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# N-acylethanolamine signaling mediates the effect of diet on lifespan in *C. elegans*

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## Abstract

Dietary restriction (DR) is a robust means of extending adult lifespan and postponing age-related disease in many species, including yeast, worms, flies and rodents[1,2]. Studies of the genetic requirements for lifespan extension by DR in the nematode *Caenorhabditis elegans (C. elegans)* have implicated a number of key players in this process[3–5], including the nutrient-sensing target of rapamycin (TOR) pathway[6] and the Foxa transcription factor PHA-4[7]. However, little is known about the metabolic signals that coordinate the organismal response to DR and maintain homeostasis when nutrients are limited. The endocannabinoid (EC) system is an excellent candidate to play such a role given its involvement in regulating nutrient intake and energy balance[8]. Despite this, a direct role for EC signaling in DR or lifespan determination has yet to be demonstrated, in part due to the apparent absence of EC signaling pathways in model organisms that are amenable to lifespan analysis[9]. N-acylethanolamines (NAEs) are lipid-derived signaling molecules, which include the mammalian EC arachidonoyl ethanolamide (AEA). Here we identify NAEs in *C. elegans*, show that NAE abundance is reduced under DR and that NAE deficiency is sufficient to extend lifespan through a DR mechanism requiring PHA-4. Conversely, dietary supplementation with the nematode NAE eicosapentaenoyl ethanolamide (EPEA) not only inhibits DR-induced lifespan extension in wild type animals, but also suppresses lifespan extension in a TOR pathway mutant. This demonstrates a role for NAE signaling in aging and suggests that NAEs represent a signal that coordinates nutrient status with metabolic changes that ultimately determine lifespan.



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**Author Contributions**

M.L., J.M.H., B.W.G., G.J.L. and M.S.G. conceived of and planned experiments. M.L., M.C.V., I.M.K., J.B.G. and M.S.G. performed experiments. M.L. and M.S.G. wrote the manuscript.

**Author Information**

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We identified a diverse set of NAEs in *C. elegans* using stable isotope dilution gas chromatography – mass spectrometry (SID-GC-MS)[10] in pseudo-multiple reaction monitoring (pMRM) mode (Fig 1a and Supplementary Fig. 1), including the C20 fatty acid containing NAEs, EPEA and AEA, the latter having been previously been identified in *C. elegans*[11]. Mammalian NAE levels are controlled through enzymatic synthesis and degradation, while their biological effects are mediated through several receptors, including the cannabinoid (CB) receptors which bind AEA[8]. Many of the upstream enzymes that regulate NAEs remain unidentified, however N-acyl phosphatidyl ethanolamine-specific phospholipase D (NAPE-PLD) catalyzes the last step in biosynthesis, whereas the hydrolytic enzyme, fatty acid amide hydrolase (FAAH), inactivates NAE molecules[12]. Although *C. elegans* lacks clear orthologs of CB receptors[9], it does have orthologs of NAPE-PLD (*nape-1)* and FAAH (*faah-1)*[13]. *nape-1* is expressed in interneurons that are in close proximity to the primary sensory neurons, and *faah-1* is expressed principally in the pharynx, suggesting that this is a major site of NAE degradation (Supplementary Fig. 2).

We hypothesized that if *C. elegans nape-1* and *faah-1* have conserved function then perturbation of their activity should alter worm NAE levels. Reducing the levels of *faah-1* through RNA interference (RNAi) or inhibiting enzyme activity with a specific chemical inhibitor increased levels of NAEs (Fig. 1b, c and Supplementary Fig. 3, 4). In contrast, no decrease in NAE levels was observed in either a *nape-1* deletion mutant or following RNAi of *nape-1* in wild type animals (data not shown), which is consistent with mammalian studies that indicate redundancy at the final step of NAE production[14]. We then reasoned that over-expression of *faah-1* would provide an alternate means of reducing NAE levels *in vivo*, and found that transgenic worm strains maintaining extra copies of the *faah-1* gene had reduced EPEA levels, as well as reductions in palmitoleoyl ethanolamide (POEA), linoleoyl ethanolamide (LOEA) and AEA (Fig. 1d and Supplementary Fig. 5). These data suggest that the function of *faah-1* as a hydrolytic enzyme involved in NAE degradation is conserved in worms.

A striking phenotype of *faah-1* over-expression in worms was a developmental delay which could be rescued by *faah-1* RNAi (Fig. 2a, b), suggesting that NAEs promote larval development. During normal growth all NAEs have a similar developmental profile, reaching the highest levels in the second larval stage (L2) and then progressively declining into adulthood (Fig. 2c and Supplementary Fig. 6). The peak in NAE levels at L2 coincides with the time at which the animal is committed to reproductive growth rather than entry into an alternate diapause stage (the dauer larva, Supplementary Fig. 6)[15]. Entry into the dauer state allows the worm to survive for extended periods of time in the absence of food and is associated with profound metabolic changes. We found that NAE levels under dauer-inducing conditions were similar to normally developing worms in L1 and early pre-dauer (L2d) but were markedly reduced during the late L2d and dauer stages (Fig. 2c and Supplementary Fig. 6). The reduction in NAE levels following commitment to dauer formation suggests that NAEs could act as signals of an altered metabolic state.

