Chaperone-mediated 26S Proteasome Remodeling Facilitates Free K63 Ubiquitin Chain Production and Aggresome Clearance*

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Background: Proteasomes facilitate HDAC6-dependent aggresome clearance by producing unanchored ubiquitin chains via Poh1 deubiquitinating enzyme.

Results: Aggresome-associated proteasomes undergo chaperone-mediated remodeling to generate free ubiquitin chains that activate HDAC6 and aggresome clearance.

Conclusion: Hsp90-mediated proteasome remodeling facilitates Poh1-dependent unanchored ubiquitin chain production and aggresome clearance.

Significance: Poh1 produces free K63 ubiquitin chains critical for aggresome processing, independent of canonical 26S proteasomes.

Efficient elimination of misfolded proteins by the proteasome system is critical for proteostasis. Inadequate proteasome capacity can lead to aberrant aggregation of misfolded proteins and inclusion body formation, a hallmark of neurodegenerative disease. The proteasome system cannot degrade aggregated proteins; however, it stimulates autophagy-dependent aggregate clearance by producing unanchored lysine (K)63-linked ubiquitin chains via the proteasomal deubiquitinating enzyme Poh1. The canonical function of Poh1, which removes ubiquitin chains en bloc from proteasomal substrates prior to their degradation, requires intact 26S proteasomes. Here we present evidence that during aggresome clearance, 20S proteasomes dissociate from protein aggregates, while Poh1 and selective subunits of 19S proteasomes are retained. The dissociation of 20S proteasome components requires the molecular chaperone Hsp90. Hsp90 inhibition suppresses 26S proteasome remodeling, unanchored ubiquitin chain production, and aggresome clearance. Our results suggest that 26S proteasomes undergo active remodeling to generate a Poh1-dependent K63-deubiquitinating enzyme to facilitate protein aggregate clearance.

The appearance of inclusion bodies is a pathological hallmark of many neurodegenerative disorders (1, 2). Inclusion bodies are mainly composed of protein aggregates resulting from misfolded proteins that have escaped proteasome-mediated degradation under pathological conditions. In cell models, protein aggregates can be actively concentrated to form a perinuclear inclusion body, termed the aggresome (1, 3, 4). Clinically, perinuclear aggresome-like inclusions, Lewy bodies, are prevalent in brain tissues from patients with Parkinson disease and dementia with Lewy bodies (1, 5). The formation of inclusion bodies in neurodegenerative disease likely reflects a coordinated effort of neurons to eliminate toxic protein aggregates.

Misfolded proteins are normally tagged by ubiquitin chains with K48-linkages and degraded by 26S proteasomes. 26S proteasomes are multi-subunit degradation machineries comprised of a barrel-shaped 20S proteolytic core flanked by one or two 19S regulatory complexes (6, 7). The 19S regulatory complex primarily functions in substrate recognition through its ubiquitin binding subunits, protein unfolding by ATPase subunits, and removal of ubiquitin tags by the deubiquitinating enzyme Poh1 (8, 9). These highly coordinated events enable the deubiquitinated substrates to enter the catalytic barrel of the 20S proteasome where they are efficiently degraded. However, the canonical ubiquitin proteasome system cannot degrade misfolded proteins once they aggregate, as these substrates are resistant to unfolding, a process essential for entering the 20S core (10). Aggregated proteins are instead cleared via autophagic machinery that sequester and deliver aggregates to lysosomes for degradation (11). Thus, the proteasome and autophagy form complementary degradative systems to maintain protein quality control and limit the buildup of toxic protein aggregates (12, 13).

