Characterizing the Molecular Switch from Proteasomes to Autophagy in Aggresome Processing

by

Priyaanka Nanduri

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________

Approved:

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Tso-Pang Yao, Supervisor

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Carol Colton

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Cynthia Kuhn

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Joel Meyer

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Dennis Thiele

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Cells thrive on sustaining order and balance to maintain proper homeostatic functions. However, the primary machinery involved in protein quality control including chaperones, ubiquitin proteasome system, and autophagy all decline in function and expression with age. Failures in protein quality control lead to enhanced protein misfolding and aggregation. Efficient elimination of misfolded proteins by the proteasome system is critical for cellular proteostasis. However, inadequate proteasome capacity can lead to aberrant aggregation of misfolded proteins and inclusion body formation, which is a hallmark of numerous neurodegenerative diseases. Due to the post-mitotic nature of neurons, they are more susceptible to the collapse in proteostasis correlated with age.

Here, we propose a cell based model of aggresome clearance using a reversible proteasome inhibitor, MG132, to identify the precise molecular machinery involved in proper processing of inclusions. It is known that once misfolded proteins are aggregated, the proteasome system can no longer degrade them. Furthermore, the continuous accumulation of aggregates often leads to aggresome formation, which results in amalgamated inclusion bodies that are simply too large for autophagosomes to engulf and degrade. Although, studies have shown that aggresomes can eventually be
cleared by autophagy, the molecular mechanisms underlying this process remain unclear.

Our research reveals that regardless of impaired proteolysis, proteasomes can still stimulate autophagy-dependent aggresome clearance by producing unanchored lysine (K)63-linked ubiquitin chains via the deubiquitinating enzyme Poh1. Unanchored ubiquitin chains activate ubiquitin-binding histone deacetylase 6, which mediates actin-dependent disassembly of aggresomes. This crucial de-aggregation of aggresomes allows autophagosomes to efficiently engulf and eliminate the protein aggregates. Interestingly, the canonical function of Poh1 involves the cleavage of ubiquitin chains en bloc from proteasomal substrates prior to their degradation by the 20S core, which requires intact 26S proteasomes. In contrast, here we present evidence that during aggresome clearance, 20S proteasomes dissociate from protein aggregates, while Poh1 and selective subunits of 19S proteasomes are retained as an efficient K63 deubiquitinating enzyme complex. The dissociation of 20S proteasome components requires the molecular chaperone Hsp90. Hsp90 inhibition suppresses 26S proteasome remodeling, unanchored ubiquitin chain production, and aggresome clearance. Ultimately, we hope to apply these molecular markers of inclusion body processing to identify the underlying lesion in aggregate prone neurodegenerative disease.
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List of Abbreviations

17-DMAG: 17-Dimethylaminoethylamino-17-demethoxygeldanamycin

3-MA: 3-Methyladenine

AD: Alzheimer’s Disease

ATG: Autophagy Related Gene

BFA: Bafilomycin-A

DMSO: Dimethyl Sulfoxide

DUB: Deubiquitinase

HDAC6: Histone deacetylase 6

HSE: Heat Shock Element

HSF: heat Shock Factor

Hsp70: Heat Shock Protein 70

Hsp90: Heat Shock Protein 90

HSPs: Heat Shock Proteins

IsoT: Isopeptidase-T

K48: Lysine-48

K63: Lysine-63

KD: Knockdown

LB: Lewy Body
MTOC: Microtubule organizing center
PD: Parkinson’s Disease
QC: Quality Control
TBSA: Tubastatin-A
Ub: Ubiquitin
UPS: Ubiquitin Proteasome System
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1. Introduction

There is a lot to be said for the benefits of a clean and organized space. For example, keeping a room or office space ordered not only enables one to find what he or she needs quicker, but also allows for one to efficiently conduct his or her activities. Similarly, if we were to consider the environment of a cell, cleanliness and organization is central to maintaining overall cell health. From the organization of proteins and organelles to their respective functions and signaling pathways, the cell thrives on having order. Thus, much of a cell’s health is dependent on the maintenance of this balance, a process termed homeostasis.

Protein homeostasis, or proteostasis, is the complex process that integrates biogenesis, folding, trafficking, and degradation of intra- and extracellular proteins to maintain a healthy and well-functioning cellular environment. Dysfunctions in proteostasis, specifically protein degradation, have been positively correlated with various age-linked progressive diseases among which neurodegenerative disorders remain most notable. While the prevalence of neurodegenerative disorders such as Parkinson’s disease and Alzheimer’s disease is increasing, effective treatments for these diseases are still lacking. Importantly, as average life expectancy increases, the demand for effective therapies for age-linked degenerative disorders has become imperative. Hence, understanding how cells maintain proteostasis is key to characterizing the origin
of these degenerative disorders, which are often associated with excessive protein misfolding and protein aggregation.

1.1 Protein quality control

Protein quality control drives the balance between protein degradation and resynthesis, an essential component of homeostasis from single cells to whole organisms. Cells possess several key mechanisms and processes to maintain protein homeostasis. Generally thought of on a spectrum, on one end, molecular chaperones modulate protein folding and repair, whereas on the other end, proteasomes and autophagy as well as other lysosome-dependent systems facilitate the degradation of dysfunctional or misfolded proteins. If perturbed, dysfunctions in proteostasis can lead to a wide variety of disease states (Nedelsky et al., 2008).

1.1.1 Chaperones

Molecular chaperones are crucial to maintaining proteostasis basally as well as in times of cellular stress. Generally, chaperones are considered as cellular folding and assembly factors. Among their numerous functions, chaperones are known to participate in the folding of newly translated and damaged polypeptides, in the transport of proteins across cellular membranes, and in the assembly of protein complexes (Hartl and Hayer-Hartl, 2002). To ensure formation of functional proteins, chaperones facilitate efficient folding by stabilizing specific folding intermediates and
preventing non-specific protein interactions, protein misfolding, and protein aggregation (Dobson, 2003). More recently, molecular chaperones have also emerged as active participants in protein degradation (Esser et al., 2004). Although chaperones comprise diverse groups of proteins, the induction of heat shock proteins (HSP), a highly conserved family of chaperones, is a hallmark of cellular stress.

Heat shock proteins constitute a large family of proteins that are often classified based on their molecular weight: Hsp10, Hsp40, Hsp60, Hsp70, Hsp90, etc. Even though HSPs were first described in response to heat shock, they are actually induced in response to a wider array of cellular stress conditions. Many members of this group perform chaperone function by stabilizing nascent proteins to ensure proper folding or by aiding to refold proteins that were damaged by the cell stress (De Maio, 1999; Powers et al., 2009). HSP expression is transcriptionally regulated by heat shock factors (HSFs) that bind to heat shock elements (HSEs) located upstream of HSP genes (Akerfelt et al., 2010; Wu, 1995). Of particular interest to this work, Hsp70 and Hsp90 among others, are two well characterized ATP-dependent chaperones that can function individually or as part of larger heterocomplexes to prevent protein aggregation (Hartl and Hayer-Hartl, 2002). Specifically, these chaperones possess the ability to promote cell survival in neurodegenerative disease by stabilizing and refolding misfolded proteins and by directly inhibiting apoptosis (Lanneau et al., 2007). However, if refolding and repair of
proteins fails, the chaperone system is impaired or overwhelmed, or if the proteins of interest simply become superfluous, then cells rely on the next line of defense in maintaining proteostasis, the ubiquitin proteasome system (UPS).

1.1.2 Ubiquitin proteasome system

The ubiquitin proteasome system is a tightly regulated and highly specific process that is considered the primary degradation machinery for misfolded, damaged, or short-lived regulatory proteins in eukaryotes (Finley et al., 2004; Hartmann-Petersen et al., 2003). A protein doomed for proteasomal degradation is first modified by the addition of a chain of ubiquitin moieties, generally linked by lysine-48 (K48), which serves as a targeting signal to the 26S proteasome. Ubiquitin modification is an ATP dependent process requiring the sequential coordination of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) enzymes (Bhattacharyya et al., 2014; Finley, 2009; Glickman and Ciechanover, 2002). Although mammalian cells generally express only a single E1 enzyme, there are approximately fifty distinct E2 enzymes and upwards of a thousand different E3 enzymes (Reinstein and Ciechanover, 2006). E3 ligases can be single- or multi-subunit enzymes that coordinate with E2s to determine substrate and ubiquitin linkage specificity (Voges et al., 1999). Among several types of ubiquitin linkages, lysine-48 (K48) linked ubiquitin are the most common in targeting substrates for proteasome mediated degradation, whereas lysine-63 (K63) chains are
reported to play a role in intracellular signaling (Hartmann-Petersen et al., 2003; Jacobson et al., 2009). Upon ubiquitin chain recognition by specific subunits on the 26S proteasome, comprised of a 19S regulatory particle and a 20S proteolytic core, the polyubiquitin chain is cleaved and recycled prior to substrate proteolysis (Bhattacharyya et al., 2014). Ultimately, the degradation process yields short peptides that can be further degraded into shorter amino acid sequences and utilized in the synthesis of new proteins.

1.1.2.1 20S Proteasome

The 20S proteasome, the primary proteolytic component of the 26S complex, is comprised of 28 subunits that form four rings of $7\alpha 7\beta 7\beta 7\alpha$ with a narrow channel through the center of the structure (da Fonseca et al., 2012). The seven non-identical alpha subunits bind with activators or regulatory particles to initiate gate opening into the 20S barrel. As for the seven distinct beta subunits, only three (i.e. $\beta 1(Y)$, $\beta 2(Z)$, and $\beta 5(X)$) are catalytically active and responsible for the various proteolytic activities of the proteasome including chymotrypsin-like, trypsin-like, and caspase-like activities (Voges et al., 1999). In order for substrates to undergo degradation in the interior of the 20S core, they must be unfolded and de-ubiquitinated, which are functions mediated by the 19S regulatory particle.
1.1.2.2 19S Proteasome

The 19S regulatory particle, also known as PA700, is a complex whose specific functions, modifications, and structural complexity are still being actively investigated. The 19S regulatory particle caps one or both ends of the 20S core particle and is crucial to ubiquitin recognition, deubiquitination, substrate unfolding, and gate opening into the 20S proteolytic chamber (Glickman et al., 1999). Not only do these functions precede substrate proteolysis by the core, they are in fact essential in facilitating substrate entry into the 20S proteasome for eventual degradation (Glickman et al., 1999). The 19S regulatory particle is comprised of 19 subunits, which can be further classified as components of the lid or base complex (Glickman et al., 1998; Lander et al., 2012). Although much of the structural work on 19S regulators has been conducted in S. cerevisiae, the subunits are homologous to those identified in preparations from a number of different laboratories using materials from various organisms.

