

20S proteasome activation promotes life span extension and resistance to proteotoxicity in *Caenorhabditis elegans*

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ABSTRACT Protein homeostasis (proteostasis) is one of the nodal points that need to be preserved to retain physiologic cellular/organismal balance. The ubiquitin-proteasome system (UPS) is responsible for the removal of both normal and damaged proteins, with the proteasome being the downstream effector. The proteasome is the major cellular protease with progressive impairment of function during aging and senescence. Despite the documented age-retarding properties of proteasome activation in various cellular models, simultaneous enhancement of the 20S core proteasome content, assembly, and function have never been reported in any multicellular organism. Consequently, the possible effects of the core proteasome modulation on organismal life span are elusive. In this study, we have achieved activation of the 20S proteasome at organismal level. We demonstrate enhancement of proteasome levels, assembly, and activity in the nematode *Caenorhabditis elegans*, resulting in life span extension and increased resistance to stress. We also provide evidence that the observed life span extension is dependent on the transcriptional activity of Dauer formation abnormal/Forkhead box class O (DAF-16/FOXO), skinhead-1 (SKN-1), and heat shock factor-1 (HSF-1) factors through regulation of downstream longevity genes. We further show that the reported beneficial effects are not ubiquitous but they are dependent on the genetic context. Finally, we provide evidence that proteasome core activation might be a potential strategy to minimize protein homeostasis deficiencies underlying aggregation-related diseases, such as Alzheimer's disease (AD) or Huntington's disease (HD). In summary, this is the first report demonstrating that 20S core proteasome up-regulation in terms of both content and activity is feasible in a multicellular eukaryotic organism and that in turn this modulation promotes extension of organismal health span and life span.—Chondrogianni, N., Georgila, K., Kourtis, N., Tavernarakis, N., Gonos, E. S. 20S

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AGING IS AN INEVITABLE natural biologic process that is defined as the time-dependent functional decline of organismal homeostasis accompanied, among others, by increased levels of damaged macromolecules (1, 2). Protein homeostasis (proteostasis) is a major node that needs to be preserved to retain organismal homeostasis (3). Recently, loss of proteostasis has been included among the hallmarks of aging (4) because chronic proteostasis imbalance exerts a direct negative impact on cellular and organismal life span (5). The various proteolytic networks are the ultimate arsenal ensuring intracellular proteostasis by eliminating damaged proteins.

The UPS is the primary site for controlled protein degradation. Specifically, normal but also abnormal, denatured, or otherwise damaged proteins are degraded by the proteasome. The 20S core proteasome is a cylinder composed of 7 different α - and 7 different β -subunits, arranged as a $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ structure. Three of the β -catalytic subunits (β_1 , β_2 , and β_5) are the enzymatic centers of the proteasome activities with chymotrypsin-like (CT-L) activity (exerted by β_5) being the major one. Attachment of 1 or 2 19S regulatory complexes at the end of the 20S core give rise to the 26S proteasome that degrades polyubiquitinated proteins in an ATP-driven process (6).

Progressive impairment of proteasome function during aging and cellular senescence is well documented (7–9), and proteasome activation has been proposed as a putative

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Abbreviations: AD, Alzheimer's disease; CT-L, chymotrypsin-like; DAF-16/FOXO, Dauer formation abnormal/Forkhead box class O; DR, dietary restriction; IIS, insulin/IGF-1 signaling; HD, Huntington's disease; HS, heat stress; HSF-1, heat shock factor-1; HSR, heat shock response; NRLB, nonreducing Laemmli buffer; *pbs-5* OE, *pbs-5* overexpression; RNAi, RNA interference; SKN-1, skinhead-1; UPS, ubiquitin-proteasome system; wt, wild type

antiaging strategy (10). So far, only enhancement of the 26S proteasome activity but not increase of the overall proteasome content has been achieved through the overexpression of components of the internal proteasome activator, namely the 19S complex (11, 12). However, it remains elusive whether enhancement of both proteasome content and activity/function is feasible in a multicellular organism and if such modulation has an effect on the organismal life span. We demonstrate, for the first time genetically mediated 20S core proteasome content up-regulation and activation in a multicellular organism, namely *Caenorhabditis elegans*, through the overexpression of the *pbs-5* 20S subunit (ortholog of the human β_5 subunit), and we provide evidence on the role of proteasome enhancement in the determination of organismal life span, stress resistance, and aggregation-related pathologies.

MATERIALS AND METHODS

Strains and genetics

We followed standard procedures for *C. elegans* strain maintenance at 20°C, unless otherwise indicated. The following strains were used: N2 [wild-type Bristol isolate (wt)], CB1370: *daf-2(e1370)III*, DR26: *daf-16(m26)I*, DR1309: *daf-16(m26)I;daf-2(e1370)III*, PS3551: *hsf-1(sy441)I*, CF1553: *muIs84[pAD76(sod-3::GFP)]*, CF1139: *daf-16(mu86)I;Is[p_{daf-16}DAF-16a::GFP]*, AM140: *rmls132[p_{unc-54}(Q35::YFP)]*, AM101: *rmls110 [pF25B3.3(Q40::YFP)]*, CL4176: *dvlS27 [pAF29(myo-3/A-Beta₁₋₄₂/letUTR)+pRF4(rol-6(su1006))]*. The following strains carrying extrachromosomal arrays were used: N2Ex[p_{let-858}PBS-5], *daf-2(e1370)Ex[p_{let-858}PBS-5]*, *daf-16(m26)Ex[p_{let-858}PBS-5]*, *daf-16(m26);daf-2(e1370)Ex[p_{let-858}PBS-5]*, *hsf-1(sy441)Ex[p_{let-858}PBS-5]*, *muIs84[pAD76(p_{sod-3}GFP)]Ex[p_{let-858}PBS-5]*, *daf-16(mu86)I;Is[p_{daf-16}DAF-16a::GFP] Ex[p_{let-858}PBS-5]* *Ex[p_{let-858}PBS-5]*, *rmls132[p_{unc-54}(Q35::YFP)]Ex[p_{let-858}PBS-5]*, *rmls110 [pF25B3.3(Q40::YFP)]Ex[p_{let-858}PBS-5]* and *dvlS27[pAF29(myo-3/A-Beta₁₋₄₂/letUTR)+pRF4(rol-6(su1006))]* *Ex[p_{let-858}PBS-5]*.

Molecular cloning

The *pbs-5* ORF plus 409 bp of 3'-UTR was PCR-amplified from genomic DNA using the 5'-ACCGGTATGTGGGGCGAGACATTGC-3' and 5'-GGGCCACGTCAACACCCAGCC-3' primers, carrying the *AgaI/ApaI* restriction sites and cloned into the L2865 vector. The construct was coinjected into the gonads of animals together with pRF4 plasmid carrying the *rol-6(su1006)* dominant transformation marker or a plasmid carrying the reporter gene *p_{unc-122}GFP*, which tags the nematode coelomocytes. Several transgenic lines were obtained and analyzed for *pbs-5* expression. Data are presented for wt line 1; *pbs-5* overexpression (*pbs-5* OE). Control worms were injected only with the coinjection marker.

Life span analysis

Synchronized young adult animals were transferred to fresh plates (100–200 individuals per experiment). D 1 of adulthood was set as $t = 0$. Animals were transferred to fresh plates every 2–3 d and examined every d for touch-provoked movement and pharyngeal pumping until death. Each survival assay was repeated at least twice. Representative assays are shown in figures; statistics refer to all performed assays. Survival curves were created using the product-limit method by Kaplan and Meier. The percentage of nematodes remaining alive is plotted against animal age. The

log-rank (Mantel-Cox) test was used to evaluate differences between survival curves and to determine P values for all independent data. N in life span figures is the number of animals that died over total where total equals the animals number that died plus the number of censored animals (due to internally hatched eggs, extruded gonad, or desiccation due to crawling off the plates). Median life span values are expressed as mean \pm SEM.

