Developmental Biology 373 (2013) 14–25

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Stress response pathways protect germ cells from omega-6 polyunsaturated fatty acid-mediated toxicity in *Caenorhabditis elegans*

Christopher M. Webster, Marshall L. Deline, Jennifer L. Watts *

School of Molecular Biosciences and Center for Reproductive Biology, Washington State University, Pullman, WA 99614-6340, USA

ARTICLE INFO

Article history:
Received 18 February 2012
Received in revised form
24 September 2012
Accepted 1 October 2012
Available online 9 October 2012

Keywords:
Omega-6 polyunsaturated fatty acid
Germ cells
Insulin/IGF signaling

ABSTRACT

Polyunsaturated fatty acids serve both structural and functional roles as membrane components and precursors for a number of different factors involved in inflammation and signaling. These fatty acids are required in the human diet, although excess dietary intake of omega-6 fatty polyunsaturated fatty acids may have a negative influence on human health. In the model nematode, *Caenorhabditis elegans*, dietary exposure to dihomo-gamma-linolenic acid (DGLA), an omega-6 fatty acid, causes the destruction of germ cells and leads to sterility. In this study we used genetic and microscopic approaches to further characterize this phenomenon. We found that strains carrying mutations in genes involved in lipid homeostasis enhanced sterility phenotypes, while mutations reducing the activity of the conserved insulin/IGF signaling pathway suppressed sterility phenotypes. Exposure to a mild heat stress prior to omega-6 fatty acid treatment led to an adaptive or hormetic response, resulting in less sterility. Mutations in *skn-1* and knockdown of genes encoding phase II detoxification enzymes led to increased sterility in the presence of dietary DGLA. Thus, detoxification systems and genetic changes that increase overall stress responses protect the germ cells from destruction. Microscopic analyses revealed that dietary DGLA leads to deterioration of germ cell membranes in the proliferative and transition zones of the developing germ line. Together, these data demonstrate that specific omega-6 polyunsaturated fatty acids, or molecules derived from them, are transported to the germ line where they disrupt the rapidly expanding germ cell membranes, leading to germ cell death.

Introduction

The role of diet and its relationship to chronic health issues has been intensely studied over the past several decades. Much of the focus on the negative health effects associated with dietary choices has been directed toward foods that contain high fats and cholesterol, without distinguishing between different types of fats. However, certain types of dietary fat promote good health. Diets rich in polyunsaturated fats, but lower in saturated fats, have numerous benefits including decreased incidence of atherosclerosis in humans (Erkkila et al., 2008; Mozaffarian et al., 2010). In *Caenorhabditis elegans*, polyunsaturated fatty acids are necessary for optimal growth and reproduction (Kniazeva et al., 2003; Watts and Browse, 2002; Watts et al., 2003).

The most common polyunsaturated fatty acids (PUFAs) are the omega-3 and omega-6 classes, which are distinguished from each other by the location of the terminal double bond in the hydrocarbon chain. Both omega-3 and omega-6 unsaturated fatty acids are essential for mammals, however, these fatty acids have distinct biological activities (Burr and Burr, 1973). Correlative studies suggest that a high ratio of omega-6 to omega-3 fatty acids in the diet may play a role in promoting diseases such as cancers and heart disease (Brown et al., 2010; Funahashi et al., 2008). Eicosanoids, which are oxygenated derivatives of PUFAs, such as prostaglandins, thromboxanes and leukotrienes, are powerful short range signaling molecules that mediate pain, inflammation, and immune responses. Eicosanoids derived from omega-6 fatty acids are generally regarded as pro-inflammatory, and may contribute to the pathogenesis of cancers and heart disease when present in excess. Omega-3 fatty acids are thought to counteract the pro-inflammatory effects of omega-6 derived eicosanoids (Funahashi et al., 2008; Simopoulos, 2008). Numerous studies point to the benefits of omega-3 fatty acids in reducing tumorigenesis in rodents as well as human cell lines (Hubbard et al., 1998; Liu et al., 2001; Mernitz et al., 2009; Yee et al., 2005). This is not always the case, however, as a positive correlation between a long-chain omega-3 fatty acid species and the incidence of high-grade prostate cancer in humans was revealed in a recent study (Brasky et al., 2011).

Key regulators of fat synthesis and fat oxidation are conserved between mammals and *C. elegans* (Watts, 2009). SBP-1 is a sterol response element binding protein (SREBP) and is required for

* Corresponding author. Fax: +1 509 335 4159.
E-mail address: jwatts@wsu.edu (J.L. Watts).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.10.002

normal lipid metabolism and needed for proper fatty acid composition and body fat deposition (Walker et al., 2011; Yang et al., 2006). NHR-49 and NHR-80 are nuclear hormone receptors of the HNF4 family that regulate fatty acid desaturation and lipid homeostasis (Brock et al., 2006; Van Gilst et al., 2005a, 2005b). The AMP-activated protein kinase senses the ratios of ATP to AMP and regulates energy homeostasis by modulating pathways involved in ATP production and consumption (Hardie, 2011). *C. elegans* requires the AAK-2 subunit of AMP kinase for optimal longevity as well as for lipid rationing that mediates survival during dauer diapause (Apfeld et al., 2004; Greer and Brunet, 2009; Narbonne and Roy, 2009). The *pept-1* gene encodes an intestinal di- and tripeptide transporter, and mutation in this gene results in a high body fat content along with increased stress resistance, decreased body size and decreased progeny production (Meissner et al., 2004; Spanier et al., 2009). The DAF-2 insulin/IGF-1 receptor and the DAF-7/TGF-β ligand are required for the regulation of dauer larvae development, aging and stress (Larsen et al., 1995; Narasimhan et al., 2011). Strains carrying mutations in the *daf-2* and *daf-7* genes have defects in sensing of environmental cues and display altered metabolism leading to high fat stores (Greer et al., 2008; Larsen et al., 1995; Ogg et al., 1997). Complex interactions among these lipid homeostatic pathways are necessary for optimal synthesis, storage, allocation, and oxidation of fats according to the developmental and reproductive needs of the organism.

C. elegans can synthesize all of its polyunsaturated fatty acids *de novo* (Hutzell and Krusberg, 1982). Unlike mammals, the nematode can directly convert omega-6 fatty acids to omega-3 fatty acids through the action of the FAT-1 omega-3 desaturase; therefore they do not require omega-6 or omega-3 fatty acids in their diet (Spychalla et al., 1997; Watts and Browse, 2002). Since its discovery, FAT-1 has been the focus of many studies and has subsequently been used in the generation of transgenic mice and pigs for the study of endogenous omega-3 fatty acids on health and fitness of the organism (Kang et al., 2004; Lai et al., 2006). While these transgenic animals appear to be less susceptible to many diseases such as cancers and colitis (Hudert et al., 2006; Weylandt et al., 2011; Xia et al., 2006), expression of the *fat-1* transgene in certain tissues can make the animal less reproductively fit and may make them more susceptible to *Mycobacterium tuberculosis* infection (Bonilla et al., 2010; Pohlmeier et al., 2011). There is clearly a need for more research into the biological functions and interactions of polyunsaturated fatty acids.

Here we investigate the effects of a specific dietary fatty acid on reproductive development in the model nematode *C. elegans*. The *C. elegans* gonad is composed of both an anterior and posterior U-shaped arm connected centrally to two proximal spermathecae, which coalesce upon a common uterus and vulva. In the distal germ line of adult worms, a self-renewing population of over 200 germ cells per gonad arm is maintained in a proliferative mitotic state through the action of a Notch receptor signaling produced in response to ligands by the Distal Tip cell (DTC) (Austin and Kimble, 1987; Hansen et al., 2004; Kimble and Crittenden, 2007; Korta and Hubbard, 2010). In addition to the Notch signaling pathway, other factors affect mitotic germ cell proliferation. For example, mutations in *pro-1* (Killian and Hubbard, 2004), ablation of the distal sheath (Killian and Hubbard, 2005) and the insulin-like ligands, INS-3 and INS-33 (Michaelson et al., 2010), influence the accumulation of larval proliferative germ cells. As germ cells proliferate, the most proximal cells transition into prophase I of meiosis. In wild type worms, meiotic germ cells have two known fates; they can either differentiate into gametes or undergo programmed cell death (Gumienny et al., 1999). Under favorable conditions *C. elegans* will produce approximately 300 self-fertilized offspring. Optimal progeny production is controlled through multiple signaling inputs, which balance germ cell apoptosis to gamete production. Physiological germ cell apoptosis is a normal occurrence during oogenesis, and serves to maintain germ cell homeostasis and resource allocation. However, excess germ cell apoptosis can be triggered though exposure to various stressors, including starvation, pathogen infection, or DNA damage (Gartner et al., 2008).

Previously, we demonstrated that dietary dihomo-gamma-linolenic acid (DGLA, C20:3n-6), an omega-6 polyunsaturated fatty acid, can lead to sterility and germ cell loss (Watts and Browse, 2006). While several omega-6 fatty acids induce sterility and germ cell loss, DGLA provided the most powerful dose response. After feeding during larval stages, before the initiation of meiosis, a dietary switch from an omega-6 fatty acid diet to a standard laboratory diet could not restore fertility. Thus, germ cell loss was a permanent consequence of DGLA feeding. In contrast, high levels of dietary omega-3 fatty acids produce no adverse effects on the germ line or fertility (Watts and Browse, 2006).

In the current study we took genetic and morphological approaches to address the role that lipid homeostasis and stress responses play in the sterility response. We found that susceptibility of germ cells to dietary DGLA is sensitive to genetic manipulation. Mutations disrupting lipid homeostasis cause increased sensitivity to DGLA, while mutations that lead to up-regulation of stress response pathways, such as those in the insulin/IGF and TGF-β signaling pathways render the worms resistant to the destructive effects of DGLA. Dietary supplementation of DGLA leads to germ cell loss though apoptosis-dependent and independent pathways and results in the destruction of both mitotic and meiotic germ cells. During DGLA feeding in adult worms, we observed membrane deterioration and the formation of multinucleated germ cells in the proliferative and transition zones of the germ line. We hypothesize that the ingestion of dietary DGLA induces a stress in the germ line, ultimately leading to membrane damage and cell death in the *C. elegans* gonad.