Since NAE levels are reduced in worms during the transition to the dauer state, we hypothesized that exogenous NAEs could prevent dauer arrest and promote reproductive growth. Only EPEA was able to completely rescue the dauer phenotype of *daf-2(e1368)*

mutants, while the less abundant AEA had a weaker effect (Fig. 2d and Supplementary Fig. 7). Importantly, EPEA concentrations in adult animals treated with 50 μM EPEA reached a similar level to those found in wild type L2 larvae (data not shown), suggesting that exogenous EPEA treatment results in physiological levels *in vivo*. Treatment with eicosapentaenoic acid, which is both a precursor and the hydrolytic breakdown product of EPEA, was unable to promote reproductive growth suggesting that EPEA itself is responsible for the dauer rescue (Supplementary Fig. 8). EPEA was also able to rescue dauer formation in other dauer-constitutive mutants that define distinct signaling pathways involved in dauer formation (Fig. 2e, f and Supplementary Table 1), suggesting that this molecule functions downstream or parallel to these primary environment sensing pathways. Several candidate NAE receptors were not required for rescue of the dauer phenotype (Supplementary Fig. 9).

These data suggest that NAEs could provide a signal of nutrient availability and energy balance. In support of this, we found that starved L1 animals showed extremely low levels of EPEA and the other NAEs, which increased dramatically following 6 h of feeding (Fig. 3a and Supplementary Fig. 10). Moreover, NAE levels were reduced in adult animals maintained under DR conditions, and re-feeding restored NAE levels back to that of well-fed controls (Fig. 3b and Supplementary Fig. 11). These results demonstrate that NAE levels in worms are responsive to nutrient availability, as has been shown in rodent studies[16,17].

Since DR reduces NAEs, we hypothesized that *faah-1* over-expression may partly mimic a DR state and confer stress resistance and longevity phenotypes. Consistent with this hypothesis, we found that animals over-expressing *faah-1* showed resistance to thermal stress (Supplementary Table 2) and increased adult lifespan (Fig. 3c and Supplementary Table 3). Over-expression of *faah-1* in the pharynx was largely sufficient for this lifespan extension (Supplementary Table 3), supporting the idea that the pharynx is a major site of NAE function.

Using a standard dietary restriction (DR) paradigm[18] we found that *faah-1* over-expression was associated with lifespan extension in the presence of abundant food but not under conditions of optimal DR (Fig. 3c-f and Supplementary Table 3). This was confirmed using a second, independent DR protocol[19] (Supplementary Table 3). The lack of an additive effect under optimal DR conditions suggests that lifespan extension resulting from *faah-1* over-expression is mechanistically equivalent to DR. This is further supported by the observations of growth delay and reduced fecundity in animals with reduced NAEs (Fig. 2a and Supplementary Fig. 12). Lifespan extension due to *faah-1* over-expression was not dependent on DAF-16, the FOXO transcription factor required for longevity in *daf-2* insulin-signaling mutants (Fig. 3g & Supplementary Table 3). However, the transcription factor *pha-4*, which is involved in the lifespan extending effects of DR[7], was required (Fig. 3h and Supplementary Table 3), indicating that low NAEs extend lifespan through a DR pathway and act upstream of PHA-4.

Since reduced NAE levels are associated with increased longevity, we hypothesized that elevated NAE levels should suppress stress resistance and lifespan extension. Consistent with this hypothesis, EPEA treatment resulted in a significant reduction in thermotolerance

(Supplementary Table 2) and lifespan (Fig. 4a, b & Supplementary Table 4). Lifespan suppression induced by EPEA was minimal under conditions of high food but was much more profound under optimal DR conditions (Fig. 4c, d and Supplementary Table 4). This is unlikely to be due to EPEA toxicity, since EPEA treatment does not adversely affect growth (data not shown), has no effect on fertility (Supplementary Fig. 13), and only has a minimal effect on lifespan under high food conditions. Instead, we propose that elevated EPEA levels under DR provide a false signal of high nutrient availability and inhibit the metabolic adaptation to reduced food availability that confers lifespan extension.

We next sought to determine whether mutations that affect either NAE biosynthesis or nutrient sensing pathways also alter NAE levels and lifespan. In rodents, the dietary availability of fatty acids has been shown to influence NAE levels[20] and thus reduced fatty acid availability could generate NAE deficiency. In *C. elegans*, the *fat-4* mutation leads to reduced arachidonic and eicosapentaenoic acid[21] and also results in reduced AEA and EPEA levels (Supplementary Fig. 14) as well as lifespan extension (Supplementary Fig. 15 and Supplementary Table 5). In addition, we found that *rsks-1* mutants, which have a defect in the worm ortholog of S6 kinase in the conserved TOR nutrient sensing pathway[6], showed a specific reduction of EPEA levels, but not the other NAEs, (Fig. 4e and Supplementary Fig. 16). Furthermore, EPEA treatment in *rsks-1* mutants completely suppressed their longevity phenotype (Fig. 4f and Supplementary Table 6). These data suggest that the TOR pathway may control NAE levels in response to nutrient availability and that the longevity of *rsks-1* mutants could be due to their inability to up-regulate EPEA in response to food.

