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Role of phospholipase D in the lifespan of *Caenorhabditis elegans*

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Abstract

We have previously shown that phospholipase D (PLD) downregulation accelerates cellular senescence, which is widely believed to play an important role in aging, by stimulating reactive oxygen species (ROS) accumulation in human cells. In this study, we examined the role of PLD in aging using the nematode *Caenorhabditis elegans*. The mRNA level of *pld-1* was found to be inversely correlated with aging. RNAi-mediated knockdown of *pld-1* expression in nematodes enhanced ROS and lipofuscin accumulation and decreased lifespan, motility, and resistance to stress compared to that in nematodes treated with control RNAi. *Pld-1* knockdown repressed the long lifespan of *age-1* and *akt-1* mutants but did not further reduce the short lifespan of *daf-16* mutants, suggesting that PLD functions between AKT-1 and DAF-16. The ROS scavenger N-acetyl-L-cysteine, a PLD effector phosphatidic acid and a possible CK2 activator spermidine attenuated the lifespan shortening and age-related biomarkers triggered by *pld-1* knockdown. *Pld-1* RNAi downregulated the expression of DAF-16 target genes such as *sod-3*, *dod-11*, and *mtl-1* in nematodes. In human cells, furthermore, PLD2 downregulation decreased the transcription of FoxO3a target genes (Cu/ZnSOD, MnSOD, catalase, thioredoxin-2, and peroxiredoxin-5), whereas ectopic PLD2 expression elevated the mRNA levels of these antioxidant genes. Taken together, these results indicated that PLD downregulation shortens longevity and induces age-related biomarkers through ROS accumulation by inhibiting the DAF-16/FoxO3a pathway in nematodes.

Introduction

The phospholipase D (PLD) lipid-signaling enzyme superfamily hydrolyzes phosphatidylcholine to generate phosphatidic acid and free choline in bacteria and eukaryotes. Phosphatidic acid plays essential roles in cellular function and contributes to membrane vesicle trafficking, anti-apoptotic signaling, malignant transformation, invasiveness, cytoskeletal reorganization, and mitogenesis as a second messenger[1,2]. PLD activity increases in response to mitogenic signals and is involved in cell proliferation and cancer[3,4]. There are five isoforms of PLD in mammalian cells: PLD1 and PLD2 in the cytoplasm, PLD3 and PLD4 in the endoplasmic reticulum, and PLD6 in mitochondria[1]. However, only one PLD gene (*pld-1*) has been reported in the nematode *Caenorhabditis elegans*, which codes for a protein with 1427 amino acids[5]. The nematode PLD-1 protein is plentiful in neurons and pharyngeal muscles, but is present to a lesser extent in muscle and epithelial cells. Viable progeny with no apparent phenotype can be produced by *pld-1* knockdown in nematodes, but the detailed impact of *pld-1* knockout has not been determined[6,7].

Aging is divided into intrinsic aging, which is genetically programmed, and extrinsic aging, which occurs due to exposure to environmental factors. The insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway is a well-known pathway that controls nematode longevity. Daf-2/IGF receptor (IGFR), Age-1/phosphoinositide 3-kinase (PI3K), and Akt-1/AKT-1/2 are components of the IIS pathway[8,9]. The transcription factor DAF-16/

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FoxO, which stimulates the expression of pro-longevity genes such as thermotolerant and antioxidant genes, functions downstream of the IIS pathway[10,11]. However, the molecular mechanism by which this signaling pathway regulates *C. elegans* longevity remains to be elucidated.

It has been reported that PLD activity is decreased in senescent cells[12,13]. We have previously shown that PLD transcription decreases during both replicative and premature senescence in human diploid fibroblast IMR-90 and colon cancer HCT116 cells. Knockdown of PLD2 causes premature senescence via the p53–$p21^{Cip1/WAF1}$ pathway by stimulating reactive oxygen species (ROS) accumulation in cells[14]. In this study, we investigated the physiological significance of PLD in nematode aging. Our results indicated that *pld-1* downregulation caused ROS accumulation, decreased longevity, and induced age-related biomarkers. Treatment with the ROS scavenger N-acetyl-L-cysteine (NAC), a putative CK2 activator spermidine, and a PLD effector phosphatidic acid, attenuated *pld-1* RNAi-mediated lifespan shortening. PLD downregulation reduced the expression of DAF-16/FoxO target genes such as superoxide dismutase (SOD). The present study suggests that PLD plays a critical role in healthy lifespan via a connection to DAF-16/FoxO-mediated expression of antioxidant proteins.

Materials and methods

Culture of nematode strains

Nematode N2 (wild-type) strain, strains carrying mutant alleles *daf-16(mu86), age-1(hx546)*, and *akt-1 (mg144)*, and reporter strains *sod-3::gfp*, *dod-11::gfp*, *dod-8::gfp*, and *mtl-1::gfp* were acquired from the Caenorhabditis Genetics Center. Nematodes were grown at 21 °C on nematode growth medium (NGM) agar plates with *Escherichia coli* strain OP50 as a food source. For some experiments (Fig. 4, Supplemental Figs. 2 and 3), nematodes were treated with NAC (Sigma-Aldrich, MO), spermidine (Sigma-Aldrich, MO), or phosphatidic acid (Sigma-Aldrich, MO).

RNAi experiments

E. coli HT115 cells expressing double-stranded *pld-1* RNA were obtained from the *C. elegans* ORFeome RNAi library. To deactivate *pld-1* function, eggs from gravid adults were placed on HT115-seeded NGM plates and allowed to hatch. Expression of double-stranded RNA was induced by treating with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Nematodes hatched from eggs were fed on HT115-seeded NGM plates until the L4 stage. To synchronize the nematodes, L4 larvae were then placed on HT115-seeded NGM plates supplemented with 5 μM 5-Fluoro-2′-deoxyuridine (FUdR; Sigma-Aldrich, MO), which prevents offspring production, and were allowed to grow to day 1 or 8 of adulthood. *E. coli* HT115 containing the empty L4440 vector was used as an RNAi control. For some experiments (Figs. 2b and 3b, Supplemental Figure 1b), we used nematodes fed with *pld-1* RNAi at the L4 larval stage for 1 day to eliminate the effect of *pld-1* downregulation on nematode development.

