Mutations in the Chaperone Hsp104 Differentially Propagate Yeast Prions

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Mutations in the Chaperone Hsp104 Differentially Propagate Yeast Prions

by

Jennifer Elizabeth Dulle

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2013

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ABSTRACT OF THE DISSERTATION
Mutations in the Chaperone Hsp104 Differentially Propagate Yeast Prions

by
Jennifer Elizabeth Dulle
Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Cell Biology)
Washington University in St. Louis, 2013
Professor Heather L. True, Chairperson

Molecular chaperones are critical elements of the protein quality control network and are responsible for protecting cells from protein misfolding and aggregation. In yeast, molecular chaperones also participate in the propagation of self-replicating, protein-only elements called prions. The AAA+ ATPase Hsp104 is a disaggregase essential for yeast prion maintenance and is responsible for fragmentation of prions to generate the transmissible prion propagons. This work focuses on further understanding the role of Hsp104 in prion propagation and protein aggregate resolubilization. To do this, I first identified novel mutations in Hsp104 which altered [PSI+] propagation and characterized their effects on Hsp104 function, protein disaggregation, and prion variant propagation. One mutant propagated a phenotypically undetectable [PSI+] phenotype that resulted from soluble oligomers of Sup35. I discovered that soluble, more SDS-sensitive oligomers of Sup35 were sufficient to transmit the prion state but were not capable of producing the nonsense suppression phenotype associated with Sup35 aggregation in the [PSI+] state. I found that these oligomers are also present in wild type [PSI+] cells and can be distinguished from the large Sup35 aggregates and still transmit the prion conformation.

Next, I characterized another set of mutations that are located in a less well-understood domain of Hsp104. I used these mutations to elucidate the function of the middle domain in prion maintenance and its affect on the biochemical activities of Hsp104. I found that this
domain mediates the disaggregation and ATPase activities of Hsp104 and has differential effects on the propagation of specific prion variants. I hypothesize that the regulation of Hsp104 function by the middle domain plays a significant role in the selective amplification of specific conformational variants.

Finally, I investigated the affect that changes in Hsp104 activity have on prion variant propagation and protein disaggregation. I utilized two novel mutations which significantly decreased the activity of Hsp104 to examine the requirements of two individual yeast prions, [PSI+] and [RNQ+], for Hsp104. I found that propagation of both prions was altered when the activity of Hsp104 was significantly decreased, although specific conformational variants of each could be maintained. I hypothesize that structural variants of yeast prions require varying amounts of Hsp104 activity for optimal propagation.

These experiments elucidate how alterations in the activity of the molecular chaperone Hsp104 affect remodeling of prions and specific prion conformational variants as well as amorphous aggregates. As an essential chaperone in yeast prion propagation, characterizing the disaggregation mechanism of Hsp104 is important for understanding the mechanism of amyloid aggregation and thus, has broad implications for both functional and disease-related amyloid models.
Chapter 1: Background and Significance
1.1 Overview: Protein Aggregation

Efficient and accurate protein folding is essential for cell function and viability. All proteins possess the inherent ability to aggregate often resulting in loss-of-function, which in many cases can lead to disease by disrupting normal cellular homeostasis.\textsuperscript{1-3} One broad class of protein conformational disorders involves the deposition of protein into insoluble and highly-structured fibrillar protein aggregates, called amyloid.\textsuperscript{4,5} Amyloid deposition is associated with multiple, severe physically and mentally debilitating neurodegenerative diseases. The mechanisms underlying the amyloid deposition associated with these diseases are still mostly unclear despite intense investigation.

There are three characterized pathways by which protein conformational disorders develop. Inherited, familial genetic mutations often enhance the likelihood of protein aggregation and thus disease progression.\textsuperscript{6-9} Alternatively, the majority of protein conformational disorders arise sporadically with age as the greatest risk factor. One possibility for the strong correlation of protein conformational disorders with age is decreased function of the protein quality control networks, specifically molecular chaperones, which regulate and protect the cells from protein misfolding and aggregation. However, the mechanisms behind sporadic amyloid aggregation as well as the effect of age on the proteostatic cellular pathways are not well understood. The third known pathway is by infection. Prion diseases are a specific class of amyloid-associated protein conformational disorders, which are capable of being transmitted between species, as evidenced by the transmission of bovine spongiform encephalopathy (or Mad Cow disease) to humans in the late 1990’s. This mode of amyloidosis development is extremely rare, but study of prion infection across species has led to significant advances in understanding the underlying mechanism of transmission.

Interestingly, recent data have demonstrated that several non-disease related proteins are capable of converting into amyloid-like fibrils.\textsuperscript{2,10-18} These data led to the hypothesis that amyloid fiber formation is a generic property of polypeptides.\textsuperscript{19} Furthermore, several examples of functional amyloid that play a role in normal protein and cellular function have been identified in a variety of organisms, including bacteria, fungi, insects, and even humans.\textsuperscript{20} The identification of functional amyloid suggests that this protein fold is evolutionarily conserved, and as such, investigation of the regulation of amyloid species by molecular
chaperones in lower organisms may provide insight into the role of the protein quality control network in the mechanism of aggregation of both functional and disease-related amyloid in higher eukaryotes.

In this chapter, I will focus on protein aggregation and its regulation by the protein quality control network, specifically molecular chaperones. First, I will examine the characteristic features of amyloid aggregates and discuss the role of amyloid as both a disease-associated and a functional protein conformation. I will describe what is known about the mechanisms behind amyloid formation, including the formation of oligomeric intermediates and their proposed role in disease pathology. I will also discuss conformational variation in amyloid structures and how this impacts both the function and toxicity associated with these amyloid structures. Next, I will examine the structure and propagation of yeast prions, specifically the [PSI+] and [RNQ+] prions, and their proposed functional roles in the cell. I will characterize how conformational variation of these prions affects both their function and their interaction with molecular chaperones, of which Hsp104 will be the focus. Subsequently, I will discuss the function of the molecular chaperone Hsp104 by first analyzing each distinct domain of Hsp104 and remarking on how they cooperate as a functional unit. I will then discuss the role of Hsp104 in yeast prion propagation and how its interaction with yeast prions differs from its other substrates. Additionally, I will discuss how other molecular chaperones cooperate with Hsp104 to both promote prion propagation as well as efficient resolution of stress-related protein aggregation. Finally, I will examine how Hsp104 has been utilized in studying mammalian protein conformational disorders.

1.2 Amyloid Structure, Mechanism, and Significance

Amyloid is generally defined as a fibrous quaternary protein structure composed of intermolecularly hydrogen-bonded β-sheets. These β-sheets laterally associate to form fibers that range from 5 to 15 nm in diameter. Investigation of these amyloid fibers by X-ray diffraction revealed a characteristic pattern of the backbone and side chains called a “cross-β” structure. This pattern is reflective of β-sheets oriented parallel to the fibril axis while their extended peptide strands are aligned perpendicularly to the fibril axis. This “cross-β” structure soon came to be recognized as a hallmark of amyloid structure. Additionally, amyloid fibers are typically long, unbranched structures formed from the entwinement of several smaller fibers, which results in a twisted or helical ultrastructural appearance.
Amyloid fibers also bind the dye congo red and display fluorescence when bound to the dye thioflavin T. These properties are common to all amyloid structures despite significant variation in the primary sequence of the amyloidogenic proteins themselves. These properties, unfortunately, also make investigation of amyloid structures challenging. Amyloid fibers are generally too large to be studied efficiently using X-ray crystallography or nuclear magnetic resonance (NMR). However, short peptides from amyloidogenic proteins can fold into the “cross-β” structure allowing characterization by crystallography and NMR. Both electron microscopy (EM) and atomic force microscopy have been useful in revealing the higher-order assembly properties of amyloid fibers. In addition, several methods have been used to elucidate the amyloid-forming regions of the protein including hydrogen-deuterium exchange (H-D exchange), solid-state NMR (ssNMR), and mutagenesis and conjugation of probes to specific residues.

Within the amyloid fold itself, variation exists in the orientation of β-strands, which comprise what is called the amyloid core. The core β-strands orient in two common forms within the β-sheet, parallel and antiparallel, with the former being the most prevalent. Parallel β-sheets can be further described by the alignment of the amino acids between the strands. For example, parallel, in-register β-sheets are composed of identical residues on different strands aligned in-register - each residue is adjacent to the identical residue on the neighboring strand – to promote hydrogen bonding between these residues. Moreover, the columns of hydrogen bonds in a β-sheet are characterized as a polar zipper, hydrogen bonds between the side chain and main chain amides, and increase stability of the sheets, adding to the overall strength and stability of the fiber. Parallel β-sheets can also be out-of-register or pseudo-register, though these structures are much less common than the parallel in-register.

Another type of amyloid fold that is different from the parallel or anti-parallel orientations is the β-solenoid structure. In these fibers, a single polypeptide strand forms the first layer then loops back around to form the second layer as well. Since the same polypeptide strand forms two or more layers, these strands cannot be in-register as the residues on the first strand will not contact the same residues on the neighboring strand. These β-solenoid structures can be pseudo-in-register though, provided the neighboring β-strands share a high degree of sequence similarity.
Additionally, the tight packing between β-sheets plays an integral role in the strength and stability of the amyloid fold. The orientation of these sheets promotes interdigitation of the side chains, such that even water is excluded from these interfaces. The close packing and interdigitation of the neighboring β-sheets is called a “steric zipper” for its ultrastructural appearance as well as its exclusion of water molecules. These “pair-of-sheets” units, as they are termed, are then able to wind around each other to form the fibrous structure. One hypothesis suggests that these “pair-of-sheets” units are the minimal structures needed for further propagation and conversion of monomer to the amyloid conformation.

Thus, amyloid fibers are highly structured and stable aggregates; they are significantly different than unstructured and amorphous aggregates that result from general protein misfolding. Fibers from amyloidogenic proteins are resistant to proteolysis and denaturation by detergents. Additionally, amyloid fibers act as a template for the conversion of monomer from a folded or disordered form to a β-sheet rich structure capable of extending the amyloid fiber.

Disease-Associated Amyloid

Several age-related neurodegenerative disorders are associated with the progressive accumulation of amyloid fibers. Alzheimer’s disease, the best known and the most prevalent, is characterized by the extracellular deposition of a proteolytic fragment of the Amyloid Precursor Protein (APP). This fragment, called Aβ, is secreted by the cell to the extracellular space via the endocytic pathway where it then forms amyloid plaques. The amyloid formation of Aβ results in widespread neuronal dysfunction and cell death. Another protein shown to play a role in the pathology of Alzheimer’s disease is the microtubule-associated protein tau. Aggregated tau is the main constituent of the neurofibrillary tangles present in the cytoplasm of neurons and in diseased tissues. Aggregated tau is commonly present in several other neurodegenerative disorders.

Nine different neurodegenerative disorders are associated with proteins containing expanded polyglutamine (polyQ) tracts (homopolymeric amino acid sequences) including Huntington’s disease and several spinocerebellar ataxias. PolyQ proteins aggregate when the length of the polyQ tract crosses a critical threshold, resulting in destabilization of the folded protein and amyloid formation. Aggregates of polyQ proteins occur both in the nucleus and the cytoplasm. Interestingly, glutamine-rich polypeptide
domains (25% or more glutamine residues) and polyQ tracts are often identified in transcription factors and proteins that play roles in DNA and RNA processing.

Another well-studied amyloidogenic protein is the membrane-bound prion protein (PrP). The mammalian prion diseases such as Creutzfeldt-Jacob disease in humans, scrapie in sheep, and chronic wasting disease (CWD) in deer and elk, are characterized by the accumulation and aggregation of PrP on the cell surface. More detail will be provided for mammalian prions in a later chapter.

Several lines of evidence suggest that amyloid aggregates, or amyloid precursors, have a clinically significant role in disease pathogenesis. Multiple hypotheses exist as to how amyloid deposits may contribute to disease pathology. Initially, the amyloid aggregates themselves were proposed to be the cause of disease through a toxic gain-of-function. However, this hypothesis is challenged by the observation of amyloid fibers in the brains of healthy individuals as well as the lack of correlative evidence between the presence of amyloid aggregates and disease severity. Another more recent hypothesis suggests that the toxic species are actually intermediates formed during the aggregation process. As a result, this hypothesis proposes that the amyloid aggregates may be benign or even, perhaps, protective as a strategy to sequester the toxic species. Toxic oligomers of several amyloidogenic proteins can be formed in vitro and shown to cause cellular toxicity and disease pathology when introduced into cell and animal models. Whether these two hypotheses are mutually exclusive or both contribute to disease progression remains to be seen.

Functional Amyloids

In addition to amyloids associated with degenerative diseases, several functional amyloids have been characterized in bacteria, fungi, insects, and now in humans. Several of these functional amyloids have been studied by EM, Congo red and thioflavin T binding, and X-ray diffraction and show similar structural patterns to the disease-related amyloids described above. Bacteria employ several structural amyloids, such as curli and the chaplins, to establish a bio-matrix that enables surface adhesion and colony formation, respectively. Fungi also express structural amyloid called hydrophobins that assemble at the air-water interface and provide stabilization for aerial growth of hyphae for spore formation. In addition, hydrophobins also provide adherence to the surface of host organisms, thereby
facilitating pathogenesis. One final example of a structural amyloid is the chorion proteins, which are present in fish and insect eggshells and protect the developing larvae from harm by stabilizing the eggshell structure.\textsuperscript{20} Thus, proteins from several different organisms that function in structural roles often adopt amyloid folds to provide rigid and stable structures for efficient functioning.

Not all functional amyloids are involved in structural roles; the amyloid prions in fungi are known to facilitate phenotypic variation through the method of protein-only inheritance in a non-Mendelian manner. The yeast prions formed from the aggregation of the proteins Sup35 and Rnq1 in \textit{Saccharomyces cerevisiae} are especially directed towards this role and are discussed in depth in the next section.\textsuperscript{43} The HET-s prion in the fungus \textit{Podospora anserina} regulates heterokaryon (multi-nucleated cell) formation important for mating and vegetative growth.\textsuperscript{44} Additionally, the structure of the HET-s prion has been identified as the β-solenoid structure.\textsuperscript{31} Non-disease related amyloids have also been proposed to exist in higher eukaryotes. In sea slugs, the cytoplasmic polyadenylation element binding protein (CPEB) may also utilize an amyloid fold to regulate synaptic changes important for learning and memory.\textsuperscript{45} Additionally, in humans, the structural protein Pmel17 forms amyloid fibers that act as a scaffold for melanin deposition in the melanosome.\textsuperscript{46} Thus, amyloids have been shown to play roles in several biological processes, which only underscore the importance of understanding the mechanisms of amyloid formation and function.

**Mechanism of Amyloid Formation**

In order for amyloid to form, proteins must first go through the thermodynamically unfavorable process of unfolding to increase exposure of amyloid-forming segments.\textsuperscript{47} Also, because of the highly structured and bonded nature of the amyloid cores, several molecules need to come together to form a stable nucleating structure capable of further propagation, in a process called nucleation.\textsuperscript{27, 48-51} For these reasons, nucleation is a rare event. However, conditions that promote exposure of these regions such as denaturing conditions, high temperatures, or high protein concentrations often enhance amyloid formation.\textsuperscript{52} In addition, interaction with polyanions, lipids, or proteoglycans can also promote amyloid fiber formation.\textsuperscript{53-58} These amyloid conformers then nucleate the amyloid aggregate by converting unfolded molecules to the amyloid form, thereby polymerizing into amyloid aggregates. The strongest
evidence in favor of the nucleated conformational conversion model is that addition of pre-formed amyloid fibers to amyloid protein monomer accelerates fiber formation, essentially by eliminating the nucleation step. This has been demonstrated both in vitro with recombinant protein and in vivo when recombinant fibers can be introduced into cell and mouse models to induce disease pathology and progression.57, 59

Following nucleation, addition of new protein molecules occurs rapidly.60 Several hypotheses exist for how the growing fiber polymerizes and what the identity of the added unit is. The pre-eminent hypothesis suggests that fiber polymerization occurs by monomer addition to the ends of fibers.61 In this model, the fiber, which has adopted the stable β-sheet rich fold, binds to monomer that has exposed amyloid-forming segments and templates these destabilized regions to β-sheets. Another hypothesis suggests that monomer do not template from amyloid aggregates directly, but form oligomeric intermediates which then polymerize to amyloid fibers. Oligomeric intermediates in the process of amyloid formation have been described for several amyloidogenic proteins. On the other hand, these oligomers may not be true intermediates of amyloid formation but may instead assemble as a result of off-pathway protein-protein interactions.62 Although these hypotheses may seem mutually exclusive, recent data have shown that the same amyloidogenic protein may assemble fibers via multiple pathways.63 For example, the human amyloid protein, β2-microglobulin can form two distinct amyloid structures, worm-like and rod-like, under different conditions at low pH, and that the pathways used to generate these two fibers are distinct and in direct competition.64–66 Further examples are observed when the mammalian prion protein PrP forms amyloid under different conditions. At acidic pH, recombinant PrP assembles into amyloid oligomers while at neutral pH, recombinant PrP converts into amyloid fibers.67–70

Interestingly, in the absence of amyloid fibers, the amyloid oligomers of such proteins as Aβ71, 72, α-synuclein73, 74, polyQ75, 76, and PrP77 can cause toxicity when introduced into cell and animal models of disease. Amyloid oligomers can encompass a range of sizes and structures78, bind the amyloid-binding dyes79, and exhibit robust β-sheet structures, similar to the larger amyloid fibers.80–82 Similar to the ubiquitous nature of the amyloid fold, amyloid oligomers of several distinct proteins share a common structure and as such may share a common method of pathogenesis.83–85 These oligomers, formed in vitro, can nucleate amyloid aggregation and cause disease pathology in disease models.86 These
findings led to the hypothesis that oligomeric intermediates are the primary toxic species. Consequently, amyloid fibers may be benign or even protective by sequestering the toxic oligomers.\textsuperscript{36, 87-89}

1.3 Prions in Mammals and Yeast

Among the amyloid-forming proteins, prions are unique. Prions, or proteinaceous infectious particles, are infectious amyloid as these aggregated proteins can be transmitted between organisms, and in some cases, between species. Prions are found in both mammals and yeast, though the protein determinants of mammalian and yeast prions are not the same. However, mammalian and yeast prions do share several features, including their amyloid fold structure, the mechanism of formation, and their ability to exist in several distinct conformations. Unlike mammalian prions, yeast prions are not toxic to the cell and instead may play a functional role. Given their mechanistic similarities, investigating yeast prions has proven beneficial to the understanding of mammalian prion propagation, infectivity, and strain formation.

Mammalian Prions

The mammalian prion protein, PrP\textsuperscript{C}, is a glycosylphosphatidyl-inositol-linked cell surface protein expressed mainly in the central nervous system.\textsuperscript{90} PrP can exist in two states, the soluble PrP\textsuperscript{C} state and the insoluble, protease-resistant aggregate PrP-res.\textsuperscript{91} The primary structure of the constitutive PrP\textsuperscript{C} is mostly α-helical with the N-terminal domain highly unstructured and containing an octapeptide-repeat region that contributes to aggregate formation.\textsuperscript{92} The aggregated PrP-res is similar to other amyloids, being highly β-sheet rich and able to bind congo red and thioflavin T.\textsuperscript{93} Recombinant PrP can form amyloid fibers under acidic conditions, perhaps alluding to conditions in the lysosome which might catalyze PrP aggregation as it travels through the secretory pathway. Interestingly, aggregated recombinant PrP or PrP-res isolated from the brains of infected animals can be used to nucleate PrP\textsuperscript{C} aggregation in an uninfected animal.

Prion populations that differ in their phenotypic or physiochemical properties are known as prion strains. Prion strains were first characterized by differences their incubation time and neuropathology.\textsuperscript{94} Prion strains have been identified in scrapie-infected sheep and goats, cattle, and humans. Differences
in prion strains can be characterized by differences in proteinase K sensitivity and cleavage pattern, stability toward denaturing agents, and glycosylation pattern. Indeed, prion strains resistant to one drug but not another could “evolve” to be resistant to the second drug suggesting that a heterogeneous population of prion conformations simultaneously exists or prion conformations are not necessarily irreversible and can continue to change.

1.32 Yeast Prions

Similar to the mammalian prion protein, some proteins in yeast can exist both in their soluble form and in a self-propagating aggregate called a yeast prion. The characterized prions in yeast are not toxic, but, instead, are epigenetically inherited, self-replicating, protein-only modes of information transfer (Figure 1.1). Prions exist in several species of yeast and are
Figure 1.1 Yeast prions are inherited epigenetically. Mating a [PRION+] haploid to a [prion-] haploid results in [PRION+] diploids. The aggregated prion proteins in the [PRION+] haploid template the monomeric prion proteins in the [prion-] haploid, resulting in aggregated prion proteins in the diploid. These aggregates are then cytoplasmically inherited by all four haploid progeny following meiosis. Thus, prion inheritance is an epigenetic, non-Mendelian mechanism.
thought to be evolutionarily conserved.\textsuperscript{98, 99} Although each prion is formed from a distinct determinant protein, heterologous interactions between the prions have been demonstrated.\textsuperscript{100-103}

Yeast prions share several characteristics that are used as criteria when identifying a new yeast prion. One is that yeast prions are reversibly curable.\textsuperscript{104} Reversible curability means that colonies can switch back and forth between the [prion-] and [PRION+] states in the absence of an associated genetic change. Secondly, yeast prions can be induced by overexpressing the protein determinant of that prion.\textsuperscript{104} Finally, prion formation of a protein mimics a null mutation of that protein.\textsuperscript{104} In that regard, aggregation of yeast prion proteins acts as a molecular switch to turn off and on the function of that protein. Additionally, prion proteins are composed of modular domains, one of which is the prion-forming domain. This domain is required for prion formation and propagation and is separable from the domain required for protein function.\textsuperscript{105, 106}

Yeast prions also share structural and mechanistic characteristics with disease-related amyloids despite vast difference in protein primary sequence. For instance, structural studies of several yeast prions suggest that the aggregated form is β-sheet rich and forms amyloid fibers in vitro, similar to PrP, Aβ, polyQ, and other amyloidogenic proteins.\textsuperscript{5} Thus, yeast prions have proven valuable as a model system to understand the effects of cellular and genetic factors on amyloid formation. One key difference between yeast prions and other amyloids is that yeast prions are maintained and actively propagated. The mechanism of prion aggregation must generate propagatable species that can be transmitted to progeny during mitosis. These propagatable species are called propagons and are proposed to be soluble, amyloid oligomers that maintain the prion conformation.\textsuperscript{107, 108} To generate these propagons, the large amyloid fibers need to be fragmented. The disaggregate chaperone Hsp104 is proposed to fragment the large prion aggregates and, as such, most of the known yeast prions require Hsp104 for propagation.\textsuperscript{109} In concert with Hsp104, yeast prions are also regulated by various Hsp70 and Hsp40 chaperones. An interesting hypothesis, then, is that environmental factors indirectly regulate prion propagation via molecular chaperones.

Another interesting phenomenon associated with yeast prions is the ability of the same primary sequence to adopt multiple prion conformations, known as prion variants.\textsuperscript{110, 111} Prion variants are similar in theory to mammalian prion strains. Prion variants have been described for at least three separate
yeast prions and have been shown to be structurally and functionally distinct. Prion variants also respond to chaperone levels differentially and may be preferentially selected for given a certain set of environmental or cellular conditions.

[PSI+]

The [PSI+] prion results from the aggregation of the translation termination factor, Sup35. Sup35 (eRF3) is a GTPase that interacts with Sup45 (eRF1) to form a translation termination complex to recognize termination codons during translation. Sup35 is composed of three modular domains termed the N domain, the M domain and the C-terminal domain. The C-terminal domain contains the GTPase domain, is mostly α-helical, and alone, is sufficient to provide the essential function of Sup35. The N domain of Sup35 constitutes the prion-forming domain of Sup35. The N domain contains a glutamine and asparagine (Q/N) rich region encompassing residues 1-40, which is crucial for nucleation of Sup35 aggregates, though some studies suggest that the sequence is less important than the amino acid composition of the domain. This suggests that proteins with Q/N rich regions, regardless of sequence similarity, might also form amyloid. Residues 41-97 of the N domain are composed of 5 and a half repeating units of the peptide (P/QGQGGYQQ/SYN) which resemble the five “octarepeat” sequences (PHGGGWGQ) present in the amino terminus of PrP. Truncation or replacement of these repeats destabilizes or eliminates [PSI+]. The M domain is highly charged, and confers stability and structure to the N domain. Structural studies suggest that while the C-terminus of Sup35 is folded, the N-terminus is unstructured allowing it to better adopt the prion conformation.

Recombinant Sup35, like other amyloid proteins, can form amyloid fibers in vitro. Both the PFD of Sup35, Sup35NM, and full-length Sup35 form fibers that are resistant to SDS, bind thioflavin T, and can be seeded by the addition of preformed fibers supporting a nucleated conformational conversion model. Analysis of Sup35 peptides suggests that these amyloid fibers are composed of parallel β-sheets. Additionally, amyloid fibers of recombinant Sup35 can be introduced into [psi-] cells to generate [PSI+] cells. Hence, amyloid fibers formed from recombinant Sup35 are infectious and can provide insight into the characteristics of infectious amyloid species.
The \([PSI+]\) prion is important to study not only for its structural implications, but also because of the unique function and phenotype it confers in cells. Since the \([PSI+]\) prion is composed of Sup35, the function of the translation termination complex is intimately connected to the presence of \([PSI+]\).\(^{132}\) When cells are \([psi-]\), Sup35 is monomeric and functional in efficient translation termination. On the other hand, when cells are phenotypically \([PSI+]\), Sup35 is aggregated and unable to fully interact with Sup45 causing an increase in nonsense suppression. Increased nonsense suppression occurring in the presence of \([PSI+]\) can result in translation of normally silent regions of the genome.\(^{133}\) \([PSI+]\) cells have been shown to have an advantage over \([psi-]\) cells while growing in certain conditions, presumably because the \([PSI+]\) state offers variation through increased nonsense suppression.\(^{134}\) This advantage is a fine balance though, as overexpression of Sup35 in \([PSI+]\) cells leads to toxicity because the increase in Sup35 aggregation sequesters Sup45 resulting in significant defects in translation termination.\(^{135}\) Thus, the \([PSI+]\) prion may be beneficial for cell adaptability and survival, but regulation of the prion phenotype is integral to preventing toxic gain-of-function.

Both intragenic and extragenic factors regulate Sup35 aggregation in vivo. Mutations in the PFD can destabilize or inhibit \([PSI+]\) while some mutations or truncations have no effect. Additionally, the propagation of \([PSI+]\) is regulated by chaperones, specifically, Hsp104.\(^{109}\) As with all yeast prions, \([PSI+]\) is cured when Hsp104 is deleted.\(^{109}\) Uniquely, \([PSI+]\) is the only prion that is also cured by the overexpression of Hsp104 suggesting that \([PSI+]\) is extremely sensitive to changes in Hsp104 activity or expression.

Variants of \([PSI+]\) were initially identified based on their functional and phenotypic differences. Two variants of \([PSI+]\) have previously been described that display distinct levels of nonsense suppression. Strong variants of \([PSI+]\) are so named because they display high levels of nonsense suppression which correlates with a very low amount of soluble Sup35.\(^{110}\) On the other hand, weak variants of \([PSI+]\) exhibit less nonsense suppression correlative with an increased pool of soluble Sup35 (Figure 1.2).\(^{136}\) When cells carrying strong and weak variants of \([PSI+]\) are mated, the progeny are strong \([PSI+]\) suggesting that strong variants of \([PSI+]\) out-compete weak variants for the available monomer pool and thus propagate more efficiently.\(^{137}\) Additionally, amyloid fibers formed in vitro have provided valuable insight about the structural characteristics of prion variants. Several studies have shown that
varying the temperature at which amyloid is assembled affects the conformation of the fiber formed as well as the resulting [PSI+] phenotype when introduced into [psi-] cells. Structural variants of Sup35 that contain a short amyloid core (~40 amino acids) display faster kinetics of aggregation, are less thermostable, and generate mostly strong [PSI+] variants when transformed into [psi-] cells. Sup35 structural variants that contain a long amyloid core (~70 amino acids) display slower aggregation kinetics, are highly thermostable, and generate mostly weak variants of [PSI+] when introduced to [psi-] cells. Thus, a hypothesis developed whereby less stable strong variants of [PSI+] are more easily fragmented and able to template monomer such that strong [PSI+] variants can out-propagate weak [PSI+] variants. This recent hypothesis is still being investigated, but does suggest that Hsp104 and other chaperones may play an important role in [PSI+] variant selection and propagation.

[RNQ+]

The [RNQ+] prion is formed from the aggregation of the protein Rnq1 whose monomeric function in the cell is still unknown. Rnq1, like Sup35, can be divided into modular domains. The N-terminal domain is folded with function unknown. The C-terminal domain is Q/N rich and is necessary and sufficient for [RNQ+] formation. Rnq1 was initially identified as a prion candidate by a BLAST search for Q/N rich proteins that were also able to aggregate in vivo. Like [PSI+], [RNQ+] is also dependent on Hsp104 as deletion of Hsp104 cures [RNQ+]. [RNQ+] is also dependent on the Hsp40 chaperone Sis1. Although the monomeric Rnq1 has no known function, the [RNQ+] prion has a function as [PIN+] or a “[PSI+] inducible” element. The [PIN+] hypothesis suggests that prions facilitate the appearance of other prions via heterologous templating. Thus, as [PIN+], the [RNQ+] prion can template monomeric
Figure 1.2 [PSI+] exists in conformationally distinct states. Strong and weak [PSI+] variants are conformationally distinct and can be distinguished by differing amounts of soluble Sup35. The amount of nonsense suppression is directly related to the amount of soluble Sup35, resulting in decreased nonsense suppression associated with weak [PSI+] variants.
Sup35 aggregation and induce the \([PSI^+]\) phenotype. Interestingly, the \([PSI^+]\) prion can also induce the formation of \([RNQ^+]\) as can the \([URE3^+]\) prion.\(^{101}\) Therefore, the \([RNQ^+]\) prion is a component of a complicated and interconnected prion network that is its own regulatory mechanism.

