SUPPLEMENTARY INFORMATION

for

"A whole organism screen identifies novel regulators of fat storage and feeding"

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SUPPLEMENTARY METHODS

Materials. S-basal and S-medium were prepared as described previously [1].

Nematode culture. Unless described otherwise *C. elegans* strains were cultured as described [2] on NGM-agar plates at 20 °C using *E. coli* OP-50 bacteria as a food source.

Compound Screen. Preparation of synchronized L1 animals: 50 ml aliquot of a mixed-stage liquid culture of *C. elegans* was centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the pellet was washed twice with 25 ml of 0.1 M NaCl. The washed pellet was resuspended in 20 ml of 0.1 M NaCl (4 °C). Ice cold 60% sucrose (20 ml) was added and mixed rapidly. After mixing, 5 ml of cold 0.1 M NaCl was overlaid. The biphasic suspension was centrifuged at 3000 rpm for 5 min. The nematodes at the interface of the sucrose/0.1 M NaCl layer were transferred to a new tube containing 20 ml of S-basal medium. The nematodes were collected by centrifugation at 3000 rpm for 3 min and washed with 20 ml of S-basal. The washed nematode pellet was suspended in 25 ml of cold alkaline-hypochlorite (8 ml of 5% NaOCl w/v, 5 ml of 5 N KOH, 12 ml of water) and rocked, end-over-end for 5-10 min at room temperature. Cold 0.1 M sodium thiosulfate (13 ml) was added and mixed thoroughly. Cold 1 M citric acid (9 ml) was then added and mixed. The suspension was then centrifuged for 5 min at 3000 rpm and then washed twice with 25 ml of S-medium. The purified eggs were then suspended in 25 ml of S-medium-B (S-medium with 0.1% PEG-8000) and allowed to hatch at 20 °C overnight.

Setting up the screen: The suspension of synchronized first larval stage (L1) animals was filtered through a 40 µM nylon filter (BD Biosciences), and centrifuged at 1500 rpm for 3 min. The nematodes were re-suspended in S-medium-B containing 0.5% low-melt agarose at a final concentration of 2-4 nematodes/µl. Aliquots of the nematode suspension were transferred to 384-well plates (5 µl per well: 10 to 20 animals). Library compounds (average concentration: 5 mM in DMSO) or controls were diluted 1:830 in complete growth medium: S-medium-B, supplemented with carbenicillin (50 µg/ml), fungizone (100 ng/ml), *E. coli* OP-50 (5 mg/ml) and Nile Red (100 nM). The diluted compounds (25 µl per well) were added to the plates containing *C. elegans*. The plates were covered with a gas permeable adhesive film (Breatheasy, Diversified Biotech) and were incubated in a humidified atmosphere for 48 hr at 20 °C.

Image acquisition and analysis: Immediately prior to image acquisition, the nematodes were anaesthetized with 20 mM NaN_3. Bright field and fluorescence images (5 independent fields per well) were acquired on a Nikon TE-2000E inverted stage automated epifluorescence microscope equipped with a 100×, 0.45 NA objective. Nile Red fluorescence images were acquired using an excitation source of 540-560 nm and an emission bandpass filter of 580-630 nm. Images from experimental wells in a given row in each plate were compared to images from the row's control (DMSO) wells. The Nile Red staining intensity of the anterior intestine of the nematodes was used to score the animals as either low fat or high fat. Only wells with highly penetrant phenotypes (>75% of the animals exhibiting a phenotype) were scored as hits. Instances were also noted where the distribution or texture of Nile Red staining lipid droplets appeared to change

(for example hazy versus punctate). Wells containing animals that exhibited either delayed or arrested development were also noted. Compounds that induced phenotypes in the primary screen were retested using Nile Red and BODIPY-FA (100 ng/ml final concentration) to confirm the activity of the compounds.

Nematode Compound treatments.

i) Liquid culture: A bacterial suspension of OP-50 (5 mg/ml) in S-medium-B containing either Nile Red (50 nM) or BOPDIPY-fatty acids (100 ng/ml) was used to dilute stock solutions of experimental compounds or vehicle controls. The diluted solution was then added to either synchronized larvae or eggs (50-60/well) in a 24-well plate. The animals were cultured for 2-3 days prior to imaging.

ii) NGM-agar: Compounds were diluted in 50 µl of a concentrated stock of *E.coli* OP-50 (100 mg/ml) containing either Nile Red (500 nM) or BOPDIPY-fatty acid (1 µg/ml). The mixture was added to wells in a 24-well plate containing 1 ml of NGM-agar medium. After the plates absorbed the liquid suspension (2-4 hr), 20-30 L1 stage nematodes or eggs were added per well. The animals were then cultured for 2-3 days prior to imaging.