ROS measurement

Synchronized (day 1 and 8 of adulthood) nematodes were washed with M9 buffer (22 mmol/L KH_2PO_4, 22 mmol/L Na_2HPO_4, 85 mmol/L NaCl, and 1 mmol/L $MgSO_4$) and then transferred to 2 mL of M9 buffer containing 6 μM dichlorofluorescein diacetate (DCFDA, Invitrogen, CA) or 3 μM dihydroethidium (DHE, Invitrogen, CA). After incubation for 60 min at 37 °C in the dark, the nematodes were rinsed with M9 buffer, and digital images were obtained with a Leica DM IRB inverted microscope (Leica Microsystems, Germany) equipped with a CoolSNAP HQ camera (Roper Scientific, NJ) operated by Metamorph Image Software (Universal Imaging Corporation, PA). Three independent experiments were performed.

Lifespan assay

Lifespan assays were performed as described previously[15]. Synchronized L4 larvae were placed on HT115-seeded NGM plates containing FUdR. Surviving nematodes were counted daily and were moved to fresh HT115-seeded NGM plates. Death was scored as the absence of a response to slight touch using a thin platinum wire. Three independent experiments were performed.

Locomotion velocity assays

The nematodes' mean velocity and maximum velocity were measured as previously described[16]. Briefly, synchronized (day 1 and 8 of adulthood) nematodes were transferred to an NGM plate without a bacterial lawn, and their movements were immediately recorded. The recording system contained a stereomicroscope (Nikon SMZ 745T), a charge-coupled device camera (TUCSEN TCH-5.0), and imaging software (TUCSEN ISCapture). The recording period was 30 s at a rate of 30 frames per second. The locomotion velocity was expressed as mm per second (the distance (mm) between displaced centroids per second). Recorded images were evaluated by ImageJ and wrMTrck (plugin for ImageJ: www.phage.dk/plugins). The locomotion velocity data were imported into an Excel worksheet. The highest locomotion velocity observed in the 30-s period was used as the maximum velocity.

Lipofuscin assay

Intestinal lipofuscin accumulation in synchronized (day 1 and 8 of adulthood) nematodes was determined by autofluorescence as previously described[17]. The

autofluorescence of lipofuscin was assessed using a fluorescence microscope (ZEISS AxioCam MRc, Germany) with excitation and emission wavelengths of 350 and 470 nm, respectively. The relative fluorescence intensity was quantified using ImageJ software (National Institutes of Health, MD) to determine lipofuscin levels.

Assays for oxidative and heat stress resistance

For oxidative stress resistance assays, synchronized (day 8 of adulthood) nematodes were transferred to HT115-seeded NGM plates containing 5 mM of H_2O_2. After 5 h of incubation, nematode survival was scored as touch-provoked movement every 4 h. For thermotolerance assays, HT115-seeded NGM plates with synchronized (day 1 and 8 of adulthood) nematodes were shifted from 21 °C to 35 °C. After the temperature shift, nematode viability was scored as touch-provoked movement every 2 h. Fifty nematodes were used in each experimental group. Data were analyzed and plotted as described for lifespan assays.

Assays for reporter gene expression

Synchronized (L4 larva) reporter nematodes *sod-3::gfp*, *dod-11::rfp*, *mtl-1::rfp*, and *dod-8::gfp* were transferred to HT115-seeded NGM plates. After 1 day, the fluorescence of GFP (green fluorescent protein) or RFP (red fluorescent protein) was measured using a fluorescence microscope (ZEISS AxioCam MRc, Germany) at excitation and emission wavelengths of 490 nm and 525 nm, respectively. The relative fluorescence intensity was quantified using ImageJ software to determine GFP levels. Three independent experiments were performed.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted fromsynchronized (day 1 or 12 of adulthood) nematodes or human HCT116 and MCF-7 cells. RNA was reverse-transcribed using gene-specific reverse primers and reverse transcriptase (Solgent, Korea), and the resulting cDNA was PCR-amplified. Primers used for assays are listed in Supplementary Table 1. Primers for act-2 and β-actin were used to normalize to RNA concentration in each sample. PCR products were resolved on a 1.5% agarose gel. Quantification of RT-PCR bands was performed using densitometry.

Statistical analysis

The statistical significance of data was analyzed by one-way ANOVA with the SPSS package program. The results were considered significant if the P value was less than 0.05. Duncan's multiple-range test was performed if differences between groups were identified as $\alpha = 0.05$.

Results

Expression level of *pld-1* inversely correlates with aging and *pld-1* RNAi promotes ROS accumulation in nematodes

We previously showed that PLD is transcriptionally repressed during senescence in human cells and PLD2 inhibition stimulates ROS formation in human cells[14]. To investigate whether PLD downregulation increases ROS levels in nematodes, we compared ROS levels in nematodes treated with *pld-1* RNAi to those in nematodes treated with control (L4440) RNAi. The mRNA level of *pld-1* was reduced in nematodes treated with *pld-1* RNAi, relative to that of nematodes treated with control RNAi (Fig. 1a). ROS quantification by DCFDA or DHE staining demonstrated that ROS levels apparently increased in *pld-1*-knockdown nematodes as compared to those in control nematodes. Consistently, an apparently higher level of ROS was observed in wild-type nematodes at day 8 of adulthood compared to wild-type nematodes at day 1 of adulthood (Fig. 1b, c). We next investigated whether the expression level of *pld-1* was related to aging in nematodes. The mRNA level of *pld-1* was decreased by 70% in nematodes at day 12 compared to that in nematodes at day 1 (Fig. 1d).

Pld-1 RNAi decreases lifespan and resistance to oxidative and heat stress in nematodes

We previously reported that PLD2 knockdown caused cellular senescence in human cells[14]. To determine if PLD knockdown reduced nematode lifespan, we determined the lifespan of *pld-1* RNAi nematodes and control nematodes. After *pld-1* RNAi treatment, the maximum lifespan of the nematodes was shortened from 24 to 18 days, and the median lifespan decreased from 18 to 12 days (Fig. 2a). To eliminate the effect of *pld-1* knockdown on nematode development, we performed lifespan assays using nematodes fed *pld-1* RNAi from the L4 larval stage and obtained similar results (Fig. 2b). Aged nematodes display reduced resistance to oxidative stress and heat stress[8]. We thus analyzed the impact of *pld-1* knockdown on resistance to oxidative stress (5 mM H_2O_2) and heat stress (35 °C) in nematodes. As shown in Fig. 2c, d, *pld-1* RNAi suppressed resistance to oxidative stress and heat stress. Collectively, these data indicate that proper PLD activity is important for longevity and stress resistance in nematodes.

