Usnic Acid extends healthspan and improves the neurodegeneration diseases via mTOR/PHA-4 signaling pathway in *Caenorhabditis elegans*
Usnic Acid extends healthspan and improves the neurodegeneration diseases via mTOR/PHA-4 signaling pathway in Caenorhabditis elegans

Yi Xiao,1,2,4 Huiling Zhang,1,2,4 Yi Sheng,1,2,4 Fang Liu,1,3 Jiajun Gao,1 Guosheng Liu,1,3 Sanhua Li,1,2 Nian Jiang,1,2 Changyan Yu,1,2 and Yun Liu1,2,3,5,*

SUMMARY
The Mammalian/mechanistic target of rapamycin (mTOR) played a central role in cellular survival and aging. Inhibition of mTOR had been proposed as a reasonable strategy to promote lifespan and delay age-related diseases in evolutionarily diverse organisms. The study showed that lifespan extension and age-related diseases improvement could be achieved by targeting evolutionarily conserved mTOR pathways and mechanisms using pharmacological interventions. Using this approach in Caenorhabditis elegans, We found that 2 μM Usnic Acid significantly extended the healthy lifespan in wild-type animals. Furthermore, via genetic screen, we showed that Usnic Acid acted on mTOR, which was followed by the activation of PHA-4/Foxa to extend the healthy lifespan. Intriguingly, Usnic Acid also delayed neurodegeneration diseases such as Alzheimer’s and polyglutamine disease through mTOR-dependent manner. Our work suggested that Usnic Acid might be a viable candidate for the prevention and treatment of aging and age-related diseases.

INTRODUCTION
Aging had been defined as a decrease in physiological and psychological function over time, accompanied by many age-related diseases, including Alzheimer’s, Parkinson’s, or Huntington disease. With the development of global population aging, the identification of a chemical or pharmacological drug that could promote a human healthy lifespan and decrease the risks of age-related diseases had become a key goal of anti-aging research. Traditional herbal medicines, including diverse natural biologically active compounds, had therapeutic efficacy with minimal adverse effects, providing sources for developing first-line anti-aging drugs.1,2 Usnic Acid was a dibenzofuran compound originating from a traditional medicine Usnea dillata Vain.3,4 Previous studies had shown that Usnic Acid was a potentially interesting candidate for such activities as anti-inflammatory, analgesic, healing, antioxidant, antimicrobial, antiprotozoal, antiviral, larvicidal and UV protection.5,6 However, whether Usnic Acid prolonged the life of the animal and the molecular mechanisms by which it extended healthspan and delayed aging-related diseases had never been examined.

C. elegans had been developed as a valuable genetic model for research on aging and aging-related diseases. Through using this tractable model, researchers revealed several signaling pathways that had important roles in promoting lifespan, such as the DAF-2/DAF-16 pathway,7 the Mammalian/mechanistic target of rapamycin mTOR pathway,8 the SKN-1/Nrf7, the mitochondrial respiratory chain ISP-1,9 the germline GLP-111 and the dietary restriction EAT-2.12

The mammalian target of rapamycin (mTOR), a key component of the molecular nutrient sensor pathway that played important homeostatic functions to regulate energy metabolism, cellular survival, aging and neurodegenerations.13,14 mTOR constituted two structurally and functionally distinct complexes, the mTOR complex 1 (mTORC1) and the mTOR complex 2 (mTORC2).13 Previous studies had shown that decreasing mTORC1 activity, for instance via caloric restriction,15 autophagy14 and through the dietary administration of rapamycin, increased lifespan in model organisms, including yeast, C. elegans, D. melanogaster, and mice.16,17 Furthermore, mTOR acted upstream of PHA-4/FOXa that was essential for lifespan-extending.18 In addition to the above physiologic roles, the mTORC1 signaling pathway also influenced the development of neurodegenerative diseases such as Alzheimer’s, Parkinson’s or Huntington.
disease through regulating protein synthesis and preventing the buildup of misfolded protein aggregates.\textsuperscript{14}

Here, we investigated the role of Usnic Acid in \textit{C. elegans} healthspan and age-related diseases such as Alzheimer’s and polyglutamine disease. We found that Usnic Acid extended healthspan and improved Alzheimer’s and polyglutamine disease by governing mTOR-dependent manner. The evolutionary conservation of the mTOR signaling pathway suggested that the Usnic Acid-prevention and treatment of aging and age-related diseases might be universal.