Triacylglyceride assay. Adapted from the method of Bligh and Dyer [3]. L4 nematodes from a liquid culture of approximately 5000 animals were washed three times by centrifugation and resuspension in 10 ml S-basal medium supplemented with 0.1% PEG-8000. The nematode population was finally suspended in approximately 200 µl of S-basal + 0.1% PEG-8000. 50 µl of this suspension was reserved for protein determination and 100 µl of suspension was diluted with 59 µl of water for lipid determination. *Lipid*

determination: Chloroform (0.2 ml) and methanol (0.4 ml) was added to the 0.159 ml aqueous suspension of nematodes and mixed by periodic vigorous vortexing over 20 min. An additional 0.2 ml of chloroform and 0.2 ml of 0.2 N HCl were then added. The mixture was mixed by vigorous vortexing over 20 min, then centrifuged at 2,500 × *g* for 5 min to separate the phases. The lower phase was washed once with 0.75 ml of the aqueous phase derived from a mixture of chloroform, methanol, 0.1 N HCl (1:1:1), then concentrated by vacuum centrifugation. The residue was dissolved in 25 µl of chloroform:methanol (1:1), 10 µl of which was applied to a thin layer chromatography plate (Merck silica gel-60), along with triglyceride standards (0.5-10 µg). The samples and standards were eluted using a hexanes-ether-acetic acid mixture (70:30:1) and the plate was developed by spraying with phosphomolybdic acid stain (Sigma) and heating in a 125 °C oven for 10 min. An image of the plate was acquired using a flat bed scanner (Epson) and the integrated density of the bands that exhibited the same elution profile as the triglyceride standards was quantified. The optical densities were then converted to TAG mass by comparison to a TAG standard curve. The mass of lipids obtained from each extraction was normalized to the total protein extractable for 5-6 independent nematode cultures per experimental condition. *Total protein determination:* 250 µl of extraction buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM tris-HCl pH 7.4, 5 mM TCEP, 1 mM EDTA) was added to the 50 µl suspension of nematodes the sample was rotated end-over-end and periodically vortexed for 1 hr at 37 °C. The sample was centrifuged (10 min at 16,500 ×*g*) and the amount of protein in the supernatant was determined by Bradford assay (Bio-Rad).

Growth assay. Synchronized L1 animals (25-30 per well) were added to 96 well plates containing S-medium with 0.1% PEG-8000 and *E. coli* OP-50 (6 mg/ml). The wells contained either compound (10 μM) or DMSO (0.1%). The animals were cultured for 60 hr then anaesthetized with NaN_3. The number of animals in each well that at each stage: gravid adult, young adult, L4, L3 were counted by visual examination using a dissection microscope.

Egg laying assay. 3-5 young adult nematodes were transferred to wells of a 24-well plate containing NGM-agar, a lawn of *E.coli* OP-50, and compounds (10 μM) . The nematodes were cultured for 14 hr. The number of eggs laid in each well was counted and normalized to the number of adults in each well.

Cell culture and antibodies. 3T3-L1 preadipocytes were cultured at 37 °C under a humidified 5% CO_2 atmosphere in DMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin. HepG2 cells were cultured in MEM supplemented with 10% FBS,1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 100 U/ml of penicillin and streptomycin. *Drosophila* Schneider S2 cells were cultured at 20 °C in Schneider's Drosophila Medium supplemented with 10% FBS and L-glutamine.

3T3-L1 adipogenic differentiation assay. 3T3-L1 pre-adipocytes were seeded in 6-well plates and grown to confluence. After 1 day of culture at confluence, the medium was changed to medium supplemented with isobutyl methyl xanthine (500 μM),

dexamethasone (1 μM), insulin (1 μg/ml)). Compounds (5-20 μM) or DMSO (0.05-0.2%) were added. After 48 hr the cell culture medium was replaced with medium supplemented with insulin (1 μg/ml) and either compounds (5-20 μM) or DMSO. After 48 hr the medium was replaced and supplemented with compounds or DMSO. After two more days of culture, the cells were washed with PBS and then fixed for 1 hr at room temperature with 8% paraformaldehyde in PBS. The fixed cells were washed twice with PBS and then stained with Oil Red O staining solution [4]. The amount of Oil Red O that was retained by the cells was determined by extracting the cells with 1 ml of isopropanol and measuring the absorption at 520 nm. Alternatively, samples intended for microscopy were counterstained with Gill's haematoxylin, mounted under a coverslip and photographed under brightfield microscopy.

LipidTOX lipid droplet staining in cultured cells. *Drosophila* Schneider S2 cells (2.7×10^5 cells/well) were treated with 1mM oleate (solubilized with 0.2 mM fatty-acid free BSA) in culture medium supplemented with 20 μM experimental compound or (0.2%) DMSO for 24 hr at 20 °C. The cells were washed once, then fixed with 4% paraformaldehyde in PBS for 1 hr. The cells were then stained overnight in LipidTOX neutral lipid stain (Invitrogen) diluted 1:1000 in PBS, then mounted with Vectashield (Vector Labs) containing DAPI. Images were acquired on a laser scanning confocal microscope (Zeiss LSM 510) equipped using a 63×, 1.4 NA oil-immersion optics. The lipidTOX neutral lipid stain was excited with the 488 nm line of an argon ion laser and 500-525 nm light was collected. The DAPI staining was imaged by two-photon excitation using a 750 nm line. Alternatively HepG2 cells were cultured on coverslips and treated

with either 25 μM F17 or 0.25 % DMSO for 24 hr, then fixed and stained as above, with the exception that a red version of LipidTOX was used and imaged using 561 nm excitation and a 580-640 nm bandpass filter.