Definitive proof for Rnq1 as a prion protein was demonstrated when recombinant Rnq1 was shown to form amyloid fibers in vitro and those fibers were capable of inducing \([RNQ^+]\) formation when transformed into \([rnq^-]\) cells.\(^{139,143,144}\) In fact, pre-formed fibers of Rnq1 amyloid can seed Rnq1 monomer demonstrating that alter Rnq1 amyloid formation follows the nucleated conformational conversion model.\(^{139}\) Similar to Sup35, mutations or truncations of both the PFD and the N-terminal non-prion domain affect Rnq1 aggregation and regulate \([RNQ^+]\) formation.\(^{145,146}\) Changes in the amino acid sequence not only regulate \([RNQ^+]\) formation, but also the ability of \([RNQ^+]\) to template Sup35.\(^{147,148}\)

Not only do mutations affect the function of \([RNQ^+]\), but changes to the conformation in the absence of mutations also differentially affect \([PSI^+]\) induction. Several variants of \([RNQ^+]\) were identified and characterized by their level of \([PSI^+]\) induction. These variants termed low, medium, high, and very high \([RNQ^+]\) for their ability to induce \([PSI^+]\) have also been differentiated by their ability to form fluorescent foci.\(^{101,137,149}\) Two fluorescent patterns were exhibited, multi-dot and single-dot fluorescence. The low, medium, high, and very high variants of \([RNQ^+]\) demonstrated single-dot fluorescence while multi-dot fluorescence was characteristic of only the high \([RNQ^+]\) variant. These variants also display differences in stability and the amount of soluble Rnq1.\(^{137,150}\)

1.4 Hsp104, an AAA+ ATPase Chaperone

Hsp104 is a AAA+ ATPase molecular chaperone in Saccharomyces cerevisiae. Hsp104 is a member of the ClpA/ClpB family of proteins, chaperones that function in protein disaggregation and degradation and is homologous to the bacterial chaperone ClpB.\(^{151}\) The heat shock protein Hsp104 is upregulated in response to stress and mediates protein disaggregation following heat shock.\(^{151,152}\) However, the disaggregase Hsp104 does not prevent protein aggregation, nor does it promote proteolysis of protein aggregates like ClpA.\(^{152}\) Instead, Hsp104 acts more like Hsp70 in that it modulates resolubilization of proteins from insoluble aggregates.\(^{152}\) Additionally, as already mentioned, Hsp104 plays an essential role in prion propagation.\(^{109}\) All known yeast prions require Hsp104 for propagation.\(^{109}\)
The pre-eminent hypothesis for the mechanism of Hsp104 action is that Hsp104 disaggregates the prion aggregates to generate the propagons for further propagation.  

In order to perform the disaggregation function, Hsp104 forms a hexamer in response to ATP binding. The hexameric state of Hsp104 can also be induced by high concentration of Hsp104 and high salt concentrations. The structure of Hsp104 was originally modeled off the structure of ClpB determined by X-ray crystallography and cryo-EM (Figure 1.3). Hsp104 is composed of five functionally distinct domains including two nucleotide-binding domains (NBD1 and NBD2) connected by a coiled-coil linker domain and flanked by N-terminal and C-terminal domains. The crystal structure of ClpB suggested that the hexamer was a three-tiered structure (the N-terminal domain, NBD1, and NBD2) with the middle domain projecting from the body of the hexamer. Despite debate over the position of the middle domain, a recent cryo-electron microscopy structure of Hsp104 strongly suggested its placement on the outside of the hexamer. As an AAA+ ATPase, Hsp104 binds and hydrolyzes ATP to generate energy for disaggregation but must form a hexamer to do so. Only ATP is capable of being hydrolyzed by Hsp104 and ADP inhibits ATP hydrolysis. Despite independent functions for each domain, all of the domains cooperate to ensure synchronization of the hexamer subunits for efficient disaggregation.

**The N-terminal Domain (NTD)**

The NTD of ClpB (residues 1-150) is mostly α-helical and highly mobile. Deletion of the NTD of ClpB was reported to maintain the same level of ATP hydrolysis and disaggregation activity as the full-length ClpB and was suggested to be unnecessary for ClpB function. However, additional studies demonstrated that deletion of the ClpB NTD prevented maximum disaggregation activity, specifically when co-chaperones were limiting suggesting that the NTD of
Figure 1.3 Modeling of hexameric Hsp104 from the cryo-EM structure. The cryo-EM model of Hsp104 (EMDatabank accession number 1630) was used to model the domains of Hsp104. Three tiers can be observed in this model corresponding to the NTD (green residue), NBD1 (blue residue), and NBD2 (red residue). The coiled coil linker domain is marked by a purple amino acid, and extends away from the body of the hexamer. The CTD is marked by a residue colored yellow and sits on the C-terminal face of Hsp104. This model was kindly provided by Dr. Francis Tsai for elucidation of Hsp104 mutants characterized in this thesis.
ClpB may provide a site of interaction for co-chaperones and may enhance disaggregation activity.\textsuperscript{161} Furthermore, mutants in the NTD of ClpB that cross-linked the NTD to the body of the hexamer to restrict mobility of the NTD reduced the disaggregation activity, substrate threading, and substrate-stimulated ATPase activity.\textsuperscript{162} Several studies have demonstrated interactions between the ClpB NTD and substrates,\textsuperscript{163} especially strongly aggregated proteins.\textsuperscript{164} Thus, the NTD of ClpB is important for interacting with both substrates and co-chaperones for efficient protein disaggregation.

In contrast, the role of the NTD in the disaggregation mechanism of Hsp104 is much less clear. Deletion of the NTD of Hsp104 does not affect prion propagation or thermotolerance.\textsuperscript{165, 166} Additionally, mutations in the NTD or deletion of the NTD can enhance [PSI+] propagation and can compensate for [PSI+] propagation defects in the presence of a mutant Hsp70.\textsuperscript{165} Thus, the NTD of Hsp104 may be similar to that of ClpB in that it enhances disaggregation activity to ensure prion propagation and is a site of interaction for Hsp70s. Interestingly, deletion of the NTD does not support elimination of [PSI+] by excess Hsp104.\textsuperscript{165} Moreover, structural studies of Hsp104 suggest that deletion of the NTD results in expansion of the central channel possibly suggesting that the NTD plays a role in preventing non-specific polypeptide binding in the channel of Hsp104.\textsuperscript{157} Therefore, despite being unnecessary for Hsp104 function, the NTD clearly still plays a role in prion and non-prion substrate disaggregation and may instead serve as a “backup” to mediate efficient disaggregation.

**Nucleotide-binding Domains (NBD1 & NBD2)**

As a member of the Hsp100/Clp subfamily of AAA+ ATPases, the energy for the protein disaggregation mechanism for Hsp104/ClpB is provided by ATP hydrolysis.\textsuperscript{167} Hsp104/ClpB has two NBDs, that in the ring-shaped hexamer, are stacked on top of each other. The ATP hydrolysis mechanism can be generally described in a few over-simplified steps resulting from numerous studies examining kinetics of several Hsp104 mutants. Within a single subunit in the Hsp104 hexamer (1) ATP binds to NBD2 and stabilizes the hexamer (2) generating a conformational change that is propagated through the middle domain to NBD1 where (3) ATP now binds while (4) ATP is hydrolyzed at NBD2 releasing ADP to (5) reverse the conformational change promoting (6) ATP hydrolysis at NBD1 to complete the cycle.\textsuperscript{168} This cycle is synchronous in the hexameric Hsp104 so that at any one time,
multiple individual subunits in the hexamer are hydrolyzing ATP. From this general model of the ATP hydrolysis mechanism, independent functions for each NBD can be recognized. For example, ATP binding and hydrolysis at NBD2 was thought primarily to promote hexamerization and provide the energy needed for the conformational change rather than significantly adding to the catalytic energy. Thus, the binding affinity of NBD2 for ATP is much higher than that of NBD1. Conversely, ATP hydrolysis at NBD1 provides the majority of the catalytic energy required for disaggregation.

NBDs are very highly conserved and have several distinct domains important for ATP hydrolysis. The Walker A and Walker B motifs contain residues that directly interact with ATP. The Walker A motif consists of the consensus sequence GXXXXGKT/S (where X is any amino acid) and the lysine (K) residue in this motif is essential for ATP binding and hydrolysis. In Hsp104, mutation of these lysines, K218T and K620T, results in loss of ATP binding and hydrolysis at that domain. The double mutant, K218T/K620T, mimics a deletion of Hsp104. The K218T mutation results in loss of function at NBD1 without altering the ability to oligomerize while the K620T mutation displays a corresponding oligomerization defect. Both of these mutations inhibit thermotolerance and [PSI+] propagation. The Walker B motif also plays a role in ATP binding. The glutamate residue in the Walker B consensus sequence, hhhhDE where h is a hydrophobic residue, is important for ATP hydrolysis. Mutation of these residues in Hsp104 (E285Q/A and E687Q/A) does not prevent ATP binding but inhibits ATP hydrolysis resulting in the commonly called “substrate trap” because substrates are bound but unable to be released. The E285Q/A mutation is especially interesting because it displays 300% ATPase activity of wild type supporting the hypothesis that NBD2 is a major contributor to the catalytic energy.

Another conserved set of domains in the NBDs are the sensor 1 and sensor 2 domains. These domains participate in ATP hydrolysis by interacting with the γ-phosphate of ATP. The sensor 1 domain contains a highly important polar residue that when mutated, impairs ATP hydrolysis. In Hsp104, mutation of these polar residues, T317A in NBD1 and N728A in NBD2, both impair ATP hydrolysis and have been used to study the kinetics of the individual NBDs. Both mutations inhibit [PSI+] propagation and thermotolerance though N728A displays a more severe effect. Thus, these mutations can impair ATP hydrolysis without impairing ATP binding. The sensor 2 motif contains a conserved arginine residue
that is also important for ATP binding and hydrolysis.\textsuperscript{167} Mutation of the arginine residue in Hsp104 NBD2, R826M, impairs ATP binding as well as prevent [PSI+] propagation and thermotolerance.\textsuperscript{175} Mutations in these two domains highlight the importance of ATP hydrolysis for the function of Hsp104. In addition to the conserved arginine residue in the sensor 2 domain, another set of conserved arginine residues are also extremely important for ATP hydrolysis and hexamer formation in response to ATP binding. These arginine residues known as “arginine fingers” extend from one subunit into the ATP binding domain of the neighboring subunit and coordinate ATP binding.\textsuperscript{167, 176} Mutation of these arginine residues impairs ATP hydrolysis and hexamer formation.\textsuperscript{176} In fact, accurate positioning of the arginine fingers has been used to validate structural models of Hsp104 as it has been shown that arginine finger residues are essential for ATPase and chaperone activities.\textsuperscript{158, 177}

The purpose of ATP hydrolysis is to drive the disaggregation mechanism of Hsp104, and therefore, both the ATPase activity and disaggregation should be coupled for maximum efficiency. Several studies have shown that substrate binding is dependent on ATP binding at NBD1 and NBD2 though more strictly regulated by NBD1.\textsuperscript{174, 178-180} As expected, then, the release of substrate is triggered by ATP hydrolysis, coupling the two cycles.\textsuperscript{174, 178, 180, 181} This coupling is optimized within the hexamer such that asymmetry of ATP binding and hydrolysis among the NBDs in the six subunits promotes continuous substrate binding and processing to prevent substrate release and incomplete disaggregation.\textsuperscript{170, 182} Additionally, substrate binding or interaction stimulates ATP hydrolysis of Hsp104/CbpB, perhaps to ensure efficient processing of the substrate.\textsuperscript{168, 183} To further complicate the disaggregation mechanism, ATP hydrolysis and substrate binding and release are also coupled to co-chaperone interactions which enhance Hsp104/CbpB functions.\textsuperscript{166, 184} Thus, regulating substrate binding and ATP hydrolysis provides optimal disaggregation activity and provides the foundation for threading of substrates as the primary method of disaggregation.

**Substrate Threading: A Mechanism of Disaggregation**

The model of the hexameric structure of Hsp104/CbpB displays a narrow channel running through the center of the hexamer that is open at both the N- and C-terminal faces of the hexamer.\textsuperscript{156-158} This channel is a conserved structural feature among the Clp proteins and Hsp100s.\textsuperscript{172, 185} The use of this
channel in protein disaggregation is most apparent in the disaggregation mechanisms of ClpA and ClpX which bind to the protease ClpP at the C-terminus to degrade substrate proteins. Translocation of substrates through the central channel positions the substrate in the protease for degradation. The application of the threading model to non-degrading chaperones like Hsp104/ClpB was made possible by the identification of conserved pore loops that did not affect hexamerization or ATP hydrolysis, but prevented aggregate refolding. These channel loops contain the conserved domain sequence GYVG where the tyrosine residue is specifically important. Mutation of the conserved tyrosine residue in these loops prevents thermotolerance by Hsp104. These loops are deep in the axial channel and function to bind the substrate and translocate the substrate through the channel through a series of conformational changes coupled to ATP binding and hydrolysis. The loops in the channel act like levers, flipping up and down in the channel to move the aggregate down to the next set of lever-like loops. Asymmetrical loop function among the hexamer subunits ensures that the substrate is constantly engaged in the channel to prevent loss of interaction between the chaperone and substrate. The disaggregation mechanism of threading was further verified for Hsp104 and ClpB when a tripeptide sequence encoding an interaction domain for ClpP was introduced into the C-terminal domain of Hsp104 and ClpB such that any substrates threaded through the central channel would be degraded by ClpP. These chimeras, termed HAP for Hsp104 and BAP for ClpB, have also been coupled with the “substrate trap” mutation to identify aggregates that get trapped in ClpP, suggesting that these aggregates are disaggregated by the threading mechanism. Use of the HAP chimera identified Sup35 from [PSI+], but not [psi-], cells as a threading substrate of Hsp104. Indeed, mutation of the pore loops either reduces or inhibits [PSI+] propagation. These data suggest that Hsp104 propagates the prion by extraction of Sup35 molecules from the aggregate.

The Middle Domain (M-domain)

The M-domain of Hsp104, composed of residues 412-532, is a coiled-coil domain inserted between NBD1 and NBD2 and is not well conserved among Hsp100/Clp chaperones. In fact, the presence of this domain loosely correlates with a function in protein disaggregation. Insertion of the coiled-coil domain into the C-terminal region of NBD1 is often limited to members of the Hsp100 family.
that function in protein disaggregation. The position of this domain in relation to hexameric Hsp104 is still unclear as multiple structural models have been proposed which show vastly different placement for this domain.\textsuperscript{156,157} From the cryo-EM reconstruction of hexameric ClpB, the M-domain was suggested to project from the body of the hexamer similar to blades in a propeller.\textsuperscript{156} Subsequent studies positioned the M-domain inside the hexamer, intercalated between the NBDs of neighboring subunits and potentially contacting substrates as they were threaded through the central channel.\textsuperscript{157} However, this model also resulted in the repositioning of the canonical arginine fingers. More recent data examining the role of the arginine fingers demonstrated that the structure that correlates best with Hsp104 function is one in which the M-domain is highly mobile and solvent-exposed.\textsuperscript{197} Furthermore, examination of M-domain residues by H/D exchange and cross-linking suggest that the M-domain interacts with NBD1 of the neighboring subunit by nestling into the body of the hexamer.\textsuperscript{198}

The model of Hsp104 function, as discussed above, suggests that the M-domain is responsible for driving hydrolysis at NBD1 through an intrasubunit signal transduction pathway.\textsuperscript{168} When peptide binds to the C-terminal domain, ATP binding and hydrolysis at NBD2 induces a conformational change in the M-domain resulting in an increase in ATP binding at NBD1.\textsuperscript{168} The peptide used for these studies was lysine-rich, a region proposed to be important for normal Hsp104 substrate recognition. As such, these data suggest that the M-domain is responsible for coupling substrate binding and ATP hydrolysis. This mechanism has important implications for disaggregation activity as deletion of the M-domain or specific mutations can abrogate protein disaggregation.\textsuperscript{172,177,192,193}

Recent evidence suggested that M-domain cycles between distinct conformational states which regulate the function of Hsp104/ClpB.\textsuperscript{198-200} In the repressed state, the M-domain interacts with the neighboring NBD1 through electrostatic interactions.\textsuperscript{198,199} Disruption of these interactions is proposed to cause the M-domain to move away from the body of the hexamer and become accessible to co-chaperones, though how the conformation switch is governed is still unknown.\textsuperscript{198,199,201} Mutations which prevent this interaction result in Hsp104 hyperactivity and are toxic to cells, especially under stressful conditions.\textsuperscript{198,199} In contrast, mutations that stabilize the interaction of the M-domain with the neighboring NBD1 repress the function of Hsp104.\textsuperscript{198,199} Though the mechanism of how the M-domain regulates
distinct activities of Hsp104 is still relatively unclear, data have shown that regulating this interaction is essential not only for Hsp104 function, but also for cell viability.\textsuperscript{198, 199, 201}

**The C-terminal Domain (CTD)**

The CTD of Hsp104 is by far the least understood domain of Hsp104. For the Clp chaperones that couple disaggregation activity with the protease activity of ClpP (ClpA and ClpX), the CTD is the interface and interaction site for ClpP. The residues that encompass the ClpP binding site (IGF) have been added to the CTD of Hsp104/ClpB to couple the protease to these chaperones indicating that the CTD is the “exit” site for disaggregated polypeptides.\textsuperscript{173, 195} However, the CTD has been proposed to be a substrate binding site.\textsuperscript{168} Additionally, the Hsp104 CTD contains an acidic motif (IDDDLD) at the extreme C-terminus, which resembles the C-terminal cofactor binding motifs characteristic of eukaryotic Hsp70 and Hsp90 (VEEVD and MEEVD).\textsuperscript{202} Through these domains, Hsp70 and Hsp90 interact with proteins that contain TPR domains suggesting that the same may be true for Hsp104.\textsuperscript{203, 204} To this end, Hsp70- and Hsp90-interacting proteins, Sti1 and Cpr7, bind to the CTD of Hsp104.\textsuperscript{205-207} In addition, deletion of the CTD abrogates Hsp104/ClpB hexamerization and some mutations can cause defects in thermotolerance.\textsuperscript{160, 202, 208}

**The Effect of GdHCl**

One of the agents that causes conversion of [PSI+] cells to [psi-] is the chaotropic agent, guanidine hydrochloride (GdHCl).\textsuperscript{209} While this effect was first demonstrated with the [PSI+] prion, it was later shown to be a common property of all yeast prions.\textsuperscript{104, 123, 139, 209} As GdHCl cures at only millimolar concentrations and after only 12-16 generations of cell growth, GdHCl is a potent inhibitor of prion propagation.\textsuperscript{209, 210} Growing [PSI+] cells in media containing GdHCl does not affect existing Sup35 aggregates and does not prevent new Sup35 from joining Sup35 aggregates.\textsuperscript{211, 212} However, GdHCl does prevent the formation of new [PSI+] propagons suggesting that this prion-curing agent may inhibit fragmentation of Sup35 aggregates. Indeed, GdHCl was shown to specifically inhibit the ATPase activity of Hsp104.\textsuperscript{213-215} A mutation of the NBD1 residue D184 can prevent GdHCl curing of [PSI+] cells suggesting that GdHCl may act by directly binding and inhibiting Hsp104 activity.\textsuperscript{214} This mechanism has

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recently been further clarified by demonstrating that GdHCl exerts dual effects on Hsp104. First, GdHCl inhibits Hsp104 by stabilizing the M-domain interaction with the hexamer and preventing efficient Hsp70 interaction. Secondly, GdHCl binding to Hsp104 prevents continuous ATP turnover by local conformational changes in the GdHCl binding site. On the other hand, there is data that GdHCl affects [PSI+] independent of Hsp104 suggesting that curing of [PSI+] by GdHCl may occur by several independent methods and clearly still needs some investigation.

**Hsp104 is essential for yeast prion propagation**

As mentioned previously, most of the known yeast prions are dependent on the function of Hsp104 as deletion or inhibition of Hsp104 prevents propagation of these yeast prions. As the essential regulator of yeast prions, much investigation has focused on the mechanism of Hsp104 interaction with prions. Furthermore, numerous mutations in all domains of Hsp104 have been characterized which affect prion propagation by modifying various Hsp104 activities. Two different models have been proposed to explain the role of Hsp104 in prion propagation that are rooted in the ability of Hsp104 to remodel protein aggregates.

The first model hypothesizes that Hsp104 is involved in remodeling prion protein monomers to catalyze formation of the amyloid conformation. The molecular basis of this mechanism is unclear, but Hsp104 could promote the amyloid conformation by unfolding the monomer via the threading mechanism. The unfolded monomer would then be primed for refolding into the altered, amyloid conformation. Kinetically, Hsp104 would reduce the transition energy required to form the amyloid nucleus or enhance joining to pre-existing aggregates. This model is supported by mainly in vitro data that showed recombinant Hsp104 catalyzed the formation of amyloid fibers of recombinant Sup35NM. This conclusion was made based on the observation that addition of Hsp104 shortened the lag phase of Sup35NM fiber formation. An alternative conclusion from this observation is that Hsp104 remolds off-pathway aggregates, which allows more efficient amyloid fiber formation. In fact, Hsp104 interacts with Sup35 but only in the [PSI+] state; an interaction between Hsp104 and monomeric Sup35 in vivo has yet to be detected. Moreover, recombinant prion protein can form amyloid fibers in the absence of Hsp104 in vitro.
The prion fragmenting hypothesis suggests that Hsp104 fragments large SDS-resistant aggregates to generate oligomeric propagons. In order to efficiently propagate, prions need to be dissociated to maintain a pool of propagons that are able to be transmitted to budding cells.\textsuperscript{222} If fragmentation does not occur, prion aggregates would both increase in size and reduce in number impairing their ability to be inherited by daughter cells.\textsuperscript{222} The disaggregation activity of Hsp104 is the ideal feature to generate propagons to ensure prion propagation. Several studies report data that supports this model as the primary role for Hsp104 in prion propagation.\textsuperscript{136, 153, 210, 213, 223} First, inhibition of Hsp104 results in an increase in aggregate size of Sup35.\textsuperscript{108, 136} As discussed, addition of GdHCl to [\textit{PSI+}] cells inhibits Hsp104 and cures [\textit{PSI+}].\textsuperscript{210, 213} [\textit{PSI+}] cells treated with GdHCl exhibit larger Sup35 aggregates, suggesting that GdHCl inhibition of Hsp104 prevents fragmentation of Sup35 aggregates.\textsuperscript{136, 153} Additionally, [\textit{PSI+}] cells grown in media containing GdHCl also decrease in propagon number as cells divide, further supporting a role for Hsp104 in prion fragmentation.\textsuperscript{136, 222} Furthermore, studies examining how inhibition of Hsp104 affects Sup35-GFP aggregation suggest that Hsp104 does not affect addition of the monomer to the aggregate, but rather, remolds existing Sup35-GFP [\textit{PSI+}] complexes to promote transmission.\textsuperscript{153} Finally, addition of recombinant Hsp104 to Sup35 amyloid fibers in vitro has demonstrated that Hsp104 is capable of disaggregating these fibers.\textsuperscript{219-221, 224, 225} Though there is some debate about how disaggregation of in vitro-made amyloid fiber occurs, these studies further support the prion fragmentation mechanism and offer new insight into the structural components of this mechanism.\textsuperscript{219-221, 224, 225}

Mutational analysis of Hsp104 suggests that optimal activity of all of the distinct properties of Hsp104, hexamer formation, ATP hydrolysis, and substrate translocation, is required for efficient prion propagation\textsuperscript{172, 218}. In general, inhibition of any one of these activities often results in prion loss. For example, the NBD mutations K218T/K620T, which abrogate ATP hydrolysis and hexamer formation, also inhibit prion maintenance and do so in the presence of wild type.\textsuperscript{109, 152} The N728A mutation, which prevents ATP hydrolysis but not hexamerization, cures [\textit{PSI+}].\textsuperscript{175, 226} Additionally, mutation of the pore loop tyrosine residues, Y257A and Y662A show a differential effect where Hsp104-Y257A only mildly destabilizes [\textit{PSI+}], while Hsp104-Y662A cures [\textit{PSI+}] cells.\textsuperscript{165, 195} Whole domain replacement with the corresponding domains from ClpB showed that NBD1, NBD2, and the M-domain are required for prion
propagation while the NTD and CTD could be deleted or replaced with no effect on \([PSI^+]\) propagation.\textsuperscript{165, 166}

Strikingly, characterized mutations that inhibit thermotolerance also inhibit prion propagation, with a few exceptional mutations in the CTD.\textsuperscript{172, 218} On the other hand, mutations that inhibit prion propagation do not always inhibit thermotolerance, suggesting that the disaggregation mechanism for prion substrates and non-prion substrates is distinct.\textsuperscript{172, 218} Additionally, these data suggest that the amount of disaggregation activity required for thermotolerance is not as strict as for prion propagation. Slight perturbations in Hsp104 function can have extreme effects on prion maintenance while having little to no effect on thermotolerance. Indeed, recent data has shown that disordered aggregates like the type that result from heat shock require less cooperative activity to be resolved than do ordered, prion aggregates.\textsuperscript{170} This study also shows that ClpB requires more cooperative activity among the hexamer to disaggregate disordered substrates than does Hsp104, perhaps demonstrating a reason why ClpB cannot substitute for Hsp104 in prion propagation.\textsuperscript{170} Thus, substrate stability and structure are contributing factors in the ability of Hsp104 to efficiently disaggregate a particular substrate.

Due to the conformational variation characteristic of individual yeast prions, one might predict that not all yeast prions are similarly regulated by Hsp104. One very interesting example is that while deletion of Hsp104 cures most of the known prions, only \([PSI^+]\) is cured by overexpression.\textsuperscript{109, 139, 217} The mechanism of \([PSI^+]\) curing by Hsp104 overexpression is still unclear as is the reason why only \([PSI^+]\) is affected. One hypothesis for the mechanism of \([PSI^+]\) curing by overexpression of Hsp104 is that an excess of Hsp104 dissolves prion aggregates to monomers.\textsuperscript{227} One observation challenging this hypothesis is that in the presence of excess Hsp104, aggregates of Sup35 increase in size.\textsuperscript{136, 228} This result contradicts the expected results if Hsp104 were to cause hyper-disaggregation of Sup35 aggregates. Another hypothesis, then, is that overexpression of Hsp104 leads to larger, non-transmissible aggregates that are "dead-end" products, though much is still unclear about this mechanism.

To further investigate the underlying cause of the increased sensitivity of the \([PSI^+]\) prion to Hsp104 expression levels, recent data investigates the regions in Sup35 that interact with Hsp104.\textsuperscript{229} These data show that a specific 20 amino acid stretch in the highly charged M domain of Sup35 (129-
148) is a site for Hsp104 interaction. The authors speculate that a region specific for Hsp104 binding, like this stretch found in Sup35, is absent in other prions, though no binding studies of Hsp104 with other prion proteins have been reported. This hypothesis suggests that prions that expose regions of high affinity for Hsp104 are more susceptible to dissociation by Hsp104. Thus, conformational variation among different yeast prions may govern the interactions with Hsp104, and as a result, the ability of Hsp104 to disaggregate individual yeast prions. Indeed, several Hsp104 mutants have been characterized which cure [PSI+] but do not cure [RNQ+], though curiously, they all localize to a similar region of Hsp104.150,230

In addition to the differences of separate yeast prions in their requirements for interaction with Hsp104, individual conformational variants of the same prion also display distinct interactions with Hsp104. For example, a weak variant of [PSI+] with a longer structural core and increased soluble pool compared to a strong [PSI+] variant, is more susceptible to curing by overexpression of Hsp104.108 Additionally, a mutation in NBD1 of Hsp104, E190K, which affects ATPase activity, can differentially affect propagation of the [RNQ+] variants.150 Moreover, a variant of [PSI+] has been characterized, which depends on excess Hsp104 for efficient propagation.231 This variant was identified as an induced [PSI+] variant when Hsp104 and Sup35 were overexpressed simultaneously.231 More recent data compare the effect of Hsp104 on conformational variants of Sup35 aggregates formed at different temperatures in vitro. Amyloid fibers of Sup35 formed at 4°C (NM4) that resulted in mostly strong [PSI+] when transformed into [psi-] cells were more readily remodeled by recombinant Hsp104 than Sup35 aggregates assembled at 37°C (NM37) that result in mostly weak [PSI+] variants after transformation into [psi-] cells.224 Remodeling of NM4 fibers by Hsp104 lead to prion-competent oligomers while the same amount of Hsp104 remodeled NM37 fibers into non-templating structures.224 Additionally, treatment of NM4 fibers with Hsp104 before transformation into [psi-] cells resulted in an increased proportion of resulting strong [PSI+] cells while treatment of NM37 fibers lead to a decrease in cells containing weak [PSI+] variants.224 These data suggest that Hsp104 selectively amplifies certain [PSI+] variants based on its ability to interact with those variants. Thus, not only does activity and expression of Hsp104 affect prion variant propagation, but can also affect induction and selection of specific prion variants.
The role of Hsp104 in asymmetric inheritance

During progressive cycles of cell division, yeast cells accumulate age-related damages that affect protein function, regulation of mitosis, and general cellular function. To prevent these age-related damages accumulating in the budding cell, including carbonylated or oxidatively damaged proteins, from being inherited by the newly generated buds, damaged proteins and other factors are confined to the mother cell in a process called asymmetric inheritance. One factor central to the mechanism of asymmetric inheritance is the sirtuin, Sir2, as deletion of Sir2, sir2Δ cells, results in an increase in the amount of carbonylated protein aggregates in the new buds and a decrease in replicative lifespan. Interestingly, overexpression of HSP104 in sir2Δ cells restores normal replicative lifespan. In fact, hsp104Δ cells also display a decrease in replicative lifespan compared to wild type cells suggesting a role for Hsp104 in promoting damage asymmetry. This process is dependent on the actin cytoskeleton and Hsp104 appears to be part of the network linking the cytoskeleton to the process of asymmetric inheritance. Co-expression of the HAP construct with ClpP displays defects in cell morphology and the actin cytoskeleton, suggesting that cytoskeletal proteins are normal substrates of Hsp104. Indeed, some evidence suggests that Hsp104 interacts with the polarisome complex and the cytokinesis machinery, and that deletion of certain components of both as well as deletion of Hsp104 can result in the symmetric distribution of damaged proteins between the aging mother cell and the new bud. The exact role of Hsp104 in promoting asymmetric inheritance is not yet fully understood but offers a new way to investigate the function of Hsp104 and perhaps add insight to its role in prion maintenance.