Materials and methods

Strains and culture conditions

C. elegans strains were maintained at 20 °C on standard Nematode Growth Media (NGM) and fed with *Escherichia coli* OP50 unless otherwise noted. The following nematode strains were used in this study: N2 (wild-type, Bristol), which was the background strain of all mutant strains tested in this study, *daf-2*(*e1370*), *daf-2*(*m577*), *daf-2*(*m579*), *daf-2*(*m41*), *daf-7*(*e1372*), *daf-1*(*m40*), *daf-3*(*e1376*), *daf-5*(*e1386*), *daf-8*(*e1393*), *daf-12*(*rh61rh411*), *daf-14*(*m77*), *pept-1*(*lg1601*), *age-1*(*hx541*), *akt-1*(*mg144*), *daf-18*(*e1375*), *daf-16*(*mu86*) and the double mutant *daf-2*(*e1370*); *daf-16*(*mgDf47*). Also tested were *ced-3*(*n717*), *ced-4*(*n1162*), *ced-9*(*n1950*), *egl-1*(*n1084n3082*), *cep-1*(*gk138*), *sbp-1*(*ep79*), *nhr-49*(*nr2041*), *nhr-80*(*tm1011*), *aak-2*(*ok524*), *daf-22*(*m130*), *dhs-28*(*ok450*), TJ356: *zIs356 [pdaf-16::daf-16::GFP; rol-6*(*su1006*)*]* (Henderson and Johnson, 2001), and JR667: *unc-119*(*e2498::Tc1*); *wIs51[unc-119*(+),*scm::gfp]* (Terns et al., 1997). Strain OD58 [*unc-119*(*ed3*) *III; itIs38* (*pAA1; pie-1::GFP::PHPLC1δ1; unc-119* (+)] carries a PH membrane domain of phospholipase-C fused to a GFP reporter all under the *pie-1* promoter (Audhya et al., 2005).

Sterility assay

The fatty acid supplemented media was previously described (Watts and Browse, 2006). Briefly, 0.1% Tergitol NP40 (Sigma Chemicals) and the sodium salts of dihomo-gamma-linolenic acid

(DGLA, C20:3n-6) or eicosapentaenoic acid (EPA, C20:5n-3) (NuChek Prep) were added to NGM media at concentrations ranging from 0.15 to 0.45 mM. *E. coli (OP50)* were seeded on plates 3 days prior to transferring *C. elegans* eggs, larvae or young adults. Fatty acid supplemented plates were stored in the dark at all times. Adult worms were scored 24–48 h after formation of the vulval slit and sterility was determined microscopically for either presence or absence of oocytes and embryos. Percent sterility was determined by counting the number of sterile worms in a total population. For sterility tests, five individual plates of each genotype and treatment were scored, each consisting of 30–50 adult nematodes.

RNA Interference

RNAi was performed on NGM plates supplemented with 100 μg/ml ampicillin and 2 mM Isopropyl-β-D-thiogalactopyranoside (Fraser et al., 2000). Plates were allowed to dry at room temperature for five days in the dark before seeding with bacteria corresponding to individual clones from the Ahringer collection [purchased from Geneservice 〈http://www.lifesciences.sourcebioscience.com〉]. On day 7, isolated embryos (purified by alkaline hypochlorite treatment of gravid adults) or synchronized L1 larva were put on plates and incubated at 20 °C. L1 synchronization was performed by washing the isolated embryos five times in M9 minimal salts buffer and allowing the embryos to hatch in M9 buffer in the absence of *E. coli* for 18 h at 20 °C prior to transferring to test plates. At adulthood, worms were scored as previously described.

Quantitative RT-PCR

Gene expression analysis was performed on a subset of genes regulated by DAF-16 or SKN-1 transcription factors. 9000 worms synchronized to the late L4 stage were collected and washed with 20 °C water, and then evenly dispersed to a non-supplemented control diet and a 0.3 mM DGLA-supplemented diet. Worms were collected after 12 h and washed three times in ice cold water and the supernatant was removed. To the worm pellet, 1 ml Trizol (Invitrogen) was added, the samples were vortexed for 1 min and frozen to −80 °C. Samples were freeze-thawed three times followed by a 1 min vortex. Chloroform was added following the protocol suggested by the manufacturer (Invitrogen). The aqueous phase containing the mRNA was further purified on a Qiagen RNeasy column and eluted into Nuclease-Free water. cDNAs were generated using SuperScript® III First Strand Synthesis for RT-PCR System kit (Invitrogen) using 5 μg of the purified RNA. All real-time PCR reactions were performed using the ABI 7500 system (Perkin-Elmer Applied Biosystems) with DyNAmo™ ColorFlash SYBR Green qPCR Kit (ThermoScientific). The thermal cycling conditions followed an initial denaturation step at 95 °C for 7 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s followed by a continuous melt curve stage at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s and 60 °C for 15 s. Each target gene data point was the average of three internal replicates and error was determined over three biological replicates. Gene-specific primers were designed using the Primer3 (v. 0.4.0) software (Rozen and Skaletsky, 2000) with product size thresholds set between 50 bp and 150 bp and spanning mRNA splice sites if possible. DAF-16 target genes assayed in this study were *sod-3* and *mtl-1* (Honda and Honda, 1999; Murphy et al., 2003) and SKN-1 target genes were *gcs*-1 and *gst-4* (Inoue et al., 2005; Kahn et al., 2008). The relative quantification in gene expression was determined by calculating the $2^{(-\Delta\Delta C(T))}$ (Livak and Schmittgen, 2001) using reference genes *tbb-2*. Fold changes were calculated in a strain specific manner on a tergitol control and the DGLA treatment in both N2 and *daf-2* animals.

Preconditioning assay

N2 and TJ356 [DAF-16::GFP (zIs356)] were used to assess the role of stress response preconditioning on the DGLA-mediated sterility. L1–L2 stage worms were preconditioned with heat stress (35 °C for 25 min, which was sufficient to drive nuclear localization of the DAF-16::GFP reporter) on NGM plates, and then were transferred to DGLA-supplemented media. Preconditioning was performed on worm larvae at the eight gonadal cell stage, as visualized by DIC microscopy. Upon reaching adulthood, worms were assessed for sterility.

Microscopy

Worm gonads were dissected in PBS buffer with 0.2 mM levamisole. Gonads were either imaged immediately on a 2% agarose pad or were washed once in PBS+0.1% Tween 20 (PBT) buffer, and then fixed in 100% methanol or 3% formaldehyde/0.1 mM H_2PO_4 for one hour on ice. Gonads were then washed twice in PBT buffer prior to either DAPI or immunofluorescence staining.

For DAPI staining (4′,6′-diamidino-2-phenylindole hydrochloride), fixed and dissected gonads were incubated in PBT buffer containing 100 ng/ml DAPI for 5–20 min in the dark. The gonads were then washed twice in PBT prior to imaging. For immunofluorescence, fixed gonads were incubated in a 30% goat serum against PBT buffer mix with a 1:200 dilution of the primary antibody (anti-green fluorescent protein, mouse IgG2a, monoclonal 3E6 [Invitrogen]) for 12 h at 4 °C. Gonads were washed 3–4 times in PBT and incubated with 1:250 dilution of Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (Invitrogen) for 8–12 h at 4 °C. Finally, gonads were washed once in PBT and stained with DAPI as described above. Confocal images were acquired using a Zeiss LSM 510 M confocal microscope (Carl Zeiss Inc., Thornwood, NY) with a 63 × 1.4 NA oil immersion objective or a 20 × 1.2 NA glycerol immersion objective. Adobe Photoshop CS4 was used in post processing to enhance contrast of images.

Apoptosis

Apoptosis measurements were performed as previously described (Watts and Browse, 2006). Briefly, worms were grown to L4 and YA stages and were put on DGLA-supplemented plates for 12–24 h. The OP50-seeded plates were then flooded with SYTO 12 (Invitrogen) for 3–6 h, which preferentially stains apoptotic germ cells in the *C. elegans* germ line. Worms were then collected in PBS, washed twice and mounted on a 2% agarose pad for imaging.

Fatty acid analysis

Fatty acid methyl esters (FAMEs) from approximately 100–150 adult nematodes were generated as previously described (Watts and Browse, 2002). FAMES were injected into an Agilent 6890 series gas chromatograph equipped with a 30 × 0.25-mm SP-2380 column (Supelco), helium as the carrier gas at 1.4 ml/min, and an Agilent 5975C mass spectrometer detector. The gas chromatograph was run at initial temperature of 120 °C for 1 min, followed by gradient flow of 10 °C/min to 190 °C, followed by an increase of 2 °C/min to 200 °C.

Results

Genetic disruption of energy and lipid homeostasis pathways lead to altered susceptibility to dietary DGLA

In order to investigate the molecular pathways leading to dietary DGLA-induced sterility, we surveyed various candidate genetic backgrounds and asked whether the response to DGLA is altered. We first tested *C. elegans* strains carrying mutations that alter lipid homeostasis and lipid storage. Mutant strains of *sbp-1*, *nhr-80*, *nhr-49* and *aak-2* showed enhanced sensitivity to DGLA-supplemented media, with all strains displaying increased sterility compared to wild type when fed 0.2 mM DGLA (Fig. 1A; Table 1). The increased sensitivity to DGLA in strains carrying mutations in these various regulatory genes demonstrates that lipid and energy homeostasis pathways are required to protect the *C. elegans* germ line from DGLA toxicity. In contrast, strains carrying mutations in the peroxisomal fatty acid oxidation genes, *dhs-28* and *daf-22*, showed moderate resistance to DGLA. Strikingly, we found that *pept-1* (peptide transporter), *daf-2* (Insulin/IGF receptor), and *daf-7* (TGF-β) mutants showed complete absence of dietary DGLA-induced sterility when compared to wild type worms, even at 0.35 mM DGLA which causes sterility in nearly all of the wild type worms (Fig. 1A; Table 1). Our results suggest that nutrient sensing signal transduction pathways and lipid homeostatic mechanisms are involved in the maintenance of germ cells in response to dietary DGLA, and peroxisomal beta-oxidation may be involved in producing a metabolite of DGLA, which is toxic to germ cells.

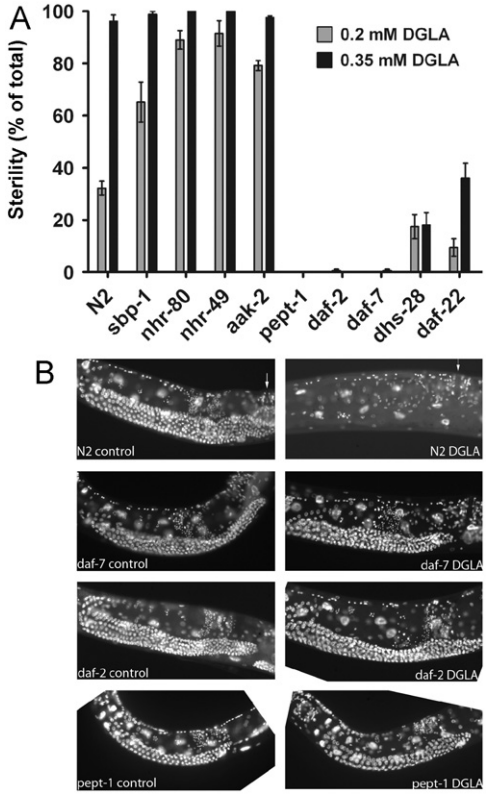

Fig. 1. Modulation of DGLA-induced sterility by lipid homeostasis mutants. (A) Supplementation of 0.2 and 0.35 mM DGLA reveals mutant strains that are more sensitive or more resistant to DGLA than wild type. Graphs display the percentage of worms in a population that are sterile when fed on 0.2 and 0.5 mM DGLA (sterility values, uptake of DGLA and statistics are shown in Table 1). Error bars, S.E.M, $^{*}p \leq 0.01$, determined by two tailed Student's t-test. (B) DAPI staining of young adult worms raised on 0.1% tergitol (control, left column) and worms raised on 0.3 mM DGLA (right column).