Pld-1 RNAi increases lipofuscin accumulation and reduces physical velocity in nematodes

Lipofuscin accumulation and decreased motility are characteristics of nematode aging[8,17]. To analyze the impact of *pld-1* on lipofuscin amount in *C. elegans*, lipofuscin levels were measured by autofluorescence. More lipofuscin was present in *pld-1* RNAi nematodes relative to levels in control RNAi nematodes (Fig. 3a and Supplemental Figure 1a). To eliminate the effect of *pld-1*

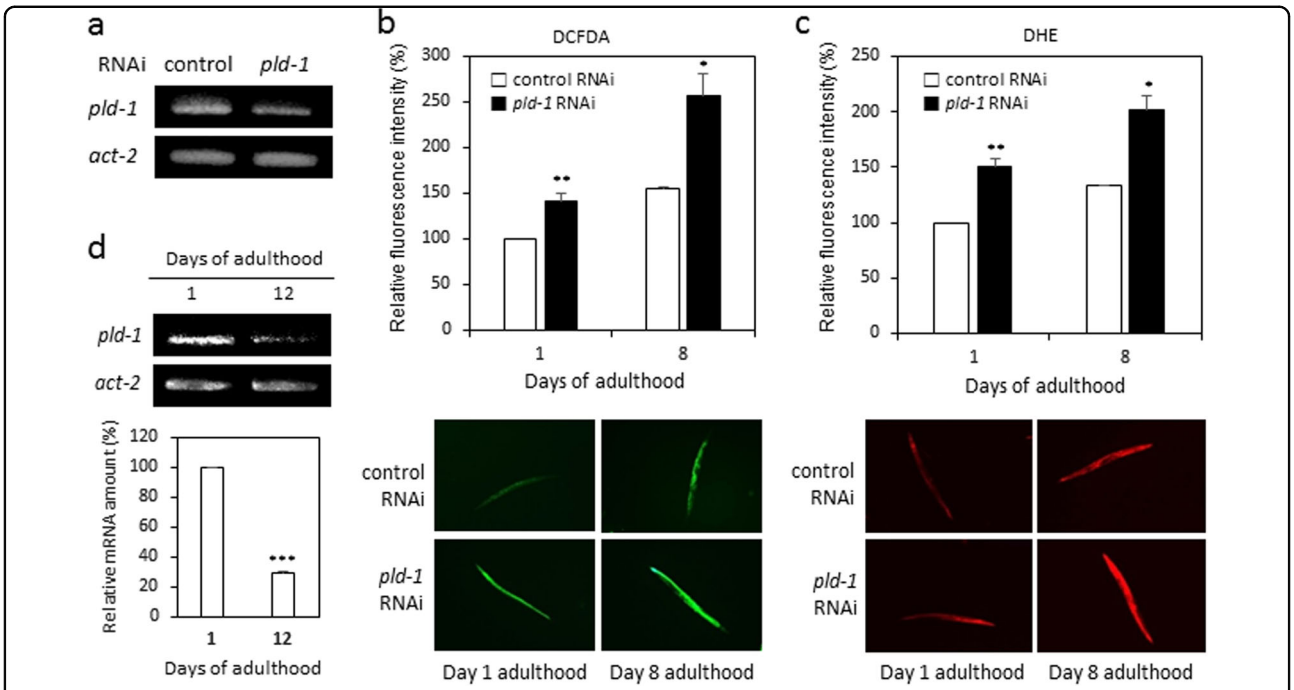

Fig. 1 *Pld-1* RNAi promotes ROS formation in nematodes and *pld-1* expression inversely correlates with aging. a Effect of *pld-1* RNAi on *pld-1* expression in nematodes. Lysates from nematodes at day 1 of adulthood fed with *pld-1* RNAi or control RNAi were utilized in RT-PCR using specific *pld-1* primers. PCR products were resolved on a 1.5% agarose gel. Representative data from three independent experiments are shown. *act-2* mRNA served as the loading control. Nematodes fed with *pld-1* RNAi or control RNAi were incubated with DCFDA (**b**) or DHE (**c**). The fluorescence intensity of nematodes at day 1 or 8 of adulthood (upper panels) was quantified using ImageJ software by determining the average pixel intensity ($n > 116$ per day 1 condition; $n > 73$ per day 8 condition). Data are shown as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Representative images of nematodes were obtained at 10× magnification (bottom panels). **d** Age-dependent reduction in *pld-1* mRNA levels. Lysates from nematodes at days 1 and 12 of adulthood were utilized in RT-PCR using specific *pld-1* primers. PCR products were resolved on a 1.5% agarose gel (upper panel). Representative data from three independent experiments are shown. *act-2* mRNA served as the loading control. The graph shows the relative mRNA level of *pld-1* (lower panel). Data are shown as the means ± SEM. $^{***}P < 0.001$

knockdown on nematode development, we investigated lipofuscin accumulation in nematodes fed *pld-1* RNAi at the L4 larval stage for 1 day and obtained similar results (Fig. 3b and Supplemental Figure 1b). We next determined nematode motility. *Pld-1* knockdown apparently reduced the average velocity (Fig. 3c) and maximum velocity (Fig. 3d) of nematodes. Therefore, these results suggest that a proper amount of PLD is required for healthy lifespan and motility in nematodes.

A ROS scavenger NAC, a possible CK2 activator spermidine, and a PLD effector phosphatidic acid rescue the lifespan shortening and age-related biomarkers mediated by *pld-1* knockdown in nematodes

To analyze the role of ROS in *pld-1* knockdown-mediated lifespan shortening, nematodes were incubated with NAC, a commonly used ROS scavenger. To determine whether NAC attenuates lifespan shortening upon *pld-1* knockdown, *pld-1*-deficient nematodes were exposed to NAC. Treatment with NAC (6 or 9 μM) counteracted the lifespan shortening mediated by *pld-1* knockdown (Fig. 4a and Supplemental Fig. 2). These findings indicate that in worms, ROS formation is one of the major upstream causes of aging triggered by PLD downregulation.

We previously reported that ectopic CK2α expression stimulates PLD2 activity and represses PLD2 knockdown-mediated senescence in human cells[14,18]. Polyamines such as spermine, spermidine, and polylysine have been known to stimulate CK2 activity in vitro[19]. Expression of ornithine decarboxylase, which catalyzes the synthesis of a polyamine precursor molecule, stimulates CK2 activity in vivo[20]. To examine whether enhanced CK2 activity abrogated the lifetime shortening induced by *pld-1* knockdown, nematodes were incubated with spermidine. Treatment with spermidine (0.2 μM) apparently counteracted the lifespan shortening mediated by *pld-1* knockdown. Furthermore, treatment with spermidine (0.2 μM) significantly extended lifespan in wild-type nematodes: the maximum and median lifespans were extended from 26 to 32 days and from 20 to 22.5 days, respectively (Fig. 4a and Supplemental Fig. 2).