NAEs have emerged as an important class of lipid mediators with a role in the response to nutrient availability in diverse organisms including mammals, non-mammalian vertebrates, and invertebrates[16,17,22–25]. In mammals, the arachidonic acid-containing NAE, AEA, elicits many of its effects through CB receptors, but can also interact with a variety of other targets. Although worms possess a number of different NAEs, including AEA and EPEA, *C. elegans*, in common with other protostomes and some primitive deuterostomes[26,27], does not possess clear orthologs of the mammalian CB receptors[9]. This suggests that there are as yet unidentified NAE receptors in nematodes that are possibly conserved mediators of NAE signaling. Taken together, our findings indicate that reduced NAE signaling mediates some of the effects of DR on lifespan extension in the nematode, and that EPEA acts as a metabolic signal that couples nutrient availability with growth and lifespan, suggesting a new role for NAE signaling in organismal aging.

## Methods Summary

*Caenorhabditis elegans* strains were maintained as previously described[28]. *C. elegans* strains containing the following alleles were used in this study: Bristol N2 (wild-type), *daf-2(e1368) III*, *daf-2(m41) III*, *daf-2(e1370) III*, *daf-2(e1371) III*, *age-1(hx546) II*, *age-1(m875) II*, *daf-7(e1372) III*, *daf-4(e1364) III*, *unc-31(e928) IV*, *unc-64(e246) III*, *daf-16(mu86) I*, *pdk-1(sa709) X*, *akt-1(ok525) V*, *daf-8(e1393) I*, *daf-11(m47) V*, *daf-28(sa191) V*, *eri-1(mg366) IV; lin-15B(n744) X*, *fat-4(wa14) IV*, *rsks-1(ok1255) III*. Generation of transgenic strains is described in Methods. The growth of worms in mass culture, lipid extractions and GC-MS are described in Methods. Dauer assays,

thermotolerance assays and lifespan analysis were performed as previously described[29,30] with exceptions detailed in Methods. Dietary restriction experiments were performed according to the method of Chen *et al*.[18].

## Methods

### Chemicals

Palmitoyl ethanolamide (PEA), oleoyl ethanolamide (OEA), linoleoyl ethanolamide (LOEA), arachidonoyl ethanolamide (AEA), PEA-$d_2$, OEA-$d_4$, and AEA-$d_4$ were obtained from Cayman Chemical (MI). Eicosapentaenoyl ethanolamide (EPEA) and palmitoleoyl ethanolamide (POEA) were obtained from Enzo Life Sciences (PA). BSTFA was from Sigma Aldrich (MO). All solvents were of GC-MS grade and all other reagents and solvents were of the highest grade available.

### Nematode strains

The following nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, University of Minnesota except *age-1(m875) II* which was obtained from D. Chen: Bristol N2 (wild-type), *daf-2(e1368) III*, *daf-2(m41) III*, *daf-2(e1370) III*, *daf-2(e1371) III*, *age-1(hx546) II*, *daf-7(e1372) III*, *daf-4(e1364) III*, *unc-31(e928) IV*, *unc-64(e246) III*, *daf-16(mu86) I*, *pdk-1(sa709) X*, *akt-1(ok525) V*, *daf-8(e1393) I*, *daf-11(m47) V*, *daf-28(sa191) V*, *eri-1(mg366) IV; lin-15B(n744) X*, *fat-4(wa14) IV*, *rsks-1(ok1255) III*.

### Nematode culture

For routine culture worms were maintained using standard culture methods[31]. For mass cultures, 10,000 worms were grown on 10 cm NGM plates seeded with 2 ml concentrated *E. coli* OP50 and starved L1s from eggs prepared by sodium hypochlorite treatment[32].

### Generation of transgenic lines

Promoter-GFP fusions were generated essentially as described by Hobert[33]. To generate the *Pnape-1::GFP* construct, approximately 3 kb of the 5’ region from the *nape-1* gene up to the 7$^{th}$ codon after the translational start (primer f1: tcacgtgagaaatagtcgctgg to primer r1:tgaggaggttgtcggtaggctcat) was fused to a *GFP::unc-54* 3’UTR fragment of pPD95.75 (gift from Andrew Fire). To generate the *Pfaah-1::GFP* construct, approximately 3 kb of the 5’ region of the *faah-1* gene up to and including the translational start (primer f1: ccatgtagagagccttccgacat to primer r1: catgatgaccttgaaatactgaaaattgaa) was fused to a *GFP::unc-54* 3’UTR sequence.

To generate the *Prps-5::faah-1* over-expressor lines, a *faah-1* genomic fragment, encompassing the entire open reading frame from the start codon to the end of the annotated 3’UTR (primer f1: atgatttttttacttggtgcttctcgttttg to primer r2: ggacaaggaatggtggacttcgg), was PCR fused[34] to the ubiquitous constitutive promoter *Prps-5*[35] (primer f1: cgaaacggtaactgtgaagaattcaata to primer r1: cctgcaaaataacaacttcagtatagt). The resulting 6 kb PCR fragment was microinjected into the gonad of young adult worms and resulting transgenic animals were identified in the next generation by their expression of a co-

injection marker. At least five independent transgenic lines were analyzed. A representative line was chosen for integration using trimethylpsoralen / UV mutagenesis. Two independent integrants were isolated and named *rfIs22[Prps-5::faah-1, Podr-1::dsRED, Punc-25::mRFP]X;* and *rfIs23[Prps-5::faah-1, Podr-1::dsRED, Punc-25::mRFP]X;* respectively. These lines were outcrossed to wild type six times before analysis.

