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Role of S5b/PSMD5 in Proteasome Inhibition Caused by TNF-α/NFκB in Higher Eukaryotes

Sang Mi Shim,[1] Won Jae Lee,[1] Youngdoo Kim,[1] Jong Wook Chang,[2] Sungmin Song,[1] and Yong-Keun Jung[1,*]
[1]Global Research Laboratory, School of Biological Science/Bio-MAX Institute, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Korea
[2]Biomedical Research Institute, MEDIPOST, Seoul 137-874, Korea
*Correspondence: ykjung@snu.ac.kr
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SUMMARY

The ubiquitin-proteasome system is essential for maintaining protein homeostasis. However, proteasome dysregulation in chronic diseases is poorly understood. Through genome-wide cell-based screening using 5,500 cDNAs, a signaling pathway leading to NFκB activation was selected as an inhibitor of 26S proteasome. TNF-α increased S5b (HGNC symbol PSMD5; hereafter S5b/PSMD5) expression via NFκB, and the surplus S5b/PSMD5 directly inhibited 26S proteasome assembly and activity. Downregulation of S5b/PSMD5 abolished TNF-α-induced proteasome inhibition. TNF-α enhanced the interaction of S5b/PSMD5 with S7/PSMC2 in nonproteasome complexes, and interference of this interaction rescued TNF-α-induced proteasome inhibition. Transgenic mice expressing S5b/PSMD5 exhibited a reduced life span and premature onset of aging-related phenotypes, including reduced proteasome activity in their tissues. Conversely, S5b/PSMD5 deficiency in *Drosophila melanogaster* ameliorated the tau rough eye phenotype, enhanced proteasome activity, and extended the life span of tau flies. These results reveal the critical role of S5b/PSMD5 in negative regulation of proteasome by TNF-α/NFκB and provide insights into proteasome inhibition in human disease.

INTRODUCTION

The ubiquitin-proteasome system (UPS) is the major mechanism for selective protein degradation in eukaryotic cells (Ciechanover, 2005). Most early studies of the UPS have focused on selective degradation of cellular proteins following their ubiquitination by E1, E2, and E3; less is known about the direct regulation of proteasome activity. A few studies have shown that proteasome expression and function can be regulated in a tissue-specific or signal-dependent manner (Gaczynska et al., 1993; Murata et al., 2007). In cells exposed to interferon-γ or viral infection, for example, immunoproteasome is induced to stimulate production of antigenic peptides (Gaczynska et al., 1993). The thymus produces a thymus-specific proteasome subunit to enhance the tissue-specific function of proteasome (Murata et al., 2007). In addition, TCF11 (long isoform of Nrf1) was identified as the major transcription factor responsible for the expression of proteasome subunits during proteasome inhibition (Radhakrishnan et al., 2010; Steffen et al., 2010).

Altered UPS function is associated with chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), which exhibit reduced activity of the degradation system and accumulation of ubiquitin conjugates and aggregation-prone proteins (Ciechanover and Brundin, 2003; Gao and Hong, 2008). Indeed, E2-25K/Hip-2, an E2 enzyme that inhibits proteasome activity, is upregulated in the brains of TG2576 mice and patients with AD (Song et al., 2003, 2008). Accumulation of hyperphosphorylated and aggregated tau, a hallmark of tauopathy, including AD, is also contributed by reduced activity of the degradation system in the brain, leading to neuronal degeneration (Ballatore et al., 2007; Dickey et al., 2006; Goldbaum et al., 2003; Shimura et al., 2004). In these diseases the reduced activity and malfunction of proteasome likely leads to the accumulation of toxic proteins, which may be the key risk factor(s) responsible for neurodegeneration (Bence et al., 2001; Berke and Paulson, 2003). However, cellular signal(s) and molecular determinant(s) responsible for the regulation of proteasome activity remain unknown.

Inflammation often contributes to the development of many diseases. Particularly, levels of inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are elevated in neurodegenerative diseases, including AD (Campbell, 2004). TNF-α contributes to Aβ-induced impairment of learning/memory; cytokine inhibition suppresses Aβ-induced neurotoxicity and reduces tau phosphorylation (Medeiros et al., 2007; Shi et al., 2011; Wang et al., 2005). Moreover, anti-TNF-α antibody treatment produces positive effects in animal models of neuronal diseases (Tweedie et al., 2007). In addition, lipopolysaccharide (LPS)-induced inflammation exacerbates the symptoms of these diseases in mouse models (Gao et al., 2003; Gao and Hong, 2008). Despite the proposed contribution of inflammation to neurodegeneration in vitro and in vivo, little is known about the molecular mechanism by which inflammatory mediators regulate neuropathology via the UPS.

In the present study we identified the TNF-α/NFκB inflammatory signal as an important regulator of proteasome activity. We show that TNF-α/NFκB inhibits 26S proteasome assembly

and thus reduces proteasome activity via abnormal expression of S5b (HGNC symbol PSMD5; hereafter S5b/PSMD5), which contributes to UPS-associated neuronal disease and tau pathology.

RESULTS

Functional Identification of the NFκB Pathway in Proteasome Regulation

To uncover signals and regulators that directly modulate proteasome activity, we established a cell-based assay using degron (CL1), which is rapidly degraded by 26S proteasome in cells (Gilon et al., 1998). By cotransfecting HEK293T cells grown on 96-well culture plates with green fluorescent protein tagged with CL1 (GFP^u) and various genes of interest, we screened 5,500 full-length cDNAs for their stimulatory effects on GFP^u fluorescence. The 5,500 cDNAs encode protein kinases, membrane proteins, and other proteins that are expressed in the brain and are associated with human diseases. Using this gain-of-functional screening method, we isolated putative positive cDNA clones that affected GFP^u fluorescence (i.e., proteasome activity).

Of the 5,500 cDNAs, 68 genes increased the fluorescence and accumulation of GFP^u by more than 2-fold, compared to control (data not shown). The 68 genes encode protein kinases (29%), proteins with unknown functions (22%), proteins functioning in NFκB activation (16%), and others (Figure 1A). Surprisingly, 11 genes encode signal mediators and protein kinases that function in the signaling pathway leading to NFκB activation (Ghosh and Karin, 2002), including TAK1, NIK, CHUK, IKKε, TRAF2, MAP3K11, T2BP, MEKK1, MAPK9, MAP2K1IP1, and MKK7. Using western blot analyses, we confirmed that ectopic expression of these clones increased GFP^u accumulation but had no effect on GFP (Figure 1B), suggesting that a signaling pathway leading to NFκB activation may inhibit proteasome activity.

TNF-α Increases S5b/PSMD5 Expression to Inhibit Proteasome Activity via NFκB

Given that the inflammatory signal triggered by TNF-α activates NFκB via TAK1, NIK, and CHUK, we examined the effect of TNF-α on proteasome activity. Treatment of TNF-α elicited an accumulation of GFP^u and ubiquitin conjugates (Figure 1C). In enzyme assays using fluorogenic substrates, TNF-α suppressed the chymotrypsin-, trypsin-, and caspase-like activities of proteasome to 70% of control levels at 6 hr (Figure 1D), paralleling the accumulation patterns of GFP^u and ubiquitin conjugates (Figure 1C). We next investigated the roles of various kinase clones in the TNF-α-induced inhibition of proteasome activity. Stable expression of an IκBα or CHUK dominant-negative mutant abolished TNF-α-induced GFP^u accumulation (Figures 1E and 1F). In addition, reduced expression of MEKK1, TAK1, NIK, CHUK, IKKβ, or IKKε by shRNAs weakened the TNF-α-induced GFP^u accumulation (Figure 1G). TNF-α thus appears to inhibit proteasome activity via the aforementioned kinases. In addition, proteasome activity was suppressed by IL-1β and LPS, both of which are known to activate NFκB (data not shown).

Because NFκB is a transcription factor, we hypothesized that the expression of cellular factor(s), including proteasome subunits, may be regulated by NFκB to modulate proteasome activity. We therefore collected 29 cDNAs encoding proteasome subunits and their associated factors, and tested their effects on GFP^u accumulation (Table S1). Expressional analyses revealed that ectopic expression of S1/PSMD1, S4/PSMC1, S7/PSMC2, or S5b/PSMD5 significantly induced GFP^u accumulation. Of these proteasome subunits and factors, only S5b/PSMD5 mRNA and protein levels increased at 6 hr after treatment with TNF-α and returned to control levels at 24 hr (Figures 2A and 2B). Like other assembly factors, immunoproteasome subunits were not induced by TNF-α (Figures S1A and S1B) (Hallermalm et al., 2001). In addition the expression of TCF11/Nrf1, which was identified as a transcriptional factor for proteasome gene expression, was not regulated by TNF-α and did not affect S5b/PSMD5 expression (Figures 2B and S1C).

