

Original Article

# Aging and SKN-1-dependent Loss of 20S Proteasome Adaptation to Oxidative Stress in *C. elegans*

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## Abstract

Aging is marked by a collapse of protein homeostasis and deterioration of adaptive stress responses that often lead to disease. During aging, the induction of stress responses decline along with protein quality control. Here, we have shown that the ability to mount an adaptive response by pretreatment with minor oxidative stress is abrogated in aged *Caenorhabditis elegans*. We have identified a defect in SKN-1 signaling sensitivity during aging and have also found an aging-related increase in basal proteasome expression and *in vitro* activity, however, adaptation of the 20S proteasome in response to stress is lost in old animals. Interestingly, increased activation of SKN-1 promotes stress resistance, but is unable to rescue declining adaptation during aging. Our data demonstrate that the aging-dependent decline in SKN-1 signaling negatively impacts adaptation of the 20S proteasome in response to acute oxidative stress.

**Keywords:** Adaptive homeostasis–Hydrogen peroxide–Proteolysis–Protein oxidation–Nrf2

A hallmark of aging is the decline in the quality control mechanisms of the cellular proteome (1). Loss of protein homeostasis results in an increase in protein oxidation, protein misfolding, and protein aggregation that can lead to aging-related diseases, including cardiovascular disease (2), ischemic stroke (3), and numerous neurodegenerative disorders (4). Several lines of evidence have indicated that oxidative stress is central to aging and the development of many diseases associated with aging (5). A major source for cellular oxidative stress is electron leakage from mitochondrial respiration, resulting in the production of superoxide and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Although not highly reactive, superoxide quickly reacts with nitric oxide radical, which leads to the formation of a potent oxidant known as peroxynitrite. In addition, H<sub>2</sub>O<sub>2</sub> can decompose to form hydroxyl radicals in the presence of metal ions (6). Thus, cellular oxidative damage is an inevitable byproduct of aerobic metabolism. Consequently, protein oxidation results in the unfolding of proteins revealing hydrophobic residues that are prone to aggregation, leading to the accumulation of protein aggregates with age. Misfolded

proteins are managed by the induction of evolutionary ancient stress response mechanisms, including the heat shock response, oxidative stress response, and proteasome-dependent degradation that triage, refold, or degrade proteins prior to aggregate formation.

Adaptation to mild stress allows cells and organisms to cope with more severe stress by triggering the production of cytoprotective genes that promote cell survival. As oxidative stress increases with age, the ability to mount an adaptive response declines, resulting in the collapse of protein homeostasis and an increase in protein aggregation (7–9). Recent work in our laboratory has shown that induction of the proteasome during oxidative stress is dependent on Nrf2, the master regulator of the oxidative stress response, and that this mechanism is conserved for the *Caenorhabditis elegans* homolog, SKN-1. H<sub>2</sub>O<sub>2</sub> increases Nrf2 translocation to the nucleus, resulting in increased expression of the 20S proteasome and the subsequent increase in proteolytic capacity. H<sub>2</sub>O<sub>2</sub>-induced proteolytic activity is blocked by the inhibition of Nrf2 in *C. elegans*, *Drosophila melanogaster*, and mouse embryonic fibroblast cell culture (10,11).

To our knowledge, there are currently no studies to address how the adaptive capacity to induce expression of the proteasome may change with age. It is also well established that adaptation to numerous stressors declines during aging, but the nature of this defect in stress response remains unclear. Therefore, we sought to characterize the decline of 20S proteasome-dependent adaptation to oxidative stress in response to SKN-1 signaling during aging in the model organism, *C. elegans*.

Due to their short life span, *C. elegans* have been used extensively in aging studies and to model aging-dependent protein aggregation, as demonstrated in neurodegenerative diseases such as Huntington's and Parkinson's disease (12). Although SKN-1 has diverged from its mammalian ortholog in regards to some aspects of its regulation, such as DNA binding and negative feedback, it has extremely similar functionality to Nrf2 and activates many of the same gene family targets. In addition to typical Nrf functions, growing evidence suggests that SKN-1 is regulated by metabolic and nutrient signaling and SKN-1 has been shown to play a central role in *C. elegans*' life span. The conserved nature of these orthologs suggests that the mechanisms that regulate SKN-1 may also be conserved in vertebrates, making *C. elegans* a useful organism to understand mammalian aging (13).

In our current study, we have found that the ability to mount an adaptive response by pretreatment with acute oxidative stress is abrogated in aged *C. elegans* and that SKN-1 signaling and adaptive proteasomal degradation decline with age. Furthermore, we have shown that increased activation of SKN-1 results in increased stress resistance and decreased protein oxidation, but is unable to restore the aging-related decline of proteasome-dependent adaptation. These results demonstrate that the aging-dependent decline in SKN-1 signaling negatively impacts adaptation of the 20S proteasome in response to oxidative stress, suggesting that maintaining tighter control of signaling for adaptive responses may partially alleviate the collapse of protein homeostasis during aging. Essentially all diseases of aging have a protein homeostasis defect, making the investigation of the regulation of proteome quality control mechanisms essential to prolonging health span.

