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Celecoxib extends *C. elegans* lifespan via inhibition of insulin-like signaling but not cyclooxygenase-2 activity

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Summary

One goal of aging research is to develop interventions that combat age-related illnesses and slow aging. Although numerous mutations have been shown to achieve this in various model organisms, only a handful of chemicals have been identified to slow aging. Here, we report that celecoxib, a nonsteroidal anti-inflammatory drug widely used to treat pain and inflammation, extends *Caenorhabditis elegans* lifespan and delays the age-associated physiological changes, such as motor activity decline. Celecoxib also delays the progression of age-related proteotoxicity as well as tumor growth in *C. elegans*. Celecoxib was originally developed as a potent cyclooxygenase-2 (COX-2) inhibitor. However, the result from a structural–activity analysis demonstrated that the antiaging effect of celecoxib might be independent of its COX-2 inhibitory activity, as analogs of celecoxib that lack COX-2 inhibitory activity produce a similar effect on lifespan. Furthermore, we found that celecoxib acts directly on 3′-phosphoinositide-dependent kinase-1, a component of the insulin/IGF-1 signaling cascade to increase lifespan.

Key words: 3′-phosphoinositide-dependent kinase-1; *Caenorhabditis elegans;* celecoxib; cyclooxygenase-2 inhibitor; insulin-like signaling; longevity; nonsteroidal anti-inflammatory drug.

Introduction

The identification of chemical interventions that can ameliorate age-related illness and degeneration has been an important aspect of current aging research. A drug that can extend lifespan by slowing down the normal aging process may also delay the progression and the onset of multiple age-related diseases. The nematode *Caenorhabditis elegans* has recently been recognized as an excellent model system for identifying genetic or pharmacological interventions which alter longevity, mainly because of its short lifespan and amenability to genetic manipulation. Many genetic pathways that were identified in *C. elegans* to regulate longevity turned out to be evolutionarily conserved. For example, mutations inhibiting the insulin/IGF-1-like signaling (IIS) have been shown to extend lifespan and delay various age-related physiological changes (Guarente & Kenyon, 2000; Garigan *et al.*, 2002; Herndon *et al.*, 2002; Kenyon, 2005). The IIS pathway is highly conserved and has been shown to influence longevity in model organisms ranging from worms to mice (Kenyon, 2010). In addition, a number of compounds have been reported to extend lifespan in worms. These include a sirtuin activator resveratrol (Wood *et al.*, 2004; Bass *et al.*, 2007), an antihyperglycemic drug metformin (Onken & Driscoll, 2010), a variety of antioxidants (e.g. vitamin E) (Harrington & Harley, 1988; Adachi & Ishii, 2000; Melov *et al.*, 2000), and several serotonin receptor antagonists (e.g. mianserin), as well as anticonvulsant medicines (e.g. ethosuximide) that affect neuronal activity (Evason *et al.*, 2005, 2008; Petrascheck *et al.*, 2007). Here, we report that the anti-inflammatory drug celecoxib and its derivatives significantly extend *C. elegans* lifespan and delay the onset of age-associated proteotoxicity and tumor growth.

Since the discovery and introduction of aspirin more than a century ago, nonsteroidal anti-inflammatory drugs (NSAIDs) have become the most widely used therapeutic agents in the treatment of conditions such as pain, fever, and inflammation. NSAIDs act primarily by inhibiting cyclooxygenase (COX), thereby blocking the formation of prostaglandins (PGs) in normal and inflamed tissues. Cyclooxygenase exists as two distinct isoforms. While COX-1 is constitutively expressed in most tissues, COX-2 is expressed in inflamed tissues in response to proinflammatory stimuli (Diaz *et al.*, 1998; Lipsky, 1999; Dannenberg *et al.*, 2001). Celecoxib (Celebrex®) (Fig. 1A) is one of the selective inhibitors of COX-2 that were originally developed as a new class of NSAID to reduce the gastrointestinal toxicities that are associated with COX-1 blockage. In addition to their potent anti-inflammatory and analgesic effects, long-term use of different NSAIDs (including celecoxib) have been reported to reduce the risk and delay the onset of various age-related diseases, including cancers (Thun *et al.*, 1991; Smalley & DuBois, 1997; Thompson *et al.*, 1997; Fukutake *et al.*, 1998; Hida *et al.*, 1998; Kismet *et al.*, 2004), Alzheimer's disease, and other neurodegenerative diseases (in t' Veld *et al.*, 2001; Aisen, 2002; Etminan *et al.*, 2003; Asanuma *et al.*, 2004). Studies in our laboratory have now further linked the drug to normal aging. Interestingly, while

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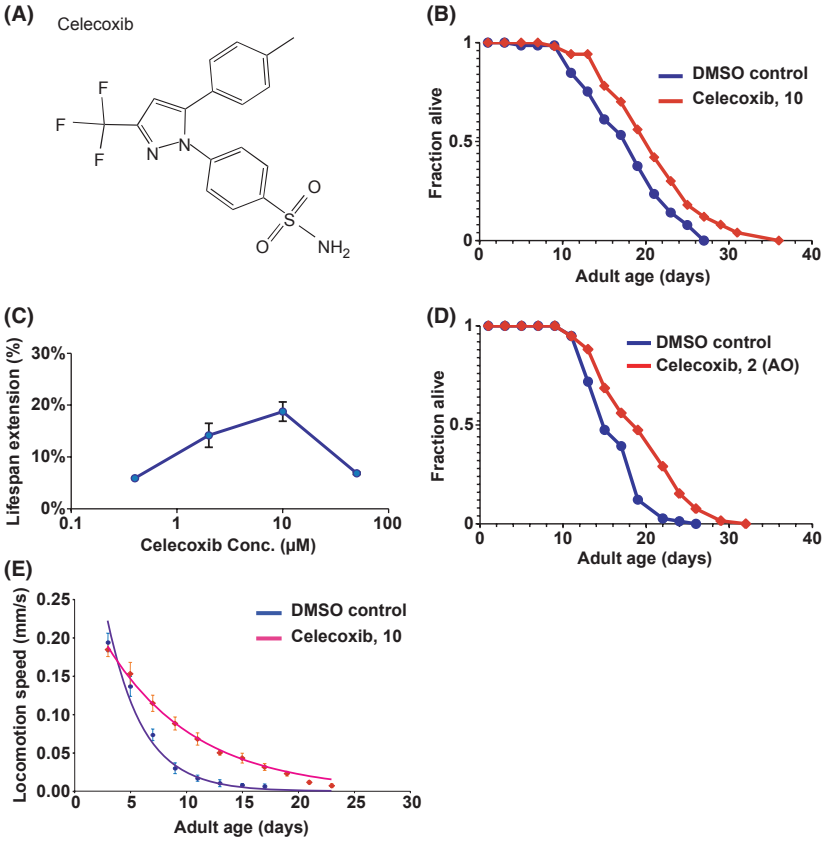

Fig. 1 Celecoxib extends adult lifespan and delays age-associated physiological changes. (A) Chemical structure of celecoxib. (B) Survival curves of wild-type (N2) animals treated with either DMSO control (blue) or 10 μM of celecoxib (red). The treatments were initiated from the time of hatching and continued until death. Statistical details and repetition of this experiment are summarized in Table S1 (Supporting information). (C) Dosage–response analysis of celecoxib (Cbx). Wild-type (N2) animals were exposed to DMSO control or 0.5, 2, 10, and 50 μM celecoxib. The average percentage change in lifespan of at least two independent experiments was plotted as a function of dosage. Statistical details and repetition of this experiment are summarized in Table S1 (Supporting information). (D) Survival curves of wild-type (N2) animals exposed to adult-only treatment of either DMSO control (blue) or 2 μM of celecoxib (red). The treatments were initiated from the first day of adulthood and continued until death. (E) The speed of spontaneous locomotion of wild-type (N2) animals treated with either DMSO control (blue) or 10 μM of celecoxib (red). Locomotion speed was quantified every other day until death as previously described (Hsu *et al.*, 2009), and the mean locomotion speed of these worms was plotted as a function of age. Error bars represent SD. Locomotion speed decayed throughout lifespan and can be best fitted by first-order exponential decay, and the rate of the decay (DMSO control, rate = 0.2686, R^2 = 0.9623; celecoxib, rate = 0.1179, R^2 = 0.9931) was calculated using the method previously described (Hsu *et al.*, 2009).

the primary target of celecoxib in clinical uses is COX-2, our results suggest that celecoxib might extend *C. elegans* lifespan via a mechanism that is independent of COX-2 but share similar phenotypic features as IIS pathway mutants. The lifespan extension resulting from celecoxib treatments requires the activity of DAF-16, the FOXO transcription factor known to regulate development and longevity in response to IIS (Lin *et al.*, 1997). Our data also suggest that celecoxib might extend lifespan by inhibiting the kinase activity of 3-phosphoinositide-dependent kinase-1 (PDK-1), a key component of the IIS cascade.