PLD catalyzes the hydrolysis of phosphatidylcholine into choline and phosphatidic acid, which functions as an

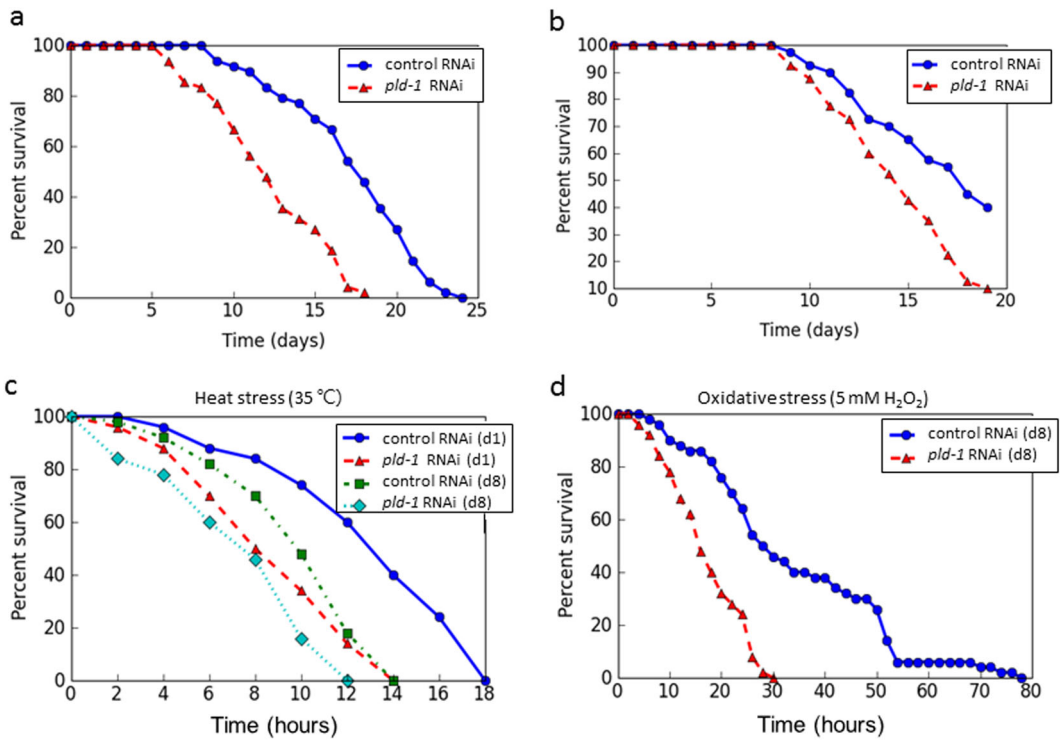

Fig. 2 *Pld-1* RNAi decreases lifespan and stress resistance in nematodes. a, **b** Effect of *pld-1* RNAi on the lifespan of nematodes. **a** Age-synchronized L4 larvae were fed on control RNAi or *pld-1* RNAi plates under standard conditions ($n = 50$ per condition). **b** Lifespan assays using nematodes fed with *pld-1* RNAi only during adulthood to exclude the effect of *pld-1* knockdown on development. Age-synchronized L4 larvae were fed on control RNAi or *pld-1* RNAi plates for 1 day ($n = 50$ per condition). **c**, **d** Effect of *pld-1* RNAi on stress resistance in nematodes. **c** Survival curves of nematodes fed with control or *pld-1* RNAi at 35 °C. Nematodes at day 1 (d1) or 8 (d8) of adulthood were scored every 2 h for viability ($n = 50$ per condition). **d** Survival curves of nematodes fed with control or *pld-1* RNAi after exposure to 5 mM H_2O_2 for 5 h. Nematodes at day 8 (d8) of adulthood were scored every 4 h for viability ($n = 50$ per condition). Viability was scored as movement away from pick touch at the indicated days or hours. Representative data from three independent RNAi experiments are shown

effector in multiple signaling pathways[1–4]. We investigated the impact of phosphatidic acid on nematode longevity. Treatment with phosphatidic acid (30 μM) successfully counteracted the shortened lifespan mediated by *pld-1* knockdown. Furthermore, treatment with phosphatidic acid (30 μM) significantly extended the lifespan of wild-type nematodes, suggesting that PLD-1 plays an important role in *C. elegans* longevity. After phosphatidic acid (30 μM) treatment, the maximum and median lifespan of the wild-type nematodes was extended from 26 to 35 days and from 20 to 28 days, respectively (Fig. 4a and Supplemental Fig. 2).

Furthermore, treatment with NAC, spermidine, or phosphatidic acid significantly attenuated lipofuscin accumulation (Fig. 4b and Supplemental Fig. 3a), decreased motility (Fig. 4c and Supplemental Fig. 3b), and susceptibility to heat and oxidative stress (Fig. 4d, e) mediated by *pld-1* knockdown. Consistently, treatment with these chemicals reduced lipofuscin accumulation (Fig. 4b and Supplemental Fig. 3a) and stimulated resistance to heat and oxidative stress (Fig. 4d, e) in wild-type nematodes.

PLD modulates the transcription of DAF-16/FoxO3a target genes in nematodes and human cells

DAF-16/FoxO is a central transcription factor that detoxifies intracellular ROS by expressing antioxidant proteins[10,11]. To determine whether *pld-1* knockdown decreased the transcriptional activity of DAF-16, we evaluated the fluorescence of several reporters that are specific for certain stress pathways, including *sod-3*, which encodes manganese superoxide dismutase (MnSOD); *dod-11*, which encodes sorbitol dehydrogenase; *mtl-1*, which encodes metallothionein; and *dod-8*, which encodes 17 β-hydroxysteroid dehydrogenase[21]. As shown in Fig. 5a, *pld-1* knockdown suppressed the transcription of *sod-3::gfp*, *dod-11::rfp*, and *mtl-1:rfp* reporter genes relative to expression upon exposure to empty vector RNAi. In contrast, *pld-1* knockdown stimulated transcription of the *dod-8::gfp* reporter gene (Fig. 5a). To examine a similar effect of PLD on transcription of FoxO3a target genes (Cu/ZnSOD, MnSOD, catalase, thioredoxin-2, and peroxiredoxin-5) in human cells, PLD2 siRNA or PLD2 expression vectors were transfected