The 19S lid complex is comprised of nine non-ATPase subunits (RPN3, 5–9, 11–12, and Sem1 in yeast) where RPN11, or Poh1 in mammals, is responsible for deubiquitination (DUB) activity associated with substrate degradation (Lander et al., 2012; Yao and Cohen, 2002). RPN11 is positioned such that it sits directly above the pore of the 20S complex and cleaves the ubiquitin chains conjugated to target substrates en bloc, thus, releasing a free ubiquitin chain (Lander et al., 2012). Importantly, the precise
positioning of ubiquitin chains for cleavage by RPN11 occurs by initially engaging an unstructured region of the target substrate into the 20S core. This initial engagement of the partially unfolded protein is necessary for the substrate to remain engaged with the 26S complex after the ubiquitin chain has been cleaved (Bhattacharyya et al., 2014). Although the precise structure of the 19S lid is still under investigation, the subunits share sequence homology with the COP9 signalosome complex, the translation initiation factor 3 complex, and the BRCA-1A complex (Glickman et al., 1998; Wang et al., 2009), which suggests a common evolutionary pathway. Furthermore, RPN11 (Poh1) has been demonstrated to have specific DUB activity toward lysine 63 (K63) linked ubiquitin chains, due to its JAMM/MPN+ domain, in the context of a mix linked ubiquitin chain with K48 linkages (Cooper et al., 2009). Although this K63 specific activity was identified in a purified 19S complex, there remains little physiological evidence for 26S independent functions of RPN11.

The 19S base contains a hetero-hexameric ring of six distinct AAA+ ATPases (RPT1-6), considered the molecular motor of the proteasome, along with four non-ATPase subunits that coordinate ubiquitin binding (RPN1-2, RPN10, RPN13) (Tomko et al., 2010). Specifically, the ATPases are said to utilize the energy of ATP binding and hydrolysis to exert a pulling force on substrate proteins, unfold them, and translocate the polypeptides through the narrow central pore of the 20S proteolytic chamber
(Gillette et al., 2008; Rabl et al., 2008; Smith et al., 2007). In the presence of ATP, the C-termini of the ATPases bind specific sites on the α-subunit ring of the 20S core particle to initiate gate opening into the 20S pore and facilitate substrate entry (Smith et al., 2007). The four non-ATPase subunits including the two structural components RPN1 and RPN2, and the ubiquitin receptors RPN10 and RPN13 are known to transiently interact with a wide variety of modifiers and assembly factors including ubiquitin ligases, DUBs, chaperones, kinases, phosphatases and ubiquitin receptors among others (Hanna and Finley, 2007; Leggett et al., 2002; Stanhill et al., 2006). For example, RPN1 is known to coordinate ubiquitin binding and deubiquitination activities not only through its interactions with RPN11 and the non-essential DUB Ubp6, but also by recruiting additional ubiquitin shuttle receptors such as Rad23, Ddi1, and Dsk2 (Rosenzweig et al., 2012). Unlike RPN11, which cleaves the proximal end of substrate conjugated ubiquitin chains, Ubp6 cleaves ubiquitins from the distal end (Hanna et al., 2006). Finally, RPN2 also aids in recruiting ubiquitinated substrates to the 26S proteasome through interactions with the ubiquitin receptor RPN13 and via transient interactions with ubiquitin ligases such as Hul5 (Besche et al., 2014; Leggett et al., 2002).

1.1.3 Autophagy

Although the ubiquitin proteasome system has garnered much attention in relation to protein quality control and neurodegenerative disease, important connections
between autophagy and neurodegeneration have also become evident. Unlike the tightly regulated processes of the UPS, autophagy refers to a mode of “bulk degradation”. Briefly, autophagy is characterized as a catabolic process that recognizes and engulfs target proteins or organelles into autophagosome vesicles that then fuse with lysosomes for degradation (Levine and Klionsky, 2004; Mehrpour et al., 2010).

Autophagy is thought to complement the ubiquitin proteasome system because unlike 26S proteasomes, autophagosomes possess the unique degradative capacity to sequester substrates of various sizes and origins. Additionally, autophagy can occur in distinct pathways such as starvation induced non-selective macroautophagy, or selective quality control autophagy wherein both processes recruit distinct cellular machinery to carry out degradation depending on the type of cellular stress.

1.1.3.1 Classical autophagy pathway

Classical autophagy, which is also referred to as starvation-induced autophagy, is mainly thought of as a means for cells to recycle their own proteins in times of depleted energy states (Murrow and Debnath, 2013). The classical autophagy pathway proceeds through a series of stages that involve the formation of a double membrane structure around cytoplasmic substrates, referred to as the autophagosome, which fuses with lysosomes to expose the contents to lysosomal hydrolases for degradation and nutrient recycling (He and Klionsky, 2009; Mehrpour et al., 2010). Due to the lack of
internal protease activity in autophagosomes, their fusion with lysosomes is critical for efficient degradation. The process starts with the nucleation of the autophagic vesicle followed by the elongation and closure of the autophagosome membrane to envelop cytoplasmic constituents (He and Klionsky, 2009). Autophagosome formation requires the coordinated activity of autophagy related genes (ATGs), which are essential in the initiation, nucleation, cycling, and the expansion/ closure of the vesicle (Homma et al., 2011). In detail, classical autophagy initiation is induced by class 3 phosphoinositide-3-kinase, Beclin-2 (Atg6), and a series of ubiquitin-like conjugation reactions involving Atg12 and Atg8 (mammalian LC3). Importantly, LC3 is modified by conjugation to phosphatidylethanolamine (PE), which is then considered LC3-II, in an Atg7- and Atg10-dependent manner (Ohsumi and Mizushima, 2004). LC3-II on the inner autophagosome membrane is degraded with the contents of the autophagosome upon fusion with the lysosome, while outer membrane LC3 and all other Atg proteins assembled on the phagophore are recycled prior to the completion of the autophagosome (He and Klionsky, 2009). Also, preceding lysosome fusion, it is possible for early and late endosomes and multivesicular bodies to fuse with autophagosomes. In addition, rather than a direct fusion with lysosomes in mammalian cells, usually an initial fusion with endosomes results in amphisome formation, which later matures to the final autolysosome state (Mizushima et al., 2008).
1.1.3.2 Quality- control autophagy

Quality control (QC) autophagy is a basal level of nutrient-independent selective autophagy that is limited to damaged organelles and unnecessary or aggregated proteins (Mizushima et al., 2008). The notion of selective autophagy was initially identified by the finding that neural-specific deletion of the essential autophagy genes Atg5 or Atg7 leads to accumulation of ubiquitin positive protein aggregates in correlation with a progressive loss of neurons in mice (Hara et al., 2006; Komatsu et al., 2006). Although there is a shared dependence on ATG machinery and a necessity to degrade cytosolic constituents via lysosomes, QC autophagy and starvation-induced autophagy are in fact distinct in their functions and substrate specificity. Ideally, the precise molecular characterization of these autophagic pathways can aid in the selective pharmacological manipulation of proteins that operate in one but not both forms of autophagy; therefore, providing effective therapeutics for diseases that may display lesions in either one of the distinct degradation pathways. QC autophagy involves specific proteins that facilitate autophagosome lysosome fusion and confer substrate specificity such as ubiquitin-binding cytoplasmic histone deacetylase 6 (HDAC6) and cytoskeletal components including the cortactin-dependent F-actin network (Lee et al., 2010a). Specifically, histone deacetylase 6 is not only unique in its modifications of cytoplasmic proteins, but also because it modulates the catalytic activity of its two distinct
deacetylase sites via the BUZ finger domain, which exclusively binds the C-terminal Gly-Gly motif of unanchored ubiquitin chains (Ouyang et al., 2011; Pai et al., 2007). Furthermore, the role of HDAC6 in proteostasis, such as the cellular management of aggregated proteins, has become a topic of great interest in recent years (Kawaguchi et al., 2003).

1.1.4 Aging and proteostasis

Aging is a process characterized by the progressive loss of cellular function resulting in increased vulnerability to environmental and physiological stress that ultimately leads to an enhanced susceptibility to disease. One of the most debilitating cellular changes an organism encounters during the aging process is a significant decline in proteostasis (Taylor and Dillin, 2011). The progressive collapse in protein homeostatic processes is not only due to chronic exposure to various physiological and environmental stressors throughout the lifespan, but also to numerous changes in the machinery that facilitate adaptation to stress. In particular, crucial protein quality control proteins including heat shock proteins and autophagy related genes display a significant decrease in expression with age (Ben-Zvi et al., 2009; Bonelli et al., 1999; Fawcett et al., 1994; Gutsmann-Conrad et al., 1998; Tonoki et al., 2009). Furthermore, research has also demonstrated an age-linked attenuation of 26S proteasomes, which are the primary degradation machinery for misfolded proteins (Tonoki et al., 2009). Protein
misfolding, which results from a multitude of challenging factors encountered throughout the lifespan such as errors in translation, the presence of polymorphisms, and stresses like oxidation leading to covalent modifications, poses a severe challenge to cells if not properly regulated (Gregersen et al., 2006). For instance, if cellular quality control machinery are unable to refold or degrade misfolded proteins to maintain proteostasis, either due to exceeded capacity or impairment, then misfolded proteins can lead to cytotoxic aggregations that interfere with normal cellular processes. In fact, the deposition of protein inclusions is a pathogenic hallmark of numerous age-linked degenerative disorders (Kopito, 2000).

1.2 Protein aggregation and aggresome formation

Protein aggregation occurs as a result of the accumulation of misfolded, mutated, or destabilized proteins that have escaped proteasome mediated degradation. Specifically, the inappropriately exposed hydrophobic regions of proteins that lack proper structure have a tendency to aggregate with the hydrophobic regions of other proteins (Taylor and Dillin, 2011). These dispersed protein aggregations can be directly cytotoxic through their disruption of membranes and interaction with cellular components, which poses a threat to cellular function and viability (Gregersen et al., 2006). Thus, to suppress cytotoxicity, cells often collect and compartmentalize dispersed...
protein aggregates into a specialized inclusion body, an aggresome, at the microtubule organizing center (MTOC) (Johnston et al., 1998; Kawaguchi et al., 2003).

Aggresome formation is a well characterized process known to involve a distinct set of proteins that aid in the recognition and transport of protein aggregates to the MTOC. This process involves the association of HDAC6 with ubiquitin positive aggregates to facilitate retrograde transport of aggregates to the MTOC via the microtubule-based motor protein, dynein (Kawaguchi et al., 2003). Disruption of the microtubule network with depolymerizing agents such as nocodazole can arrest aggresome formation (Fig 1). In addition to the concentration of HDAC6, dynein, and ubiquitin chains of various linkages, aggresomes also display accumulation of Hsp70 and ubiquitin-binding scaffold protein p62, and are encaged by the intermediate filament vimentin (Johnston et al., 1998; Kopito, 2000).

**Figure 1: Nocodazole disrupts aggresome formation**

Aggresomes are formed by treating A549 cells with the reversible proteasome inhibitor MG132 for 24hrs. The addition of nocodazole, a microtubule depolymerizing agent,
blocks the transport of aggregates to the MTOC. Aggresomes are stained with anti-ubiquitin (red), and the nucleus is stained with DAPI (blue). Scale bar = 10µm.

