MOLECULAR BIOLOGY

N6-adenosine methylation of ribosomal RNA affects lipid oxidation and stress resistance

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During stress, global translation is reduced, but specific transcripts are actively translated. How stress-responsive mRNAs are selectively translated is unknown. We show that METL-5 methylates adenosine 1717 on 18S ribosomal RNA in C. elegans, enhancing selective ribosomal binding and translation of specific mRNAs. One of these mRNAs, CYP-29A3, oxidizes the omega-3 polyunsaturated fatty acid eicosapentaenoic acid to eicosanoids, key stress signaling molecules. While metl-5−/− deficient animals grow normally under homeostatic conditions, they are resistant to a variety of stresses. metl-5 mutant worms also show reduced bioactive lipid eicosanoids and dietary supplementation of eicosanoid products of CYP-29A3 restores stress sensitivity of metl-5 mutant worms. Thus, methylation of a specific residue of 18S rRNA by METL-5 selectively enhances translation of cyp-29A3 to increase production of eicosanoids, and blocking this pathway increases stress resistance. This study suggests that ribosome methylation can facilitate selective translation, providing another layer of regulation of the stress response.

INTRODUCTION

Ribosomal modifications help specify which transcripts are translated under different environmental conditions (1, 2), providing an additional layer of control to gene regulation. Ribosomal RNAs (rRNAs; 28S, 18S, 5.8S, and 5S in eukaryotes) are encoded by many copies of ribosomal DNA throughout the genome, which display tissue-specific expression patterns (3), raising the possibility that different combinations of rRNAs and ribosomal proteins could form ribosomes that are specialized for translation of subsets of mRNAs (4). rRNAs are heavily modified during production and maturation. Some of these modifications (2′-O-methylation and pseudouridinylation) occur in substoichiometric frequency (5), suggesting that specialized ribosomes might regulate differential translation of distinct transcripts. Ribosomes are bound to different transcripts in response to different stresses (6–8), and rRNA methylation has been shown to regulate ribosome assembly on a cohort of specific transcripts (9, 10). However, which specific enzymes regulate rRNA methylation events, what the effects of each of these methylation events are, which specific transcripts are translated, and what the functional consequences of most of the rRNA modifications are remain unclear.

Fatty acids and their oxidized signaling molecules, termed eicosanoids, regulate many physiological processes, including inflammation (11), immune responses (11), embryo implantation (12), the perception of pain (13), cell growth (14), blood flow (14), and tumorigenesis (14). Whether rRNA methylation can control these physiological processes by regulation of fatty acid production and oxidation is unknown. Here, we identify METL-5 as a novel, highly specific 18S rRNA N6-methyladenosine (m6A) methyltransferase of adenosine 1717. We find that METL-5 inhibits the stress response in Caenorhabditis elegans by selectively increasing the translation of CYP-29A3, a cytochrome P450 enzyme that oxidizes ω-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) to eicosanoids that increase the lethality of stress in wild-type (WT) worms. Together, this work mechanistically demonstrates how the complexity of rRNA modifications can regulate specific stress responses.

RESULTS

METL-5 methylates 18S rRNA on the N6 position of adenosine 1717 in vivo

To identify putative rRNA methyltransferases in C. elegans, we performed a directed RNA interference (RNAi) screen. We knocked down 13 genes with homology to the methyltransferase like (METTL) family, a family of proteins that methylate DNA and RNA in a variety of species (15). We then assessed general RNA methylation by mass spectrometry (MS). This approach will favor RNA methylation given that >80% of the total RNA in a cell (16) is rRNA. We extracted RNA from worms fed with bacteria expressing an empty vector (E.V.) or double-stranded RNA against each of the 13 METTL genes. We performed ultrahigh-performance liquid chromatography (LC) coupled with triple quadrupole tandem MS (UHPLC-MS/MS) to look for changes in methylation of adenosine at the N6 position (m6A) or cytosine at the N3 or C5 positions [N3-methylcytidine (m3C) or C5-methylcytidine (m5C), respectively] (Fig. 1A). Knockdown of C38D4.9/metl-5, a gene that has closest homology to human METTL5, caused a ~40% decrease in m6A levels in total RNA (Fig. 1B). To test whether the change in RNA m6A methylation was due to metl-5 knockdown rather than an off-target effect of the small interfering RNA, we examined RNA methylation in the two available metl-5 mutant strains: strain tm4561, which contains a large deletion of exon 2 and also leads to a frameshift, and strain gk747459, which contains a cytosine to thymine point mutation, which converts a glutamine to a stop codon immediately after the predicted catalytic domain of METL-5 (Fig. 1C). Both of these mutant strains displayed a ~50% reduction in m6A levels in
replicates performed in duplicate. **
P were C. elegans causes a decrease in m6A levels on total RNA without any significant effects on m5C or m3C levels, as members in this study. aa, amino acid; Nt, N terminus; Ct, C terminus. (A) Schematic of metl-5 genomic DNA (gDNA), cDNA, and protein indicating the location of the catalytic domain and the mutations used in this study. gk747459 Q to ochre stop 139 tm4561 deletion 61–98 NPPF catalytic residues 125–128. to separate 28S, 18S, and 5.8S/5S rRNA. We performed UHPLC-MS/MS on each population of RNA and found no discernable change in mRNA m6A methylation (97% of WT; fig. S1A) or in m6A levels on 28S or 5.8/5S rRNA in metl-5 mutant strains (Fig. 1E). However, N6-adenosine methylation of 18S rRNA in both mutant strains was reduced by an order of magnitude compared to WT worms (Fig. 1E). To rule out the possibility that the change in 18S rRNA methylation could be due to contamination with bacterial 16S rRNA, we measured the degree of N6-adenosine methylation of bacterial 16S RNA (fig. S1B). Although total Escherichia coli RNA contained 0.35% m6A, only 0.06% of bacterial 16S rRNA was methylated on adenosine, about a third of the total RNA with no discernable changes in m5C and m3C (Fig. 1D). These results suggest that both of these mutant strains are putative null metl-5 strains and that METL-5 can methylate m6A on RNA. Since RNA makes up >80% of the total RNA in a cell (16), these results suggest that METL-5 methylates adenosine on rRNA.

To determine which RNAs METL-5 modifies, we electrophoresed on agarose gels total RNA from WT and metl-5 mutant worms to separate 28S, 18S, and 5.8S/5S rRNAs. mRNA was isolated by two successive rounds of polyadenylation selection, followed by rRNA depletion. We performed UHPLC-MS/MS on each population of RNA and found no discernable change in mRNA m6A methylation (97% of WT; fig. S1A) or in m6A levels on 28S or 5.8/5S rRNA in metl-5 mutant strains (Fig. 1E). However, N6-adenosine methylation of 18S rRNA in both mutant strains was reduced by an order of magnitude compared to WT worms (Fig. 1E). To rule out the possibility that the change in 18S rRNA methylation could be due to contamination with bacterial 16S rRNA, we measured the degree of N6-adenosine methylation of bacterial 16S RNA (fig. S1B). Although total Escherichia coli RNA contained 0.35% m6A, only 0.06% of bacterial 16S rRNA was methylated on adenosine, about a third of the...
level in WT *C. elegans* 18S rRNA (Fig. 1E). Thus, any contaminating 16S rRNA in our 18S rRNA preparations would have a negligible effect on overall methylation levels. The decrease in 18S rRNA in metl-5 mutant strains was specific to m^6^A since m^5^C levels on ribosomal subunits did not significantly change in the two mutant strains (Fig. 1E). A recent paper demonstrated that human METTL5 required a methyltransferase activator protein, TRMT112, to maintain stability (17). However, we found that knockdown of the TRMT112 ortholog, C04H5.1, had no effect on 18S rRNA m^6^A levels (fig. S1C). Together, these data suggest that METL-5 controls 18S methylation on the N6 position of adenosines.