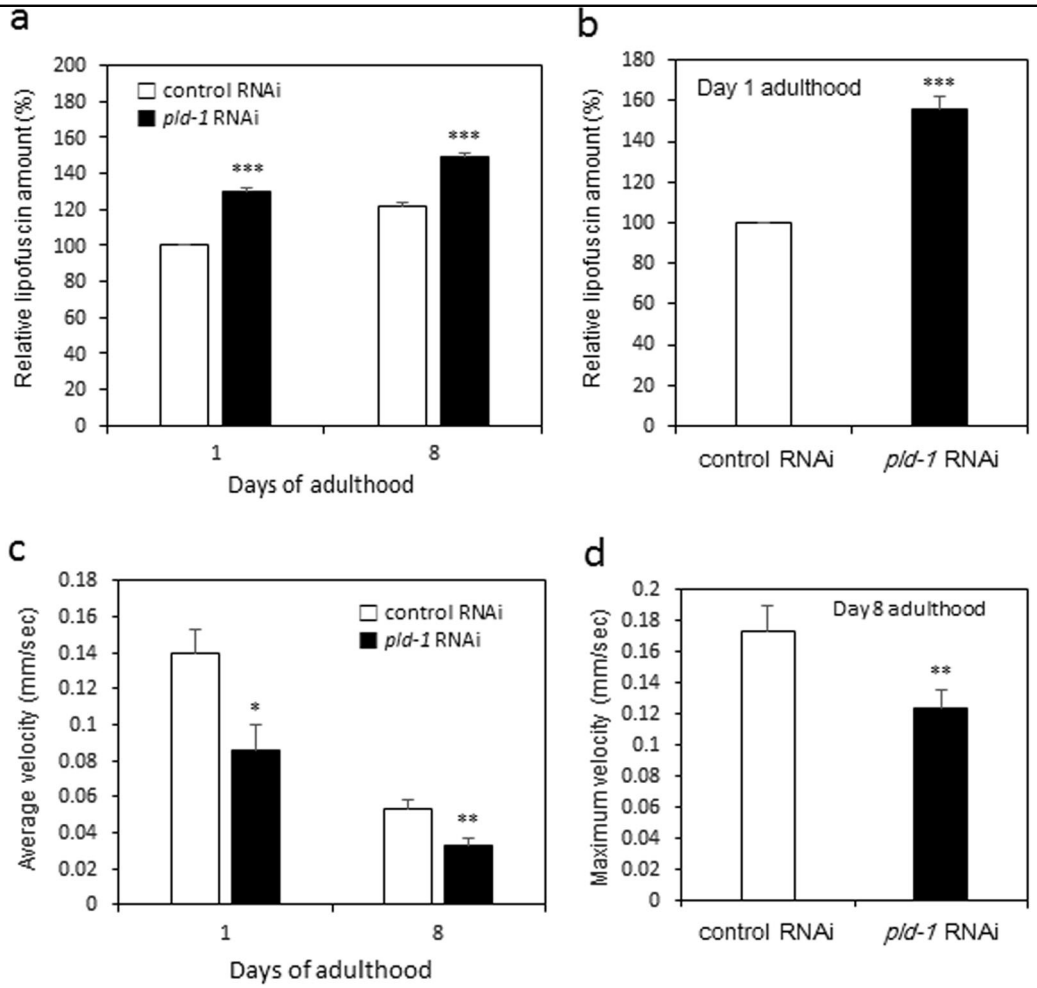

Fig. 3 *Pld-1* RNAi increases lipofuscin accumulation and reduces the physical velocity of nematodes. a, **b** Effect of *pld-1* RNAi on lipofuscin accumulation. **a** Age-synchronized L4 larvae were fed on control RNAi or *pld-1* RNAi plates under standard conditions. **b** Effects of *pld-1* RNAi on lipofuscin accumulation in nematodes fed *pld-1* RNAi for 1 day at the L4 larval stage, to eliminate the effect of *pld-1* knockdown on development. The autofluorescence images were obtained with a fluorescence microscope. The fluorescence intensity was quantified using ImageJ software by determining the average pixel intensity ($n > 134$ per day 1 condition; $n > 103$ per day 8 condition). The average velocity (**c**) and maximum velocity (**d**) of individual nematodes were measured at day 1 or 8 of adulthood ($n = 20$ per condition). Data are shown as the means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

into HCT116 and MCF-7 cells. PLD2 knockdown decreased mRNA levels of FoxO3a target genes in HCT116 cells and MCF-7 cells (Fig. 5b). Conversely, ectopic PLD2 expression increased the mRNA levels of these antioxidant genes in these cells (Fig. 5c). Thus, the present data suggested that PLD increases intracellular antioxidant levels through downregulating DAF-16/FoxO3a activity in nematodes and human cells.

Pld-1 regulates longevity between AKT-1 and DAF-16 in nematodes

We next investigated the relationship between DAF-16 and PLD-1 in nematodes using the *daf-16(mu86)* mutant. As shown in Fig. 6a, the longevity of *daf-16(mu86)* mutant nematodes was not synergistically reduced by *pld-1* RNAi treatment, suggesting that PLD-1 and DAF-16 are positioned in the same pathway for lifespan control. Because AGE-1 and AKT-1 lie upstream of DAF-16 in the IIS pathway[8–11], we investigated the role of AGE-1 and AKT-1 in PLD-mediated longevity regulation using the *age-1(hx546)* and *akt-1(mg144)* single mutants. Even though the lifespans of both mutants were drastically increased compared with lifespans of control nematodes, *pld-1* RNAi completely repressed the longevity extension effect of the *age-1(hx546)* and *akt-1(mg144)* mutations (Fig. 6b, c). We next examined whether the mRNA level of *pld-1* was upregulated in the *age-1(hx546)* and *akt-1 (mg144)* mutant nematodes. The mRNA level of *pld-1* was increased by 170% in these mutants compared to that in wild-type N2 nematodes at day 12 of adulthood (Fig. 6d). Collectively, these data suggest that AGE-1 and AKT-1 are upstream regulators of PLD in nematodes.

