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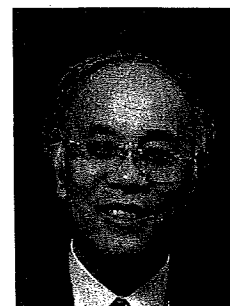
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Profile

Keiji Tanaka was born in 1949 and started his research career in 1972 with studies on the amino acid and protein metabolism in the Institute of Enzyme Research, after graduating from the Faculty of Medicine (School of Nutrition) at The University of Tokushima. He received his Ph.D. from The University of Tokushima in 1980, working on the hepatic protein metabolism. He was promoted to assistant professor in 1976 and associate professor in 1995 at the Institute for Enzyme Research at The University of Tokushima, and head of the Department of Molecular Oncology in 1996 and Vice-Director in 2002 at The Tokyo Metropolitan Institute of Medical Science. He is an acting director at The Tokyo Metropolitan Institute of Medical Science since 2006. Over the past 25 years, he focused on elucidating the structure and molecular/physiological functions of the proteasome. The discoveries of proteasomes in 1988, immunoproteasomes in 1994, hybrid proteasomes in 2000, and thymoproteasomes in 2007 are the highlights of his study. His current research interests include intracellular proteolysis mediated by the proteasome, ubiquitin, and autophagy system in eukaryotes in general. He was awarded the Naito Memorial Foundation Prize in 2003, the Asahi Culture Prize and the Uehara Prize in 2004, and the Toray Science Technology Prize in 2007. At present he is a guest professor of Ochanomizu Woman's University, Tokyo Medical and Dental University, The University of Tokyo Graduate School of Frontier Sciences, Juntendo University School of Medicine, and Niigata University School of Medicine.



Genetic Evidence Linking Age-Dependent Attenuation of the 26S Proteasome with the Aging Process^{∇†}

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The intracellular accumulation of unfolded or misfolded proteins is believed to contribute to aging and age-related neurodegenerative diseases. However, the links between age-dependent proteotoxicity and cellular protein degradation systems remain poorly understood. Here, we show that 26S proteasome activity and abundance attenuate with age, which is associated with the impaired assembly of the 26S proteasome with the 19S regulatory particle (RP) and the 20S proteasome. In a genetic gain-of-function screen, we characterized *Rpn11*, which encodes a subunit of the 19S RP, as a suppressor of expanded polyglutamine-induced progressive neurodegeneration. *Rpn11* overexpression suppressed the age-related reduction of the 26S proteasome activity, resulting in the extension of flies' life spans with suppression of the age-dependent accumulation of ubiquitinated proteins. On the other hand, the loss of function of *Rpn11* caused an early onset of reduced 26S proteasome activity and a premature age-dependent accumulation of ubiquitinated proteins. It also caused a shorter life span and an enhanced neurodegenerative phenotype. Our results suggest that maintaining the 26S proteasome with age could extend the life span and suppress the age-related progression of neurodegenerative diseases.

Ubiquitin-conjugated, misfolded protein aggregates are observed in the brain during normal aging and in late-onset human neurodegenerative diseases, such as Alzheimer's, Parkinson's, and polyglutamine diseases (e.g., Huntington's disease or spinocerebellar ataxias) (9). Many of the mutations that cause dominantly inherited neurodegenerative diseases dramatically increase the amount of protein aggregates in vitro and in vivo, supporting the widely accepted hypothesis that proteotoxicity caused by the aggregates underlies the pathogenesis of many neurodegenerative diseases (32). Proteotoxicity can have many effects, including disruption of microtubule-dependent axonal transport (10), perturbation of membrane permeability (23), and impaired function of the ubiquitin-proteasome system (UPS) (1, 17). Aggregation-mediated toxicity has also been suggested in normal aging, because recent reports show that the impairment of autophagy in the central nervous system causes accumulation of ubiquitinated proteins and leads to neurodegenerative diseases (12, 21). These observations suggest that the continuous clearance of misfolded proteins through cellular degradation systems, including the UPS and autophagy, is important for preventing aggregation-mediated proteotoxicity both in age-related neurodegenerative diseases and in normal aging.

Clinical symptoms of neurodegenerative diseases generally do not appear or progress until advanced ages, not only in sporadic forms but also in inherited forms of neurodegenerative diseases (26). These observations suggest that aggregation-mediated toxicity appears in a late-onset manner both in normal aging and in neurodegenerative diseases. Furthermore, a link between the aging process and aggregation-mediated proteotoxicity has been suggested by evidence that Huntington's disease-associated proteotoxicity was ameliorated when the aging process slowed, that is, the life span extension via decreased insulin/insulin growth factor-1-like signaling in *Caenorhabditis elegans* (13, 31).

A possible mechanism for the late onset of aggregation-mediated toxicity is age-related impairment of the UPS, because loss-of-function mutations in genes encoding UPS components can enhance the cytotoxicity of protein aggregation in dominantly inherited neurodegenerative diseases (4, 5, 18). In addition, an age-related decline of proteasome activity has been observed in the tissues of humans and other mammals (8) and in aged flies (36). Considering the role of the proteasome in neuroprotection and the age dependence of most neurodegenerative diseases, the age-related decline of proteasome activity could well be a key factor both in normal aging and in the late onset and/or progression of neurodegenerative diseases. However, the mechanism underlying the age-related decline of proteasome activity remains to be elucidated, and there is no direct genetic evidence showing that the age-related decline of proteasome activity causes age-related aggregation-mediated toxicity in normal aging and in age-related neurodegenerative diseases.

Here, we studied the age-related decline of proteasome ac-

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tivity by using *Drosophila melanogaster* and found age-related attenuation of the 26S proteasome activity and abundance that was associated with impaired assembly of the 26S proteasome with the 19S regulatory particle (RP) and the 20S proteasome. In a genetic gain-of-function screen, we identified *Rpn11*, which encodes one of the lid subunits in the 19S RP, as a suppressor of the age-dependent progression of a polyglutamine-induced neurodegenerative phenotype. The overexpression of *Rpn11* prevented the age-related reduction of the 26S proteasome activity, which suppressed the age-dependent accumulation of ubiquitinated proteins and extended the life span. On the other hand, the loss of function of *Rpn11* enhanced the age-related reduction of 26S proteasome activity, leading to a shorter life span, a premature age-dependent accumulation of ubiquitinated proteins, and an early onset of a neurodegenerative phenotype. Our results demonstrate for the first time that the age-related reduction of the 26S proteasome activity is a key factor in the induction of certain age-related biological changes and in the increased risk for the onset or progression of neurodegenerative diseases. Our findings imply that improving the amount and/or activity of the 26S proteasome by overexpressing a lid subunit, such as *Rpn11*, could provide an extension to the mean life span and prevent the age-dependent onset or progression of neurodegeneration.

MATERIALS AND METHODS

Fly stocks and generation of transgenic flies. Flies were raised on standard *Drosophila* medium at 25°C, and transgenic strains were generated as described previously (22). The *w¹¹¹⁸* strain was used as the wild-type strain. The *UAS-LacZ*, *tub-GAL80^Δ*, *GMR-GAL4*, and *da-GAL4* (Bloomington *Drosophila* Stock Center), *UAS-MJDT-Q78* (2), *GMR-huntingtin 120Q* (16), and *UAS-LacZIR* (20) fly strains were used in this study. The gene search (GS) system alleles were gifts from T. Aigaki. Using the temporal and regional gene expression targeting (TARGET) system, we raised flies at the restrictive temperature (18°C) before eclosion to suppress the activity of *GAL4*. One day after eclosion, the flies were moved to the permissive temperature (29°C) (28). For the longevity assay, more than 200 males of each genotype were collected and cultured in vials containing 20 males each. The flies were maintained at 29°C from day 2 and transferred to vials with fresh food every 3 to 4 days, and deaths were scored every day. Statistical significance was defined as a *P* value of <0.0001 by log-rank test.

Screening methods. To identify the suppressor for neurodegeneration-induced cell death, we adapted the P element-based GS system (33). The GS system vector contains two copies of the upstream activating sequence (UAS) enhancer adjacent to a core promoter: there is one copy near the terminal inverted repeats at each end of the vector, and each is oriented to direct transcription outward. This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS vector at various locations. For the first screen, we performed a dominant-modifier screen for heat-shocked *GAL4* (*hs-GAL4*)-mediated *Reaper* (a proapoptotic gene in *Drosophila*)-induced lethality to identify a strong suppressor of cell death. We identified 5 suppressor alleles from 1,600 alleles. Expecting that this collection of suppressor alleles would include suppressors of neurodegeneration, in the second screen, we performed a dominant-modifier screen for *GMR-GAL4*-mediated polyglutamine-induced neurodegenerative cell death. One allele, named *DANC* (defender against neural cell death), was identified as such a suppressor.

Immunoblotting. Whole flies or fly heads were prepared, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted as described previously (22). All samples were separated by 10% SDS-PAGE, and the blots were probed with antihemagglutinin (anti-HA) Y11 (1:500; Santa Cruz Biotechnology), mouse antiubiquitin (1:500; Stressgen Biotechnologies), anti- β -tubulin (1:500; Chemicon), anti- α 2 (1:1,000) (11), or anti-Rpn11 (1:300) primary antibody and with anti-mouse immunoglobulin G-horseradish peroxidase (1:1,000; Promega) or anti-rabbit horseradish peroxidase-linked immunoglobulin G (1:1,000; Cell Signaling) secondary antibody. For the glycerol density gradient analyses, 200 μ l of each fraction was precipitated with acetone and subjected to SDS-PAGE. The relative amount of each protein was determined by densitometric analysis using Image Gauge software (Fujifilm) (22).

Quantitative data were obtained as the ratio of the indicated protein signal to that of the loading control for each immunoblot and were plotted as a ratio graph.

Histology and immunohistochemistry. The heads of adult flies from each genotype were fixed and embedded in Epon resin for the preparation of semithin horizontal sections (1 μ m). The sections were stained with toluidine blue and examined under a light microscope as described previously (18). Immunohistochemistry treatment of eye discs from third-instar larvae was carried out as described previously (22). The following antibodies were used: rabbit anti-HA (1:500; Santa Cruz Biotechnology) and rat anti-ELAV 7E8A10 (1:20; Hybridoma Bank). Alexa Fluor 488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes and Jackson ImmunoResearch and used at 1:100. All fluorescently labeled samples were examined with an LSM5 model confocal microscope (Carl Zeiss).