Results

Celecoxib treatment extends *C. elegans* lifespan and delays the age-related decline of motor activity

One goal of aging studies is to identify drugs that can slow aging and delay age-related illness and degeneration. To identify compounds that might slow aging and extend lifespan in *C. elegans*, we assayed a panel of randomly selected compounds that have known effects on human physiology. Animals grown on agarose plates were exposed to each drug at two different concentrations from hatching. To ensure that the drugs retained their potency throughout the entire experiment, animals were transferred to fresh plates with drugs every 2–4 days. Among those examined, celecoxib (Fig. 1A) had the greatest effect on longevity, extending adult mean lifespan by up to 20% (Fig. 1B; Table S1, Supporting information). Results from our dose–response analysis indicated that animals treated with external concentrations of 10 and 2 μM celecoxib displayed the largest lifespan extension at 20 °C (Fig. 1C; Table S1, Supporting information). Animals exposed to either higher or lower concentrations of celecoxib exhibited a smaller or an insignificant lifespan extension (Fig. 1C; Table S1, Supporting information). Moreover, the longevity effect of celecoxib was not temperature sensitive. Celecoxib caused similar increases on lifespan at 15 °C

(Table S1, Supporting information). The rate of age-associated motor activity decline has been shown to be a prominent predictor of lifespan as well as a great physiological parameter of animals' healthiness (Hsu *et al.*, 2009).Thus, we examined the locomotion speed of celecoxib-treated animals throughout the entire lifespan. Our result showed that the rate of the motor activity decay (DMSO control, rate = 0.2686; celecoxib, rate = 0.1179) is significantly reduced in celecoxib-treated worms (Fig. 1E), indicating that both healthspan and lifespan are increased when worms are exposed to long-term celecoxib treatments.

It has been reported that *C. elegans* lifespan can be extended by feeding the worms with dead bacteria, which would reduce their susceptibility to bacterial infections (Garigan *et al.*, 2002). Therefore, it is possible that celecoxib may exert its longevity effect by killing the bacteria, instead of acting directly on the worms. To test this possibility, we examined the effects of celecoxib on the growth of two commonly used bacteria strains, OP50 and HT115. The results indicate that the growth of OP50 and HT115 was completely unaltered when exposed to celecoxib (Fig. S1, Supporting information).

In *C. elegans*, signaling pathways that regulate longevity have been suggested to have different temporal requirements to regulate longevity. For instance, the IIS functions during early adulthood to regulate longevity (Dillin *et al.*, 2002a). Conversely, mitochondrial respiration functions during larval development to influence longevity (Dillin *et al.*, 2002b). We found that the lifespan-extending effect of celecoxib treatment initiated at the first day of adulthood is comparable to those initiated from hatching (Fig. 1D; Table S1, Supporting information). This finding suggests that exposure to celecoxib only during adulthood is sufficient to produce the antiaging effect.

Celecoxib further extends the lifespan of animals with reduced food uptake and mitochondrial respiration

To determine whether celecoxib extends lifespan via biological processes previously known to modulate aging in *C. elegans*, we next tested the effect of combining celecoxib treatment and various mutations that alter lifespan. Dietary restriction (DR) is known to extend lifespan in a wide range of species (Masoro, 2005) and can be mimicked by the mutations of *eat-2* gene that is required for pumping food into the pharynx (Lakowski & Hekimi, 1998). Therefore, we first examined whether the lifespan of *eat-2(ad1116)* mutants can be further extended by celecoxib. Treatments with celecoxib significantly extended the lifespan of *eat-2* mutants by 17% (Fig. 2A; Table S1, Supporting information). The FoxA transcription factor PHA-4 has been previously shown to be required for *eat-2* mutations to extend lifespan in worms (Panowski *et al.*, 2007). Treatments with celecoxib also extended the lifespan of *pha-4(zu225)* mutants to a similar extent (Table S1, Supporting information). Moreover, the rate of pumping (eating) was not affected in celecoxib-treated animals [Day 1 adult pumping rate: control, 282 ± 22 min^{-1}; celecoxib, 276 ± 24 min^{-1}; $n = 15$; $P = 0.54$], indicating that celecoxib may not exert its effects via changes in appetite or food limitation. Together, our results suggest that DR is not the primary mechanism underlying the antiaging effect of celecoxib.

Reduction in the mitochondrial respiration by RNA interference (RNAi) also extends lifespan in *C. elegans* (Dillin *et al.*,

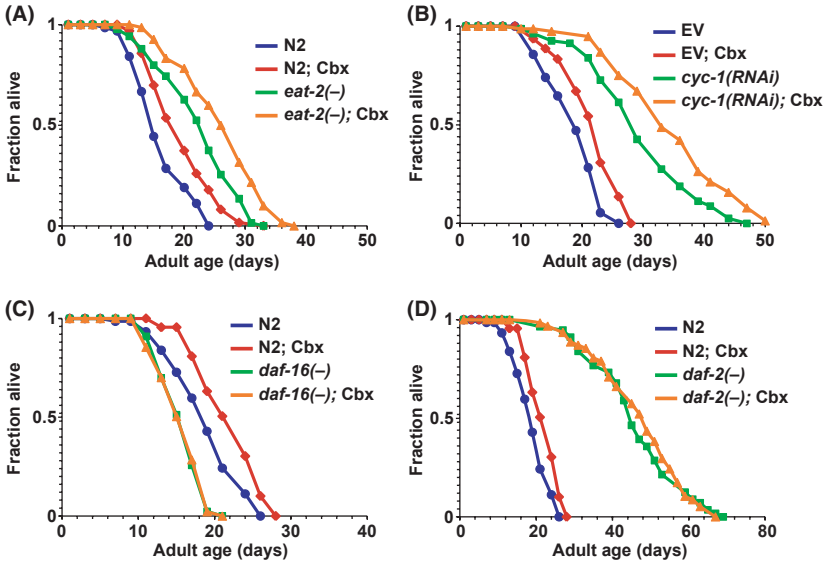

Fig. 2 Celecoxib extends adult lifespan in a *daf-16*-dependent manner. Survival curves of N2 animals and (A) *eat-2(ad1116)*, (B) *cyc-1(RNAi)*, (C) *daf-16(mu86)*, or (D) *daf-2(e1370)* mutants grown on plates containing DMSO control (blue or green) or 2 μM of celecoxib (red or orange). All drug treatments were initiated from the first day of adulthood and continued until death, except *cyc-1(RNAi)*. In all cases, these data represent the results of a single trial. Repetition of this experiment and statistical details are summarized in Table S1 (Supporting information).

2002b). To investigate whether celecoxib plays a role in the mitochondrial respiration to influence longevity, we treated the worms grown on *cyc-1* RNAi bacteria with celecoxib. Exposure to celecoxib further extended the lifespan of *cyc-1 (RNAi)* animals by 17% (Fig. 2B; Table S1, Supporting information). This finding suggests that celecoxib may not influence longevity by reducing mitochondrial respiratory chain activity.