\section*{RESULTS

\textbf{Usnic acid extended the \textit{C. elegans} lifespan and healthspan}}

We tested the effects on the worm lifespan of Usnic Acid. Usnic Acid at 1, 2, and 4 \textmu{}M increased mean lifespan by 14\%, 22\%, and 10\% (Figure 1A; Table S1). These results suggested that Usnic Acid exhibited a saturating effect on longevity, maximal at 2 \textmu{}M drug, and declining at 4 \textmu{}M drug (Figure 1A; Table S1). As they age, \textit{C. elegans} exhibited muscle deterioration and locomotion rate decline, used as a marker for health and rate of aging in \textit{C. elegans}.\textsuperscript{19–21} 2 \textmu{}M Usnic Acid treatment increased the locomotory ability (determined by the average bends of the worm body per 60 s) and decreased the content of by 50\% age pigments in \textit{C. elegans} (Figures 1B and 1C). However, we did not observe any reduction in the fertility of nematodes treated with or without 2 \textmu{}M Usnic Acid (Figure 1D). Taken together, these results indicated that Usnic Acid not only extended lifespan, but also had pronounced health-beneficial effects for the \textit{C. elegans}.
Usnic acid promoted lifespan through the mTOR signaling pathway

To investigate the molecular mechanisms by which the Usnic Acid extended lifespan, we screened several signaling pathways, which controlled lifespan in *C. elegans*, such as EAT-2, DAF-2, SKN-1/Nrf, ISP-1, and LET-363/mTOR. We found that 2 μM Usnic Acid failed to increase the lifespan in *let-363* RNAi worms, compared to empty vector (EV) worms (Figure 2A and Table S1). However, 2 μM Usnic Acid extended lifespan in *eat-2(ad1116)*, *daf-2(e1370)*, *skn-1(zu67)*, and *isp-1(qm150)* mutants (Figures 2B–2F and Table S1). Taken together, these results suggested that Usnic Acid acted on the mTOR signaling pathway to promote lifespan in *C. elegans*.

Usnic acid extended healthspan through inhibition the mTOR signaling pathway

Next, we tested the core components of the mTOR signaling, the mTOR effector kinases (S6 kinase RSKS-1), which acted downstream of mTOR. We found that 2 μM Usnic Acid could not increase the lifespan in *rsks-1(ok1255)* (Figure 3A and Table S1). Similar results also observed in *rsks-1* RNAi worms (Figure 3B and Table S1). As they age, *C. elegans* exhibited muscle deterioration and locomotion rate decline, used as a marker for health and rate of aging in *C. elegans*. 2 μM Usnic Acid treatment failed to decrease age pigments and increase the locomotory ability (determined by the average bends of the worm body per 60 s) in the *let-363* and *rsks-1* RNAi worms (Figures 3C and 3D), indicating that Usnic Acid improved fitness depending on the mTOR signaling in *C. elegans*. Furthermore, our results found that 2 μM Usnic Acid treatment significantly decreased the levels of S6K phosphorylation, suggesting the inhibition of the TORC1 pathway. Notably, *let-363* knockdown also significantly reduced the levels of S6K phosphorylation. However, 2 μM Usnic Acid treatment did not further decrease the levels of S6K phosphorylation in *let-363* RNAi worms (Figure 3E). Taken together, these results suggested that Usnic Acid promoted healthspan through the inhibition of the TORC1 pathway in *C. elegans*.

