DAF-18 is required for the age-dependent increase in DAF-16 activity in

Caenorhabditis elegans

Kali Carrasco and Matthew J. Youngman

Department of Biology, Villanova University, Villanova, PA 19085

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Corresponding author:

Matthew J. Youngman

Department of Biology

Villanova University

800 E. Lancaster Ave., Mendel Hall Rm. 191C

Villanova, PA 19085 USA

(610) 519-3916

matthew.youngman@villanova.edu

ABSTRACT

The insulin/insulin-like growth factor signaling (IIS) pathway modulates growth, survival, and lifespan by regulating FOXO transcription factors. In *Caenorhabditis elegans*, IIS maintains DAF-16/FOXO in an inactive state unless animals are challenged by environmental stress. Recent evidence suggests that DAF-16 becomes activated as part of normal aging in *C. elegans*, yet the regulatory module responsible for this phenomenon is largely undefined. Embedded within IIS is phospholipid signaling in which PIP₃ produced by the PI3 kinase AGE-1 is an upstream event in DAF-16 inhibition. Countering AGE-1 is DAF-18, an ortholog of human PTEN phosphatase that dephosphorylates PIP₃. Although it is required for normal lifespan in *C. elegans*, functional characterization of DAF-18 has primarily focused on its roles during development in the germline and neurons. In this study we asked whether DAF-18 plays a role in the age-dependent activation of DAF-16, and specifically in DAF-16-mediated immunity. Our data show that DAF-18 is expressed in multiple tissues during adulthood. We found that DAF-18 contributes to host defense in adult animals by functioning in the neurons and intestine, likely working through DAF-16 which acts in those same tissues to confer immunity. Supporting this possibility, DAF-18 was required for increased DAF-16 transcriptional activity during aging. Post-translational modifications including ubiquitination and sumoylation appear to be required for the function of DAF-18 during aging in *C. elegans*, indicating that strategies to modulate PTEN activity are evolutionarily conserved. Our results establish an important role for DAF-18 later in life and imply that it is a critical component of a neuroendocrine signaling circuit that governs the dynamic activity of DAF-16.

INTRODUCTION

In diverse animal species, the insulin and insulin-like growth factor signaling pathway (IIS) regulates lifespan and integrates environmental cues to control growth and development (Kenyon *et al.* 1993; Tamemoto *et al.* 1994; Kimura *et al.* 1997; Tatar *et al.* 2001; Yuan *et al.* 2009). While eliminating insulin signaling reduces the size of some organisms, lifespan extension is a benefit in common to animals with low IIS activity. This was demonstrated first in the roundworm *Caenorhabditis elegans* but has since been shown to hold true in vertebrates as well (Kenyon *et al.* 1993). For example, female mice heterozygous for mutations in the insulin-like growth factor receptor *IGF1R* live 33% longer than their wildtype counterparts (Holzenberger *et al.* 2003). In dogs, a nonsynonymous SNP in *IGF1R* that may reduce the ability of the receptor to bind its ligand is found in tiny breeds, which tend to live longer, and is not present in large breeds that die comparatively younger (Hoopes *et al.* 2012). In humans, mutations in *IGF1R* associated with reduced IGF1R activity are found in centenarians (Suh *et al.* 2008). Regulatory mechanisms that attenuate insulin signaling could therefore be of particular importance to preserving normal lifespan.

In *C. elegans*, the basis for the effect of IIS on influencing longevity is its regulation of the ultimate downstream target of the pathway, the forkhead box class O (FOXO) transcription factor DAF-16. When bound by agonist insulin-like peptides, the kinase activity of the insulin receptor DAF-2 is activated and it phosphorylates the PI3 kinase AGE-1. AGE-1 phosphorylates PIP₂ to produce PIP₃, which is necessary to activate PDK-1, which, in turn, activates the kinase AKT-1. AKT-1 is the component of the IIS pathway that directly interacts with and inhibits DAF-16. When phosphorylated

by AKT-1, DAF-16 is expelled from the nucleus and sequestered in the cytosol, physically separated from its transcriptional targets. Mutations in *daf-2* or *age-1* that disrupt the upstream steps of the IIS pathway extend the lifespan of *C. elegans* by 2- to 3-fold (Kenyon *et al.* 1993; Dorman *et al.* 1995). This extraordinary phenotype is suppressed by loss-of-function mutations in *daf-16*, indicating that its activity is what is responsible for augmenting the longevity of *daf-2* and *age-1* mutants (Kenyon *et al.* 1993). The transcriptional targets of DAF-16 are well-defined, and they span broad functional categories including metabolism, detoxification, genome maintenance, proteostasis, and innate immunity (McElwee *et al.* 2003; Murphy *et al.* 2003; Tepper *et al.* 2013; Li *et al.* 2019). Many DAF-16 targets play important roles in the response to environmental insults, and so activation of DAF-16 is correlated with resistance to ultraviolet light, oxidative stress, and microbial infection (Henderson and Johnson 2001; Wolff *et al.* 2006). By upregulating repair and maintenance pathways, DAF-16 improves cellular health, limits cellular damage, buffers organisms from stress and thus increases healthspan—the period of life spent in youthful vigor and without disease.

When wildtype *C. elegans* are maintained under stress-free conditions and their food source is unlimited, constitutive inhibition through IIS prevents DAF-16 from being activated. Until recently, environmental insults were the only inputs known to shift DAF-16 into an active state. Three reports now challenge that notion by suggesting that aging is sufficient to upregulate DAF-16 expression and to induce its transcriptional activity (Bansal *et al.* 2014; Li *et al.* 2019; McHugh *et al.* 2020). The regulatory mechanism that governs the dynamic activity of DAF-16 during aging is, at this point, largely undefined.

The series of successive activation events in the IIS pathway that lead to DAF-16 inhibition is reminiscent of similar modules in other signaling pathways that are subject to redundant regulation. One example is the familiar phosphorylation cascade within MAPK signaling pathways. Kinases within these pathways may be inactivated through dephosphorylation by members of three different classes of protein phosphatases. For example, ERK1/2 is the sole substrate of three dual-specificity phosphatases of the MKP family, but it is also regulated by an additional six phosphatases that dephosphorylate it and at least one other type of MAPK (Kondoh and Nishida 2007). There are considerably fewer phosphatases that act to inhibit IIS components. These proteins are attractive candidates for putative regulators of the age-dependent activation of DAF-16. Perhaps because of its broad spectrum of substrates, AKT-1 is the most tightly regulated IIS kinase, being prone to dephosphorylation by two tyrosine phosphatases, EAK-6 and SDF-9, and one serine/threonine kinase (of the PP2A family) (Hu *et al.* 2006; Padmanabhan *et al.* 2009). In contrast, the PI3 kinase AGE-1 is regulated by only a single phosphatase, but through a different mechanism. Instead of dephosphorylating AGE-1 itself, DAF-18 dephosphorylates the product of AGE-1's catalysis, PIP₃ (Ogg and Ruvkun 1998). DAF-18 is uniquely positioned to oppose insulin signaling by intersecting with one of the first steps of the pathway. Since neuroendocrine signals, including insulins, limit lifespan, we hypothesized that counteracting these signals may be crucial for the age-dependent activation of DAF-16, and so we investigated the role of DAF-18 in regulating DAF-16 during aging in *C. elegans*.

DAF-18 is the *C. elegans* ortholog of mammalian PTEN, a tumor suppressor that is frequently mutated in sporadic cancers (Milella *et al.* 2015). Although it functions primarily as a lipid phosphatase, PTEN can also dephosphorylate protein substrates. When localized to the cytosol, PTEN acts to inhibit processes that are of particular relevance to cancer, including progression through the cell cycle, cell growth, and cell migration. PTEN can also localize to the nucleus where it contributes to genome integrity by activating DNA repair pathways, and it plays a role in chromatin remodeling (Milella *et al.* 2015). Similar to PTEN, DAF-18 also inhibits cell proliferation primarily by depleting PIP₃ levels and thus negatively regulating AKT-1. For example, during L1 diapause DAF-18 prevents germ cell proliferation that is mediated by AKT activation of the TOR complex and it also blocks division of Q neuroblasts promoted by AKT activation of MPK-1 (Fukuyama *et al.* 2006, 2012; Watanabe *et al.* 2008; Zheng *et al.* 2018). In the germline, DAF-18 activates the serine/threonine kinase PAR-4 to inhibit oocyte growth, maturation, and ovulation (Narbonne *et al.* 2015, 2017). In this capacity, DAF-18 acts locally within one gonad arm to oppose systemic IIS signaling that promotes oocyte growth. Notably, in none of the aforementioned roles does DAF-18 act by regulating DAF-16.

While capable of acting independently of DAF-16, prior to our study several lines of evidence suggested that DAF-18 plays a key role in determining lifespan by functioning through DAF-16. First, mutations in *daf-18* suppress the extended lifespan of *daf-2* mutants, similar to mutations in *daf-16* (Dorman *et al.* 1995). Along those lines, restoring *daf-18* expression in *daf-2(e1370);daf-18(mg198)* mutants rescues the lifespan extension phenotype of *daf-2* mutants in a *daf-16*-dependent manner (Masse

et al. 2005). Third, *daf-18* is necessary for the enrichment in nuclear localization of DAF-16 in *daf-2* mutants (Larsen *et al.* 1995; Dorman *et al.* 1995; Lin *et al.* 2001). Finally, both *daf-16* and *daf-18* are required for lifespan extension through hormetic heat conditioning (Cypser *et al.* 2006). Taken together, these results strongly suggest that DAF-18 plays a role in aging, but specific functions for DAF-18 in adult animals have been established only in axon regeneration and associative learning (Tomioka *et al.* 2006; Byrne *et al.* 2014).

Here we show that the age-dependent increase in DAF-16 transcriptional activity requires DAF-18. We find substantial functional overlap between DAF-18 and DAF-16 during aging, suggesting that the two proteins act in concert. In particular, in postreproductive *C. elegans* both proteins contribute to innate immunity. Each confers resistance to microbial infection by functioning during adulthood in both the neurons and the intestine. DAF-18 appears to be expressed in chemosensory amphid neurons in adult *C. elegans*. Combined with our functional data, this suggests that it may be part of a neuroendocrine signaling axis between the neurons and the intestine to coordinate DAF-16 function as animals age. Finally, we uncover evidence to suggest that during aging DAF-18 may be deubiquitinated and sumoylated, two modifications that would promote its association with the plasma membrane to anchor it near its lipid substrates.

MATERIALS AND METHODS

***C. elegans* strains and maintenance**

Worms were maintained using standard techniques as previously described (Brenner 1974). The *C. elegans* strains used in this study are as follows: Bristol wildtype N2, GR1329 [*daf-16(mgDf47)*], RB712 [*daf-18(ok480)*], VIL001 *mjyls001 [plys-7::GFP]*, DAF-18::GFP zhEx343 [*daf-18 genomic::gfp*], pCFJ90 [*Pmyo-2::mCherry*], NR350 [*rde-1(ne219); kzs20 [hlh1p::rde-1 + sur-5p::NLS::GFP]*], VP303 [*rde-1(ne219); kbIs7 [nhx-2p::rde-1 + rol-6(su1006)]*], TU3401 [*sid-1(pk3321); uls69 [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*]. To avoid possible confounding effects from UV-induced lesions at loci other than *daf-18*, RB712 was outcrossed 3 times prior to functional characterization

Generation of age-matched cohorts of *C. elegans*

Synchronized populations of worms were obtained by sodium hypochlorite treatment of gravid adult animals to harvest eggs followed by hatching L1 larvae in M9 buffer in the absence of a bacterial food source for 16-20h at 22°C. Approximately 2000 L1 larvae were plated on to NGM media seeded with either *E. coli* OP50 or *E. coli* HT115 strains harboring RNAi constructs, depending on the experiment. To age animals to Day 6 of adulthood, worms were allowed to develop to the L4 larval stage before transferring them to new NGM plates containing 25 ug/mL 5-fluorodeoxyuridine (FUdR) and seeded with *E. coli*.