## Methods

### *C. elegans* Strains Utilized in This Study

Strains were cultured at 20°C using standard techniques (14). All experiments were performed on age-matched hermaphrodites synchronized by egg lay. Day 3 corresponds to 3 days post egg lay and represents the first day of adulthood. The wild-type control strain was N2 Bristol. The SKN-1::GFP strain was provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. The *skn-1* gain-of-function (*gf lax120*) strain was a gift from the Sean Curran laboratory. The following chromosomal arrays were also used: *P.pbs-5::GFP* and *P.pbs-5(ARE)::GFP*. Due to recent evidence that the DNA synthesis inhibitor fluorodeoxyuridine, commonly used to block egg laying in aging experiments, induces adaptation and promotes cytoprotection in response to stress (15–17), we chose to transfer adults to new plates on a daily basis to avoid confounding the current generation with its progeny. We also chose to avoid the use of sterile mutants as studies have found that blocking germ line development promotes protein homeostasis (18).

### Transgenic Animals

The genomic *pbs-5* promoter fragment was amplified by polymerase chain reaction (PCR) from mixed-stage genomic DNA. The

*P.pbs-5(ARE)::GFP* mutant was produced using PCR site-directed mutagenesis, thereby interchanging a span of 10 nucleotides including two adjacent putative antioxidant response element (ARE) motifs into a PSTI endonuclease restriction site. For a detailed list of all primers used for molecular cloning, refer to [Supplementary Table 1](#). Chromosomal arrays were generated by injecting N2 animals with the expression construct RR10 or 11 (10 ng/μL) and co-injection marker KP708 (*Pttx-3-rfp*, 40 ng/μL). Microinjection was performed using standard techniques (19).

### Oxidative Stress Assays

For survival assays, animals were treated with a H<sub>2</sub>O<sub>2</sub> bolus in M9 buffer for 20 minutes and allowed to recover on seeded nematode growth medium (NGM) plates for 2–3 hours. Animals were scored for survival with a platinum pick. For tolerance assays, animals were pretreated with H<sub>2</sub>O<sub>2</sub> as indicated for 20 minutes and allowed to adapt on seeded NGM plates for 16 hours. The next day, animals were treated with an LD<sub>50</sub> dose of H<sub>2</sub>O<sub>2</sub> determined by the survival assay. Experiments were performed in 3–7 replicates with *N* = 30–90 animals. The stability of H<sub>2</sub>O<sub>2</sub> in M9 was validated via spectrophotometry by measuring absorbance at 240 nm and extrapolating with an extinction coefficient of 43.6 M<sup>-1</sup> cm<sup>-1</sup> ([Supplementary Figure 1](#)).

### Microscopy and Analysis

Fluorescence microscopy experiments were performed essentially as previously described (20). Day 3 and Day 10 animals were paralyzed using 30 mg/mL 2,3-butanedione monoxime and mounted on 2% agarose pads for imaging. Images were acquired with a Nikon eclipse 90i microscope equipped with a Nikon PlanApo 60× or 100× objective (NA = 1.4) and a Photometrics Coolsnap ES2 camera controlled by Metamorph 7.0 software (Universal Imaging/Molecular Devices). For quantitative analysis, each animal was outlined and the average fluorescence was quantified using Metamorph. At least 30–70 animals were imaged for all experiments. To provide standardization for images acquired on different days, fluorescence values were normalized to the values of 0.5 μM FluoSphere yellow-green beads (Life Technologies, cat. F-8813) acquired during each imaging session.

### Quantitative RT-PCR

Approximately 100 animals of the indicated genotypes and age group were collected, washed in M9 buffer, and homogenized in TRIzol reagent (Invitrogen, cat. 15596-026). RNA was extracted according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (Life Technologies, cat. N8080234) and quantitative PCR was performed using iTaq SYBR Green (Bio-Rad, cat. 172–5121). Samples were normalized to the relative mRNA expression of untreated N2 animals at Day 3 and calculated from five biological replicates. Primers utilized in this study are listed in [Supplementary Table 1](#).

### Chromatin Immunoprecipitation

SKN-1::GFP animals were treated at Day 3 and Day 10 with ±2-mM H<sub>2</sub>O<sub>2</sub> for 20 minutes and collected immediately for chromatin immunoprecipitation (ChIP) with a GFP antibody. ChIP was performed as described previously with modifications as specified in the [Supplementary Methods](#) (21). Immunoprecipitated DNA was purified and quantitative PCR (qPCR) was performed using primers specific to ARE elements within the *pbs-5* and *gst-4*

promoter, in addition to upstream or downstream nonspecific sites (Supplementary Table 1).

### Western Blotting

N2 animals were collected in M9 and sonicated in RIPA buffer (50-mM Tris pH 8, 150-mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail) until fragmented (approximately three 10-second pulses at 30% amplitude). Twenty micrograms of lysate was run on a 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel (Biorad, cat. 456-1034) and blotted to a PVDF membrane (Biorad, cat. 162-0177) prior to immunostaining with PAS-7 (Developmental Studies Hybridoma Bank, cat. CePAS7-c) and ACTIN (Santa Cruz, cat. sc-1616 HRP) antibodies. Western blots were performed in 2-3 replicates and quantified using Image J.

### Carbonyl Assay

Oxidatively damaged proteins were measured by the detection of protein carbonyl groups. Animals were collected as described for Western blotting, and the supernatant was then used for protein carbonyl assays using reagents from the Oxyblot kit (Millipore, cat. S7150) per manufacturer's instructions. Briefly, 10 µg of protein was brought to a 5-µL volume with RIPA buffer, and 5 µL of 12% SDS was added for a total concentration of 6% SDS. Samples were incubated with 10 µL of 2,4-dinitrophenylhydrazine for 20 minutes, after which 7.5 µL of neutralization buffer was added to halt the derivatization reaction. Samples were reduced with 1.5-µL β-mercaptoethanol prior to running on an 8% hand cast SDS-PAGE gel. Carbonyl blots were performed in 2-4 replicates and quantified using Image J.