Interestingly, although proteasomes cannot directly degrade protein aggregates, they are concentrated at protein inclusion bodies and are, in fact, required for their clearance (13). Using the aggresome as a model, it was shown that the clearance of protein inclusions involves an elaborate process of active de-aggregation followed by autophagy-dependent degradation (13). The disassembly and disposal of aggresomes requires a proteasomal deubiquitinating enzyme, Poh1. Poh1 normally cleaves off ubiquitin conjugates from proteasomal substrates at the proximal end prior to their degradation in the 20S core (14, 15). This unique cleavage mode releases ubiquitin chains en bloc. It was proposed that Poh1 similarly acts on ubiquitinated protein aggregates and produces unanchored free ubiquitin chains (13). In this context, released ubiquitin chains are bound by the protein deacetylase HDAC6 through an unusual ubiquitin-binding zinc finger (BUZ finger) that specifically recognizes C-terminal

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Gly-Gly residues of unanchored ubiquitin chains (16, 17). Binding to the ubiquitin chains activates HDAC6, leading to its dissociation from the aggresome and induction of an actinomyosin network that facilitates the de-aggregation of aggresomes and subsequent degradation by autophagy (13, 18).

Canonical K48-linked ubiquitin chains are normally coupled to 26S proteasome-mediated substrate degradation (19); curiously, however, Poh1 specifically produces K63-linked chains in the context of aggresome clearance (13). In fact, in Poh1-deficient cells, aggresome clearance can be effectively restored by exogenous free K63-linked, but not K48-linked, ubiquitin chains (13). These findings indicate that Poh1 produces ubiquitin chains of different linkages in 26S proteasome- and autophagy-mediated degradation. How Poh1 achieves these findings raise an interesting possibility that Poh1 could function as a K63-specific DUB activity was characterized biochemically and found to reside in a 19S proteasome complex (14). These findings raise an interesting possibility that Poh1 could function as a K63-specific DUB independent of the 26S proteasome. However, the physiological relevance of such a Poh1-K63-DUB complex is not known.

In this study, we have further investigated the mechanism underlying the Poh1-mediated cleavage of K63-linked ubiquitin chains from protein aggregates. We report here that during the process of aggresome disassembly and clearance, the protein aggregate-associated proteasomes undergo apparent reorganization. This remodeling involves the dissociation of the 20S proteasome as well as ATPase subunits of the 19S proteasome from the aggresome, whereas Poh1 and select 19S subunits remain associated with protein aggregates. We found that this change in proteasome composition requires the molecular chaperone Hsp90. Importantly, inhibition of Hsp90 also suppresses K63-linked ubiquitin chain production, HDAC6 activation and subsequent aggresome clearance. These findings suggest that Hsp90-dependent proteasome remodeling liberates Poh1 from the canonical 26S proteasomes to function as a K63-deubiquitinating enzyme, thereby activating HDAC6- and autophagy-dependent aggresome clearance.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following antibodies and reagents were used in this study: anti-HDAC6 (H-300), anti-p62/SQSTM1 (n=3), anti-RPN1, and anti-P4D1 (Santa Cruz Biotechnology); K63-specific anti-ubiquitin (APU3) and K48-specific anti-ubiquitin (APU2) (Millipore); anti-PSMA2 (Cell Signaling); anti-PSMB5 (Novus Biologicals); anti-PRN7, anti-RPN8, anti-RPN9, anti-RPN2 (Bethyl Labs); anti-Hsp70, anti-RPT6, anti-Hsp90 (16-F1) (Enzo); anti-Poh1/PSMD14 (Epitomics); anti-PSMC4/RPT3 (Abgent); anti-Alpha4/PSMA4 (Biomol); anti-RPN10/PSMD4 (ProteinTech); anti-β actin (Sigma). The following compounds were used: nocodazole, 17-AAG, PU-H71 (Sigma), MG132 (EMD Millipore), 17-DMAG (Cayman Chemicals), Tubastatin-A (Bio-Vision), HS-10 (gift from Dr. Timothy Haystead at Duke University).

**Cell Culture and Treatments**—Human lung carcinoma cells (A549) were grown at 37 °C with 5% CO₂ in DMEM (Gibco) (high glucose, high l-glutamine, high sodium pyruvate) supplemented with 10% FBS and 1% (50 μg/ml) penicillin-streptomycin (Life Technologies). For induction of aggresomes, cells were grown to 70% confluency prior to treatment with 5 μM MG132 for 24 h. To initiate aggresome clearance, MG132 was washed out by rinsing twice with equal volume of MG132-free culture media. Additionally, depending upon the experiment, the following compounds were added after MG132 washout: 17-DMAG (2 μM), Novobcin (500 μM), 17-AAG (2 μM), PU-H71 (2 μM), HS-10 (1 μM), Tubastatin-A (10 μM), nocodazole (5 μM).