To generate worm lines with tissue specific expression of *faah-1* we used the *faah-1* genomic fragment described above and discretely PCR spliced it to promoter fragments that drive expression specifically in pharyngeal muscle (*Pmyo-2*), body wall muscle (*Pmyo-3*) and neurons (*Punc-119*). Primers for *Pmyo-2* (f1:ggtggtggacagtaactgtctgt, r1:catttctgtgtctgacgatcgagg). Primers for *Pmyo-3* (f1: cacttccggcgccctgaatctaa, r1:catttctagatggatctagtggtcgtggg). Primers for *Punc-119* (f1: ggctccaatcggaaacgcgaaca, r1:catatatgctgttgtagctgaaaattttggggattatggg).

## Mass culture for GC-MS of larval stages

For larval cultures of N2 and *daf-2(e1368)* for GC-MS analysis, the NGM plates included 5 μg/ml lathosterol to facilitate reproductive growth and prevent dauer formation of *daf-2(e1368)* when grown in mass culture. To generate larval cultures N2 and *daf-2(e1368)* were grown at 15°C on NGM plates to generate $1x10^7$ arrested L1s which were then grown in liquid culture at 25°C until the appropriate larval stage. Supplementary Figure 17 shows that NAE levels are not different when worms are grown on solid media and liquid culture suggesting that liquid culture conditions supplemented with the appropriate amount of food do not induce dietary restriction. Arrested L1s were inoculated into 2 L glass flasks (no more than $1×10^6$ worms per flask) containing 90 ml S-medium (+ cholesterol, final concentration 5 μg/ml) and 10 ml of concentrated *E. coli* OP50. The liquid cultures were then incubated in a shaking incubator at 25°C, 150 rpm. The number of animals at each larval stage required to yield 0.7 g wet weight was calculated using data from Knight *et al.*[36] and flasks were inoculated accordingly (Larval stage, time of harvest, number of worms: N2: L1, 6 h, $5×10^6$ worms; L2, 15 h, $2.5×10^6$ worms; L3, 21 h $1×10^6$ worms; L4, 28 h, $5×10^5$ worms; YA, 36 h, $2.5×10^5$ worms; GA, 48 h, $2.5×10^5$ worms; *daf-2(e1368):* L1, 6 h, $5×10^6$ worms; L2, 15 h, $2.5×10^6$ worms; L2d, 25 h, $2.5×10^6$ worms; dauer, 48 h $1x10^6$ worms). Worms were harvested by low speed centrifugation and washed 3 times with S-basal before the worm pellet was snap frozen in liquid nitrogen.

To compare NAE levels in fed and starved L1s, N2 animals were grown on 10 cm NGM plates. Eggs harvested by sodium hypochlorite treatment were allowed to hatch in S-basal in the absence of food and maintained at 20°C for 24 h. At this point arrested L1s were inoculated into 2 L glass flasks (no more than $1×10^6$ worms per flask) containing 100 ml S-medium (+ cholesterol, final concentration 5 μg/ml) or 90 ml S-medium + 10 ml of concentrated *E. coli* OP50 for starved and fed cultures respectively. The liquid cultures were then incubated in a shaking incubator at 20°C, 150 rpm for 6 h after which worms were harvested by low speed centrifugation and washed 3 times with S-basal before the worm pellet was snap frozen in liquid nitrogen.

### Mass cultures with FAAH inhibitor URB597

URB597 (Cayman Chemical, MI) was re-suspended in DMSO to a final concentration of 20 mM and further diluted in S-basal for spotting onto plates. 10 cm NGM + *E. coli* plates were inoculated with 10,000 arrested L1s and grown at 20°C for 48 h. At this point animals were harvested and transferred to NGM + *E. coli* plates containing either DMSO vehicle or 10 μM URB597 and incubated for a further 24 h at 20°C before harvesting.

### Mass culture for DR and re-feeding in adult animals

To generate mass cultures under DR conditions we used a modification of the protocol described by Mair *et al.*[37]. 10 cm NGM plates were inoculated with 10,000 arrested L1s and grown at 20°C for 48 h. At this point animals were washed off and transferred to plates containing 10 μg/ml FUDR to inhibit progeny production. After a further 24 h at 20°C adult worms were harvested and inoculated into 2 L flasks (75,000 worms per flask) containing S-medium (+ 5mg/ml cholesterol, 100 μg/ml FUDR, 50 μg/ml carbenicillin). Flasks were supplemented with concentrated OP50 to give $OD_{600nm}$ = 2.5 for fed conditions and $OD_{600nm}$ = 0.2 for DR conditions. The liquid cultures were then incubated in a shaking incubator at 20°C, 150 rpm for either 12 or 24 h after which worms were harvested by low speed centrifugation and washed 3 times with S-basal before the worm pellet was snap frozen in liquid nitrogen. For re-feeding experiments, worms were incubated for 12 h under DR conditions and then harvested and re-inoculated into flasks containing *E. coli* OP50 at $OD_{600nm}$ = 2.5 for an additional 12 h before harvesting.