### Proteasome Activity Assays

N2 and *lax120* animals were treated at Day 3 and Day 10 with ±1 or 10 µM of H<sub>2</sub>O<sub>2</sub> for 20 minutes and allowed to adapt for 16 hours on seeded NGM plates. Animals were collected in M9 and sonicated in proteolysis buffer (50-mM Tris, 20-mM KCL, 5-mM MgAc, 0.5-mM dithiothreitol, pH 7.5) until obliterated. Samples were normalized to 5 µg and diluted in 100 µL of proteolysis buffer in a 96-well plate. Either 20-µM Suc-LLVY-AMC substrate or 1-µg oxidized [<sup>3</sup>H] hemoglobin were added, and samples were incubated in a plate shaker at 37°C for 4 and 2 hours, respectively. Degradation of Suc-LLVY-AMC substrate was measured at 355 nm/444 nm ex/em, and degradation of oxidized [<sup>3</sup>H] hemoglobin substrate was measured using a scintillation counter.

## Results

### Adaptation to Oxidative Stress Declines During Aging and Is Dependent on SKN-1

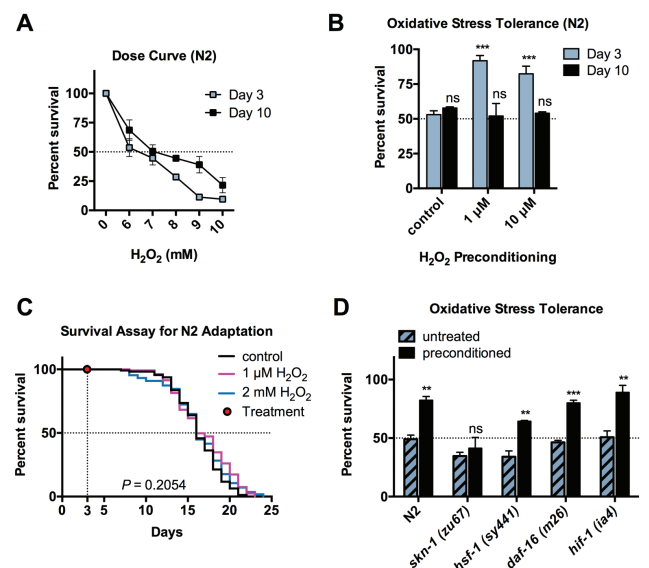
Adaptation occurs for a broad spectrum of stressors at both mild and moderate intensity. For our aging studies, we chose Day 10 as an old age time point, at which more than 90% of the population has survived, thus eliminating selection bias by only examining an older, geriatric population. In support of this, microarray analysis has shown that postreproductive *C. elegans* at this age undergo considerable transcriptional changes and therefore represent a fundamentally different life stage than young adult animals (22).

In order to determine the amount of H<sub>2</sub>O<sub>2</sub> to induce 50% survival, we performed a dose curve for Day 3 and Day 10 animals

(Figure 1A). Interestingly, older animals tolerated a slightly higher dose of H<sub>2</sub>O<sub>2</sub> compared with younger animals. Therefore, a median lethal dose (LD<sub>50</sub>) of 6- and 7-mM H<sub>2</sub>O<sub>2</sub> was used for Day 3 and Day 10 animals, respectively. Next, we evaluated stress resistance, both before and after H<sub>2</sub>O<sub>2</sub> preconditioning. As expected, adaptation to oxidative stress resulted in cytoprotection for Day 3 animals exposed to a median lethal dose of H<sub>2</sub>O<sub>2</sub> (Figure 1B). Interestingly, low (1 µM) and moderate (2 mM) doses of acute H<sub>2</sub>O<sub>2</sub> were equally sufficient to induce cytoprotection, indicating that survival after adaptation is not dose dependent (Supplementary Figure 2). In contrast, Day 10 animals were unable to adapt after pretreatment with any dose of H<sub>2</sub>O<sub>2</sub>. These data suggest that adaptation to acute oxidative stress declines with age.

In order to determine if adaptation that occurs early in life has a lasting effect on organismal life span or if moderate doses of H<sub>2</sub>O<sub>2</sub> would have a negative effect, we conducted life-span assays for both conditions. We found no significant impact on longevity for animals treated with 1- or 2-mM adaptive doses of H<sub>2</sub>O<sub>2</sub> at Day 3 (Figure 1C).

Preconditioning with a stressor may engage multiple adaptive mechanisms that could result in the activation of more than one transcription factor. To test this idea, we examined the requirement of key stress response regulators for adaptation to oxidative stress. Using the *skn-1* (*zu67*) deletion mutant, we verified our model that SKN-1 is necessary for adaptation to H<sub>2</sub>O<sub>2</sub> (Figure 1D). In addition, neither HSF-1, which mediates intrinsic thermotolerance (23), nor DAF-16, which mediates stress resistance induced by the downregulation of insulin-like signaling (24), is required for H<sub>2</sub>O<sub>2</sub> adaptation (Figure 1D). Furthermore, HIF-1, which regulates the hypoxic stress