Unlike in HEK293T cells, in primary cultured cortical and hippocampal neurons exposed to TNF-α, S5b/PSMD5 protein began to accumulate at 6 hr and remained high until 24 hr (Figure 2C). In neurons the kinetics of proteasome activity inhibition was similar to that of S5b/PSMD5 induction. Likewise, incubation of mouse embryo fibroblasts (MEFs) with TNF-α increased S5b/PSMD5 protein level and suppressed proteasome activity. However, TNF-α failed to induce S5b/PSMD5 expression and did not inhibit proteasome activity in NFκB $p50^{-/-}$ and $p65^{-/-}$ MEFs (Figure 2D). Furthermore, ectopic expression of p65 or p50 increased S5b/PSMD5 level and inhibited proteasome activity (Figure 2E). Together, these results suggest that TNF-α increases S5b/PSMD5 expression and inhibits proteasome activity via NFκB.

To assess the role of S5b/PSMD5 in TNF-α-induced inhibition of proteasome activity, we targeted the expression of S5b/PSMD5 using five different shRNAs and two siRNAs (Figures S2A and S2B). Downregulation of S5b/PSMD5 expression enhanced proteasome activity and abolished TNF-α-induced GFP^u accumulation and proteasome inhibition (Figures 3A, 3B, and S2A–S2C). Consistently, ectopic expression of S5b/PSMD5 reduced proteasome activity and increased the fluorescence of GFP^u as well as that of another short-lived GFP Ub^{G76V}-GFP (Dantuma et al., 2000) (Figures 3B and S3A–S3D). Also, S5b/PSMD5 overexpression rescued the S5b/PSMD5 knockdown effect on GFP^u accumulation (Figure S2D). In addition, proteasome activity was further inhibited by the increasing concentrations of MG132, a potent inhibitor of proteasome, in S5b/PSMD5-overexpressing cells (Figure S3E). We confirmed that S5b/PSMD5 regulated GFP^u accumulation by affecting protein degradation but not transcription (Figures S3F and S3G). Interestingly, incubation of cell extracts with purified S5b/PSMD5 protein caused both accumulation of ubiquitin conjugates and inhibition of proteasome activity (Figures 3C and 3D), indicating that S5b/PSMD5 plays an essential role in the proteasome inhibition. These findings support our hypothesis that surplus S5b/PSMD5 functions as a negative regulator of proteasome activity. Unlike S5b/PSMD5, expression or knockdown of other 19S proteasome assembly factors, such as p27/PSMD9 and p28/PSMD10, did not significantly affect proteasome activity (Figure 3E).

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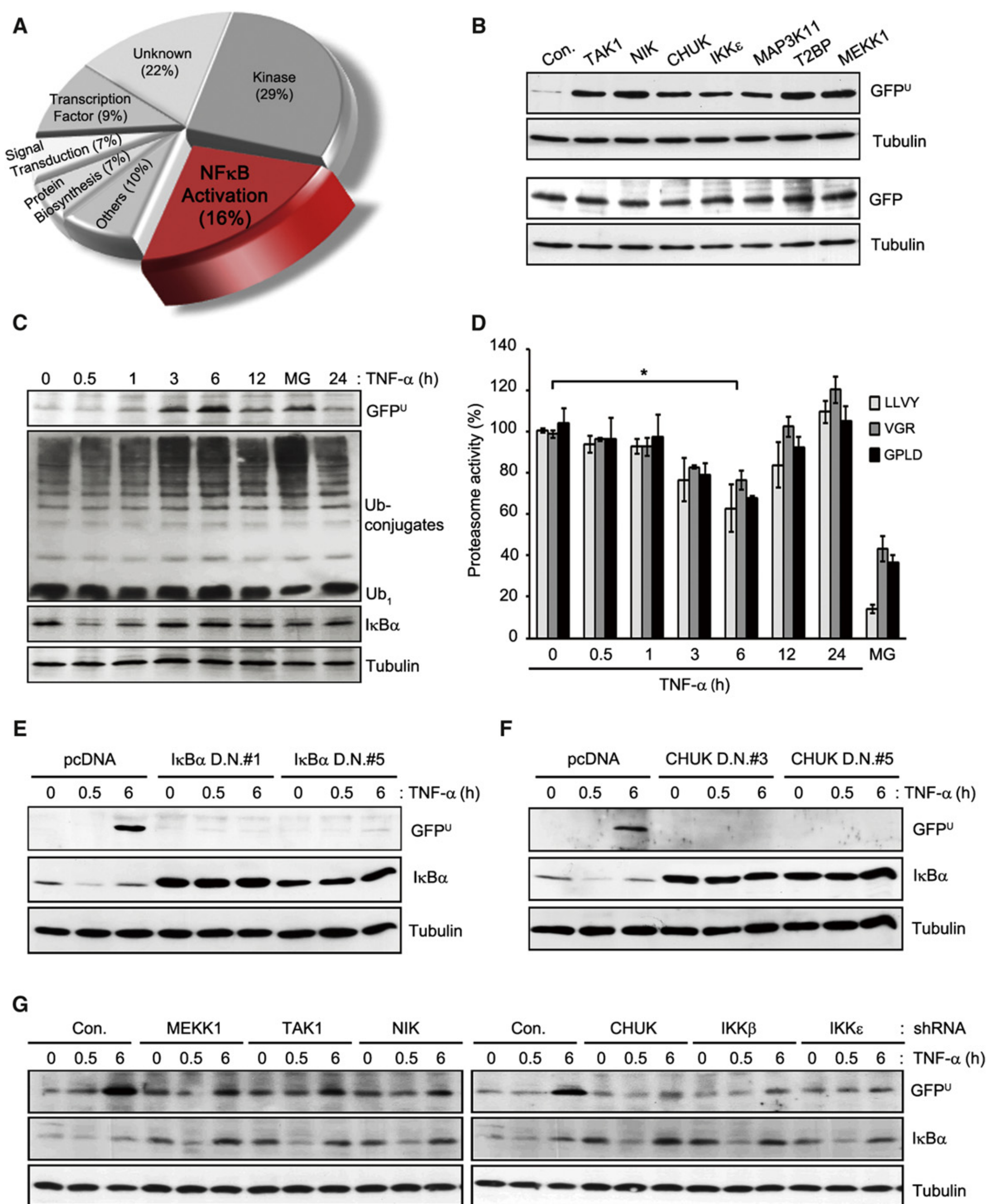

Figure 1. Identification of the TNF-α/NFκB Signaling Pathway as a Proteasome Inhibitory Pathway by Gain-of-Functional Screening

(A) Classification of the 68 putative positive clones based on their cellular functions.

(B) HEK293T cells were cotransfected for 36 hr with GFP^u and pcDNA (Con) or the indicated clones. Cell extracts were prepared and analyzed by western blotting. A control plasmid encoding EGFP (GFP) was included in every transfection.

(C) HEK293T cells were transfected with GFP^u for 24 hr and then left untreated or treated with TNF-α (50 ng/ml) for the indicated times or with MG132 (MG, 1 μM) for 12 hr. Cell extracts were then analyzed by western blotting.

(D) HEK293T cells were treated with TNF-α or MG132 as in (C). Cell extracts were prepared as described in Extended Experimental Procedures, and three types of proteasome activities were measured using Suc-LLVY-AMC (white box), Bz-VGR-AMC (gray box), and Ac-GPLD-AMC (black box). Error bars represent mean ± SD from at least three independent experiments. *$p < 0.001$.

(E and F) HEK293T cells stably expressing pcDNA3, IκBα-DN (#1 and #5) (E), or CHUK-DN (#3 and #5) (F) were transfected with GFP^u for 36 hr and then treated with TNF-α for the indicated times. Western blot analyses were performed.

(G) HEK293T cells were cotransfected for 42 hr with GFP^u and shRNAs targeted to MEKK1, TAK1, NIK, CHUK, IKKβ, or IKKε and exposed to TNF-α for the indicated times. GFP^u levels in the cell extracts were examined by western blot analysis as in (E).