**Immunofluorescence Analysis**—A549 cells cultured on glass coverslips were rinsed twice with PBS and fixed with either 4% paraformaldehyde (PFA) for 15 min at room temperature or ice cold methanol for 20 min at −20 °C. Antibodies requiring methanol fixation are P4D1, PSMA2, Poh1, and PSMC4/RPT3, and all others were stained using PFA fixation. Cells fixed in PFA were rinsed with PBS and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min. Following permeabilization, cells were rinsed with PBS, blocked with 5% BSA in PBS containing 0.1% Triton X-100 for 1 h, and incubated with antibodies, as described previously (4). Cells fixed in methanol were not permeabilized under 0.5% Triton in PBS, but all other incubation steps were the same as for PFA fixation. Additionally, for Hsp90 staining, cells were first incubated in 0.05% saponin (Sigma) in PBS at 4 °C for 5 min to reduce cytoplasmic background, then rinsed with PBS and fixed in PFA. Cells were imaged on a Leica SP5 confocal microscope with ×40 oil objective. 488, 561, and 633 laser lines were used for sequential excitation. Final images were acquired with z-stacks of 0.97 microns sections and line-averaging of 4. Quantification of signal intensity was determined using ImageJ software. Association of each subunit with the aggresome was analyzed by calculating the ratio of average signal intensity per unit area at the aggresome (marked by ubiquitin or p62) to the intensity observed in the cytoplasm. For each condition, data were quantified by averaging over 50 cells per experiment from three separate experiments.

**Aggresome Separation and Western Blotting**—Cells were lysed in Triton buffer containing 50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, 1 mM PMSE, 10 mM N-Ethylmaleimide (NEM), a mixture of protease inhibitors (Sigma 1:200) and phosphatase inhibitors (Sigma 1:200), and mixed for 20 min at 4 °C. Next, lysates were centrifuged at 16,000 × g for 30 min at 4 °C. The supernatants were used as detergent-soluble fractions. The pellets were suspended in Triton buffer (same as above) containing 1% SDS, sonicated at amplitude 30 for 10 s, heated at 100 °C for 3 min, and analyzed as detergent-insoluble fractions. All samples were normalized for protein concentration using BCA reagent assay, and analyzed using Western blot analysis. Quantitative evaluation of proteins was determined by ImageJ densitometry analysis using actin as an internal control.
siRNA Interference—The following siRNAs were used in this study: as previously reported Poh1 (13), PSMA2 siRNA obtained from Invitrogen (HSS108661); Rpn10 (NM_002810) and PSMB4 (00040457) siRNA obtained from Sigma. For knockdown, A549 cells were transfected with 20 nM of a negative control siRNA or the siRNA specific for the target protein using RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Additionally, a second transfection of these same siRNAs was done 24 h later to achieve higher knockdown efficiency.

Analysis of HDAC6-associated Free Ubiquitin Chains—This procedure was done as previously reported (13) with the following modifications. The stable FLAG-HDAC6-expressing A549 cells were lysed in NETN buffer (170 mM NaCl, 20 mM Tris-Cl pH 8.0, 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40, 1 mM Na3VO4) supplemented with phosphatase and protease inhibitors (Sigma), 10 mM N-ethylmaleimide (NEM), and 10 µM MG132 as previously reported. Next, lysates were centrifuged at 16,000 x g for 15 min at 4 °C and the supernatant was used to immunoprecipitate FLAG-HDAC6 and associated proteins. The supernatant was normalized for protein concentration across conditions, and 1 mg was incubated with 40 µl of anti-FLAG M2 agarose beads (Sigma) followed by mixing at 4 °C for 45 min. Next, 5 mM DTT was added to the mixture to quench the NEM and mixed for an additional 15 min at 4 °C. The beads were then centrifuged at 400 x g for 5 min, followed by three washes with 1 ml of 150 mM NETN buffer. The remaining wash buffer was aspirated, and beads were incubated in 40 µl of Buffer F (20 mM HEPES-KOH at pH 7.0, 10% (v/v) glycerol, and 0.02% (w/v) CHAPS) at 72 °C for 5 min. Following centrifugation at 11,000 x g for 10 min at 25 °C, the supernatant was incubated with or without 100 nM Isopeptidase-T at 30 °C for 30 min, resolved on a 4–20% gradient SDS-PAGE, transferred to nitrocellulose membranes and probed with an ubiquitin antibody (UG9510 Enzo). The quantification of the relative ubiquitin signal was obtained by scanning blot densitometry.

