Aggresomal sequestration and STUB1-mediated ubiquitination during mammalian proteaphagy of inhibited proteasomes

Won Hoon Choia,b, Yejin Yuna,b, Seoyoung Parka,c, Jun Hyoung Jeona,b, Jeeyoung Leea,b, Jung Hoon Leea,b, Su-A Yangd, Nak-Kyoon Kima, Chan Hoon Jungb, Yong Tae Kwonb, Dohyun Hanf, Sang Min Limf, and Min Jae Leea,b,c

*Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, 03080 Seoul, Korea; aDepartment of Biomedical Sciences, Seoul National University Graduate School, 03080 Seoul, Korea; bNeuroscience Research Institute, Seoul National University College of Medicine, 03080 Seoul, Korea; cScience Division, Tomonube, 34109 Danjeon, Korea; dConvergence Research Center for Diagnosis, Korea Institute of Science and Technology, 02792 Seoul, Korea; and eProteomics Core Facility, Biomedical Research Institute, Seoul National University Hospital, 03080 Seoul, Korea

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The 26S proteasome, a self-compartmentalized protease complex, plays a crucial role in protein quality control. Multiple levels of regulatory systems modulate proteasomal activity for substrate hydrolysis. However, the degradation mechanism of mammalian proteasomes is poorly understood. We found that inhibited proteasomes are sequestered into the insoluble aggresome via HDAC6- and dynenine-mediated transport. These proteasomes colocalized with the autophagic receptor SQSTM1 and cleared through selective macroautophagy, linking aggresomal segregation to autophagic degradation. This proteaphagic pathway was counterbalanced with the recovery of proteasomal activity and was critical for reducing cellular proteostress. Changes in associated proteins and polyubiquitulation on inhibited 26S proteasomes participated in the targeting mechanism to the aggresome and autophagosome. The STUB1 E3 Ub ligase specifically ubiquitylated purified human proteasomes in vitro, mainly via Lys63-linked chains. Genetic and chemical inhibition of STUB1 activity significantly impaired proteasome processing and reduced resistance to proteostress. These data demonstrate that aggresomal sequestration is the crucial upstream event for proteasome quality control and overall protein homeostasis in mammals.

**Intracellular proteolysis not only regulates various homeostatic pathways responding to environmental changes and nutritive conditions for survival but also contributes to protein quality control (PQC) by removing defective, misfolded, and potentially harmful proteins from the cell. Two machineries, the ubiquitin (Ub)–proteasome system (UPS) and autophagy–lysosome system (henceforth referred to as autophagy), are responsible for controlled protein degradation in eukaryotes. The UPS accounts for the majority of cellular proteolysis, continuously regulating the clearance of most short-lived proteins with polyubiquitin (polyUb) chains assembled in different linkage conformations (1). In contrast, autophagy operates at a basal level under normal conditions, but is stimulated during various pathological and physiological states (2).

...Gargoyles subjected to autophagic degradation. This proteaphagic pathway was counterbalanced with the recovery of proteasomal activity and was critical for reducing cellular proteostress. Changes in associated proteins and polyubiquitination on inhibited 26S proteasomes participated in the targeting mechanism to the aggresome and autophagosome. The STUB1 E3 Ub ligase specifically ubiquitylated purified human proteasomes in vitro, mainly via Lys63-linked chains. Genetic and chemical inhibition of STUB1 activity significantly impaired proteasome processing and reduced resistance to proteostress. These data demonstrate that aggresomal sequestration is the crucial upstream event for proteasome quality control and overall protein homeostasis in mammals.

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**Significance**

The ubiquitin–proteasome system and autophagy are two major intracellular proteolytic pathways, and both remove misfolded and proteotoxic proteins from eukaryotic cells. This study describes the detailed regulatory pathway of proteasome degradation by autophagy for its own quality control. We discovered that a portion of inhibited proteasomes is actively sequestered into the aggresome, an insoluble fraction of the mammalian cell. The aggresome functions as a triage point for proteasome recovery and autophagic degradation. This mainly distinguishes protease quality control in mammals from that in other organisms. STUB1/CHIP E3 Ub ligase has a critical role in targeting inhibited proteasomes into the aggresome. These results provide strong insights into protein catabolism in various pathological conditions originating from impaired proteasomes.

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The authors declare no competing interest.

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1To whom correspondence may be addressed. Email: minjie@snu.ac.kr.

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proteasome inhibition results in the compensatory autophagy induction (10–12). Conversely, proteasomal activity up-regulation leads to delayed autophagic flux, mainly due to the defective fusion between autophagosome and lysosome (13). If proteasomes are chronically inhibited, excess aberrant proteins concentrate into a protei-neaceous inclusion body termed the aggresome. Aggresome formation is generally believed to represent a cytoprotective mechanism by which toxic aggregates are sequestered and their autophagic clearance is facilitated (14–16). Although a variety of proteins have been detected within aggresomes, the nature and fate of proteasomes found in the aggresome remain essentially unknown.