**Figure 1.** Adaptation to H<sub>2</sub>O<sub>2</sub> diminishes during aging and is dependent on SKN-1. (A) Dose survival curve for N2 animals exposed to H<sub>2</sub>O<sub>2</sub>. The LD<sub>50</sub> dose was determined to be 6 and 7 mM for Day 3 and Day 10 animals, respectively. (B) Oxidative stress tolerance for N2 animals adapted to 1- to 10-µM H<sub>2</sub>O<sub>2</sub> preconditioning at Day 3 and Day 10 and challenged with LD<sub>50</sub> determined in (A). (C) Survival assay for N2 animals exposed to 1-µM or 2-mM H<sub>2</sub>O<sub>2</sub> for 20 minutes at Day 3. Significance determined by log-rank (Mantel-Cox) test. *p* = .2054, not significant. (D) Oxidative stress tolerance at 6 mM for untreated versus preconditioned (1-µM H<sub>2</sub>O<sub>2</sub>) *skn-1*(*zu67*), *hsf-1*(*sy441*), *hif-1*(*ia4*), and *daf-16*(*m26*) animals. Error bars denote standard error of the mean values. \**p* < .05, \*\**p* < .01, \*\*\**p* < .001 relative to control using Student's *t* test.

response, is also not necessary for adaptation to  $H_2O_2$  (Figure 1D). Based on these findings, we conclude that SKN-1 specifically promotes survival after preconditioning with  $H_2O_2$ .

### Aging Results in a Loss of SKN-1 Recruitment to the *Pbs-5* Promoter During Aging

We next characterized changes in regulation of the proteasome during the adaptive response in young compared with old animals. The proteasome is composed of two  $\beta$ -rings that make up the internal enzyme complex of the proteasome, flanked by  $\alpha$ -rings on either end that manage substrate entry. Each ring is composed of seven subunits known as proteasome beta or alpha subunits (PBS or PAS), which share significant homology to their mammalian counterparts (Figure 2A).

Several proteasome subunits contain AREs within their promoters, which recruit the transcription factor Nrf2/SKN-1 (10). There are two SKN-1 binding consensus sites at position -253 in the promoter of the *pbs-5* operon. The sequences and positions of these sites are closely conserved between *C. elegans* and other nematode species (Figure 2B). Both of these sites have the sequence TTTGTCAT, which matches the consensus ARE motif (WWTRTCAT) (25,26). Due to the close proximity of these conserved ARE sites, we chose to focus on the *pbs-5* subunit to further investigate regulation of the proteasome.

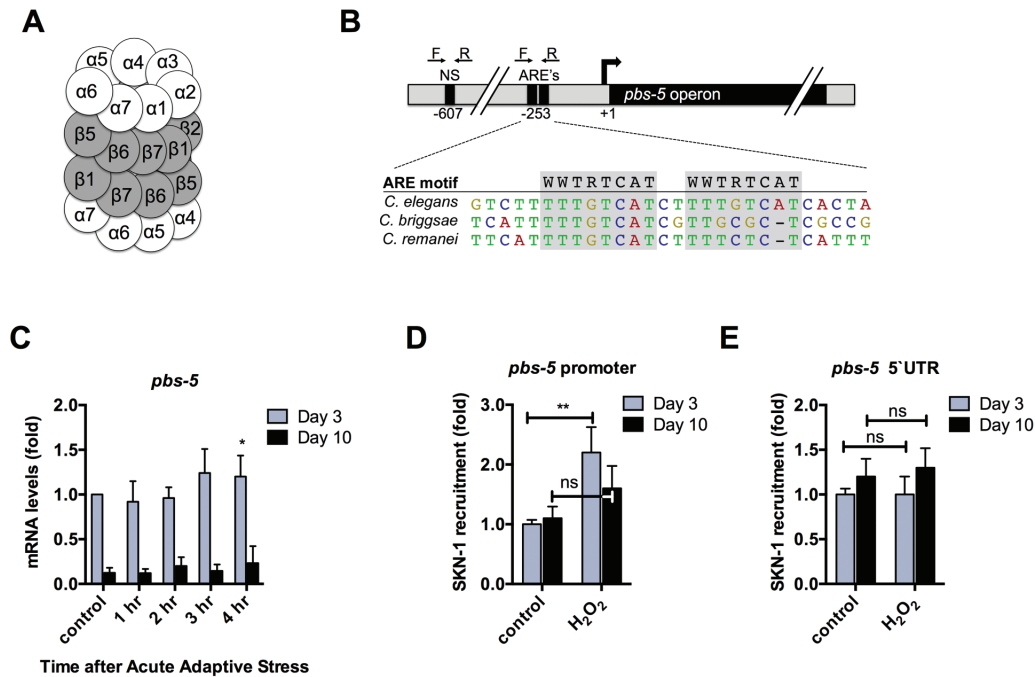
We performed qPCR and found that adaptive  $H_2O_2$  treatment resulted in a significant increase in the levels of *pbs-5* transcripts at Day 3, but no change in levels for Day 10 (Figure 2C). The proteasome comprises 1% of total cellular proteins and has an abundance of transcripts similar to that of a housekeeping gene (27). However, at Day 10, we found a marked decrease in *pbs-5* transcripts compared

with Day 3 (Figure 2C), indicating that induction of *pbs-5* declines with age.