### Lipid extraction

Lipid extracts were generated by a modification of the method of Sultana *et al.*[38]. 600 mg frozen worm pellets were thawed on ice in 4 ml methanol in 50 ml glass centrifuge tubes and then subjected to 4 x 1 min sonication on ice. After sonication 30 ng of the internal standards PEA-$d_2$, OEA-$d_4$ and AEA-$d_4$ were added followed by 8 ml chloroform and 4 ml 0.5 M KCl / 0.08 M $H_3PO_4$ to a final ratio of 1:2:1. Samples were vortexed and then sonicated in an ultrasonic water bath for 15 min. After vortexing for 2 x 1 min samples were centrifuged for 10 min at 2000 g to separate the phases. The lower phase was collected into a clean glass tube, dried under nitrogen and re-suspended in 4 ml hexane.

### Solid phase fractionation

The total lipid extracts were fractionated on Sep-Pak Classic silica columns (Waters Corporation, MA) to collect the fatty acid amide and monoglyceride fraction using a modification of the Sultana *et al*[38]. Sequential elutions were performed using 2 ml 99:1 hexane: acetic acid v/v, 15 ml 90:10 hexane: ethyl acetate v/v, 10 ml 80:20 hexane:ethyl acetate v/v, 5 ml 70:30 hexane: ethyl acetate v/v with the final elution of 5 ml 2:1 choroform:isopropanol v/v containing the fatty acid amide / monoglyceride fraction. This sample was dried under nitrogen and re-suspended in 1.5 ml hexane. The volumes for the solvent cuts were optimized by analyzing each fraction by thin layer chromatography.

### Gas chromatography – mass spectrometry

Samples were analyzed by GC-MS using GC conditions based on those described by Hardison *et al*[10]. Samples were analyzed in duplicate, corresponding to at least 200 mg worms per run, following derivatization with BSTFA for 1 h. Just before GC-MS analysis, samples were dried under $N_2$ and re- suspended in 1 μL hexane for injection. GC-MS analysis was performed using a Varian 2100T ion-trap GC/MS/MS with a 3900 GC (Varian Inc, Walnut Creek, CA) operating in splitless mode with a VF-5ms capillary column (30 m X 0.25 mm i.d., 5% phenyl-95% methyl polysiloxine, 0.25 μm film thickness; Varian, Inc., Walnut Creek, CA). GC conditions: Injector 250°C. Initial column temperature was 150°C for 1 min and then ramped at 20°C per min to 300°C and held for 15 min. MS conditions: Analytes were chemically ionized using acetonitrile vapor. Data was collected using the MRM mode in Varian System Control software v6.40. Precursor ions were isolated using an isolation window of 3. MS/MS fragmentation was performed in the ion trap using an excitation storage level of 164.0, excitation amplitude of 80, and non-resonant collision energy for all analytes. Three analysis segments were performed in each run with the endogenous NAEs and labeled internal standard with equivalent carbon atoms analyzed during each segment. The C16 NAEs (POEA, PEA, and PEA-$d_4$) were typically analyzed for the first 7.5 min of each run, the C18 NAEs (LOEA, OEA, and OEA-$d_2$) segment was from 7.5 to 8.0 min, and the C20 NAEs (EPEA, AEA, and AEA-$d_4$) were analyzed for the rest of the run. The MS scans cycled between each analyte continuously with a total cycle time of 0.72 sec for three analytes per segment.

Peak areas were generated by manually integrating the extracted ion chromatogram for the MS/MS fragment ion of interest (POEA *m/z* 370, 280; PEA *m/z* 372, 282; PEA-$d_4$ *m/z* 376, 286; LOEA *m/z* 396, 306; OEA *m/z* 398, 308; OEA $d_2$ *m/z* 400, 310; EPEA *m/z* 418, 328; AEA *m/z* 420, 330; AEA $d_4$ *m/z* 424, 334) using Varian MS Data Review v6.4. The fragment ion chosen for each transition corresponds to the loss of O-TMS ([M + H - 90]+) which was the base peak in all MS/MS spectra and had the maximum signal to noise and dynamic range.

### Dauer assays

Dauer assays were performed as previously described[29]. NAEs were resuspended in ethanol to a final concentration of 10 mM. For dose range experiments serial dilutions were made to yield 10, 5, 2, 1 and 0.5 mM. 15 μL of each working solution of NAEs were added to 135 μL S-basal before being spotted onto a 3 ml NGM. Equal distribution of the NAE throughout the agar was assumed to yield final concentrations of 50, 25, 10, 5 and 2.5 μM. Plates were then scored for the number of animals at each developmental stage (dauers, L3, L4, young adults and gravid adults).

### Thermotolerance assays

Thermotolerance assays were performed as previously described[30]. For experiments with the *faah-1* over-expression lines, first day adults were transferred from the growth temperature (20°C) to a 35°C heat shock incubator. For experiments with EPEA, worms were transferred to plates containing 50 μM EPEA at the L4 stage and after 24 h were shifted to the 35°C incubator.