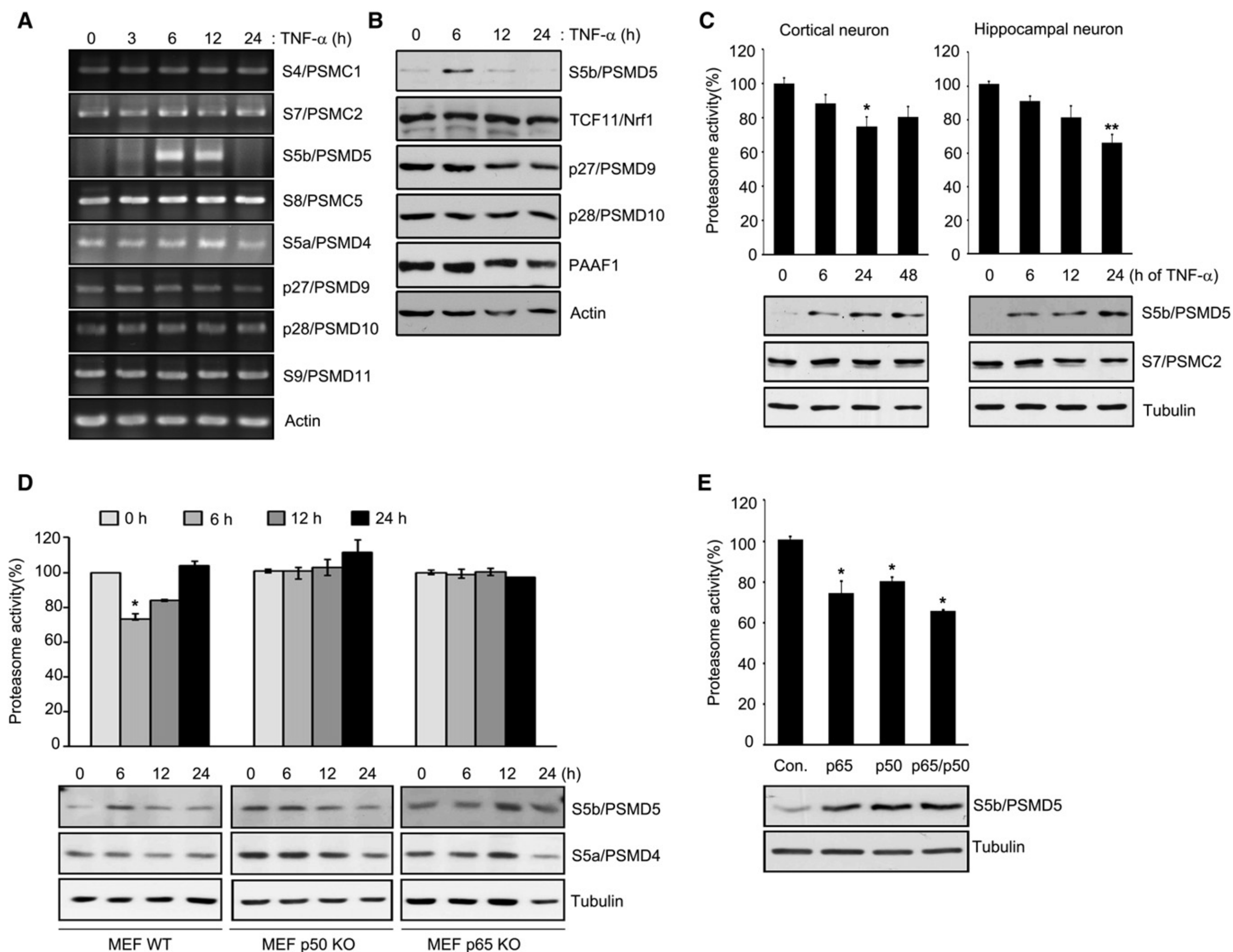

Figure 2. Increased S5b/PSMD5 Expression and Proteasome Inhibition by TNF-α/NFκB

(A and B) HEK293T cells were treated with TNF-α (50 ng/ml) for the indicated times, and total RNA and proteins were isolated and analyzed by RT-PCR (A) and western blotting (B), respectively. See also Figure S1 and Table S1.

(C) Cells were treated with TNF-α (50 ng/ml) for the indicated times, and cell extracts of primary rat cortical (left) and hippocampal neurons (right) were examined for proteasome activity (top) and analyzed by western blotting (bottom). Error bars represent mean ± SD. $^*p < 0.01$, $^{**}p < 0.0001$.

(D) WT, NFκB $p50^{-/-}$, and NFκB $p65^{-/-}$ MEFs were treated with TNF-α (50 ng/ml) for the indicated times, after which cell extracts were prepared for measurement of chymotrypsin-like activity using Suc-LLVY-AMC (top) and for western blot analyses (bottom). Error bars represent mean ± SD (n = 3). $^*p < 0.005$. KO, knockout.

(E) HEK293T cells were transfected for 36 hr with pcDNA (Con), NFκB p50, p65, or p50+p65 subunits, after which chymotrypsin-like activity was measured in cell extracts using Suc-LLVY-AMC (top). S5b/PSMD5 expression was assessed by western blotting (bottom). Error bars represent mean ± SD. $^*p < 0.001$.

Surplus S5b/PSMD5 Inhibits 26S Proteasome Assembly

Next, to determine the mechanism by which S5b/PSMD5 inhibits proteasome activity, we performed in vitro enzyme assays and native gel analyses using purified 26S proteasome. Addition of purified S5b/PSMD5 protein apparently reduced the purified 26S proteasome activity in a dose-dependent manner, whereas the S5b/PSMD5 RE mutant protein that is defective in S7/PSMDC2 binding (Le Tallec et al., 2009) did not exhibit an inhibitory effect (Figure 4A, left and middle; Figure S4A). On the other hand, addition of other 19S proteasome assembly factors, including p27/PSMD9 and p28/PSMD10, did not inhibit the purified 26S proteasome activity (Figure S4B). In native gel analyses, addition of S5b/PSMD5 protein appeared to inhibit the assembly and activity of 26S proteasome (RP_2-CP and RP-CP) and increased the activity of 20S proteasome and the amounts of 20S core particle (CP) and S5a/PSMD4 (regulatory particle [RP]) (Figure 4A, right). On the other hand, the purified S5b/PSMD5 R184E mutant protein did not exhibit any inhibitory effect on the assembly and activity of 26S proteasome (Figure 4A, right). Thus, it appears that excess S5b/PSMD5 directly inhibits the assembly and activity of 26S proteasome in vitro.

We further performed gel filtration (FPLC) analysis using Superose 6 and examined cellular proteasome assembly in detail. TNF-α treatment shifted the 19S and 20S core subunits

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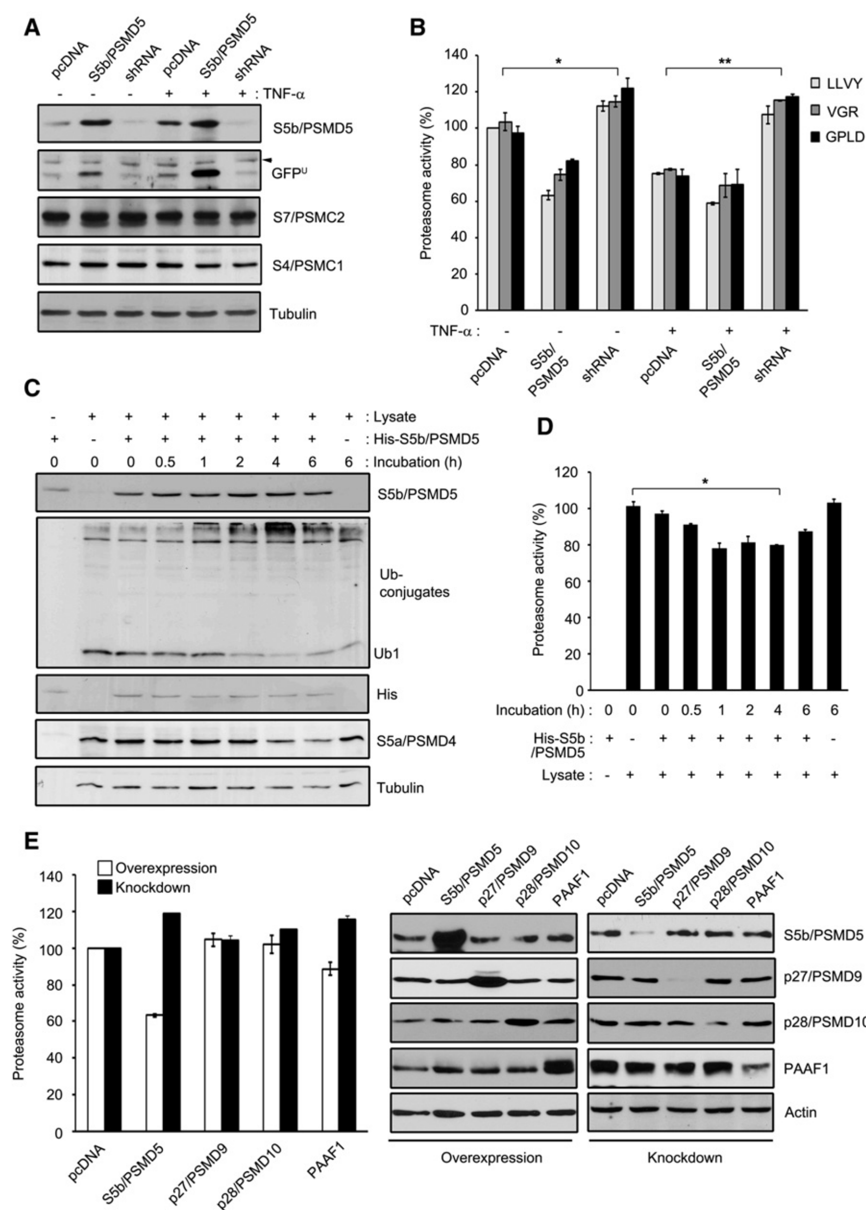

Figure 3. Inhibition of Proteasome Activity by TNF-α via S5b/PSMD5

(A and B) HEK293T cells were cotransfected for 42 hr with GFP^u and pcDNA, S5b/PSMD5 cDNA, or S5b/PSMD5 shRNA#2, after which they were left untreated or incubated with TNF-α (50 ng/ml) for 6 hr. Cell extracts were analyzed by western blotting (A). The arrowhead indicates a nonspecific signal. Fluorogenic substrates were used to measure three types of proteasome activities in the same cell extracts (B). Error bars represent mean ± SD (n = 3) *$p < 0.05$, **$p < 0.0001$. See also Figures S2 and S3.