RNAi treatments. *NGM/agar based treatments:* HT115 bacteria containing an RNAi vector targeting *sbp-1* (Y47D3B.7) [5] or a control vector were grown overnight, induced with 4 mM IPTG for 1 hr at 37 °C, pelleted and re-suspended in 0.5 culture volumes of NGM medium containing 2 μM Nile Red, 100 μg/ml ampicillin and 4 mM IPTG. Compounds or DMSO were diluted in 50 μl of the HT115 mixture and added to 24 well plates containing 1 ml of NGM agar, 6 mM IPTG, and 25 mg/ml carbenicillin. After equilibration overnight at 37 °C the plates were cooled to 20 °C and 20-30 synchronized first larval stage nematodes were added to each well and incubated at 20 °C for 3 days. The Nile Red fluorescence of gravid adults was then quantified by integrating the fluorescence intensity of the two anterior-most pairs of intestinal cells.

Liquid culture RNAi suppressor screening [6]: Arrayed HT115 bacterial clones containing transcription factors and kinases derived from a genome-wide library [5] were replica-plated into 96-well plates and cultured in 0.2 ml of LB medium, on a rotary shaker overnight at 37 °C, under ampicillin selection (100 μg/ml). The next day, the bacterial cultures were treated for 2 hr at 37 °C with 4 mM IPTG. The bacteria were then concentrated by centrifugation (3,500 × *g*, 10 min) and resuspended in 0.1 ml of S-medium B supplemented with peptone (3 g/l), ampicillin (100 μg/ml), IPTG (4 mM) and Nile Red (100 nM). The bacterial clones were arrayed in duplicate, in adjacent wells of 384-well plates (20 μl of suspension/well). 5-10 L1 nematodes were added per well in 5

μl aliquots and the animals were cultured for 2 d at 20 °C. Then F17 (5 μl of a 60 μM solution) was added to every other column of clones, and DMSO (5 μl of a 0.6% solution of DMSO/well) was added to the adjacent columns. The nematodes were cultured for 1 d at 20 °C and then candidate RNAi suppressors of F17's Nile Red reducing effects were identified by visual screening of the plates.

Liquid culture based RNAi treatments for *fat-7* (F10D2.9), or K08F8.2 were performed similar to the protocol described for suppressor screening except that more animals (25-50) were cultured in larger format (24-well) plates in larger volumes of liquid medium (0.25 ml). After the third day of culture, the animals were washed free of bacteria, anaesthetized with NaN_3 and imaged. The Nile Red fluorescence of gravid adults was then quantified by integrating the fluorescence intensity of the two anterior-most pairs of intestinal cells.

Assay of ACC and AMPK phosphorylation. HepG2 cells were treated with either 30 μM F17, 30 μM F21, 0.3% DMSO, 30 μM F17 + 20 μM Compound C (Calbiochem) for 16 hr or with AICAR (2 mM) or DMSO (0.2%) for 2 hr. Cells were washed, then lysed at 4 °C in lysis buffer (25 mM Tris-HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM NaF, 2 mM EDTA, protease and phosphatase inhibitors (Complete, Roche). The crude lysate was clarified by centrifugation at 16,000 × *g* for 10 min, separated by SDS-PAGE and blotted on PVDF membranes. Membranes were probed with rabbit polyclonal antibodies directed against either phospho-S79-ACC or phospho-T172-AMPK, followed by a horseradish peroxidase conjugated secondary goat anti-rabbit IgG. The chemiluminescence of each

blot was quantified using a cooled-CCD imaging system (LSM-3000, Fuji) The blots were then stripped and re-probed with antibodies directed against ACC and AMPK-α. The blots were developed and imaged as for the phospho-specific antibodies. The relative phosphorylation levels of each lane were quantified by measuring the integrated intensity using ImageJ, and normalized to the intensity from the DMSO control lane. The relative levels of phosphorylation were then normalized to the relative levels of the target protein for each condition.

Statistical Analysis. A two-tailed student's *t*-test was used to calculate the significance levels for feeding rate, FAT-7 expression, vital dye staining and ACC/AMPK phosphorylation levels.