Proteasome activity. Whole flies or heads alone were homogenized in buffer B (25 mM Tris-HCl [pH 7.5], 2 mM ATP, 5 mM MgCl₂, and 1 mM dithiothreitol). Proteasome peptidase activity in the lysates was measured with a synthetic peptide substrate, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-AMC) (Sigma).

Glycerol density gradient analysis. The whole bodies of 70 flies of the appropriate genotypes were homogenized in buffer B. The lysates were clarified by centrifugation at 20,000 \times g, and the lysates (1 mg of protein) were subjected to 8 to 32% (vol/vol) linear glycerol density gradient centrifugation (22 h, 83,000 \times g). The gradient was separated into 32 fractions (11), and proteasome peptidase activity in 10 μ l of each fraction was measured with Suc-LLVY-AMC activity.

Plasmid construction. The expressed sequence tag clone RE07468 was purchased from Invitrogen as full-length *Rpn11* cDNA. The sequence of the clone was confirmed, and an *Rpn11* fragment was amplified with the following PCR primers: 5'-CGG GGT ACC ACC ATG GAT CGT CTG CTA CGT CTT GGA-3' and 5'-GCT CTA GAT CAC TTA AAG ACT ATG GTG TCC A-3'. The amplicon was then inserted into the KpnI-XbaI sites of the *pUAST* vector to generate *pUAST-Rpn11*. For the RNA interference experiment, a fragment containing the first 500 bp of the *Rpn11* open reading frame was inserted into *pUAST-R57* (a gift from R. Ueda), as previously described (22).

Anti-Rpn11 antibody. The N-terminally His₆-tagged recombinant Rpn11 (His-RPN11) protein was produced in the *Escherichia coli* strain BL21(DE3) pLysS (Novagen) using *pRSET-His-Rpn11* by incubating the bacteria for 20 h at 20°C. The recombinant His-Rpn11 protein expressed in *E. coli* BL21(DE3) was purified on a Ni²⁺ column and used to immunize rabbits (Hokudo Co.).

RT-PCR analysis. For reverse transcription-PCR (RT-PCR) analysis of the genes surrounding the *DANC* allele or of other GS system fly lines, flies expressing *DANC* or other GS system genes with *hs-GAL4* were treated or not with heat shock (twice at 37°C for 30 min, with a 30-min interval between treatments), and their total RNA was prepared 3 h later by using Trizol (Invitrogen). The RNA was reverse transcribed and subjected to PCR analysis (26 cycles) using the following primers: *Rpn11* (5'-ACT TAA AGA CTA TGG TGT CCA-3' and 5'-TGG ATC GTC TGC TAC GTC TT-3'); *Rpn9* (5'-CGG GGT ACC ACC ATG TCC AAT CCT CAG CC-3' and 5'-GCT CTA GAC TAA TTG GTG AGG ATT TCG GC-3'); *Rpn5* (5'-CGG GGT ACC ACC ATG GAC ACC TAT TTG TT-3' and 5'-GCT CTA GAT TAA TCC TCG ACA GCA CAC AT-3'); *Rpn2* (5'-ATG AGT CTT ACG TCC GCC GCG-3' and 5'-GAT GCA ACT TTT CAA CCT CGT T-3'); $\alpha 6$ (5'-ATG TTT CGC AAC CAG TAC GAT AG-3' and 5'-CTA TGG ACG CTG CTC GGT TGC AA-3'); and *GAPDH* (5'-CCA CTG CCG AGG AGG TCA ACT A-3' and 5'-GCT CAG GGT GAT TGC GTA TGC A-3').

RESULTS

Proteasome activity decreases and ubiquitinated proteins accumulate with age. To gain insight into the cause of the age-related proteasomal dysfunction, we assessed the peptidase activities of the proteasome with age in *Drosophila*. The peptidase activities of the proteasome in lysates of wild-type fly heads were measured by Suc-LLVY-AMC-hydrolyzing activity (chymotrypsin-like activity), which cleaves peptide bonds after hydrophobic amino acids. Lysates of wild-type fly heads showed gradually decreased proteasome activity with age (Fig. 1A). The proteasome activity remarkably decreased in flies that were 20 to 30 days posteclosion, the time when age-related symptoms, such as memory impairment, are first

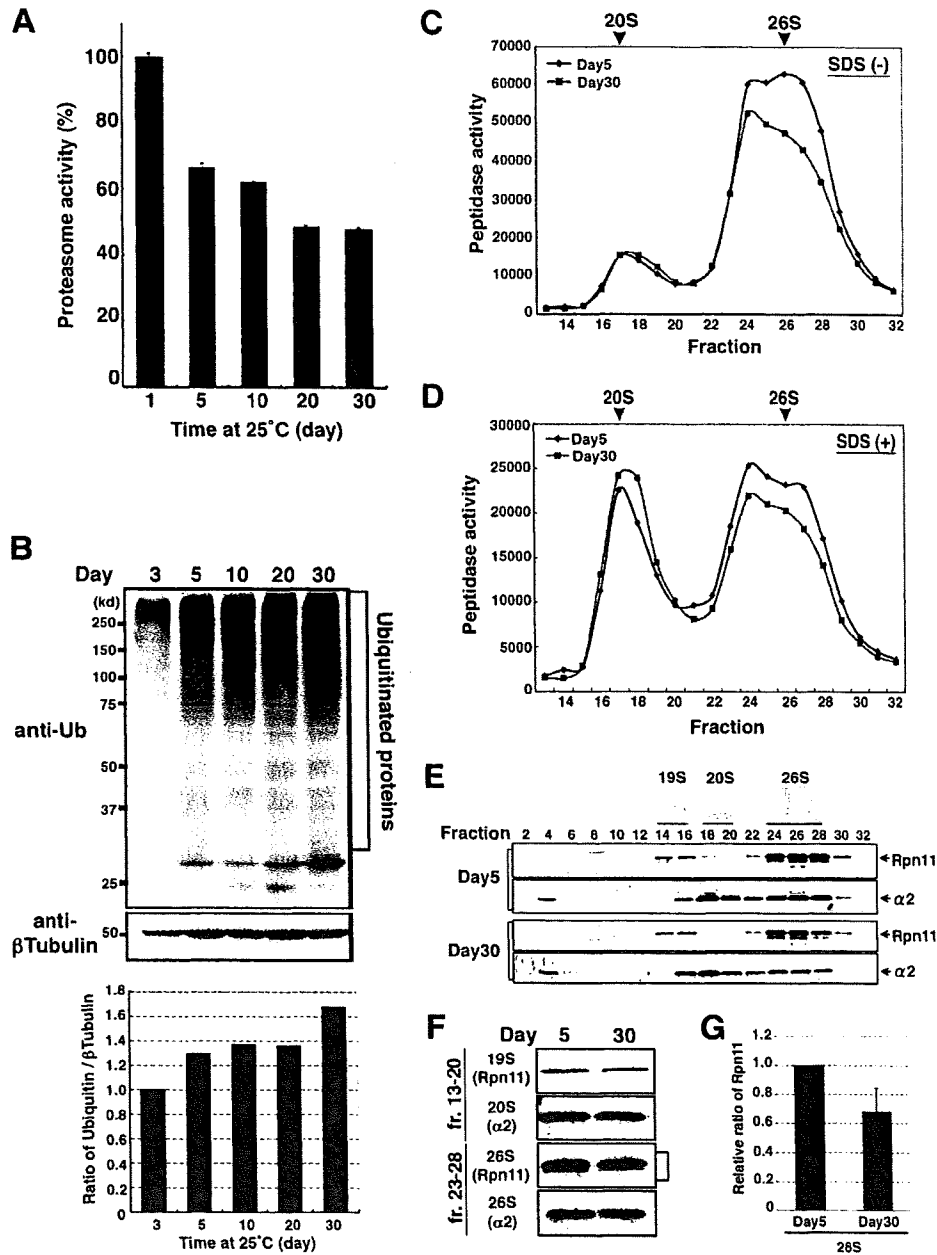


FIG. 1. The 26S proteasome activity decreases and ubiquitinated proteins accumulate with age. (A) Proteasome activity in wild-type flies decreased with age. The proteasome activity in wild-type fly heads was measured by hydrolysis of Suc-LLVY-AMC (chymotrypsin-like activity) on the indicated day posteclosion. (B) Accumulation of ubiquitinated proteins with age in wild-type flies. Wild-type fly head extracts were immunoblotted with antiubiquitin (anti-Ub) or anti- β -tubulin antibodies on the indicated day posteclosion. (C) The 26S proteasome activity in aged flies was significantly lower than that in young flies. Extracts of day 5 or day 30 flies were fractionated by glycerol density gradient centrifugation, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (D) Suc-LLVY-AMC hydrolysis activities of the same lysates shown in panel C were measured with the addition of 0.01% SDS [SDS(+)], a potent artificial activator of the 20S proteasome. The peptidase activity of the 20S proteasome did not significantly change between day 5 and day 30. (E) Immunodetection of the Rpn11 or the α 2 subunit revealed a decrease in the level of this subunit with age in the 26S-containing fractions. Immunoblot analysis shows the even fractions probed with antibodies against Rpn11 and α 2. (F) Fractions (fr. 13 to 20 (19S or 20S included) or fractions 23 to 28 (26S included) shown in panel E were pooled and immunoblotted with antibodies against Rpn11 or α 2. (G) The protein level of the 26S proteasome is lower in the aged fly than in the young fly. The amount of Rpn11 protein (the amount of the band indicated on panel F by a line) was determined by densitometric analysis. The graph shows the relative ratio of the amounts of Rpn11 in fractions 23 to 28 (26S included) at day 30 to that at day 5 from three individual experiments.

detectable (the normal *Drosophila* life span is 60 to 70 days) (40). This finding indicated that the reduction of proteasome activity was age dependent, as expected.

Because the proteasome maintains cellular homeostasis, we examined whether the age-related decline of proteasome activity caused the accumulation of unfolded proteins, represented by polyubiquitinated proteins. Extracts were made from wild-type fly heads or whole bodies and immunoblotted with an antiubiquitin antibody. Polyubiquitinated proteins, which showed a high-molecular-weight smear, gradually increased with the age of the flies, in both head (Fig. 1B) and whole-body extracts (see Fig. 5A). These results indicate that the reduction of proteasome activity and the accumulation of ubiquitinated proteins are observed with age.