RNAi treatment

E. coli HT115 strains carrying plasmids encoding dsRNA were thawed from glycerol stocks stored at -80°C and grown overnight at 37°C on LB plates

supplemented with ampicillin and tetracycline. Single colonies were used to inoculate 200 mL of LB + ampicillin and incubated with shaking overnight at 37°C. To concentrate cells, cultures were centrifuged for 10 minutes at 3000xg and the cell pellet was resuspended in 20 mL of fresh ampicillin-supplemented LB. Approximately 1 mL of the concentrated cell cultures was used to seed two sets of NGM plates: one containing carbenicillin, and 2M IPTG (referred to as “RNAi plates”) and a second set containing carbenicillin, 2M IPTG, and 25ug/mL FUdR; referred to as “RNAi + FUdR plates”). Seeded plates were incubated in the dark at 22°C for at least 3 days before animals were added to them for knockdown experiments.

Synchronized populations of worms were subjected to RNAi treatments at one of two points during aging, depending on the experiment. For experiments involving a brief pulse of RNAi to knockdown *daf-18*, *daf-16*, or *age-1*, wild type N2 L1 larvae harvested from hypochlorite treatment of gravid adult hermaphrodites were maintained on NGM plates with lawns of *E. coli* OP50 at 20°C as described above until Day 4 of adulthood. Approximately 48 hours prior to challenging these animals with *P. aeruginosa*, they were transferred to RNAi +FUdR plates seeded with the *daf-18*, *daf-16*, or *age-1* RNAi clone (or L4440 vector control clones) and incubated at 20°C. For all other RNAi experiments, after hatching overnight in M9 buffer wild type N2 L1 larvae harvested from hypochlorite treatment of gravid adult hermaphrodites were cultivated on RNAi plates seeded with concentrated *E. coli* RNAi and maintained at 20°C. Upon reaching the L4 stage, these animals were transferred by chunking to RNAi + FUdR plates where they were separated from the agar chunk before it was removed from the

plate. *C. elegans* were aged on these plates at 20°C until Day 6 of adulthood when they were harvested or used for other assays.

Transgenic strains of *C. elegans* were used to knock down *daf-18* and *daf-16* in a tissue-specific manner. In particular, to target these genes by RNAi in only the intestine or the muscle, in an *rde-1(ne219)* mutant background *rde-1* is supplied in an integrated transgene that drives its expression by a promoter that is specific to either the intestine (strain VP303) or the muscle (strain NR350). For neuron-specific RNAi, *sid-1* expression is restored only in neurons under the *unc-119* promoter in a *sid-1(pk3321)* background (strain TU3401).

Fluorescence microscopy

To monitor the expression of *Plys-7::GFP* during aging, worms on RNAi or RNAi + FUDR plates were inspected under a Zeiss Stemi SV11 APO Stereo Microscope equipped with a Zeiss digital camera. In each of three independent biological replicates at least 200 animals spread over 4 separate plates per condition were scored for *Plys-7::GFP* expression levels at each time point. Depending on their pattern of fluorescence animals were assigned to one of three categories, as follows: 1) High—bright GFP signal observed throughout entire length of intestine, 2) Medium—comparatively weaker GFP signal that tended to be brighter in the anterior intestine than in the posterior intestine, 3) Low—dim GFP signal in all or only a segment of the intestine. To generate micrographs of the expression of the *Plys-7::GFP* reporter during aging, *C. elegans* maintained on NGM plates seeded with OP50 or subjected to feeding-based RNAi were mounted onto agarose pads (10% agarose in M9 buffer)

seated on glass slides and immobilized in a slurry of 0.1 μ m polystyrene beads (Polysciences, Warrington, PA).

The expression of DAF-18::GFP was assessed by confocal microscopy using a Leica TCS SP8 inverted confocal microscope equipped with both HyD and standard PMTs (Leica). Day 6 adult *C. elegans* that had been maintained on NGM and (after reaching the L4 stage) NGM + FUdR plates seeded with *E. coli* OP50 were mounted on to agarose pads on glass slides as described above. Both the 488 nm and 552 nm laser lines were used to illuminate samples during image acquisition. Z-stacks consisting of 25-40 optical sections were collected for each region of interest at a resolution of 1024x1024 pixels at a magnification of either 400 or 630x. Composite images were initially processed with Leica LASX software, and further adjustments were made using NIH ImageJ.

***Pseudomonas aeruginosa* infection**

Larval stage and adult *C. elegans* were infected with the bacterial pathogen *Pseudomonas aeruginosa* as described (Youngman *et al.* 2011). Briefly, approximately 100 animals of the indicated ages were transferred from plates containing *E. coli* OP50 or *E. coli* HT115 (for RNAi treatments) and distributed as three groups of ~30 worms each onto plates seeded with *P. aeruginosa* strain PA14 which were then incubated at 25°C. At intervals of 12-24 hours, the survival of *C. elegans* on the *P. aeruginosa* plates was assessed and dead animals were removed at each time point until all of the worms in the assay had died. At least three independent biological replicates of each infection experiment were performed.

Calculation of median survival times and statistical analysis

To calculate the median survival time (LT_{50}) of *C. elegans* infected with *P. aeruginosa*, the fraction of animals alive at each time point during an assay was first plotted as a function of time in Excel, accounting for animals that were inadvertently overlooked or that escaped between time points. These data were then imported into SigmaPlot (Systat Software, San Jose, CA) and a three parameter sigmoidal curve was fit according to the general equation $y=a/(1+e^{-(x-x_0)/b})$. This equation was used to determine the point at which 50% of the animals in the assay had died (Efron 1987). The average fold difference between the LT_{50} of mutant strains or experimental RNAi treatments and control animals was calculated, and the statistical significance of that difference was assessed using a one-sample t-test after first applying a Shapiro-Wilk test for normality.

RNA isolation

To harvest *C. elegans* for RNAi isolation, approximately 10,000-20,000 animals were washed off of NGM plates in M9 buffer and allowed to settle to the bottom of a 15 mL conical tube by gravity. After removing the supernatant the worms were washed with 10 mL of fresh M9 and again allowed to settle to the bottom of the tube. All but 1-2 mL of buffer were removed and used to resuspend the worms before transferring them to cryovials where more buffer was removed, leaving behind sufficient volume to cover the worm pellet. After adding 300 μ L of Trizol reagent (ThermoFisher Scientific, Waltham, MA), the worms were vortexed in a series of 30 second intervals over a period of 5 minutes interspersed by brief rest periods and then transferred to -80°C for storage. To prepare total RNA from worm pellets, frozen animals in Trizol were thawed at room temperature and then briefly vortexed before pelleting in a microcentrifuge at

16,000xg for 5 min to remove worm carcasses and debris. Following a phenol/chloroform extraction, total RNA was precipitated in isopropanol, washed in 70% ethanol, and resuspended in RNase-free H₂O. RNA was then reisolated over a column using the RNeasy kit (Qiagen, Germantown, MD). The absorbance of samples at 260 nm was measured by Nanodrop (ThermoFisher Scientific, Waltham, MA) and used to calculate the concentration of RNA.

qRT-PCR

To measure gene expression levels by qRT-PCR, cDNA was reverse transcribed from 1 µg samples of total RNA using Superscript IV Vilo RT(Invitrogen). Diluted cDNA was used as a template in PCR reactions carried out in Power SYBR Green master mix (Applied Biosystems, Foster City, CA). Previously described primer pairs (Troemel *et al.* 2006) were used to amplify sequences from *lys-7*, *sod-3*, *mtl-1*, and *cpr-2* transcripts with *tba-1* as the reference, and fluorescence was quantified using BioRad CFX96 thermocycler (BioRad, Hercules, CA). Relative gene expression levels were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). This analysis was performed on a total of three biological replicates, representing three independently generated cohorts of aging worms.

Data availability

Files uploaded as supplemental material to figshare are described below. Table S1 contains the raw data from manual scoring of *plys-7::GFP* expression in three independent biological replicates corresponding to the data shown as bar graphs in Fig. 2 B-D. A separate section of the table shows the raw scores converted to fractions so that the proportion of animals in each category may be compared. Table S2 lists

raw Ct values and calculated $\Delta\Delta\text{Ct}$ for *mtl-1* and *sod-3* expression levels in L4 larvae and Day 6 adults treated with RNAi targeting *daf-18*, *daf-16*, or *age-1*. These data correspond to the bar graph in Fig. 2E. Table S3 lists raw Ct values and calculated $\Delta\Delta\text{Ct}$ for *cpr-2* and *lys-7* expression levels in L4 larvae and Day 6 adults treated with RNAi targeting *daf-18*, *daf-16*, or *age-1*. These data correspond to the bar graph in Fig. 2E. Table S4 lists compiled fold differences in expression of *mtl-1*, *sod-3*, *lys-7*, and *cpr-2* between L4 and Day 6 in animals treated with RNAi targeting *daf-16*, *daf-18*, or *age-1* for three independent biological replicates. Also listed are average fold differences in expression between Day 6 and L4 with standard deviations and standard error. These data correspond to the bar graph in Fig. 2E. Table 5 lists experimental details and statistical analyses corresponding to each survival curve in Figs. 3-5. Figure S1 shows confocal micrographs of *daf-18::GFP*-expressing worms treated with RNAi targeting GFP. Figure S2 shows additional replicates of the *P. aeruginosa* infection assay. Figure S3 shows additional replicates of the RNAi experiment to investigate the timing requirement of *daf-18* in *C. elegans* immunity. Figures S4 and S5 depict results from two additional replicates of the experiment in which RNAi was used to knock down *daf-18* expression in specific tissues before worms were infected with *P. aeruginosa*. Figure S6 shows an additional replicate of the experiment in which worms treated with RNAi targeting candidate DAF-18 regulators were infected with *P. aeruginosa*.

RESULTS

Expression pattern of DAF-18 in adult *C. elegans*

In *C. elegans* larvae a *daf-18* transcriptional reporter is expressed in a number of tissues, including the intestine, body wall muscles, seam cells, hypodermis, and neurons (Masse *et al.* 2005). The expression of endogenous DAF-18 in wildtype animals is most evident in the Z2/Z3 germline precursor cells and in oocytes, yet when overexpressed or in a *daf-2* mutant background, DAF-18 is detected in the intestine, the ventral nerve cord, and in head amphid neurons (Brisbin *et al.* 2009; Liu *et al.* 2014). We asked whether DAF-18 is still found in these same tissues in postreproductive adult *C. elegans* and so we examined the expression of DAF-18::GFP in adult animals six days after they had reached the L4 larval stage (hereafter referred to as Day 6 adults). At lower magnification, DAF-18::GFP signal was observed in two locations: near the procorpus and terminal bulb of the pharynx and, more faintly, within two broad parallel segments near the animal's posterior (Fig. 1A). The substantial amount of autofluorescence in the intestine from lipofuscin precluded our ability to make conclusive observations regarding the expression of DAF-18 in that tissue in adult *C. elegans*. Closer examination at higher magnification revealed that DAF-18::GFP appears to be expressed in head neurons whose dendrites traverse the isthmus of the pharynx and extend toward the mouth, reminiscent of its expression in amphid neurons in younger animals (Fig. 1B; (Brisbin *et al.* 2009)). Surprisingly, at the posterior, what appeared as a diffuse pattern at lower magnification was actually discrete rows of puncta. While the specific identity of these structures awaits further confirmation, we note that their pattern is strikingly similar to the appearance of fragmented mitochondria in the body wall muscle of animals subjected to acute thermal stress (Fig. 1C (Momma *et al.* 2017)). Our imaging studies show that in postreproductive adult *C.*

C. elegans DAF-18 continues to be expressed in many of the same tissues in which it is found in younger animals, suggesting that its role in those tissues is not limited to developmental processes or other functions that are exclusive to larvae.

DAF-18 is required for the age-dependent increase in the transcriptional activity of DAF-16

Similar to the effect of mutations in *daf-16*, both reduced-function and loss-of-function mutations in *daf-18* suppress the constitutive dauer and extended lifespan phenotypes of *daf-2* mutants (Dorman *et al.* 1995). This suggests that even in the absence of a repressive signal through the insulin receptor, DAF-18 is necessary for the full activation of DAF-16, which is responsible for the stress resistance and exceptional longevity of *daf-2* animals (Ogg and Ruvkun 1998). In wildtype larvae, DAF-16 appears to only be activated in response to acute environmental insults, remaining sequestered in the cytosol under nutritionally replete, stress-free conditions (McHugh *et al.* 2020). Shortly after animals transition to adulthood, however, *daf-16* expression is upregulated despite the absence of overt stressors (Bansal *et al.* 2014). This is met with a commensurate increase in DAF-16 transcriptional activity toward a subset of its targets, including those that confer resistance to bacterial pathogens (Li *et al.* 2019; McHugh *et al.* 2020). To determine whether DAF-18 regulates the age-dependent increase in DAF-16 transcriptional activity, we first asked whether DAF-18 was necessary for the increased expression of *plys-7::GFP*, an *in vivo* reporter in which the promoter of the DAF-16 transcriptional target gene *lys-7* drives GFP expression in the intestine (Alper *et al.* 2007).

