A Potential Mechanism for Targeting Aggregates With Proteasomes and Disaggregases in Liquid Droplets

Emma Mee Hayes1,2, Liina Sirvio1,2 and Yu Ye1,2*

1 Division of Neuroscience, Department of Brain Sciences, Imperial College London, London, United Kingdom, 2 UK Dementia Research Institute at Imperial College London, London, United Kingdom

Insoluble protein deposits are hallmarks of neurodegenerative disorders and common forms of dementia. The aberrant aggregation of misfolded proteins involves a complex cascade of events that occur over time, from the cellular to the clinical phase of neurodegeneration. Declining neuronal health through increased cell stress and loss of protein homeostasis (proteostasis) functions correlate with the accumulation of aggregates. On the cellular level, increasing evidence supports that misfolded proteins may undergo liquid-liquid phase separation (LLPS), which is emerging as an important process to drive protein aggregation. Studying the reverse process of aggregate disassembly and degradation has only recently gained momentum, following reports of enzymes with distinct aggregate-disassembly activities. In this review, we will discuss how the ubiquitin-proteasome system and disaggregation machineries such as VCP/p97 and HSP70 system may disassemble and/or degrade protein aggregates. In addition to their canonically associated functions, these enzymes appear to share a common feature: reversibly assembling into liquid droplets in an LLPS-driven manner. We review the role of LLPS in enhancing the disassembly of aggregates through locally increasing the concentration of these enzymes and their co-proteins together within droplet structures. We propose that such activity may be achieved through the concerted actions of disaggregate machineries, the ubiquitin-proteasome system and their co-proteins, all of which are condensed within transient aggregate-associated droplets (TAADs), ultimately resulting in aggregate clearance. We further speculate that sustained engagement of these enzymatic activities within TAADs will be detrimental to normal cellular functions, where these activities are required. The possibility of facilitating endogenous disaggregation and degradation activities within TAADs potentially represents a novel target for therapeutic intervention to restore protein homeostasis at the early stages of neurodegeneration.

Keywords: liquid droplet, protein aggregation, ubiquitin proteasome pathway, disaggregate machinery, neurodegeneration, liquid-liquid phase separation, protein misfolding
INTRODUCTION

Protein aggregates are observed in a range of neurodegenerative disorders, including Alzheimer’s, Parkinson’s, Huntington’s disease and amyotrophic lateral sclerosis ALS (ALS). Alzheimer’s disease is the most common form of dementia and is characterized by the presence of amyloid-β (Aβ) plaques (Knopman et al., 2021) and tau-containing neurofibrillary tangles (Goedert and Spillantini, 2017). Parkinson’s disease is a movement disorder, hallmarked by insoluble neuronal protein deposits called Lewy bodies that mainly consist of aggregated α-synuclein (αS) (Poewe et al., 2017). Huntington’s disease is typically characterized by chorea and psychiatric symptoms, in which expansion of repeat sequences (poly-glutamine or poly-Q) in the huntingtin protein (HTT) drives its aggregation (Tabrizi et al., 2020). The aggregation of several other proteins, such as TDP-43 and C9orf72, has been associated with ALS, a rare progressive neuromuscular disorder (Balendra and Isaacs, 2018).

Interestingly, although these neurodegenerative disorders have distinct clinical presentations and are associated with specific proteins, the aggregation of misfolded proteins appears to be a common pathological mechanism (Soto and Pritzkow, 2018).

Compared with protein aggregation, the reverse process of aggregate disassembly by disaggregases and proteasomes has only recently gained attention. In this review, we will discuss the function of disaggregases and the ubiquitin (Ub)-proteasome system in restricting protein aggregation. We will examine how these components may assemble into liquid droplets, through a physical phenomenon called liquid phase condensation or liquid-liquid phase separation (LLPS), to potentially enhance their local activity. We propose that these enzymes and their co-proteins participate in the formation of putative “transient aggregate-associated droplets” (TAADs) which function as disaggregating/proteolytic centers to drive the disassembly of misfolded protein deposits. Enhancing the activity in such centers at the early stages of protein aggregation may represent a novel route to impede the progression of neurodegenerative disorders.

PROTEIN AGGREGATION

Misfolded proteins lack defined tertiary structures and tend to accumulate together, leading to protein aggregation. For example, both tau and αS are inherently disordered and lack defined tertiary structural features upon dissociation from their physiological binding partners (Vasili et al., 2019). Similarly, the expansion of repeat sequences, such as poly-Q in the HTT and poly-GA in the C9orf72 gene, introduces unfolded protein sequences that form a critical feature in enabling their respective aggregation (Nonaka et al., 2018; Bonfanti et al., 2019). Other proteins, such as TDP-43 assemble into aggregates only upon specifically altered cellular conditions leading to protein misfolding, including LLPS-driven mechanisms (Watanabe et al., 2020).