Oxidative stress resistance assays

Nematodes at L4 stage were exposed to 2 mM paraquat, and survival was scored on d 5 [for wt and *daf-16(m26)*] and on d 10 [for *daf-2(e1370)*] of exposure. Nematodes at d 1 of adulthood were exposed to freshly made 80 mM juglone for 45 min at 20°C and survival was scored after 24 h recovery.

Heat stress resistance assays

Synchronized animals were grown at 20°C until d 1 of adulthood. Worms were then transferred at 35°C for 8 h and scored for survival after 24 h recovery at 20°C. Nematodes were scored as dead if they failed to respond to heat or tactile stimuli.

RNA isolation and real-time PCR analysis

RNA was extracted from synchronized young adult animals and analyzed by standard methods. Primers are summarized in **Table 1**. Data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method and are presented as the x -fold difference in mRNA transcript abundance in transgenic or RNA interference (RNAi)-subjected animals, normalized to the endogenous *pmp-3* gene, relative to control animals. Use of *cdc-42* as normalizer produced similar results. Both genes were validated for quantitative gene expression studies using the GeNorm application, in agreement with previous studies (13).

RNA interference

The *pbs-5* RNAi construct is from the Ahringer library (14) (Source Bioscience LifeSciences, Nottingham, United Kingdom). Synchronized young adult animals (120 individuals per experiment) were transferred on NGM plates seeded with HT115(DE3) bacteria transformed with either the empty pL4440 vector or the RNAi-encoding plasmid. Isopropyl- β -D-1-thiogalactopyranoside (2 mM) induced the double-stranded RNA expression.

Immunoblot analysis and detection of oxidized proteins

Synchronized young adult animals (unless otherwise stated) were collected and boiled in nonreducing Laemmli buffer (NRLB) for 10 min. SDS-PAGE and immunoblotting was performed as described previously (8). Actin was used as a loading control (MP Biomedicals, Santa Ana, CA, USA; clone C4). Immunoblot detection of carbonyl groups into proteins (oxidized proteins) was performed with Oxyblot protein oxidation kit (Chemicon International, Temecula, CA, USA). Each immunoblot was repeated at least twice. Quantification of the ratio of each detected protein to actin and normalization to control appear under each representative immunoblot.

Dot blot Analysis

Equal numbers of synchronized animals were collected 24 h after temperature upshift from 16 to 25°C, washed, resuspended,

TABLE 1. Primers used for real-time PCR analysis

| Gene | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|-----------------|--------------------------|--------------------------|
| <i>pbs-1</i> | TGTGCCGTTCTGGATCTG | TGTAGAGGAATTGGCGGA |
| <i>pbs-2</i> | GCCCACATCTTTATATGTGCTC | TCTCGGCTTCATCTTTCCGT |
| <i>pbs-5</i> | CCACTTATCGGGATTCTGG | GGCAAACCTCGTACCACAG |
| <i>pas-2</i> | GGAATACGAAATGATGTACGG | CGAACACCACCAGATTGAG |
| <i>pas-5</i> | GCATTGGAATCAAGACGAG | GAGTCGGCAATCAAACCT |
| <i>rpn-6</i> | GATGGAGATATTAAGGTTGGAC | TCATTATAGAGGCGAACAAAGAC |
| <i>rpn-11</i> | TACTCCATCCCAATCGCC | TGCTCCTTGTCTGTTCCAC |
| <i>rpt-5</i> | GCAGTCATTAAGACTTCCACTC | ATCCTTATTGACACCAACGA |
| <i>rpt-6</i> | GGTTCCTGATTCAACATACGA | TCCAAAGAGCAAGACTCCT |
| <i>gst-4</i> | AAGTTGTTGAACCAGCCC | AATCACAATATCAGCCCCAAGTC |
| <i>sod-3</i> | GCAGATTTGGAACCTGTAAT | AGCCTCGTGAAGTTTCTC |
| <i>daf-16</i> | GCACAAGTTTACGAATGGA | CGAGAATGAAGAGACAGATTG |
| <i>skn-1</i> | GAGACGAGACGATAAACGA | CAGATGAATATGGACGCACACTC |
| <i>hsp-16.2</i> | CTTTACCACTATTTCCGTCCA | TAACAATCTCAGAAGACTCAGA |
| <i>pmp-3</i> | GTTCCCGTGTTCATCACTCAT | ACACCGTCGAGAAGCTGTAGA |
| <i>cdc-42</i> | CTGCTGGACAGGAAGATTACG | CTCGGACATTCTCGAATGAAG |

and homogenized in M9 using ultrasounds. The proteins were precipitated with methanol/chloroform (3:1:vol:vol) and resuspended in NRLB. The same volume of total protein was spotted onto 0.2 μ m nitrocellulose membrane. Immunoblotting was performed using 6E10 antibody (Covance, Madison, WI, USA; recognizes total A β protein species) or an anti-amyloid oligomer antibody (Rbx; Merck Millipore, Darmstadt, Germany; recognizes all kinds of amyloid oligomers). Part of the lysates was subjected to SDS-PAGE, and actin was used as a loading control. Each dot blot was repeated at least twice. Quantification of the ratio of each detected protein to actin and normalization to control appear under each representative dot blot.

Proteasome peptidase assay

Synchronized young adult animals were homogenized in proteasome activity lysis buffer (1 M Tris-HCl, pH 7.6, 100 mM ATP, 3 M KCl, 0.1 M EDTA, 1 M DTT, 0.2% Nonidet P-40, 10% glycerol, 10 μ g/ml aprotinin, and 10 mM PMSF) using ultrasounds. CT-L activity was assayed with hydrolysis of the fluorogenic peptide LLVY-AMC (Enzo Life Sciences, Lausen, Switzerland), for 30 min at 37°C. Specific activity was determined in the presence of 20 μ M MG132 or 20 μ M lactacystin. Fluorescence was measured in a fluorescence spectrophotometer.

Native gel electrophoresis for proteasome complexes

Synchronized animals were collected, washed, resuspended in proteasome activity lysis buffer, and homogenized using ultrasounds. Protein (40 μ g) was used for native gel electrophoresis for proteasome complexes followed by immunoblot analysis as described previously (15). Antibodies against selected 19S or 20S subunits were applied to detect 26S and 20S fully assembled proteasomes. Part of the lysates was subjected to SDS-PAGE, and actin was used as a loading control. Each blot was repeated at least twice. Quantification of the ratio of each detected proteasome complex to actin and normalization to control appear under each representative native gel.

Microscopy

Animals were mounted on 2% agarose pads, anesthetized with 5 mM sodium azide and observed at room temperature. Endogenous gut fluorescence and GFP levels were examined using

a fluorescence Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) with the Endow GFP filter (EX BP 470/40, BS FT 495, EM BP 525/50). Images were acquired using an Olympus DP71 Digital camera (Olympus, Tokyo, Japan) and Olympus CELL-A software. Emission intensity was measured on gray scale images with an 8 bit pixel depth (256 shades of gray). At least 30 images per condition from 3 independent trials were processed with the NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). For DAF-16a::GFP localization, synchronized control and *pbs-5* OE animals, expressing the p_{daf-16} DAF-16a::GFP transgene reporter fusion in *daf16(mu86)* genetic background (CF1139), were grown at 20°C, mounted on 2% agarose pads, and immobilized with 5 mM levamisole. The localization of DAF-16a::GFP was examined at d 1 of adulthood. Worms were classified into 3 groups, according to the extent of nuclear-cytoplasmic GFP distribution in the intestinal cells.