The expression of *plys-7::GFP* increases in an age-dependent manner, and this requires *daf-16* (McHugh *et al.* 2020). As previously reported, we observed three major categories of *plys-7::GFP* expression levels within the intestines of age-matched cohorts of *C. elegans* (Fig. 2A). Animals in the “low” category showed weak *plys-7::GFP* expression that appeared to be confined to only the anterior-most portion of the intestine. “Medium” levels of *plys-7::GFP* expression were characterized by prominent GFP signal in the anterior intestine with weaker expression in posterior segments. *C. elegans* with robust *plys-7::GFP* expression throughout the entire length of the intestine without interruption were classified as “high” expressors. To test the possible role for DAF-18 in activating DAF-16 during aging, we examined the effect of knocking down *daf-18* on the expression of *plys-7::GFP* expression at three points: the L4 larval stage, Day 3 of adulthood, and Day 6 of adulthood. For comparison, *daf-16* and *age-1* were also targeted by RNAi. At each time point, animals were examined by fluorescence microscopy and were scored as belonging to one of the three categories described above, according to the level of *plys-7::GFP* expression in their intestine. If DAF-16 is regulated by DAF-18 during aging, we expected that RNAi targeting *daf-18* would have the same effect on *plys-7::GFP* expression during adulthood as knocking down *daf-16*. This is, in fact, what we found. In L4 larvae, nearly all animals (more than 90% in all cases) exhibited low expression of *plys-7::GFP*, regardless of what gene was targeted by RNAi (Fig. 2B). By Day 3 of adulthood, *plys-7::GFP* expression had increased in control animals fed *E. coli* harboring an empty RNAi vector (L4440), with animals being split almost evenly between the “high” and “low” expression categories (Fig. 2C). There was not an equivalent increase in *plys-7::GFP* expression when either

daf-18 or *daf-16* was knocked down. Instead, while medium levels of *plys-7::GFP* expression were observed in about 30% of animals, the majority of worms in both of these treatment groups still showed low levels of *plys-7::GFP* expression. By contrast, RNAi targeting *age-1* enhanced the age-dependent increase in *plys-7::GFP* expression, resulting in a larger proportion of Day 3 *C. elegans* being assigned to the “high” category. This same general pattern persisted through Day 6 of adulthood. At that point, almost all *C. elegans* in the control group showed high levels of *plys-7::GFP* expression, similar to animals treated with RNAi targeting *age-1* (Fig. 2D). RNAi of *daf-18* and *daf-16* largely suppressed the increased expression of *plys-7::GFP* in Day 6 animals. Compared to L4 larvae, the fraction of Day 6 animals with medium levels of *plys-7::GFP* expression still increased when *daf-18* or *daf-16* were knocked down, yet fewer than 10% had high *plys-7::GFP* expression levels and in about half of the animals its expression was low. Together, the results of our *in vivo* reporter assay suggest that DAF-18 is necessary for the activation of DAF-16 transcriptional activity that occurs during aging in *C. elegans*.

To validate the results of our studies with *plys-7::GFP*, we measured the levels of endogenous transcripts of *lys-7* along with the transcripts of three other genes whose expression is regulated by DAF-16, *mtl-1*, *sod-3*, and *cpr-2*. (Troemel *et al.* 2006; Kwon *et al.* 2010). qRT-PCR was used to compare the expression levels of the four DAF-16 transcriptional targets in L4 larvae to their expression levels in Day 6 adults (Fig. 2E). Each of the genes that we tested was more highly expressed in adult *C. elegans* than in larvae, although the magnitude by which they were upregulated differed. For example, *lys-7* was upregulated by almost 100-fold in Day 6 adults

compared to L4 animals, while the expression of *sod-3* was only about 2-fold greater. To test whether *daf-18* is necessary to bring about the changes in expression of DAF-16 transcriptional targets over time that we observed, we used RNAi to knock down its expression and evaluated the effect relative to knocking down *daf-16* or *age-1*. Knockdown of *daf-18* caused substantial reductions in the expression of DAF-16 targets, phenocopying the effect of knocking down *daf-16* (Fig. 2E). Targeting either *daf-18* or *daf-16* with RNAi decreased the expression of *mtl-1* and *sod-3* in Day 6 adults to levels that were lower than at the L4 larval stage and reduced *lys-7* and *cpr-2* expression by nearly 10-fold. In contrast, knocking down *age-1* increased the expression of the DAF-16 transcriptional targets in Day 6 *C. elegans* by two to three orders of magnitude. These results are consistent with our observations with the *plys-7::GFP* reporter, providing additional evidence that DAF-18 regulates the age-dependent increase in DAF-16 transcriptional activity. Further, they imply that DAF-18 and AGE-1 play opposing roles during aging in wildtype *C. elegans*.

DAF-18 functions in innate immunity in adult *C. elegans*

We previously reported that one functional consequence of the age-dependent increase in DAF-16 transcriptional activity is that DAF-16 contributes to innate immunity in adult but not larval stage *C. elegans* (McHugh *et al.* 2020). Since we found that in adults the elevated levels of two DAF-16 targets with potential functions as immune effectors, *lys-7* and *cpr-2*, require DAF-18 we asked whether DAF-18 contributes to host defense during aging. To test this possibility, the survival of *daf-18(ok480)* mutants infected with the human opportunistic pathogen *Pseudomonas aeruginosa* at either the L4 larval stage or at Day 6 of adulthood was compared to the

survival of age-matched infected *daf-16(mgDf47)* or N2 wild type animals. When challenged with *P. aeruginosa* as L4 larvae, *daf-18(ok480)* mutants died at a similar rate as wildtype animals, as did *daf-16(mgDf47)* mutants (Fig. 3A; Table S5). On the other hand, when the infection was initiated at Day 6 of adulthood, *daf-18(ok480)* mutants and *daf-16(mgDf47)* animals both died more rapidly than wildtype worms (Fig. 3B; Table S5). Stated in more quantitative terms, the infected adult *daf-18(ok480)* and *daf-16(mgDf47)* mutants had median lifespans (LT₅₀) of ~70 hours, whereas the LT₅₀ of infected N2 wildtype adults was ~120 hours. These data indicate that, similar to DAF-16, DAF-18 functions in innate immunity in adult *C. elegans*.

That *daf-18(ok480)* phenocopies the enhanced pathogen susceptibility of *daf-16(mgDf47)* implies that DAF-18 cooperates with DAF-16 to confer resistance to infection during aging. If this is the case, then both genes should be functioning in innate immunity simultaneously. Said another way, the timing requirements for *daf-18* and *daf-16* in host defense should be the same. While it is ubiquitously expressed during larval development and for the duration of life in *C. elegans*, DAF-16 functions exclusively during adulthood to fulfill its roles in innate immunity and in determining lifespan (Dillin *et al.* 2002; McHugh *et al.* 2020). For these two aspects of DAF-16's function its temporal requirement was defined by initiating RNAi to reduce its expression only during discrete intervals instead of according to more commonly used regimens that involve continuous knockdown, beginning at an early larval stage or in the parental generation.

We employed a similar strategy to determine the timing requirement of DAF-18 in host defense. Specifically, we compared the effect of sustained RNAi treatment that

knocked down *daf-18* expression starting at the L1 larval stage and continuing through adulthood to a brief treatment in which RNAi was delayed until Day 4 of adulthood. Animals treated according to the latter scheme would therefore be allowed to develop and complete the reproductive period in the presence of normal *daf-18* expression levels. If the timing requirements for DAF-18 and DAF-16 were the same, then we anticipated that waiting until Day 4 of adulthood to knock down *daf-18* expression would be sufficient to recapitulate the enhanced pathogen susceptibility phenotype that we observed when *daf-18* RNAi treatment began at L1, just as it is for bringing about the *daf-16* pathogen susceptibility phenotype (McHugh *et al.* 2020).

We found that the sustained RNAi treatment accurately reproduced the phenotype of the *daf-18(ok480)* loss-of-function mutant. That is, knockdown of *daf-18* from the L1 stage did not cause L4 larvae to be more susceptible to *P. aeruginosa* infection than untreated control (L4440) animals (Fig. 3C). At Day 6 of adulthood, however, animals subjected to this RNAi regimen targeting *daf-18* died from bacterial infection at rate that was similar to when the RNAi was directed against *daf-16*, and both sets of *C. elegans* died faster than infected control worms treated with an empty RNAi vector (Fig. 3D). Delivering just a short pulse of RNAi only to adult worms turned out to have this same effect. When *daf-18* was knocked down for two days during adulthood, from Day 4 until *P. aeruginosa* infection was initiated at Day 6, animals were still more susceptible to the infection than untreated controls and died at a similar rate to worms subjected to the sustained RNAi treatment (Fig 3D). Moreover, the LT₅₀ of Day 6 animals subjected to the brief RNAi treatment targeting *daf-18* closely approximated the LT₅₀ of animals in which *daf-16* was knocked down using the same

regimen, corroborating our previous observations (Fig. 3D; (McHugh *et al.* 2020)).

These results indicate that DAF-18 and DAF-16 have identical timing requirements for their roles in innate immunity and that their contributions to host defense are not the products of having acted earlier in the worm's life but instead are made at the point just prior to when animals were challenged with *P. aeruginosa*. That is, both proteins function during adulthood to protect adult *C. elegans* from bacterial infection.

Within the IIS pathway, DAF-18 counteracts the function of the AGE-1 kinase that is immediately downstream of the DAF-2 insulin receptor by dephosphorylating PIP₃, thus depriving PDK-1 of a lipid that promotes its activation. Considering the antagonistic relationship between DAF-18 and AGE-1, abrogating the function of one protein should have the opposite effect of when the function of the other is compromised. This is what we observed in our analysis of DAF-16 transcriptional target expression levels in Day 6 adults when *daf-18* or *age-1* were knocked down (Fig. 2D, E). Based on those results, we predicted that whereas *daf-18* promotes innate immunity during aging, *age-1* might act to inhibit it. To test this possibility, we subjected worms to sustained RNAi treatment targeting *age-1* beginning at the L1 stage of larval development. When challenged with *P. aeruginosa* as L4 larvae, the survival of these animals was similar to that of untreated control worms (Fig. 3C). Yet when the infection was initiated at Day 6 of adulthood, *age-1* knockdown caused worms to become more resistant to the pathogen, extending the median survival of infected *C. elegans* by nearly two-fold (Fig. 3D; Table S5). This result confirmed our hypothesis and suggests that *age-1* functions to limit host defense in postreproductive animals but not in larvae. When we investigated the timing requirement for *age-1* by

treating worms with RNAi to knock down *age-1* beginning at Day 4 of adulthood before infecting them with *P. aeruginosa* two days later, these animals died at the same rate as untreated controls, suggesting that AGE-1 functions prior to Day 4 to regulate host defense in adults (Fig. 3D).

DAF-18 and DAF-16 function in the intestine and neurons to protect adult *C. elegans* from bacterial pathogens

Our functional characterization of DAF-18 during aging supports the possibility that it acts together with DAF-16 to modulate innate immunity in adult *C. elegans*. Since DAF-16 is downstream of DAF-18 in the IIS pathway, this could mean that the two proteins function in the same tissues. Although it is expressed in many other tissues, the sites of action of DAF-16 for its role in lifespan determination are the intestine and neurons (Wolkow *et al.* 2000; Libina *et al.* 2003). We asked about the site of action of both DAF-18 and DAF-16 for their roles in host defense by knocking down their expression only in individual tissue types through the use of tissue-specific RNAi strains of *C. elegans*. We expected that inhibiting *daf-16* or *daf-18* expression in the particular tissue in which they are required for innate immunity should be sufficient to recapitulate the effect of systemic RNAi that reduces their expression in all tissue types (Fig. 4A and B; Table S5). Although DAF-18::GFP appears to be expressed in body wall muscle (Fig. 1C), muscle-specific RNAi of *daf-18* had no effect on the susceptibility of either L4 larvae or Day 6 adults to *P. aeruginosa* infection (Fig. 4C and D). Similarly, knockdown of *daf-16* in the muscle did not affect the survival of animals infected as either L4 larvae or Day 6 adults. Inhibiting the expression of *daf-18* and *daf-16* in either the intestine or the neurons, however, caused Day 6 adults to be just as sensitive to *P.*

aeruginosa infection as when *daf-18* or *daf-16* were knocked down in all tissues (Fig. 4E-H). These results indicate that DAF-18 and DAF-16 act in both the intestine and neurons in postreproductive adult worms and that their functions in those tissue promote host defense.

