Delaying aging and the aging-associated decline in protein homeostasis by inhibition of tryptophan degradation

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Toxicity of aggregation-prone proteins is thought to play an important role in aging and age-related neurological diseases like Parkinson and Alzheimer's diseases. Here, we identify tryptophan 2,3-dioxygenase (*tdo-2*), the first enzyme in the kynurenine pathway of tryptophan degradation, as a metabolic regulator of age-related α-synuclein toxicity in a *Caenorhabditis elegans* model. Depletion of *tdo-2* also suppresses toxicity of other heterologous aggregation-prone proteins, including amyloid-β and polyglutamine proteins, and endogenous metastable proteins that are sensors of normal protein homeostasis. This finding suggests that *tdo-2* functions as a general regulator of protein homeostasis. Analysis of metabolite levels in *C. elegans* strains with mutations in enzymes that act downstream of *tdo-2* indicates that this suppression of toxicity is independent of downstream metabolites in the kynurenine pathway. Depletion of *tdo-2* increases tryptophan levels, and feeding worms with extra L-tryptophan also suppresses toxicity, suggesting that *tdo-2* regulates proteotoxicity through tryptophan. Depletion of *tdo-2* extends lifespan in these worms. Together, these results implicate *tdo-2* as a metabolic switch of age-related protein homeostasis and lifespan. With TDO and Indoleamine 2,3-dioxygenase as evolutionarily conserved human orthologs of TDO-2, intervening with tryptophan metabolism may offer avenues to reducing proteotoxicity in aging and age-related diseases.

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Maintaining a stable proteome is critical for an organism's survival, and cells have evolved several mechanisms to cope with misfolded and aggregation-prone proteins (1). Conditions such as environmental stress or aging increase protein damage, and at the same time, protein quality control systems are thought to be compromised, resulting over time in the accumulation of toxic aggregation-prone proteins (2). Toxicity of such aggregation-prone proteins is thought to play an important role in aging and age-related diseases, such as Parkinson, Alzheimer's, and Huntington disease (3–5). Although aging seems to be the greatest risk factor for developing neurodegenerative diseases, little is known about the metabolic and molecular processes that drive the toxicity of aggregation-prone proteins during aging.

Studies in the nematode *Caenorhabditis elegans* have identified evolutionarily conserved genetic pathways that regulate protein homeostasis as well as aging, including the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS), hypoxic response, and dietary restriction pathways (6–13).

Here, we describe the identification of the tryptophan-converting enzyme tryptophan 2,3-dioxygenase (TDO-2) as a metabolic regulator of age-related protein toxicity and lifespan in *C. elegans*.

Results

Knockdown of *tdo-2* Suppresses Toxicity of α-Synuclein in *C. elegans*. α-Synuclein is an aggregation-prone protein that is involved in Parkinson disease and other synucleinopathies (14, 15). We have previously identified 80 genes that modify α-synuclein inclusion formation in a *C. elegans* model (16).

Toxicity of α-synuclein in this model can be measured by a progressive decline in the worms' motility during aging (17). To search for modifiers of α-synuclein toxicity, we measured the motility of animals in which each of the 80 genes was individually knocked down by RNAi (Dataset S1). We identified 10 genes that increased toxicity and 3 genes that decreased toxicity on knockdown (Fig. 1*A* and Dataset S1). Our data are in line with other studies showing that the presence of inclusions does not necessarily correlate with toxicity (18). The most potent suppressor of toxicity identified in this screen was *C28H8.11* (NCBI accession number NM_065883.3), which resulted in a 2.5-fold increase in motility in day 4 adults (Fig. 1*A*). We named this gene *tdo-2* based on its homology to human TDO (NCBI accession number NP_005642.1) (Fig. S1*A*). *tdo-2* encodes TDO-2, which catalyzes the first and rate-limiting step in the kynurenine pathway of tryptophan degradation (Fig. 1*B*). Analysis of a transcriptional reporter strain expressing GFP under the control of the *tdo-2* promoter suggests that *tdo-2* is mainly expressed in body wall muscle cells and the hypodermis and therefore, that it may regulate proteotoxicity through these tissues (Fig. S1*B*) (19).