### Lifespan analysis

Lifespan analysis was performed as previously described[30]. On the first day of adulthood animals were transferred to plates containing 10 μg/ml FUDR, to inhibit progeny production, with each condition assayed in duplicate with 50 worms per plate. Worms were transferred to new plates every two days and transferred to plates without FUDR at day 8. Animals that crawled off the plate, experienced internal hatching or exhibited vulval protrusion were scored as censored data. Survival analysis was performed using Prism 4 software (Graphpad Software, Inc.); Kaplan-Meier survival curves were plotted for each lifespan assay and compared using the Log-rank test. For DR experiments we used the method of Chen *et al*.[18], with animals transferred to DR plates as first day adults. To examine the effect of EPEA on lifespan, worms from a synchronous lay were transferred to plates containing 50 μM EPEA at the L4 stage. After 24 h worms were transferred to plates containing FUDR. During the lifespan worms were transferred to fresh EPEA every 2 days. For DR lifespans in the presence of EPEA, worms from a synchronous lay were transferred to EPEA plates at the L4 stage and moved to the DR plates + 10 μg/ml FUDR as first day adults. Worms were transferred every day for the first 8 days to prevent food depletion in the DR condition and every 2 days thereafter. For *rsks-1* lifespans with EPEA worms were moved to EPEA plates at L4 and moved to FUDR plates 24 h later. Worms were transferred to fresh EPEA plates every day for the first 8 days and every 2 days thereafter.

### RNA interference

RNA interference by feeding and by soaking was performed as previously described[39,40]. For mass culture RNAi by soaking approximately 2,500 L4 worms were incubated for 24 h in 1 ml of M9 buffer with 0.5 μg/μL of dsRNA synthesized *in vitro* with the megascript kit (Ambion Inc., TX). Soaking was performed in 50 ml glass tubes with slight shaking. The resulting P0 young adults were dispersed onto mass culture plates and allowed to produce F1 offspring. The mass cultures were harvested for analysis before the F1 produced eggs. The *faah-1 (*B0218.1), *nape-1 (Y37ARE11.4)* and *pha-4* (F38A6.1) RNAi clones were from the *C. elegans* ORF-RNAi Library (Open Biosystems, AL).

### Length measurement

Worms were grown on either control RNAi or *faah-1* RNAi for two generations. Eggs from a 15 min synchronous lay were collected and incubated at 20°C. Each day 20 larvae were mounted on a glass slide and anesthetized with 0.1% sodium azide. Brightfield images were captured on an Olympus IX70 inverted microscope using an Olympus DP70 digital camera. Body size was measured using Image J software (NIH) and calibrated against a stage micrometer.

### Fertility assays

Synchronous populations of worms were cultured at 20°C until the L4 stage. Individual worms were then transferred and maintained each on a separate plate and transferred daily. Progeny were left to develop for 24 h before counting. For fertility in the presence of EPEA, eggs were transferred to control or 50 μM EPEA plates and after 48 h single worms were transferred daily to individual plates containing vehicle or EPEA.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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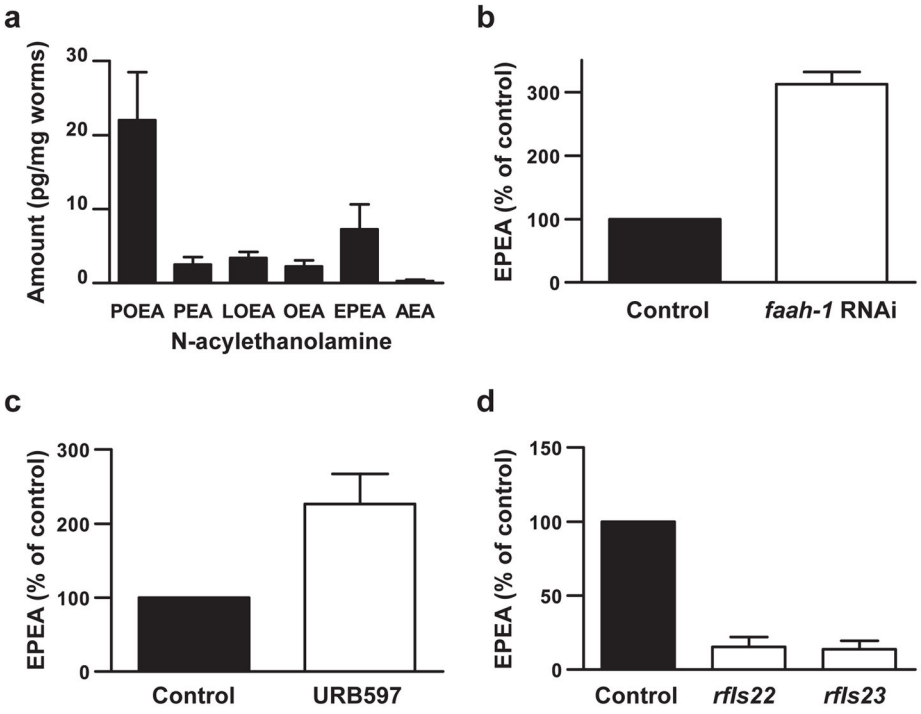