Several studies have focused on proteasome synthesis, assembly, and activity regulation, but our understanding of their destruction mechanism is incomplete as proteasomes are generally thought to be highly abundant and stable (17, 18). Only recently, studies on Arabidopsis and Saccharomyces cerevisiae have uncovered selective protea phagic routes (19–24), and mammals appear to utilize a similar clearance process (25). In the current study, we discovered the adaptive response of mammalian cells upon proteasome inhibition, where purging inhibited proteasomes from the cell is preceded by its segregation into insoluble aggresomes via microtubule-based transport. Autophagy inhibition resulted in elevated proteasome levels in the insoluble fraction and a number of speckled puncta in the cytoplasm, indicating autophagic clearance of proteasomes in the aggresome. Targeting proteasomes to the aggresomes appeared to be associated with the attachment of Lys63-linked polyUb chains by STUB1. A biophysical screen identified a small molecule, which efficiently inhibited STUB1-mediated polyubiquitylation and aggresomal formation of inhibited proteasomes in vitro and in vivo. Considering the constant load of proteins being degraded and need for amino acids for de novo protein synthesis, nonfunctional proteasomes should be replaced to cope with proteotoxic stress on a global scale. Our findings demonstrate that STUB1-mediated proteasome ubiquitylation, aggresomal sequestration, and subsequent autophagic degradation are the major survival strategies for mammalian proteaphagy under proteotoxic stress.

Results

Inhibited Proteasomes Accumulate in the Insoluble Fraction and the Aggresome. During our initial studies on proteasome homeostasis, autophagic induction (by amino acid or glucose starvation) resulted in reduced levels of proteasome subunits in whole-cell extracts (WCEs), but chemical or genetic inhibition of autophagy (via an bafilomycin A1 [BafA1] treatment or Atg5 knockout) did not alter this tendency (SI Appendix, Fig. S1A). These data suggest that, unlike in nitrogen-starved yeast and plants (19–21), bulk autophagy might not be the major route for 26S proteasomes in mammalian cells (Fig. 1A and B). Consistent with its direct interaction with inhibited proteasomes, endogenous HDAC6 was found to strongly colocalize with aggresome-sequestered proteasomes (SI Appendix, Fig. S3A). However, prolonged proteasome inhibitor treatment led to the significant transcriptional up-regulation of many proteasome subunit-, Ub-, and autophagy-related genes (SI Appendix, Fig. S3B). These results imply that sequestration might be linked to an autophagic clearance and compensatory de novo synthesis of active proteasomes for the maintenance of the cellular proteasome pool. Collectively, we confirm that inhibited 26S proteasomes accumulate into the aggresome with the autophagic receptor SQSTM1. We speculate that aggresomal segregation is crucial for the subsequent elimination of nonfunctional proteasomes or their recovery to the soluble and active state (see below).

Inhibited Proteasomes Are Delivered to the Aggresome through HDAC6- and Dynine-Mediated Transport. To gain insight into the transport of proteasomes to the aggresome, we cotreated the cells with cycloheximide, a translation inhibitor, and MG132 for 12 h. In yeast and plants under carbon-deprived conditions (28, 29), these puncta strongly overlapped with immunostained SQSTM1, Ub, cystic fibrosis transmembrane conductance regulator-AF508 (misfolded protein), or vimentin in the MG132-treated cells (Fig. 1C and SI Appendix, Fig. S2 C–E). The characteristics and colocalization pattern of these proteins strongly indicated that proteasomal inclusions observed on the cytoplasmic side of the perinuclear region are aggresomes. Both RP and CP subunits showed similar enlarged juxtanuclear signals (SI Appendix, Fig. S2F).

Label-free holotomographic images, based on the differences in the refractive index (RI) among cellular organelles, uncovered a distinct juxtanuclear inclusion body with exceptionally high RI, concentration, and sphericity (Fig. 1 D and E and SI Appendix, Fig. S2G). When combined with fluorescence immunostaining, the RI images revealed that the aggresomal puncta were surrounded by an SQSTM1-positive inclusion body (Fig. 1D). Although the aggresome occupies a considerable volume near the nucleus, this rearrangement did not result in cell cycle arrest, mitotic failure, or apoptosis (SI Appendix, Fig. S3A). However, prolonged proteasome inhibitor treatment led to the significant transcriptional up-regulation of many proteasome subunit-, Ub-, and autophagy-related genes (SI Appendix, Fig. S3B). These results imply that sequestration might be linked to an autophagic clearance and compensatory de novo synthesis of active proteasomes for the maintenance of the cellular proteasome pool. Collectively, we confirm that inhibited 26S proteasomes accumulate into the aggresome with the autophagic receptor SQSTM1. We speculate that aggresomal segregation is crucial for the subsequent elimination of nonfunctional proteasomes or their recovery to the soluble and active state (see below).

