Extended Data

A lipid droplet-peroxisome network drives longevity by monounsaturated fatty acids via modulating ether lipid synthesis and ferroptosis

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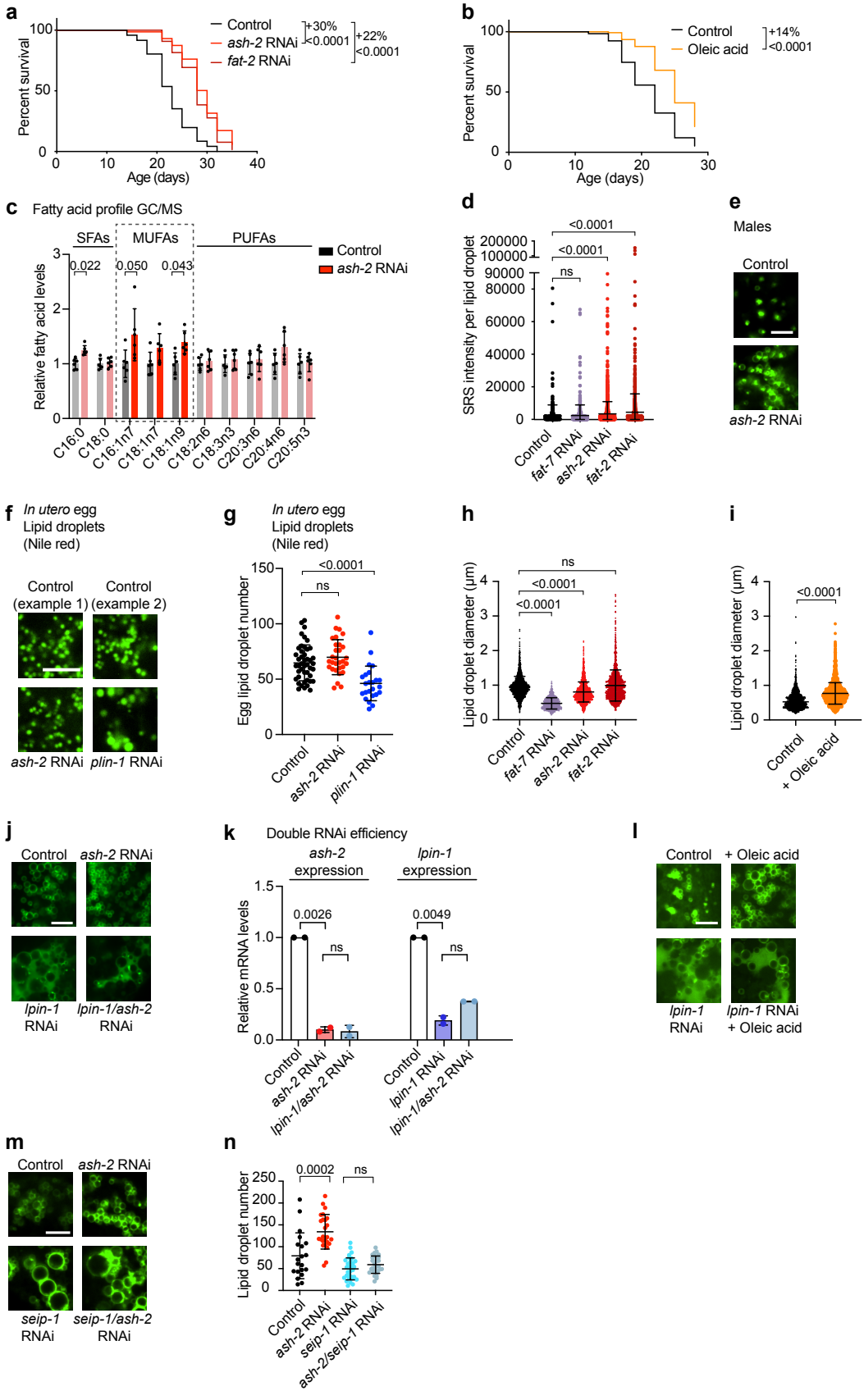
Extended Data Figure 1



Extended Data Figure 1.