1.5 The Chaperone Network: Hsp104, Hsp70, Hsp40

In order to cover the variety of protein misfolding and aggregation issues, cells have evolved an elaborate and highly cooperative network of molecular chaperones. In yeast and bacteria, a bichaperone network composed of Hsp104/ClpB and the Hsp70 system are responsible for processing disordered and stress-induced aggregates as well as highly structured and stable prion aggregates. The Hsp70 system is composed of Hsp70s and Hsp40s in yeast and DnaK and DnaJ in bacteria, each with distinct functions in protein folding and aggregate resolubilization.
**Effects of Hsp40s on yeast prions**

The primary functions of the Hsp40 chaperones are to direct the interaction of Hsp70s with aggregate and misfolded substrates by binding directly to these substrates as well as stimulate ATP hydrolysis in their Hsp70 partners. In yeast, there are 13 Hsp40 proteins and 41 in humans. Hsp40s share a highly conserved J-domain critical for Hsp70 ATPase stimulation. In addition to the J-domains, Hsp40s possess variable extra domains which allow targeting of Hsp70 chaperones to diverse cellular targets. The two most well-characterized Hsp40s in yeast are Ydj1 and Sis1, which each display distinct substrate preferences as well as very different activities on prion propagation. Ydj1 and Sis1 both physically associate with Sup35 aggregates, but only Sis1 is essential for [PSI+] propagation. Additionally, Sis1 is required for [RNQ+] and [URE3] propagation. However, Ydj1 overexpression cures [URE3] and some variants of [RNQ+]. These data suggest opposing models in prion propagation for the two Hsp40s, highlighting the distinct roles each plays in the cell. Additionally, Ydj1 and Sis1 bind Rnq1 when it is in the [RNQ+] state though each has a separate binding site. Thus, one hypothesis is that binding of each Hsp40 to [RNQ+] targets different Hsp70s to the prion resulting in either propagation or curing. Interestingly, Sis1 was recently shown to enhance [PSI+] curing in the presence of overproduced Hsp104. How individual prions are regulated by differential Hsp40 binding and how the Hsp40s have differential effects is still relatively unclear.

**Effects of Hsp70s on yeast prions**

Hsp70s are conserved and essential chaperones that directly facilitate protein refolding by binding to exposed hydrophobic regions of incompletely folded proteins. Binding to these hydrophobic regions typically prevents aggregation and promotes efficient folding. Hsp70 functions by cycling between ATP- and ADP-bound states to bind and release substrates. In the ATP-bound state, Hsp70 has low substrate affinity and rapid exchange while in the ADP-bound state, affinity for substrate is high. Hsp70 has a slow intrinsic rate of ATP hydrolysis which can be stimulated by binding Hsp40s. At one time, Hsp40s present bound substrate to Hsp70 and stimulate Hsp70 ATP hydrolysis to initiate Hsp70 binding. Yeast express six cytosolic Hsp70s, four are the essential Ssa subfamily (Ssa1-4) and two are the ribosome-associated Ssb subfamily (Ssb1 & 2). Efficient Hsp70 function also requires
nucleotide exchange factors (NEFs) to accelerate ADP release and subsequent rebinding of ATP. The major NEFs in yeast are Fes1 and Sse1 (Hsp110).\textsuperscript{250}

The Ssa and Ssb Hsp70s have both been demonstrated to associate with Sup35 in the $[\text{PSI}^+]$ state where Ssa1 is a major component (1 Ssa1:2 Sup35),\textsuperscript{240} however, the two chaperones demonstrate opposite effects on the $[\text{PSI}^+]$ prion. For example, increased levels of Ssa1 prevent $[\text{PSI}^+]$ curing by excess Hsp104 and with some variants of $[\text{PSI}^+]$, increase phenotypic expression.\textsuperscript{251} Conversely, excess Ssb1 increases $[\text{PSI}^+]$ curing by excess Hsp104 and inhibits phenotypic expression of some $[\text{PSI}^+]$ variants.\textsuperscript{252} Excess Ssa1 is proposed to result in an increase in the size of Sup35 aggregate, which then counteracts the disaggregating effect of Hsp104.\textsuperscript{253} Moreover, excess Ssa1 on its own can cure $[\text{PSI}^+]$ and lead to an increase in aggregate size of specific $[\text{RNQ}^+]$ variants.\textsuperscript{254} On the other hand, a mutation in Ssa1 (Ssa1-21; L483W) decreases the number of $[\text{PSI}^+]$ propagons, thus destabilizing the $[\text{PSI}^+]$ prion. Interestingly, several mutations in the NTD of Hsp104 can overcome the defect imposed by Ssa1-21 and restore normal $[\text{PSI}^+]$ propagation. Clearly, the functions of Hsp70 and Hsp104 in prion propagation are interdependent as mutations or changes in one can be compensated for by mutations changes in the other. However, it is unclear if this mechanism holds true for prions in general as these mutations have only been tested with $[\text{PSI}^+]$.

Not only do Hsp70s play a role in prion propagation, but the NEFs associated with Hsp70 also show interesting prion phenotypes. For example, increased expression of the Hsp110 Sse1 strongly increased de novo $[\text{PSI}^+]$ formation whereas deletion of SSE1 severely inhibited it.\textsuperscript{255} Additionally, overproduction of Sse1 led to very strong variants of $[\text{PSI}^+]$ while depletion of Sse1 resulted in only weak $[\text{PSI}^+]$ variants.\textsuperscript{255} Furthermore, the Hsp90 co-chaperone, which can also interact with Hsp70 and has been shown to bind Hsp104, destabilized $[\text{PSI}^+]$ when overexpressed and stabilized $[\text{PSI}^+]$ when depleted.\textsuperscript{256} Undoubtedly, the mechanism of prion regulation by chaperone is complicated, resulting in highly varied phenotypes among different Hsp70:Hsp40:NEF interactions and more work is needed to understand these mechanisms.

The Bichaperone network: Hsp104, Hsp70, and Hsp40
Many studies have shown that the cooperation of the Hsp70 system and Hsp104/ClpB is required for efficient solubilization of protein aggregates. Disaggregation of heat-aggregated proteins by the yeast chaperone Hsp104 required the presence of Ssa1 and Ydj1 and similar data was shown for ClpB, DnaK, and DnaJ in bacteria.\textsuperscript{257, 258} In both organisms, Hsp70/DnaK is proposed to act upstream of Hsp104/ClpB, promoting disaggregation by binding to aggregated substrates.\textsuperscript{166, 259} The engineered constructs, HAP and BAP, were able to degrade aggregated substrates in the presence of ClpP, but strictly in an Hsp70/DnaK-dependent manner.\textsuperscript{191, 195} These data suggested that Hsp70/DnaK were required for the initial substrate binding and translocation by Hsp104/ClpB. Consistent with this hypothesis, binding of substrates to the N-terminal pore loop of ClpB required DnaK\textsuperscript{190} and DnaK binding to the substrate required DnaJ.\textsuperscript{260}

Increasing evidence supports the binding of Hsp70/DnaK to the M-domain of Hsp104/ClpB during the disaggregation process.\textsuperscript{261} Indeed, a recent structural study using NMR has also shown specific contacts between DnaK and the M-domain of ClpB.\textsuperscript{262} Mixing domains of Hsp104 and ClpB demonstrated that the M-domain from Hsp104 is required for interaction with Ssa1 and Ydj1, but replacement with the M-domain from ClpB was able to couple Hsp104 to DnaK and DnaJ.\textsuperscript{166, 177, 263} Surprisingly, Hsp104 and Ssa1 can also function with DnaJ, suggesting that the interface between Hsp104 and Hsp70 is the species-specific factor.\textsuperscript{257} Thus, the current proposed mechanism for the bichaperone network is that Hsp40/DnaJ recruits Hsp70/DnaK to the aggregated substrate whereby Hsp70/Hsp40/substrate then binds to the M-domain of Hsp104 and stimulates disaggregation of the substrate by Hsp104/ClpB. Additionally, Hsp70 stimulation of the M-domain to depends on the conformation of the M-domain such that the M-domain must be accessible and in the de-repressed conformation for binding and stimulation to occur.\textsuperscript{201, 264} Although the mechanism of protein disaggregation by the bichaperone network is not entirely clear, recent structural studies have provided a deeper understanding of this interaction and its effects on aggregated substrates.

If this bichaperone mechanism is true for protein aggregates, then, can it also be applied to the mechanism of prion propagation? Initial studies suggest that, indeed, prion disaggregation may occur in a similar fashion. One study showed that incorporation of Hsp70 and Hsp40 into amyloid fibers in vitro made the fibers a better substrate for Hsp104 disaggregation.\textsuperscript{265} Additionally, amyloid fibers that were
disaggregated by Hsp104 in the presence of Ssa1 retained more infectivity than fibers disaggregated by Hsp104 alone.\textsuperscript{265} Furthermore, disaggregation of fibers in the presence of Ssb1 decreased the infectivity of the fibers.\textsuperscript{265} This in vitro data mimics the [$PSI^+$]-promoting (Ssa1) and [$PSI^+$]-antagonizing (Ssb1) phenotypes observed in vivo. More recently, Ssa1 was shown to be required for Hsp104 fragmentation of Rnq1 fibrils in vivo.\textsuperscript{266} In the absence of Ssa1, Hsp104 was able to bind Rnq1 fibrils, but was incapable of remodeling them.\textsuperscript{266} Additionally, excess Hsp104 outcompeted Ssa1 for binding to Rnq1 fibrils in vivo suggesting a mechanism for the observed antagonism of excess Hsp104 curing of [$PSI^+$] by excess Ssa1.\textsuperscript{266}

1.6 Hsp104 in Models of Protein Aggregation Disorders

To date, no clear metazoan homolog or analog of Hsp104 has been identified. Initial attempts to isolate a disaggregase by cellular fractionation have proven mostly unsuccessful. One study shows that crude whole lysates of \textit{Caenorhabditis elegans} are able to disaggregate and degrade Aβ fibers, but the factor(s) responsible have yet to be identified.\textsuperscript{267} It appears as if metazoan proteostasis focuses on clearing aggregates by proteolysis and autophagy instead of disaggregation and renaturation. Given the common structure of amyloid fibers and oligomers and the ability of Hsp104 to rapidly and efficiently remodel yeast prions, the use of Hsp104 to remodel the mammalian disease-associated amyloid structures is an attractive therapeutic model to reverse amyloid aggregation.\textsuperscript{30, 83, 172} Hsp104 has previously been expressed in mammalian cell lines and has been shown to collaborate with human Hsp70 and Hsp40 chaperones to refold thermally denatured firefly luciferase.\textsuperscript{268} Several different disease models have been generated to examine the ability of Hsp104 to interact with and disaggregate disease-associated amyloid substrates.

Expression of expanded polyQ tracts in yeast results in polyQ aggregation that is dependent on the [$RNQ^+$] prion.\textsuperscript{269, 270} When cells are [$RNQ^+$], expanded polyQ can aggregate and cause toxicity. Overexpression of Hsp104 in these cells resulted in significantly reduced toxicity potentiated by small heat shock proteins Hsp26.\textsuperscript{269-271} As such, Hsp104 may be able to cooperate with small heat shock proteins to disaggregate amyloid in other systems. Interestingly, small heat shock proteins have also been shown to suppress Aβ toxicity suggesting that an interaction between Hsp104 and the small heat
shock proteins may be applicable to more than just polyQ disorders.\textsuperscript{272} The co-expression of expanded polyQ and Hps104 in \textit{C. elegans} resulted in reduced inclusion formation and alleviation of developmental defects.\textsuperscript{273} Most remarkably, expression of Hsp104 in mouse models of Huntington’s disease prolonged life span of these mice by 20\% though the physical symptoms were not improved.\textsuperscript{274} Additionally, Hsp104 caused a significant decrease in the number of inclusions.\textsuperscript{275}

As another model for a disease-associated protein, overexpression of human α-synuclein in yeast leads to toxicity and formation of cytoplasmic foci.\textsuperscript{276} Unlike the effects on expanded polyQ, overexpression of Hsp104 has no effect on α-synuclein toxicity.\textsuperscript{271} However, the cytoplasmic foci that form from aggregation of α-synuclein may not be amyloid-like aggregates. Similarly, in a \textit{C. elegans} model of Parkinson’s disease, Hsp104 also had no effect on toxicity.\textsuperscript{277} In a rat model, however, when Hsp104 and α-synuclein A30P were simultaneously expressed, Hsp104 reduced inclusion formation and prevented neurodegeneration.\textsuperscript{278} Nevertheless, several challenges remain to fully validate Hsp104 as a potential therapy for Parkinson’s disease.

Finally, Hsp104 can interact with Aβ42 and can prevent formation of Aβ42 fibers in an ATP-independent manner.\textsuperscript{279} However, Hsp104 is unable to disaggregate Aβ42 fibers in vitro. Although these data show promising results for a therapeutic interaction between Hsp104 and Aβ42, no complementary in vivo approaches have been reported. More recently, aggregates of mutant SOD1 were shown to be disaggreagted by Hsp104, and these observations may provide insight into the mechanism of amyotrophic lateral sclerosis amyloid formation and disease progression.\textsuperscript{280}

\textbf{1.7 Summary and Significance}

Protein aggregation and amyloid deposition are common features of several protein conformational disorders and investigating the mechanisms of amyloid aggregation has contributed significantly to our understanding of these diseases. As non-toxic, epigenetic amyloid conformers, the use of yeast prions has allowed investigation of the genetic and environmental factors which contribute to amyloid formation. One class of amyloid regulating factors is the molecular chaperones, whose normal function in preventing protein aggregation and misfolding make them ideal partners to ensure prion propagation. Indeed, amyloid-associated protein conformational disorders are age-related and suggest
that changes in the chaperone network over time may contribute to cellular dysfunction and disease pathology. Unfortunately, the very nature of the disease does not allow efficient investigation or treatment of the factors promoting amyloid formation. In humans, detection of disease symptoms is often too late to treat the disease and studying normal mouse models without artificially speeding up the aggregation process would take years. Therefore, yeast prions and their regulation by the bichaperone network provide an easily manipulated system in which to investigate amyloid aggregation and chaperone function.

References


Chapter 2: Soluble Oligomers are Sufficient for Transmission of a Yeast Prion but do not Confer Phenotype

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**Author Contributions** J.E.D., R.E.B., and H.L.T. designed the experiments. J.E.D. carried out all the experiments except for thermotolerance assays and fluorescence imaging, which was performed by R.E.B., and the HAP-R830S spotting which was carried out by L.A.U. J.E.D. and H.L.T. analyzed the data. J.E.D. and H.L.T. wrote the manuscript.
Abstract

Amyloidogenic proteins aggregate through a self-templating mechanism that likely occurs via oligomeric or prefibrillar intermediates. For disease-associated amyloidogenic proteins, such intermediates have been suggested to be the primary cause of cellular toxicity. However, isolation and characterization of these oligomeric intermediates has proven difficult, sparking controversy over their biological relevance in disease pathology. Here, we describe an oligomeric species of a yeast prion protein in cells that is sufficient for prion transmission and infectivity. These oligomers differ from the classic prion aggregates as they are soluble and less resistant to SDS. We found that the large SDS-resistant aggregates are required for the prion phenotype but soluble, more SDS-sensitive oligomers contain all the information necessary to transmit the prion conformation. Thus, we identified distinct functional requirements of two types of prion species for this endogenous epigenetic element. Furthermore, the non-toxic, self-replicating amyloid conformers of yeast prion proteins have again provided valuable insight into the mechanisms of amyloid formation and propagation in cells.

Introduction

The amyloid hypothesis proposes that large, protease-resistant amyloid fibers underlie the toxicity associated with several neurodegenerative diseases (Caughey and Lansbury, 2003; Chiti and Dobson, 2006). A definitive link between the amyloid aggregate and toxicity and neurodegeneration has not been established (Haass and Selkoe, 2007). A recent alternative proposal posits that an intermediate in the amyloid pathway is the primary toxic agent, while the large, insoluble aggregates may sequester oligomers and perhaps aid in cell survival (Kirkland et al., 2002). Soluble oligomers of several amyloidogenic proteins including amyloid-β, huntingtin, α-synuclein, and PrP have been detected both from analysis of amyloid-forming recombinant proteins and in cell and mouse models (Conway et al., 1998; Lasmezas et al., 1997; Lesne et al., 2006; Sajnani et al., 2012; Sanchez et al., 2003; Silveira et al., 2005; Tzaban et al., 2002). These oligomers, characterized as putative intermediates in amyloid formation, encompass a variety of sizes and structures that cause toxicity when introduced into disease models (Klyubin et al., 2005; Sajnani et al., 2012; Silveira et al., 2005). Isolation of these dynamic, soluble
oligomers has remained largely elusive and, as such, investigation of their role in amyloid formation has proven challenging.

The yeast prion protein Sup35 forms self-perpetuating amyloid conformers that are transmissible and infectious (Patino et al., 1996; Paushkin et al., 1996; Serio et al., 2000). To propagate the [PSI+] prion, Sup35 aggregates must undergo remodeling by the chaperone Hsp104, which facilitates monomer addition by severing amyloid structures to generate transmissible species, or propagons (Chernoff et al., 1995; Kryndushkin et al., 2003; Satpute-Krishnan et al., 2007; Shorter and Lindquist, 2006). Until now, in vivo studies have primarily reported on the role of Hsp104 in aggregate fragmentation (Ness et al., 2002; Satpute-Krishnan et al., 2007). Interestingly, Hsp104 has also been shown to catalyze amyloid formation in vitro, specifically impacting the formation of amyloid oligomers (Shorter and Lindquist, 2004). Moreover, recent in vitro evidence identified Sup35 oligomers as intermediates during amyloid formation under some conditions (Ohhashi et al., 2010). Here, we identify soluble, more SDS-sensitive oligomers of Sup35 as prion propagons and show that Hsp104 plays a role in their maintenance.

**Results and Discussion**

We performed a screen to identify cellular changes that rescued cells from toxic overexpression of SUP35 in [PSI+] cells (Vishveshwara et al., 2009). As this toxicity is dependent on [PSI+], we expected to uncover factors that affected Sup35 aggregation. One mutation identified, *hsp104-R830S*, caused [PSI+] cells to appear phenotypically [psi-] by a readout of translation termination efficiency of a reporter that is indicative of the functional, soluble state of Sup35 (Fig. 2.1A). Fluorescent imaging showed that Sup35 in *hsp104-R830S* cells appeared diffuse (Fig. 2.1B), and by semi-denaturing detergent-agarose gel electrophoresis (SDD-AGE), only monomeric Sup35 was detected in *hsp104-R830S* cells (Fig. 2.1C).

Strikingly, mating *hsp104-R830S* cells that appeared [psi-] to wild type HSP104 [psi-] cells produced many diploids that were phenotypically [PSI+], as seen by increased nonsense suppression (Fig. 2.2). Furthermore, sporulation of these diploids produced two [PSI+] and two [psi-] haploid progeny (Fig. 2.1D), thereby indicating that prion-competent propagons had been maintained in *hsp104-R830S* cells that resulted in cryptic [PSI+] (phenotypically undetectable). Indeed, transformation of *hsp104-R830S* cell
lysates into wild type [psi-] cells demonstrated that these propagons were infectious and produced [PSI+] cells. Out of 1,154 cells transformed, 45 were [PSI+] (3.9% infectivity). Control transformations of [psi-] lysates into [psi-] cells resulted in only red colonies. Of the thousands of red colonies resulting from these control transformations, over 400 were further analyzed and confirmed to be [psi-]. Thus, the infectious propagon in hsp104-R830S cells is unable to cause observable nonsense suppression, but re-establishes and maintains the [PSI+] phenotype in the presence of wild type HSP104.

Hsp104 normally functions to disaggregate non-prion aggregates and promote cellular recovery from stress (Glover and Lindquist, 1998). Therefore, we tested the activity of the Hsp104-R830S mutant with other known substrates. Interestingly, the thermotolerance of hsp104-R830S cells resembled that of wild type cells (Fig. 2.3A). Moreover, hsp104-R830S cells efficiently resolubilized heat-aggregated luciferase (Fig. 2.3B). Hsp104 threads substrates through a central channel as a mechanism of disaggregation (Tessarz et al., 2008). The Hsp104 variant, HAP, has been used to investigate threading activity by coupling Hsp104 to the ClpP protease so that threaded substrates are degraded, resulting in decreased viability (Tessarz et al., 2008). We created the HAP-R830S variant and found that the mutant maintained threading activity (Fig. 2.3C). Hsp104 is an AAA+ ATPase. Mutations that inhibit ATP hydrolysis or hexamerization typically prevent [PSI+] propagation (Glover and Lum, 2009). We purified Hsp104-R830S and measured a reduced initial rate of ATP hydrolysis of 0.1659±0.0308 nmol P\textsubscript{i}·µg\textsuperscript{-1}·min\textsuperscript{-1}, as compared to the initial rate of wild type Hsp104 of 0.2975±0.412 nmol P\textsubscript{i}·µg\textsuperscript{-1}·min\textsuperscript{-1}, which is comparable to previously published data (Tkach and Glover, 2004). We next analyzed the distribution of Hsp104-R830S, relative to wild type Hsp104, using glycerol gradients and size-exclusion chromatography. These analyses revealed that Hsp104-R830S forms unstable hexamers (Fig. 2.3D & 2.4). Together, these data reveal that the reduced activity of Hsp104-R830S impairs the maintenance of the [PSI+] phenotype.

Next, we assessed the state of Sup35 in hsp104-R830S cryptic [PSI+] cells. We first performed sedimentation analysis with hsp104-R830S cryptic [PSI+] lysates and found that Sup35 was soluble (Fig. 2.5A). Next, we subjected lysates of hsp104-R830S cryptic [PSI+] and HSP104 [psi-] cells to sucrose
gradient fractionation. Monomeric Sup35 in [psi-] cells remained in the top fractions, while Sup35 from \textit{hsp104-R830S} lysates was detected further down the gradient, demonstrating the existence of some oligomeric species (Fig. 2.5B). To understand how these oligomers relate to the large SDS-resistant aggregates associated with the [PSI+] phenotype, we created a system to examine the effect of \textit{hsp104-R830S} on pre-existing Sup35 aggregates. We covered \textit{hsp104-R830S} cryptic [PSI+] with wild type \textit{HSP104} expressed from a glucose-repressible promoter such that cells grown in non-repressing galactose allowed the propagation of [PSI+] and maintained SDS-resistant aggregates (Fig. 2.5C, Gal). We then shifted the cells to glucose to repress wild type \textit{HSP104} (Fig. 2.6A) and performed SDD-AGE to monitor the effect of \textit{hsp104-R830S} on SDS-resistant Sup35 aggregates. Within twelve hours of wild type \textit{HSP104} repression, monomeric Sup35 was apparent in \textit{hsp104-R830S} cells. Within twenty-four hours, SDS-resistant aggregates had disappeared (Fig. 2.5C). As expected, when \textit{hsp104-R830S} pGAL-\textit{HSP104} cells were switched from glucose back to galactose to derepress wild type \textit{HSP104}, they became phenotypically [PSI+] (Fig. 2.6B). Therefore, despite the loss of SDS-resistant aggregates, the propagons remained. Thus, the large SDS-resistant Sup35 aggregates are not strictly required for transmission of the prion.

We therefore reasoned that a less stable subpopulation of Sup35 might be capable of prion transmission. Indeed, that the addition of SDS to Sup35 prion aggregates results in smaller SDS-resistant polymers suggests that some less SDS-resistant species exists within the large prion aggregates(Kryndushkin et al., 2003). We investigated whether the soluble oligomers in \textit{hsp104-R830S} cryptic [PSI+] cells were less resistant to SDS, as has been reported for another prion (Taneja et al., 2007). We again performed SDD-AGE on \textit{hsp104-R830S} cryptic [PSI+] lysates but decreased the SDS concentration, and only then did we observe the presence of oligomeric species (Fig. 2.5D). To determine how these species related to prion propagons and the cryptic [PSI+] phenotype, we repeated the wild type \textit{HSP104} repression time course with \textit{hsp104-R830S} cells using a low SDS SDD-AGE. We observed the maintenance of less SDS-resistant, oligomeric species throughout the time course (Fig. 2.5E), suggesting that the soluble, less SDS-resistant oligomers are the transmissible propagons in \textit{hsp104-R830S} cryptic [PSI+] cells.
Although we observed the unstable, soluble oligomers in the absence of insoluble aggregates, it is likely that both species exist in wild type cells, and the more SDS-sensitive, soluble oligomers are more readily detectable in cells lacking insoluble aggregates. Therefore, we separated the Sup35 species in both hsp104-R830S cryptic [PSI+] and wild type [PSI+] cells by sucrose gradient and performed protein transformation to determine whether the soluble fractions contained infectious propagons. Both hsp104-R830S and HSP104 cells contained soluble Sup35 oligomers that were infectious to [psi-] cells (Fig. 2.7A). While much of the Sup35 species from wild type lysates migrated to the bottom of the gradient (35% of the total Sup35 which correspond to 54% of the infectivity), there were highly infectious oligomers in wild type [PSI+] cells that did not migrate with the heavy-sedimenting aggregates. This soluble pool of Sup35 contains 46% of the infectivity in wild type [PSI+] lysates. Curiously, the high-molecular weight aggregates observed in hsp104-R830S cells were not very infectious, suggesting that these aggregates are not the same high-molecular weight aggregates present in wild type [PSI+] cells and are not efficient [PSI+] propagons. We then performed a simple solubility assay to separate wild type [PSI+] lysates into soluble and insoluble fractions (see Fig. 2.5A) and then performed infectivity assays and SDD-AGE analysis. Strikingly, we found much of the infectivity in the soluble fraction (32.8% of the total Sup35 which corresponds to 30% of the infectivity) from sedimentation analysis (Fig. 2.7B). Additionally, by SDD-AGE, we found that there were no Sup35 oligomers resistant to 2% SDS in the soluble fraction (Fig. 2.7C). As such, these data support our hypothesis that insoluble aggregates are not strictly required for prion transmission.

Propagons contain the variant-specific properties required for inheritance and propagation of distinct prion phenotypes (Satpute-Krishnan and Serio, 2005; Tanaka et al., 2004). Therefore, the soluble oligomers in hsp104-R830S cryptic [PSI+] cells should retain the properties necessary to propagate the parental strong [PSI+] phenotype. If hsp104-R830S altered the prion structure, instead of cryptic [PSI+], weakened nonsense suppression and enhanced mitotic loss could be expected. After crossing hsp104-R830S cryptic [PSI+] cells to [psi-] cells, we compared the [PSI+] meiotic progeny to the parental strong [PSI+] variant used initially in the screen. We found that the [PSI+] progeny were indeed strong [PSI+] phenotypically (Fig. 2.7D). We then performed SDD-AGE analysis of the resulting [PSI+] haploids as
compared to the strong $[PSI^+]$ and a weak $[PSI^+]$ variant (Bagriantsev and Liebman, 2004; Derkatch et al., 1996; Kryndushkin et al., 2003). The SDS-resistant Sup35 aggregates in the $[PSI^+]$ progeny appear the same as strong $[PSI^+]$ (Fig. 2.7E). We also noted that all the $[PSI^+]$ transformants from infection of $hsp104-R830S$ lysates into $[psi^-]$ cells were strong $[PSI^+]$. Thus, $Hsp104-R830S$ did not alter the properties of the original variant phenotypically or biochemically, suggesting that there was no change in prion structure.

We have shown, for the first time, the presence of soluble, prion-competent, less SDS-resistant oligomers of Sup35 in vivo. The less SDS-resistant, soluble oligomers are sufficient for transmission of the prion conformation, but are not sufficient to produce the nonsense suppression phenotype associated with $[PSI^+]$. We have also shown that the insoluble aggregates that characterize $[PSI^+]$ cells are not required for prion transmission or infectivity. Furthermore, we have uncovered two novel features of yeast prion propagons. First, they can be more SDS-sensitive, where previously only SDS-resistant aggregates of Sup35 were shown to be infectious (Bagriantsev et al., 2008). This sensitivity parallels the previously described PK-sensitive PrP species which can also transmit the prion (Sajnani et al., 2012). Second, soluble oligomers were able to act as propagons in vivo. Oligomers of several amyloidogenic proteins have been described and can cause toxicity in the absence of large aggregates (Haass and Selkoe, 2007). Indeed, our data correlate well with previous reports that visible Sup35 aggregates are lost in the mother cell and yet the prion is faithfully propagated (Taguchi and Kawai-Noma, 2010). Perhaps the SDS-resistant, insoluble Sup35 aggregates function as a reservoir for the continued renewal of transmissible propagons. This role for SDS-resistant yeast prion aggregates mirrors the proposed role for amyloid aggregates as traps for oligomers (Kirkitadze et al., 2002). As such, we clearly show separate functions of two distinct aggregate species that are structurally-related components of the same prion propagation pathway. A mutant in Hsp104 enabled us to initially tease out these species. We favor a model whereby the decreased activity of $hsp104-R830S$ results in reduced fragmentation of aggregates but our data do not exclude the possibility that Hsp104 normally plays a role in generating the large insoluble aggregates.
The soluble oligomers that exist in many conformational disorders are challenging to characterize due to their dynamic and metastable nature. In addition, conflicting reports on the properties and structure of oligomers complicate the elucidation of the important oligomeric species (Haass and Selkoe, 2007). The properties and mechanisms associated with yeast prion propagons may be similar to those associated with amyloidogenic proteins involved in self-propagating protein conformational disorders. Therefore, investigating these features may lead to a better understanding of the function and structure of soluble, self-templating oligomers, as well as the role of amyloid in disease.

Materials and Methods

Strains and Yeast cultivation

The yeast strains used in this study are derivatives of *Saccharomyces cerevisiae* 74-D694 and were grown using standard culture techniques. YPD is rich yeast medium whereas SD is synthetic medium lacking amino acids as needed to select for maintenance of plasmids or screen for a nonsense suppression phenotype (SD-Ade). The strain used in the screen, 74-D694 [PSI+] can1Δ::MFA1pr-HIS3-MFa1p r-LEU2 ade1-14 ura3-52 lys2Δ::KanMX4 pRS315CUP1-SUP35, was transformed with ten independent plasmid pools of the mini-transposon (3XHA/lacZ(mTn3)) mutagenized library (kindly provided by M. Snyder) and selected on media containing 350µM copper sulfate to induce expression of SUP35. Candidates that rescued toxicity associated with increased expression of SUP35 were passaged a second time on copper sulfate media and were examined for the effect on [PSI+] phenotypically by color and genetically by mating to unmutagenized 74-D694 [PSI+] and [psi-] isogenic strains. Several mutants, not linked to the transposon, were analyzed and determined to be in Hsp104 thru genetic analysis. The mutant characterized here, *hsp104-R830S*, was recreated and these strains were used for further characterization. Phenotypic assays are based on the amount of functional Sup35 to terminate the premature stop codon in *ade1-14*, which results in the accumulation of a red-pigmented intermediate in the adenine biosynthesis pathway (Liebman and Derkatch, 1999).