Paralysis assay

Synchronized AM140 and the relative *pbs-5* OE animals were grown at 20°C. Synchronized CL4176 and the relative *pbs-5* OE animals were grown at 16°C for 2 d before temperature up-shift to 25°C for transgene induction. Paralysis scoring was initiated at d 1 of adulthood for AM140 and 16 h following temperature up-shift for CL4176. The percentage of paralyzed animals is plotted against animal age for AM140 strain and against the hours post-temperature up-shift for CL4176 strain. Nematodes were scored as paralyzed if they failed to move, exhibited “halos” of cleared bacteria around their heads, accumulated eggs close to their bodies, or failed to respond to a touch-provoked movement. Animals that died were excluded. The log-rank (Mantel-Cox) test was used to evaluate differences between paralysis curves and to determine *P* values for all independent data. *N* in paralysis figures is the number of animals that paralyzed over total where total equals the number of paralyzed animals plus the number of dead and censored. Median paralysis values are expressed as mean \pm SEM.

Motility assay

Animals were grown at 20°C until L4 stage and then transferred at 25°C. RNAi-treated strains were transferred at d 1 of adulthood to HT115-seeded plates, containing an empty control vector (pL4440) or expressing double-stranded RNAi of the *pbs-5* gene. Motility of each animal (number of body circles) was monitored for 1 min at d 5.

Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software Incorporated, La Jolla, CA, USA) and Microsoft Office 2003 Excel (Microsoft Corporation, Redmond, WA, USA) software packages. Mean values were compared using unpaired *t* tests. Data in all assays are the mean of 3 independent experiments, unless otherwise indicated.

RESULTS

Overexpression of *pbs-5* catalytic proteasome subunit confers enhanced proteasome content, assembly, and activity

To assess whether enhancement of proteasome content and activity is possible in a multicellular organism, we initially established stable transgenic lines overexpressing the *pbs-5* gene in wt *C. elegans*. *pbs-5* is the ortholog of the human β_5 subunit that is responsible for the main proteasome activity, namely CT-L activity, and has been shown to be the core rate-limiting subunit for proteasome activation in human primary fibroblasts (16). It is noteworthy that *pbs-5* was the only 20S subunit that was found up-regulated in *glp-1* mutants, which display increased proteasome activity (12). RNA expression analysis for *pbs-1*, *pbs-2*, *pbs-5*, *pas-2*, and *pas-5* subunits (Fig. 1A) revealed up-regulation of both α - (*pas*)- and β - (*pbs*)-type subunits in *pbs-5* OE animals. Protein expression analysis for PBS-1 and PBS-5 subunits (Fig. 1B) verified the overexpression of both subunits. The enhanced expression levels of proteasome subunits were also translated to increased functional proteasome levels, because we detected enhanced levels of CT-L activity by ~1.8-fold in *pbs-5* OE animals (Fig. 1C; the conditions used for the activity assay favored the detection of both 20S and 26S proteasome activity). Proteasome inhibitors (MG132 and lactacystin) blocked the activities in both extracts from control and *pbs-5* OE animals, thus verifying the specificity of the measurements (Supplemental Fig. 1A). Enhanced proteasome activity was also detected in a second *pbs-5* OE line (Supplemental Fig. 1B), thus eliminating a line-specific effect. Analysis of proteasome complexes revealed more 20S and 26S particles in *pbs-5* OE animals (Fig. 1D) and lower polyubiquitinated protein levels (Fig. 1E). To exclude that *rpn-6* overexpression is responsible for this activation as suggested by Vilchez *et al.* (12), we have also tested the protein expression levels of RPN-6 in *pbs-5* OE animals that was not found up-regulated (Supplemental Fig. 1C). Overall, these results suggest that *pbs-5* OE leads to increased proteasome content, assembly, and activity in *C. elegans*, by analogy to previous studies in human primary fibroblasts upon overexpression of the human β_5 subunit (16). This is the first report for genetically mediated 20S core proteasome activation (in both terms of activity and content) in a multicellular organism.

Proteasome activation extends the life span of wt animals

Proteasome function has been shown to decline during aging and senescence of different cells, tissues, and organisms (3) and 20S core proteasome activation has

been shown to extend cellular life span in human primary fibroblasts (16). We therefore sought to determine whether 20S activation affects *C. elegans* longevity. Life span analysis showed that *pbs-5* OE resulted in significant extension of animal life span (Fig. 2A; results were confirmed in a second line, Supplemental Fig. 2A). Immunoblot analysis of PBS-5 in d 5 verified that *pbs-5* OE is maintained during animal life span (Supplemental Fig. 1D). In support, knock-down of *pbs-5* by RNAi (for RNA and protein analysis following RNAi, see Supplemental Fig. 3A and B) abolished the extended life span of *pbs-5* OE animals (Supplemental Fig. 3C). Interestingly, knock-down of *pbs-5* promoted an up-regulation in the expression of other 20S subunits, probably as a compensatory mechanism to the decreased levels of the crucial *pbs-5* subunit (Supplemental Fig. 3D) in accordance with previous studies in *C. elegans* (12, 17) and *Drosophila* (18). We also examined whether the extended life span is coupled with physiologic and metabolic changes related to deceleration of the aging process. Despite valuable criticism regarding lipofuscin accumulation in aged *C. elegans* (19, 20), age pigment (intestinal lipofuscin and advanced glycation end-products) accumulation is a known feature of aging in various species that has been used as a reporter of nematode health span (21, 22). To this end, we found significantly lower levels of age pigment autofluorescence in *pbs-5* OE compared with control animals at different time points (Fig. 2B, C). In conclusion, proteasome activation through *pbs-5* OE results in wt animal life span extension and increased health span.

We also examined *pbs-5* OE animals for phenotypic differences as these characteristics are altered in long-lived *C. elegans* mutants (feeding behaviors, fecundity, developmental abnormalities, movement defects, and anatomic alterations; Table 2). We did not observe any severe physiologic alterations between *pbs-5* OE and control animals. Notably, we detected selected signs that have been causally linked with longevity of long-lived mutants (23, 24), such as slightly slower developmental timing and defecation rhythms and increased capacity to enter the stress-resistant dauer larvae form. These measurements suggest that although *pbs-5* OE animals do not exhibit pronounced physiologic alterations, the observed differences contribute to life span extension.

pbs-5 OE-mediated life span extension is dependent on the DAF-16/FOXO, SKN-1, and HSF-1 transcription factors

Aging in *C. elegans* is mainly regulated by the conserved endocrine insulin/IGF-1 signaling (IIS) pathway that involves the IGF receptor DAF-2 and DAF-16/FOXO transcription factor (25). First, we investigated whether *pbs-5* OE might influence life span via DAF-16. *pbs-5* OE further extended the life span of long-lived *daf-2* mutants (Fig. 3A; results were confirmed in a second line, Supplemental Fig. 2B). However, this extension was abolished in the *pbs-5* OE *daf-16* mutants (Fig. 3B). Moreover, loss of DAF-16 fully suppressed the longevity conferred by *pbs-5* OE in *daf-2* mutants [*daf-16(m26);daf-2(e1370)*; Fig. 3C]. These results suggest that *pbs-5*-mediated life span extension requires DAF-16 activity and DAF-16 acts downstream of PBS-5.