Proteasomes Deposited in the Aggresome Are Cleared by Autophagy. To examine the fate of proteasomes deposited in the aggresome, MG132-treated cells were returned to the inhibitor-free
Fig. 1. Treatment with proteasome inhibitors led to the accumulation of proteasomes in the insoluble aggresome via microtubule-based transport. (A) Accumulation of proteasome subunits in the insoluble fraction of mouse embryonic fibroblasts (MEFs) after treatment with 5 μM MG132 for the indicated periods (0 to 24 h). Whole cell extracts (WCEs) were separated into Triton X-100-soluble (soluble) and pellet fractions (insoluble) and subjected to SDS/PAGE/immunoblotting (IB). (B) As in A, except that the cells were treated with various concentrations of MG132 for 12 h. (C) Representative images of AS49 cells treated with MG132 (10 μM, 12 h). Immunofluorescent staining (IFS) with anti-SQSTM1 (green) and anti-PSMB5 (red) antibodies. Nuclei were counterstained with DAPI (blue). (D) Refractive index (RI)-based holotomographic images of MG132 (5 μM, 12 h)-treated cells. Distinct juxtanuclear inclusion bodies from RI images are colocalized with SQSTM1-positive signals (green) from IFS. (E) Quantitative analysis of tomographic images of the aggresomes (mean RI and sphericity) compared with those of nucleoli from the identical cells. A box-and-whisker plot with n = 29. (F) Inhibition of aggresome formation in A549 cells by cotreatment with nocodazole (2 μM) and MG132 (5 μM) for 12 h. (G, Top) A549 cells were treated with 5 μM along with indicated final concentrations of nocodazole for 12 h. Insoluble fractions were fractionated from WCEs and analyzed by SDS/PAGE/IB. (H) Direct interaction between HDAC6 and a proteasome was stronger with MG132 treatment. HEK293 cells stably expressing biotin-tagged CP subunit PSMB2 were treated with MG132 (5 μM) for 6 h or 12 h. Human proteasomes were affinity-purified from WCEs and then analyzed by SDS/PAGE/IB. (I) A549 cells were transfected with 20 nM siRNA for silencing HDAC6 (siHDAC6) or with scrambled (control) siRNA (siControl) for 48 h and then treated with 5 μM MG132 for 12 h. The cells were fixed and subjected to co-IFS with anti-PSMB5 (green) and anti-SQSTM1 (red) antibodies. (Scale bars: 10 μm.) (J) GFP-tagged dynein was transiently overexpressed in HEK293-PSMB2-biotin cells in the presence of 5 μM MG132 for 12 h and/or 100 nM bafilomycin A1 (BafA1) for 4 h. WCEs (Left) were subjected to affinity purification of proteasomes with streptavidin (Right), followed by IB with indicated antibodies.
(normal) medium. The levels of both CP and RP subunits accumulated in the insoluble fraction gradually diminished during the normal washout process (Fig. 2A and SI Appendix, Fig. S4A). Correspondingly, the aggresomal structure dissipated from the outside and completely dissolved after ~24 h of MG132 washout (Fig. 2B and SI Appendix, Figs. S2A and B). A possible explanation for this observation is the reversion of inhibited proteasomes to active and soluble proteasomes in the cytosol. However, we found that cellular autophagic flux inhibition by adding either BafA1 or chloroquine led to a drastic delay in the elimination of proteasome subunits from the insoluble fraction during the washout process (Fig. 2A). Similarly, washing the drug out with a medium supplemented with autophagy inhibitors led to aggresome clearance failure and drastically increased the number of small cytoplasmic puncta (Fig. 2C), in sharp contrast to the results from normal washout (Fig. 2B). The proteasome-positive puncta colocalized with SQSTM1, and the lysosomal marker LAMP1, with the BafA1-containing washout media, both in the presence and absence of MG132, suggesting that the dissipating proteasomes are probably autolysosomes (SI Appendix, Figs. S4A and E). These results strongly indicate that autophagic degradation is actively involved in the postaggresomal processing of inhibited proteasomes, while other mechanisms, such as recovery of aggresomal proteasomes and lagged-phase segregation, may still be active.