18S rRNA has one known N6-adenosine methylation site in both *Xenopus laevis* (adenosine 1789) and *Homo sapiens* (adenosine 1832) (18). These sites correspond to adenosine 1717 in *C. elegans* 18S rRNA. This residue is predicted to interact with mRNA on the basis of its location in the crystal structure of *Thermus thermophilus* (19). However, this residue was reported to be unmodified in unicellular organisms (20). To test whether adenosine 1717 is methylated by METL-5 in *C. elegans*, 18S rRNA purified from WT and metl-5 mutant worms was analyzed for methylation by directed RNA cleavage, followed by 32P labeling and thin-layer chromatography (21). Adenosine 1717 on 18S rRNA was methylated ~98% of the time in WT *C. elegans* but was unmethylated in both metl-5 mutant strains (Fig. 1F). Together, these data suggest that metl-5 is responsible for the majority of 18S rRNA methylation at site 1717.

**METL-5 directly methylates 18S rRNA on the N6 position of adenosine 1717**

To determine whether METL-5 directly methylates 18S rRNA, we expressed a glutathione S-transferase (GST)–tagged metl-5 in bacteria, purified METL-5 to a single band (Fig. 2A), and analyzed its ability to methylate 18S rRNA from metl-5 mutant worms. Recombinant METL-5 methylated 18S rRNA from metl-5 mutant, but not WT, worms, confirming that METL-5 is an 18S rRNA methylase that is active without any cofactors and that WT 18S rRNA is constitutively and uniformly methylated (Fig. 2B). To verify further that METL-5 is an 18S rRNA methylase, the putative catalytic site AspProProPhe (NPPF) sequence of METL-5 (22) was mutated to AlaProProAla (APPA). Mutation of the catalytic site to APPA ablated the N6-adenosine methyltransferase activity on 18S rRNA from metl-5 mutant worms (Fig. 2B). To determine whether METL-5 could methylate adenosine 1717 within the 18S sequence, we performed in vitro methylation assays with recombinant METL-5 using 26- and 46-nucleotide synthetic oligonucleotides consisting of adenosine 1717 and flanking nucleosides from the 18S rRNA sequence (Fig. 2C). We found that WT, but not catalytically dead, METL-5 methylated both oligonucleotides. This methylation was specific to adenosine 1717, as there was no methylation detected in in vitro methylation assays using the same oligos where adenosine 1717 had been replaced by a guanosine (Fig. 2C).

**metl-5 mutant worms are more resistant to a variety of stresses via a translation-dependent process**

To identify the biological role of metl-5, we compared the life span of WT and metl-5 mutant strains of *C. elegans*. Under normal growth conditions, metl-5 mutant strains did not exhibit any gross morphological abnormalities and showed no significant difference in survival or fecundity compared to WT worms (Fig. 3A and table S1). metl-5 mutant strains were also similarly susceptible to the pathogenic bacterium *Pseudomonas aeruginosa* (PA14) [metl-5(gk747459), *P* = 0.70; metl-5(tm5461), *P* = 0.65; fig. S2A and table S1] (23, 24). However, metl-5 mutants were more resistant to the oxidant paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) [metl-5(gk747459), *P* = 0.01; metl-5(tm5461), *P* < 0.01; fig. S2B and table S1] (25), 37°C heat shock [*P* < 0.05 by one-way analysis of variance (ANOVA); metl-5(gk747459), *P* = 0.0001; Fig. 3, B and C, and table S1], cold stress [metl-5(gk747459), *P* = 0.0013; metl-5(tm5461), *P* = 0.009; Fig. 3D and table S1] (26), ultraviolet (UV) irradiation [metl-5(gk747459), *P* < 0.0001; metl-5(tm5461), *P* < 0.0001; fig. 3E and table S1] (27), and a combination of hypoxia and mild heat stress (*P* < 0.0001 by two-way ANOVA; fig. S2C) (28). To determine whether the increased survival in response to hypoxia and mild heat stress was due to resistance to hypoxia or heat stress or both, metl-5 mutant worm survival to either stress was assessed independently (fig. S2, D to F). Mutant worms had no change in survival compared to WT worms after 6 days of growth at 0.1% oxygen (fig. S2D) or after 3 weeks of growth at mildly elevated temperatures (25°C and 28°C) (fig. S2, E and F, and table S1), suggesting that they were only protected from the combined stress. In addition, we also found that metl-5 mutant worms displayed increased survival after 37°C heat shock and UV irradiation not only when stressed at the L4 larval stage but also if the stress was applied immediately after reproduction stopped (day 7) or in middle age (day 10) (fig. S2, G and H). Unsurprisingly, as C04H5.1 knockdown had no effect on 18S rRNA m^6^A methylation levels (fig. S1C), knockdown similarly had no effect on 37°C heat shock resistance (fig. S2I). Thus, metl-5 mutant worms were resistant to a variety of stresses (oxidant, cold, heat shock, and UV irradiation) but were not more resistant to *P. aeruginosa* infection or milder heat stresses. To determine whether this increased stress resistance was due to METL-5 activity, we generated transgenic rescue strains of WT or APPA mutant metl-5 driven by the ubiquitous *elt-3* promoter in a metl-5(tm4561) mutant background (P*elt-3:w:metl-5* WT and P*elt-3:w:metl-5* APPA) (fig. S2J). Three independent P*elt-3:w:metl-5* WT lines but not three independent P*elt-3:w:metl-5* APPA lines rescued the 37°C heat shock and 18S rRNA m^6^A levels compared to three independent control rescue lines (Fig. 3, F and G). These results indicate that METL-5 catalytic activity is required for both the 18S rRNA methylation and the stress resistance phenotype.

To investigate whether the increased stress resistance of metl-5 mutant worms is due to changes in N6-adenosine methylation, we subjected WT worms to heat stress, paraquat, hypoxia, and starvation and measured the effects of stress on m^6^A levels. None of these stresses had any discernable effect on m^6^A levels in total RNA (fig. S3, A to D) or 18S rRNA (fig. S3, E to H). Similarly, heat stress had no discernable effect on m^6^A levels or location, as assessed by immunofluorescence of whole worms (fig. S3, I and J). Thus, the increased stress resistance of metl-5 mutant worms was unlikely to be due to stress-induced differences in 18S rRNA methylation.

Next, considering that this is a rRNA modification, we decided to explore the premise that increased stress resistance is dependent on translation. We examined the effect of cycloheximide (29), a translation inhibitor, on heat shock and UV irradiation resistance of metl-5 mutant worms. Cycloheximide treatment eliminated the increased survival of metl-5 mutant worms to heat shock and UV irradiation but had no effect on the survival of WT worms (Fig. 3, H and I). Thus, the beneficial effects of metl-5 mutation seem to depend on translation.

