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Protein disaggregation machineries in the human cytosol

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Abstract

Proteins carry out the vast majority of functions in cells, but can only do so when properly folded.

Following stress or mutation, proteins can lose their proper fold, resulting in misfolding, inactivity, and aggregation—posing a threat to cellular health. In order to counteract protein aggregation, cells have evolved a remarkable subset of molecular chaperones, called protein disaggregases, which collaboratively possess the ability to forcibly untangle protein aggregates. Here, we review the different chaperone disaggregation machineries present in the human cytosol and their mechanisms of action. Understanding, how these disaggregases function, is both universally and clinically important, as protein aggregation has been linked to multiple, debilitating neurodegenerative diseases.

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Protein disaggregation, molecular chaperones, amyloid fibers, protein refolding, Hsp70, J-domain proteins (JDPs), Hsp110.

Introduction

The process of folding is a seminal event in the life of a protein, converting a newly synthesized polypeptide into the functional three-dimensional conformation thermodynamically encoded by its amino acid sequence. Incorrect folding or misfolding, can not only lead to the loss of this function but also to the accumulation of protein aggregates—insoluble deposits of polypeptides. Aggregates are a hallmark of stressed, aged, and disease-state cells and pose a danger not only after they have accumulated but also during the process of their formation. Cell health therefore heavily depends on the ability to prevent protein aggregation.

It is unsurprising, then, that a number of distinct biological processes have evolved to protect cells from the deleterious effects of aggregates. These involve aiding proper folding, sequestering misfolded and aggregation-prone species, and even the dissolution and clearance of already-formed protein aggregates, carried out by a specialized group of proteins termed molecular chaperones [1,2]. Here we will focus on recent advancements in our mechanistic understanding of protein disaggregases—the chaperone machines that can reverse aggregation, restoring proteins to their native structure, function, and localization.

Human disaggregation system

Bacteria, protists, fungi, and plants possess a robust bi-chaperone disaggregation system consisting of ring-shaped ClpB/Hsp100 AAA+ ATPases and the Hsp70 chaperone system [3]. Working together, these chaperones efficiently dissolve protein aggregates by actively unfolding and threading individual aggregate-trapped polypeptides through the central channel of the Hsp100 AAA+ ATPases [4]. Interestingly, though, metazoans lack any Hsp100 orthologs [5], sparking a decades-long search for disaggregase activity in the human cytoplasm.