RESULTS

20S Proteasomes and ATPase Subunits Dissociate from the Aggresome during Its Clearance—We have previously reported that subunits of both 19S (Poh1) and 20S (PSMA2) proteasomes are concentrated at aggresomes induced by a proteasome inhibitor, MG132 (13). To investigate the status of pro-
teasomes during aggresome clearance, we removed MG132 (washout) and determined Poh1 and PSMA2 localization by immunostaining. This analysis revealed that Poh1, the DUB subunit of the 19S proteasome, remains concentrated at de-aggregated aggresomes targeted for autophagic degradation after 12 h of MG132 washout (Fig. 1A). Unexpectedly, under the same condition, little PSMA2, an /H9251-/H9255-subunit of the 20S proteasome complex, was found at de-aggregated aggresomes (Fig. 1B compare 24 h MG132 and 12 h Wash). Quantification of relative signal intensity demonstrated a significant reduction of PSMA2, but not Poh1, associated with de-aggregated aggresomes (Fig. 1C). Biochemical fractionation confirmed that Poh1 remained enriched in Triton X-100 insoluble protein aggregate fractions, while the abundance of PSMA2 was clearly reduced (Fig. 1D). Marked reduction in protein aggregate fractions was similarly observed for additional 20S proteasome α-subunit 4 (PSMA4), and β-subunits 4 and 5 (PSMB4, PSMB5, Fig. 1D). As the overall abundance of proteasome subunits is not affected by MG132 washout (data not sown), these results indicate that 20S proteasomes dissociate from protein aggregates during aggresome clearance while Poh1, a component of the 19S proteasome, remains concentrated.

The 19S proteasome can be further divided into two subcomplexes: the Poh1-containing lid complex and the ATPase-containing base complex, which unfolds and threads peptides through the 20S core proteasome for degradation. We found that the 19S lid subunits RPN7, RPN8, and RPN9 behaved similarly to Poh1 and were retained in Triton X-100-insoluble (aggresome) fractions. In contrast, the 19S ATPase subunits, RPT3 (Fig. 2A and C) and RPT6 (Fig. 2C) were largely dissociated from the protein aggregates. Interestingly, the non-ATPase subunits of the 19S base, RPN1, RPN2 (Fig. 2C) and RPN10 (Fig. 2B and C), all remained. Collectively, these data reveal a reorganization of the 26S proteasome during aggresome clearance where Poh1 and selective components of the 19S proteasome, but not those of 20S proteasomes, are retained at protein aggregates targeted for degradation.

![FIGURE 2. 19S ATPase dissociate while non-ATPase subunits remain with de-aggregated aggresomes. A, immunolocalization of 19S-associated ATPase RPT3 (red), and (B) non-ATPase RPN10 (red), at 24 h MG132 and 12 h Wash. Both subunits were co-stained with either ubiquitin or p62 (green) as the aggresome marker, respectively. Note that RPN10 displays weak permeabilization of the aggresome upon formation at 24 h MG132, but staining improves upon 12 h MG132 wash off, which may indicate ease of antibody accessibility upon proteasome remodeling. ATPase subunit RPT3 displays clear dissociation from de-aggregated aggresomes. Boxed regions of the images are zoomed to highlight proteasome associations with the aggresome. Scale bar indicates 15 μm. C, immunoblotting of 19S lid (Poh1, RPN7, RPN8, and RPN9), 19S base non-ATPase (RPN1, RPN2, and RPN10) and ATPase (RPT6 and RPT3) subunits separated by detergent-soluble and insoluble (aggresome) fractions. These bands were developed on the same gel as Fig. 1 to clearly allow comparison of relative abundance of 19S and 20S subunits during formation and clearance. Indicated densitometry values reflect ratio of each band density to the density of 24h MG132 condition and normalized to actin. D, densitometry data displayed in C were quantified for the insoluble fraction and averaged over three separate experiments. Error bars indicate ± S.E., * p < 0.01.]