In agreement with the results of chemical inhibition of autophagy, a knockdown of SQSTM1 using siRNA caused a significant delay in proteasome clearance from the insoluble fraction after the washout, nearly as effectively as BafA1 treatment (Fig. 2D). Notably, cells deficient in SQSTM1 were unable to form the aggresome under proteasome inhibition conditions (SI Appendix, Fig. S4G). This finding implies that impaired autophagic flux may counter-regulate aggresomal assembly of inhibited proteasomes, but the underlying feedback mechanism is yet to be determined. Moreover, we observed that autophagosome formation suppression by ATG5 and ATG7 knockdown caused a significant accumulation of proteasome subunits in the washed-out cells, as seen after SQSTM1 knockdown (Fig. 2D and SI Appendix, Fig. S5A), further confirming the defective proteasome clearance when autophagy is impaired. We then upregulated cellular autophagy by removing amino acids from the media while washing out MG132. As shown in SI Appendix, Fig. S5B, significantly lower levels of proteasome components, SQSTM1, and LC3-II were observed in the insoluble fraction, as compared with the normal washout group. These results underscore the role of autophagy in the clearance of inhibited proteasomes from the aggresome.

Furthermore, we found that significant proteasomal activity could be recovered after MG132 washout when measured with suc-LLVY-AMC as reporter substrates (Fig. 2E). Both inhibitor dissociation from proteasomes and transcriptional bounce-back mechanism are probably responsible for this recovery in activity. In contrast, when cells were treated with the irreversible inhibitors epoxomycin and carfilzomib (31, 32), there was much less recovery of proteasome function after the drugs were washed out (7.5- and 2.2-fold lower cellular proteasome activity, respectively, compared with that after MG132 washout; Fig. 2E). Furthermore, in stark contrast to MG132-treated cells, juxtanuclear aggresomes in the epoxomycin-treated cells were not cleared out, but increased in size after the washout (Fig. 2F). These results indicate that only a minor proportion of inhibited proteasomes in the cell can be concentrated in the aggresome, and defective proteasomes in the cytoplasm are still actively transported to the aggresome (lagged-phase segregation) until proteasomal activity is restored. These rationales are plausible owing to the abundance of proteasomes (>0.5% of the total cellular proteins) (17, 18). Consistent with this, in a washout experiment using cotreatment with MG132 and nocodazole, the cytoplasmic speckles in the nucodazole-treated cells appeared to be transported to the juxtenuclar aggresomes after ~12 h of washout (Fig. 2G). Although MG132 alone did not affect cell viability, cotreatment with nucodazole and MG132 resulted in substantially elevated cytotoxicity (SI Appendix, Fig. S5C). Therefore, segregating inhibited proteasomes in the aggresome promotes cell survival during prolonged proteasome inhibition.

These results indicate that the spontaneous MG132 dissociation from the inhibited proteasome might be a slow process, and aggresomal sequestration of these proteasomes could be a faster responding mechanism in mammals than direct recovery to active status. In addition, these data establish aggresomal segregation as a cellular checkpoint, where decisions are made on whether to recover incompetent proteasomes (when the inhibitors are removed) or salvage them by autophagy (e.g., during chronic inhibition) as an effective survival strategy in mammals. Despite the existence of many mechanistic differences, this proteasome dynamic appears to be parallel to the observation in yeast and plants. For example, while PSGs reversibly accommodate inactive proteasomes under energy-limited conditions, more severe stress may result in sequestration of dysfunctional proteasomes in the insoluble protein deposit (IPD)-like structure and subsequent degradation via autophagy (23, 33, 34).

Proteasome Structure and Interacting Proteins Are Actively Modified during the Sequestration Process. To investigate the molecular mechanism underlying the translocation of inhibited proteasomes to the aggresome, we first performed a sucrose-gradient ultracentrifugation analysis to monitor proteasome assembly. Following immunoblotting analysis of the fractionated WCEs, we noticed that a significant proportion of the CP and RP subunits shifted from the free form to the 26S species (31, 32), there was much less segregation of the CP and RP subcomplexes during MG132 treatment was also observed in size-exclusion chromatography (SI Appendix, Fig. S6B). Next, we performed label-free quantitative mass spectrometry (MS) based on the intensity-based absolute quantification (iBAQ) algorithm, which uses the sum of peptide intensities of a protein of interest divided by the theoretically observable peptide numbers as an accurate proxy for protein amounts (35). Proteasomes and their associated proteins after prolonged MG132 treatment were affinity-purified from three independent cultures and subjected to the iBAQ analysis (SI Appendix, Fig. S7A). This method showed significant reproducibility (as evaluated by means of intragroup components and gene ontology clustering; SI Appendix, Table S1), without requiring pre- or posttreatment of samples, e.g., isotope labeling. According to our criteria (1.5-fold change and P < 0.05), 118 differentially associated proteins (DAPs) were identified; 81 and 87 proteins significantly changed their interaction with the proteasome after 6 and 12 h of MG132 treatment, respectively, compared to that in time 0 (SI Appendix, Fig. S7B). Most of the responding proteins, including the 70-kDa heat shock protein (HSP70) family, have previously been identified as proteasome-associated proteins (36), further validating this method (SI Appendix, Fig. S7C).