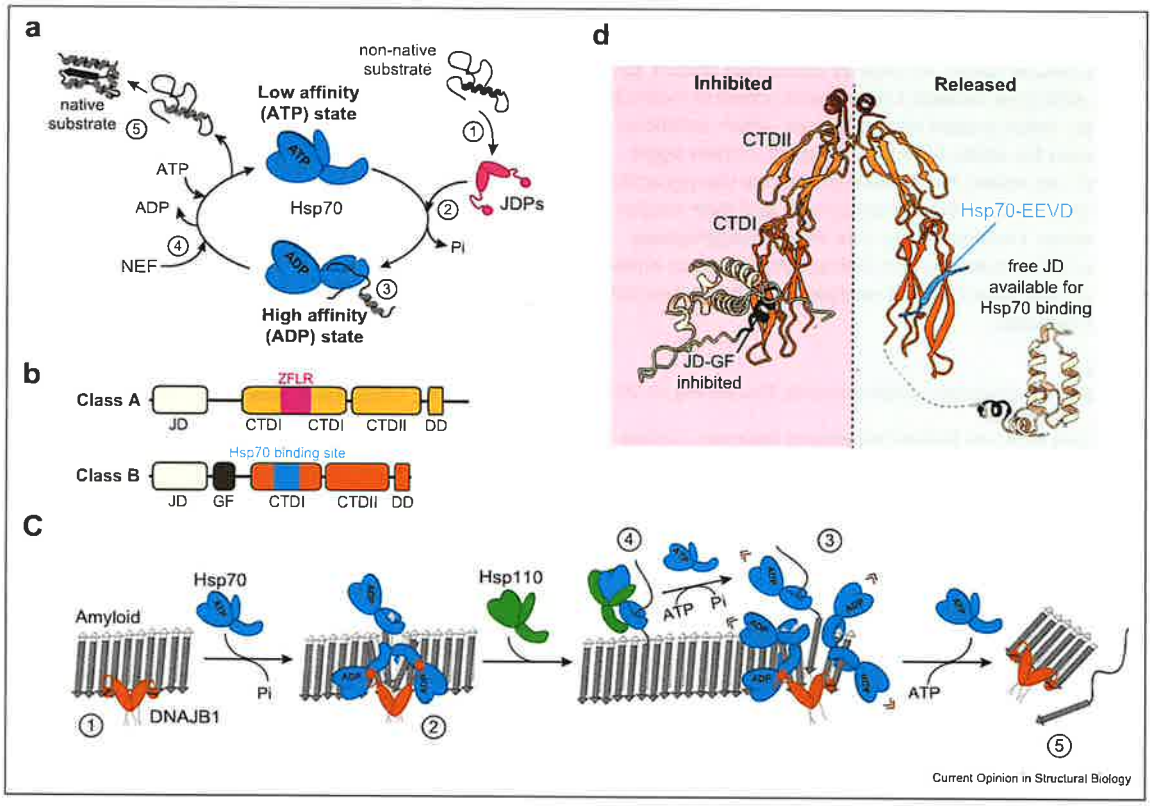
Such human disaggregation activity was ultimately identified and attributed to the Hsp70 chaperone, working in collaboration with a specific subset of class B J-domain proteins (JDPs or Hsp40s), as well as a Hsp110 nucleotide-exchange factor (NEF) [5,6]. This tri-chaperone system was observed, at least *in vitro*, to be efficient in the disaggregation of ordered amyloid fibers such as those associated with Parkinson's (α-synuclein) [7,8], Huntington's (HTT) [9], and recombinant and patient-derived amyloid fibers of the Alzheimer's associated tau protein [10,11]. However, compared to the bacterial disaggregation system, these human

disaggregases had reduced activity in the disaggregation of amorphous cytoplasmic and nuclear aggregates [6,12].

Disaggregation of amyloid fibers

A mechanistic understanding of how the Hsp70 chaperone system disassembles stable amyloid structures came from a seminal study by Wentink et al. [8], which revealed how the adenosine triphosphate (ATP)-dependent conformational cycle of Hsp70 chaperones was uniquely adapted for protein disaggregation. In the canonical cycle (Figure 1a), Hsp70 chaperones remodel their client proteins through regulated cycling between an "open" ATP-bound conformation and a "closed" adenosine diphosphate (ADP)-bound state, where substrates are effectively trapped by Hsp70. Iterative binding cycles result in the partial unfolding of the substrate, allowing spontaneous refolding upon release. The activity of Hsp70s is allosterically regulated by two auxiliary factors: the JDP co-chaperones and nucleotide exchange factors (NEFs). JDPs trigger Hsp70 ATP