The aggregation process has been extensively studied for a range of misfolded proteins (Figure 1). Usually, misfolded protein monomers are thought to interact with each other and form globular oligomers that remain soluble (Breydo and Uversky, 2015). Oligomers can further aggregate and through structural rearrangements assemble into stable and highly organized filamentous aggregates (fibrils) or form amorphous aggregates that have no distinct higher-order structure (Figure 1A). Critically, these misfolded proteins and oligomers may act as templates or “seeds” for further aggregation to occur inside the cell (Goedert and Spillantini, 2017; Vasili et al., 2019). These aggregate seeds can also pass on to naive cells, thus contributing to the spreading of pathology through the brain (Pecho-Vrieseling et al., 2014; Rauch et al., 2018, 2020).

Studies in cellular and mouse models of pathology suggest that, compared with stable aggregates and fibrils, oligomers may be the more toxic aggregate species (Wegmann et al., 2016; De et al., 2019b), which easily penetrate lipid bilayers and cause cell stress (De et al., 2019a; Klenerman et al., 2021; Lobanova et al., 2021). The formation of aggregated fibrils, such as in neurofibrillary tangles and Lewy bodies, is reasoned likewise to be a potentially protective cellular mechanism to sequester the toxic oligomers (Kopeikina et al., 2012; Li and Haney, 2020).

In many cases, the aggregation of misfolded proteins is associated with post-translational modifications including phosphorylation and ubiquitination (Figure 1B). The role of phosphorylation, ubiquitination and many other post-translational modifications on tau and αS, such as sumoylation, nitration, O-GlcNacylation, acetylation and methylation, have been reviewed extensively for their roles in regulating the function of misfolded proteins through altering their physiochemical properties, turnover, structural conformation or interaction with their binding partners (reviewed in e.g., Zhang et al., 2019; Alquezar et al., 2020). Alterations in the phosphorylation status of misfolded proteins is believed to contribute to aggregate pathology. For example, hyperphosphorylation can cause tau to dissociate from microtubules, leading to microtubule destabilization and loss of neuronal cytoskeletal structure (Alonso et al., 2018). Additionally, tau phosphorylation at residues S202/T205 is considered a key pathological event of its aggregation (Simic et al., 2016). In Parkinson’s disease, αS is heavily phosphorylated at residue S129 (Fujiwara et al., 2002) and this modification is thought to accelerate aggregation of the protein (Karampatsou et al., 2017). Recent high-resolution cryo-EM studies have further found a high level of both ubiquitination and phosphorylation events on fibrils derived from post-mortem tauopathy and synucleinopathy donor samples (Arakhamia et al., 2020; Schweighauser et al., 2020), suggesting that both types of modifications are associated with aggregates.

A second aspect that may influence protein aggregation is LLPS, which describes a physical process whereby a macromolecular solution assumes a dense phase that resembles liquid droplets coexisting within a more dilute phase (Alberti et al., 2019). Demixing of intracellular macromolecules and assembly of liquid droplets may be influenced by the concentration and nature of the macromolecules and of the solution, all of which are factors that may determine whether phase separation occurs (principles of LLPS reviewed in
Hyman et al., 2014). The phase state of droplets ranges from the “liquid-like” phase that readily fuse, coalesce and disassemble, to denser “gel-like” phases that are limited in exchange with the dilute phase but remain reversible. This phase can further progress into solid-like phases of hydrogels containing amyloid-like fibrils (review of phases in Peran and Mittag, 2020 and LLPS-relevant investigation techniques in Alberti et al., 2019). These hydrogels can be difficult to reverse without changes to the surrounding environment or interventions from other substrates. Droplets that do not readily exchange with the dilute phase or that are irreversible have been associated with a range of diseases (reviewed in Shin and Brangwynne, 2017; Alberti and Dormann, 2019). LLPS allows for the formation of membrane-less compartments (such as the nucleolus or centrosome) and enables cells to concentrate biomolecules at regions of interest, thus bringing relevant proteins in contact in a fluid and dynamic way (Hyman et al., 2014; Alberti and Hymannd, 2021). This process can also reduce variability and noise in protein concentration in cells (Klosin et al., 2020) and influence protein functions—for example, being in a more liquid phase would allow for increased molecular interactions while being in a more solid-like phase, although difficult to reverse, would sequester proteins from the cytosol (Hernandez-Vega et al., 2017; Olzmann and Carvalho, 2019). Emerging roles of...
LLPS in RNA processing and translation and neuroinflammation suggest that this physical process has important regulatory functions in biology (reviewed in Wolozin and Ivanov, 2019; Zbinden et al., 2020; Pakravan et al., 2021; Wang et al., 2021).