To evaluate recruitment of SKN-1 to the *pbs-5* promoter in response to stress during aging, we performed ChIP of SKN-1 from SKN-1::GFP-expressing animals. We monitored SKN-1 occupancy by PCR using primers that flank the ARE sites within the promoter (Figure 2B). For Day 3 animals, SKN-1 occupancy at the *pbs-5* AREs increased approximately twofold after treatment with  $H_2O_2$  (Figure 2D). This increase is similar to that observed with other SKN-1 targets (28). However, there was no increase in SKN-1 recruitment in response to stress in Day 10 animals. Furthermore, we found no significant change in the association of SKN-1 to a nearby nonspecific region within the *pbs-5* promoter in response to stress (Figure 2E). These findings suggest that the decline of adaptation during aging may be due to a reduction in proteasome gene transcription by SKN-1.

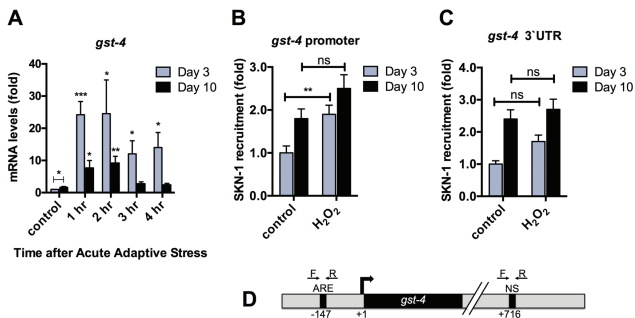
### SKN-1 Induction of *gst-4* Decreases With Age

Phase II detoxification is an element of glutathione metabolism and is a cellular response to a multitude of stressors, including xenobiotics, electrophiles, and reactive oxygen species (29–31). Because this response is also mediated by SKN-1, we next investigated whether induction of *gst-4* by  $H_2O_2$  also declines during aging. We found that an acute adaptive dose of 2-mM  $H_2O_2$  induces the transcription of *gst-4* (Figure 3A). Whereas Day 3 animals exhibited an increase in transcription following oxidative stress, induction of *gst-4* in Day 10 animals was reduced (Figure 3A). Furthermore, induction of phase II genes *gcs-1* and *gst-10* in response to  $H_2O_2$  was also reduced during aging (Supplementary Figure 3). Interestingly, basal *gst-4* levels are higher at Day 10 suggesting that the increased tolerance for  $H_2O_2$



**Figure 2.** SKN-1 recruitment to the *pbs-5* promoter declines during aging. (A) Schematic representation of the  $\alpha$  and  $\beta$  subunits of the 20S proteasome. (B) Representation of the *pbs-5* putative promoter and the ARE-specific and nonspecific (NS) primers used for qPCR of SKN-1::GFP ChIP samples. This region is closely conserved for two other nematode species. (C) qPCR analysis of *pbs-5* for Day 3 and Day 10 N2 animals in response to 2-mM  $H_2O_2$ . Expression levels were adjusted to N2 Day 3 control animals. (D) ChIP showing that SKN-1 is recruited to the *pbs-5* promoter in response to  $H_2O_2$  for Day 3, but not for Day 10 animals. (E) SKN-1 occupancy does not change in response to stress at a nonspecific site upstream of the *pbs-5* promoter. Error bars denote standard error of the mean values. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  relative to control using Student's  $t$  test.





**Figure 3.** Induction of phase II detoxification gene *gst-4* declines in response to  $H_2O_2$  in aged animals. (A) qPCR analysis of *gst-4* for Day 3 and Day 10 N2 animals in response to 2-mM  $H_2O_2$ . Expression levels were adjusted to N2 Day 3 control animals for each gene. (B) SKN-1 is recruited to the *gst-4* promoter in response to 2-mM  $H_2O_2$  for Day 3, but not for Day 10 animals. (C) SKN-1 occupancy does not change in response to stress at a nonspecific site downstream of the *gst-4* promoter. Error bars denote standard error of the mean values. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  relative to control using Student's *t* test. (D) Representation of the *gst-4* putative promoter and the ARE-specific and nonspecific (NS) primers used for qPCR of SKN-1::GFP ChIP samples.

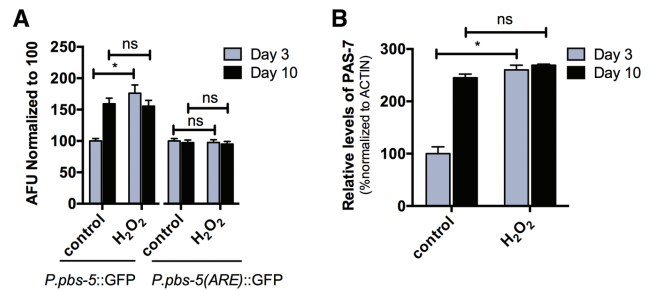
observed in older animals (Figure 1A) may be due to elevated detoxification enzymes during aging (Figure 3A).

Similar to Figure 2D, we also examined promoter occupancy of *gst-4* by SKN-1. We found that upon treatment with  $H_2O_2$ , SKN-1 is significantly recruited to the *gst-4* promoter for Day 3 animals, but not for Day 10 animals (Figure 3B). To verify that an increase in SKN-1 binding to DNA was specifically due to recruitment to an ARE motif, we also performed qPCR using primers to a nonspecific site in the downstream untranslated region for *gst-4* and found no significant change between the control and  $H_2O_2$ -treated animals (Figure 3C and D). Together, the results shown in Figures 2 and 3 suggest a defect in SKN-1 signaling sensitivity in response to oxidative stress during aging.

