Published in final edited form as:

Curr Pharm Biotechnol. 2014 ; 15(11): 1008–1018.

Yeast Prions Help Identify and Define Chaperone Interaction Networks

Michael Reidy* and **Daniel C. Masison**

Building 8, Room 225, 8 Center Drive, Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Proteins in the cell experience various stressful conditions that can affect their ability to attain and maintain the structural conformations they need to perform effectively. Protein chaperones are an important part of a cellular protein quality control system that protects the integrity of the proteome in the face of such challenges. Chaperones from different conserved families have multiple members that cooperate to regulate each other's activity and produce machines that perform a variety of tasks. The large numbers of related chaperones with both functionally overlapping and distinct activities allows fine-tuning of the machinery for specific tasks, but presents a daunting degree of complexity. Yeast prions are misfolded forms of cellular proteins whose propagation depends on the action of protein chaperones. Studying how propagation of yeast prions is affected by alterations in functions of various chaperones provides an approach to understanding this complexity.

Keywords

Amyloid; chaperones; Hsp70; Hsp40; Hsp104; Hsp90; yeast prions

BACKGROUND

In order to function properly, proteins must assume and maintain specific structural conformations. While the information that determines a protein's shape is encoded within the primary sequence, factors such as temperature, pH, molecular crowding, and the osmotic and reductive environments in a cell have a huge impact on whether polypeptide chains reach and retain their native conformations. Some proteins are also faced with the need to traverse membranes or assemble into higher order complexes, and irreparably damaged proteins must be eliminated. The molecular chaperones are part of an essential protein quality-control system that evolved to deal with these challenges [1]. By assisting the folding of nascent polypeptides and the unfolding/re-folding of mature and denatured

*Address correspondence to this author at the Building 8, Room 225, 8 Center Drive, Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA; Tel: (301) 594-1142; reidy@mail.nih.gov.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

proteins, these highly conserved proteins provide stability to the proteome and reduce the ravages of a frequently inhospitable environment. Chaperones were first identified as proteins whose expression increased in response to elevated temperature, and are thus commonly referred to as heat-shock proteins (HSPs) [2]. In general, their approximate molecular weights are used to define the various conserved HSP families, which are conserved universally in all forms of life (Table 1).

In the budding yeast *Saccharomyces cerevisiae*, the activities that molecular chaperones use in their many important roles in the cell also associate them intimately in the propagation of prions, which are infectious entities that exist devoid of nucleic acids. The “protein-only” or prion hypothesis (first used to refer to the infectious agent of the transmissible spongiform encephalopathies in mammals [3]) was shown by Reed Wickner in 1994 to explain certain long-known non-Mendelian yeast phenotypes [4]. Soon thereafter it was discovered that the propagation of these yeast prions, which are composed of misfolded cytoplasmic proteins that form amyloid fibers, requires the action of the molecular chaperones [5]. Protein chaperones act in prion propagation by fragmenting the prion fibers, which is essential for the replication of yeast prions.

Amyloid-based yeast prions share a structure defined by a parallel, in-register beta-sheet core often containing a region rich in glutamine/asparagine (Q/N) residues [6–10]. Misfolding and aggregation of the prion proteins into the prion state reduces cellular fitness due to depletion of the proteins that lead to loss of function phenotypes. Prion aggregates also can sequester other proteins that interact with the prion proteins specifically or with aggregates in general [11–13]. Accordingly, the presence of prions can have deleterious effects on growth, especially when protein chaperone systems are compromised [14–16]. Table 2 lists the most studied yeast prions and their protein determinants, the normal cellular functions of the proteins and the phenotypes caused by sequestration of the proteins into insoluble prion fibers. Due to their stringent dependency on the chaperone machinery for their propagation, amyloid-based yeast prions that have readily monitored phenotypes provide a valuable system with which to study functions and interactions of molecular chaperones.

Over the years many laboratories have established a multitude of *S. cerevisiae* strains harboring prions composed of natural and artificially constructed proteins. An interesting and important observation that arose from this work was that prions composed of the same protein may exist in several “variants” that exhibit differences in the relative strength of their phenotypes [17–21]. The molecular basis for such variation is thought to be due primarily to small differences in the amyloid structure itself [21]. These variations in turn probably affect how the prions interact with the chaperones, adding another level of complexity and thusly expanding the toolkit for studying the functions of chaperones using the prion system.

CHAPERONES

The primary roles of protein chaperones are to help other proteins attain and maintain their native conformations and to protect the integrity of the proteome. As the range of biochemical reactions necessary for life are conserved throughout evolution, so too are the

suite of molecular chaperones that keep the machinery of the cell in working order. At the center of chaperone-associated processes is Hsp70, which has critical roles in protein translation, transport across membranes, degradation and complex assembly and disassembly [22, 23]. The central roles Hsp70 plays in these fundamental cellular processes make it crucial for viability and it is found in all three domains of life.