a, Kaplan Meier survival curve of worms treated with control (empty vector) RNAi, and *ash-2* or *fat-2* RNAi (MUFA-enriched). n≥ 92 worms for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentage of median lifespan extension and *P*-values are indicated on the right. *P*-values log-rank Mantel-Cox test.

b, Kaplan Meier survival curve of worms with oleic acid supplementation (MUFA-enriched). n≥ 135 worms for each condition. Representative of three independent experiment (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values as in a.

c, Fatty acid profile by gas-chromatography coupled to mass spectrometry (GC/MS) of MUFA-enriched worms treated with control (empty vector) or *ash-2* RNAi. Fatty acid levels were normalized to the control condition. Data are mean ± s.d of two independent experiments each with three biological replicates (see Source Data Extended Data Figure 1 for all experiment data and statistics). *P*-values: two-tailed Mann-Whitney test with Benjamini-Hochberg test for multiple hypothesis correction. Only significant *p*-values shown. Saturated fatty acids (SFAs) and poly-unsaturated fatty acids (PUFAs) are displayed with lighter filling.

d, Lipid droplet intensity, as assessed by SRS, in worms treated with control (empty vector) RNAi, *fat-7* RNAi (MUFA-depleted), or *ash-2* or *fat-2* RNAi (MUFA-enriched). Data are mean ± s.d. SRS intensity of n≥ 420 lipid droplets in ≥ 18 worms for each condition. Each dot represents the SRS signal intensity of one lipid droplet. Lower segment of y-axis displays values from 0 - 90 000. Upper segment of y-axis from 90 000 - 200 000. See Source Data Extended Data Figure 1 for all experiment data and statistics. *P*-values: two-tailed Mann-Whitney test.

e, Intestinal lipid droplets in MUFA-enriched males measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line treated with control (empty vector) or *ash-2* RNAi. Zoomed-in fluorescent images of the mid-intestine area. Scale bar = 5 μm. Lipid droplet number quantified in Fig. 1f.

f-g, Lipid droplets of eggs (*in utero*) measured by Nile red fluorescence in MUFA-enriched worms. **f**, Fluorescent images of lipid droplet in eggs of worms treated with control (empty vector), *ash-2,* or *plin-1* RNAi. Scale bar = 5 μm. **g,** Quantification of lipid droplet number in worms treated as in f. *n* ≥ 24 eggs in ≥ 15 worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 1 for all experiment data and

statistics). Data are mean ± s.d. Each dot represents the lipid droplet number in a 16 x 16 μm^2 area of an individual egg. *P*-values: two-tailed Mann-Whitney test.

h-i, Lipid droplet size, measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line, in MUFA-enriched worms. **h,** Quantification of lipid droplet diameter of worms treated with control (empty vector) RNAi, *fat-7* RNAi (MUFA-depleted), or *ash-2* or *fat-2* RNAi (MUFA-enriched). Diameter of $n \geq 875$ lipid droplets in ≥ 14 worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 1 for all experiment data and statistics). Data are mean ± s.d. Each dot represents the diameter of one lipid droplet. *P*-values: two-tailed Mann-Whitney test. **i,** Quantification of lipid droplet diameter upon dietary oleic acid supplementation. Diameter of $n \geq 1299$ lipid droplets in ≥ 20 worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 1 for all experiment data and statistics). Data and *P*-values as in **h**.

j, Intestinal lipid droplets, measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line, in MUFA enriched and lipid droplet depleted worms. Zoomed-in fluorescent images of the mid-intestine area of worms treated with control (empty vector), *ash-2*, *lpin-1*, or *ash-2/lpin-1* RNAi. Scale bar = 5 µm. See Fig. 1n for lipid droplet quantification.

k, Double RNAi is efficient in conditions using two RNAi constructs. RT-qPCR on RNA extracted from whole worms treated with control (empty vector), *ash-2*, *lpin-1* or *ash-2/lpin-1* RNAi. The mRNA levels of target genes relative to *act-1* mRNA levels were normalized to the empty vector controls. Data are mean ± s.d. of 2 independent experiments, with 3 replicates each (see Source Data Extended Data Figure 1 for all experiment data and statistics). *P*-value: two-tailed Mann-Whitney test.