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metl-5 mutant worms display reduced translation of the P450 gene cyp-29A3

We next sought to unbiasedly understand the translation-dependent mechanism responsible for increased stress resistance in metl-5 mutant worms. Ribosome methylation has been proposed to regulate ribosomal binding to particular transcripts during specific conditions (9, 10). To examine whether 18S rRNA N6-adenosine methylation alters translation, we evaluated 35S-methionine incorporation into newly synthesized proteins in WT and metl-5 mutant strains grown for 3 hours at 20° or 37°C (30). As expected, heat shock altered the pattern of translated proteins on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4A). However, no consistent difference in the profile of newly synthesized proteins, as assessed by phosphor imaging of 35S incorporation, or in the total amount of synthesized protein, as assessed by scintillation counting, was apparent at either temperature in metl-5 mutant relative to WT worms (Fig. 4, A and B). Similarly, although heat shock transitioned all ribosomes to monosomes, the polysome profile of metl-5 mutant worms was similar to WT worms at both temperatures (Fig. 4C). Thus, 18S rRNA N6-adenosine methylation does not globally affect polysome profiles and the translation status in C. elegans. To determine whether 18S rRNA N6-adenosine methylation alters ribosome binding or association levels of specific transcripts, we sequenced ribosome-bound RNAs and total cellular polyadenylated selected RNA (31) in three independent biological replicates from WT and metl-5 mutant worms grown at 20°C or heat-shocked at 37°C for 3 hours. We observed a high degree of variability in biological replicates, presumably due to slight differences in the cohorts of worms from experiment to experiment and the heterogeneity of RNAs in different cell types. Although heat shock caused marked shifts in which transcripts were translated and bound by ribosomes, metl-5 mutation did not cause significant shifts in translation efficiency (Fig. 4D). We therefore examined actively translated transcripts in WT and metl-5 mutant worms (Fig. 4E and fig. S4). Only a small number of transcripts were significantly changed in metl-5 mutants compared to WT worms at either temperature. A single transcript, cyp-29A3 encoding for a cytochrome P450 enzyme, stood out as greatly reduced (more than 10-fold) for ribosomal binding under both conditions in metl-5 mutant worms compared to WT worms (Fig. 4F). Because of its relatively low abundance, cyp-29A3 was excluded from our RNA sequencing analysis (fig. S5). We therefore performed quantitative real-time polymerase chain reaction (qRT-PCR) to directly examine the expression levels of cyp-29A3. We found that cyp-29A3 displayed equal levels of expression at 20°C in WT and metl-5 mutant worms, and cyp-29A3’s expression was elevated to an equivalent extent when heat-shocked at 37°C (Fig. 4G). Together, these data suggested that cyp-29A3 has lower ribosome occupancy in metl-5 mutant worms. To examine this more directly, we extracted RNA from polysome profile fractions of WT and metl-5 mutant worms at 20° and 37°C and performed qRT-PCR of cyp-29A3 and the control housekeeping gene act-1. We found that act-1 distribution was similar between WT and metl-5 mutant strains and did not change substantially with heat stress (fig. S4D). Conversely, cyp-29A3 mRNA levels were higher in the polysome fractions after heat shock, indicating an increase in active translation of CYP-29A3. However, the levels of
Fig. 3. metl-5 mutant worms are resistant to several stresses. (A) Two metl-5 mutant strains display a nonsignificant trend toward fewer progeny than WT worms grown at 20° or 25°C. Each bar represents the mean ± SEM of one to six independent experiments performed with three plates of 10 worms each, ns, not significant as assessed by two-way ANOVA and Tukey’s multiple comparisons test of one-way ANOVA. (B) Two metl-5 mutant strains display increased survival after L4 worms were placed at 37°C for the time indicated, then grown at 20°C, and assessed for survival after 24 hours. This graph represents the mean ± SEM of five independent experiments. Bar: *P < 0.05, as assessed by unpaired t test, and **P < 0.05 and ***P < 0.005, as assessed by two-way ANOVA. (C) metl-5(gk747459) mutant worms display a 156% increase in average life span relative to WT worms when L4 worms were exposed to a 37°C heat shock for 5 hours and then grown at 20°C for the remainder of the assay. This graph represents one experiment of five and was performed with three plates of at least 30 worms per plate. Significance and replicate experiments are presented in table S1. ****P < 0.0001, as assessed by log-rank Mantel-Cox survival analysis. (D) Two metl-5 mutant strains display increased survival after young adults were placed at 2°C for 6 hours before being returned to 20°C for the remainder of their life. This graph represents one experiment with three plates of 30 worms per plate, which was performed in triplicate. Statistics and replicate experiments are presented in table S1. **P < 0.005, as assessed by log-rank Mantel-Cox survival analysis. (E) Two metl-5 mutant strains display increased survival after young adults were exposed to 0.8 Joules and then grown at 20°C for the remainder of their life. This graph represents one experiment with three plates of 30 worms per plate, which was performed in triplicate. Statistics and replicate experiments are presented in table S1. ****P < 0.0001, as assessed by log-rank Mantel-Cox survival analysis. (F) metl-5 WT but not the catalytically inactive mutant APPA overexpression lines in metl-5(tm4561) mutant worms rescues 185 rRNA m6A methylation levels, as assessed by UHPLC-MS/MS. Each bar represents the mean ± SEM of two to three independent lines. ****P < 0.0001, as assessed by log-rank Mantel-Cox survival analysis. (G) metl-5 WT but not the catalytically inactive mutant APPA overexpression lines in metl-5(tm4561) mutant worms reverses 185 rRNA m6A methylation levels, as assessed by UHPLC-MS/MS. Each bar represents the mean ± SEM of two to three independent lines. ****P < 0.0001, as assessed by log-rank Mantel-Cox survival analysis. (H) Increased heat shock survival of two metl-5 mutant strains was eliminated by inhibition of translation by cycloheximide treatment. This graph represents the mean ± SEM of three independent experiments of three plates containing 20 to 35 worms each. Bar: *P < 0.05, **P < 0.005, and ***P < 0.0005, as assessed by one-way ANOVA relative to control-injected lines. (I) Increased heat shock survival of two metl-5 mutant strains was eliminated by inhibition of translation by cycloheximide treatment. This graph represents the mean ± SEM of three independent experiments of three plates containing 20 to 35 worms each. Bar: *P < 0.05 and **P < 0.005, as assessed by Tukey’s multiple comparisons test of one-way ANOVA; strain: **P < 0.005 and ***P < 0.0005, as assessed by two-way ANOVA. (J) Increased survival of two metl-5 mutant strains exposed to 0.8 Joules was eliminated by inhibition of translation by cycloheximide treatment. This graph represents the mean ± SEM of three independent experiments of three plates containing 20 to 35 worms each. Bar: *P < 0.05, as assessed by one-way ANOVA with Tukey’s multiple comparisons test. *P < 0.05 and **P < 0.005, as assessed by two-way ANOVA.
Fig. 4. *metl-5* has no global effect on translation but regulates ribosome occupancy of cyp-29A3. (A) The profile of newly translated proteins changes in *C. elegans* in response to 3 hours at 37°C, but this is unaffected by *metl-5(tm4561)* mutation, as assessed by phosphor imaging of 35S incorporation. This blot is representative of three independent experiments. (B) Absolute 35S incorporation was unaffected by 3 hours at 37°C or *metl-5(tm4561)* mutation, as assessed by scintillation counting. Control (ctl) lanes represent worms that were treated with 35S-methionine for 1 min. Each bar represents the mean ± SEM of three independent experiments performed in duplicate. (C) Polysome profiles of *metl-5(tm4561)* mutant worms were indistinguishable from WT worms grown at 20°C or when profiles shifted to monosomes after 2 hours at 37°C. This graph is a representative experiment where UV absorbance at OD254 (optical density at 254 nm) is monitored continuously. (D) Volcano plot of translation efficiency as calculated by ribosome occupancy relative to mRNA transcript of WT and *metl-5(tm4561)* mutant worms grown at 20°C (top) or heat-shocked for 3 hours at 37°C (bottom) revealed no significant change in translation efficiency in mutant worms. (E) Ribosome sequencing clusters of WT and *metl-5(tm4561)* mutant worms grown at 20°C or heat-shocked for 3 hours at 37°C revealed global changes in transcripts bound by ribosomes in response to heat shock with no global change in response to *metl-5* mutation. (F) Volcano plot of ribosome sequencing reveals that ribosomes are significantly differentially bound to the normalized transcripts of only cyp-29A3 and *metl-5* after correction for multiple hypothesis testing. Ribosome sequencing was normalized by values within the replicate. (G) cyp-29A3 mRNA is at similar levels in WT and *metl-5* mutant worms grown at 20°C and is increased to a similar extent in WT and *metl-5* mutant worms in response to heat shock at 37°C for 5 hours. Each bar represents the mean ± SEM of two independent experiments performed in duplicate. (H) cyp-29A3 mRNA is higher in polysome fractions after heat shock, but this increase is blunted in the *metl-5* mutant worms relative to WT worms, as assessed by polysome fractionation followed by qRT-PCR. cyp-29A3 expressions relative to the spiked-in internal standard of firefly luciferase were examined at each fraction of the polysome profile by qRT-PCR of WT and *metl-5* mutant worms grown at 20°C (left) and 37°C for 5 hours (right). Relative expression is normalized to fraction #1 to demonstrate the relative change in mRNA presence. Fractions #1 and #2 represent the free RNA, fractions #4 to #6 represent the monosomes, and fractions #7 to #11 represent the polysomes.