1.3 Inclusion bodies in neurodegenerative disease

Pathological inclusions containing disease-specific misfolded proteins are a common feature of numerous neurodegenerative diseases including Parkinson’s disease (PD), Dementia with Lewy Bodies (DLB), Alzheimer’s disease (AD), Huntington’s disease (HD), Spinocerebellar Ataxias (SCA), and Amyotrophic Lateral Sclerosis (ALS) (Ross and Poirier, 2004). Many neurodegenerative disorders are marked by proteinaceous inclusions because unlike cells that are able to dilute cytotoxic misfolded proteins through cell division, neurons are post-mitotic; therefore, they are particularly sensitive to the decline in protein quality control during aging (Ross and Poirier, 2004).

Specifically, many aggregate prone neurodegenerative diseases display an impairment of the ubiquitin proteasome system, which may be due to the attenuation of 26S proteasomes with age, mutations in enzymes that are essential to the UPS pathway, a direct impairment of the 20S core particle by partially digested aggregated proteins, or a combination of all three (Bence et al., 2001; Bennett et al., 2005).

One of the most noted and well characterized aggresome-like inclusions are Lewy Bodies (LBs), which primarily occur in late-onset Parkinson’s disease and Dementia with Lewy Bodies. Although Lewy Bodies are primarily comprised of misfolded α-synuclein protein, they display similar aggresome-like features including
concentration of HDAC6, ubiquitin, p62, and Hsp70, and are encaged by vimentin (McNaught et al., 2002). Additionally, an interesting component of aggresomes and Lewy Bodies is the proteasome, which is also concentrated to the inclusions despite its inability to degrade proteins once aggregated (McNaught et al., 2002; Wigley et al., 1999).

1.4 The role of aggresomes in disease pathogenesis

The precise contribution of aggresome-like inclusion bodies to disease pathogenesis has been a long standing debate. In fact, the precise nature and fate of protein aggregates in eukaryotic cells has been poorly understood. Numerous pathological studies have focused on defining and refining the correlation between protein deposits, neuronal dysfunction, neuronal loss and severity of symptoms at time of death. However, due to the fact that studies of human tissue rely on postmortem samples, it is difficult to glimpse the early stages of disease during which the triggering species is most likely to be observable. Most recently, a protective action of aggresome formation, as opposed to an immediately pathogenic role, has been increasingly supported because the formation of inclusions is a means to contain cytotoxicity. However, the fact that these inclusion bodies precede neuronal loss is a clear indication of lesions in protein quality control preceding cell death. In addition, there is evidence that these fibrillar protein inclusions can indeed be processed and secreted from cells,
which leads to cell to cell transmission of these disease associated species (Masuda-Suzukake et al., 2014; Masuda-Suzukake et al., 2013). Establishing an understanding of how these aggresome-like inclusions are processed may be key to unraveling the precise contribution of these inclusions to disease pathogenesis.

1.5 Aggresome processing

There is significant evidence demonstrating that aggresomes can indeed be cleared from cells (Fortun et al., 2003; Kopito, 2000). Initial studies suggested that the ubiquitin-proteasome pathway might be the primary mode of removal of aggregation-prone species due to their usual reactivity with anti-ubiquitin antibodies; however, more recent studies have indicated that aggregated proteins present a particular challenge to 26S proteasomes due to their inability to unfold (Holmberg et al., 2004; Snyder et al., 2003). Furthermore, proteasome function may even be impaired by the improper digestion of aggregated proteins by the 20S core (Bence et al., 2001; Bennett et al., 2005). Thus, it has become increasingly apparent that aggresomes are processed through autophagy (Fortun et al., 2003; Kopito, 2000). Additionally, studies have also implied the involvement of HDAC6 in the eventual clearance of aggresomes, which suggests a connection between HDAC6 and autophagy; however, the precise contribution remains unclear (Iwata et al., 2005; Pandey et al., 2007). Curiously, autophagosomes are significantly smaller in size than an aggresome, approximately at least five times less in
volume, so how then are autophagosomes still able to efficiently degrade the inclusions through autophagic means? What exactly are the contributions of HDAC6 to eventual clearance of aggresomes? Finally, why are proteasomes concentrated at aggresomes if they are unable to degrade aggregated proteins?

1.6 Questions to be addressed

Age-linked aggregate-prone neurodegenerative disorders are increasing in prevalence, yet the production of effective therapies is still lacking. Despite a clear indication of a pathogenic role of aggresomes in disease, there is a definite dysfunction of protein quality control mechanisms implied by the continuous accumulation of inclusion bodies in the disease state. Previous studies have shown that aggresomes can be cleared by autophagy, so why then are inclusions such as Lewy Bodies still prevalent? How are aggresomes processed by autophagy considering the significantly smaller size of autophagosomes compared to the inclusion body? Additionally, how does HDAC6, the ubiquitin-binding protein required for quality control autophagy, contribute to aggresome processing? Is there a functional role for proteasomes that are actively concentrated to the aggresomes despite their inability to degrade aggregated proteins? The ultimate goal of the work presented here is to characterize the molecular machinery and mechanisms underlying efficient aggresome processing, which not only enables us to better identify the lesion underlying aggregate-prone disorders, but also
aids in determining the eventual fate of the aggresome and their possible contribution to disease pathogenesis. Ultimately, we aim to elucidate how cells adapt to proteotoxic stress, and how they coordinate communication among the various protein quality control systems to ensure cell homeostasis.
2. Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains

2.1 Introduction

Protein misfolding is a result of physiological and environmental stressors that continuously challenge proteins throughout the aging process. Generally, misfolded proteins are poly-ubiquitinated and degraded by the ubiquitin proteasome system. However, when proteasome capacity is exceeded, misfolded proteins tend to accumulate and lead to formation of toxic aggregates (Tran and Miller, 1999). Aggregated proteins cannot be properly unfolded to pass through the proteolytic barrel of the proteasome and can, in fact, inhibit proteasome activity. Impairment of the proteasome results in further buildup and aggregation of misfolded proteins, a pathological feature of numerous neurodegenerative diseases (Bennett et al., 2007; Pandey et al., 2007; Snyder et al., 2003). Aggregated proteins that cannot undergo degradation by the UPS are instead processed by autophagy where they are sequestered by autophagosomes and delivered to lysosomes for clearance (Holmberg et al., 2004; Venkatraman et al., 2004; Webb et al., 2003). Thus, autophagy acts as a compensatory degradation system when the proteasome system is impaired. How these two

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complementary degradative systems communicate and coordinate to dispose of toxic protein aggregates remains poorly understood.

Aggresome formation, which involves microtubule-dependent dynein motors and histone deacetylase 6 (HDAC6), often occurs as a means to suppress cytotoxicity and concentrate dispersed protein aggregates to the microtubule organizing center (MTOC). At the MTOC, the aggresome is eventually processed by autophagy (Kopito, 2000; Yao, 2010). The formation of aggresomes, which are related to Lewy Bodies prevalent in neurodegenerative disease such as Parkinson’s and Dementia with Lewy Bodies, is proposed to facilitate the autophagic clearance of toxic protein aggregates at the MTOC, where autophagosomes and lysosomes are concentrated (Iwata et al., 2005; Lee et al., 2010a; McNaught et al., 2001). Curiously, even though 26S proteasomes cannot degrade aggregated proteins, they are still accumulated at aggresomes and Lewy Bodies (McNaught et al., 2002; Wigley et al., 1999). There is no evidence elucidating why proteasomes are concentrated at the aggresome, or whether these inclusion body-associated proteasomes contribute to aggresome processing. However, the fact that autophagy and proteasomes converge at these inclusion bodies suggests an interesting possibility of a functional interaction between these two degradative systems.

Aggresome formation is a well characterized event that requires several ubiquitin-binding proteins (Yao, 2010). Among the ubiquitin-dependent machinery, cytoplasmic protein deacetylase, HDAC6, plays a central role in facilitating both the
concentration as well as the autophagic clearance of protein aggregates (Iwata et al., 2005; Kawaguchi et al., 2003; Lee et al., 2010a). Specifically, using a Drosophila model, researchers demonstrated that transgenic expression of HDAC6 suppresses toxicity caused by proteasome deficiency, which implies the involvement of HDAC6 in the compensatory autophagy mechanism when proteasomes are impaired (Pandey et al., 2007). The precise mechanism of how HDAC6 might connect proteasomes and autophagy machinery is still unknown. Interestingly, research has suggested that the unique ubiquitin-binding BUZ finger domain of HDAC6 is required for association with and clearance of ubiquitinated protein aggregates (Kawaguchi et al., 2003; Lee et al., 2010a). The BUZ finger domain is unusual in that it binds the C-terminal Gly-Gly (GG) residues of ubiquitin, whereas most other ubiquitin-binding domains interact with the hydrophobic core (Ouyang et al., 2011; Pai et al., 2007). Importantly, this structural feature indicates that HDAC6 exclusively binds free ubiquitin chains but not ubiquitinated proteins (Ouyang et al., 2011). This finding raises an interesting possibility that unanchored ubiquitin chains could in fact regulate HDAC6-dependent ubiquitinated protein aggregate processing.

Here, we present a novel finding that the proteasome system is indeed an integral component of the autophagy-dependent clearance of aggregated protein. Specifically, we demonstrate that the 19S proteasome associated de-ubiquitinating (DUB) enzyme subunit Poh1 stimulates aggresome clearance by producing free K63
linked ubiquitin chains that bind and activate HDAC6. The activation of HDAC6 is essential in initiating the actinomyosin-dependent de-aggregation of aggresomes, which is necessary for their eventual autophagic clearance. Ultimately, our findings establish a critical signaling function of proteasomes associated with the aggresome and ascertain the unique role of unanchored ubiquitin chains in connecting the proteasome and autophagy pathway.

2.2 Results

2.2.1 Aggresomes disassemble prior to clearance via autophagy

Although aggresome formation has been extensively characterized, the mechanism of how a large inclusion body is processed and becomes accessible to autophagosomes for clearance remains largely unknown. Thus, to examine the mechanism of aggresomes processing, we utilized a reversible proteasome inhibitor (MG132) to induce aggresomes. This method of aggresome induction was selected due to the overwhelming evidence suggesting impairment of UPS in aggregate prone neurodegenerative diseases (Bence et al., 2001; Kopito and Sitia, 2000). To monitor aggresome clearance, we removed MG132 by washing out the media, and imaged aggresomes at various time points using an anti-ubiquitin or anti-p62 antibody. As displayed in Fig 2, aggresomes first appeared fragmented at 12 hours of washout, and were no longer visible by 24 hours of MG132 washout. Therefore, our data suggest that aggresomes were first disassembled into smaller micro-aggregates prior to their
clearance (Fig 2 at 12h), which suggests a critical “de-aggregation” step that precedes final clearance by autophagy.

Figure 2: Aggresomes are disassembled prior to clearance
After formation of aggresomes with MG132 for 24hr in A549 cells, the inhibitor was washed off for a duration of 12hrs and 24hrs to observe aggresome clearance. Aggresomes undergo a fragmentation and de-aggregation step at 12hrs before complete clearance from the cell at 24hr wash off. Aggresomes are stained with anti-ubiquitin (red), nucleus stained with DAPI (blue). Scale Bar = 10µm.