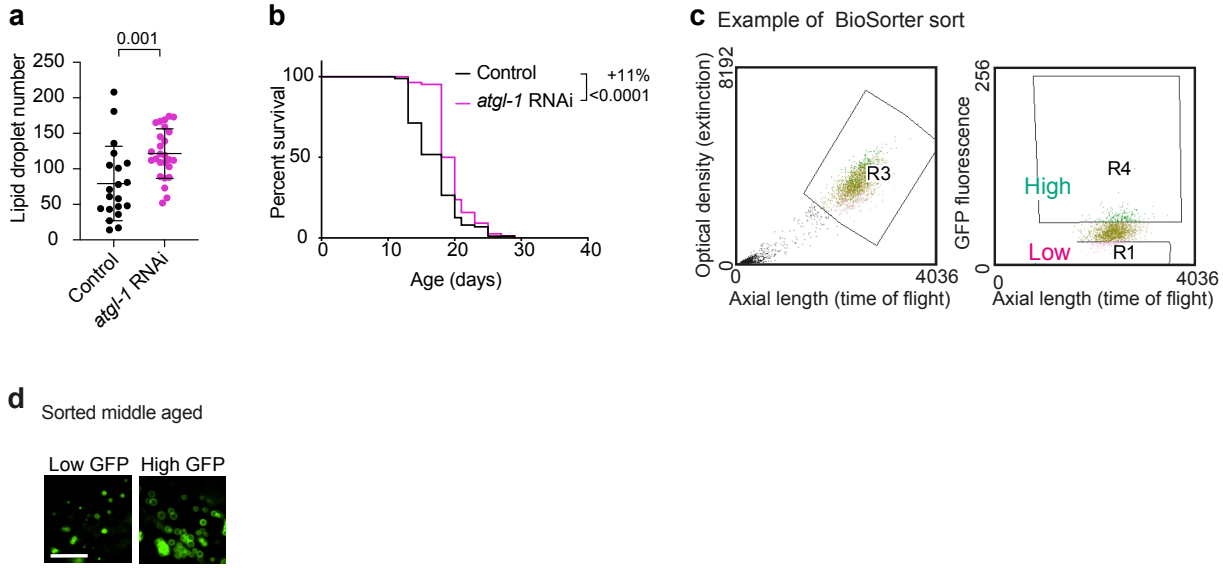
l, Intestinal lipid droplets, measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line, in MUFA enriched and lipid droplet depleted worms. Zoomed-in fluorescent images of the mid-intestine area of worms treated with control (empty vector) or *lpin-1* RNAi or upon dietary oleic acid. Scale bar = 5 µm. See Fig. 1o for lipid droplet quantification.

m-n, *seip-1* is required for lipid droplet increase upon *ash-2* knock-down. Intestinal lipid droplet number, measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line, in MUFA-enriched worms and lipid droplet depleted worms. **m,** Lower panels: Zoomed-in fluorescent images of the mid-intestine area in worms treated with control (empty vector), *ash-2*, *seip-1,* or *ash-2/seip-1* RNAi. Scale bar = 5 µm. **n,** Quantification of lipid droplet number in worms treated as in m. $n \geq 20$ worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 1 for all experiment data and statistics). Data are mean ± s.d.

Each dot represents the lipid droplet number in a 26 x 26 μm^2 area in the intestine of an individual worm. *P*-values: two-tailed Mann-Whitney test.

All experiment data and statistics are in Source Data Extended Data Figure 1. All lifespan data and statistics are in Extended Data Table 1.

Extended Data Figure 2



Extended Data Figure 2.