Figure 1



The Hsp70 chaperone system. (a) The canonical chaperone cycle of Hsp70s. (1) J-domain proteins (JDP) cochaperones (~50 family members; pink) recruit client proteins and mediate their delivery to Hsp70-ATP. (2) Combined, the JDP-Hsp70 and client-Hsp70 interactions synergistically trigger Hsp70's ATP hydrolysis and transition to the high-affinity ADP-bound state. (3) Client remodeling. (4) Nucleotide-exchange factors (NEFs) induce ADP dissociation and rebinding of ATP, converting Hsp70 into the low-affinity state and consequently leading to substrate release. (5) The released substrate can then fold into its native state or rebind to Hsp70. **(b)** Domain organization of class A and class B JDPs. Class A JDPs are comprised of the conserved J-domain, which is essential for Hsp70-binding (light yellow); two client binding domains, client-binding domain (CTD)I and CTDII (yellow), with a zinc finger-like region embedded in CTDI (pink); and a dimerization domain (DD, yellow). Class B JDPs are structurally similar to class A but lack the zinc-finger-like region (ZFLR). The class B CTDI instead contains a binding site for the Hsp70 EEVD tail. In addition, class B JDPs have long GF regions that contain the inhibitory helix 5. **(c)** Amyloid disaggregation by the DNAJB1, Hsp70, and Hsp110 chaperones. (1) DNAJB1 (orange) specifically recognizes the fiber form of α-synuclein (gray) through multivalent interactions with the fiber's flexible C-terminal tails. (2) Autoinhibition of DNAJB1 (black line) is released upon interaction with Hsp70, resulting in the local, catalytic recruitment of multiple Hsp70s (blue) to the amyloid surface. (3) Owing to its high molecular mass, Hsp110 (green) is sterically excluded from locally concentrated Hsp70 clusters and thus preferentially binds and dissociates Hsp70s that have not been clustered by DNAJB1. (4) This activity biases the growth of larger and denser Hsp70 clusters, conducive to generating entropic pulling forces that destabilize the amyloid fibril, resulting in fibril fragmentation and/or monomer extraction. **(d)** Structural model of the DNAJB1 regulatory mechanism. **(left)** The DNAJB1 protomer is shown in an autoinhibited state, where the J-domain (light orange) is docked onto CTDI (orange) and the inhibitory helix (black) is blocking the Hsp70 binding sites. **(right)** Binding of the Hsp70 C-terminal EEVD tail (blue) to the DNAJB1 CTDI causes the detachment of the J-domain from CTDI, thereby releasing the autoinhibition. The J-domain is then free to recruit and activate Hsp70 chaperones.

hydrolysis inducing tight substrate binding, while NEFs promote ADP dissociation, allowing ATP rebinding and resetting of Hsp70 to the "open" state, resulting in substrate release [2] (Figure 1a).

In addition to Hsp70 activation, JDPs also act as the substrate recognition and targeting factors of the Hsp70 system. The human cytoplasm contains approximately 50 different JDPs that have historically been divided into 3 classes (A, B, and C), based on shared architectural features (Figure 1b) and substrate preferences. This diversity of JDP chaperones drives both the functional versatility and substrate specificity of the Hsp70 system. Protein disaggregation is initiated by a specific subset of JDPs from class B (such as DNAJB1 or DNAJB4), which play a crucial role in protein disaggregation by first interacting with amyloid fibers with nanomolar affinity [8]. This is achieved through multivalent interactions with flexible sequences protruding from the core of the fibers (α-synuclein or Huntingtin Exon 1; Figure 1c step 1, light gray) [8,10,13]. Such a binding mode ensures high coverage of these chaperones on the fibers, estimated at one JDP unit every 5 nm [7]. Recent nuclear magnetic resonance (NMR) and crosslinking studies have mapped the amyloid binding site to the second client-binding domain (CTDII) within DNAJB1 [13–15], allowing each DNAJB1 dimer to bind multiple flexible sequences, increasing the chaperone's affinity for the fibers through an avidity effect.

The fiber-bound JDPs then recruit Hsp70 chaperones, and this simultaneous interaction with both the JDPs and the amyloids stimulates the ATP hydrolysis rates of the Hsp70 machinery, resulting in a conformational change in the Hsp70s and their tight binding to the amyloids [7,8,16]. The interaction of class B JDPs with the fiber is surprisingly long-lived, allowing JDPs to recruit multiple rounds of Hsp70s to the same site [8,16] (Figure 1c step 2). This reaction differs from the canonical Hsp70 cycle, where JDPs deliver clients to Hsp70 and then leave the ternary complex, presumably to allow the substrates to fold [2] (Figure 1a, step 2).

Binding of Hsp70s close to the amyloid core leads to a significant entropy loss due to excluded volume effects. The random motion of Hsp70s generates collisions with the fiber wall and adjacent Hsp70s, creating a net directional vector away from the aggregate. This then pulls at the bound polypeptide, gradually releasing it from the tangle while increasing the freedom of movement (and therefore entropy) of the Hsp70 chaperones [8,16–18] (Figure 1c, step 3). This favorable entropy change can generate pulling forces (up to 10–20 pN) necessary to release the Hsp70-bound peptide from the amyloid [8,16–18]. In order to generate the loss of entropy and subsequent force required for disaggregation, Hsp70 chaperones must be tightly clustered onto the fibers, achieved through the combined effort of the class B JDPs and the Hsp110 NEF.