We confirmed that aggresomes are indeed cleared via autophagy by inhibiting autophagy via bafilomycin A1 (BFA), or 3-methyladenine (3-MA). Both inhibitors resulted in arrested aggresome clearance and the accumulation of micro-aggregates at the perinuclear region (Fig 3).

Figure 3: Bafilomycin A1 and 3-methyladenine inhibit aggresome clearance
Aggresomes were initially induced with MG132 (5 µM) for 24hrs at which point MG132 was washed out with the addition of DMSO, Bafilomycin A1 (BFA), or 3- methyladenine (3-MA) for an additional 24h. Both BFA and 3-MA inhibit clearance of aggresomes. This figure was generated by Rui Hao.

2.2.2 HDAC6 is required for aggresome disassembly and clearance

Considering that HDAC6 has been strongly associated with the processing of ubiquitin-positive protein aggregates, we proceeded to further characterize its role in aggresome disassembly and processing. Interestingly, while HDAC6 was initially concentrated at the aggresome, as previously reported (Kawaguchi et al., 2003), we observed that it was no longer associated with the ubiquitin-positive structures once aggresomes de-aggregated (Fig 4).

**Figure 4: HDAC6 dissociates from aggresomes during clearance**

Representative images of HDAC6 (green) in relationship to the aggresome labeled with anti-p62 (red) illustrate localization of HDAC6 during aggresome clearance, a process
initiated by MG132 washout. Bottom panels display zoomed areas (white squares). Note that protein aggregates are no longer positive for HDAC6 after 12h washout.

Using western-blotting techniques, we confirmed that HDAC6 protein levels remained stable over this time course (data not shown), which indicates that HDAC6 dissociates from the aggresome rather than being degraded upon aggresome disassembly.

Furthermore, by use of a highly selective HDAC6 inhibitor, Tubastatin A (TBSA) (Butler et al., 2010), we probed the potential role of HDAC6 in aggresome processing. As displayed in Fig 5A, TBSA treatment completely inhibited the de-aggregation and clearance of the aggresome, while inhibitors targeting other HDAC members (MS275, NaBut- Sodium Butyrate) had little to no effect. Interestingly, TBSA treatment also prevented the dissociation of HDAC6 from protein aggregates (Fig 5C), suggesting that HDAC6 is released from protein aggregates following activation. These results show that HDAC6 activity is required for the disassembly and clearance of the aggresome.

Figure 5: HDAC6 specific inhibition prevents aggresome clearance and HDAC6 dissociation from aggresomes
(A) A549 cells were treated with an HDAC6-selective inhibitor, Tubastatin A (TBSA, 10 µM), class I HDAC inhibitors, MS275 (10 µM), or sodium butyrate (NaBut, 1mM) during 24hrs of MG132 washout. (B) The presence of aggresomes was analyzed over three independent experiments and quantified. Error bars show ± S.E.M. (C) Representative images of HDAC6 (green) and the ubiquitin positive aggresome (ub) (red) in TBSA-treated cells. Scale bar = 25 µm. Figure by Hao R and Nanduri P.

2.2.3 Poh1 is required for aggresome disassembly and clearance

Previous studies have demonstrated that the BUZ finger domain of HDAC6, which interacts exclusively with free ubiquitin chains, is required for its association with and clearance of protein aggregates. Therefore, we investigated the source of ubiquitin chain production at the aggresome. In principle, free ubiquitin chains can be produced by Poh1 (RPN11), a JAMM/MPN-domains containing deubiquitinating enzyme that cleaves ubiquitin chains en bloc from substrates targeted to the proteasome (Verma et al., 2002; Yao and Cohen, 2002). Poh1 is an integral subunit of the 19S regulatory particle, which along with the proteolytic core (20S proteasome), are concentrated at the aggresome (Fig 6, (Wigley et al., 1999)).

Figure 6: 19S and 20S proteasomes are concentrated at the aggresome
After 24hrs of MG132 treatment to induce aggresomes, A549 cells were stained for 19S proteasome DUB (Poh1) and 20S proteasome α-subunit (PSMA2). Each subunit was co-stained with anti-ubiquitin (red) to visualize aggresomes.

We examined if Poh1 is required for aggresome clearance by siRNA-mediated knockdown of Poh1. As hypothesized, the knockdown (KD) of Poh1 significantly inhibited disassembly and clearance of MG132-induced aggresomes (Fig 7A).

Importantly, the aggresome clearance defect in Poh1 KD cells was effectively reversed by the re-introduction of wild type Poh1, but not a catalytically inactive (H113A/H115A) mutant Poh1 expressed at similar levels (Fig 7B) (Gallery et al., 2007). Although Poh1 is a stoichiometric subunit of 19S proteasomes required for proper 26S proteasome assembly and function (Verma et al., 2002), the Poh1 mutant (H113A/H115A) has been shown to fully support proteasome assembly (Verma et al., 2002). The inability of mutant Poh1 to restore aggresome clearance in Poh1 KD cells is a clear indication that the deubiquitinase function of Poh1 is indeed necessary for aggresome clearance. Additionally, we examined if the accumulation of aggresomes in Poh1 KD cells was due to a defect in aggresome clearance, or a consequence of the continuous accumulation of new aggregates, which may result from compromised proteasome function. The transport of nascent aggregates to the MTOC can be efficiently blocked by the addition of nocodazole, a microtubule polymerizing agent (Fig 1). In support of a clearance defect, we observed that the retention of aggresomes in Poh1 KD cells was not affected by inhibiting microtubule-dependent delivery of nascent cytoplasmic aggregates to the
MTOC by nocodazole during the MG132 washout period (Fig 7C). Thus, the aggresome accumulation in Poh1 KD cells is mainly due to a defect in aggresome clearance instead of an increase in aggregate formation.

**Figure 7: The deubiquitinase function of Poh1 is required for aggresome clearance**

(A) A549 cells were transfected with control or Poh1-specific siRNA and treated with MG132 (5 μM, 24h) to induce aggresome or followed by MG132 washout (24h) as indicated. Aggresomes (marked by arrows) were detected by anti-ubiquitin (red) and quantified. Values represent the mean ± S.E.M. n = 3. (B) A549 cells stably expressing shRNA-resistant wild type (wt) or catalytically inactive (CI) H113/H115A mutant Poh1 were infected with Poh1-shRNA lentivirus. The percentage of cells containing an aggresome after MG132 washout was analyzed and quantified. (C) Poh1 knockdown (KD) cells were treated with MG132 to induce aggresomes followed by 24h MG132 washout in the presence of DMSO or 2.5 μM of nocodazole (Noc). Cells containing aggresomes were quantified and averaged from three independent experiments in the histogram. Note that nocodazole treatment disrupted microtubule networks (μ-tubulin, green) but had no effect on aggresome clearance. Figure by Hao R.
2.2.4 Unanchored K63 ubiquitin chains produced by Poh1 activate HDAC6 and aggresome disassembly

2.2.4.1 Poh1 activates aggresome clearance by producing unanchored K63 ubiquitin chains

Considering the significant defect in aggresome clearance under Poh1 KD, we hypothesized that if Poh1 promotes aggresome processing by producing unanchored ubiquitin chains, then the introduction of exogenous free ubiquitin chains should restore aggresome clearance in Poh1 KD cells. To test this hypothesis, we first treated stable Poh1 KD cells with MG132 to induce aggresome formation. Then, after washout of MG132, we microinjected the cells with either K63- or K48-linked free ubiquitin chains, as both types of ubiquitination were associated with detergent-insoluble protein aggregates (Fig 8A and B).

(A549 cells were cultured with or without MG132 (5 µM) for 24hrs and lysed in a 1% non-ionic Triton X-100 buffer for separation into soluble (S) and insoluble (I) components using centrifugation. Insoluble fraction contains the detergent resistant

Figure 8: Aggresomes contain both K63 and K48 linked ubiquitin chains

(A) A549 cells were cultured with or without MG132 (5 µM) for 24hrs and lysed in a 1% non-ionic Triton X-100 buffer for separation into soluble (S) and insoluble (I) components using centrifugation. Insoluble fraction contains the detergent resistant
aggresomes which display concentration of both K48- and K63-linked ubiquitin. (B) Aggresomes induced with 24h MG132 treatment were stained for K48- or K63- linked ubiquitin chains (green) and co-stained with pan-ubiquitin (red).

In addition, we also injected ubiquitinated protein conjugated with K63-linked ubiquitins as a control, which do not bind HDAC6 in vitro (data not shown). As displayed in Fig 9, after 24h of MG132 washout, non-injected Poh1 KD cells retained aggresomes, as expected (white arrowheads). However, aggresomes retained in Poh1 KD cells were efficiently processed and eliminated from cells following microinjection of free K63-linked ubiquitin chains. In contrast, K48-linked free ubiquitin chains and K63-linked ubiquitin conjugated protein failed to promote aggresome clearance. These results support the conclusion that Poh1 promotes aggresome clearance primarily by producing K63-linkage specific unanchored ubiquitin chains.

![Figure 9: Microinjection of free K63 ubiquitin chains rescues aggresome clearance in Poh1 KD cells](image)

(A) Poh1 KD cells were pre-treated with MG132 to induce aggresome formation. 3h after MG132 was removed, cells were microinjected with indicated ubiquitin species or BSA
mixed with fluorescence-conjugated dextran. The presence of aggresomes was analyzed 21 h post-injection. Injected cells were identified by dextran (green, top panels) and marked by white dotted lines (bottom panels). Aggresomes were identified by staining with an antibody specific for K48-linked ubiquitin (red) and marked by white arrowheads in injected cells. Note that only Poh1 KD cells injected with the K63-linked ubiquitin chains display aggresome clearance. Non-injected cells retained aggresomes under all conditions (white arrowheads) and served as an internal control. (B) This graph shows the quantification from three independent experiments. 50 to 100 injected cells were scored in each experiment. *, p < 0.01. Error bars indicate ± S.E.M. Scale bar = 25 μm. Figure generated by Hao R.

2.2.4.2 Unanchored ubiquitin chains bind and activate HDAC6

Similar to the effect of Tubastatin A, we observed that HDAC6 did not dissociate from aggresomes in Poh1 KD cells after MG132 washout, which suggests that Poh1 is in fact required for HDAC6 activation (Fig 10A). As HDAC6 binds unanchored ubiquitin chains in vitro (data not shown), we further investigated if HDAC6 activation is mediated by Poh1 deubiquitinase activity. To test our hypothesis, we first confirmed if HDAC6 binds endogenous free ubiquitin chains during aggresome clearance. We modified a binding assay that takes advantage of two unique properties of free ubiquitin chains (Zeng et al., 2010): resistance to heat and sensitivity to isopeptidase T (IsoT), which digests unanchored poly-Ub chains by binding to the C-terminal tail of the ubiquitin (Reyes-Turcu et al., 2006). As shown in Fig 10B, immuno-precipitation of FLAG-HDAC6 indeed pulled down heat-resistant and IsoT-sensitive ubiquitin species, which are particularly prominent 12h post MG132 washout (Fig 10B, compare lane 1 and 3). Most importantly, the free ubiquitin chains associated with HDAC6 were greatly
diminished in Poh1 KD cells (Fig 10C), which indicates that Poh1 is required for the production of free ubiquitin chains that bind and activate HDAC6.