References

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4. Stotz, H. *et al.* Oil red O: Comparison of staining quality and chemical components as determined by thin layer chromatography. *Stain Technol*. **61**, 187-190 (1986).
5. Kamath, R.S. *et al.* Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature*. **421**, 231-7 (2003).
6. Lehner, B.,Tischler, J. & Fraser, A.G. RNAi screens in Caenorhabditis elegans in a 96-well liquid format and their application to the systematic identification of genetic interactions. *Nat Protoc. 2006;1(3):1617-20.*

SUPPLEMENTARY RESULTS

Supplementary Figures S1-S7

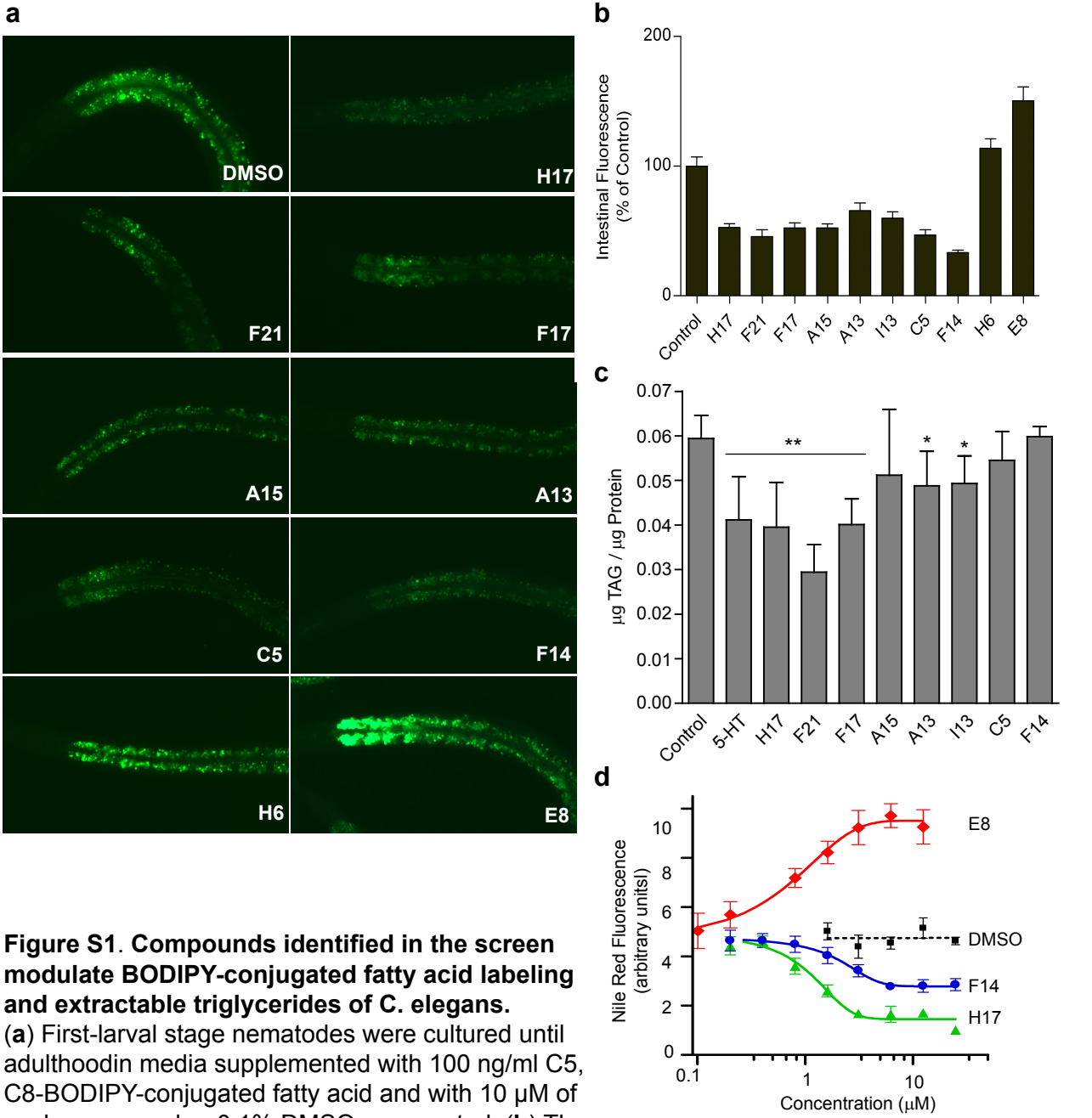
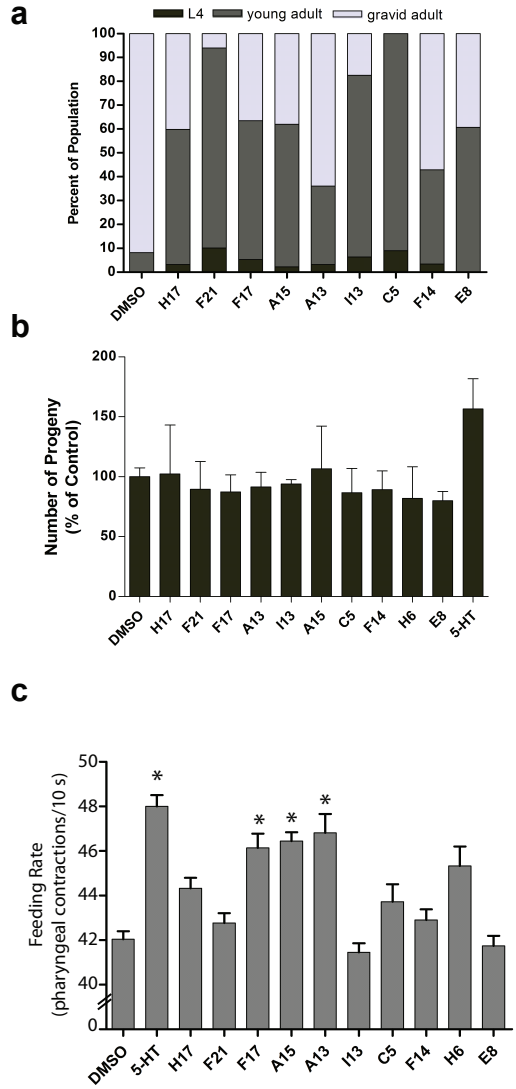