The unique ability of the class B JDPs (DNAJB1 and DNAJB4) to cluster Hsp70 [8,14] can be attributed to the existence of two unique structural features. First, class B JDPs (DNAJB1 and DNAJB4) contain an autoinhibitory element positioned in their glycine-phenylalanine (GF) rich region (Figure 1d, black) that regulates Hsp70 binding and is key for efficient targeting of Hsp70s close to the surface of the α-synuclein amyloids [14]. Secondly, the JDPs possess two distinct Hsp70s binding sites: the canonical J-domain and the regulatory interaction of CTDI with the C-terminal EEVD tail of Hsp70, which releases the autoinhibition, thus allowing the simultaneous recruitment of multiple Hsp70s (Figure 1d). The combination of these two traits enables JDPs such as DNAJB1 to induce densely packed Hsp70 clusters at the aggregate surface, which in turn generate entropic-pulling forces strong enough to destabilize the amyloid fold [8,14,17,19]. Conversely, the lack of such features in other classes of JDPs may explain their inability to substitute for DNAJB1 and its counterparts in disaggregation [7,10,14].

The action of the class B JDPs is necessary, but not sufficient, to enable efficient disaggregation, and the function of Hsp110 is also required for this process. Among the human NEFs, the Hsp110 family is seemingly unique in its ability to support Hsp70-dependent protein disaggregation [6–8]. The canonical function of NEFs is to stimulate the release of ADP from Hsp70 and promote the rebinding of ATP, inducing the opening of the substrate-binding pocket and dissociating substrates from Hsp70 [2] (Figure 1a, step 4). Hsp110, however, performs an additional role vital to the disaggregation process. Hsp110 is a large (95 kDa) bulky protein and therefore cannot readily interact with the tightly clustered Hsp70s to induce their release from the fibers. It therefore selectively binds and releases only nontightly-packed Hsp70s, which do not contribute to the entropic pulling forces [8,18] (Figure 1c, step 4). This, then, recycles Hsp70 molecules, ensuring a steady supply of these chaperones to more crowded locations, greatly increasing the overall efficiency of the disaggregation reaction as a whole.

Interestingly, recent cryo-electron tomography and total internal reflection fluorescence microscopy found that such JDP and Hsp110-induced clustering may preferentially occur at the ends of the fibers [20]. Real-time *in vitro* disaggregation reactions of α-synuclein fibers using atomic force microscopy (AFM) detected rapid bursts of disaggregation occurring preferentially from the fiber ends, preceded by the initial thickening of these fiber segments, potentially corresponding to local chaperone binding [16,20,21]. These reactions

happen in a stepwise manner, with bursts of fibril disassembly occurring at a rate of ~4 monomers per second [20]. Such a mechanism is quite different from the consecutive disaggregation reaction reported for the Hsp100 disaggregation machinery, indicating that the human disaggregation machinery does not continuously extract monomers from one end of a fiber to the other but disaggregates larger fiber segments in a highly cooperative manner [21].

To summarize, while Hsp70 chaperones are the engines directly responsible for the generation of the entropic pulling forces and subsequent amyloid disaggregation, this is only made possible through the combination of the unique architecture of class B JDPs that drive the aggregate specificity [14] and the uniquely high molecular mass of the Hsp110 NEF [8], which together regulate this powerful disaggregation mechanism.