SDD-AGE protein analysis

Cells were lysed by beadbeating in Buffer A (25mM Tris-HCl pH 7.5, 50mM KCl, 10mM MgCl₂, 1mM EDTA, 10% Glycerol plus mini EDTA-free protease inhibitors (Roche), Aprotinin (Sigma) and PMSF
(Sigma)). Samples were treated at room temperature for 7 minutes in sample buffer containing 2% SDS then electrophoresed through a 0.1% SDS, 1.5% agarose gel. The gels were subjected to western blot with anti-Sup35 antibody. The experiments done with lower SDS concentrations contained 0.01% SDS in the gel and between 0.05 and 0.1% SDS in the sample buffer, as indicated. Molecular weight markers were not used because non-denatured protein aggregates were being analyzed.

Fluorescence Microscopy

Cells were transformed with pRS426CUP1-SUP35NM-GFP and grown in media lacking uracil. 50µM CuSO₄ was added to logarithmically growing cells and grown for four hours to induce expression of SUP35NM-GFP. Images of cells expressing Sup35NM-GFP, in water and at room temperature, were captured on an Olympus Bmax-60F microscope containing a 1.35NA 100X UPlanApo objective lens, spinning disc Confocal Scanner Unit (CSU10), and a Stanford Photonics XR-Mega10 ICCD camera. Images were acquired using QED software and analyzed by Image J.

Protein Transformation

Recipient [psi-] cells from logarithmically growing cultures were spheroplasted with lyticase. Samples from cell lysis, sedimentation analysis, and sucrose gradient fractionation were added to the spheroplasts in 1M sorbitol along with a vector containing the URA3 gene for selection on media lacking uracil. The cells were incubated at room temperature then recovered at 30°C. Media containing glucose, sorbitol, and agar was added to the cells before plating the cells on sorbitol plates lacking uracil. Transformants were replica plated to YPD plates, and individual colonies were picked from those YPD plates and spotted onto YPD and both media lacking adenine and containing GdHCl for quantification of infectivity.

Purification of Hsp104

Hsp104 was purified as previously described(Lum et al., 2004). Recombinant Hsp104 tagged with a 6x-Histidine tag was expressed in E. coli cells and affinity purified using a Ni²⁺-sepharose column. Next, the 6x-Histidine tag was removed by cleavage with the TEV protease and the untagged Hsp104 was separated from the uncleaved, tagged Hsp104 by affinity purification. The untagged Hsp104 was further purified using anion exchange via a Q-sepharose column. The pure Hsp104 collected from the Q-sepharose column was then separated on an S-300 gel filtration column to isolate monomeric Hsp104.
Purified, monomeric Hsp104 was pooled, concentrated, and frozen at -80°C. Multiple purification preparations were used for all assays in repeat experiments.

ATP Hydrolysis Assays

ATP hydrolysis was monitored by the Malachite Green assay. 2µg purified Hsp104 was equilibrated in buffer (40mM TrisHCl pH7.5, 175mM NaCl, 5mM MgCl\(_2\), 0.02% Triton X-100) at 37°C before adding 5mM ATP (Sigma). During incubation of Hsp104 with ATP at 37°C, samples were taken and free phosphate was measured by addition of Malachite green dye (Sigma). Color development occurred over one minute and was terminated by the addition of 34% citric acid (Sigma). Absorbance was measured at 650nm.

Glycerol Gradients

Purified protein at 75ug was incubated in buffer (20mM Tris pH 8, 100mM NaCl, 10mM MgCl\(_2\), 2mM EDTA, 10% glycerol) +/- ATP for 5 minutes on ice before loading onto linear glycerol gradients (15-35%). The gradients were spun at 34,000 rpm for 18 hours in an SW55 rotor. Gradients were fractionated and the fractions analyzed by SDS-PAGE and western blot with anti-Hsp104 antibody.

Size Exclusion Chromatography

Purified protein at 1mg/mL was incubated in buffer (50mM Tris pH7.5, 200mM KCl, 10mM MgCl\(_2\), 2mM DTT, 2mM EDTA) +/- ATP for five minutes. The sample was applied to an S-300HR column and fractions were collected. The concentration of Hsp104 was measured by Bradford analysis (Bio-Rad).

Thermotolerance Assays

Cells were grown to mid-log phase at 30°C. An equal number of cells from each sample were aliquoted and pre-treated at 37°C for thirty minutes. Samples were heat shocked at 50°C for the indicated amounts of time and then diluted five-fold before plating onto solid rich medium.

In Vivo Luciferase Refolding Assay

In vivo luciferase refolding assays were performed as previously described (Tipton et al., 2008). Briefly, cells carrying pRS316GPD-Lux (kindly provided by B. Bukau) were pretreated at 37°C for one hour to induce expression of HSP104, then heat shocked at 44°C for an hour. After fifty minutes of heat shock, cycloheximide was added to prevent new protein synthesis. After heat shock, the cells recovering
at 30°C were aliquoted at fifteen minute intervals and D-luciferin (Sigma) was added. Luminescence was measured and plotted as a percentage of total luciferase activity before heat shock.

Hsp104 Shutoff Experiments

The diploid strains, HSP104/hsp104Δ::leu2 and HSP104/hsp104-R830S were transformed with pRS416GAL-HSP104, sporulated, and dissected on CSM-Ura + 0.25% Galactose + 2% raffinose. Low levels of galactose were used to avoid curing [PSI+] by overexpression of HSP104. Progeny were selected that contained the plasmid and were pink in color as evidence that [PSI+] was maintained. Cells were grown in CSM-Ura + 0.25% galactose + 2% raffinose for 48 hours before switching the cells to glucose media lacking uracil to repress expression of the HSP104 plasmid. At six hour intervals, aliquots of the sample were taken and both pelleted for SDD-AGE and plated on CSM-Ura + 0.25% galactose + 2% raffinose plates. SDD-AGE was performed as described above. Gels were transferred to PVDF membrane and probed with anti-Sup35 antibody.

Sup35 Solubility Assays

Cells were lysed by beadbeating in buffer (10mM NaPO4 pH 7.5, 250mM NaCl, 2% SDS, 1% Triton X-100 plus mini EDTA-free protease inhibitors (Roche), and PMSF (Sigma)). Lysates were subjected to ultracentrifugation at 100,000rpm in a Beckman TLA-100 rotor for one hour. The supernatant was collected and the pellet resuspended in lysis buffer. Total, supernatant, and pellet fractions were subjected to SDS-PAGE and western blot with anti-Sup35 antibody as well as protein transformation.

Sucrose Gradients

Cells were lysed by beadbeating in Buffer A. Total protein was normalized to 1mg/mL and loaded onto a 4mL linear sucrose gradient (15-60%) and centrifuged at 32,000rpm for either three or 22 hours. In order to compare infectivity of soluble and insoluble Sup35 from HSP104 [PSI+] by sucrose gradient, we increased the time of centrifugation to a time that reproducibly showed a large fraction of pelletable Sup35. Thus, after a 22 hour spin, we are able to distinguish the soluble Sup35 and the insoluble Sup35 which migrates to the bottom of the gradient. Gradients were fractionated and the fractions were analyzed by SDS-PAGE, western blot, and protein transformation.
References


Figure 2.1 *hsp104-R830S* propagates cryptic [*PSI*+].

A, [*PSI*+], [*psi*], and *hsp104-R830S* cells were spotted on media containing CuSO₄ to induce toxic overexpression of Sup35, media lacking adenine (SD-Ade) to assess nonsense suppression of the premature stop codon in *ade1-14*, and rich media (YPD). B, *HSP104 [*PSI*+] and *hsp104-R830S* cells containing pSup35NM-GFP were imaged by fluorescence microscopy. Scale bar represents 10µm. C, Western blot of an SDS-containing agarose gel (SDD-AGE) shows Sup35 aggregate status in lysates of indicated strains. This blot is one representative of three individual experiments. D, An example of two tetrads where mating *hsp104-R830S* to [*psi*] cells, both containing the *ade1-14* mutation, resulted in tetrads with two red (efficient translation termination, *hsp104-R830S*) and two light pink (increased nonsense suppression, [*PSI*+] *HSP104*) haploids.
Figure 2.2 hsp104-R830S haploids crossed to [psi-] haploids results in [PSI+] diploids.

The presence of phenotypically undetectable [PSI+] prion propagons was apparent when red hsp104-R830S haploids were mated to wild type red [psi-] haploids. Though the hsp104-R830S haploids appear [psi-] (red in color due to efficient translation termination of ade 1-14), prion propagons are still present that can efficiently template soluble Sup35 in the presence of wild type HSP104 to produce mostly pink [PSI+] HSP104:hsp104-R830S heterogeneous diploids. Diploids from the cross were spotted onto YPD plates and [PSI+] and [psi-] controls are labeled in the upper left corner of the plate.
Figure 2.3 Analysis of Hsp104-R830S activity reveals the mechanism of altered activity.

A, Growth on YPD plates of the strains heat shocked at 50°C for times indicated. Untreated cells were spotted before heat shock. B, HSP104 (black), hsp104-R830S (red), and hsp104Δ (orange) cells expressing heat-aggregatable luciferase were heat shocked at 44°C while blocking new protein synthesis. Luminescence during recovery at 30°C was plotted as a fraction of luminescence before heat shock. Error bars represent the standard deviation. C, hsp104Δ cells carrying pRS315CUP1-ClpP, covered by the indicated plasmids, were spotted on media containing CuSO₄ to induce expression of ClpP. D, Hsp104 (upper) and Hsp104-R830S (lower) incubated +/- ATP (red/black) separated on a glycerol gradient by ultracentrifugation. Top of the gradient is fraction number 1. Fractions were analyzed by SDS-PAGE and western blot. All experiments were repeated at least three times.
Figure 2.4 Hsp104-R830S does not form efficient hexamers.

Recombinant Hsp104 (upper panel) and Hsp104-R830S (lower panel) were incubated either with ATP (♦) or without ATP (◊) and applied to an S-300 size exclusion column. Fractions of the eluate were collected and Bradford analysis (absorbance at 595nm) was performed to quantify the amount of Hsp104 protein in each fraction. Both recombinant Hsp104 and Hsp104-R830S without ATP migrate mainly as monomers or dimers. Incubation of wild type Hsp104 with ATP causes hexamers to form, but Hsp104-R830S incubated with ATP is distributed across several fractions, suggesting an inability to efficiently hexamerize (or maintain stable hexamers) in response to ATP binding. Proteins of known molecular weights, thyroglobulin (669kDa) and aldolase (158kDa), were also applied to the column and their elution peaks are labeled for reference.
Figure 2.5 *hsp104-R830S* propagates SDS-sensitive soluble oligomers of Sup35.

A, Ultracentrifugation separated lysates into total (T), soluble (S) and pellet (P) fractions. B, Fractionation of *[psi−]* and *hsp104-R830S* cryptic *[PSI+]* lysates separated by a three hour centrifugation through a linear sucrose gradient. C, *Hsp104* and *hsp104-R830S* cells carrying p416GAL-*HSP104* grown in galactose were switched to glucose to repress p416GAL-*HSP104* and p416GAL-*HSP104* was efficiently repressed by 12 hours in glucose as shown by western blot in an *hsp104Δ* control (Fig. S3). Aliquots were taken from galactose and between 12 and 72 hours in glucose and subjected to SDD-AGE. The shift in aggregate distribution occurs when changing carbon sources. D, Lysates of the indicated strains were analyzed by SDD-AGE. The *HSP104 [PSI+]* and *[psi−]* and *hsp104-R830S* samples were incubated in sample buffer containing the indicated percentages of SDS. E, Same as (D) but samples were subjected to SDD-AGE containing 0.01% SDS. All experiments were repeated at least three times and protein molecular weight markers (kD) are indicated.
Figure 2.6 Glucose represses expression of wild type HSP104 but [PSI+] propagons persist in the absence of SDS-resistant aggregates.

(A) In order to establish a system whereby we could determine the effect of hsp104-R830S on pre-existing [PSI+] aggregates, we transformed a plasmid expressing wild type HSP104 driven by the galactose promoter (pRS416-GAL-HSP104) into heterozygous diploids expressing either wild type HSP104 or hsp104-R830S and hsp104Δ on the chromosome. In order to determine if switching the cells expressing pRS416-GAL-HSP104 to glucose efficiently repressed wild type HSP104, we performed a western blot to detect the amount of Hsp104 expressed while growing in galactose (plasmid-borne wild type HSP104 is expressed, Gal) and for various amounts of time after switching the cells to glucose (plasmid-borne wild type HSP104 is repressed, 6-48 for wild type and hsp104-R830S and 6-24 for hsp104Δ). We compared wild type HSP104 and hsp104-R830S cells containing pRS416-GAL-HSP104 to hsp104Δ cells containing pRS416-GAL-HSP104. After only six hours in glucose, we could detect no Hsp104 in the hsp104Δ cells. This suggests that over the time course of our assay (Fig. 3C), wild type HSP104 from the plasmid was efficiently repressed while growing in glucose. Total protein loading was assessed by membrane stain.

(B) We investigated the effect of hsp104-R830S on pre-existing aggregates of Sup35 by both SDD-AGE (Fig. 3C & 3E) and by nonsense suppression phenotype (using ade1-14) after repression of wild type HSP104. Briefly, cells (hsp104-R830S or hsp104Δ) carrying galactose-inducible wild type HSP104 on a plasmid were grown in low galactose (0.25%) to maintain [PSI+]. Cells were then switched to glucose media to repress wild type HSP104, grown for various times in liquid glucose media (hours post shift to glucose), and then plated on media plates lacking uracil and containing galactose (0.25%) to derepress wild type HSP104. The restoration of wild type HSP104 allowed for assessment of whether the cells contained any species capable of propagating [PSI+]. Throughout the time course tested, the hsp104-R830S cells were phenotypically [PSI+] on galactose plates while the hsp104Δ cells had truly lost the prion phenotype (red in color indicating efficient translation termination).
Figure 2.7 Soluble oligomeric \( [PSI^+] \) propagons are infectious and maintain the prion variant structure.

A, Fractions of \( [PSI^+] \) and \( hsp104-R830S \) cryptic \( [PSI^+] \) lysates separated on a linear sucrose gradient by a 22 hour ultracentrifugation step were analyzed by western blot and the amount of Sup35 (presented as the percent of soluble Sup35) in each fraction was quantified by Image J and is indicated below each fraction in the western blot. Equal volumes of each fraction were transformed into \( [psi^-] \) cells. The fraction of infected \( [PSI^+] \) cells obtained from each gradient fraction is indicated for \( hsp104-R830S \) (black) and \( HSP104 \) (grey) cells. The fraction of infected \( [PSI^+] \) cells was generated by compiling the infectivity data from four \((HSP104)\) or five \((hsp104-R830S)\) separate sucrose gradients and transformations. Sup35 in \( hsp104-R830S \) lysates appears to be more susceptible to proteolysis and frequently shows degradation products. Protein molecular weight markers (kD) are indicated. B, Total, soluble, and insoluble fractions of \( HSP104 [PSI^+] \) lysates from sedimentation analysis were transformed into \( [psi^-] \) cells. The relative infectivity of the total, soluble, and insoluble fractions was calculated as above and is indicated. Inset: A western blot for Sup35 in Total (T), Soluble (S), and Insoluble (I) fractions from one sedimentation assay in this experiment with protein molecular weight marker (kD). Of the thousands of red colonies resulting from transformation of the soluble and insoluble fractions from wild type \( [psi^-] \) cells as a negative control, further analysis verified that 48 from each fraction were \( [psi^-] \) in each of three independent experiments. C, Total (T), Soluble (S), and Insoluble (I) fractions of \( HSP104 [PSI^+] \) lysates from sedimentation analysis were analyzed by SDD-AGE. The soluble fraction is from the top of the supernatant to prevent contamination from the pellet. This experiment was repeated three times. D, Two representative tetrads from the mating of \( hsp104-R830S \) cryptic \( [PSI^+] \) to \( [psi^-] \) were spotted on YPD (upper) and SD-Ade plates (lower) to assess the level of nonsense suppression. Strong \( [PSI^+] \) (Control A), weak \( [PSI^+] \) (Control B), and \( [psi^-] \) (Control C) strains were spotted for reference. E, The lysates of the four white haploids from the tetrads in (C) were analyzed by SDD-AGE and compared to strong (St) and weak (Wk) \( [PSI^+] \) controls.
Chapter 3: Regulation of the Hsp104 M-Domain Activity is Critical for Prion Propagation

Jennifer Dulle and Heather True
Abstract

Molecular chaperones play a significant role in preventing protein misfolding and aggregation in order to prevent toxicity and protein loss-of-function. Indeed, protein conformational disorders resulting from the misfolding and aggregation of proteins and have been linked to changes in the chaperone network. In yeast, molecular chaperones also play a role in prion propagation. Yeast prions are self-replicating amyloid aggregates, which are proposed to play a functional role in yeast cell biology. Yeast prion propagation requires the molecular chaperone Hsp104 to fragment prion aggregates to generate propagons which can transmit the prion conformation to soluble prion protein monomers. Hsp104 is an AAA+ ATPase involved in disaggregating stress-induced protein aggregates that is essential for yeast prion propagation. Here, we show that the coiled-coil middle domain of Hsp104 is an integral part of the prion propagation mechanism. We have generated mutations in the M-domain of Hsp104 which are predicted to stabilize either the repressed or de-repressed conformation of the M-domain and have characterized the effect of these mutations on Hsp104 activity. We show that mutations predicted to stabilize the repressed conformation of the M-domain inhibit general chaperone activity. On the other hand, de-repressed mutations have differential effects on ATP hydrolysis and disaggregation suggesting that the M-domain is involved in coupling these two mechanisms. Furthermore, we show that changes in the M-domain have more of an effect on weak [PSI+] variants whereas strong [PSI+] variants are less susceptible to de-regulation of the middle domain. Additionally, changes in the M-domain differentially affect of [PSI+] and [RNQ+] propagation, further supporting the hypothesis that these two prions vary in their dependence and interaction with Hsp104. Thus, we provide evidence that regulation of the M-domain of Hsp104 is critical for efficient prion propagation and that elucidating the role of the M-domain in the mechanism of prion propagation is important for understanding propagation of individual prions as well as prion variants.

Introduction

Protein aggregates pose a considerable challenge to cellular homeostasis. As such, protein misfolding and aggregation are guarded against by molecular chaperones, which act as the cell’s first line of defense by promoting proteostasis. In bacteria, fungi, and plants, the Hsp100 chaperones, together
with Hsp70 and Hsp40 chaperones, are responsible for disaggregating protein aggregates and promoting cell survival in response to cell stress[1,2]. The AAA+ ATPase Hsp104 is the primary disaggregate in the yeast Saccharomyces cerevisiae[3,4]. Hsp104 is essential for cell survival post-heat stress (thermotolerance) as well as recovery from various other stresses[4,5]. Like its bacterial homolog, ClpB, the hexameric Hsp104 threads aggregated protein substrates through a central channel to be refolded by Hsp70 chaperones[6,7].

In addition to its role in protein disaggregation, Hsp104 is also essential for yeast prion propagation[8]. Prions in yeast are self-replicating, cytoplasmically inherited protein aggregates proposed to play a role in cellular adaptation and survival[9,10]. Prions are amyloidogenic in nature, and the self-propagating templates are cross β-sheet structures that are highly stable and resistant to high temperature and detergents[11]. One of the best characterized yeast prions is formed from the reversible aggregation of the translation termination factor, Sup35[12,13,14]. This prion, called [PSI+], has been proposed to play a role in creating genetic diversity by promoting translation of normally silent regions of the genome[15]. Interestingly, the de novo formation of [PSI+] is regulated by another yeast prion, [RNQ+], resulting from the aggregation of the Rnq1 protein[16,17,18,19]. The proposed function for the prion [RNQ+] is to act as an amyloid template for the conversion of monomeric Sup35 to the prion conformation[16,18]. The formation of [PSI+] has been shown to increase cell viability under various stresses suggesting that the formation and propagation of [PSI+] is an important biological process and must be highly regulated[10,15,20]. As such, prion propagation and maintenance in yeast cells require efficient fragmentation to generate prion-competent oligomers, or propagons, that can be transmitted to daughter cells. Hsp104 is proposed to remodel prion aggregates to produce propagons, which as a result generates more free fiber ends that appear to be required for additional monomer templating[21,22,23,24]. Furthermore, Hsp104 has been implicated in the selection and propagation of prion variants, or conformationally-distinct aggregates of the same protein sequence[25,26,27].

Alterations in the stability of prions, in the different variant structures, are proposed to govern their interaction and fragmentation by Hsp104[25,28,29].

Structurally, Hsp104 can be divided into five functionally-distinct, yet cooperative, domains. The N-terminal domain is not required for either prion propagation or thermotolerance, but has been proposed
to be a site for substrate binding, as well as an interaction site for the Hsp70s and Hsp40s[30,31]. Two nucleotide-binding domains, NBD1 and NBD2, bind and hydrolyze ATP to catalyze the disaggregation mechanism and stabilize hexamer formation[32]. The role of the C-terminal domain is still not well understood as it is unnecessary for prion propagation and thermotolerance yet both activities are affected by mutations in this domain[30,33,34]. Finally, the linker region, or middle domain (M-domain), is proposed to regulate both ATP hydrolysis and substrate disaggregation by coordinating the individual actions of NBD1 and NBD2[30,35,36].

The M-domain is a coiled-coil insertion between NBD1 and NBD2 and is characteristic of Hsp100 chaperones that function as disaggregases, including the bacterial homolog, ClpB[37,38]. Biochemical, genetic, and structural studies with both Hsp104 and ClpB suggest that the M-domain projects from the body of the hexamer and makes contact with the NBD1 of neighboring subunits[39,40,41]. In both Hsp104 and ClpB, the M-domain regulates ATP hydrolysis[40,42,43], is essential for substrate disaggregation[44,45,46], and mediates the interaction between Hsp104 and the Hsp70 chaperones[44,47,48]. Recent data suggests that the M-domain of ClpB can occupy two distinct functional states, repressed and de-repressed[43]. In the repressed state, the M-domain is nestled against the body of the hexamer, maintaining contact with a neighboring NBD1. Interaction with Hsp70 is proposed to promote a shift of the M-domain away from NBD1 to the de-repressed conformation, thereby increasing the ATPase activity and, in turn, promoting substrate disaggregation[36,43]. ClpB mutations that stabilize the M-domain in the repressed state prevent ATPase stimulation by substrate and decrease substrate disaggregation[43]. On the other hand, mutations in ClpB that prevent binding of the M-domain to NBD1 result in hyperactivity and cause toxicity in vivo[36,43]. Thus, the mobility of the M-domain plays a significant role in regulating the activity of ClpB, and is presumed to play a similar role in Hsp104. As such, elucidating the function of the M-domain in each aspect of Hsp104 activity is critical to understanding how Hsp104 is able to disaggregate a broad range of substrates.

In the present study, we generated mutations in the M-domain of Hsp104 analogous to the repressed and de-repressed mutations in ClpB and investigated their effect on thermotolerance and yeast prion propagation. We found that M-domain mutants predicted to repress the mobility of the M-domain prevent thermotolerance and prion propagation. Strikingly, mutations we hypothesized would de-repress
Hsp104 M-domain function also result in prion elimination. Our data show that the M-domain regulates Hsp104 disaggregate activity and suggest that changes in the mobility of the M-domain have significant consequences for prion propagation.

Results

Hsp104 M-domain mutant, *hsp104-V426I*, causes sectoring [PSI+] phenotype

We performed a genetic screen to identify factors important for aggregation of the translation termination factor Sup35, and the resulting propagation of the [PSI+] prion. To identify candidates, we used a color-based phenotypic assay established to follow [PSI+] propagation. In this assay, a premature termination codon is present in the ADE1 gene, ade1-14, which prevents completion of the adenine biosynthesis pathway. Disruption of adenine biosynthesis causes the accumulation of a red-pigmented intermediate and prevents these cells from growing on media lacking adenine. Suppression of the premature termination codon leads to completion of the adenine biosynthesis pathway resulting in cells that are phenotypically light pink or white when grown on rich media (YPD) and are able to grow on media lacking adenine. When Sup35 is monomeric and functional (in non-prion containing [psi-] cells), translation termination is efficient, and the ade1-14 cells appear red in color and do not grow on media lacking adenine. Conversely, when Sup35 is in a prion state, it is aggregated and less functional, and the [PSI+] colonies are ADE+ (light pink in color on YPD and able to grow on media lacking adenine). From our screen, we identified a candidate that caused the [PSI+] cells to change from a light pink phenotype to a sectoring color phenotype (Fig. 3.1A). When a fraction of the cells in a colony do not inherit [PSI+] propagons, those cells become [psi-] and phenotypically red. All of the progeny resulting from those [psi-] cells will also be [psi-] and this pattern of inheritance can result in a sectoring color phenotype. By genetic testing, we discovered that this phenotype resulted from a point mutation in Hsp104. We sequenced HSP104 in this mutant strain and identified the mutation as *hsp104-V426I*.

To determine whether this Hsp104 mutant was affecting the aggregation of Sup35 in [PSI+] cells, we transformed the *hsp104-V426I* mutant strain with Sup35NM-GFP and analyzed the Sup35 aggregation pattern by fluorescence imaging. In *hsp104-V426I* cells, we observed cells that contained fluorescent foci indicative of Sup35 aggregates as well as cells that displayed diffuse fluorescence similar
to \([psi^{-}\) cells (Fig. 3.1B). Interestingly, the \(hsp104-V426I\) cells with fluorescent foci contained a single or a few large fluorescent foci, unlike the wild type \([PSI^{+}\) cells, which contained multiple, small fluorescent foci (Fig. 3.1B). Thus, we conclude that the mutant \(hsp104-V426I\) affects the aggregated state of the prion determinant Sup35 reflecting the changes in the \([PSI^{+}\) phenotype.

**Hsp104-V426 is located in the coiled-coil M-domain**

To determine how this mutation may be affecting Hsp104 function, we examined the structural models of Hsp104 to determine where this residue is located\[39,49\]. We discovered that V426 appears to be located in the first helix of motif 1 of the M-domain of Hsp104 and is analogous to the L424 residue in ClpB. Recently, data on the structure and function of the M-domain of ClpB suggested that the L424 residue plays a role in mediating the mobility and position of the coiled-coil M-domain by contributing to the interaction between the M-domain and NBD1 of the neighboring subunit. Another residue in the M-domain of ClpB, Y503, has also been shown to regulate M-domain mobility\[43\]. The Y503 residue interacts with NBD1, though it does so intramolecularly rather than interacting with the neighboring subunit like L424. Mutation of this residue, Y503D, led to a pronounced decrease in KJE-dependent (DnaK-DnaJ-GrpE, KJE) ClpB disaggregation activity\[42\]. More recently, ClpB-Y503D was shown to increase the rate of substrate-stimulated ATP hydrolysis and cause toxicity when expressed in bacteria grown at high temperatures\[43\]. Thus, the Y503D mutation in ClpB was proposed to be hyperactive by stabilizing the de-repressed conformation of the M-domain. As such, we wondered if the Hsp104-V426I mutation we identified in our screen might be disrupting the function of the Hsp104 M-domain in a similar fashion. To further test this, we referred to two classes of ClpB M-domain mutants, repressed and de-repressed, that had varying effects on the function of ClpB\[43,50\]. In a recent study, two repressed mutants, ClpB-E432A and ClpB-D480C, and two de-repressed mutants, ClpB-K476C and ClpB-Y503D, were analyzed for their affect on ClpB ATPase activity, disaggregation activity, and cell growth\[43\]. We created the analogous mutations in the M-domain of Hsp104 to determine if the effects of these mutants on disaggregate function are conserved between the chaperones. The analogous repressed Hsp104 mutations are predicted to be Hsp104-D434A and Hsp104-D484C. Likewise, Hsp104-K480C and Hsp104-Y507D are analogous to the de-repressed mutations. We also generated Hsp104-V426C
analogous to the ClpB-L424C mutation used to characterize the interaction of the M-domain with NBD1. We first analyzed the biochemical properties and disaggregation activities of these mutants to determine if they display similar functional effects as their counterparts in ClpB. Then, we analyzed the effect of these mutants on the propagation of yeast two prions - [PSI+] and [RNQ+].

M-domain mutants display varying levels of ATPase activity and hexamer formation

One proposed function of the M-domain is to regulate ATPase activity by interacting with NBD1 of the neighboring subunit in the hexamer and coordinating ATP binding and hydrolysis between NBD1 and NBD2[40,42,51]. Both the repressed and de-repressed ClpB mutants showed basal levels of ATP hydrolysis similar to wild type ClpB[43]. However, the de-repressed ClpB mutants had significantly higher substrate-stimulated ATPase activity[43]. To determine if the analogous M-domain mutants in Hsp104 had a similar impact on the ATPase activity, we purified recombinant wild type Hsp104 and the M-domain mutants and measured both the basal and substrate-stimulated ATP hydrolysis rates by the Malachite Green assay[32]. Interestingly, Hsp104-V426I, the mutant identified in our screen that altered [PSI+] propagation, maintained wild type rates of both basal and substrate-stimulated ATP hydrolysis (Fig. 3.2). Hsp104-D434A, Hsp104-D484C, and Hsp104-V426C exhibited decreased basal levels of ATPase activity as compared to wild type, while Hsp104-K480C and Hsp104-Y507D displayed higher rates of basal ATPase activity (Fig. 3.2). Additionally, wild type Hsp104, Hsp104-V426I, Hsp104-V426C, Hsp104-K480C, and Hsp104-Y507D all exhibited increased rates of ATP hydrolysis in the presence of substrate (Fig. 3.2). Conversely, addition of substrate did not increase the ATP hydrolysis rate above the basal level for Hsp104-D434A or Hsp104-D484C.