The age-related reduction of proteasome activity results from the attenuation of the 26S proteasome. Polyubiquitinated proteins are recognized and degraded by the 26S proteasome. The 26S proteasome is a huge protein complex composed of one proteolytically active 20S proteasome and two 19S RPs, each attached to one end of the 20S proteasome (37). To compare the biochemical nature of proteasomes in young with that in aged flies, we performed an 8 to 32% glycerol density gradient centrifugation analysis and analyzed each fraction for chymotrypsin-like activity, using a peptide substrate (Suc-LLVY-AMC). The peptidase activity, particularly in the 26S proteasome-containing fractions (Fig. 1C, fractions 24 to 28) of the aged flies, was significantly lower than that of the young flies (Fig. 1C). On the other hand, the peptidase activity of the 20S proteasome, which was assayed in the presence of 0.01% SDS, a potent artificial activator of the latent 20S proteasome, did not significantly change with age (Fig. 1D).

Next, we tested the protein levels of each proteasome. Each fraction was subjected to immunoblotting with an anti-Rpn11 antibody to detect the 19S RP and the 26S proteasome or with an antibody against the $\alpha 2$ subunit to detect the 20S and 26S proteasomes. The immunodetection of Rpn11 and $\alpha 2$ revealed that the protein level of the 26S proteasome was lower in the aged fly than that in the young fly; however, the protein levels of the 20S proteasome and the 19S RP remained essentially stable with age (Fig. 1E, F, and G). These data indicate that the age-related reduction of proteasome activity results from the attenuation of the activity and amount of the assembled 26S proteasome.

Identification of *Rpn11*, a component of the 19S RP, as a suppressor of progressive neurodegeneration. Previously, we showed that the inhibition of proteasome function in flies expressing expanded polyglutamine enhances both the accumulation of ubiquitin conjugates and polyglutamine-induced neural degeneration (18). To examine whether the age-related attenuation of the 26S proteasome causes the age-dependent progression of neurodegenerative diseases, we focused on a progressive phenotype of neurodegenerative diseases. Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3, is the most common dominantly inherited progressive ataxia caused by polyglutamine expansion. Expression of a truncated form of the human MJD protein with an expanded polyglutamine domain (MJDtr-Q78) causes progressive neural degeneration in *Drosophila* (39). The expression of MJDtr-Q78 driven by the eye-specific promoter *GMR* caused

only a slight disruption of the regular, external lattice of the eye in young flies (Fig. 2A); however, the eye morphology of these flies showed progressive degeneration over time, with significant loss of pigmentation by day 15 (Fig. 2B).

To identify the suppressor of neurodegeneration-induced cell death, we adapted the P element-based GS system (33). This system combined with the "local hops" technique (34) enabled us to perform an efficient gain-of-function screen using alleles containing the GS system vector, a P element-based vector with UAS enhancers, at various locations. In the course of performing a gain-of-function screen for suppressors of neural cell death, using fly alleles with the GS vector, we identified 1 of 1,600 alleles, which we called the *DANC* allele, that suppressed the progression of the MJDtr-Q78-induced degenerative phenotype (Fig. 2C and D, and see Materials and Methods). We used the inverse PCR method to determine the insertion site for the GS vector in the *DANC* strain; this vector can induce transcription bidirectionally from its insertion site (Fig. 2I). The PCR amplified a predicted gene, *Rpn11*, the expression level of which was elevated in a GAL4-dependent manner (Fig. 2J). Rpn11 is one lid component of the multiple subunits that make up the 19S RP. The 19S RP can be divided into two subcomplexes, known as the "base" and "lid" subcomplexes (see Fig. S2N in the supplemental material). Among the lid subunits, Rpn11 functions as a metalloprotease that cleaves the isopeptide bonds between a ubiquitinated substrate and the most proximal ubiquitin of the polyubiquitin chain (35, 41). We also examined another GS fly line, encoded by *GS13423*, in which the GS vector, inserted in the 5' untranslated region of *Rpn11*, could induce unidirectional transcription (Fig. 2I) and drive *Rpn11* expression in a GAL4-dependent manner (Fig. 2K). *GS13423* also suppressed the progression of the MJDtr-Q78-induced rough eye phenotype (Fig. 2E and F). To confirm directly that *Rpn11* was responsible for the suppression of the MJDtr-Q78-induced phenotype in the *DANC* fly line, we generated the *UAS-Rpn11* transgenic fly lines and overexpressed *Rpn11* in the fly eye with MJDtr-Q78. As with the *DANC* or *GS13423* allele, the overexpression of *Rpn11* suppressed the MJDtr-Q78-induced progressive loss of pigmentation (Fig. 2G and H) without affecting *GAL4* expression by the overexpression of *Rpn11* or with age (see Fig. S1 in the supplemental material).

We next examined whether overexpression of *Rpn11* could suppress another model of expanded polyglutamine disease, the human Huntingtin (*Htt*) gene (16). The photoreceptor neurons in the wild-type fly eye are arranged in a series of repeating trapezoids visible as seven rhabdomeres within each ommatidium (Fig. 2L and M). When *Htt* peptides were expressed in the eye, although almost seven rhabdomeres were observed within each ommatidium at day 2 (Fig. 2N and R), the loss of rhabdomeres in each ommatidium was observed at day 15 (Fig. 2O and R). However, when *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly reduced (Fig. 2P, Q, and R). These data indicate that the overexpression of *Rpn11* suppresses the age-related progression of neural degeneration induced by polyglutamine.

Overexpression of *Rpn11* ameliorates the toxicity of expanded polyglutamine. A number of studies have focused on the strong linkage between the pathogenesis of neurodegenerative diseases and protein aggregation (32). We next ad-

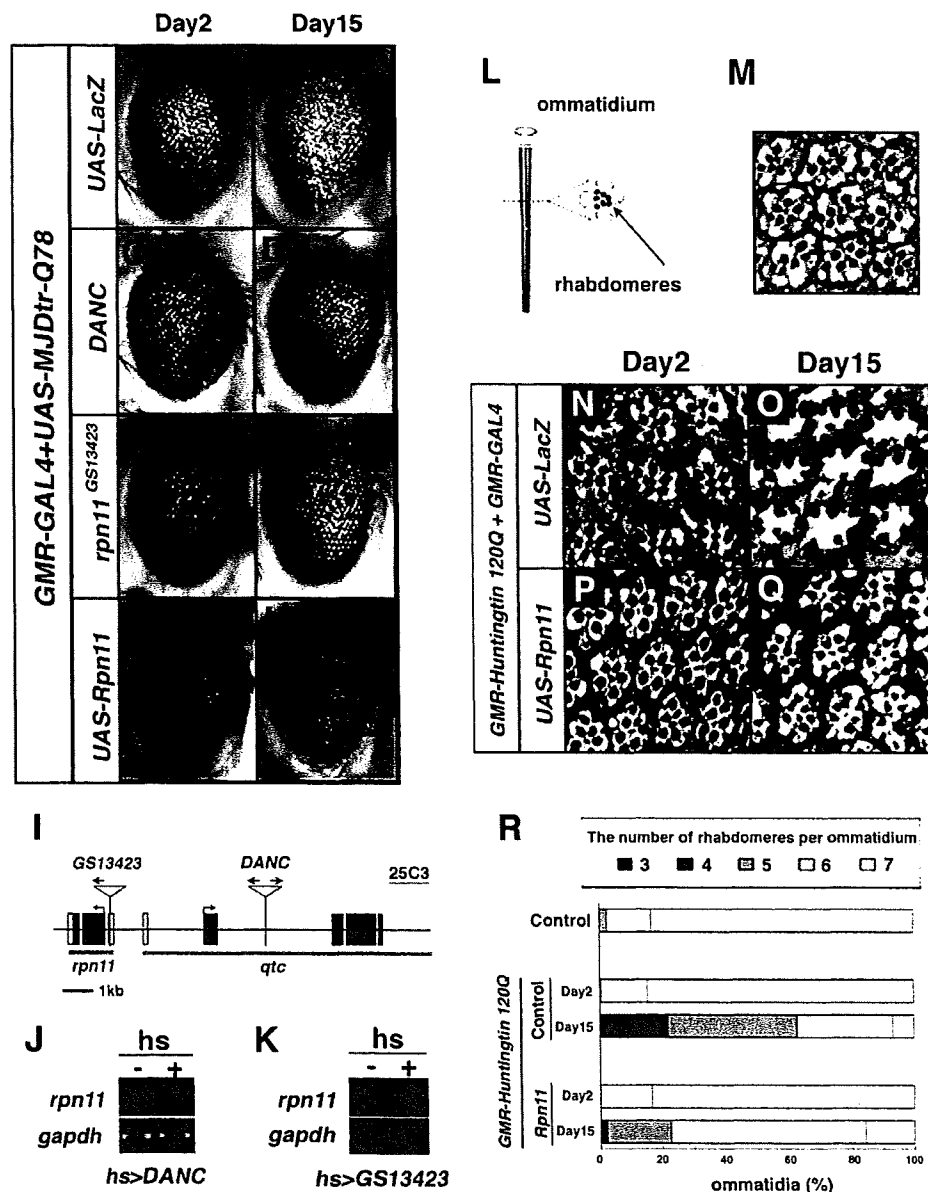


FIG. 2. Identification of *Rpn11* as a suppressor of age-related polyglutamine-induced progressive neurodegeneration. (A to H) Light photomicrographs of fly eyes expressing an expanded polyglutamine protein product of *MJDtr-Q78*, along with the indicated transgene. (A and B) Flies of genotype *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78/+* are shown at day 2 (A) and day 15 (B) posteclosion. These flies show eye degeneration, with a progressive loss of external pigment. (C to H) The progressive pigment loss phenotype was suppressed in flies expressing both *MJDtr-Q78* and *DANC* (C and D), *GS13423* (E and F), or *Rpn11* (G and H). The fly genotypes shown are *w*; *GMR-GAL4/GS13423*; *UAS-MJDtr-Q78/+* (E and F) and *w*; *GMR-GAL4/UAS-Rpn11*; *UAS-MJDtr-Q78/+* (G and H). (I) The genomic structures around the *DANC* and *GS13423* alleles are shown. (J and K) The *DANC* and *hs-GAL4* flies (J) and the *GS13423* and *hs-GAL4* flies (K) were heat shocked (*hs+*) or not (*hs-*), and the expression levels of the surrounding genes, as well as that of *GAPDH*, were analyzed by RT-PCR. Note that *rpn11* was upregulated in a *GAL4*-dependent manner. (L) Schematic of a single *Drosophila* ommatidium showing the regular trapezoidal arrangement of seven visible rhabdomeres within the photoreceptor neuron. (M) Representative semithin sections are shown of compound eyes from wild-type flies 2 days after eclosion. Normal ommatidia contain seven visible rhabdomeres at a given plane of the section. A representative single ommatidium is circled. (N to Q) Semithin sections of compound eyes from flies expressing *GMR-Huntingtin 120Q*, with *LacZ* as a control (N and O), or with *Rpn11* (P and Q). When *Rpn11* was coexpressed with *Htt*, the age-related loss of rhabdomeres was significantly improved (N to Q). The following genotypes are shown: *w*; *UAS-LacZ/GMR-GAL4*; *GMR-Huntingtin120Q/+* (N and O) and *w*; *UAS-Rpn11/GMR-GAL4*; *GMR-Huntingtin120Q/+* (P and Q). (R) Quantification of the number of rhabdomeres per ommatidium. More than 100 ommatidia per eye section were counted, and at least four eyes were sectioned for each fly line. The mean number of rhabdomeres per ommatidium \pm SD for the control flies was 6.7 ± 0.5 ; that for the *Htt* plus control *LacZ* flies on day 15 was 5.2 ± 0.9 ; and that for the *Htt* plus *Rpn11* flies on day 15 was 5.9 ± 0.6 . Differences between the *Htt* plus control *LacZ* flies on day 15 and the *Htt* plus *Rpn11* flies on day 15 are significant: $P < 0.0001$ (Student's *t* test).

