JBC Papers in Press. Published on August 25, 2020 as Manuscript RA120.013760
The latest version is at https://www.jbc.org/cgi/doi/10.1074/jbc.RA120.013760

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

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Running title: Protein lipoylation in *C. elegans*

Keywords: *Caenorhabditis elegans*, development, energy metabolism, fatty acid metabolism, inborn error of metabolism, lipoic acid, mitochondrial metabolism, oxidative stress, post-translational modification, RNA interference

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ABSTRACT

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 encodes 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 this nematode could be a valuable model to dissect the role of protein mislipoylation and to develop new therapies.

INTRODUCTION

Lipoic acid (LA) is a sulfur-containing cofactor derived from octanoic acid that is required for the function of several key multienzymes 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 it was first purified and

characterized in the early 1950s (1), the mechanisms of protein lipoylation in animals are 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, acetoin 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 TPP as a cofactor while P subunits require PLP; H proteins lack the catalytic role of E2 subunits; and DH complexes catalyze irreversible reactions while 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). Besides, 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 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 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 while 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 atoms (15). Afterwards, LipL is required to transfer the lipoyl moiety from GcvH to E2s 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), since 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; suffer from severe respiratory deficiency and extreme muscle weakness. Reported clinical pictures of patients with mutations in *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

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disorders such as neonatal-onset epilepsy and encephalopathy (19–24). Presently, treatments for patients with LA deficiency are only restricted to alleviate 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 since 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 (Δ*lipA* Δ*lplA*) or TM136 (Δ*lipB* Δ*lplA*) that can neither synthesize LA nor take it from the environment (11, 12). When synchronized wild type 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 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*, 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 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, while 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. As the commonly used dsRNA producing bacterium, *E. coli* HT115, is a LA prototroph, we replaced its LA synthase gene (*lipA*) by a kanamycin resistance cassette. The resulting strain, AL100, is

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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, this 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 blot analysis of total proteins from these arrested animals confirmed that they have reduced level 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). Besides, 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 non-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, had gonad arms healthy in appearance but had a much reduced capacity for developing embryos. In contrast to control worms, at 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 a high magnification microscopy, it could be appreciated that most of them were unable to develop embryos, while others had few embryos compared with control worms (Fig.3G-H). A typical 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 which are structurally distinct from those of other animals, as monomethyl branched-chain fatty acids (mmBCFAs) containing species has been reported (36). Moreover, the mmBCFAs C15ISO and C17ISO, play an essential role in *C. elegans* development since suppression of their biosynthesis results in larval arrest (37). As BKDH, the enzyme that catalyzes the synthesis of the precursors of mmBCFAs is a lipoate-dependent complex we quantified fatty acid content by gas chromatography/mass spectrometry (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±11.21 % compared to 51.07± 3.95 % in control worms), possibly to overcome the deficiency

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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 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 LIAS 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). As 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).

Altogether, these results support the idea that worms are unable to ligate exogenous LA to its proteins and that *de novo* synthesized LA attached to its 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 blot 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 lipA* double mutant (Fig.S5).

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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 demonstrate 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, which 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 (K157) necessary for the transferase activity. This gene has been annotated as *lpl-1*, since it was supposed to encode a lipoate ligase. Octanoyltransferases, amidotransferases and lipoate ligases are members of the same enzyme family, as 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 Δ*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, due to the essentiality of PDH and ODH enzyme activities (Fig.6B). We found that growth of Δ*lip2* yeasts in a glycerol-containing medium was completely rescued by a plasmid that allows expression of ZC410.7 (Fig.6B). In addition, Western blot analysis of Δ*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 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 renamed it as LIPT2.

C45G3.3 encodes the worm amidotransferase

C45G3.3 is composed of 289 amino acids in length. 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 (K141) present in the active site of the members of the cofactor transferases enzyme family, which includes lipoyl and biotinyl ligases, octanoyl and amidotransferases (43). As 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 Δ*lip3* in a glycerol-supplemented medium (Fig.7A). Furthermore, lipoylation profiles were restored in yeast Δ*lip3* complemented with C45G3.3 (Fig.7B).

When we expressed C45G3.3 in a *B. subtilis lipM lplJ* double mutant, that neither synthesize nor ligate LA, cells were unable to utilize free LA provided in the medium (Fig.7C), indicating that this protein is

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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, due to 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 filling this gap, we decided to determine how *C. elegans* assembles LA in their 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, in neither worm strain collection (CGC or National Bioresource Project for the Experimental Animal "Nematode *C. elegans*") there are submitted mutants deficient in the candidate genes involved in LA metabolism that we studied in this work.

According to protein databases (UniProtKB/Swiss-Prot, 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 is 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 powers 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 downregulation of *M01F1.3*, *dlat-1* or *dlst-1* by RNAi produces multiple developmental and embryonic defects (Fig.3 and S1). We also found that downregulation of *M01F1.3* by RNAi resulted in a decrease in mmBCFAs synthesis (Fig.4A). The E2 subunit of BKDH, 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 mmBCFAs-derived glycosyl ceramides are required for post-embryonic development in *C. elegans*. Further analysis indicated that these mmBCFAs-derived glycosyl ceramides promote intestinal TORC1 activity (36). *C. elegans* loss-of-function mutants in the *dbt-1* gene have deficiency in mmBCFAs and 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 since, 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 hidroxylases (47). In line with this possibility, it has been described that *C. elegans* mutants devoid of HIF-prolyl hydroxylase activity, 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

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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 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 being homologues 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 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 µg ml^{-1} ampicillin, 10 µg ml^{-1} tetracycline, 25 µg ml^{-1} kanamycin, 50 µg ml^{-1} spectinomycin, 0.5 µg ml^{-1} erythromycin and 12.5 µg ml^{-1} lincomycin.

Yeast strains were grown at 30ºC 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 sulphate, 2% glucose) with supplements added according to Sherman et al., lacking uracil (52). 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 µg ml^{-1} cholesterol (M9sup). Isovaleric acid 100 mM (C5ISO) or LA 25 µM was 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).

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Transformation of *B. subtilis* was accomplished by the method of Dubnau and Davidoff-Abelson (55).

Worm cDNA was obtained using TRI REAGENT® (MRC) and Direct-zol™ MiniPrep Kit (Zymo Research). All *C. elegans* genes were amplified using worm cDNA as template. *B. subtilis* chromosomal DNA was obtained using "Wizard® Genomic DNA Purification Kit" (Promega). For yeast gene amplification, one BY4741 colony was boiled for 10 minutes in NaOH 50 mM and 5 µl 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), 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 crossover 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 *Xba*I and *BamH*I sites of pBluescriptSK(+) to render pSK469. The Spectinomycin resistance determinant, which was obtained by digesting pJM134 with *Eco*RV, was inserted into the *Sma*I 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 *Cla*I and *Hinc*II sites of the previously generated plasmid. The plasmid obtained, pCM24, was linearized with *Sca*I 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 (56). 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). In order to confirm that AL100 strain was still able to produce interference, we performed a *let-767* RNAi experiment as its resulting phenotype is very well characterized in literature (57). Indeed, we obtained uncoordinated, transparent and morphologically abnormal worms as described.

To construct a 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 *Sma*I and *Xba*I sites of vector pLarC1, which allows expression of mM01F1.3 under a xylose inducible promoter (58). The resulting plasmid was named pAL14.

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

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includes an *E. coli* ribosome binding site, and M01F1.3_Hin_REV. The resulting 1070 bp fragment was inserted into *Xba*I and *Hind*III 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 (58), rendering plasmid pAL18.

The coding sequences 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* (60). 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 (58), rendering plasmid pAL24.

lip2 and *lip3* sequences were amplified from *S. cerevisiae* BY4741 genomic DNA. The pair of oligonucleotides used was 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. 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 22h of growth. The samples were centrifuged and the pellets were washed with buffer [20 mM Tris-Hcl (pH 8.0), 150 mM NaCl]. They were resuspended in 180 µl of lysis buffer [50 Tris-Hcl (pH 8.0), 1 mM phenymethylsulfonyl fluoride (PMSF)] per OD_{600} unit. Resuspended cells were disrupted by incubation with lysozyme (100 µg 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 and washed three times, and resuspended in 500 µl of M9 buffer with 1 mM PMSF. Samples were sonicated and protein content was measured according to the method of Lowry (61). Samples were boiled in the presence of loading buffer.

S. cerevisiae protein extracts were obtained based on the protocol published by Sattlegger et al. (62). Briefly, yeast cells were grown overnight in CM-ura medium, collected and resuspended in breaking buffer [20 mM TrisHCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 1 mM PMSF]. 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 x 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 immunoglobulin G 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). In samples from RNAi experiments the same blots were probed with an antibody

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against actin to serve as loading control (α-actin, Santa Cruz Biotechnology).