The ATPase activity of Hsp104 depends on the oligomeric state of the chaperone. Hsp104 mutants that inhibit hexamer formation also inhibit ATP hydrolysis[32]. Indeed, the M-domain has also been implicated in hexamer formation[40]. We reasoned that the decreased rates of ATP hydrolysis for a subset of the M-domain mutants might correlate with inefficient hexamer formation or a change in stability of the hexameric state. To test this, we incubated the purified Hsp104 M-domain mutants with ATP and then subjected the samples to ultracentrifugation on a linear glycerol gradient. Both Hsp104-D434A and Hsp104-V426C, which displayed decreased rates of ATP hydrolysis, also displayed a decrease in stable
hexamer formation (Fig. 3.3A). Hsp104-D484C appeared in two distinct populations: one that migrates like wild type Hsp104 that represents the hexameric pool, and one that migrates similar to the low-molecular weight species observed for Hsp104-V426C and Hsp104-D434A (Fig. 3.3A). Thus, the apparent lack of efficient hexamer formation of these three mutants, Hsp104-D434A, Hsp104-D484C, and Hsp104-V426C, likely contributes to the observed decrease in their ATPase activity. Alternatively, Hsp104-V426I, Hsp104-K480C, and Hsp104-Y507D all form hexamers and sediment on the gradient like wild type Hsp104 (Fig. 3.3B). Therefore, Hsp104-D434A, Hsp104-D484C, and Hsp104-V426C appear to decrease the ability of the M-domain to regulate ATPase activity and hexamer formation, while Hsp104-K480C and Hsp104-Y507D appear to cause hyperactivity, resulting in increased basal ATPase activity and an apparent de-repressed state.

**Hsp104-K480C and Hsp104-Y507D cause cell toxicity in a temperature-dependent manner**

Since the repressed and de-repressed ClpB mutants showed a difference in cell viability at high temperatures[43], we next tested if any of the Hsp104 M-domain mutants showed temperature-dependent growth defects. We grew the mutant strains described above on media that selected for the plasmid at 25, 30, and 37°C. At 25 and 30°C, all the mutant strains grew as well as the wild type HSP104 cells (Fig. 3.4). At 37°C, however, both *hsp104-K480C* and *hsp104-Y507D* were unable to grow (Fig. 3.4). This defect in growth at high temperature is similar to that of the analogous ClpB mutants, ClpB-K476C and ClpB-Y503D, which were hypothesized to be hyperactive mutants that result in cellular toxicity [43,50]. For comparison, a vector-only control was also plated, and this strain shows normal cell growth. Therefore, the toxicity associated with these mutant strains is not due to a lack of Hsp104 or a simple loss-of-function, but suggests a toxic gain-of-function for these mutants that impairs cell growth. As this toxicity is observed at 37°C which induces Hsp104 expression, we hypothesize that constitutive expression of these two mutants is detrimental to cellular homeostasis resulting in decreased viability.
M-domains mutants present varying levels of thermotolerance and non-prion aggregate disaggregation

Hsp104 is required for cell viability following heat shock (thermotolerance)[5]. To confer thermotolerance, Hsp104 must disaggregate non-prion substrates that aggregate as a consequence of the heat stress. The M-domain of Hsp104 (and ClpB) is proposed to affect the disaggregation of substrates by providing a site for an interaction with co-chaperones (Hsp70 and Hsp40 in yeast, DnaK and DnaJ in bacteria)[42,47]. Thus, mutations in the M-domain which abrogate interaction with co-chaperones may have a negative effect on the ability of the chaperone to disaggregate substrates. Furthermore, as the ATPase activity and disaggregation activity are interdependent, mutations in the M-domain that affect the regulation of ATPase activity may also affect the disaggregation mechanism. Therefore, we investigated the disaggregation activity of the Hsp104 M-domain mutants in vivo by analyzing their ability to confer thermotolerance to yeast, as well as their ability to disaggregate heat-aggregated luciferase.

We first transformed an hsp104Δ strain with a plasmid expressing each of the M-domain mutant Hsp104 genes, wild type HSP104, or an empty vector control. We then grew these strains to mid-logarithmic phase at 30°C, moved them to 37°C to induce expression of Hsp104, then heat shocked the strains at 50°C for various amounts of time before plating the cells to assess viability. We found that, like the hsp104Δ strain, hsp104-D434A and hsp104-D484C cells were not thermotolerant (Fig. 3.5A). Alternatively, both hsp104-V426I and hsp104-V426C cells maintained wild type thermotolerance ability (Fig. 3.5A). Interestingly, the two mutants with the highest ATPase activity, hsp104-K480C and hsp104-Y507D, presented an intermediate level of thermotolerance, where the amount of thermotolerance fell between that of wild type HSP104 and hsp104Δ strains (Fig. 3.5A).

We next tested the ability of the M-domain mutants to disaggregate heat-aggregated luciferase, which has previously been shown to be a substrate of Hsp104[30]. The strains described above, containing a plasmid expressing either wild type or mutant Hsp104, were transformed with a plasmid expressing luciferase. These strains were grown for an hour at 37°C to induce Hsp104 expression then heat shocked for an hour at 44°C to induce luciferase aggregation. After heat shock, the cells were allowed to recover at 30°C and we took samples periodically to quantify the relative amount of luminescence as a measure of the amount of luciferase resolubilized by Hsp104. As we saw in the
thermotolerance assays, hsp104-D434A and hsp104-D484C cells resembled the hsp104Δ strain in that there appeared to be no increase in the amount of resolubilized luciferase over time (Fig. 3.5B). In contrast, hsp104-V426I, hsp104-V426C, hsp104-K480C, and hsp104-Y507D cells exhibited luciferase recovery at about half the rate as that observed in wild type HSP104 cells. Thus, these data correlate with the thermotolerance data and suggest that Hsp104-D434A, Hsp104-D484C, Hsp104-K480C, and Hsp104-Y507D have defects in disaggregation. Alternatively, Hsp104-V426I and Hsp104-V426C appear to affect the disaggregation of specific substrates.

**Hsp104 M-domain mutants vary in their ability to propagate variants of the [PSI+] prion**

Given the varying effects of the M-domain mutants on ATPase activity and disaggregation ability, we next sought to ascertain the effect of the M-domain mutants on [PSI+] propagation. We first demonstrated that Hsp104-V426I caused a defect in the propagation of one [PSI+] variant, strong [PSI+], and resulted in sectoring colonies (Fig. 3.1A). To investigate the effect of the remaining M-domain mutants on strong [PSI+] propagation, we transformed a strong [PSI+] heterozygous diploid containing wild type HSP104 and hsp104Δ with a plasmid containing either wild type HSP104 or the M-domain mutants. Instantly, we noticed that Hsp104-D434A was dominant and cured the [PSI+] diploids to generate red [psi-] diploids (Fig. 3.6A). Next, the diploids were sporulated, and we selected haploids harboring hsp104Δ and each wild type or mutant Hsp104 plasmid and analyzed them phenotypically to assess [PSI+] propagation. By nonsense suppression, hsp104-V426I cells appeared to sector while hsp104-V426C, hsp104-K480C, and hsp104-Y507D appeared darker pink, indicative of a weaker nonsense suppression phenotype (Fig. 3.6A). In contrast, hsp104-D434A, and hsp104-D484C, appeared to resemble the vector control, suggesting that these mutations do not propagate strong [PSI+] (Fig. 3.6A). We next performed SDD-AGE analysis on the haploids to determine the effect of the mutants on Sup35 aggregate distribution and to confirm our analyses of the color phenotype. By SDD-AGE analysis, hsp104-V426I, hsp104-V426C, and hsp104-K480C cells maintained aggregates of Sup35 while hsp104-D434A, hsp104-D484C, and hsp104-Y507D cells did not (Fig. 3.6B).

The distribution of Sup35 aggregates in hsp104-V426C and hsp104-K480C cells is shifted compared to wild type HSP104 strong [PSI+] cells, suggesting that cells expressing these mutants may
be propagating a weak variant of [PSI+] in correlation with the decreased nonsense suppression phenotype[24,52]. Therefore, we next tested if any of the mutants were capable of propagating a weak [PSI+] variant. Using the same approach as for strong [PSI+], we transformed weak [PSI+] heterozygous diploids expressing wild type HSP104 and hsp104Δ with plasmids expressing either wild type HSP104 or the M-domain mutants. Similar to our observations with the strong [PSI+] diploid, hsp104-D434A dominantly cured diploids carrying a weak [PSI+] variant (Fig. 3.6C). Since hsp104-D434A dominantly cures two distinct variants of [PSI+], we propose that this mutation inhibits wild type Hsp104 function in mixed hexamers. Interestingly, diploids expressing hsp104-K480C also appeared to decrease nonsense suppression suggesting that hsp104-K480C might have a dominant curing effect on weak [PSI+] (Fig. 3.6C). Next, we sporulated the diploids and isolated hsp104Δ haploids expressing the wild type or mutant HSP104 to assess the color phenotype. We observed that none of the Hsp104 M-domain mutants were able to propagate the weak variant of [PSI+] (Fig. 3.6C). To confirm this phenotypic observation, we performed SDD-AGE analysis and surprisingly found that hsp104-V426C cells contained Sup35 aggregates (Fig. 3.6D).

Interestingly, despite several attempts to generate strong or weak [PSI+] haploids expressing hsp104-Y507D, we were only able to isolate a single haploid expressing hsp104-Y507D from the strong [PSI+] heterozygous diploid (Fig. 3.6B). In fact, this single haploid was unable to further grow beyond the initial isolation (Fig. 3.6B). In addition to sporulating diploids, we also attempted to replace the wild type HSP104 in a strong [PSI+] strain with hsp104-Y507D by co-expressing both wild type HSP104 and hsp104-Y507D and then eliminating the wild type HSP104 plasmid. This method also proved unsuccessful at isolating [PSI+] cells expressing hsp104-Y507D. From these data, we propose that hsp104-Y507D is highly toxic in the presence [PSI+]. Consequently, we would predict that [PSI+] cells that lost the prion and became [psi-] would still be viable in the presence of hsp104-Y507D. Intriguingly, we were even unable to isolate any [psi-] cells expressing hsp104-Y507D after sporulation, suggesting that the combination of either strong or weak [PSI+] and hsp104-Y507D is so potent that toxicity occurs before cells have a chance to reverse the prion phenotype. Furthermore, expression of hsp104-Y507D in hsp104Δ [psi-] [rnq-] cells showed no similar toxicity, suggesting that toxicity is dependent on Sup35 aggregation. Similar toxicity in the presence of [PSI+] has been observed for another M-domain mutant,
hsp104-A503V, suggesting that prion-dependent toxicity is not specific for this one residue, but may be caused by a deregulation of M-domain function[53].

M-domain mutants are able to propagate distinct variants of [RNQ+]

We next examined the ability of the M-domain mutants to propagate several variants of the [RNQ+] prion. Similar to [PSI+], the [RNQ+] prion is also sensitive to changes in Hsp104 activity and variants of [RNQ+] are differentially affected by changes in Hsp104 activity[54,55]. Several variants of [RNQ+] exist that are characterized by their ability to induce the [PSI+] prion phenotype and the aggregate pattern observed in cells by fluorescence[16,56]. [RNQ+] variants display either a single-dot (s.d.) or multi-dot (m.d.) pattern of fluorescence describing the appearance of Rnq1-GFP aggregates in [RNQ+] cells[56]. [RNQ+] variants that display s.d. fluorescence can facilitate the induction of [PSI+] at low, medium, high, and very high rates upon Sup35 overexpression. On the other hand, the only established m.d. variant of [RNQ+] exhibits a high rate of [PSI+] induction. We utilized these five characterized [RNQ+] variants to ascertain the effect of M-domain mutants on conformational variants of [RNQ+].

We utilized a plasmid shuffle technique to determine the effect of the M-domain mutants on the [RNQ+] variants. Strains containing each [RNQ+] variant harbor a chromosomal hsp104Δ and are complemented by the expression of wild type HSP104 from a plasmid. We transformed the plasmids containing the M-domain mutants into these strains, then, selected for cells that eliminated the wild type HSP104 by growing them on counter-selection media to determine the effect of the mutants on the pre-existing [RNQ+] variants. Interestingly, we observe a differential effect of these mutants on the propagation of the [RNQ+] variants. Of the M-domain mutants, hsp104-V426I and hsp104-K480C cells were able to maintain all of the tested [RNQ+] variants (Fig.3.7). On the other hand, cells expressing hsp104-D434A were [rnq-] in all of the tested variants of [RNQ+], suggesting that this mutant is a general prion inhibitor. Interestingly, cells expressing hsp104-V426C and hsp104-Y507D were able to propagate all but the s.d. medium and s.d. very high [RNQ+] variants, while hsp104-D484C cells propagate only the s.d. low variant of [RNQ+]. Furthermore, all M-domain mutants, excepting hsp104-V426I, were
characterized by a shift in the aggregate distribution of Rnq1 aggregates in all of the tested [RNQ+] variants.

Discussion

Here, we present analysis of six Hsp104 M-domain mutants, which have differential effects on chaperone function and cell viability. Initially, we identified Hsp104-V426I from a screen for factors that affected [PSI+] propagation. We observed that hsp104-V426I cells had defects in [PSI+] propagation that manifested as a sectoring [PSI+] phenotype. We have observed this phenotype before from other Hsp104 mutants that have varying effects on Hsp104 structure and function (unpublished data) but this was the only mutation we identified that is located in the M-domain. The coiled-coil M-domain of Hsp104 is proposed to regulate ATPase activity, substrate disaggregation, and co-chaperone interactions[38]. We noted that the V426 residue in Hsp104 is analogous to the recently characterized L424 residue in ClpB, which was shown to play a role in regulating the position and mobility of the M-domain in ClpB[43]. Previously, it was shown that the stability of the coiled-coil M-domain of ClpB depends on the leucine zipper-like interactions between leucine and isoleucine residues and that mutation of these residues to alanine caused significant changes in chaperone activity, ATP hydrolysis, and hexamer formation[57]. Perhaps, then, mutation of the valine at residue 426 to an isoleucine disrupts the normal isoleucine-leucine interactions, resulting in slight destabilization of the M-domain. Although we do not have direct evidence that the V426I mutation in the M-domain of Hsp104 alters the interaction of the M-domain with the neighboring NBD1 domain, previous data from ClpB suggest that this residue may contribute to M-domain positioning, and that mutation of this residue could disrupt interactions of the M-domain with neighboring subunits within the hexamer.

In order to elucidate the effect of the V426I mutation on the function of the M-domain of Hsp104, we examined the rates of ATP hydrolysis, hexamer formation, and thermotolerance and disaggregation. We also examined these same properties among a set of mutations in the M-domain that in ClpB were proposed to stabilize the repressed or de-repressed conformation of the M-domain resulting in changes in the regulation of overall chaperone activity[43,50]. We generated the analogous mutations in Hsp104 and characterized their ability to form hexamers, hydrolyze ATP, and disaggregate protein substrates.
We found that, in general, the M-domain mutants had similar effects on the activity of Hsp104 as they displayed in ClpB.

Both Hsp104-D434A and Hsp104-D484C decreased overall ATPase activity and disaggregation activity suggesting that prolonged occupation of the repressed state inhibits Hsp104 activity[43]. The M-domain mutants Hsp104-K480C and Hsp104-Y507D increased the overall rate of ATP hydrolysis and caused toxicity when cells were grown at higher temperatures indicative of the proposed effects of the de-repressed conformation on M-domain function. Interestingly, our data for the rates of ATP hydrolysis of Hsp104-K480C differed from a recent report in that we found an increase in the basal ATPase activity compared to wild type Hsp104[41]. Interestingly, from biochemical characterization, both Hsp104-V426I and Hsp104-V426C appear to stabilize neither the repressed nor the de-repressed conformation, as those effects are characterized by the other analyzed mutants. Instead, we propose that the Hsp104-V426I and Hsp104-V426C mutations result in a semi-repressed conformation that only moderately affects the regulatory function of the M-domain. The difference in the ATP hydrolysis rates of Hsp104-V426I and Hsp104-V426C suggest that the biochemical properties associated with the side chain of this residue are important. A range of phenotypes associated with the identity of the mutated residue is also observed for the Hsp104 M-domain residue D484. In a previously published report, mutation of D484 to a lysine (D484K) was characterized by cellular growth defects and increased ATPase and chaperone activity, which is the opposite of our data for Hsp104-D484C[36]. Interestingly, this study suggests that disruption of the ionic interactions associated with Hsp104-D484 leads to hyperactivity while we show that disruption of these ionic interactions can also lead to reduced activity[36]. Thus, the M-domain is finely tuned to regulate various functions of Hsp104 and disruption of this balance can lead to severe consequences for Hsp104 function.

Although several studies have examined the role of the M-domain in the regulation of protein disaggregation and ATPase activity, much less is known about the effect of the Hsp104 M-domain regulatory function on yeast prion propagation. Here, we show that mutations that disrupt M-domain function also inhibit prion propagation. The repressed mutants, Hsp104-D434A and Hsp104-D484C, inhibit propagation of both strong and weak [PSI+] variants. The M-domain mutant Hsp104-D434A is even more pronounced as it inhibits wild type Hsp104 function to cause dominant curing of both variants.
of [PSI+]. Additionally, the de-repressed mutants Hsp104-K480C and Hsp104-Y507 appear to have distinct effects on [PSI+] propagation despite similar biochemical properties. Hsp104-K480C is able to propagate strong [PSI+] but has a semi-dominant curing effect on weak [PSI+]. These data correlate well with observations that overexpression of Hsp104 cures weak [PSI+] variants more efficiently than strong variants, though the mechanism of [PSI+] elimination by excess Hsp104 is not well understood[8,58,59].

Another hypothesis to explain the differences observed between weak and strong [PSI+] is that weak [PSI+] variants are more dependent on Hsp70s and Hsp40s for efficient propagation, as varying levels of Hsp70 expression can have greater effects on weak [PSI+] variants than strong variants[60]. Indeed, Hsp104 acts in concert with Hsp70s and Hsp40s and the balance of this complex is important[1,61,62].

As the de-repressed M-domain mutants of ClpB were shown to have reduced interaction with the KJE chaperones, and Hsp104-K480C is deficient in thermotolerance and luciferase refolding, perhaps a reduced interaction of Hsp104-K480C with co-chaperones is responsible for the curing of the weak [PSI+] variants.

Our biochemical data for Hsp104-V426I as a semi-repressed mutant correlate well with the sectoring [PSI+] phenotype observed in hsp104-V426I cells. Unlike the repressed mutants, Hsp104-D434A and Hsp104-D484C, which cure [PSI+], the semi-repressed Hsp104-V426I and Hsp104-V426C maintain strong [PSI+], albeit inefficiently, and inhibit propagation of weak [PSI+]. Recently, a decreased interaction of Hsp104 with weak [PSI+], as compared to strong [PSI+], was demonstrated in vitro[25], and we have recently shown that decreased Hsp104 activity is sufficient to propagate strong but not weak variants of [PSI+] (Dulle and True, unpublished data). Therefore, changes in the regulatory function of the M-domain alter the ability of Hsp104 to stably propagate distinct [PSI+] variants.

In addition to changes in [PSI+] propagation, we also found differential effects of the M-domain mutants on the propagation of conformational variants of the [RNQ+] prion. The repressed M-domain mutants, Hsp104-D434A and Hsp104-D484C cannot propagate any tested variant of [RNQ+]. Although we have previously characterized a mutant of Hsp104 that displays decreased activity but is still able to propagate specific variants of [RNQ+], clearly a threshold of activity exists that is required for [RNQ+] propagation[54]. Interestingly, the M-domain mutants Hsp104-Y507D and Hsp104-V426C are able to propagate the two variants of [RNQ+] (s.d. medium and very high) but not the others while Hsp104-
D484C could propagate the s.d. low variant of [RNQ+]. We have previously characterized mutations in Hsp104, which showed differential propagation among the [RNQ+] variants[54]. One hypothesis for differential prion variant propagation suggests that the stability of the prion variant dictates the interaction with Hsp104[25]. However, the s.d. [RNQ+] variants have been shown to have similar stabilities, suggesting that stability is only one contributing factor toward Hsp104 interaction[63]. Another contributing factor may be the amount of soluble Rnq1 as the variants were shown to differ in the amount of soluble Rnq1 with s.d. very high and s.d. low [RNQ+] containing the most[63]. However, our data also suggest that medium [RNQ+], which contains less soluble Rnq1 than low and very high but more than high, is also propagated differentially among the M-domain mutants. Curiously, the semi-repressed mutant, Hsp104-D484C, shows a similar pattern of [RNQ+] variant propagation as the de-repressed Hsp104-Y507D mutant. How these two mutants with different biochemical properties show the same pattern of [RNQ+] propagation is as yet unclear. Furthermore, our data clearly demonstrates the complexity of prion variant propagation and illustrates the need for further investigation to understand the mechanism of interaction between chaperones and conformationally distinct prion variants.

The structure and function of the Hsp104/ClpB M-domain has been a subject of much investigation and controversy in recent years. Various structural studies of ClpB and Hsp104 have proposed significantly different models for M-domain position in relation the hexameric structure[39,49,64]. More recent data suggest that the true placement of the M-domain may be a combination of previous models. Specific residues in the M-domain are protected, suggesting that at least part of the M-domain is tightly packed into the body of the hexamer[43,47,49]. Additionally, cross-linking and fluorescence quenching experiments suggest that the M-domain contacts residues in the NBD1 either in the neighboring subunit or in the same subunit[43]. Furthermore, the flexibility of the M-domain to break and re-form these contacts is integral to regulation of the chaperone function by the M-domain[36,43,50]. The data in our study do not lend direct support any one structural model, but do suggest that the M-domain of Hsp104 plays a key role in regulating the disaggregation of both prion and non-prion substrates.

The M-domain has clearly been determined to play a significant role in regulating Hsp104/ClpB function. Characterization of mutations in the M-domain demonstrates that this coiled-coil region affects
all of the distinct activities that Hsp104/ClpB possesses, suggesting that this domain may be the master regulator of Hsp104/ClpB function[35,36,42,43,44,47,50,53]. As such, elucidation of the M-domain regulatory mechanism is vital to our understanding of the disaggregation mechanism of Hsp104/ClpB.

Materials and Methods

Strain and Plasmid Construction

All *S. cerevisiae* strains were derivatives of 74-D694 and were grown using standard culture techniques. Strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic media lacking amino acids with correlated with plasmid selection (0.67% yeast nitrogen base, 2% glucose).

Hsp104 mutants were cloned into the pRS313 vector for expression in vivo. Point mutations in *HSP104* were generated by bridge PCR (oligonucleotides listed in Supplementary Table One) using pRS313-pha-HSP104 (kindly provided by B. Bukau) as the template. Bridge PCR and pRS313-pha-HSP104 were digested with EcoRI and Bsu36I and ligated together. Hsp104 mutants were also cloned into pProEx-HTb-HSP104 (kindly provided by J. Glover[34]) by the same digestion and ligation. The pRS313-pha-hsp104-V426I plasmid was generated by PCR amplifying HSP104 from the EMS mutagenized strain, then digesting and ligating as described.

The Lindquist lab characterized and kindly provided the strong [PSI+] variant[8,65]. The Liebman lab characterized and kindly provided the weak [PSI+] variant[65]. The Sc4 and Sc37 [PSI+] variants, made by transforming Sup35 fibers assembled at 4°C and 37°C into [psi-] cells, were made and kindly provided by the Weissman lab[66]. To analyze the mutant pRS313-pha-HSP104 plasmids in the [PSI+] variants, each variant was mated to an *hsp104Δ* (*hsp104::leu2*) strain and diploids were selected. The mutant pRS313-pha-HSP104 plasmids were transformed into the heterozygous diploids, the diploids were sporulated, and haploids were selected on media lacking histidine and leucine. Colonies were verified as haploids by plating on A and α strains to determine mating type.

The [RNQ+] variant strains were kindly provided by the Liebman lab. To create strains carrying both the mutant Hsp104 plasmids and the [RNQ+] variants, we created an *HSP104* plasmid shuffle strain. First, pRS316-pha-HSP104 (kindly provided by J. Weissman) was first transformed into each of the [RNQ+] variants. *HSP104* on the chromosome was deleted by transforming in the knock-out cassette.
pAG32-HygromycinB that had been PCR amplified to contain flanking sequences to \textit{HSP104} (oligonucleotide sequences in Supplementary Table One). Deletion strains were confirmed first by selection on media lacking uracil and containing Hygromycin B (Invitrogen) then by colony PCR. These strains were then transformed with each of the mutant pRS313-\textit{hsp104} plasmids, selected on media lacking histidine and uracil, grown overnight in liquid media lacking just histidine, and then plated on media lacking histidine and containing 5'Fluoroorotic acid (US Biologicals) to select for cells that had dropped the pRS316-\textit{phs-HSP104} plasmid. Colonies that grew on media lacking histidine but not uracil were selected.

\textbf{EMS mutagenesis screen}

The strong $[PSI^+]$ yeast strain was subjected to EMS mutagenesis as previously described\cite{54}. Two cultures with viabilities of about 17\% were plated to determine changes in color. Candidates were selected based on color phenotype and were initially identified as mutations in \textit{HSP104} by back-crossing to an \textit{hsp104Δ} strain and analyzing the progeny for segregation of the prion-dependent nonsense suppression phenotype. Genomic DNA was PCR amplified and sequenced to identify the point mutations in \textit{HSP104}.

\textbf{SDD-AGE}

Cells were lysed by disruption of the membranes with glass beads in Sup35 PEB buffer (25mM Tris-HCl pH 7.5, 50mM KCl, 10mM MgCl$_2$, 1mM EDTA, 10\% Glycerol plus mini EDTA-free protease inhibitors (Roche), Aprotinin (Sigma) and PMSF (Sigma)) or Rnq1 PEB buffer. Samples were incubated in sample buffer at room temperature for seven minutes then separated on a 1.5\% agarose gel. The protein distribution was analyzed by western blot with anti-Sup35 antibody or antibody against Rnq1.

\textbf{Hsp104 Purification}

Recombinant Hsp104 was expressed and purified from \textit{E. coli} cells as previously described\cite{67}. After purification, the pool of recombinant Hsp104 was separated on an S-300 gel filtration column to isolate Hsp104 monomers. Purified, monomeric Hsp104 was concentrated and frozen at -80°C.

\textbf{ATP Hydrolysis Assays}
The Malachite green assay was used to measure the rates of ATP hydrolysis[32]. Purified protein (2µg) was incubated with 5mM ATP in buffer (40mM TrisHCl pH7.5, 175mM NaCl, 5mM MgCl₂, 0.02% Triton X-100) at 37°C. At each minute over a time course of 12 minutes, Malachite green dye was added to the sample and the reaction stopped by the addition of 34% citric acid. The absorbance was measured at 650nm and the concentration of free phosphate calculated based on a standard of KH₂PO₄ and normalized to the sample containing no Hsp104.

**Glycerol Gradients**

Purified Hsp104 was centrifuged at 34k rpm for 18 hours through a 4mL linear (10-35%) glycerol gradient. The gradients were fractionated and equal volumes of each fraction were analyzed by SDS-PAGE and western blot using anti-Hsp104 antibody. Individual bands from each fraction were quantified using Image J and reported as a percent of total Hsp104.

**Thermotolerance**

An equal number of cells from cultures of HSP104, hsp104-V426I, hsp104-V426C, hsp104-K480C, hsp104-Y507D, hsp104-D434A, hsp104-D484C, and hsp104Δ were treated at 37°C for 30 minutes to induce HSP104 expression, then heat-shocked at 50°C. At 10, 15, 20, 25, and 30 minutes during heat shock, samples were taken and spotted media lacking histidine in a five-fold dilution.