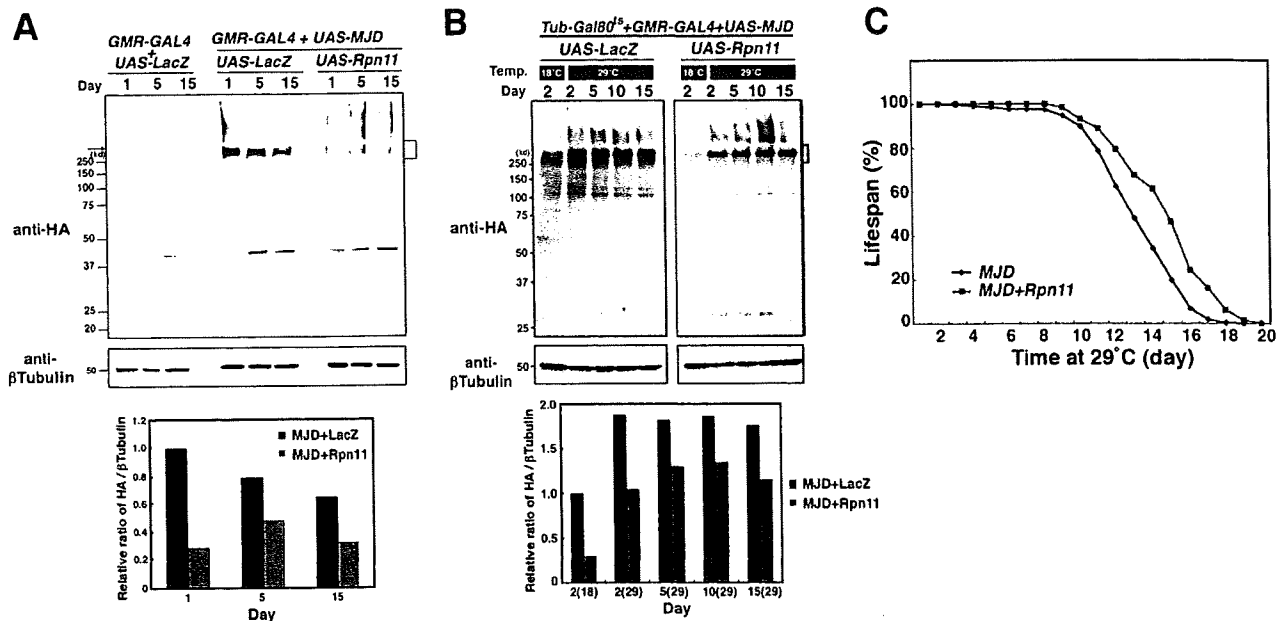


FIG. 3. Overexpression of *Rpn11* ameliorates the toxicity caused by expanded polyglutamine. (A) The coexpression of *Rpn11* with *MJDtr-Q78* (*MJD+Rpn11*) significantly decreased the polyglutamine-induced aggregation. The expanded polyglutamine protein in flies ran as an SDS-insoluble complex in the stacking gel and at the top of the separating gel and was detected by immunoblot analysis with an anti-HA antibody, with which *MJDtr-Q78* was tagged. Fly heads from each line of the indicated genotype were subjected to immunoblotting with an anti-HA antibody and an anti- β -tubulin antibody as a loading control. Fly genotypes were *w*; *GMR-GAL4/UAS-LacZ*; *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDtr-Q78/+* and *w*; *GMR-GAL4/UAS-Rpn11*; *UAS-MJDtr-Q78/+*. (B) The overexpression of *Rpn11* after eclosion suppressed the aggregation induced by the eye-specific expression of the polyglutamine protein. Fly genotypes were *w*; *tub-GAL80^{ts}/UAS-LacZ*; *GMR-GAL4/UAS-MJDtr-Q78/+* and *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *GMR-GAL4/UAS-MJDtr-Q78/+*. Graphs show the ratio of HA to β -tubulin (A and B). The relative amounts of each protein were determined by densitometric analysis. For the anti-HA antibody, the band indicated on the figure by a line was analyzed. (C) The coexpression of *Rpn11* and *MJDtr-Q78* in adult flies partially suppressed the short life span of *MJDtr-Q78* flies (the mean life span \pm SD for *MJD* flies was 12.3 ± 0.1 days, $n = 640$; that for *MJD* plus *Rpn11* flies was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$). Fly genotypes were *w*; *tub-GAL80^{ts}/+*; *da-GAL4/UAS-MJDtr-Q78* and *w*; *tub-GAL80^{ts}/UAS-Rpn11*; *da-GAL4/UAS-MJDtr-Q78*.

dressed whether the suppression of polyglutamine-induced neural degeneration by *Rpn11* was accompanied by a change in polyglutamine protein aggregate formation. To do this, we performed an immunoblot analysis of HA-tagged *MJDtr-Q78*. The expanded polyglutamine protein ran as an SDS-insoluble complex at the top of the separating gel and in the stacking gel; however, the amount of SDS-insoluble complex was significantly decreased in flies expressing *MJDtr-Q78* with *Rpn11* (Fig. 3A).

We next determined whether *Rpn11* expression is required during development or only during adulthood to suppress the polyglutamine protein aggregation. We adapted the TARGET system to control gene expression temporally (28). In this system, the GAL4-UAS system is conditionally regulated by a temperature-sensitive allele of *GAL80*. At a restrictive temperature (18°C), the activity of GAL4 is repressed, whereas this repression is relieved by a temperature shift to the permissive temperature (29°C). When *MJDtr-Q78* was expressed after eclosion, using the TARGET system, the SDS-insoluble complex was clearly detected from day 2 to day 15. However, when *Rpn11* was coexpressed after eclosion, the SDS-insoluble complex was significantly reduced (Fig. 3B).

To address whether *Rpn11* suppressed neurodegeneration by modulating the toxicity of the aggregated polyglutamine proteins, we assessed the life span of *MJDtr-Q78* flies overex-

pressing *Rpn11*. The coexpression of *Rpn11* with *MJDtr-Q78* during adulthood in the whole bodies of adult flies partially suppressed the *MJDtr-Q78*-induced shortening of the life span (the mean life span \pm standard deviation [SD] for flies expressing *MJDtr-Q78* alone was 12.3 ± 0.1 days, $n = 640$; that for flies expressing *MJDtr-Q78* plus *Rpn11* was 13.8 ± 0.16 days, $n = 260$; log-rank test, $P < 0.0001$) (Fig. 3C). Thus, the overexpression of *Rpn11*, even occurring only in the adult flies, reduced the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins.

Other lid subunits, but not subunits of the base or the 20S proteasome, may suppress the polyglutamine-induced phenotype. Next, we assessed whether the suppressive effect of *Rpn11* on the progression of the polyglutamine-induced neurodegenerative phenotype was unique among proteasome components. For this experiment, we used GS fly lines that expressed different components of the 26S proteasome in a GAL4-dependent manner (see Fig. S2K to M in the supplemental material). We found that the overexpression of several lid subunits (see Fig. S2N in the supplemental material), *rpn9* (see Fig. S2C and D in the supplemental material) and *rpn5* (see Fig. S2E and F in the supplemental material), as well as *rpn11*, suppressed the progression of the polyglutamine-induced neurodegenerative phenotype. However, neither the *rpn2* base subunit (see Fig. S2G and H in the supplemental material) nor the 20S $\alpha 6$