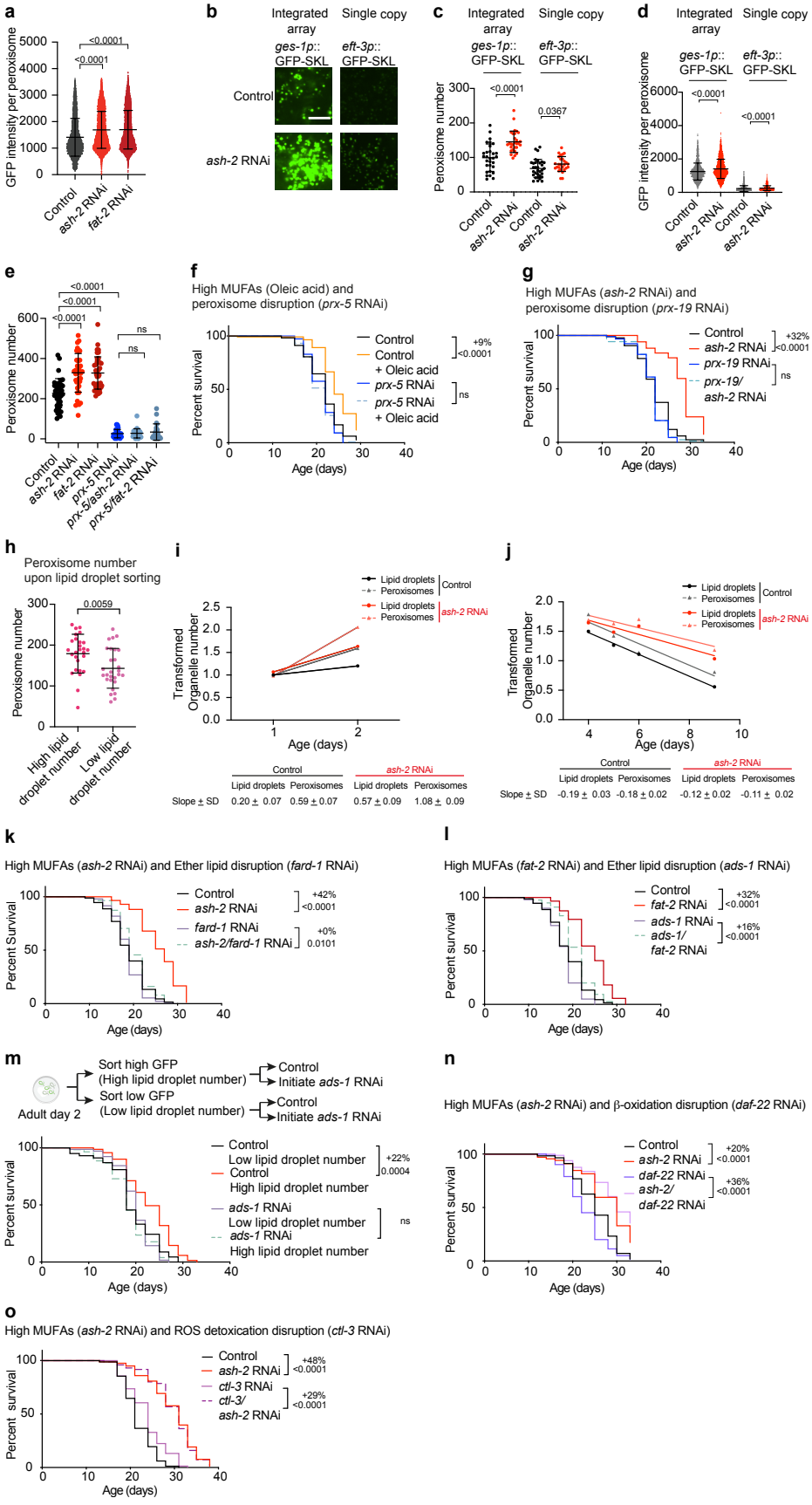
a, *atgl-1* knockdown leads to increased lipid droplet number. Intestinal lipid droplet number, measured by fluorescence in the *dhs-3p::dhs-3::GFP* transgenic line, in lipid droplet degradation depleted worms. Quantification of lipid droplet number in worms treated with control (empty vector) or *atgl-1* RNAi. $n \geq 20$ worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 2 for all experiment data). Data are mean ± s.d. Each dot represents the lipid droplet number in a 26 x 26 μm^2 area in the intestine of an individual worm. *P*-values: two-tailed Mann-Whitney test.

b, *atgl-1* knockdown extends lifespan. Kaplan Meier survival curve of wildtype worms treated with control (empty vector) or *atgl-1* RNAi. n ≥ 108 for each condition (see Extended Data Table 1 for all lifespan experiments and statistics). The percentage of median lifespan extension are indicated on the right. *P*-values: log-rank Mantel-Cox test.