### Proteasome Expression Increases During Aging, But Is Not Induced During Adaption to Oxidative Stress

To further evaluate declining SKN-1 signaling during aging on the 20S proteasome, we examined the impact of adaptive  $H_2O_2$  pretreatment on *pbs-5* expression. We generated a *P.pbs-5::GFP* chromosomal array that expresses GFP driven by 557bp of the *pbs-5* promoter, which contains the entire 5' intergenic region between *pbs-5* and the neighboring gene (Supplementary Figure 4). We found that *P.pbs-5::GFP* expression increased after pretreatment with oxidative stress for Day 3 animals (Figure 4A and Supplementary Figure 7A). We also found that Day 10 animals exhibited increased basal levels of *P.pbs-5::GFP*, but did not show an increase in expression after pretreatment with  $H_2O_2$  (Figure 4A and Supplementary Figure 7A).

The *pbs-5* gene is part of an operon (CEOP1752), which includes the *jagunal 1* (*K05C4.2*) and *sol-2* (*K05C4.11*) genes. We mutated the two ARE sites within the *pbs-5* operon promoter by scrambling six of the nucleotides within the motif and generated transgenic animals expressing these mutated AREs driving expression of GFP (*P.pbs-5(ARE)::GFP*, Supplementary Figure 4). We found that mutating these ARE sites completely blocked the increase in expression of *P.pbs-5(ARE)::GFP* after treatment with  $H_2O_2$  (Figure 4A and Supplementary Figure 7A). In addition, we found that the expression of *P.pbs-5(ARE)::GFP* did not change despite age or stress.



**Figure 4.** Defective SKN-1 signaling impacts proteasome expression in response to stress. (A) Quantification of fluorescence for animals in response to stress. AFU normalized to 100 in comparison with each control.  $N = 65-91$  animals. (B) Densitometry of Western blots described in (A). Error bars denote standard error of the mean values. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  relative to control using Student's *t* test.

These results indicate that the aging-dependent increase in proteasome expression is also dependent on SKN-1. It should be noted that the *P.pbs-5::GFP* and *P.pbs-5(ARE)::GFP* strains were generated by microinjection of DNA encoding the desired transgene, which may have been incorporated unequally and thus cannot be directly compared. Therefore, we compared the fold change in fluorescence after exposure to  $H_2O_2$ .

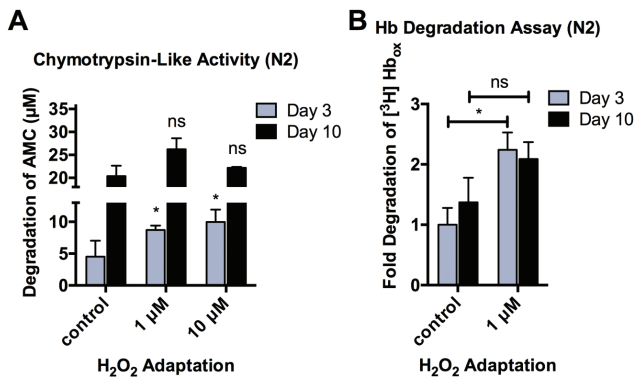
Although there is no commercially available antibody for the PBS-5 subunit of the proteasome, there is one available for PAS-7. We found that  $H_2O_2$  adaptation results in an increase in PAS-7 expression for Day 3 animals (Supplementary Figure 7B). However, consistent with our data for *P.pbs-5::GFP* (Figure 4A and Supplementary Figure 7A), Day 10 animals exhibited increased basal levels of PAS-7, but no adaptive increase in expression. Because proteasome transcript levels are at least four times lower in Day 10 animals compared with Day 3 animals (Figure 2C), the increase in PAS-7 expression during aging may indicate a passive accumulation of proteasomes that do not become degraded. Accumulation of proteasomes during aging may also account for the slight increase in median lethality observed for Day 10 animals (Figure 1A).

### Proteolytic Activity Increases During Aging, But Is Not Induced During Adaptation to Oxidative Stress

The decrease in the adaptive expression of proteasome subunits with age suggested that there might be a corresponding defect in proteasome function. Therefore, we measured proteasome function with two in vitro proteolytic activity assays. First, we characterized the capacity for the 20S proteasome to degrade a Suc-LLV-AMC fluorogenic proteasome substrate in vitro (Figure 5A). For Day 10 animals, we found that basal proteolytic capacity increased significantly, but adaptation to  $H_2O_2$  was lost with age. An increase in the in vitro capacity for proteolytic activity at Day 10 is consistent with the increase in basal proteasome expression observed in Figure 4 and Supplementary Figure 7.

Second, we measured the proteolytic capacity to degrade oxidized  $[H^3]$  hemoglobin in vitro (Figure 5B). As in Figure 5A, we found that basal proteasome activity increased with age, but Day 10 animals lost the capacity to adapt to oxidative stress.

For both small peptide and hemoglobin degradation assays, inhibition of proteasomal catalytic activity using epoxomicin, lactacystin, or MG132 resulted in nearly complete inhibition of proteolysis, indicating that other cellular proteases did not significantly contribute to substrate degradation (Supplementary Figure 5).



**Figure 5.** Proteasome adaptation is lost during aging, despite an increase in proteolytic activity. (A) Pretreatment with 1- to 10-µM H<sub>2</sub>O<sub>2</sub> results in an increase in adaptive proteolytic activity for Day 3 animals, but not for Day 10 animals. Proteasome peptidase activity was measured based on the degradation of a Suc-LLVY-AMC fluorogenic proteasome substrate measured at 355 nm/444 nm ex/em. (B) Pretreatment with 1-µM H<sub>2</sub>O<sub>2</sub> results in an increase in adaptive proteolytic activity for Day 3 animals, but not for Day 10 animals. Proteolytic capacity to degrade oxidized [<sup>3</sup>H] hemoglobin was measured by release of acid-soluble counts (liquid scintillation). Error bars denote standard error of the mean values. \**p* < .05, \*\**p* < .01, \*\*\**p* < .001 relative to control using Student's *t* test.