Figure S1. **Compounds identified in the screen modulate BODIPY-conjugated fatty acid labeling and extractable triglycerides of C. elegans.** (**a**) First-larval stage nematodes were cultured until adulthoodin media supplemented with 100 ng/ml C5, C8-BODIPY-conjugated fatty acid and with 10 µM of each compound or 0.1% DMSO as a control. (**b**) The integrated fluorescence intensity of the two anterior-most pairs of intestinal cells was quantified for 5-7 animals per condition. (**c**) First-larval stage nematodes were cultured in media supplemented with10 µM compound, 5 mM serotonin (5-HT) as a positive control or 0.1% DMSO as negative control.Chloroform extracts of fourth-larval stage animals were separated by thin layer chromatography, and quantified by image densitometry comparison to a triglyceride (TAG) standards titration. Total protein in urea extracts from a reserved aliquot was quantified by Bradford assay. The TAG to protein ratio was determined for 5-6 independent nematode cultures per condition. Error bars represent the standard deviation. ** $p < 0.001$, * $p < 0.02$ two-tailed t-test. (**d**) The dose-dependence of the Nile Red phenotype in animals treated with different concentrations of each compound. The integrated intensities of the anterior intestinal cells of 7-10 animals per condition were quantified. Error bars represent the standard error of the mean.

Figure S2. Effects on growth, fecundity and feeding of compounds that modulate Nile Red staining. (**a**) Developmentally synchronized L1 C. elegans were cultured in liquid medium at 20 °C with 10 μM of each compound or 0.1% DMSO as a control. The percentage of animals of each developmental stage at 60 hr of development time is shown: black: L4, dark grey: young adult, light grey: gravid adult. (**b**) The number of eggs laid per adult animal under each condition was scored manually and normalized to that of the DMSO treated control. 5 mM 5-hydroxytryptamine (5HT) was used as positive control. Error bars represent the standard error of the mean for three independent experiments. (**c**) To measure feeding rates, first larval stage animals were cultured until adulthood on NGM plates containing either 10 μM compound, 5 mM 5-hydroxy-tryptamine, (5-HT, positive control) or DMSO control. The number of pharyngeal contractions in a 10 s interval was recorded for 10-15 animals per condition. Error bars represent the standard error of the mean.* $p < 0.001$ two-tailed t-test.