RNA interference by feeding

The RNAi feeding vectors were obtained from the *C. elegans* whole genome Ahringer RNAi feeding library (Source BioScience). 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 (57) with modifications. Briefly, cells were grown ON in LB with the corresponding antibiotics, collected, washed and resuspended in M9 supplemented with acetate and succinate. Induction was performed ON at room temperature in liquid culture containing 1 mM isopropyl 1-thio-μ-D-galactopyranoside (IPTG) and ampicillin. The cells were collected, mixed with 1 mM IPTG 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 minutes. Plates were prepared the day before of the experiment. Six experiments were performed independently, by triplicates.

Longevity assays

Lifespans were assayed at 20ºC. Data was collected from three independent trials, using an initial number of 50 worms per treatment per trial. 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, in order 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 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 Bligh and Dyer method (63). 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 μg/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 by 0.25 mm in diameter) of 100% poliethilenglycol (Supelco WAX-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 hours. Adults were removed and progeny was scored two days later. Data was collected from 4 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 Andorclara camera. Images were processed and analyzed with Nis Elements and ImageJ.

Bioinformatics analyses

Protein sequences searches were carried out using UniProtKB/Swiss-Prot (http://www.uniprot.org/) (The UniProt Consortium, 2014) and WormBase (www.wormbase.org). Homologous

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proteins were identified using BLASTP (http://www.uniprot.org/) (64), protein alignments were generated with T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/index.html) (65) and formatted with BoxShade Server (https://embnet.vital-it.ch/software/BOX_form.html).
Mitochondrial localization predictions were performed with MitoProtII (http://ihg.gsf.de/ihg/mitoprot.html) (31) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) (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 (66). For brood size assays and lipid profiles, data are presented as means ± SD. 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 the article.

Acknowledgements

We gratefully acknowledge Silvia Altabe for technical help with GC/MS data processing. We thank John E. Cronan, Jr. for the gift of strain QC145 and Antonio Uttaro for providing yeast mutants. We thank D. Krapf for the of α-actin antibody. We thank Marina Avecilla and Viviana Villalba for technical support.

Funding and additional information

A. Lavatelli was a Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), D. de Mendoza and M.C. Mansilla are Career Investigators of the same institution. This work was supported by grants from CONICET (P-UE 2016-IBR) and Ministerio de Ciencia, Tecnología e Innovación Productiva (EULACH 16/T02-0161).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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Abbreviations

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; LDs, lipoylable domains; mmBCFAs, monomethyl branched-chain fatty acids; NGM, Nematode Growth Medium; ODH, oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase.

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Table 1. Strains and Plasmids used in this work

	Relevant characteristics[a]	Source or reference
Strains		
Escherichia coli		
OP50	*ura*$^-$	(53)
TM131	*rpsL lipA150*::Tn*1000*dKm *lplA148*::Tn*10*dTc	(12)
TM136	*rpsL lipB182*::Tn*1000*dKm *lplA148*::Tn*10*dTc	(11)
QC145	*lplA::*FRT::*Km lipB::*FRT*::cat*	(67)
HT115	F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase) Tc^r	(68)
AL100	HT115 *lipA150*::Tn*1000*dKm	This study
AL103	HT115 *lplA::*FRT::*Km lipB::*FRT*::cat*	This study
Bacillus subtilis		
JH642	*trpC2 pheA1*	Laboratory stock
CM37	JH642 *lipA*::Sp^r	This study
NM65	JH642 *lplJ::Sp*^{r} *lipM::Km*^{r}	(13)
Saccharomyces cerevisiae		
BY4741	*S. cerevisiae* MATa *his3 leu2 met15 ura3*	EUROSCARF
Δ*lip2*	*S. cerevisiae* MATa *his3 leu2 met15 ura3 lip2*	(69)
Δ*lip3*	*S. cerevisiae* MATa *his3 leu2 met15 ura3 lip3*	(69)
Plasmids		
pBluescriptSK(+)	*E. coli* cloning vector, Amp^r	Stratagene

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pBAD33	Arabinose inducible expression vector	(59)
pCR2.1-TOPO	TOPO TA cloning vector, Km^r	ThermoFisher Scientific
pSK469	pBluescriptSK(+) containing a region upstream *lipA*	This study
pJM134	Integrational vector; Sp^r	Perego, unpublished
pCM24	pBluescriptSK(+) containing *lipA* regions interrupted with spectinomycin cassette	This study
pNM48	pJM134 containing *lplJ* interrupted by a spectinomycin cassette	(13)
pLarC1	pHT115 containing P*xylA* and *xylR*, MCL^r	(58)
p426-GPD	Vector for constitutive protein expression in yeast driven by glyceraldehyde 3-phosphate dehydrogenase promoter. Amp^r	(60)
pAL6	p426-GPD containing C45G3.3 coding sequence	This study
pAL10	p426-GPD containing wild type copy of *lip2* gene	This study
pAL11	p426-GPD containing wild type copy of *lip3* gene	This study
pAL14	pLarC1 containing the mature coding sequence of *M01F1.3*	This study
pAL15	Mature coding sequence of *M01F1.3* 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	(70)

[a] Amp, Tc, Cm, Km, MCL and Sp denote ampicillin, tetracycline, chloramphenicol, kanamycin, macrolides and spectinomycin resistance cassettes, respectively.

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Table 2. Oligonucleotides primers used in this work

Primer name	Sequence (5´-3´)[b]
LipHind	CGTGTAAGCTTCGATTTTATGATATAATTC
LipBam	ATTAGGATCCATTCGGGCTTTCTGAG
LipA3up	GCAATCGATGCCCGTTCACAACA
LipAfill	CGCCAGTTAACCTGGCGTTTTGG
mlipAsRBSSma	TACCCGGGAAGGAAGGTCTTAAAATGGCAGCCACAAAGAAAAAACC
mlipAREVXba	CGTCTAGATTAATTTTGTCTATTTCTCAACACATTTTTC
mlipAcRBSXba	ATTCTAGAGAAGGAGATATACCATGGCAGCCACAAAGAAAAAACC
M01F1.3_Hind_REV	CGAAGCTTATTAATTTTGTCTATTTCTCAACAC
C45G3.3sRBSSma	TACCCGGGAAGGAAGGTCTTAAAATGTGTTCTATATCACG
C45G3.3REVXba	AATCTAGACTAAAGATCCGGCGAGAAT
C45G3.3BamFOR	TGGGATCCTATGTGTTCTATATCACGC
C45G3.3EcoREV	AGGAATTCCCTAAAGATCCGGCGAG
lip2BamFOR	CAGGATCCATGTCGAGGTGTATTCGC
lip2stHinREV	ATAAGCTTGCTCACGGATTCTTTTTCAAAATATTGACG
lip3BamFOR	GCGGATCCATGTCTATGATGCTAAGTAATTGGG
lip3stHinREV	GCAAGCTTTATGTGTAACTGTCAATATTCTCC
ZC410.7bBamFOR	TTGGATCCATGCGTCTCTCCTCAC
ZC410.7bHinREV	ATAAGCTTTTAACTAGAAACAACATTCG
ZC410.7bsubSma	ATTCTAGAGAAGGAGATATACCATGATGCGTCTCTCCTCAC
ZC410.7bXbaREV	AATCTAGATTAACTAGAAACAACATTCG

[b] Restriction sites are underlined.

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Figure Legends

Figure 1. The LipB-LipA pathway, present in *E. coli*, is the simplest pathway characterized, as it only requires two enzyme activities to get all proteins lipoylated (octanoyltransferase and lipoate synthase). On the contrary, 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 catalize the transfer reaction to E2 or GcvH subunits. In the case of *B. subtilis*, this reaction is restricted to GcvH and ODH-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, Δ*lplA* Δ*lipA* (TM131) and Δ*lplA* Δ*lipB* (TM136). Pictures were taken at 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 blot using antibodies against LA. DLAT-1, DLST-1 and DBT-1 are the lipoylated E2 subunits of PDH, ODH and BKDH, respectively.

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-F) or grown in NGM and fed with HT115 (E). Photographs were taken with a 100X magnification and correspond to the fourth day of the second-generation animals. Scale bars represent 100 μm. C) Protein extracts from N2 control animals and from worms of the first and second generation treated with *M01F1.3* RNAi were analyzed by Western blot using antibodies against LA. The same blot was probed with an antibody against actin to serve as loading control. G-H) Nomarski photographs taken at the fourth day of 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 μm.

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 average ± 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 minutes and represented as percentage of survival 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 lane) or the *M01F1.3* dsRNA producing vector (black solid lane). *M01F1.3* RNAi treatment was also performed in the presence of 25 μM LA (gray solid lane). Worms were transferred to fresh plates when necessary in order to avoid generation mixes. 50 worms were used for each treatment in each assay. Data was 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 hours. Progeny was scored after two days. Each graph is the average ± S.D from four independent experiments. *** $P<0.001$; NS denotes no significance 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 a 25X magnification at the fourth day of RNAi treatment in strain XE1581. Scale bars represent 1 mm.