c, BioSorter sorting example separating an age-synchronized population of the *dhs-3p::dhs-3::GFP* transgenic line according to their GFP fluorescence intensity (as a proxy for lipid droplet number). Left panel: Optical density (extinction) and axial length (time of flight) of all particles measured by the sorter. Gate R3 was used to exclude bacterial debris and eggs and enrich for adult hermaphrodite individual worms. Right panel: individual worms from Gate R3 were sorted by GFP fluorescence intensity to separate the highest 10% fluorescent worms (Gate R4) from the lowest 10% fluorescent worms (Gate R1).

d, When sorted at middle age, high fluorescent DHS-3::GFP worms have a higher intestinal lipid droplet number than low fluorescent worms. Lipid droplet number was assessed after manual sorting of a synchronized population of middle-aged worms (adult day 6) of the *dhs-3p::dhs-3::GFP* transgenic line. Zoomed-in fluorescent images of the mid-intestine area. Scale bar = 5 μm. Quantification of lipid droplet number in worms shown in Fig. 2g.
All experiment data and statistics are in Source Data Extended Data Figure 2. All lifespan data and statistics are in Extended Data Table 1.

Extended Data Figure 3



Extended Data Figure 3.

a, Increased GFP intensity in peroxisomes upon MUFA enrichment. Intestinal peroxisomes, measured by fluorescence in the *ges-1p::GFP-SKL* transgenic line, in MUFA-enriched worms. Quantification of peroxisome localized GFP intensity in worms treated with control (empty vector) RNAi or *ash-2* or *fat-2* RNAi (MUFA-enriched). GFP intensity of $n \geq 6061$ peroxisomes in ≥ 35 worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 3 for all experiment data and statistics). Data are mean ± s.d. Each dot represents the GFP intensity of one peroxisome. *P*-values*:* two-tailed Mann-Whitney test. **b-d,** Intestinal peroxisomes measured by fluorescence in the *ges-1p::GFP-SKL* transgenic line and the single-copy knock-in[1] *eft-3p::GFP-SKL* line. **b,** Zoomed-in fluorescent images of the last intestinal cell in worms treated with control (empty vector) or *ash-2* RNAi. Scale bar = 5 μm. **c,** Quantification of peroxisome number in worms treated as in b. $n \geq 27$ worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 3 for all experiment data and statistics). Data are mean ± s.d. Each dot represents the peroxisome number in a 13 x 13 μm^2 area in the intestine of an individual worm. A smaller region of interest was chosen to not count hypodermal peroxisomes in the *eft-3p::GFP-SKL* line*. P*-values*:* two-tailed Mann-Whitney test. **d,** Quantification of peroxisome-localized GFP intensity in worms treated with control (empty vector) or *ash-2* RNAi. GFP intensity of $n \geq 1537$ peroxisomes in ≥ 22 worms for each condition. Representative of two independent experiments (see Source Data Figure 3 for all experiment data and statistics). Data are mean ± s.d. Each dot represents the GFP intensity of one peroxisome. *P*-values*:* two-tailed Mann-Whitney test.

e, *prx-5* is required for peroxisome number increase in MUFA-enriched worms. Intestinal peroxisomes measured by fluorescence in the *ges-1p::GFP-SKL* transgenic line. Quantification of peroxisome number in worms treated with control (empty vector), *ash-2, fat-2, prx-5, ash-2/prx-5,* or *fat-2/prx-5* RNAi. $n \geq 22$ worms for each condition. Representative of three independent experiments (see Source Data Extended Data Figure 3 for all experiment data and statistics). Data are mean ± s.d. Each dot represents the peroxisome number in a 26 x 26 μm^2 area in the intestine of an individual worm. *P*-values*:* two-tailed Mann-Whitney test.

f, *prx-5* is necessary for longevity upon MUFA enrichment by dietary oleic acid. Kaplan Meier survival curve of worms treated with control (empty vector) or *prx-5* RNAi upon dietary oleic acid. n ≥ 120 for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan

extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *prx-5* RNAi and oleic acid significantly interact with each other using the Cox proportional hazard test (*P*-value= 8.26e-06).