Posttranslational modifications modulate the activity of DAF-18 during aging

Similar to PTEN in mammals, DAF-18 is regulated at several levels in *C. elegans*. For example, the HLH-25 transcription factor inhibits *daf-18* expression and thus appears to be a functional ortholog of HES1, which negatively regulates PTEN in human cells and in mice (Chou *et al.* 2015). At the protein level, in general phosphorylation inhibits DAF-18 by decreasing its stability. For example, DAF-18 is phosphorylated by the Eph receptor tyrosine kinase VAB-1, and *vab-1* mutants have higher levels of DAF-18 protein than wildtype animals (Brisbin *et al.* 2009). Interestingly, a human PTEN allele with threonine-to-alanine substitutions that destroy phosphorylation sites was able to rescue the short lifespan phenotype of *daf-2(e1370);daf-18(mg198)* mutants more effectively than the wildtype PTEN allele, suggesting that the lack of phosphorylation made PTEN more active (Solari *et al.* 2005). The scenario during aging that is supported by our functional analysis implies that DAF-18 activation increases in adult *C. elegans*. Since most previous investigations of DAF-18 regulation have identified or characterized negative regulators, to search for potential DAF-18 activators, we selected *C. elegans* orthologs of three positive regulators of PTEN—*smo-1*, *math-33*, and *cpb-3*-- for functional analysis studies (Bermúdez Brito *et al.* 2015). *smo-1* encodes the sole *C. elegans* ortholog of mammalian SUMO, a ubiquitin-like moiety that regulates protein function. In mammals,

sumoylation of PTEN influences its subcellular localization ((Huang *et al.* 2012). MATH-33 is an ortholog of human USP7 that deubiquitinates target proteins in a thiol-dependent manner. CBP-3 has predicted histone acetyltransferase activity and is an ortholog of human CREB binding protein (CBP). Depending on the specific nature of the modification, ubiquitination either destabilizes PTEN or promotes its localization to the nucleus and so MATH-33 would be expected to influence those same properties of DAF-18 (Chen *et al.* 2018). For comparison, we also characterized one putative negative regulator of DAF-18, SUP-17. With predicted metallopeptidase activity, SUP-17 is an ortholog of human ADAM10 which, by proteolytically activating Notch or receptor tyrosine kinases (such as HER2), may inhibit either the expression or catalytic activity of PTEN (Wen *et al.* 1997).

To determine whether our candidate genes might regulate DAF-18 during aging, we assessed their roles in host defense in L4 larvae and in Day 6 adults. We reasoned that knocking down genes that encode putative activators of DAF-18 would phenocopy the knockdown of *daf-18*, curtailing the survival of adult *C. elegans* infected with *P. aeruginosa*, but having no effect on the ability of L4 larvae to resist bacterial infection. Conversely, RNAi targeting potential DAF-18 inhibitors should lead to the opposite phenotype, conferring resistance to bacterial infection during adulthood. None of the genes that we tested appear to influence innate immunity in L4 worms, since RNAi treatments targeting each gene individually failed to modify the survival of animals infected at that stage of development (Fig. 5A, Table S5). When animals were challenged with *P. aeruginosa* at Day 6 of adulthood, RNAi targeting *cbp-3* did not produce a phenotype. Knockdown of *smo-1* or *math-33*, however, both reduced the

median survival of infected animals to a similar extent as RNAi targeting *daf-18* or *daf-16* when compared to untreated animals (Fig. 5B). This agrees with our prediction for the phenotype of possible positive regulators of the age-dependent function of DAF-18, and it is consistent with the established roles for the orthologs of SMO-1 and MATH-33 in PTEN regulation. These data suggest that during aging in *C. elegans* the activity of DAF-18 may be regulated by post-translational modifications, including sumoylation and ubiquitination.

DISCUSSION

Considering its critical, evolutionarily conserved roles in lifespan determination and stress resistance, understanding the mechanism by which DAF-16 is regulated in *C. elegans* has the potential to reveal paradigms governing the activity of FOXO transcription factors in other species. While DAF-16 has been known to be activated by acute stress for some time, more recent evidence has revealed that the transcriptional activity of DAF-16 increases as animals age even without the trigger of an environmental insult (Li *et al.* 2019; McHugh *et al.* 2020). This implies that there is a regulatory mechanism inherent to the aging process that governs DAF-16 activity once animals reach adulthood. So far the only putative regulator of the age-dependent activation of DAF-16 to be identified is SMK-1, a component of the PP4 phosphoprotein phosphatase complex that, based on homology, modulates the activity of the catalytic subunit (McHugh *et al.* 2020). Here we tested the possibility that DAF-18, the *C. elegans* ortholog of mammalian PTEN that antagonizes PI3 kinase in the IIS pathway, also plays a role in regulating DAF-16 during adulthood. Although DAF-18 functions in adult animals to inhibit axon regeneration and associative learning,

both processes are at least partially independent of DAF-16 (Tomioka *et al.* 2006; Byrne *et al.* 2014). Our data suggest an additional regulatory function for DAF-18 during aging that directly impacts the transcriptional activity of DAF-16 and, consequently, its role in innate immunity. We found that the age-dependent increase in expression of DAF-16 targets requires DAF-18 and that without *daf-18* host defense in postreproductive adult animals is compromised, enhancing their susceptibility to lethal infection by bacterial pathogens, just as the loss of functional *daf-16* does. Our results suggest that as *C. elegans* age an inhibitory signal through the IIS must be counteracted by DAF-18 in order to initiate a basal level of DAF-16 transcriptional activity that buffers older animals against anticipated but yet to be encountered environmental stress. We anticipate that our findings will have implications for PTEN-mediated regulation of aging processes in evolutionarily diverse species, including humans.

Prior to our work, a connection between *daf-18* and aging had been established by studies examining its role as a suppressor of the extended lifespan of *daf-2* mutants (Larsen *et al.* 1995; Dorman *et al.* 1995; Ogg and Ruvkun 1998; Mihaylova *et al.* 1999; Honda and Honda 2002; Patel *et al.* 2008). Significantly, in the absence of any other genetic lesions, mutations in *daf-18* shorten lifespan while *daf-18* overexpression extends lifespan, indicating that DAF-18 contributes to longevity when IIS signaling is intact in wildtype animals (Dorman *et al.* 1995; Mihaylova *et al.* 1999; Honda and Honda 2002; Masse *et al.* 2005; Brisbin *et al.* 2009). It is under these same conditions that DAF-16 becomes activated during the course of aging, and this is why we decided to investigate a possible role for DAF-18 in regulating DAF-16 during adulthood. A

direct output of DAF-16 activation is a change in gene expression, and we measured this in two ways in adult animals in the presence and absence of DAF-18. Both our qualitative assessment of DAF-16 transcriptional target expression using the *in vivo* *plys-7::GFP* reporter and our quantitative measurement of endogenous transcript levels showed that increased DAF-16 transcriptional activity during adulthood depends on DAF-18. While transcriptomic analysis would provide a more complete picture of the full extent of DAF-18's influence on the transcriptional output of DAF-16, we found DAF-18 to be required for the age-dependent increase in the expression of representative detoxification enzymes and immune effectors. Accordingly, our functional analysis revealed a role for DAF-18 in the innate immunity of adult *C. elegans*. While DAF-18 had no bearing on the ability of larval stage animals to resist bacterial pathogens, postreproductive adults lacking functional DAF-18 died from *P. aeruginosa* infection more rapidly than their wildtype counterparts. This same age-dependent enhanced pathogen susceptibility is a characteristic of animals lacking functional DAF-16 (this study and McHugh *et al.* 2020). Our results therefore support the possibility that DAF-18 is necessary to activate DAF-16 during aging and that it contributes to DAF-16-mediated host defense in adult *C. elegans*.

PTEN is tightly regulated in mammals, yet comparatively few DAF-18 regulators have been identified in *C. elegans*. Since DAF-18 has not been extensively characterized during aging, our studies gave us an opportunity to search for possible mechanisms of DAF-18 regulation that had not previously been reported in worms. The results of our reverse genetic candidate gene approach suggest that in order to perform its functions in innate immunity during adulthood, DAF-18 must be

deubiquitinated by MATH-33 and it must be sumoylated through conjugation to SMO-

1. Based on their effects on PTEN, both of these modifications may be necessary to efficiently target DAF-18 to the plasma membrane. Monoubiquitination of PTEN promotes its translocation to the nucleus, and polyubiquitinated PTEN is degraded (Chen *et al.* 2018). Therefore, regardless of which ubiquitin moiety is removed from the protein, the result is to promote its activity in the cytosol. Binding of PTEN to the plasma membrane is associated with its sumoylation on two adjacent lysine residues (Huang *et al.* 2012). If the effects of these posttranslational modifications are evolutionarily conserved, de-ubiquitinated, sumoylated DAF-18 should be closely positioned to its lipid substrate. This provides additional evidence to suggest that DAF-18 functions as a lipid phosphatase during aging. One caveat of our data is that the knockdown of *math-33* and *smo-1* likely affects posttranslational modifications of multiple proteins and not only DAF-18. A notable case in point is that DAF-16 is a known substrate of MATH-33 under conditions of low IIS (Heimbucher *et al.* 2015). Additional studies are needed to confirm that the enhanced pathogen susceptibility phenotype caused by knockdown of *smo-1* and *math-33* in Day 6 adults is attributable solely to aberrant modification of DAF-18 that prevents DAF-16 activation. Should this be confirmed, then MATH-33 and the SUMO conjugating enzyme UBC-9 will represent new components of the regulatory mechanism governing DAF-18 function during aging.

Although DAF-18 is capable of dephosphorylating protein substrates, its role in lifespan determination requires its lipid phosphatase activity (Solari *et al.* 2005; Brisbin *et al.* 2009; Liu *et al.* 2014). This strongly suggests that the mechanism by which DAF-

18 regulates DAF-16 is by dephosphorylating PIP₃ produced by AGE-1, the PI3 kinase that is immediately downstream of the DAF-2 insulin receptor, thereby preventing activation of the inhibitory AKT-1 kinase. The requirement for DAF-18 to activate DAF-16 during adulthood therefore implies that there is a repressive signal through IIS at that time that must be opposed by DAF-18. If this is the case, then manipulations to relieve such repression should cause hyperactivation of DAF-16 and thus bring about phenotypes associated with its constitutive activity, including increased stress resistance. Indeed, we found that RNAi knockdown of *age-1* caused Day 6 adult but not larval stage *C. elegans* to become more resistant to bacterial infection. Similarly, knocking down *daf-2* beginning at the L1 stage improves the resistance of Day 6 animals to *P. aeruginosa* infection (McHugh *et al.* 2020). When we probed the specific timing requirements for *age-1*, *daf-18*, and *daf-16* in innate immunity using short pulses of RNAi, we found that we could delay the knockdown of *daf-16* and *daf-18* until the fourth day of adulthood and still recapitulate the effect of longer RNAi treatments targeting those genes but that this was too late to bring about the *age-1* knockdown phenotype. This indicates that by Day 4 of adulthood, AGE-1 has already completed its regulatory function whereas DAF-18 and DAF-16 remain active. Therefore, inhibition through IIS may be restricted to the first few days of adulthood. Supporting this possibility, the latest point at which *daf-2* knockdown can be initiated and produce the same magnitude of lifespan extension as in *daf-2* mutants is Day 3 of adulthood (Dillin *et al.* 2002). By Day 8 of adulthood, RNAi targeting *daf-2* has no effect on longevity. Why, then, is DAF-18 required after upstream steps in the IIS pathway have apparently stopped functioning? The consensus of several genetic analyses conducted by

different teams of investigators is that *daf-18* mutations in fact only partially suppress mutations in *daf-2* and *age-1* (Larsen *et al.* 1995; Ogg and Ruvkun 1998; Honda and Honda 2002; Patel *et al.* 2008). One explanation for this phenomenon is that in the absence of PIP₃ produced by AGE-1, a parallel compensatory pathway may be induced to activate PDK-1 and this, too, must be counteracted by DAF-18 (Ogg and Ruvkun 1998). Perhaps this is the scenario at later stages of adulthood once the IIS-mediated inhibition of DAF-16 has dissipated.