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Figure 5. M01F1.3 complements a *lipA* mutant in *B. subtilis*. A) Growth of *B. subtilis* WT (JH642) or Δ*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 37ºC for 2 days. B) Protein extracts from strain CM37 transformed with pAL14 and grown with or without xylose were analyzed by Western blot with antibodies against LA. BCFAP, branched chain fatty acid precursors.

Figure 6. Characterization of ZC410.7. A) N2 worms grown in M9sup and fed with *E. coli* Δ*lipA* strain (AL100). Photographs were taken with a 100X magnification and correspond to the fourth day of the second-generation animals. Scale bars represent 100 μm. B) Yeast strains were grown overnight in synthetic glucose medium lacking uracil, and upon standarize by OD_{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 wild type copy of *lip2* or *C. elegans ZC410.7* coding sequence. C) Protein extracts obtained from these cultures were incubated with antibodies against LA.

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 standarize by OD_{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 wild type 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 Δ*lipM* Δ*lplJ* (NM65) strains transformed with either the empty vector (EV) or pAL18, a plasmid which 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 37ºC for 2 days. BCFAP, branched chain fatty acid precursors.

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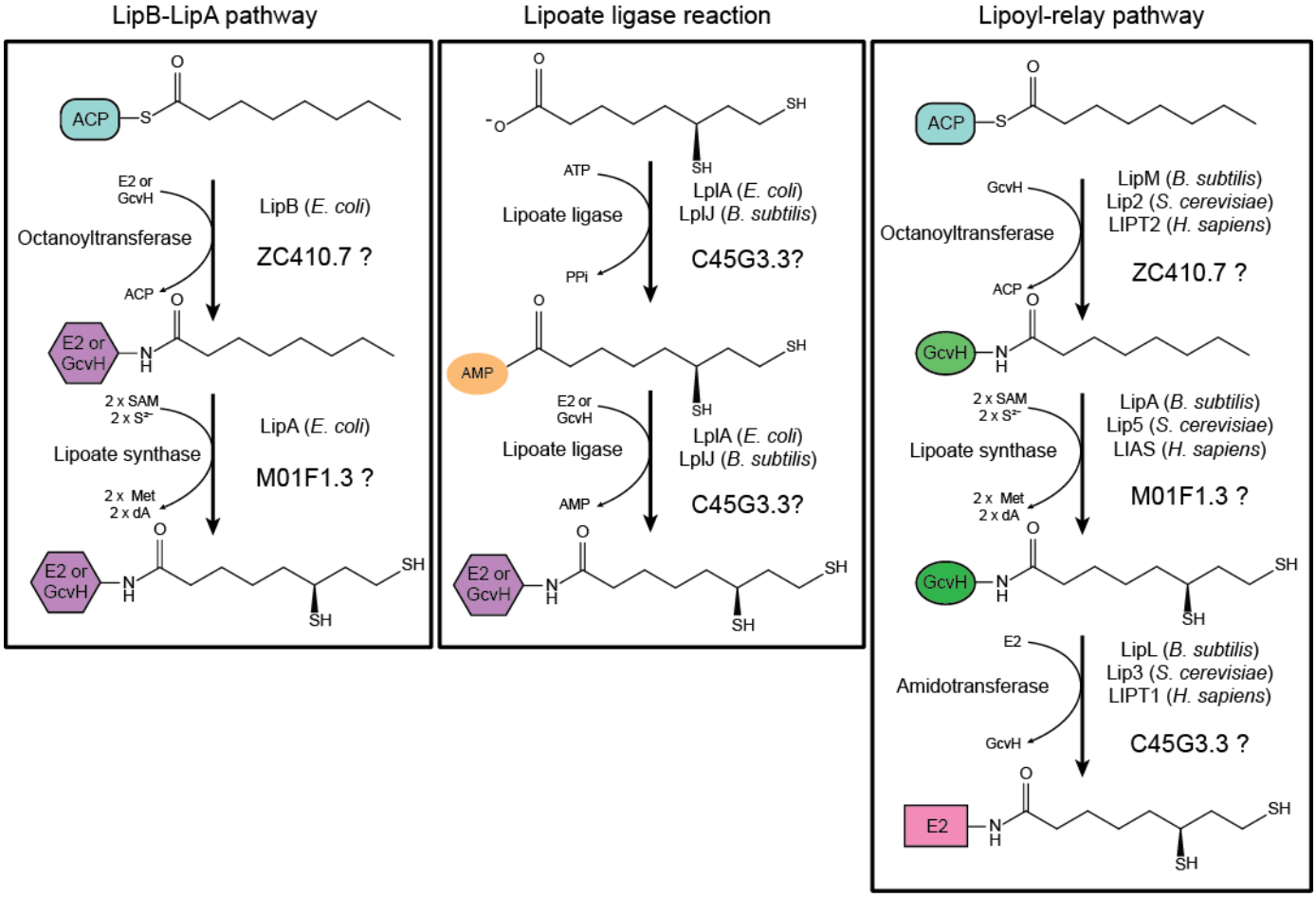

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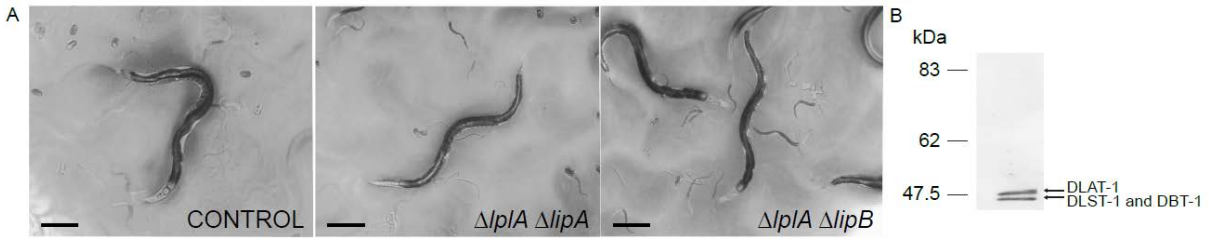

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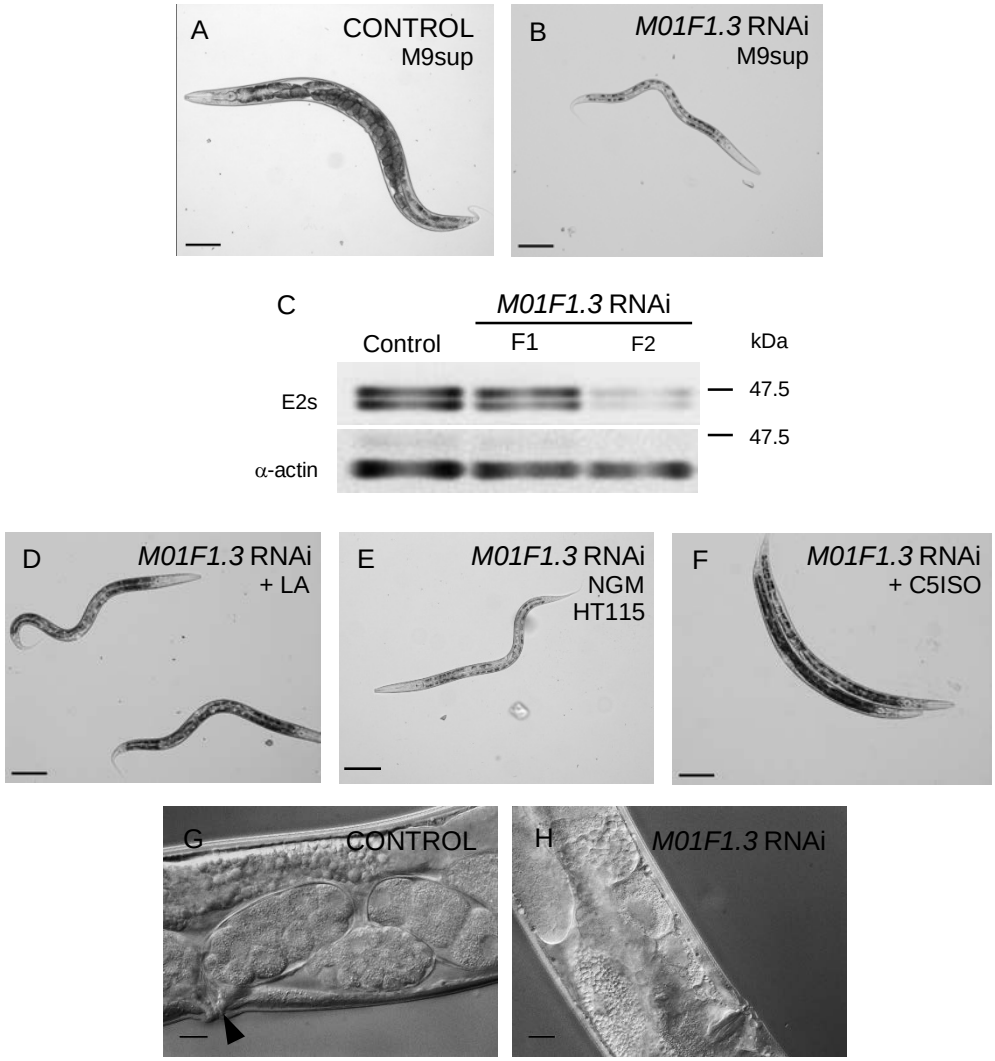