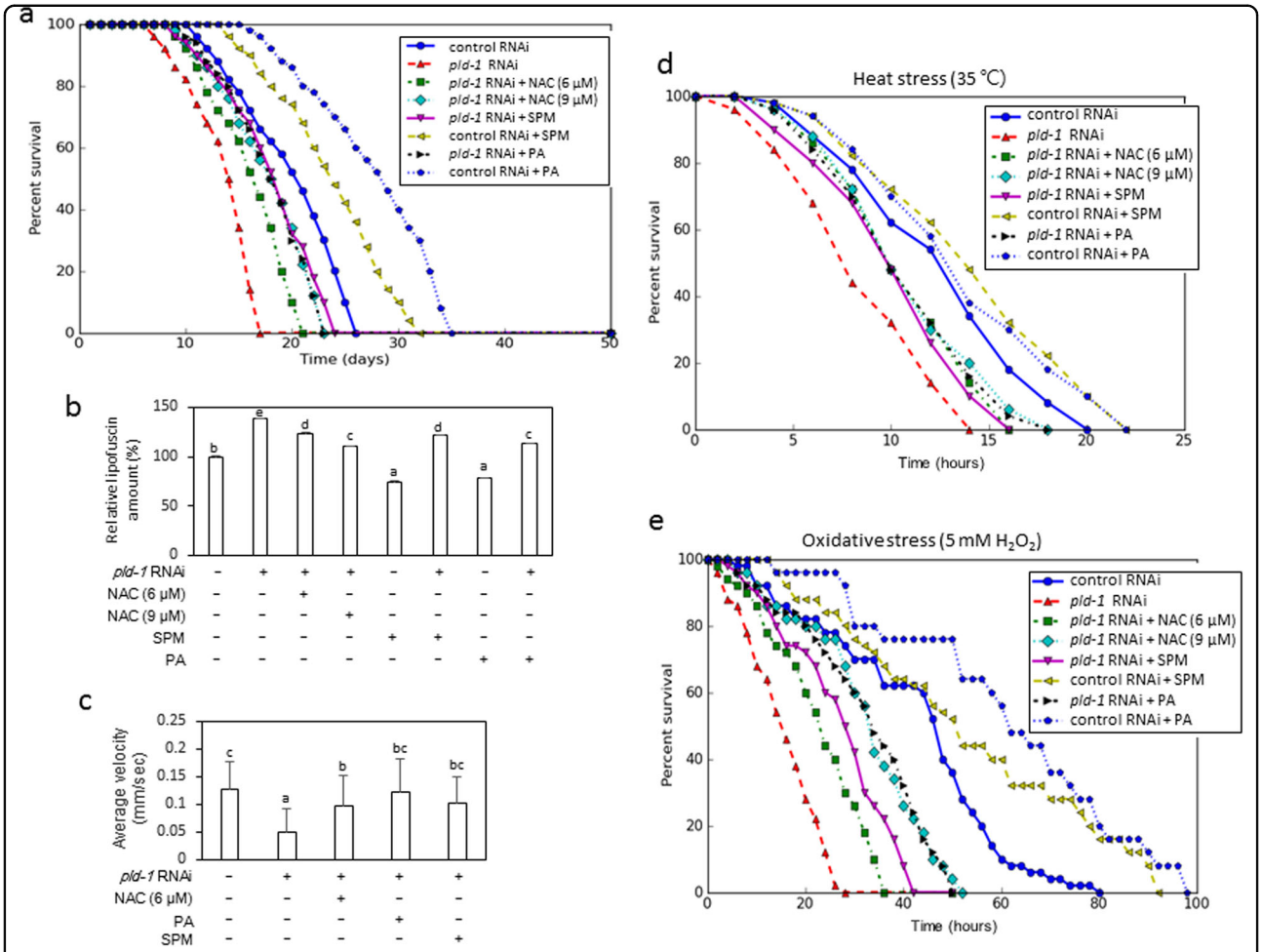

Fig. 4 The ROS scavenger NAC, a putative CK2 activator spermidine, and a PLD effector phosphatidic acid counteract the lifespan shortening and age-related biomarkers induced by *pld-1* knockdown. Age-synchronized L4 larvae were fed on control RNAi or *pld-1* RNAi plates containing NAC (6 or 9 μM), spermidine (SPM, 0.2 μM), or phosphatidic acid (PA, 30 μM) under standard conditions. **a** Viability was scored as movement away from pick touch at the indicated days. Representative data from three independent RNAi experiments are shown ($n = 50$ per condition). **b** The fluorescence intensity was quantified using ImageJ software by determining the average pixel intensity ($n = 50$ per condition). Data are shown as the means ± SEM. Bars that do not share a common letter (a, b, c, d, e) are significantly different among groups at $P < 0.05$. **c** The average velocity of individual nematodes were measured at day 1 of adulthood ($n = 28$ per condition). Data are shown as the means ± SEM. Bars that do not share a common letter (a, b, c) are significantly different among groups at $P < 0.05$. **d** Survival curves of nematodes at 35 °C. Nematodes at day 1 of adulthood were scored every 2 h for viability ($n = 50$ per condition). **e** Survival curves of nematodes after exposure to 5 mM H_2O_2 for 5 h. Nematodes at day 1 of adulthood were scored every 4 h for viability ($n = 50$ per condition). Viability was scored as movement away from pick touch at the indicated hours

Discussion

We previously demonstrated that PLD expression decreases during replicative senescence in IMR-90 cells and PLD2 knockdown triggers premature senescence in HCT116 and MCF-7 cells, suggesting that decreased PLD activity is tightly linked to the senescence process[14]. Because nematodes are broadly used as an animal model for studying aging, we examined the role of *pld-1* in nematode aging. The present study showed that *pld-1* knockdown shortened longevity (Fig. 2a, b), reduced resistance to heat and oxidative stress (Fig. 2c, d), increased lipofuscin accumulation (Fig. 3a, b, Supplemental Fig. 1), and decreased motility (Fig. 3c, d) in nematodes. Because reduced resistance to oxidative stress and heat stress, lipofuscin accumulation, and declined velocity of body movement are distinctive features of aging[8,16,17], these results collectively suggest that *pld-1* knockdown causes a range of age-related biomarkers in nematodes. Consistently, phosphatidic acid, which acts as a downstream effector of PLD, successfully counteracted the *pld-1* knockdown-mediated lifespan shortening and age-related biomarkers, including retardation of locomotion, increased lipofuscin accumulation, and reduced resistance to heat and oxidative stress (Fig. 4, Supplemental Figs. 2 and 3). In addition, phosphatidic acid considerably extended lifespan

and reduced age-related biomarkers in wild-type nematodes (Fig. 4, Supplemental Figs. 2 and 3a). Notably, the mRNA level of *pld-1* inversely correlated with aging in nematodes (Fig. 1d). This is consistent with results of a previous study showing that PLD is transcriptionally repressed during senescence in human cells[14]. Based on these data, we propose that sufficient PLD activity is necessary for extending healthy lifespan in nematodes.

