plate or burrowed into the medium.

GC/MS determination of fatty acid methyl-ester content

Lipid extraction was performed using the Bligh and Dyer method (67). Briefly, second-generation N2 worms fed with AL100 transformed with either the empty vector (at L3 larval stage) or the *M01F1.3* dsRNA-producing vector (at the arrested stage) were harvested and washed three times with M9 buffer to eliminate excess bacteria. The worm pellet was transferred to a glass tube and 3 ml of a chloroform:methanol 1:2 mixture was added. Dibutylhydroxytoluene was added as an antioxidant at a final concentration of 5 μ g/ml. The samples were incubated at -20°C for at least 12 h. The fatty acid methyl esters were prepared by transesterification of glycerolipids with 0.5 M sodium methoxide in methanol and then analyzed in a Shimadzu GC-2010 Plus gas chromatograph-mass spectrometer, on a capillary column (30 mm $\times$ 0.25 mm in diameter) of 100% polyethylene glycol (SUPELCOWAX 10, Sigma-Aldrich).

Brood size assays

Four adult worms treated with *M01F1.3* RNAi were placed in fresh plates and allowed to lay eggs for 4.5 h. Adults were removed and progeny was scored 2 days later. Data were collected from four independent trials.

Microscopy

Morphologic phenotypes were observed using an Olympus MVX10 microscope and pictures were taken with an Olympus DP72 camera. Microphotographs were taken with a Nikon Eclipse 800 microscope and an Andor Clara camera. Images were processed and analyzed with NIS-Elements and ImageJ.

Bioinformatics analyses

Protein sequence searches were carried out using UniProtKB/Swiss-Prot (<http://www.uniprot.org>) (The UniProt Consortium, 2014) and WormBase (RRID:SCR_003098). Homologous proteins were identified using BLASTP (<http://www.uniprot.org>) (68), and protein alignments were generated with T-Coffee (RRID:SCR_019024) (69) and formatted with Box-Shade Server (RRID:SCR_007165). Mitochondrial localization predictions were performed with MitoProtII (RRID:SCR_019023) (31) and TargetP (RRID:SCR_019022) (30).

Statistical analyses

All assays were performed in at least triplicate. Mean survival days, standard error, intervals of mean survival days with 95% confidence, and equality *p* values to compare averages were calculated by log rank and Kaplan–Meier tests using OASIS 2 Online Application for Survival Analysis (70). For brood size assays and lipid profiles, data are presented as means $\pm$ S.D. Statistical significance was determined using Student's two-tailed *t* test. *p* values of <0.05 were taken to indicate statistical significance.

Data Availability

All data are contained within this article and in the supporting information.

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Abbreviations—The abbreviations used are: ACP, acyl carrier protein; BKDH, branched-chain α -ketoacid dehydrogenase; GCS, glycine cleavage system; GcvH, H subunit of GCS; LA, lipoic acid; LB, Lysogeny broth; LD, lipoylable domain; mmBCFAs, monomethyl branched-chain fatty acids; NGM, Nematode Growth Medium; ODH, oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; DH, dehydrogenase; SAM, S-adenosylmethionine; C5ISO, isovaleric acid.

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