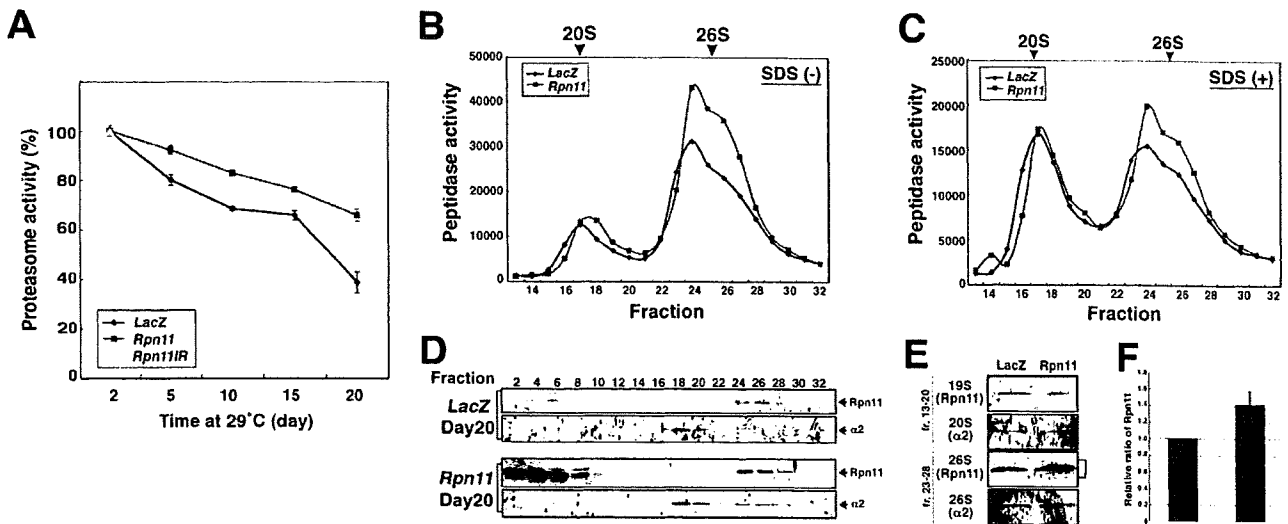


FIG. 4. Overexpression of *Rpn11* suppresses the age-related reduction of the 26S proteasome activity. (A) Proteasome activity in whole *Drosophila* with overexpression or knockdown of *rpn11* after eclosion. Fly genotypes were *w; tub-GAL80^{ts}/UAS-LacZ; da-GAL4/+; w; tub-GAL80^{ts}/UAS-Rpn11; da-GAL4/+*, and *w; tub-GAL80^{ts}/UAS-Rpn11; da-GAL4/UAS-Rpn11IR*. (B) The peptidase activity in the 26S proteasome fraction of 20-day-old flies expressing *Rpn11* was significantly higher than that of control flies. Extracts of flies overexpressing *LacZ* or *Rpn11* at day 20 were fractionated, and the Suc-LLVY-AMC hydrolysis activities were measured. The experiments were repeated three times. (C) The peptidase activity of the 20S proteasome in flies expressing *LacZ* was not different from that of flies expressing *Rpn11*. Suc-LLVY-AMC hydrolysis activities of the lysates used for panel B were measured with the addition of 0.01% SDS [SDS(+)]. (D) The amount of Rpn11 in the 26S proteasome fraction was significantly greater in flies overexpressing *Rpn11* at day 20 than that in control flies. Immunoblot analysis for the even fractions shown in panel B was performed with anti-Rpn11 or anti- $\alpha 2$ antibody. (E) Fractions (fr.) 13 to 20 or fractions 23 to 28 in panel D were pooled and immunoblotted with anti-Rpn11 or anti- $\alpha 2$. (F) The amount of Rpn11 in the 26S fractions was significantly greater in flies overexpressing *Rpn11*. The amount of Rpn11 protein (the amount of the band shown in panel E by a line) was determined by densitometric analysis. The graph shows the ratio of the amount of Rpn11 in fractions 23 to 28 (26S included) in flies expressing *Rpn11* at day 20 to the amount of Rpn11 in fractions 23 to 28 in flies expressing *LacZ* at day 20 from three individual experiments.

subunit (see Fig. S2I and J in the supplemental material) could do so. Whereas GS system-based gene overexpression might induce expression of genes that are not closed to the GS system insertion site, these results suggest that the additional expression of at least some lid subunits suppresses the progression of expanded polyglutamine-induced degeneration, which implies that they might also have the ability to suppress the age-dependent impairment of proteasome activity.

The age-related reduction of proteasome activity is suppressed by the overexpression of *Rpn11*. To assess whether the ameliorative effect of *Rpn11* on the progression of expanded polyglutamine-induced degeneration was caused by preventing the age-related attenuation of the proteasome, we analyzed proteasome activity in flies expressing *Rpn11* with age. We used the TARGET system to express *Rpn11* only after eclosion, because *Rpn11* expression only in the adult was sufficient to reduce the polyglutamine-induced toxicity by suppressing the accumulation of expanded polyglutamine proteins (Fig. 3B and C) and because the additional expression of proteasome subunits during development might have affected development and reproduction. With the TARGET system, *Rpn11* expression was prevented when flies were raised at 18°C and was permitted when the flies were switched to 29°C at 1 day posteclosion (28). The overexpression of *Rpn11* after eclosion significantly prevented the age-related reduction of proteasome activity seen in the *LacZ* control, especially at day 20 (Fig. 4A).

To examine how the overexpression of *Rpn11* prevented the

age-related reduction of proteasome activity, extracts of 20-day-old flies expressing *Rpn11* were fractionated by 8 to 32% glycerol density gradient centrifugation. When *Rpn11* was overexpressed, the peptidase activity in the 26S proteasome-containing fraction of 20-day-old flies was higher than that in the control flies (Fig. 4B). However, the peptidase activity of the 20S proteasome did not significantly change (Fig. 4C). The immunodetection of Rpn11 or $\alpha 2$ revealed that the amount of Rpn11 in the 26S proteasome fractions was significantly greater in flies overexpressing *Rpn11* (Fig. 4D, E, and F), even though the protein levels of the 19S RP and the 20S proteasome showed almost no changes (Fig. 4E). These results suggest that the ectopic expression of *Rpn11* after eclosion helped to maintain the activity and amount of the 26S proteasome with age.

Overexpression of *Rpn11* suppresses the age-related accumulation of ubiquitinated proteins and extends the life span. Next, to examine whether the maintenance of the 26S proteasome by the ectopic expression of *Rpn11* could also suppress the age-dependent accumulation of misfolded proteins, as it did with the accumulation of polyglutamine proteins (Fig. 3A and B), fly extracts were immunoblotted with the antiubiquitin antibody. Although the amount of polyubiquitinated proteins gradually increased with age in the control flies, this age-dependent accumulation was significantly suppressed when *Rpn11* was overexpressed after eclosion (Fig. 5A). If the age-dependent accumulation of polyubiquitinated proteins is associated with proteotoxicity in vivo, it is possible that the age-

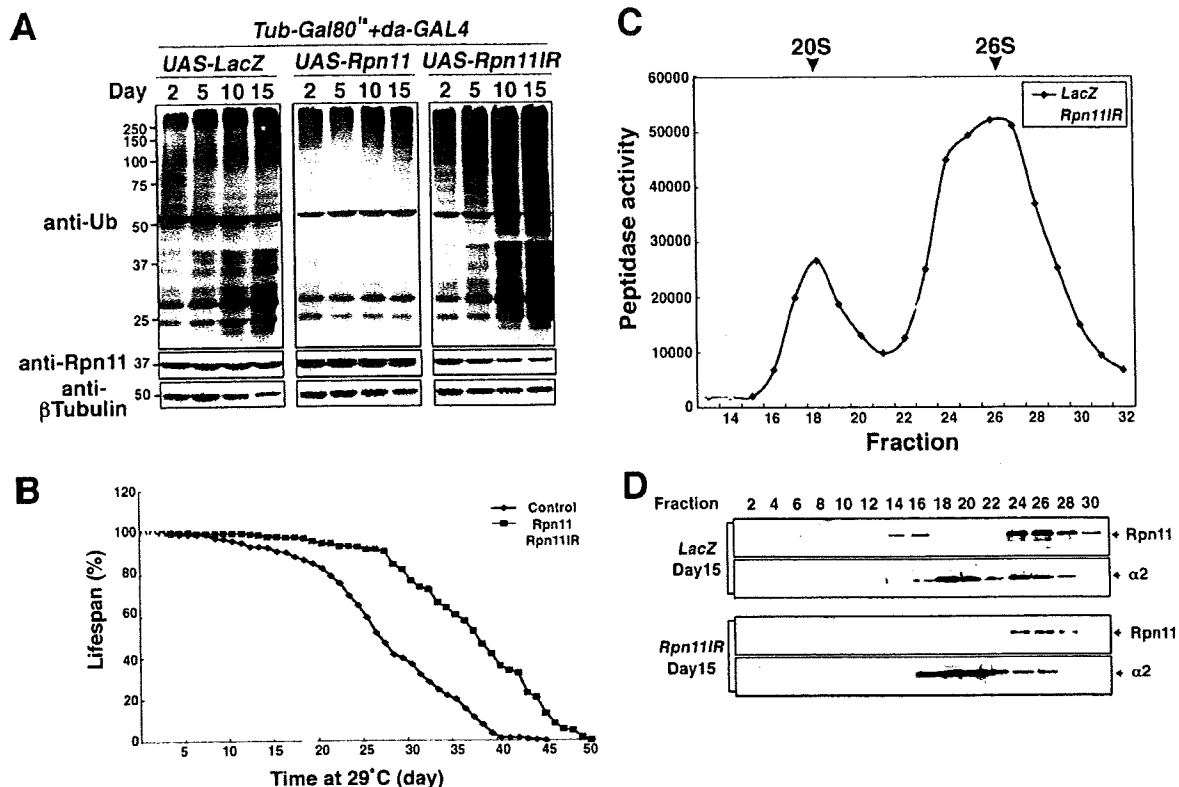


FIG. 5. *Rpn11* is required to suppress the age-dependent accumulation of ubiquitinated proteins and to extend the life span. (A) The accumulation of ubiquitinated proteins with age in the adult body was suppressed by the overexpression of *Rpn11* after eclosion and enhanced by the knockdown of *rpn11*. Whole flies from each line of the indicated genotype at the indicated day posteclosion were subjected to immunoblotting with antiubiquitin (anti-Ub), anti-Rpn11, and anti-β-tubulin antibodies. Fly genotypes were *w; tub-GAL80^{ts}/UAS-LacZ; da-GAL4/+; w; tub-GAL80^{ts}/UAS-Rpn11; da-GAL4/+*, and *w; tub-GAL80^{ts}/+; da-GAL4/UAS-Rpn11IR*. Numbers at left are molecular masses (in kDa). (B) The overexpression of *Rpn11* significantly extended the life span compared with that of control flies (the mean life span \pm SD of control flies was 27.2 ± 0.56 days, $n = 220$), and for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, $n = 240$; log-rank test, $P < 0.0001$). On the other hand, the ubiquitous expression of *Rpn11IR* in adult flies reduced their life span (the mean life span \pm SD for control flies was 27.2 ± 0.56 days, $n = 220$; and for the *rpn11* knockdown line was 11.1 ± 0.18 , $n = 320$; log-rank test, $P < 0.0001$). Fly genotypes were *w; tub-GAL80^{ts}/UAS-GFP; da-GAL4/+; w; tub-GAL80^{ts}/UAS-Rpn11; da-GAL4/+*, and *w; tub-GAL80^{ts}/+; da-GAL4/UAS-Rpn11IR*. (C) Knockdown of *rpn11* led to a decrease in the 26S proteasome activity. Extracts of flies expressing *LacZ* or *Rpn11IR* ubiquitously for 15 days after eclosion were fractionated by 8 to 32% glycerol gradient centrifugation. Genotypes shown are *w; tub-GAL80^{ts}/UAS-LacZ; da-GAL4/+* and *w; tub-GAL80^{ts}/+; da-GAL4/UAS-Rpn11IR*. (D) Immunodetection of Rpn11 or $\alpha 2$ revealed that the knockdown of *rpn11* inhibited the assembly of the 26S proteasome. The levels of Rpn11 and $\alpha 2$ in the fractions containing the 26S proteasome (fractions 24 to 30) were significantly decreased. Immunoblot analysis was performed for each fraction, using antibodies against Rpn11 or $\alpha 2$.

dependent accumulation of polyubiquitinated proteins affects the life span. Therefore, we compared the life span of control flies with that of flies expressing *Rpn11* after eclosion. The overexpression of *Rpn11* during adulthood significantly extended the mean life span compared with that of control flies (mean life span \pm SD for control flies was 27.2 ± 0.56 days, $n = 220$; life span for *Rpn11*-overexpressing animals was 36.8 ± 0.53 days, $n = 240$; log-rank test, $P < 0.0001$) (Fig. 5B). Thus, the overexpression of *Rpn11* suppresses the age-dependent accumulation of ubiquitinated proteins and extends the mean life span in flies. These results imply that the maintenance of the 26S proteasome with age prevents the age-related aggregation-mediated toxicity.