**Figure 1. NAE levels in *C. elegans* are modulated by FAAH activity**

**a,** Levels of NAEs in first day adult wild type N2 worms measured by SID-GC-MS (mean +s.d., n=5). POEA – palmitoleoyl ethanolamide; PEA– palmitoyl ethanolamide; LOEA – linoleoyl ethanolamide; OEA – oleoyl ethanolamide; EPEA eicosapentaenoyl ethanolamide; AEA – arachidonoyl ethanolamide. **b,** EPEA levels are elevated in first day *eri-1(mg366) IV; lin-15B(n744)* adults after exposure to *faah-1* dsRNA by soaking (mean+s.d., n=2). **c,** EPEA levels are elevated in first day wild type N2 adults after 24 h exposure to 10 μM URB597, a chemical inhibitor of mammalian FAAH (mean+s.d., n=5, $p<0.05$, Wilcoxon Signed Rank Test). **d,** Over-expression of *faah-1* results in reduced EPEA levels in first day wild type N2 adults (mean+s.d., N2 n=9, r*fIs22* n=7 and *rfIs23* n=8, $p<0.05$ for both *rfIs22* and *rfIs23*, Wilcoxon Signed Rank Test).

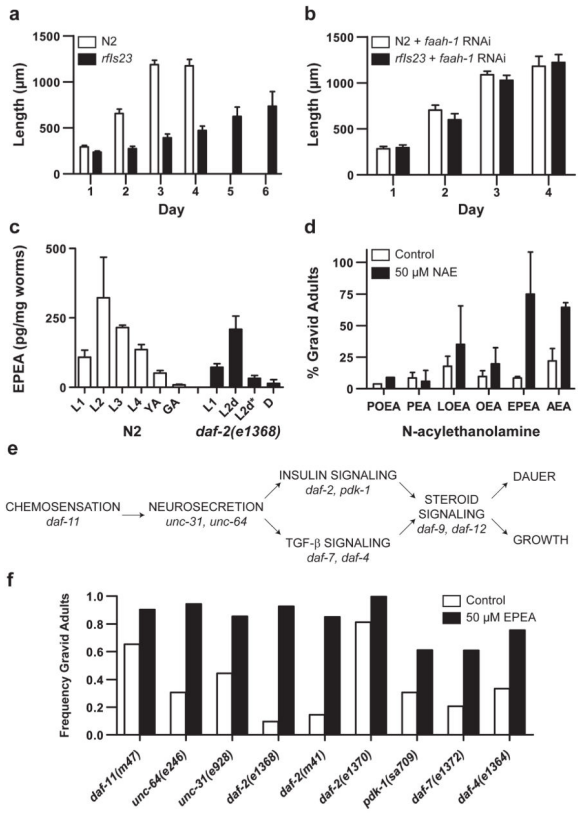


**Figure 2. NAEs affect reproductive growth and dauer formation**

**a,** *faah-1* over-expression results in developmental delay (mean+s.d., N2 n=54, *rfIs23* n=76). **b,** *faah-1* RNAi rescues the growth delay of *faah-1* over-expressors (mean+s.d., N2 n=59, *rfIs23* n=53). **c,** Levels of EPEA during development in N2 and *daf-2(e1368)* animals grown at 25°C (mean+s.d., n=2). Abbreviations: L1 – 1st larval stage; L2 – 2nd larval stage; L3 – 3rd larval stage; L4 – 4th larval stage; YA – young adults; GA – gravid adults; L2d – alternate L2 stage preceding the dauer molt; L2d* – later time point in L2d; D – dauer. **d,** Effect of treatment with exogenous NAEs on reproductive growth in *daf-2(e1368)* mutants at 24°C (mean+s.d., n=2). **e,** Scheme illustrating genes and pathways involved in dauer formation in *C. elegans*. **f**, EPEA rescues dauer formation in multiple dauer constitutive mutants (all p<0.0001, Chi-squared test, additional data in Supplementary Table 1).