In the proteomic profile, we found that, among the 49 proteins shared between two time points, DAPs with >10-fold changes include E3 Ub ligases, proteasome adaptors, and autophagy-related proteins (SI Appendix, Fig. S7E). Multiple E3 Ub ligases, such as UBE3A, UBR4, and RNF181, accumulated during the treatment of proteasomes with MG132 for 6 h (SI Appendix, Fig. S7F). Components of the BAG6–BAT3 complex, which participate in the cytosolic POC of misfolded proteins and proteasome substrates (37), were also significantly up-regulated (SI Appendix, Table S2).
Appendix, Fig. S7G), suggesting that many of these DAPs reflect the cellular responsive mechanisms against long-term proteasome inhibition. In the native gel profile of purified MG132-treated proteasomes (SI Appendix, Fig. S7H), ECM29 and HDAC6 manifested a significant upshift into higher-molecular-weight species, which is consistent with the strong association of these proteins with
inhibited proteasomes also suggesting structural remodeling (38, 39). The exact mechanism of the binding of these proteins to the nonfunctional proteasome remains to be determined. However, the biochemical and MS data strongly suggest that inhibition at the active site allosterically transforms the proteasome holoenzyme, such as a stronger CP–RP association and changes in various interacting proteins. In yeast and plant proteaphagy, the CP and RP are degraded at similar rates (20, 24). Therefore, the structural change of mammalian proteasomes may facilitate the sequestration of inhibited 26S proteasomes as well as subsequent autophagic degradation.

**STUB1 Is Essential for Ubiquitylation and Aggresomal Formation of Inhibited Proteasomes.** Due to the association of multiple E3 Ub ligases with inhibited proteasomes, we postulated that direct ubiquitylation might be involved in selective regulation during mammalian proteaphagy. To test this hypothesis, we used siRNA to knock down the E3 enzymes but found that silencing BIRC6 or UBE3A had little effect on cellular aggresome formation (Fig. 3 A and B and SI Appendix, Fig. S8A). Knocking down RNF181 or UBR4 delayed the formations of both PSMB5-positive and SQSTM1-positive aggresomes (SI Appendix, Fig. S8A). In contrast, we observed that STUB1, which recognizes and ubiquitylates HSP70 substrates responding to various kinds of cellular stress, is essential for the early phase of aggresomal segregation (Fig. 3 A and B). SQSTM1 localization in the aggresome of STUB1-deficient cells raised the possibility that STUB1-mediated polyubiquitylation might contribute to the selective transport of inhibited proteasomes without affecting the formation of aggresomes per se. This hypothesis was supported by our findings that, upon MG132 treatment, proteasome subunits were less efficiently deposited in the insoluble fraction when STUB1 was knocked down (SI Appendix, Fig. S8B). A pulldown assay revealed a significantly increased interaction between inhibited proteasomes and STUB1, and HSP70 members such as HSPA1A and HSPA6 as well, in the presence of MG132 (Fig. 3C and SI Appendix, Figs. S7D and S9G). Although further studies are required to determine the possible contribution of other Ub E3 ligases in this process, our data suggest that STUB1 is a major regulator of proteasome ubiquitylation and aggresomal deposition.

To find whether STUB1 directly polyubiquitylates the 26S proteasome, we carried out an in vitro reconstitution assay with different combinations of recombinant wild-type Ub, UBE1 (as E1), UBE2D2 (E2), STUB1 (E3), and purified human 26S proteasomes. STUB1 generated the nondegradable and heterogeneous forked Ub linkages (43) on the proteasome. After treating the cells with MG132, elevated levels of proteasome ubiquitylation were observed (Fig. S9H and SI Appendix, Fig. S8 E and F), but HSP70 inhibitors potently blocked this process (SI Appendix, Fig. S8 F and G). This could be because of the direct interaction between the proteasome and STUB1 in the presence of MG132 (Fig. 3C); however, whether HSP70 family members are involved is yet to be determined.