cyp-29A3 mRNA were reduced in the heavier polysome fractions in the *metl-5* mutant strain relative to WT worms (Fig. 4H). These data therefore suggest that cyp-29A3 is preferentially translated in response to heat stress, and this induction is blunted in *metl-5* mutant worms.

Eicosanoid synthesis, which cyp-29A3 regulates, is reduced in *metl-5* mutant worms

The ω-3 and ω-6 fatty acids, α-linolenic acid and linoleic acid, respectively, are essential fatty acids, which humans cannot synthesize (32). α-Linolenic acid and linoleic acid are precursors for longer and
more unsaturated fatty acids, termed PUFAs, including the \( \omega-3 \) PUFA EPA and the \( \omega-6 \) PUFA arachidonic acid (AA) that increase cell membrane flexibility and selective permeability (32). These PUFAs are oxidized to create the signaling molecules termed eicosanoids, which include prostaglandins, thromboxanes, and leukotrienes (33). Some PUFAs are metabolized to eicosanoids by cytochrome P450 enzymes (34). *C. elegans* express all genes required for the synthesis of PUFAs (32) and 75 cytochrome P450 genes (35). CYP-29A3 oxidizes the omega-3 PUFA EPA to produce three types of eicosanoids termed epoxygenosatetraenoic acids (EETs): 17,18-epoxy eicosatetraenoic acid (17,18-EpETE), 17,18-dihydroxyeicosatetraenoic acid (17,18-DiHETE), and 20-hydroxyeicosapentaenoic acid (20-HEPE) (35). Eicosanoids are hormone-like signaling lipids implicated in a variety of processes including inflammation (11), embryo implantation (12), and tumorogenesis (14). In *C. elegans*, these three EETs play a role in pharyngeal pumping and food uptake (36). To determine whether the change in cyp-29A3 accounts for the stress resistance of metl-5 mutant worms, we examined the heat shock sensitivity of a genetic mutant strain of cyp-29A3, gk827495, in which tryptophan-89 is converted to an amber stop codon. cyp-29A3 mutant worms at the L4 larval stage were more resistant to heat stress than WT worms (P < 0.01, by unpaired t test; Fig. 5A). We also found that cyp-29A3 mutant worms displayed increased survival after 37°C heat shock immediately after reproduction stopped (day 7) and in middle age (day 10) (fig. S2G). Furthermore, metl-5;cyp-29A3 double mutant worms did not survive any better than metl-5 or cyp-29A3 mutants alone (fig. S6A), suggesting that the products of these two genes act in the same pathway. To test whether CYP-29A3 expression was sufficient to rescue the metl-5 stress-resistant phenotype, we generated transgenic rescue strains of cyp-29A3 driven by the ubiquitous eft-3 promoter in a metl-5(tm4561) mutant background (P\(_{\text{eft-3}}\)::cyp-29A3). Overexpression of cyp-29A3 in three independent P\(_{\text{eft-3}}\)::cyp-29A3 lines (fig. S6B) had no effect on 18S rRNA \( \text{m}^{\text{A}} \) methylation levels, as assessed by UHPLC-MS/MS (fig. S6C); however, overexpression did rescue the 37°C heat shock resistance phenotype compared to three independent control rescue lines (Fig. 5B), indicating that CYP-29A3 is sufficient to rescue the stress resistance phenotype. Knockdown of cyp-29A3 reduces levels of the lipid eicosanoid EETs 17,18-EpETE, 17,18-DiHETE, and 20-HEPE (35). To determine whether metl-5 mutant worms, which show decreased ribosome occupancy on cyp-29A3 mRNA (Fig. 4H), also have decreased lipid content, we stained metl-5 mutant worms for total lipid content with Oil Red O (ORO) (37, 38). metl-5 and cyp-29A3 mutant worms had decreased lipid abundance to a similar extent (P < 0.0001, by one-way ANOVA; Fig. 5C). Furthermore, to examine whether metl-5 mutant worms specifically had decreased eicosanoid EETs synthesized by CYP-29A3, we compared eicosanoid levels in whole WT, cyp-29A3, and metl-5 mutant worms by gas chromatography coupled with MS (GC-MS). metl-5 and cyp-29A3 mutant worms had significantly decreased levels of the 17,18-EpETE, 17,18-DiHETE, and 20-HEPE eicosanoid EETs (Fig. 5D and fig. S7, A and B), while metl-5 mutant worms showed no change in EPA, AA, or other fatty acids (Fig. 5D and fig. S7C).

We hypothesized that the reduced cyp-29A3 translation and CYP-29A3 synthesis of eicosanoids could lead to the increased heat stress resistance in metl-5 mutant worms. To test whether changes in these eicosanoids were responsible for the increased heat stress resistance of metl-5 and cyp-29A3 mutant worms, the diets of WT, cyp-29A3(gk827495), and metl-5(gk747459) mutant strains were supplemented with the CYP-29A3 substrates, the omega-6 and omega-3 PUFAs, AA or EPA, respectively, and the CYP-29A3 products, the eicosanoid EETs 17,18-EpETE, 17,18-DihETE, and 20-HEPE. Although dietary supplementation of the CYP-29A3 PUFAs substrates had no effect on heat shock resistance of WT, cyp-29A3, or metl-5 mutant worms, addition of the CYP-29A3 products 17,18-EpETE, 17,18-DiHETE, and 20-HEPE reverted the heat shock resistance of cyp-29A3 and metl-5 mutant worms to that of WT worms after 5 hours of 37°C heat shock (Fig. 5, E and F). These results suggest that reduced eicosanoid synthesis by CYP-29A3 is responsible for the increased stress resistance of metl-5 mutant worms.