Mixed JDP based disaggregation of amorphous aggregates

While remarkably efficient at the disaggregation of amyloid fibers *in vitro*, the tri-chaperone disaggregase system finds a greater challenge in highly heterogeneous nonamyloid aggregates compared to the bacterial and yeast bi-chaperone systems [5,6,22]. To fully unlock its ability to dissolve stress-induced amorphous aggregates, human Hsp70 requires the functional cooperation of class B JDPs with class A JDPs [22–24] (Figure 1b). The synergy of the two JDP classes in disaggregation appears to stem from the ability to engage a broader range of protein aggregate structures through the combination of different substrate specificities of the individual JDPs [22–24]. The specific substrate preferences of the different JDPs remain poorly understood. On a functional level, class B JDPs are reported to preferably act on larger oligomeric and aggregate assemblies, while class A JDPs are primarily implicated in the resolution of smaller aggregates and individual misfolded proteins [22,23,25]. Together, they would efficiently recruit Hsp70 to a broad range of heterogeneous protein aggregates, assembling into higher-order chaperone complexes of JDPs, Hsp70, and Hsp110 that presumably combine features favorable for disaggregation, such as the clustering ability of class B JDPs, with the Hsp70 refolding and aggregation prevention activity promoted by class A JDPs.

In cells, the specific combination of the JDPs DNAJA1 and DNAJB1 was shown to be particularly powerful in clearing persistent aggregates that accumulate after heat stress [25]. DNAJA1/DNAJB1-based disaggregase assemblies specifically accumulated in response to protein misfolding stress, following increased expression levels of the JDPs as part of the heat shock response transcriptional program [25]. This interclass cooperation of JDPs was shown to be important to the organismal health and resilience against heat stress in *Caenorhabditis elegans* [24] and to counteract proteotoxicity in human cells [25].

Noteworthy is that Hsp70 disaggregases, based on interclass JDP cooperation, were found to assemble at late stages of stress recovery, when a significant fraction of aggregates have already been disassembled [25]. This points to the existence of an additional, highly efficient cellular disaggregase activity that complements the Hsp70 disaggregation system.

The VCP/p97 disaggregase

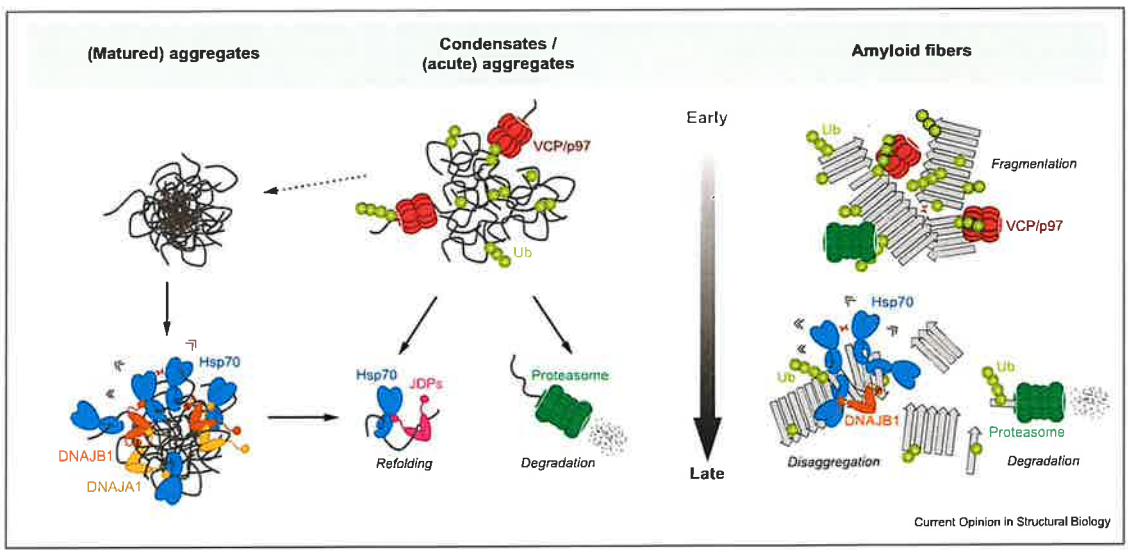
Recent work has put a spotlight on the AAA+ ATPase valosin-containing protein (VCP)/p97 as an alternative source of disaggregation activity in the human cytosol. Best known for its key role in retro-translocation of misfolded proteins across the Endoplasmic reticulum (ER) membrane, VCP/p97 uses the power of ATP hydrolysis to exert a mechanical pulling force that can drive protein transport, the disassembly of protein complexes, or indeed the solubilization of protein aggregates [26,27].