Usnic acid extended healthspan through the mTOR/PHA-4 signaling pathway

The FOXA transcription factor PHA-4 acted downstream of mTOR to increase the lifespan. Whether PHA-4 was downstream of the mTOR and extended the healthspan of *C. elegans* after Usnic Acid treatment remained unclear. We found that 2 μM Usnic Acid failed to extend the lifespan in *pha-4* RNAi, *pha-4(zu225);smg-1(cc546ts)* worms (Figures 4A and S1A; Table S1), indicating that Usnic Acid extended lifespan dependent on *pha-4*. RNAi of *pha-4* shortened the long lifespan of *rsks-1(ok1255)* animals and the
pha-4(zu225); smg-1(cc546ts) allele shortened the long lifespan of let-363 RNAi-treated animals. However, 2 μM Usnic Acid failed to promote the lifespan in rsks-1(ok1255); pha-4 RNAi and pha-4(zu225); smg-1(cc546ts); let-363 RNAi worms, compared with WT worms (Figures 4A and S1B; Table S1), indicating that Usnic Acid extended lifespan via mTOR/PHA-4 dependent manner. Furthermore, 2 μM Usnic Acid treatment did not decrease age pigments and increase the locomotory ability (determined by the average bends of the worm body per 60 s) in the pha-4 RNAi, rsks-1(ok1255) and rsks-1(ok1255); pha-4 RNAi worms (Figures 4B and S2), indicating that Usnic Acid improved fitness depending on the mTOR/PHA-4 signaling in C. elegans. Quantitative real-time PCR analysis demonstrated that the mRNA levels of pha-4 were up-regulated in Usnic Acid treatment animals compared with control (Figure 4C). However, Usnic Acid failed to enhance their expression in the rsks-1(ok1255) worms (Figure 4C). In addition, we detected the expression of PHA-4 through using the transgenic worms expressing PHA-4::GFP. We observed higher levels of GFP in Usnic Acid treatment animals, but not in the rsks-1(ok1255) worms (Figure 4D). In conclusion, these findings indicated that Usnic Acid extends healthspan through the mTOR/PHA-4 signaling pathway.

Usnic acid protected from polyglutamine aggregation via mTOR-dependent manner

As the aging, protein aggregates, such as proteins containing polyglutamine (polyQ) repeats would be accumulated. In C. elegans, the strains AM44 and AM140 were model of polyglutamine neurodegenerative diseases such as Huntington disease, in which polyQ proteins were expressed throughout the nematode nervous system. Worm motility was significantly decreased by the aggregation of polyQ expression in neurons, with a pathogenic threshold at a length of 35-40 glutamines. Next, we tested whether Usnic Acid could have beneficial effects in polyglutamine (polyQ) disease model. Notably, 2 μM Usnic Acid substantially improved motility and reduced toxicity of worms expressing PHA-4::GFP. We observed higher levels of GFP in Usnic Acid treatment animals, but not in the rsks-1(ok1255) worms (Figure 4D). In conclusion, these findings indicated that Usnic Acid extends healthspan through the mTOR/PHA-4 signaling pathway.

**Figure 3.** Usnic Acid extended healthspan through inhibition mTOR signaling pathway

(A) Survival of rsks-1(ok1255) treated with 2 μM UA and the untreated controls. (log-rank test). (B) Survival of rsks-1 RNAi treated with 2 μM UA and the untreated controls. (log-rank test). (A and B) See Table S1 for lifespan data. (C) Quantification of “aging pigments” by measuring lipofuscin fluorescence normalized to autofluorescence in 2 μM UA-treated empty vector (EV), let-363 RNAi and rsks-1 RNAi; versus the control (n ≥ 20). (unpaired t-test). (D) Locomotion as determined by the number of body bends per min in 2 μM UA-treated empty vector (EV), let-363 RNAi and rsks-1 RNAi; versus the control (n ≥ 20). (unpaired t-test). (E) Western blot analysis of the phosphorylated S6K levels when worms were exposed to 2 μM UA versus the control. (*p < 0.05, unpaired t-test). Error bars represent mean ± SEM of 3 independent biological replicates. NS, no significance.
the polyQ aggregate levels in let-363 RNAi and rsks-1 RNAi worms (Figure 5D). Taken together, these results suggested that Usnic Acid protected from polyglutamine aggregation through mTOR signaling.

Usnic acid extended the lifespan of the model for Alzheimer’s disease via mTOR-dependent manner

Additionally, in a set of C. elegans strains used as a model for Alzheimer’s disease,25,26 we found that 2 μM Usnic Acid had beneficial effects on the lifespan of the control strain CL802 (Figure 6A), the rapid paralysis strain CL4176 (Figure 6B), and the slow paralysis strain CL2006 (Figure 6C), which expressed human Aβ1-42 under control of a muscle-specific promoter and maintained at 15°C and studied the lifespan at 20°C. However, 2 μM Usnic Acid did not further prolong lifespan in let-363 RNAi and rsks-1 RNAi mutants (Figures 6D–6I). In conclusion, these findings indicated that Usnic Acid extended the lifespan of the model for Alzheimer’s disease through the mTOR signaling pathway.