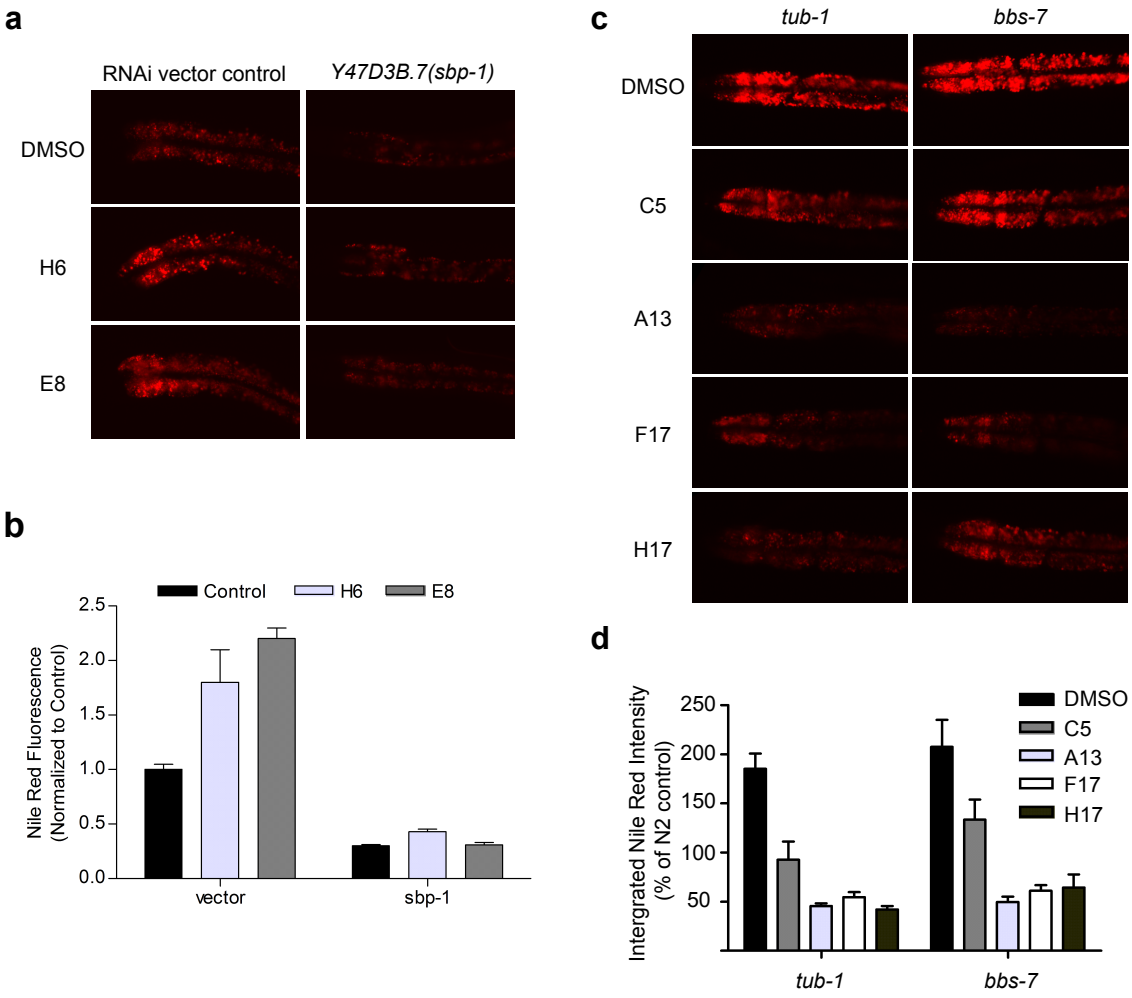


Figure S3. The high Nile Red-staining phenotypes induced by E8 and H6 are dependent on the C. elegans ortholog of SREBP (*sbp-1*) and compounds that reduce Nile Red staining in wild-type animals similarly reduce staining in nematodes that have mutations in homologs of human obesity-linked genes. (**a**) *C. elegans* cultured on HT115 E .coli expressing either a control double stranded RNA or double stranded RNA targeting sbp-1 were concurrently treated with Nile Red and either 10 μM compound or 0.1% DMSO as a control. Animals were imaged as adults. (**b**) The integrated fluorescence intensity of the two-anterior most pairs of intestinal cells was quantified for 5-7 animals per condition. Error bars represent the standard error of the mean. (**c**) Gravid adult *tub-1* and *bbs-7* animals were cultured for 2 days on 10 μM compound or DMSO (0.1%) in medium supplemented with Nile Red. (**d**) Quantification of Nile Red intensity in the anterior intestinal cells of compound-treated *tub-1* and *bbs-7* animals normalized to DMSO treated wild-type nematodes (not shown). Relative to control treated animals, C5 reduced staining by 51% in wild-type, 50% in *tub-1* and 35% *bbs-7*. A13 reduced staining by 82% in wild-type, 76% in *tub-1*, and 76% in *bbs-7* mutants. F17 reduced staining by 85% wild-type, 71% in *tub-1*, and 70% in *bbs-7* mutants. H17 reduced staining by 95% in wild-type, 78% in *tub-1* and 69% in *bbs-7* mutants. The fluorescence intensity of the two anterior pairs of intestinal cells was quantified for 5-7 animals per condition. Error bars represent the standard error of the mean.