We then examined the fate of the cytoplasmic speckles formed in STUB1 knockdown cells after MG132 treatment. Unlike PSMB5-positive aggresomes in control cells, which dissolved after the 24-h drug washout under normal conditions, the proteasome speckles in the STUB1-knockdown cells showed much delayed reduction during the washout (SI Appendix, Fig. S8H). Therefore, STUB1 activity appears to be required for the proper segregation and disposal of inhibited proteasomes both in vitro and in vivo. Although other E3 Ub ligases deserve further investigation, our results collectively indicate that the direct ubiquitylation of 26S proteasomes by STUB1 is a major prerequisite for the selective segregation of nonfunctional proteasomes. The results also suggest that STUB1-regulated proteasome homeostasis might antagonize the cellular stress caused by aberrant UPS function. We found that genetic inactivation of STUB1 in AS49 cells resulted in a significant increase in cytotoxicity only in the presence of MG132 and the PSMB5-degrading agent in STUB1-null (STUB1−/−) mouse embryonic fibroblasts (MEFs; Fig. 3J). Consistent with this observation, STUB1−/− MEFs elicited a stronger apoptotic response to MG132-mediated proteasomal stress as compared to wild-type cells (Fig. 3J).

**A STUB1 Inhibitor Effectively Blocks Proteasome Polyubiquitylation and Aggresomal Sequestration.** To further validate the function of STUB1 in proteasome ubiquitylation in the cell, we screened ~500 curated fragments (<300 Da) based on NMR spectrum alteration induced by STUB1 interaction (Fig. 4A and SI Appendix, Fig. S9 A–D). Four hits, mostly having a six-membered ring, were identified as the true binding molecules of STUB1. Using the screened molecules as starting compounds, we synthesized various heterodimers and derivatives to strengthen the binding affinities. When the compounds were functionally analyzed in vitro, compound 153 virtually completely prevented ubiquitylation of purified human proteasomes (IC50 = 85.44 μM; Fig. 4B and C). We further studied and confirmed that compound 153 effectively blocked the ubiquitylation of proteasome subunits, but did not influence proteasome activity or proteasomal DUB activity (Fig. 4C and SI Appendix, Fig. S9 E and F). Future studies on obtaining cocrystal structures of STUB1 with these compounds will aid in understanding the nature of the interactions and help in designing effective inhibitors.