DISCUSSION

Understanding the role played by common compounds that substantially affected the aging process would be critical for predicting and interpreting the outcome of introducing new interventions. Usnic acid had been incorporated for years in cosmetics, perfumery, and traditional medicines, which had shown different biological and physiological activities that might have a great relevance in pharmacology and clinics.27,28 However, whether Usnic acid influenced aging and aging-related diseases remained unknown. In this study, we had shown that Usnic acid extended the healthspan and delayed neurodegeneration diseases such as Alzheimer’s and polyglutamine disease. We revealed that 2 μM Usnic acid treatment increased the locomotory ability and decreased age pigments in C. elegans, which used as a marker for health and rate of aging in C. elegans. Taken together, these results indicated that Usnic acid not only extended lifespan but also had pronounced health-beneficial effects for the C. elegans. Furthermore, through the
genetic screen, we found that Usnic acid promoted healthspan dependent on mTOR/PHA-4 dependent manner.

Perhaps the other intriguing therapeutic value of Usnic Acid was its potential to reduce the risk and delay the onset of age-associated neurodegenerative disease. In this study, we expanded upon these findings to show that 2 μM Usnic acid was capable of delaying pathology in the worm model of polyglutamine disease and Alzheimer’s disease via mTOR signaling. The kinase mammalian target of rapamycin (mTOR) was a major modulator of autophagy, which was a cellular recycling process required to prevent the buildup of misfolded protein aggregates that contributed to the development of neurodegenerative diseases. This data suggested the therapeutic potential of Usnic Acid to manage certain age-related diseases, such as neurodegeneration. Based on the observations that Usnic Acid was capable of increasing lifespan in worms and considering its mechanism of action extensive evolutionary conservation, our work suggested that Usnic Acid might yield significant health benefits by delaying aging and preventing specific age-associated pathologies in mammals.

Limitations of the study

We had shown that Usnic Acid extended the healthspan and improved neurodegeneration diseases via the mTOR/PHA-4 signaling pathway in C. elegans. However, it remained to be determined whether Usnic Acid in mammals also influenced healthspan and improved neurodegeneration diseases.

STAR*METHODS

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Figure 5. Usnic Acid protected from polyglutamine aggregation via mTOR-dependent manner
(A–C) Mobility analysis of poly Q67 worms in 2 μM UA-treated empty vector (EV), let-363 RNAi, and rxs-1 RNAi on day 1 (A), day 3 (B), and day 5 (C). (*p < 0.05, unpaired t-test). Error bars represent mean ± SEM of 3 independent biological replicates.
(D) Western blot analysis of the poly Q67::GFP levels when worms were exposed to 2 μM UA and let-363 RNAi versus the control. (*p < 0.05, unpaired t-test). Error bars represent mean ± SEM of 3 independent biological replicates. NS, no significance.
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105539.