Knocking down of *rpn11* enhances the age-related accumulation of ubiquitinated proteins and shortens the life span, accompanied by reduced 26S proteasome. To examine whether impairment of the 26S proteasome activity enhanced the

age-related aggregation-mediated toxicity, we assessed the accumulation of ubiquitinated proteins and the life span in the *rpn11* knockdown flies. We generated transgenic flies bearing an inverted repeat (IR) fragment of the *Rpn11* cDNA in the *pUAST* vector (*Rpn11IR*) that would specifically inhibit *rpn11* expression in a GAL4-dependent manner via a mechanism of RNA interference. When *rpn11* was knocked down in whole-body extracts after eclosion by an *Rpn11IR*, the age-dependent reduction of proteasome activity was enhanced (Fig. 4A), along with the attenuation of the activity and amount of the 26S proteasome (Fig. 5C and D), suggesting that the assembly of the 26S proteasome was impaired. In the *rpn11* knockdown flies, the accumulation of ubiquitinated proteins was clearly enhanced with age (Fig. 5A). In addition, the mean life span in the *rpn11* knockdown flies was severely impaired (mean life span \pm SD for control flies was 27.2 ± 0.56 days, $n = 220$; and for the *rpn11*

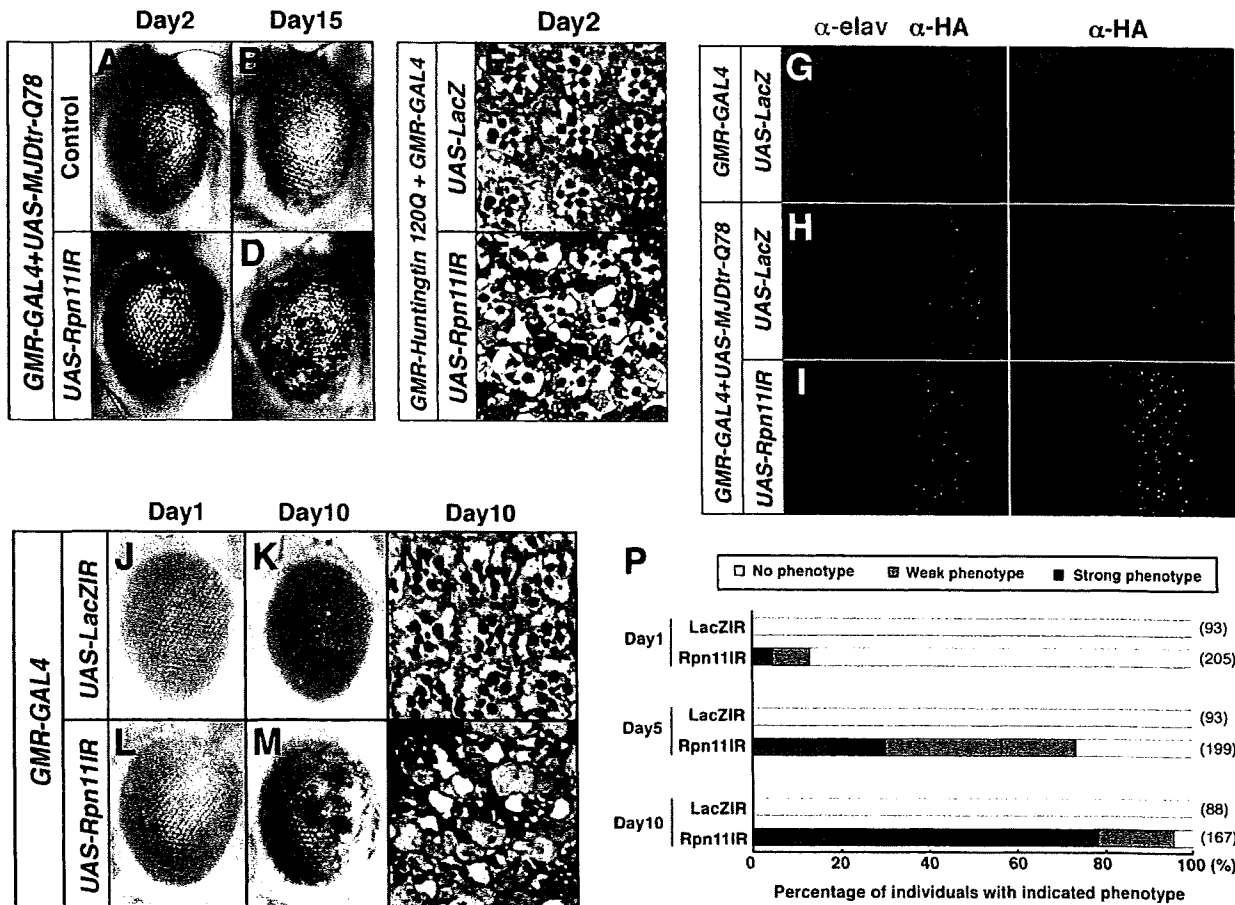


FIG. 6. Knocking down *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. (A to F) Knocking down *rpn11* clearly enhances the phenotype of polyglutamine-induced neurodegeneration. Light microscopy (A to D) and semithin-section (E and F) images of the compound eyes are shown. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDir-Q78/+* (A, B) and *w*; *GMR-GAL4/+*; *UAS-MJDir-Q78/UAS-Rpn11IR* (C and D), *w*; *UAS-LacZ/GMR-GAL4*; *GMR-Huntingtin120Q/+* (E), and *w*; *GMR-GAL4/+*; *GMR-Huntingtin120Q/UAS-Rpn11IR* (F). (G to I) Polyglutamine aggregation was significantly enhanced when *rpn11* was knocked down. Eye imaginal discs of wandering third-instar larvae were immunostained with anti-ELAV (green) and anti-HA (magenta), which *MJDir-Q78* was tagged with. ELAV is expressed in all photoreceptor neurons. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZ* (G), *w*; *GMR-GAL4/UAS-LacZ*; *UAS-MJDir-Q78/+* (H), and *w*; *GMR-GAL4/+*; *UAS-MJDir-Q78/UAS-Rpn11IR* (I). (J to O) Knocking down *rpn11* caused the severe neural degeneration. Light microscopy (J to M) and semithin-section (N and O) images of fly eyes expressing *Rpn11IR* (L, M, and O) or control protein (J, K, and N) are shown. The following genotypes are shown: *w*; *GMR-GAL4/UAS-LacZIR* at day 1 (J) and day 10 (K and N), respectively, and *w*; *GMR-GAL4/UAS-Rpn11IR* at day 1 (L) and day 10 (M and O), respectively. (P) The *Rpn11IR*-induced neural degeneration is progressive. Quantification of the appearance of black dots on the surface of the eye with age is shown. A weak or strong phenotype is categorized as the appearance of one black dot or the appearance of more than two black dots, respectively. Numbers of individuals counted are shown at the right. Progressive phenotypes are seen in flies in which *rpn11* was knocked down.

knockdown fly line, life span was 11.1 ± 0.18 days, $n = 320$; log-rank test, $P < 0.0001$) (Fig. 5B).

Knocking down of *rpn11* enhances the toxicity of expanded polyglutamine and causes the age-related onset of a neurodegenerative phenotype. Next, we examined whether the impairment of the 26S proteasome activity enhances the toxicity of expanded polyglutamine. When *rpn11* was knocked down in the *MJDir-Q78*-expressing fly eye, the *MJDir-Q78*-induced progressive loss of pigmentation was clearly enhanced (Fig. 6A to D). In addition, when *Rpn11IR* was coexpressed with *Htt*, the loss of rhabdomeres was severe even at day 2 (Fig. 6E and F).

To determine whether knocking down of *rpn11* enhanced

polyglutamine aggregation, we examined polyglutamine aggregation formation in developing eye discs of larvae expressing polyglutamine. Polyglutamine aggregation was observed in eye discs of larvae expressing expanded polyglutamine protein, which was detected with antibody to the HA tag (Fig. 6H). When *rpn11* was knocked down, polyglutamine aggregation was significantly enhanced (Fig. 6I).

Next, we examined whether the impairment of the 26S proteasome activity leads to a neurodegenerative phenotype. While the eye-specific knockdown of *rpn11* resulted in an almost normal phenotype on day 1 after eclosion (Fig. 6L and P), by day 10 after eclosion, black dots progressively appeared on the surface of the eye with age (Fig. 6M and P). In addition,

retinal sections showed that knocking down *rpn11* caused the severe loss of rhabdomeres (Fig. 6N and O). These data indicate that impairment of the 26S proteasome leads to retinal degeneration in otherwise wild-type individuals and to early onset of polyglutamine-induced defects.

DISCUSSION

Although aggregation-mediated toxicity is involved in both normal aging and neurodegenerative disease, the reason that aggregation-mediated toxicity emerges late in life has not been elucidated. Our results demonstrate for the first time that an age-related reduction of the 26S proteasome activity affects longevity and could underlie the induction of certain effects of aging and the age-dependent increased risk of the onset and/or progression of neurodegeneration *in vivo*.

In this study, we demonstrated that the age-related reduction of proteasome activity results from the attenuation of both the activity and amount of the 26S proteasome, which is associated with impaired assembly of the 26S proteasome from the 19S RP and the 20S proteasome *in vivo*. Age-related proteasome dysfunction has been reported in studies on humans, other mammals, and flies (3, 19). Thus, the age-related reduction of proteasome activity is evolutionarily conserved. Importantly, our data indicated that the reduction of the 26S proteasome activity with age might result from a defect in its assembly. This indication is supported by our data that the lysates from the young flies showed two peaks of peptidase activity (Fig. 1C, fractions 24 and 26) among the 26S proteasome fractions, which represented the 26S proteasome with one or two RP caps, but aged flies showed only one peak (Fig. 1C, fraction 24), which indicates that the 26S proteasome with one cap was the predominant form (Fig. 1C).