Knockdown of *tdo-2* suppressed α-synuclein toxicity from day 1 of adulthood on without affecting expression levels of α-synuclein (Fig. 1*C* and Fig. S1 *C* and *D*). A decline in muscle function is one of the first features that can be observed during aging in many organisms (20–23), and decreased protein quality control or the expression of aggregation-prone proteins can accelerate this age-dependent decline in muscle function (6, 24). We, therefore, tested whether knockdown of *tdo-2* would suppress the decline in motility during aging of control animals as well. Indeed,

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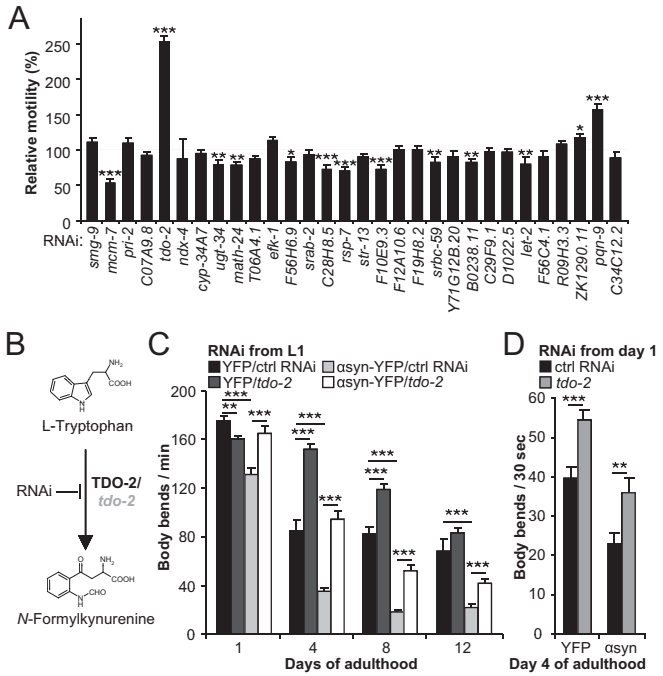

Fig. 1. Depletion of *tdo-2* suppresses age-related proteotoxicity in *C. elegans*. (*A*) Relative motility of α-synuclein animals grown on the indicated RNAi foods compared with α-synuclein animals on control RNAi. We show 30 genes that were selected after a first round of screening and retested for two more rounds in multiple experiments. Bars represent the combined average of three independent experiments per RNAi food. (*B*) TDO-2 catalyzes the first and rate-limiting step of tryptophan degradation. (*C*) Number of body bends per minute in animals expressing α-synuclein–YFP or YFP only grown on control or *tdo-2* RNAi from L1 measured over time. The graph shows one experiment, which is representative for three independent experiments ($n = 15$). (*D*) Number of body bends per 30 s in α-synuclein animals grown on control or *tdo-2* RNAi starting from day 1 of adulthood. The graph shows one experiment, which is representative for three independent experiments ($n = 15$). Unless indicated otherwise, motility was measured on day 4 of adulthood. In all panels, error bars represent the SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

from day 4 on, knockdown of *tdo-2* suppressed the decline in motility in control animals expressing YFP (Fig. 1*C*).

To exclude that knockdown of *tdo*-2 regulates α-synuclein toxicity by interfering with a developmental program, we fed worms on *tdo-2* RNAi starting from the last larval stage (L4) as well as day 1 of adulthood. In both cases, we observed that knockdown of *tdo-2* increased motility of animals expressing α-synuclein by 1.7- and 1.6-fold on day 4 of adulthood compared with an increase of 1.2- and 1.4-fold in motility of animals expressing YFP only (Fig. 1*D*, Fig. S1*E*, and Table S1).

Together, our data suggest that depletion of *tdo-2* suppresses α-synuclein toxicity and the age-related decline of motility in general and that this suppression is independent of its role in development.

Effect Upstream of *tdo-2* Regulates Toxicity of α-Synuclein. To explore whether modulation of α-synuclein toxicity by *tdo-2* depends on metabolites downstream of *tdo-2*, we inactivated enzymes downstream in the pathway by RNAi and by crossing α-synuclein worms with mutant strains (Fig. 2*A*). We performed liquid chromatography–tandem MS (LC-MS/MS) to measure levels of kynurenines (25).