To determine if the suppression is simply due to a failure to uptake dietary DGLA, we performed GC/MS analysis to measure the incorporation of DGLA into the supplemented worms. While most strains accumulated DGLA from their diet to a similar extent as wild type animals, our analysis of the worm fatty acid composition revealed that strains carrying mutations in *sbp-1* and *nhr-49* accumulated higher levels of supplemented DGLA than wild type or other mutant strains (Table 1). This was especially evident on plates containing 0.35 mM DGLA, where the wild type worms showed an accumulation of DGLA such that 13.9% of the total fatty acids consisted of DGLA, while the *sbp-1* mutant accumulated DGLA to 18.3% and *nhr-49* accumulated DGLA to 24% of total fatty acids. On the other hand, our fatty acid composition measurements did not show evidence of increased uptake of DGLA in *pept-1* mutants (Table 1), though it has been proposed that this strain absorbs higher amounts of free fatty acids (Spanier et al., 2009). Even so, the strong resistance to DGLA-induced sterility in *pept-1* mutants suggests that other homeostatic changes may occur in these mutants, for example, fewer dietary resources may be allocated to germ cells.

To examine germ cells and gametes in the resistant strains, we DAPI stained young adult worms grown on dietary DGLA. We found that wild type worms raised on the DGLA diet lacked germ cells, sperm, and oocytes, consistent with our previous observations (Watts and Browse, 2006) (Fig. 1B). In contrast, the germ cells and gametes in *daf-2* and *daf-7* mutants grown on

Table 1
Sterility and DGLA uptake in *C. elegans* strains exposed to dietary DGLA.

Strain	Control		0.2 mM DGLA			0.35 mM DGLA		
	% Sterility[a]	% DGLA[b]	% Sterility[a]	*p*-value[c]	% DGLA[b]	% Sterility[a]	*p*-value[c]	% DGLA[b]
N2	0.0	2.8	32.0 ± 32.6	–	8.4	96.0 ± 2.4	–	13.9
daf-2	0.0	2.3	0.7 ± 0.7	1.9×10^{-4}	6.6	0.0 ± 0.0	2.5×10^{-6}	11.5
daf-7	0.0	2.6	0.0 ± 0.0	2.9×10^{-4}	9.1	0.7 ± 0.7	6.9×10^{-7}	14.4
daf-22	0.0	2.7	9.3 ± 3.4	9.43×10^{-4}	8.8	36.0 ± 5.6	1.1×10^{-4}	16.5
dhs-28	0.0	3.8	17.3 ± 4.6	3.2×10^{-2}	8.8	17.9 ± 4.8	7.6×10^{-6}	12.2
aak-2	0.0	2.8	79.3 ± 1.9	1.47×10^{-6}	8.2	97.3 ± 0.7	0.62	12.9
sbp-1	0.0	2.2	65.3 ± 7.6	9.3×10^{-3}	8.4	98.8 ± 1.2	0.34	18.3
nhr-49	0.0	4.3	91.7 ± 4.8	1.4×10^{-4}	10.5	100.0 ± 0.0	0.18	24.0
nhr-80	0.0	2.9	89.2 ± 3.4	1.1×10^{-5}	8.2	100.0 ± 0.0	0.18	14.9
pept-1	0.0	2.1	0.0 ± 0.0	2.9×10^{-4}	6.4	0.0 ± 0.0	2.5×10^{-6}	10.0
OP50	–	0	–	–	6.7	–	–	8.7

[a] % Sterility monitors the percentage of worms in a population that become sterile and experience complete germ cell loss when fed their entire life of DGLA. ($\pm$S.E.M.)
[b] % DGLA measurements of total worm lipids. Fatty acid methyl esters generated from pooled total worm lipids from sterility assay.
[c] *P*-values based on sterility comparison to wild-type N2 animals. Type 3, two tailed t-test performed to generate statistical significance

DGLA were indistinguishable from the untreated controls. The *pept-1* worms grown on DGLA were also indistinguishable from untreated *pept-1* worms, although we noticed that all *pept-1* mutants displayed germ lines that were considerably smaller than wild type, consistent with the observation of (Korta et al., 2012). While wild type worms have >300 mitotic and meiotic germ cells total, *pept-1* mutants had 109 ± 20 (Fig. 1B). Therefore, it appears that mutation of *daf-2*, *daf-7* or *pept-1* offers the germ line protection against the toxic effects of dietary DGLA.

Insulin and TGF-β pathway mutants affect DGLA resistance

Because of the strong resistance to dietary DGLA by *daf-7* and *daf-2* mutants, we further investigated the conserved insulin/IGF and the TGF-β signaling pathways. The DAF-2 insulin/IGF-1 receptor signals through a conserved PI3-kinase/AKT pathway that ultimately represses the DAF-16/FOXO transcription factor by phosphorylation and cytoplasmic retention (Lin et al., 2001) (Fig. 2A). In addition to the *daf-2* (*e1370*) mutant, suppression was also seen in both *age-1* and *akt-1* mutants (Fig. 2B). Mutations in this pathway ultimately lead to nuclear localization of DAF-16/FOXO, which is responsible for numerous transcriptional changes that regulate developmental and metabolic cues, confer long lifespan, and activate resistance genes to pathogen infection, as well as stress resistance (Furukawa-Hibi et al., 2002; Lee et al., 2003; Pinkston-Gosse and Kenyon, 2007). Consistent with this, a *daf-16* mutant was more sensitive to DGLA than wild type worms (Fig. 2B). Furthermore, a mutation in the DAF-18/PTEN phosphatase should result in accumulation of PIP_3 and ultimately lead to constitutive activation of AKT-1 and nuclear exclusion of DAF-16. Indeed, the data demonstrates that the *daf-18* mutant is more sensitive to DGLA-induced sterility (Fig. 2B). Finally, to determine if the resistance to DGLA in *daf-2* mutants depends on DAF-16, we tested sterility of the *daf-2;daf-16* double mutant. We found that the double mutant strain had higher levels of sterility than the *daf-2* mutant, consistent with a role for DAF-16/FOXO in conferring resistance (Fig. 2B).

Reduction-of-function *daf-2* mutants can be divided into class 1 (weakest) and class 2 (more severe) mutations (Gems et al., 1998; Patel et al., 2008). To further examine whether DGLA resistance recapitulates the *daf-2* allele classes, two representative alleles of each class were investigated. We found strains carrying class 2 alleles of *daf-2* (*m579* and *e1370*) were the most resistance to DGLA, though the class 1 alleles (*m577* and *m41*) were still more resistant than wild-type worms (Fig. 2C). Thus, as observed for other *daf-2* phenotypes, the stronger allele classes conferred the greatest DGLA resistance. Strong loss-of-function alleles in *daf-2* affect early development and were not examined in this study (Gems et al., 1998; Patel et al., 2008).

Parallel to the insulin IGF-1 signaling pathway, the transforming growth factor β (TGF-β) pathway has been shown to control the dauer decision in response to sensory cues (Patterson and Padgett, 2000). Under favorable conditions, the DAF-7 TGF-β ligand is neuronally expressed and can activate target DAF-1 and DAF-4 receptors in several tissues to transduce its signal to the R-Smads DAF-8 and DAF-14. Once phosphorylated they serve to negatively regulate the DAF-3 Co-Smad, which when active, helps to promote dauer formation (Fig. 2A).

We found that mutations in the upstream TGF-β signaling pathway resulted in resistance to the sterility-inducing effects of dietary DGLA. The most powerful resistance was seen in *daf-7*, *daf-1*, *daf-8* and *daf-14* mutants when compared to the wild type control (Fig. 2D). As expected, the *daf-3* mutants showed increased sensitivity to dietary DGLA (Fig. 2D). The nuclear

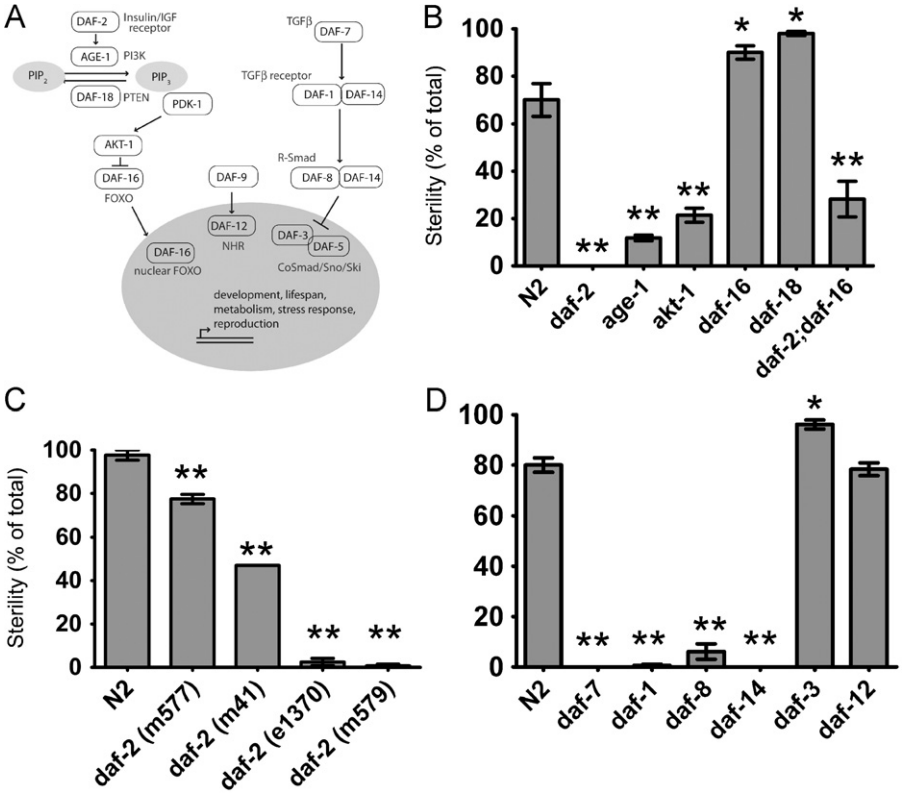

Fig. 2. Activation of stress response pathways suppresses germ cell loss in response to DGLA feeding. (A) Schematic of the conserved insulin/IGF-1 and TGF-β signaling pathways in *C. elegans*. (B, C, and D) Mutations in the insulin signaling pathway and the TGF-β signaling pathway modulate the sterility responses. Graphs display the percentage of worms in a population that are sterile. Plates containing 0.15mM DGLA were used for the assays shown in panels B and D. For the assay shown in panel C, 0.3mM DGLA was used. Error bars, S.E.M, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, determined by two tailed Student's t-test.

hormone receptor DAF-12 is another key regulator of the decision between dauer and reproductive growth (Antebi et al., 2000). We found that *daf-12* worms displayed sensitivity to DGLA similar to wild type, and therefore does not appear to be important to play a role in the response to DGLA. Taken together, the data from the genetic analysis of the insulin and TGF-β pathways suggests that temporal activation of a stress response leads to resistance to dietary DGLA.