g, *prx-19*, a gene involved in peroxisome function, is necessary for longevity upon MUFA enrichment by *ash-2* depletion. Kaplan Meier survival curve of worms treated with control (empty vector), *ash-2, prx-19*, or *ash-2/prx-19* RNAi. n ≥ 95 for each condition (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *ash-2* and *prx-19* RNAi significantly interact with each other using the Cox proportional hazard test (*P*-value= 2.76e-07).

h, Peroxisome number correlates with lipid droplet number. Intestinal peroxisomes measured by fluorescence in the *dhs-3p::dhs-3::GFP*, *vha-6p::mRFP-SKL* transgenic line. Quantification of peroxisome number in worms sorted based on high and low lipid droplet number using the BioSorter at middle age (adult day 6). Representative of two independent experiments (see Source Data Extended Data Figure 3 for all experiment data and statistics). $n \geq 27$ worms for each condition. Data are mean ± s.d. Each dot represents the organelle number normalized in a 26 x 26 μm^2 area in the intestine of an individual worm.

i-j, Peroxisome number and lipid droplet number increase and decrease with similar dynamics. Intestinal peroxisomes and lipid droplets measured by fluorescence in the *dhs-3p::dhs-3::GFP*, *vha-6p::mRFP-SKL* transgenic line. Quantification of organelle numbers in worms treated with control (empty vector) or *ash-2* RNAi. $n \geq 23$ worms for each condition. Representative of two independent experiments (see Source Data Extended Data Figure 3 for all experiment data and statistics). Data are mean ± s.d. normalized to young adult control worms. Each dot represents the mean organelle number in a 26 x 26 μm^2 area in the intestine of all worms imaged for this condition normalized to control worms. **i,** organelle number increase at younger ages and **j,** organelle number degradation at older ages. A linear regression line is fitted with the slope describing the directionality and effect size.

k, *fard-1*, a gene involved in ether lipid synthesis, is necessary for longevity upon MUFA enrichment by *ash-2* depletion. Kaplan Meier survival curve of worms treated with control (empty vector), *ash-2, fard-1*, or *ash-2/fard-1* RNAi. n ≥ 92 for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *ash-2* and *fard-1* RNAi significantly interact with each other

using the Cox proportional hazard test (*P*-value= 1.35e-06). The controls for this lifespan curve are shared with Fig. 3m.

l, *ads-1*, a gene involved in ether lipid synthesis, is necessary for longevity upon MUFA enrichment by *fat-2* depletion. Kaplan Meier survival curve of worms treated with control (empty vector), *fat-2, ads-1*, or *fat-2/ads*-1 RNAi. n ≥ 94 for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *fat-2* and *ads-1* RNAi do not significantly interact with each other using the Cox proportional hazard test (*P*-value= 0.097). The controls for this lifespan curve are shared with Fig. 3m.