The formation of tau droplets is favored by hyperphosphorylation and pathological mutations such as P301S, P301L and A152T, which increase the likelihood of liquid-to-gel-like transition (Wegmann et al., 2018). While liquid droplets formed from tau proteins could sometimes be a physiological process to assist with microtubule polymerization in neurons (Hernandez-Vega et al., 2017), the increased density of tau also facilitates its aberrant aggregation (Ambadipudi et al., 2017). For HTT, it was shown that its aggregation-prone exon 1 fragment forms liquid droplets that became progressively resistant to 1,6-hexanediol, a compound that disrupts LLPS over time, indicative of the transition to a gel-like aggregate resistant to 1,6-hexanediol, a compound that disrupts LLPS (Wegmann et al., 2018). For HTT, it was shown that its aggregation-prone exon 1 fragment forms liquid droplets that became progressively resistant to 1,6-hexanediol, a compound that disrupts LLPS over time, indicative of the transition to a gel-like aggregate structure (Peskett et al., 2018). For αS-containing liquid droplets, a similar liquid-to-gel-like transition has been observed both in vitro and in vivo. The familial Parkinson's disease mutations A53T and E46K in αS and S129-phosphorylated αS accelerate this transition, suggesting that LLPS may be a critical step in aggregation (Ray et al., 2020). The important next step is to unveil the molecular features of LLPS-driven aggregation of misfolded proteins in a physiological context.

REVERSING PROTEIN AGGREGATION

Disaggregases, such as the metazoan VCP (also known as p97), and the 70 kDa heat shock proteins (HSP70) disaggregate machinery, are a group of enzymes that utilize ATP to reverse protein aggregation, refolding aggregates back into monomers in their native conformations (Desantis and Shorter, 2012). These enzymes may target aggregates alone or associate with additional co-proteins that assist with enzymatic functions. VCP/p97 belongs to the AAA+ ATPase superfamily (Puchades et al., 2020), which form characteristic hexameric ring complexes that couple ATP hydrolysis with mechanical work (Figure 2A). Other members of this superfamily include subunits Rpt1-6 of the 19S proteasome regulatory particle, prokaryotic ClpB and its yeast ortholog Hsp104 (Figure 2B), whose disaggregation activity was reported nearly three decades ago (Parsell et al., 1994). A recent report further reveals a metazoan AAA+ ATPase disaggregase, Skd3, a mitochondrial protein that is able to solubilize αS aggregates and is distantly related to ClpB (Cupo and Shorter, 2020). The ability to restrict and reverse protein aggregation appears to be an activity that has been conserved in evolution.

VCP/p97 and Disaggregation

VCP/p97 was found to associate with the HSP70 disaggregate machinery (discussed below) and engage with tau and HTT aggregates in post-mortem brain tissues (Hirabayashi et al., 2004; Darwich et al., 2020). VCP/p97 plays a pivotal role in endoplasmic reticulum (ER)-associated degradation by pulling misfolded proteins from ER membranes for proteasomal degradation (Wolf and Stolz, 2012). Apart from assisting with degradation of misfolded proteins, VCP/p97 also contributes to other cellular processes including chromatin remodeling, transcriptional regulation and the DNA damage response (Vaz et al., 2013).

Previous studies found a very high level of expression of VCP/p97 in mammalian cells, underscoring its importance in proteostasis functions (Peters et al., 1990; Zeiler et al., 2012). Compared with other members of the AAA+ ATPase superfamily, VCP/p97 lacks motifs within the catalytic domain that facilitate binding and guidance of substrates through the central pore (Hanzelmann and Schindelin, 2017; Saffert et al., 2017). Instead, VCP/p97 associates with co-factors Ufd1 and Npl4, which aid in the recognition, binding and transfer of ubiquitinated substrates to VCP/p97 (Blythe et al., 2017; Bodnar and Rapoport, 2017). Mutations on VCP have been linked to several forms of neurodegenerative disorders (Watts et al., 2004; Johnson et al., 2010; Meyer and Weihl, 2014; reviewed in Sun and Qiu, 2020) and endogenous VCP associates with protein inclusions in both Huntington's disease and Lewy body dementia (Hirabayashi et al., 2001).

The VCP/Ufd1/Npl4 machinery targets ubiquitinated substrates and is linked with proteasomal responses to aggregated proteins. For instance, knocking out components of the VCP/p97 machinery shows impeded degradation of ubiquitinated HTT (Higgins et al., 2020). Similarly, proteasome-mediated degradation of TDP-43 aggregates is impeded by the VCP inhibitor NSM-873 (van Well et al., 2019). A recent study further demonstrates that wild-type VCP/p97 is capable of disassembling tau aggregates derived from Alzheimer’s disease donors, but a frontotemporal dementia-linked pathological D395G mutation in VCP showed markedly reduced disaggregation activity (Darwich et al., 2020). This provides evidence for the direct involvement of VCP/p97 activity in a clinically observed neurodegenerative disorder. A strong association exists between the VCP machinery and the proteasome system to enable unfolding-degradation of ubiquitinated aggregates, presumably in a sequential manner. The highly specialized roles of VCP, Ufd1, Npl4 and the proteasome may provide better control and specificity for each of the successive steps in targeting the aggregates for removal.