Figure 10: HDAC6 activation is mediated by binding free ubiquitin chains produced by Poh1

(A) Representative image of HDAC6 and aggresome (ub) in Poh1 KD cells 24h after MG132 washout. (B) FLAG-HDAC6 was immuno-precipitated under the following conditions: 1) no treatment, 2) 5μM MG132 for 24h, 3) 12h MG132 washout, 4) 24h MG132 washout. The immune complexes were subjected to heating, and the eluates were treated with Isopeptidase T (IsoT) as indicated (see Methods for details). Samples were analyzed by immuno-blotting with antibodies for ubiquitin or HDAC6. Note that MG132 washout (conditions 2 to 3) led to a decrease in total ubiquitinated protein levels in whole cell lysate (WCL), but an increase in HDAC6-associated free ubiquitin chains. (C) Free ubiquitin chains associated with HDAC6 in wild type and Poh1 KD cells were assessed 12h after MG132 washout. Note that Poh1 KD led to the accumulation of total ubiquitinated proteins (left panel) but a reduction of HDAC6-associated unanchored ubiquitin chains (right panel). Figure by Hao R.

As Poh1-dependent aggresome clearance specifically involves the generation of K63-linked ubiquitin chains (Fig 9), we classified the nature of HDAC6-associated unanchored ubiquitin chains by linkage-specific ubiquitin antibodies. As shown in Fig 11, unanchored ubiquitin chains bound by HDAC6 were positive for the K63-linkage but
not the K48-linkage. Collectively, these findings support the idea that HDAC6 binds K63-linked free ubiquitin chains produced by Poh1 during aggresome clearance.

Figure 11: HDAC6 binds free K63 linked ubiquitin chains during aggresome clearance

Free ubiquitin chains released from immunoprecipitated FLAG-HDAC6 were immunoblotted using a pan-ubiquitin antibody (pan-ub), K63-specific ubiquitin antibody (K63-ub) and K48-specific ubiquitin antibody (K48-ub) sequentially. Recombinant K63- or K48-linked poly-ubiquitin chains were loaded into adjacent wells to validate the specificity of the linkage-specific ubiquitin antibodies. Figure by Hao R.

2.2.5 Poh1 and HDAC6 promote de-aggregation and clearance of aggresomes via an actinomyosin system

The data thus far have demonstrated the significance of HDAC6 activation in promoting aggresome disassembly and clearance; however, we have yet to examine the mechanics of this process. Our previous studies have demonstrated that HDAC6 induces the formation of a cortactin-dependent actin network around the aggresome (Lee et al., 2010a). Accordingly, we observed that both the inhibition of HDAC6
activation by TBSA and the siRNA mediated knockdown of cortactin, an essential component of F-actin remodeling promoted by HDAC6, significantly inhibited F-actin network formation at the aggresome during aggresome disassembly (12h MG132 washout, Fig 12A). Importantly, Poh1 KD also suppressed F-actin remodeling, thereby phenocopying HDAC6 inactivation (Fig 12A). Therefore, these results support the model wherein Poh1 activates HDAC6- and cortactin-dependent F-actin remodeling required for aggresome clearance. Specifically, F-acting remodeling is most prominent during aggresome de-aggregation, where the F-actin network appeared to become interspersed among the protein aggregates (Fig 12B). These data raise the possibility that local actin cytoskeleton-dependent forces might actually be the drivers of aggresome disassembly. Also, considering that Type II non-muscle myosin 9 (IIA) and 10 (IIB) have been established as the main motors associated with the actin cytoskeleton (Vicente-Manzanares et al., 2009), we examined whether these actin-dependent motors are involved in aggresome clearance using siRNA-mediated knockdown. As shown in Fig 12C, we observed that only knockdown of myosin 10 dramatically inhibited aggresome de-aggregation and clearance, whereas knockdown of myosin 9 had no effect. Importantly, in contrast to Poh1 KD cells, the aggresomes that accumulated in myosin 10 KD cells were largely devoid of HDAC6 after MG132 washout, which implies that HDAC6 activation is upstream of myosin 10 activity (Fig 12D). Similarly, HDAC6 was not retained on aggresomes in cortactin KD cells (Fig 12D). Collectively, these results are
consistent with a model where HDAC6 acts as an effector downstream to Poh1, but upstream to the actinomyosin system. We conclude that proteasome-associated Poh1 activates HDAC6-dependent actinomyosin machinery to facilitate the de-aggregation and clearance of the aggresome.

**Figure 12: Disassembly of aggresomes occurs through cortactin and myosin10 dependent actin network**

A549 cells were pre-treated with MG132 (5 µM) for 24h to induce aggresome formation. (A) Representative images of the aggresome (ubiquitin, green) and F-actin (red, Rhodamine-Phalloidin) 12h after MG132 washout in cells with indicated condition. In the panels of F-actin, the location of the aggresome is marked by an arrowhead. Right Panel: Cells exhibiting F-actin punctae around the aggresome were quantified from three experiments. Error bars indicate ± S.E.M. *, p < 0.01. (B) Representative staining of
F-actin and a de-aggregated aggresome 12h post MG132 washout. The right panel shows the zoomed areas marked by white squares. (C) A549 cells transfected with the indicated siRNA were imaged 0h or 24h after MG132 washout. Aggresomes (arrows) were detected with anti-ubiquitin antibody (red) and percentage of cells retaining an aggresome was quantified from three independent experiments. Error bars show S.E.M. (D) Co-staining of HDAC6 (green) and ubiquitin (red) in cells expressing indicated siRNA 24h after MG132 washout. Arrows mark aggresomes. The right panel shows the quantification of aggresomes that are positive for HDAC6 (n=3). Error bars show ± S.E.M. *, p < 0.01. Data generated by Nanduri P in collaboration with Hao R.

2.3 Discussion

Our study established two important findings regarding aggresome processing. First, we revealed that aggresomes undergo a crucial de-aggregation step in order to become accessible to autophagosomes for eventual autophagy dependent clearance. Second, we have discovered a novel signaling role of 26S proteasomes in initiating autophagy, thus explaining why they are actively concentrated to aggresomes regardless of their inability to degrade aggregated protein. We provided evidence that the proteasome system actively facilitates aggresome disassembly by producing unanchored K63 linked ubiquitin chains that bind and activate HDAC6. Supporting previous implications of HDAC6 involvement in eventual autophagic elimination of protein aggregates (Iwata et al., 2005), we confirmed that HDAC6 activation is indeed crucial in promoting actinomyosin dependent disassembly of the large aggresome, which results in smaller fragments that are easily engulfed by autophagosomes. Therefore, our collective findings indicate a critical function of inclusion body-associated proteasomes
and identified unanchored ubiquitin chains as signaling molecules that connect and coordinate proteasomes and autophagy to eliminate toxic protein aggregates.

Our studies identified the proteasomal deubiquitinating enzyme, Poh1, as a critical factor that controls the disassembly and degradation of the aggresome. We revealed that in Poh1 deficient cells, aggresome clearance failed (Fig 7). Although Poh1 is best characterized for its essential role in 26S proteasome-dependent proteolysis, several lines of evidence indicate that Poh1 activates aggresome clearance mainly by producing free K63 ubiquitin chains. First, while Poh1 inactivation likely affects multiple biological processes, microinjection of unanchored K63-linked chains sufficiently restored aggresome clearance in Poh1 knockdown cells. These results indicate that the production of unanchored K63-linked chains is key to Poh1-dependent aggresome clearance (Fig 9). Second, previous studies have established a K63 specific DUB activity of proteasomal Poh1, where Poh1 specifically cleaves K63 linkages within a mix linked ubiquitin chain containing both K63- and K48- linkages (Cooper et al., 2009). Thus it is plausible that this selective endo-protease activity could allow Poh1 to specifically cleave and release free K63-linked ubiquitin chains from protein aggregates, which are conjugated with both K48- and K63-linked ubiquitin chains (Fig 8). Lastly, consistent with this proposition, during aggresome clearance HDAC6 becomes associated with and activated by K63-linkage containing free ubiquitin chains, whose production depends upon Poh1 (Fig 10 and 11). Although these data do not exclude the involvement of the
canonical proteasomal function of Poh1, collectively they strongly suggest that the production of unanchored ubiquitin chains is the main function of proteasomal Poh1 in promoting aggresome clearance. In this context, the proteasome does not function as classical proteolytic machinery; rather, it acts as a signaling complex that produces unanchored ubiquitin chains with regulatory activities.

Our study has established a critical role of HDAC6, a component of QC autophagy (Lee et al., 2010a), as the key regulatory effector of Poh1-generated ubiquitin chains. Specifically, we demonstrate that HDAC6 binds unanchored K63-linked ubiquitin chains during aggresome clearance in wild type but not Poh1 deficient cells (Fig 10 and 11). Additionally, the activation of HDAC6 stimulates remodeling of the cortactin and myosin dependent F-actin network to actively disassemble the aggresome and facilitate autophagic clearance. Thus, the regulation of HDAC6 by unanchored ubiquitin chains provides a simple model explaining how proteasomes might communicate with autophagy machinery if their activity is no longer adequate and challenged by protein aggregates. We propose that proteasomal Poh1 serves to stimulate compensatory autophagy activity via HDAC6. Accordingly, Poh1 deficiency, similar to HDAC6 inhibition, leads to defects in aggresome de-aggregation and clearance (Fig 7).

We have identified a critical role of free K63 linked ubiquitin chains in initiating aggresome clearance, which suggests that these unique ubiquitin species likely have a much broader regulatory function. Although our data support K63-linked ubiquitin
chains as the key mediators in activating HDAC6 and aggresome disassembly, the involvement of other ubiquitin linkages cannot be excluded. We do not know how exactly the binding of K63-linked or other ubiquitin chains modulates HDAC6 activity; therefore, further structural characterization of HDAC6 with various ubiquitin chains would be crucial in answering this question. Additionally, further studies would also be required to determine whether the length of the ubiquitin chain is critical in modulating HDAC6 activity. However, it is worth noting that ubiquitin chains with different linkages show differential activities in activating RIG-1 and TAK1 (Xia et al., 2009; Zeng et al., 2010). Thus, unanchored ubiquitin chains with different linkages could have distinct regulatory activities.