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AUTHOR CONTRIBUTIONS
Yun Liu and Yi Xiao conceptualized and designed the study, aided in acquiring and analyzing data, drafted, and critically revised the article. Yi Xiao, Huiling Zhang, Yi Sheng, Fang Liu, Jiajun Gao, Guosheng Liu,
Sanhua Li, Nian Jiang, Changyan Yu participated in experiments and the data analysis. Yi Xiao wrote the article. All authors read and approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit p-S6K antibody | CST    | Cat# 9205  |
| Rabbit anti-S6K antibody | CST    | Cat# 2708  |
| Rabbit anti-GFP antibody | CST    | Cat# 2956  |
| Rabbit anti-beta actin antibody | Abcam | Cat# ab227387 |
| **Bacterial and virus strains** |    |            |
| *E.coli* OP50 | Yun Nan University | N/A |
| **Chemicals, peptides, and recombinant proteins** |    |            |
| Usnic Acid | Sigma-Aldrich | CAS:7562-61-0 |
| TRIzol Reagent | Invitrogen | Cat#15596026 |
| IPTG | Sigma-Aldrich | CAS:367-91-1 |
| **Experimental models. Organisms/strains** |    |            |
| DA1116 eat-2(ad1116) | Caenorhabditis Genetics Center | WB Strain: DA1116 |
| CB1370 daf-2(e1370) | Caenorhabditis Genetics Center | WB Strain: CB1370 |
| EU1 skn-1(p627)IV/nT(n754) let-7[nIV;IV] | Caenorhabditis Genetics Center | WB Strain: EU1 |
| MQ887 isp-1(qm150) | Caenorhabditis Genetics Center | WB Strain: MQ887 |
| RB1206 nks-1(ok1255) | Caenorhabditis Genetics Center | WB Strain: RB1206 |
| OP37 PHA-4::GFP | Caenorhabditis Genetics Center | WB Strain: OP37 |
| PD8120 smg-1(cc546) | Caenorhabditis Genetics Center | WB Strain: PD8120 |
| SM190 smg-1(cc546);pha-4[p225] | Caenorhabditis Genetics Center | WB Strain: SM190 |
| AM44 rmIs190[F2S83.3p::Q67::CFP] | Caenorhabditis Genetics Center | WB Strain: AM44 |
| AM140 rmIs132 [unc-54p::Q35::YFP] | Caenorhabditis Genetics Center | WB Strain: AM140 |
| CL802 smg-1(cc546) I rol-6(su1006) II | Caenorhabditis Genetics Center | WB Strain: CL802 |
| CL4176 smg-1(cc546) I dvlIs27 [myo-3p::A-Beta(1–42);let-851 3′UTR] + rol-6 (su1006) X | Caenorhabditis Genetics Center | WB Strain: CL4176 |
| CL2006 dvlIs2 [pCL12(unc-54/human Abeta peptide 1–42 minigene) + pRF4] | Caenorhabditis Genetics Center | WB Strain: CL2006 |
| **Software and algorithms** |    |            |
| GraphPad Prism | GraphPad | https://www.graphpad.com/scientific-software/prism/ RRID: SCR_002798 |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |

RESOURCE AVAILABILITY

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yun Liu (liuyunzmu@126.com).

**Materials availability**
All data generated or analyzed in this study are included in this published article.

**Data and code availability**
Data reported in this paper will be shared by the lead contact upon request.
This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experiments were performed in compliance with the guidelines of Guizhou Provincial College-based Key Lab for Tumor Prevention and Treatment with Distinctive Medicines of Zunyi Medical University.

Nematode strains and maintenance

Worms were maintained and propagated under standard conditions.30,31 The following nematodes strains were provided from the Caenorhabditis Genetics Center (CGC at the University of Minnesota, USA). N2 Bristol wild-type, DA1116 eat-2(ad1116), CB1370 daf-2(e1370), EU1 skn-1(zu67)/IV/nT1[unc-754] let-7/IV; V, MQ887 isp-1(qm150), RB1206 rsk-1(ok1255), OP37 PHA-4::GFP, PD8120 smg-1(cc546), SM190 smg-1(cc546);pha-4(zu225), AM44 rmls190 [F25B3.3p::Q67::CFP], AM140 rmls132 [unc-54p::Q35::YFP], CL802 smg-1(cc546); rol-6(su1006) II, CL4176 smg-1(cc546); dvl-27 [myo-3p::A-Beta(1–42);let-851 3’UTR] + rol-6 (su1006) X, and CL2006 dvl-2 [pCL12(unc-54/human Abeta peptide 1–42 minigene) + pRF4]. C. elegans mutants were backcrossed three times into the WT strain (N2) and used in the laboratory.

METHOD DETAILS

RNA Interference

The E. coli strains for RNAi were obtained from the Ahringer library.32 RNAi feeding experiments were performed on synchronized L1 to L2 larvae at 20°C. Briefly, dsRNA-expressing E. coli strain HT115(DE3) was cultured overnight in LB medium containing 100 μg/mL ampicillin at 37°C, and then spread to NKM plates containing 100 μg/mL ampicillin and 5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The RNAi-expressing bacteria were kept overnight at 25°C. Synchronized L1 to L2 larvae were placed in RNAi dishes until mature at 20°C. Unc-22 RNAi served as a positive control in all experiments to explain RNAi efficiency.