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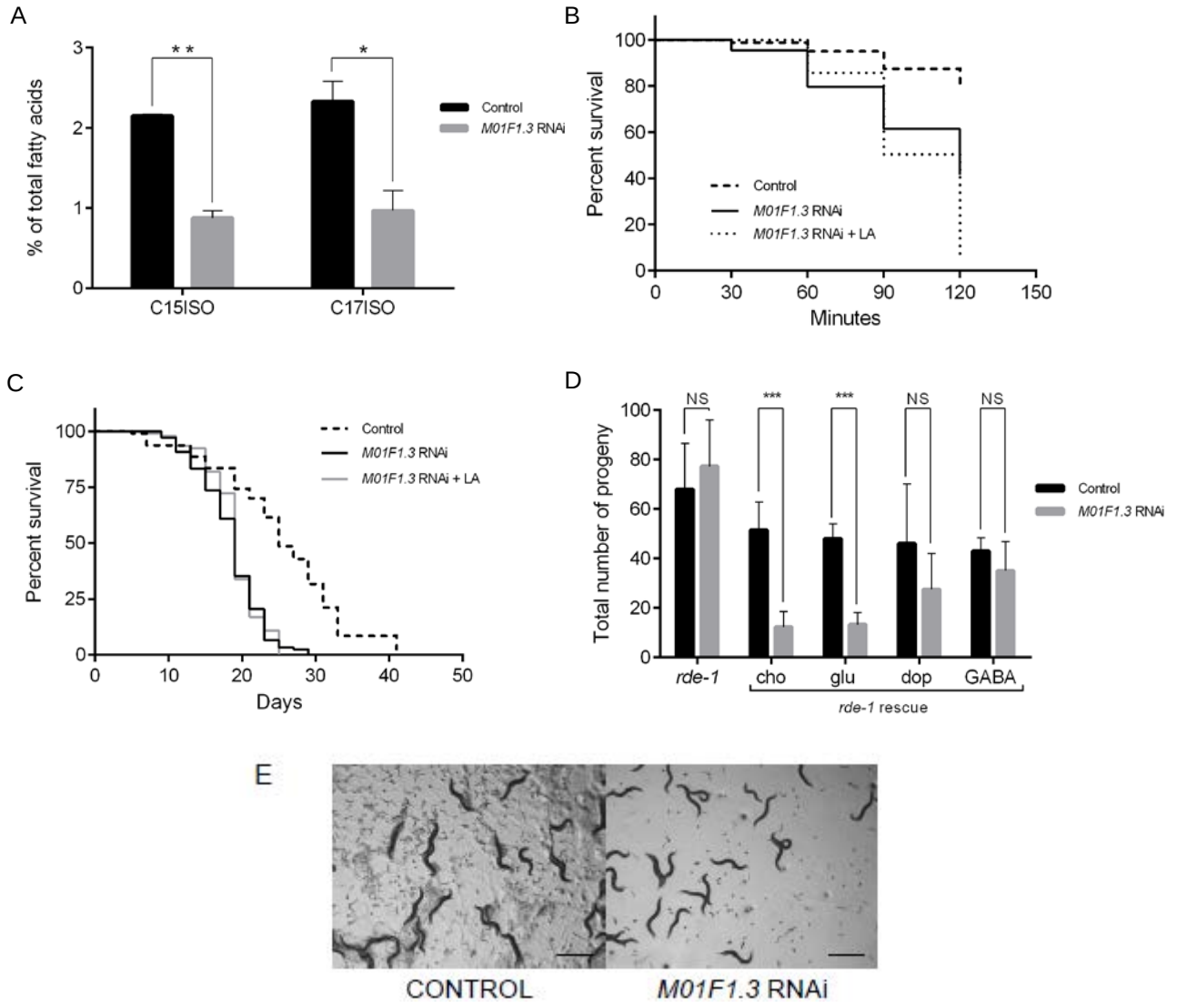

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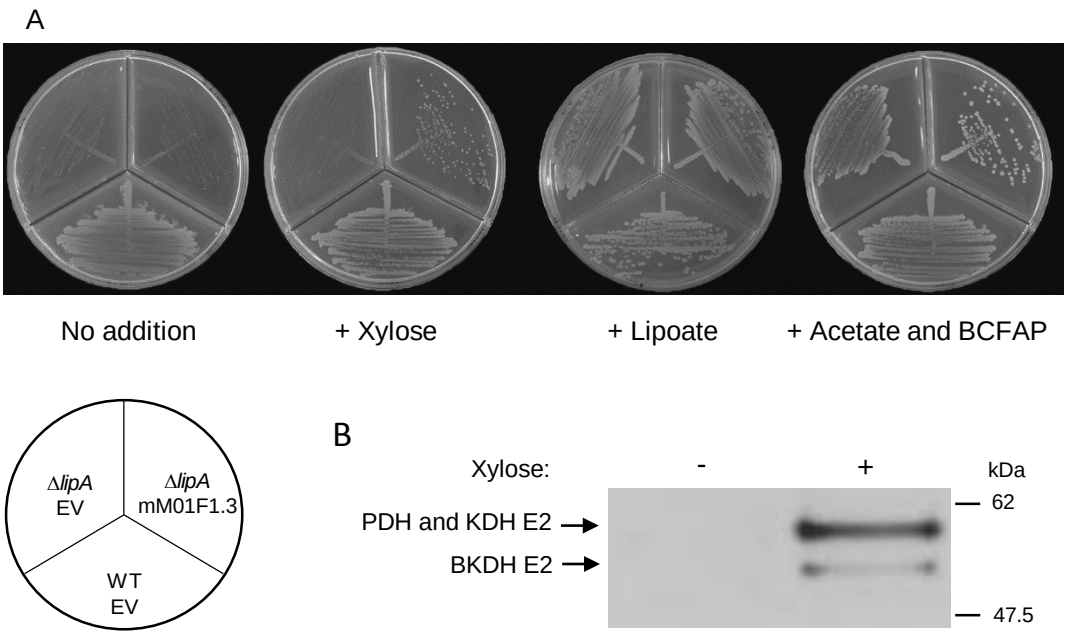

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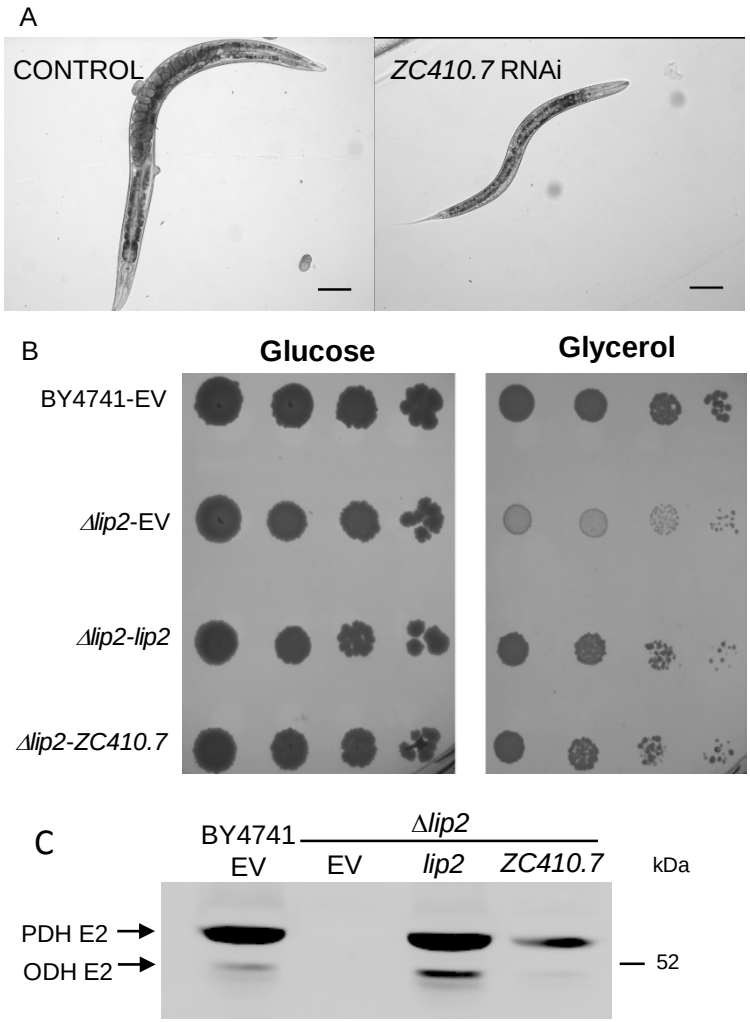

