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Insulin/IGF-1 Signaling Regulates Proteasome Activity through the Deubiquitinating Enzyme UBH-4

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SUMMARY
The proteasome plays an important role in proteostasis by carrying out controlled protein degradation in the cell. Impairments in proteasome function are associated with severe and often age-related diseases. Here, we have characterized a molecular mechanism linking insulin/IGF-1 signaling (IIS) to proteasome activity. We show that decreased IIS, which promotes proteostasis and longevity, increases proteasome activity through the FOXO transcription factor DAF-16 in C. elegans. Furthermore, we reveal that DAF-16 represses expression of the proteasome-associated deubiquitinating enzyme ubh-4, which we suggest functions as a tissue-specific proteasome inhibitor. Finally, we demonstrate that proteasome activation through downregulation of the ubh-4 human ortholog uchl5 increases degradation of proteotoxic proteins in mammalian cells. In conclusion, we have established a mechanism by which the evolutionarily conserved IIS contributes to the regulation of proteasome activity in a multicellular organism.

INTRODUCTION
Maintenance of protein homeostasis is essential to all living organisms. As an important part of the proteostasis network, the ubiquitin-proteasome system (UPS) executes most of the controlled protein degradation in the cell. In the UPS, the substrate protein targeted for degradation becomes polyubiquitinated via the actions of the ubiquitin-activating E1 enzyme, conjugating E2 enzymes, and ligating E3 enzymes (Hershko and Ciechanover, 1998). The polyubiquitinated substrate is then recognized and degraded by the 20S proteasome, a large multisubunit protein complex consisting of a 20S core capped by one or two regulatory 19S lid particles. The 20S core is a barrel-shaped complex of 28 subunits (14 α and 14 β subunits), and it contains the proteolytic activities of the proteasome, whereas the 19S regulatory particle, responsible for capture, deubiquitination, and unfolding of the polyubiquitinated substrates, forms from 19 different subunits. In addition, there are multiple proteasome-associated proteins possessing essential activities, such as ubiquitin ligases and deubiquitinating enzymes (DUBs), which regulate proteasome function (Finley, 2009; Hanna and Finley, 2007). The 20S can also be activated by the 11S complexes and PA200/Blm10 to promote degradation, probably mainly in an ubiquitin-independent manner (Stadtmueller and Hill, 2011). Thus, the proteasome is not a static proteolytic machine; on the contrary, its function is highly regulated (Finley, 2009; Hanna and Finley, 2007). However, it remains an open question how proteasome activity is regulated in a multicellular organism.

Dysfunction of the proteasome has been detected in many severe disorders, such as Alzheimer’s, Parkinson’s, and Huntington’s diseases (Dahlmann, 2007), which usually emerge at the later stages of life. Accordingly, it has been postulated mainly based on in vitro proteasome activity assays that proteasomal degradation decreases upon aging. This might be due to reduced expression of proteasome subunits, a decrease in proteasome assembly, modifications of proteasome subunits, and/or inhibitory effects of oxidized and crosslinked substrates (Vernace et al., 2007). Additionally, the association and function of different proteasome regulators might change upon aging. Interestingly, an inhibitory mechanism seems to be part of the proteasome regulation and not only a physiological effect caused by aging, as the proteasome-associated DUB USP14 and its yeast ortholog Ubp6 have been shown to inhibit the proteasome (Hanna et al., 2006; Lee et al., 2010).

We have previously shown that proteasome activity changes upon aging in C. elegans (Hamer et al., 2010). This led us to ask whether signaling pathways regulating longevity also affect proteasome activity. Insulin/IGF-1 signaling (IIS) is an evolutionarily conserved signaling cascade regulating lifespan in worms, flies, rodents, and possibly humans (Kenyon, 2005, 2010). In C. elegans, reduced IIS caused by mutations in DAF-2 (insulin/IGF-1 receptor ortholog) leads to long-lived and stress-resistant worms (Kenyon et al., 1993), mediated through negative regulation of the FOXO transcription factor DAF-16 (Lin et al., 1997, 2001; Ogg et al., 1997). DAF-16 controls expression of multiple genes regulating diverse processes such as metabolism, stress responses, longevity, and development (Lee et al., 2003; Murphy et al., 2003). Decreased IIS also affects protein homeostasis during the normal aging process (Ben-Zvi et al., 2009; David et al., 2010) and under disease-related conditions (Cohen et al., 2006, 2009; Hsu et al., 2003; Morley et al., 2002).

In the current study, we demonstrate that proteasome activity is modified through the IIS and the proteasome-associated DUB...
UBH-4. Our data show that DAF-16 negatively affects ubh-4 expression. In line with being one of the many DAF-16 targets genes, our data reveal that ubh-4 has a mild effect on C. elegans longevity. Furthermore, our results demonstrate that knockdown of uch15, the mammalian ortholog of ubh-4, leads to increased degradation of proteins linked to proteotoxicity. Hence, we have established a molecular mechanism connecting the evolutionarily conserved IIS pathway to DUB-regulated proteasome activity.

RESULTS

IIS-Deficient daf-2 Mutants Display Higher UPS Activity than Wild-Type Animals

Aging causes a decline in protein homeostasis leading to accumulation of damaged and unfolded proteins. An increasing body of evidence suggests that the same signaling pathways that are involved in controlling aging, including IIS, also regulate the mediators of proteostasis, such as metabolic enzymes, stress proteins, and chaperones (Balch et al., 2008). Here, we investigate whether IIS also contributes to regulation of the UPS by using the well-established aging model C. elegans. We have previously developed an in vivo reporter system for UPS activity based on the photoconvertible UbG76V-Dendra2 reporter protein (Hamer et al., 2010; Li et al., 2011), which allows us to measure the rate of proteasomal degradation in live animals. Because the intestine is an important tissue for DAF-16-mediated longevity in C. elegans (Libina et al., 2003), we initially imaged UPS activity in intestinal cells. The photoconverted UbG76V-Dendra2 signal decreased by approximately 30% in 6 hr in wild-type animals compared to an approximately 2-fold faster degradation in the long-lived daf-2(e1370) mutants (Figures 1A and 1B; Tables S1 and S2) without an effect on the control Dendra2 (Figures S1A and S1B). RNAi against proteasome 19S subunit rpn-2 decreased the degradation of UbG76V-Dendra2 in the intestine of daf-2(e1370) mutants (Figure 1C), whereas RNAi of autophagy component <i>lgl-1</i> did not affect reporter degradation (Figure S1D), demonstrating that the reporter was proteasomally degraded in these animals. The enhanced UPS activity detected upon decreased IIS was DAF-16 dependent, as the effect was diminished in the daf-16(m26);daf-2(e1370) double mutant (Figures 1A and 1B). The short-lived daf-16(m26) or daf-16(mgDF50) mutants, deficient in five or all eight DAF-16 isoforms (Kwon et al., 2010; Lin et al., 1997; Ogg et al., 1997), showed similar reporter degradation as wild-type animals (Figure 1B). Together, these results demonstrate that under conditions of reduced IIS, active DAF-16 is mediating the enhanced UPS activity in the intestine.