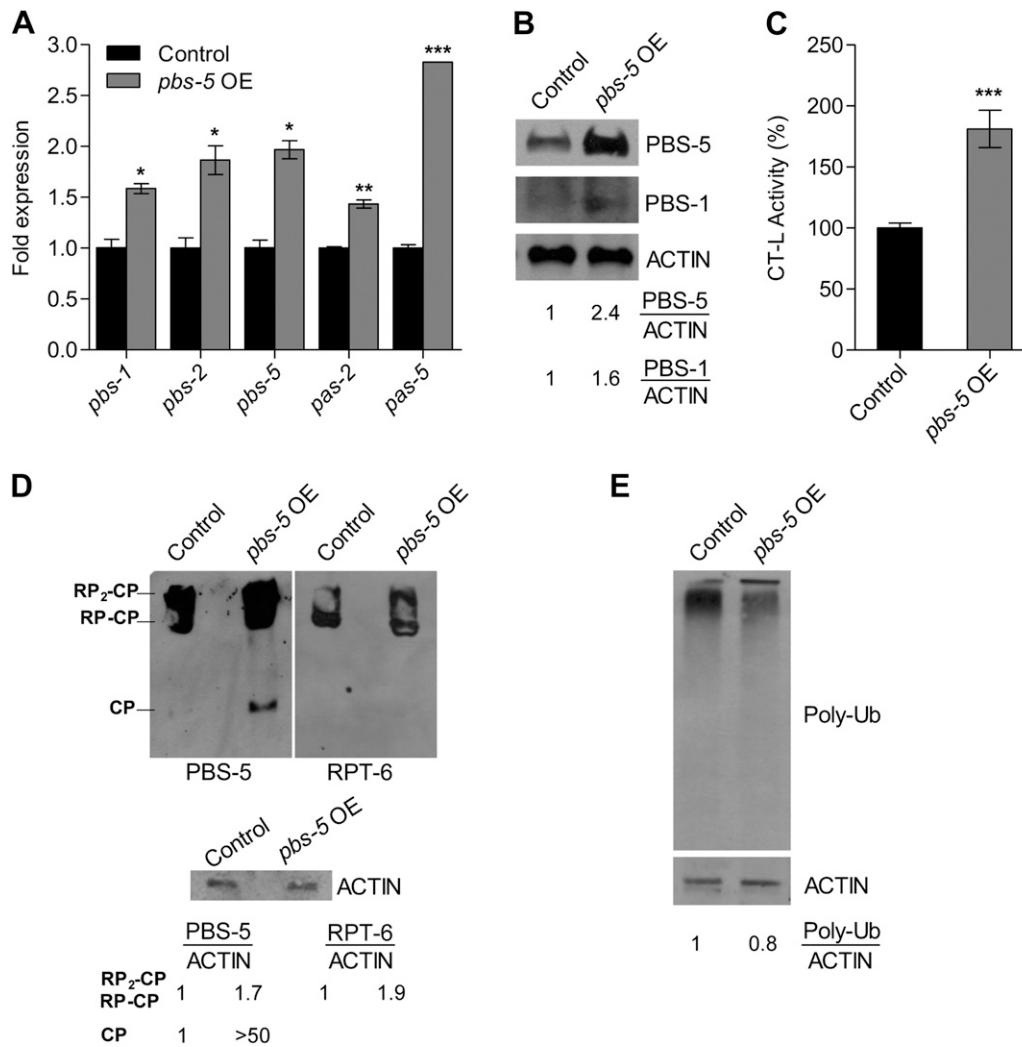


Figure 1. *pbs-5* OE induces proteasome expression and function in wt *C. elegans*. A) Real-time PCR analysis of *pbs-1*, *pbs-2*, *pbs-5*, *pas-2*, and *pas-5* subunits in wt control and *pbs-5* OE animals. Expression levels of each gene were arbitrarily set to 1 in control animals. *pmp-3* gene expression was used as normalizer. B) Immunoblot analysis of PBS-1 and PBS-5 subunits in wt control and *pbs-5* OE animals. C) Proteasome activity in *pbs-5* OE animals is expressed as percentage (%) with mean value of CT-L activity in control animals set to 100%. Native gel electrophoresis followed by immunoblot analysis against PBS-5 (20S subunit) and RPT-6 (19S subunit) (D) and immunoblot analysis of polyubiquitinated (Poly-Ub) proteins in wt control and *pbs-5* OE animals (E). Actin was used as a loading control. Error bars denote \pm SEM. CP, core particle; RP, regulatory particle (single capped), RP₂, regulatory particle (double capped). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

To investigate whether DAF-16 transcriptional activity is modified upon *pbs-5* OE, we monitored the expression of the reporter $p_{sod-3}GFP$ in *pbs-5*-overexpressing animals. $p_{sod-3}GFP$ is transcriptionally up-regulated in a DAF-16-dependent manner (26), and *sod-3* is a usual reporter for DAF-16 activity (27, 28). We found that *pbs-5* OE produced a significant increase in $p_{sod-3}GFP$ expression in various tissues at all examined ages (Fig. 3D, E). Consistent with this observation, mRNA levels of endogenous *sod-3* gene were markedly elevated in wt *pbs-5* OE animals and *pbs-5* OE *daf-2* mutants, whereas *sod-3* induction was lost in *pbs-5* OE *daf-16* mutants (Supplemental Fig. 4A). Moreover, *pbs-5* knockdown abolished *sod-3* induction in *pbs-5* OE animals (Supplemental Fig. 4B). Finally, we neither recorded any changes of the endogenous *daf-16* mRNA levels (Supplemental Fig. 4C) nor detected any significant changes in DAF-16 intracellular localization using a

reporter fusion of DAF-16 ($p_{daf-16}DAF-16a::GFP$; Fig. 3F) upon *pbs-5* OE. Therefore, we suggest that *pbs-5* OE may affect DAF-16 function by promoting its nuclear activity but not its translocation. Such regulation has been previously shown for various DAF-16 regulators [i.e., HCF-1 (29), EAK-7 (30), and SMK-1 (31)]. Moreover, up-regulation of DAF-16 target survival genes, such as *sod-3* could, at least in part, account for the *pbs-5*-mediated longevity.

The transcription factor *skn-1* is also prominent in life span regulation (32, 33) and oxidative stress resistance (34), and it also responds to proteasome perturbations (17, 34–36). Therefore, we have also tested its levels and activity in *pbs-5* OE animals. We did not detect changes of the endogenous *skn-1* mRNA levels upon *pbs-5* OE, implying that they are not affected *per se* by this overexpression (Supplemental Fig. 4D). Knock-down of *skn-1* (for RNA analysis of *skn-1* and its target gene *gst-4* following *skn-1*