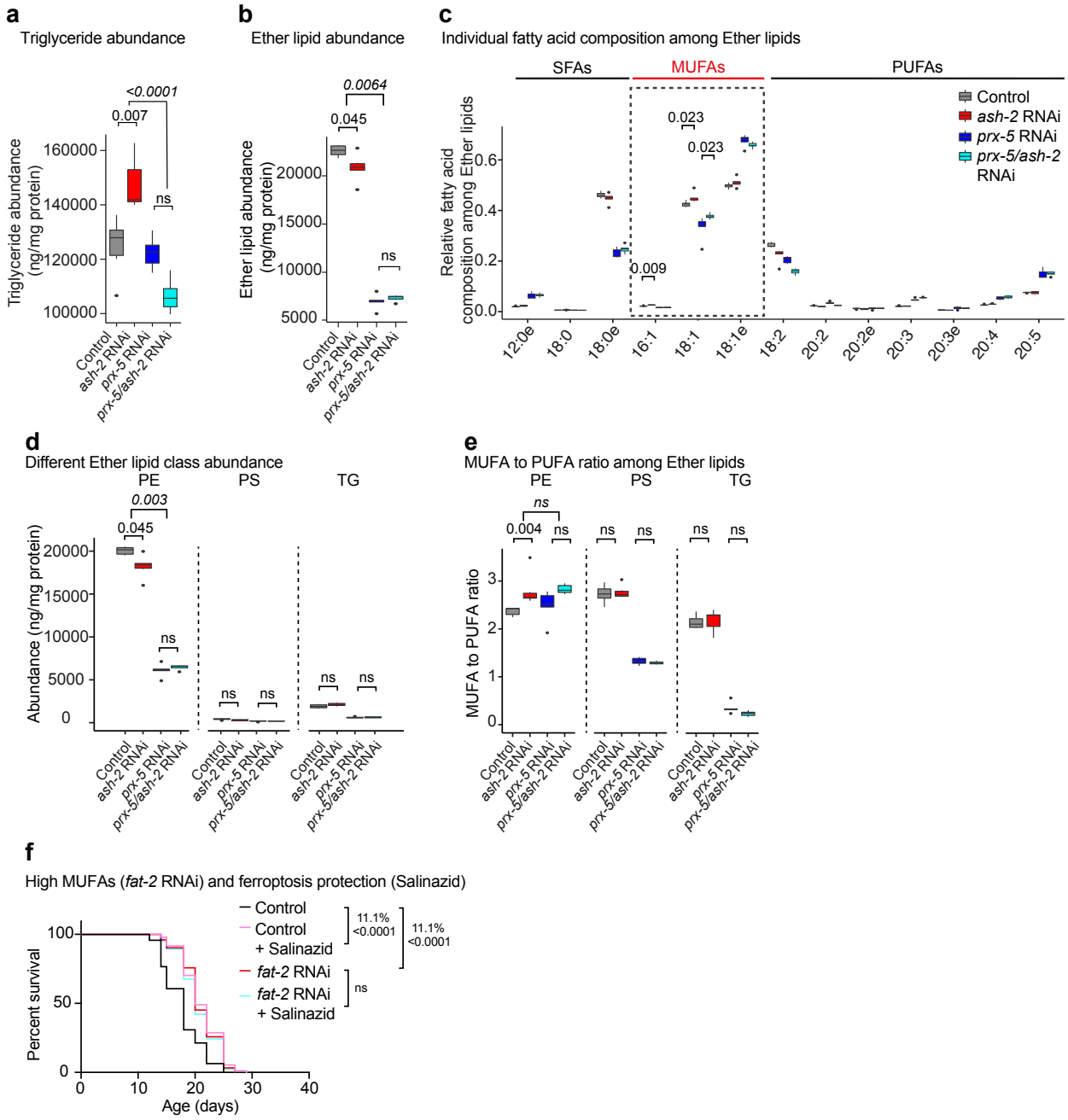
m, *ads-1*, a gene involved in ether lipid synthesis, is necessary for longevity upon high lipid droplet number. Kaplan Meier survival curve of worms manually sorted into high and low lipid droplet number and subsequently treated with control (empty vector) or *ads-1* RNAi. n ≥ 77 for each condition (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. Lipid droplet number and *ads-1* RNAi significantly interact with each other using the Cox proportional hazard test (*P*-value= 0.027830).

n, *daf-22*, a gene involved in b-oxidation, is not required for longevity upon MUFA enrichment by *ash-2* RNAi. Kaplan Meier survival curve of worms treated with control (empty vector), *ash-2, daf-22*, or *daf-22/ash-2* RNAi. n ≥ 66 for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *ash-2* and *daf-22* RNAi significantly interact with each other using the Cox proportional hazard test (*P*-value= 0.002560).

o, *ctl-3*, a gene involved in reactive oxygen species detoxification, is not required for longevity upon MUFA enrichment by *ash-2* RNAi. Kaplan Meier survival curve of worms treated with control (empty vector), *ash-2, ctl-3*, or *ctl-3/ash-2* RNAi. n ≥ 83 for each condition. Representative of two independent experiments (see Extended Data Table 1 for all lifespan experiments and statistics). Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *ash-2* and *ctl-3* RNAi significantly interact with each other using the Cox proportional hazard test (*P*-value= 0.009083).

All experiment data and statistics are in Source Data Extended Data Figure 3. All lifespan data and statistic are in Extended Data Table 1.