**DISCUSSION**

Our study identified METL-5 as a novel 18S rRNA N6-adenosine methyltransferase at adenosine 1717 in vivo and in vitro. We found that this enzyme is important for modulating the stress response in *C. elegans*. We show that N6-adenosine methylation by METL-5 regulates the binding of ribosomes to the transcript of CYP-29A3, a cytochrome P450 enzyme that oxygenates EPA to produce eicosanoid EETs 17,18-EpETE, 17,18-DiHETE, and 20-HEPE, decreased levels of which are critical for the heat stress resistance of metl-5 mutant worms (Fig. 5G). How eicosanoid EETs can regulate the heat stress response is still unknown. Because eicosanoids have been implicated broadly in acting as small-molecule signaling mediators for inflammation, tissue homeostasis, immunity, and cancer (11–14), it will be important in future studies to determine how these signaling lipids can regulate the stress response in *C. elegans*. In *C. elegans*, cytochrome P450 enzymes and eicosanoids have been implicated in regulating the response to hypoxia (39), pharyngeal pumping and food uptake (36), sperm guidance (40), germ cell death and sterility (41), development, dauer formation, and longevity (42–44).

The interesting finding that deletion of metl-5 and cyp-29A3 increased stress resistance raises the question as to what evolutionary pressure would cause *C. elegans* to retain this detrimental pathway. We hypothesize that antagonistic pleiotropy could explain this finding and that some beneficial consequences of suppressing this pathway will be revealed under alternative conditions, as has been seen in other examples in *C. elegans* (45).

Our work suggests that METL-5 regulates the translation of CYP-29A3 to control stress resistance. It is still unclear how, on a molecular level, 18S rRNA methylation could specifically regulate the translation of CYP-29A3. Whether the 18S rRNA methylated on adenosine 1717 or alternatively unmethylated uniquely interacts with cyp-29A3 mRNA and other transcripts is still unclear. We also have not ruled out whether METL-5 could methylate other substrates that are much less abundant than 18S RNA.

METTL5, the mammalian homolog of METL-5, was recently identified as an 18S rRNA methyltransferase in humans. Whether METTL5 methylates the same residue on the 18S and whether it plays a conserved role in regulating the stress response and translation of cytochrome P450 enzymes remain to be seen. Human METTL5 seems to require binding of the methyltransferase activator TRMT112 to be stabilized (17). However, we found that the worm ortholog of TRMT112, C04H5.1, was dispensable for METL-5 stability and activity. Whether an alternative binding protein is required for METL-5 activity in *C. elegans* remains to be seen.

rRNA modifications provide a rich new layer of regulation of translation, and it will be exciting in the future to determine the
OH20-HEPE

\[
\begin{align*}
\text{C. elegans cyp-29A3 transcripts, which, in turn, controls the oxidation of EPA to eicosanoid EETs, which regulate stress resistance in} \\
\text{mutant worms after 5 hours of 37°C heat shock. Each column represents the mean ± SEM of three independent experiments of three plates containing 20 to 35 worms each. *P < 0.05, as assessed by one-way ANOVA.} \\
&
\text{Dietary supplementation with eicosanoid EETs 17,18-EpETE, 17,18-DiHETE, and 20-HEPE reverts the increased stress resistance of cyp-29A3(gk827495) mutant worms after 5 hours of 37°C heat shock. Each column represents the mean ± SEM of three independent experiments of three plates containing 20 to 54 worms each. *P < 0.05 and **P < 0.01, as assessed by one-way ANOVA.} \\
\text{METL-5 can methylate the 18S rRNA of the 40S ribosomal subunit, which regulates binding to cyp-29A3 transcripts, which, in turn, controls the oxidation of EPA to eicosanoid EETs, which regulate stress resistance in C. elegans.}
\end{align*}
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extent to which these heavily modified RNAs can help specify appropriate translation. Two different methylations of 28S rRNA, N6-adenosine methylation of adenosine 4220 (46) and C5 cytosine methylation of cytosine 2381 (10), have been shown to affect poly-some profiles, affect translation of specific mRNAs, and regulate cell proliferation and tumorigenesis (46) or longevity (10), highlighting the regulatory capacity of rRNA methylation for determining specific cellular responses. Further studies will be required to determine

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how these rRNA methylation events can specify the ribosome to a distinct subset of target messenger RNAs. While more experiments are required to identify how 18S rRNA N6-adenosine methylation regulates ribosome occupancy on cyp-29A3 mRNA and how the reduced eicosanoids in metl-5 mutant worms control the heat stress response, the conservation of adenosine 1717 methylation on 18S rRNA and the eicosanoid synthesis pathway between worms and mammals suggest that this signaling pathway could regulate stress responses in other species, including mammals.

**MATERIALS AND METHODS**

**Strains used and RNAi**

The N2 Bristol strain was used as the WT background. Worms were grown on *dam* *dem* bacteria [New England Biolabs (NEB), C2925] on standard nematode growth medium (NGM) plates (47) in all experiments save for RNAi experiments. Bacteria expressing double-stranded RNA of METTL family members and C04H5.1 were obtained from the Ahringer and Vidal libraries (a gift from T. K. Blackwell). Bacteria were grown at 37°C and seeded on NGM plates containing ampicillin (100 mg ml⁻¹) and isopropylthiogalactoside (IPTG; 0.4 mM). Each vector was sequenced to verify the presence of the appropriate gene of interest. (IPTG; 0.4 mM). PCRs were performed using the following primers: metl-5 tm4561 (forward), 5′-GTGGAATAATTTTCTCAAATGTGCCTGAGA-3′; metl-5 tm4561 (reverse), 5′-ACAGCCACGTCGAATGTGCC-3′; metl-5 gk747459 (forward), 5′-GGCTCAAAATTTGAGGTAGTAGAAAATCAG3′-; metl-5 gk747459 (reverse), 5′-GGCTCAAAATTTGAGGTAGTAGAAAATCAG3′-; cyp-29A3 (forward), 5′-TCACTG-GAAGGCCTAGAAGAAAGTGC-3′; and cyp-29A3 (reverse), 5′-ATCGCTGAAATATTTGATGTTCC-3′. PCRs for metl-5(gk747459) were digested with HpyCH4V to distinguish WT from mutant genotype, and PCRs for cyp-29A3 were digested with Hae III to distinguish WT from mutant genotype. PCRs were performed according to the manufacturer’s protocol (Invitrogen, Platinum PCR SuperMix), and PCRs were resolved on agarose gels.

**Ribonuclease H site-specific cleavage directed by 2′-O-methyl RNA-DNA chimeras**

18S RNA was subjected to ribonuclease H (RNase H) site-specific cleavage directed by 2′-O-methyl RNA-DNA chimeras. Gel-purified 18S RNA (300 ng) was mixed with 5 pmol chimeric oligo in 30 mM tris (pH 8.3), 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin (w/v), and proteinase K (60 mg ml⁻¹) and incubated at ~80°C for 1 hour, 60°C for 1 hour, and then 95°C for 15 min.