70 kDa Heat Shock Proteins Family and Disaggregases

The 13-member HSP70-family of molecular chaperones participates in the folding of newly synthesized proteins, assists with the translocation of polypeptides into organelles, and the disassembly of stable complex structures. Many of the cellular homeostatic protein folding functions are thought to be carried out by the constitutively expressed HSP70-family member HSC70, while expression of the HSP70 protein is induced by stress and accumulation of misfolded proteins (Rosenzweig et al., 2019). HSP70 recognizes misfolded proteins by binding to hydrophobic protein stretches such as the tetra-repeat domain of tau (Kundel et al., 2018) or the N-terminal region of αS (Tao et al., 2021), inhibiting their aggregation propensity (Klucznik et al., 2004; Dedmon et al., 2005; Kundel et al., 2018; Tao et al., 2021). The binding of HSP70 to αS and HTT oligomers is also
FIGURE 2 | Molecular representations of enzyme complexes with protein unfolding/refolding activity. (A) Metazoan VCP/p97 (pdb-id 5C1A) (Hanzelmann and Schindelin, 2016), a homohexamer of the same superfamily and colored in different shades of blue, shown next to the six ATPases of the 19S proteasome subparticle (see Figure 3), a heterohexamer consisting of Rpt1-6 (pdb-id 5GJR) (Huang et al., 2016). (B) Prokaryotic ClpB (pdb-id 6QS4) (Deville et al., 2019) and its yeast ortholog Hsp104 (pdb-id 5KNE) (Yokom et al., 2016) are homohexamers of the AAA+ ATPase superfamily. For clarity, identical subunits within the same complex are colored with different shades of the same color scheme. The positions of substrate channeling pores are indicated with dotted lines. (C) A model of the HSP70 disaggregase machinery, where HSP70 work together with DNAJA, DNAJB1, and nucleotide exchange factor (NEF) to target aggregates of various sizes. Double-headed arrows represent reversible interactions between proteins.

shown to prevent further aggregation, though HSP70 alone is unable to disassemble Aβ fibrils (Muchowski et al., 2000; Evans et al., 2006).

Chaperones of the HSP70-family form a disaggregate system in metazoans by recruiting additional co-chaperones called J-proteins and a nucleotide exchange factor (NEF), such as Apg2 and HSP110 (Rosenzweig et al., 2019). This HSP70/J-protein/NEF combination is hereafter referred to as the HSP70 disaggregation machinery (Figure 2C). By engaging different classes of J-proteins, the HSP70 chaperone forms transient complexes that disassemble aggregates of a range of sizes with assistance from the NEF (Nillegoda et al., 2015). Similarly, the HSC70 chaperone could disassemble αS fibrils in vitro by associating with a J-protein (DNAJB1) and a NEF (Apg2), decreasing aggregate toxicity by ~10% (Gao et al., 2015). However, more recent data shows that disagggregation of brain-derived tau filaments via the HSC70/DNAJB1/Apg2 machinery produced oligomeric tau that was more seeding-competent in HEK293 cells (Nachman et al., 2020). These results indicate that whether toxicity increases or decreases following the disassembly activity may depend on how completely the disaggregation occurs.

C-Terminus of Hsc70-Interacting Protein and Aggregate Degradation

The HSP70 system interacts with C-terminus of Hsc70-interacting protein (CHIP), a Ub E3 ligase (see Figure 3A), and targets aggregates for proteasomal degradation. CHIP facilitates substrate ubiquitination through its C-terminal catalytic domain, while its N-terminal domain enables interactions with HSP70 (Zhang et al., 2020). Interactions with the disaggregation machinery appears critical for CHIP activity, as aggregate-bound chaperones are required for efficient ubiquitination (Qian et al., 2006; Tetzlaff et al., 2008; Kalia et al., 2011). Our recent in vitro study further suggests that the binding of misfolded proteins such as tau may directly enhance the activity of CHIP (Zuo
et al., 2020). Whether misfolded proteins and aggregates are refolded or degraded is thought to be dependent on the ratio of the disaggregation machinery to CHIP. HSP70 and other heat shock proteins are expressed at much higher levels than CHIP (Meacham et al., 2001), suggesting a potential cellular preference for refolding over degradation. However, upon elevated CHIP expression or prolonged substrate-HSP70 interactions, a larger fraction of substrates become ubiquitinated by CHIP, tagging them for degradation (Qian et al., 2006).

Many misfolded proteins and their aggregates are substrates of CHIP. It was shown that CHIP ubiquinates HSP70-bound αS and accelerates the degradation of αS aggregates, leading to reduced aggregate toxicity (Shin et al., 2005; Tetzlaff et al., 2008). CHIP-mediated tau ubiquitination and degradation could be augmented in HeLa cells and in a mouse model of tauopathy, upon induced endogenous HSP70 expression through inhibition of HSP90 (Petrucelli et al., 2004; Dickey et al., 2007). Toxicity induced by Aβ and HTT is reduced by CHIP ubiquitination and subsequent proteasomal degradation in mammalian cells and mouse disease models (Jana et al., 2005; Miller et al., 2005; Kumar et al., 2007). In a rodent Huntington’s disease model, the loss of CHIP accelerates pathological phenotypes such as HTT aggregation and neurodegeneration (Miller et al., 2005). It therefore appears that increasing CHIP activity would be beneficial for restricting protein aggregation in cells.