(C and D) HEK293T cell lysates (100 μg) were incubated with purified His-S5b/PSMD5 protein (3 μg) for the indicated times. Western blot analyses were then performed (C), and proteasome activities were examined using Suc-LLVY-AMC (D). Error bars represent mean ± SD (n = 3). *$p < 0.01$.

(E) HEK293T cells were transfected for 48 hr with S5b/PSMD5, p27/PSMD9, p28/PSMD10, and PAAF1 cDNAs (overexpression) or siRNAs (knockdown), and cell lysates were examined for proteasome activity using Suc-LLVY-AMC (left) and analyzed by western blotting (right). Error bars represent mean ± SD.

(three fractions) toward low molecular weight fractions (Figure 4B, middle; Figure S4C). A similar shift was detected in cells overexpressing S5b/PSMD5 (Figure 4B, bottom; Figure S4C). Enzyme assays of each fraction showed that the activities in 26S proteasome fractions were significantly suppressed by TNF-α and more markedly inhibited in cells overexpressing S5b/PSMD5. In contrast, enzymatic activities in the 20S proteasome fractions were increased by TNF-α or S5b/PSMD5 overexpression in the presence of SDS (Figures 4C and S4E). Moreover, immunoprecipitation assays revealed that the interactions of 20S core subunits with the 19S subunits were inhibited by TNF-α and S5b/PSMD5 overexpression, but not by S5b/PSMD5 R184E overexpression (Figure 4D). Taken together, these results suggest that TNF-α and S5b/PSMD5 overexpression suppress the assembly of 26S proteasome.

Interaction of Excess S5b/PSMD5 with S7/PSMC2 Is Critical for Proteasome Inhibition

S5b/PSMD5 is known to interact with S4/PSMC1, S7/PSMC2, and S2/PSMD2 in vitro (Gorbea et al., 2000). We also found that S5b/PSMD5 interacted with S7/PSMC2 in the brain tissue of S5b/PSMD5 transgenic (*Tg*) mice (Figure S5A). Interestingly, in cells overexpressing HA-S5b/PSMD5 and His-S7/PSMC2, TNF-α enhanced the interaction of HA-S5b/PSMD5 with S7/PSMC2 without affecting the total cellular levels of S7/PSMC2 (Figure 5A, top). On the other hand, TNF-α had no effect on the interaction of S5b/PSMD5 with either S4/PSMC1 or S2/PSMD2, and S5b/PSMD5 did not interact with S5a/PSMD4 (Figure 5A, middle and bottom; Figure S5B), indicating that TNF-α enhances the interaction of S5b/PSMD5 with S7/PSMC2. We further examined the interaction of endogenous S5b/PSMD5 with S7/PSMC2 in the proteasome and nonproteasome fractions obtained from gel filtration (FPLC) analysis. Unlike the other 19S subunits, which were detected in the proteasome fractions (12–24) from FPLC analysis, the majority of S5b/PSMD5 was detected in the nonproteasome fractions (33–46) of control cells (Figures 4B and 5B, top; Figure S4D). Consistently, TNF-α significantly increased the amounts of S7/PSMC2 and S5b/PSMD5 in the nonproteasome fractions (Figure 5B, middle). We therefore pooled the nonproteasome fractions (30–45) and performed immunoprecipitation analyses. Compared to control, TNF-α

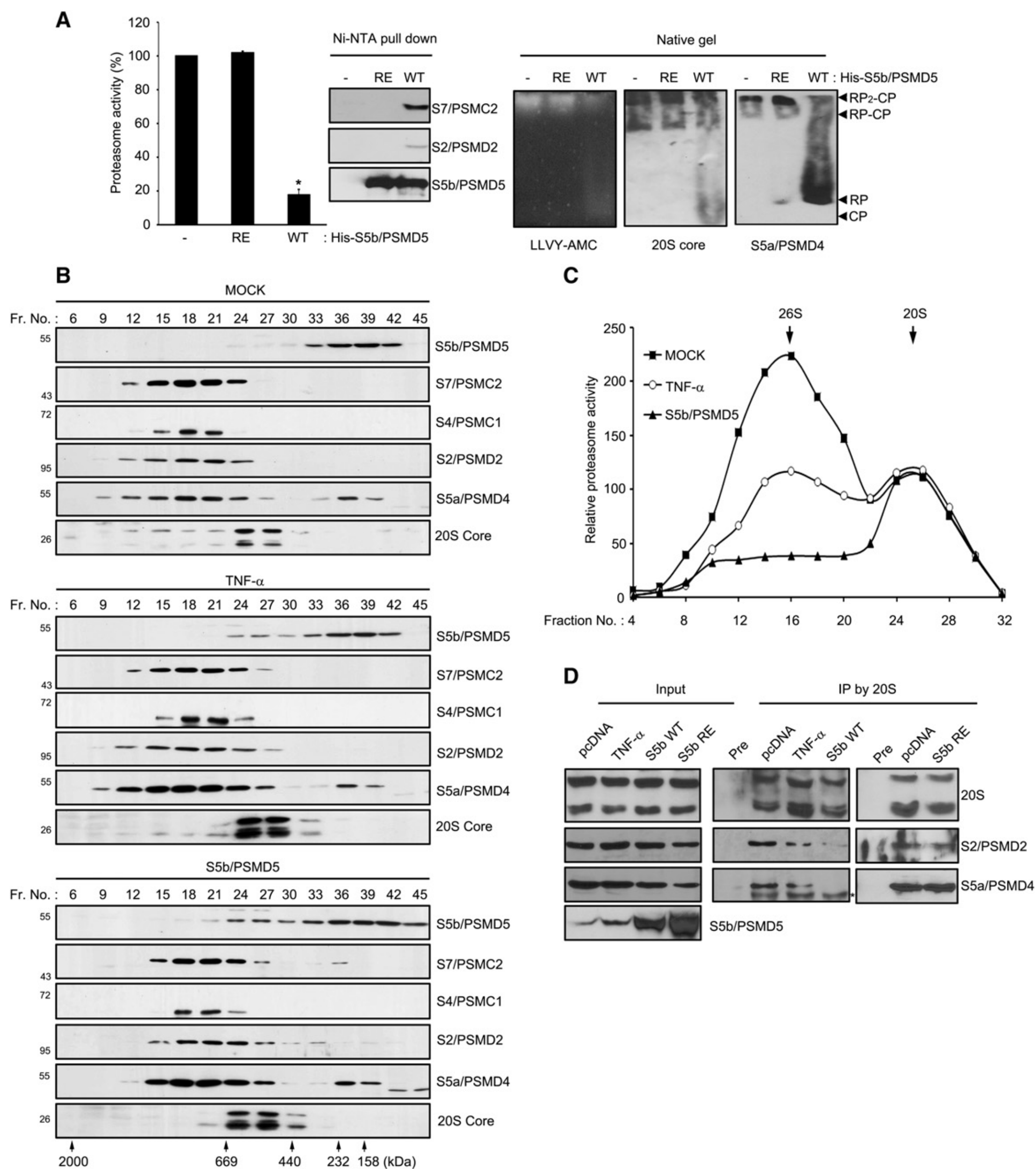

Figure 4. TNF-α and Excess S5b/PSMD5 Inhibit 26S Proteasome Assembly and Activity

(A) Purified 26S proteasome (3 μg) was incubated with His-S5b/PSMD5 (WT) or His-S5b/PSMD5 R184E (RE) protein (5 μg) for 3 hr, and the reaction mixtures were subjected to proteasome activity analysis using Suc-LLVY-AMC (left) and pull-down analyses with Ni-NTA resin and western blotting (middle). The samples were also resolved on native gel (right), and the gel was then overlaid with Suc-LLVY-AMC (Native gel, left) before transfer to a nitrocellulose membrane for western blotting (Native gel, middle and right). The RP and catalytic particle (CP) of proteasome complexes are shown. Error bars represent mean ± SD. *$p < 0.0001$.

(B and C) HEK293T cells were exposed to TNF-α (50 ng/ml) for 6 hr or transfected with S5b/PSMD5 for 36 hr. Cell extracts were fractionated by gel filtration using a Superose 6 column, and the respective locations of 26S and 20S in the fractions are indicated by arrows. Sets of three fractions were analyzed by western blotting using the indicated antibodies against proteasome subunits (B). Blue dextran (2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) were used as molecular weight markers. Chymotrypsin-like proteasome activity was measured in each fraction using Suc-LLVY-AMC (C). Fr. No., fraction number. See also Figure S4.

(D) HEK293T cells were transfected with pcDNA, S5b/PSMD5 WT, or S5b/PSMD5 R184E for 36 hr or treated with TNF-α for 6 hr. Cell extracts were prepared and subjected to immunoprecipitation (IP) analyses using preimmune serum (Pre) or anti-20S core antibody (20S). Whole-cell lysates (Input) and the immunoprecipitates were analyzed by western blotting.