**Luciferase Refolding**

An hsp104Δ strain containing plasmids expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-K480C, hsp104-Y507D, hsp104-D434A, hsp104-D484C, and an empty vector control were transformed with pRS316-GPD-luciferase, a plasmid expressing luciferase (kindly provided by B.Bukau)[6]. Cells were grown at 37°C for one hour, then heat-shocked at 44°C for one hour. Fifty minutes into the heat shock, cycloheximide (Sigma) was added to the culture to block protein synthesis. At various times during recovery at 30°C, 100 µL samples were taken and 50µL of 1mM beetle luciferin (Promega) was added. Luminescence was measured on a Sirius luminometer. The resolubilization of luciferase was calculated by dividing the measured luminescence at each time point by the measured luminescence prior to heat shock and normalized to the luminescence measured immediately after heat shock.
References


36. Lipinska N, Zietkiewicz S, Sobczak A, Jurczyk A, Potocki W, et al. (2013) Disruption of ionic interactions between the nucleotide binding domain 1 (NBD1) and middle (M) domain in Hsp100


Figure 3.1 A point mutation in Hsp104 destabilizes [PSI+]. A, Cells expressing hsp104-V426I or HSP104 were plated onto rich media to illustrate the destabilizing effect this mutation has on [PSI+]. In the presence of hsp104-V426I, [PSI+] is lost by a fraction of the buds, generating sectors of [psi-] (phenotypically red) cells in the [PSI+] colony. B, The copper-inducible fluorescent protein, Sup35NM-GFP, was transformed into hsp104-V426I and wild type [PSI+] and [psi-] cells. Fluorescence imaging was done on an Olympus confocal microscope.
Figure 3.2 Hsp104 M-domain mutants affect ATPase activity. The ATPase activity of recombinant Hsp104, Hsp104-V426I, Hsp104-V426C, Hsp104-D434A, Hsp104-D484C, Hsp104-K480C, and Hsp104-Y507D was measured by the Malachite Green assay after incubation with 5mM ATP either in the absence (black) or presence (grey) of 0.25mg/mL β-casein. The amount of free inorganic phosphate in each sample was calculated by comparison with a standard phosphate solution. For each protein, the average initial rate of ATP hydrolysis is plotted. Each protein was measured in quadruplicate from two separate purification preparations and error bars reflect standard deviation between the samples.
Figure 3.3 The M-domain plays a role in hexamer formation. The oligomeric distribution of recombinant Hsp104 (dark blue, A & B) and Hsp104-V426I (red), Hsp104-K480C (yellow), and Hsp104-Y507D (green) (A) and Hsp104-V426C (orange), Hsp104-D434A (purple), and Hsp104-D484C (grey) (B) was analyzed by ultracentrifugation through a linear glycerol gradient in the presence of 5mM ATP. Equal fractions from the gradients were collected and analyzed by western blotting with anti-Hsp104 antibody. The amount of Hsp104 in each fraction was quantified by Image J and graphed as a fraction of the total Hsp104. The gradients were repeated twice with recombinant protein from two separate purification preparations.
Figure 3.4 Hsp104-K480C and Hsp104-Y507D are toxic at high temperatures. Hsp104Δ strains expressing wild type HSP104, hsp104-V426I, hsp104-V426C, hsp104-K480C, hsp104-Y507D, hsp104-D434A, and hsp104-D484C from a HIS3-expressing plasmid were plated on media lacking histidine and grown at 25, 30 or 37°C to assess temperature-dependent growth defects. A vector-only control is also plated.
A

No Heat

HSP104  hsp104Δ

No Heat

hsp104-V426I  hsp104-V426C  hsp104-K480C  hsp104-Y507D

No Heat

hsp104-D434A  hsp104-D464C

B

Fraction Luciferase Recovered

0.0 0.02 0.04 0.06 0.08 0.1 0.12 0.14 0.16

0 15 30 45 60

Time (min)
Figure 3.5 M-domain mutants have differing affects on the ability to disaggregate non-prion substrates. A, hsp104Δ strains expressing wild type HSP104, hsp104-V426l, hsp104-V426C, hsp104-K480C, hsp104-Y507D, hsp104-D434A, and hsp104-D484C from a HIS3-expressing plasmid were heat-shocked to measure the mutants’ ability to function in thermostolerance. The mutant and wild type cells as well as an empty vector control (Vector) were heat-shocked at 50°C for various amounts of time then plated on media lacking histidine to assess viability. The spottings represent a five-fold serial dilution. B, The same hsp104Δ strains containing a plasmid expressing luciferase and expressing wild type HSP104 (red), hsp104-V426l (purple), hsp104-V426C (light blue), hsp104-K480C (yellow), hsp104-Y507D (green), hsp104-D434A (orange), hsp104-D484C (pink), or an empty vector control (dark blue) were grown at 37°C to induce Hsp104 expression then heat-shocked at 44°C for an hour to induce luciferase aggregation. At fifty minutes into heat shock, cycloheximide was added to the cells to block new protein synthesis. At various times during recovery, samples were taken, luciferin was added, and the luminescence was measured. The graph represents the amount of luciferase recovered as a fraction of the total luciferase before heat shock. Three separate samples for each mutant were analyzed and error bars reflect standard deviation between the samples.
Figure 3.6 M-domain mutants differentially affect propagation of strong and weak variants of [PSI+]. A, Strong [PSI+] diploids or haploids were transformed with plasmids expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-D434A, hsp104-D484C, hsp104-K480C, hsp104-Y507D or an empty vector control (EV) and spotted on media selecting for the plasmid. Each column is a five fold dilution. B, Strong [PSI+] haploids expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-D434A, hsp104-D484C, hsp104-K480C, or hsp104-Y507D, as indicated, were subjected to SDD-AGE analysis and western blot with antibody against Sup35. This is one representative of three separate experiments. C, Weak [PSI+] diploids or haploids were transformed with plasmids expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-D434A, hsp104-D484C, hsp104-K480C, hsp104-Y507D or an empty vector control (EV) and spotted on media selecting for the plasmids. Each column is a five fold dilution. D, Weak [PSI+] haploids expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-D434A, hsp104-D484C, hsp104-K480C, hsp104-Y507D, or an empty vector control were subjected to SDD-AGE analysis and western blot with antibody against Sup35. This is one representative of two separate experiments.
Figure 3.7 M-domain mutants differentially propagate [RNQ+] variants. Strains containing the [RNQ+] variants, s.d. low, medium, high, and very high and m.d. high, expressing HSP104, hsp104-V426I, hsp104-V426C, hsp104-D434A, hsp104-D484C, hsp104-K480C, hsp104-Y507D, or an empty vector control (EV) were subjected to SDD-AGE analysis and western blot with antibody against Rnq1. Each SDD-AGE is on representative of at least three separate experiments.
Chapter 4: Low Hsp104 Activity Sufficient for Propagation of Strong Variants of the [PSI+] Prion

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Abstract

The molecular chaperone network plays a critical role in the formation and propagation of self-replicating yeast prions. Not only do individual prions differ in their requirements for certain chaperones, but structural variants of the same prion can also display distinct dependences on the chaperone machinery, specifically Hsp104. The AAA+ ATPase Hsp104 is a disaggregase required for the maintenance of most known yeast prions. As a key component in the propagation of prions, understanding how Hsp104 differs in its interactions with specific variants is crucial to understanding how prion variants may be selected or evolve. Here, we investigate two novel mutations in Hsp104, hsp104-G254D and hsp104-G730D, which allow us to elucidate some mechanistic features of Hsp104 disaggregation and its requirement for activity in propagating specific prion variants. Both Hsp104 mutants propagate the [PSI+] prion to some extent, but show a high rate of prion loss. Both Hsp104-G254D and Hsp104-G730D display reduced biochemical activity, yet differ in their ability to efficiently resolubilize disordered, heat-aggregated substrates. Additionally, both mutants impair weak [PSI+] propagation, but are capable of propagating the more stable strong [PSI+] variant to some extent. One of the Hsp104 mutants also has the ability to propagate one variant of the [RNQ+] prion. Thus, our data suggest that changes in Hsp104 activity limit substrate disaggregation in a manner that depends more on the stability of the substrate than the nature of the aggregated species.

Introduction

Prions are self-templating, amyloidogenic protein aggregates. In mammals, prions are associated with several neurodegenerative diseases, including scrapie in sheep, chronic wasting disease in deer and elk, and Creutzfeldt-Jacob disease in humans.¹ ² In the budding yeast Saccharomyces cerevisiae, prions are non-toxic, epigenetic elements of inheritance, but they do share many characteristics with mammalian prions and other disease-related amyloidogenic proteins.³ ⁵ The yeast prion [PSI+] results from a self-propagating aggregated state of the translation termination factor Sup35.⁶ Aggregation of Sup35 results in partial loss-of-function that leads to an increase in global nonsense suppression.⁷ Consequently, the ability to generate [PSI+] cells, coupled with the reversible or metastable nature of this prion mechanism, and has been proposed to be advantageous due to the ability
to alter phenotypes through the [PSI+] -dependent translation of normally silent regions of the genome.\textsuperscript{9,10} Interestingly, the appearance of the [PSI+] prion appears to be regulated by the presence of another yeast prion, called [RNQ+] (or [PIN+]) which is the aggregated form of the Rnq1 protein.\textsuperscript{11-15} Though the soluble protein Rnq1 (in [rnq] -cells) has no known function, the prion state of Rnq1, [RNQ+], functions to promote the de novo induction of [PSI+], and is often required for the aggregation of other amyloidogenic proteins in yeast.\textsuperscript{16}

Although the prion conformation is self-templating, the maintenance of prions in yeast relies on the molecular chaperone network to produce aggregates that can be transmitted from mother to daughter cells. The AAA+ ATPase chaperone Hsp104 is a disaggregase required for propagation of both [PSI+] and [RNQ+], as well as all other recognized yeast prions.\textsuperscript{11,17-19} Hsp104, in concert with Hsp70 and Hsp40 chaperones, also functions to resolubilize proteins that aggregate as a consequence of various environmental stresses.\textsuperscript{20,21} Hsp104 has five distinct domains and is functional as a hexamer that contains a central pore used for threading substrates.\textsuperscript{22-24} The N-terminal domain is proposed to play a role in substrate recognition and may also be a site for interaction of co-chaperones, though the N-terminal domain is not required for either thermotolerance or prion propagation.\textsuperscript{25} The two ATP-binding domains (NBD1 and NBD2), connected by a coiled-coil linker domain, both bind and hydrolyze ATP to power the disaggregation mechanism.\textsuperscript{26,27} Lastly, the function of the fifth modular domain, the C-terminal domain, is still mostly unclear, though some data have implicated this domain as having a role in substrate interaction and processing.\textsuperscript{28,29}

The proposed role of Hsp104 in prion propagation is to fragment prion aggregates in order to generate the smaller heritable species, or propagons.\textsuperscript{30-32} Consequently, deletion of Hsp104 eliminates both [PSI+] and [RNQ+], and several mutants in each domain of Hsp104 have been characterized that affect [PSI+] and [RNQ+] propagation.\textsuperscript{12,17,33-36} Interestingly, [PSI+] is especially sensitive to changes in the Hsp104 system as the overexpression of Hsp104 eliminates [PSI+] to generate [psi-] cells, whereas Hsp104 overexpression does not eliminate [RNQ+].\textsuperscript{12,17,37,38} Hsp104 has also been shown to catalyze amyloid formation of prion proteins in vitro.\textsuperscript{37,39,40} Furthermore, maintenance of prions by Hsp104 is aided by Hsp70 and Hsp40 co-chaperones, and overexpression or deletion of these chaperones can affect prion propagation.\textsuperscript{41-47}
Yeast prions exist as an array of self-propagating structures, known as prion variants (analogous to “prion strains” in mammalian prion nomenclature). Although formed from the same protein sequence, prion variants maintain distinct structures having varying stabilities and causing different cellular phenotypes, much like mammalian prion strains result in variation in disease pathology. Prion variants have been described for both [PSI+] and [RNQ+]. [PSI+] variants are characterized by the amount of nonsense suppression they confer, which is related to the ability of the aggregate to template monomeric Sup35. Weak variants of [PSI+] maintain an increased pool of monomeric Sup35 relative to strong [PSI+] variants. As such, weak [PSI+] variants show less nonsense suppression (more folded, soluble Sup35 means more translation termination). Additionally, distinct Sup35 amyloid structures have been generated using denatured, recombinant protein (usually using just the prion-forming domain, also called “NM”) by simply changing the temperature at which amyloid fibers are formed. From structural studies of in vitro formed amyloid, Sup35 aggregates with a shorter protected amyloid core are less stable and give rise to a strong [PSI+] phenotype when [psi-] cells are infected with these aggregates, while fibers with longer, more stable cores tend to give rise to weak [PSI+] phenotypes upon infection. Recent data suggest that the longer, more stable core associated with the weaker [PSI+] variants is more refractory to Hsp104 activity or interaction. In contrast, the shorter core of strong variants is more labile to Hsp104 disaggregation, resulting in the generation of more propagons in strong [PSI+] cells, and thus increased conversion of monomeric Sup35. As such, strong [PSI+] variants present a stronger nonsense suppression phenotype as compared to weak [PSI+] variants, and are better able to propagate when challenged with fluctuations in Hsp104 activity.

Variants of [RNQ+] were first identified in vivo and were characterized by their differential ability to facilitate formation of the [PSI+] prion. These variants were named low, medium, high, and very high [PIN+] to indicate their rate of [PSI+] induction. These [PIN+]/[RNQ+] variants were further described by their patterns of fluorescence when Rnq1 was tagged with GFP. Two distinct phenotypes were observed: cells that contained multiple, small fluorescent foci termed multi-dot (m.d.), and cells that contained a single, large fluorescent focus termed single-dot (s.d.). [RNQ+] variants that induced [PSI+] at low, medium, high, and very high rates were all found within the microscopic s.d. pattern, while only one m.d. [RNQ+] variant was characterized and it induced [PSI+] at a high rate. Further investigation of
these established [RNQ+] variants showed that a distinct region of the Rnq1 protein was required for propagation of certain variants, suggesting that [RNQ+] variants, like [PSI+], may also differ in their core region. Similar to Sup35, Rnq1 fibers display distinct characteristics when generated under various conditions in vitro, though structural studies to elucidate the amyloid core of these [RNQ+] variants have not yet been done. Additionally, much less is understood about the interaction between [RNQ+] and Hsp104 and how changes in Hsp104 activity affect propagation of specific [RNQ+] variants.

Here, we characterize two novel mutations in Hsp104 that we identified as causing a defect in [PSI+] propagation. We show that these mutations decrease select activities of Hsp104, resulting in diminished disaggregation activity of specific substrates. Finally, we find that defects in [PSI+] and [RNQ+] propagation are specific for certain prion variants, thereby providing further evidence for the hypothesis that Hsp104 may interact more efficiently with less stable [PSI+] variants than more stable variants, and that this may also hold true for variants of [RNQ+].

Results

**Point mutations in HSP104 cause a defect in the maintenance of [PSI+].** We performed a mutagenesis screen in order to identify factors required for the propagation of a specific variant of the [PSI+] prion. To identify such factors, we used a colony color based phenotypic assay commonly used to track [PSI+] prion propagation. In this colorimetric assay, a premature stop codon in the ADE1 gene, ade1-14, blocks the completion of the adenine biosynthesis pathway and results in the accumulation of a red-pigmented intermediate. Disruption of the pathway at this point also results in the inability of these cells to grow on media lacking adenine. Cellular changes that afford an increase in nonsense suppression of the premature stop codon in ade1-14 can result in production of sufficient Ade1 protein to generate light pink or white colonies and allow for growth on media lacking adenine. Such an increase in nonsense suppression can be generated by reduced function of the translation termination complex (eRF1 and eRF3, or Sup45 and Sup35, respectively). This can occur either by mutation of either termination factor or by conversion of wild type Sup35 into a prion state. When Sup35 is monomeric and fully functional in [psi-] cells, the premature stop codon in ade1-14 is recognized, resulting in colonies that are red in color on rich media and cannot grow on media lacking adenine. When Sup35 is aggregated in
[\textit{PSI}+] cells, however, the nonsense codon is suppressed and the [\textit{PSI}+] cells appear white on rich media and can grow on media lacking adenine.

In addition, structural variants of the [\textit{PSI}+] prion present a range of color phenotypes indicative of the amount of soluble Sup35 characteristic of those variants. For example, because of the relative increase in soluble Sup35, weak [\textit{PSI}+] strains harboring \textit{ade 1-14} are pink in color on rich media. In our screen, we expected to find mutants that affected [\textit{PSI}+] propagation by a change in color phenotype from the light pink indicative of strong [\textit{PSI}+] in our parent 74-D964 strain. Interestingly, in addition to mutations that completely inhibited [\textit{PSI}+] propagation, we found a subset of mutations that partially inhibited [\textit{PSI}+] inheritance. These mutants displayed a sectoring color phenotype on rich media (Fig. 4.1A). Color sectoring within a [\textit{PSI}+] colony results when a fraction of the budding daughter cells lose the prion, thereby producing sections of the colony that are [\textit{psi}-] and phenotypically red. We identified two point mutations in the chaperone Hsp104 that demonstrated an interesting effect on the inheritance of [\textit{PSI}+] unlike the curing phenotype that is often characterized. These mutants, which we sequenced to identify as \textit{hsp104-G730D} and \textit{hsp104-G254D}, presented varying levels of sectoring and [\textit{PSI}+] loss (Fig. 4.1A). We recreated both mutants in an unmutagenized [\textit{PSI}+] 74-D694 strain by double homologous recombination to verify the phenotypic effects and used these strains for all subsequent analyses.

In order to determine how the mutants affected the biochemical properties of Sup35 in [\textit{PSI}+] cells, we first performed SDD-AGE (semi-denaturing with detergent agarose gel-electrophoresis)\textsuperscript{63} and analyzed the SDS-resistant Sup35 species. In \textit{hsp104-G254D} and \textit{hsp104-G730D} cell lysates, we found that the size of the Sup35 aggregate distribution was increased, as was the amount of Sup35 monomer (Fig. 4.1B). From these data, one could hypothesize that these Hsp104 mutants are unable to efficiently fragment Sup35 aggregates, resulting in larger aggregates that cannot be as easily passed on to daughter cells. In addition, less propagons would also result in decreased monomer addition and a larger pool of monomeric Sup35. Alternatively, the mutants could be propagating a weak variant of [\textit{PSI}+] which would be predicted to show the same change in aggregate pattern.

\textbf{Strong [\textit{PSI}+] propagons are maintained in both \textit{hsp104-G254D} and \textit{hsp104-G730D}.} The SDD-AGE analysis of the mutant strains (Fig. 4.1B) showed an increase in both high-molecular weight species and
Sup35 monomer, which is characteristic of weak [PSI+] variants. However, the original [PSI+] variant in the screen was strong [PSI+]. Thus, we used a genetic test and phenotypic test to analyze whether the mutants appeared to be propagating a different variant of [PSI+] or if the variant propagated in the sectoring colonies still appeared to be strong [PSI+]. As the mutants are recessive, we could readily analyze the properties of the variant by mating the sectoring hsp104-G254D and hsp104-G730D cells to wild type [psi-] cells, sporulating diploids, and analyzing resultant haploid progeny phenotypically and biochemically. Mating [PSI+] hsp104-G254D and [PSI+] hsp104-G730D cells to wild type [psi-] cells resulted in diploids that were light pink, and more similar to the strong [PSI+] parent than the mutant haploids (Fig. 4.2A). Sporulation of the diploids resulted in tetrads with two stable [PSI+] haploids and two sectoring/ weaker [PSI+] haploids. For both heterozygous diploids, the two haploid progeny that expressed wild type HSP104 resembled strong [PSI+] phenotypically, by both color and growth on media lacking adenine (Fig. 4.2B). Finally, we performed SDD-AGE analysis on cell lysates from full tetrads obtained from the heterozygous diploids and compared the aggregate distribution of the wild type HSP104 haploids to lysates from control cells containing the parental strong [PSI+] variant. The biochemical analyses correlated with the colorimetric phenotypes: all the wild type haploids propagated Sup35 aggregates that resembled those from control strong [PSI+] cell lysates (Fig. 4.2C). In sum, our phenotypic and biochemical analyses suggest that the underlying structure of Sup35 aggregates is retained in [PSI+] cells harboring the hsp104-G254D and hsp104-G730D mutations; however, when these mutations are present as the only copy of Hsp104 in the cell they are unable to efficiently propagate the prion to maintain the strong [PSI+] phenotype.

**Hsp104 missense mutant proteins are defective in ATP hydrolysis and efficient hexamer formation.** As the increased size of the Sup35 aggregates in hsp104-G254D and hsp104-G730D cells could suggest a fragmentation defect of Hsp104, we next investigated whether these mutations affected ATP hydrolysis. We purified recombinant wild type Hsp104, Hsp104-G254D, and Hsp104-G730D proteins from *E. coli* cells. We then tested the ability of Hsp104-G254D and Hsp104-G730D to hydrolyze ATP by monitoring release of inorganic phosphate using the Malachite Green assay. The G254D mutation is in NBD1 and G730D is in NBD2 and as such, may be involved in ATP binding or hydrolysis.
As compared to wild type Hsp104, both mutants had significantly reduced ATPase activity (Table 4.1). Surprisingly, these results contradict previous results in the literature, which demonstrate that mutants of Hsp104 that display similarly low levels of ATP hydrolysis often cure \([\text{PSI}^+]\).\(^{17,33}\)

As ATP hydrolysis is dependent on the ability of Hsp104 to form hexamers,\(^{64}\) we next investigated whether these mutants were capable of oligomerizing in the presence of nucleotide.\(^{65}\) Using glycerol gradient fractionation in the presence of ATP, we found that Hsp104-G254D and Hsp104-G730D do form hexamers, though appear to do so less efficiently than wild type, as there was a slight shift in the peak of Hsp104-G254D and Hsp104-G730D toward the top of the gradient (Fig. 4.3). In the absence of ATP, wild type Hsp104 and the mutant proteins remain monomeric (data not shown). Thus, the reduced ATP hydrolysis of both Hsp104-G254D and Hsp104-G730D may correlate with their inefficient hexamerization ability.

We reasoned that if the majority of the mutant were in the hexameric state, we might observe wild type levels of ATPase activity from the mutants. Hsp104 has previously been shown to hexamerize even in the absence of ATP when incubated in a buffer containing low salt (<20mM).\(^{64}\) Therefore, we again measured hydrolysis of ATP by the Malachite green assay, this time in a low salt (15mM NaCl) buffer to promote hexamerization. In low salt buffer, we found that the NBD2 mutant, Hsp104-G730D, displayed ATPase activity similar to wild type (Table 4.1). Hsp104-G254D, however, did show an increase in ATP hydrolysis in low salt buffer, but not to the same extent as Hsp104 or Hsp104-G730D, suggesting the hexamer defect of Hsp104-G254D may not be the only cause of the reduced rate of ATP hydrolysis.

**Low activity mutations in HSP104 vary in their ability to disaggregate non-prion substrates.** In addition to its role in prion propagation, Hsp104 is also necessary for cell survival following acute heat shock.\(^{21}\) With the aid of Hsp70 and Hsp40 co-chaperones, Hsp104 resolubilizes proteins that aggregate as a result of heat or other stresses and is crucial for cellular recovery from such stresses.\(^{20}\) Therefore, we tested the ability of Hsp104-G254D and Hsp104-G730D to provide thermotolerance. We first pre-treated cells at 37°C to induce Hsp104 expression, and then heat-shocked \(HSP104\), \(hsp104\Delta\), \(hsp104\)-G254D, and \(hsp104\)-G730D cells at 50°C for various times as indicated, before plating on rich media to determine the relative viability. We found that both wild type \(HSP104\) and \(hsp104\)-G254D were
thermotolerant, but \textit{hsp104-G730D} cells were not, and resembled the \textit{hsp104\Delta} strain (Fig. 4.4). Thus, despite having lower ATPase activity, Hsp104-G254D was more active in thermotolerance assays than Hsp104-G730D, suggesting that the mechanism of disaggregation is de-coupled from the ATP hydrolysis activity for these two mutants.

\textit{hsp104-G254D} and \textit{hsp104-G730D do not propagate weak [PSI\textsuperscript{+}].} Recently, it has been shown that some mutations in Hsp104 that reduce ATPase activity can impair the propagation of weak [PSI\textsuperscript{+}] variants, but not strong [PSI\textsuperscript{+}] variants.\textsuperscript{66} Accordingly, we next tested the ability of \textit{hsp104-G254D} and \textit{hsp104-G730D} to propagate weak [PSI\textsuperscript{+}] variants. We mated \textit{hsp104-G254D [psi\textsuperscript{-}]} and \textit{hsp104-G730D [psi\textsuperscript{-}]} cells to two previously characterized weak [PSI\textsuperscript{+}] variants and examined the diploids and haploid progeny phenotypically and biochemically.\textsuperscript{49,53} In both weak [PSI\textsuperscript{+}] variants, the heterozygous diploids displayed decreased nonsense suppression as compared to the wild type weak [PSI\textsuperscript{+}] parent (Fig. 4.5A). These data suggest that both \textit{hsp104-G254D} and \textit{hsp104-G730D} were inhibiting weak [PSI\textsuperscript{+}] propagation, even in the presence of wild type \textit{HSP104}. After sporulation, we observed two distinct patterns of inheritance in the haploid progeny. Resultant tetrads were either all red and [psi\textsuperscript{-}], or segregated 2:2 weak [PSI\textsuperscript{+}]:[psi\textsuperscript{-}] (Fig. 4.5B) where the [PSI\textsuperscript{+}] progeny segregated with the wild type \textit{HSP104}. Loss of the [PSI\textsuperscript{+}] phenotype, even in the wild type haploids, is consistent with the increased mitotic loss of weak [PSI\textsuperscript{+}] we observed in the diploids. Presumably, the reduced activity of Hsp104-G254D and Hsp104-G730D prevent weak [PSI\textsuperscript{+}] propagation and also partially inhibit wild type Hsp104 from stably propagating weak variants. Next, we chose tetrads that maintained weak [PSI\textsuperscript{+}] in the wild type haploids for SDD-AGE analysis and found that the variant propagated resembled the weak [PSI\textsuperscript{+}] parent (Fig. 4.5C). Thus, \textit{hsp104-G254D} and \textit{hsp104-G730D} do not irreversibly decrease the ability of wild type \textit{HSP104} to propagate weak [PSI\textsuperscript{+}] variants and do not appear to alter the prion variant structure by phenotypic assays.

\textit{hsp104-G730D propagates a known variant of [RNQ\textsuperscript{+}].} Like [PSI\textsuperscript{+}], the [RNQ\textsuperscript{+}] prion is also dependent on Hsp104 for its maintenance.\textsuperscript{12} To determine if the prion propagation defect observed in \textit{hsp104-G254D} and \textit{hsp104-G730D} cells was specific to [PSI\textsuperscript{+}], we tested the ability of both mutants to
propagate several established [RNQ+] variants. After mating [psi−] hsp104-G254D and [psi−] hsp104-G730D cells to the s.d. low, medium, high, very high, and m.d. high variants of [RNQ+] and sporulating the diploids, we performed SDD-AGE on the hsp104-G254D and hsp104-G730D haploids to determine the [RNQ+] state of the cells. Interestingly, we found one variant of [RNQ+], the m.d. high variant, that was propagated, at least to some extent, in hsp104-G730D cells (Fig. 4.6). None of the s.d. variants could be propagated by hsp104-G730D, and hsp104-G254D did not propagate any of the [RNQ+] variants tested (data not shown). Thus, similar to recent reports for [PSI+], these data may suggest that the m.d. [RNQ+] variant is less stable than the s.d. [RNQ+] variants and thus more refractory to changes in the activity or interaction with Hsp104.

**Discussion**

Here, we identify two novel mutations in Hsp104, Hsp104-G254D and Hsp104-G730D, which affect Hsp104 activity in a substrate- and prion variant-specific manner. Cells carrying the NBD1 mutant, hsp104-G254D, or the NBD2 mutant, hsp104-G730D, display high mitotic loss of the strong variant of [PSI+]. Phenotypically, hsp104-G730D displays a high degree of sectoring while hsp104-G254D exhibits fewer sectoring colonies but more completely [psi−] (red) colonies. Despite both showing marked reductions in ATP hydrolysis activity under physiological conditions, the mutants differ in their ability to disaggregate a broad range of substrates, though both are unable to propagate weak variants of [PSI+]. The hsp104-G254D mutant is active in thermotolerance, but is defective in the propagation of all tested variants of [RNQ+]. On the other hand, Hsp104-G730D expressing strains are unable to resolve disordered aggregates but, curiously, can propagate at least one variant of the [RNQ+] prion to some extent.

Manipulation of the chaperone network has long been known to regulate prion propagation and formation. Hsp104 is a general prion regulator, deletion of Hsp104 eliminates all of the known yeast prions and dependence on Hsp104 is often one of the criteria used when identifying new yeast prions. Therefore, we were not surprised when several candidates from our mutagenesis screen for factors involved in [PSI+] propagation were identified as mutations in Hsp104. Several interdependent features of Hsp104 contribute to its overall function in substrate disaggregation, and
understanding the effect these features have on each other is not simple. For example, as an AAA+ ATPase, hydrolysis of ATP provides the primary energy for the disaggregate ability of Hsp104 and yet we identified two mutations, Hsp104-G254D in NBD1 and Hsp104-G730D in NBD2, that still disaggregate substrates despite having significantly lower rates of ATP hydrolysis. Interestingly, reducing the ATPase activity of Hsp104 was previously shown to enhance substrate disaggregation. However, the increased disaggregation ability extended only to disordered substrates; decreased ATPase activity instead impaired prion remodeling. On the other hand, a highly characterized mutation in the Walker B motif in NBD1, E285A/Q, hydrolyzes ATP at a rate much higher than wild type (300-500%), but fails to support either thermotolerance or prion propagation. Therefore, ATP hydrolysis is not always a reliable indicator for substrate disaggregation ability.

One hypothesis proposed suggested that disordered substrates are less stable and thus require less overall force by Hsp104 to be disaggregated. High temperature and other stresses result in the increased exposure of hydrophobic regions in proteins, leading to the formation of unstable, heterogeneous protein aggregates. Unlike these stress-induced aggregates, prions are highly ordered, stable protein aggregates that are typically resistant to denaturation by detergents and high temperatures. Mutations in Hsp104 that promote thermotolerance but are not sufficient for prion propagation have been previously described. Interestingly, hsp104-G730D cells can propagate strong [PSI+] and m.d. high [RNQ+], but are unable to function in thermotolerance. Additionally, mutations in the C-terminal domain including K774E, L814S, L840Q, and 22 or 38 residue deletions of the C-terminus cause a loss of thermotolerance but these mutants are able to propagate [PSI+] to some extent. These previously characterized mutants are similar to Hsp104-G730D in that they exhibit reduced ATP hydrolysis and show defects in hexamerization. As yet, no function has been ascribed to the Hsp104 C-terminal domain, though some data suggest it is a site of substrate interaction.

The hypothesis that Hsp104 is better able to remodel less stable aggregates can also be applied to the broad range of prion variants. Despite having the same sequence, prion variants compose distinct structures that vary in stability and hence, may vary in their interaction with Hsp104. Hsp104 has previously been shown to play a role in prion variant selection. Continued expression of high levels of Hsp104 resulted in propagation of a [PSI+] variant dependent on overexpression of Hsp104.
Additionally, recent data show that, like with disordered aggregates, less stable variants of [PSI+] are more efficiently remodeled by Hsp104 despite equal binding affinities for Hsp104. At low concentrations, Hsp104 catalyzed the generation of prion-competent seeds of strong variants but was unable to remodel weak variants. Therefore, the decreased activity of our Hsp104 mutants may specifically inhibit weak variants of [PSI+] in vivo due to the inability to interact with or remodel this specific amyloid structure or any structure that is more stable. Moreover, expressing the mutant concurrently with wild type, as seen in the diploids, caused mitotic loss of weak [PSI+] variants, thereby supporting previous results indicating that weak variants require a high level of cooperativity between hexameric subunits to propagate.

In addition to causing unstable strong [PSI+] propagation, hsp104-G730D is also able to propagate m.d. high [RNQ+] to some extent. The m.d. high [RNQ+] variant has been shown to be less thermal-stable than the s.d. [RNQ+] variants. We have previously published another mutation in Hsp104, E190K, which also had a differential effect on the propagation of the [RNQ+] variants. Like hsp104-G730D, hsp104-E190K was unable to maintain the s.d. variants, but was able to propagate m.d. high [RNQ+]. Additionally, both hsp104-G730D and hsp104-E190K showed defects in both [PSI+] propagation and thermostolerance. Our data from these two mutants suggest that variants of the [RNQ+] prion may be regulated by Hsp104 in a manner similar to the mechanisms elucidated for [PSI+]. Interestingly, the m.d. [RNQ+] variant that is the most resistant to alterations in Hsp104 activity also facilitates a high rate of [PSI+] induction. It’s interesting to theorize that changing environmental conditions may cause fluctuations in Hsp104 activity that in turn control the appearance and disappearance of prions, and as such, the environment may influence prion-dependent phenotypic variation, adaptability and survival in different conditions. Therefore, discerning Hsp104’s mechanism of disaggregation, as it applies to distinct prion variants, is critical to understanding how prions appear, disappear, and evolve.

As our understanding of self-propagating prions continues to develop, so too does the knowledge that the biological phenomenon of protein-only heritability is highly complex. In addition to mutations in the primary sequence affecting aggregation and propagation, we now understand that the same primary sequence can adopt multiple conformations, and these conformations differ in stability, heritability, and
function. How a single protein sequence can adopt multiple conformations is a biological phenomenon that is still relatively unclear. Just as gene expression is regulated by an intricate network of transcription factors and promoter elements, prion variant propagation is regulated by a network of chaperones and in some cases, other prions. Understanding how changes in the chaperone network regulate prion variant formation and propagation will broaden our understanding of the mechanisms of prion variant generation, selection, and evolution.