Hsp70 is a modular protein containing an ATPase domain that regulates substrate interactions of an adjacent peptide-binding domain with a movable lid structure. Hsp70 acts in its various roles via a two-step cycle of binding and releasing exposed hydrophobic portions of substrate proteins that is mediated allosterically by the nucleotide state, as depicted in (Fig. 1A). In the ATP-bound form, an alpha-helical lid structure is positioned away from the open substrate-binding pocket, which has affinity for solvent-exposed hydrophobic stretches of proteins [24, 25]. Hydrolysis of ATP to ADP causes the lid to clamp down onto the substrate-binding pocket, essentially trapping the bound polypeptide [26]. Release of ADP allows rebinding of ATP, which causes the lid to re-open thereby promoting release of the substrate and resetting Hsp70 to the low-affinity mode and the start of the cycle. The released substrate is free to re-fold or undergo further bind-release cycles.

The extremely low intrinsic ATP turnover rate of roughly one ATP hydrolyzed in twenty minutes [27–30] and its two-step cycle make Hsp70 ideally suited to complex and specific regulation. Accordingly, a large cohort of cochaperones has evolved to fine-tune its activities for specific tasks and to recruit Hsp70 to where it is needed [31–33]. Interaction with any of the many different co-chaperones of the Hsp40 (J-protein) and tetratricopeptide repeat (TPR) classes stimulates hydrolysis of ATP [29, 34, 35]. Hsp40s bind hydrophobic substrates with specificity overlapping Hsp70 [36] and can deliver substrates to Hsp70 concomitant with this stimulation, which helps ensure ATP hydrolysis is associated with substrate binding. Nucleotide exchange factors promote the release of ADP, facilitating return to the open conformation [37–41]. The decision to degrade hopelessly unfolded proteins occurs through processes specified by other Hsp70-interacting chaperones [42, 43].

Specific co-factors are involved in numerous and varied cellular processes that require Hsp70's bind-release activity. For example, nascent polypeptide chains are bound by specialized and dedicated ribosome-associated Hsp70/40 partners that protect them from misfolding and promote their proper folding. Upon release from the ribosome, sub-cellular localization of intact proteins to a compartment such as the mitochondria will require unfolding to traverse membranes and refolding upon arrival. This process is facilitated by other localized or specialized Hsp70 and Hsp40s on both sides of the membranes. Proteins synthesized into the ER are received by the dedicated ER versions of Hsp70 and Hsp40, but when they are targeted for degradation they must be unfolded to pass back to the cytosol for destruction, a process aided by Hsp70 and yet other specialized co-chaperones [44–46]. Cytosolic yeast proteins that are buffeted by a harsh environment or are intrinsically weak in the stability of their folds may be acted upon numerous times by Hsp70 and the major Hsp40 Ydj1. As discussed below, a different Hsp40, Sis1, mediates interaction of the chaperone machinery and the prions, in addition to its unknown essential role in the cell.

The yeast cytosol contains four Hsp70s of the essential Ssa subfamily. They are highly homologous and functionally interchangeable, but they display functional distinctions [47–51]. Because they possess similar intrinsic activity, these distinctions are presumed to be due to subtle differences in their interactions with co-chaperones [51]. Combining this variety with the extensive array of combinations of cochaperones that interact with Hsp70, the chaperone with its mundane cycle of substrate binding and release common to all Hsp70 reactions becomes a useful multi-purpose machine that can perform efficiently in many different tasks.

Another major chaperone, Hsp90, is required for the maturation of folding, and hence activity, of certain transcription factors, kinases and other signaling factors that provide key regulatory functions in the cell cycle [52]. Although expression of Hsp90 is induced by stress, processes known to require Hsp90 typically respond to exogenous cues other than stress, such as the presence of hormones or changes in the available nutrient pool. Hsp90 orthologs in prokaryotes are not essential, but in eukaryotes Hsp90 is essential for life and present in large quantities. In yeast the two paralogs of Hsp90 together make up to 1–3% of the total protein in the cell [53].

Hsp90 binds substrates as a dimer and its reaction cycle is similar to that of Hsp70 in that it undergoes ATP-regulated conformational changes that govern the binding and release of substrate “clients”. Also like Hsp70, the ATP state of Hsp90 is highly regulated by co-chaperones. However, the number and structural diversity of regulators of Hsp90 is even higher than those of Hsp70. Practically every step of the Hsp90 cycle is influenced by one or more co-chaperones that can have both overlapping and distinct functions depending on the client being acted on or other factors such as environmental stimuli [54]. In addition, Hsp90 can be modified post-translationally in complex ways including phosphorylation, acetylation, nitrosylation and ubiquitylation [55]. In the context that such modifications impact interaction with co-chaperones, and thus Hsp90 activities, the modifiers themselves can be viewed as Hsp90 co-chaperones.

Hsp70 and Hsp40 play a vital role in the Hsp90 reaction cycle [56]. Some client proteins are first bound by Hsp40 and Hsp70, which deliver them to Hsp90 via interaction with the TPR protein Sti1 (Hop in humans). Sti1/Hop pauses the Hsp90 cycle in the high substrate affinity form and by physically bridging Hsp90 and Hsp70 allows the substrate to be transferred from Hsp70 to Hsp90. In addition to the cell cycle regulators indicated above, the Hsp90 system in yeast is also thought to be directly involved in modifying chromatin of promoter regions to make it more accessible for initiation of transcription [57]. Because of the diversity of Hsp90’s clientele, perturbation of the Hsp90 system has pleiotropic effects on the cell and probably accounts for its indispensability.

Stresses that are severe enough to cause indiscriminant protein denaturation, such as high temperature, result in the formation of aggregated proteins that must be cleared or reactivated in order for the cell to survive the assault. A variety of cells that have been tested possess measurable protein disaggregation activity, usually determined as the amount of a specific enzymatic activity, such as luciferase or malate dehydrogenase, that can be recovered after heat or chemical inactivation by either a defined protein complex or cell

lysate [58]. In prokaryotes and non-metazoan eukaryotes, a dedicated disaggregation motor performs the brunt of this duty. These remarkable proteins, the Hsp100 family of molecular chaperones, perform a function that has been compared to unscrambling an egg [59, 60]. The Hsp100 motor is a hexameric AAA+ ATPase that forms a barrel-shaped structure with an axial pore that extrudes individual denatured polypeptides from the mass of aggregated protein [61, 62]. In order to function in this way it requires the help of Hsp70 and Hsp40 [63–65]. Hsp40 and Hsp70 act first on the aggregate and recruit the Hsp100 to the substrate for extrusion [66]. Hsp70/40 also are likely to be important for helping extracted proteins refold.

While the Hsp100s are not present in metazoans, these organisms have not lost the ability to disaggregate proteins. Interestingly, it seems that in higher eukaryotes such as mammals, modified forms of Hsp70 known as the Hsp110 family (not to be confused with the Hsp100 family), in concert with Hsp70, possess the disaggregation activity [58, 67]. Hsp110s also exist in yeast, but their major role is thought to be more as a nucleotide exchange factor for Hsp70 [39–41]. Whether the Hsp110 in yeast has bona fide chaperone functions apart from its NEF activity is the subject of ongoing study [68–70]. In the budding yeast *Saccharomyces cerevisiae* it is the disaggregation machinery, a complex that has evolved to clear deadly aggregates from stressed cells, that is absolutely required for the replication and persistence of infectious amyloid particles known as prions.

While these examples illustrate a few of the more well-understood processes utilizing the core molecular chaperone reaction cycle, a bioinformatical survey of chaperone genes across all sequenced species provides a glimpse that there are many more processes whose reaction mechanisms have yet to be worked out. With that in mind, although yeast and humans possess a similar number of cytosolic paralogs of Hsp70, the number of J-proteins has amplified from twenty-two in yeast to over forty in humans [71, 72]. The numbers of specialized J-proteins in other higher eukaryotes, such as plants, are even greater [73]. These observations have led to a view that the extent of divergence of intrinsic Hsp70 activities might be diminishing and that the amplification of Hsp70 co-chaperones that cooperate with it and regulate its functions has expanded and diversified the ways Hsp70 can perform as needed in cells of increasingly complex organisms [72].

YEAST PRIONS

Whether one regards prions in yeast as a disease [74], an artifact of unnatural laboratory selection pressure, or an inheritable non-Mendelian “phenotypic switch” [75], all sides can probably agree that the presence of prions expands the possibilities of what can be learned using the powerful yeast system. The intimate association of prions and protein chaperones has made this benefit most readily seen by the many contributions to the study of protein quality control, and molecular chaperones in particular.

The most widely studied yeast prions are [*PSI*⁺], [*URE3*] and [*PIN*⁺] (also known as [*RNQ1*⁺]), which are composed of the misfolded, amyloid forms of Sup35, Ure2 and Rnq1, respectively (see Table 2). Sup35 is a ribosomal release factor. When Sup35 is in the prion state, sequestration of much of the protein into prion aggregates leads to a nonsense

suppressor phenotype. In strains containing either the *ade2-1* or *ade1-14* nonsense allele [*psi⁻*] cells are red and unable to grow without adenine. The nonsense suppression caused when [*PSI⁺*] is present in such cells restores adenine prototrophy and white/pink colony color. A similar red/white adenine auxotrophy phenotype has been established for monitoring [URE3], but here the phenotype is linked to the function of Ure2 as a transcriptional regulator. Ure2 normally represses expression of genes involved in nitrogen catabolism including *DAL5*, which encodes the allantoin transporter. Placing the *ADE2* open reading frame under the control of the *DAL5* promoter allows the presence of [URE3] to be diagnosed by the activation of expression of *ADE2* due to sequestration of Ure2 into prion fibers [18, 20]. As with [*PSI⁺*], [ure-o] cells are red and Ade⁻, while [URE3] cells are white and Ade⁺. The *DAL5* promoter system has also been expanded to use *CAN1*, *kanMX* and *URA3* genes to monitor and select for [URE3] [76]. Deletion of Rnq1, the determinant of the [*PIN⁺*] (Psi-INDucible) prion, has no selectable phenotype [17, 77]. However, cells that are [*PIN⁺*] convert to a [*PSI⁺*] state at a frequency orders of magnitude above [*pin⁻*] cells when Sup35 or its N-terminal domain is overexpressed [17]. The presence of [*PIN⁺*] also can be determined by observing fluorescent foci in cells expressing an Rnq1-GFP fusion.

The experimental conditions used to determine whether a phenotype is a bona-fide prion reflect what is known about prion biology, as generally yeast prions share certain characteristics. These characteristics have been put forth as requirements in order to be called a prion [4]. Firstly, the prion should depend on the production of the protein determinant. Since the prion is in essence an alternative conformation of a certain protein, then if that protein is no longer expressed then the prion should also disappear. Secondly, the phenotype should be related to depletion of that protein's function. There are exceptions to this criterion, such as the [*PIN⁺*] prion, which represents a gain-of-function phenotype by virtue of the fact that cells become [*PSI⁺*] much more efficiently if [*PIN⁺*] is present. Thirdly, because prions propagate in the cytoplasm, the phenotype in question must be infectious in cytoductions and dominant in crosses and not follow the classical Mendelian rules of inheritance after meiosis. That is, the phenotype will be present in all progeny. A fourth criterion for confirming a bona-fide prion is that the phenotype should be reversibly curable. In other words, the prion state should be able to be turned "on" and "off". Proteins that have the ability to form prions can convert spontaneously to the prion state rarely, and this conversion usually can be elevated by over-expression of the prion protein. Curing of prions can occur by interrupting the production of the prion protein or by inhibiting cellular processes important for prion propagation. Prions can again arise in cured cells as long as the prion protein is being produced. The efficient curing of prions by disruption of the molecular chaperone disaggregation machinery not only identified this machinery as a critical aspect of prion propagation in yeast, but also opened a unique window into studying cellular chaperone machinery.

CHAPERONE FUNCTIONS REQUIRED FOR AMYLOID-BASED YEAST PRIONS

In order for prions to persist in an expanding yeast population they must grow, replicate and be transmitted to daughter cells. Growth occurs through the recruitment of soluble determinant protein at the ends of the amyloid fibers and its conversion to the same misfolded prion form as it joins the fiber [78–80]. The in-register parallel beta-sheet structure of the amyloid core templates the misfolding of the newly incorporated monomer [81]. Such growth is thought to occur spontaneously since purified prion proteins assemble into amyloid readily *in vitro* and the process continues as long as there is a supply of the soluble form of the protein. Transmission also is thought to be passive, occurring as prion seeds are carried with cytoplasm when cells divide or when they fuse during mating [82]. The replication step, however, is crucially dependent upon the molecular chaperones. Specifically, action on prion fibers by the AAA+ disaggregase Hsp104, assisted by Hsp70 and its Hsp40 and nucleotide exchange factor (NEF) regulators [5, 83–85], leads to fragmentation of amyloid fibers, thereby creating both new ends for growth and new seeds that can be transmitted to daughter cells (see Fig. 1B).

Hsp104

The disaggregase Hsp104 was the first chaperone identified as playing a crucial role in the maintenance of prions [5]. Yury Chernoff, then working in the laboratory of Susan Lieberman, reported that elevated Hsp104 eliminated the [*PSI⁺*] prion. Perplexingly, it was shown in the same study that depleting Hsp104 also caused [*PSI⁺*] to disappear, leading to the conclusion that the prion required an intermediate level of the chaperone. Since then, Hsp104 has been shown to be generally required for propagation of amyloid-based yeast prions.

The discovery of the role of Hsp104 in prion propagation also led to the explanation of an old observation that milli-molar amounts of guanidine-hydrochloride (Gdn-HCl) in the growth medium caused cells to lose their [*PSI⁺*] prions [86]. Many years after the initial observation it was established that Gdn-HCl acted by blocking prion replication [87]. Subsequent work identified the target of Gdn-HCl as Hsp104 [88–90], implicating Hsp104 as a key factor for the replication step of prion propagation. Inactivating Hsp104 arrests replication of prion seeds, which then become diluted among cells of a growing population until the number of cells is greater than the number of prions. Cells arising beyond that point will be “cured” as they fail to inherit prion seeds, or propagons [91].

Although it is a central protein remodeling motor of the molecular chaperone system, Hsp104 is dispensable for growth, even at elevated temperatures. However, Hsp104 is essential for acquired thermotolerance, which is survival after transient exposure to lethal heat. Although some Hsp104 amino acid residues are more important for prion propagation than for thermotolerance [83, 92, 93], it was soon appreciated that the mutations of Hsp104 that impair its ability to resolubilize proteins from aggregates also negatively impact its ability to perform the replication step in prion propagation. For example, conserved tyrosines that line the inner pore of the Hsp104 motor that are believed to act as ratchets that both pull substrates through the pore and prevent their back-slipping are required for both

processes [83, 84, 94–96]. Alterations of these residues that inhibit resolubilization of protein to varying degrees have identical proportional effects on propagation of prions [83].

Curiously, although all amyloidogenic prions depend on Hsp104 for propagation, overexpressing Hsp104 does not cure cells of other prions in the same highly efficient way that it cures cells of $[PSI^+]$. Curing of $[PSI^+]$ by elevated Hsp104 also depends on or is considerably affected by many factors other than Hsp104 [16, 83, 97–100]. Thus, any explanation for the mechanism of how overexpressed Hsp104 cures prions must account for the involvement of these factors. This complexity presents a difficulty in deciphering the cellular events that underlie the curing process. Although it has been almost two decades since overexpressing Hsp104 was found to cure cells of $[PSI^+]$, no explanation for a specific mechanism for the curing has gained universal acceptance.

At first, it was sensibly theorized that since Hsp104 breaks $[PSI^+]$ fibers, so as to allow them to replicate and expand in the population, then additional breaking caused by elevating Hsp104 would eliminate $[PSI^+]$ by re-solubilizing the prion-form of Sup35 until no propagons remain. Biochemical support for such complete disaggregation by Hsp104 alone has been reported [101], but the *in vitro* system in that study lacked many components that are required for the curing *in vivo* and the findings have yet to be confirmed by others. Furthermore, it was shown that $[PSI^+]$ amyloids actually increase in size when Hsp104 is over expressed *in vivo* [102], demonstrating that brute force breakage of $[PSI^+]$ propagons into oblivion is not occurring in the cell as implied by the various *in vitro* studies. Additionally, there is very much evidence implying that the way Hsp104 acts with regard to its role in replication of all amyloidogenic prions is distinct from how it acts in curing cells of $[PSI^+]$. It was then discovered that the N-terminal domain of Hsp104, which does not have a clearly defined role, is dispensable for thermo tolerance and for prion propagation, but absolutely required for curing by Hsp104 over expression [83]. Moreover, if curing is due to mass action effects of Hsp104 acting alone, then it would be expected to occur independently of cell division, but it doesn't [99].

Perhaps the most puzzling aspect of Hsp104 over expression curing is that it requires Hsp90 and its cofactors [99, 100]. Deletions of Sti1 or mutations that affect its ability to bind to Hsp90 disrupt curing, as does sub-lethal concentrations of the Hsp90-specific inhibitor radicicol [99]. In contrast, components of the Hsp90 system other than those shared with the disaggregation machinery are not involved in prion propagation [103]. Elucidating the mechanism of this phenomenon is further complicated by the fact that mutations that disrupt the ubiquitin-proteasome system (UPS) also negatively impact the curing of $[PSI^+]$ by increased levels of Hsp104 [104]. Unpublished work from our group suggests that a decrease in the free ubiquitin pool is the likely culprit. Accordingly, increasing the cellular level of ubiquitin improves the rate of curing in otherwise wild-type cells, an effect that is mediated through the Hsp90 system. This suggested that these two processes are linked in some way [99]. Perhaps Hsp90 mediates the turnover of an unidentified factor, which in turn promotes prion loss in response to increased Hsp104 levels, but this explanation is speculative at best.

Recent studies from three different groups have shed much light on this phenomenon. Helsen and Glover reported in 2012 the existence of an Hsp104 binding site in the middle domain of Sup35, which is crucial for the curing but dispensable for normal Hsp104-mediated propagation [105]. This finding was remarkable since it provided a possible explanation for why overexpressed Hsp104 cures only $[PSI^+]$. In the same year a study by the Bukau group provided substantial evidence supporting an explanation that overexpressing Hsp104 cures $[PSI^+]$ by disrupting stoichiometry of disaggregation machinery components that reduces its ability to fragment prion fibers, which is the same way that impairing Hsp104 activity reduces replication of prions [66]. The authors found that overexpressed Hsp104 out-competed Ssa1 for binding to Sup35 NM amyloid *in vivo*, and that this competition depended on the N-terminal domain of Hsp104. Remarkably, they also found that inhibiting Hsp90 with radicicol or deletion of Sti1 abolished overexpressed Hsp104 disruption of Ssa1 binding to NM. However, the kinetics of loss of $[PSI^+]$ prions observed when Hsp104 is overexpressed are very different than those seen when Hsp104 activity is impaired by guanidine or by overexpression of a dominant negative Hsp104 mutant. Owing to the fact that typical $[PSI^+]$ cells carry on the order of 200 propagons [91, 106], arresting prion replication by guanidine treatment causes $[psi^-]$ cells to arise only after several cell divisions [87], after which the proportion of $[psi^-]$ cells essentially doubles for every cell division. In stark contrast, $[psi^-]$ cells appear very shortly after induction of Hsp104 overexpression, and this curing continues at a linear rate of 10–15% per cell division [99, 100].

The most recent attempt to explain this the curing was reported by the laboratory of Lois Greene in 2014 [107]. Using GFP-labeled full-length Sup35, the authors showed that overexpression of Hsp104 caused the disappearance of foci that were earlier shown to represent the heritable $[PSI^+]$ propagons [108]. Based on their fluorescence data, the authors describe two different activities of Hsp104: “severing” (the activity responsible for breaking the fibers which is needed for normal propagation) and “trimming” (removal of material from the ends of the fibers). They reported that the curing by overexpression is due more to the trimming activity than the severing activity. In their model the amyloid fibers are trimmed to a “core” particle, which is disposed of by the UPS. Their data do not support the model that curing is due to Hsp104 inhibition, and moreover they rule out asymmetric segregation as a possible mechanism. They therefore concluded that “dissolution” of the prion seeds is the mechanism by which elevated Hsp104 causes curing of $[PSI^+]$. More work is likely needed to resolve the discrepancies in the various theories, and this continues to be a subject of intense study.

Hsp70 System

Isolation of a mutant in the Hsp70 Ssa1, of the essential cytosolic Ssa subfamily, identified Hsp70 as being involved in $[PSI^+]$ propagation [109]. Subsequently, a direct role for Hsp70 in prion replication was provided by studies showing physical interactions between Ssa1 and Sup35 [12, 66, 110]. The Ssa1 mutant containing the L483W substitution, termed Ssa1–21, weakened the $[PSI^+]$ phenotype and caused frequent mitotic loss of $[PSI^+]$. Biochemical analysis revealed that this mutation had significantly elevated basal ATPase activity and a structurally altered substrate-binding domain that suggested the mutation promoted substrate

binding [30]. In agreement with this interpretation, second-site mutations that suppressed L483W and restored stable propagation of [*PSI⁺*] were expected to reduce affinity for substrates in general [111]. How a general increase in Hsp70 substrate-binding activity translates to impaired prion propagation has not yet been clarified.

Additional second-site suppressing mutations altered sites of Hsp70 that physically interact with Hsp40 and TPR proteins, implicating these Hsp70 system co-chaperones as acting with Hsp70 in its role in prion propagation [111]. These findings implied that prion propagation could be impaired in a similar way by altering Hsp70 function directly by mutation or indirectly by altering factors that regulate its activity. Accordingly, promoting the substrate-binding step or slowing substrate release, by elevating factors that stimulate ATP hydrolysis (e.g. Sti1) or depleting factors that promote nucleotide exchange (e.g. Fes1), respectively, have the same effect as the L483W alteration [103]. In contrast, depleting Sti1 and elevating abundance of Fes1 reduce the anti-prion effect of Ssa1–21, as expected.

Analysis of extragenic suppressors of the Ssa1–21 pheno-type led to the discovery that proteins that regulate Hsp70 are required for both anti-prion effects of Ssa1–21 and the curing of [*PSI⁺*] by over-expressed Hsp104 [16, 99]. The effect of Ssa1–21 on [*PSI⁺*] is thus strongly related to Hsp104 curing, even though Hsp104 is expressed at normal levels in Ssa1–21 mutant cells.

All of the four Ssa family proteins have been found to affect other processes of prion biology. Increasing abundance of any Ssa Hsp70 allows a higher frequency of [*PSI⁺*] induction by overexpressed Sup35, alters the size distribution of prion fibers, and protects [*PSI⁺*] from the curing effect of overexpressed Hsp104 [97, 110]. Although in these instances [*PSI⁺*] responds similarly to the different Ssa proteins, when [*PSI⁺*] propagates in cells expressing individual Hsp70s as the only Ssa protein in the cell, it displays markedly different phenotypes [49, 50]. [URE3] also propagates differently depending on the Ssa protein available, but the dependency of [URE3] on specific Ssa proteins differs from that of [*PSI⁺*] [49, 112]. For example, while propagation of [*PSI⁺*] is supported best by Ssa1, [URE3] is supported best by Ssa2 and is unstable when only Ssa1 is present. These differences show not only that the highly homologous and functionally redundant Ssa Hsp70s possess distinct activities, but also that different prions depend on distinct Hsp70 functions for optimal propagation. These differences likely have their basis in variations in ways the Hsp70s interact with cofactors that cooperate with them and regulate their activity.

Hsp40s and NEFs are the major regulators of the Hsp70 ATP cycle, and both strongly influence prion propagation. The obligate Hsp40s are the most abundant and varied Hsp70 co-chaperones [31, 72]. The Ydj1 and Sis1 cytosolic Hsp40s influence prion propagation in different ways. Over-expressing Ydj1 cures cells of [URE3], apparently though interaction with Hsp70, but does not effect [*PSI⁺*] [14, 113]. Depleting Sis1 causes rapid and efficient loss of [URE3] and [*PIN⁺*], but affects [*PSI⁺*] weakly and only after a very long delay, indicating [*PSI⁺*] prions depend less strictly on Sis1 than the other prions [114]. Moreover, all regions of Sis1 except its J-domain are at least individually dispensable for [*PSI⁺*] propagation, implying other Hsp40s can provide some Hsp40 functions necessary for [*PSI⁺*] propagation [16]. Another Hsp40, Apj1, was identified by its ability when over-expressed to

cure cells of a hybrid Sup35 prion containing the prion-determining domain from *P. methanolicus* Sup35 [115]. The NEFs Sse1 and Fes1 both are required for stable [URE3] propagation and can strongly influence induction and propagation of [PSI⁺] in different conditions [103, 116–118]. Additionally, overexpression of the N-terminal domain of the BAG-1 homolog Snl1, another NEF, cures [URE3] in an Hsp70-dependent manner [119]. Where tested, the ability of the Hsp40s and NEFs to interact with or regulate Hsp70 activity is required for them to have their influence on prion propagation, indicating the effects these co-chaperones have on prions is mediated through Hsp70.

Hsp90

The Hsp90 chaperone system is distinct from the disaggregation machinery, yet shares several of the same components. Hsp90 is very highly regulated throughout its reaction cycle by many co-chaperones, such as TPR proteins, peptidyl-prolyl isomerases and Hsp70/40. Chemical inhibition of the Hsp90 ATPase does not have any overt effect on propagation of [PSI⁺] prions and, likewise, altering Hsp90 function by depleting or overexpressing different Hsp90 cochaperones does not significantly affect [PSI⁺] phenotype [103]. These observations suggest that Hsp90 is not needed for prion replication. Nevertheless, Hsp90 is required for prion curing by overexpressing Hsp104 [85], again pointing to a mechanistic difference in the way Hsp104 acts in the processes of prion propagation and elimination.

PRIONS HELP US UNDERSTAND CHAPERONE MACHINERY INTERACTIONS, COOPERATION AND REGULATION.

Because propagation of yeast prions can be noticeably perturbed by even subtle alterations in the abundance or function of any of a diverse mix of chaperones and cochaperones, they provide a sensitive system for studying various aspects of how chaperones function and cooperate. For example, compared with the stable [URE3] phenotype of cells expressing Ssa2 as the only Ssa Hsp70 in the cell, [URE3] is lost frequently from cells expressing only Ssa1 [49]. These distinct phenotypes provide a useful genetic system for uncovering the molecular basis underlying the functional distinction between these nearly identical Hsp70s [51]. Creating Ssa1/Ssa2 hybrid proteins by swapping their NBD, SBD and CTD structural domains identified the NBD as the region responsible. Individually swapping the only four amino acids in this region that differ between Ssa1 and Ssa2 showed that glycine residue 83 of Ssa2 (alanine in Ssa1) is responsible for the Ssa2 character. Although Ssa2^{G83A} is identical to Ssa1 except for the very subtle change of glycine to alanine at this one residue, it behaves like Ssa1. Similarly, Ssa1^{A83G} behaves like Ssa2.

Ssa2, but not Ssa1, also supports Hsp70 function necessary for a defined vacuolar import and degradation (VID) pathway [48] and, remarkably, this same residue directs the Hsp70 functional distinction of Ssa1 and Ssa2 in this protein turnover pathway. The VID pathway is functional in cells expressing only Ssa2 or Ssa1^{A83G}, but not in cells expressing only Ssa1 or Ssa2^{G83A} [51]. Disrupting the VID pathway does not affect [URE3] stability, indicating that the specific dependency of [URE3] on Ssa2 does not involve VID function. Thus, [URE3] prions provide a facile approach to study how nearly identical Hsp70 chaperones can function distinctly not only in prion propagation, but also in an unrelated cellular protein

degradation process. The fact that the structural difference that determines the functional differences resides in the NBD indicates that it is the regulation of substrate binding, rather than substrate binding *per se*, that specifies the distinction in Hsp70 functions. These observations are in line with the view that the expansion of Hsp70 functions to perform additional roles in more highly evolved organisms occurs by expansion in the numbers of Hsp70 regulators rather than Hsp70 isoforms [54].

The Hsp100-based disaggregation machinery evolved to refold cellular protein components that become misfolded or aggregated after exposure to stresses, such as transient high temperature. It is conserved in non-metazoan organisms including higher eukaryotes such as plants, but a barrier to collaboration across species exists among some of the eukaryotic and prokaryotic machinery components. For example, although constitutively expressed versions of Hsp70s from fungal, plant and primate species can function in place of the essential Ssa family to support growth and prion propagation in yeast, their inducible counterparts can not complement these functions ([50, 120], our unpublished observations). Likewise, the Ssa Hsp70s cannot function with the *E. coli* Hsp100 (ClpB) to propagate prions in yeast [121]. Nevertheless, *Schizosaccharomyces pombe* Hsp104 functions with the *Saccharomyces cerevisiae* Hsp70 system to propagate prions [122] and to provide thermotolerance [123]. Likewise, *Sa. cerevisiae* Hsp104 can work with Hsp70s from *Sc. pombe*, and the *Sc. pombe* NEF Fes1 can replace *Sa. cerevisiae* Fes1 in this system [122].

By swapping the coiled-coil Middle (M) domain of Hsp104 with that of *E. coli* ClpB (Hsp100), it was found that the cross-species Hsp100 and Hsp70 system components could now collaborate to support both prion propagation and thermo tolerance [85, 124]. Here, prions helped identify structural elements that direct inter-chaperone interactions and showed the M-region of the Hsp100 chaperone mediates functional interaction specifically with the cognate Hsp70 system. In contrast to such specificity, Hsp40s are more promiscuous and they do not exhibit this degree of interspecies barrier, presumably due to a less diverged J-protein-Hsp70 interaction [63, 85].

The dependency of yeast prions on the Hsp100 disaggregation machinery was also exploited to define the specific components of this machinery that are important for its role in prion propagation. ClpB, which was thought to lack amyloid remodeling activity on the basis of its inability to act on amyloid *in vitro*, was shown to be able to propagate [*PSI*⁺] in place of Hsp104 (i.e. in *hsp104* cells) [85]. However, this ability required co-expression of both *E. coli* DnaK and its cognate nucleotide exchange factor GrpE. Although the *E. coli* J-protein DnaJ was not needed in this system, ClpB failed to propagate prions when a DnaK mutant specifically defective in interacting with J-proteins was expressed in place of DnaK. This finding confirmed that a J-protein is a required component of the Hsp100 machinery for propagating prions and implied that one of the yeast Hsp40s was cooperating with the bacterial chaperones. The yeast J-protein was identified by independently expressing mutant versions of Sis1, Ydj1, and DnaJ that each had a compensatory mutation that restored interaction with the mutant DnaK. Even though DnaJ is the cognate J-protein for DnaK, only Sis1 cooperates with the *E. coli* machinery to support [*PSI*⁺] propagation, showing that this process requires a specific J-protein function of Sis1. The same system was also used to show that ClpB, DnaK and GrpE can protect yeast from exposure to lethally elevated

temperature and that Ydj1 is the J-protein that worked best with these chaperones to provide this thermotolerance. An attractive feature of this system is that it allows monitoring interactions between specifically targeted chaperones even in the presence of native counterparts.

These findings show that the basic Hsp100 disaggregation reaction requires the Hsp70 system consisting minimally of Hsp70 with its Hsp40 (J-protein) and NEF partners, and that functions of this disaggregation machinery are conserved from bacteria to yeast. The findings also support a view that the Hsp40 component is the primary factor that directs the specificity of the machine's activities in different cellular processes, which is consistent with earlier work indicating that Hsp40 and the Hsp70 system initiate the disaggregation reaction [36, 64, 65]. Whether the basis of this specificity is due to preferences of different Hsp40s for aggregated substrates that are highly ordered, amorphous or exhibit particular amino acid elements, or due to differences in abundance of other protein quality control factors under different environmental conditions, remains to be worked out.

To date we know that human HspA8 (Hsc70) also supports prion propagation in place of Ssa Hsp70 and the human homologs DnaJA1 and DnaJB1 function in place of Ydj1 and Sis1, indicating that conservation of functional interactions of these chaperones with yeast Hsp104 and Hsp70 systems extends to humans ([120, 125], our unpublished results). The ability of HspA8 to support viability of yeast in place of Ssa Hsp70 also means it must function in all process that rely on Ssa Hsp70, and, by implication, all of the cofactors necessary for these processes. It is expected that other chaperones and co-chaperones from humans and other species will function well enough in yeast to allow this system to continue to be exploited as a sensitive system for identifying and characterizing the complex physical and functional interactions among chaperones in defined physiological processes.

ACKNOWLEDGEMENTS

We thank our NIH colleagues for insightful discussions and critical reading of the manuscript.

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Fig. (1).

(A) Hsp70 bind/release cycle. In the ATP-bound form (left), Hsp70 (blue) is poised for interaction with substrate. Hsp40 delivers substrate (black) to Hsp70 while concomitantly stimulating its ATPase. Hydrolysis of ATP causes the closing of the lid structure over the peptide-binding pocket of the substrate-binding domain through an allosteric mechanism, trapping the substrate (right). Exchange of ADP for ATP is facilitated by dedicated nucleotide exchange factors (NEF), which causes the re-opening of the lid, substrate release and a resetting of the cycle. **B. The prion replication reaction.** In yeast, amyloid prion fibers (represented here by gray stacked blocks) are acted upon by the disaggregation machinery, which is composed of Hsp104 (red), Hsp70s of the Ssa family (blue), the Hsp40 Sis1 (black) and nucleotide exchange factors such as Fes1 and/or Sse1 (green). Extrusion of a single prion protein molecule through the Hsp104 motor's pore causes the fiber to break, thereby creating new ends for continued growth of the fiber and new propagons for dissemination to daughter cells.

The major molecular chaperone families and general functions, with their names in *E. coli*, *S. cerevisiae*, and *H. sapiens* listed. For *S. cerevisiae*, only the major cytosolic chaperones are listed. For *H. sapiens*, the nomenclature proposed by Kampinga, *et al.* is used [126].

Table 1.

Family	Function	<i>E. coli</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>
Hsp100	Disaggregate motor; rescues insoluble protein; ATPase	ClpB	Hsp104	-
Hsp90	Maturator of specific clients; some refolding activity <i>in vitro</i> ; ATPase	HtpG	Hsc82, Hsp82	HspC (5 members)
Hsp70	"Holdase"; foldase; unfoldase; disaggregate; ATPase	DnaK	Ssa1 - 4; Ssb1, Ssb2	HspA (13 members)
Hsp40	Activator of Hsp70 ATPase; substrate recognition and modulator of specific chaperone process	DnaJ	Ydj1, Sis1, 20 others	DnaJA (4 members) DnaJB (14 members) DnaJC (32 members)
Hsp110 (Diverged Hsp70 family)	Nucleotide exchange factor for Hsp70, may bind substrate; in mammals has disaggregate activity (in concert with Hsp70)	-	Sse1	HspH (4 members)
NEF's	Nucleotide exchange factors	GrpE	Fes1, Ssl1	HspBP, Bag proteins

Table 2.

The three most characterized prions of *S. cerevisiae*. Listed are the determinants of each prion along with a brief description of their functions. Their phenotypes and most common methods of diagnosing their presence in cells are also listed.

Name	Determinant/Function	Prion phenotype/Assay
[<i>PSI</i> ⁺]	Sup35; ribosomal release factor	Suppresses nonsense mutations: <i>ade1-14</i> or <i>ade2-1</i> in adenine biosynthetic pathway. [<i>PSI</i> ⁺] colonies are white, [<i>psi</i> ⁻] colonies are red.
[URE3]	Ure2; negative regulator of genes involved in nitrogen catabolism	Relieves repression of <i>DAL5</i> promoter: <i>P_{DAL5}-ADE2</i> reporter. [URE3] colonies are white, [ure-o] colonies are red.
[<i>PIN</i> ⁺]	Rnq1; function not yet determined	[<i>PIN</i> ⁺] increases ability to induce the [<i>PSI</i> ⁺] prion upon overexpression of Sup35, causes fluorescent foci in cells expressing Rnq1-GFP fusion protein. Rnq1-GFP is diffuse in [<i>pin</i> ⁻] cells.