Deletions of *kmo-1* encoding kynurenine 3-monooxygenase, *flu-2* encoding kynureninase, and *haao-1* encoding 3-hydroxyanthranilic acid oxygenase, blocked the kynurenine pathway, which was measured by increases in kynurenine, anthranilic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid (Fig. 2*E* and Table S2). Deletions in *kmo-1* and *haao-1* did not affect the motility of α-synuclein worms (Fig. 2*B* and Table S1), nor did a deletion in *afmd-1* encoding formamidase (Fig. S2*A*). Deletion or RNAi inactivation of *flu-2* increased the motility of α-synuclein animals by 1.3-fold (Fig. 2 *C* and *D* and Table S1). When we then depleted *tdo-2* in *afmd-1*, *kmo-1*, and *haao-1* mutant animals, we observed a strong increase in motility comparable with the increase observed in WT α-synuclein animals on *tdo-2* RNAi (Fig. 2*B*, Fig. S2*A*, and Table S1). Similarly, depletion of *tdo-2* increased motility in *flu-2* mutant animals from 1.3- up to 1.8-fold (Fig. 2*C* and Table S1). Knockdown of *tdo-2* almost fully blocked the kynurenine pathway in all mutants, increasing the tryptophan levels by more than fivefold (Fig. 2*E* and Table S2). This finding showed that, in WT animals, at least 80% of tryptophan was degraded by TDO-2. Knockdown of *tdo-2* reduced the levels of all other metabolites close to background levels (Fig. 2*E* and Table S2). Kynurenic acid (KA) was detected only in *kmo-1* and *flu-2* mutant animals, and its level was unaltered by knockdown of *tdo-2* (Fig. 2*F*). Moreover, supplementing the worms' feed with kynurenine did not affect α-synuclein toxicity, and when we depleted *tdo*-2 in animals grown on kynurenine-supplemented plates, we still observed the strong suppression of α-synuclein toxicity (Fig. S2*B* and Table S1). Together, these data show that variations in the levels of kynurenine, anthranilic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, or KA could not account for the changes in α-synuclein toxicity.

Suppression of α-Synuclein Toxicity by *tdo-2* Can Be Independent of Serotonin Synthesis. The only other route known to use tryptophan as a substrate is the pathway synthesizing the neurotransmitter serotonin. To test whether serotonin is required for suppressing α-synuclein toxicity after *tdo-2* depletion, we deleted *tph-1* encoding tryptophan hydroxylase, which is necessary for serotonin synthesis (Fig. 2*A*), in α-synuclein animals. Previous studies reported that no detectable levels of serotonin were measured in *tph-1* mutant animals (26). Although deletion of *tph-1* resulted in an ∼60% decrease in motility (Fig. 2*G* and Table S1), which could be explained by the fact that *tph-1* mutants have been shown to be severely impaired in initiation and maintenance of swimming (27), depletion of *tdo-2* still resulted in an ∼2.6-fold increase in motility, similar to the relative increase that we observed in control animals (∼2.5-fold increase). However, suppression of toxicity by *tdo-2* depletion was more variable in the *tph-1* mutants than WT animals (Table S1). Together, these results indicated that, although serotonin could, in part, be responsible, regulation of proteotoxicity by *tdo-2* can occur independently of its presence.

Increasing Tryptophan Concentrations Inhibits Toxicity. L-tryptophan is an essential amino acid that can only be taken up through the diet in both humans and *C. elegans* (28). To test whether the accumulation of tryptophan was involved in regulating proteotoxicity, we supplemented the nematodes' feed with increasing amounts of L-tryptophan. We observed a dose-dependent suppression of α-synuclein toxicity (Fig. 2*H*, Fig. S2*C*, and Table S1). Supplementation with similar amounts of the essential amino acid L-threonine did not affect α-synuclein toxicity (Fig. S2*C*), whereas we measured similar fold increases in their abundance by LC-MS/MS (Fig. S2 *D* and *E*). These results suggest that inhibition of TDO-2 regulates proteotoxicity by increasing the levels of tryptophan.