Materials and Methods

Strain and Plasmid Construction

S. cerevisiae strains used in this study were derivatives of 74-D694 and were grown and manipulated using standard techniques. Yeast strains were either grown in rich media (1% yeast extract, 2% peptone, 2% glucose) or synthetic media lacking amino acids corresponding to plasmid selection (0.67% yeast nitrogen base, 2% glucose) or nonsense suppression analyses. Diploids were generated by mating haploids containing selectable plasmids and were verified by growth on minimal media and plating on haploid tester strains. Haploid progeny from diploids were isolated by micromanipulation and verified by phenotypic assays and mating type testing.

Hsp104 mutants from the original mutagenized strains were PCR amplified and cloned into the pRS306 integrating vector. The mutants were integrated into a clean 74-D694 background by the pop-in/pop-out method and verified by DNA sequencing. The pProEx-Htb-Hsp104 purification plasmid has previously been described. Hsp104 mutations were cloned into pProEx-Htb-Hsp104 by restriction digest with BamHI and Bsu36I followed by ligation and sequencing to verify the mutation.

The “strong” and “weak” [PSI+] variants were characterized and kindly provided by the Chernoff and Liebman labs. The Sc37 [PSI+] variant was made by transforming Sup35NM fibers generated at 37°C into [psi-] cells. These strains were made and kindly provided by the Weissman lab. The [RNQ+] variant strains were characterized as specific [PIN+] strains initially, but later confirmed to be [RNQ+], and were kindly provided by the Liebman lab. To analyze the Hsp104 mutants in both [RNQ+] and [PSI+] variants, each variant was mated to both hsp104-G254D [psi-][rnq-] and hsp104-G730D [psi-][rnq-] cells and diploids were selected. The diploids were sporulated and dissected by micromanipulation.
EMS Mutagenesis Screen

A strong [PSI+] yeast strain was subjected to EMS mutagenesis as previously described. Two cultures with viabilities of about 17% were plated to determine changes in color. Candidates were selected based on color phenotype and were initially identified as mutations in HSP104 by back-crossing to an hsp104Δ strain and analyzing the progeny for segregation of the phenotype. Genomic DNA was PCR amplified and sequenced to identify the point mutations in HSP104.

SDD-AGE

SDD-AGE analysis was performed as previously described. Cells were lysed by beadbeating in PEB buffer (25mM Tris-HCl pH 7.5, 50mM KCl, 10mM MgCl₂, 1mM EDTA, 10% Glycerol plus mini EDTA-free protease inhibitors (Roche), Aprotinin (Sigma) and PMSF (Sigma)). Samples were incubated in sample buffer at room temperature for seven minutes then separated on a 1.5% agarose gel. The protein distribution was analyzed by western blot with anti-Sup35 antibodies or anti-Rnq1 antibodies.

Hsp104 Purification

Recombinant Hsp104 was expressed in E. coli cells and purified as previously described with one further separation step added. Briefly, Hsp104, tagged with a 6xHis tag on the N-terminus, was first isolated on a Nickel-sepharose column, the 6xHis tag was cleaved off using the TEV protease, and Hsp104 was re-applied to the Nickel-sepharose column to separate the untagged Hsp104. Untagged Hsp104 was then applied to an anion exchange Q-sepharose column followed by an S-300 size exclusion column to isolate monomeric Hsp104 from any aggregated Hsp104 species. Untagged, monomeric Hsp104 was stored at -80°C.

ATP Hydrolysis Assays

ATP hydrolysis was measured by the Malachite green assay as previously described. Briefly, 2µg of purified protein was incubated in buffer (40mM TrisHCl pH7.5, 175mM NaCl, 5mM MgCl₂, 0.02% Triton X-100) with 5mM ATP at 37°C. At various times, Malachite green dye was added to the sample for one minute and the reaction was stopped by the addition of 34% citric acid. The dye absorbance was determined at 650nm and the amount of free phosphate calculated based on a standard of KH₂PO₄.

Glycerol Gradients
Purified Hsp104 was applied to a 4mL linear (10-35%) glycerol gradient and centrifuged at 34k rpm for 18 hours in a SLA-600 rotor. Gradients were fractionated and equal volumes of each fraction were analyzed by SDS-PAGE and western blot using an anti-Hsp104 antibody. Individual bands from each fraction were quantified using Image J and reported as a percent of total Hsp104.

**Thermotolerance**

An equal number of cells from cultures of HSP104, hsp104-G254D, hsp104-G730D, and hsp104Δ grown to mid-log phase were pre-treated at 37°C to induce HSP104 expression then heat-shocked at 50°C. At the indicated time intervals, samples were taken and spotted on rich media in a five-fold serial dilution.

**References**


15. Derkatch IL, Bradley ME, Hong JY, Liebman SW. Prions affect the appearance of other prions: the story of [PIN(+)]. Cell 2001; 106:171-82.


43. Sharma D, Masison DC. Functionally redundant isoforms of a yeast Hsp70 chaperone subfamily have different antiprion effects. Genetics 2008; 179:1301-11.


Figure 4.1. *hsp104-G254D* and *hsp104-G730D* strains show inefficient [PSI+] propagation.

(A) *hsp104-G254D* and *hsp104-G730D* cells display a sectoring [PSI+] phenotype as a result of inefficient [PSI+] inheritance. (B) Lysates of sectoring *hsp104-G254D* and *hsp104-G730D* cells with strong [PSI+] cells ([PSI+]) and [psi-] cells ([psi-]) as controls were analyzed by SDD-AGE analysis followed by western blot and blotting for Sup35. These results were reproduced at least three times. An example of the shift is shown. The general loss of the lower aggregate species and the decrease in aggregated Sup35 is reproducible. The appearance of monomeric Sup35 on SDD-AGE western blots is more variable, even with controls, for unknown reasons.
Figure 4.2 *hsp104-G254D* and *hsp104-G730D* cells propagate the original strong [PSI+] variant.

(A) *hsp104-G254D* and *hsp104-G730D* cells (G254D and G730D, respectively) and diploids from the mating of *hsp104-G254D* and *hsp104-G730D* to wild type [psi-] cells (WT/G254D and WT/G730D) were spotted on rich media (YPD) and media lacking adenine (SD-Ade). Strong [PSI+] and [psi-] cells are spotted for comparison. The second spot in each row is a five-fold dilution of the first spot. (B) Full tetrads from the sporulation of the diploids in (A) were spotted for each mutant as indicated. Each haploid progeny in the tetrad is labeled, A-D. Strong [PSI+] and [psi-] are spotted for color comparison. The second spot in each row is a five-fold dilution of the first spot. (C) A representative tetrad from (B) was analyzed by SDD-AGE analysis and western blot. The letters A-D correspond to the haploids with the same label in (B) and weak [PSI+] (Weak), strong [PSI+] (Strong) and [psi-] were analyzed for comparison. SDD-AGE analysis was performed four times with haploids from both *hsp104-G254D* and *hsp104-G730D* cells.
Table 4.1 Hsp104 mutants display defects in ATP hydrolysis under physiological salt conditions

<table>
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<tr>
<th></th>
<th>Physiological*</th>
<th>Low†</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.225 ± 0.0397</td>
<td>0.732 ± 0.0543</td>
</tr>
<tr>
<td>G254D</td>
<td>0.0325 ± 0.00177</td>
<td>0.211 ± 0.00177</td>
</tr>
<tr>
<td>G730D</td>
<td>0.00583 ± 0.00439</td>
<td>0.677 ± 0.0321</td>
</tr>
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Numbers represent the average initial rate (nmol·µg⁻¹·min⁻¹) of ATP hydrolysis for WT Hsp104, Hsp104-G254D, and Hsp104-G730D. ATPase assays were measured by the Malachite Green assay. Rates were calculated from three separate experiments from two different preparations of purified protein.

*Buffer contains 150mM NaCl
†Buffer contains only 15mM NaCl
Figure 4.3 Hsp104-G254D and Hsp104-G730D mutant proteins form hexamers in vitro.

Hsp104 (blue), Hsp104-G254D (green), and Hsp104-G730D (red) were incubated with 5mM ATP for 10 minutes then subjected to ultracentrifugation through a linear (10-35%) glycerol gradient. Equal volume fractions were collected and the amount of Hsp104 protein in each fraction was analyzed by SDS-PAGE and western blot. Individual bands were quantified and the amount of Hsp104 in each fraction was plotted as a percent of the total Hsp104 protein. The graph shows the data from one assay. This assay was performed three times and all gave similar results.
Figure 4.4 *hsp104-G254D* and *hsp104-G730D* display different levels of non-prion disaggregation.

The thermotolerance of *HSP104*, *hsp104Δ*, *hsp104-G254D*, and *hsp104-G730D* cells was tested. Cells were first grown at 37°C in liquid culture to induce *HSP104* expression, heat-shocked at 50°C for 10 to 30 minutes as indicated, and then spotted on rich media. Control cells (No Heat) were plated without heat shock. Thermotolerance assays were repeated four times and all showed similar results.
Neither hsp104-G254D nor hsp104-G730D can propagate weak [PSI+].

(A) Cured hsp104-G254D [psi-] and hsp104-G730D [psi-] cells, weak [PSI+], the weak [PSI+] variant Sc37 (from transformation of [psi-] cells with Sup35NM fibers formed at 37°C), and the diploids from the matings of the weak [PSI+] cells to the mutants were spotted on YPD. The second spot in each row is a five-fold dilution of the first spot. (B) Diploids of the mutants crossed to weak [PSI+] (Weak/Mut in (A)) were sporulated and 16 tetrads dissected. Two representative tetrads on YPD are shown. Each haploid is labeled A-D. (C) Diploids of weak [PSI+] crossed to the mutants (Weak/Mut in (A)) and a representative tetrad from these heterozygous diploids were subjected to SDD-AGE and western blot analysis to determine Sup35 aggregate distribution. SDD-AGE analysis was performed twice using four distinct tetrads.
Figure 4.6 hsp104-G730D can propagate multi-dot high [RNQ+].

hsp104-G730D cells were mated to s.d. low (low), medium (med), high (high), very high (v.high) and m.d. high (m.d. high) [RNQ+] and then sporulated. The hsp104-G730D haploid progeny from four separate tetrads of each variant mating, along with the unmated [RNQ+] variants as controls (WT), were analyzed by SDD-AGE and western blot.
Chapter 5: Conclusions and Future Directions
5.1 Hsp104-R830S and soluble Sup35 oligomers future directions

Summary

Yeast prions are self-replicating, protein-only epigenetic elements that are non-toxic.¹ To be efficiently propagated, prions must be fragmented to generate smaller, prion-competent propagons that are easily transmissible to the budding cells.²,³ Propagons must also maintain the structural information of the prion in order to faithfully propagate the prion to the soluble prion protein in the newly budded cell. The generation of these propagons requires the AAA+ ATPase chaperone Hsp104.²,⁴ In Chapter 2, I investigate a point mutant of Hsp104 that propagates soluble, more SDS-sensitive oligomers of Sup35 that are sufficient for transmission of the prion state but are incapable of conferring the nonsense suppression phenotype associated with [PSI+]. The novel properties of the soluble, prion-competent Sup35 oligomers can provide insight into the mechanisms of prion formation and propagation. The following section will describe some experiments designed to utilize them.

Structural analysis of soluble Sup35 oligomeric propagons

Our findings in chapter 2 represent the first identification of soluble oligomers of a prion protein in vivo. From protein transformation experiments where we transformed [psi-] cells with these soluble oligomers, we discovered that these oligomers could transmit the prion state to a soluble pool of monomer. Several studies have hypothesized that propagons in yeast are a soluble oligomeric species, but until now, these structures have always been transient and have yet to be characterized structurally. One future experiment is to characterize these amyloid oligomers by several structural methods to investigate how these structures differ from the large aggregates that confer the [PSI+] phenotype. For example, structural data of the amyloid aggregate is difficult to achieve due to its large bulky structure. However, we have shown from matings of hsp104-R830S cells containing soluble Sup35 oligomers to [psi-] cells that these oligomers retain the same amyloid core as the parental [PSI+] strain. Perhaps, now, given their soluble nature and small size these structures are more amenable to various structural techniques such as NMR. Furthermore, these oligomers be analyzed by EM and AFM to determine if they share ultrastructural features with the intermediate protofilaments identified of Aβ aggregation.
These oligomers could also be used in single-molecule studies to investigate the templating or joining mechanism.

In addition to the correct structure, propagons also need to interact with molecular chaperones for efficient propagation. One area of chaperone involvement that is the least characterized is in prion formation. When prion monomers template into the β-sheet rich conformation, they first have to unfold and many prion proteins have natively unfolded or destabilized domains already present. One very important job of molecular chaperones is to bind unfolded or exposed domains to prevent aggregation. Interestingly, overexpression of the Hsp70 chaperone Ssa1 leads to increased [PSI+] production, while the overproduction of another Hsp70, Ssb1, results in increased [PSI+] loss from excess Sup35. What these mechanisms are and why they are opposing is still unclear. We should use the oligomers to probe the role of chaperones in the formation of prions by looking at chaperone binding, oligomeric stimulation of chaperone activity, and specific chaperone domains or activities that may be required for an interaction.

**Analyze the role of oligomeric intermediates for the [RNQ+] prion**

In order to determine if oligomeric intermediates are a general property of yeast prions, future experiments should examine the role of oligomers in the propagation of the [RNQ+] prion. In our experiments, we only closely looked at the Sup35 species in hsp104-R830S cells. By solubility assays, hsp104-R830S cells were able to maintain [RNQ+]. Similar to wild type [PSI+] cells, wild type [RNQ+] cells may also contain oligomeric species sufficient for transmission of the [RNQ+] state. Wild type [RNQ+] cells should be separated by sedimentation and the soluble and insoluble fractions transformed into [rnq-] cells to determine if any infectious species exist in the soluble fraction. Subsequently, wild type [RNQ+] lysates should be separated by sucrose gradient fractionation and analyzed by western blot and protein transformation into [rnq-] cells. Since [RNQ+] does not have an easily observable phenotype like [PSI+] does, a Rnq1 reporter developed in our lab should be used to quantify the infectivity of specific soluble or oligomeric species. The RRP construct is a chimera of the Rnq1 prion-forming domain and the middle and C-terminal domains of Sup35 and can be used as a phenotypic readout of the [RNQ+] state.
Analyze the role of oligomeric intermediates in the propagation of conformational variants

Recombinant Sup35 can form distinct amyloid structures in vitro when assembled under different temperatures. Structural variation is also the basis for prion variants observed in cells, such as weak and strong [\(\text{PSI}^+\)] and the low, medium, high, and very high variants of [\(\text{RNQ}^+\)]. One hypothesis is that the formation of amyloid oligomers is also conformation-dependent and may only occur under certain conditions, as seen for Sup35 and PrP. Therefore, the presence of amyloid oligomers in variants of the [\(\text{PSI}^+\)] and [\(\text{RNQ}^+\)] prions should be assessed. Again, this could be done by separating soluble oligomers from SDS-resistant aggregates by sucrose gradients and sedimentation assays and using the species isolated to infect [\(\psi^i\)] or [\(\text{rrp}^-\)] cells and quantify the amount of infectivity. By SDD-AGE and solubility assays, weak [\(\text{PSI}^+\)] maintains an increased soluble pool of Sup35 compared to strong [\(\text{PSI}^+\)].

Perhaps this soluble pool is not simply functional monomer, but a mix of oligomeric species, of which some may be highly infectious.

Determine the domains of Sup35 involved

Although we were able to separate the soluble oligomeric species from large aggregates in wild type \(\text{HSP104}\) cells, we originally identified these propagons in a point mutant of Hsp104. We could use this mutant again to help identify domains and regions of Sup35 important for oligomer formation. For example, we have seen that full-length Sup35 is capable of forming soluble propagons but what about the PFD of Sup35 alone? Investigating the mechanism of Hsp104-R830S on distinct Sup35 domain deletion or truncations mutants not only provides information about Sup35 regions involved in amyloid formation, but also gives insight into the interaction between Hsp104 and Sup35. I would propose using a plasmid shuffle technique to replace full-length Sup35 with various Sup35 deletions and truncations and measuring the amount of soluble oligomers. Additionally, measuring the amount of infectious soluble oligomers by protein transformation may provide insight into which regions of Sup35 are important in forming infectious structures.
Investigation of a “dead-end” substrate

In Figure 2.4, the sucrose gradient of lysates from hsp104-R830S cells showed that Sup35 species migrated just as far as Sup35 from the wild type cells. The difference between these species was in their levels of infectivity. High molecular weight species from hsp104-R830S cells were not infectious suggesting they might be examples of “dead-end” substrates or non-heritable amyloids. I propose that these structures should be isolated as well and compared by structural techniques like EM or sensitivity to denaturants or proteases to their counterparts in the wild type cells to determine differences between infectious aggregates and non-infectious aggregates.

Conclusions

We found that soluble, prion-competent oligomers exist in wild type [PSI+] cells and are highly infectious. These oligomers have mostly remained elusive because of their transient and unstable nature. Here, we have identified a mutation in Hsp104 which propagates these oligomers allowing a more stable system within which to investigate the structure and mechanism of these structures.

5.2 Hsp104 M-domain Future Directions

Summary

Hsp104 is composed of five functionally distinct domains that each contribute to the overall function of the disaggregate chaperone. The middle domain (M-domain) is a coiled-coil whose position in the hexamer has been a matter of much debate.\textsuperscript{16-18} Recent studies have suggested that this domain plays a role in regulating the function of the whole chaperone. The M-domain of ClpB was proposed to alternate between two conformational states, repressed and de-repressed.\textsuperscript{19} The repressed M-domain is stabilized against the body of the hexamer through an interaction with the NBD1 of the neighboring subunit. The de-repressed state, however, is unattached from the body of the hexamer and accessible to co-chaperones. We found that mutations that were predicted to promote the repressed state inhibited Hsp104 function. These mutations inhibited ATPase activity, disaggregation activity, and were not able to form efficient hexamers. Conversely, mutations proposed to induce the de-repressed state stimulated ATPase activity but exhibited reduced disaggregation activity. Interestingly, the mutants exhibited
variation in their ability to propagate prions, suggesting that de-regulation of the function of the M-domain can cause defects in yeast prion propagation.

The Interaction of the M-domain with Co-chaperones

One of the proposed roles for the M-domain of Hsp104 and ClpB is as a site for co-chaperone interaction. Hsp104 and the co-chaperones, Hsp40 and Hsp70, often have opposing effects on [PSI+] propagation. Perhaps, then, reducing or increasing the levels of co-chaperones may complement the defects in [PSI+] propagation observed from these M-domain mutants. A comprehensive genetic experiment to delete (or reduce in the case of the essential chaperones) or overexpress each co-chaperone with each mutant would enhance our knowledge of the interactions between the M-domain and the co-chaperones as well as the role of Hsp70 chaperones in prion propagation.

Structural Studies of M-domain mutants

As yet, no evidence has been reported on the movement of the M-domain of Hsp104 and whether it can actually alternate between the repressed and de-repressed states similar to what has been reported for ClpB. One way to investigate this question would be to create cysteine mutants of the predicted pairs and perform both cross-linking and FRET studies. Both methods would provide information on the interactions between the predicted residues and would provide evidence for the model of alternating repressed and de-repressed conformations.

Hsp104Y507D and [PSI+]

Despite repeated efforts, I was unable to isolate more than one cell that contained both Hsp104-Y507D and [PSI+]. Clearly, antagonistic interactions of Hsp104-Y507D and [PSI+] led to toxicity but how this occurs is unknown. One way to investigate this mechanism is by using a shutoff system, much like the one we employed in Chapter 2. Expression of hsp104-Y507D in [PSI+] cells containing wild type HSP104 is not toxic. Therefore, a plasmid with wild type HSP104 driven by a repressible promoter could be used to maintain viability before adding the repressing agent, such as glucose for the galactose promoter or doxycycline for the tetracycline-repressible promoter. As wild type Hsp104 is repressed, the
activity of Hsp104-Y507D and [PSI+] in the cell could be investigated by numerous methods to look at the Sup35 aggregates themselves or protein aggregation or defects in translation. Thus, this system could be used to understand how co-expression of Hsp104-Y507D and [PSI+] are toxic.

**Conclusions**

From our data, we find that the function of the M-domain is very important for proper Hsp104 function. De-regulation of this domain can either cause a loss-of-function or a toxic gain-of-function. Investigating the function of the M-domain will elucidate the mechanisms of disaggregation and ATPase substrate stimulation in addition to co-chaperone interactions.

**5.3 Low Hsp104 Activity Mutants Future Directions**

**Summary**

The optimal activity of the molecular chaperone Hsp104 is dependent on several variables including ATPase activity, hexamer formation, and ability to bind and translocate substrates. Mutations in Hsp104 that inhibit these features often inhibit chaperone activity. As Hsp104 is essential for yeast prion propagation, changes in Hsp104 expression or activity can have severe consequences for prion maintenance. However, several conformational variants exist which are not as sensitive to changes in Hsp104 activity. For example, the [PSI+] prion is cured by overexpression of Hsp104, but the [RNQ+] prion is not. Clearly conformational features of the prion substrates play a role in their interaction with Hsp104.

Here we show two novel mutants of Hsp104 which inhibit Hsp104 activity but are still capable of propagating certain variants of both [PSI+] and [RNQ+]. These mutants have very low ATPase activity and do not form stable hexamers and appear not to be functional. However, enough activity is maintained for unstable prion propagation. What are the differences in requirements for prion and non-prion substrates in their interaction with Hsp104? Furthermore, how are specific conformations maintained but not others? The ability of Hsp104 to efficiently propagate some prion variants and not others is a mechanism not well understood.
Changes in Hsp104 activity alter propagation of specific variants

Classically, much of the mutational analysis done with Hsp104 has been focused on the [PSI+] prion. And yet, the [PSI+] prion cannot form without the presence of [RNQ+] suggesting that the interaction of Hsp104 with the [RNQ+] prion is as equally important. Many of the mutations that affect Hsp104 ATPase activity or disaggregation activity should be re-evaluated in the context of [RNQ+] and variants of [RNQ+] which show differences in their ability to induce [PSI+]. Furthermore, variants of [PSI+] exist that are more susceptible to changes in Hsp104 activity and the interaction of Hsp104 with these weak variants should be further investigated.

Conclusions

Investigating how different aggregated structures respond to changes in Hsp104 activity will elucidate mechanisms of protein disaggregation. Furthermore, structural studies of the substrates of Hsp104 will provide insight into why some structures appear dominant or more likely to aggregate. All in all, investigating the interaction between Hsp104 and various substrates will provide insight into the physical characteristics of protein folding.

References

Appendix A: Requirements of Hsp104p activity and Sis1p binding for propagation of the [RNQ+] prion

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Requirements of Hsp104p activity and Sis1p binding for propagation of the \([RNQ^+]\) prion

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Key words: \([RNQ^+], [PSI^+], \) Hsp104p, Sis1p, mutagenesis

Abbreviations: RRP, \([RNQ^+]\) reporter protein; NBD, nucleotide binding domain; M, middle domain; EMS, ethyl methyl sulfonate; GdnHCl, guanidine hydrochloride; PrD, prion-forming domain

The formation and maintenance of prions in the yeast \(Saccharomyces cerevisiae\) is highly regulated by the cellular chaperone machinery. The most important player in this regulation is Hsp104p, which is required for the maintenance of all known prions. The requirements for other chaperones, such as members of the Hsp40 or Hsp70 families, vary with each individual prion. \([RNQ^+]\) cells do not have a phenotype that is amenable to genetic screens to identify cellular factors important in prion propagation. Therefore, we used a chimeric construct that reports the \([RNQ^+]\) status of cells to perform a screen for mutants that are unable to maintain \([RNQ^+]\). We found eight separate mutations in Hsp104p that caused \([RNQ^+]\) cells to become \([mq^+]\). These mutations also caused the loss of the \([PSI^+]\) prion. The expression of one of these mutants, Hsp104p-E190K, showed differential loss of the \([RNQ^+]\) and \([PSI^+]\) prions in the presence of wild type Hsp104p. Hsp104p-E190K inefficiently propagated \([RNQ^+]\) and was unable to maintain \([PSI^+]\). The mutant was unable to act on other in vivo substrates, as strains carrying it were not thermostolerant. Purified recombinant Hsp104p-E190K showed a reduced level of ATP hydrolysis as compared to wild type protein. This is likely the cause of both prion loss and lack of in vivo function. Furthermore, it suggests that \([RNQ^+]\) requires less Hsp104p activity to maintain transmissible protein aggregates than Sup35p. Additionally, we show that the L94A mutation in Rnq1p, which reduces its interaction with Sis1p, prevents Rnq1p from maintaining a prion and inducing \([PSI^+]\).

Introduction

Prions are proteins that can form a self-propagating aggregated state. In mammals, prions are a causative agent of neurodegenerative disease.\(^1\) In yeast, however, prions act as an epigenetic mode of inheritance. The prion-forming proteins in yeast are involved in a variety of cellular processes, including protein degradation, translation termination and chromatin modification.\(^2-4\) One of the yeast prions, \([PSI^+]\), provides a potential mechanism to respond to environmental stresses.\(^3,4\) Interestingly, \([PSI^+]\) is regulated by another prion, \([RNQ^+]\), whose presence increases the rate of \([PSI^+]\) appearance.\(^5-12\) However, \([RNQ^+]\) is not required for the maintenance of \([PSI^+]\). Thus, the maintenance of \([RNQ^+]\) is critical for the formation of \([PSI^+]\) and its adaptive effect on the cells.

The maintenance of yeast prions is regulated by the cellular chaperone machinery.\(^13,14\) A crucial player in this regulation is the AAA-\(^\alpha\) ATPase Hsp104p that is essential for the survival of acute heat shock.\(^15,16\) Hsp104p is a disaggregator and has been shown to dissociate aggregated proteins both in vivo and in vitro.\(^15,17,18\)

Like its bacterial homolog ClpB, the active form of Hsp104p is a ring-shaped hexamer with a pore in the center.\(^19\) Interestingly, Hsp104p can be divided up into five functionally distinct domains. Although the N terminus is generally dispensable for function, the amino and carboxy termini are likely involved in substrate binding.\(^20,21\) The two nucleotide binding domains (NBDs) coordinate to hydrolyze ATP to ADP and this enzymatic activity is required for Hsp104p function in prion maintenance.\(^22-25\) Finally, the M domain lies between the two NBDs and is thought to coordinate the activities of the NBDs by propagating a conformational change in response to binding or hydrolysis of ATP.\(^23,26,27\) Due to the discrete nature of the domains, a variety of mutations have been described in each domain of Hsp104p that affect prion propagation.\(^20,25,28-31\)

One model of Hsp104p function in prion biology posits that Hsp104p has two roles in prion maintenance: generation of transmissible material (seeds) from prion aggregates and conversion of monomer into a prion-competent form.\(^17,32-34\) A complete loss of Hsp104p activity eliminates (cures) all known yeast prions.\(^3,4,11,25,35\) However, increasing Hsp104p activity by
overexpressing the disaggregase cures only the [PSI+] prion and does not affect [RNQ+] or any other yeast prion. This indicates that Hsp104p has both general effects on all known yeast prions and specific effects on the [PSI+] prion, at least when overexpressed.

Other members of the cellular chaperone machinery also show differential effects on prion propagation. For example, overexpression of the Hsp70 family member Ssa1p cures [URE3] but not [PSI+]. Excess Ssa1p does, however, reduce the curing of [PSI+] by Hsp104p overexpression and promote [PSI+] formation. Overexpression of the Hsp40 member Ydj1p cures the [URE3] prion but affects only certain variants of [PSI+] and [RNQ+]. Deletion of either of two different Ssa1p nucleotide exchange factors, SSE1 or FES1, cures [URE3] but not [PSI+], though the deletion of SSE1 weakens [PSI+]–mediated nonsense suppression. Moreover, Hsf1p, which regulates the expression of heat shock proteins, influences [PSI+] formation. Hsf1p contains two activation domains and deletion of the N-terminal activation domain inhibits [PSI+] formation while deletion of the C-terminal activation domain promotes [PSI+] formation. This suggests two classes of interaction with [PSI+] by proteins regulated by Hsf1p.

Rnq1p has been shown to interact with both Ssa1p and the Hsp40 family member Sis1p. The propagation of [RNQ+] can be abolished by mutations within Sis1p, albeit with varying efficiency, which may indicate different requirements for its activity in prion maintenance.

Here, we describe a screen for cellular factors that affect the propagation of the [RNQ+] prion. Using a chimeric reporter for the [RNQ+] status of the cell, we found several novel alleles of HSP104 that are unable to propagate both [RNQ+] and [PSI+]. Interestingly, one of the alleles shows differential rates of curing of [RNQ+] and [PSI+] in the presence of wild type Hsp104. Additionally, we show that Rnq1p-L94A, which has a decreased interaction with Sis1p, aggregates non-specifically and cannot support [PSI+] induction.

**Results**

EMS mutagenesis reveals genes necessary for the maintenance of [RNQ+]. We developed a system to identify cellular factors important in the propagation of [RNQ+]. The phenotypes associated with [RNQ+], insolubility of the Rnq1p protein and an increase in the induction rate of [PSI+], are not amenable for use in a high-throughput screen. Therefore, we used the RRP reporter to assay the [RNQ+] status of the cells. As described previously, RRP consists of a fusion of the Rnq1p prion forming domain (PrD) and the C-terminal translation termination domain of Sup35p. In [rnq+] cells, RRP is soluble and able to promote faithful termination of translation. In [RNQ+] cells, RRP aggregates and is unable to promote faithful translation termination. We used a strain that has the ade1-14 allele for a sensitive readout of nonsense suppression. This allele harbors a premature stop codon that is read through when RRP is aggregated. Therefore, [rnq+] cells expressing RRP are adenine auxotrophs and appear red on rich medium (YPD) due to the accumulation of an intermediate in the adenine biosynthesis pathway. [RNQ+] cells expressing RRP are adenine prototrophs and appear light pink on YPD.

We used EMS to create an unbiased set of mutants that are unable to maintain [RNQ+]. A strain that was light pink due to expression of RRP in a [RNQ+] background was mutagenized with EMS to approximately 14% viability and plated on YPD. Following plating on YPD, some of the colonies turned red, indicating a deficiency in adenine biosynthesis, possibly as a consequence of the loss of RRP-associated nonsense suppression (Fig. 1A). Of the approximately 150,000 colonies screened, 312 colonies turned red on YPD. These 312 colonies were re-plated onto YPD to determine if the red phenotype was stable. 40 of these colonies remained red following two passages on YPD, indicating a stable phenotype.