The unique dependence on K63-linked ubiquitin chains in aggresome clearance is intriguing, since this modification normally does not target substrates for proteasome-mediated degradation. However, we found that MG132 treatment led to a dramatic increase in K63-linked ubiquitination associated with protein aggregates (Fig 8). Thus, protein aggregates are likely tagged by mixed ubiquitin chains of both K48- and K63-linkage. These observations are of potential significance, as JAMM/MPN-containing deubiquitinating enzymes, including Poh1, can cleave K63- but not K-48 linked ubiquitin chains in vitro (Cooper et al., 2009). We speculate that the addition of K63-linked ubiquitin chains to aggregated proteins creates substrates for Poh1, which subsequently cleaves and produces unanchored K63-linked ubiquitin chains that
activate HDAC6 and autophagic-dependent aggresome clearance. Thus, it is possible that additional K63 modification of protein aggregates, which were originally tagged with K48 linked ubiquitin, constitutes a new ubiquitin code to initiate aggresome-associated autophagy. Identifying the ligase responsible for K63-linked ubiquitination on protein aggregates and determining how Poh1 activity is regulated toward these unusual substrates are two critical issues to be addressed in the future.

The general assumption surrounding the biogenesis of inclusion bodies in neurodegenerative disease involves an impaired proteasomal system that fails to degrade misfolded proteins. However, our study suggests that proteasome deficiency may also lead to a failure in proper processing and removal inclusion bodies. This dual mechanism could explain the prevalence of inclusion bodies in Parkinson’s disease, where proteasome deficiency has been documented (McNaught et al., 2001). Our study also shows that exogenous free ubiquitin chains can stimulate aggresome clearance in Poh1-deficient cells. The possibility that free ubiquitin chain-mimetics might activate inclusion body clearance offers a potential therapeutic solution for neurodegenerative disease.
3. Chaperone-mediated proteasome remodeling facilitates free K63 ubiquitin chain production and aggresome clearance

3.1 Introduction

The appearance of inclusion bodies is a pathological hallmark of many neurodegenerative disorders (Kopito, 2000; Soto, 2003). 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 (Johnston et al., 1998; Kawaguchi et al., 2003; Kopito, 2000). Clinically, perinuclear aggresome-like inclusions, Lewy Bodies, are prevalent in brain tissues from patients with Parkinson’s disease and Dementia with Lewy Bodies (Kopito, 2000; McNaught et al., 2002). 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 (Bhattacharyya et al., 2014; da Fonseca et al., 2012). The 19S

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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 (Glickman et al., 1999; Voges et al., 1999). 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 (Kopito and Sitia, 2000). Aggregated proteins are instead cleared via autophagic machinery that sequester and deliver aggregates to lysosomes for degradation (Hyttinen et al., 2014). Thus, the proteasome and autophagy form complementary degradative systems to maintain protein quality control and limit the buildup of toxic protein aggregates (Hao et al., 2013; Pandey et al., 2007).

Interestingly, although proteasomes cannot directly degrade protein aggregates, they are concentrated at protein inclusion bodies and are, in fact, required for their clearance (Hao et al., 2013). 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 (Hao et al., 2013). 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 (Cooper et al., 2009; Yao and Cohen, 2002).
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 (Hao et al., 2013). 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 Gly-Gly residues of unanchored ubiquitin chains (Ouyang et al., 2011; Pai et al., 2007). 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 (Hao et al., 2013; Lee et al., 2010a).

Canonical K48-linked ubiquitin chains are normally coupled to 26S proteasome-mediated substrate degradation (Glickman and Ciechanover, 2002); curiously, however, Poh1 specifically produces K63-linked chains in the context of aggresome clearance (Hao et al., 2013). In fact, in Poh1-deficient cells, aggresome clearance can be effectively restored by exogenous free K63-linked, but not K48-linked, ubiquitin chains (Hao et al., 2013). These findings indicate that Poh1 produces ubiquitin chains of different linkages in 26S proteasome- and autophagy-mediated degradation. How Poh1 achieves these distinct biochemical functions is not known. Interestingly, although the canonical deubiquitinase (DUB) activity of Poh1 requires the intact 26S proteasome (Verma et al., 2002; Yao and Cohen, 2002), a Poh1-dependent K63-specific DUB activity was characterized biochemically and found to reside in a 19S proteasome complex (Cooper et
al., 2009). 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.

3.2 Results

3.2.1 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
(Hao et al., 2013). To investigate the status of proteasomes 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 hours of MG132 washout (Fig 13A). Unexpectedly, under the same condition, minute levels of PSMA2, an α-subunit of the 20S proteasome complex, was found at de-aggregated aggresomes (Fig 13B compare 24h MG132 and 12h Wash). Quantification of relative signal intensity demonstrated a significant reduction of PSMA2, but not Poh1, associated with de-aggregated aggresomes (Fig 13C). Biochemical fractionation confirmed that Poh1 remained enriched in Triton X-100 insoluble protein aggregate fractions, while the abundance of PSMA2 was clearly reduced (Fig 13D). 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 13D). 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.
Figure 13: 20S proteasomes dissociate from the aggresome during de-aggregation

(A) Immunolocalization of 19S associated deubiquitinase Poh1 (red) and (B) 20S associated PSMA2 (red), both co-stained with ubiquitin (green) as the aggresome marker. Proteasome subunits were observed upon aggresome formation at 24h of MG132 treatment and 12h of MG132 washout when aggresomes undergo de-aggregation. Boxed regions of the image are zoomed to highlight proteasome associations with the aggresome. Scale bar indicates 15 µm. (C) Immunostaining results from (A) and (B) were quantified at 24h MG132 and 12h wash for both subunits. Refer to experimental methods for image analysis. Significant loss of PSMA2 is observed during 12h wash, whereas Poh1 signal remains stable. Error bars indicate ± S.E.M. *, p < 0.01. (D) Immunoblotting of 19S DUB (Poh1) and 20S core subunits (PSMA2, PSMA4, PSMB4, and PSMB5) separated by detergent-soluble and insoluble (aggresome) fractions. Actin is provided as a loading control and detergent resistant Hsp70 and Hsp90 reflect level of insoluble aggresome. Indicated densitometry values for each protein reflect the density ratio: (band density for a particular treatment condition / band density at 24h MG132). All ratios were normalized to actin. (E) Densitometry data displayed in (D) were
quantified for the insoluble fraction at 24h MG132 and 12h wash averaged over three separate experiments. Error bars indicate ± S.E.M. *, p < 0.01.

The 19S proteasome can be further divided into two sub-complexes: 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 fractions after MG132 washout (Fig 14C). In contrast, the 19S ATPase subunits, RPT3 (Fig 14A, C) and RPT6 (Fig 14C) were largely dissociated from the protein aggregates. Interestingly, the non-ATPase subunits of the 19S base, RPN1, RPN2 (Fig 14C) and RPN10 (Fig 14B, 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 14: 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 24h MG132 and 12h 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 24h MG132, but staining improves upon 12h 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 Figure 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.M. *, p < 0.01.

### 3.2.2 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 (Hao et al., 2013), aggresomes induced by MG132 treatment were cleared ~24hrs after the proteasome inhibitor was removed, and this process was suppressed by Poh1 KD (Fig 14A). We found that PSMA2 or PSMB4 KD also led to the presence of aggresomes 24hrs 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 (Hao et al., 2013). 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 (Johnston et al., 1998), whereas aggresome clearance proceeds normally in the presence of a microtubule depolymerizing agent, nocodazole (Hao et al., 2013). As shown in Fig 14A, 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 (Hao et al., 2013). In stark contrast, in PSMA2 and PSMB4 KD cells, the addition of nocodazole during the 24hr MG132 washout period led to prominent accumulation of dispersed...
micro-aggregates instead of a large aggresome (Fig 15A). 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 15A).

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-aggregated aggresomes on their way to degradation (Hao et al., 2013; Kawaguchi et al., 2003). 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 15C), 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-aggregated aggresomes, showed phenotypes similar to those of Poh1 KD (Fig 15A), 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.
Figure 15: 20S proteasomes are not essential for aggresome clearance

(A) Aggresomes labeled with ubiquitin (red) 24h post MG132 wash with or without the addition of nocodazole under non-targeting siRNA control (Control KD), Poh1 KD, RPN10 KD, PSMA2 KD or PSMB4 KD. Nocodazole sensitive PSMA2 KD aggregates reflect aggregates undergoing aggresome formation. Knockdown efficiency is displayed by immunoblotting adjacent to each knockdown condition. (B) Quantification of aggresomes and dispersed aggregates displayed in (A) over three different experiments. Error bars indicate ± S.E.M. *, p < 0.01. (C) HDAC6 associates with micro-aggregates in PSMA2 KD cells under 24h wash with nocodazole, which classifies those dispersed aggregates as pre-aggresomal. Scale bar for all image data indicates 15 µm.

3.2.3 Hsp90 facilitates 20S proteasome dissociation and aggresome clearance

The molecular chaperone Hsp90 was reported to interact with and regulate 26S proteasome assembly (Imai et al., 2003). We found that Hsp90 is enriched at the
aggresomes induced by MG132 treatment (Fig 16A). 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 16B-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 12hrs post MG132 washout.

Figure 16: Hsp90 facilitates 26S remodeling at the aggresome

(A) Immunolocalization of Hsp90 at the aggresome upon formation. (B) Immunostaining of 19S (Poh1) and (C) 20S (PSMA2) upon aggresome formation (24h
MG132) and 12h of MG132 washout with Hsp90 inhibitor 17-DMAG (2 µM). Aggresomes are marked with ubiquitin (green). PSMA2 does not display a significant dissociation from aggresomes in the presence of 17-DMAG during MG132 washout. Boxed regions of the image are zoomed to highlight proteasome associations with the aggresome. These experiments were conducted at the same time as Figure 1 A and B to minimize variations in staining between experiments. Scale bar indicates 15 µm. (D) Quantification of signal intensity for data displayed in (B) and (C). Refer to experimental methods for image analysis. No significant reduction in PSMA2 was observed under 17-DMAG. (E) Immunoblotting of 19S (Poh1, RPN7, RPN8, RPN9, RPN10, RPN1, RPN2, RPT3, and RPT6) and 20S (PSMA2, PSMA4, PSMB4, and PSMB5) subunits separated by detergent soluble and insoluble fractions. Note that compared to 12h wash, 20S and ATPase subunits do not dissociate in the presence of 17-DMAG. Actin is provided as a loading control and detergent-resistant Hsp70 and Hsp90 reflect the level of insoluble aggresome. Hsp70 expression has been previously reported to increase under Hsp90 inhibition. Densitometry values reflect ratios of band density for each condition to the intensity of 24h MG132 treatment. All ratios are normalized to actin. (F) Densitometry data for results displayed in (E) quantified over three separate experiments. Error bars indicate ± S.E.M. *, p < 0.01.