18S rRNA (300 ng) was mixed with 5 pmol chimeric oligo in 30 mM tris-Cl (pH 7.5) in a total volume of 5 μl. The resulting mixture was heated for 3 min at 95°C, followed by cooling to room temperature for 3 min. RNase H (5 U; NEB), recombinant shrimp alkaline phosphatase (1 U; NEB), and RNasin (20 U; Promega) were added in a total volume of 10 μl in 1× T4 polynucleotide kinase (PNK) buffer (NEB), and the mixture was incubated for 1 hour at 44°C, followed by heat inactivation for 5 min at 75°C. Radioactive end-labeling was performed with the addition of T4 PNK (20 U; NEB) and 2 μl of [γ-32P]ATP (adenosine triphosphate) (6000 Ci/mmol) at 37°C for 1 hour in a total volume of 20 μl in 1× T4 PNK buffer, followed by heat inactivation for 5 min at 75°C. The free [γ-32P]ATP was removed by the use of Bio-Spin 6 column (Bio-Rad) according to the manufacturer’s instructions. The radioactive-labeled 18S fragments were subjected to tris-borate EDTA (TBE)–urea gel electrophoresis, followed by staining with SYBR Gold. The band that corresponded to the 38–base pair (bp) 5′ end-radiolabeled 3′ half 18S RNA fragment was excised, and RNA was eluted for 1 hour at 37°C in 300 μl of RNA extraction buffer [300 mM sodium acetate (pH 5.5), 1 mM EDTA, and 0.25% (v/v) SDS], followed by ethanol precipitation. The purified RNA was resuspended in diethyl pyrocarbonate–treated water and was digested with Nuclease P1 (2 U; Wako, USA) in 10 mM ammonium acetate (pH 5.2) and 2 mM ZnCl₂ for 2 hours at 60°C in a total volume of 20 μl. Digested nucleotide mixture (2.5 μl) was analyzed by thin-layer chromatography on a glass-backed polyethyleneimine–cellulose plate (Merck Millipore) in a buffer containing isopropanol/HCl/water (70:15:15). Signal acquisition and quantification of the radiolabeled adenosine and m6A were carried out using a BAS storage phosphor screen (GE Healthcare Life Sciences) at 200-μm resolution using the ImageQuant TL software (GE Healthcare Life Sciences).
Methyltransferase assays
The coding sequence of metl-5 was cloned as an in-frame fusion to the GST-tagged vector pGEX-4T1. The catalytic site was mutated through site-directed mutagenesis. The recombinant proteins were expressed in E. coli BL21. Overnight induction of protein expression was carried out with 1 mM IPTG at 18°C. Bacteria were harvested at 4000 rpm and 4°C and resuspended in 10 ml of protein purification lysis buffer [50 mM tris-HCl (pH 7.5), 0.25 M NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), and protease inhibitors]. After freezing the pellet at −80°C for 1 hour, the lysate was sonicated with a Bioruptor for 5 min on high level with 30-s on and 30-s off. Proteins were purified with Glutathione Sepharose 4B beads (GE Healthcare). Proteins and beads were washed three times with protein purification lysis buffer before incubating the beads with elution buffer [12 mg/ml; glutathione (GoldBio) in protein purification lysis buffer (pH 8.0)] for 30 min. Eluates were dialyzed overnight at 4°C with dialysis buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 20% glycerol]. Bradford assays and SDS-PAGE gel electrophoresis, followed by Coomassie staining, were performed to determine integrity and quantity of purified proteins.

In vitro methylation reactions assaying METL-5 activity against 18S rRNA purified from metl-5 mutant worms were performed in a buffer containing 50 mM tris (pH 8.0), 1 mM EDTA, 1 mM DTT, 5% glycerol, 160 μM d3-S-adenosyl methionine (SAM) using 200 nM GST–METL-5 protein, and 0.5 to 2 μg of 18S rRNA for 1 hour at 37°C. Then, reactions were incubated for 20 min at 65°C, followed by clean up and buffer exchange with Bio-Spin P-30 columns (Bio-Rad). RNA was digested to nucleosides with 20 U of S1 nuclease (Thermo Fisher Scientific) at 37°C for 2 hours, followed by treatment with Fast Alkaline Phosphatase (Thermo Fisher Scientific) for 1 hour at 37°C. Samples were diluted two times with Milli-Q water, and 5 μl was used for UHPLC-MS/MS analysis. Similar reactions were performed with synthesized 18S RNA oligos of the following sequences:

- short 18S A1717, GUAAAGUCGUAAACAGGUAGCUGUA;
- short 18S A1717–G, GUAAAGUCGUAGCAAGGUAGCUGUA;
- long 18S A1717, GGUUUGAACGUGUAAAGUGUAAAGGUAGGUAGCUAGGUAGGUAACC, and long 18S A1717–G, GGUUUGAACGUGUAAAGUGUAGCAAGGUAGCUAGGUAGGUAACC.

Longevity assays
Worm life-span assays were performed at 20°, 25°, and 28°C. Worm populations were synchronized by placing young adult worms on NGM plates seeded with dam′ dcm′ bacteria for 6 hours and then removed. The hatching day was counted as day 1 for all life-span measurements. Worms were changed every other day to new plates to eliminate confounding progeny and were marked as dead or alive. Worms were scored as dead if they did not respond to repeated prods with a platinum pick. Worms were censored if they crawled off the plate or died from vulval bursting. For each life-span assay, ~90 worms were used in three plates (30 worms per plate). The data were plotted with the Kaplan-Meier survival curve, and statistical significance was determined by log-rank (Mantel-Cox) tests. Life-span assays were repeated at least once and showed similar trends in relative life-span effects. Representative Kaplan-Meier survival curves are shown in fig. S2 (D and E). Means, SEMs, and P values are shown in table S1.

PA14
NGM plates (35 mM) with 0.35% peptone rather than 0.25% peptone were spotted with 15 μl of PA14 from overnight cultures. This was spread over the entire plate. Worms were plated at the L4 stage on three plates per condition with 30 worms per plate (90 worms per assay). They were grown at 25°C and assessed every 6 to 12 hours for survival as in (24).

Paraquat
L4 or young adults were synchronized as described in the longevity assay, and five to seven worms were placed in 96-well plates in sextuplicate (at least 40 worms) in S medium containing 200 mM methyl viologen dichloride hydrate (paraquat). Worms were probed with a platinum pick every 45 min and were scored as alive as in the longevity assay in (49).

UV stress assays
Young adults were synchronized as described in the longevity assay on three plates per condition with 30 worms per plate (90 worms per assay). They were exposed to 0.8 J, then grown at 20°C, assessed every 24 hours for survival, scored as alive as in the longevity assay in (27).

Cold shock assays
Young adults were synchronized as described in the longevity assay on three plates per condition with 30 worms per plate (90 worms per assay). They were placed at 2°C for 6 hours and then assessed every 12 to 24 hours for survival and were scored as alive as in the longevity assays in (26). Note that a large number of the worms were censored because of bagging in these experiments. Censored worms were included in the statistical tests but were excluded from the figure shown in Fig. 3D.

Hypoxia and heat stress assays
Gravid day 1 adults were synchronized as described in the longevity assay on three plates per condition with 30 worms per plate (90 worms per assay). Replicate plates were prepared for six time points (0, 24, 48, 72, 96, and 120 hours) and left to grow at 28°C and 0.1% O2 for the indicated length of time before being allowed to recover for 24 hours at 20°C and scored as alive as in the longevity assay. This is an adaptation of the protocol used in (28).