A repressive signal that acts through the IIS pathway to inhibit DAF-16 would originate with the production of insulins that act as DAF-2 agonists. There are 40 genes in the *C. elegans* genome that encode insulin-like peptides (ILPs), and they can either stimulate or block IIS (ref). While some insulins are expressed in intestinal cells, the majority are expressed in neurons. The prevailing model for lifespan determination in *C. elegans* is that DAF-2 agonist insulins secreted by chemosensory neurons in the worm's head bind to DAF-2 in target tissues, including the intestine, to activate the IIS pathway. As a result, AKT-1 adds an inhibitory phosphate to DAF-16, sequestering it in the cytosol. Without the robust expression of DNA repair machinery, chaperones, detoxification enzymes, immune effectors, and other DAF-16 transcriptional targets important for defending against stress or mitigating its effects, the capacity of animals to withstand challenges is hobbled, leaving them vulnerable to cellular damage that leads to functional decline. Our data indicate that while this may be the case very early in adulthood, inhibition of DAF-16 is swiftly reversed potentially through an analogous neuroendocrine route.

In Day 6 adult animals we detected DAF-18::GFP in head neurons and body wall muscle. Although we could not empirically identify the specific neurons in which DAF-18 is expressed, based on their morphology and previous characterizations of DAF18, they are almost certain to be chemosensory amphid neurons that detect either volatile or soluble environmental cues (Tomioka *et al.* 2006; Brisbin *et al.* 2009). Using tissue-specific RNAi, we confirmed that neuronal DAF-18 is indeed functional and that it contributes to innate immunity in adult *C. elegans*. Those studies also established the intestine as a site of action for DAF-18 during adulthood, even though we were unable to detect its expression there by microscopy. Significantly, our data indicate that DAF-16 functions in the same tissues as DAF-18 in Day 6 animals. The neurons and intestine are notable in the context of aging for two reasons. First, there is an axis of communication between neurons (the brain) and the intestine, as described above, that may control organismal aging. The so-called gut-brain axis (GBA) is evolutionarily conserved and is implicated in some age-related diseases (Houser and Tansey 2017). Second, the activity of DAF-16 in the intestine or the neurons alone is sufficient to affect lifespan. Specifically, genetic manipulations in the *daf-2* mutant background to activate or inactivate DAF-16 exclusively in either the neurons or the intestine but no other tissues partially recapitulates or partially suppresses the *daf-2* longevity phenotype, respectively (Wolkow *et al.* 2000; Libina *et al.* 2003). Our data suggest that DAF-18 and DAF-16 cooperate in chemosensory neurons and the intestine during adulthood. We therefore envision a revised model for the neuroendocrine control of lifespan in *C. elegans*. In adult animals, DAF-18 in the neurons may be required for DAF-16 to upregulate the expression of a DAF-2 antagonist. This would oppose

inhibition through IIS in distal tissues, including the intestine, leading to the activation of DAF-16 in a cell non-autonomous manner. This is consistent with the results of tissue-specific rescue experiments demonstrating that DAF-18 in the neurons can promote DAF-16 nuclear localization in other tissues (Masse *et al.* 2005). Within the intestine, DAF-18 may be required to reduce PIP₃ levels produced by AGE-1 or by other compensatory pathways, permitting DAF-16 to upregulate the genes that preserve homeodynamic space and thus assure normal lifespan.

Proto-oncogenes are typically regarded as one of the best examples of such genes, as many are necessary for cell cycle progression earlier in life, yet their activity in older animals can lead to tumor progression. Tumor suppressors, on the other hand, play the opposite role. During development these genes prevent overgrowth of cells and limit organ size, and their absence causes developmental abnormalities or embryonic lethality (Pomerantz and Blau 2013). True to their name, later in life tumor suppressors stop aberrant cell division, thus helping to prevent cancer. Unlike proto-oncogenes, whose inactivation during aging would promote longevity by reducing the chances of developing life-threatening disease, organisms benefit from tumor suppressors being active both early and later in life. Our work on DAF-18, the *C. elegans* ortholog of the human tumor suppressor PTEN, demonstrates that this is the case even in animals that do not maintain mitotic cell populations during adulthood. While DAF-18 is important for the development of several tissues and controls cell proliferation in the germline, we find that it is required specifically during adulthood to protect animals from bacterial infection that would otherwise shorten their lifespan. This suggests that there is an evolutionarily conserved requirement for tumor

suppressors to preserve lifespan, not just to prevent diseases of aging, and that their functional repertoire may expand later in life.

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FIGURE LEGENDS

Figure 1. DAF-18 is primarily expressed in head neurons in adult *C. elegans*. Day 6

adult animals of strain zhEx343 expressing DAF-18::GFP were imaged by confocal microscopy. (A) Autofluorescence from lipofuscin in the intestinal cells is the predominant signal in images of whole adult animals. Comparatively weaker GFP expression is observed in the head (arrowhead) near the pharynx (red) and toward the posterior. (B) Higher magnification of the head region reveals that DAF-18::GFP is expressed in a presumptive amphid neuron whose dendrite crosses the isthmus of the pharynx (arrow) and extends to the mouth. (C) Higher magnification of the region bounded by the rectangle in (A) shows DAF-18::GFP expression in body wall muscle.

Figure 2. The age-dependent increase in the transcriptional activity of DAF-16

requires DAF-18. (A) Expression of the *Plys-7::GFP* reporter in the intestine of Day 6

adult *C. elegans*. Based on the expression level of *Plys-7::GFP*, individual animals within a population may be classified as either low, medium or high expressers.

Micrographs show representative animals belonging to each category. (B-D) N2 wild type animals treated with an empty RNAi vector (L4440) or with RNAi targeting *daf-16*, *daf-18*, or *age-1* were scored according to the degree of *Plys-7::GFP* expression at the L4 stage (B), Day 3 of adulthood (C) or Day 6 of adulthood (D). At each time point, bars corresponding to a particular RNAi treatment are color coded to represent the fraction of the population scored as having high (green), medium (blue), or low (black) *Plys-7::GFP* expression. Shown is the average number of animals in each category over three independent biological replicates. In each replicate at least 200 animals from

each RNAi treatment group were scored. (E) Endogenous transcript levels of DAF-16 transcriptional targets *mtl-1*, *sod-3*, *lys-7*, and *cpr-2* were measured in Day 6 N2 wild type animals treated with an empty RNAi vector (L4440) or with RNAi targeting *daf-16*, *daf-18*, or *age-1*. The mRNA abundance of each transcript was normalized to the levels of the housekeeping gene *tba-1* and is reported relative to its abundance in L4 larvae subjected to the same RNAi treatments. Error bars, standard deviation.

Figure 3. DAF-18 contributes to innate immunity during adulthood in *C. elegans*.

(A and B) Age-matched cohorts of N2 wildtype animals, *daf-16(mgDf47)*, or *daf-18(ok480)* mutants were infected with *P. aeruginosa* at the L4 larval stage (A) or at Day 6 of adulthood (B) and their survival was recorded over time. Plotted in each panel is the fraction of animals alive as a function of time after the infection was initiated. (C and D) Beginning at the L1 larval stage N2 wildtype animals were treated with an empty RNAi vector (L4440, black) or with RNAi targeting *daf-16* (dark blue), *daf-18* (dark green), or *age-1* (red) and then infected with *P. aeruginosa* at either the L4 larval stage (C) or at Day 6 of adulthood (D). In another group of animals, RNAi knocking down *daf-16* (light blue), *daf-18* (light green), or *age-1* (orange) was initiated at Day 4 and worms were infected with *P. aeruginosa* at Day 6 (D). Plotted in each panel is the fraction of animals alive as a function of time after the start of the infection.

Figure 4. DAF-18 functions in the intestine and neurons of adult animals to protect them from bacterial pathogens. Beginning at the L1 larval stage N2 wild type animals (A and B) along with mutants in which RNAi-mediated gene knockdowns are

restricted to the muscle (C and D), the intestine (E and F) or the neurons (G and H) were treated with an empty RNAi vector (L4440, black) or with constructs targeting *daf-18* (green) or *daf-16* (blue). Animals were infected with *P. aeruginosa* at either the L4 larval stage (A, C, E, G) or at Day 6 of adulthood (B, D, F, H) and their survival was recorded over time. In each panel, the fraction of worms alive is plotted as a function of time since the infection was initiated. The data shown are representative of three independent replicates.

Figure 5. The age-dependent function of DAF-18 in innate immunity may be regulated by post-translational modifications. Following RNAi treatment beginning at the L1 larval stage to knock down the candidate DAF-18 regulators *sup-17* (purple), *cbp-3* (pink), *smo-1* (red), or *math-33* (orange), N2 wild type *C. elegans* were challenged with *P. aeruginosa* as either L4 larvae (A) or as Day 6 adults (B). Their survival was monitored over time and was compared to that of age-matched infected animals in which *daf-18* (green) or *daf-16* (blue) were targeted by RNAi or animals that were treated with an empty RNAi vector (L4440, black). In each panel, the fraction of worms alive is plotted as a function of time since the infection was initiated. The data shown are representative of three independent replicates.

Figure S1. GFP expression in the head neurons of transgenic adult *C. elegans* is specific to DAF-18::GFP. *C. elegans* expressing the DAF-18::GFP translational fusion were subjected to feeding-based RNAi beginning at the L1 larval stage. At Day 6 of adulthood, animals treated with an empty vector (A) or with RNAi targeting either *daf-18*

(B) or *gfp* (C) were imaged by confocal microscopy. Images are representative of animals examined during three independent biological replicates.

Figure S2. Additional replicates of *Pseudomonas aeruginosa* infection of larval and adult *daf-18* mutants. The survival of *daf-18(ok480)* mutants (blue) was compared to that of *daf-16(mgDf47)* animals (green) and to wildtype N2 animals (*black*) after infection with *P. aeruginosa* at either the L4 stage (A and C) or at Day 6 of adulthood (B and D). The fraction of animals alive is plotted as a function of time. Data from two independent biological replicates (A and B; C and D) are shown.

Figure S3. Additional replicates of RNAi experiment to investigate the timing requirement for *daf-18* in *C. elegans* innate immunity. Beginning at the L1 larval stage N2 wildtype animals were treated with an empty RNAi vector (L4440, black) or with RNAi targeting *daf-16* (dark blue), *daf-18* (dark green), or *age-1* (red) and then infected with *P. aeruginosa* at either the L4 larval stage (A, C) or at Day 6 of adulthood (B, D). Another group of animals was allowed to age on their normal *E. coli* food source until Day 4 of adulthood when RNAi targeting *daf-16* (light blue), *daf-18* (light green), or *age-1* (orange) was initiated. These animals were then infected with *P. aeruginosa* at Day 6 and their survival was monitored over time (B, D). Plotted in each panel is the fraction of animals alive as a function of time after the start of the infection. Data from two independent biological replicates (A and B; C and D) are shown.

Figure S4. Second biological replicate of tissue-specific RNAi experiment to determine the site of action of *daf-18* in innate immunity during adulthood. N2 wild type animals (A and B) along with mutants in which RNAi-mediated gene knockdowns are restricted to the muscle (C and D), the intestine (E and F) or the neurons (G and H) were treated with an empty RNAi vector (L4440, black) or with constructs targeting *daf-18* (green) or *daf-16* (blue) starting at the L1 larval stage. *P. aeruginosa* infection was initiated at either the L4 larval stage (A, C, E, G) or at Day 6 of adulthood (B, D, F, H) and the survival of infected animals was recorded over time. In each panel, the fraction of worms alive is plotted as a function of time after the start of the infection.

Figure S5. Third biological replicate of tissue-specific RNAi experiment to determine the site of action of *daf-18* in innate immunity during adulthood. N2 wild type animals (A and B) along with mutants in which RNAi-mediated gene knockdowns are restricted to the muscle (C and D), the intestine (E and F) or the neurons (G and H) were treated with an empty RNAi vector (L4440, black) or with constructs targeting *daf-18* (green) or *daf-16* (blue) starting at the L1 larval stage. *P. aeruginosa* infection was initiated at either the L4 larval stage (A, C, E, G) or at Day 6 of adulthood (B, D, F, H) and the survival of infected animals was recorded over time. In each panel, the fraction of worms alive is plotted as a function of time after the start of the infection.