### Increased Activity of SKN-1 Does Not Rescue Adaptation During Aging

We next asked if increased activity of SKN-1 could rescue proteasome function during aging. In order to evaluate increased SKN-1 activity, we utilized a mutant expressing *skn-1* gain-of-function (gf) allele *lax120* (32), which encodes S245L, to determine if increased activation of SKN-1 can rescue adaptation during aging.

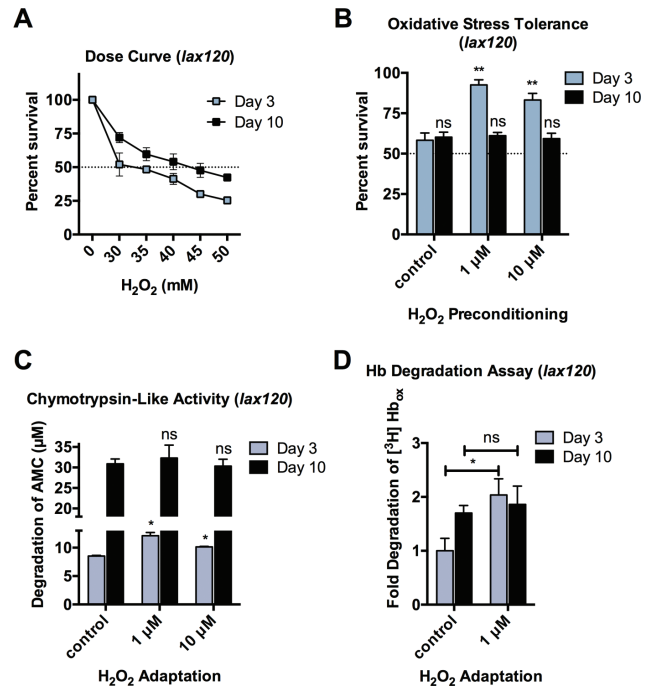
We performed a survival assay and found that increased activation of SKN-1 results in a 5-fold increase in stress resistance compared to N2 animals (Figures 1A and 6A). While young *lax120* animals showed adaptation similar to wild-type, increased SKN-1 activation did not rescue adaptation for Day 10 animals at the adjusted median lethal dose (Figure 6B). Therefore, while increased SKN-1 activity directly impacts SKN-1 gene target levels and oxidative stress tolerance, it was not able to rescue the aging-related decline of adaptation.

We next asked if increased activation of SKN-1 could rescue proteasome functional adaptation during aging. We performed a chymotrypsin-like activity assay and found that SKN-1 gain-of-function does not rescue 20S proteasome-dependent adaptation during aging (Figure 6C). In addition, increased SKN-1 activity did not rescue adaptation of proteolytic capacity to degrade oxidized [<sup>3</sup>H] hemoglobin in Day 10 animals (Figure 6D).

### Adaptive Proteolytic Degradation Declines During Aging In Vivo

In order to evaluate proteasome capacity in vivo, we measured total protein carbonylation, an irreversible consequence of oxidative damage that often leads to loss of protein function (33). In accordance with other studies (34–36), we found that protein carbonylation increases with age. We also found that *C. elegans* exhibit an increase in carbonylation as early as Day 5 (Supplementary Figure 6).

We next sought to evaluate the impact of H<sub>2</sub>O<sub>2</sub> pretreatment and SKN-1 gain-of-function on protein carbonylation during aging.

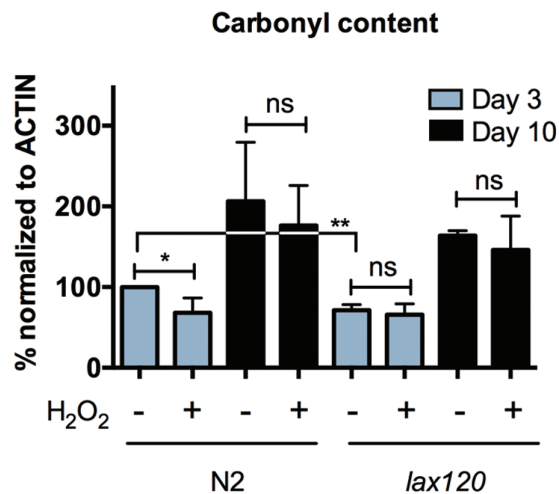


**Figure 6.** Increased SKN-1 activation does not rescue adaptive proteolytic capacity during aging. (A) Animals with increased SKN-1 activity (*lax120*) have an increased tolerance for H<sub>2</sub>O<sub>2</sub> compared with N2. The LD<sub>50</sub> dose was determined to be 30 and 50 mM for Day 3 and Day 10 animals, respectively. (B) Oxidative stress tolerance for *lax120* animals adapted to 1- to 10-µM H<sub>2</sub>O<sub>2</sub> preconditioning at Day 3 and Day 10 and challenged with LD<sub>50</sub> determined in (A). (C) Increased activity of SKN-1 (*lax120*) does not rescue proteolytic capacity to degrade Suc-LLVY-AMC for Day 10 animals. (D) Increased activity of SKN-1 (*lax120*) does not rescue proteolytic capacity to degrade oxidized [<sup>3</sup>H] hemoglobin for Day 10 animals. Error bars denote standard error of the mean values. \**p* < .05, \*\**p* < .01, \*\*\**p* < .001 relative to control using Student's *t* test.