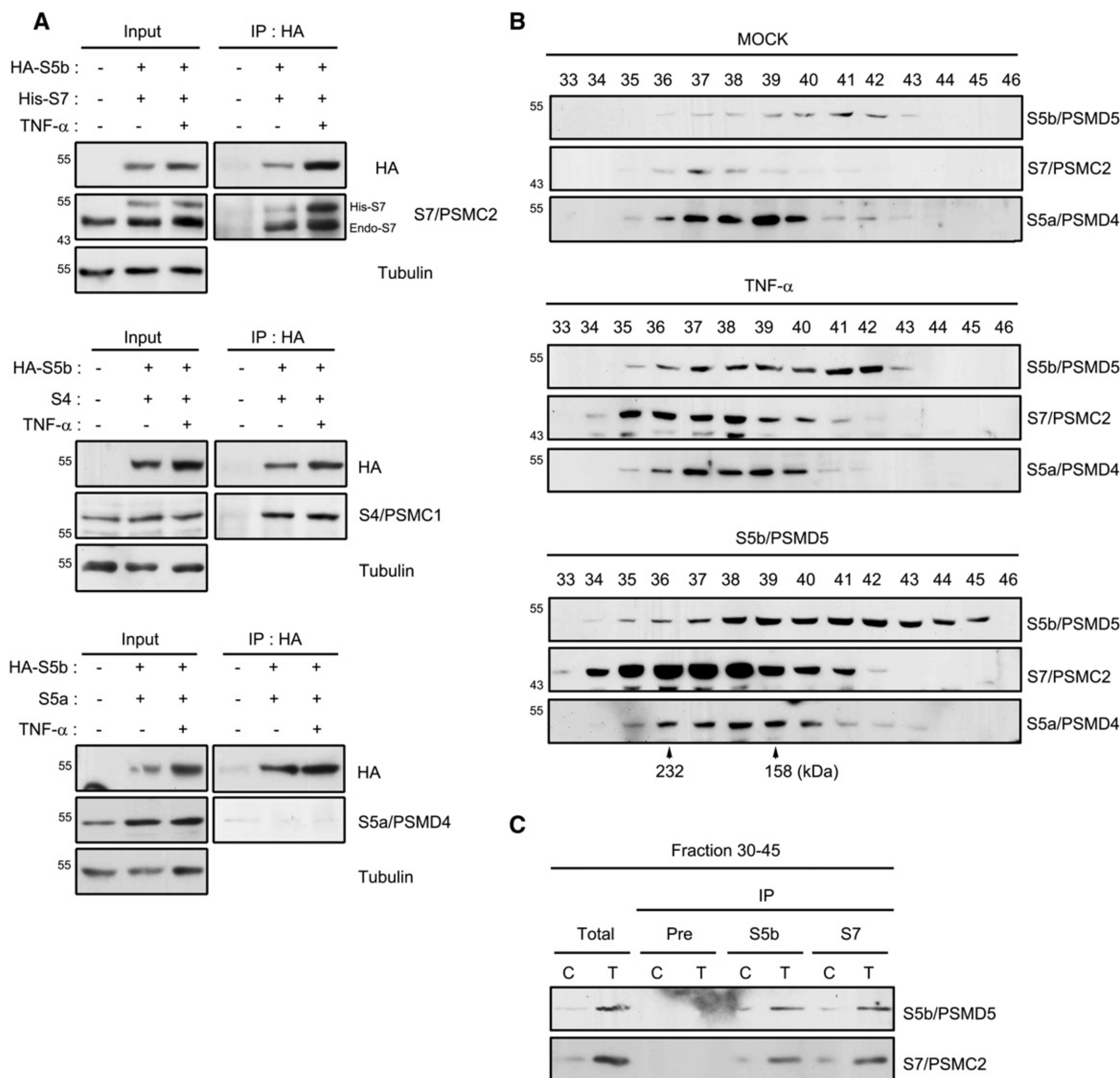

Figure 5. TNF-α Enhances the Interaction between S5b/PSMD5 and S7/PSMC2 in Nonproteasome Complexes

(A) HEK293T cells were cotransfected for 36 hr with HA-S5b/PSMD5 and His-S7/PSMC2, S4/PSMC1, or S5a/PSMD4, and then left untreated or treated with TNF-α (50 ng/ml) for 6 hr. Following immunoprecipitation (IP) using anti-HA antibody, western blot analyses were performed. See also Figure S5.

(B) The nonproteasome fractions (33–46) in Figure 4B were analyzed by western blotting.

(C) Fractions 30–45 of Mock (lane C) or TNF-α-treated cell lysates (lane T) in (B) were pooled (Total) and subjected to IP assays using preimmune serum (Pre), anti-S5b/PSMD5, or anti-S7/PSMC2 antibody. Western blot analyses were then performed.

enhanced the interaction between S5b/PSMD5 and S7/PSMC2 in the nonproteasome fractions (Figure 5C).

To identify the S5b/PSMD5 domain that interacts with S7/PSMC2 and inhibits proteasome activity, we constructed serial deletion mutants based on the predicted secondary structure of S5b/PSMD5 (Figure 6A). Immunoprecipitation analyses revealed that the S5b/PSMD5 N3 mutant interacted with both His-S7/PSMC2 and endogenous S7/PSMC2, whereas the other S5b/PSMD5 mutants failed to do so (Figure 6B). Among the S5b/PSMD5 mutants, only the S5b/PSMD5 N3 mutant induced GFP^u accumulation (Figure 6C). The observations suggest that the interaction between S5b/PSMD5 and S7/PSMC2 may be critical for the inhibition of proteasome activity.

We then explored the contribution of the S5b/PSMD5-S7/PSMC2 interaction to proteasome inhibition. We then synthesized Tat-tagged S5b/PSMD5 peptide (amino acid residues 181–188) to target the interaction between S5b/PSMD5 and S7/PSMC2 (Figure 6A). When added to cells, the Tat-S5b (181–188) peptide rescued TNF-α-induced GFP^u accumulation in a dose-dependent manner (Figure 6E, left; Figure 6F) and inhibited the interaction between S5b/PSMD5 and S7/PSMC2 in cells (Figure 6E, right). In contrast the Tat-S5b (181–188) R184E peptide failed to exhibit these activities. Collectively, these observations strongly suggest that the interaction between S5b/PSMD5 and S7/PSMC2 is critical for TNF-α-induced inhibition of proteasome activity.

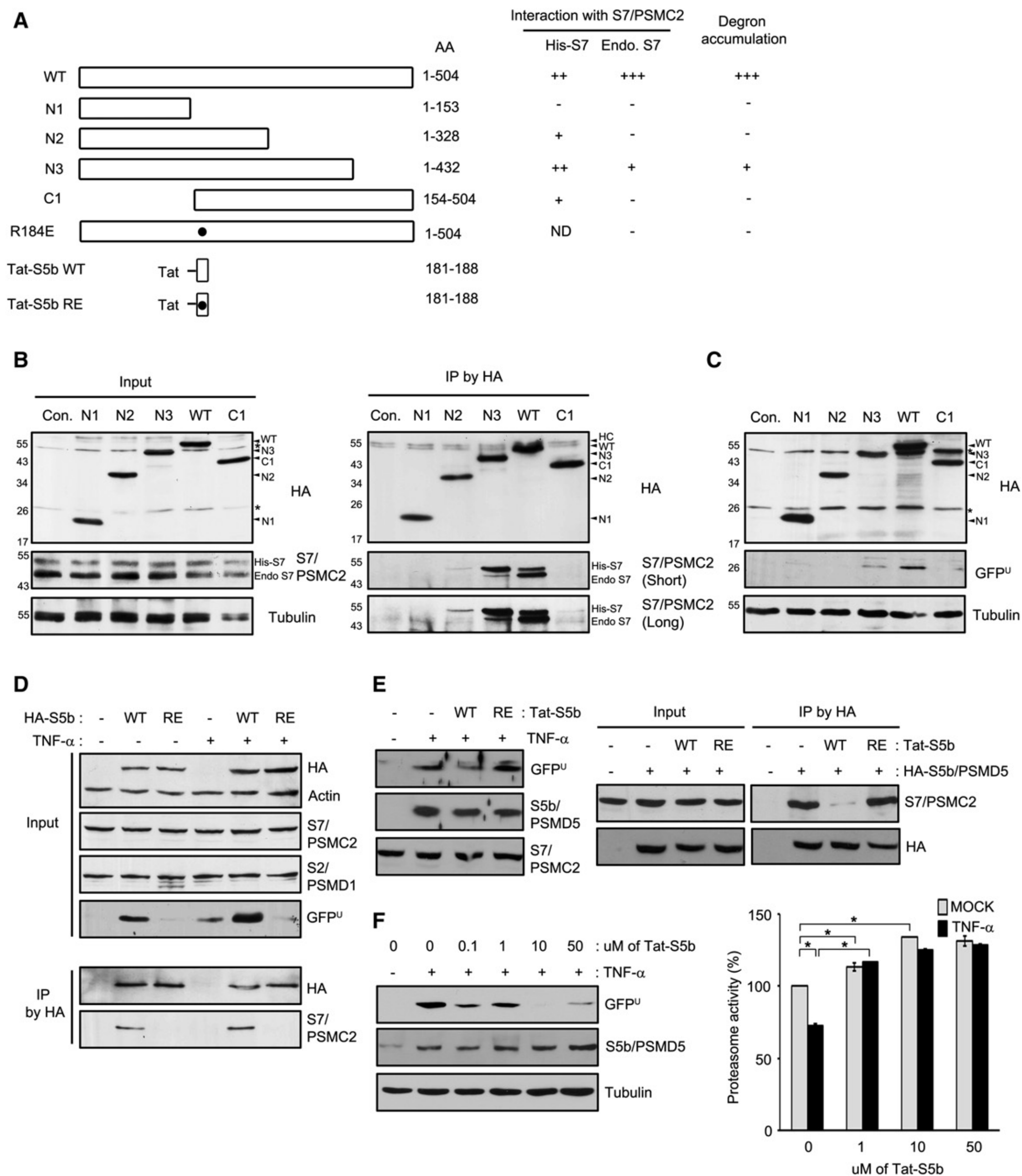

Figure 6. The Interaction between S5b/PSMD5 and S7/PSMC2 Is Critical for Proteasome Inhibition