To determine whether suppression of STUB1 activity would affect aggresome formation in vivo, we treated HEK293 cells with compound 153 and MG132 simultaneously. Analysis of the insoluble fraction showed reduced levels of various proteasome subunits, along with polyUb conjugates, SQSTM1, and HDAC6, but little effects on these proteins in the soluble fraction (Fig. 4D). Consistently, compound 153 significantly reduced the levels of polyubiquitylated PSMD14 (SI Appendix, Fig. S9C). Furthermore, treatment with the STUB1 inhibitor significantly suppressed aggresomal sequestration of proteasomes after MG132 treatment, while SQSTM1-positive inclusion bodies were comparably observed (Fig. 4E and SI Appendix, Fig. S9F). The similarity between outcomes after treatments of STUB1 siRNA and compound 153 (Figs. 3J and 4E) signifies the effect of the small molecule and importance of STUB1 on proteasome quality control. When cells were cotreated with MG132 and 100 μM compound 153 for 12 h, synergistic cytotoxicity was observed, while the structurally closely related compound 154...
Fig. 3. STUB1 is critical for the direct polyubiquitylation of inhibited proteasomes and for their aggresomal assembly. (A) Impaired aggresomal deposition of inhibited proteasomes when A549 cells were transfected with siSTUB1 but not when transfected with siControl or siBIRC6. The cells were analyzed by IFS with anti-PSMB5 (green) and anti-SQSTM1 (red) antibodies in the presence of DMSO (Upper) or MG132 (5 μM, 12 h; Lower). (B) Quantitation of A. Percentage of cells with aggresomes were counted and plotted as mean ± SD of three independent experiments with ∼500 cells (***P < 0.001, one-way ANOVA followed by the Bonferroni post hoc test). (C) Strong accumulation of STUB1 in the insoluble fraction and among purified proteasomes with MG132 treatment. Myc-tagged STUB1 was transfected into HEK293-PSMB2-biotin cells, which were then treated with MG132 (5 μM, 12 h). Soluble and insoluble fractions of WCEs were prepared and subjected to SDS/PAGE/IB along with affinity-purified proteasomes. (D) In vitro polyubiquitylation of human proteasomes by STUB1. The 26S proteasomes were affinity-purified and incubated with recombinant Ub, UBE1, UBE2D2, and STUB1 proteins in different combinations. The ubiquitylation of 26S proteasomes was monitored with SDS/PAGE/IB using anti-Ub antibodies. Note that autoubiquitylation of STUB1 produced distinct polyUb species of proteasome subunits. (E) As in D, except that the reaction lacks UBE2D2 and produces no ubiquitylation. (F) The RP subunits were decorated with polyUb chains only when all of the components were added in the reaction. (G) Different polyUb-conjugates were formed with UbK63R after in vitro ubiquitylation of 26S proteasomes, whereas UbK11R and UbK48R produced polyUb chains comparable to those of wild-type Ub. (H) In vitro polyubiquitylation reactions were conducted with recombinant proteins Ub, UBE1, UBE2D2, and STUB1 and purified proteasomes from MG132-treated cells. Proteasome subunits were examined by SDS/PAGE/IB with the indicated antibodies. (I) Comparison of cell survival in wild-type (++) and STUB1−/− MEFs and A549 cells, which were transfected with either siControl or siSTUB1 for 48 h. MG132 was administered at 5 μM concentration for 12 h. Values are presented as mean ± SD (n = 3; *P < 0.01, two-tailed Student’s t test). (J) The ++ and STUB1−/− MEFs were treated with 5 μM MG132 for 12 h before harvesting the WCEs, which were subjected to immunoblotting with indicated antibodies.
Fig. 4. STUB1 inhibitors identified via biophysical screening specifically inhibit proteasome ubiquitylation in vitro and aggresome formation in vivo. (A) An overview of the STUB1 inhibitor identification process, which largely consists of the NMR-based fragment screenings using saturation transfer difference (STD), synthesis of lead compounds, and functional analysis for their inhibitory effects (both in vitro and in vivo). (B) Compounds (1 mM) derived from the screened fragments were subjected to in vitro ubiquitylation assays of the purified proteasomes. Compound 153 potently inhibited the ubiquitylation of proteasome subunits. (C) As in B, except that graded doses (0, 250, 500, and 1,000 μM) of compound 153 were used. Chemical structure of compound 153 is shown. (D) Reduced levels of proteasome subunits in the insoluble fraction of HEK293 cells after treatment with 5 μM MG132 with 0, 10, 25, 50, and 100 μM compound 153 for 12 h. Triton X-100–soluble and pellet fractions from WCEs were isolated and subjected to SDS/PAGE/IB analysis. For PSMD1, PSMC2, PSMA4, and HDAC6 from the soluble and insoluble fractions, short and long exposed images were shown respectively due to distinctively different protein levels. (E) Representative images of A549 cells treated with MG132 (5 μM, 12 h) in the absence or presence of compound 153 (75 μM, 12 h). IFS with anti-SQSTM1 (red) and anti-PSMB5 (green) antibodies. Nuclei were counterstained with DAPI (blue). Arrows indicate the aggresomes.
had little effect on cell viability, regardless of the presence of MG132 (SI Appendix, Fig. S9I). Based on these data together with those of previous in vitro experiments, compound 153 appears to antagonize the upstream quality control cascade of nonfunctional proteasomes by blocking STUB1-mediated polyubiquitylation. Therefore, STUB1 may play a significant role in relieving cellular stress and promoting cell survival by mediating proteasome quality control.

**Discussion**

In this study, we report the pervasive but unique molecular mechanism of mammalian proteaphagy, which includes the extensive polyUb modification by STUB1, sequestration of inhibited proteasomes into the aggresome, and subsequent autophagic degradation (Fig. 5). However, the morphological and functional similarity between the aggresome (in mammals) and PSGs (in yeast and plants) suggests that the quality control mechanisms for nonfunctional proteasomes in eukaryotes are essentially conserved in evolution. The PSG may serve as a transient platform for the recovery of proteasomes from their incompetent state. The aggresome-mediated sequestration of defective proteasomes in mammals (Fig. 1) may be the unique feature of mammalian proteasome quality control possibly because yeast and plants lack some of the key structural features or constituents of dynein motors (45–47).

For delivery to the aggresome, proteasomes need to be extensively decorated with polyUb moieties (Fig. 3), which are also key determinants in yeast for depositing misfolded proteins along with proteasomes to the juxtanuclear quality control compartments (JUNO) (34, 48). The presence of proteasomes along with unprocessed substrates in the aggresome has been regarded as a collateral deposition, but our data imply that the aggresome may be an active player in PQC and act as a temporary storage place for excess amounts of proteins destined for proteasomal degradation. Therefore, the spatial exchange of proteasomes under inhibitory conditions is critical for not only proteasome dynamics but also global proteostasis. Notably, the components in the JUNO were highly fluidic through the cytosolic compartments, while those in the IPODs showed less mobility (34). PolyUb chains on inhibited proteasomes may trigger spontaneous and reversible phase separation in the aggresome (49, 50). In contrast to the proteins in the insoluble aggregates, which lack structural integrity and intrinsic activity, proteins in liquid droplets are functional and can reversibly diffuse to the cytoplasm. If the inhibition is persistent, then the nonfunctional proteasomes are degraded by autophagy (Fig. 2). Therefore, our findings lead to the conclusion that UPS and autophagy are alternative pathways but tightly connected in a compensatory feedback circuit, potentially involving phase transition.