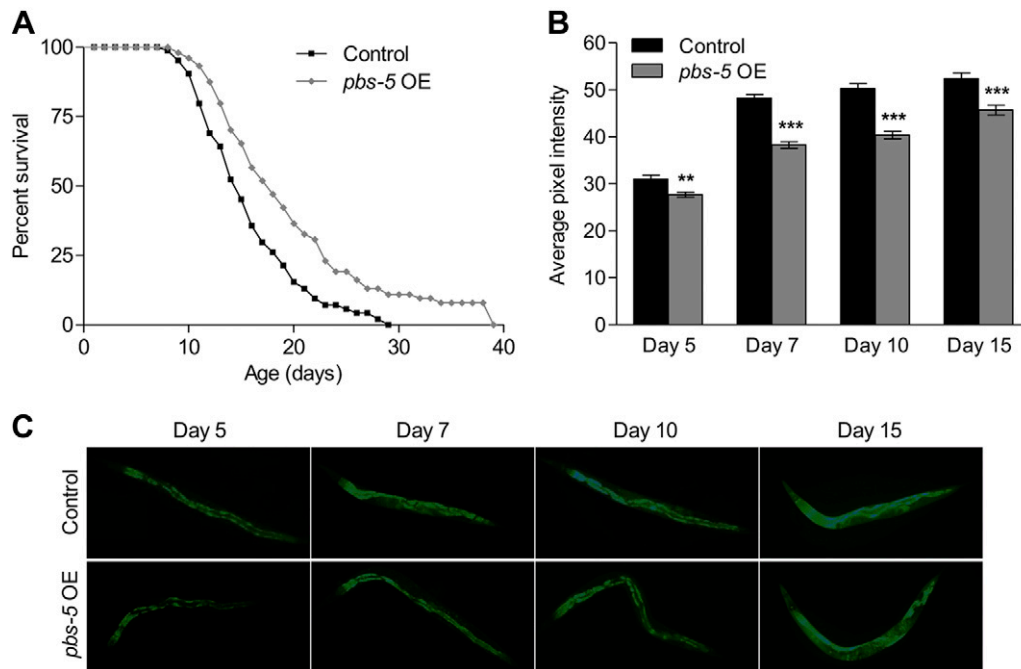


Figure 2. *pbs-5* OE extends the life span and health span of wt *C. elegans*. **A**) Survival curves of wt control and *pbs-5* OE animals. Control: mean = 14.0 ± 0.4 , $n = 497/604$, *pbs-5* OE: mean = 17.7 ± 0.7 , $n = 546/620$, $P < 0.0001$. **B**) Quantification of intestinal autofluorescence and **C**) representative images of wt control and *pbs-5* OE animals at d 5, 7, 10, and 15 of adulthood. Three independent experiments, 30 animals per strain per time point. Error bars denote \pm SEM. ** $P < 0.01$, *** $P < 0.001$.

RNAi, see Supplemental Fig. 4E, F) in *pbs-5* OE animals abolished their extended life span (Fig. 4A). This abolishment was not due to a direct, reduced transcription of proteasome subunits because the mRNA levels of different subunits were not significantly decreased in animals subjected to RNAi for *skn-1* gene (Fig. 4B). The mRNA levels of *gst-4* target gene were increased in *pbs-5* OE animals, thus revealing enhanced SKN-1 transcriptional ability upon *pbs-5* OE (Fig. 4C). These results imply that *pbs-5*-mediated life span extension also requires SKN-1 activity.

We also examined the requirement of the heat-shock transcription factor *hsf-1*, a key player of the proteostasis network, on *pbs-5*-mediated life span extension. We found that this extension is also dependent on HSF-1 (Fig. 4D); *pbs-5* OE shortens the life span of *hsf-1* mutants. Given that collapse of proteostasis is an early molecular event (37) and that protein aggregates can lead to global UPS impairment (38), we assume that the *pbs-5*-mediated proteasome activation remains insufficient in mutants with highly impaired proteostasis, such as *hsf-1* mutants. Moreover, we have performed thermal stress in *pbs-5*-overexpressing *hsf-1* mutants and we did not record any resistance (Fig. 4E). We also observed a significant down-regulation of *hsp-16.2* (an

hsf-1 target gene) mRNA levels in *pbs-5* OE *hsf-1* mutants, thus further advocating for the lack of resistance upon heat stress (HS; Supplemental Fig. 4G). Supportingly, PBS-5 was recently shown to be a negative heat shock response (HSR) regulator (39). Accordingly, *pbs-5* OE in wt animals neither extended their life span under conditions of mild HS at 25°C (Supplemental Fig. 4H) nor conferred any resistance during acute heat stress at 35°C (Supplemental Fig. 4I). In total, these experiments suggest that *pbs-5* OE-mediated life span extension is dependent on DAF-16/FOXO, SKN-1, and HSF-1 transcription factors.

Proteasome activation confers resistance to oxidative stress

Given that the proteasome is activated upon oxidative stress (40), we investigated the possible resistance of *pbs-5* OE animals to different oxidants, namely paraquat and juglone. Paraquat is a generator of superoxide anions, whereas juglone is a reactive oxygen-generating naphthoquinone and a producer of damaging adducts with proteins. *pbs-5* OE animals were significantly more resistant

TABLE 2. Phenotypic analysis of wt *pbs-5* OE animals

| Animals | Pharyngeal pumping ^a | Defecation ^b | Body size ^c | Fecundity ^d | Embryonic lethality ^e | Developmental timing ^f | Dauer formation ^g |
|-----------------|---------------------------------|-------------------------|------------------------|------------------------|----------------------------------|-----------------------------------|------------------------------|
| Control | 222 \pm 25 | 40 \pm 3 | 1.2 \pm 0.01 | 303 \pm 21 | 0.2 \pm 0.4 | 55 \pm 1 | 1.0 \pm 0.5 |
| <i>pbs-5</i> OE | 230 \pm 15 | 49 \pm 5* | 1.1 \pm 0.01 | 366 \pm 64* | 1.2 \pm 1.3 | 57 \pm 1* | 10.0 \pm 6.2 [†] |

All assays were performed at 20°C unless noted otherwise. ^aPumps per minute. ^bDuration of defecation cycle in seconds. ^cAdult body length in millimeters. ^dNumber of eggs laid per worm. ^ePercentage of eggs that failed to hatch. ^fDuration of postembryonic development (hours from egg hatching to L4 molt). ^gPercentage of animals that become dauer larvae at 27°C. * $P < 0.001$. [†] $P < 0.05$.