We have previously shown that UPS reporter degradation is relatively slow in body-wall muscle cells (Hamer et al., 2010), and in line with this observation both the wild-type and daf-2(e1370) animals showed similar UbG76V-Dendra2 degradation of about 10% at 6 hr after photoconversion (data not shown). However, in 24 hr body-wall muscle cells of the daf-2(e1370) mutants exhibited a significantly faster UPS reporter degradation (approximately 1.2-fold) compared to wild-type animals (Figures 1D and S1C), which was not dependent on autophagy (Figure S1E). In contrast to the degradation in intestinal cells, the daf-16(m26);daf-2(e1370) or daf-16(mgDF47);daf-2(e1370) double mutations did not significantly decrease the reporter degradation rate (Figure 1D), suggesting that the enhanced degradation does not require active DAF-16 in body-wall muscle cells. Interestingly, daf-16(m26) mutants (deficient in five DAF-16 isoforms) displayed similar reporter degradation in body-wall muscle cells as the daf-2(e1370) mutants (Figure 1D). This appeared to be a muscle-specific function of the DAF-16 isoforms, because in daf-16(mgDF50) mutants (deficient in all DAF-16 isoforms) degradation was comparable to wild-type animals (Figure 1D). Altogether, reduced IIS leads to a minor increase in UPS activity also in body-wall muscle cells, but in a DAF-16-independent manner.

The proteasomal degradation of the UbG76V-Dendra2 UPS reporter is likely affected by specific ubiquitin ligase(s). Therefore, we have developed a fluorescent reporter that responds to changes in proteasome activity by detecting the pool of polyubiquitinated proteins in vivo. The reporter takes advantage of a commercially available short-lived fluorescent ZsProSensor fusion protein, which consists of ZsGreen fused to the C-terminal part of the mouse ornithine decarboxylase, a protein domain mediating proteasomal degradation in an ubiquitin-independent manner (Murakami et al., 1992). In line with being a proteasomal substrate, expression of the ZsProSensor in intestinal cells did not produce a fluorescent signal, suggesting that it is rapidly degraded in C. elegans (data not shown). We hypothesized that fusing the ZsProSensor to the ubiquitin interaction motifs (UIMs) of the proteasome 19S subunit RPN-10 would create a reporter that binds polyubiquitinated proteins via its UIM motifs, resulting in stabilization of the reporter and detectable fluorescence. Approaches using the polyubiquitin-binding domains have been reported for capturing the cellular pool of polyubiquitin chains from mammalian cell lysates (Bennett et al., 2007) and for visualizing polyubiquitinated proteins in mammalian cells (Sims et al., 2012). Expression of our polyubiquitin reporter in the C. elegans intestine resulted in visible fluorescent foci with a colocalizing pattern to immunostained polyubiquitinated proteasome proteins (Figure S2A). As we predicted, the polyubiquitin reporter also responded to proteasome dysfunction, as RNAi against rpn-2 generated 2.6-fold higher fluorescent signal compared to control RNAi-treated animals (Figure S2B), thus validating the function of this in vivo polyubiquitin reporter system. The change in fluorescence intensity is likely dependent on both the UIM domains and the ZsProSensor degron, as upon proteasome impairment we would expect less degron-mediated degradation of the reporter resulting in increased fluorescence, as well as an increase in cellular polyubiquitinated proteins, which would create more binding sites for the polyubiquitin reporter and result in increased reporter fluorescence. Thus, the polyubiquitin reporter stability is affected by the balance between proteasomal degradation and binding to polyubiquitinated proteins. By using the polyubiquitin reporter approach, we were able to confirm the importance of DAF-16 in enhancing proteasomal degradation in the intestine, as daf-16 RNAi increased the fluorescent intensity in the daf-2(e1370) background, but not in wild-type animals (Figure 2). These results further demonstrate that IIS has a crucial role in regulating proteasome activity in vivo.
Figure 1. Reduced IIS Increases UPS Activity In Vivo

(A) Fluorescence micrographs of wild-type, daf-2(e1370), and daf-16(m26);daf-2(e1370) animals expressing UbG76V-Dendra2 in intestinal cells before, after, and 6 hr after photoconversion.

(B) Quantification of UbG76V-Dendra2 degradation in intestinal cells of wild-type and IIS mutant animals.

(C) Quantification of UbG76V-Dendra2 degradation in intestinal cells of daf-2(e1370) animals after control or rpn-2 RNAi.

(D) Quantification of UbG76V-Dendra2 degradation in body-wall muscle cells of wild-type and IIS mutant animals. All graphs show the average percentage of green or red fluorescence relative to the initial fluorescence intensity (before photoconversion) or the intensity at the point of photoconversion (0 hr after conversion), respectively.

Scale bar 20 μm. Error bars, SEM, *p < 0.05 and **p < 0.01, Table S2. See also Figure S1.
To complement the in vivo approaches, we also investigated proteasome activity in whole-animal lysates (Figure 3A) using the well-known native in-gel proteasome activity assay with suc-LLVY-AMC-substrate (Elsasser et al., 2005), which enables visualization of active proteasome complexes. We observed that daf-2(e1370) mutants displayed approximately 1.6-fold higher proteasome activity compared to wild-type animals (Figure 3B), which is similar to the in vivo UPS reporter data (Figures 1A and 1B). The increase in activity was diminished in daf-16(m26);daf-2(e1370) double mutants (Figure 3B), further demonstrating that activation of DAF-16 through decreased IIS enhances proteasome activity. Proteasome activity was slightly increased in lysates from daf-16(m26) mutants (Figure 3B), which parallels the observation in body-wall muscle cells (Figure 1D) and show that the in vitro assay reflects the sum of proteasome activity. Taken together, both our in vivo and in vitro results demonstrate that the reduced IIS in long-lived daf-2(e1370) mutants enhances proteasome activity. Notably, a recent article (Vilchez et al., 2012b) stated that reduced IIS does not upregulate proteasome activity based on in vitro proteasome peptidase assay with whole-animal lysates from the sterile daf-2(mu150);fer-15(b26);fem-1(hc17) mutant strain. Our result is in agreement with previous microarray and quantitative mass spectrometry data, showing that proteasome subunits are not more highly expressed in daf-2 mutants than in wild-type animals (Dong et al., 2007; McElwee et al., 2003; Murphy et al., 2003; Zarse et al., 2012). Thus, the enhanced UPS activity detected upon decreased IIS in C. elegans is not derived from changes in proteasome levels, but rather from differences in the regulation of its function.