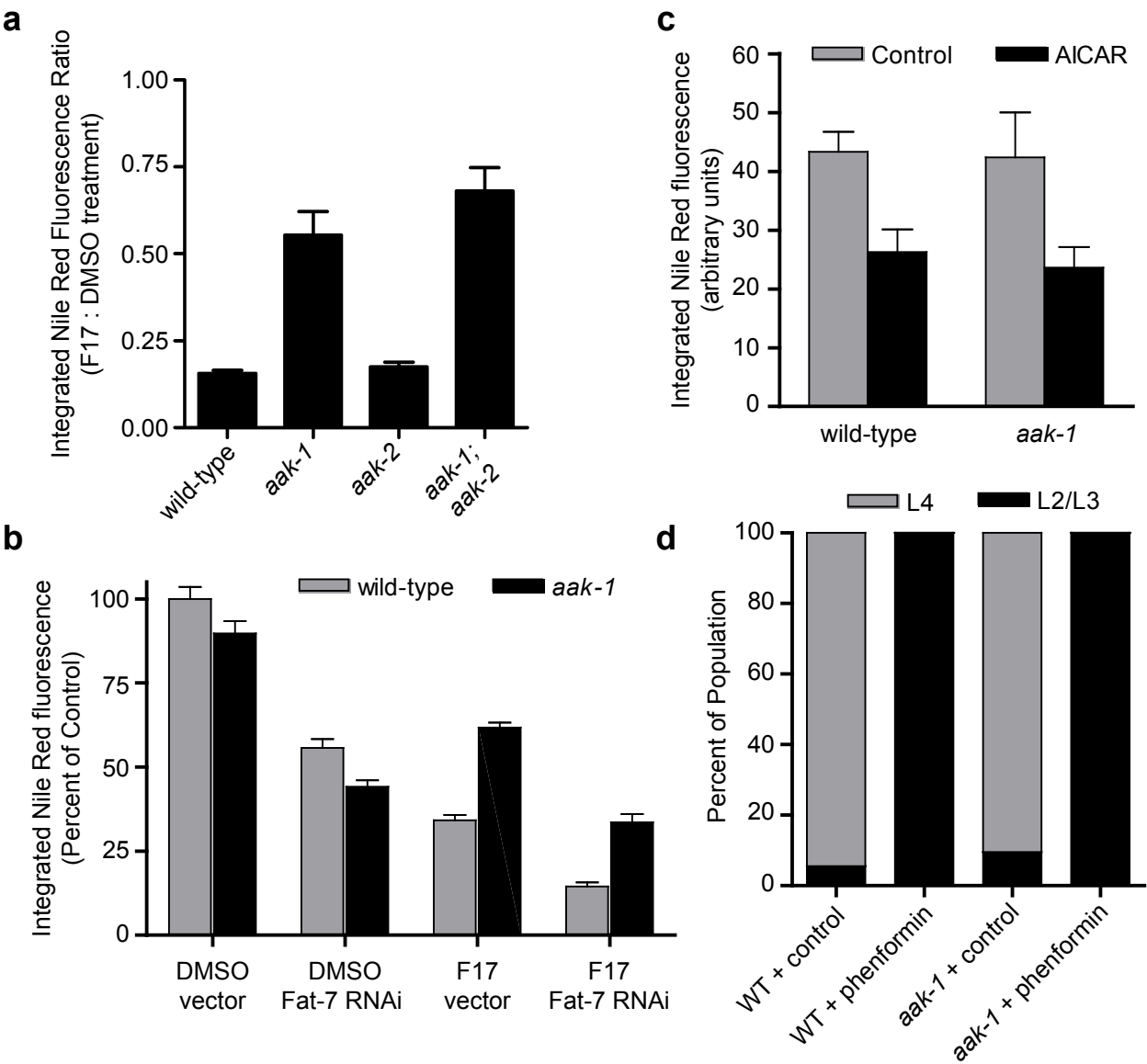


Figure S4. Fat epistasis controls. (**a**) The Nile Red fluorescence of the anterior intestine of F17 treated wild-type *aak-1*, *aak-2*, and *aak-1; aak-2* double mutant animals normalized to DMSO treated control in each background. Error bars represent the standard error of the mean. The difference between *aak-1* and *aak-1;aak-2* double mutant animals is statistically insignificant ($p > 0.1$: two-tailed t-test). (**b**) Integrated Nile Red staining intensity of the anterior intestinal cells of wild-type (grey bars) or *aak-1* mutants (black bars) that were cultured with Nile Red on *E. coli* HT115 expressing either a control RNAi construct (vector) or a construct targeting fat-7. After two days of culture at 20 °C, the nematodes were treated for 24 hr with either 10 μM F17 or 0.1% DMSO. Error bars represent the standard error of the mean Nile Red fluorescence of 8-10 animals per condition. (**c**) Integrated Nile Red staining intensity of young adult wild-type and *aak-1* mutant nematodes treated with either AICAR (1 mM) or 0.1% DMSO as a control. The fluorescence intensity of the two anterior pairs of intestinal cells was quantified for 5-7 animals per condition. Error bars represent the standard error of the mean. (**d**) Synchronized L1 wild-type (WT) and *aak-1* mutants were treated with 7.5 mM phenformin or S-medium (control) and cultured for 45 hr at 20 °C. The percentage of the population at each developmental stage is indicated. 18-20 animals per condition were assayed.

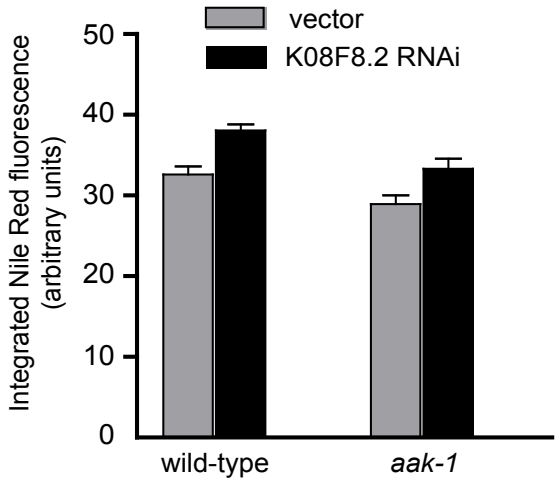


Figure S5. Characterization of K08F8.2 RNAi on Nile Red accumulation. Synchronized L1 animals were cultured on HT-115 bacteria harboring either a K08F8.2 RNAi expression plasmid or a vector control plasmid in media supplemented with Nile Red and 0.1% DMSO. The integrated Nile Red fluorescence of 7-10 animals per condition of the anterior intestinal cells is quantified. Error bars represent the standard error of the mean.