Transgenic strain creation
Expression vectors for creating transgenic strains were based on pDS1 plasmid vector (a gift from W. Mair and S. Dutta) that contains the ubiquitous eft-3 promoter and unc-54 3′ untranslated region. metl-5 and the metl-5 APPA catalytic mutant were amplified from the pGEX-4T1 constructs and cyp-29A3 from cDNA, followed by restriction-free cloning into the pDS1. Germline transformation experiments were performed as described (48). For the metl-5 rescue experiments, injection mixtures contained pDS1::metl-5 or pS-D1::metl5 APPA plasmids at 50 ng/μl, pTG96 (20 ng/μl; Psur-5′:gfp) as a cotransformation marker, and 1-kb DNA ladder (80 ng/μl; Invitrogen) as carrier DNA. For the overexpression of cyp-29A3 in metl-5 (tm4561), the injection mixture contained pDS1::cyp-29A3 at 5 ng/μl, pTG96 (20 ng/μl; Psur-5′:gfp) as a cotransformation marker, and 1-kb DNA ladder as carrier DNA (80 ng/μl). For the control lines, the injection mixture contained pTG96 (20 ng/μl; Psur-5′:gfp) as a cotransformation marker and 1-kb DNA ladder (80 ng/μl) as carrier DNA.
Heat stress assays
Synchronized L4 worms were placed at 37°C for the time indicated and then grown at 20°C for the remainder of the assay. Survival was assessed every 6 to 24 hours. For cycloheximide treatment, cycloheximide was diluted to 1 or 0.05 mg/ml (29), and 100 μl of cycloheximide was added on top of spotted food and allowed to soak in for 1 hour before synchronized L4 worms were placed on plates. Worms were allowed to crawl on plates for 45 min before they were placed at 37°C for 5 hours and returned to 20°C for the remainder of the assay. Survival was assessed after 24 hours.

Whole-mount immunocytochemistry
For whole-worm immunostaining, worms were washed several times to remove bacteria, resuspended in fixing solution [160 mM KCl, 40 mM NaCl, 20 mM Na2EGTA, 10 mM spermidine HCl, 30 mM Pipes (pH 7.4), 50% methanol, 2% β-mercaptoethanol, and 2% formaldehyde], and subjected to two rounds of snap-freezing in liquid N2. The worms were fixed at 4°C for 30 min and washed two times briefly in T buffer [100 mM tris-HCl (pH 7.4), 1 mM EDTA, and 1% Triton X-100] before a 1-hour incubation in T buffer supplemented with 1% β-mercaptoethanol at 37°C. The worms were washed with borate buffer [25 mM H3BO3 and 12.5 mM NaOH (pH 9.5)] and then washed in 1× phosphate-buffered saline (PBS) to equilibrate pH for RNase treatment. Worms were incubated at 37°C with a 1:100 RNase A/T1 Mix (Thermo Fisher Scientific) in PBS for 2 hours. Worms were washed in borate buffer and then incubated in borate buffer containing 10 mM DTT for 15 min. Worms were washed with borate buffer and then incubated in borate buffer containing 0.3% H2O2. Worms were washed in borate buffer briefly, blocked in PBST (PBS, 1% bovine serum albumin, 0.5% Triton X-100, 5 mM sodium azide, and 1 mM EDTA) for 1 hour, and incubated overnight with α6mA antibody (1:100 in PBST; Synaptic Systems, 202 003). Worms were washed four times for 25 min in PBST and then incubated with Alexa Fluor 588 secondary antibody (1:50 in PBST). Worms were washed four times for 25 min in PBST. 4′,6-Diamidino-2-phenylindole (2 mg ml⁻¹) was added to visualize nuclei. The worms were mounted on a microscope slide and visualized using a Zeiss LSM 700 confocal system.

Fertility assays
From days 3 to 8 after hatching, 10 worms were placed on NGM plates and visualized using a Zeiss LSM 700 confocal system.

Polysome profiling and qRT-PCR
Worms were synchronized by bleaching, and L4 worms were flash-frozen. Worm pellets were lysed and homogenized in lysis buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl2, cycloheximide (50 μg/ml), 1 mM DTT, protease and phosphatase cocktails (Sigma-Aldrich), 1% Tween 20, and 0.25% DOC] using pellet pestles for 1.7-m1 tubes. SUPERase-In (Invitrogen) was added to each sample at 1 U/μl. RNA (~320 μg/ml) was loaded on a sucrose gradient (10 to 50%) prepared in SW 41 ultracentrifuge tubes (Beckman) using the Bioimp Gradient Maker. Samples were centrifuged for 2 hours at 39,000 rpm at 4°C in a SW 41 rotor (Beckman). Gradients were profiled at 1 ml/min using the Brandel gradient fractionation system coupled with the Bio-Rad UV detector, which continually monitored OD 254 (optical density at 254 nm) values.

For qRT-PCR from polysome fractions, RNA (~160 to 800 μg/ml) was loaded on the sucrose gradients. One milliliter of fractions was collected every minute and flash-frozen. Equal volumes were taken from each fraction for RNA extraction and supplemented with a spike-in of 50 pg of firefly luciferase RNA (Promega). RNA was extracted using TRIzol LS and Direct-zol RNA MiniPrep Plus kit (Zymo). Eluted RNA was precipitated with the addition of GlycoBlue (Invitrogen) as a carrier. Equal volumes were taken for reverse transcription using SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed using iQaq Universal SYBR Green Supermix on a CFX real-time detection system (Bio-Rad). The results were expressed as 2^[-(gene of interest number of cycles – firefly luciferase number of cycles)] using the following primers: cyp-29A3 (forward), CCACCTGCTCATCCGATTTTTTG; cyp-29A3 (reverse), TGACCTTTTCTCTACCGTTCCTACTG; Fluc (forward), CGAGGGGATGATAAACC CGG; Fluc (reverse), CCAGATCCACAACCTTCGCT; act-1 (forward), ACGACGATCCGGCCCATCC; and act-1 (reverse), GAAAGCTGTTGTGACGATGTT.

Ribosome profiling
Ribosome profiling was performed according to published protocol (50) with modifications according to published protocols (51). Flash-frozen worm pellets were lysed and homogenized in lysis buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 5 mM MgCl2, cycloheximide (50 μg/ml), 1 mM DTT, protease and phosphatase cocktails (Sigma-Aldrich), 1% Tween 20, and 0.25% DOC] using pellet pestles for 1.7-m1 tubes. SUPERase-In (Invitrogen) was added to each sample at 1 U/μl.

**35S-methionine feeding experiments**
Bacteria were heat-shocked at 70°C for 30 min to kill the bacteria and prevent the bacteria from producing any 35S-labeled proteins. 35S-methionine (10 μCi; PerkinElmer) was added to 100 μl of heat-killed bacteria per condition. L4-staged WT and metl-5(tm4561) mutant worms were grown in mixture of heat-killed bacteria with 35S-methionine. Worms were washed four times in M9 buffer (22 mM KH2PO4, 34 mM K2HPO4, 86 mM NaCl, and 1 mM MgSO4), followed by one wash in 70% ethanol and two additional washes in M9 buffer. Worm lysis buffer [115 μl; 20 mM NaPO4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (DOC), 0.5% SDS, and 2 mM EDTA] was added to each sample, and they were flash-frozen in liquid N2. Six freeze-thaws were performed in liquid N2, and samples were placed in a Bioruptor for 5 min with 30-s on and 30-s off. Protein concentration was calculated by performing a Bradford assay, and equivalent concentrations of protein were placed in scintillation vials for measuring the translation of newly synthesized proteins. Samples were also run on SDS-PAGE gels, and gels were fixed in 30% (v/v) methanol and 10% glacial acetic acid while rocking at room temperature for 1 hour. Gels were washed two times in methanol for 45 min before briefly washing with water and drying the gels on Whatman paper. Newly synthesized proteins were detected by exposure of dried gels to phosphor screen for 2 weeks, followed by Storm phosphor imaging. Each experiment was performed with duplicate samples in triplicate.
Ten percent of each lysate (used for total RNA extraction and mRNA sequencing) was flash-frozen in 1 ml of TRIzol LS (Invitrogen). Lysate (~320 μg/ml) was incubated at 23°C for 1 hour with RNase T1 (20 U/40 μg). Treated lysates were run on sucrose gradients (10 to 50%), and the monosome peak was collected. RNA from the monosome fraction was extracted using TRIzol LS and a Direct-zol kit (Zymo). The RNA was loaded on a Novex 15% TBE-urea gel (Life Technologies), and a fragment between 26 and 33 bp was excised and extracted from the gel. The library was prepared using the TruSeq Small RNA kit (Illumina) according to published protocol (50). The PCR product was then loaded on a Novex 6% TBE gel (Life Technologies), and a band around 150 bp was excised from the gel. The DNA was eluted from the gel and sent for quality assurance and sequencing at the Bauer Core Facility at Harvard University. RNA for mRNA sequencing was extracted using Direct-zol and sent for polyadenylate selection, library preparation, and sequencing at Novogene.