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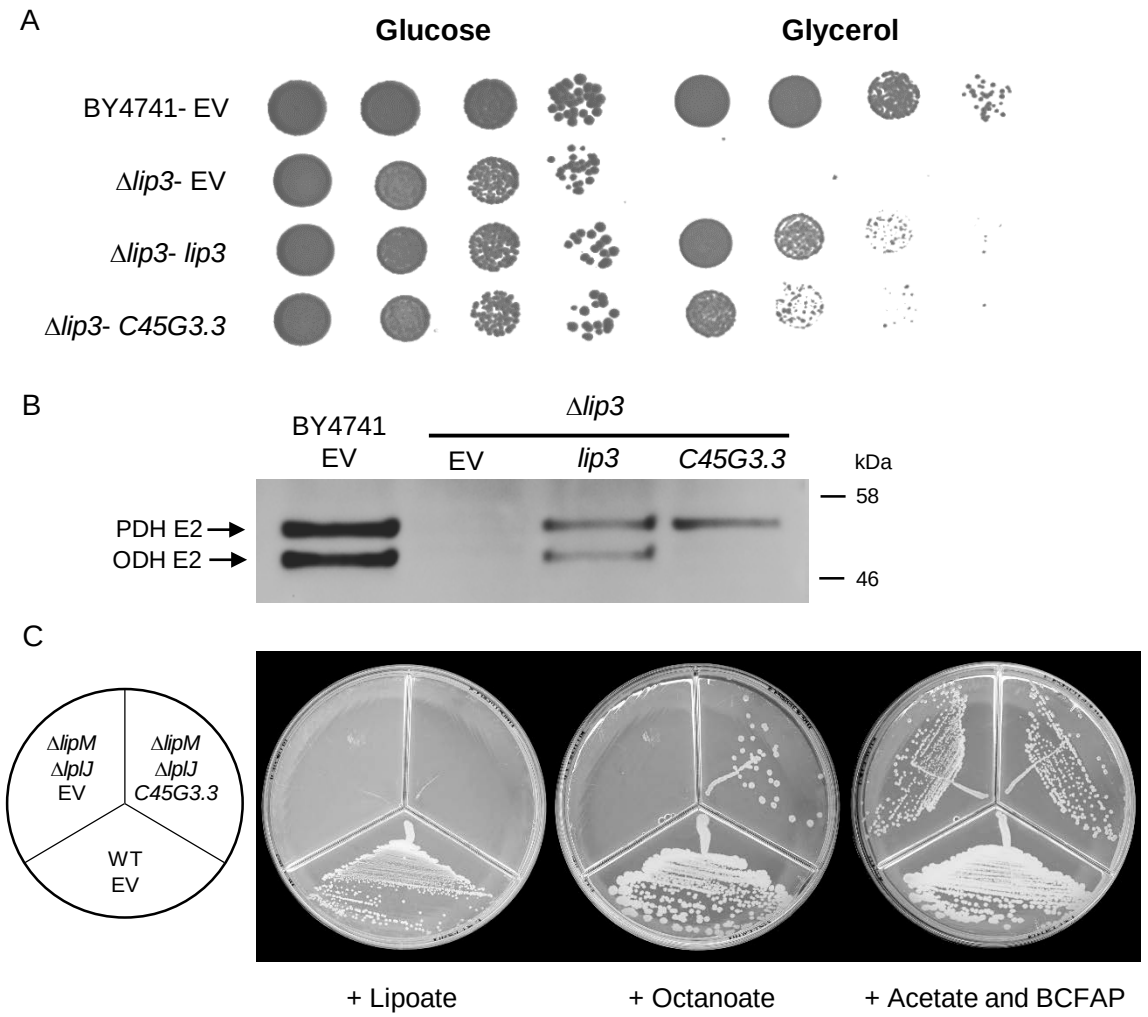

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Defining *Caenorhabditis elegans* as a model system to investigate lipoic acid metabolism

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J. Biol. Chem. *published online August 25, 2020*

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SUPPORTING INFORMATION

The lipoyl-relay pathway is essential for development in *Caenorhabditis elegans*

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Running title: Protein lipoylation in *C. elegans*

Keywords: *Caenorhabditis elegans*, development, energy metabolism, fatty acid metabolism, lipoic acid, mitochondrial metabolism, oxidative stress, post-translational modification, RNA interference

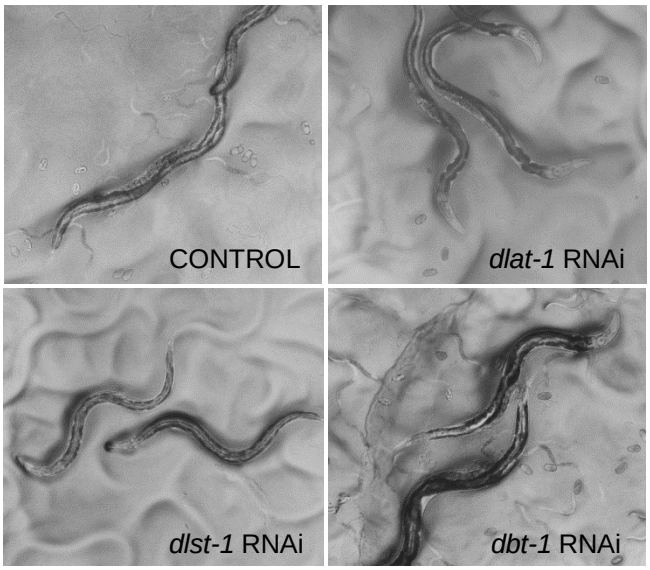
Table S1. *M01F1.3* RNAi treatment significantly alters membrane fatty acid composition

	Percentage of fatty acid type [a]	
Fatty acid	Empty vector	*M01F1.3 RNAi*
14:0	1.03 ± 0.17	1.68 ± 0.80
14:1(n-5)	0.26 ± 0.03	0.41 ± 0.04
15:0 iso	2.15 ± 0.02	0.88 ± 0.09
15:0 anteiso	0.03 ± 0.01	0.10 ± 0.06
15:0	0.08 ± 0.01	0.11 ± 0.08
15:1 (n-5)	0.64 ± 0.21	0.37 ± 0.23
16:0 iso	0.03 ± 0.03	0.42 ± 0.39
16:0	6.58 ± 0.51	10.45 ± 1.36
16:1	2.81 ± 0.14	6.24 ± 1.85
17:0 iso	2.33 ± 0.25	0.97 ± 0.25
16:2 (n-4)	0.25 ± 0.04	0.22 ± 0.10
17:0	0.21 ± 0.01	0.15 ± 0.09
16:3	0.04 ± 0.01	0.02 ± 0.02
17 cyclo	16.55 ± 1.15	10.18 ± 0.11
18:0 DMA	1.63 ± 0.16	1.62 ± 0.01
18:0	7.61 ± 0.26	8.39 ± 0.31
18:1 (n-9)	5.77 ± 0.30	5.86 ± 0.75
18:1 (n-7)	10.64 ± 1.04	18.04 ± 2.86
19:0 iso	0.10 ± 0.06	0.01 ± 0.01
18:2 (n-6)	9.08 ± 0.39	11.66 ± 0.25
18:3 (n-6)	2.21 ± 0.27	0.75 ± 0.49
19:0	0.04 ± 0.00	0.05 ± 0.05
18:3 (n-3)	0.41 ± 0.01	0.39 ± 0.09
19 cyclo	9.11 ± 1.20	2.49 ± 0.34
20:0	0.92 ± 0.09	0.54 ± 0.02
20:1 (n-9)	0.36 ± 0.06	0.42 ± 0.24
20:2 (n-6)	1.50 ± 0.04	1.00 ± 0.69
20:3 (n-6)	2.90 ± 0.33	2.60 ± 1.04
21:0	0.06 ± 0.00	0.04 ± 0.04
20:4 (n-6)	0.80 ± 0.12	1.15 ± 0.51
20:3 (n-3)	0.44 ± 0.08	0.08 ± 0.05
20:4 (n-3)	3.55 ± 0.69	2.64 ± 1.32
20:5 (n-3)	9.41 ± 0.17	9.53 ± 0.68
22:0	0.49 ± 0.04	0.61 ± 0.08
UFAs	51.07 ± 3.95	61.38 ± 11.21
UFAs/SFAs[b]	1.04	1.59

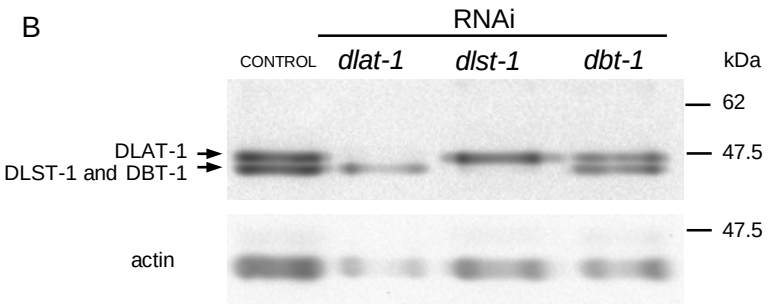
[a] Relative percentage values of fatty acids measured by GC/MS analysis of N2 worms grown on control conditions (empty vector) or *M01F1.3* RNAi. Values are the means of the results of three independent experiments (mean ± standard deviation). DMA, dimethylacetal; 17 cyclo, cis-9-10 methylene-hexadecanoic acid; 19 cyclo, cis-11-12 methylene-octadecanoic acid.