Phase II detoxification involved in generating resistance to DGLA

Mutations leading to reduced insulin signaling are known to activate many transcription factors associated with longevity and detoxification (McElwee et al., 2003, 2004; Tullet et al., 2008). One such factor is SKN-1/NRF, which plays a key role in the regulation of genes involved in detoxification (An et al., 2005; Oliveira et al., 2009; Park et al., 2009; Tullet et al., 2008). In order to test whether detoxification systems are involved with the DGLA response, we performed RNAi against *skn-1* and its target genes *gcs-1* (gamma-glutamine cysteine synthetase) and *ugt-22* (UDP-glucuronosyl/UDP-glucosyltransferases), which encode a gene product required for glutathione biosynthesis and conjugation (An et al., 2005; Inoue et al., 2005). We found that knockdown of *skn-1* in wild type worms enhances the sterility response of DGLA (Fig. 3A). Furthermore, knockdown of either *gcs-1* or *ugt-22* results in powerful enhancement of sterility in response to DGLA feeding. Since phase II detoxification enzymes are important for remediation of many lipophilic molecules, this data supports the hypothesis that a toxic metabolite of DGLA is likely responsible for the sterility effect.

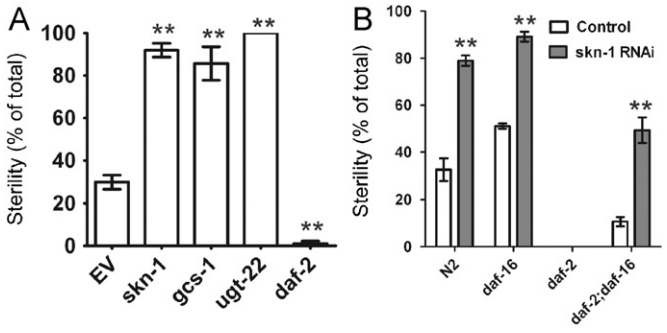

Fig. 3. Involvement of SKN-1 and Phase II detoxification systems in resistance to DGLA. (A) RNAi knockdown of *skn-1*, *gcs-1* and *ugt-22* lead to increased susceptibility to DGLA (0.15mM) as compared to the empty vector (EV) control. Error bars, S.E.M, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, determined by two tailed Student's t-test. (B) SKN-1 is required for resistance to dietary DGLA. RNAi knockdown of *skn-1* in *daf-16*, and *daf-16*;*daf-2* double mutants are more susceptible to DGLA-induced sterility than the empty-vector controls (0.12 mM DGLA), *daf-2*(*e1370*) were completely resistant. Error bars, S.E.M, $^{**}p \leq 0.01$, determined by two tailed Student's t-test.

We next performed an epistasis analysis to determine the role of SKN-1 in mutants in the IIS pathway (Fig. 3B). We found that the reduction of SKN-1 in the wild type, *daf-16*, and *daf-2*; *daf-16* strains leads to increased sterility. However, *daf-2* worms remained fertile when treated with *skn-1*(*RNAi*) under all DGLA concentrations tested thus far, even when DGLA was elevated to 0.3 mM in the media (data not shown). This demonstrates that even under conditions of reduced SKN-1 abundance, the *daf-2* mutants are able to maintain their germ cells in the presence of DGLA. This may be occurring through the activation of a SKN-1 independent detoxification pathway, although we cannot rule out that detoxification occurs due to residual SKN-1 activity that is not eliminated by the RNAi treatment.

Preconditioning with heat stress leads to DGLA resistance

Hormetic responses are those in which a mild stress preconditions a cell or organism to tolerate a much greater subsequent stress. To determine whether sterility caused by DGLA is affected by a hormetic response, we applied a mild heat stress to wild-type worms at the end of the L1 larval stage, and subsequently transferred the worms to plates containing DGLA for the rest of their development. We observed a significant reduction in sterility in the preconditioned worms compared to the control animals that were not exposed to the heat stress (Fig. 4A). This suggests that animals can be preconditioned to resist the toxicity effects of DGLA. We next asked whether the preconditioning or hormetic effect was dependent on DAF-18 and DAF-16. Indeed, we found these hypersensitive mutants to DGLA feeding were resistant to the preconditioning effects during the early stages of germ line development, suggesting that the preconditioning response requires activated DAF-16/FOXO (Fig. 4A).

To determine if dietary DGLA invokes a stress response in *C. elegans*, we performed an acute (12 h) and chronic (L1 through adulthood) exposure to DGLA. We observed that dietary DGLA did not induce DAF-16::GFP nuclear localization (Fig. 4B and data not shown). Furthermore, we examined transcription levels of several genes induced by DAF-16 and SKN-1 by qRT-PCR, and found that none were induced after 24 h of DGLA exposure (Fig. 4C). Taken together, these data suggest that dietary DGLA itself does not activate a stress response. However, mild stress treatment during

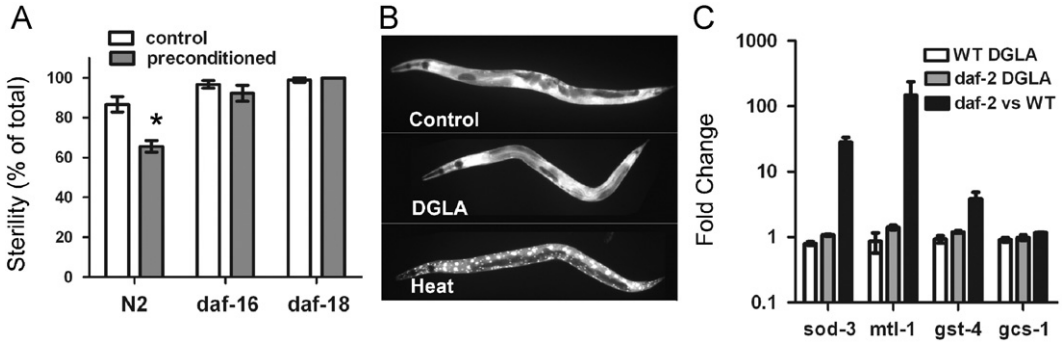

Fig. 4. Preconditioning suppresses germ cell loss. (A) Synchronized L1 worms were raised on standard NGM plates to the 8-cell gonad stage, then preconditioned with a mild heat stress of 35 °C for 25 min and plated on 0.25 mM DGLA-supplemented plates until worms reach adulthood. Graph displays the percentage of sterile worms in the population. (B) DAF-16::GFP transgenic worms show no noticeable GFP nuclear localization when fed a DGLA-supplemented diet (0.3mM) from L1 through adulthood (DGLA), indistinguishable from the unsupplemented control. Heat stress preconditioning positive control (Heat) shows strong nuclear localization of DAF-16::GFP. (C) Quantitative real time RT-PCR analysis of targets of DAF-16 (*mtl-1* and *sod-3*) and targets of SKN-1 (*gcs-1* and *gst-4*). WT DGLA and *daf-2* DGLA represent the fold change of worms when grown on 0.3 mM DGLA compared to the same strains grown on unsupplemented plates. Error bars, S.E.M.

the L1 larval stage confers resistance to the effects of DGLA, and this resistance is likely mediated by DAF-16/FOXO.

DGLA induces sterility independently of the DNA damage-induced apoptosis pathway

Previously, we demonstrated that feeding *C. elegans* a diet supplemented with DGLA leads to sterility when worms are exposed for their full life or in early larval stages. Because germ cell loss in adult worms fed DGLA correlated with increases in programmed germ cell death (Watts and Browse, 2006), we sought to examine this pathway further. Based on our previous studies, we reasoned that during metabolism, DGLA may undergo a specific oxidative reaction, perhaps in the peroxisome, to produce a reactive metabolite that could damage DNA, culminating in excess germ cell apoptosis (Gumienny et al., 1999). In *C. elegans*, DNA damage-induced apoptosis depends on the BH3-only protein EGL-1 and the ortholog of the human p53 tumor suppressor gene, *cep-1* (Gartner et al., 2008) (Fig. 5A). EGL-1 feeds into the core apoptosis pathway by inhibiting CED-9. We examined the effects of dietary DGLA in *C. elegans* mutant backgrounds defective in the DNA damage apoptotic response pathway. Strains carrying mutations in *cep-1* and *egl-1* showed similar susceptibility to DGLA as control worms (Fig. 5B). This indicates that sterility and the associated germ cell loss is likely a CEP-1/p53 independent process, and argues against DNA damage as the trigger leading to germ cell apoptosis. Examination of apoptotic corpses by SYTO 12 staining in worms treated with DGLA revealed a similar increase in apoptotic corpses in the *cep-1* and *egl-1* mutants (Fig. 5C). In agreement with our previous studies (Watts and Browse, 2006), we found that *ced-3*(-), *ced-4*(-), and *ced-9*(*gf*) showed a decrease in the number of sterile worms compared to controls, demonstrating a role for physiological germ cell apoptosis in DGLA-induced sterility (Fig. 5B). However, even in worms lacking CED-3 and CED-4 activity, many of the DGLA-fed worms became sterile and lacked germ cells. This suggests that germ cell loss is also occurring independently of the core apoptotic pathway, and led us to examine whether DGLA causes destruction of cells in the mitotic region of the germ line.