The free 20S proteasome is almost inactive and cannot degrade multiubiquitinated proteins because the pores leading into the catalytic chamber are closed. The opening of these gates is triggered by the 19S RP attached to one end of the 20S proteasome (6). The assembly of the 26S proteasome is known to be ATP dependent, and a recent report indicates that ATP binding is sufficient to promote the assembly of the 26S proteasome from the 19S RP and the 20S proteasome (25). That report's data indicate that no additional assembly factors are required *in vitro* (25). Therefore, we at first suspected that an age-associated decline in ATP levels caused the disassembly of the 26S proteasome. However, no decline in ATP levels could be detected, even in flies that were 30 days posteclosion (data not shown), despite the obvious decline of proteasome activity (Fig. 1A). Thus, specific regulators besides ATP may affect the assembly of the 26S proteasome *in vivo*.

Previous reports have identified extraproteasomal proteins that promote the assembly of the 26S proteasome. For example, Hsp90, an ATP-regulated chaperone, promotes the assembly and maintains the stability of the 26S proteasome in *Saccharomyces cerevisiae* (14). Ecm29 has also been identified as a stoichiometric binding partner for the yeast 26S proteasome and is proposed to enhance its stability by binding to both the 20S proteasome and the 19S RP (24). It is possible that these specific regulators of the assembly or

stability of the 26S proteasome are affected by age and cause the age-related decline of the 26S proteasome activity.

Our results demonstrate that an age-related reduction of the 26S proteasome activity could be key to the age-related accumulation of misfolded or unfolded proteins and the duration of the life span. This idea that the homeostasis of general protein folding is important during aging is supported by recent findings for the role of chaperones in promoting longevity in *C. elegans* and other organisms. For example, overexpression of either the HSP70 or HSP16 chaperone, which resists protein misfolding, increases the life span (38, 42), whereas reduced chaperone expression by *heat shock factor-1* (*hsf-1*) knockdown shortens the life span (13). Although the direct effects of the proteasome function on life span have been less well explored, a recent report suggests that AIRAP (arsenic-inducible proteasomal 19S regulatory particle-associated protein), which associates tightly with the 19S proteasome, is involved in regulating the life span. The report showed that *C. elegans* lacking *aip-1*, a homologue of mammalian AIRAP, exhibit a shortened life span (43). Taken together with our findings, these results support our idea that maintenance of the 26S proteasome activity with age should promote longevity, implying that the age-related decline of the 26S proteasome activity is an important element in determining longevity.

Although genetic variants, behaviors, and environmental factors have been associated with increased risk of disease (27), our genetic evidence indicates that the age-related decline of the 26S proteasome activity could be a key risk factor for the progression or late onset of neurodegenerative diseases, because the overexpression of *Rpn11* suppressed the progression of polyglutamine-induced neurodegeneration with the maintenance of the 26S proteasome activity during aging. It is possible that Rpn11 functions as a metalloprotease and suppresses the polyglutamine-induced neurodegeneration by upregulation of the deubiquitinating activity. However, because it is reported that Rpn11 could not work by itself (35, 41), it is unlikely that Rpn11 functions as a metalloprotease. In fact, we measured the deubiquitinating activity in flies overexpressing *Rpn11*, but it did not change significantly compared with that of the control flies (data not shown).

Although further study is required to determine the precise details of how *Rpn11* suppresses the age-related impairment of proteasome activity, our data indicate that this mechanism could involve the promotion of 26S proteasome assembly. We revealed that GS system-based overexpression of some lid subunits, such as *Rpn11*, -9, and -5, suppressed the progression of polyglutamine-induced neurodegenerative phenotypes (see Fig. S2 in the supplemental material). Some reports indicate the existence of a core interaction cluster composed of the lid components Rpn11, -9, -5, and -8 (7, 15). It is possible that the expression of any given lid subunit could promote assembly of the lid complex, which could conceivably drive the assembly of the 26S proteasome by increasing the pool of 19S RPs. Although it is conceivable that the overexpression of *Rpn11* could lead to the upregulation of other proteasome components, our quantitative PCR data showed that overexpression of *Rpn11* did not affect the expression of other proteasome components or of the autophagy-related gene *atg5* (see Fig. S3 in the sup-

plemental material). These lines of evidence suggest that Rpn11 could promote the assembly of the 26S proteasome from free subunits without the upregulation of other lid subunits.

We have examined age-related attenuation of the 26S proteasome as one component in a process of protein degradation. Because the network of protein homeostasis is a complex process, it is possible that chaperone and autophagy could also gradually decrease and play an important role in the late onset or progression of neurodegenerative diseases (29, 30). Given the importance of maintaining the 26S proteasome activity, the mechanism for the late onset or progression of neurodegenerative diseases may be revealed by future studies of specific regulators of the 26S proteasome, for example, the 19S lid subunits such as Rpn11, some unidentified lid assembler, or a specific assembler for the 26S proteasome. This knowledge might be translated into the development of valuable therapeutic tools for the treatment of progressive age-related neurodegenerative diseases, including polyglutamine-induced disease.

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Stress management by p62

Peroxisomal translocon uncovered

The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1

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Impaired selective turnover of p62 by autophagy causes severe liver injury accompanied by the formation of p62-positive inclusions and upregulation of detoxifying enzymes. These phenotypes correspond closely to the pathological conditions seen in human liver diseases, including alcoholic hepatitis and hepatocellular carcinoma. However, the molecular mechanisms and pathophysiological processes in these events are still unknown. Here we report the identification of a novel regulatory mechanism by p62 of the transcription factor Nrf2, whose target genes include antioxidant proteins and detoxification enzymes. p62 interacts with the Nrf2-binding site on Keap1, a component of Cullin-3-type ubiquitin ligase for Nrf2. Thus, an overproduction of p62 or a deficiency in autophagy competes with the interaction between Nrf2 and Keap1, resulting in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes. Our findings indicate that the pathological process associated with p62 accumulation results in hyperactivation of Nrf2 and delineates unexpected roles of selective autophagy in controlling the transcription of cellular defence enzyme genes.

Macroautophagy (hereafter referred to as autophagy) is a highly conserved bulk protein degradation pathway responsible for the turnover of long-lived proteins, the disposal of excess or damaged organelles, and the clearance of aggregation-prone proteins. Isolation membranes engulf the cytoplasmic constituents, and the resulting autophagosomes fuse with lysosomes, resulting in complete degradation of the sequestered cytoplasmic components by lysosomal hydrolases¹. Thus, inactivation of autophagy leads to cytoplasmic protein inclusions, which are composed of degenerated proteins, and the

excess accumulation of deformed organelles, leading to liver injury², diabetes^{3,4}, heart disease⁵ and neurodegeneration^{6,7}.

Although autophagy has generally been considered non-selective, growing lines of evidence indicate the selectivity of autophagy in sorting vacuolar enzymes such as aminopeptidase I and α -mannosidase⁸ and in the removal of aggregation-prone proteins⁹, unwanted organelles^{10,11}, and microbes¹². Such selectivity by autophagy enables various methods of cellular regulation, as is well known for the ubiquitin proteasome pathway. The protein p62, which binds ubiquitin and LC3 (refs 13–15) and is a selective substrate for autophagy, regulates the formation of protein aggregates. Genetic ablation of p62 suppressed the appearance of ubiquitin-positive protein aggregates in autophagy-deficient mice¹⁶ and flies¹⁷, indicating that p62 is important in the formation of inclusion bodies. Moreover, loss of p62 markedly attenuated liver injury accompanied by the robust induction of antioxidant proteins resulting from autophagy deficiency¹⁶. This implies that impaired turnover of p62 is a major cause of the pathogenic changes seen in the livers of autophagy-deficient mice. Importantly, excess accumulation of p62 and inclusion bodies containing both ubiquitylated proteins and p62 have been identified in several human disorders, especially in neurodegenerative diseases¹⁸, liver injuries¹⁹ and hepatocellular carcinoma²⁰. However, the molecular functions of p62 in autophagy-deficient conditions and its pathophysiological roles in human disorders are still unknown.

The Nrf2–Keap1 system is currently recognized as one of the main cellular defence mechanisms against oxidative and electrophilic stresses^{21–23}. Under quiescent conditions, the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) is constitutively degraded through the ubiquitin–proteasome pathway because its binding partner Keap1

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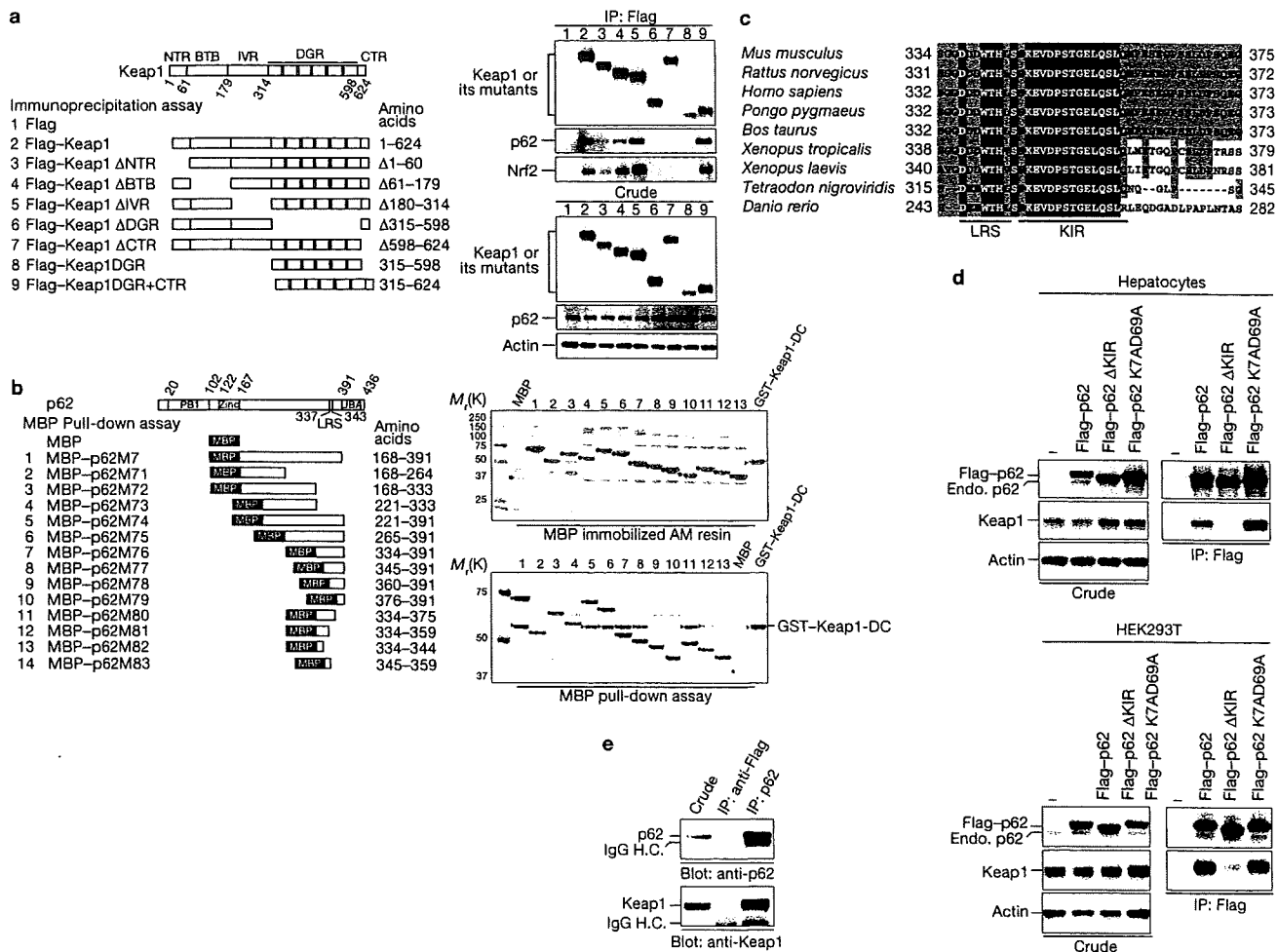