UBH-4 Is a Proteasome-Associated DUB in C. elegans

We hypothesized that there might be differences in the composition of the proteasome regulatory complexes between wild-type and daf-2(e1370) animals. We therefore analyzed RP2-core particle (CP) and RP-CP complexes isolated from native gel with LC-MS-MS and detected most of the 19S regulatory particles in both wild-type and daf-2(e1370) samples (Table S3). Variations in 19S RP composition or in the associated proteins may lead to differences in proteasome activity. Interestingly, the deubiquitinating enzyme (DUB) UBH-4 was detectable in wild-type, but not in daf-2(e1370) animals. We therefore analyzed RP2-core particle (CP) and RP-CP complexes isolated from native gel with LC-MS-MS and detected most of the 19S regulatory particles in both wild-type and daf-2(e1370) samples (Table S3). Variations in 19S RP composition or in the associated proteins may lead to differences in proteasome activity. Interestingly, the deubiquitinating enzyme (DUB) UBH-4 was detectable in wild-type, but not in daf-2(e1370) samples. UBH-4 has previously been shown to interact with the 19S subunit RPN13 by yeast two-hybrid experiment (Li et al., 2004) and the mammalian homolog UCHL5 to associate with RPN13 (Hamazaki et al., 2006; Qiu et al., 2006; Yao et al., 2006). To investigate the in vivo interaction in C. elegans, we expressed His- and streptavidin-binding-protein (SBP)-tagged UBH-4 and HA-tagged RPN-13 under their own promoters. Immunoprecipitation of UBH-4 in daf-2(e1370) mutants could arise from differences in proteasome abundance. However, no difference in the proteasome amount was detected between wild-type and IIS mutant animals by western blotting against the proteasome 20S alpha subunits (Figures 3C and 3D). Our result is in agreement with previous microarray and quantitative mass spectrometry data, showing that proteasome subunits are not more highly expressed in daf-2 mutants than in wild-type animals (Dong et al., 2007; McElwee et al., 2003; Murphy et al., 2003; Zarse et al., 2012). Thus, the enhanced UPS activity detected upon decreased IIS in C. elegans is not derived from changes in proteasome levels, but rather from differences in the regulation of its function.

Figure 2. IIS Affects the Stability of the Intestinal Polyubiquitin Reporter

(A) Fluorescence micrographs of control or daf-16 RNAi-treated wild-type animals expressing the polyubiquitin reporter (Pvha-6::UIM2-ZsPro-Sensor) in the intestine.

(B) Quantification of the fluorescent intensity of the intestinal polyubiquitin reporter in wild-type animals after control or daf-16 RNAi.

(C) Fluorescence micrographs of control or daf-16 RNAi-treated daf-2(e1370) animals expressing the polyubiquitin reporter (Pvha-6::UIM2-ZsPro-Sensor) in the intestine. Exposure time used for green fluorescent channel is longer in (A) than in (C).

(D) Quantification of the fluorescent intensity of the intestinal polyubiquitin reporter in daf-2(e1370) animals after control or daf-16 RNAi. Graphs show average fold change in fluorescence compared to the control RNAi (set as 1). Error bars, SEM, **p < 0.01, Table S2. See also Figure S2.
His-SBP-UBH-4, but not His-SBP-GFP, brought down HA-RPN-13 (Figure 4A), demonstrating that UBH-4 and RPN-13 interact in *C. elegans*.

To confirm the DUB function of UBH-4 in *C. elegans*, we downregulated UBH-4 by RNAi and analyzed the proteasome-associated DUB activity with in-gel activity assay. To specifically detect the proteasome-associated DUB activity, only the part of the native gel containing the RP2-CP and RP-CP complexes was incubated with the Ub-AMC substrate. A clear reduction in fluorescence, i.e., substrate hydrolysis, which reflects decreased deubiquitinating activity, was detected in lysates of *ubh-4* RNAi-treated animals compared to control lysates, establishing that UBH-4 functions as a DUB in *C. elegans* (Figure 4B). Additionally, we observed accumulation of polyubiquitinated proteins after *ubh-4* RNAi (Figures 4C and 4D), suggesting that the deubiquitinating activity of UBH-4 is required to maintain protein homeostasis.

Expression of *ubh-4* Is Negatively Regulated by DAF-16

As we detected UBH-4 in lysates from wild-type, but not *daf-2(e1370)* mutant animals in the mass spectrometry analysis, we speculated that the expression of *ubh-4* may be regulated through IIS. To examine the transcriptional regulation of *ubh-4*, we expressed GFP under the *ubh-4* promoter and 3’ UTR. GFP was detected in several tissues such as the muscles and neurons, with the highest expression being in the intestine (Figure 5A). The *ubh-4* expression decreased upon aging but without notable changes in the expression pattern (Figures S3A and S3B).

Interestingly, a clear difference between wild-type and *daf-2(e1370)* mutants was a high GFP expression in the intestine of young adult wild-type animals (Figure 5A, left panels), hardly detectable in the *daf-2(e1370)* mutants (Figure 5A, middle left panels). In turn, *daf-2(e1370)* mutants displayed higher GFP expression in embryos than the wild-type animals (Figure 5A, middle left panels). *daf-16(m26) and daf-16(m26);daf-2(e1370) mutants showed similar expression pattern as the wild-type animals with a strong GFP signal in the intestine (Figure 5A, right middle and right panels, respectively). Taken together, these data suggest that DAF-16 negatively regulates *ubh-4* expression in the intestine. Because different DAF-16 isoforms regulate expression of partly distinct sets of genes (Kwon et al., 2010), we also investigated the expression of *ubh-4* in *daf-16* deletion mutants in addition to the *daf-16(m26) point mutation. In both *daf-16(m26)* and *daf-16(mG374);daf-2(e1370) deletion mutants, the GFP expression pattern resembled wild-type with high intestinal expression (Figure S4A). To further confirm the DAF-16-dependent regulation of *ubh-4* expression in the intestine, we downregulated DAF-16 by RNAi in *daf-2(e1370) animals expressing Pubh-4::gfp. *daf-16* RNAi clearly increased the GFP signal in the intestine of *daf-2(e1370) mutants (Figure S4B), demonstrating that *ubh-4* expression is negatively regulated by DAF-16 in the intestine of *C. elegans*. The DAF-16a and DAF-16d/f isoforms have been connected to lifespan regulation (Kwon et al., 2010; Lee et al., 2001; Lin et al., 2001), and we therefore performed
DAF-16 isoform-specific RNAi. Both downregulation of daf-16a and daf-16d/f induced an upregulation of Pubh-4::gfp expression in the intestine of daf-2(e1370) mutants (Figure S4C), suggesting that multiple DAF-16 isoforms are regulating ubh-4 expression. We identified one canonical DAF-16 binding site (GTAAACA, Furuyama et al., 2000) on the proximal promoter (531–525 bp upstream from first coding codon) of ubh-4. This site is also contained within the modENCODE reported DAF-16 binding region defined by chromatin immunoprecipitation sequencing (ChIP-seq) of a DAF-16::GFP fusion protein (Celniker et al., 2009) (Figure S4D). We mutated the canonical DAF-16 binding site on Pubh-4::gfp expression vector and observed a clear increase in intestinal GFP expression in daf-2(e1370) animals (Figure 5B), suggesting that DAF-16 regulates ubh-4 expression from its canonical binding site on the ubh-4 promoter.