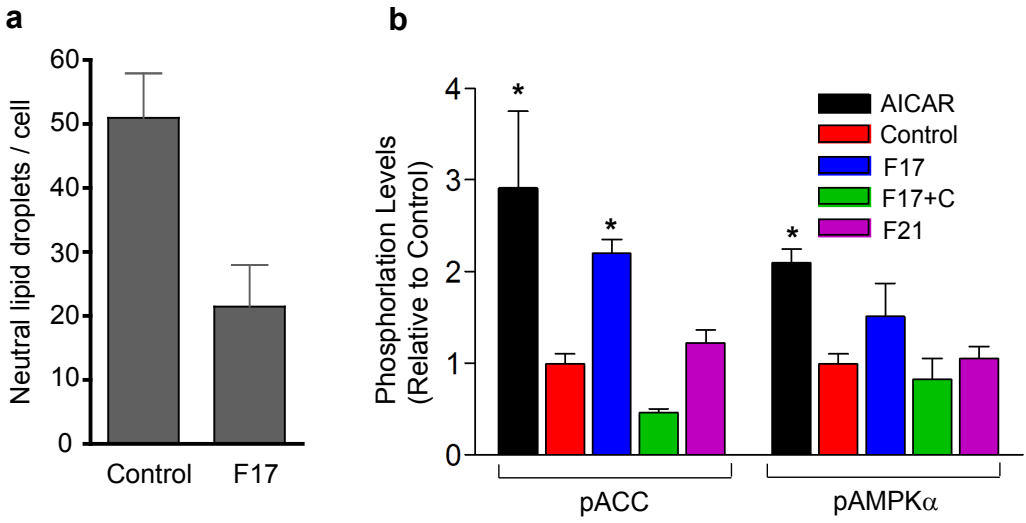


Figure S6. Quantification of HepG2 neutral lipids, as well as ACC/AMPK phosphorylation in response to F17. (**a**) Total lipid droplets in maximum intensity z-projections were quantified and normalized to the number of nuclei in each field. Error bars represent the standard error of the mean of 4 independent fields per condition. (**b**) Relative phosphorylation levels normalized to ACC and AMPKα total protein were quantified from three independent experiments. Error bars represent the standard error of the mean. * $p < 0.05$ relative to control (two-tailed t-test).

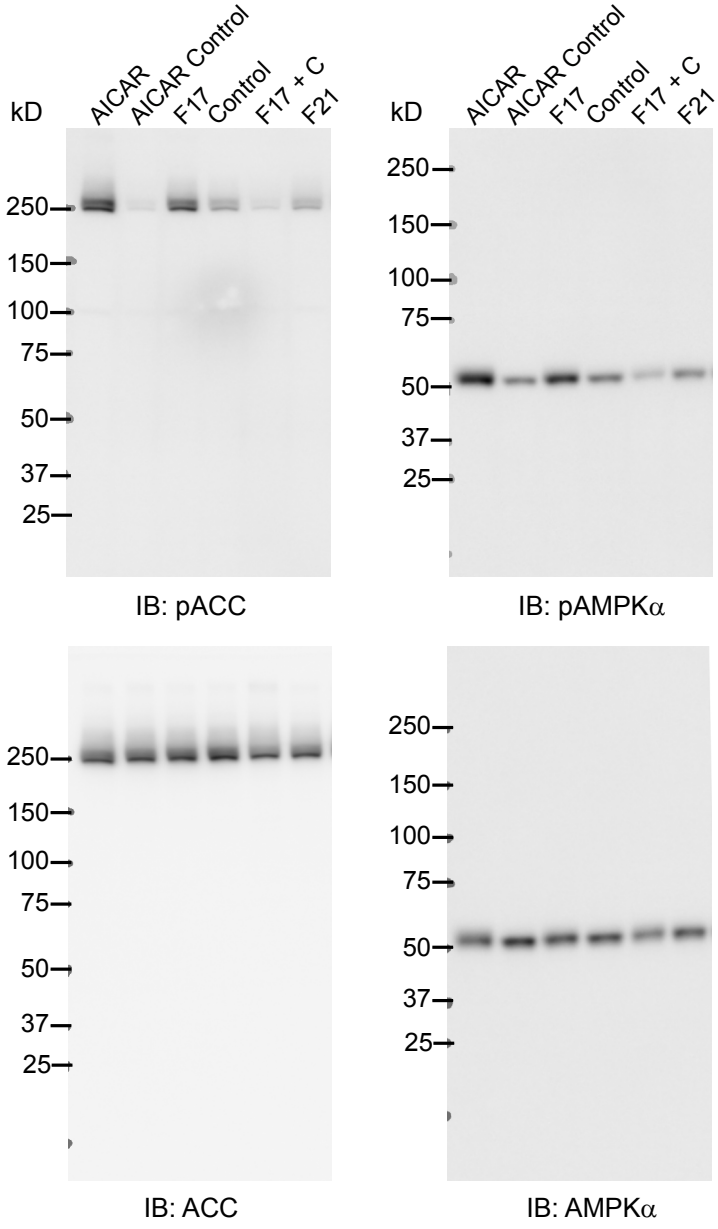


Figure S7. Uncropped western blots used to produce Figure 7.