Celecoxib extends lifespan in a *daf-16*-depedent manner

Mutations affecting the IIS pathway have been shown to influence *C. elegans* lifespan (Kenyon *et al.*, 1993; Larsen *et al.*, 1995; Morris *et al.*, 1996; Kimura *et al.*, 1997; Lin *et al.*, 1997; Ogg *et al.*, 1997; Ogg & Ruvkun, 1998; Paradis & Ruvkun, 1998; Paradis *et al.*, 1999). For example, animals carrying reduction-of-function mutations in *daf-2*, a homolog of human insulin/IGF-1 receptor, or mutations in components of its downstream PI3K/PDK-1/AKT signaling pathway, are significantly long-lived (Panowski & Dillin, 2009; Kenyon, 2010). The FOXO transcription factor DAF-16 is required for IIS mutations to extend lifespan (Kenyon *et al.*, 1993; Lin *et al.*, 1997; Ogg *et al.*, 1997). Thus, we next examined whether *daf-16* is also required for celecoxib to influence longevity. We found that treatment with celecoxib failed to extend the lifespan of animals carrying a null mutation of *daf-16* (Fig. 2C; Table S1, Supporting information), suggesting that celecoxib may influence longevity by inhibiting a component of the IIS pathway upstream of DAF-16 or by activating DAF-16 directly. We have also tested the effect of celecoxib on *daf-2*mutants and found no significant lifespan extension (Fig. 2D; Table S1, Supporting information). Again, this supports the idea that celecoxib may extends lifespan by modulating the IIS pathway activity.

Celecoxib extends lifespan via a mechanism independent of its COX-2 inhibitory activity

Although celecoxib was originally developed as a potent COX-2 inhibitor, our finding that celecoxib extends lifespan in a *daf-16*-dependent manner raises the possibility that the longevity effect of celecoxib may be independent of its COX-2 inhibitory activity. The reason is twofold. First, in mammalian models, celecoxib is known to have additional cellular targets. For instance, several studies have suggested that celecoxib might inhibit tumor growth, at least in part, by acting on a COX-2-independent mechanism, when treated at a higher dosage (e.g. IC_{50} = 40 nM for COX-2 inhibition; > 20 μM for apoptosis induction) (Hsu *et al.*, 2000; Kismet *et al.*, 2004; Zhu *et al.*, 2004). More importantly, no COX isoforms have been identified in unicellular organisms, the plant kingdom, insects, and nematodes, including *C. elegans* (Simmons *et al.*, 2004).

To investigate whether the longevity activity of celecoxib can be dissociated from its COX-2 inhibitory activity, we analyzed the lifespan of animals exposed to OSU-03012, a close structural analog of celecoxib (Fig. 3A) that exhibits no detectable COX-2

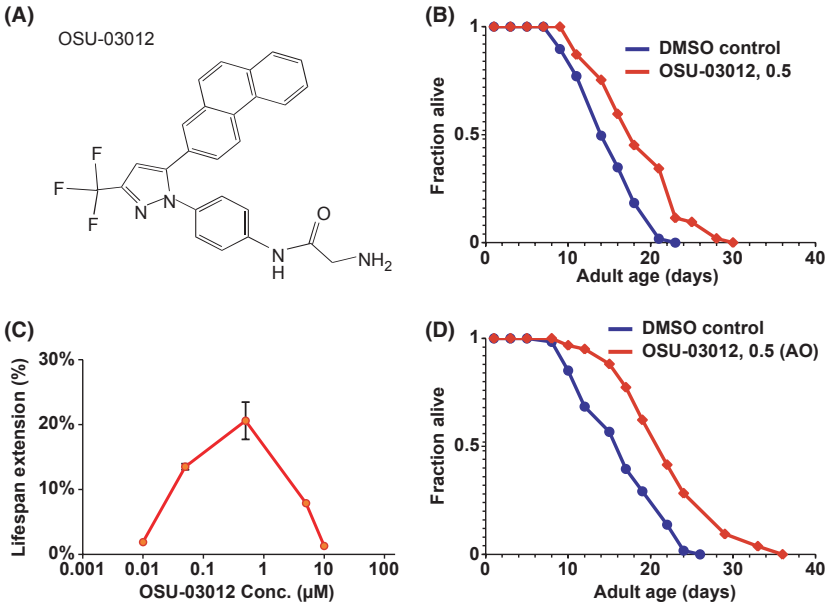

Fig. 3 OSU-03012, a derivative of celecoxib, also extends lifespan. (A) Chemical structure of the celecoxib derivative, OSU-03012. (B) Survival curves of wild-type (N2) animals treated with either DMSO control (blue) or 0.5 μM of OSU-03012 (red). The treatments were initiated from the time of hatching and continued until death. Statistical details and repetition of this experiment are summarized in Table S2 (Supporting information). (C) Dosage–response analysis of OSU-03012 (OSU). Wild-type (N2) animals were treated with DMSO control or 0.01, 0.05, 0.5, 5, and 10 μM OSU-03012. The average percentage change in lifespan was plotted as a function of dosage. Statistical details and repetition of this experiment are summarized in Table S2 (Supporting information). (D) Survival curves of wild-type (N2) animals exposed to an adult-only treatment of either DMSO control (blue) or 0.5 μM of OSU-03012 (red). The treatments were initiated from the first day of adulthood and continued until death.

inhibitory activity up to 50 μM (Zhu *et al.*, 2004). Treatment with OSU-03012 significantly extends worm lifespan to an extent similar to that of celecoxib when initiated from hatching (Fig. 3B; Table S2, Supporting information). Wild-type animals treated with 0.5 μM OSU-03012 displayed the largest lifespan extension (Fig. 3C; Table S2, Supporting information). Adult-only treatment of 0.5 μM OSU-03012 displayed an even greater lifespan extension (35%) (Fig. 3D; Table S2, Supporting information). Similar to what we have observed with celecoxib, exposure to OSU-03012 further extends the lifespan of *eat-2(ad1116)* and *cyc-1 (RNAi)* mutants, but not the lifespan of *daf-16(mu86)*and *daf-2(e1370)* mutants (Fig. 4A–D; Table S2, Supporting information). As OSU-03012 exhibits no detectable COX-2 inhibitory activity, our findings strongly suggest that celecoxib and its derivative OSU-03012 act on a target other than COX-2 to modulate longevity in *C. elegans*. It should be noted that we could not rule out the possibility that different mutants may exhibit varied sensitivity to the drugs. However, this is unlikely to be the case, as *daf-16(mu86)* mutants failed to respond to all three different concentrations of OSU-03012 we have examined (Table S2, Supporting information).

Celecoxib and OSU-03012 might extend lifespan by inhibiting PDK-1 activity

Among all the potential secondary targets reported to date [e.g. PDK-1, Bcl-2, PPAR-δ (Kismet *et al.*, 2004)], inhibition of PDK-1, a known IIS pathway component upstream of DAF-16, by celecoxib is particularly intriguing. It has been reported that celecoxib and a number of its derivatives exhibit different degrees of inhibitory activity against human PDK-1 (e.g. celecoxib, IC_{50} = 48 μM; OSU-03012, IC_{50} = 5 μM) (Zhu *et al.*, 2004). Given the strong antagonistic activity of OSU-03012 on human PDK-1 both *in vitro* and *in vivo* (Zhu *et al.*, 2004), we have also tested the effect of OSU-03012 on *pdk-1* mutants' lifespan. Treatment with OSU-03012 failed to extend the lifespan of either the long-lived loss-of-function *pdk-1(sa680)* mutants or the short-lived gain-of-function *pdk-1(mg142)* mutants (Paradis *et al.*, 1999) (Fig. 4E; Table S2, Supporting information).