DGLA treatment reduces the size of the proliferative zone

To examine the fate of mitotic germ cells during DGLA exposure in adults, we first allowed the worms to establish a robust population of germ cells by growing on an unsupplemented diet during early larval stages. We commenced feeding DGLA at late L4 stage or young adult worms and maintained the worms on DGLA for 16 and 24 h. We then stained the worms with DAPI and measured the distance in cell diameters from the distal tip cell (DTC) to the transition zone. The transition zone is characterized by the appearance of crescent-shaped nuclei indicating differentiation into leptotene/zygotene of meiotic prophase I. Under normal conditions a wild-type adult worm has approximately 22–23 germ cell diameters (CD) between the DTC and the

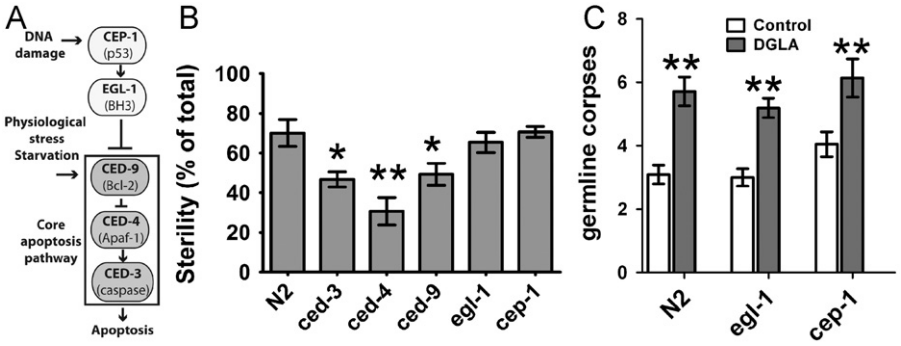

Fig. 5. DGLA supplementation leads to the destruction of proliferating germ cells. (A) Schematic of the conserved apoptosis pathway. (B) Evaluation of adult worm sterility in response 0.15 mM dietary DGLA. Strains carrying mutations in the proapoptotic genes *ced-3* and *ced-4* show moderate resistance to DGLA. A gain-of-function mutation in *ced-9* had moderate suppression where the tumor suppressor mutant *cep-1* and BH3 ortholog *egl-1* show no significant change compared to N2. (C) Average number of SYTO 12 positive cells per gonad in N2, *egl-1*, and *cep-1* strains grown for 24 h at the young adult stage on unsupplemented plates (control) or 0.3 mM DGLA plates (DGLA), $n=22$ for each strain. Error bars, S.E.M, $^{*}p \le 0.05$, $^{**}p \le 0.01$, determined by two tailed Student's t-test.

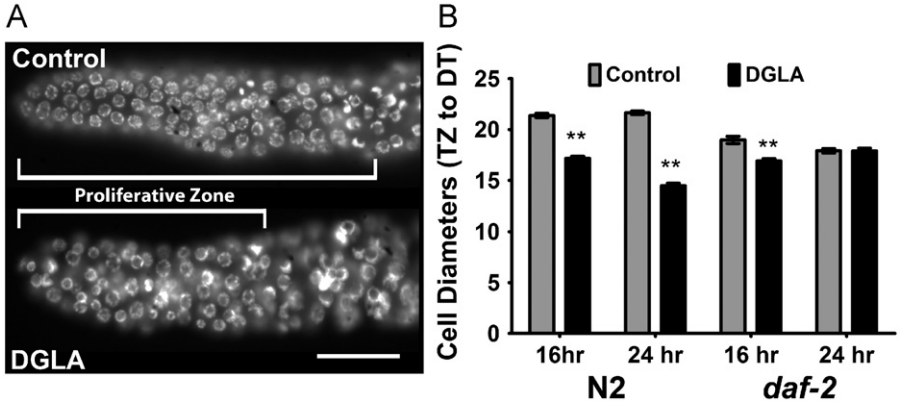

Fig. 6. Short term DGLA exposure leads to a decrease in the mitotic proliferative zone. (A) DAPI stained dissected gonads of young adult N2 worms grown on plates containing DGLA (0.45mM) for 24 h show a decreased mitotic proliferative zone when compared to the unsupplemented control. Scale bar is 20 μm. (B) Measurement of numbers of germ cell diameters (CD) from the distal tip of the gonad to the proliferative zone/transition zone border in adult N2 and *daf-2* (*e1370*) worms fed DGLA or control diet for 16 and 24 h. Error bars, S.E.M, $^{*}p \le 0.05$, $^{**}p \le 0.01$, determined by two tailed Student's t-test.

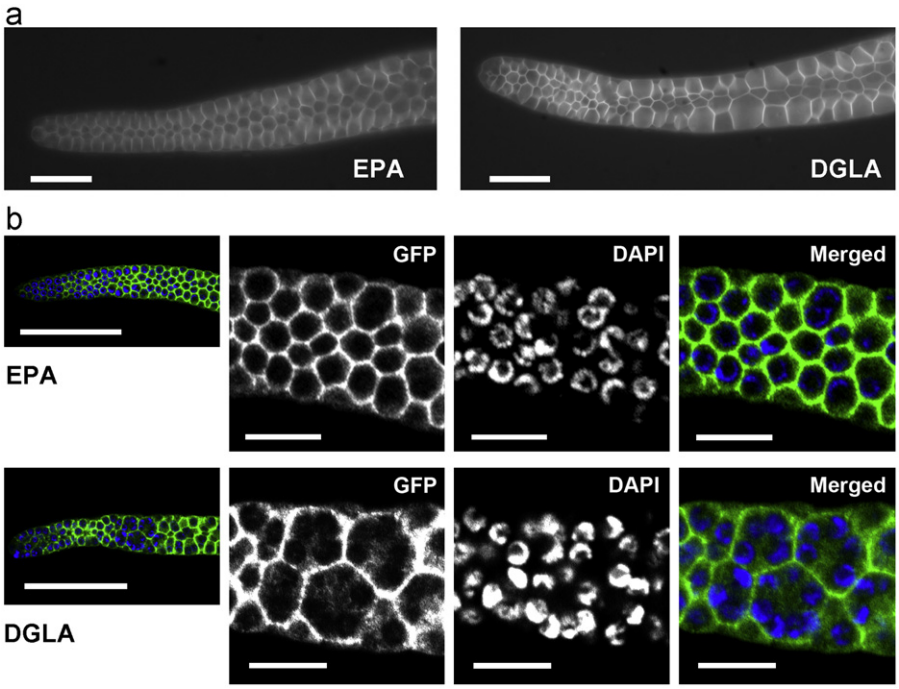

Fig. 7. DGLA feeding leads to membrane deterioration and multinucleated cells. (A) OD58 [PH::GFP(PLC1δ1)] gonads of 24 h DGLA-fed (0.45mM), EPA-fed (0.45mM), or unsupplemented control worm were dissected and visualized by fluorescence microscopy. Scale bars are 20 μm. Gonads from EPA-fed worms were indistinguishable from gonads of unsupplemented control worms (B) Gonads dissected and fixed from 24 h adult DGLA-fed and EPA-fed worms. Fixed gonads were stained with anti-GFP and DAPI and imaged with confocal microscopy. Left panels show merged GFP and DAPI images of adult gonad regions from the distal tip cell to transition zone. Scale bar on left panels is 50 μm. Subsequent panels show zoomed images depicting membrane organization (GFP), nuclear morphology (DAPI) and merged images of cells in the transition zone regions of the same gonad. Scale bars on the zoomed images are 10 μm.

appearance of a small population of leptotene/zygotene nuclei (Crittenden et al., 1997). We found that the proliferative mitotic zone decreased from approximately 22 cell diameters (CD) in the control worms down to 14–15 CDs in the 24-hour feeding experiment (Fig. 6). This represents approximately a 32% decrease in the proliferative zone length as measured in cell diameters between the distal tip and the transition zone. This decrease demonstrates that dietary DGLA treatment may result in a time-dependent reduction of germ cell diameters in the mitotic region. Furthermore, the DGLA-treated germ cell nuclei lacked uniformity with some nuclei having a condensed morphology while other nuclei appeared expanded (Fig. 6A). We included the *daf-2*(*e1370*) mutant in our proliferative zone analysis and consistent with our sterility results; we saw little change in the size of the mitotic zone between treatments, albeit the number of germ cell diameters is slightly reduced in the *daf-2* mutant (Michaelson et al., 2010). These findings demonstrate that dietary DGLA adversely affects germ cells in the proliferative and transition zones of the germ line.

To test if DGLA affects post-embryonic cell divisions in tissues outside the germ line, we examined the proliferation of seam cells during larval development using the JR667 (scm_p::GFP) strain that allows for the visualization of seam cells (Terns et al, 1997). We examined greater than 50 DLGA treated worms and found wild type numbers of seam cells in all worms exposed 0.15 and 0.3 mM DGLA. Therefore, while dietary DGLA has drastic effects on proliferating germ cells, it does not affect the proliferation seam cells during larval development. Dietary DGLA is incorporated into all worm lipids thus far tested (Watts and Browse, 2006). Because the mitotic region is in a constant state of proliferation, there is a high demand for the lipid precursors for the synthesis of new membranes. We therefore examined membrane integrity after DGLA feeding starting at late L4 worms or young adult stage. Using the reporter strain, OD58 (*pie-1*::GFP::PH(PLC1δ1)) we were able to visualize the membranes and membrane integrity in the germ cells. After a 24-hour feeding of DGLA or eicosapentaenoic acid (EPA), a 20-carbon omega-3 PUFA, adult worm gonads were dissected and visualized by fluorescence microscopy. OD58 control and EPA treated gonads show a uniform hexagonal membrane structure (Fig. 7A). After DGLA feeding, the germ cell membranes show severe lack of uniformity with cell sizes ranging from small to very large and vacuous (Fig. 7A). Confocal imaging of the dissected gonad reveals these larger cells to be multinucleated and in some cases possessing upwards of five nuclei when compared to the control worms (Fig. 7B). We observed disrupted membranes in worms treated for as little as 6 h on DGLA. The appearance of disrupted gonad membranes prior to the appearance of altered nuclear morphology suggests that DGLA, or an oxidized metabolite, may be causing membrane damage in the rapidly growing germ cell membranes, leading to destruction of mitotic as well as meiotic germ cells.

Discussion

Our studies show that the irreversible germ cell death and sterility induced by dietary DGLA is very sensitive to genetic background. We found that strains defective in lipid homeostasis pathways are highly sensitive to DGLA, while strains that are defective in nutrient sensing that are generally resistant to stresses, are resistant to DGLA. In addition, strains carrying mutations in peroxisomal beta-oxidation genes, and a strain with a mutation in the *pept-1* peptide transporter also show resistance to dietary DLGA. These findings, along with microscopic analyses, allow us to form a model to begin to explain the dramatic destruction of germ cells that occur when worms consume DGLA.