The VCP/p97 disaggregase clients characterized to date cover the full scope of cellular condensate and aggregate structures, ranging from stress granules [28,29] to stress-induced aggregates [30], and even amyloid fibers of tau [31,32] and Huntingtin Exon 1 [33]. The disaggregation activity of VCP/p97 reported is, however, limited to poly-ubiquitinated clients [34] that are selectively recognized by cofactor proteins such as the UFD1-NPLOC4 complex [35,36].

Structurally, VCP/p97 is a hexameric ring-like complex formed by two ATPase domains (D1 and D2) in each protomer, arranged around a central cavity through which substrates are threaded in an ATP-dependent manner [37–40], by a mechanism analogous to the ClpB/Hsp100 AAA+ ATPase of the bi-chaperone disaggregation system [41]. In addition, each of the six VCP/p97 subunits contains a regulatory N-terminal domain that acts as a docking site for cofactor proteins [37–39]. Rather than binding specific sequences in substrates, these cofactors primarily recognize the polyubiquitin tag of aggregated proteins, thus allowing the targeting and unfolding of a large range of cellular protein species [38].

Interestingly, VCP/p97 disaggregation activity further depends on functional cooperation with the proteasome, as evident by proteasomal inhibition blocking most disaggregase functions of VCP/p97 [31]. It is unclear whether the proteasome actively participates in the disaggregation process, as has been reported for tau amyloid fibrils *in vitro* [42], or primarily serves to rapidly degrade liberated polypeptides to prevent their reaggregation.

Figure 2



Sequential action of human disaggregases VCP/p97 acts upstream of Hsp70 to dissolve protein condensates and aggregates formed in response to acute proteotoxic stress (left) or to clear persistent, amyloid-type aggregates (right). **(left)** Polyubiquitinated aggregate substrates are recognized by VCP/p97 (red), which mechanically unfolds these clients by threading them through its central pore. Released polypeptides can then be either targeted to the proteasome (green) or refolded by downstream chaperones such as Hsp70 (blue) in cooperation with a class A J-domain proteins (JDP) (yellow). Aggregates that escape processing by valosin-containing protein (VCP)/p97 are targeted by the Hsp70 disaggregase in a second phase of the stress response. To effectively recognize and solubilize such amorphous aggregates, Hsp70 relies on the functional cooperation of both class A and B JDPs. In human cells, the cooperation of DNAJA1 and DNAJB1 dominates the late stress response by efficiently disaggregating residual aggregated proteins, which can then be either refolded or degraded. **(right)** Ubiquitinated deposits of amyloid fibers are prime targets for VCP/p97, which cooperates with the proteasome to initiate disaggregation. Fragmentation is a dominant process during the disaggregation of amyloid fibers by VCP/p97, creating smaller fiber species that can be recognized and processed by the tri-chaperone DNAJB1/Hsp70/Hsp110 disaggregase. Released polypeptides may either recover native function or be degraded by the proteasome.

Sequential action of human disaggregases

While the interplay between the two human disaggregase systems remains to be elucidated, early results indicate VCP/p97 disaggregation activity is dominant in the earlier stages of the cell's response to external stressors, breaking down aggregates for degradation or further processing by the Hsp70 disaggregase (Figure 2). The Hsp70 system, on the other hand, appears to be primarily responsible for the disaggregation of smaller aggregates and those not recognized by VCP/p97 [25,31].

The preprocessing of aggregates by VCP/p97 was shown to be important to the clearance of stable aggregates such as amyloid fibers of tau, from human cells [31]. VCP/p97 is essential to break down ubiquitinated fibers into smaller fragments or oligomers, but complete clearance of the resulting smaller aggregates is achieved only in the presence of active Hsp70. Both chaperone systems, acting in a sequential manner, are ultimately required to dissolve pre-existing amyloid aggregates [31]. In the case of heat-induced amorphous aggregates, however, the two disaggregase machines work in parallel, each targeting specific aggregate subpopulations. VCP/p97 targets ubiquitinated condensates and aggregates during the early stages of heat shock recovery, while Hsp70 solubilizes nonubiquitinated and more persistent aggregates [25]. The disaggregation of such aggregates by Hsp70 is facilitated by upstream-acting chaperones such as the small heat shock protein HSPB1, which maintains amorphous aggregates in a soluble state that is more amenable to efficient disaggregation [43].