THE UB-PROTEASOME SYSTEM IN REVERSING PROTEIN AGGREGATION

The Proteasome System

Aberrant loss of proteasomal degradation activity is observed in Alzheimer’s, Parkinson’s and Huntington’s disease (Keller et al., 2000; Furukawa et al., 2002; McNaught et al., 2002; Seo et al., 2004; Diaz-Hernandez et al., 2006; Thibaudeau et al., 2018). Substrates destined for degradation are normally conjugated with multiple Ub moieties through a sophisticated enzymatic cascade involving E1, E2, and E3 enzymes (Figure 3A; Komander and Rape, 2012). The concerted actions of these enzymes ensure that virtually all biological processes involve protein ubiquitination events at some point (Swatet and Komander, 2016; van Wijk et al., 2019). The diversity in cellular outcomes is believed to be dictated by distinct forms of ubiquitination of the substrate (reviewed by e.g., Komander and Rape, 2012; Swatet and Komander, 2016; Oh et al., 2018; van Wijk et al., 2019). Both tau and αS have been observed with typical Ub modifications that include mono-, multiple- and polyubiquitination (Zhang et al., 2019; Alquezar et al., 2020). Enzymes of the UPS, along with Ub moieties and chaperones, are abundantly detected in insoluble aggregate structures, such as Lewy bodies and neurofibrillary tangles (Garcia-Sierra et al., 2012; Zhang et al., 2019; Arabhamia et al., 2020; Schweighauser et al., 2020), suggesting an important role for the Ub-proteasome system in protein aggregation. In fact, several other E3 enzymes of the Ub system have been implicated in neurodegeneration (reviewed in Zheng et al., 2016; Upadhyyay et al., 2017). Canonically, ubiquitinated proteins are recognized by the 26S proteasome holoenzyme (Collins and Goldberg, 2017; Marshall and Vierstra, 2019). This holoenzyme consists of a barrel-shaped 20S core particle capped on one or both ends by 19S regulatory particles (Figure 3B; Budenholzer et al., 2017; Bard et al., 2018). Unfolded proteins are processed by subunits with proteolytic activities that reside in the 20S core particle (Rennella et al., 2020; Coleman et al., 2021). The regulatory particle is responsible for substrate recognition, deubiquitination, and ATP-dependent substrate unfolding that enables subsequent translocation through the channel pore (Lu et al., 2017). The base of the regulatory particle, including the hexameric ATPase, is suggested to have some chaperone activity (Braun et al., 1999).

Early works found that aggregates impaired protein degradation via the UPS in HEK293 cells (Bence et al., 2001; Bennett et al., 2005). More recent studies have shown that oligomers in particular have a strong inhibitory effect on the activity of the proteasome by e.g., preventing substrate translocation and protein degradation (Kristiansen et al., 2007; Mykyu et al., 2016; Thibaudeau et al., 2018). In agreement, engineered proteasomes with a constitutively open channel pore that facilitates access to the core particle increases tau aggregate degradation and cell survival to toxic insults (Choi et al., 2016). We have recently demonstrated that the proteasome holoenzyme possesses a novel fibril-fragmenting activity, capable of breaking stable fibrils into smaller, more toxic fragments (Cliffe et al., 2019). In a follow-up study, we further show that a single Ub modification at the N-terminus of tau or αS, similar to ubiquitination events catalyzed by the E2 enzyme UBE2W, enables their oligomers to be potently degraded by the proteasome holoenzyme (Ye et al., 2020), while unmodified oligomers are resistant to degradation (Iliina et al., 2016). It may therefore be plausible that proteasomes first break fibrils into smaller entities and that additional enzymes are recruited to assist the removal of oligomers in a Ub-dependent manner (Figure 3C). Similar to observations in disaggregases, it is plausible that proteasome activity may aggravate or decrease toxicity depending on how completely aggregate degradation occurs.

Proteasome-Interacting Proteins in Mediating Aggregate Degradation

Several studies have indicated that the proteasome interacts with components of HSP70 and VCP disaggregate machineries (Guerrero et al., 2008; Besche et al., 2009). A study of proteasome-associated proteins isolated from the human brain suggests that proteasomes bind to both HSP70 and VCP (Tai et al., 2010). Possibly, the interaction between proteasomes and disaggregases facilitates degradation of terminally misfolded proteins and aggregates through the sequential action of disaggregation-degradation (Figure 3C). Similarly, co-proteins known to interact with the proteasome, such as UBQLN2 and UBE3C (discussed below), may also assist aggregate removal in a concerted manner.