Optimal regulation of lipid homeostasis protects worms from dietary DGLA

We found that mutations in *sbp-1*, *nhr-49*, *nhr-80* and *aak-2* showed increased sensitivity to DGLA (Fig. 1A). This indicates that lipid homeostatic pathways regulated by SBP-1, nuclear receptors, and AMPK act in wild type worms to partially mitigate the toxicity of DGLA, and in their absence DGLA is detrimental to developing germ cells at much lower concentrations. Strains carrying mutations in *sbp-1*, fatty acid desaturases, and *nhr-49* produce fewer viable progeny than wild type, reflecting their defects in lipid homeostasis (Brock et al., 2007; Van Gilst et al., 2005a, 2005b; Watts et al., 2003; Yang et al., 2006). NHR-49 is particularly important during the starvation response for the activation of beta-oxidation genes and it also enables adult worms to survive starvation by regulating the process of adult reproductive diapause, in which most germ cells undergo apoptosis during food scarcity (Angelo and Van Gilst, 2009). While *nhr-80* mutants have normal progeny production, NHR-80 plays an important role in the regulation of lifespan extension in animals lacking a germ line (Goudeau et al., 2011).

Lipid and energy homeostasis is often challenged by physiological stressors, which through a network of signaling mediators and cross talk, can lead to regulation of metabolic homeostasis by controlling lipid synthesis and degradation, energy sensing, and the distribution of nutrients to tissues (Mailloux et al., 2007; Van Gilst et al., 2005a; Watts, 2009; Zuryn et al., 2010). In *C. elegans*, the most proliferative tissue during larval development is the germ line, which expands from four cells in a newly hatched worm to thousands of gametes in an adult worm. During both gametogenesis and embryogenesis a complex series of signaling and nutrient allocation events take place in order to ensure the survival of genetic material. Thus, the germ line is arguably the most sensitive tissue with regards to changes in nutrition during development.

Interestingly, mutation in the peptide transporter gene, *pept-1*, confers strong DGLA resistance. We demonstrate that this strain incorporates DGLA at similar levels as wild type, yet germ cells do not undergo destruction as they do in wild type worms. Our observations, together with others (Korta et al., 2012), reveal that *pept-1* worms have a smaller germ line than wild type (Fig. 1B), raising the possibility that the defective uptake of peptides results in fewer resources that are allocated to the germ line in these animals.

Peroxisomal beta-oxidation promotes sterility caused by DGLA

We found that mutations in *dhs-28* and *daf-22* showed moderate resistance to dietary DGLA. These genes are part of the peroxisomal beta-oxidation pathway and are required for the production of the dauer pheromone as well as for the detoxification and oxidation of long chain fatty acids (Butcher et al., 2009; Joo et al., 2009). Because of the role of the peroxisome in the detoxification of long chain fatty acids, we had predicted that strains carrying mutations in *dhs-28* and *daf-22* genes would be more sensitive to DGLA. However, the fact that these strains show resistance to DGLA supports the hypothesis that a toxic metabolite may be produced from DGLA, and that the production of this metabolite requires chain shortening in the peroxisome.

Genetic and physiological activation of a stress response protects against DGLA toxicity

Mutations that lead to reduced insulin/IGF and TGF-β signaling conferred robust resistance to dietary DGLA. Strikingly, strains carrying *daf-2* and *daf-7* mutations maintain a normal cohort of germ cells under conditions that lead to complete germ cell loss in wild type (Fig. 1). The insulin/IGF and TGF-β signaling pathways have been shown to be involved in regulating development, lifespan, metabolism, stress responses and the balance of proliferation and differentiation in the *C. elegans* germ line (Baumeister et al., 2006; Dalfo et al., 2012; Fielenbach and Antebi, 2008; Kenyon, 2010; Michaelson et al., 2010). Thus, mechanisms that allow *daf-2* mutants to resist heat and oxidative stress may be relevant to resisting the toxic effects of dietary DGLA.

Resistance to various stresses in *daf-2* mutants depends to a large extent on DAF-16/FOXO, which becomes localized to the nucleus. Remarkably, in spite of its toxic effects, dietary DGLA in wild type animals did not seem to activate a robust stress response, as DAF-16 did not become nuclear localized, nor did DAF-16 target genes become induced after DGLA feeding (Fig. 4). This suggests that the insulin/IGF-1 pathway was maintained in an active state, and DGLA nutrient sensory pathways may not recognize DGLA as a toxin.

Interestingly, physical activation of a stress response through a preconditioning heat stress allowed wild type L1 worms to resist the effects of DGLA (Fig. 4A). This type of hormetic response has been characterized in a number of organisms in response to toxins and other stressors, leading to protection from later insults (Cypser and Johnson, 2002; Ristow and Schmeisser, 2011). We found that direct or indirect preconditioning was largely dependent on the DAF-16 FOXO transcription factor, because both *daf-16* and *daf-18* mutants did not respond to heat stress preconditioning. These results corroborate our hypothesis that induction of a stress response, either physically through a heat shock, or genetically through alteration of the insulin/IGF pathway, restore germ line development and fertility in worms exposed to DGLA.

Intact phase II detoxification systems resist DGLA toxicity

RNAi knockdown of *skn-1* as well as *gcs-1* and the phase II conjugation gene, *ugt-22* resulted in increased sensitivity to DGLA (Fig. 3A), indicating phase II detoxification systems protect the germ line from dietary DGLA. In animals, phase II detoxification/conjugation systems are activated mostly through SKN-1/Nrf (Park et al., 2009; Przybysz et al., 2009; Wang et al., 2010). Toxic molecules are removed from cells by way of conjugation reactions that include glucuronidation, glutathiolation and sulfation, which will ultimately lead to a more water-soluble product available for excretion. The transcriptional up-regulation of a glutathione biosynthesis gene, *gcs-1*, is a hallmark of phase II detoxification under the control of SKN-1 in *C. elegans* (Ferguson et al., 2010; Kell et al., 2007; Park et al., 2009; Przybysz et al., 2009). Our observations that knockdown of *skn-1*, *gcs-1* and *ugt-22* leads to increased sterility in the presence of dietary DGLA supports the model that sterility induced by dietary DGLA may occur through a toxic lipid metabolite that requires activation of detoxification systems for removal.

Dietary DGLA disrupts germ cell membranes

Previously we demonstrated that dietary DGLA leads to the loss of germ cell nuclei and increased apoptosis. Because *ced-4* mutant worms that are defective in germ cell apoptosis still become sterile when fed DLGA (Watts and Browse, 2006), we examined germ cells in earlier stages of development. We found that DGLA affects mitotic and meiotic germ cells at the distal end of the gonad, and the earliest effects of DGLA feeding that we observe occur near the transition zone, the area in contact with the S1 sheath cell (Killian and Hubbard, 2005). The region may be particularly critical for the import of lipids to support the high

demand for membrane expansion in the early meiotic germ cells. Evidence that nutrient allotment and proliferation in this region is a regulated process comes from a study demonstrating that proliferation of mitotic germ cells during the L3 and L4 larval stages depends on insulin signals (Michaelson et al., 2010).

Our microscopic examinations indicate that the initial stages of germ cell degradation begin at the membrane. We observed membrane degradation after six hours of DGLA supplementation (Fig. 7A) while germ cell nuclear morphology did not look abnormal until 24 h after supplementation. These data are consistent with a model that DGLA, or a toxic metabolite, is transferred from the intestine to the germ line along with other nutrients to support the rapid growth of the germ line. The toxic lipid may interfere with membrane growth or cytokinesis, leading to multinucleate germ cells which are ultimately destroyed by apoptosis or other death pathways.

Biology of omega-6 fatty acids

A high ratio of omega-6 to omega-3 polyunsaturated fatty acids in the diet has been associated with numerous disease states (Brown et al., 2010; Funahashi et al., 2008; Wang et al., 2009). It has been implied that humans likely evolved on a diet where the omega-6:omega-3 ratio was 1:1, whereas modern, Western diets are composed of ratios greater than 15:1 omega-6 to omega-3 fatty acids (Simopoulos, 2003). While a complete mechanistic understanding of omega-6 fatty acids in health and disease is elusive, it appears that much of their toxicity is associated with the formation of inflammatory molecules. For example, a strong association was found between DGLA levels in phospholipids and inflammatory and endothelial activation markers in overweight and obese patients (Steffen et al., 2012). Recently some of the anti-inflammatory roles of omega-3 fatty acids were shown to have a mechanism that was receptor mediated, resulting in increased insulin sensitivity (Oh et al., 2010). The adverse health effects of excess dietary omega-6 PUFAs may be mediated by eicosanoids, which are powerful short-lived signaling molecules produced from 20-carbon polyunsaturated fatty acids (PUFAs) through a series of oxidation reactions. In response to certain stimuli, arachidonic acid and other PUFAs are released from phospholipids for conversion into eicosanoids such as prostaglandins (Fonteh et al., 1995).

Although *C. elegans* homologs of cyclooxygenase and lipoxygenase enzymes are not apparent in the genome, a class of F-series prostaglandin molecules essential for sperm guidance have been described in *C. elegans* (Edmonds et al., 2010; Kubagawa et al., 2006). The biosynthetic pathway of these prostaglandins has not been elucidated, but the molecules appear to be regulated in part by the insulin/IGF pathway (Edmonds et al., 2010). Eicosanoid signaling molecules can also be generated by cytochrome P450 monooxygenase activity on 20-carbon PUFAs (Arnold et al., 2010; Kulas et al., 2008). *C. elegans* possess approximately 80 genes encoding cytochrome P450s (CYPs) (Menzel et al., 2001). One of the gene products, CYP33E2, has been shown to produce epoxy- and hydroxyl-derivatives of eicosapentaenoic acid (20:5, EPA) and, to a lesser extent, of arachidonic acid (20:4, AA) (Kulas et al., 2008). Future studies are needed to determine whether DGLA is enzymatically modified to produce an oxidized product capable of causing damage to the developing membranes of the germ line.

Model and conclusions

Our current model predicts that DGLA, or a specific oxidized metabolite derived from DGLA, is transported to the distal gonad as part of a nutrient transport pathway to supply lipids for the developing germ line. Fatty acid homeostasis regulators, such as SBP-1, AAK-2, NHR-49, and NHR-80 ensure that the proper lipids are available for transport into the germ line to support the membrane generation and the massive energy requirements for the production of offspring. Excess dietary DGLA appears to overwhelm the homeostatic systems, and the omega-6 fatty acid or its oxidized derivative, interferes with the growth or maintenance of cell membranes in the distal germ line, leading to germ cell death and ultimately to sterility. Basal levels of GCS-1 and UGT-22 act to detoxify the DGLA to a certain extent, however, a robust stress response toward DGLA is not initiated, perhaps because DGLA is a membrane component as well as a nutrient, and therefore may bypass the stress responses that are induced by non-nutrient toxins. However, if a stress response is initiated such that DAF-16 becomes localized to the nucleus, germ cells are protected from the DGLA assault. Future genetic and biochemical studies will focus on elucidation of the molecular nature of the DGLA metabolite. A more thorough understanding of the effects of dietary DGLA in the germ line promises to provide insights into the cellular responses to dietary omega-3 and omega-6 fatty acids in animals. Furthermore, the potential for a dietary lipid to destroy germ cells may have far-reaching implications, ranging from cancer biology and infertility in humans to the control and remediation of pathogenic nematodes in agricultural products.