Furthermore, 17-DMAG treatment also inhibited aggresome clearance after MG132 washout (Fig 17A, 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 17C, D). Additionally, novobiocin, an Hsp90 inhibitor that targets the C-terminal domain of Hsp90 (Marcu et al., 2000a; Marcu et al., 2000b), also suppressed aggresome clearance (Fig 17A). Other Hsp90 inhibitors also displayed a similar defect in aggresome clearance (Blagg and Kerr, 2006; Fadden et al., 2010). These data show that Hsp90 activity facilitates proteasome remodeling and aggresome clearance.
Figure 17: Hsp90 is required for efficient aggresome clearance

(A) Hsp90 inhibition by 17-DMAG (2 µM), Novobcin (500 µM), 17-AAG (2 µM), PU-H71 (2 µM), HS-10 (1 µM) prevents aggresome clearance 24h post MG132 washout. Aggresomes are labeled with ubiquitin (red). (B) The presence of aggresomes was quantified among three separate experiments and compared to DMSO as a control. Error bars indicate ± S.E.M. *, p < 0.01. (C) Aggresomes remain intact even in the presence of nocodazole during 24h wash with 17-DMAG, which reflects a clear defect in aggresome clearance. (D) Aggresomes remaining under nocodazole wash with or without 17-DMAG are quantified among three separate experiments. Error bars indicate ± S.E.M. *, p < 0.01. Scale bars indicates 15 µm.

3.2.4 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 (Hao et al., 2013). 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 18). These data indicate that 26S proteasome remodeling precedes HDAC6 activation.
Figure 18: HDAC6 inhibition does not affect 20S proteasome dissociation

A) Immunostaining of 19S (Poh1) and 20S (PSMA2) upon aggresome formation (24h MG132) and 12h of washout with HDAC6 inhibitor Tubastatin-A (10 µM) (TBSA). PSMA2 displays significant dissociation from aggresomes in the presence of TBSA during MG132 washout. Boxed regions of the image are zoomed to highlight proteasome associations with the aggresome. These experiments were done at the same time as Figure 1A and B to minimize variations in staining between experiments. B) Image data from (A) are quantified among three separate experiments. Refer to experimental methods for image analysis. Error bars indicate ± S.E.M. *, p < 0.01. Scale bars indicates 15 µm. C) Immunoblotting of Poh1 and PSMA2 in the detergent-soluble and insoluble fractions at 24h MG132, 12h wash, and 12h wash with TBSA. Addition of TBSA does not affect the dissociation of PSMA2 from the aggresome. Densitometry readings reflect ratio of band density for each condition to 24h MG132. All ratios are normalized to actin.

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 19A, B), a process dependent upon Poh1 (Hao et al., 2013). 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 (Hao et al., 2013; Xia et al.,
2009). We found that 17-DMAG treatment significantly reduced the amount of
unanchored ubiquitin chains bound by HDAC6 during MG132 washout (Fig 19C, D).
Further supporting this conclusion, 17-DMAG prevented HDAC6 dissociation from the
aggresome, indicating that HDAC6 was inactive (Fig 19E, 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.
Figure 19: Hsp90 facilitates the efficient production of free K63 ubiquitin chains and HDAC6 activation

(A) Immunoblotting of K63 linked ubiquitin in the detergent-soluble and insoluble fractions during conditions of DMSO, 24h MG132, 12h wash, and 12h wash with 17-DMAG. There is a clear retention of K63 ubiquitin on the aggresome under inhibition of Hsp90. (B) Densitometry quantification of data displayed in (A) calculated by the ratio of band density for each condition to band density at 24h MG132. Data quantified over three separate experiments, error bars indicate ± S.E.M. *, p < 0.01. (C) FLAG-HDAC6 was immunoprecipitated under the following conditions: (1) no treatment, (2) 5 µM MG132 for 24h, (3) 12h MG132 washout, and (4) 12h MG132 washout in the presence of 2 µM 17-DMAG. The immune complexes were subjected to heating, and the eluates were treated with isopeptidase T (IsoT) as indicated (see the Experimental Procedures for details). Samples were analyzed by immunoblotting with antibody for ubiquitin. Note that MG132 washout (condition 2 versus 3) led to a decrease in total ubiquitinated protein levels in whole cell lysate (input) but an increase in HDAC6-associated free ubiquitin chains. This increase was suppressed under the addition of 17-DMAG (condition 4). (D) Densitometry quantification of data displayed in (C). There is a significant reduction in the amount of free ubiquitin chains bound by HDAC6 in the
presence of 17-DMAG. Data reflect the ratio of density of each condition to the density at 24h MG132 treatment across three separate experiments. Error bars indicate ± S.E.M. *, p < 0.01. (E) Immunolocalization of HDAC6 upon aggresome formation (24h MG132), de-aggregation (12h Wash), and washout under Hsp90 inhibition (12h 17-DMAG). Note that HDAC6 dissociation is blocked under 17-DMAG reflecting a state of inactivation. Boxed regions were zoomed and displayed below each condition. Scale bar indicates 15 μm. (F) Imaging data from (E) is quantified among three separate experiments. Error bars indicate ± S.E.M. *, p < 0.01. Refer to experimental methods for image analysis.

3.3 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 (Hao et al., 2013). 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 (Hao et al., 2013). 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 (Lander et al., 2012; Yao and Cohen, 2002). 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 (Hao et al., 2013). Despite the presence of both forms of ubiquitin linkages on protein aggregates, Poh1 appears to mainly produce K63-linked ubiquitin chains during aggresome clearance (Hao et al., 2013). 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 (Lander et al., 2012), 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 (Cooper et al., 2009). Thus, in contrast to Poh1-mediated deubiquitination associated with proteosomal substrate degradation, which requires intact 26S
proteasomes (Verma et al., 2002; Yao and Cohen, 2002), K63-targeted deubiquitination activity of Poh1 does not require 20S core proteasome components and can function as an independent entity (Cooper et al., 2009).

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 (Chandra et al., 2010). RPN10, along with 19S base-associated RPN13, which interacts with RPN2, directly bind ubiquitinated peptides delivered to proteasomes (Finley, 2009). Structural data for RPN13 reveal its preference for K48-linked ubiquitin (Schreiner et al., 2008), whereas mammalian RPN10 has the capacity to bind both K48 and K63 chains (Wang et al., 2005). Upon proteasome inhibition, RPN13 becomes poly-ubiquitinated, and as a result, its ubiquitin-binding activity diminishes (Besche et al., 2014). 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 15).

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 (Figure 14). Interestingly, the 19S lid is homologous to another K63-specific deubiquitinating enzyme complex, the BRCA1-A complex (Wang et al., 2009), where Poh1-related BRCC36 encodes for the catalytic subunit (Cooper et al., 2009). Similar to Poh1, the BRCA1-A complex does not require ATPase activity to catalyze K63-linked deubiquitination (Cooper et al., 2009). 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 (Breusing and Grune, 2008; Hanna et al., 2007; Kurepa and Smalle, 2008; Livnat-Levanon et al., 2014; Stanhill et al., 2006; Wang et al., 2010). For example, 20S core particles become dissociated from 19S proteasomes in response to mitochondrial and oxidative stress (Breusing and Grune, 2008; Kurepa and Smalle, 2008; Livnat-Levanon et al., 2014; Wang et al., 2010). The dissociated 20S proteasomes appear to be more effective than 26S proteasomes in degrading proteins damaged by the
oxidative stress (Davies, 2001). 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 (Stanhill et al., 2006). 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 (Besche et al., 2014). 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 (Hanna and Finley, 2007). Whether proteasome remodeling is impaired in neurodegenerative disease or other disorders would be of great interest in the future.
4. Experimental methods

4.1 Antibodies and reagents

The following antibodies and reagents were used in this study: anti-HDAC6 (H-300), anti-p62/ SQSTM1 (D-3), anti-RPN1, and anti-P4D1 (Santa Cruz Biotechnology Inc.); K63-specific anti-ubiquitin (APU3) and K48-specific anti-ubiquitin (APU2) (Millipore); anti-PSMA2 (Cell Signaling); anti-PSMB5 (Novus Biologicals); anti-RPN7, 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-Beta4/PSMB4, 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 (BioVision), HS-10 (gift from Dr. Timothy Haystead at Duke University).

4.2 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 24hrs. 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), Novobicin (500 μM), 17-AAG (2 μM), PU-H71 (2 μM), HS-10 (1 μM), Tubastatin-A (10 μM), nocodazole (2.5 μM).

4.3 Immunofluorescence analysis

A549 cells cultured on glass coverslips were rinsed twice with PBS and fixed with either 4% paraformaldehyde (PFA) for 15 mins 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-X100 in PBS for ten minutes. Following permeabilization, cells were rinsed with PBS, blocked with 5% BSA in PBS containing 0.1% Triton-X100 for 1 hr, and incubated with antibodies, as described previously (Kawaguchi et al., 2003). 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. F-actin staining was performed by incubating cells with Rhodamine-conjugated Phalloidin in PBS for 30 min (Gao et al., 2007). Images were taken using a Leica SP5 confocal microscope. 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.

4.4 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-X100, 1 mM EDTA, 1 mM DTT, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 10 mM N-Ethylmaleimide (NEM), a cocktail 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 sec, 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.

4.5 siRNA Interference

The following siRNAs were used in this study: as previously reported Poh1(Hao et al., 2013) and 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 24hrs later to achieve higher knockdown efficiency.

### 4.6 Analysis of HDAC6-associated free ubiquitin chains

This procedure was done as previously reported (Hao et al., 2013) 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] NP-40, 1 mM Na$_3$VO$_4$) 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 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 1mg was incubated with 40 µl 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 g for 5 min followed by three washes with 1mL 150 mM NETN buffer. The remaining wash buffer was aspirated and beads were incubated in 40 µl 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 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.

### 4.7 Microinjection of free ubiquitin chains

Microinjection was performed on an Olympus IX-70 inverted microscope equipped with an Eppendorf FemtoJet and TransferMan NK2 microinjection system. A549 cells transfected with Poh1 siRNA were cultured on poly-L-lysine coated glass coverslips, treated with 5 μM MG132 for 24 hours followed by three hours of MG132-free fresh medium prior to the injection. PBS solution containing 2 μg/μl of BSA control, K63-linked poly-ubiquitin chains, K63-ubiquitinylated protein, or K48-linked poly-ubiquitin chains was microinjected into the cytoplasm using holding pressure. About 0.2 pg proteins were injected per cell. Injected cells were incubated in fresh full growth medium at 37°C for another 21 hours before analysis. 70 to 100 cells were injected in each experiment with three independent experiments.
5. Conclusions and future direction

5.1 Conclusion

The general assumption when considering protein degradation is that the three major systems for protein quality control, chaperones, proteasomes, and autophagy work in parallel to maintain proteostasis. Thus, if one fails, then on to the next. However, it has become more and more apparent over recent years that these systems in fact work interdependently to coordinate necessary processes for efficient protein quality control. Here, we identified a key line of communication under proteotoxic stress where unanchored K63 linked ubiquitin chains, generated by proteasomal Poh1 upon chaperone-mediated 26S proteasome remodeling, activates HDAC6-dependent quality control autophagy. Successful elimination of aggresomes from cells is only achieved upon efficient functioning of all three quality control systems. Hence, our work indicates a highly coordinated and complementary relationship between these degradation systems that becomes critical in times of cellular stress.