TDO-2 Is a General Regulator of Proteotoxicity. To test whether TDO-2 had a general role in the regulation of proteotoxicity, we analyzed the effects of *tdo-2* depletion on toxicity of other aggregation-prone disease proteins, including β-amyloid peptide

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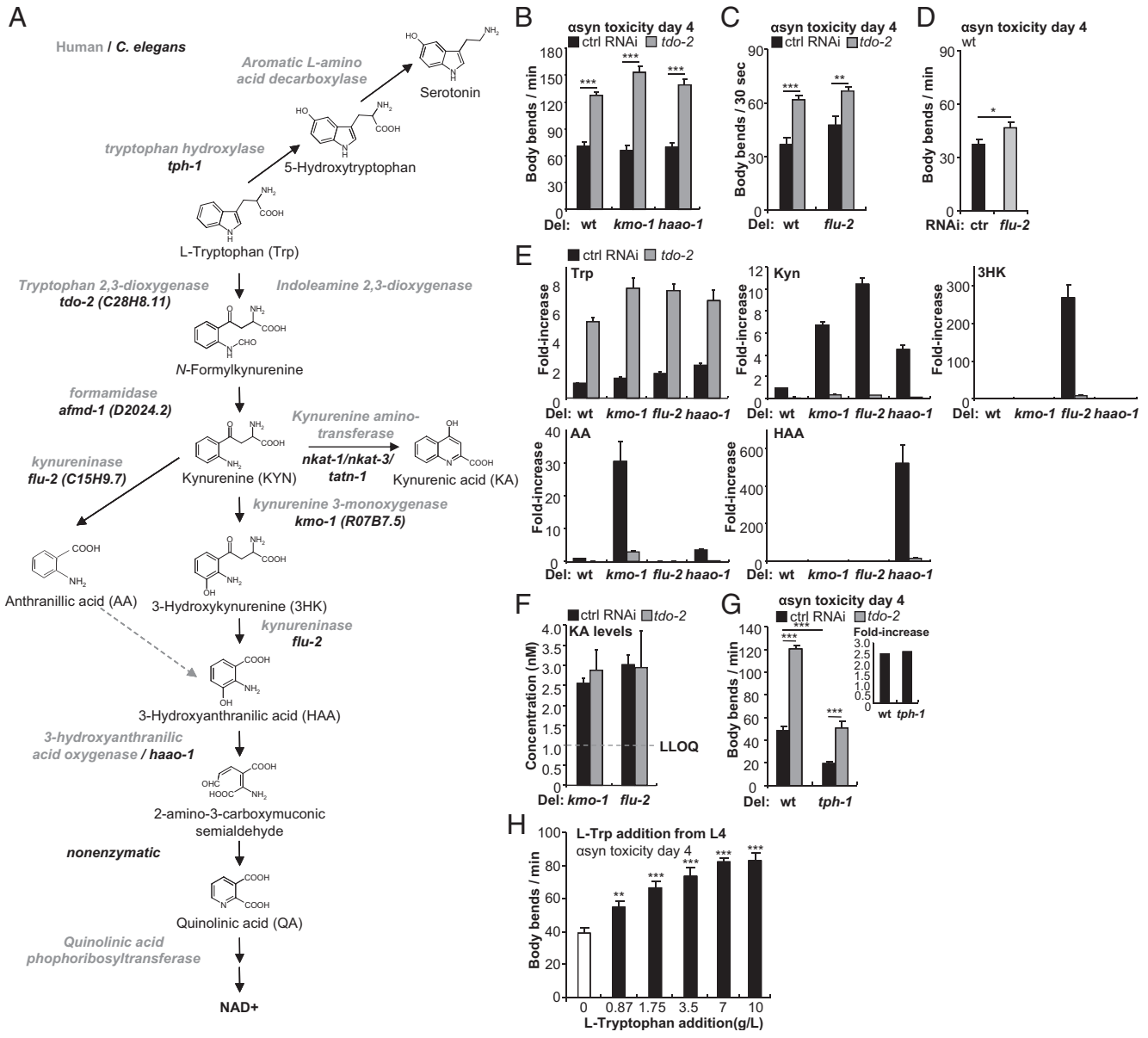