UBQLN2 is a proteasome shuttling factor, which contains an N-terminal domain that interacts with the proteasome, and a C-terminal domain to which ubiquitinated and misfolded
FIGURE 3 | The ubiquitin-proteasome system. (A) Protein ubiquitination is initiated by an E1 activating enzyme, which charges Ub to its active site cysteine residue in an ATP-dependent manner. This activated Ub is then transferred to the active site cysteine of an E2 conjugating enzyme, which with support from an E3 ligating enzyme, results in protein ubiquitination. Ubiquitinated proteins are recognized by proteasomes, leading to the unfolding and degradation of the substrate. (B) Schematic representation of the 26S proteasome holoenzyme. This enzyme complex consists of a 20S core particle (CP), capped by 19S regulatory particles (RP). Both the CP and RP may exist as free particles or assembled into a holoenzyme. Rpt1-6 of the RP are colored in shades of green as in Figure 2A. A number of proteins interact reversibly to assist with various proteasomal functions as required by the host cell. (C) In addition to protein degradation, the proteasome holoenzyme is also able to fragment stable fibrils into smaller fragments (< 500 nm) such as oligomers (Cliffe et al., 2019); as well as capable of degrading oligomers that are ubiquitinated at their N-termini by e.g., UBE2W (Ye et al., 2020).

proteins may bind (Finley, 2009). The interaction between proteasome and UBQLN2 appears critical for neurons, as deletion of its N-terminal domain increases UBQLN2 punctate formation and neuronal toxicity (Sharkey et al., 2018). Mutations in UBQLN2 are implicated in familial ALS and have been found to decrease proteasome-mediated aggregate degradation (Deng et al., 2011; Chang and Monteiro, 2015; Hjerpe et al., 2016). Interestingly, UBQLN2 appears to preferentially associate with larger protein aggregates over monomers (Zhang et al., 2021). This seems consistent with the observation that deletion of dsk2, the yeast homolog of UBQLN2, has no effect on early HTT aggregation but was required once intracellular protein deposits of aggregated proteins known as inclusion bodies have formed (Chuang et al., 2016).

Interactions between protein aggregates and HSP70 are facilitated by UBQLN2. Previous studies show that UBQLN2 is activated by HTT or poly-GA aggregates binding to HSP70 (Hjerpe et al., 2016; Zhang et al., 2021). This interaction is abolished in the presence of ALS/frontotemporal dementia-linked mutations in UBQLN2 or knock-down of CHIP (Zhang et al., 2021). Plausibly, aggregates are targeted by CHIP-HSP70-UBQLN2 in an ordered manner to ensure their efficient degradation at the proteasome. As HSP70 can coat protein aggregates (Whiten et al., 2018; Nachman et al., 2020), recruiting UBQLN2 could provide a mechanism for HSP70 to deal with larger ubiquitinated and disassembly-resistant aggregates.

UBE3C is a Ub E3 ligase that is recruited to the proteasome when protein misfolding is induced (Gottlieb et al., 2019). Hul5,
its yeast ortholog, targets misfolded proteins following heat shock ubiquitination response and removes low-solubility proteins and aggregates during stress (Fang et al., 2011). Overexpressing Hul5 partially rescues the accumulation of protein aggregates when the protein quality control network is compromised (Eisele et al., 2021), suggesting an important role for Hul5’s ubiquitination activity at the proteasome in impeding protein aggregation. The proteasome quality control function of Hul5 is recapitulated in human UBE3C, which is recruited to aggresomes via HSP70 interaction and promotes degradation of its substrates (Mishra et al., 2009). A number of mutations on UBE3C that either render the ligase inactive or lead to reduced enzymatic activity are linked to autism spectrum disorder (Ambrozkiewicz et al., 2020; Singh and Sivaraman, 2020). The activity of UBE3C assists proteasomal degradation by enhancing the complete removal of partially degraded proteins (Chu et al., 2013) and prevents ubiquitinated substrates from binding stalled proteasomes (Besche et al., 2014).

Perhaps with some redundancy, UBE3A and UBE3B are also members of the UBE3 ligase family and interact reversibly with the proteasome to assist its function. UBE3A mutations affecting proteasomal binding or activity have been linked to Angelman syndrome (Yi et al., 2017; Kuhnle et al., 2018). In neurons, UBE3A is found at axon terminals and dendritic spines (Burette et al., 2018), in agreement with its role in assisting proteasome functions in regulating neural processes (Hsu et al., 2015; Kim et al., 2016). Similarly, UBE3B is found to regulate the branching of dendrites and metabolic processes, and knock-down of UBE3B renders cells sensitive to oxidative stress (Svilar et al., 2012). It is plausible that evolution has created partial redundancy and partial specificity in UBE3 ligases to enable distinct outcomes for ubiquitination at the proteasome during stress.