To determine whether the activity of *C. elegans* PDK-1 could indeed be inhibited by celecoxib and OSU-03012 *in vivo*, we analyzed the phosphorylation status of SGK-1 (serum- and glucocorticoid-induced kinase 1), a known substrate of PDK-1, in

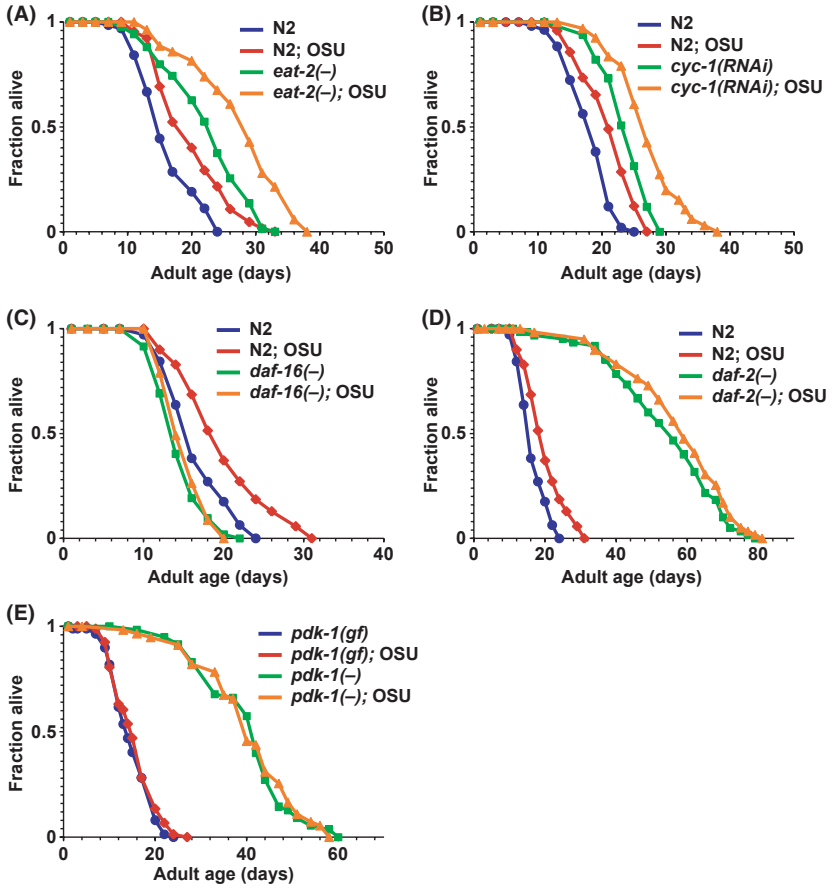

Fig. 4 OSU-03012 extends lifespan via a DAF-16 and 3′-phosphoinositide-dependent kinase-1 (PDK-1)-dependent mechanism. Survival curves of N2 animals and (A) *eat-2(ad1116)*, (B) *cyc-1(RNAi)*, (C) *daf-16(mu86)*, (D) *daf-2(e1370)*,(E) *pdk-1(mg142), or pdk-1(sa680)* mutants grown on plates containing DMSO control (blue or green) or 0.5 μM of OSU-03012 (red or orange). *pdk-1(mg142)* is a gain-of-function (gf) mutation of *pdk-1*, whereas *pdk-1(sa680)* is a loss-of-function (–) mutation of *pdk-1*. In all cases, these data represent the results of a single trial. Repetition of this experiment and statistical details are summarized in Table S2 (Supporting information).

animals exposed to both drugs. It has been reported that the Thr^{256} residue in the activation loop of human SGK1 is phosphorylated by PDK1 (Tessier & Woodgett, 2006), whereas the Ser^{422} residue in the hydrophobic motif might be phosphorylated by mTOR (Hong *et al.*, 2008). The phosphorylation status of SGK-1 is assessed by immunoprecipitating SGK-1::Green Fluorescence Protein fusion proteins from drug-treated BR2773 animals (i.e. a transgenic strain–expressing SGK-1::GFP) and blotting with anti-phospho-Thr, anti-phospho-Ser, or anti-phospho-(Ser/Thr)PDK-1 docking motif antibodies. We found that treatments with both drugs significantly reduce Threonine phosphorylation of SGK-1 by PDK-1, while serine phosphorylation of SGK-1 remains basically unaltered (Fig. 5). Thus, our findings strongly suggest that celecoxib and OSU-03012 might act directly on PDK-1 or a component upstream of PDK-1 in the IIS pathway to increase longevity in worms.

Celecoxib and OSU-03012 treatments enhance DAF-16 activity

Previous studies have shown that DAF-16 accumulates in the nucleus when the activity of its upstream kinases is reduced (Henderson & Johnson, 2001; Lin *et al.*, 2001). To further examine the idea that celecoxib and OSU-03012 might act on a component of the IIS pathway upstream of DAF-16, likely PDK-1, to influence longevity, we examined the nuclear localization of DAF-16 using a GFP reporter strain (TJ356) (Henderson & Johnson, 2001). In agreement with our model, we found an increased level of nuclear-localized DAF-16::GFP fusions after 72 h of treatment with celecoxib or OSU-03012 (Fig. 6A–B), indicating that celecoxib and OSU-03012 treatments might promote DAF-16 activation. Interestingly, we have also observed a higher level of nuclear-localized DAF-16::GFP in the anterior end compared with the posterior end of the animals (Fig. 6A). This may presumably be because of the way worms absorb the drugs (i.e. pumping via pharynx). Because it is possible that the nuclear-localized DAF-16 is not fully activated, we also measured the expression level of *sod-3*, a known *daf-16* target gene involved in stress responses, by qRT-PCR (Honda & Honda, 1999). The expression of *sod-3* significantly increased in animals exposed to celecoxib or OSU-03012 for 72 h (Fig. 6C).

In addition to regulating longevity, the IIS and DAF-16 also control entry into an alternative developmental state, named dauer in *C. elegans* (Morris *et al.*, 1996; Kimura *et al.*, 1997; Lin *et al.*, 1997). The dauer is a growth-arrested, stress-resistant alternative larval stage that is induced by food limitation, high temperatures, and crowding. Strong *daf-2* alleles enter dauer stage without any environmental cues, while weak *daf-2* alleles (e.g. e1370) enhance dauer formation only at high temperature (Fig. 6D). To examine the ability of OSU-03012 to promote dauer formation, we exposed the *daf-2(e1370)* mutants to the drug from hatching and analyzed the dauer formation. We found that OSU-03012 treatment increased dauer formation of *daf-2(e1370)* mutants at 22.2 °C from 35% of untreated animals to 62% (Fig. 6D), suggesting that treatment of OSU-03012 can further lower the IIS pathway activity in a sensitized *daf-2(e1370)* background. Together, our results are consistent with a model that celecoxib and OSU-03102 act on a component of the IIS pathway, most likely PDK-1, to extend *C. elegans* lifespan.