Proteasome Remodeling Facilitates Aggresome Clearance
The Intact 20S Proteasome Is Not Essential for Aggresome Clearance—The apparent dissociation of 20S proteasome subunits from the de-aggregated aggresomes led us to investigate whether the 20S proteasome is required for aggresome clearance. We analyzed the effect of siRNA-mediated knockdown (KD) of two essential 20S core subunits, PSMA2 and PSMB4. As shown previously (13), aggresomes induced by MG132 treatment were cleared 24 h after the proteasome inhibitor was removed, and this process was suppressed by Poh1 KD (Fig. 3A). We found that PSMA2 or PSMB4 KD also led to the presence of aggresomes 24 h post MG132 washout. We have previously shown that the retention of aggresomes post-MG132 washout could either be a failure in clearance, or the result of a continuous accumulation of nascent aggregates (13). To distinguish between these possibilities, we took advantage of findings that an intact microtubule network is required for the concentration of nascent protein aggregates to form an aggresome (3), whereas aggresome clearance proceeds normally in the presence of a microtubule depolymerizing agent, nocodazole (13). As shown in Fig. 3A, a single large aggresome was retained in Poh1 KD cells during MG132 washout in the presence of nocodazole, consistent with a defect in aggresome clearance (13). In stark contrast, in PSMA2 and PSMB4 KD cells, the addition of nocodazole during the 24 h MG132 washout period led to prominent accumulation of dispersed micro-aggregates instead of a large aggresome (Fig. 3A). These results indicate that the aggresome accumulation in PSMA2 and PSMB4 KD cells is mainly due to an increase in the influx of nascent protein aggregates rather than a clearance defect (Fig. 3A).

To further confirm that the micro-aggregates observed in 20S KD cells are pre-aggresomal, we examined the status of HDAC6, which is specifically enriched at pre-aggresomal aggregates, but not at de-aggresomal aggregates on their way to degradation (4, 13). In PSMA2 KD cells, the nocodazole-induced micro-aggregates during MG132 washout (labeled by the ubiquitin-binding protein p62), were indeed positive for HDAC6 (Fig. 3C), supporting the notion that they are pre-aggresomal nascent protein aggregates. In contrast to PSMA2 and PSMB4 KD, knockdown of 19S-associated RPN10, which remains concentrated at de-aggresomal aggresomes, showed phenotypes similar to those of Poh1 KD (Fig. 3A), indicating a critical role of RPN10 in aggresome clearance. All together, these data show that intact 20S proteasomes are not essential for aggresome disassembly and clearance.

Hsp90 Facilitates 20S Proteasome Dissociation and Aggresome Clearance—The molecular chaperone Hsp90 was reported to interact with and regulate 26S proteasome assembly (21). We found that Hsp90 is enriched at the aggresomes induced by MG132 treatment (Fig. 4A). To investigate whether Hsp90 also plays a role in proteasome remodeling associated with aggresome clearance, we applied an N-terminal ATPase-targeting Hsp90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), during aggresome clearance. As shown in Fig. 4, B–F, immunostaining and immunoblotting assays showed that 17-DMAG significantly decreased the dissociation of 20S (PSMA2, PSMA4, PSMB4, and PSMB5) and ATPase subunits (RPT3 and RPT6) from the aggresome 12 h post MG132 washout. Furthermore, 17-DMAG treatment also inhibited aggresome clearance after MG132 washout (Fig. 5, A and B). Importantly, the effect of 17-DMAG on aggresome clearance is insensitive to nocodazole treatment, indicating an inhibition of clearance, rather than enhanced formation of new aggregates (Fig. 5, C and D).
Additionally, novobiocin, an Hsp90 inhibitor that targets the C-terminal domain of Hsp90 (22, 23), also suppressed aggresome clearance (Fig. 5A). Other Hsp90 inhibitors also displayed a similar defect in aggresome clearance (24, 25). These data show that Hsp90 activity facilitates proteasome remodeling and aggresome clearance.