**Cross-Talk Between Proteasomal and Autophagosomal Degradation**

A second route for degradation of eukaryotic proteins is through the autophagy-lysosome pathway, which shares links to the UPS (Dikic, 2017; Varshavsky, 2017). The flux of this pathway is stimulated upon induction of specific cellular stress conditions such as starvation or protein aggregation (Chen et al., 2019). Substrates targeted by the autophagy-lysosome pathway are enclosed in vesicles known as autophagosomes and are degraded following their fusion with lysosomes (Runwal et al., 2019). Selective degradation of protein aggregates via autophagy,
known as aggrephagy, plays a critical role in restricting their accumulation in cells (Lamark and Johansen, 2012). Like proteasome activity, there is evidence of autophagic dysfunction in neurodegenerative disorders (Menzies et al., 2017). There is significant cross-talk between these two systems (reviewed by e.g., Korolchuk et al., 2010; Bustamante et al., 2018)—for example in SH-SY5Y neuroblastoma cells, proteasome inhibition via MG132 activates the transcription factor EB, a master regulator of autophagy that increases the expression of genes involved in autophagy activation and increases autophagosome biogenesis (Li et al., 2019). In HEK293 cells, loss of HSP70 or its cofactor HSP110 is found to induce autophagy and reduce proteasome activity (Felecciano et al., 2019).

The scaffold protein p62 is known to mediate cross-talk between the UPS and the autophagy-lysosome pathway (Bustamante et al., 2018). p62 serves as a signaling hub for diverse cellular processes and functions as an autophagy receptor to enable degradation of ubiquitinated proteins (Katsuragi et al., 2015). The UBA domain of p62 interacts with LC3, a key protein involved in autophagosome formation and thereby bridging between substrate ubiquitination and autophagosomal degradation (Pankiv et al., 2007). An early study in HeLa cells demonstrates p62 co-localizes with ubiquitinated protein aggregates and along with LC3 forms a shell surrounding HTT protein aggregates (Bjorkoy et al., 2005). Expectedly, loss of p62 increases HTT aggregation-mediated cell death. More recently, it is found that the loss of two Ub receptors of the proteasome led to the accumulation of ubiquitinated proteins and activates autophagy via p62/LC3 mechanisms (Demishtein et al., 2017). This suggests existence of a balance between proteasomal and autophagosomal degradation activities, which is mediated by p62.

THE ROLE OF LIQUID-LIQUID PHASE SEPARATION IN PROTEIN AGGREGATION

The Proteasome System and Disaggregases Are Involved in Liquid Droplets

Proteasomes are highly abundant in mammalian cells and are distributed throughout the cytosol and the nucleus (e.g., Brooks et al., 2000; Pack et al., 2014; Zhang et al., 2018). Previous studies have shown that starvation and nutrient depletion in yeast and plant cells induce proteasomes to assemble into storage granules (Gu et al., 2017; Marshall and Vierstra, 2018). In mammalian cells, osmotic shock induces the accumulation of proteasomes into foci structures (Lafarga et al., 2002). Our data show that such cellular bodies are assembled promptly upon stress and do not appear to be dependent on cytoskeletal transport (Zhang et al., 2018). Recently, Yasuda et al. (2020) demonstrated that these stress-induced proteasome foci are assembled through LLPS and contain denser degradation activity than the surrounding cellular environment. These foci also stain positive for VCP/p97, UBE3A and Ub-modified substrates, and are together suggested to form individual proteolytic centers in the cell (Yasuda et al., 2020). Inhibition of VCP/p97 increases foci size, whereas inhibition of ubiquitination activity prevents foci formation, and knock-out of UBE3A reduces foci number. VCP/p97 and proteasomes have also been reported to co-localize in stress granules induced by oxidative stress (Turakhia et al., 2018).

Molecular chaperones and components of the disaggregate machinery also appear to assemble into or associate with cellular bodies that act under LLPS principles. UBQLN2 co-localizes with stress granules and undergoes LLPS under in vitro conditions as well as in HeLa cells, where its HSP70 interaction domain is critical for liquid droplet formation (Hjerpe et al., 2016). The VCP machinery contributes to clearing stress granules, with mutations on VCP/p97 delaying the disappearance of stress granules and stress recovery (Buchan et al., 2013). In yeast cells, Hsp70 and its co-chaperone Hsp104 co-localize with stress granules and are essential for post-stress recovery (Cherkasov et al., 2013). In HeLa cells, accumulated TDP-43 was found to be held in cytoprotective nuclear bodies by HSP70 (Gu et al., 2021). Similarly, HSP70 associates with and maintains ribonuclear granules in a liquid-like state (Ganassi et al., 2016; Mateju et al., 2017). In an elegant study by Frottin et al. (2019), misfolded proteins were found to enter LLPS-dependent granules to prevent irreversible aggregation. HSP70 was required to recover proteins from the stress-induced granules to allow their refolding or degradation.

A recent study showed that proteasome particles, Ub E1, E2, E3 enzymes and deubiquitinases are found in liquid droplets in the nucleus, as was p62, the essential component of these droplets (Fu et al., 2021). These LLPS features were dependent on the interaction between p62 and polyubiquitin chains as well as p62 self-interaction (Sun et al., 2018; Zaffagnini et al., 2018). The binding of ubiquitinated substrates appears to heavily influence the ability of p62 to promote protein phase separation (Zaffagnini et al., 2018). Upon proteolytic stress in HeLa cells, HSP70, CHIP and HSP90, another heat shock protein, is recruited to the p62-essential nuclear foci enriched in proteasome particles to enhance the degradation of ubiquitinated substrates (Fu et al., 2021). These foci are suggested to be catalytic centers for degradation of misfolded proteins resulting from heat and oxidative stress. Together, the multiple protein interactions involving ubiquitinated substrates, HSP70, proteasomes and their co-proteins may provide a rationale for how liquid droplets coordinate to maintain proteostasis in response to aggregation of misfolded proteins.