Fig. 2. Increased levels of tryptophan regulate toxicity of α-synuclein. (*A*) Schematic representation of the kynurenine pathway of tryptophan degradation and serotonin synthesis in human and *C. elegans*. Human genes are shown in gray, with the ortholog *C. elegans* genes in black. (*B*) Number of body bends per minute of α-synuclein animals carrying mutations in *kmo-1* or *haao-1* on control or *tdo-2* RNAi from L1. The graph shows one experiment, which is representative for two independent experiments ($n = 15$). (*C*) Number of body bends per 30 s of α-synuclein animals with a deletion in *flu-2* on control or *tdo-2* RNAi from L1. The graph shows one experiment, which is representative for three independent experiments ($n = 15$). (*D*) Number of body bends per minute of α-synuclein animals on control or *flu-2* RNAi from L1. The graph shows one experiment, which is representative for three independent experiments ($n = 15$). (*E*) Quantification of the indicated metabolites in α-synuclein animals carrying the indicated mutations on control or *tdo-2* RNAi by LC-MS/MS. Bars show the average fold increase relative to α-synuclein animals on control RNAi and represent the average of three independent experiments. (*F*) Quantification of KA levels in *kmo-1* and *flu-2* mutant animals. KA levels were determined in a separate experiment, but the same samples were used as in *E*. Bars represent the average of three independent experiments. LLOQ, lower limit of quantification. (*G*) Number of body bends per minute in α-synuclein and α-synuclein; *tph-1(del)* animals on control or *tdo-2* RNAi. The graph shows one experiment, which is representative of three independent experiments ($n = 15$). (*H*) Number of body bends per minute in α-synuclein animals grown on plates containing different amounts of L-tryptophan starting from L4. The graph shows one experiment, which is representative of three independent experiments ($n = 15$). Unless indicated otherwise, motility was measured on day 4 of adulthood. In all panels, error bars represent the SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and polyglutamine (Q40/128Q) in muscle cells and mechanosensory neurons, and on functions of endogenous metastable proteins, which are frequently used as indicators of general protein homeostasis (6, 12, 17, 29–31). Depletion of *tdo-2* resulted in an ∼30% decrease in the number of paralyzed Aβ-worms over a 14-d period (Fig. 3*A*). Similarly, depletion of *tdo-2* increased the motility of Q40 animals by 1.5- and 2.1-fold in 8- and 12-d-old animals, respectively (Fig. 3*B* and Table S1), without affecting their expression levels (Fig. S3 *A* and *B*).

Next, we tested whether *tdo-2* could also regulate the misfolding of metastable proteins in strains carrying mutations in *unc-52* (*e669su250*) and *unc-54* (*e1157*), encoding perlecan and a myosin heavy chain protein, which result in the temperature-sensitive misfolding of these muscle-specific proteins. When we moved