Pretreatment with H<sub>2</sub>O<sub>2</sub>, followed by a 16-hour recovery, resulted in a significant decrease in total protein carbonylation for young animals (Figure 7 and Supplementary Figure 8). SKN-1 gain-of-function also resulted in a significant decrease in carbonylation compared with the untreated N2 control, though pretreatment did not impact carbonylation for *lax120* animals. Moreover, for both Day 10 N2 and *lax120* animals, protein carbonylation did not decrease after pretreatment indicating that SKN-1 activation is beneficial for young animals, but does not alleviate detrimental phenotypes associated with decreased proteasome utility in older animals. Therefore, these results demonstrate that while young *lax120* mutants were able to increase proteolytic function in response to the adaptive H<sub>2</sub>O<sub>2</sub> treatment, old animals did not exhibit adaptation in any of these assays.

### Discussion

The overall decline of adaptive stress responses is thought to be a major factor in organismal aging and aging-related disease. Acute stress leads to the induction of transcriptional programs evolved to promote cellular homeostasis. Several studies have indicated that aging correlates with a defect for induction of these adaptive mechanisms (37). In parallel, another key to adaptation is the immediate response to stress marked by changes in affinity of existing cytoprotective proteins that play a role in proteome quality control. Our



**Figure 7.** Adaptation to oxidative stress reduces total protein carbonylation for Day 3, but not for Day 10 animals. Densitometry of total protein carbonylation for N2 and *lax120* animals  $\pm$  adaptation to H<sub>2</sub>O<sub>2</sub>. Error bars denote standard error of the mean values. \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  relative to control using Student's *t* test.

results demonstrate dynamic changes for adaptive signaling and proteolytic capacity during aging. These findings highlight the complexity of the proteasome system and the decline of transcriptional signaling in aging animals.

Many cell culture studies have found that proteasome function declines with replicative senescence (38–42) and that inhibition of the proteasome can induce senescence (43). These findings rely on *in vitro* proteolytic activity assays and Western blot analysis to evaluate ubiquitylation and expression of proteasome subunits. In addition to immortalized cell culture studies, primary cells cultured from old tissues have also exhibited a decrease in proteasome function for cells propagated from both human patients (44–46) and mammalian models (47). A caveat for these findings is that cells grown in culture become progressively closer to replicative senescence with each passage, which may not be the best parallel to model normal progressive physiological aging in an organism.

Interestingly, mammalian animal studies have indicated tissue-specific variability in proteasome function during aging. For instance, it was found that proteasome expression and proteolytic activity decline in the spinal cord of aged rats (48). However, despite increased oxidative stress, there was no change in proteolytic activity found in the cortex, cerebellum, or hippocampus during aging (49). In contrast, several studies have found aging-related increases in 20S expression and activity in skeletal muscle (50–54). Furthermore, Altun and colleagues found that expression of ubiquitin ligases and deubiquitylating enzymes also increase during aging, indicating a role for both the 20S and 26S conformations of the proteasome in muscle wasting (51). In addition to muscle tissue, increased 20S proteasome function has been noted in the liver (55) and spleen (56), whereas decreased function has been noted in the heart (47) and lung (57). Therefore, the regulation of proteasome activity is likely tissue dependent and varies depending on the aging continuum. Because our study used whole animal lysate for all biochemistry analyses, it is possible that the major proteasome pool is representative of muscle and intestine, which constitute the largest group of tissues in *C. elegans*. Our findings add to the field of proteasome research and help to further elucidate the dynamic regulation of 20S proteasome regulation during aging.

However, despite an increase in 20S proteasome activity, protein oxidation also increased with age. It should be noted that assays for proteolytic capacity are *in vitro* and it is not yet feasible to measure 20S proteasome activity *in vivo*. Therefore, it is possible that, although 20S proteasomes from aged cells have the capacity for proteolytic degradation, they may not actively degrade proteins *in vivo*. Proteins that are heavily oxidized have been shown to inhibit the proteasome (41,58–60). In addition, a variety of proteome quality control machinery are sequestered at protein aggregates, which increase during aging (61–63). Consistent with our data, a recent study in yeast found that enhancing protein disaggregation restores proteasome activity in replicatively aged cells, indicating that *in vivo* inactivation of proteasome function is reversible (64). Thus, increased protein oxidation during aging may be attributed to the accumulating, yet reversible, inactivation of the 20S proteasome.

We propose that the aging-related decline in SKN-1 signaling sensitivity in response to increasing stress contributes to the accrual of dysfunctional 20S proteasomes and the subsequent loss of adaptation during aging. Although enhanced SKN-1 activity increases stress tolerance, it does not rescue adaptation or result in an increase in life span. However, recent work has shown that direct overexpression of proteasome expression has a positive impact on health span and life span in *C. elegans* (65).

Our results indicate that understanding the balance between proteasome function and oxidatively damaged aggregated proteins is important in managing the collapse of protein homeostasis during aging. Further work is necessary to identify methods to alleviate declining adaptation of the 20S proteasome to resolve aging-dependent protein oxidation and aggregation.

## Supplementary Material

Supplementary material can be found at: <http://biomedgerontology.oxfordjournals.org/>

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