Acknowledgments

We thank Xiaoju Zou and Xun Shi for technical assistance, Nate Tucker and Ryan Middleton for assistance with confocal microscopy, and Kwan Hee Kim and Tracy Vrablik for critical reading of the manuscript. Funding was provided by NIH R01 DK74114 to JLW. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

References

An, J.H., Vranas, K., Lucke, M., Inoue, H., Hisamoto, N., Matsumoto, K., Blackwell, T.K., 2005. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. Proc. Natl. Acad. Sci. USA 102, 16275–16280.

Angelo, G., Van Gilst, M.R., 2009. Starvation protects germline stem cells and extends reproductive longevity in *C. elegans*. Science 326, 954–958.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., Riddle, D.L., 2000. daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes. Dev. 14, 1512–1527.

Apfeld, J., O'Connor, G., McDonagh, T., DiStefano, P.S., Curtis, R., 2004. The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*. Genes. Dev. 18, 3004–3009.

Arnold, C., Konkel, A., Fischer, R., Schunck, W.H., 2010. Cytochrome P450-dependent metabolism of omega-6 and omega-3 long-chain polyunsaturated fatty acids. Pharmacol. Rep. 62, 536–547.

Audhya, A., Hyndman, F., McLeod, I.X., Maddox, A.S., Yates 3rd, J.R., Desai, A., Oegema, K., 2005. A complex containing the Sm protein CAR-1 and the RNA helicase CGH-1 is required for embryonic cytokinesis in *Caenorhabditis elegans*. J. Cell Biol. 171, 267–279.

Austin, J., Kimble, J., 1987. glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. Cell 51, 589–599.

Baumeister, R., Schaffitzel, E., Hertweck, M., 2006. Endocrine signaling in *Caenorhabditis elegans* controls stress response and longevity. J. Endocrinol. 190, 191–202.

Bonilla, D.L., Fan, Y.Y., Chapkin, R.S., McMurray, D.N., 2010. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. J. Infect. Dis. 201, 399–408.

Brasky, T.M., Till, C., White, E., Neuhouser, M.L., Song, X., Goodman, P., Thompson, I.M., King, I.B., Albanes, D., Kristal, A.R., 2011. Serum phospholipid fatty acids and prostate cancer risk: results from the prostate cancer prevention trial. Am. J. Epidemiol. 173, 1429-1439.

Brock, T.J., Browse, J., Watts, J.L., 2006. Genetic regulation of unsaturated fatty acid composition in *C. elegans*. PLoS Genet. 2, e108.

Brock, T.J., Browse, J., Watts, J.L., 2007. Fatty acid desaturation and the regulation of adiposity in *Caenorhabditis elegans*. Genetics 176, 865–875.

Brown, M.D., Hart, C., Gazi, E., Gardner, P., Lockyer, N., Clarke, N., 2010. Influence of omega-6 PUFA arachidonic acid and bone marrow adipocytes on metastatic spread from prostate cancer. Br. J. Cancer. 102, 403–413.

Burr, G.O., Burr, M.M., 1973. A new deficiency disease produced by the rigid exclusion of fat from the diet. Nutr. Rev. 31, 248–249, Nutrition classics from: The Journal of Biological Chemistry 82, 345–367 (1929).

Butcher, R.A., Ragains, J.R., Li, W., Ruvkun, G., Clardy, J., Mak, H.Y., 2009. Biosynthesis of the *Caenorhabditis elegans* dauer pheromone. Proc. Natl. Acad. Sci. USA 106, 1875–1879.

Crittenden, S.L., Rudel, D., Binder, J., Evans, T.C., Kimble, J., 1997. Genes required for GLP-1 asymmetry in the early *Caenorhabditis elegans* embryo. Dev. Biol. 181, 36–46.

Cypser, J.R., Johnson, T.E., 2002. Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. J. Gerontol. A Biol. Sci. Med. Sci. 57, B109–B114.

Dalfo, D., Michaelson, D., Hubbard, E.J., 2012. Sensory regulation of the *C. elegans* germline through TGF-beta-dependent signaling in the niche. Curr. Biol. 22, 712–719.

Edmonds, J.W., Prasain, J.K., Dorand, D., Yang, Y., Hoang, H.D., Vibbert, J., Kubagawa, H.M., Miller, M.A., 2010. Insulin/FOXO signaling regulates ovarian prostaglandins critical for reproduction. Dev. Cell. 19, 858–871.

Erkkila, A., de Mello, V.D., Riserus, U., Laaksonen, D.E., 2008. Dietary fatty acids and cardiovascular disease: an epidemiological approach. Prog. Lipid Res. 47, 172–187.

Ferguson, A.A., Springer, M.G., Fisher, A.L., 2010. skn-1-Dependent and -independent regulation of aip-1 expression following metabolic stress in *Caenorhabditis elegans*. Mol. Cell. Biol. 30, 2651–2667.

Fielenbach, N., Antebi, A., 2008. *C. elegans* dauer formation and the molecular basis of plasticity. Genes. Dev. 22, 2149–2165.

Fonteh, A.N., Samet, J.M., Chilton, F.H., 1995. Regulation of arachidonic acid, eicosanoid, and phospholipase A2 levels in murine mast cells by recombinant stem cell factor. J. Clin. Invest. 96, 1432–1439.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., Ahringer, J., 2000. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature 408, 325–330.

Funahashi, H., Satake, M., Hasan, S., Sawai, H., Newman, R.A., Reber, H.A., Hines, O.J., Eibl, G., 2008. Opposing effects of n-6 and n-3 polyunsaturated fatty acids on pancreatic cancer growth. Pancreas 36, 353–362.

Furukawa-Hibi, Y., Yoshida-Araki, K., Ohta, T., Ikeda, K., Motoyama, N., 2002. FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. J. Biol. Chem. 277, 26729–26732.

Gartner, A., Boag, P.R., Blackwell, T.K., 2008. Germline survival and apoptosis. WormBook, 1–20.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., Riddle, D.L., 1998. Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. Genetics 150, 129–155.

Goudeau, J., Bellemin, S., Toselli-Mollereau, E., Shamalnasab, M., Chen, Y., Aguilaniu, H., 2011. Fatty acid desaturation links germ cell loss to longevity through NHR-80/HNF4 in *C. elegans*. PLoS Biol. 9, e1000599.

Greer, E.L., Brunet, A., 2009. Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. Aging Cell 8, 113–127.

Greer, E.R., Perez, C.L., Van Gilst, M.R., Lee, B.H., Ashrafi, K., 2008. Neural and molecular dissection of a *C. elegans* sensory circuit that regulates fat and feeding. Cell Metab. 8, 118–131.

Gumienny, T.L., Lambie, E., Hartwieg, E., Horvitz, H.R., Hengartner, M.O., 1999. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Development 126, 1011–1022.

Hansen, D., Hubbard, E.J., Schedl, T., 2004. Multi-pathway control of the proliferation versus meiotic development decision in the *Caenorhabditis elegans* germline. Dev. Biol. 268, 342–357.

Hardie, D.G., 2011. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev. 25, 1895–1908.

Henderson, S.T., Johnson, T.E., 2001. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. 11, 1975–1980.

Honda, Y., Honda, S., 1999. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J. 13, 1385–1393.

Hubbard, N.E., Lim, D., Erickson, K.L., 1998. Alteration of murine mammary tumorigenesis by dietary enrichment with n-3 fatty acids in fish oil. Cancer Lett. 124, 1–7.

Hudert, C.A., Weylandt, K.H., Lu, Y., Wang, J., Hong, S., Dignass, A., Serhan, C.N., Kang, J.X., 2006. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. Proc. Natl. Acad. Sci. USA 103, 11276–11281.

Hutzell, P.A., Krusberg, L.R., 1982. Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comp. Biochem. Physiol. 73B (3), 517–520.

Inoue, H., Hisamoto, N., An, J.H., Oliveira, R.P., Nishida, E., Blackwell, T.K., Matsumoto, K., 2005. The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. Genes Dev. 19, 2278–2283.

Joo, H.J., Yim, Y.H., Jeong, P.Y., Jin, Y.X., Lee, J.E., Kim, H., Jeong, S.K., Chitwood, D.J., Paik, Y.K., 2009. Caenorhabditis elegans utilizes dauer pheromone biosynthesis to dispose of toxic peroxisomal fatty acids for cellular homoeostasis. Biochem. J. 422, 61–71.

Kahn, N.W., Rea, S.L., Moyle, S., Kell, A., Johnson, T.E., 2008. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in *Caenorhabditis elegans*. Biochem. J. 409, 205–213.

Kang, J.X., Wang, J., Wu, L., Kang, Z.B., 2004. Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids. Nature 427, 504.

Kell, A., Ventura, N., Kahn, N., Johnson, T.E., 2007. Activation of SKN-1 by novel kinases in *Caenorhabditis elegans*. Free Radic. Biol. Med. 43, 1560–1566.

Kenyon, C.J., 2010. The genetics of ageing. Nature 464, 504–512.

Killian, D.J., Hubbard, E.J., 2004. *C. elegans* pro-1 activity is required for soma/germline interactions that influence proliferation and differentiation in the germ line. Development 131, 1267–1278.

Killian, D.J., Hubbard, E.J., 2005. *Caenorhabditis elegans* germline patterning requires coordinated development of the somatic gonadal sheath and the germ line. Dev. Biol. 279, 322–335.

Kimble, J., Crittenden, S.L., 2007. Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. Annu. Rev. Cell Dev. Biol. 23, 405–433.

Kniazeva, M., Sieber, M., McCauley, S., Zhang, K., Watts, J.L., Han, M., 2003. Suppression of the ELO-2 FA elongation activity results in alterations of the fatty acid composition and multiple physiological defects, including abnormal ultradian rhythms, in *Caenorhabditis elegans*. Genetics 163, 159–169.

Korta, D.Z., Hubbard, E.J., 2010. Soma-germline interactions that influence germline proliferation in *Caenorhabditis elegans*. Dev. Dyn. 239, 1449–1459.

Korta, D.Z., Tuck, S., Hubbard, E.J., 2012. S6K links cell fate, cell cycle and nutrient response in *C. elegans* germline stem/progenitor cells. Development 139, 859–870.

Kubagawa, H.M., Watts, J.L., Corrigan, C., Edmonds, J.W., Sztul, E., Browse, J., Miller, M.A., 2006. Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment in vivo. Nat. Cell Biol. 8, 1143–1148.