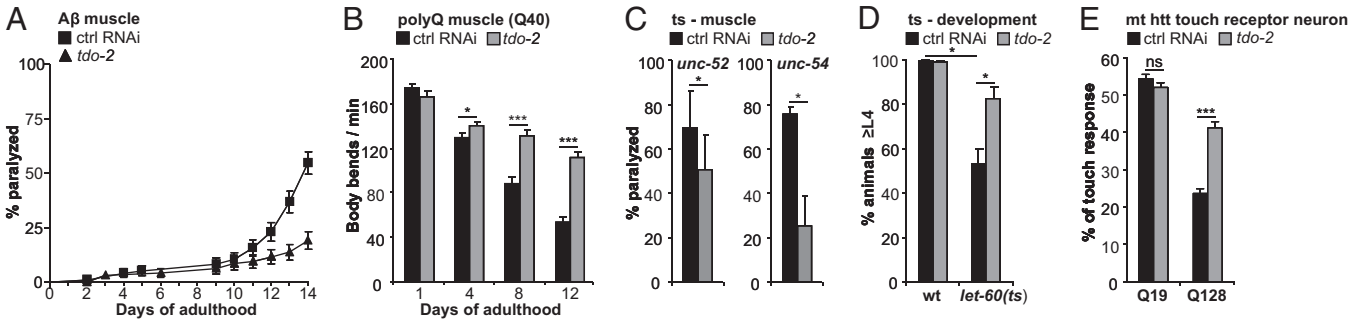

Fig. 3. TDO-2 is a general regulator of proteotoxicity. (*A*) The percentage of paralyzed Aβ-animals on control or *tdo-2* RNAi starting from L4 measured for 14 d. One of four independent experiments is shown; $n = 100$ for each independent experiment. (*B*) Number of body bends per minute in animals expressing Q40 in the muscle on control or *tdo-2* RNAi starting from L4 measured over time. The graph shows one experiment, which is representative of three independent experiments ($n = 15$). (*C*) The percentage of paralyzed *unc-52(e669su250)* and *unc-54(e1157)* animals on control or *tdo-2* RNAi from the embryonic stage on after ∼42 h at the restrictive temperature. Bars represent the average of three independent experiments, and $n = 100$ for each independent experiment. (*D*) The percentage of *let-60(ga89)* and WT animals that reached the L4 stage or older 48 h after egg-laying by animals grown on control or *tdo-2* RNAi from L1 stage on. Bars represent the average of three independent experiments. (*E*) Touch response assays on animals expressing mt htt in the touch receptor neurons grown on control or *tdo-2* RNAi. Three independent experiments were performed using a total of more than 150 individuals per strain and treatment. In all panels, error bars represent the SEM. $*P < 0.05$, $***P < 0.001$.

these strains to a restrictive temperature (25 °C) for ∼42 h, resulting in the temperature-sensitive misfolding of these muscle-specific proteins, ∼70% of the control animals became paralyzed. Depletion of *tdo-2* reduced this paralysis phenotype by ∼20% in *unc-52* animals and ∼50% in *unc-54* animals (Fig. 3*C*). We then tested whether *tdo-2* could suppress a temperature-sensitive phenotype independent of motility. Animals carrying a mutation in *let-60* (*ga89*), the *C. elegans* homolog of ras-1, present a developmental defect at the restrictive temperature. Depletion of *tdo-2* resulted in a rescue of this developmental defect in the offspring of *let-60* animals grown at the restrictive temperature (25 °C), which was measured by the number of hatched animals reaching L4 after ∼48 h (Fig. 3*D*).

Finally, we tested whether suppression of proteotoxicity by *tdo-2* depletion could be extended to other cell types. To do so, we knocked down *tdo-2* in RNAi-sensitive animals expressing the first exon of mutant huntingtin with an expanded polyQ tract (128Q) in touch-receptor neurons (Fig. S3*C*). Touch response is greatly impaired in 128Q animals but only moderately impaired in 19Q control animals (32, 33). Depletion of *tdo-2* reduced neuron dysfunction in 128Q animals, whereas it did not affect the touch response in 19Q animals (Fig. 3*E* and Fig. S3 *D* and *E*).

These data indicate that TDO-2 functions as a general regulator of proteotoxicity.

TDO-2 Regulates Lifespan. Because many of the known regulators of proteotoxicity play a role in regulating lifespan (7, 10, 12, 34, 35), we investigated whether down-regulation of *tdo-2* would also affect lifespan. Expression of α-synuclein in the body wall muscles cells did not affect the animals' lifespan (Fig. 4*A* and Table S3). Knockdown of *tdo-2* in both WT animals and animals expressing α-synuclein resulted in an extension of both the median and mean lifespan of ∼15% (Fig. 4*A* and Table S3). Related to this finding, knockdown of *tdo-2* in WT animals affected reproductive lifespan as well by causing a delay and an extension in the production of progeny without affecting the total amount of progeny (Fig. S4*A*). Furthermore, knockdown of *tdo-2* in animals without germ lines could still extend longevity (Fig. S4*B* and Table S3), showing that *tdo-2* does not exert its effects through the germ line.

Moreover, depletion of *tdo-2* could extend both mean and median lifespan even more in long-lived, serotonin-deficient *tph-1* mutant animals (Fig. 4*B* and Table S3), indicating that lifespan extension by *tdo-2* does not depend on serotonin.

Together, these data show that *tdo-2* regulates both the chronological lifespan as well as the reproductive span of *C. elegans*.