The red phenotype created by the EMS mutagenesis could result not only from the loss of the [RNQ+] prion but also from a variety of different mutations affecting adenine biosynthesis or translation termination. Therefore, we wanted to select those red mutants that had, in fact, converted from [RNQ+] to [rnq+]. The solubility of the Rnq1p protein was analyzed by western blot following high speed centrifugation of cell lysates. In [rnq+] cells, Rnq1p remains in the soluble fraction while in [RNQ+] cells Rnq1p is found in the insoluble fraction. The solubility of Rnq1p in the 40 stable red colonies was assayed. Of these 40 mutants, 14 showed soluble Rnq1p, indicating that [RNQ+] had been cured (Fig. 1B and data not shown).
Genetic linkage indicates the \([-\text{rnq}]\) phenotype is caused by single mutations. Next, we assessed the genetic properties of the red mutants. We mated the 14 \([-\text{rnq}]\) mutants to a wild type \([-\text{RNQ}^+]\) strain. We then assayed the solubility of Rnq1p in the resulting diploids as described above. Three of the diploids showed soluble Rnq1p, indicating that the diploids were \([-\text{rnq}]\) and that the mutant phenotype was dominant over wild type (data not shown). The remaining eleven mutants had insoluble Rnq1p in the diploids, indicating that \([-\text{RNQ}^+]\) was propagating in the cells and that the mutations were recessive (data not shown).

To determine if the loss of \([-\text{RNQ}^+]\) was caused by a single genetic lesion we employed a genetic test. The \([-\text{RNQ}^+]\) diploids generated above were sporulated to obtain haploid progeny. Single mutations should segregate in a 2:2 ratio and produce two \([-\text{rnq}]\) cells and two \([-\text{RNQ}^+]\) cells. Multiple mutations would create a variety of ratios due to random segregation. The haploid progeny acquired from the sporulation of the recessive mutants were analyzed for their \([-\text{RNQ}^+]\) status using the solubility assay described above. Eight of the recessive mutants generated the 2:2 ratio of \([-\text{rnq}]\) to \([-\text{RNQ}^+]\) cells, indicating a single mutation (Fig. 2A and data not shown). The remaining three mutants showed other ratios of \([-\text{RNQ}^+]\) to \([-\text{rnq}]\) haploid progeny, suggesting that multiple mutations had possibly been acquired.

Due to the ability of the three dominant mutations to cure \([-\text{RNQ}^+]\) in the diploid, all four haploid progeny will be \([-\text{rnq}]\), regardless of whether the phenotype is caused by one or more loci. However, the haploids can then be backcrossed to a wild type, \([-\text{RNQ}^+]\), haploid strain and the \([-\text{RNQ}^+]\) status of the resulting diploids analyzed. Single gene traits should produce a 2:2 ratio of \([-\text{RNQ}^+]\) diploids to \([-\text{rnq}]\) diploids. The haploid progeny from the dominant mutants were analyzed in this manner. Only one of the dominant mutations produced the 2:2 ratio indicative of a single gene trait (Fig. 2B). One of the dominant mutants failed to sporulate and, therefore, could not be analyzed in this manner, while the third produced variable ratios indicative of a multi-locus trait. We chose to focus on the mutants whose phenotypes were caused by alteration of a single locus.

**Missense mutations in HSP104 cure \([-\text{RNQ}^+]\).** We set out to identify the specific mutations that cured the \([-\text{RNQ}^+]\) prion. Since inactivation of the cellular disaggregase Hsp104p is known to cure all yeast prions it was an obvious candidate for our analysis.

**Figure 3.** The EMS-induced mutations that cure \([-\text{RNQ}^+]\) are in HSP104. (A) HSP104 was deleted in the mutant strains as indicated in the methods. The resulting \(\Delta hsp104\) strains were mated to a strain carrying wild type Rnq1p in the \([-\text{RNQ}^+]\) state. The resulting diploids were sporulated and the solubility of Rnq1p from individual haploids determined by well trap assay as described in Materials and Methods, transferred to PVDF and probed with an anti-Rnq1p antibody. Two representative tetrads are shown. A minimum of four tetrads were tested for each mutant. (B) Diagram of mutations in hsp104p that cure \([-\text{RNQ}^+]\). The general domain structure of Hsp104p is indicated above the diagram. NTD, N-terminal domain; NBD1, nucleotide binding domain one; M domain, middle domain; NBD2, nucleotide binding domain two; CTD, C-terminal domain. Mutations found in strains cured of \([-\text{RNQ}^+]\) are indicated below the diagram.
To determine if our mutants were in Hsp104p, we deleted the HSP104 gene in each of our mutants. The resulting Δhsp104 mutants were then mated to a [RNQ⁺] strain. These diploids were sporulated and the [RNQ⁺] status of the haploid progeny was assessed by a Rnq1p well-trap assay (Fig. 3A and data not shown). For this assay, lysates from the haploid cells were resuspended in sample buffer containing 1% SDS and incubated at room-temperature. Insoluble Rnq1p in [RNQ⁺] strains resists solubilization by SDS and does not enter the separating gel when subjected to electrophoresis, but soluble Rnq1p enters the gel and can be detected by western blot. All of the HSP104 deletions produced a 2:2 ratio of [RNQ⁺] to [rnq⁻] haploids, indicating that the mutations caused by EMS were in HSP104.

We sequenced HSP104 from each of the mutant strains and found that eight of the single gene mutations carried a single missense mutation in the gene while one carried a nonsense mutation (Fig. 3B). The missense mutations found were as follows: A178T, E190K, A297T, A502T, G619D, A660T, E689K and T726I. The G619D mutant was dominant for the curing of [RNQ⁺]. Of these mutations, T726I, has been previously described. Additionally, a different mutation in G619, G619V, has also been described and both T726I and G619V have been shown to affect prion maintenance. The other mutations found in this screen are novel.

**HSP104 missense mutations cure [PSI⁺].** Because Hsp104p is also required for the propagation of [PSI⁺], we tested whether the mutations also affected the [PSI⁺] prion. In order to isolate haploids containing both the HSP104 mutation and SUP35, we mated the original mutant strain which contained the mutant HSP104 and RRP to a [RNQ⁻] strain that contained wild type HSP104 and SUP35. We used western blot analysis to identify spores containing wild type Sup35p that had become [rnq⁻] due to the presence of the mutant HSP104 (data not shown). RRP is recognized by polyclonal antibodies raised against Sup35p and can easily be distinguished from wild type Sup35p by its larger size. The [rnq⁻] strains carrying wild type Sup35p will also carry the HSP104 mutation. We then backcrossed these strains to a [PSI⁺] [RNQ⁻] strain. We dissected the resulting diploids and analyzed the degree of nonsense suppression of their haploid progeny in order to determine their [PSI⁺] status (Fig. 4 and data not shown). Each of the mutants that cured [RNQ⁻] was also able to cure [PSI⁺], indicating that the mechanism of curing by the mutants is not specific to [RNQ⁻] but likely affects prion propagation in general. Surprisingly, Hsp104p-E190K, which was recessive for the curing of [RNQ⁻], appeared to be dominant for the curing of [PSI⁺] (Fig. 4). All of the other mutations showed the same dominant or recessive effects on the curing of both [RNQ⁻] and [PSI⁺].

Hsp104p-E190K cures [RNQ⁻] slowly in the presence of wild-type Hsp104p. To further investigate the E190K mutation, the mutant strain was recreated by the pop-in/pop-out method in an unmutagenized version of 74-D694. Interestingly, initial results indicated that HSP104-E190K was dominant for curing [PSI⁺] but recessive for curing [RNQ⁻]. Further analysis, however, suggested that [RNQ⁻] was cured in a dominant fashion as well. We hypothesized that Hsp104p-E190K was less efficient at curing [RNQ⁻] than [PSI⁺] in the presence of wild type Hsp104p. Two other mutations of Hsp104p, P557L and L6462R, are known to cure [PSI⁺] but not [RNQ⁻]. Of these two, Hsp104p-L6462R looked cured by the lack of Rnq1p-GFP fluorescent foci but was confirmed [RNQ⁻] by SDD-AGE and mating to [rnq⁻]. Therefore, Hsp104p-L6462R was suggested to weaken or destabilize [RNQ⁻] but not cure it. To determine if Hsp104p-E190K was similar to either of these two mutants, we transformed haploid [RNQ⁻] cells expressing wild type Rnq1p with a plasmid that expresses Hsp104p-Hsp104p-E190K. We then tested the transformants for the [RNQ⁻] prion by well-trap assay. Of the 24 transformants tested, all but two had become [rnq⁻] (Fig. 5A). The two remaining [RNQ⁻] transformants were grown in selective medium for 22 generations and then retested for soluble Rnq1p (Fig. 5B). Both had become [rnq⁻]. This is consistent with our hypothesis that Hsp104p-E190K shows inefficient curing of [RNQ⁻] in the presence of wild type Hsp104p.

In order to gain more insight into the slow curing of [RNQ⁻] by Hsp104p-E190K, we looked at the effect of Hsp104p-E190K on prion strain variants of [RNQ⁻]. Three strain variants of [RNQ⁻] have been identified that induce [PSI⁺] with differing frequencies. The three [RNQ⁻] variants we used were previously characterized as low, medium and high [PIN⁺] ([PSI⁺] inducibility) and are so named for their increasing ability to induce [PSI⁺] in the presence of moderate overexpression of Sup35p. We transformed haploid cells of low, medium and high [PIN⁺] with a plasmid expressing Hsp104p, Hsp104p-E190K, or a vector control. We then immediately analyzed the transformants that grew for [RNQ⁺] variants. We then immediately analyzed the transformants that grew for [RNQ⁺] variants. We were able to cure [RNQ⁺] variants in all of the transformants. As expected, overexpression of Hsp104p did not cure [RNQ⁻] in any of the variants. Interestingly, the expression of Hsp104p-E190K in the...
presence of wild type Hsp104p resulted in significant Rnq1 protein in the soluble pools of the three variants. In the low [PIN+] strain, the majority of the protein had shifted to the soluble pool while in the high [PIN+] strain, the majority of Rnq1p stayed in the insoluble fraction. In the medium [PIN+] strain, the distribution between soluble and insoluble was approximately equal. Thus, the efficiency of [RNQ+] curing by Hsp104p-E190K is dependent on the strain variant of [RNQ+].

Hsp104p-E190K does not support thermotolerance. Since our data suggest that there is a difference in handling various substrates by Hsp104p-E190K, we next asked whether the mutant was defective in recognition and processing of other substrates in vivo. As Hsp104p is required for resistance to lethal heat shock, we tested whether the strain carrying the Hsp104p-E190K mutant was thermotolerant. Strains expressing either wild type Hsp104p, Hsp104p-E190K, or no Hsp104p at all were first exposed to a sublethal heat stress (37°C) to induce HSP104 expression and then exposed to a lethal heat stress (50°C). Strains expressing wild type Hsp104p were able to recover from a 20 minute heat shock while the Δhsp104 strains showed significant cell death (Fig. 6). The strain expressing Hsp104p-E190K was also unable to survive lethal heat stress and appeared similar to the deletion (Fig. 6). This indicates that the in vivo function of Hsp104p in resolubilizing essential proteins aggregated by heat stress is compromised by the E190K mutation.

E190K forms hexamers but has a defect in ATP hydrolysis. As the maintenance of prions is closely tied to the levels of Hsp104p, we wanted to determine if the steady state expression of the Hsp104p-E190K mutant is similar to that of wild type. Lysates from logarithmically growing cells were analyzed by western blot using an antibody against Hsp104p. Hsp104p-E190K was expressed to a similar level as wild type Hsp104p (Fig. 7A).

To determine the reason why Hsp104p-E190K is unable to support prion propagation and is defective in protecting the cells from heat stress, we examined two biochemical properties of Hsp104p, hexamer formation and ATPase activity. To test the ability of Hsp104p-E190K to form hexamers, we expressed and purified recombinant wild type Hsp104p and Hsp104p-E190K from E. coli. Purified protein was analyzed by size exclusion chromatography under conditions that promote hexamer formation.

The mutant protein eluted in a similar volume as both the wild type Hsp104p protein and a 670 kDa standard, indicating that under these conditions, the mutant is able to form hexamers (Fig. 7B and data not shown).

Given that Hsp104p-E190K forms hexamers, we wanted to see whether it is able to hydrolyze ATP as efficiently as the wild type protein. To test this, the purified protein was incubated with a fixed concentration of ATP. At various time points, the amount of free P, produced by the hydrolysis of ATP to ADP was measured. While Hsp104p-E190K did show hydrolysis of ATP, it did not hydrolyze ATP as well as wild type Hsp104p (Fig. 7C). This provides a likely explanation for its defect in both thermostolerance and prion propagation.

Rnq1p-L94A is unable to propagate the [RNQ+] prion. Our screen uncovered novel mutations in Hsp104p but failed to uncover other novel factors required for the maintenance of the [RNQ+] prion. Had our screen been to saturation, we would have predicted it to reveal point mutations in SIS1, which is required for [RNQ+] propagation, as well as mutations in RNQ1 itself that abolish prion propagation. The domains of Sis1p required for the propagation of [RNQ+] have been described.

We recently performed a screen for mutations in Rnq1p that disrupt prion propagation with the same RRP reporter and [RNQ+] dependent phenotype used here. Interestingly, we identified mutations that allow [RNQ+] to propagate but affect the ability of [RNQ+] to induce [PSF]. None of those mutations affected the interaction with Sis1p, however, as detected by co-immunoprecipitation (Bardill JP and True HL, unpublished data). Recently, one binding site for Sis1p on Rnq1p was identified and a mutation in Rnq1p that severely reduces the interaction with Sis1p has been...
We created this mutation in Rnq1p, L94A, to ask whether the alteration in the interaction with Sis1p at this site would affect the propagation of the [RNQ+] prion. We created the L94A mutation in a Rnq1p-expressing plasmid and analyzed its ability to maintain the [RNQ+] prion. Expression from the native RNQ1 promoter was undetectable by western blot, so we used a GPD promoter which produced Rnq1p-L94A at steady state levels approximately equal to wild type Rnq1p (data not shown). This plasmid was used to replace wild type Rnq1p by plasmid shuffle in either a [RNQ+] or [rnq-] cell. The solubility of the Rnq1p-L94A mutant was then assayed. Wild type Rnq1p fractionates into either the insoluble or soluble fraction when shuffled through a [RNQ+] or [rnq-] strain, respectively (Fig. 8A). Rnq1p-L94A also fractionated into the insoluble fraction when shuffled through a [RNQ+] strain. However, the mutant was also insoluble when shuffled through a [rnq-] strain, indicating that the aggregation of Rnq1p-L94A is non-specific (Fig. 8A). Furthermore, we crossed the strain carrying Rnq1p-L94A to a [rnq-] strain and found that the haploid progeny carrying the wild type RNQ1 remained [rnq-] (data not shown). This suggests that the aggregates from the mutant L94A strain were unable to transmit the aggregate structure and are thus not prion-like. We also tested the ability of Rnq1p-L94A to induce [PSI+]. Following overexpression of Sup35p, cells containing Rnq1p-L94A showed a severe reduction in the ability to induce [PSI+] (Fig. 8B). These data indicate that the interaction of Rnq1p with Sis1p is important for the maintenance of the [RNQ+] prion as well as [PSI+] induction.

Discussion

Here we describe six novel mutations within HSP104 that cure both the [RNQ+] and [PSI+] prions. Five of these mutations are located in one of the nucleotide binding domains and one was found in the M domain. One of the Hsp104p mutants, E190K, displays differential kinetics for curing [PSI+] and [RNQ+] in the presence of wild type Hsp104p. Furthermore, Hsp104p-E190K shows varying kinetics within [RNQ+] variants that suggests Hsp104 may function differently in different prion strain variants. Hsp104p-E190K is unable to support thermotolerance on its own and shows reduced ATPase activity, suggesting that [RNQ+] requires less Hsp104p activity to propagate the prion state than [PSI+].

To our knowledge this represents the first screen for cellular factors that affect [RNQ+] propagation. The phenotypes typically described. We created this mutation in Rnq1p, L94A, to ask whether the alteration in the interaction with Sis1p at this site would affect the propagation of the [RNQ+] prion.
associated with the [\textit{RNQ\textsuperscript{+}}] prion, aggregation of Rnq1p and [\textit{PSI\textsuperscript{+}}] inductions, are not readily amenable to large scale screens. However, as the nonsense suppression of RRP is dependent on the cells being [\textit{RNQ\textsuperscript{+}}],\textsuperscript{50} it constitutes an excellent system to screen for factors that affect [\textit{RNQ\textsuperscript{+}}] propagation.

Our screen for mutants that are unable to propagate [\textit{RNQ\textsuperscript{+}}] only uncovered mutations within \textit{HSP104}. Hsp104p is the only known protein required for the propagation of all known yeast prions.\textsuperscript{3,4,11,25,35} Furthermore, over thirty different mutations have been discovered in Hsp104p that affect prion propagation.\textsuperscript{20,25,28-31} These mutations are found throughout the protein and may interfere with Hsp104p activity at a variety of different steps including substrate recognition, hexamer formation and nucleotide hydrolysis. Thus, there are many targets within Hsp104p that can be altered and cause the loss of prion propagation. Generally, the mutations that cure [\textit{PSI\textsuperscript{+}}] cause the cells to lose the thermotolerance phenotype. However, this is not always a complete loss of thermotolerance.\textsuperscript{28} This indicates that thermotolerance and prion propagation are genetically separable.

The majority of the mutants found in this screen were in either NBD1 (A178T, E190K, A297T) or NBD2 (G619D, A660T, E689K, T726I). A loss of ATPase activity from either of these domains or the inability to form hexamers generally results in defects in the propagation of [\textit{PSI\textsuperscript{+}}]. Of the two domains, NBD1 appears to provide the majority of the ATPase activity required for disaggregation activity.\textsuperscript{23,39} While NBD2 has a low rate of hydrolysis, nucleotide binding to NBD2 regulates the formation of hexamers as well as the rate of hydrolysis of NBD1.\textsuperscript{22,24} As an example of the importance of these two domains, the K218T and K620T mutations which lie within the Walker A motifs of NBD1 and NBD2, respectively, have been shown previously to impair ATPase activity.\textsuperscript{54} Thus, in our screen the mutants in NBD1 and NBD2 likely alter the ATPase activity or oligomerization ability of the protein to produce the loss of prion phenotypes. This conclusion is supported by the decreased rate of ATP hydrolysis of Hsp104p-E190K as compared to wild type Hsp104p.

The final mutation we identified was in the M domain (A502T). Deletions within this middle domain abrogate the function of Hsp104p\textsuperscript{59} as this domain likely plays a role in transducing the allosteric signal from NBD2 to NBD1. Thus, the A502T mutation we identified likely disrupts the coordination between the domains, reducing the ATPase activity and inhibiting the overall disaggregation ability of the protein. Interestingly, the mutation A503V has differential effects on [\textit{PSI\textsuperscript{+}}] and [\textit{RNQ\textsuperscript{+}}].\textsuperscript{60} It causes toxicity in [\textit{PSI\textsuperscript{+}}] strains and affects aggregate size but does not affect [\textit{RNQ\textsuperscript{+}}] propagation.\textsuperscript{60}

Due to its differential effects on curing [\textit{RNQ\textsuperscript{+}}] and [\textit{PSI\textsuperscript{+}}] in the presence of wild type Hsp104, we extended our analysis of the E190K mutant. While it is competent to form hexamers, it shows significantly decreased ATPase activity in vitro as compared to wild type Hsp104. The in vivo function of the mutant is also compromised as it cannot support thermotolerance. Given that it appeared to have no defect in hexamer formation, the mutant likely forms mixed hexamers with wild type Hsp104p. This mixed hexamer may have enough activity to temporarily propagate [\textit{RNQ\textsuperscript{+}}] but not [\textit{PSI\textsuperscript{+}}]. Our data indicate that aggregates of Rnq1p may require less Hsp104p activity to propagate than aggregates of Sup35p. Interestingly, too much Hsp104p activity cures [\textit{PSI\textsuperscript{+}}] but not [\textit{RNQ\textsuperscript{+}}].\textsuperscript{11,25} Thus, [\textit{RNQ\textsuperscript{+}}] appears to persist through a broader range of Hsp104 activities. This may be one reason why [\textit{RNQ\textsuperscript{+}}] cells are found in nature while [\textit{PSI\textsuperscript{+}}] cells are not.\textsuperscript{61} Allowing [\textit{RNQ\textsuperscript{+}}] to persist over a broader range of conditions may also benefit the yeast as the presence of the [\textit{RNQ\textsuperscript{+}}] prion dramatically increases the appearance of [\textit{PSI\textsuperscript{+}}].\textsuperscript{10-12,62} Further investigations into the different regulation of yeast prions will lead to a better understanding of their biological function.

In further characterizing the effect of E190K on [\textit{RNQ\textsuperscript{+}}] by looking at the high, medium and low [\textit{PIN\textsuperscript{+}}] variants, we found that the mixed hexamers of wild type and Hsp104p-E190K differ in their ability to recognize and/or propagate the [\textit{RNQ\textsuperscript{+}}] variants. Previous data suggests that medium and low [\textit{PIN\textsuperscript{+}}] have different aggregate structures than high [\textit{PIN\textsuperscript{+}}].\textsuperscript{63} While Hsp104p-E190K is not the first Hsp104 mutant to show differential curing of [\textit{PSI\textsuperscript{+}}] and [\textit{RNQ\textsuperscript{+}}],\textsuperscript{29} it is the first Hsp104 mutant that demonstrates a difference in recognition between the variants of [\textit{RNQ\textsuperscript{+}}]. Interestingly, the overexpression of the Hsp40 co-chaperone Ydj1p showed differential curing of the
[RNQ⁺] strain variants, suggesting that the whole chaperone network is involved in recognizing distinct structures.⁴⁰

Another Hsp40, Sis1p, is essential for the propagation of [RNQ⁺] and interactions between Sis1p and Rnq1p have been proposed to generate the infectious seeds required for [RNQ⁺] propagation.³¹,³² The Rnq1p-L94A mutation dramatically reduces the ability of Rnq1p to bind Sis1p but does not completely abolish binding.²⁶ Our data indicate that the reduction in Sis1p binding prevents the protein from effectively maintaining a prion. It appears that Rnq1p-L94A aggregates non-specifically, indicating that the interaction with Sis1p promotes the formation of ordered aggregates. These non-specific aggregates are unable to induce [PSI⁺], suggesting the aggregation of Rnq1p alone is not sufficient to promote [PSI⁺] induction.

**Materials and Methods**

**Strain and plasmid construction.** All *S. cerevisiae* strains were derived from 74-D694. Standard culturing and manipulation techniques were used for both yeast and *E. coli.*⁴⁰ Yeast strains were grown in either a rich medium, YPD (1% yeast extract, 2% peptone, 2% glucose), or a synthetic medium (0.67% yeast nitrogen base, 2% glucose) lacking the appropriate amino acids to select for plasmids. Haploid spores were generated from diploid parents and isolated by micromanipulation.

RRP has been described previously.⁴⁰ pPROEx Htb Hsp104 has previously been described.²¹ A fragment containing the E190K mutation was amplified using the oligonucleotides 5'-TTC TTT CCA AGG CAC CAT CGC and 5'-CGG GAT CCA CCC TTG. This product was digested with BglII and EcoRI and ligated into a BglII/EcoRI fragment of pPROEx Htb Hsp104 to generate pPROEx Htb Hsp104-E190K. HSP104 was cloned into pRS316.²¹ by digesting pPROEx Htb Hsp104 with BamHI and XhoI and ligated into a BamHI/XhoI fragment of pRS316. Sequence carrying the E190K mutation was excised from pPROEx Htb Hsp104-E190K on a BamHI fragment and ligated into a cognate fragment of pRS316-Hsp104 or pRS306-Hsp104 to create pRS316-Hsp104-E190K and pRS306-Hsp104-E190K, respectively. Single mutants were also cloned with bridge PCR. The N-terminus of RNQI was amplified with the oligonucleotide 5'-GGG GAT ATC ATG GAT ACG GAT AAG CAA ATC CTC GGC TCC GTC GAC ATG ATC AGC GAT AAG CAA ATC TCA GAT GAG G-3' and an oligonucleotide specific to the mutant. The reverse complement of the mutant specific oligonucleotide was then used along with the oligonucleotide 5'-CCC GTC GAC TCA GTA GGC GTT CTT CTT GGC G-3' to amplify the C-terminus of RNQI. This produced full length RNQI carrying the desired mutation which was digested with EcoRV and SalI and ligated into pRS313 that contained the RNQI promoter on an EcoRV/EcoRI fragment and the RNQI terminator on a SalI/XhoI fragment. For GPD-Rnq1-L94A, an EcoRV/SalI fragment was removed from the pRS313-Rnq1-L94A mutant and cloned into a EcoRV/SalI fragment of p413-GPD.

**EMS mutagenesis.** Overnight cultures of [RNQ⁺] cells expressing RRP were washed twice in 50 mM potassium phosphate buffer pH 7.0 and then resuspended in buffer. The cells were normalized to 5 x 10⁷ cells/ml and incubated with ethyl methyl sulfonate (EMS) at a final concentration of 3% (v/v). Mutagenesis was halted at various time points by the addition of an equal volume of 10% Na₂SO₃. The treated cells were extensively washed with H₂O and their viability was determined by plating assay. A culture with approximately 14% viable cells was plated on YPD to assess color.

**Analysis of [RNQ⁺] status.** The [RNQ⁺] status of the cells was biochemically assessed in one of two ways.²² For a full solubility assay, yeast cells were lysed with glass beads in buffer containing 100 mM Tris-HCl pH 7, 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM glycerol, 0.5 mM dithiothreitol (DTT), 50 mM N-ethylmaleimide (NEM), 3 mM phenylmethylsulfonylfluoride (PMSF) and complete Mini protease inhibitor cocktail (Roche). Following lysis, an equal volume of RIPA buffer (50 mM Tris-HCl pH 7, 200 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) was added to the lysate and cell debris was cleared by a brief centrifugation. This cleared lysate is the total protein. Insoluble protein was pelleted by centrifugation at 80,000 RPM (27,000 xg) in a Beckman TLA-100 rotor for 30 minutes. The supernatant containing the soluble protein was removed and the pellet was resuspended in a 1:1 mix of lysis buffer and RIPA buffer. Total, supernatant and pellet fractions were subjected to SDS-PAGE, transferred to PVDF membranes and probed with an anti-Rnq1p antibody.

Well trap assays were performed as previously described.²² Cells were lysed with glass beads in buffer containing 50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 5% glycerol supplemented with an anti-protease solution (Sigma), 10 mM PMSF, 50 mM NEM. Lysate was incubated in sample buffer (100 mM Tris-Cl pH 6.8, 1% SDS, 10% glycerol, 0.1% bromophenol blue) for seven minutes at room temperature. Treated lysates were subjected to SDS-PAGE, transferred to PVDF and blotted with an anti-Rnq1p antibody.

**Thermotolerance assays.** Thermotolerance assays were performed as previously described.²² Equal numbers of yeast cells in logarithmic growth were resuspended in liquid medium. The resuspended cells were pretreated at 37°C for 30 minutes and then shifted to 50°C for various periods of time. Following heat shock, the cells were incubated on ice for 2 minutes. The cells were then serially diluted and spotted onto YPD.

**Hsp104p purification.** His₆-Hsp104p and His₆-Hsp104p-E190K were expressed and purified from *E. coli* BL21(DE3) as previously described.²² Polyhistidine-tagged Hsp104p and Hsp104p-E190K were isolated by affinity chromatography on Ni-NTA sepharose. The histidine tag was cleaved with TEV protease and the untagged protein further purified by anion exchange chromatography. The fractions were analyzed by SDS-PAGE, pooled and frozen at -80°C.

**ATPase activity assay.** Characterization of Hsp104 ATPase activity was performed in Buffer A (40 mM Tris, pH 7.5, 175 mM NaCl, 5 mM MgCl₂, 0.02% Triton X-100) as reported.⁵⁴ The reactions were performed at 37°C with either 1 μg Hsp104p or 5 μg Hsp104p-E190K and 5 mM ATP (Sigma). At various time points during the reaction, Malachite Green Reagent was added to the reaction tube to quantify the amount of free Pi, and the
color development was stopped by the addition of citric acid. The A_{600} of the sample was determined and the absorbance units calibrated against a standard of known concentrations of KH_{2}PO_{4}. Each time point was done three independent times and the mean ± standard deviation was calculated.

**Gel filtration chromatography.** Gel filtration was performed as previously described. Two milligrams of purified Hsp104p or Hsp104p-E190K were incubated for 5 minutes on ice with Buffer B (40 mM Tris-HCl, pH 8.0, 15 mM NaCl, 5 mM MgCl_{2}, 10 mM ATP) before loading onto Sephacyr S-300 High Resolution resin (Amersham Biosciences). The column was equilibrated in Buffer B and was run at 4°C at a flow rate of 0.05 mL/min. Fractions (5 mL) were collected and analyzed for the presence of Hsp104 by western blot using an antibody probe against Hsp104p. Resolved bands were quantified using Image J. Molecular weights of Hsp104p and Hsp104p-E190K were estimated by comparison of their elution profiles with the following standards: thyroglobulin M, 670,000; catalase M, 250,000; bovine serum albumin M, 66,000.

**[PSF] induction assays.** [PSF] induction assays were performed as described previously. Plasmid shuffled strains containing the Rnq1p mutants were transformed with a Ura-marked plasmid carrying SUP35 (pSUP2,56) and plated on selective media for both plasmids. Individual transformants were grown in selective media to OD_{600} -1.6 and plated on YPD. After five days of growth, [PSF] colonies were counted as any colony with a light pink sector. Representative colonies were checked for curing on plates containing 3 mM GdnHCl. The vast majority (>95%) of colonies with light pink sectors were curable on 3 mM GdnHCl (data not shown). Overexpression of Sup35p in a [RNQ'] strain has also been shown to create non-heritable amyloids of Sup35p that cause nonsense suppression similar to [PSF]. Since this nonsense suppression is dependent on the overexpression of Sup35p, we selected cells with light pink sectors and spotted them onto medium containing 5-FOA. Cells that converted to [PSF] remained light pink on 5-FOA medium, while cells with non-heritable amyloids reverted back to red. Experiments with both wild type Rnq1p and the mutants revealed that, on average, about 12% of colonies with light pink sectors were the result of non-heritable amyloids while the rest were bona fide [PSF] (data not shown). This proportion is in line with the frequency of non-heritable amyloid induction previously reported.

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**References**

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**References**