Celecoxib and OSU-03012 treatments ameliorate age-associated illness

An intriguing question in biology is how the normal aging process is coupled to age-related diseases. Could a drug that extends lifespan also delay the onset of age-related diseases? Reducing IIS pathway activity has been shown to ameliorate the onset and severity of progressive age-related neuronal degener-

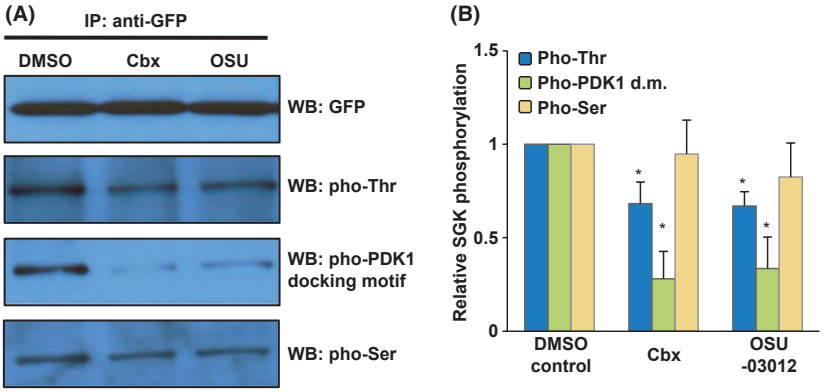

Fig. 5 Effect of celecoxib and OSU-03012 on 3′-phosphoinositide-dependent kinase-1 (PDK-1) activity. (A–B) The *in vivo* kinase activity of PDK-1 was assessed by measuring the phosphorylation status of SGK-1, a known substrate of PDK-1. Whole worm extracts were prepared from transgenic animals–expressing SGK-1::GFP fusion proteins (BR2773) treated with DMSO control, 10 μM celecoxib, or 0.5 μM OSU-03012 from hatching. Extracts were made from synchronized Day 1 adult worms and subjected to immunoprecipitation using anti-GFP antibodies. The immunoprecipitates were then immunoblotted using anti-GFP, anti-phospho-Thr, anti-phospho-Ser, or anti-phospho-(Ser/Thr) PDK-1 docking motif antibodies. The blots shown in (A) represent the result of a single experiment. The average of three independent experiments was quantified and presented in (B). The relative phosphorylation status of SGK-1 was analyzed by densitometry using ImageJ 1.37 software (NIH, Bethesda, MD, USA). *Significant changes ($P < 0.05$). *P* values were calculated by unpaired Student's *t*-test.

ation associated with aberrant protein aggregations (e.g. Alzheimer's and Huntington's disease) as well as tumor growth in *C. elegans* disease models (Morley *et al.*, 2002; Hsu *et al.*, 2003; Cohen *et al.*, 2006; Pinkston *et al.*, 2006). For instance, YFP fusions containing 35 glutamine repeats (Q35-YFP) expressed in the body wall muscle cells form aggregates and cause mobility loss as the animals age (Morley *et al.*, 2002). Both the formation of polyQ aggregates and its proteotoxicity are delayed in long-lived IIS pathway mutants and accelerated in short-lived IIS pathway mutants (Morley *et al.*, 2002; Hsu *et al.*, 2003). To test whether OSU-03012 treatment can also ameliorate the aggregation-associated proteotoxicity by inhibiting the IIS pathway in a worm model, we first analyzed the age-dependent formation of polyQ aggregates in drug-treated animals. We found that the formation of polyQ aggregates in animals exposed to OSU-03012 is delayed compared to the control animals (Fig. 7A). The aggregation-associated proteotoxicity is also significantly ameliorated in OSU-03012-treated animals (Fig. 7B).

Mutations in *gld-1*, a tumor suppressor gene required for oocyte development in *C. elegans*, causes lethal germ line tumors (Francis *et al.*, 1995). The growth of these tumors is

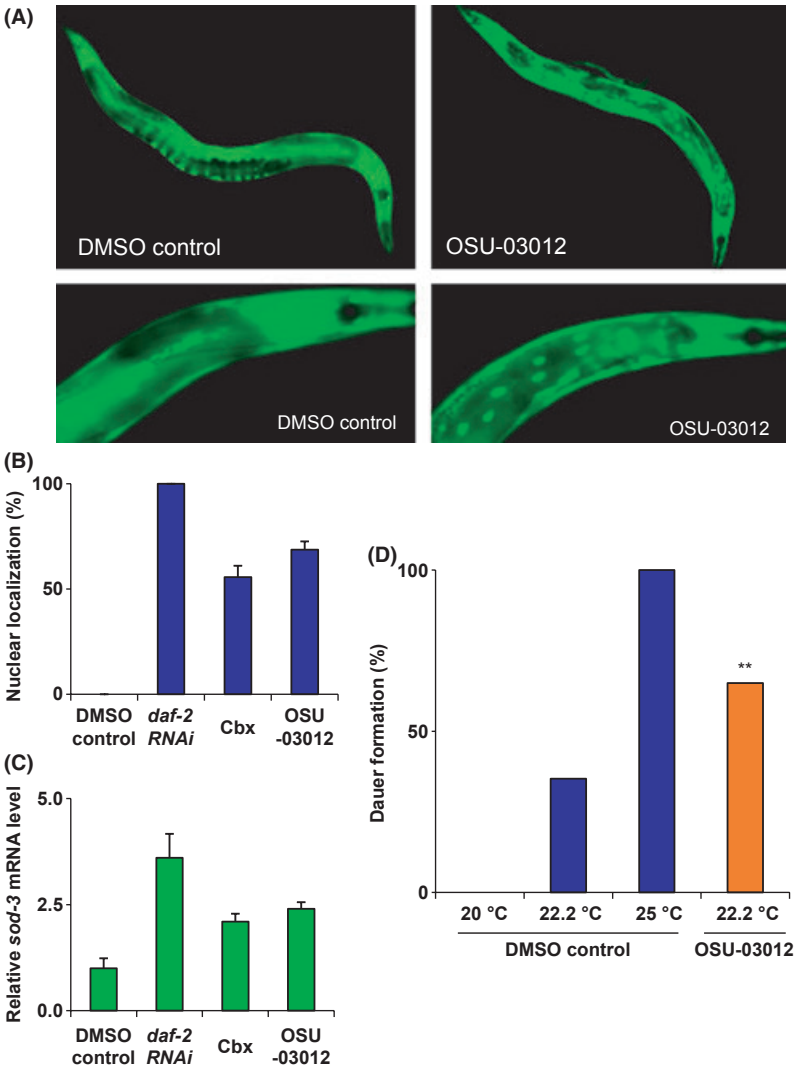

Fig. 6 OSU-03012 alters the nuclear localization and the activity of DAF-16. (A) Nuclear accumulation of DAF-16 induced by OSU-03012 treatment. Images of TJ356 animals, which carry an integrated *daf-16::gfp* array in a wild-type background, exposed to either DMSO control or 0.5 μM of OSU-03012 for 72 h. (B) Quantification of the nuclear accumulation of DAF-16::GFP in response to 10 μM celecoxib and 0.5 μM OSU-03012 treatments. Worms were scored for the presence or absence of GFP accumulation within the intestinal nuclei at the first day of adulthood ($n \geq 120$ for each treatment). An animal was scored as having nuclear DAF-16 if more than one intestinal nucleus contained DAF-16-GFP. RNA interference treatment by feeding of *daf-2* was also performed as a positive control. Lifespan following each drug treatment were determined to confirm the effectiveness of the drug treatment. (C) Effects of celecoxib and OSU-03012 on DAF-16 transcriptional activity. Wild-type N2 animals were exposed to DMSO control, 10 μM celecoxib, or 0.5 μM OSU-03012 from hatching. Relative mRNA levels of *sod-3* of these animals were measured by quantitative RT-PCR, and the mean of three different sample sets is shown. The relative mRNA levels were normalized against *act-1* (beta-actin) levels. Error bars: ±SD. (D) *daf-2(e1370)* mutants (P_0) were exposed to DMSO control or 0.5 μM OSU-03012 at 20 °C. The F_1 eggs were then moved to the different temperatures indicated for 72 h before being scored for dauer formation. Each bar represents combined data of three independent experiments per condition ($n \geq 150$ for each treatment). Asterisks indicate significant changes (*$P < 0.005$). *P* values were calculated by Pearson's chi-square test.