Next, we determined whether lifespan extension by depletion of *tdo-2* depended on the IIS, dietary restriction, or hypoxia pathways, which had previously been implicated in protein homeostasis (7, 10–12, 36). To that end, we depleted *tdo-2* in strains with inactivating mutations in *hsf-1* and *daf-16*, which act in the IIS pathway, and *hif-1*, which acts in the hypoxia stress response pathway. Depletion of *tdo-2* in *daf-16* deletion mutants resulted in a small extension of the median lifespan (Fig. 4*C* and Table S3) and affected the mean lifespan in one of three independent experiments (Table S3). It was noteworthy that knockdown of *tdo-2* in a DAF-16::GFP reporter strain prevented a decline in DAF-16::GFP levels in old worms, supporting a role for DAF-16 in regulating lifespan by *tdo-2* (Fig. S4 *C*–*E*). Depletion of *tdo-2* in *hif-1* mutant animals resulted in an extension of median and mean lifespan as well but not to the same extent as for WT animals. This finding suggests that lifespan extension by *tdo-2* depletion may, in part, depend on *hif-1* (Fig. 4*E* and Table S3). In contrast, depletion of *tdo-2* resulted in an extension of both median and mean lifespan in *hsf-1* loss-of-function mutant animals, which carry a truncating mutation in the transactivation domain preventing the heat induction of HSF-1 target genes (37). This increase was similar to the increase seen in WT animals (Fig. 4*D* and Table S3). Similarly, when we depleted *tdo-2* in long-lived *eat-2* mutants, which are often used to mimic dietary restriction, this depletion resulted in an even larger increase in both median and mean lifespan (Fig. 4*F* and Table S3). Moreover, we did not observe any change in pharyngeal pumping behavior (Fig. S4*F*). This finding indicates that *tdo-2* does not act through dietary restriction, although we cannot exclude that other forms of dietary restriction play a role.

Interestingly, the regulation of proteotoxicity was independent of *hsf-1*, *eat-2*, and *daf-16* (Fig. S5). This finding showed that the regulation of lifespan by *tdo-2* is either downstream or independent of its role in regulating proteotoxicity.

Together, our data suggest that *daf-16* is involved in lifespan regulation by *tdo-2* but that *tdo-2* acts in parallel to pathways regulating dietary restriction and hypoxia.

Discussion

Our data suggest that *tdo-2* plays a role in regulating general protein homeostasis during aging by increasing tryptophan levels. Previously, inhibition of the kynurenine pathway has been shown to suppress toxicity of neurodegenerative disease proteins by