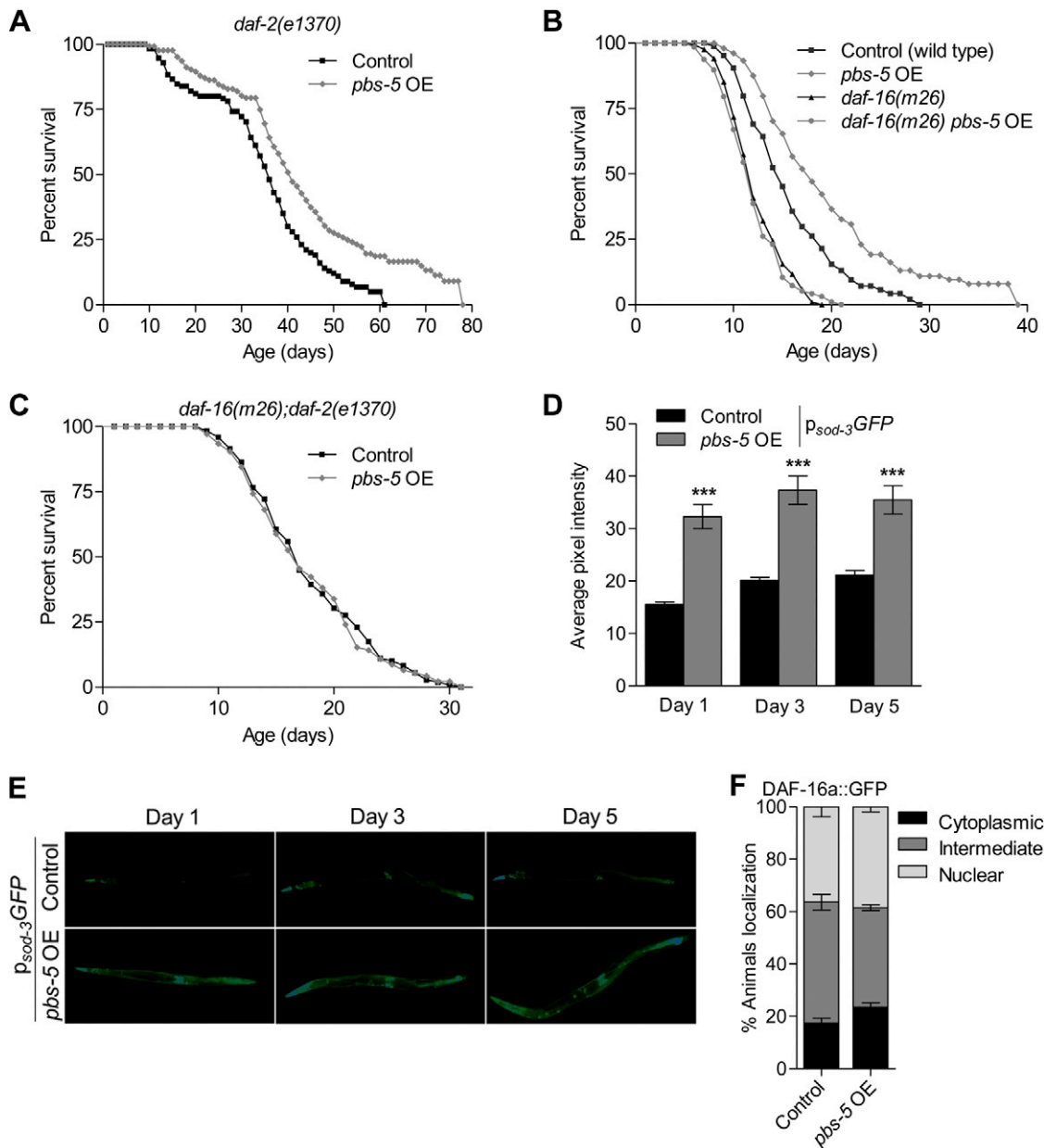


Figure 3. *pbs-5* OE-mediated life span extension is dependent on the DAF-16/FOXO transcription factor. Survival curves of control and *pbs-5* OE (A) *daf-2(e1370)*. Control: mean = 35.5 ± 0.5 , $n = 198/289$, *pbs-5* OE: mean = 42.5 ± 1.5 , $n = 205/313$, $P < 0.0001$. B) *daf-16(m26)* (including wt control and *pbs-5* OE animals values, which appear in Fig. 2). Control: mean = 13.2 ± 0.6 , $n = 421/554$, *pbs-5* OE: mean = 12.2 ± 0.2 , $n = 418/575$, not significant (NS). C) *daf-16(m26);daf-2(e1370)*. Control: mean = 18.0 ± 1.0 , $n = 216/259$, *pbs-5* OE: mean = 17.0 ± 0.0 , $n = 191/254$, NS. D and E) Quantification of fluorescence intensity (D) and representative images (E) of control and *pbs-5*-overexpressing animals carrying the DAF-16/FOXO transcriptional reporter $p_{sod-3}GFP$ at d 1, 3, and 5 of adulthood. Four independent experiments, 30 animals per strain. F) Percentage of cytosolic, nuclear, or intermediate accumulation of DAF-16a::GFP in control and *pbs-5* OE animals, expressing the $p_{daf-16}DAF-16a::GFP$ transgene reporter fusion in *daf-16(mu86)* genetic background. Six independent experiments, 150 animals per strain per time point. Error bars denote \pm SEM. *** $P < 0.001$.

to both stressors (Fig. 5A). It has been shown before that carbonylated proteins, which are by-products of oxidative stress (41), can be degraded by the proteasome (42). We detected decreased levels of low to middle molecular weight carbonylated proteins (30 to 70 kDa) in *pbs-5* OE animals (Fig. 5B). These decreased levels may be due to the enhanced levels of proteasome activity and, in turn, may contribute to the observed increased stress resistance.

To assess whether oxidative stress resistance is also dependent on DAF-16/FOXO factor, we exposed *pbs-5*-overexpressing *daf-2* and *daf-16* mutants to paraquat. *pbs-5* OE *daf-2* mutants were more resistant to oxidative stress (Fig. 5C), although this resistance was eliminated in *pbs-5* OE *daf-16* mutants (Fig. 5D). We conclude that DAF-16 factor is required for the increased oxidative stress resistance of *pbs-5* OE animals.

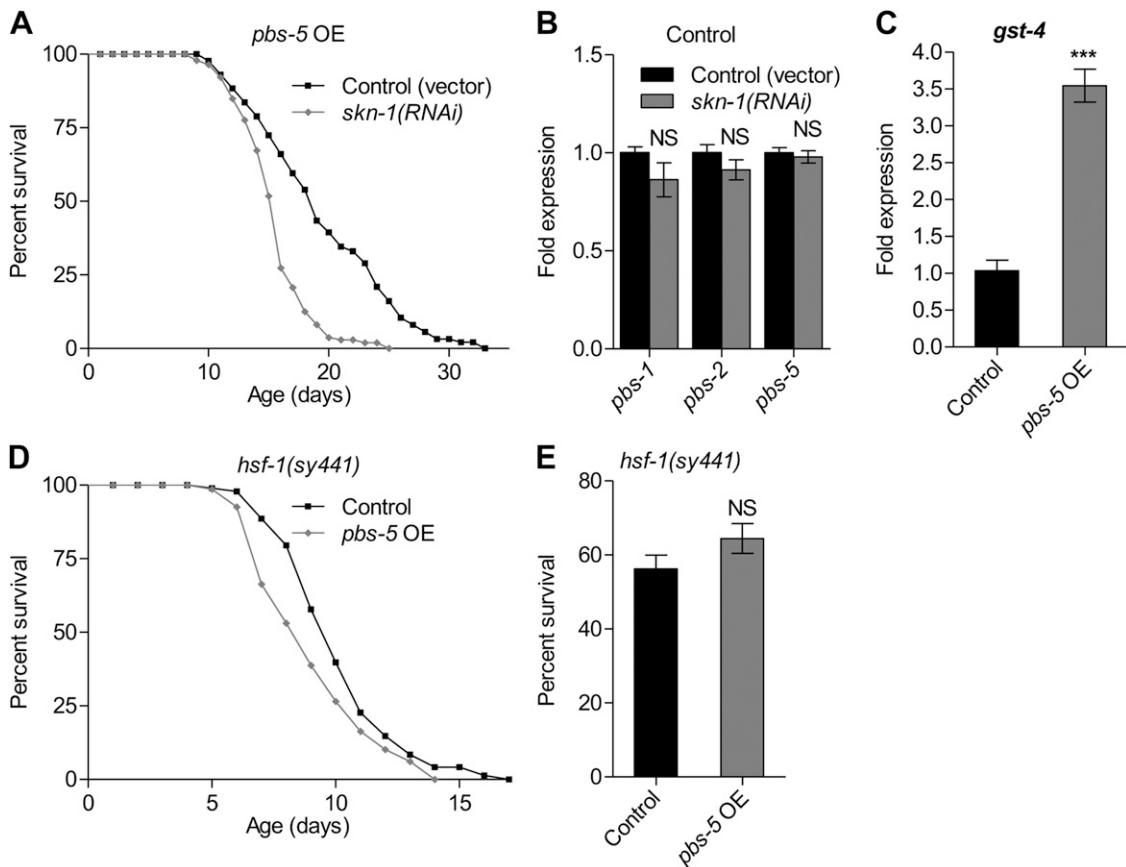


Figure 4. *pbs-5* OE-mediated life span extension is dependent on the SKN-1 and HSF-1 transcription factors. *A*) Survival curves of *pbs-5* OE animals subjected to RNAi for *skn-1* or the empty vector. Control (vector): mean = 19.5 ± 0.5, $n = 123/148$, *skn-1(RNAi)*: mean = 16 ± 1.0, $n = 134/151$, $P < 0.0001$. Real-time PCR analysis of (*B*) *pbs-1*, *pbs-2*, and *pbs-5* subunits and (*C*) *gst-4* gene in (*B*) control animals subjected to RNAi for *skn-1* or the empty vector and (*C*) wt control and *pbs-5* OE animals. Expression levels of each gene were arbitrarily set to 1 in control animals. *pmp-3* gene expression was used as normalizer. *D*) Survival curves of control and *pbs-5* OE *hsf-1(sy441)* animals. Control: mean = 10.0 ± 0, $n = 184/370$, *pbs-5* OE: mean = 9.0 ± 0, $n = 103/390$, $P < 0.0001$. *E*) Survival of control and *pbs-5* OE *hsf-1(sy441)* animals subjected to HS. Six independent experiments, 700 animals per strain. Error bars denote ± SEM. *** $P < 0.001$.