Numerous reports have demonstrated that increased intracellular oxidative stress might be a critical mechanism for aging development[22,23]. We also previously showed that PLD2 knockdown triggers cellular senescence via ROS formation in human cells[14]. The present results also demonstrated that *pld-1* knockdown significantly increased ROS accumulation in nematodes (Fig. 1b, c). Moreover, the antioxidant NAC apparently abrogated the longevity shortening and age-related biomarkers triggered by *pld-1* knockdown, indicating that ROS is an important upstream trigger of aging induced by PLD downregulation in nematodes (Fig. 4, Supplemental Figs. 2 and 3). We examined the role of DAF-16/FoxO3a, a major transcription factor for antioxidant genes, in PLD knockdown-mediated ROS accumulation. The present results showed that the transcriptional activity of DAF-16 on the promoters of *sod-3* and *dod-11* decreased upon *pld-1* knockdown in nematodes (Fig. 5a). These data suggest that PLD knockdown-mediated downregulation of SOD-3/MnSOD, which detoxifies ROS[24], and DOD-11/sorbitol dehydrogenase, which reduces the nicotinamide adenine dinucleotide levels in the conversion of sorbitol to fructose[25], can cause ROS accumulation in nematodes. Our present experiments using human PLD2 also confirmed this phenomenon; downregulating human PLD2 decreased the transcription of antioxidant genes (Cu/ZnSOD, MnSOD, catalase, thioredoxin-2, and peroxiredoxin-5) in HCT116 and MCF-7 cells, whereas ectopic PLD2 expression increased the transcription of antioxidant genes in these cells (Fig. 5b, c). The fact that the longevity of the *daf-16(mu86)* mutant was not synergistically reduced by *pld-1* RNAi indicates that PLD-

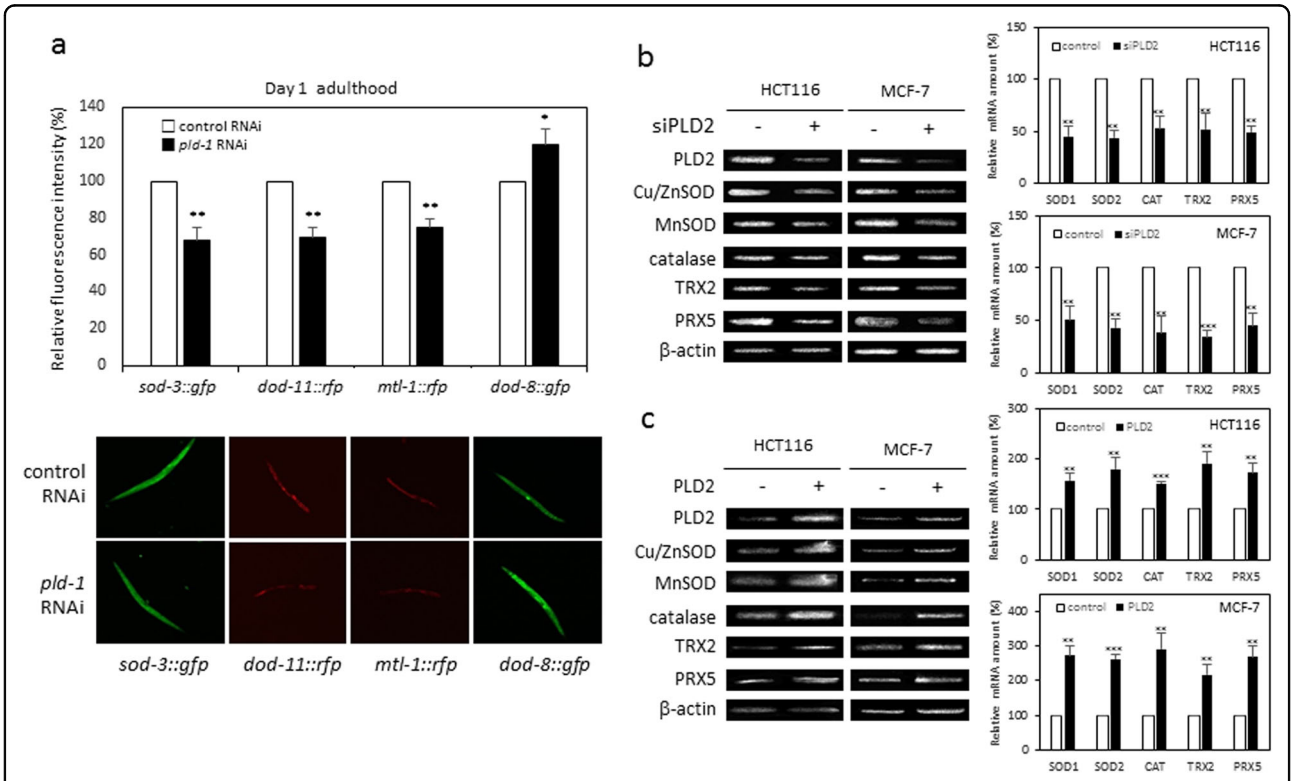

Fig. 5 PLD modulates the transcription activity of DAF-16/FoxO in nematodes and human cells. a Nematodes with integrated *sod-3::gfp*, *mtl-1::rfp*, *dod-11::rfp*, and *dod-8::gfp* reporter constructs were fed with control RNAi or *pld-1* RNAi at L4 stage for 1 day. Fluorescence intensity of GFP and RFP (upper panel) was quantified using ImageJ software by determining the average pixel intensity ($n > 111$ per condition). Data are shown as the means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. Representative fluorescence images at day 1 of adulthood were obtained at 10× magnification (bottom panel). **b**, **c** PLD2-modulated transcriptional activity of FoxO3a in human cells. HCT116 and MCF-7 cells were treated with PLD2 siRNA (**b**) or pcDNA-PLD2 (**c**) for 48 h. Total RNA was extracted from the cells, and then RT-PCR was performed using specific primers for PLD2, Cu/ZnSOD (SOD1), MnSOD (SOD2), catalase, thioredoxin-2 (TRX2), and peroxiredoxin-5 (PRX5). PCR products were resolved on a 1.5% agarose gel (left panel). β-actin was used as an internal control. Representative data from three independent experiments are shown. The graphs show the relative transcription levels of these antioxidant genes (right panel). Data are shown as the means ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$