Extended Data Figure 4



Extended Data Figure 4.

a, Triglycerides increase upon MUFA-enrichment (*ash-2* RNAi), which is dependent on functional peroxisomes (*prx-5* and *prx-5/ash-2* RNAi). Triglyceride abundance among all lipids in middle-aged worms (adult day 6) treated with control (empty vector), *ash-2*, *prx-5*, or *ash-2/prx-5* RNAi. Six biological replicates of ~500 individual worms per condition. Box and whisker plot, with median (central line), 25th and 75th percentile (outer lines), and minimum and maximum within 1.5 times the interquartile range (whiskers). Values beyond these are

considered outliers and plotted individually. *P*-values: Unpaired two-samples Wilcoxon test with Benjamini-Hochberg test for multiple hypothesis correction. Italic *P*-values: two-way ANOVA Complete list of all detected lipid classes and statistics is in (Source Data Extended Data Figure 4).

b, Ether lipid abundance upon MUFA enrichment and upon inhibition of functional peroxisomes. Ether lipid abundance in middle aged worms (adult day 6) treated as in a. Box and whisker plot, with median (central line), 25th and 75th percentile (outer lines), and minimum and maximum within 1.5 times the interquartile range (whiskers). Values beyond these are considered outliers and plotted individually. *P*-values: Unpaired two-samples Wilcoxon test with Benjamini-Hochberg test for multiple hypothesis correction. Italic *P*-values: two-way ANOVA. Complete list of all detected ether lipid classes, their sum and statistics is in (Source Data Extended Data Figure 4).

c, Relative abundance of individual fatty acids in ether lipids upon MUFA enrichment and inhibition of functional peroxisomes. Relative fatty acid composition in middle-aged worms (adult day 6) treated as in a. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Box and whisker plot, with median (central line), 25th and 75th percentile (outer lines), and minimum and maximum within 1.5 times the interquartile range (whiskers). Values beyond these are considered outliers and plotted individually. *P*-values: Unpaired two-samples Wilcoxon test with Benjamini-Hochberg test for multiple hypothesis correction. *P*-values for MUFAs are displayed all *P*-values are in (Source Data Extended Data Figure 4). Only abundant fatty acids with a relative abundance >0.0045 are plotted and only significant *p*-values for MUFAs are shown. All fatty acids in ether lipids and statistics are in (Source Data Extended Data Figure 4).

d, Phosphatidylethanolamine (PE) is the most abundant ether-lipid class. Abundance of different ether-lipid classes in worms treated as in a. Phosphatidylserine (PS), triglyceride (TG). Only abundant ether lipid classes with an abundance >250 are plotted. Box and whisker plot, with median (central line), 25th and 75th percentile (outer lines), and minimum and maximum within 1.5 times the interquartile range (whiskers). *P*-values: Unpaired two-samples Wilcoxon test with Benjamini-Hochberg test for multiple hypothesis correction. Italic *P*-values: two-way ANOVA. All experiment data and statistics are in (Source Data Extended Data Figure 4).

e, MUFA to PUFA ratio among different ether-lipid classes in worms treated as in a. Phosphatidylethanolamine (PE), phosphatidylserine (PS), triglyceride (TG). Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Box

and whisker plot, with median (central line), 25th and 75th percentile (outer lines), and minimum and maximum within 1.5 times the interquartile range (whiskers). *P*-values: Unpaired two-samples Wilcoxon test with Benjamini-Hochberg test for multiple hypothesis correction. Italic *P*-values: two-way ANOVA. All experiment data and statistics in (Source Data Extended Data Figure 4).

f, Ferroptosis protection does not further increase MUFA-mediated longevity. Kaplan Meier survival curve of worms treated with control (empty vector) or *fat-2* RNAi upon Salinazid. n ≥ 110 for each condition. Percentages of median lifespan extension and *P*-values are indicated on the right. *P*-values: log-rank Mantel-Cox test. *fat-2* RNAi and Salinazid significantly interact with each other using the Cox proportional hazard test (*P*-value= 2.53e-05). Representative of one independent experiment (see Extended Data Table 1 for all lifespan experiments and statistics). Controls are also plotted in Fig. 4j.

All experiment data and statistics are in Source Data Extended Data Figure 4. All lifespan data and statistics are in Extended Data Table 1.

References

1 Silva-García, C. G. *et al.* Single-Copy Knock-In Loci for Defined Gene Expression in Caenorhabditis elegans. *G3 (Bethesda)* **9**, 2195-2198, doi:10.1534/g3.119.400314 (2019).