Figure S6. Additional replicates of RNAi experiment to functionally characterize putative regulators of DAF-18. RNAi treatment to knock down the candidate DAF-18

regulators *sup-17* (purple), *cbp-3* (dark green), *smo-1* (red), or *math-33* (yellow) was initiated in N2 wildtype *C. elegans* at the L1 larval stage. For comparison, other animals were treated with an empty vector (L4440, black) or with RNAi constructs targeting *daf-18* (light green) or *daf-16* (blue). Animals were then infected with *P. aeruginosa* as either L4 larvae (A, C) or as Day 6 adults (B, D), and the fraction of worms alive was recorded at regular intervals until all individuals had died. Data from two independent biological replicates (A and B; C and D) are shown.

Figure 1

Figure 2

A

B

C

D

E

Figure 3

Figure 4

Figure 5

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Table S1. Tabulation of scoring data from manual assessment of *plys-7::GFP* expression shown in Fig. 2. The numbers of animals assigned to each category of *plys-7::GFP* expression (low, medium, or high) are reported according to age and RNAi treatment for three independent biological replicates. Separate tables show the total number of worms in each category across all three replicates and the proportions of animals in each category (labeled as "converted to fractions").

Worm totals

Replicate 1

Larval Stage 4

	Low	Med	High	
L4440	194	6	0	
daf-16	195	5	0	
daf-18	195	4	1	
age-1	197	3	0	

Day 3 of Adulthood

	Low	Med	High	
L4440	9	88	103	
daf-16	153	44	3	
daf-18	126	72	2	
age-1	0	10	190	

Day 6 of Adulthood

	Low	Med	High	
L4440	0	13	187	
daf-16	91	99	10	
daf-18	90	104	6	
age-1	0	11	189	

Replicate 2

Larval Stage 4

	Low	Med	High	
L4440	183	17	0	
daf-16	192	8	0	
daf-18	185	15	0	
age-1	198	2	0	

Day 3 of Adulthood

	Low	Med	High	
L4440	31	75	94	
daf-16	177	23	0	
daf-18	168	32	0	
age-1	5	17	178	

Day 6 of Adulthood

	Low	Med	High	
L4440	0	14	186	
daf-16	151	47	2	
daf-18	113	83	4	
age-1	0	4	196	

Replicate 3

Larval Stage 4

	Low	Med	High	
L4440	195	5	0	
daf-16	189	10	1	
daf-18	191	9	0	
age-1	190	9	1	

Day 3 of Adulthood

	Low	Med	High	
L4440	21	107	72	
daf-16	159	40	1	
daf-18	164	34	2	
age-1	2	12	186	

Day 6 of Adulthood

	Low	Med	High	
L4440	3	31	164	
daf-16	138	53	9	
daf-18	131	56	13	
age-1	0	5	195	

Totals from all three replicates

Larval Stage 4

	Low	Med	High	
L4440	572	28	0	
daf-16	576	23	1	
daf-18	571	28	1	
age-1	585	14	1	

Day 3 of Adulthood

	Low	Med	High	
L4440	61	270	269	
daf-16	489	107	4	
daf-18	458	138	4	
age-1	7	39	554	

Day 6 of Adulthood

	Low	Med	High	
L4440	3	58	537	
daf-16	380	199	21	
daf-18	334	243	23	
age-1	0	20	580	

Standard Deviations:

Larval Stage 4

	Low	Med	High	
L4440	6.658328	6.658328	0	
daf-16	3	2.516611	0.57735	
daf-18	5.033223	5.507571	0.57735	
age-1	4.358899	3.785939	0.57735	

Day 3 of Adulthood

	Low	Med	High	
L4440	11.01514	16.09348	15.94783	
daf-16	12.49	11.15049	1.527525	
daf-18	23.18045	22.53886	1.154701	
age-1	2.516611	3.605551	6.110101	

Day 6 of Adulthood

	Low	Med	High	
L4440	1.732051	10.11599	13	
daf-16	31.56475	28.44878	4.358899	
daf-18	20.55075	24.06242	4.725816	
age-1	0	3.785939	3.785939	

Converted to Fractions**

Larval Stage 4

	Low	Med	High	
L4440	0.97	0.03	0	
daf-16	0.975	0.025	0	
daf-18	0.975	0.02	0.005	
age-1	0.985	0.015	0	

Day 3 of Adulthood

	Low	Med	High	
L4440	0.045	0.44	0.515	
daf-16	0.765	0.22	0.015	
daf-18	0.63	0.36	0.01	
age-1	0	0.05	0.95	

Day 6 of Adulthood

	Low	Med	High	
L4440	0	0.065	0.935	
daf-16	0.455	0.495	0.05	
daf-18	0.45	0.52	0.03	
age-1	0	0.055	0.945	

Larval Stage 4

	Low	Med	High	
L4440	0.915	0.085	0	
daf-16	0.96	0.04	0	
daf-18	0.925	0.075	0	
age-1	0.99	0.01	0	

Day 3 of Adulthood

	Low	Med	High	
L4440	0.155	0.375	0.47	
daf-16	0.885	0.115	0	
daf-18	0.84	0.16	0	
age-1	0.025	0.085	0.89	

Day 6 of Adulthood

	Low	Med	High	
L4440	0	0.07	0.93	
daf-16	0.755	0.235	0.01	
daf-18	0.565	0.415	0.02	
age-1	0	0.02	0.98	

Larval Stage 4

	Low	Med	High	
L4440	0.975	0.025	0	
daf-16	0.945	0.05	0.005	
daf-18	0.955	0.045	0	
age-1	0.95	0.045	0.005	

Day 3 of Adulthood

	Low	Med	High	
L4440	0.105	0.535	0.36	
daf-16	0.795	0.2	0.005	
daf-18	0.82	0.17	0.01	
age-1	0.01	0.06	0.93	

Day 6 of Adulthood

	Low	Med	High	
L4440	0.015	0.155	0.82	
daf-16	0.69	0.265	0.045	
daf-18	0.655	0.28	0.065	
age-1	0	0.025	0.975	

Averages:

Larval Stage 4

	Low	Med	High	
L4440	0.953333	0.046667	0	
daf-16	0.96	0.038333	0.001667	
daf-18	0.951667	0.046667	0.001667	
age-1	0.975	0.023333	0.001667	

Day 3 of Adulthood

Standard Deviations:

Larval Stage 4

	Low	Med	High	
L4440	0.033292	0.033292	0	
daf-16	0.015	0.012583	0.002887	
daf-18	0.025166	0.027538	0.002887	
age-1	0.021794	0.01893	0.002887	

Day 3 of Adulthood

	Low	Med	High		Low	Med	High
L4440	0.101667	0.45	0.448333	L4440	0.055076	0.080467	0.079739
daf-16	0.815	0.178333	0.006667	daf-16	0.06245	0.055752	0.007638
daf-18	0.763333	0.23	0.006667	daf-18	0.115902	0.112694	0.005774
age-1	0.011667	0.065	0.923333	age-1	0.012583	0.018028	0.030551
Day 6 of Adulthood				Day 6 of Adulthood			
	Low	Med	High		Low	Med	High
L4440	0.005	0.096667	0.895	L4440	0.00866	0.05058	0.065
daf-16	0.633333	0.331667	0.035	daf-16	0.157824	0.142244	0.021794
daf-18	0.556667	0.405	0.038333	daf-18	0.102754	0.120312	0.023629
age-1	0	0.033333	0.966667	age-1	0	0.01893	0.01893

Table S4. Compiled fold differences in expression of *mtl-1*, *sod-3*, *lys-7*, and *cpr-2* between L4 and Day 6 in animals treated with RNAi targeting the indicated genes for three independent biological replicates. These data correspond to the bar graph in Fig. 2E. Average fold difference in expression between Day 6 and L4 with standard deviations and standard error are shown below.

<i>mtl-1</i>	L4440	<i>daf-16</i>	<i>daf-18</i>	<i>age-1</i>
Rep 1	11.3137085	0.30707579	0.47521974	79.5248222
Rep 2	4.60542192	0.32458465	0.37892914	29.8570557
Rep 3	5.00487256	0.29185695	0.44647976	69.8731689
Av	6.97466766	0.30783913	0.43354288	59.7516822
st dev	3.76302362	0.0163772	0.04943169	26.3354347

<i>sod-3</i>	L4440	<i>daf-16</i>	<i>daf-18</i>	<i>age-1</i>
Rep 1	1.81923679	0.0900373	0.14899474	218.779437
Rep 2	2.17849731	0.29456678	0.37371231	144.340599
Rep 3	1.49484925	0.20073072	0.21563551	219.285509
Av	1.83086112	0.1951116	0.24611419	194.135181
st dev	0.34197224	0.10238046	0.11541754	43.124116

<i>lys-7</i>	L4440	<i>daf-16</i>	<i>daf-18</i>	<i>age-1</i>
Rep 1	128.296085	10.1026836	14.6213032	543.699868
Rep 2	77.171701	8.39773347	12.1257325	705.906519
Rep 3	121.937664	10.362738	9.42613711	639.145241
Av	109.13515	9.6210517	12.0577243	629.583876
st dev	27.8631284	1.0673742	2.59825067	81.5249298

<i>cpr-2</i>	L4440	<i>daf-16</i>	<i>daf-18</i>	<i>age-1</i>
Rep 1	12.2950015	3.03844533	3.24900959	112.465082
Rep 2	25.9320916	2.73208051	4.0278222	144.007486
Rep 3	15.5984777	4.00925265	6.36429187	129.487288
Av	17.9418569	3.25992616	4.54704122	128.653285
st dev	7.11414972	0.66677019	1.62124558	15.7877321

Averages	<i>mtl-1</i>	<i>sod-3</i>	<i>lys-7</i>	<i>cpr-2</i>
L4440	6.97466766	1.83086112	109.13515	17.9418569
<i>daf-16</i>	0.30783913	0.1951116	9.6210517	3.25992616
<i>daf-18</i>	0.43354288	0.24611419	12.0577243	4.54704122
<i>age-1</i>	59.7516822	194.135181	629.583876	128.653285

Standard Deviations	<i>mtl-1</i>	<i>sod-3</i>	<i>lys-7</i>	<i>cpr-2</i>
L4440	3.76302362	0.34197224	27.8631284	7.11414972
<i>daf-16</i>	0.0163772	0.10238046	1.0673742	0.66677019
<i>daf-18</i>	0.04943169	0.11541754	2.59825067	1.62124558
<i>age-1</i>	26.3354347	43.124116	81.5249298	15.7877321

Standard Err	<i>mtl-1</i>	<i>sod-3</i>	<i>lys-7</i>	<i>cpr-2</i>
L4440	2.1725827	0.19743776	16.0867847	4.10735626
<i>daf-16</i>	0.00945538	0.05910938	0.61624878	0.38495995
<i>daf-18</i>	0.0285394	0.06663635	1.50010072	0.93602657
<i>age-1</i>	15.2047703	24.89772	47.0684402	9.11505139

Table S5. Experimental details and statistical analyses of *P. aeruginosa* infection assays corresponding to survival curves shown in Figs. 3-5. For each survival curve depicted in the figures, supporting information regarding the number of animals in each replicate, their maximum lifespan and their median lifespan (LT50) is provided. Relative LT50 was calculated by dividing the LT50 of an experimental group (mutant or RNAi-treated animals) by the LT50 of the corresponding control group. Compiled data for all replicates for a particular experiment are provided in the Averages section of each entry, which also includes statistical analyses. Lifespan and LT50 are presented in units of hours. p-values were determined using one-sample t-test. Std. dev., standard deviation; Q1, first quartile; Q3, third quartile; IQR, interquartile range

Figure 3A data

daf-16(mgDf47), daf-18(ok480) vs N2 at L4

Replicates						
	daf-16(mgDf47)		daf-18(ok480)		N2	
Trial 1	animals	102	animals	105	animals	173
	plates	3	plates	3	plates	3
	lifespan	145	lifespan	145	lifespan	145
	LT50	94.92	LT50	96.43	LT50	102.04
	relative LT50	0.930223	relative LT50	0.945		
Trial 2	animals	127	animals	134	animals	114
	plates	3	plates	3	plates	3
	lifespan	146	lifespan	156	lifespan	156
	LT50	103.03	LT50	104.45	LT50	104.86
	relative LT50	0.983	relative LT50	0.996		
Trial 3	animals	105	animals	124	animals	121
	plates	3	plates	3	plates	3
	lifespan	118	lifespan	118	lifespan	132
	LT50	73.856	LT50	73.858	LT50	79.73
	relative LT50	0.926	relative LT50	0.926		