[b] Ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA)

A



B



C

	control	*dlat-1* RNAi	*dlst-1* RNAi	*dbt-1* RNAi
DLAT-1	128	ND	232	136
DLST-1 and DBT-1	175	261	ND	174
actin	100	100	100	100

Fig. S1. Effect of blocking E2 subunits expression. A) N2 worms grown in M9sup and fed with *E. coli* Δ*lplA* Δ*lipA* strain (AL100) transformed with either the empty vector (control) or the vectors which allow to knock down the expression of the different E2 subunits. B) Protein extracts from control animals and from worms treated with *dlat-1*, *dlst-1* or *dbt-1* RNAi were incubated with antibodies against LA (upper panel). DLAT-1, DLST-1 and DBT-1 are the lipoylable E2 subunits of PDH, ODH and BKDH, respectively. The same blot was probed with an antibody against actin to serve as loading control (lower panel). C) Intensity of lipoylated bands from (B) expressed as percentages relative to the actin signal (considered as 100%). Intensities were quantified using Gel-Pro analyzer 4.0. ND: not detected

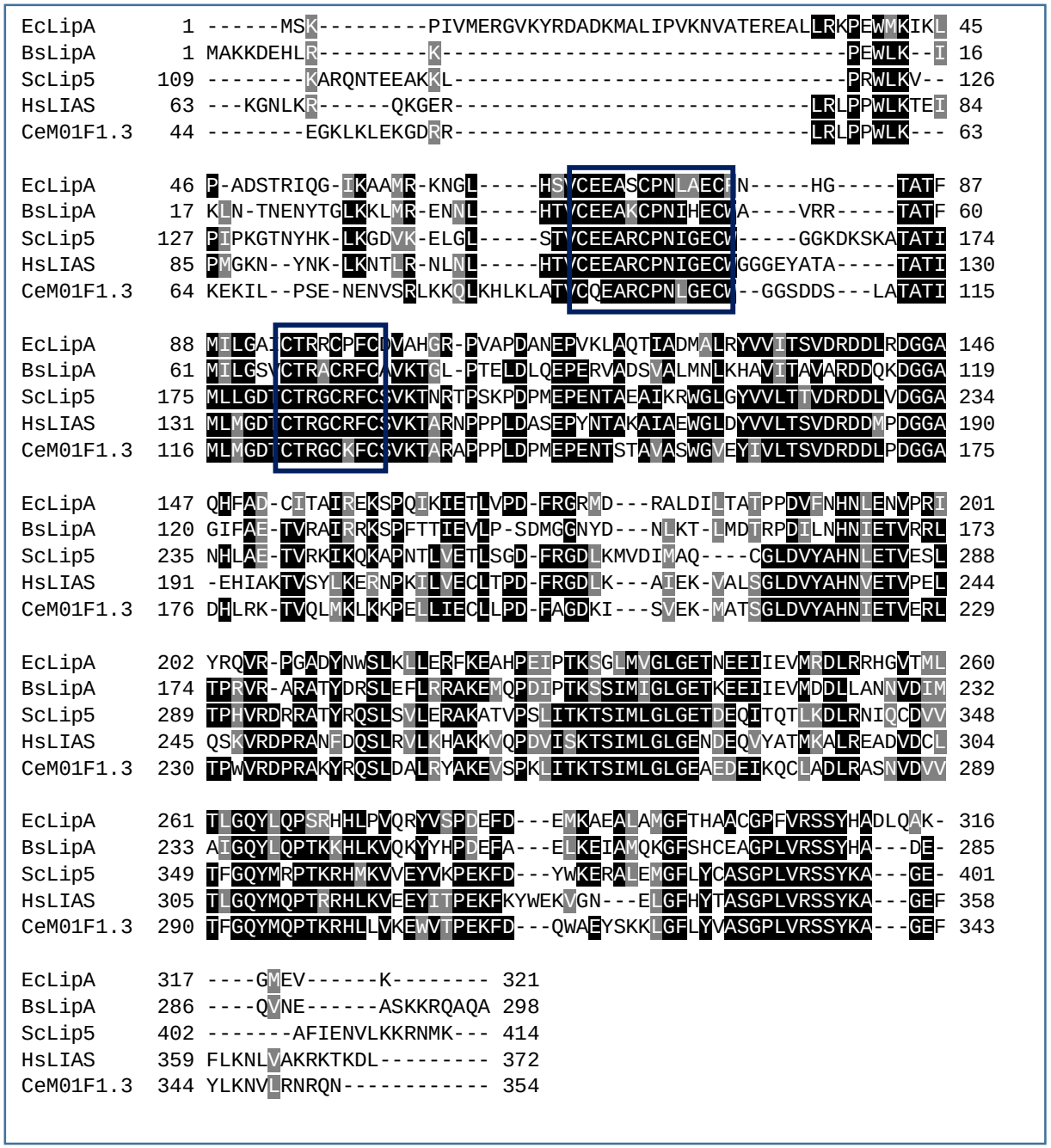


Figure S2. Alignment of *C. elegans* M01F1.3 against characterized lipoate synthases. Black shading denotes identical residues whereas gray shading denotes residues of similar properties. Cysteine motifs are boxed. The alignment was made using T-Coffee software and was formatted with BoxShade. *Ec, E. coli*; *Bs, B. subtilis*; *Sc, S. cerevisiae*; *Hs, H. sapiens*; *Ce, C. elegans*.