Hsp90 Regulates the Production of Unanchored Ubiquitin Chains—We next explored the potential function of Hsp90-dependent proteasome remodeling. Aggresome clearance requires the ubiquitin-binding deacetylase HDAC6, which is activated by unanchored K63 ubiquitin chains produced by Poh1 (13). We found that pharmacological inhibition of HDAC6 by Tubastatin-A (TBSA) prevented aggresome clearance; however, it did not affect the dissociation of the 20S proteasome (PSMA2) (Fig. 6). These data indicate that 26S proteasome remodeling precedes HDAC6 activation.

We next determined if Hsp90 inhibition affects the production of K63 ubiquitin chains and subsequent HDAC6 activation. We observed that 17-DMAG treatment prevented reduction of protein aggregate-associated K63-linked ubiquitination after MG132 washout (Fig. 7, A and B), a process dependent upon Poh1 (13). This finding suggests that Poh1 activity might be impaired upon Hsp90 inhibition. To directly determine if Hsp90 inhibitor treatment decreased Poh1-mediated production of free ubiquitin chains that bind and activate HDAC6, we immunoprecipitated HDAC6 and analyzed the abundance of free ubiquitin chains bound by HDAC6. Unanchored ubiquitin chains are characterized by resistance to heat-induced denaturation, but sensitivity to Isopeptidase-T (IsoT)-mediated degradation (13, 26). We found that 17-DMAG treatment significantly reduced the amount of unanchored ubiquitin chains bound by HDAC6 during MG132 washout (Fig. 7, C and D). Further supporting this conclusion, 17-DMAG prevented HDAC6 dissociation from the aggresome, indicating that HDAC6 was inactive (Fig. 7, E and F). Taken together, these data show that Hsp90 is required for efficient proteasome remodeling, the production of unanchored ubiquitin chains, HDAC6 activation, and eventual aggresome clearance.
DISCUSSION

The proteasome system is primarily responsible for efficient degradation of unnecessary, misfolded, and damaged proteins. Although it lacks the intrinsic capacity to degrade aggregated proteins, the proteasome system can facilitate the disposal of protein aggregates by autophagy (13). Both forms of proteasome-dependent degradation require the deubiquitinating enzyme, Poh1. However, the canonical proteasome-mediated substrate degradation mainly involves the cleavage of K48-linked ubiquitin chains whereas autophagy-dependent aggregate degradation requires K63-linkages (13). The distinct types of ubiquitin linkages suggest that Poh1 processes proteasomal and autophagic substrates by different mechanisms. This study has provided evidence that aggresome-associated 26S proteasomes undergo changes in their composition, enabling Poh1 to cleave K63-linked ubiquitin chains from protein aggregates and activate the clearance of aggresomes. We show that Hsp90 is required for 26S proteasome remodeling and the production of free ubiquitin chains that stimulate aggresome clearance. How Hsp90 facilitates 26S proteasome disassembly would require further study.