Averages					
daf-16(mgDf47)		daf-18(ok480)		N2	
Lifespan		Lifespan		Lifespan	
Average	136.3333	Average	139.6667	Average	144.3333
Std. Dev.	15.885	Std. Dev.	19.55335	Std. Dev.	12.01388
LT50		LT50		LT50	
Average	90.6	Average	91.579	Average	95.544
Median	94.92	Median	96.433	Median	102.041
Q1	73.856	Q1	73.858	Q1	79.728
Q3	103.026	Q3	104.445	Q3	104.863
IQR	29.17	IQR	30.587	IQR	25.135
Relative LT50		Relative LT50			
Average	0.946	Average	0.956		
Std. Dev.	0.0313	Std. Dev.	0.036		
p value	0.0974	p value	0.168		

Figure 3B data

daf-16(mgDf47), daf-18(ok480) vs N2 at Day 6

Replicates						
	daf-16(mgDf47)		daf-18(ok480)		N2	
Trial 1	animals	112	animals	117	animals	139
	plates	3	plates	3	plates	3
	lifespan	56	lifespan	72	lifespan	131
	LT50	32.37	LT50	31.7	LT50	66.03
	relative LT50	0.490232	relative LT50	0.480		
Trial 2	animals	113	animals	121	animals	129
	plates	3	plates	3	plates	3
	lifespan	86	lifespan	55.5	lifespan	108
	LT50	25.13	LT50	24.02	LT50	62.4
	relative LT50	0.403	relative LT50	0.385		
Trial 3	animals	124	animals	114	animals	129
	plates	3	plates	3	plates	3
	lifespan	71	lifespan	71	lifespan	120
	LT50	25.77	LT50	25.97	LT50	61.77
	relative LT50	0.417	relative LT50	0.420		

Averages					
daf-16(mgDf47)		daf-18(ok480)		N2	
Lifespan		Lifespan		Lifespan	
Average	71	Average	66.16667	Average	119.6667
Std. Dev.	15	Std. Dev.	9.251126	Std. Dev.	11.50362
LT50		LT50		LT50	
Average	27.739	Average	27.23	Average	63.4
Median	25.718	Median	25.966	Median	63.404
Q1	25.125	Q1	24.022	Q1	61.769
Q3	32.375	Q3	31.702	Q3	66.026
IQR	7.25	IQR	7.68	IQR	4.257
Relative LT50		Relative LT50			
Average	0.426	Average	0.428		
Std. Dev.	0.0472	Std. Dev.	0.0481		
p value	0.00233	p value	0.00235		

Figure 3C data

RNAi Timing Experiments - L4

Replicates								
	<i>daf-16</i>		<i>daf-18</i>		<i>age-1</i>		<i>L4440</i>	
Trial 1	animals	87	animals	122	animals	143	animals	123
	plates	3	plates	3	plates	3	plates	3
	lifespan	116	lifespan	116	lifespan	152	lifespan	128
	LT50	69.81	LT50	70.05	LT50	81.69	LT50	75.99
	relative LT50	0.919	relative LT50	0.922	relative LT50	1.075		
Trial 2	animals	105	animals	97	animals	100	animals	95
	plates	3	plates	3	plates	3	plates	3
	lifespan	137	lifespan	145	lifespan	162	lifespan	162
	LT50	84.13	LT50	90.76	LT50	104.6	LT50	96.49
	relative LT50	0.872	relative LT50	0.941	relative LT50	1.084		
	animals	113	animals	95	animals	109	animals	113

Trial 3	plates	3	plates	3	plates	3	plates	3
	lifespan	123	lifespan	141.5	lifespan	141.5	lifespan	141.2
	LT50	66.67	LT50	66.34	LT50	73.88	LT50	71.13
	relative LT50	0.937	relative LT50	0.933	relative LT50	1.039		

Averages							
daf-16		daf-18		age-1		L4440	
Lifespan		Lifespan		Lifespan		Lifespan	
Average	125.3333	Average	134.1667	Average	151.8333	Average	143.733
Std. Dev.	10.69268	Std. Dev.	15.82982	Std. Dev.	10.25102	Std. Dev.	17.141
LT50		LT50		LT50		LT50	
Average	73.538	Average	75.7126	Average	86.724	Average	81.204
Median	69.807	Median	70.047	Median	81.691	Median	75.994
Q1	66.673	Q1	66.339	Q1	73.876	Q1	71.131
Q3	84.134	Q3	90.762	Q3	104.605	Q3	96.486
IQR	17.461	IQR	24.423	IQR	30.729	IQR	25.355
Relative LT50		Relative LT50		Relative LT50		Relative LT50	
Average	0.909	Average	0.932	Average	1.066		
Std. Dev.	0.0336	Std. Dev.	0.00949	Std. Dev.	0.0241		
p value	0.0429	p value	0.00638	p value	0.0418		

Figure 3D data

Replicates																				
	daf-16			PT daf-16			daf-18			PT daf-18			age-1			PT age-1			L4440	
Trial 1	animals	116	animals	123	animals	136	animals	109	animals	119	animals	120	animals	9						
	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3						
	lifespan	74	lifespan	96	lifespan	74	lifespan	131	lifespan	204	lifespan	106	lifespan	13						
	LT50	15.54	LT50	20.65	LT50	17.95	LT50	22.46	LT50	90.67	40.57	LT50	40.2							
	relative LT50	0.865738	relative LT50	1.150	relative LT50	0.446	relative LT50	0.558	relative LT50	2.253	relative LT50	0.000								
Trial 2	animals	117	animals	110	animals	113	animals	93	animals	93	animals	97	animals	9						
	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3						
	lifespan	75	lifespan	62	lifespan	75	lifespan	90	lifespan	149	lifespan	110	lifespan	9						
	LT50	20.12	LT50	18.36	LT50	20.99	LT50	25.67	LT50	86.72	LT50	38.13	LT50	46.7						
	relative LT50	0.431	relative LT50	0.875	relative LT50	0.449	relative LT50	0.550	relative LT50	1.857	relative LT50	0.816								
Trial 3	animals	128	animals	129	animals	137	animals	122	animals	132	animals	107	animals	14						
	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3						
	lifespan	91	lifespan	98	lifespan	91	lifespan	91	lifespan	188	lifespan	127.5	lifespan	127						
	LT50	31.83	LT50	34.42	LT50	27.53	LT50	32.55	LT50	76.65	LT50	49.11	LT50	47.5						
	relative LT50	0.669	relative LT50	0.000	relative LT50	0.579	relative LT50	0.684	relative LT50	1.611	relative LT50	1.032								

Averages							
daf-16		PT daf-16		daf-18		PT daf-18	
Lifespan		Lifespan		Lifespan		Lifespan	
Average	22.49667	Average	19.505	Average	22.15667	Average	26.8933
Std. Dev.	8.401038	Std. Dev.	1.619275	Std. Dev.	4.895399	Std. Dev.	5.15504
LT50		LT50		LT50		LT50	
Average	22.498	Average	24.81	Average	22.157	Average	26.895
Median	20.12	Median	20.654	Median	20.991	Median	25.668
Q1	15.541	Q1	19.358	Q1	17.946	Q1	22.464
Q3	31.832	Q3	34.416	Q3	27.533	Q3	32.553
IQR	16.291	IQR	15.058	IQR	9.587	IQR	10.089
Relative LT50		Relative LT50		Relative LT50		Relative LT50	
Average	0.495	Average	0.550	Average	0.491	Average	0.597
Std. Dev.	0.152	Std. Dev.	0.158	Std. Dev.	0.0757	Std. Dev.	0.0754
p value	0.029	p value	0.0387	p value	0.0073	p value	0.0115

Figure 4A data

N2: daf-16, daf-18 vs L4440 at L4

Replicates				
	daf-16	daf-18	L4440	
Trial 1	animals	103	animals	102
	plates	3	plates	3
	lifespan	159	lifespan	159
	LT50	78	LT50	82.68
	relative LT50	0.943396	relative LT50	0.929
Trial 2	animals	90	animals	108
	plates	3	plates	3
	lifespan	165	lifespan	121.5
	LT50	89.58	LT50	90.85
	relative LT50	0.986	relative LT50	0.982
Trial 3	animals	111	animals	117
	plates	3	plates	3
	lifespan	182.5	lifespan	182.5
	LT50	117.14	LT50	125.56
	relative LT50	0.933	relative LT50	0.905

Averages			
daf-16		daf-18	
Lifespan		Lifespan	
Average	0.954119	Average	0.938936
Std. Dev.	0.028118	Std. Dev.	0.039223
LT50		LT50	
Average	94.905	Average	93.25
Median	89.575	Median	89.225
Q1	77.996	Q1	76.842
Q3	117.143	Q3	113.684
IQR	39.147	IQR	36.842
Relative LT50		Relative LT50	
Average	0.954	Average	0.939
Std. Dev.	0.0281	Std. Dev.	0.0392
p value	0.15	p value	0.114

Figure 4B data

N2: daf-16, daf-18 vs L4440 at Day 6

Replicates	
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Figure 4C data

Muscle-specific: daf-16, daf-18 vs L4440 at L4

Averages					
daf-16		daf-18		L4440	
Lifespan		Lifespan		Lifespan	
Average	0.909681	Average	0.940648	Average	80.97667
Std. Dev.	0.044827	Std. Dev.	0.024546	Std. Dev.	8.336572
LT50		LT50		LT50	
Average	73.869	Average	76.302	Average	80.978
Median	68.562	Median	70.925	Median	76.367
Q1	66.138	Q1	70.205	Q1	75.963
Q3	86.908	Q3	87.775	Q3	90.603
IQR	20.77	IQR	17.57	IQR	14.64
Relative LT50		Relative LT50			
Average	0.909	Average	0.941		
Std. Dev.	0.0454	Std. Dev.	0.0245		
p value	0.0741	p value	0.0524		

Figure 4D data

Muscle-specific: daf-16, daf-18 vs L4440 at Day 6

Averages	
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20
21	21
22	22
23	23
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25	25
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45	45
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70	70
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72	72
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79	79
80	80
81	81
82	82
83	83
84	84
85	85
86	86
87	87
88	88
89	89
90	90
91	91
92	92
93	93
94	94
95	95
96	96
97	97
98	98
99	99
100	100

<i>daf-16</i>		<i>daf-18</i>		L4440	
Lifespan		Lifespan		Lifespan	
Average	0.980549	Average	0.961802	Average	29.08
Std. Dev.	0.043292	Std. Dev.	0.058529	Std. Dev.	4.054368
LT50		LT50		LT50	
Average	28.55	Average	28.127	Average	29.083
Median	27.31	Median	25.363	Median	27.027
Q1	24.642	Q1	24.31	Q1	26.463
Q3	33.698	Q3	34.707	Q3	33.758
IQR	9.056	IQR	10.397	IQR	7.295
Relative LT50		Relative LT50			
Average	0.980	Average	0.962		
Std. Dev.	0.0427	Std. Dev.	0.0583		
p value	0.501	p value	0.374		

Figure 4E data

Intestinal-specific: daf-16, daf-18 vs L4440 at L4

Replicates					
	<i>daf-16</i>		<i>daf-18</i>		L4440
Trial 1	animals	112	animals	112	animals
	plates	3	plates	3	plates
	lifespan	159	lifespan	159	lifespan
	LT50	64.18	LT50	65.67	LT50
	relative LT50	0.859285	relative LT50	0.879	
Trial 2	animals	112	animals	140	animals
	plates	3	plates	3	plates
	lifespan	165	lifespan	165	lifespan
	LT50	71.29	LT50	67.63	LT50
	relative LT50	1.019	relative LT50	0.967	
Trial 3	animals	112	animals	128	animals
	plates	3	plates	3	plates
	lifespan	126	lifespan	117	lifespan
	LT50	63.66	LT50	60.6	LT50
	relative LT50	1.032	relative LT50	0.982	