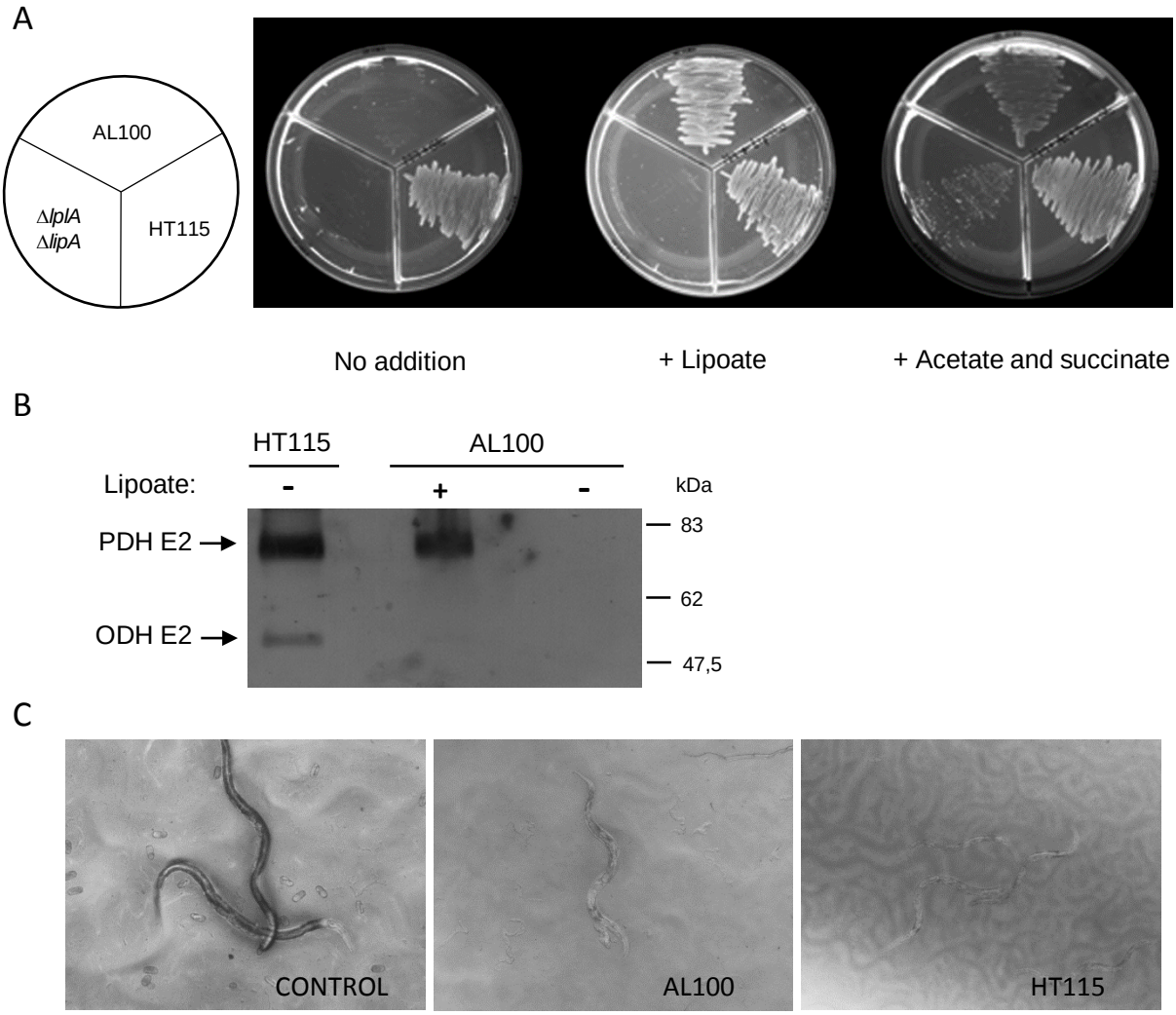


Fig. S3. Testing AL100 strain. A) Growth of the HT115-derived lipoate auxotroph strain AL100 (Δ*lipA*) in M9 minimal medium with the indicated supplements. HT115, which is prototroph for lipoate, was used a positive growth control while Δ*lplA* Δ*lipA* (TM131) strain, as a negative growth control. B) Protein extracts obtained from *E. coli* strains HT115 and AL100 grown in M9 minimal medium supplemented with acetate and succinate, in the presence or absence of exogenous lipoate, were incubated with anti-lipoate antibodies. E2 PDH, pyruvate dehydrogenase E2 subunit (82 kDa); E2 ODH, 2-oxogluatarate E2 subunit (51 kDa). C) Comparison of the *let-767* RNAi phenotypes obtained by feeding worms with either HT115 or AL100. AL100 transformed with the empty vector was used as a control.

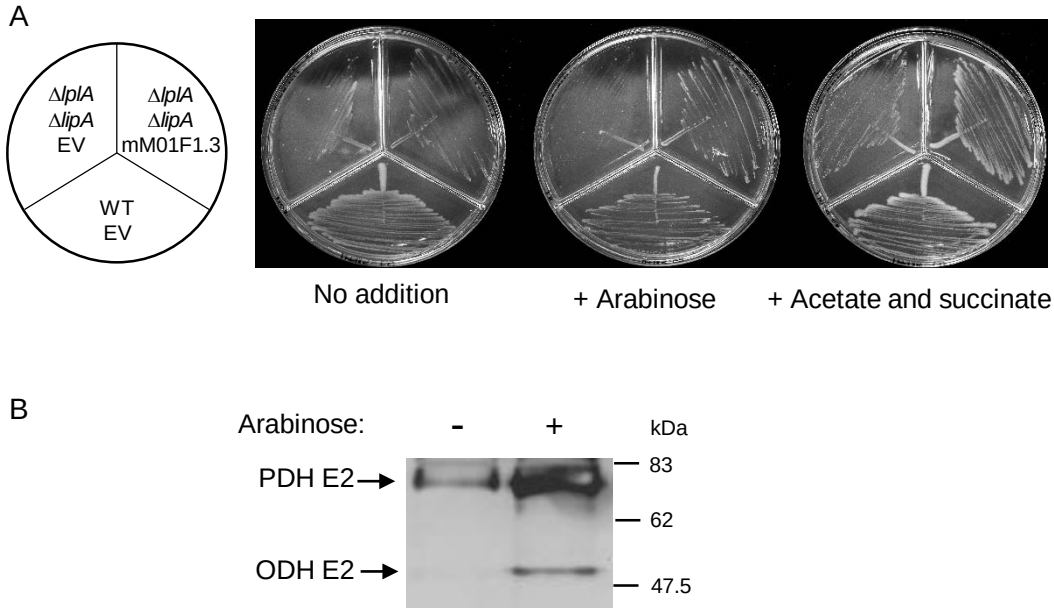


Fig. S4. A) Growth of *E. coli* Δ*lplA* Δ*lipA* (TM131) transformed with plasmid pAL15, that contains the cDNA coding for the mature form of M01F1.3 protein downstream an arabinose inducible promoter. Strains TM131 and HT115 were transformed with the empty vector (EV) as controls. Strains were streaked onto LB plates with the indicated supplements. The plates were incubated at 37ºC for 3 days. B) Protein extracts from strain TM131 transformed with the plasmid expressing M01F1.3 (pAL15), grown in the presence or the absence of the inductor were analyzed by Western blot with antibodies against LA.

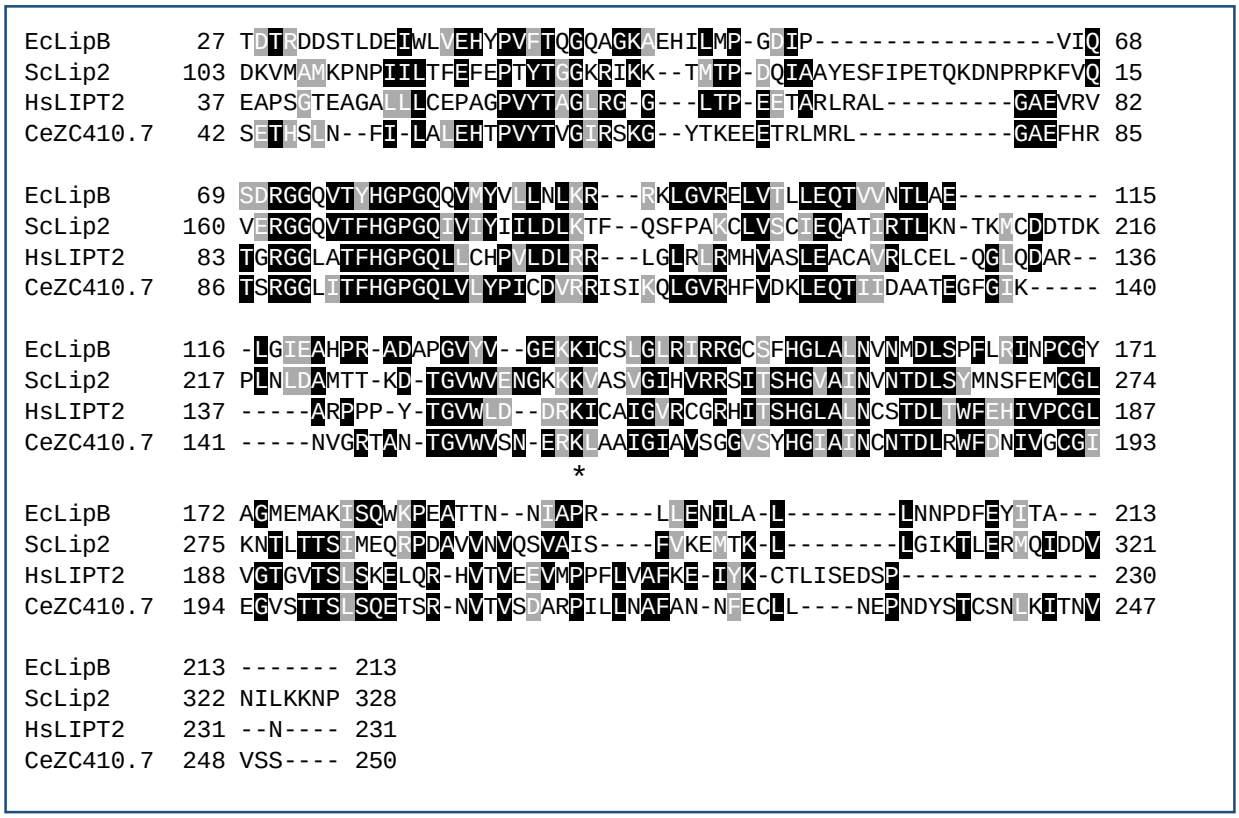


Figure S5 Alignment of *C. elegans* ZC410.7 against octanoyltransferases. Black shading denotes identical residues whereas gray shading denotes residues of similar properties. The conserved lysine residue is marked with an asterisk. The alignment was made using T-Coffee software and was formatted with BoxShade. *Ec, E. coli*; *Sc, S. cerevisiae*; *Hs, H. sapiens*; *Ce, C. elegans*.