(A) Schematic diagram of S5b/PSMD5 mutants and Tat-S5b peptides (left) and summary of their abilities to interact with S7/PSMC2 and modulate degron accumulation (right). The numbers indicate amino acid residues (AA) of human S5b/PSMD5.

(B) HEK293T cells were cotransfected with S5b/PSMD5 deletion mutants and His-S7/PSMD5, and immunoprecipitation (IP) assays were performed with anti-HA antibody. Total cell lysates (Input) (left) and the immunoprecipitates (IP) (right) were analyzed by western blotting.

(C) HEK293T cells were cotransfected with S5b/PSMD5 deletion mutants and GFP^{u} for 36 hr and cell extracts analyzed by western blotting.

(D) HEK293T cells were transfected with pcDNA, HA-S5b/PSMD5 (WT), or HA-S5b/PSMD5 R184E (RE) for 36 hr and left untreated or treated with TNF-α for 6 hr, after which cell lysates were subjected to an IP assay to examine the interaction with endogenous S7/PSMC2. Western blot analyses were performed.

(E) HEK293T cells were transfected with GFP^{u} for 24 hr, left untreated or pretreated with 10 μM Tat-S5b (181–188) (WT) or Tat-S5b (181–188) R184E (RE) peptide for 18 hr, and then exposed to TNF-α (50 ng/ml) for 6 hr. Cell extracts were prepared and analyzed by western blotting (left). After transfection with HA-S5b/PSMD5 for 24 hr, HEK293T cells were incubated with 50 μM Tat-S5b (181–188) (WT) or Tat-S5b (181–188) RE peptide for an additional 24 hr. Cell extracts were immunoprecipitated using anti-HA antibody (right).

(F) HEK293T cells were transfected with GFP^{u} for 24 hr and then incubated with the indicated concentrations of Tat-S5b WT peptide. Western blot analyses were performed (left), and proteasome activities were measured (right). Error bars represent mean ± SD (n = 3). *$p < 0.001$.

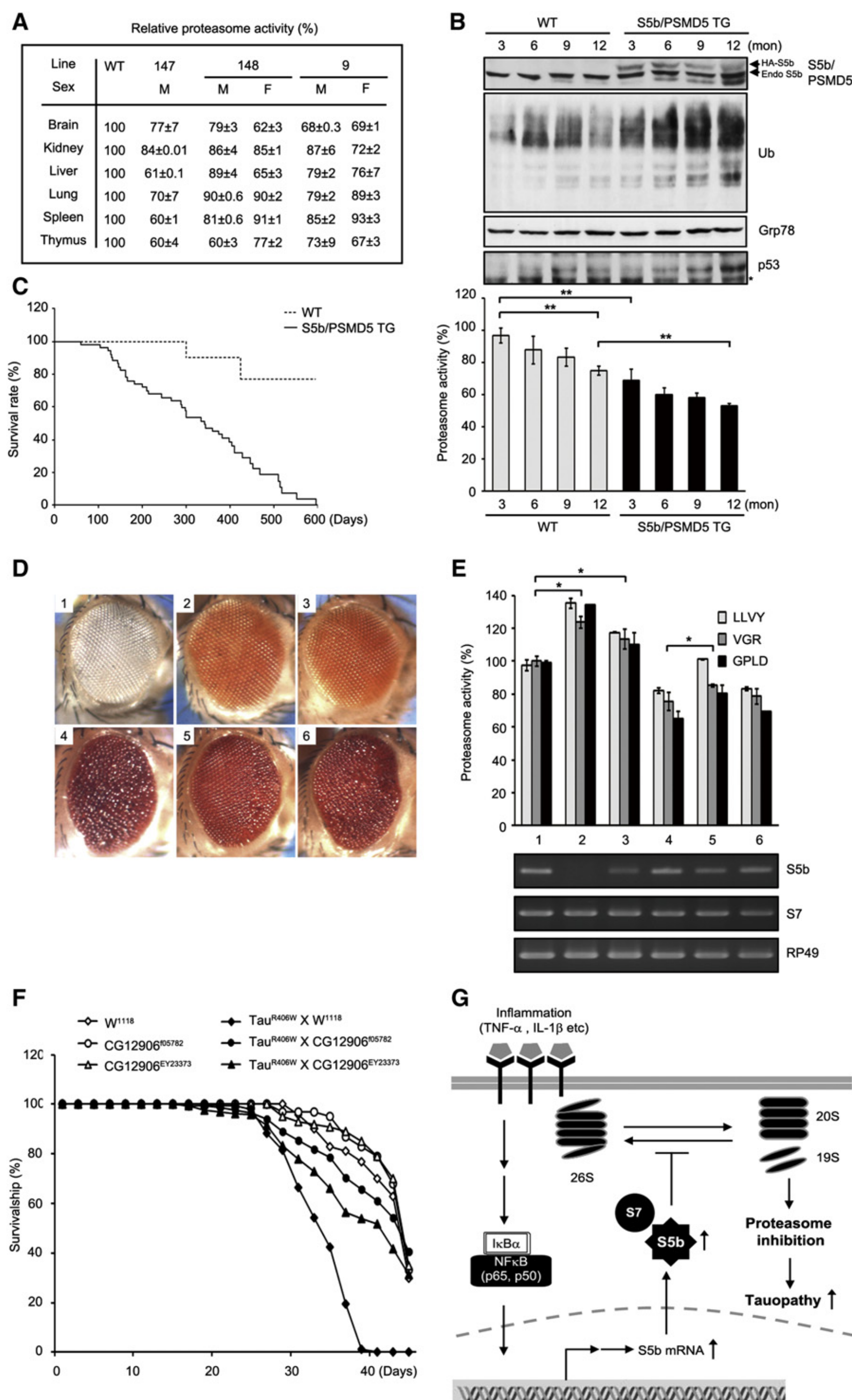

Figure 7. Expression Level of S5b/PSMD5 Regulates Proteasome Activity and Tauopathy in Animal Models

(A) Mouse tissues were collected from 4-week-old WT and S5b/PSMD5 *Tg* mice (lines 147, 148, and 9), and tissue extracts were analyzed for proteasome activities using Suc-LLVY-AMC. Proteasome activity in WT tissue was assigned a value of 100, and activity in the S5b/PSMD5 *Tg* tissue is expressed relative to that of the WT. F, female; M, male. Error bars represent mean ± SD (n = 3).

(B) Cortical tissues were collected from 3-, 6-, 9-, and 12-month-old S5b/PSMD5 *Tg* mice and their littermates, tissue extracts were analyzed by western blotting (top), and proteasome activity was measured using Suc-LLVY-AMC (bottom). Error bars represent mean ± SD. $^{**}p < 0.0001$. See also Figure S6.

(C) Life span of WT (n = 30) and S5b/PSMD5 *Tg* (n = 54) mice.

(D) WT (1, w^{1118}) and S5b/PSMD5 mutant (2, $CG12906^{f05782}$; 3, $CG12906^{EY23373}$) flies were crossed to generate tau rough eye/WT (4, gl-tau$^{2.1}$ X w^{1118}), tau rough eye/S5b/PSMD5 heterozygote (5, gl-tau$^{2.1}$ X $CG12906^{f05782}$), and tau rough eye/S5b/PSMD5 heterozygote (6, gl-tau$^{2.1}$ X $CG12906^{EY23373}$) lines. Typical fly eyes are shown.

(E) Fly heads were collected, and tissue extracts were analyzed for proteasome activities (top). Error bars represent mean ± SD. Total RNA was isolated and analyzed by RT-PCR (bottom). The fly genotypes are (1) w^{1118}, (2) $w^{1118}CG12906^{f05782}$, (3) $y^1w^{67c23}CG12906^{EY23373}$, (4) w^{1118}; *gl-tau*$^{2.1}$/+, (5) $w^{1118}CG12906^{f05782}$/+; *gl-tau*$^{2.1}$/+, and (6) $y^1w^{67c23}CG12906^{EY23373}$/+; *gl-tau*$^{2.1}$/+. $^{*}p < 0.001$.