The overall ubh-4 expression was downregulated by 20% in daf-2(e1370) mutants compared to wild-type animals when analyzed by quantitative PCR (qPCR) (Figure 5C). However, daf-16(m26) and daf-16(m26);daf-2(e1370) double mutants showed similar levels of ubh-4 mRNA compared to wild-type animals (Figure 5C), providing further support for the observation that DAF-16 negatively regulates ubh-4 expression. To test whether we could mimic the ubh-4 expression level detected in daf-2(e1370) mutants, we performed ubh-4 RNAi dilution experiments. ubh-4 RNAi produced an approximately 80% decrease in ubh-4 mRNA levels (Figure 5D). By diluting ubh-4 RNAi bacteria to a final concentration of 1% with bacteria carrying an empty plasmid (pL4440), we were able to decrease ubh-4 mRNA levels by 20%, which is comparable to the level observed in daf-2(e1370) mutants (Figures 5C and 5D). Furthermore, diluted ubh-4 RNAi reduced the expression of UBH-4::GFP mainly in the intestine, whereas undiluted ubh-4 RNAi resulted in an almost total loss of UBH-4::GFP in all tissues (Figure 5E). Thus, experiments with diluted ubh-4 RNAi provide an opportunity to investigate proteasome function under similar ubh-4 expression conditions as in the long-lived daf-2(e1370) animals.

UBH-4 Regulates Proteasome Activity
As the long-lived daf-2(e1370) mutants have less UBH-4 and higher proteasome activity, we hypothesized that UBH-4 could function as a proteasome inhibitor. It has been previously shown that the human ortholog UCHL5 delays degradation of lightly polyubiquitinated substrates (Lam et al., 1997b) and that knockdown of uchl5 in HeLa cells leads to faster degradation of the UPS reporter protein Ub-R-GFP, as well as to a reduced amount of polyubiquitinated proteins (Koulich et al., 2008). In addition, it has been reported that another proteasome-associated DUB USP14 and its yeast homolog Ubp6 attenuate proteasomal degradation (Hanna et al., 2006; Lee et al., 2010). To address if UBH-4 regulates proteasome activity in C. elegans, we investigated how downregulation of ubh-4 by RNAi affected UPS activity in different tissues. Excitingly, both undiluted and diluted ubh-4 RNAi treatment resulted in higher UPS activity in the intesti...
Figure 5. Expression of *ubh-4* Is Regulated in a DAF-16-Dependent Manner

(A) Expression of *Pubh-4::gfp* reporter in wild-type and IIS mutants. i, intestine; n, nerve ring; p, pharynx; g, gonad; e, embryo; m, muscle; s, spermatheca. The exposure time varies between strains.

(B) Expression of *Pubh-4(D531-525+6bp)::gfp* reporter in *daf-2(e1370)* animals. Arrows point intestinal expression.

(C) Relative fold change in *ubh-4* mRNA levels.

(D) Relative fold change in *ubh-4* mRNA levels.

(E) *Pubh-4::ubh-4::gfp* (*rrf-3(pk1426)*) reporter in *daf-2(e1370)* animals. Arrows point intestinal expression.

(legend continued on next page)
intestine (Figures 6A and S5A). Accordingly, ubh-4 RNAi treatments decreased the polyubiquitin reporter signal in the intestine by 0.15- to 0.18-fold (Figure 6C). The enhanced proteasome activity detected in the intestine after ubh-4 RNAi is interesting considering that we observed accumulation of polyubiquitinated proteins in whole-animal extracts after the same treatment. This could be due to the accumulation of polyubiquitinated proteins in other tissues, because in muscle cells diluted ubh-4 RNAi did not affect UPS activity, and undiluted ubh-4 RNAi even caused decreased reporter degradation compared to control RNAi animals (Figures 6B and S5B). The decreased UPS activity detected in body-wall muscle cells after undiluted ubh-4 RNAi could stem from a starting decline in general physiology, as the animals looked sick when undergoing these imaging experiments. On the whole-animal level, ubh-4 RNAi treatments increased proteasome activity by 1.5- to 1.7-fold, as measured with proteasome in-gel activity assay in RNAi-sensitive rf-3(pk1426) animals (Figures 6D and 6E). N2 animals displayed a similar trend in upregulation of proteasome activity after ubh-4 RNAi (Figure S5C), but the effect was milder, which is probably due to the lower RNAi efficacy. The increase in proteasome activity detected in the in-gel assay after ubh-4 knockdown did not originate from general 19S disruption, because the proteasome complexes were intact (Figure 6D) and disruption of the 19S by RPN-2 RNAi resulted in decreased proteasome activity (Figures S5D and S5E). In addition, ubh-4 knockdown did not affect the levels of proteasome 20S alpha subunits (Figures S5F and S5G), revealing that UBH-4 directly regulates proteasome activity and not abundance.