We observed that most of the proteasome-interacting proteins showed increased interaction when proteasomes were inhibited (SI Appendix, Fig. S7). Some of these DAPs are related with the PQC, likely reflecting the consequence of a cellular effort to eliminate defective polypeptides (by BAG-6) (37, 51) or cytosolic misfolded proteins (by UBR proteins) (52, 53). Previous studies have identified that Ub receptors Rpn10/PSMD4 and Cue5/Tollip are involved in proteaphagy in yeast and plants, respectively (19, 20). Similarly, our MS analysis identified that many Ub receptors associated with mammalian proteasomes, such as PSMD4, Tollip, UBQLN2, SQSTM1, and RAD23, were highly up-regulated (SI Appendix, Table S1). The role of these Ub receptors in mammalian proteaphagy remains to be further characterized. Using in vitro reconstitution and small-molecule inhibitors, we also demonstrated that STUB1 directly mediates polyubiquitylation of inhibited proteasomes. Therefore, the function of STUB1 as a pivotal E3 Ub ligase for degrading nonnative proteins expands to proteasome homeostasis. Structural remodeling or posttranslational modifications may enable STUB1 to specifically recognize nonfunctional proteasomes. Since many other E3 Ub ligases interact with proteasomes, it is possible that proteasome quality control is mediated by different E3 enzymes in other cell types. Overall, it appears that the upstream regulatory cascade governing proteaphagy starts from the inhibition of active sites and ends in the polyubiquitylation of proteasomes.

It is conceivable that the adaptive mechanism for proteasome homeostasis has strong pathological implications. Under mild stress conditions, excessive amounts of misfolded proteins may clog the translocation channel or entry pore of the RP, thereby accumulating incompetent proteasomes in the insoluble aggresome and initiating de novo proteasome synthesis as a compensatory mechanism. However, chronic stress may impair the proteasome–aggresome–autophagy circuit and result in the development of proteopathies gradually over time. Our study provides a framework for integrating proteasome quality control mechanisms in mammals and other eukaryotes, suggesting that maintaining a functional proteasome pool in the cell has cytotoxic effects upon numerous proteopathic challenges. Further understanding of this pathway will provide effective therapeutic strategies to reduce the levels of aberrant proteins in cells. Specifically, a strategy to improve this regulatory circuit could potentially attenuate drug resistance to proteasome inhibition therapy in patients with multiple myeloma.
Absence of trypsin inhibitors. The cell lysates were then centrifuged at 16,000 g for 10 min at 4 °C. The supernatants were designated as detergent-soluble fraction. The pellets were washed with lysis buffer and resuspended in lysis buffer supplemented with 1% of SDS, then sonicated for 10 s with a microsonicator, heated at 100 °C for 10 min, and then analyzed as a detergent-insoluble fraction. Equal volumes of each insoluble and soluble fraction were boiled for 10 min in SDS sample buffer and analyzed by IB.

Purification and In Vitro Ubiquitination of 26S Proteasomes. Human proteasomes were purified as previously described (54). For ubiquitination of the purified proteasomes, 50 μM Ub, 150 nM UBE1, and 1 μM UBE2D2 were incubated for 10 min at room temperature. Then, 50 nM recombinant STUB1 and 60 nM purified proteasome were added. The reactions were allowed to proceed for 4 h at 37 °C and stopped by the addition of SDS sample buffer.

Quantitative LC-MS/MS Analysis. LC-MS/MS analysis was performed on a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer was performed as previously described. The signal intensity for the protein or peptide of interest was considered statistically significant if its fold change was greater than 1.5 and if it had a P value <0.05. For a comparison of proteomes, two-sided t tests were performed with a significance level of 5%. All statistical analyses were performed in the Perseus software.

Hydrogen-1 NMR Screening and Compound Synthesis. In order to identify compounds that can bind to STUB1 protein, we employed 1H ligand-observed NMR screening methods like the saturation-transfer difference experiment with second-generation BIOMER Premium Fragment Library. The STD NMR experiments were carried out at 293 K on an 800-MHz Bruker NMR spectrometer. Preliminary NMR screenings were performed with a mixture of five different fragments. For fragments presenting the binding potential, secondary STD NMR experiments were carried out. Detailed synthesis methods for compound 153 are available in SI Appendix.

Data Availability Statement. All protocols and source data are presented in the main text or the SI text. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019193. The plasmids constructs and reagents used in this study are available on request from the corresponding author.

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