In the context of 26S proteasome-mediated degradation, substrate unfolding by the 19S ATPase subunits orients Poh1
such that it can readily cleave off the K48-linked ubiquitin chains, enabling substrates to enter the 20S catalytic barrel (15, 27). We have previously shown that K63-linked ubiquitin chains were added to aggregated proteins in response to proteasome inhibition, resulting in substrates that are doubly tagged by K48- and K63-linked ubiquitin chains (13). Despite the presence of both forms of ubiquitin linkages on protein aggregates, Poh1 appears to mainly produce K63-linked ubiquitin chains during aggresome clearance (13). The exact nature of how and where K63-linked ubiquitin chains are conjugated onto an aggregated protein remains unknown. However, as aggregated peptides cannot be readily unfolded and moved toward the entrance of the 20S proteasome where Poh1 is located (27), topographically it is unlikely for Poh1 to access K63-linked ubiquitin chains in the 26S proteasome configuration. Our analyses indicate that proteasomes associated with the aggresome lose the 20S proteasome subunits during clearance. Accordingly, this remodeling might liberate Poh1 from the 26S proteasomes trapped by ubiquitinated protein aggregates, and thereby enable its access to the K63-linked ubiquitin chains. Interestingly, a previous study has demonstrated a Poh1-dependent, K63-specific cleavage activity in a purified 19S complex (14). Thus, in contrast to Poh1-mediated deubiquitination associated with proteasomal substrate degradation, which requires intact 26S proteasomes (15, 20), K63-targeted deubiquitination activity of Poh1 does not require 20S core proteasome components and can function as an independent entity (14).

Interestingly, we also found evidence that ATPase subunits of the 19S proteasome dissociate from the de-aggregated aggresome. Because ATPase subunits mainly serve to unfold 26S proteasomal substrates for their entry through the 20S catalytic barrel, it is expected that they would be dispensable for the clearance of protein aggregates. Unlike ATPase subunits, however, three other subunits of the 19S base complex, RPN1, RPN2, and RPN10, remain associated with the aggresome during its clearance. RPN10 physically associates with RPN1 as well as RPN11, the yeast homolog of Poh1 (28). RPN10, along with

![Figure 7](image_url)
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19S base-associated RPN13, which interacts with RPN2, directly bind ubiquitinated peptides delivered to proteasomes (29). Structural data for RPN13 reveal its preference for K48-linked ubiquitin (30), whereas mammalian RPN10 has the capacity to bind both K48 and K63 chains (31). Upon proteasome inhibition, RPN13 becomes poly-ubiquitinated, and as a result, its ubiquitin binding activity diminishes (32). Under this condition, RPN10 would be expected to become the dominant ubiquitin-binding protein on the proteasome. We speculate that this arrangement and the physical proximity of RPN10 to Poh1 might allow RPN10 to better present ubiquitinated protein aggregates to Poh1 for deubiquitination. Consistent with this possibility, we found that RPN10 is required for clearance of aggresomes (Fig. 3A).

Although the detailed composition of this Poh1-dependent deubiquitinating enzyme complex associated with aggregate clearance would require future investigation, our findings indicate that the 19S lid subunits would be its main constituents (Fig. 2). Interestingly, the 19S lid is homologous to another K63-specific deubiquitinating enzyme complex, the BRCA1-A complex (33), where Poh1-related BRCC36 encodes for the catalytic subunit (14). Similar to Poh1, the BRCA1-A complex does not require ATPase activity to catalyze K63-linked deubiquitination (14). Thus, we propose that a Poh1-containing 19S-lid complex, assisted by ubiquitin-binding RPN10 and likely other proteasomal subunits, can function as a K63-specific deubiquitinating enzyme that facilitates autophagy-dependent aggregate degradation.

Changes in proteasome subunit composition have been noted under several pathophysiological conditions (34–39). For example, 20S core particles become dissociated from 19S proteasomes in response to mitochondrial and oxidative stress (34, 36, 38, 39). The dissociated 20S proteasomes appear to be more effective than 26S proteasomes in degrading proteins damaged by the oxidative stress (40).Arsenic challenge increases misfolded proteins and induces a proteasome-associated protein, AIRAP. The AIRAP-associated proteasomes show different activity and stability profiles from those of canonical 26S proteasomes (37). Additionally, direct inhibition of 20S proteasome catalytic activity, similar to MG132 treatment used in this study, led to selective enrichment of several ubiquitin ligases at the proteasomes and RPN13 ubiquitination (32). Thus, proteasomes challenged or poisoned by pathologically or pharmacologically generated misfolded protein aggregates do not sit idly, but rather they undergo active remodeling. This adaptive remodeling probably enables impaired proteasomes to adjust their own activity or relay the stress signal to activate compensatory autophagy function (41). Whether proteasome remodeling is impaired in neurodegenerative disease or other disorders would be of great interest in the future.

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