**UBH-4 Slightly Affects Lifespan and Brood Size in C. elegans**

Because ubh-4 knockdown resulted in enhanced proteasome activity, we investigated whether it also causes phenotypic effects such as changes in lifespan and fertility. A systematic RNAi screen previously reported that ubh-4 RNAi slightly increases lifespan (Hamilton et al., 2005). When we performed experiments with undiluted ubh-4 RNAi started from the L1 stage in RNAi-sensitive rf-3(pk1426) animals, we did not detect an increase in lifespan, but instead consistently observed a minor decrease (Table S4). However, by using diluted ubh-4 RNAi, a slight but significant increase in lifespan was detected (Figure 6F; Table S4). Similar to the lifespan of rf-3(pk1426) animals, the lifespan of daf-16(mgDf47);rf-3(pk1426) mutants was shortened by undiluted ubh-4 RNAi, but no major changes were detected after diluted ubh-4 RNAi treatment (Figure 6F; Table S4). The lifespan of long-lived rf-3(pk1426);daf-2(e1370) mutants was not affected by either of the ubh-4 RNAi treatments (Figure 6F; Table S4), indicating that a further decrease in the ubh-4 mRNA level in daf-2 mutants does not provide beneficial effects on longevity. In support of our lifespan analysis, mortality calculation revealed that diluted ubh-4 RNAi decreased mortality of rf-3(pk1426) animals (Figure 6G), but did not affect mortality of long-lived rf-3(pk1426);daf-2(e1370) or short-lived daf-16(mgDf47);rf-3(pk1426) mutants (Figure S6A). Knockdown of ubh-4 by intestine- or muscle-specific ubh-4 RNAi did not reproduce the lifespan effect in animals with normal IIS (data not shown), indicating that some additional tissue(s) including the intestine is required for the ubh-4-mediated lifespan extension. Importantly, ectopic expression of ubh-4 from an intestinal promoter, which does not contain a canonical DAF-16 binding site, resulted in minor decrease in lifespan and increased mortality of daf-2(e1370) mutants when compared to control animals expressing intestinal Dendra2 (Figures 6H and 6I; Table S4).

We next tested whether ubh-4 RNAi affects brood size. We detected decreased brood size in rf-3(pk1426) animals after undiluted ubh-4 RNAi (Figure S6B; Table S5), which is in line with an earlier study reporting that ubh-4 RNAi causes embryonic lethality (Maeda et al., 2001). However, diluted ubh-4 RNAi did not significantly decrease brood size (Figure S6B; Table S5). Brood sizes of rf-3(pk1426);daf-2(e1370) or daf-16(mgDf47);rf-3(pk1426) mutants were not affected by ubh-4 RNAi treatments (Figure S6B; Table S5). Together, our data suggest that ubh-4 is one of the many DAF-16 target genes affecting cumulatively longevity, and that there might be a threshold in the UBH-4 level for the beneficial effects on lifespan without affecting other physiological processes, such as reproduction.

**ubh-4 RNAi Does Not Induce DAF-16 or SKN-1 Activation**

To investigate whether the ubh-4 RNAi-induced enhancements of proteasome activity and lifespan were derived from DAF-16-mediated effects, we measured the expression of selected DAF-16 target genes by qPCR after undiluted or diluted ubh-4 RNAi treatments. Although we noticed some fluctuation in mRNA levels after ubh-4 RNAi, no major changes in expression of DAF-16 target genes were observed (Figure S6C). Next, we studied the effects of ubh-4 RNAi on expression of SKN-1 target genes, as SKN-1, like DAF-16, is an IIS-regulated transcription factor that promotes longevity and stress responses (Tullet et al., 2008). ubh-4 RNAi has been reported to induce SKN-1::GFP translocation into the nuclei (Kahn et al., 2008). When we performed ubh-4 RNAi experiments, no major changes in the expression of the selected SKN-1 target genes were detected (Figure S6D). Together, our data show that the increases in longevity and proteasome activity caused by ubh-4 knockdown do not likely originate from activation of DAF-16 or SKN-1.

Recent reports have demonstrated that increased expression of a 19S subunit encoding gene rpn-6.1 and its human homolog PSMD11 are required for enhanced proteasome activity in germ-line-deficient C. elegans and human embryonic stem cells, respectively (Vlčech et al., 2012a, 2012b). To test whether the

(C) Expression of ubh-4 in wild-type and IIS mutants examined with qPCR. Graph shows the average fold changes in ubh-4 mRNA levels compared to the wild-type (set as 1). Results are averaged from three independent experiments. Error bars, SD.

(D) Expression of ubh-4 in rf-3(pk1426) animals examined with qPCR after diluted (1%) and undiluted ubh-4 knockdown. Graph shows the average fold changes in ubh-4 mRNA levels compared to the control RNAi (set as 1). Results are average from four independent experiments. Error bars, SD.

(E) Expression of Pubh-4::ubh-4::gfp in rf-3(pk1426) animals after diluted (1%) and undiluted ubh-4 RNAi. Arrows point intestinal expression.

See also Figures S3 and S4.
Figure 6. UBH-4 Regulates Proteasome Activity and Affects Lifespan

(A and B) (A) Quantified degradation of UbG76V-Dendra2 in intestinal cells and (B) in body-wall muscle cells of wild-type animals after control, diluted (1%), or undiluted ubh-4 RNAi. Graphs in (A) and (B) show the average percentage of green or red fluorescence relative to the initial fluorescence intensity (before photoconversion) or the intensity at the point of photoconversion (0 hr after conversion), respectively. Error bars, SEM, **p < 0.01, Table S2.

(C) Quantification of fluorescent intensity of intestinal polyubiquitin reporter in wild-type animals treated with control, diluted, or undiluted ubh-4 RNAi. Graph shows average fold change in fluorescence compared to the control RNAi (set as 1). Error bars, SEM, **p < 0.01, Table S2.

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*rpn-6.1* expression levels could be involved in the detected increase in proteasome activity reported within this study, we examined *rpn-6.1* expression in wild-type and *daf-2(e1370)* animals, as well as in *ubh-4* RNAi-treated animals. However, *daf-2(e1370)* animals and *ubh-4* RNAI-treated animals did not display an increase in *rpn-6.1* expression (Figures S6E and S6F), demonstrating that the upregulation of proteasome activity caused by reduced IIS or *ubh-4* knockdown is not mediated by changes in *rpn-6.1* expression.

**uchl5 Knockdown Increases the Degradation of the Aggregation-Prone Ataxin3 Mutant**

To examine if the regulation of *ubh-4* by the IIS pathway is evolutionarily conserved, we examined the expression of *uchl5* in human osteosarcoma (U-2 OS) cells under conditions of decreased IIS. By treating the cells with the PI(3)K inhibitor LY-294002, we detected approximately 30% decrease in *uchl5* mRNA levels as measured with qPCR (Figure 7A), indicating that human *uchl5* is also regulated by the IIS pathway. Next, we addressed whether UCHL5 affects proteasome activity. Knockdown of *uchl5* (Figure S7A) slightly increased degradation of UbG76V-GFP in U-2 OS cells (Table S6) without decreasing the level of the control GFP (data not shown). This result is in agreement with a previous report showing that *uchl5* small interfering RNA (siRNA) increases UPS activity in HeLa cells (Koulich et al., 2008).