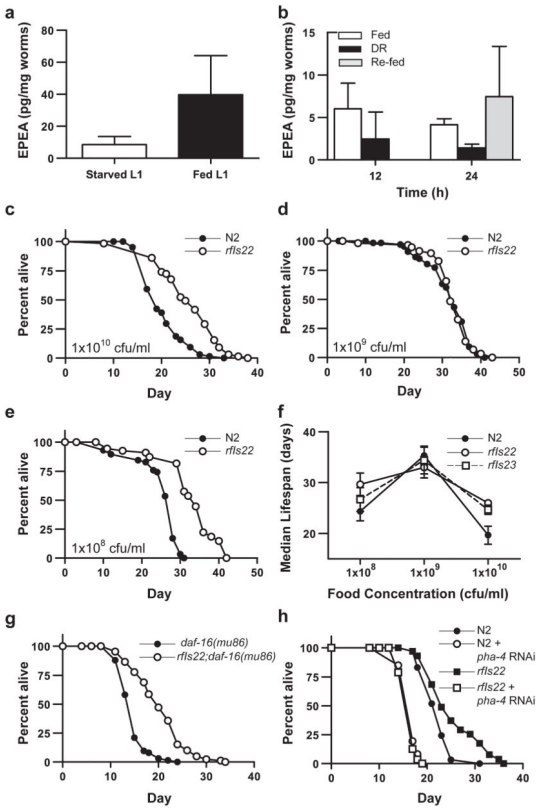


**Figure 3. Reduced NAE levels are associated with DR and are sufficient to confer lifespan expression**

**a,** EPEA levels are reduced in starved L1 larvae and increase after 6 h of exposure to food (mean+s.d., n=3). **b,** EPEA levels are altered in response to food availability in adult wild type N2 animals (mean+s.d., Mann-Whitney U test: 12 h Fed (n=6) vs DR (n=12) $p<0.05$; 24 h Fed (n=7) vs DR (n=7) $p<0.001$; 24 h DR vs Re-fed (n=6) $p<0.005$; 24 h Fed vs Re-fed p=n.s.). **c,** *faah-1* over-expression extends lifespan in N2 wild type animals under fed conditions ($1x10^{10}$ cfu/ml *E. coli*, $p<0.0001$, Log-Rank test). **d,** Lifespan is not different between N2 and a *faah-1* over-expressing line under conditions of optimal DR ($1\times10^{9}$ cfu/ml *E. coli*). **e,** *faah-1* over-expression extends lifespan in N2 wild type animals under conditions of sub-optimal DR conditions ($1\times10^{8}$ cfu/ml *E. coli*, $p<0.0001$, Log-Rank test). **f,** *faah-1* over-expression affects lifespan in a nutrient dependent manner (mean lifespan + s.d., n=3). **g,** *faah-1* over-expression extends lifespan in a *daf-16* mutant ($p<0.0001$, Log-Rank test). **h,** Lifespan extension resulting from *faah-1* over-expression requires the Foxa transcription factor PHA- 4 (N2 Control vs N2 + *pha-4* RNAi, $p<0.0001$; *rfIs22* Control vs *rfIs22* + *pha-4* RNAi $p<0.0001$, *rfIs22* Control vs N2 Control p=0.0014, Log-Rank test).

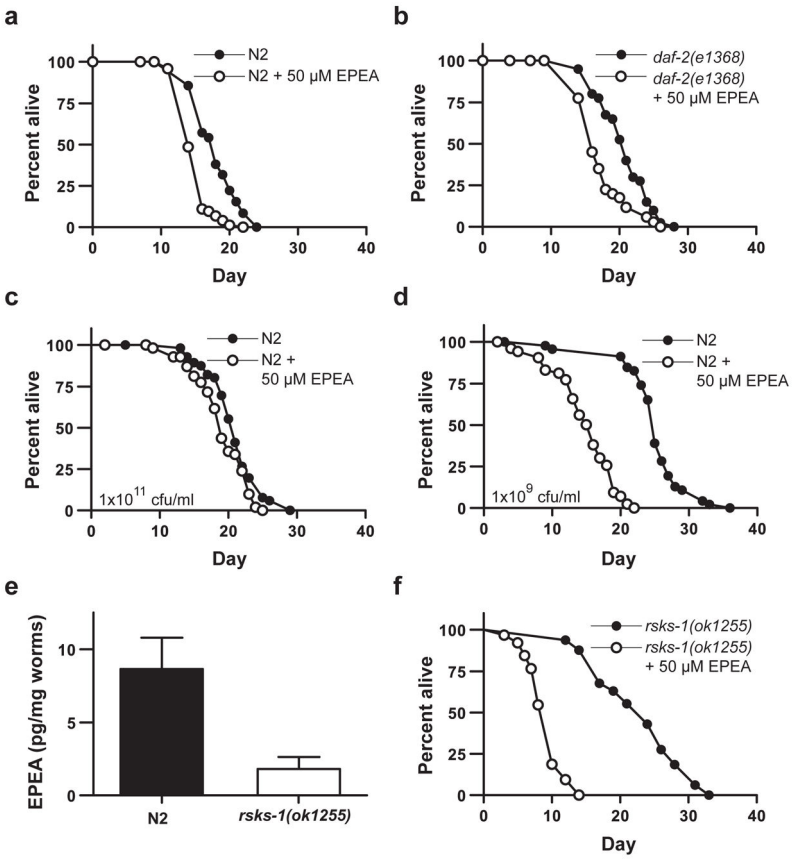


**Figure 4. EPEA suppresses the effects of dietary restriction on lifespan**

**a,** EPEA treatment reduces lifespan in wild type N2 animals on control RNAi bacteria ($p<0.0001$, Log-Rank test). **b,** EPEA treatment reduces lifespan in *daf-2(e1368)* mutants on control RNAi bacteria ($p=0.0005$, Log-Rank test). **c**, EPEA has a minimal effect on N2 lifespan in the presence of high food concentrations ($1\times10^{11}$ cfu/ml *E. coli*, $p<0.0001$, Log-Rank test). **d,** EPEA treatment completely suppresses the effect of optimal DR on wild type N2 lifespan ($1\times10^{9}$ cfu/ml *E. coli*, $p<0.0001$ Log-Rank test). **e**, EPEA levels are reduced in *rsks-1(ok1255)* mutants, a genetic model of DR (mean+s.d., n=4, $p<0.05$, Mann-Whitney U test). **f,** EPEA treatment suppresses lifespan extension in *rsks-1(ok1255)* mutants ($p<0.0001$, Log-Rank test).