Fluorescence microscopy

Synchronized L1 worms of the PHA-4::GFP strain were transferred to agar plates supplemented with or without 2 μM Usnic Acid. The images were obtained using a Zeiss Axioskop 2 plus fluorescence microscope (Carl Zeiss, Jena, Germany) with a digital camera, the microscope magnification 40(objective) ×10(eyepiece). Fluorescence intensity was quantified by using the ImageJ software (NIH).29 Three plates of about 40 animals per plate were tested per assay and all experiments were conducted independently for 3 times.

Lifespan assays

All lifespan assays were performed at 20°C according to standard protocols.33 Briefly, synchronized L1 to L2 larvae were placed on NGM plates until they reached maturity at 20°C. After L4 around 120 nematodes were manually transferred to fresh incubation plates containing the Usnic Acid (0, 1, 2, 4 μM). Nematodes that did not respond to mild stimuli were marked as dead. Animals that climbed down from plates or showed unnatural deaths, especially due to internal hatching of animals were censored.34 Three plates were tested per assay and all experiments were performed three times independently.

Locomotion assays

Nematodes were synchronized and treated with or without 2 μM Usnic Acid starting at L4 larvae stage. On days 5 and 10 of life, 30 individuals from the control and experimental plates were measured for body bend rate in liquid. Briefly, worms were placed in 20 μL M9 buffer on a glass slide and filmed with a Zeiss Imager M2 microscope. Body bends were counted by reviewing each frame of the 60 s film.20

Age pigment fluorescence detection

Nematodes were synchronized and treated for 10 days with or without 2 μM Usnic Acid starting at L4 larvae stage. Worms were mounted onto an agarose pad attached to a glass slide and photographed with Zeiss Imager M2 microscope. Fluorescence intensity was measured by ImageJ.21
Fertility assay
Nematodes were synchronized and treated with or without 2 μM Usnic Acid starting at L4 larvae stage. Worms were transferred onto individual plates inoculated and subsequently onto fresh plates every day of the reproductive period. Eggs were counted manually.

Quantitative real-time PCR
Nematodes were synchronized and treated for 1 day with or without 2 μM Usnic Acid starting at L4 larvae stage. Total RNA was extracted from worms with TRIzol Reagent (Invitrogen). Total RNA samples were reversely transcribed by Super-Script II (Invitrogen) to generate random primer cDNA. QPCR analysis was performed using SYBR Premix-Ex TagTM (Takara, Dalian, China) on Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Using pmp-3 for an internal control. The following primers were used for this study:

**pmp-3 primers:**
- pmp-3-F: TGGATTGTCAATTGGCGTCG
- pmp-3-R: GTTGTCGCAGAGTGGTGTTT

**pha-4 primers:**
- pha-4-F: TCAAAGAGGAGCCAGAGTCG
- pha-4-R: ACACCTGAAGCAGCCGACGA.

Western blotting
Nematodes were synchronized and treated for 1 day with or without 2 μM Usnic Acid starting at L4 larvae stage. After the worms were homogenized in liquid nitrogen, they were ice cleaved in pyrolysis buffer (BioTeKe) for 60 min. The total protein were loaded (40 μg per well) and separated on a 10% SDS polyacrylamide gel. Then, proteins were transferred to immobilon-PSQ transfer PVDF membrane (Millipore, Bedford, MA). Primary antibodies against p-S6K (1:1,000 dilution, CST, 9205), S6K(1:1,000 dilution, CST, 2708), GFP (1:1,000 dilution, CST, 2956), and beta-actin (1:1,000 dilution; Abcam, ab227387) used. The secondary antibody was a peroxidase-coupled anti-rabbit IgG (1:20000 dilution; Abmart). Supersignal chemiluminescence substrate (Pierce) was used for imprinting. ImageJ software was used to measure band intensity.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data were presented as mean ± SEM. Statistical analyses for all data except for lifespan assays was carried out using Student’s t-test (unpaired, two-tailed) or ANOVA after testing for equal distribution of the data and equal variances within the dataset. Survival data were analyzed by using the log rank (Mantel-Cox) test. p < 0.05 was considered significant.