(F) Newly eclosed adults were collected, and more than 200 flies were placed in each culture vial. Flies were maintained at 25°C and moved to a humidified temperature-controlled environmental chamber at 29°C for life span measurement. Every 2 days, flies were transferred to new vials, and the dead flies were enumerated. The fly genotypes are w^{1118}, $w^{1118}CG12906^{f05782}$, $y^1w^{67c23}CG12906^{EY23373}$, w^{1118} *elav-gal4; UAS-tau*^{R406W}/+, $w^{1118}CG12906^{f05782}/w^{1118}$ *elav-gal4; UAS-tau*^{R406W}/+, and $y^1w^{67c23}CG12906^{EY23373}/w^{1118}$*elav-gal4; UAS-tau*^{R406W}/+.

(G) Proposed model for the role of S5b/PSMD5 in TNF-α/NFκB-mediated proteasome inhibition and tauopathy pathogenesis. S5b/PSMD5 protein expression is induced by TNF-α/NFκB and interferes with the assembly and, thus, activity of 26S proteasome via its increased interaction with S7/PSMC2, leading to accumulation of ubiquitin conjugates and p-Tau in the affected neurons for neurodegeneration. See also Figure S7.

Proteasome Regulation by S5b/PSMD5 in Animal Models and Its Contribution to Tau Pathology

To assess the effect of S5b/PSMD5 upregulation on proteasome activity in vivo, we generated S5b/PSMD5 *Tg* mice using the CMV/β-actin promoter (pCAGGS), which drives the ubiquitous expression of HA-S5b/PSMD5 (Niwa et al., 1991). HA-S5b/PSMD5 was expressed in the brain and almost all other tissues of 4-week-old S5b/PSMD5 *Tg* mice (line 147) (Figure 7B, top; Figure S6A). In enzyme assays, proteasome activities were downregulated by 30%–40% in most of the tissues of all mouse lines (147, 148, and 9) examined (Figures 7A and S6B). Particularly, compared to age-matched littermates, accumulation of ubiquitin conjugates was evident, and proteasome activity was reduced by more than 30% in

the cortex of 3- to 12-month-old S5b/PSMD5 *Tg* mice (Figure 7B).

The S5b/PSMD5 *Tg* mice (line 147) were apparently smaller than the age-matched littermates, and their body weights were significantly decreased to 40% and 25% of control in 9-month-old female and male mice, respectively (Figures S6C–S6E). In addition, skeletal changes, such as curvature of the upper back, and loss of hair glossiness were evident in 9-month-old S5b/PSMD5 *Tg* mice (line 147) (Figure S6C). Furthermore, tumor suppressor p53 was upregulated in the cortex of S5b/PSMD5 *Tg* mice compared to the littermates (Figure 7B, top). S5b/PSMD5 *Tg* mice exhibited a shorter life span (average 343 days) than the wild-type (WT) littermate (average 782 days), as evaluated by a Kaplan-Meier survival curve (Figure 7C). These observations are consistent with earlier reports showing a strong association of UPS inhibition and inflammation with the premature aging phenotype (Hamer et al., 2010; Liu et al., 2011; Min et al., 2008).

The *CG12906* gene has been identified as a fly homolog of the human S5b/PSMD5 gene (Le Tallec et al., 2009); therefore, we measured proteasome activity in *CG12906* mutant flies. Although two lines of S5b/PSMD5 mutant flies (*CG12906^{f05782}* and *CG12906^{EY23373}*) showed no significant morphological defects in their eyes (Figure 7D, top), enzyme assays revealed that the proteasome activity was significantly enhanced in the S5b/PSMD5 mutant flies, particularly in the S5b/PSMD5-deficient *CG12906^{f05782}* fly (Figure 7E, top). RT-PCR analyses revealed that S5b/PSMD5 was not expressed in the *CG12906^{f05782}* fly but was expressed to a small degree in the *CG12906^{EY23373}* fly (Figure 7E, bottom), showing a correlation between the expression level of S5b/PSMD5 and the proteasome activity in these flies.

Tau, a microtubule-binding protein, is known to be highly phosphorylated and degraded by the UPS (Dickey et al., 2006). Therefore, we assessed the role of S5b/PSMD5 in tau phosphorylation in S5b/PSMD5 *Tg* mice. Hyperphosphorylated tau (p-Tau) increased in the cortex of S5b/PSMD5 *Tg* mice compared to age-matched control littermates (Figure S7A). Similarly, the level of p-Tau highly increased in cells overexpressing S5b/PSMD5 or exposed to either TNF-α or MG132 (Figure S7B). These observations indicate that the increased expression of S5b/PSMD5 enhances p-Tau accumulation in cells and *Tg* mice.

Using the tauopathy fly model (gl-tau), which exhibits the rough eye phenotype (Jackson et al., 2002), we further addressed the role of S5b/PSMD5 in the regulation of tau-mediated neuronal degeneration. When the tau fly was crossed with the S5b/PSMD5 mutant flies, the tau rough eye phenotype was rescued in the *CG12906^{f05782}* fly and, to a lesser degree, in the *CG12906^{EY23373}* fly (Figure 7D, bottom). In addition we crossed the S5b/PSMD5 mutant flies with the tau *Tg* fly, which expressed tau^{R406W} in the neurons, and compared their life spans. The life span of S5b/PSMD5 mutant flies, *CG12906^{f05782}* and *CG12906^{EY23373}*, exhibited no apparent defects and were similar to that of WT flies (average 43 days); the tau *Tg* fly exhibited a shorter life span (average 33 days) (Figure 7F). When crossed together, the life span of the tau *Tg* fly was significantly rescued by S5b/PSMD5 deficiency (average 41 days in *CG12906^{EY23373}* and average 43 days in *CG12906^{f05782}*). Thus, these observations indicate that S5b/PSMD5 deficiency in the fly model neither alters normal life span nor induces abnormal morphology but suppresses neuronal toxicity of tau with enhanced proteasome activity. We therefore propose that S5b/PSMD5 is a critical regulator of proteasome activity in higher eukaryotic cells and animal models and contributes to the pathogenesis of UPS-associated neuronal disease, such as tauopathy.

DISCUSSION

S5b/PSMD5 Upregulated by TNF-α/NFκB Is a Proteasome Inhibitor in Higher Eukaryotes

S5b/PSMD5 was initially isolated in two forms: acidic (S5a) and basic (S5b) (Deveraux et al., 1995). Recently, yeast Hsm3 was proposed as a homolog of human S5b/PSMD5 and as one of the chaperones supporting 19S base assembly (Le Tallec et al., 2009). From our genome-wide screening and subsequent characterization, we found that excess S5b/PSMD5 inhibited proteasome activity, probably via S7/PSMC2. S7/PSMC2 is required for substrate unfolding and opening of the α ring channel of CP (Murata et al., 2009). Mutation of S7/PSMC2 elicits a severe growth defect in yeast (Rubin et al., 1998), and knockdown of S7/PSMC2 expression in fly leads to incomplete 26S proteasome assembly (Szabó et al., 2007). Likewise, downregulation of S7/PSMC2 led to GFP^{u} accumulation in mammalian cells (Figure S2E). Given that the half-life of S7/PSMC2 is less than 2 hr (Figures S5C and S5D), there is a possibility that the S5b/PSMD5 in nonproteasome fractions inhibits proteasome activity by binding to newly synthesized S7/PSMC2 and sequestering it into a nonproteasome complex, thereby causing a partial deficiency of unstable S7/PSMC2 in 26S proteasome. The release of S7/PSMC2 from S5b/PSMD5 in the nonproteasome complex by the Tat-S5b peptide would rescue proteasome activity.

In cells exposed to TNF-α, a small amount of S5b/PSMD5 was also detected in proteasome complexes (fractions 24–27) in which the CP and RP subunits were detected. An inherent function of S5b/PSMD5 may be to maintain the RP complex as an assembly factor (Funakoshi et al., 2009; Park et al., 2009; Saeki et al., 2009; Le Tallec et al., 2009). These observations raise another possibility that excess S5b/PSMD5 may inhibit the association of RP with CP by binding to the C terminus of S7/PSMC2. This region of S7/PSMC2 is important for the binding of RP to the α ring of CP (Smith et al., 2007; Le Tallec et al., 2009). If so, the assembly of 26S complex could be delayed by the binding of excess S5b/PSMD5 to the RP complex because assembly would only be completed once S5b/PSMD5 is dissociated from the complex. In addition, other possibilities, such as S5b/PSMD5 modification by other proteins present in the S5b/S7 complex, remain to be addressed.