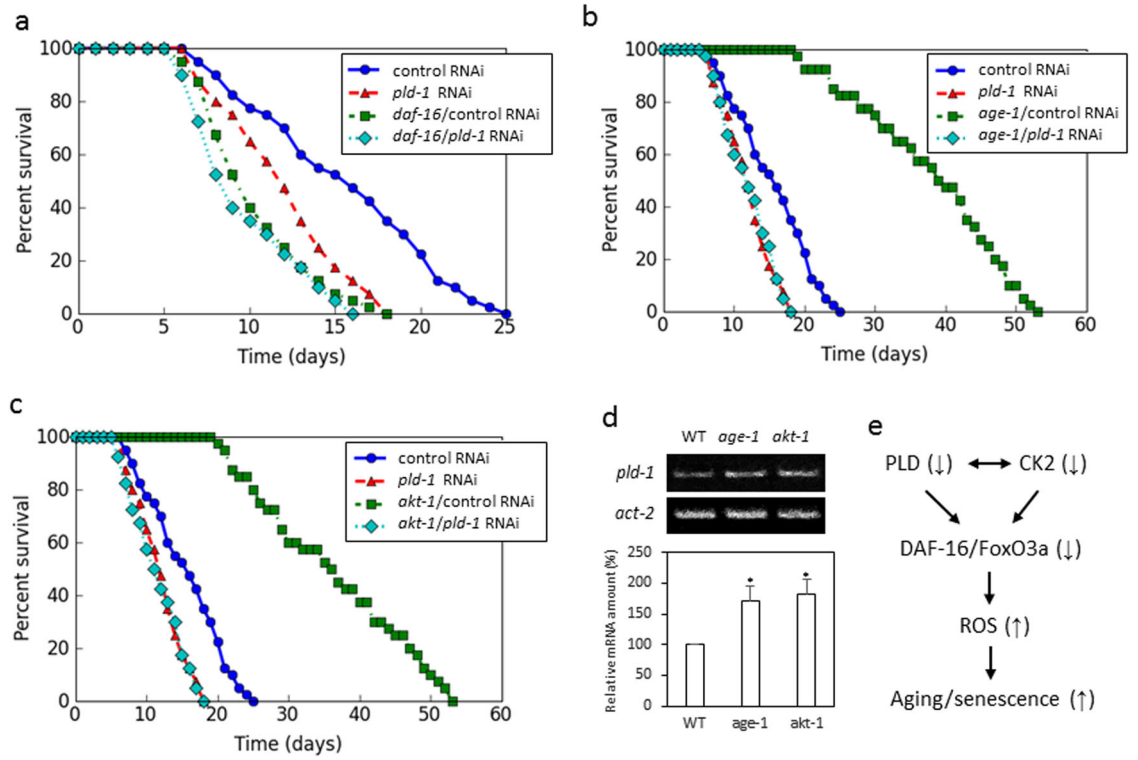

Fig. 6 Effect of *pld-1* RNAi on the lifespan of *daf-16*, *age-1*, and *akt-1* single-mutant nematodes. *Daf-16(mu86)* (**a**), *age-1(hx546)* (**b**), and *akt-1 (mg144)* (**c**) single-mutant nematodes were fed on control RNAi or *pld-1* RNAi plates under standard conditions ($n = 40$ per condition). Viability was scored as movement away from pick touch at the indicated days. Representative data from three independent RNAi experiments are shown. **d** Effect of *age-1(hx546)* or *akt-1(mg144)* mutation on *pld-1* expression. Lysates from nematodes at days 12 of adulthood were utilized in RT-PCR using specific *pld-1* primers. PCR products were resolved on a 1.5% agarose gel (upper panel). Representative data from three independent experiments are shown. *act-2* mRNA served as the loading control. The graph shows the relative mRNA level of *pld-1* (lower panel). Data are shown as the means ± SEM. $^*P < 0.05$. **e** Possible model illustrating the role of DAF-16/FoxO3a in aging and senescence induced by PLD downregulation in *C. elegans* and human cells

1 and DAF-16 are positioned in the same pathway for lifespan control (Fig. 6a). Collectively, the present study demonstrated that DAF-16/FoxO3a plays a key role in PLD downregulation-mediated ROS accumulation in nematodes and human cells (Fig. 6d). In addition, the fact that *pld-1* knockdown decreased the transcriptional activity of DAF-16 on the *mtl-1* promoters while increasing the transcriptional activity on the *dod-8* promoter suggests that PLD downregulation may reduce stress resistance and increase fat accumulation through controlling DAF-16 activity in nematodes (Fig. 5a). MTL-1/metallothionein is a heavy metal-binding protein that is involved in metal homeostasis[26], and DOD-8/17 β-hydroxysteroid dehydrogenase is localized to lipid droplets, which are highly specialized for lipid storage[27].

We previously showed that *kin-10* (the ortholog of CK2β) downregulation shortened nematode longevity and induced several age-related biomarkers (slowed motility, increased lipofuscin levels, decreased resistance to stress, and increased ROS levels) and that *kin-10* knockdown decreased or increased the transcriptional activity of DAF-16 depending on the promoters of the target genes, demonstrating that CK2 regulates lifespan via the AGE-1-AKT-1-DAF-16 pathway in nematodes[28]. Similar to results of a previous study[28], shortened lifespan and increased age-related biomarkers were also observed by *pld-1* downregulation in this study. The present study showed that like CK2, PLD also regulates lifespan downstream of AGE-1 and AKT-1 (Fig. 6b, c) and upstream of DAF-16 in nematodes (Fig. 6a). All these data suggested the possibility that *pld-1* knockdown may promote aging through a pathway identical to that of the *kin-10* knockdown-mediated nematode aging. This possibility was also proposed by the fact that spermidine, which can act as a CK2 activator, successfully counteracted the lifespan shortening and age-related biomarkers mediated by *pld-1* knockdown in nematodes (Fig. 4, Supplemental Figs. 2 and 3). This is consistent with the fact that PLD and CK2 positively regulate each other for the following reasons. First, CK2 downregulation, like PLD downregulation, induces cellular senescence via activating the ROS-p53-$p21^{Cip1/WAF1}$ pathway by downregulating FoxO3a in human cells[29–32]. Second, enhanced CK2 activity strongly represses PLD2 knockdown-

mediated senescence in human cells, and vice versa[14]. Finally, PLD2 stimulates CK2 activity through activating protein kinase C[18], whereas CK2 stimulates PLD activity via PLD phosphorylation in human cells[33]. Taken together, the previous and present studies strongly suggest that a positive regulation loop between PLD and CK2 may play a critical role in modulating longevity and senescence in nematode and human cells (Fig. 6e). A more complete understanding of the PLD-CK2-DAF-16/FoxO3a network will deliver an improved understanding of organism aging and cellular senescence.

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Conflict of interest
The authors declare that they have no conflict of interest.

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