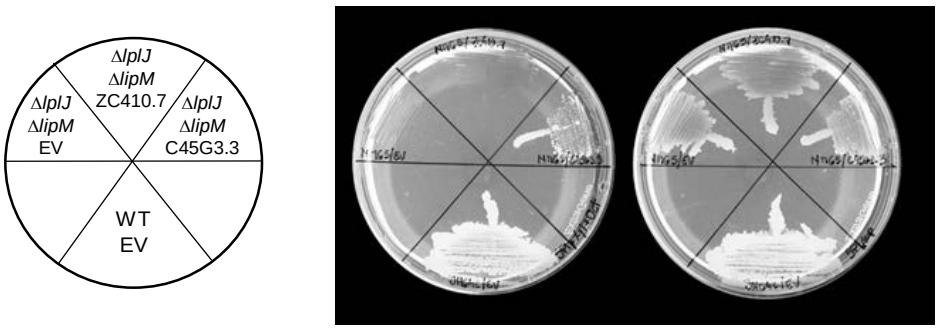


Figure S6. ZC410.7 lacks octanoyl-ligase or octanoyl-CoA transferase activity. Growth of *B. subtilis* WT (JH642) or Δ*lipM* Δ*lplJ* (NM65) strains transformed with either the empty vector (EV), pAL18 or pAL24, which allow expression of *C. elegans* C45G3.3 or ZC410.7 proteins under the control of a xylose inducible promoter, respectively. Strains were streaked onto minimal medium plates containing xylose and casamino acids vitamin-free, with the indicated supplements. Plates were incubated at 37ºC for 2 days. BCFAP, branched chain fatty acid precursors.

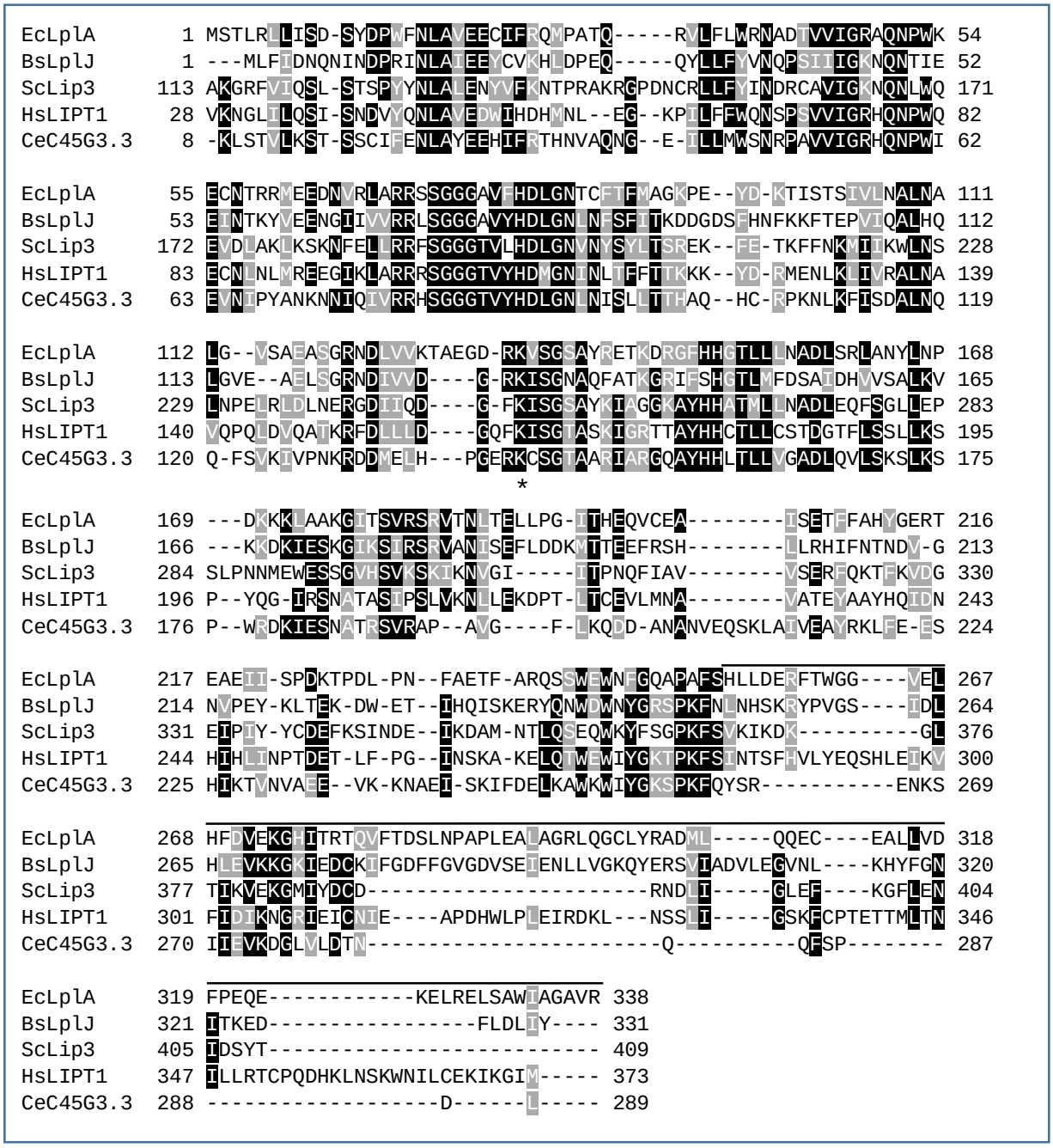


Figure S7. **Alignment of *C. elegans* C45G3.3 against lipoate ligases and amidotransferases.** Black shading denotes identical residues whereas gray shading denotes residues of similar properties. The conserved lysine residue is marked with an asterisk. The overline denotes the accessory domain which allows to differentiate between octanoyltransferases and lipoate ligases. The alignment was made using T-Coffee software and was formatted with BoxShade. *Ec, E. coli*; *Bs, B. subtilis; Sc, S. cerevisiae*; *Hs, H. sapiens*; *Ce, C. elegans*.

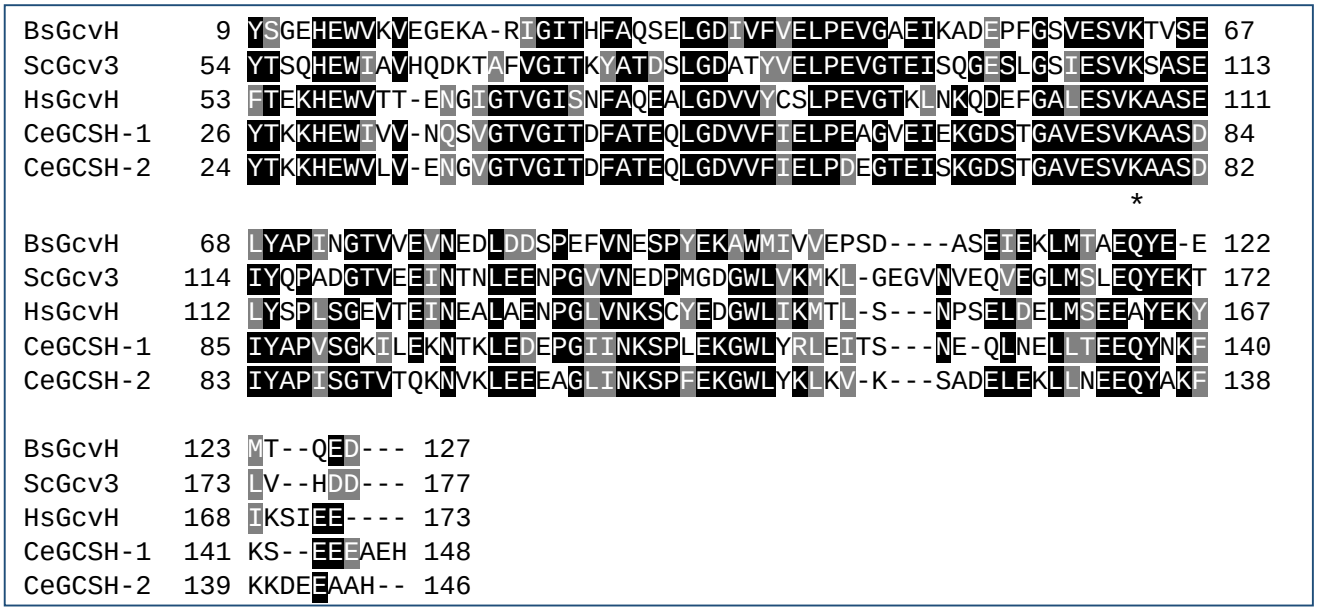


Figure S8. **Alignment of *C. elegans* GCSH-1 and GCSH-2 against H subunits of GCS.** Black shading denotes identical residues whereas gray shading denotes residues of similar properties. The conserved lysine residue is marked with an asterisk. The alignment was made using T-Coffee software and was formatted with BoxShade. *Bs, B. subtilis*; *Sc, S. cerevisiae*; *Hs, H. sapiens*; *Ce, C. elegans*.