Figure 1 Interaction between Keap1 and p62. (a) Diagrams of the deletion–mutation constructs of Keap1 (left) and the corresponding immunoprecipitation assays (right). Each Flag-tagged mouse Keap1 and mutant was expressed in HEK293T cells. At 22 h after transfection, lysates were prepared and immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were subjected to SDS–PAGE and analysed by immunoblotting with anti-Flag, anti-p62, anti-Nrf2 and anti-actin antibodies. Data are representative of three individual experiments. (b) Diagrams of the deletion–mutation constructs of p62 (left) and the corresponding input (upper right) and pull-down assay (lower right). The MBP-tagged mouse p62 deletion mutants conjugated to amylose (AM) resins were incubated with purified GST-tagged mouse Keap1-DC. The pulled-down complexes with the MBP–p62 mutants were subjected to SDS–PAGE and revealed by staining with Coomassie brilliant blue. The bands corresponding to MBP–p62 and its mutants are indicated by black dots. Red arrowheads indicate the band corresponding to GST–Keap1-DC. For details of construct 14 see Supplementary Information, Fig. S3. (c) Alignment of the Keap1-interacting regions (KIR; red line) and the LC3-recognition sequences

(LRS; green line) of p62 homologues in various species. Black and grey boxes indicate identical amino acid residues with complete and partial conservation, respectively. (d) Immunoprecipitation assays. Flag-tagged p62, KIR-deleted p62 (p62 Δ KIR) and a p62 mutant defective in oligomerization (p62 K7AD69A) were expressed in primary mouse hepatocytes by the adenovirus system (left) or in HEK293T cells by transfection (right). Cell lysates were immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were subjected to SDS–PAGE and analysed by immunoblotting with anti-p62 and anti-Keap1 antibodies. The bands corresponding to Flag–p62, endogenous p62, Keap1 and actin are indicated. The data shown are representative of three separate experiments. (e) Interaction of endogenous p62 with Keap1. Lysates prepared from the human hepatocellular carcinoma cell line Huh-1 were immunoprecipitated with anti-p62 antibody or anti-Flag antibody (negative control) followed by immunoblotting with antibodies against p62 and Keap1. The bands corresponding to endogenous p62, Keap1 and IgG heavy chain (IgG H.C.) are indicated. The data shown are representative of three separate experiments. Uncropped images of blots are shown in Supplementary Information, Fig. S11.

(kelch-like ECH-associated protein 1) is an adaptor of the ubiquitin ligase complex^{24–27}. Exposure to electrophiles, reactive oxygen species and nitric oxide instigates the modification of the cysteine residues of Keap1, leading to its inactivation^{28–30}. As a result, Nrf2 becomes stabilized and translocates to the nucleus to induce the transcription of numerous cytoprotective genes through its heterodimerization with small Maf proteins^{31–33} (see Supplementary Information, Fig. S10). In this study we found that p62 acts to stabilize Nrf2 in autophagy-deficient mouse

livers and subsequently induces the expression of various cytoprotective enzymes. This sustained activation of Nrf2 seems to be a major cause of toxicity in autophagy-impaired livers.

RESULTS

Identification of Keap1 as a p62-interacting protein

Previous genetic studies on the autophagy-essential protein Atg7 in the mouse showed that loss of autophagy caused a marked accumulation

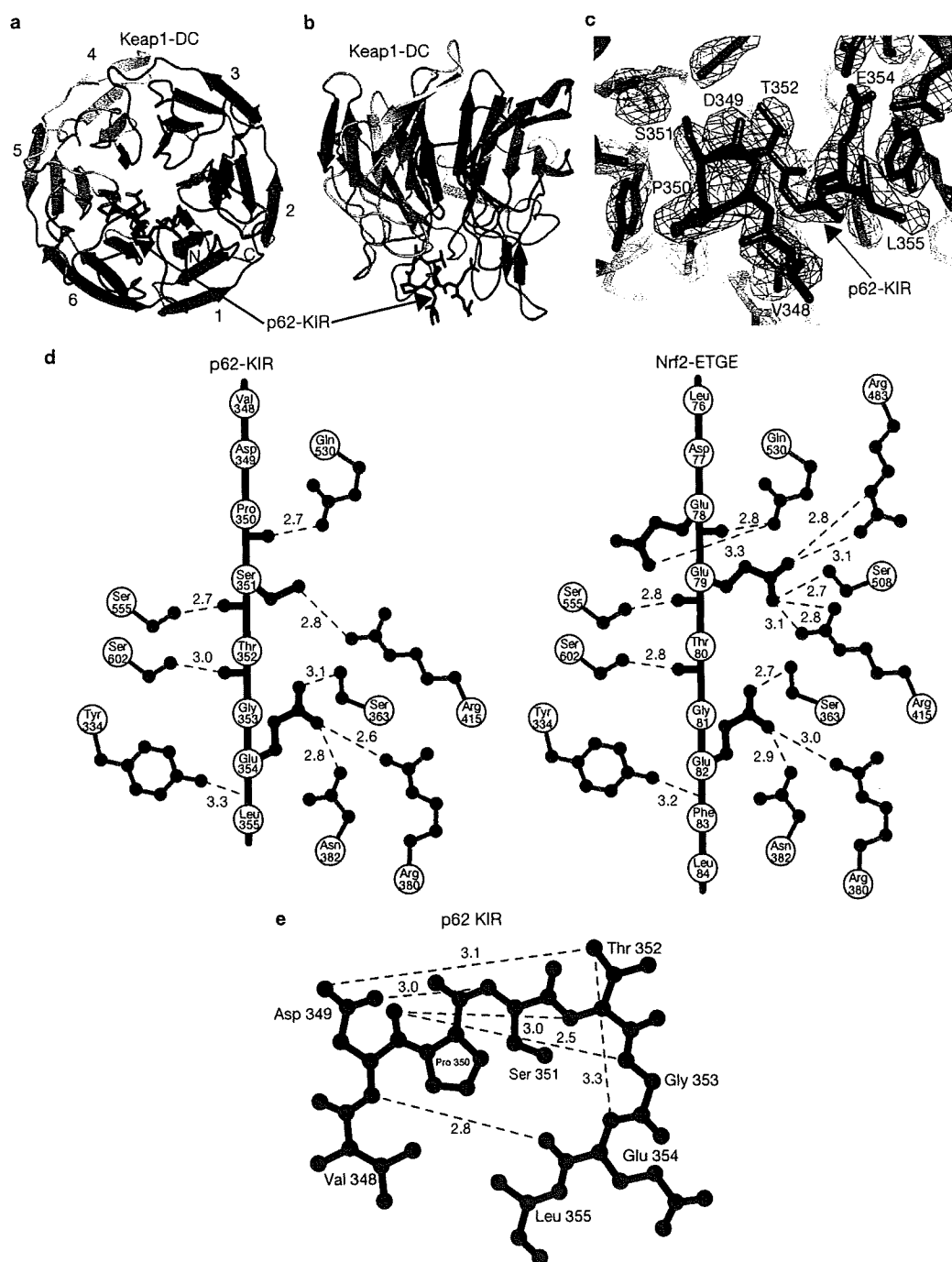


Figure 2 Crystal structure of Keap1-DC in complex with p62-KIR. (a, b) Bottom view (a) and side view (b) of the complex structure. The ribbon model represents Keap1-DC and the stick model shows p62-KIR. Each β -propeller blade is numbered from 1 to 6. (c) The simulated-annealing $F_o - F_c$ omit map is contoured at 3σ . p62-KIR (pink) was omitted from the calculation. The electron density of the peptide-bound region V348

to L355 of p62-KIR was unambiguously visible. (d) Intermolecular hydrogen bonds of Keap1-DC in complex with p62-KIR (left; PDB ID 3ADE) and in complex with the Nrf2-ETGE region (right; PDB ID 1x2r). (e) Intra-peptide hydrogen bonds of p62-KIR in the Keap1-DC complex. Hydrogen bonds (green broken lines) and their distances (Å) are displayed in d and e.

of p62 along with robust induction of antioxidant proteins, including NAD(P)H dehydrogenase quinone 1 (Nqo1) and glutathione *S*-transferase (GST)¹⁶. A battery of such detoxifying and antioxidant genes is regulated by the transcription factor Nrf2, which is activated by oxidative and electrophilic stresses^{31,32} (see Supplementary Information, Fig. S10). A prominent accumulation of Nrf2 in the nucleus was

observed in livers deficient in *Atg7*, but this was ameliorated by the additional loss of p62 (ref. 16). We therefore postulated that in autophagy-deficient livers, oxidative stresses occur in a p62-dependent manner. However, treatment of *Atg7*-deficient hepatocytes with the antioxidant reagent *N*-acetylcysteine did not affect the nuclear accumulation of Nrf2 or the high-level expression of antioxidant enzymes (Supplementary