To address our initial questions, how do aggresomes undergo autophagic processing considering their remarkably large size, and how exactly do proteasomes and HDAC6 contribute to this processing, we have characterize distinct molecular machinery including HDAC6 and proteasomal Poh1 that directly facilitate disassembly of large inclusions and make them accessible to autophagosomes for elimination. However, the discovery of Poh1 involvement in the generation of free K63 ubiquitin
chains begged the question how does Poh1 function to deubiquitinate protein aggregates when they are resistant to unfolding, a necessary criteria for 26S proteasome mediated functions. While characterizing the various proteasomal subunits involved in this process, we uncovered that 26S proteasomes undergo significant remodeling during aggresome clearance. This local remodeling event, mediated by Hsp90, allows for Poh1 and select 19S non-ATPase subunits to liberate from the canonical 26S complex and efficiently deubiquitinate K63 chains conjugated to aggresomal proteins. The generation of unanchored K63 chains is critical to initiate HDAC6 mediated actin remodeling, where the cytoskeletal components including actin, cortactin, and myosin 10 generate the physical motor required to disassemble the large inclusion into fragments that are easily engulfed by autophagosomes for elimination.

5.2 What is the fate of processed aggresomes?

However, the question remains, what is the fate of these processed inclusions? Are they degraded intracellularly, or are they secreted into the extracellular matrix, where they can potentially propagate to neighboring cells? Recent developments in studies of fibrillar α-synuclein oligomers demonstrate the capacity of these pathogenic fibrils to propagate from cell to cell over time (Lee et al., 2010b; Masuda-Suzukake et al., 2014). Specifically, recent studies conducted in laboratory of Dr. Virginia Lee have demonstrated that inoculation of pathological α-synuclein fibrils via intracerebral injections in asymptomatic α-synuclein transgenic mice display Lewy-like inclusions
that propagate well beyond the injection site and reduce survival (Luk et al., 2012). It is of interest to test whether inhibition of aggresome processing through HDAC6 inhibitors (Tubastatin A) or Hsp90 inhibition can prove beneficial in halting propagation of pathogenic fibrils. Although both inhibitors have shown neuroprotective effects (Butler et al., 2010; Luo et al., 2010), the issue of a valid animal model that displays a progressive loss of neurons paired with the development of inclusion bodies similar to human disease has not been established. However, it will be highly beneficial to test if transgenic mice directly injected the pathogenic fibrils display reduced propagation of disease and increased survival with the administration of HDAC6 inhibitors or Hsp90 inhibitors.

### 5.3 Clearance of chronic stress-induced aggresomes

An important study to further expand our current aggresome processing pathway is to characterize aggresome clearance using a chronic stress induced aggresome model. Mitochondrial dysfunction is a pathological hallmark of aggregate-prone neurodegenerative disorders (Abou-Sleiman et al., 2006; Breusing and Grune, 2008; Taylor and Dillin, 2011). Mitochondria are a source of oxidative stress leading to enhanced protein misfolding, dysfunctional 26S proteasomes, and accumulation of pathogenic aggregates (Wang et al., 2010). In fact, mitochondrial toxins such as rotenone and paraquat have been correlated with the occurrence of Parkinson’s disease leading to the prevalence of Lewy Bodies (Nistico et al., 2011; Spivey, 2011; Tanner et al., 2011;
Uversky, 2004). It is of high importance to test whether our proposed model of autophagy initiation and proteasome remodeling can be applied to aggresomes induced via chronic indirect proteotoxic stress where the proteasome machinery is not directly inhibited by our treatment. Research has shown that aggresomes can be induced with low dose chronic treatment of the mitotoxin rotenone in SHSY5Y cells overexpressing α-synuclein (Shaikh and Nicholson, 2008). Not only does rotenone provide a disease relevant model due to its correlation with development of Parkinson’s disease, but rotenone is also a reversible mitochondrial complex I inhibitor. This reversible binding allows us to study the clearance machinery and specifically proteasome remodeling in aggresomes induced without direct proteasomal stress. Are these aggresomes similar to MG132 aggresomes in composition? Do they display a similar 26S proteasome remodeling/ Poh1 production of K63 chains/ HDAC6 initiation of an actin dependent autophagic clearance mechanism? These studies will allow us to apply our model of aggresome processing to various stress induced inclusions and possibly identify multiple mechanisms involved in aggresome processing, which may very well vary by the type of cellular stress.

5.4 Identify the K63 ligase

Unanchored K63-linked ubiquitin chains are crucial to the initiation of HDAC6 dependent aggresome processing. Generally, misfolded proteins are initially conjugated with K48 linked ubiquitin chains for proteasome mediated degradation; however, if
degradation fails or if misfolded proteins are aggregated, rendering them resistant to proteasomal degradation, then an additional K63 chain is conjugated for further processing of substrates. The additional ubiquitination of a previously ubiquitinated substrate requires the activity of an E4 ligase (Hoppe, 2005). For instance, Hul5 (human Ube3C) is a proteasome associated E4 ligase that interacts with RPN2 of the 19S regulatory particle. Hul5 has been reported to increase association with 26S proteasomes upon proteasome inhibition (Besche et al., 2014). In vitro, it was observed that Hul5 can elongate previously conjugated ubiquitin chains with K63 linked ubiquitin (Crosas et al., 2006). Additionally, it was recently reported that Hul5 polyubiquitinates the proteasome bound ubiquitin receptor RPN13 in response to proteasome inhibition rendering it unable to bind ubiquitin chains for proteasomal degradation (Besche et al., 2014).

Considering that Hul5 plays a vital role in regulating proteasome function under conditions of proteotoxic stress, I investigated if Hul5 could mediate K63 ubiquitination of substrates targeted to the proteasome that cannot undergo proteasomal degradation. Although the experiment was not successful, due to the fact only one siRNA can show this outcome, we did produce a striking phenotype that associates K63 ubiquitination to aggresome formation (Fig 20).
Figure 20: Hul5 mediated K63 ubiquitination mediates aggresome formation

(A) A549 cells were cultured with MG132 (5 µM) for 24hrs under control knockdown (cont KD) or Hul5 knockdown and lysed in a 1% non-ionic Triton X-100 buffer for separation into soluble (S) and insoluble (I) components using centrifugation. Insoluble fraction contains the detergent resistant aggresomes. Hul5 KD cells display a significant decrease in K63 ubiquitination as well as ubiquitin binding p62. Hsp70 reflects total levels of insoluble aggregated proteins and actin is displayed as a loading control. (B) Aggresomes were induced with MG132 for 24h under control KD or Hul5 KD and stained with antibody specific for K63 linked ubiquitin (green) and co-stained with pan-ubiquitin (Red). Hul5 KD cells display decreased K63 ubiquitination as well as significant impairment in aggresome formation.

It is highly possible that other siRNAs, which also demonstrate efficient knockdown of Hul5, do not produce a similar phenotype because of functional redundancy of other aggresome associated K63 ligases. In addition to Hul5, several E3 ligases have been identified on the proteasome that accumulate upon proteasome inhibition. It is possible that these ligases, which may have functional redundancy with Hul5, are indirectly targeted via the initial Hul5 siRNA. Therefore, one strategy would be to target several ligases at once using a siRNA pool, which may aid us in determining if both K63 ligase
and K63 deubiquitination activities are coordinated by the proteasome in response to proteotoxic stress. This discovery will unravel another crucial layer of communication that is essential in connecting the proteasome and autophagy pathway.

5.5 Molecular composition of Lewy Bodies

Ultimately, further research to test whether the proposed proteasome remodeling and aggresome processing model is relevant to actual Lewy Bodies identified in post-mortem PD and DLB patients will be of utmost importance. Thus, by analyzing the molecular composition of Lewy Bodies, we may be able to pinpoint the molecular lesion underlying their dysfunctional processing. Specifically, by utilizing our findings of aggresome processing as a biomarker guide to proper aggresome processing, it would be highly beneficial to determine the concentration of various proteasome subunits, as well as HDAC6 and K63 ubiquitin in Lewy Bodies. However, we must consider the possibility of a heterogeneous distribution of Lewy Bodies. Variations in Lewy Body composition may arise from distinct cellular stresses endured throughout the course of the disease, or inclusions formed due to propagation of pathogenic protein aggregates, which may vary in composition from the original stress-induced inclusions. Thus, to investigate the possibility of a heterogeneous distribution of Lewy Bodies, it will be best to obtain tissue samples from the brainstem as well as the cortex. Analysis of different brain regions may elucidate the state of inclusion body processing as a factor of time as well as the type of stress.
Due to our molecular understanding of aggresome processing, it may be possible to identify the underlying lesion leading to the prevalence of inclusions. For example, if Lewy Bodies are positive for all 26S proteasome subunits as well as K63 ubiquitin and HDAC6, the results would suggest that remodeling was not initiated. This could occur due to the engagement of substrates that may be partially digested the 20S proteolytic core, in which case the proteasome will remain locked in a 26S state. Additionally, oxidative stress can dissociate 26S proteasome into 19S and 20S components (Wang et al., 2010). PD patients often display a high level of reactive oxygen species (ROS) and oxidative damage (Abou-Sleiman et al., 2006), which may result from mitochondrial dysfunction; therefore, it is possible to have improper recruitment of proteasomes to the inclusion body due to their dissociated state. In principle, if the necessary subunits of the 26S proteasome are not available to initiate proper K63 deubiquitination activities, it may result in the retention of Lewy Bodies. Finally, 26S proteasome have been reported to deplete with increasing age (Tonoki et al., 2009). Therefore, age-dependent attenuation in 26S proteasomes may also result in the loss of crucial 19S subunits required for K63 chain production and inclusion body clearance. Overall, the important molecular events we have characterized in this study to initiate proper aggresome processing should serve as tools to probe the underlying lesion in aggregate-prone neurodegenerative disease and pave the way for effective pharmacological therapies.
References


Biography

Priyaanka Nanduri was born on April 2, 1987 in Chennai, India and immigrated to the United States when she was eight years of age. She attended the University of Denver in Denver, CO from 2005-2009 where she pursued her Bachelor of Science, magna cum laude, with a triple major in Molecular Biology, Biochemistry, and Psychology. Additionally, she received a concentration in Cognitive Neuroscience with distinction in Psychology. After her Bachelor’s, Priyaanka was employed for one year as a professional research assistant in the lab of the late Dr. George Eisenbarth at the Barbara Davis Center for Childhood Diabetes. She matriculated at Duke University in the Pharmacology Program in the fall semester of 2010 and earned a Doctor of Philosophy degree in 2015. Her graduate research was conducted in the laboratory of Dr. Tso-Pang Yao.

Priyaanka received an Honorable Mention for the NSF Graduate Research Fellowship Program in 2012 and won a poster presentation award at the Duke Pharmacology and Cancer Biology annual retreat the same year. Additionally, with the support of the Duke University Graduate School Travel Fellowship in 2013, she presented her work at the national NINDS satellite meeting for Mechanisms of Misfolded Protein Propagation in Neurodegenerative Diseases. Priyaanka is a member of the American Association for the Advancement of Science.

Priyaanka has published the following articles:


