The metazoan protein disaggregase and amyloid depolymerase system
Hsp110, Hsp70, Hsp40, and small heat shock proteins

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A baffling aspect of metazoan proteostasis is the lack of an Hsp104 ortholog that rapidly disaggregates and reactivates misfolded polypeptides trapped in stress induced disordered aggregates, preamyloid oligomers, or amyloid fibrils. By contrast, in bacteria, protozoa, chromista, fungi, and plants, Hsp104 orthologs are highly conserved and confer huge selective advantages in stress tolerance. Moreover, in fungi, the amyloid remodeling activity of Hsp104 has enabled deployment of prions for various beneficial modalities. Thus, a longstanding conundrum has remained unanswered: how do metazoan cells renature aggregated proteins or resolve amyloid fibrils without Hsp104? Here, we highlight recent advances that unveil the metazoan protein-disaggregase machinery, comprising Hsp110, Hsp70, and Hsp40, which synergize to dissolve disordered aggregates, but are unable to rapidly solubilize stable amyloid fibrils. However, Hsp110, Hsp70, and Hsp40 exploit the slow monomer exchange dynamics of amyloid, and can slowly depolymerize amyloid fibrils from their ends in a manner that is stimulated by small heat shock proteins. Upregulation of this system could have key therapeutic applications in various protein-misfolding disorders. Intriguingly, yeast Hsp104 can interface with metazoan Hsp110, Hsp70, and Hsp40 to rapidly eliminate disease associated amyloid. Thus, metazoan proteostasis is receptive to augmentation with exogenous disaggregases, which opens a number of therapeutic opportunities.

Introduction

Controlling the "quality" of proteins beyond translation is key in assuring correct cellular functioning and preserving organismal health.[1] For a nascent polypeptide chain, folding into the correct native structure and maintaining the correct native form is challenging within the crowded environment of a cell.[2-4] Thus, proteins occasionally assume defective, nonfunctional conformations. To circumvent this fundamental problem of cell biology, nature has devised several mechanisms to prevent or resolve protein misfolding in the cell. For instance, molecular chaperones block protein aggregation and actively help proteins reach their native conformations.[5] If a protein has been damaged beyond repair, it can be cleared by specialized degradation systems.[6-8] Alas, protein misfolding and aggregation can overcome these systems, especially upon environmental stress, which can even elicit aging and disease.[9] Indeed, protein misfolding underpins several devastating neurodegenerative diseases, including Alzheimer disease, Parkinson disease, Creutzfeldt-Jakob disease, and Huntington's disease.[10,11]

In the 1990s, Susan Lindquist and coworkers unequivocally defined a new branch in protein quality control: protein disaggregation coupled to protein reactivation.[12-14] Although it had been speculated that protein disaggregation and reactivation might occur,[15] it had never been convincingly demonstrated. Lindquist and colleagues discovered a new heat shock protein (Hsp) in *Saccharomyces cerevisiae*,

Hsp104, which was found to have a key role in allowing cells to survive severe stress after heat treatment (thermotolerance).[14] In subsequent investigations, Hsp104 was found to solubilize large protein aggregates resulting from severe heat stress and recover enzymatically active proteins from these aggregates.[12] Accordingly, yeast cells lacking Hsp104 were no longer able to rapidly solubilize and reactivate proteins from an aggregated state following thermal stress.[13,16]

Since then, we have learned much about the way Hsp104 functions. Hsp104 is a ring-shaped homohexamer with two AAA+ nucleotide-binding domains (NBDs) per subunit that couple ATP binding and hydrolysis to protein disaggregation.[17-22] Hsp104 is thought to drive protein disaggregation by threading substrates through its central channel to solution.[23-26] Hsp104 disaggregates a diverse array of structures, ranging from stable amyloid to less stable disordered aggregates.[12,13,27-33] Hsp104 hexamers adapt different mechanisms of intersubunit collaboration to disaggregate stress-induced aggregates vs. amyloid.[27,34] Hsp104 acts alone or in concert with other molecular chaperones to rescue aggregated polypeptides.[12,20,28,32,34-36] In particular, Hsp70, Hsp40, and small heat shock proteins (Hsp26 and Hsp42) can synergize with Hsp104 to promote the reactivation of protein aggregates.[35,37,38] Hsp104 is also essential for the formation and propagation of several yeast prions; protein-based genetic elements comprised of amyloid fibers that can confer advantageous self-perpetuating changes in protein structure and function.[32,39-46]

Hsp104 is highly conserved in eubacteria and eukaryotes. Inexplicably, however, Hsp104 has no exact homolog or ortholog in metazoa.[47] NBD2, but not other parts of Hsp104, appears to be partially conserved in the four ER-resident AAA+ proteins: torsin A, B, 2A, and 3A, as well as the mitochondrial AAA+ protein, SKD3.[48-50] This deficiency of Hsp104 in animals is puzzling, as a protein that reverses protein aggregation and restores protein function would be pivotal in combating aberrant protein aggregation.[17,47] The reason underlying the loss of Hsp104 is unknown, and is even more baffling because Hsp104 is

well tolerated and even neuroprotective in animal systems.[30,51-55] For example, Hsp104 rescues α-synuclein aggregation and dopaminergic neurodegeneration in a rat model of Parkinson disease.[30] Whether mammals boast an equivalent protein disaggregase has endured as a persistent unanswered question.

Hsp110, Hsp70, and Hsp40 as a novel protein disaggregase system

We have recently shed some light on this issue and have identified the mammalian disaggregase system via biochemical fractionation of mammalian cytosol and reconstitution with pure components.[56] The mammalian disaggregase system is comprised of an Hsp110 (Apg-2), an Hsp70 (Hsc70 or Hsp70), and an Hsp40 (Hdj1 or Hdj2).[56] The combination of these three proteins was found to establish an active disaggregase system in the mammalian cytosol prepared from rat liver or sHeLa cells.[56] Hsp110, Hsp70, and Hsp40 were able to refold proteins from large chemically or thermally denatured protein aggregates.[56] Using pure proteins, we established that Hsp70 and Hsp40 alone are not sufficient for robust disaggregase activity, but must be supplemented with Hsp110.[56] Hsp110 homologs are found in all eukaryotes and contribute toward thermotolerance in mammalian cells.[57,58] Hsp110 can serve as a nucleotide exchange factor (NEF) for Hsp70 but also displays chaperone activity.[59-64] We established that Hsp110-Hsp70-Hsp40 disaggregase activity was most effective against disordered, amorphous aggregates.[56] Indeed, Hsp110, Hsp70 and Hsp40 were unable to rapidly disaggregate Sup35 prions or amyloid forms of α-synuclein.[56] Disaggregase activity was conserved to the yeast homologs.[56] Thus, Sse1 (Hsp110), Ssa1 (Hsp70), and Sis1 or Ydj1 (Hsp40) could synergize to rescue proteins from large disordered aggregates.[56] This activity was slow in comparison to Hsp104- catalyzed protein disaggregation, which might help explain why minimal disaggregase activity is observed in yeast lacking Hsp104 immediately after heat shock.[13] In yeast, Sse1 contributes to prion propagation[65-67] and might also be involved in the dissolution

of 'Q-bodies' or 'stress foci': punctate cytoplasmic structures where misfolded proteins are collected prior to maturation into larger inclusions.[68-70] Using a series of Sse1 mutants,[61] we determined that Sse1 must engage both substrate and Hsp70, promote nucleotide exchange on Hsp70, and bind and hydrolyze ATP itself to promote disaggregation of disordered aggregates. Thus, simply providing Hsp70 with a NEF, such as Fes1 or Snl1ΔN, in place of Sse1 was insufficient to promote protein disaggregation.[56] Likewise, using a series of Ssa1 mutants, we determined that Hsp70 must engage substrate and Hsp110, and hydrolyze ATP for protein disaggregation.[56] Hsp40 must harbor a functional J domain to promote protein disaggregation, but the J domain alone is insufficient.[56] Optimal disaggregase activity was achieved when the Hsp40 could stimulate Hsp110 and Hsp70 ATPase activity.[56] Finally, while Hsp110, Hsp70 and Hsp40 were unable to rapidly resolve amyloid conformers directly, they enhanced disaggregation of Sup35 prions and α-synuclein amyloid fibrils by Hsp104.[56]

About a year later, a subsequent study confirmed the metazoan disaggregation activity exerted by Hsp110 (Apg-1, Apg-2, or Hsp105), Hsp70 (Hsc70 or Hsp70), and Hsp40 (Hdj1 or DNAJA2) in vitro.[71] Curiously, under the in vitro conditions employed the ATPase activity of Hsp110 was not required to promote protein disaggregation.[71] Hsp110 appeared to contribute primarily by acting as a nucleotide exchange factor (NEF) for Hsp70. Mild aggregation conditions were even established where Hsc70 and the alternative Hsp40, DNAJA2, could disaggregate substrates if provided with the Hsp70 NEFs Bag-1 or Snl1ΔN instead of Hsp110.[71] Under these circumstances, stimulation of Hsp70 nucleotide exchange was sufficient for disaggregation.[71]

Using *C. elegans* as a model system, knockdown of Hsp110 in briefly heat-shocked *C. elegans* resulted in persistent luciferase-YFP aggregates and a drastically reduced lifespan.[71] The persistence of protein aggregates and lifespan reduction could reflect a requirement for Hsp110 in the solubilization of protein aggregates in vivo.[71]

Alternatively, it might point to a role for Hsp110 in the inhibition of ongoing aggregation after the transient heat shock. Unfortunately, the experiments performed could not differentiate between these two possibilities, as the expression of luciferase-YFP was not shut down after the transient heat shock. Thus, it is unclear whether the persistence of luciferase-YFP aggregates reflects a failure to disaggregate pre-existing luciferase-YFP or whether newly synthesized luciferase-YFP continued to aggregate after the transient heat shock (perhaps due to seeding by preformed aggregates).

Originally, to convincingly establish the disaggregase activity of Hsp104 in vivo, it was necessary to stringently shut down protein synthesis using cycloheximide immediately after the heat shock.[13] In this way, one could be absolutely certain that any protein reactivation that occurred was due to recovery of previously aggregated protein and not due to the accumulation of newly synthesized material.[13] A similar strategy has been employed to demonstrate that Hsp104 can solubilize amyloid in vivo.[29] Thus, in the study by Rampelt et al. one cannot be certain what proportion of the soluble luciferase-YFP observed in wild-type *C. elegans* after a 12h or 24h recovery from heat shock represents newly synthesized protein or bona fide resolubilized protein.[71] A similar issue arises in more recent experiments that also aimed to demonstrate in vivo disaggregase activity of Hsp110 using *Drosophila* S2 cells, but again the critical cycloheximide control was also omitted.[72] As such, although the foregoing experiments provide compelling indications,[71,72] we still await an unequivocal in vivo demonstration of Hsp110, Hsp70, and Hsp40 disaggregase activity.

More recently, Goloubinoff and colleagues corroborated that the cytosol and the endoplasmic reticulum of mammalian cells contain Hsp110 and Hsp70 machineries that can unfold and solubilize stably misfolded and aggregated protein.[62] Outstandingly, Hsp110 (Hsp105) was found to be an ATP-dependent unfoldase that can prevent aggregation, catalyze the unfolding of misfolded polypeptides, and favor their conversion into native protein on its own.[62] Thus, Hsp110 can act as a
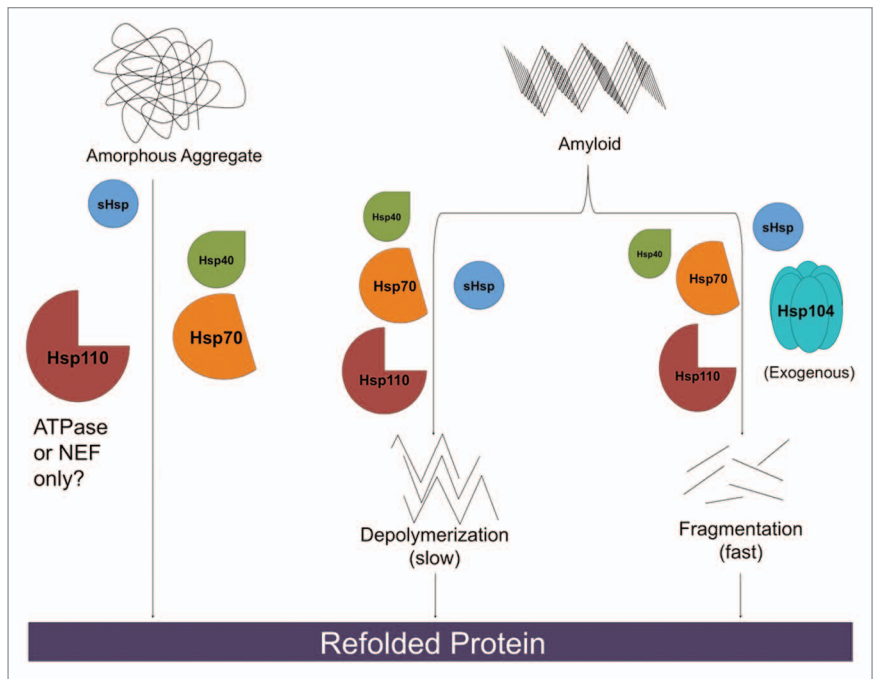

Figure 1. Hsp110, Hsp70, Hsp40, and sHsps are a disaggregation system in metazoan cells. Heat shock proteins Hsp110, Hsp70, and Hsp40 are capable of dissolving disordered aggregates. For labile aggregates, Hsp110 may only need to operate as a nucleotide exchange factor (NEF) for Hsp70, whereas for more stable aggregates it may need to serve as a NEF for Hsp70, engage substrate, and bind and hydrolyze ATP. Hsp110, Hsp70, and Hsp40 can also slowly depolymerize ordered amyloid substrates from their ends. Rapid amyloid dissolution can be achieved by supplementing Hsp110, Hsp70 and Hsp40 with exogenous Hsp104. Here, fibrils can be fragmented and monomers extracted from anywhere in the fibril (not just the ends), which leads to more rapid dissolution. sHsps can stimulate all of these protein disaggregation reactions, but are not absolutely required.

bona fide chaperone with unfolding activity, and does not simply serve as a NEF for Hsp70.[62] Furthermore, titration of the ATP- and Hsp40-dependent refolding activity in the presence of various amounts of Hsp110 and Hsp70 showed optimal disaggregation activity at a 1:1 ratio.[62]

Intriguingly, even without ATP, Hsp110 promotes the release of a prebound substrate from Hsp70, and Hsp70 could activate the release of a prebound substrate from Hsp110.[62] Together with our study,[56] these findings conflict with the notion that the only function for Hsp110 in protein disaggregation is as a NEF for Hsp70.[62] Indeed, it should also be noted that several key in vivo functions of Sse1 require its ATPase activity.[73,74]

Collectively, these results from three different groups independently corroborate the existence of a disaggregase system, consisting of Hsp110, Hsp70, and Hsp40, that couple protein disaggregation to protein renaturation in metazoa (**Fig. 1**). Discrepancies in the details of the system

regarding the requirement for Hsp110 ATPase activity in disaggregation and the relative level of activity of the system are likely the result of different experimental conditions. For instance, we used equal concentrations of Hsp70 and Hsp110,[56] while Rampelt and colleagues used a 1:10 ratio of Hsp110 to Hsp70.[71] Analogously to our work, Goloubinoff and coworkers identified an optimal 1:1 ratio for the concentrations of Hsp110 and Hsp70, which might even suggest an Hsp110:Hsp70 heterodimer that co-operatively drives disaggregation via synergistic entropic pulling.[56,62,75-77] Indeed, Sse1 has been co-crystallized in 1:1 complex with the Hsc70 nucleotide-binding domain, suggesting that a 1:1 complex could be critical.[78]

Moreover, Ssa1 and Sse1 display high affinity for different peptides,[79] indicating that they might interact and exert force on different regions of the polypeptide to cooperatively drive disaggregation. In each of these studies,[56,62,71] different conditions were used to generate the protein

aggregates studied. For instance, in our study, more severe chemical or thermal denaturation was used to generate aggregates.[56] Hence, it is highly probable that different aggregated conformers were studied in each case. We suggest that proteins can adopt a wide variety of conformations in the aggregated state, some of which are more labile and do not require the full chaperone repertoire of Hsp110 for disaggregation (NEF activity is sufficient), whereas others are more recalcitrant and require the full complement of Hsp110 activities encompassing: substrate binding, Hsp70 binding, promotion of nucleotide exchange on Hsp70, and ATP binding and hydrolysis. Additional studies are required to explore this hypothesis further. However, the Hsp70 NEF, Snl1ΔN, could not substitute for Hsp110 under our more stringent aggregation conditions,[56] whereas it could under much milder aggregation conditions.[71] An interesting parallel may be drawn with Hsp104, which employs distinct mechanisms to dissolve labile aggregates vs. stable amyloid.[27] Hsp104 subunits within the hexamer can function independently to resolve disordered aggregates.[27] Thus, even a single subunit within the hexamer can drive disaggregation of disordered aggregates.[27] By contrast, multiple Hsp104 subunits must work together within the hexamer in a co-operative manner to drive amyloid dissolution.[27] Some very stable amyloid conformations even require direct co-operation between two Hsp104 hexamers.[27,56] By analogy, the Hsp110-Hsp70-Hsp40 system might also exhibit mechanistic plasticity in protein disaggregation. We suggest that more stable aggregated structures might necessitate the full repertoire of Hsp110 modalities, whereas the NEF activity might suffice for more facile conformers.

Amyloid Disaggregation in Metazoa

In addition to amorphous aggregates, misfolded proteins in the cell can form amyloids and prions.[10,80,81] Amyloids are self-templating protein conformers.[10,80,81] They form long, stable fibers by self-replicating their 'cross-β' conformation at their growing ends and by converting other copies of the same protein to the 'cross-β' amyloid form.[10,80,81] When amyloid fibers become infectious, they are termed prions.[10,80,81] Initially, we found Hsp110, Hsp70 and Hsp40 were unable to rapidly remodel amyloid in the absence of Hsp104.[56] However, in a later study, we found Hsp110, Hsp70 and Hsp40 especially in conjunction with small heat shock proteins (sHsps), can very slowly depolymerize amyloid fibers (Sup35 prions or α-synuclein fibrils) from their ends,[35] providing a pathway for amyloid disaggregation in metazoans in the absence of Hsp104. Amyloid depolymerization is a lengthy process that occurs on a similar timeframe to molecular recycling within amyloid fibers (days).[35,82,83] The disaggregase system involving Hsp110, Hsp70, Hsp40, and sHsps might exploit this process to slowly eliminate amyloid by accelerating monomer dissociation or by capturing released monomers or by sealing off fibril ends once a monomer has been released thereby preventing monomer reassociation.[35] While newly released monomers could hypothetically collect into toxic oligomers, the chaperone system would likely prevent any toxic oligomer formation. Remarkably, we found this activity to be conserved in humans.[35] Thus, Hsp110 (Apg-2), Hsp70 (Hsc70), Hsp40 (Hdj1) and a small heat shock protein (HspB5) slowly depolymerized α-synuclein fibrils, which are connected to Parkinson disease.[19] These data suggest that in metazoa, which lack an Hsp104 homolog, Hsp110, Hsp70, and Hsp40 can slowly eliminate amyloid forms by specifically hijacking their intrinsic monomer recycling process.[35,82,83]

Treating Neurodegenerative Disease: Can we give Hsp110 a boost?