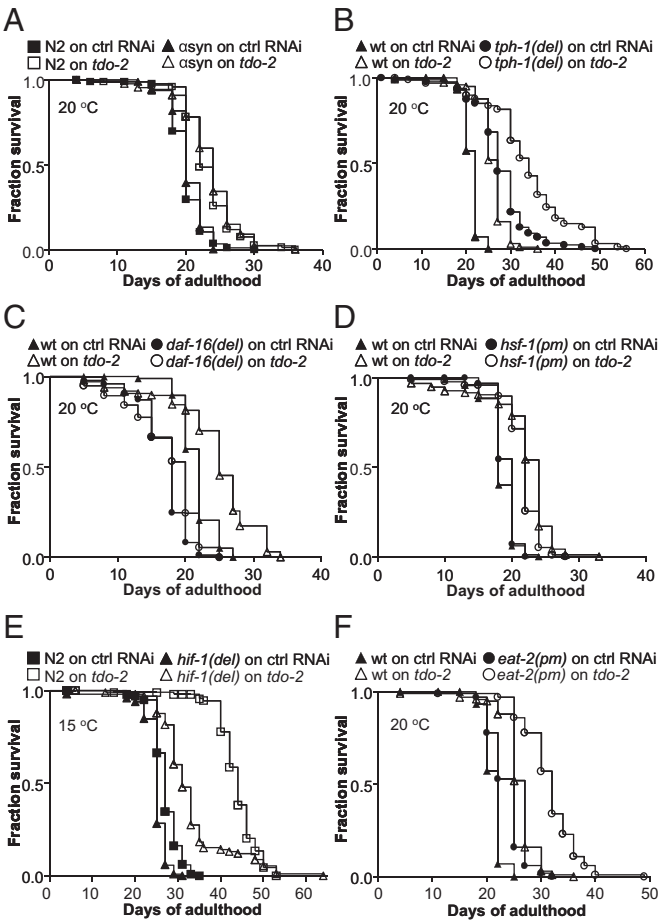

Fig. 4. Depletion of TDO-2 enhances longevity. (*A*) Survival curves of N2 and α-synuclein animals on control or *tdo-2* RNAi. (*B*) Survival curves of α-synuclein and α-synuclein;*tph-1(mg280)* animals on control or *tdo-2* RNAi. (*C*) Survival curve of α-synuclein and α-synuclein;*daf-16(mu86)* animals on control or *tdo-2* RNAi. (*D*) Survival curve of α-synuclein and α-synuclein;*hsf-1(sy441)* animals on control or *tdo-2* RNAi. (E) Survival curve of N2 animals and *hif-1(ia4)* animals on control or *tdo-2* RNAi. (*F*) Survival curve of α-synuclein and α-synuclein;*eat-2 (ad1116)* animals on control or *tdo-2* RNAi. Results in *B* and *F* represent one experiment. In all panels, one representative survival curve is shown. All of the survival experiments were performed three times (Table S3).

changing the relative abundance of the downstream metabolites 3-hydroxykynurenine, which is neurotoxic, and kynurenic acid, which is neuroprotective (38–40). Our data suggest yet another role for the kynurenine pathway in neurodegeneration, namely as a regulator of age-related protein toxicity. We show that this role is independent of downstream metabolites. The fact that the protective effect of *tdo-2* inhibition is upstream of the kynurenine pathway is supported by several of our findings. First, deletion of individual enzymes downstream of *tdo-2* in the kynurenine pathway never resulted in a similar suppression of α-synuclein toxicity as depletion of *tdo-2*. Second, in strains in which downstream enzymes were deleted, depletion of *tdo-2* always suppressed proteotoxicity to a similar extent as *tdo-2* depletion in WT animals, independent of relative changes in the metabolite levels. Finally, when we fed worms with increasing amounts of tryptophan, it also suppressed α-synuclein toxicity.

Although our results suggest that *tdo-2* regulates toxicity through tryptophan, tryptophan does not seem to act directly on α-synuclein aggregation (Fig. S2*F*). One possibility is that tryptophan or tryptophan derivatives play a role by acting on other signaling molecules that influence proteotoxicity.

In mammals, TDO or its functional homolog indoleamine 2,3-dioxygenase can be induced by various internal and external stimuli, such as hormones (41), stress (42, 43), and immune activation (44), leading to the breakdown of tryptophan. Together with the recent finding that inhibition of TDO reverses tumoral immune resistance as well (45), our findings implicate TDO-2 as a general metabolic regulator of age-related pathologies and lifespan.

Because *tdo-2* expression naturally increases during aging (46) and in experimental models expressing α-synuclein (47, 48), this process may contribute to the age-dependent decline in protein homeostasis. Our results suggest that inhibiting TDO may delay this age-dependent process. Moreover, they provide insight into the role of tryptophan metabolism in the biology of animal aging. Because TDO and indoleamine 2,3- dioxygenase are evolutionary highly conserved orthologs of TDO-2, tryptophan metabolism may offer targets for therapeutic intervention in the early toxic molecular events of aging-associated neurodegenerative diseases.

Methods

Media and Strains. Standard conditions were used for *C. elegans* propagation at 20 °C (49). Information on strains, RNAi experiments, RT-PCRs, immunoblotting, DAF-16::GFP localization, motility, paralysis, temperature sensitivity, touch response, lifespan, fecundity, and pharyngeal pumping assays can be found in *SI Methods*.

LC-MS/MS. Samples were prepared as described in *SI Methods*. Tryptophan and the kynurenine pathway metabolites were measured by LC–electrospray ionization–MS/MS as described previously (25) and in detail in *SI Methods*.

Supplementation with L-Tryptophan, L-Threonine, or L-Kynurenine. Different amounts of L-tryptophan (T-0254; Sigma) and L-threonine (T-8625; Sigma) were added to NGM medium before autoclaving. Different amounts of L-kynurenine (K8625; Sigma) were dissolved in H_2O and added to the Nematode Growth Medium (NGM) after autoclaving. Animals were grown on the supplemented plates either from L1 or L4 as indicated. Motility was measured on day 4 of adulthood. Threonine levels were measured by HPLC-MS/MS and are described in more detail in *SI Methods*.

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