The preference of VCP/p97 for polyubiquitinated substrates suggests a tight link between the disaggregation and cellular degradation pathways. Nevertheless, studies consistently demonstrate a bias towards the refolding of disaggregated substrates over their degradation, which circumvents the need to resynthesize depleted proteins and is thus most energetically efficient for the cell [12,44]. This, then, raises the question of what the downstream fate of substrates liberated by VCP/p97 is and how this fate (refolding vs degradation) is determined.

The sequence of action and different roles of the disaggregation systems are likely to be enforced by

multiple factors, including different substrate specificities, changes in the abundance of key players in response to stress or disease state, and other cellular demands on these key chaperone systems.

The double-edged nature of disaggregation

The ability to dissolve protein aggregates formed as the result of stress or mutation is key to the cellular quality control network and is essential for cellular and organismal health. Through disaggregation, chaperones reduce the burden on autophagy, prime substrates for proteasomal degradation, and allow the potential recovery of misfolded proteins while reducing the cytotoxicity associated with protein aggregates. Mutations in VCP/p97 or the Hsp70 machinery, in particular its JDP partner DNAJB4, are therefore linked to devastating diseases such as myopathies [45,46], amyotrophic lateral sclerosis (ALS) [47], and frontotemporal dementia (FTD) [48,49].

Nevertheless, an emerging body of evidence suggests that disaggregation may not always be beneficial. This is particularly evident for amyloid substrates. While disaggregation reduces this aberrant protein conformation and is thus seemingly protective, disaggregation by both Hsp70 and VCP/p97 has been shown to result in fragmentation of amyloid fibers, increasing the overall number of fibers that can then serve as templates for further protein aggregation [7,10,31,50,51].

Indeed, when isolated by centrifugation, the products of chaperone-mediated disaggregation in the form of fragments or oligomers have the ability to trigger protein aggregation and toxicity in naive cells [7,10,31]. In *C. elegans*, the knockdown of the sole Hsp110, disabling the disaggregation activity of the Hsp70 system, resulted in slower aggregation, less toxicity, and less cell-to-cell transmission of α-synuclein [52].

However, upregulation of the Hsp70 disaggregation machinery by overexpression of DNAJB1 or Hsp110 was shown to reduce amyloid load and the associated toxicity in cells and in a Parkinson's mouse model [9,53]. Taken together, these findings suggest that disaggregation activity is generally beneficial. However, under circumstances where disaggregation capacity is limited or not effectively coupled to refolding or degradation, chaperone-mediated fragmentation of amyloid-type aggregates can contribute to accelerating amyloid replication and the spreading of the disease pathology [50,54]. Such circumstances can occur when key proteostasis components are mutated or when protein quality control capacity declines as a consequence of aging [1].

Strategies aiming to exploit disaggregation activity for therapeutic purposes will thus need to reinstate a favorable balance without significant disruption to other protein quality control activities. Such therapeutics should aim for high substrate and functional specificity, as illustrated by recent studies where an artificial J-domain protein was developed to selectively recruit Hsp70 to dissolve protein condensates [55] or the substrate specificity and activity of yeast Hsp104 were increased to work alongside endogenous chaperones to potentiate amyloid disaggregation and reduce neurodegeneration in *C. elegans* [56]. It will therefore be important to further improve our understanding of the delicate balance between the protection offered by the disaggregases and their potentially harmful contribution to the spreading of amyloid-type aggregates, as well as their synergies with other branches of the protein quality control network within the cellular and organismal context.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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This study demonstrates the potential therapeutic benefits associated with increased substrate specificity of chaperone disaggregases.