Previously, it has been shown that the proteasome-associated DUB USP14 prevents degradation of several proteins that cause neurotoxicity (Lee et al., 2010). We investigated if *uchl5* knockdown can also affect the levels and aggregation of the neurotoxic protein ataxin3, which is known to undergo proteosomal degradation. For this purpose, we expressed ataxin3(Q28)-GFP and ataxin3(Q84)-GFP (Chai et al., 2002) and knocked down *uchl5* in U-2 OS cells. To analyze the microscopy images of the transfected cells, we developed a method to measure cellular fluorescence intensities (representing protein levels), number of fluorescent foci (representing possible aggregates) and intensity of the foci (Figure S7B; Table S6; Experimental Procedures). *uchl5* siRNA decreased the fluorescent signal in both ataxin3(Q28)-GFP- and ataxin3(Q84)-GFP-transfected cells, indicating that degradation of the transfected proteins was increased (Figure 7C; Table S6). We also detected a slight decrease in the number and a clear decrease in the intensity of the fluorescent foci in ataxin3-GFP-transfected cells after *uchl5* siRNA (Figure S7C; Table S6), implying that decreased levels of the transfected proteins affect the formation of aggregates. Together, these results show that, similar to the IIS-regulated UCHL5 in *C. elegans* (Figure 7D), a decreased level of UCHL5 increases UPS activity and enhances degradation of aggregation prone proteins in human cells.

**DISCUSSION**

The data we present establish an in vivo mechanism by which IIS regulates proteasome activity through the FOXO transcription factor DAF-16 target *ubh-4*. Together with Dr. Blackwell’s group, we have previously shown that the IIS is involved in regulating proteasome activity through the transcription factor SKN-1 in *C. elegans* (Li et al., 2011). Studies by other groups have also reported links between UPS and IIS by showing that FOXO transcription factors regulate the expression of ubiquitin ligases MAFbx and MuRF1, which contribute to muscle atrophy (Sandri et al., 2004; Sitt et al., 2004). Recent studies have also highlighted the roles of DAF-16 and FOXO4 in regulation of proteasome activity in germline-lacking *C. elegans* and human embryonic stem cells, respectively (Vilchez et al., 2012a, 2012b). In addition, DAF-16 and possibly some other IIS components are under proteasomal control, which affects lifespan in *C. elegans* (Kuhlbrodt et al., 2011; Li et al., 2007). Here, we have identified the DUB *ubh-4* as differentially expressed in wild-type and long-lived *daf-2* IIS mutants.

The increase in UbG76V-Dendra2 degradation and decrease in accumulation of polyubiquitinated proteins in the intestine, which we observed upon *ubh-4* RNAi, may stem from reduced ubiquitin chain trimming by UBH-4. In accordance, previous studies have demonstrated that proteasomal degradation of ubiquitinated substrates can be enhanced by deleting or inhibiting the ubiquitin-chain-trimming DUBs UCHL5 (UBH-4 ortholog) or USP14 (Koulich et al., 2008; Lam et al., 1997a, 1997b; Lee et al., 2010). It is likely that *ubh-4* also regulates proteasome activity in a noncatalytic manner, perhaps by inhibiting substrate entry to the proteolytic core, as we observed that *ubh-4* knockdown increases proteasome activity also in the ubiquitination independent in-gel activity assay. Interestingly, the yeast homolog of USP14, Ubp6, has been shown to inhibit substrate degradation in a manner independent of its deubiquitinating activity (Hanna et al., 2006). On the other hand, binding of polyubiquitinated proteins to USP-14 and UCHL5 can also activate proteasomal ATPases (Peth et al., 2013). Thus, proteasome-associated DUBs appear to exert their regulatory effects through multiple mechanisms.
Surprisingly, we detected increased accumulation of polyubiquitinated proteins in whole-animal extracts after *ubh-4* RNAi, although proteasome activity increased according to the in-gel proteasome activity assay and the intestinal fluorescent reporters. This could be a consequence of proteasome inhibition in other tissues, such as body-wall muscle, in which we detected decreased proteasome activity after *ubh-4* RNAi. The tissue-specific effects of *ubh-4* knockdown could derive from variable expression levels of *ubh-4* between tissues, as well as from differential expression of, for example, the proteasomal polyubiquitin receptor *rpn-13*. In mammalian cells, association of UCHL5 with RPN13 has been reported to activate UCHL5 (Lam et al., 1997b). In this scenario, deubiquitination may lead to substrate dissociation from the proteasome. Therefore, in our case, knockdown of ubiquitin-chain-trimming UBH-4 may lead to preferred and faster degradation of lightly ubiquitinated substrates and to the accumulation of heavily polyubiquitinated proteins, which can then be detected in the polyubiquitin western blot.

We have previously shown that the IIS-regulated SKN-1 affects proteasome activity mainly in the intestine (Li et al., 2011). The role of DAF-16 as a proteasome activity modulator through UBH-4 is also most prominent in the intestine, where *ubh-4* expression is downregulated in a DAF-16-dependent manner. Recent reports suggest a high degree of complexity in regulation of DAF-16-mediated processes with different isoforms crosstalking, regulating partly distinct sets of genes, and showing tissue specificity (Kwon et al., 2010). Additionally, the growth factor IGF-1, which inhibits FOXO activity, increases proteasome activity in mouse brain frontal cortex lysates (Crowe et al., 2009), whereas overexpression of constitutively active FOXO3 slightly increases proteasomal degradation in myotubes (Zhao et al., 2007). Together with these reports, our results establish that proteasome activity is under cell-type-specific regulation by the IIS.

Enhancement of proteasome activity has been shown to extend lifespan in yeast and *Drosophila* (Kruegel et al., 2011; Tonoki et al., 2009), and high proteasome activity has been observed in lysates from long-lived naked mole-rats and...
fibroblasts derived from centenarians (Chondrogianni et al., 2000; Pérez et al., 2009). Additionally, a recent study reported that ectopic expression of rpn-6 in C. elegans resulted in upregulated proteasome activity and extended lifespan during mild heat stress (Vichez et al., 2012b). We suggest that the mild effect of UBH-4 on C. elegans lifespan could derive from its regulation of proteasome activity. The minor lifespan effect of ubh-4 downregulation is in agreement with the cooperative action of the DAF-16 target genes on longevity (Kenyon, 2010). Because both activation of the proteasome and the slight increase in lifespan are attained after an approximate 20% decrease in total ubh-4 mRNA, we speculate that a subpopulation of proteasome complexes is lacking the associated UBH-4. Under these conditions, the proteasome-associated deubiquitinating activity would not be downregulated to an extent causing negative physiological effects, emphasizing the fine balance in gene expression required to control cellular processes.