Averages					
<i>daf-16</i>		<i>daf-18</i>		L4440	
Lifespan		Lifespan		Lifespan	
Average	0.970229	Average	0.942899	Average	68.77
Std. Dev.	0.096294	Std. Dev.	0.055678	Std. Dev.	6.583441
LT50		LT50		LT50	
Average	66.37	Average	64.635	Average	68.768
Median	64.18	Median	65.668	Median	69.937
Q1	63.661	Q1	60.604	Q1	61.676
Q3	71.268	Q3	67.634	Q3	74.69
IQR	7.607	IQR	7.03	IQR	13.014
Relative LT50		Relative LT50			
Average	0.970	Average	0.943		
Std. Dev.	0.0962	Std. Dev.	0.0558		
p value	0.645	p value	0.218		

Figure 4F data

Intestinal-specific: daf-16, daf-18 vs L4440 at Day 6

Replicates					
	<i>daf-16</i>		<i>daf-18</i>		L4440
Trial 1	animals	129	animals	128	animals
	plates	3	plates	3	plates
	lifespan	92.5	lifespan	92.5	lifespan
	LT50	31.34	LT50	32.98	LT50
	relative LT50	0.606072	relative LT50	0.638	
Trial 2	animals	188	animals	225	animals
	plates	3	plates	3	plates
	lifespan	77.5	lifespan	77.5	lifespan
	LT50	22.37	LT50	21.48	LT50
	relative LT50	0.540	relative LT50	0.519	
Trial 3	animals	157	animals	110	animals
	plates	3	plates	3	plates
	lifespan	74	lifespan	74	lifespan
	LT50	22.52	LT50	25.04	LT50
	relative LT50	0.543	relative LT50	0.603	

Averages					
<i>daf-16</i>		<i>daf-18</i>		L4440	
Lifespan		Lifespan		Lifespan	
Average	0.56302	Average	0.586667	Average	44.87
Std. Dev.	0.037302	Std. Dev.	0.061208	Std. Dev.	5.923825
LT50		LT50		LT50	
Average	26.053	Average	26.028	Average	44.87
Median	24.449	Median	23.625	Median	41.499
Q1	22.373	Q1	21.477	Q1	41.405
Q3	31.338	Q3	32.982	Q3	51.706
IQR	8.965	IQR	11.505	IQR	10.301
Relative LT50		Relative LT50			
Average	0.579	Average	0.575		
Std. Dev.	0.0341	Std. Dev.	0.0598		
p value	0.00218	p value	0.00655		

Figure 4G data

Neuronal-specific: daf-16, daf-18 vs L4440 at L4

Replicates					
	daf-16		daf-18		L4440
Trial 1	animals	112	animals	129	animals
	plates	3	plates	3	plates
	lifespan	159	lifespan	159	lifespan
	LT50	63.17	LT50	64.12	LT50
Trial 2	relative LT50	0.903332	relative LT50	0.917	relative LT50
	animals	176	animals	140	animals
	plates	3	plates	3	plates
	lifespan	121.5	lifespan	165	lifespan
Trial 3	LT50	68.18	LT50	70.54	LT50
	relative LT50	0.911	relative LT50	0.942	relative LT50
	animals	119	animals	126	animals
	plates	3	plates	3	plates
Trial 3	lifespan	117	lifespan	117	lifespan
	LT50	60.38	LT50	61.27	LT50
	relative LT50	0.907	relative LT50	0.920	relative LT50
	animals	119	animals	126	animals

Averages					
	daf-16		daf-18		L4440
Lifespan			Lifespan		Lifespan
Average	0.907079		Average	0.926573	Average
Std. Dev.	0.003779		Std. Dev.	0.013831	Std. Dev.
LT50			LT50		LT50
Average	63.896		Average	65.312	Average
Median	63.167		Median	64.121	Median
Q1	60.338		Q1	61.274	Q1
Q3	68.184		Q3	70.541	Q3
IQR	7.846		IQR	9.267	IQR
Relative LT50			Relative LT50		
Average	0.907		Average	0.927	
Std. Dev.	0.00389		Std. Dev.	0.0139	
p value	0.000582		p value	0.0117	

Figure 4H data
Neuronal-specific: daf-16, daf-18 vs L4440 at Day 6

Replicates					
	daf-16		daf-18		L4440
Trial 1	animals	150	animals	177	animals
	plates	3	plates	3	plates
	lifespan	92.5	lifespan	75.5	lifespan
	LT50	28.03	LT50	26.06	LT50
Trial 2	relative LT50	0.644664	relative LT50	0.599	relative LT50
	animals	201	animals	231	animals
	plates	3	plates	2	plates
	lifespan	77.5	lifespan	85	lifespan
Trial 3	LT50	32.87	LT50	38.59	LT50
	relative LT50	0.567	relative LT50	0.665	relative LT50
	animals	134	animals	127	animals
	plates	3	plates	3	plates
Trial 3	lifespan	90	lifespan	90	lifespan
	LT50	23.52	LT50	25.04	LT50
	relative LT50	0.515	relative LT50	0.549	relative LT50
	animals	134	animals	127	animals

Averages					
	daf-16		daf-18		L4440
Lifespan			Lifespan		Lifespan
Average	0.575613		Average	0.604488	Average
Std. Dev.	0.065064		Std. Dev.	0.058461	Std. Dev.
LT50			LT50		LT50
Average	28.141		Average	29.898	Average
Median	28.031		Median	26.063	Median
Q1	23.525		Q1	25.039	Q1
Q3	32.868		Q3	38.593	Q3
IQR	9.343		IQR	13.554	IQR
Relative LT50			Relative LT50		
Average	0.581		Average	0.609	
Std. Dev.	0.0732		Std. Dev.	0.0585	
p value	0.01		p value	0.00738	

Figure 5A data
DAF-18 Regulators - L4

Replicates											
	daf-16		daf-18		sup-17		cbp-3		smo-1		L4440
Trial 1	animals	140	animals	128	animals	113	animals	130	animals	125	animals
	plates	3	plates	3	plates	3	plates	3	plates	3	plates
	lifespan	138	lifespan	115.5	lifespan	138	lifespan	138	lifespan	188.5	lifespan
	LT50	61.31	LT50	62.68	LT50	76.58	LT50	67.67	LT50	64.2	LT50
Trial 2	relative LT50	0.878	relative LT50	0.898	relative LT50	1.097	relative LT50	0.970	relative LT50	0.920	relative LT50
	animals	127	animals	151	animals	121	animals	123	animals	136	animals
	plates	3	plates	3	plates	3	plates	3	plates	3	plates
	lifespan	125.75	lifespan	146	lifespan	125.75	lifespan	194	lifespan	146	lifespan
Trial 3	LT50	82.43	LT50	88.78	LT50	90.52	LT50	85.07	LT50	86.54	LT50
	relative LT50	0.924	relative LT50	0.995	relative LT50	1.014	relative LT50	0.953	relative LT50	0.970	relative LT50
	animals	130	animals	155	animals	119	animals	141	animals	157	animals
	plates	3	plates	3	plates	3	plates	3	plates	3	plates
Trial 3	lifespan	162	lifespan	162	lifespan	162	lifespan	162	lifespan	141	lifespan
	LT50	86.87	LT50	85.56	LT50	89.73	LT50	93.85	LT50	88.89	LT50
	relative LT50	0.958	relative LT50	0.944	relative LT50	0.990	relative LT50	1.035	relative LT50	0.981	relative LT50
	animals	130	animals	155	animals	119	animals	141	animals	157	animals

Averages						
	daf-16	daf-18	sup-17	cbp-3	smo-1	math-33
Lifespan						

Average	0.920126	Average	0.945569	Average	1.033791	Average	0.98603	Average	22.05007	Average	0.742529	Average	83.23
Std. Dev.	0.040017	Std. Dev.	0.048328	Std. Dev.	0.056328	Std. Dev.	0.04346	Std. Dev.	36.50291	Std. Dev.	0.368082	Std. Dev.	#DIV/0!
LT50		LT50		LT50		LT50		LT50		LT50		LT50	
Average	76.87	Average	79.005	Average	85.612	Average	82.199	Average	79.879	Average	78.646	Average	83.233
Median	82.433	Median	85.555	Median	89.732	Median	85.071	Median	86.542	Median	83.394	Median	89.253
Q1	61.312	Q1	62.679	Q1	76.582	Q1	67.672	Q1	64.203	Q1	63.018	Q1	69.795
Q3	86.866	Q3	88.78	Q3	90.524	Q3	93.854	Q3	88.892	Q3	89.525	Q3	90.652
IQR	25.554	IQR	26.101	IQR	13.942	IQR	26.182	IQR	24.689	IQR	26.507	IQR	20.857
Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50	
Average	0.920	Average	0.946	Average	1.034	Average	0.986	Average	0.957	Average	0.942		
Std. Dev.	0.04	Std. Dev.	0.00484	Std. Dev.	0.0563	Std. Dev.	0.0435	Std. Dev.	0.0323	Std. Dev.	0.0536		
p value	0.0743	p value	0.19	p value	0.408	p value	0.633	p value	0.0731	p value	0.201		

Figure 5B data

DAF-18 Regulators - Day 6

Replicates													
daf-16		daf-18		sup-17		cbp-3		smo-1		math-33		L4440	
Trial 1	animals	102	animals	116	animals	124	animals	139	animals	116	animals	138	
	plates	3	plates	33	plates	3	plates	3	plates	3	plates	3	
	lifespan	125	lifespan	99.25	lifespan	125	lifespan	175	lifespan	99.25	lifespan	125	
	LT50	27.5942	LT50	36.0454	LT50	63.3517	LT50	65.703	LT50	31.4247	LT50	37.8622	
	relative LT50	0.397	relative LT50	0.518	relative LT50	0.911	relative LT50	0.945	relative LT50	0.452	relative LT50	0.545	
Trial 2	animals	145	animals	121	animals	123	animals	113	animals	115	animals	157	
	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3	
	lifespan	109	lifespan	83.75	lifespan	109	lifespan	131.5	lifespan	109	lifespan	109	
	LT50	31.6603	LT50	33.2127	LT50	56.8947	LT50	46.4154	LT50	32.2423	LT50	36.0297	
	relative LT50	0.455	relative LT50	0.676	relative LT50	1.159	relative LT50	0.945	relative LT50	0.657	relative LT50	0.734	
Trial 3	animals	142	animals	141	animals	129	animals	138	animals	132	animals	169	
	plates	3	plates	3	plates	3	plates	3	plates	3	plates	3	
	lifespan	96.5	lifespan	118.75	lifespan	141.25	lifespan	150.25	lifespan	96.5	lifespan	96.5	
	LT50	31.1875	LT50	30.9917	LT50	46.1264	LT50	45.9346	LT50	30.0329	LT50	29.1432	
	relative LT50	0.449	relative LT50	0.612	relative LT50	0.911	relative LT50	0.907	relative LT50	0.593	relative LT50	0.575	

Averages													
daf-16		daf-18		sup-17		cbp-3		smo-1		math-33		L4440	
Lifespan		Lifespan		Lifespan		Lifespan		Lifespan		Lifespan		Lifespan	
Average	0.433604	Average	0.602231	Average	0.993505	Average	0.93238	Average	0.56718	Average	0.617896	Average	56.42687
Std. Dev.	0.031983	Std. Dev.	0.079389	Std. Dev.	0.142946	Std. Dev.	0.022	Std. Dev.	0.104709	Std. Dev.	0.101457	Std. Dev.	#DIV/0!
LT50		LT50		LT50		LT50		LT50		LT50		LT50	
Average	30.147	Average	33.417	Average	55.458	Average	52.684	Average	31.233	Average	34.345	Average	56.47
Median	31.187	Median	33.213	Median	56.895	Median	46.415	Median	31.425	Median	36.03	Median	50.645
Q1	27.594	Q1	30.992	Q1	46.126	Q1	45.935	Q1	30.033	Q1	29.143	Q1	49.108
Q3	31.66	Q3	36.046	Q3	63.352	Q3	65.703	Q3	32.242	Q3	37.862	Q3	69.527
IQR	4.066	IQR	5.054	IQR	17.226	IQR	19.768	IQR	2.209	IQR	8.719	IQR	20.419
Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50		Relative LT50	
Average	0.552	Average	0.620	Average	0.994	Average	0.932	Average	0.567	Average	0.618		
Std. Dev.	0.136	Std. Dev.	0.0794	Std. Dev.	0.143	Std. Dev.	0.022	Std. Dev.	0.105	Std. Dev.	0.101		
p value	0.0292	p value	0.013	p value	0.994	p value	0.0335	p value	0.019	p value	0.0227		