Kulas, J., Schmidt, C., Rothe, M., Schunck, W.H., Menzel, R., 2008. Cytochrome P450-dependent metabolism of eicosapentaenoic acid in the nematode *Caenorhabditis elegans*. Arch. Biochem. Biophys. 472, 65–75.

Lai, L., Kang, J.X., Li, R., Wang, J., Witt, W.T., Yong, H.Y., Hao, Y., Wax, D.M., Murphy, C.N., Rieke, A., Samuel, M., Linville, M.L., Korte, S.W., Evans, R.W., Starzl, T.E., Prather, R.S., Dai, Y., 2006. Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat. Biotechnol. 24, 435–436.

Larsen, P.L., Albert, P.S., Riddle, D.L., 1995. Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics 139, 1567–1583.

Lee, S.S., Kennedy, S., Tolonen, A.C., Ruvkun, G., 2003. DAF-16 target genes that control *C. elegans* life-span and metabolism. Science 300, 644–647.

Lin, K., Hsin, H., Libina, N., Kenyon, C., 2001. Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. 28, 139–145.

Liu, G., Bibus, D.M., Bode, A.M., Ma, W.Y., Holman, R.T., Dong, Z., 2001. Omega 3 but not omega 6 fatty acids inhibit AP-1 activity and cell transformation in JB6 cells. Proc. Natl. Acad. Sci. USA 98, 7510–7515.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Mailloux, R.J., Beriault, R., Lemire, J., Singh, R., Chenier, D.R., Hamel, R.D., Appanna, V.D., 2007. The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. PLoS One 2, e690.

McElwee, J., Bubb, K., Thomas, J.H., 2003. Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. Aging Cell 2, 111–121.

McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H., Gems, D., 2004. Shared transcriptional signature in *Caenorhabditis elegans* dauer larvae and long-lived *daf-2* mutants implicates detoxification system in longevity assurance. J. Biol. Chem. 279, 44533–44543.

Meissner, B., Boll, M., Daniel, H., Baumeister, R., 2004. Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans. J. Biol. Chem. 279, 36739–36745.

Menzel, R., Bogaert, T., Achazi, R., 2001. A systematic gene expression screen of *Caenorhabditis elegans* cytochrome P450 genes reveals CYP35 as strongly xenobiotic inducible. Arch. Biochem. Biophys. 395, 158–168.

Mernitz, H., Lian, F., Smith, D.E., Meydani, S.N., Wang, X.D., 2009. Fish oil supplementation inhibits NNK-induced lung carcinogenesis in the A/J mouse. Nutr. Cancer. 61, 663–669.

Michaelson, D., Korta, D.Z., Capua, Y., Hubbard, E.J., 2010. Insulin signaling promotes germline proliferation in *C. elegans*. Development 137, 671–680.

Mozaffarian, D., Micha, R., Wallace, S., 2010. Effects on coronary heart disease of increasing polyunsaturated fat in place of saturated fat: a systematic review and meta-analysis of randomized controlled trials. PLoS Med. 7, e1000252.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., Kenyon, C., 2003. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature 424, 277–283.

Narasimhan, S.D., Yen, K., Bansal, A., Kwon, E.S., Padmanabhan, S., Tissenbaum, H.A., 2011. PDP-1 links the TGF-beta and IIS pathways to regulate longevity, development, and metabolism. PLoS Genet. 7, e1001377.

Narbonne, P., Roy, R., 2009. *Caenorhabditis elegans* dauers need LKB1/AMPK to ration lipid reserves and ensure long-term survival. Nature 457, 210–214.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., Ruvkun, G., 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature 389, 994–999.

Oh, D.Y., Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M., Olefsky, J.M., 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142, 687–698.

Oliveira, R.P., Porter Abate, J., Dilks, K., Landis, J., Ashraf, J., Murphy, C.T., Blackwell, T.K., 2009. Condition-adapted stress and longevity gene regulation by *Caenorhabditis elegans* SKN-1/Nrf. Aging Cell 8, 524–541.

Park, S.K., Tedesco, P.M., Johnson, T.E., 2009. Oxidative stress and longevity in *Caenorhabditis elegans* as mediated by SKN-1. Aging Cell 8, 258–269.
Patel, D.S., Garza-Garcia, A., Nanji, M., McElwee, J.J., Ackerman, D., Driscoll, P.C., Gems, D., 2008. Clustering of genetically defined allele classes in the *Caenorhabditis elegans* DAF-2 insulin/IGF-1 receptor. Genetics 178, 931–946.
Patterson, G.I., Padgett, R.W., 2000. TGF beta-related pathways. Roles in *Caenorhabditis elegans* development. Trends Genet. 16, 27–33.
Pinkston-Gosse, J., Kenyon, C., 2007. DAF-16/FOXO targets genes that regulate tumor growth in *Caenorhabditis elegans*. Nat. Genet. 39, 1403–1409.
Pohlmeier, W.E., Hovey, R.C., Van Eenennaam, A.L., 2011. Reproductive abnormalities in mice expressing omega-3 fatty acid desaturase in their mammary glands. Transgenic Res. 20, 283–292.
Przybysz, A.J., Choe, K.P., Roberts, L.J., Strange, K., 2009. Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of *Caenorhabditis elegans* to the xenobiotic juglone. Mech. Ageing Dev. 130, 357–369.
Ristow, M., Schmeisser, S., 2011. Extending life span by increasing oxidative stress. Free Radic. Biol. Med. 51, 327–336.
Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.
Simopoulos, A.P., 2003. Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. World Rev. Nutr. Diet. 92, 1–22.
Simopoulos, A.P., 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med. (Maywood) 233, 674–688.
Spanier, B., Lasch, K., Marsch, S., Benner, J., Liao, W., Hu, H., Kienberger, H., Eisenreich, W., Daniel, H., 2009. How the intestinal peptide transporter PEPT-1 contributes to an obesity phenotype in *Caenorhabditis elegans*. PLoS One 4, e6279.
Spychalla, J.P., Kinney, A.J., Browse, J., 1997. Identification of an animal omega-3 fatty acid desaturase by heterologous expression in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 94, 1142–1147.
Steffen, B.T., Steffen, L.M., Tracy, R., Siscovick, D., Hanson, N.Q., Nettleton, J., Tsai, M.Y., 2012. Obesity modifies the association between plasma phospholipid polyunsaturated fatty acids and markers of inflammation: the Multi-Ethnic Study of Atherosclerosis. Int. J. Obes. (Lond) 36, 797-804.
Terns, R.M., Kroll-Conner, P., Zhu, J., Chung, S., Rothman, J.H., 1997. A deficiency screen for zygotic loci required for establishment and patterning of the epidermis in *Caenorhabditis elegans*. Genetics 146, 185–206.
Tullet, J.M., Hertweck, M., An, J.H., Baker, J., Hwang, J.Y., Liu, S., Oliveira, R.P., Baumeister, R., Blackwell, T.K., 2008. Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*. Cell 132, 1025–1038.
Van Gilst, M.R., Hadjivassiliou, H., Yamamoto, K.R., 2005a. A *Caenorhabditis elegans* nutrient response system partially dependent on nuclear receptor NHR-49. Proc. Natl. Acad. Sci. USA 102, 13496–13501.
Van Gilst, M.R., Hadjivassiliou, H., Jolly, A., Yamamoto, K.R., 2005b. Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in *C. elegans*. PLoS Biol. 3, e53.
Walker, A.K., Jacobs, R.L., Watts, J.L., Rottiers, V., Jiang, K., Finnegan, D.M., Shioda, T., Hansen, M., Yang, F., Niebergall, L.J., Vance, D.E., Tzoneva, M., Hart, A.C., Naar, A.M., 2011. A conserved SREBP-1/phosphatidylcholine feedback circuit regulates lipogenesis in metazoans. Cell 147, 840–852.
Wang, J., Robida-Stubbs, S., Tullet, J.M., Rual, J.F., Vidal, M., Blackwell, T.K., 2010. RNAi screening implicates a SKN-1-dependent transcriptional response in stress resistance and longevity deriving from translation inhibition. PLoS Genet. 6, e10001048.
Wang, S., Wu, D., Matthan, N.R., Lamon-Fava, S., Lecker, J.L., Lichtenstein, A.H., 2009. Reduction in dietary omega-6 polyunsaturated fatty acids: eicosapentaenoic acid plus docosahexaenoic acid ratio minimizes atherosclerotic lesion formation and inflammatory response in the LDL receptor null mouse. Atherosclerosis 204, 147–155.
Watts, J.L., 2009. Fat synthesis and adiposity regulation in *Caenorhabditis elegans*. Trends Endocrinol. Metab. 20, 58–65.
Watts, J.L., Browse, J., 2002. Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 99, 5854–5859.
Watts, J.L., Browse, J., 2006. Dietary manipulation implicates lipid signaling in the regulation of germ cell maintenance in *C. elegans*. Dev. Biol. 292, 381–392.
Watts, J.L., Phillips, E., Griffing, K.R., Browse, J., 2003. Deficiencies in C20 polyunsaturated fatty acids cause behavioral and developmental defects in *Caenorhabditis elegans* fat-3 mutants. Genetics 163, 581–589.
Weylandt, K.H., Krause, L.F., Gomolka, B., Chiu, C.Y., Bilal, S., Nadolny, A., Waechter, S.F., Fischer, A., Rothe, M., Kang, J.X., 2011. Suppressed liver tumorigenesis in fat-1 mice with elevated omega-3 fatty acids is associated with increased omega-3 derived lipid mediators and reduced TNF-{alpha}. Carcinogenesis 32, 897-903.
Xia, S., Lu, Y., Wang, J., He, C., Hong, S., Serhan, C.N., Kang, J.X., 2006. Melanoma growth is reduced in fat-1 transgenic mice: impact of omega-6/omega-3 essential fatty acids. Proc. Natl. Acad. Sci. USA 103, 12499–12504.
Yang, F., Vought, B.W., Satterlee, J.S., Walker, A.K., Jim Sun, Z.Y., Watts, J.L., DeBeaumont, R., Saito, R.M., Hyberts, S.G., Yang, S., Macol, C., Iyer, L., Tjian, R., van den Heuvel, S., Hart, A.C., Wagner, G., Naar, A.M., 2006. An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. Nature 442, 700–704.
Yee, L.D., Young, D.C., Rosol, T.J., Vanbuskirk, A.M., Clinton, S.K., 2005. Dietary (n-3) polyunsaturated fatty acids inhibit HER-2/neu-induced breast cancer in mice independently of the PPARgamma ligand rosiglitazone. J. Nutr. 135, 983–988.
Zuryn, S., Kuang, J., Tuck, A., Ebert, P.R., 2010. Mitochondrial dysfunction in *Caenorhabditis elegans* causes metabolic restructuring, but this is not linked to longevity. Mech. Ageing Dev. 131, 554–561.