In addition to our results showing that the IIS/DAF-16 pathway regulates proteasome activity, epidermal growth factor signaling has been reported to affect proteasomal degradation and longevity (Liu et al., 2011). Modulation of proteasome activity through multiple lifespan regulating pathways would provide flexibility in meeting the proteostasis requirements upon aging. Similar to many of the cellular processes in metazoans, we show that the enhancement of proteasome activity through downregulation of ubh-4 is evolutionarily conserved. Affecting the IIS in a cell-type-specific manner could create opportunities to modulate the proteasome in age-related disorders exhibiting dysfunctional proteasome.

**EXPERIMENTAL PROCEDURES**

**Nematodes**

C. elegans strains were grown under standard conditions (Brenner, 1974) at 20°C. N2 (Bristol) strain was used as the wild-type. N2, CB1370: daf-2(e1370)III, DR26: daf-16(m26), GR1307: daf-16(md540), DR1309: daf-16(m26);daf-2(e1370)III, GR1309: daf-16(md474);daf-2(e1370)III, CF1814: rrf-3(pk1426)II, rde-1(ne213)V; kbIs7, ubh-4

**Lifespan Assays and Progeny Counts**

All lifespan experiments were performed at 20°C. Synchronized animals were plated on RNAi feeding plates as L1 larvae (day 1). Animals were transferred to a new plate every second day and, once they stopped producing offspring, every few days. Animals were checked every day and classified as dead when they failed to respond to a gentle prod with a platinum pick. Animals that crawled off the plate, died from an extruded gonad or had internally hatched offspring were censored. Experiments were repeated three to five times.

**Plasmids and Generation of Transgenic Lines**

Cloning of Dendra2 and UbG76V-Dendra2 expression vectors has been described earlier (Hamer et al., 2010; Li et al., 2011). pPubh-4::gfp expression vector was created by amplifying 1375 bp promoter region upstream and 264 bp downstream of the ubh-4 coding region using PCR (see Table S7 for all primers used for cloning in this study). The PCR product amplified upstream of ubh-4 coding region was digested with PciI and AgeI, and the downstream amplified PCR product was digested with NcoI. Sequences were cloned into the pPD30.38 expression vector containing GFP coding sequence between inserted AgeI and NcoI restriction sites (the unc-54 promoter and enhancer were replaced).

To create the pPubh-4::Dendra2 vector, the pPubh-4::gfp expression vector was amplified with PCR using oligonucleotides having Smal restriction site in place of DAF-16 consensus sequence (gtaaca). To create the pPubh-4::ubh-4::gfp expression vector, the ubh-4 coding region amplified with PCR from C. elegans cDNA was digested with AgeI and cloned into the pPubh-4::gfp expression vector.

To create pPubh-4::His-gfp expression vector, the sequence encoding His-SBP tag was amplified with PCR (from Gateway Drosophilin expression vector, kind gift of Dr. Jussi Taipale), digested with AgeI and ligated into PciI (blunted with Klenow fragment) and AgeI-digested pPubh-4::gfp expression vector. For pPubh-4::His-ubh-4::gfp expression vector, GFP was replaced between Nhel and AgeI sites with ubh-4 coding region amplified with PCR from C. elegans cDNA. Expression vector pPphpn-13::HA-RPN-13 was created by amplifying with PCR 1,100 bp promoter sequence upstream of rpn-13 coding region. The PCR product was digested with EcoRV and AgeI and ligated into PciI (blunted with Klenow fragment) and AgeI-digested pPubh-4::gfp expression vector to replace ubh-4 promoter. GFP was replaced with rpn-13 coding region, which was amplified with PCR from C. elegans cDNA. PCR product was digested with AgeI and Acc65I. The sequence encoding HA tag was amplified with PCR (from Gateway Drosophilin expression vector, kind gift of Dr. Jussi Taipale), and the product was digested with AgeI and ligated in front of the rpn-13 coding sequence. To create vector for intestinal ubh-4 overexpression (pPvh-6::ubh-4), the ubh-4 coding region, amplified with PCR from C. elegans cDNA (digested with AgeI and Nhel andblunted with Klenow fragment), was used to replace the ZsGreen coding sequence in Nhel- and NotI-digested, Klenow-fragment-blunted pPvh-6::ZsGreen expression vector (not used in this study). All transgenic lines were created by microinjection (Mello et al., 1991). Of the strains carrying extrachromosomal arrays, four independent lines expressing transcriptional or translational ubh-4 reporters, two independent lines of Dendra2 or UbG76V-Dendra2 transgenic strains, and one line of both strains used in immunoprecipitation experiments were isolated and analyzed. Results shown are representative of all lines.

To create ZsProSensor expression vector for intestinal cells, ZsProSensor coding sequence was cut from pZsProSensor-1 vector (Clontech) with Nhel and NotI and ligated into the Nhel- and NotI-digested pPD30.38 expression vector (Addgene, Fire Lab vector kit) containing unc-54 promoter. To create polyubiquitin reporter (U14M-2-ZsProSensor) expression constructs for intestinal cells, ZsGreen-MDDC coding sequence was amplified from PCR from pZsProSensor-1 vector (Clontech). PCR product was digested with AgeI and NotI. Coding sequence for polyubiquitin binding motifs (ULMs, amino acids 195–313) of the RPN-10 was amplified with PCR from C. elegans cDNA, and the product was digested with Nhel and AgeI. PCR fragments were ligated into the Nhel- and NotI-digested pPD30.38 expression vector containing unc-54 promoter, the vha-6 promoter was amplified with PCR from Pvh-6::HSP1 expression vector (Morley and Morimoto, 2004) (kind gift of Dr. Richard Morimoto). The PCR product was digested with PciI and Nhel and used to replace unc-54 promoter in both ZsProSensor and polyubiquitin reporter expression vectors. The Polyubiquitin reporter extrachromosomal array was integrated using gamma irradiation and backcrossed five times with wild-type. One line carrying integrated transgene was used in experiments. The integrated line carrying polyubiquitin reporter transgene in daf-2(e1370) background was created by crossing.