Proteasome activation confers deceleration of aggregation-related pathologies and maintenance of proteostasis

Neurodegenerative disorders such as AD or HD are characterized by protein homeostasis perturbations and have been directly linked to proteasome malfunction (43, 44). Therefore, we tested the effects of *pbs-5* OE in relation to these diseases. To this end, we exploited 2 nematode polyglutamine (polyQ) disease models, in which expression of polyQ protein in the body wall muscles (AM140 strain) (45) and in neurons (AM101 strain) (46) causes proteotoxic aggregation that results in age-dependent paralysis and motility defects. Remarkably, *pbs-5* OE in animals expressing 35 glutamine residues (Q35) in body wall muscle delayed the observed paralysis (Fig. 6A; results were confirmed in a second line, Supplemental Fig. 2C) and significantly improved motility of worms expressing Q40 in neurons (Fig. 6B). Accordingly, RNAi of *pbs-5* had a detrimental effect on animal motility (Fig. 6C). Likewise, in CL4176 strain (an AD model where human $A\beta_{1-42}$ is expressed in a temperature-inducible manner in muscle thus leading to paralysis) (47), *pbs-5* OE significantly

delayed the paralysis phenotype (Fig. 6D; results were confirmed in a second line, Supplemental Fig. 2D). It has already been shown in another AD model (CL2006 strain) that increase of the $A\beta$ oligomeric/toxic species follows $A\beta$ expression in paralyzed worms (48). Notably, we revealed that total but also oligomeric $A\beta$ levels were reduced in *pbs-5* OE CL4176 animals as compared with the control animals (Fig. 6E, Supplemental Fig. 2E), presuming that this reduction is due to the elevated levels of proteasomal degradation of *pbs-5* OE animals. In total, *pbs-5* OE appears to protect against the aggregate-related pathology progression and polyQ or $A\beta$ proteotoxicity.

DISCUSSION

Increasing evidence suggests that positive modulation of proteostasis-related key players, including proteolytic mechanisms like the UPS, confer high proteome protection and maintenance and contribute to the extended life span mediated by several pathways, such as IIS and dietary restriction (DR). This study demonstrates that overexpression of a single 20S core proteasome subunit,

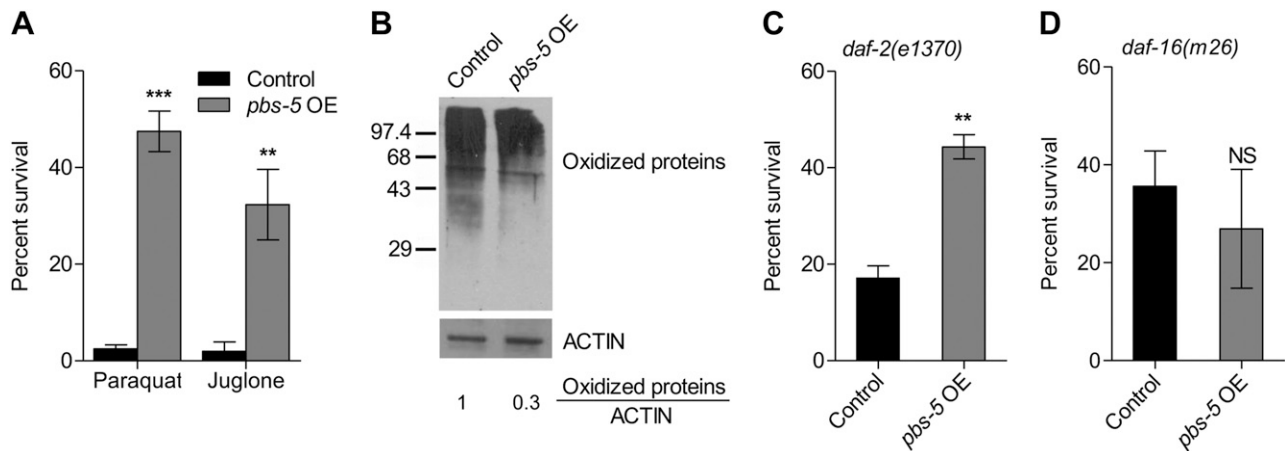


Figure 5. *pbs-5* OE increases oxidative stress resistance in animals with intact DAF-16/FOXO transcription factor. A) Survival of wt control and *pbs-5* OE animals treated with paraquat (3 independent experiments, 350 animals per strain) and juglone (8 independent experiments, 700 animals per strain). B) Oxyblot analysis of carbonylated proteins in wt control and *pbs-5* OE young adult animals. MW markers containing 1-3 DNP residues/molecule are shown on the left. Actin was used as a loading control. Survival of control and *pbs-5* OE (C) *daf-2(e1370)* and (D) *daf-16(m26)* animals treated with paraquat at d 10 and 5 of exposure, respectively; 350 animals per strain. Error bars denote \pm SEM. NS, not significant. ** $P < 0.01$, *** $P < 0.001$.

namely *pbs-5*, leads to enhancement of proteasome content and activity of both 20S and 26S complexes in a multicellular organism. This activation extends the life span of wt nematodes and confers resistance to oxidative stress. Our findings suggest that these ameliorated characteristics are, at least in part, the result of a complex equilibrium between DAF-16/FOXO, SKN-1, and HSF-1 factors. Moreover, we show that proteasome activation confers resistance to proteotoxic stress, thus nominating this complex as a potential candidate to minimize aggregation-related negative outcomes. This is the first report of proteostasis enhancement through 20S proteasome core activation that promotes longevity and stress/proteotoxic resistance in an intact multicellular organism.

E3 ligases are the first UPS components that have been modulated in multicellular organisms (35, 49–52). Their modulation has been shown to alter nematode life span and stress resistance through the selectively altered proteolysis of their target proteins. More recently, enhancement of 26S activity (but notably not content) has been achieved via overexpression of different 19S activator proteasome subunits in *Drosophila* (11) and in *C. elegans* (12), and the role of UBH-4 deubiquitinating enzyme on proteasome activity has been also revealed in *C. elegans* (53). In the current study, we have interfered with the last effector of the UPS system, the 20S proteasome, thus enhancing the general proteasome content and function. Our results indicate a common regulation between proteasome subunits because overexpression of *pbs-5* has resulted in up-regulation of both β - and α -type subunits. A similar co-regulation at least between β -type subunits has been shown in human (8, 16, 54, 55) and murine (56) cells. These studies indicated β -type subunits as the “rate-limiting” factors for the determination of total 20S proteasome levels. Native gel electrophoresis reveals that there are more 20S and 26S proteasomes in *pbs-5* OE animals. Consistent with enhanced 26S proteasome activity in our lines, we found decreased levels of polyubiquitinated proteins. These results support that proteasome up-regulation

in terms of content, assembly, and function is feasible in a higher organism.

Proteasome activation confers increased life span and health span maintenance in wt animals, in agreement with previous studies in human fibroblasts (16) or yeast cells (57, 58). Animals overexpressing *pbs-5* possess decreased levels of carbonylated proteins, suggesting a possible increased clearance by the proteasome. These diminished levels could also be linked to the enhanced longevity of wt *pbs-5* OE animals. In support, previous studies revealed a negative correlation between protein carbonylation levels and life span, suggesting that high levels may contribute to a shortened life span in *daf-16* mutants, and lower levels are found in long-lived *age-1* and *daf-2* mutants (59). These findings along with the observed lower rates of age pigments accumulation reveal a pivotal role of the proteasome on proteostasis and organismal life span.