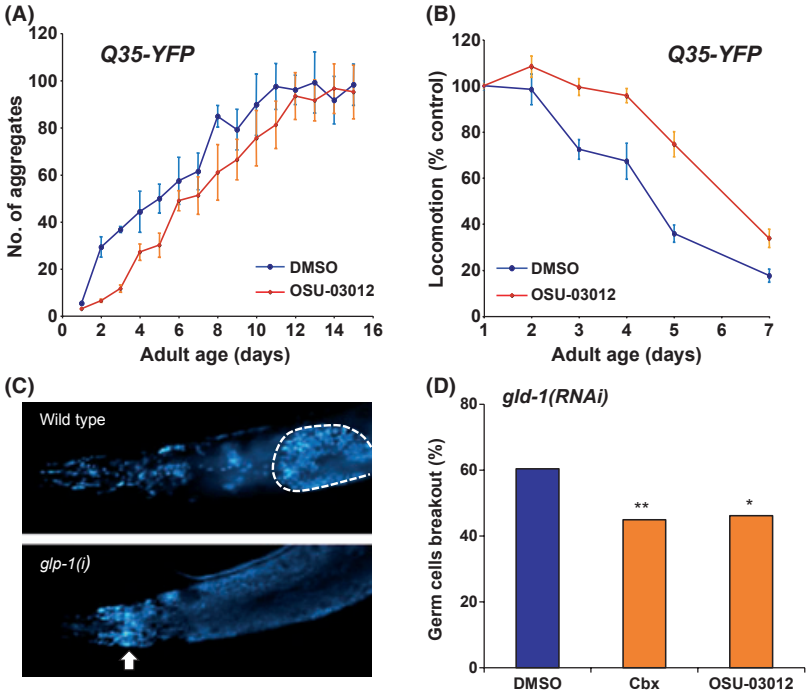

Fig. 7 OSU-03012 delays the progression of age-related diseases. (A) OSU-03012 delays the accumulation of polyQ aggregates. The number of polyQ aggregates in Q35 animals treated with DMSO control (blue) or 0.5 μM of OSU-03012 (red) was scored and plotted as a function of age. Under each condition, 10–15 worms were randomly selected from a pool of > 200 worms and counted for aggregates every day. Data are mean ± SD. (B) Relative locomotion speed of Q35 animals treated with DMSO control (blue) or 0.5 μM of OSU-03012 (red). Locomotion speed was quantified every day as previously described, and the mean locomotion speed was plotted as a function of age. Data are mean ± SD as a percentage of day 1 DMSO control. (C) Images of day 6 adult wild types (left panel) or *gld-1(RNAi)* (right panel) animals stained with the DNA-intercalating dye 4′,6′-diamidino-2-phenylindole (DAPI) using standard method. The gonad of wild-type animal was outlined by white dashed line. The white arrows indicate the presence of undifferentiated germ cells outside of the gonad in *gld-1(RNAi)* mutants. (D) Worms exposed to DMSO control, 10 μM celecoxib, or 0.5 μM OSU-03012 from hatching were scored for the presence or absence of undifferentiated germ cells outside of the gonad as shown in the right panel of (C) at day 6 of adulthood ($n \geq 125$ for each treatment). The percentage of animals with undifferentiated germ cells found in the head is plotted here. Each bar represents combined data of 2–4 independent experiments per condition. Mean ± SD = 60.4 ± 3.3, 45.0 ± 1.7, and 46.2 ± 2.6 for DMSO, cbx, and OSU-03012, respectively. Asterisks indicate significant changes (**$P < 0.005$, *$P < 0.05$). P values were calculated by Pearson's chi-square test.

significantly suppressed in long-lived IIS pathway mutants (Pinkston *et al.*, 2006). To examine whether the progression of germ line tumor growth in *gld-1* mutants could be delayed by celecoxib and OSU-03012, we monitored the growth of germ line tumors in drug-treated mutants. As predicted, the growth of the germ line tumors in *gld-1* mutants is inhibited by celecoxib and OSU-03012 treatments, presumably by inhibiting PDK-1 activity (Fig. 7C–D). The inhibitory effects of these drugs on the proliferation of germ line cells appears to occur only when *gld-1* is mutated, because both the brood size and the length of the reproductive period in N2 animals are not altered by celecoxib treatment (Fig. S2, Supporting information). Interestingly, both of these compounds have been proposed as a cancer chemoprevention drug. Our findings have demonstrated that celecoxib, a compound widely used as an anti-inflammatory drug in humans, extends lifespan and delays the progression of age-related proteotoxicity and tumor growth in *C. elegans*.

Discussion

In this study, we report that celecoxib, a NSAID, extends both mean and maximum lifespan in *C. elegans*. Furthermore, the physical healthiness, as indicated by the age-associated decay rate of motor activity (Hsu *et al.*, 2009), is significantly improved in celecoxib-treated animals. The effect of celecoxib on aging is not a result of a change in the nutritional value of the bacteria, because celecoxib has no effect on bacteria growth (Fig. S1, Supporting information). These findings prompt one critical question: What is the mechanism of action by which celecoxib extends lifespan? Celecoxib was originally developed as a selective COX-2 inhibitor for the treatments of pain and inflammation. Therefore, one might naturally predict celecoxib to extend lifespan via a mechanism involving reduced COX activity. However, several lines of evidence suggest that the lifespan-extending effect of celecoxib is independent of its COX-2 inhibitory activity. First, no homolog of mammalian COXs have been identified in unicellular organisms, the plant kingdom, insects, and nematodes, including *C. elegans* (Simmons *et al.*, 2004). We have also performed our own search for a *C. elegans* homolog of mammalian COXs using bioinformatics approaches based on sequence homology and failed to identify any COX isoforms in *C. elegans*. Secondly, results from our structural–activity analysis demonstrated that the antiaging effect of celecoxib is likely to be independent of its COX-2 inhibitory activity, as a structural

analog of celecoxib that completely lacks COX-2 inhibitory activity produces a similar effect on lifespan (Fig. 3–4). Finally, celecoxib is known to affect the activity of other proteins at a higher dosage in the mammalian system. For instance, several studies have suggested that celecoxib might induce apoptosis and inhibit tumor growth, at least in part, by acting on a COX-2-independent mechanism (Hsu *et al.*, 2000; Kismet *et al.*, 2004; Zhu *et al.*, 2004). However, the dosage required to induce apoptosis is significantly higher than the dosage required for COX-2 inhibition (i.e. IC_{50} = 40 nM for COX-2 inhibition; > 20 μM for apoptosis induction). Nevertheless, in the absence of its primary target (i.e. COXs), it is plausible that celecoxib acts on one of the secondary targets to produce the longevity effects in *C. elegans*.

In *C. elegans*, a number of environmental and physiological signals have been shown to influence longevity (Kenyon, 2010). Reduction in food intake, mitochondrial respiration activity, IIS, and signals from the germ line cells have all been reported to extend worm lifespan (Kenyon, 2010). The results of our genetic studies shown here have revealed the relationship between celecoxib and these known pathways. First, our results indicate that celecoxib and its derivative OSU-03012 do not influence longevity by acting on the mechanism that mediates DR response (Figs 2A and 4A). It also appears that celecoxib and its derivatives do not influence longevity by altering the mitochondrial respiratory chain activity (Figs 2B and 4B). Interestingly, we found that, in modulating *C. elegans* lifespan, celecoxib and its derivatives are completely dependent on the activity of the FOXO transcription factor DAF-16 (Figs 2C and 4C). Consistently, we have found that worms exposed to celecoxib or OSU-03012 exhibit an increased level of nuclear-localized DAF-16, increased expression of DAF-16 target genes, and enhanced dauer formation (Fig. 6). Together, these findings strongly suggest that chronic treatments of celecoxib and its derivatives may extend lifespan by modulating the IIS pathway and DAF-16 activity.