The Hsp110-Hsp70-Hsp40 disaggregase system might prove to be an advantageous therapeutic target against the numerous neurological disorders connected to protein misfolding and aggregation.[10] Indeed, co-expression of Hsp110 and Hsp40 in *Drosophila melanogaster* suppresses the cytotoxicity of polyglutamine aggregation.[84] Importantly, the ATPase activity of Hsp110 was critical for this rescue.[84] A feasible explanation for this effect invokes Hsp110 and Hsp40 interfacing with members of the Hsp70 family to disassemble polyglutamine aggregates and thus reduce the cellular toxicity of protein aggregation. Polyglutamine aggregation is connected to several neurodegenerative diseases, including Huntington's disease and Spinocerebellar Ataxias.[85] Similarly, Hsp110 was found to completely reverse a vesicle transport defect produced by a mutant (G85R) of Superoxide Dismutase 1 (SOD1) associated with amyotrophic lateral sclerosis (ALS) in the isolated axoplasm from the giant axon of the squid *Loligo pealei*.[86] Hsp110 appears to either directly bind to the mutant SOD1, or to associate with the mutant protein via Hsp70, and occlude binding surfaces that would otherwise interact with endogenous proteins leading to a gain of toxic function. Further studies are warranted to determine whether Hsp110, Hsp70, and Hsp40 can also disaggregate misfolded SOD1 conformers connected to ALS or polyglutamine fibrils and oligomers connected to Huntington's disease and Spinocerebellar Ataxias. Treatment of several neurodegenerative disorders could entail the activation of the Hsp110-Hsp70-Hsp40 disaggregase system. As Hsp110, Hsp70 and Hsp40 enhanced amyloid remodeling by Hsp104,[56] one alternative possibility to achieve such activation would be to supplement metazoan cells with Hsp104.[47] For instance, Hsp104 prevented the aggregation and toxicity of polyglutamine in *C. elegans*.[54] In mouse and rat, Hsp104 expression resulted in extension the animal's lifespan and rescue of striatal dysfunction respectively.[53,55] We have recently introduced Hsp104 into *Drosophila* models of Spinocerebellar Ataxia Type-3.[51] Notably, Hsp104 suppressed toxicity of a C-terminal ataxin-3 fragment when expressed even after the onset of polyglutamine-induced degeneration.[51] This constitutes the first disaggregase treatment that halts disease progression after the start of pathogenic degeneration.[51] Notably, induction of Hsp70 after polyglutamine-mediated degeneration had already initiated was unable to significantly mitigate disease progression.[51] It is possible that simultaneous induction of not only Hsp70, but also

Hsp110 and Hsp40 is necessary to achieve toxicity suppression after degeneration and aggregation have initiated.[84]

Another possibility to enhance clearance of harmful amyloids would be to boost sHsp levels or activity to facilitate the action of endogenous human Hsp110, Hsp70, and Hsp40. Thus, small molecules that induce the expression of these proteins without compromising other components of the stress response could be critical.[11,87,88] For example, the ability to stimulate the dissolution of α-synuclein fibers in patients with Parkinson disease might provide an unprecedented therapeutic leap in the treatment of this disease. Although released monomers could theoretically reassemble into toxic oligomers, the proteostasis network would likely prevent this situation. Lastly, direct pharmacological activation of Hsp110 or Hsp70 is another attractive possibility.[89] While

pharmaceutical discovery efforts generally focus on protein inhibition, protein activation is an emerging field.[90]

In conclusion, the metazoan disaggregase machinery is comprised of the heat shock proteins Hsp110, Hsp70, and Hsp40, which dissolve disordered aggregates.[56,62,71] Hsp110, Hsp70, and Hsp40 exploit the exchange dynamics of amyloid, and can slowly depolymerize amyloid fibrils from their ends.[35] This amyloid depolymerase activity as well as the disaggregation of disordered aggregates is stimulated by sHsps.[35,71] Augmentation of this disaggregase network could have key applications in various neurological disorders linked to protein misfolding. Fascinatingly, this metazoan disaggregation network is amenable to augmentation with exogenous disaggregases, which opens several exciting avenues for potential treatments.[47]

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