Could Liquid-Liquid Phase Separation Be a Novel Mechanism to Concentrate Disaggregation and Degradation Activities?

Given that components of the disaggregate machineries, proteasomes and their co-proteins have all been found in liquid droplets, it is tempting to speculate that a “transient aggregate-associated droplet” (TAAD) is assembled in response to accumulating misfolded proteins (Figure 4). These TAADs may be initiated through the presence of misfolded protein condensates, to which chaperones, proteasomes or their co-proteins would nucleate. Additional components and
interacting partners of disaggregase and proteasome systems would also be recruited. The existence of such droplets would facilitate the concerted actions of ubiquitination, disaggregation, refolding/unfolding and degradation to take place locally, ensuring a high density of disassembly- and proteolytic activity to occur without compromising homeostatic cellular processes outside the TAAD. An elegant cryo-electron tomography study in nematodes reports that poly-GA aggregates recruit proteasomes to form foci structures with aggregates (Guo et al., 2018). Since these aggregates do not affect 26S proteasome expression, it follows that proteasomes are depleted from other cellular regions—indeed proteasome concentration is ~30 fold higher within poly-GA aggregates than the cellular surroundings. Others and we have further reported the super-resolution imaging of proteasome foci in live cells (Gu et al., 2017; Zhang et al., 2018), describing a high local density of these molecular degradation machines under stress. Similar recruitment of proteasomes to aggresomes (Hao et al., 2013) and granules (Frottin et al., 2019) has been observed, further underscored by our in vitro experiments that demonstrated direct proteasomal actions on both large fibrils and small Ub-modified oligomers (Cliffe et al., 2019; Ye et al., 2020).

The TAADs may only assemble briefly as a result of stress-related emergencies, since the prolonged engagement of disaggregases and proteasomes would neglect their canonical roles in proteostasis. As mentioned in the second section, aggresomes and other large, end-stage aggregates and fibrils may be neuroprotective and less toxic than oligomers. However, it is possible that fibrils could indirectly affect disaggregases and the proteasome system by depleting their enzymatic activity from elsewhere in the cell, so that they cannot fulfill their normal function in proteostasis. It may further be possible that a phase change to a more gel-like state could trap these enzymes in the TAADs, which would further aggravate protein aggregation. Current methods using fluorescence recovery after photobleaching (FRAP) to determine how rapidly proteins found within the droplet exchange with those outside, combined with determining droplet fluidity by measuring how stable they are in the presence of 1,6-hexanediol treatment, will enable examination of the phase of TAADs.

CONCLUDING REMARKS

We have discussed the ability of the disaggregase and the proteasome systems to assemble into liquid droplets, proposing the concept of TAADs to allow these systems to act in a concerted manner to remove aggregates. These putative droplets may provide an “emergency solution” to cell stress, temporarily redistributing enzymes needed for proteostasis in the cell. Aggregation-prone proteins and disaggregases appear to act in an LLPS-driven manner, enabling cells to enhance the disassembly of aggregates within droplet structures. A critical next step is to quantitatively examine the early stages of neurodegeneration for changes in droplet size, fluidity, composition and the local disaggregate/degradation activity.

Recent technical developments such as “optoDroplet,” takes advantage of optogenetics to precisely control and study phase transitions within cells (Shin et al., 2017). The construct in optoDroplet consists of an intrinsically disordered protein region fused to a light-sensitive protein that self-interacts upon light exposure, thus enabling light intensity to tune droplet formation in cells. Potentially, a protein of interest acting under LLPS principles may be fused to the optoDroplet construct and brought into droplet formation in a photo-controllable manner. This technique adequately complements the canonical methods for assessing LLPS in cells such as 1,6-hexanediol (McSwiggen et al., 2019), FRAP (Alberti et al., 2019) or alteration of buffer conditions (Wei et al., 2017; Schuster et al., 2018). Furthermore, combining optoDroplet with CRISPR technology (Nihongaki et al., 2017) would allow researchers to perform genome-wide screens to further identify proteins that act under LLPS principles. Currently, it is difficult to determine whether disaggregate and proteasome droplets are beneficial for aggregate removal or detrimental to overall cell proteostasis. However, by utilizing both established and novel techniques for investigating LLPS and its contribution to protein aggregation, therapeutic interventions may be developed to restore protein homeostasis at the early stages of neurodegeneration.

AUTHOR CONTRIBUTIONS

EMH and LS performed the literature review. YY conceptualized the manuscript and supervised the project. EMH and YY wrote the original draft. All authors contributed manuscript writing and editing.

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