In mammals, it has been shown that celecoxib inhibits mammalian PDK-1 activity, a known IIS pathway component, at higher dosages (Zhu *et al.*, 2004). A number of celecoxib derivatives, including OSU-03012, have also been reported to exhibit different degrees of inhibitory activity against mammalian PDK-1, while lacking COX-2 inhibitory activity (Zhu *et al.*, 2004). In *C. elegans*, PDK-1 is known to function in the IIS pathway to control longevity, development, and metabolism (Paradis *et al.*, 1999). A reduction-of-function mutation in *pdk-1* results in increased lifespan (Paradis *et al.*, 1999). Therefore, given the known role of PDK-1 in IIS and lifespan regulation, it has emerged to be the most likely physiological target of celecoxib and OSU-03012 in influencing worm aging. Indeed, treatments with OSU-03012 failed to extend the lifespan of both *pdk-1(sa680)* and *pdk-1(mg142)* mutants (Fig. 4E), suggesting that these drugs may exert their effects by altering PDK-1 activity. Thus, when PDK-1 is mutated, the longevity effects of these compounds are compromised. At the molecular lever, both sa680 and mg142 alleles contain a missense mutation located in the kinase domain of PDK-1, in close proximity to one another [i.e. sa680, G255R; mg142, A263V (Paradis *et al.*, 1999)]. The PDK1 kinase domain has at least three ligand-binding sites; the ATP-binding pocket, the peptide substrate-binding site, and a groove in the N-terminal lobe that binds its kinase substrates. Many inhibitors of PDK-1 were designed or screened to target these sites to compete with either the substrates or ATP (Bobkova *et al.*, 2010). In fact, celecoxib and OSU-03012 have been proposed to inhibit mammalian PDK-1 through competition with ATP (Zhu *et al.*, 2004). Thus, mutations such as mg142 or sa680 that would likely change the tertiary structure of the kinase domain may very well alter the PDK-1 inhibitory activity of a compound. Furthermore, we have shown that *in vivo* PDK-1 activity is significantly reduced in celecoxib- or OSU-03012-treated animals (Fig. 5). Together, our findings support the model that celecoxib and OSU-03012 function as PDK-1 inhibitors to increase longevity in worms. Alternatively, celecoxib may act on a component upstream of PDK-1 in the IIS pathway or act on an unknown target that indirectly alters IIS and PDK-1 activity. However, the latter model is less preferable because it has been shown that celecoxib and OSU-03012 can directly inhibit mammalian PDK-1 activity *in vitro* (Zhu *et al.*, 2004).

In addition to longevity and dauer formation, the IIS pathway has been shown to influence many other aspects of worm physiology, such as developmental timing, reproduction, feeding rate, and fat storage (Kenyon *et al.*, 1993; Kimura *et al.*, 1997; Gems *et al.*, 1998). However, we have observed no significant differences in fecundity (Fig. S2, Supporting information) and developmental timing [egg to egg time: control, 70.0 ± 1.4 h; celecoxib, 70.2 ± 1.5 h; $P = 0.68$] between the drug-treated and control animals. Furthermore, it has been reported that the lifespan of the hypomorphic *daf-2(e1370)* mutants can be further extended by *daf-2* RNAi (Arantes-Oliveira *et al.*, 2003). However, only a small (7–9%), but not statistically significant, extension of lifespan has been observed in *daf-2(e1370)* mutants when treated with OSU-03012 (Fig. 4D; Table S2, Supporting information). Similarly, compared with the robust effects of mutations in the IIS pathway (e.g. *daf-2* or *pdk-1*) on longevity (> 100%), the effects of celecoxib and OSU-03012 are rather small (~20%). The reason why these drug treatments did not completely phenocopy *daf-2* or *pdk-1* mutations may be twofold. First, the maximum level of inhibition of IIS activity that could be achieved by the drug treatments may be limited by other detrimental secondary effects associated with these compounds (e.g. secondary targets), as exposure of the animals to high doses of these drugs do cause lethality (Figs 1C and 3C). For instance, the external concentration of OSU-03012 that produced the optimal longevity effect (i.e. 0.5 μM) is much lower than the reported IC_{50} of the compound for PDK-1 inhibition (i.e. 5 μM) (Zhu *et al.*, 2004). The internal concentration of the drug is likely to be even lower. Therefore, the effects of the drugs we observed on worm physiology may be suboptimal compared with other IIS mutants or RNAi. Second, as we men-

tioned earlier, we have observed a higher level of nuclear-localized DAF-16::GFP in the anterior end compared with the posterior end of the animals (Fig. 6A). This may presumably be because of the way worms absorb the drugs (i.e. pumping via pharynx). Therefore, it is possible that the effects we observed with these drugs are limited by the number and the types of cells they can reach. This may explain why certain aspects of worm physiology are not affected by the drug treatments.

It has been proposed that mild stresses early in life may causes a lifespan extension by enhancing the existing damage repair mechanisms (Rattan, 2008). This effect, which is often referred to as hormesis, has also been observed in worms, as mild heat-shock and oxidative stress result in a small but significant extension in lifespan (Lithgow *et al.*, 1995; Cypser & Johnson, 2002). Therefore, while our results strongly suggest that celecoxib and its derivatives might extend lifespan by inhibiting PDK-1 activity, we cannot rule out the possibility that the increased longevity is the result of a hormetic effect induced by the cytotoxicity of celecoxib, because high doses of celecoxib do cause lethality.

It is noteworthy that the external concentrations of celecoxib that extend lifespan (2–10 μM) are very close to the maximum serum concentration (1.8 ± 0.6 μM) found in osteoarthritis patients who were orally administered 200 mg celecoxib (Itthipanichpong *et al.*, 2005). The internal concentration of celecoxib that extends lifespan in worms, however, is likely to be ten- to 100-fold lower than external concentrations (Rand & Johnson, 1995; Evason *et al.*, 2005).

In addition to its use as an anti-inflammatory drug for the treatment of rheumatoid arthritis and osteoarthritis, celecoxib has been shown to exert potent anticancer activities as well. Several epidemiological, preclinical, and clinical studies have shown that regular use of celecoxib significantly reduce the risk of multiple cancers, including colorectal, pancreatic, lung, skin, and breast cancers [reviewed in (Kismet *et al.*, 2004)]. For instance, it has been shown in recent clinical trials that celecoxib is very effective in preventing colorectal adenomatous polyps (Arber *et al.*, 2006; Bertagnolli *et al.*, 2006). In addition to its role in cancer prevention, celecoxib appears to be effective in treating tumors that have already formed [reviewed in (Gasparini *et al.*, 2003)]. Despite these ongoing clinical investigations, the molecular mechanism underlying celecoxib-mediated antitumor effects *in vivo* remains unclear. While celecoxib can inhibit COX-2 and can cause cell cycle arrest and apoptosis in certain cancer cells, accumulating evidence suggest that inhibition of COX-2 may not play a dominant role in this drug's anticancer effects. For instance, it has been shown that the antitumor effect of celecoxib can be obtained in cancer cells that do not express COX-2 (Grosch *et al.*, 2001; Chuang *et al.*, 2008). Furthermore, a structure–function analysis of several dozens of celecoxib analogs reveals that the antitumor potency is not dependent on its COX-2 inhibitory activity (Zhu *et al.*, 2002, 2004). Interestingly, these findings are consistent with our observations in *C. elegans*, as celecoxib also delays the progression of tumor growth, likely in a COX-2-independent manner. Considering recent studies associating celecoxib use with a higher risk of cardiovascular events (Solomon *et al.*, 2005), celecoxib derivatives such as OSU-03012 that target PDK-1 specifically may be a more suitable candidate for future development of anticancer or even antiaging drugs.

Celecoxib or OSU-03012 treatment in worms also delays the onset of polyQ-mediated protein aggregation and proteotoxicity (Fig. 7A–B). This beneficial effect may be the result of a direct inhibition of a mechanism that normally promotes aging (e.g. IIS pathway) by the drugs. Thus, the age-dependent progression of polyQ proteotoxicity is delayed when the rate of aging is reduced. Alternatively, celecoxib may act on a specific target that independently controls the development of aggregate-mediated proteotoxicity. In humans, epidemiological studies have shown that long-term use of NSAIDs reduces the risk and delays the onset of Alzheimer's disease as well as other neurodegenerative diseases (Aisen, 2002; Asanuma *et al.*, 2004). Recent studies have reported that a subset of NSAIDs can lower the production of amyloidigenic Aβ42 or Aβ40 peptides, potentially independent of its COX inhibitory activity (Weggen *et al.*, 2001; Gasparini *et al.*, 2004). However, nonselective NSAIDs appear to be more effective than selective COX-2 inhibitors in protection from Aβ toxicity. Moreover, clinical trails so far have failed to show any beneficial effect of celecoxib in AD (Firuzi & Pratico, 2006).