For mammalian cell culture experiments, expression vectors pEGFP-C1-Ataxin3Q28 (Addgene plasmid 22122), pEGFP-C1-Ataxin3Q84 (Addgene plasmid 22123) (Chai et al., 2002), and UbG76V-GFP (Addgene plasmid 11841) (Dantuma et al., 2000) were purchased from Addgene. pEGFP-C2 expression vector (kind gift of Dr. Marikki Laiho) was used to express GFP in mammalian cells.
Mammalian Cell Culturing
Human osteosarcoma (U-2 OS) cells were cultured in Dulbecco’s modified Eagle’s medium with 15% fetal bovine serum (GIBCO). For siRNA experiments, FlexiTube GeneSolution for uch5i (QIAGEN) and AllStars Negative Control siRNA (QIAGEN) were used with HiPerFect Transfection Reagent (QIAGEN). Cells were incubated for 3 days with medium containing siRNAs. Expression vectors for atax3(Q28), atax3(Q84), and UbG76V-GFP were transfected with Fugene 6 (Roche) a day after siRNA transfection. Cells were fixed with 3.5% paraformaldehyde in PBS. For Pi(3)K inhibition, U-2 OS cells were incubated for 7 hr with 20 μM LY-294002 (Enzo Life Sciences).

C. elegans RNA Interference
Unless otherwise indicated, RNAi was performed using the feeding protocol as described earlier (Timmons et al., 2001). E. coli strain HT115 carrying the empty pL4440 expression vector was used as a control in experiments with RNAi clones from J. Ahhringer library.daf-2[e1370] mutants carrying intestinal UbG76V-Dendra2 were placed on rpn-2 RNAi (C23G10.4, J. Ahhringer library) as L3 larvae 2 days before photoconversion. For proteasome in-gel activity assays, rfn-3/pk(1426) animals were placed on rpn-2 RNAi as L4 larvae and harvested 2 days later. Animals expressing polyubiquitin reporter in the intestine were maintained on control RNAi bacteria and transferred to rpn-2 RNAi at L4 larvae and imaged 3 days later. Bacteria carrying empty RNAi vector (pL4440) was used to dilute ubh-4 RNAi bacteria. In imaging experiments, ubh-4 and lgg-1 (C08B11.7 and C32D5.9, respectively, J. Ahhringer library) RNAi were initiated from hatching. ubh-4 RNAi was initiated from L1 stage in proteasome in-gel activity assays, lifespan experiments, and progeny counts. For daf-16 RNAi (pAD43) and pD12 (control RNAi for daf-16) (kind gifts of Dr. Andrew Dillin), 10 mM isopropl (β-D-1-thiogalactopyranoside (IPTG) was used to induce production of double-stranded RNA, and, before seeding the plates, IPTG concentration was increased to 20 mM. L4440-daf-16a (Addgene plasmid 31503) and L4440-daf-16 daf (Addgene plasmid 31505) RNAi expression vectors (Kwon et al., 2010) were purchased from Addgene. daf-16 RNAi was initiated from hatching in all experiments and imaged at first day of adulthood.

C. elegans Immunofluorescence
An unsynchronized population of animals expressing polyubiquitin reporter was fixed and permeabilized as described previously (Finney and Ruvkun, 1990). Polyubiquitinated proteins were immunostained with FK1 antibody (Enzo) and visualized with Alexa Fluor 594 (Invitrogen).

Microscopy, Image Analysis, Equipment, and Settings
For UbG76V-Dendra2 and Dendra2 imaging, young adult (4 and 5 days old with transgenic animals in daf-2[e1370] background) animals were imaged. Dendra2 imaging was done as described earlier (Hamer et al., 2010). Briefly, animals were mounted on an agarose pad on glass slides and immobilized using 0.5 mM levamisole in M9 (22 mM KH2PO4, 41 mM NaH2PO4, 8.5 mM NaCl, and 19 mM NH4Cl). Animals were recovered on feeding plates in between imaging steps. For confocal imaging, a motorized Zeiss Axio Observer Z1 inverted microscope with LSM 5 Live line scanner and LSM 710 software Rel. 4.2 was used. Images were acquired with 63 × 1.4 numerical aperture (NA) plan-achromat objective. Photoconversion was carried out using a diode 405 nm (50 mW). Diode-pumped solid-state (DPSS) (488 nm; 100 mW) and 543 nm helium-neon (1 mW) lasers. Cells were incubated for 3 or 6 hr using 40 or 20 mA as a constant, respectively. Developing buffer with 80 μM of suc-LLVY-AMC (Bachem) was used to develop the gel. Coomassie staining was done with Colloidal Blue Staining Kit (Invitrogen). For deubiquitinating assay on native gel, electrophoresis was done as described above. Development of the gel was done in developing buffer (Elssasser et al., 2005) supplied with 1 mM Ub-AMC (Boston Biochem) for 1 hr at 30°C. Gels were imaged with Multilimage Light Cabinet using FluoroChem (3.04B program (Alpha Innotech Corporation). Signal levels were adjusted using Photoshop 9.0 (Adobe Systems) and quantified analyzed using Fiji. The quantification of the proteasome activity is the sum of fluorescent signal within the bands representing RP2-CP, RP-CP, and CP. As a technical point, we noticed that the proteasome activity measurements using the in-gel activity assay produce sometimes inconsistent results, and therefore multiple repeats were performed.

Mass Spectrometry Analysis
The proteins were separated using native gel and visualized with Coomassie staining. For protein identification, gel lanes containing RP2–CP and RP-CP proteasome complexes were extracted followed by in-gel trypsin digestion.
Peptides were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Ultimate 3000 nano-LC (Dionex) and a QSTAR Elite hybrid quadrupole TOF-MS (Applied Biosystems/MDS Sciex) with nano-ESI ionization as described previously (Ohman et al., 2010). The LC-MS/MS data were searched with in-house Mascot version 2.2 through ProteinPilot 3.0 interface against the NCBInr 20100513 database (10,987,230 sequences; 3,739,173,051 residues, taxonomy: C. elegans [28,762 sequences]). All of the reported protein identifications are statistically significant (p < 0.05).

**Immunoprecipitation and Western Blotting**

His-tag immunoprecipitation was done with Protoni Ni-NTA agarose (Macherey-Nagel). Western blot samples were separated on a polyacrylamide gel and blotted onto a nitrocellulose membrane using a semidry blotting system (Bio-Rad). FK1 antibody for polyubiquitinated proteins (Enzoproteasome 20S α1, 2, 3, 5, 6, and 7 subunits antibody (Enzo), anti-α-tubulin antibody (Sigma), streptavidin-HRP (Dako), and anti-HA antibody (Covance) were used in blotting. Signal levels were adjusted using Photoshop 9.0 (Adobe Systems) and quantified using Fiji.

**Statistical Analysis**

With Dendra2 and polyubiquitin reporter imaging, the statistical difference of relative protein degradation between different strains or RNAi treatments was determined by applying the Student’s t test (two-tailed). Lifespan counts, the statistical difference between different RNAi treatments was significant (p < 0.05).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.05.012.

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