In yeast, by contrast, Hsm3 deletion shows defective proteasome function (Le Tallec et al., 2009). Although the function of S5b/PSMD5 as a proteasome chaperone is well studied in yeast, a study in mammalian cells did not show any obvious defects in the proteasome assembly in S5b/PSMD5 knockdown cells (Kaneko et al., 2009). Also, a recent report showed that Hsm3 overexpression inhibited proteasome assembly (Barrault et al.,

2012). These observations raise a possibility that there may be a functional difference in the role of S5b/PSMD5 in yeast and higher eukaryotes, such as *D. melanogaster* and mouse, which are functionally more complex than yeast (e.g., they have an immune system). Furthermore, the sequence identity between yeast Hsm3 and human S5b/PSMD5 is very low (less than 20%), whereas that of S7/PSMC2 is over 70% (http://www.ebi.ac.uk/Tools/sequence.html, Needle program).

Rpn14 has been reported to act as a chaperone with Hsm3 during base assembly; its deletion causes a defect in base assembly and inhibits 26S proteasome activity in yeast (Funakoshi et al., 2009; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). In contrast, knockdown of PAAF1 (human homolog of yeast Rpn14) expression in mammalian cells enhances proteasome activity, and PAAF1 overexpression inhibits it (Lassot et al., 2007; Park et al., 2005), whereas the effects were not as big as S5b/PSMD5. In higher eukaryotes, S5b/PSMD5 and PAAF1 may have distinct functions, though only the expression of S5b/PSMD5 is regulated by TNF-α/NFκB. Thus, in addition to their proposed inherent function in base assembly, levels of the 19S proteasome assembly chaperones (e.g., S5b/PSMD5) may also be an important determinant of proteasome activity.

Significance of Proteasome Inhibition by TNF-α/S5b/PSMD5 in UPS-Associated Neuronal Degeneration

Reduced proteasome activity or increased inflammation has been reported to decrease longevity and accelerate aging (Hamer et al., 2010; Liu et al., 2011; Min et al., 2008). The S5b/PSMD5 *Tg* mice were apparently smaller than age-matched littermates, and their body weights were significantly decreased with the premature onset of aging-associated phenotypes. On the other hand, the UPS impairment hypothesis in neurodegenerative diseases remains controversial. In some studies, global UPS impairment was not observed in HD model mice (Maynard et al., 2009; Valera et al., 2007). Others observed that proteasome inhibition caused the aggregation of misfolded proteins, which was associated with perturbation of cellular functions and various human disorders (Tyedmers et al., 2010). Our data from tau protein support the latter that proteasome inhibition is one of the major factors involved in the pathogenesis of neurodegenerative diseases, depending on the nature of aggregation-prone proteins. Therefore, UPS impairment is believed to be associated with chronic neurodegenerative diseases (Ciechanover and Brundin, 2003).

Moreover, neuroinflammation is an important pathophysiological feature of neurodegenerative diseases (Gao and Hong, 2008). Consistently, *Tg* mice expressing TNF-α in their brains show chronic inflammation and neurodegeneration (Probert et al., 1997). Thus, inhibition of the inflammation alleviates the progression of neurodegenerative diseases (He et al., 2007; Tweedie et al., 2007). Although many reports have suggested that neuroinflammation mediates the pathology of neurodegenerative diseases, most studies have mainly focused on the role of oxidative stress (Boje and Arora, 1992). On the other hand, we propose that TNF-α inhibits proteasome activity/assembly in higher eukaryotic cells. Thus, enhancement of proteasome activity in neuronal cells and tissues can rescue tau-mediated neuronal degeneration, as seen in our tau rough eye model with S5b/PSMD5 deficiency. Consistently, TNF-α potently inhibited proteasome activity via S5b/PSMD5 to increase the number of mutant huntingtin exon1-containing expanded polyglutamine (n = 120) aggregates in neuronal cells (data not shown). These findings suggest that in general, proteasome inhibition resulting from upregulated S5b/PSMD5 may link the neuroinflammation signal to neuronal degeneration in the aforementioned diseases (e.g., AD and HD).

Unlike MG132 and in vitro analysis using purified S5b/PSMD5 protein and 26S proteasome, TNF-α and S5b/PSMD5 overexpression partially inhibited proteasome activity, ranging from 15% to 25% in cultured cells and 30%–40% in mouse tissues. We believe that the relative ratio in the amount of S5b/PSMD5 protein to proteasome in cells under inflammation is not as high as that in the in vitro analysis using purified proteins. In addition, action mechanisms of MG132 and S5b/PSMD5 in the proteasome inhibition are distinct. Given that most neurodegenerative diseases are chronic and associated with aging, the partial inhibition of proteasome activity triggered by TNF-α, IL-1β, or LPS may contribute to the pathogenesis of diseases caused by chronic inflammation. We would like to define this kind of proteasome regulation exhibited by S5b/PSMD5-deficient flies and S5b/PSMD5 *Tg* mice as "chronic inhibition of proteasome activity." In conclusion we propose that S5b/PSMD5 plays an important role in a tauopathy model and in TNF-α/NFκB-mediated proteasome inhibition and has therapeutic potential in neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Cell-Based Functional Screening

HEK293T cells were grown in a 96-well culture plate for 24 hr and cotransfected with GFP^u and various cDNAs for 36 hr. Thereafter, cells were observed under a fluorescence microscope, and relative fluorescence intensities were compared to that of control (pcDNA). A monomeric RFP (mRFP), included in every transfection, served as an internal transfection control. The cDNA expression plasmids were constructed in pcDNA by PCR and analyzed by DNA sequencing.

Cell Culture and DNA Transfection

HEK293T and other cells were obtained from the American Type Culture Collection and grown in DMEM or RPMI containing 10% fetal bovine serum and penicillin/streptomycin at 37°C under 5% CO_2 (v/v). NFκB $p65^{-/-}$ and $p50^{-/-}$ MEFs were kindly provided by Dr. A. Hoffmann (University of California, San Diego, La Jolla, CA, USA) (Hoffmann et al., 2003). Cells were transfected with the appropriate vectors using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Antibodies

The following antibodies were used: anti-S5b/PSMD5 (Novus); anti-S7/PSMC2, anti-S4/PSMC1, anti-20S core antibodies (Biomol); anti-S2/PSMD2 antibody (Abcam); anti-GFP, anti-Tubulin, anti-Ub, anti-IκBα antibodies (Santa Cruz Biotechnology); anti-p27/PSMD9, anti-p28/PSMD10, anti-PAAF1, anti-TCF11/Nrf1 antibodies (Sigma-Aldrich); and anti-His antibody (Santa Cruz Biotechnology). Anti-PHF-1 antibody was kindly provided by Dr. P. Davies (Albert Einstein College of Medicine, Bronx, NY, USA).

Native-PAGE

Proteasome assembly was examined by native PAGE, as previously described by Elsasser et al. (2005). Cell lysates were separated on 4% (w/v) native-PAGE at 4°C. The gel was overlaid with buffer containing Suc-LLVY-AMC with or

without 0.001% SDS, or transferred to nitrocellulose membranes for western blot analysis.

Preparation of Cell Extracts and Separation of Proteasome Complexes by Gel Filtration

Cells were lysed in a lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 2 mM ATP), lysates were centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was filtered through a 0.2 μm membrane (Sartorius). Gel filtration was carried out using a Superose 6 FPLC column (AKTA; GE Healthcare), and 0.25 ml fractions were collected.

Proteasome Activity Assays

After preparation of cell lysates in retic buffer, proteasome activities were measured using the fluorogenic substrates Suc-LLVY-AMC, Bz-VGR-AMC, and Ac-GPLD-AMC (Biomol) and a fluorometer (FlexStation 3 Microplate Reader; Molecular Devices). The excitation and emission wavelengths were 380 and 460 nm, respectively. Purified 26S proteasome was purchased from Boston Biochem.

In Vitro Ubiquitination

HEK293T cell lysates (100 μg) were incubated with purified His-S5b/PSMD5 (3 μg) at 37°C in ubiquitination buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 10% glycerol, 1 mM ATP, 1× ATP regeneration system, 1× protease inhibitor cocktail). Cell lysates were then subjected to SDS-PAGE for western blotting or assayed for proteasome activity using Suc-LLVY-AMC.

Preparation of Tat-S5b Peptide

S5b/PSMD5 (181–188) (VRYRVYEL) and S5b/PSMD5 R184E (VRYEVYEL) peptides were synthesized and coupled to a membrane-permeant Tat-derived sequence (YARAAARQARA) (Peptron, Daejeon, South Korea).

Tauopathy Flies

D. melanogaster was raised in the dark at 25°C. The WT (W^{1118}) and S5b/PSMD5 mutant ($CG12906^{f05782}$ and $CG12906^{EY23373}$) strains used in this study were obtained from the Bloomington Drosophila Stock Center at Indiana University. Various genetic combinations were generated using standard crosses.

Generation of *Tg* Mouse

The pCAGGS-HA-S5b/PSMD5 vector was injected into mouse embryos with a C57BL genetic background, and three independent founders (F_0) were identified by PCR analysis using synthetic oligonucleotides (forward, TG S5b-F; reverse, S5b N1-R) (Macrogen, Seoul, Korea). The primer sequences are listed in Table S2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.07.013.

LICENSING INFORMATION

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