Overall, our results support a model that celecoxib and its derivative OSU-03012 act through PDK-1, a conserved component of the IIS pathway, to extend lifespan in *C. elegans*. These findings may serve as a starting point for developing new therapeutics combating various aging-related diseases.

Experimental procedures

Strains

All strains used were maintained and handled as described previously (Brenner, 1974). CF1037: *daf-16(mu86)*I, DA1116: *eat-2(ad1116)*II, CF1041: *daf-2(e1370)*III, JT9609: *pdk-1(sa680)*X, GR1318: *pdk-1(mg142)*X; TJ356: *zIs356* [Ex(*daf-16::gfp* + *rol-6*)], BR2773: *byEx*[*sgk-1::gfp*],AM140: *rmIs132*[*unc-54p*::*Q35::yfp*].

Pharmacological compounds

Celecoxib was extracted from Celebrex capsules obtained from Amerisource Health (Malvern, PA, USA) with ethyl acetate followed by recrystallization from a mixture of ethyl acetate and hexane. 2-amino-N-[4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-phenyl] acetamide (OSU-03012) was synthesized by Dr. Chen's laboratory as described previously (Zhu *et al.*, 2004). These compounds were dissolved in DMSO for storage and diluted in water before use. Compounds at various concentrations were added to the NG plates approximately 8–12 h before transferring animals onto these drug-containing

plates. The final DMSO concentration was kept at 0.1% after adding drugs to the plates.

RNA interference experiments

HT115 bacteria transformed with RNAi vectors (L4440) expressing dsRNA of the genes indicated were grown at 37 °C in LB with 10 μg mL^{-1} tetracycline and 50 μg mL^{-1} carbenicillin, then seeded onto NG-carbenicillin plates, and supplemented with 100 μL of 0.1 M IPTG. Eggs were added to plates and transferred to new plates every 3–6 days.

Lifespan analysis

Lifespan analysis was conducted at 15 or 20 °C as described previously (Kenyon *et al.*, 1993; Apfeld & Kenyon, 1999). Strains were grown at 20 °C for at least two generations without starvation before used in lifespan analysis. At least 60 worms were used for each experiment. In all experiments, the prefertile period of adulthood was used as $t = 0$ for lifespan analysis. Statview 5.01 (SAS Institute) software was used for statistical analysis to determine the means and percentiles. In all cases, *P* values were calculated using the log-rank (Mantel–Cox) method. For a typical drug treatment experiment, unless indicated otherwise, parental worms were cultured in the presence of the drug, and progeny were selected at the L4 stage to start the experiments. Thus, these worms were exposed to the drug from fertilization until death. To ensure that the drugs retain its potency throughout the entire experiment, animals were transferred to fresh plates with the same drugs every 2–4 days.

DAF-16 nuclear localization assay

For quantification of DAF-16::GFP localization, synchronized eggs from TJ356 animals (i.e. transgenic animals–expressing DAF-l6::GFP) were seeded onto either DMSO control or relevant drug plates. The GFP expression was then analyzed using an Olympus BX61 (Olympus America Inc., Center Valley, PA, USA) fluorescent microscope at 40× or 100× magnifications. Using a blind assay, worms were scored for the presence or absence of GFP accumulation within the intestinal nuclei as 1-day-old adult ($n = 120$ or greater for all treatments). An animal was scored as having nuclear GFP if more than one intestinal nuclei contained DAF- 16-GFP. Lifespans following each treatment were analyzed to confirm the effectiveness of each drug treatment.

Quantitative RT-PCR analysis

To measure the mRNA level of *sod-3* in drug-treated animals, total RNA was isolated from approximately 5000 Day 1 adult worms grown on either control or drug-containing NG plates. cDNA was then prepared from 4 μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). TaqMan real-time PCR experiments were then performed in using the Chromo 4 system (MJ Research). Relative mRNA level of *sod-3* was calculated and normalized against the internal control (*act-1,* the β-actin). Primer and probe sequences are available upon request.

Immunoprecipitation and Western blotting analysis

Worm extracts were prepared from Day1 adult BR2773 (sgk-1::gfp) worms grown on either control or drug-containing HG plates. Animals were harvested and washed two times with cold M9 buffer. Animals were then washed once with homogenization buffer (HB buffer, 20 mM Hepes, pH 7.6, 100 mM NaCl, 10 mM KCl, 1.5 mM, $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 44 mM Sucrose, 0.5% Trion X-100). The worm pellet was resuspended in 3× volume of HB buffer with 1.5 mM NaF, 2 mM Na_2VO_4, and protease inhibitors mix (Roche, Basel, Switzerland). The worm pellet was then lysed by applying to the freeze-and-thaw cycle twice. The lysate was transferred into a Dounce homogenizer and stroked 30 times with a B pestle. The lysate was collected and spun at 14 000 *g* for 20 min. The supernatant was collected, and protein concentration was measured by Bradford assay (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

For immunoprecipitation experiments, 2.5 mg of total protein was first incubated with rabbit polyclonal anti-GFP antibody (Abcam, Cambridge, UK #6556) at 1:500 dilution for 3–5 h at 4 °C. Forty microliters of 50% protein A-agarose slurry was then added to the extract and incubated for another 3–5 h at 4 °C. The beads were washed three times with TNTG buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) with protease inhibitors. After the final wash, the beads were boiled with SDS sample buffer for Western blotting analysis using respective antibodies. The mouse monoclonal anti-phospho (Ser/Thr)-PDK-1 docking motif antibody (#9634) and the rabbit polyclonal anti-phosphothreonine antibody (#9381) were purchased from Cell Signaling Technology, Danvers, MA, USA . The mouse monoclonal anti-phosphoserine antibody was purchased from Sigma-Aldrich, St. Louis, MO, USA (#P3430).

polyQ aggregation quantitation

Approximately 200 synchronized eggs of Q35-yfp-expressing animals were placed on plates containing OSU-03012 or DMSO control. Animals were then transferred to fresh plates with the same drugs every 3–4 days. From each group, 10–15 worms were randomly selected to be scored for aggregates every day. Animals selected were viewed at 100× magnification with a stereomicroscope equipped for epifluorescence. Images of these animals were taken, and the number of aggregates in each animal was blindly counted by three independent observers after all the images have been collected. Aggregates were defined as discrete structures with clear boundaries on all sides.

Mobility assay

The spontaneous locomotion speed was measured using an automated worm tracking system as previously described (Hsu *et al.*, 2009).

DAPI staining for *gld-1(–)* tumor formation

Day 6 adult worms grown on *gld-1* RNAi bacteria and treated with different drugs were collected and fixed with cold methanol at –20 °C for 30 min. The fixed samples were then incubated with 100 ng mL^{-1} of DNA-intercalating dye 4′,6′-diamidino-2-phenylindole (DAPI) for 20 min. After washing by M9 buffer, the breaking out of the germ cells from gonads was visualized and scored using fluorescence microscopy.

Dauer formation assay

*daf-2 (e1370)*animals were grown on plates containing DMSO, celecoxib, or OSU-03012 at 20 °C, F1 eggs were incubated at 22.5 or 25 °C, and animals were scored for dauer arrest 72 h later.

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Author contributions

The author(s) have made the following declarations about their contributions: TTC, CSC, and ALH conceived and designed the experiments; TTC and WCC performed the experiments; TTC, WCC, and ALH analyzed the data; CSC contributed reagents/materials/analysis tools; and TTC and ALH wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. Celecoxib does not affect bacteria growth.

Fig. S2. Celecoxib does not decrease progeny production.

Table S1 The effect of celecoxib on lifespan.

Table S2 The effect of OSU-03012 on lifespan.

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