Transcriptome and ribosome profiling sequencing and analysis
Transcriptomes and ribosome profiling libraries were sequenced on the Illumina NovaSeq 6000 and NextSeq 500 platforms. mRNA libraries were sequenced in a paired-end mode with each read being 150 nucleotides long. Ribosome profiling libraries were sequenced in a single-end mode with 51-nucleotide read length before adapter trimming. Adapters were removed with Cutadapt software (52), and short read alignment and counting were performed with STAR aligner (53). Differential gene expression was evaluated with the DESeq2 package in the R programming environment (54). Gene set enrichment analysis (GSEA) was done with GSEA stand-alone software [Broad Institute; (55)] using a collection of *C. elegans* gene lists derived from the gene2go annotation data at the National Center for Biotechnology Information (NCBI). They are analogous to the Gene Ontology-based series of human-only collections available from the Molecular Signatures Database, a source gene list collection used in the original implementation of GSEA software by Broad Institute. Detailed analytical pipeline and all custom scripts are openly available on GitHub (https://github.com/germaximus/Liberman_2019). Raw data and gene counts tables are available from the NCBI Gene Expression Omnibus repository under accession number GSE131269.

Quantitative real-time polymerase chain reaction
Five hundred worms were synchronized by egg laying and grown to the L4 stage at 20°C. Worms were washed three times with M9 buffer (22 mM KH₂PO₄, 34 mM K₂HPO₄, 86 mM NaCl, and 1 mM MgSO₄). Worm pellets were resuspended in TRIzol LS and Direct-zol kit (Zymo). The RNA was loaded on a Novex 15% TBE-urea gel (Life Technologies), and a fragment between 26 and 33 bp was excised and extracted from the gel. The library was prepared using the TruSeq Small RNA kit (Illumina) according to published protocol (50). The PCR product was then loaded on a Novex 6% TBE gel (Life Technologies), and a band around 150 bp was excised from the gel. The DNA was eluted from the gel and sent for quality assurance and sequencing at the Bauer Core Facility at Harvard University. RNA for mRNA sequencing was extracted using Direct-zol and sent for polyadenylate selection, library preparation, and sequencing at Novogene.

GO staining
Briefly, worms were synchronized by egg laying and grown to the L4 stage at 20°C. Worms were washed off plates in 2 ml of PBST solution and spun at 560g for 1 min, and supernatant was removed. Worms were washed three times in PBST, and all supernatant save for 100 μl was removed. A total of 600 μl of 40% isopropanol was added, and worms were rocked at room temperature for 3 min. Worms were spun at 560g for 1 min, and all but 100 μl of supernatant was removed. ORO working solution (600 μl) was added to each tube. To make a working solution of ORO, initially, 500 mg of ORO powder was added to 100 ml of 100% isopropanol; this stock solution was diluted in water to 60% isopropanol and filtered through a 0.2-μm filter. Worms and working solution of ORO were rocked for 2 hours at room temperature. Tubes were spun at 560g for 1 min, and supernatant was removed. Pellets were resuspended in 600 μl of PBST and rotated at room temperature for 30 min. Tubes were centrifuged at 560g for 1 min, and supernatant was removed. Five microliters of worms was placed on microscope slides, and a coverslip was added. Slides were imaged on a Zeiss Discovery V8 fluorescent scope, and ORO staining was quantified in ImageJ.

GC-MS quantification of eicosanoids
GC-MS was performed by the Harvard Small Molecule Mass Spectrometry Facility. Briefly, 50 μl of 2-μl 20-HETE-d6 (Cayman Chemical) in methanol was added to each frozen sample as an internal standard to ensure that extractions were equivalent in each sample. Hydrolysis solution (1 ml; 90% methanol in water and 0.3 N KOH) was added to each sample, which was thawed and vortexed. Samples were transferred to bead beater vials with 100 μl of 0.1-mm zirconia beads. Samples were homogenized in a TissueLyser LT at 50 Hz for 15 min. The homogenate (without the beads) was transferred to glass vials and incubated at 80°C for 1 hour before being transferred to Eppendorf tubes and centrifuged at maximum speed for 5 min. The supernatant was transferred to new glass vials and evaporated under nitrogen flow, and then, samples were resuspended in 2 ml of 0.1 M ammonium acetate (pH 7.0) and 5% methanol in water and purified by solid-phase extraction (SPE) purification. The hydrolyzed samples were brought to a pH of 6.0 with 25 μl of acetic acid. SPE cartridges (SiliCycle SiliaPrep C8/SAX; 200 mg) were conditioned with 2 ml of methanol followed by 2 ml of 0.1 M ammonium acetate (pH 7.0) and 5% methanol in water. Samples were passed on the cartridges, which were then washed with 2 ml of 50% methanol in water. Samples were eluted with 2 ml of hexane:ethyl acetate 75:25 and 1% acetic acid. Samples were reduced to 1 ml by N₂ flow and transferred to 1.5-ml vials before being evaporated to dryness under N₂ flow. Samples were resuspended in 50 μl of methanol and transferred to glass microinserts. Hydrolysis was adapted from (56), and SPE purification was adapted from (57). Samples were run on a Thermo Fisher Scientific Q Exactive Plus with a Kinetex 2.6-μm C18 100 Å, 150 × 2.1 mm. Sample (5 μl) was injected, and the column was maintained at 25°C.
lipids are detected, but their identification was projected on the basis of the accurate mass rather than the running of standards, as was performed for 20-HEPE, 17,18-EpETE, 17,18-DiHETE, and 100 μM for 17,18-EpETE, 17,18-DiHETE, and 100 μM for AA and EPA and were recovered, while 50 to 60% of EPA and AA were recovered. Therefore, comparisons were only performed between the same compounds between samples. Other major fatty acids were detected, but their identification was projected on the basis of the accurate mass rather than the running of standards, as was performed for 20-HEPE, 17,18-EpETE, 17,18-DiHETE, and 20-HETE-d6, AA, and EPA.

Lipid supplementation
NGM plates were prepared as in (58). Briefly, NGM plates were prepared with the addition of 0.1% Tergitol dissolved in water, and eicosanoids or PUFAs were added to the media when it cooled to 55°C. Final concentrations of 1 mM AA, 1 mM EPA, 100 μM 17,18-EpETE, 100 μM 17,18-DiHETE, and 100 μM 20-HEPE were used after initial optimization experiments were performed with concentrations ranging from 10 μM to 1 mM for AA and EPA and 10 to 100 μM for 17,18-EpETE, 17,18-DiHETE, and 20-HEPE based on an examination of lipid concentrations used in previously published papers. Plates were kept in the dark and spotted 24 to 48 hours before L4 worms were added. Worms were grown on supplemented plates for 4 hours before heat shock at 37°C was performed as above. Tergitol alone plates were used as a control.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/17/eaa4370/DC1

View/request a protocol for this paper from Bio-protocol.

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