Many manipulations that increase nematode longevity also increase resistance to various acute stressors. We also report a protective effect of proteasome activation against oxidative stress, in accordance with studies in mammalian (16, 55, 56) and yeast cells (57, 58). In support, animals with impaired UPS function present oxidative-mediated damage (60). We show that enhanced proteasome activity does not confer resistance to all kinds of stress, in accordance with previous reports (12). Concerning HS, proteasome activation does not seem to promote survival in accordance with a recent study showing that *pbs-5* negatively regulates HSR (39). Although there are studies that challenge the link between oxidative damage accumulation and *C. elegans* longevity (61, 62), the possible deleterious effects of accumulated specific stress-damaged proteins due to dysfunctional proteasomal degradation cannot be ignored.

Metabolic signaling pathways that regulate the aging process, mediated by IIS (with the involvement of both DAF-16 and HSF-1 stress responsive factors) or SKN-1 (32, 33) can modulate the proteostasis machinery to maintain a youthful proteome for longer (5). Our results indicate that *pbs-5*-mediated life span extension is a complex trait

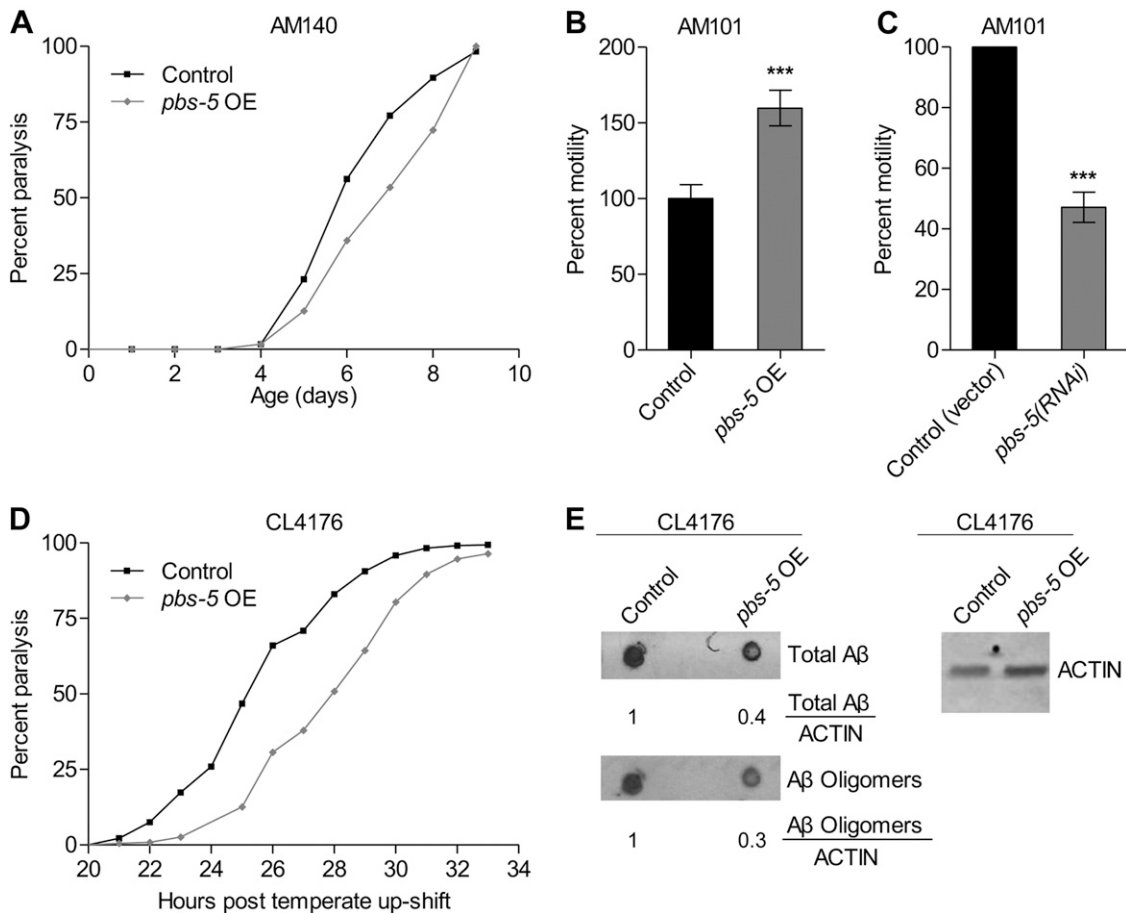


Figure 6. *pbs-5* OE reduces polyglutamine and A β toxicity. *A*) Paralysis curves of control and *pbs-5* OE animals, expressing Q35::YFP in body wall muscle cells (AM140 strain). Control: mean = 6 \pm 0, n = 220/303, *pbs-5* OE: mean = 7.5 \pm 0.5, n = 135/292, P < 0.0001. Percent motility of *B*) control and *pbs-5* OE animals expressing Q40::YFP in neurons (AM101 strain), and *C*) animals expressing Q40::YFP in neurons subjected to RNAi for *pbs-5* or the empty vector at d 5 of adulthood. Mean value of motility of control animals set to 100%; 80 animals per strain. *D*) Paralysis curves of control and *pbs-5* OE animals expressing human A β ₁₋₄₂ peptide (CL4176 strain), Control: mean = 25.67 \pm 1.2, n = 485/491, *pbs-5* OE: mean = 28 \pm 2, n = 334/349, P < 0.0001. *E*) Dot blot analysis of total A β and A β oligomers in control and *pbs-5* OE animals expressing human A β ₁₋₄₂ peptide (CL4176 strain) 24 h post-temperature up-shift. Equal numbers of worms were lysed and spotted for dot blot analysis or run in SDS-PAGE. Actin was used as a loading control. Error bars denote \pm SEM. *** P < 0.001.

among DAF-16/FOXO, SKN-1, and HSF-1 factors and, at least in part, the regulation of their downstream targets. Proteasome modulation through multiple trivial pathways associated with life span is apparently necessary to ensure an adequate cellular response during the aging progression and its effects in proteostasis. Nonetheless, our results also suggest that proteasome activation does not always exert beneficial effects on longevity. The genetic background dictates whether such enhancement will turn out to be beneficial, neutral, or even toxic. For example, in mutants like *hsf-1* where proteostasis collapse is overwhelming, we do not observe a positive effect of proteasome activation. Moreover, HSF-1 is necessary during larval development for proteotoxicity protection (63), and it sets the needed conditions during early developmental stages that enable DAF-16 to promote longevity later on (64). Therefore, hypomorphic *hsf-1* mutants are more aggravated (in terms of proteotoxic insults) compared with wt animals from the beginning of their lives and thus *pbs-5* OE and its positive outcomes might not be enough to rescue this heavily problematic phenotype.

Finally, we show a PBS-5-mediated increased resistance to proteotoxic stress in AD and HD nematode models. Therefore, proteasome activation appears to be a potential protective player in the battle against disorders that are linked to proteostasis failure. We suggest that this is directly related to the proteasome-mediated proteolysis of toxic oligomers. In support, worms with decreased proteasome activity due to lack of an inducible 19S subunit are unable to combat proteotoxic insult (65), and overexpression of the same subunit reduces A β accumulation thus reducing β -amyloid peptide toxicity (66). Additionally, 26S proteasome activation has been shown to reduce aggregated polyQ proteins (12). In conclusion, the modulation of cellular degradation systems is an emerging field of potential therapeutic intervention in aggregation-related pathologies (67).

Aging is accompanied by the gradual accumulation of damaged macromolecules due to defects of both repair and elimination. Our results suggest that the enhancement of the core proteasome content and activity endows the organism with an elevated capacity of removing oxidized and misfolded/aggregated proteins, thus preserving

proteostasis. As the current work demonstrates that genetically mediated core proteasome up-regulation and activation in a multicellular organism promotes longevity, future anti-aging studies should emphasize on the combined and highly balanced activation of the various UPS nodes. **FJ**

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